# Role of PTEN nuclear translocation in excitotoxic and ischemic neuronal injuries following stroke

by

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# ABSTRACT

Stroke is a leading cause of death and disability in developed countries. About 87% of stroke cases results from blood vessel(s) occlusion in the brain, leading to neuronal death and neurological impairment (Lloyd-Jones et al., 2010). The ischemic progression likely involves multiple events, and increasing evidence showed that the ischemic neuronal death is caused, at least in part, by over-activation of N-methyl-D-aspartate subtype glutamate receptors (NMDARs) (Rothman, 1983, Rothman, 1984, Simon et al., 1984). A large number of pre-clinical studies showed that NMDAR antagonists have strong neuroprotective effects against ischemic insults (Park et al., 1988b, Bullock et al., 1990). However, none of the following human clinical trials have succeeded yet (Muir, 2006). Several explanations have been suggested (Gladstone et al., 2002), including the following two major reasons that may be overcome by the novel therapeutics proposed here. First, the stroke patient inclusion periods (>6 hours) used by most clinical trials are beyond the therapeutic time window (<1 hour) of NMDAR antagonists. Second, NMDAR antagonists, when used at the concentration required to block excitotoxicity, also abolish the pro-survival downstream cascades of NMDARs, thus leading to intolerable side effects. Therefore, instead of aiming at the receptor *per se*, my current study turns to focus on identifying NMDAR's downstream death signals, which may permit a much wider therapeutic time window and are solely involved in pro-death signaling pathways of NMDARs. My results suggest that PTEN (phosphatase and tensin homolog deleted on chromosome TEN) is such a candidate. Both NMDA stimulation on cultured neurons (*in vitro*) and ischemic insults in rats (*in vivo*) can trigger ubiquitination-

dependent PTEN translocation into the nucleus. Inhibition of PTEN nuclear translocation using an interfering peptide (Tat-K13) can significantly reduce the excitotoxic/ischemic neuronal injuries both *in vitro* and *in vivo*. Most excitingly, the intervention time window of PTEN nuclear translocation (6 hours post-ictus) is much wider than that of the only clinically available anti-stroke drug named tissue plasminogen activator (3 hours) (NINDS, 1995). These findings strongly suggest that PTEN nuclear translocation is a critical step leading to excitotoxic/ischemic neuronal injuries, and Tat-K13 represents a potential therapeutic strategy for stroke treatment.

# PREFACE

This work was designed by Dr. Yu Tian Wang, Dr. Changiz Taghibiglou and me. The experiments and data analysis were mainly performed by me, except work in Fig.3E was performed by Dr. Changiz Taghibiglou (Dr. Yu Tian Wang lab); the double-blinded counting in Fig. 5 was performed by Ida Wang (Dr. Yu Tian Wang lab); MCAO surgery and peptide injection in Figure 10 and Figure 11 were performed by Dr. Woei-cherng Shyu (Dr. Woei-cherng Shyu lab), Dr. Zhifang Dong (Dr. Yu Tian Wang lab) and Ted Lai (Dr. Yu Tian Wang lab), while the nuclear fractionation and western blotting were done by me; work in Figure 12, 13 and 14 was all performed by Dr. Woei-Cherng Shyu (Dr. Woei-cherng Shyu lab).

The animal studies have been approved by UBC research ethics board. The animal care certificates are as follows: A08-0247, A06-0356, and A08-0807.

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# LIST OF ABBREVIATIONS

Akt/PKB - protein kinase B

AMPAR - alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype glutamate receptor

FDG-PET - [$^{18}$F] fluoro-2-deoxyglucose positron emission tomography

GFP - Green Fluorescent Protein

HDACs - histone deacetylases

HIV-1 - human immunodeficiency virus-type 1

Hsp90 - heat shock protein 90

MCA - middle cerebral artery

MCAO - middle cerebral artery occlusion

MRI - magnetic resonance image

NEDD4-1 - neural precursor cell expressed, developmentally downregulated-4-1

NLS - nuclear localization signal

NMDAR - N-methyl-D-aspartate subtype glutamate receptor

NR2AR - NR2A-containing NMDAR

NR2BR - NR2B-containing NMDAR

PDK1 - phosphatidylinositol-dependent kinase 1

PH domain - pleckstrin homology domain

PI(3,4,5)P$_3$ - phosphatidylinositol(3,4,5)- triphosphate

PI(4,5)P$_2$ - phosphatidylinositol(4,5)- bisphosphate

PI3K - phosphatidylinositol-3-kinase

PSD - postsynaptic density

PTEN - phosphatase and tensin homolog deleted on chromosome 10

RasGRF1 - Ras–guanine nucleotide–releasing factor 1

RasGRF2 - Ras–guanine nucleotide–releasing factor 2

TBP - TATA-binding protein

tPA - tissue plasminogen activator

XIAP - X-linked inhibitor of apoptosis protein

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# 1 INTRODUCTION

## 1.1 Stroke

Stroke is the third leading cause of death and the leading cause of disability in developed countries (Lloyd-Jones et al., 2010). There are around 795,000 stroke cases per year, and about 80% of stroke patients survive. However, among these survivors, 50% had some hemiparesis, 30% were unable to walk without some assistance, 26% were dependent in activities of daily living and 26% were institutionalized in a nursing home (Lloyd-Jones et al., 2010). The estimated direct and indirect cost of stroke for 2010 is $73.7 billion. Therefore, development of effective therapeutics is critical in relieving human suffering and economic burden.

Stroke results from sudden or gradually progressing disturbance in the blood flow to the brain, leading to a rapidly developing loss of brain functions. The blood supply could be hampered in two ways: 1) ischemic stroke, caused by blood clots formed inside the vessels; or 2) hemorrhagic stroke, in which the vessels rupture, resulting in blood leaking into the brain. The ischemic stroke accounts for about 87% of all stroke cases, while the hemorrhagic stroke only represents the rest 13% (Green and Shuaib, 2006, Lloyd-Jones et al., 2010).  Therefore, in my current study, I focus on investigating the ischemic type of stroke.

## 1.1.1 Core and penumbra of ischemic stroke

In the ischemic stroke, the affected brain areas can be divided into ischemic core, penumbral, and extrapenumbral regions, based on the degree of blood flow reduction during ischemic insults (Nedergaard et al., 1986, Duverger and MacKenzie, 1988, Ginsberg and Pulsinelli, 1994, Back et al., 1995). In the core ischemic regions, the blood flow is less than 15% of baseline level, while, in the penumbral regions, the blood supply is between 15% and 40%. However, in the extrapenumbral regions, the blood flow is maintained above 40%. This penumbra concept is of considerable interest because it is the conceptual basis for the therapeutic reversal of ischemic damage arising from stroke (Hossmann, 1994).

Besides the measurement of blood flow, there are other major biochemical and physiological differences between the core and penumbra. During an ischemic insult, the core undergoes severe ATP-depletion (about 25% of basal level) which results in anoxic depolarization (Sun et al., 1995). This rapid depolarization is invariably accompanied by a dramatic increase in extracellular $K^+$ caused by the failure of $Na^+/K^+$-ATPase (Gido et al., 1997), as well as a significant decrease in extracellular $Ca^{2+}$ due to the opening of calcium-permeable channels (Harris and Symon, 1984). In contrast, the penumbra has a preserved ATP level of 50%-70% of basal value during ischemic challenges (Welsh et al., 1991, Folbergrova et al., 1992), and only experiences a sporadic transient depolarization which has the characteristic form of spreading depression (Ginsberg and Pulsinelli, 1994). Unlike the anoxic depolarization in the core, the spreading depression in penumbra could be significantly reduced by N-methyl-D-aspartate subtype glutamate receptor (NMDAR)

antagonist MK-801, suggesting that this spreading depression may originate from glutamate release in the core ischemic regions (Iijima et al., 1992, Mies et al., 1994). Another major difference between core and penumbra is the free radical changes (Solenski et al., 1997). The ischemic core has very little free radical increase, probably due to the low oxygen level of core region during ischemic insult and early reperfusion. In contrast, the free radical increases dramatically in the penumbra because blood flow in the penumbra is maintained at a moderate level during ischemic challenges and undergoes a prompt restoration in the early reperfusion.

These differences trigger different cell death cascades in the ischemic core and penumbra. One study showed that the ratio between apoptotic cells and necrotic cells is 1:1 in the ischemic core, but 9:1 in the penumbra at 4 hours following a 2-hour transient ischemia (Charriaut-Marlangue et al., 1996). Therefore, it is almost impossible to rescue the core damage when ischemia lasts more than 2 hours, whereas the injuries in the penumbra are reversible and thus become the major therapeutic targets for almost all current stroke research.

## 1.1.2 Progression of ischemic infarct

The cell death in ischemic stroke is usually measured by quantifying the volume of infarct, which represents a contiguous mass of damaged brain tissue. The infarct first appears in the core region and expands into penumbra as the ischemia is prolonged. 10-20 minutes ischemia only produces scattered cell death in the core (Li et al., 1995), while 1 hour ischemia causes infarct in the core (Kaplan et al., 1991). When ischemia lasts for more than 6 hours, the infarct develops fully into the penumbra

and becomes part of the irreversibly damaged area (Hata et al., 2000).

The progression of ischemic infarct could be divided into three phases: acute, sub-acute and delayed phases (Hossmann, 2006). During the acute phase, the ischemia-induced ATP depletion (Welsh et al., 1991) and anoxic depolarization (Nedergaard and Hansen, 1993) are believed to be the initiators of ischemic damage. These injuries are usually established within a few minutes of stroke onset. In the sub-acute phase, the infarct expands from core into penumbra which may take several hours to several days to mature depending on the duration of ischemia. The main mechanisms of this phase include spreading depression (Pettigrew et al., 1996) and a variety of cellular damages such as glutamate excitotoxicity (Simon et al., 1984), calpain over-activation (Vosler et al., 2008), mitochondria dysfunction (Kluck et al., 1997), free radical toxicity (Eliasson et al., 1999), and endoplasmic reticulum (ER) dysfunction (Paschen and Frandsen, 2001). The sub-acute phase is when the major increase in cell death occurs. The final stage is called delayed phase because it could last for several days or even weeks. Brain edema and inflammation are the major contributors to this slowly progressing damage (DeGraba, 1998, Badaut et al., 2002, Griesdale and Honey, 2004).

The primary events during ischemia are inhibition of electron transport chain and subsequent drop in ATP production triggered by the abrupt disruption in blood flow. The major consequence of this energy depletion is failure of $Na^+/K^+$-ATPase, which in turn causes accumulation of extracellular $K^+$ (~60mM) and hence anoxic depolarization (Nedergaard and Hansen, 1993). This anoxic depolarization is characterized by a large (10-30mV) extracellular negative direct-current shift and

massive changes in ion gradients such as decreased extracellular $Na^+$ (~50mM), $Cl^-$ (~90mM) and $Ca^{2+}$ (~0.1mM) (Jiang et al., 1992, Martin et al., 1994).

It is suggested that the anoxic depolarization-induced $K^+$ and glutamate release from core region is responsible for the spreading depression-like depolarization in the penumbra (Nedergaard and Hansen, 1993, Hossmann, 1996). Compared to the anoxic depolarization, the spreading depression is less drastic and is dependent on the ionotropic glutamate receptors, particularly NMDA receptor subfamily (Iijima et al., 1992, Mies et al., 1994). The causative role of spreading depression in ischemic injury progression is supported by the linear relationship between the number of depolarization and the infarct size. With each wave of spreading depression, the infarct volume increases by more than 20% (Mies et al., 1993). What's more, artifactually increasing the number of spreading depression by KCl (Busch et al., 1996) or electrical stimulation (Back et al., 1996) increases the infarct volume by 30~100% in ischemic tissues, while, in contrast, a similar artifactual spreading depression in healthy tissues is harmless. The explanation is most likely to be that the ischemic tissues have a mis-relationship between the reduced blood supply and the increased energy demand of ion exchange pumps, leading to the expansion of infarct into the penumbra area; on the other hand, in healthy tissues, the enhanced metabolic workload could be compensated by an increase in blood flow, and thus the spreading depression is not damaging (Lipton, 1999).

Since the infarct evolves even after the blood flow is restored, the progression of ischemia cannot be simply explained by energy impairment. Collectively, the disturbances that follow the initial metabolic changes are referred to as the

"molecular injuries", which interconnect with each other in such a complex way that it's hard to predict their relative pathogenic importance in ischemic progression. These "molecular injuries" mainly include glutamate excitotoxicity (Simon et al., 1984), calpain over-activation (Vosler et al., 2008), mitochondria dysfunction (Kluck et al., 1997), free radical toxicity (Eliasson et al., 1999), and endoplasmic reticulum (ER) dysfunction (Paschen and Frandsen, 2001).

Glutamate excitotoxicity has been intensively studied and debated ever since the finding of protective effect of NMDA receptor antagonist for forebrain ischemia in rat by Simon et al. in 1984 (Simon et al., 1984). Normal extracellular glutamate concentration measured by microdialysis is around 1-5μM, whereas, during ischemia, extracellular glutamate increases progressively to a toxic level of 30-80μM and remains at its maximum for as long as 3 hours (Wahl et al., 1994). Blocking the over-activation of glutamate receptors, particularly NMDA receptors, showed strong protective effects against ischemic injuries in several animal models, no matter whether the antagonists were administered before or up to 1 hour post-ischemia onset (Park et al., 1988b, a, Bullock et al., 1990, Nishikawa et al., 1994, Sauer et al., 1995). However, the underlying mechanisms are not well understood. Whether the protective effects of glutamate/NMDA receptor antagonists are mediated through decreasing ischemic core temperature (Corbett et al., 1990), increasing blood flow in the infarct area (Buchan et al., 1992), or preventing $Ca^{2+}$ influx (Choi, 1987) into neurons is hotly debated in different studies. Although various results still need to be satisfactorily explained, some well-controlled studies persuasively demonstrated that NMDA antagonists significantly reduce the ischemic infarct volume with no measurable changes in either core temperature (Takizawa et al., 1991, Frazzini et

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al., 1994) or blood flow (Park et al., 1989, Dezsi et al., 1992), strongly suggesting that the protective effects of NMDA blockers are most likely via blocking $Ca^{2+}$ entry into neurons through NMDA receptors.

The $Ca^{2+}$ overload caused by over-activation of NMDA receptors leads to many cellular damages including $Ca^{2+}$-activated protease calpain and mitochondrial dysfunction. The intracellular concentration of $Ca^{2+}$ during ischemia could reach micromolar range that is required for activation of calpain, a neutral cysteine protease (Silver and Erecinska, 1992). Following $Ca^{2+}$ stimulation, calpain precursors are autocatalytically cleaved into activated fragments, which then attack many critical cytoskeletal proteins, such as MAP2 (Pettigrew et al., 1996), spectrin (Hong et al., 1994) and tubulin (Saido et al., 1994), leading to ischemic cell death (Vosler et al., 2008). In addition to activating calpain, the elevated $Ca^{2+}$ also contributes to ischemic injuries by causing opening of mitochondrial permeability transition (MPT) pores (Siesjo and Siesjo, 1996, Dubinsky and Levi, 1998). The MPT pores increase permeability of the inner mitochondrial membrane and hence result in the imbalance in electrochemical gradients. The disturbance in ion gradient equilibration not only interferes with the electron transport system/ATP production, but also enhances swelling of mitochondria. This swelling may eventually cause rupture of the outer mitochondrial membrane and release of pro-apoptotic proteins, particularly cytochrome *c*, leading to initiation of apoptotic cell death cascade (Kluck et al., 1997, Reed, 1997, Green and Kroemer, 2004).

Under normal conditions, the rates of free radicals generation and elimination are equal, hence maintaining a relatively constant concentration that is tolerable by the

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cells. During ischemia, the net production rate of free radicals is greatly enhanced due to over-activation of cyclooxygenase (COX) pathway (Kukreja et al., 1986) or disturbance of mitochondrial electron transport system (Piantadosi and Zhang, 1996). Moreover, the reaction between enhanced free radicals and nitric oxide (NO), which is generated by the neuronal NO synthase (nNOS), leads to the formation of an even more toxic molecular called peroxynitrite (ONOO$^-$) (Eliasson et al., 1999). Together, the free radicals and peroxynitrite produce profound oxidative damages to the cells by causing endoplasmic reticulum (ER) stress, mitochondrial disturbances, fragmentation of DNA and so on (Hossmann, 2006). In a large number of animal studies, agents that prevent free radical/NO buildup have been proved to be strongly neuroprotective against ischemic stroke (Kinouchi et al., 1991, Umemura et al., 1994, Yang et al., 1994). But very surprisingly, in two recently completed human clinical trials (SAINT 1 and 2), the free radical scavenger (NXY-058) only had marginal positive effects and failed to meet treatment criteria (Wang and Shuaib, 2007, Kimelberg, 2008). For future therapeutic development, it would be very helpful to resolve the conflicting results between animal studies and human clinical trials.

Endoplasmic reticulum (ER) dysfunction is also suggested to contribute to ischemic cell death. During ischemia, the stress of energy failure, excitotoxicity and free radicals induce depletion of ER $Ca^{2+}$ store and hence result in disturbance in $Ca^{2+}$ homeostasis (Paschen and Frandsen, 2001). The major consequences of the ER dysfunction are expression of ER stress proteins, such as DNA-damage-inducible gene 153 (gadd153) (Onoue et al., 2005), and suppression of global protein synthesis (Paschen and Doutheil, 1999). The latter is suggested to be caused by activation of protein kinase R (PKR) which phosphorylates and inhibits eIF-2a, an

initiation factor for protein synthesis (Paschen and Doutheil, 1999).

The delayed ischemic damage is primarily mediated by brain edema and inflammation. The brain edema induced by increased intracellular osmolarity and breakdown of blood-brain barrier is considered as one of the most dangerous complications of stroke (Hossmann, 2006). Some studies showed that the ischemic brain edema could be significantly reduced by blocking the aquaporin channels, which facilitates water flux through the plasma membrane (Badaut et al., 2002, Griesdale and Honey, 2004). However, as the driving force of cell swelling is the osmotic gradient across the plasma membrane, blocking aquaporin channels simply delays the edema formation and hence its therapeutic importance is limited. Inflammation is another factor contributing to the slow progressing ischemic damages. Up-regulation of two major pro-inflammatory cytokines, IL-1β and TNF-α, are suggested to be responsible for inflammation response following ischemic insults (DeGraba, 1998).

In conclusion, the progression of ischemic stroke is a complex process that evolves in time and space. The injuries initiate from the infarct core within a few minutes of ischemia onset and expand into penumbra during the following hours, days and even weeks after restoration of blood flow. This slow development of ischemic damages provides a relatively wide time window for therapeutic intervenes. Thus, understanding the underlying mechanisms, especially those involved in delayed cell injuries, are critical for developing effective treatments for ischemic stroke (Hossmann, 2006).

## 1.1.3 Treatments for ischemic stroke

Current treatments for ischemic stroke include two major approaches. One is vascular-based therapeutics that aims to improve blood reperfusion after stroke onset. The other is neuroprotection-based therapeutics that aims to interfere with the signaling cascades that lead to ischemic cell death.

In the first class, the only clinically available drug is tissue-plasminogen activator (tPA) (NINDS, 1995, Wardlaw et al., 1997). However, the use of tPA is limited due to several factors. First, tPA is protective only when given within 3 hours of stroke onset (NINDS, 1995, Hacke et al., 1998). Given that the median time for patients to get to hospital is about 6 hours, tPA is not a choice in most stroke cases. Second, computed tomography (CT) scan is required to rule out any signs of hemorrhage before tPA application, because of the adverse effects of tPA on hemorrhagic stroke (Green and Shuaib, 2006). Hence, the need of CT scan further delays the administration of tPA to the patients. Third, tPA increases the chance of hemorrhagic transformation in the stroke patients (Wardlaw et al., 1997, Brinker et al., 1999), possibly through increasing the level of matrix metalloproteinase-9 (MMP-9), and therefore enhancing the hemorrhage rate (Lapchak et al., 2000). All together, these limitations of tPA result in its administration in less than 5% of stroke patients (Green and Shuaib, 2006).

The second class of treatments is called neuroprotection-based therapeutics, because the working mechanism is to block the signaling cascades that lead to ischemic cell death. In the animal studies, a large number of drugs, such as NMDA

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receptor antagonists, have been proven to be neuroprotective (Park et al., 1988b, Bullock et al., 1990). However, none of them has passed the human clinical trials yet (Muir, 2006). The discrepancies between the animal results and the clinical outcomes are explained as follows (Hunter et al., 1995, Green and Shuaib, 2006). First of all, experimental animals (particularly rodents) are different from human stroke patients in many ways. For example, unlike human brains, rodent brains are lissencephalic and have very little white matter, which may suggest different mechanisms of injuries and neuroprotection after stroke. Also, the rodents used in the animal studies are generally young and healthy, while human stroke patients usually have coexisting diseases, such as age and diabetes (Gladstone et al., 2002). Hence, extra caution should be taken when interpreting results of animal studies. Second, the poor clinical feasibility of preclinical results is also suggested to be associated with many methodological issues, including problems with blinding, randomization, reporting bias and use of anaesthetic agents that have intrinsic neuroprotective activities (Macleod et al., 2004, Muir, 2006). Third, some drugs simply delay the ischemic injury process rather than provide a sustained neuroprotection (Valtysson et al., 1994, Corbett and Nurse, 1998, Gladstone et al., 2002). Hence, they failed the long-term human clinical trials, in spite of their promising protective effects in the short-term animal studies. Fourth, most compounds have significant dose-limiting toxicity. They produced intolerable adverse effects (e.g. sedation and ataxia) in human stroke patients, when used at the same doses that have the maximal neuroprotective effects in animal studies (Prass and Dirnagl, 1998). Last, most drugs show protective effects only when administered within the first hour of stroke onset in animal studies, whereas the clinical trials usually use inclusion periods of >6 hours for patient recruitment, hence resulting in

lack of efficacy (McCulloch, 1991, Scatton, 1994, Prass and Dirnagl, 1998). In all, the above reasons explain the difficulties in transferring the positive therapeutic outcomes of animal experiments into clinical studies.

Besides the above two major categories of stroke treatments, other therapies that are currently under evaluating include promoting arteriogenesis of collateral vessels by granulocyte-macrophage colony-stimulating factor (GM-CSF) (Buschmann et al., 2003), as well as enhancing regeneration of neurons by transplantation of immortalized neuroepithelia cells (Modo et al., 2002), neural stem cells (Chu et al., 2004) and stem cells derived from fetal brain tissue (Borlongan et al., 1997), bone marrow (Grabowski et al., 1995) or umbilical cord blood (Willing et al., 2003).

To make the future drug investigation more relevant to clinical situations, animal studies should adhere to better quality controls including double-blinding, complete randomization, minimizing usage of anesthetic agents (e.g. ketamine) that have marked intrinsic neuroprotective activities, cerebral blood flow monitoring, wider therapeutic time window evaluation, long-term benefit assessment, et al. (STAIR, 1999).

**1.1.4 Animal models of ischemic stroke**

Although animal models cannot exactly resemble human stroke conditions, they provide a valuable means for careful dissection of mechanisms of injuries and neuroprotection after ischemic insults, and for pre-clinically testing new anti-stroke therapies. Therefore, animal models form the fundamental basis of current stroke

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studies. Large animals (e.g. monkey), compared to small animals (e.g. rat), are generally believed to be better models for human stroke research, because larger animals have gyrencephalic brains, which are anatomically and functionally closer to those of humans (Traystman, 2003). Yet, experiments with larger animals are very expensive, labor intensive, as well as involve public animal welfare concerns. Therefore, most labs still choose to use small animals, especially rodents, which are of low costs and less likely to promote animal welfare issues (Traystman, 2003). However, rodent brains do have some major differences from human brains. First, unlike human brains, rodent brains are lissencephalic and have very little white matter, which may suggest different mechanisms of injuries and neuroprotection after stroke (Yam et al., 1998, Dewar et al., 1999). Second, rodent stroke models produce very large infarcts, ranging from 5%-50% of the ipsilateral hemisphere, with most studies having infarcts between 21 and 45% of the ipsilateral hemisphere; on the other hand, human stroke are mostly small in size, ranging from 4.5%-14% of the ipsilateral hemisphere (Carmichael, 2005). Because of this unique characteristic of rodent stroke, it is important to monitor the functional recovery of specific neural circuits, such as motor/sensory cortex maps, in addition to the conventional emphasis on minimizing infarct size. Third, human stroke are usually complicated by the coexisting diseases, such as age, diabetes, hypertension, and arrhythmia (Traystman, 2003). Using young and healthy animals may not exactly mimic the real conditions of human stroke, and results in difficulties in transferring the positive therapeutic outcomes of animal experiments into clinical studies. Therefore, it will be useful to incorporate these complications into animal models by, for example, using spontaneously hypertensive and diabetic rats (Sakakibara et al., 2002). All, in spite of the above problems, rodent models still form the fundamental basis of the current

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preclinical studies. In fact, as long as the researchers take particular caution about the experimental design and data interpretation, rodent models will still be great tools for studying the mechanisms of injuries and neuroprotection after stroke.

To induce ischemic stroke, the most widely-used method is middle cerebral artery (MCA) occlusion, because the majority of human strokes results from an occlusion of this artery (Garcia, 1984). The transient occlusion of MCA models the ischemia and reperfusion, while the permanent occlusion of MCA mimics the prolonged vessel blockade (Ringelstein et al., 1992). There are several commonly-used ways to occlude the MCA. The proximal MCA can be occluded by using intraluminal filament. This method produces a relatively large infarct area, including a core region in the lateral portion of the caudate-putamen and parietal/somatosensory cortex, and a penumbra area in most other regions of the neocortex and entorhinal cortex (Bolander et al., 1989, Nagasawa and Kogure, 1989, Lipton, 1999). To resemble human stroke more closely, a more restricted damage could be produced by occluding the distal MCAO with neurosurgical clip/suture. This distal model forms an infarct core in most of the frontal and parietal cortex, and a penumbra area in adjacent temporal, frontal, and cingulate cortex (Tamura et al., 1981, Chen et al., 1986, Carmichael, 2005). But the disadvantage of this model is that it requires craniotomy. Besides using neurosurgical clip/suture, branches of distal MCA can also be occluded by either photothrombosis, or local injection of thromboembolic clots or endothelin-1. The photothrombosis can be induced by laser irradiation of blood vessels following intravenous administration of photosensitizing dye (Watson et al., 1985). The major advantage of photothrombosis is that it can place a small size of infarct within a functionally distinct cortical area, such as barrel field or

hindpaw somatosensory cortex. However, the disadvantages of photothrombosis stem from microvascular injuries. This technique produces relatively little ischemic penumbra, regions of local collateral flow and reperfusion, compared to those seen in human stroke. Also, it induces simultaneous vasogenic edema (leaky vessels) and cytotoxic edema (swollen cells), which more closely mimics human traumatic brain injuries rather than focal stroke (Albensi et al., 2000, Schneider et al., 2002, Provenzale et al., 2003). Occlusion of distal MCA can also be achieved by directly injecting spontaneously formed blood clots into MCA (Beech et al., 2001). This model closely resembles human stroke, but the location and size of infarcts are not consistent, which make the results of neural repair experiments difficult to analyze (Carmichael, 2005). The last alternative to occlude distal MCA is local injection of endothelin-1, which is a potent and long-acting venous and arterial vasoconstrictor (Fuxe et al., 1997). This approach produces localized stroke within specific brain circuits (Adkins-Muir and Jones, 2003). However, the effects of endothelin-1 on facilitating astrocytosis and axonal sprouting make the interpretation of outcomes of neuroprotective treatments difficult (Carmichael, 2005).

Different animal models capture different elements of human stroke. With attention to the specific research purposes, different animal models can be selected to study the major targets of stroke therapies, such as improving blood reperfusion, interfering with cell death cascades and repairing the functional circuits (Carmichael, 2005).

## 1.2 NMDA receptors

Glutamate mediates fast synaptic transmission at most excitatory synapses in the mammalian brain (Dingledine et al., 1999). Glutamate primarily acts on two major subfamilies of postsynaptic, ligand-gated glutamate receptors: alpha-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid subtype glutamate receptor (AMPA receptor or AMPAR) and N-methyl-D-aspartate subtype glutamate receptor (NMDA receptor or NMDAR). AMPA receptors are responsible for the primary depolarization in glutamate-mediated neurotransmission and play key roles in synaptic plasticity (Man et al., 2000, Man et al., 2003, Keifer and Zheng, 2010). In contrast, NMDA receptors mainly contribute to certain forms of synaptic plasticity (e.g. LTP and LTD) under physiological conditions (O'Dell et al., 1991, Lu et al., 1998, Liu et al., 2004), but are regarded as the main mediator for glutamate-induced excitotoxicity under pathological insults such as ischemic stroke (Arundine and Tymianski, 2004, Cull-Candy and Leszkiewicz, 2004). Given that the main purpose of my present study is to develop effective anti-stroke treatments, only NMDA receptor will be discussed here.

### 1.2.1 Characteristics of NMDA receptors

NMDA receptors are ionotropic receptors that are permeable to $Na^+$, $K^+$, and $Ca^{2+}$ (Hardingham and Bading, 2003). Under physiological conditions, their high $Ca^{2+}$ permeability contributes to activation of intracellular signaling pathways that are critically important in mediating certain forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD) (Collingridge et al., 2004). In

contrast, the pathological conditions, such as ischemic stroke, lead to over-activation of NMDA receptors that results in an excessive influx of $Ca^{2+}$ and hence triggers excitotoxic cell death cascades (Rothman, 1983, Rothman, 1984, Simon et al., 1984, Arundine and Tymianski, 2004). Besides the high permeability to $Ca^{2+}$, NMDA receptors are distinguished from other ligand-gated channels by their sensitivity to extracellular $Mg^{2+}$ blockade at resting membrane potential (Kutsuwada et al., 1992, Monyer et al., 1992, Monyer et al., 1994). As the $Mg^{2+}$ blockade could only be relieved at depolarized potentials, NMDA receptors act as a coincidence detector of both pre- and post-synaptic excitation. Upon activation, NMDA receptors (NMDAR-EPSC) exhibit exceptionally slow kinetics with a rise time of about 10 ms and a decay time of about 100 ms (Vicini et al., 1998, Cull-Candy and Leszkiewicz, 2004).

To date, seven NMDA receptor subunits have been identified: the ubiquitously expressed NR1 subunit, four NR2 subunits (A-D), and two NR3 subunits (A and B) (Cull-Candy and Leszkiewicz, 2004). Most native NMDA receptors appear to be tetramers, composed of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits (Laube et al., 1998, Dingledine et al., 1999). The two NR2 subunits in each NMDAR could be identical or different, giving rise to di-heteromer (e.g. NR1/NR2A) or tri-heteromer (e.g. NR1/NR2A/NR2B), respectively. Activation of NMDA receptors requires full occupation of the two independent glycine sites and the two independent glutamate sites (Benveniste and Mayer, 1991, Clements and Westbrook, 1991).

NMDA receptor subunits all share a common membrane topology, characterized by an extracellular N-terminus, three trans-membrane domains (M1, M3 and M4), a

channel-lining domain (M2), and an intracellular C-terminal tail (Mayer, 2006). Part of the N-terminal forms a S1 region, while the extracellular loop between M3 and M4 constitutes a S2 region. Together, the S1 and S2 regions from the same NR1 subunit form a binding site for glycine, whereas the S1 and S2 regions from the same NR2 subunit form a binding site for glutamate (Paoletti and Neyton, 2007). The channel-lining region (M2) is a re-entrant "pore loop" that enters the membrane from the intracellular surface (Beck et al., 1999). A critical asparagine (N) residue located on the M2 is responsible for the properties of NMDA receptors for $Mg^{2+}$ blockade and $Ca^{2+}$ permeability (Burnashev et al., 1992, Kawajiri and Dingledine, 1993). This asparagine site is directly homologous to the Q/R (glutamine-arginine) site in AMPA receptor subunits. The C-terminal tail of NMDA receptors contains binding sites for various kinases and structural proteins that function as channel property modulators or receptor stabilizers (Sheng and Sala, 2001, Mayer and Armstrong, 2004).

NR1 has eight functional isoforms originating from a single gene (Hollmann et al., 1993, Dingledine et al., 1999). The type of NR1 splice variant is important in influencing certain pharmacological properties of NMDA receptors, such as inhibition by protons ($H^+$) (Traynelis et al., 1995).

NR2 subunits are critical in determining many key properties of NMDA receptors, including sensitivity to $Mg^{2+}$, channel conductance, decay time, developmental expression and regional distribution. NR2A- and NR2B- containing receptors are of high conductance (~40 to 50pS) and high sensitivity to $Mg^{2+}$ blockade, compared with NR2C- (~22 to 35 pS) and NR2D- (~16 to 35 pS) containing receptors which are less sensitive to $Mg^{2+}$ (Cull-Candy and Leszkiewicz, 2004). Also, the decay time of

diheteromeric NMDA receptors (NR1/NR2) spans a ~50 folds range, with NR1/NR2A the fastest and NR1/NR2D the slowest. Roughly, their time constants of deactivation in response to a 1ms pulse of 1mM glutamate are as follows: NR2A, 100ms; NR2B, 250ms; NR2C, 250ms; NR2D, 4s (NR2A < 2C = 2B << 2D) (Vicini et al., 1998). Because of the difference in decay time, it has been proposed that the fast NR2A-containing receptors may be better coincidence detectors of pre- and post- synaptic excitation, whereas the slow NR2B-containing receptors are more efficient at excitatory postsynaptic potential (EPSP) summation, bringing neurons more rapidly to firing threshold (Kumar and Huguenard, 2003, Cull-Candy and Leszkiewicz, 2004). Besides the biophysical differences, the four NR2 subunits also show distinct patterns of developmental expression and regional distribution in the central nervous system (Monyer et al., 1994, Watanabe et al., 1994). In early development, NR2A subunits are not the major subtypes, but they gradually become abundant and ubiquitously expressed in adult brain. In contrast, NR2B subunits are the major NMDA receptor subtypes in immature brain, but decrease substantially and are restricted to the forebrain (including hippocampus and cortex) in adult. There are not many NR2C during early developmental stages, but they become highly enriched in the cerebellum in adult brain. NR2D subunits are more prominent in immature brain than in adult and their locations are confined to the diencephalon and midbrain (Monyer et al., 1994, Watanabe et al., 1994).

NR3 subunits have distinct properties from other NMDA receptor subtypes. The NR1/NR3 assemblies function as glycine-gated cation-permeable ion channels, which are impermeable to $Ca^{2+}$, resistant to $Mg^{2+}$ blockade, and unaffected by glutamate or NMDA receptor antagonists (Dingledine et al., 1999, Chatterton et al.,

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2002, Sasaki et al., 2002). NR3 subunits have restricted spatio-temporal distributions. NR3A subunits are predominantly expressed during development, although their presence in certain neuronal population persists in adult. NR3B are restricted to somatic motoneurons of the brainstem and spinal cord (Nishi et al., 2001).

In addition to neurons, NMDA receptors are also found in astrocytes (Krebs et al., 2003) and oligodendrocytes (Karadottir et al., 2005, Lalo et al., 2006). In neurons, NMDA receptors are present at both pre- and post- synaptic sites (Tzingounis and Nicoll, 2004). In the postsynaptic membrane of excitatory neurons, the density of NMDA receptors is higher in dendritic spines, particularly within the postsynaptic density (PSD), than that in the dendritic shaft and somatic membrane (Kohr, 2006).

Specific antagonists, particularly the subunit-selective blockers, are important tools in studying NMDA receptor-related physiological and pathological processes. Two commonly used experimental NMDA receptor antagonists that have no subunit-selectivity are MK-801 and D-AP5. MK-801 blocks NMDA receptors by binding deep within the channel pore in an activity-dependent manner (Wong et al., 1986), whereas D-AP5 competitively inhibits NMDA receptors by targeting the glutamate binding site of NR2 subunit (Feng et al., 2005). A moderately selective blocker for NR2A-containing receptors is NVP-AAM007, which works on glutamate binding site of NR2 subunit in a competitive manner (Liu et al., 2004, Neyton and Paoletti, 2006). In contrast, ifenprodil and its derivatives selectively inhibit NR1/NR2B heteromers over other NR1/NR2 assemblies (Williams, 1993). They are non-competitive blockers that target the polyamine site of NR2B subunit. Notably, in the presence of a small amount of glutamate (e.g. low intensity synaptic stimulation), ifenprodil and

its derivatives actually potentiate, rather than inhibit, the NMDA receptor currents (Kew et al., 1996). To date, the pharmacological tools for discriminating between NMDA receptor subunits are still very limited and none of them are truly selective. Therefore, the generation of more efficient and selective NMDA receptor antagonists is highly demanded for future investigation of NMDA receptor functions.

## 1.2.2 Signaling pathways downstream of NMDA receptors

To date, the clinical trials of NMDAR antagonists in treating stroke patients have all been proved unsuccessful because of poor tolerance and efficacy (Prass and Dirnagl, 1998). The failures suggest that global blockade of NMDARs might be as toxic as their over-activation. Indeed, increasing evidence showed that disruption of physiological level NMDAR activities triggers widespread apoptosis in developing brains (Gould et al., 1994, Ikonomidou et al., 1999) and aggravates neurodegeneration in mature brains that are subject to pathological insults such as traumatic injuries (Ikonomidou et al., 2000). The above evidence, together with the results from NMDA excitotoxicity studies (Simon et al., 1984, Bullock et al., 1990, Sauer et al., 1995), suggests that NMDARs are involved in both pro-survival and pro-death signaling pathways, and hence too little and too much of NMDAR activities are both harmful.

Accordingly, the downstream pathways of NMDARs could be roughly divided into two types, pro-survival and pro-death (Fig. 1). The major pro-survival pathways, induced by physiological level of NMDAR activation, are generally believed to be the phosphatidylinositol-3-kinase (PI3K)-Akt cascade (Lafon-Cazal et al., 2002, Papadia

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et al., 2005), cAMP-responsive element binding protein (CREB)-regulated gene expression (Wu et al., 2001), and nuclear export of the class II histone deacetylases (HDACs) (McKinsey et al., 2000, Chawla et al., 2003). On the other hand, the pro-death pathways, triggered by pathological level of NMDAR activation, include mitochondrial dysfunction (Dubinsky and Levi, 1998) and activation of various cell death signals, such as DAPK1 (Tu et al., 2010), nNOS (Aarts et al., 2002, Sun et al., 2008), p38/JNK MAK kinases (Cao et al., 2005, Centeno et al., 2007, Soriano et al., 2008, Lai et al., 2011), calpain (Bi et al., 1996, Bi et al., 1998, Xu et al., 2007), SREBP1 (Taghibiglou et al., 2009) and PTEN (Gary and Mattson, 2002, Ning et al., 2004, Chang et al., 2010, Jurado et al., 2010).


***Pro-survival signaling pathways downstream of NMDA receptors***


The acute phase of NMDAR's neuroprotective effect is primarily mediated by the PI3K/Akt signaling cascade. PI3K inhibitors, as well as transfected catalytically inactive mutant of Akt, strongly reduce the pro-survival effect of NMDARs in cultured cerebellar granule neurons (Lafon-Cazal et al., 2002) and hippocampal neurons (Lafon-Cazal et al., 2002, Papadia et al., 2005). Upon physiological stimulation of NMDARs, the influx of $Ca^{2+}$ results in the activation of PI3K in a $Ca^{2+}$/calmodulin-dependent manner (Joyal et al., 1997). The activated PI3K then catalyzes the phosphorylation of Phosphatidylinositol(4,5)- bisphosphate $(PI(4,5)P_2)$ to Phosphatidylinositol(3,4,5)- triphosphate $(PI(3,4,5)P_3)$ (Liu et al., 2009). In turn, $PI(3,4,5)P_3$ brings phosphatidylinositol-dependent kinase 1 (PDK1) and protein kinase B/Akt into close proximity and enables the phosphorylation of Akt by PDK1 (Vivanco and Sawyers, 2002, Papadia et al., 2005). The phosphorylated Akt then

translocates to the nucleus and phosphorylates two types of downstream targets: the pro-death and pro-survival substrates (Mocanu and Yellon, 2007). The pro-death substrates, such as forkhead box O (FOXO) transcription factors (Brunet et al., 1999, Soriano et al., 2006), glycogen synthase kinase-3-beta (GSK3β) (Cross et al., 1995), and Bcl-2-associated death promoter (BAD) (Jonassen et al., 2001), become inactive after phosphorylation. On the contrary, the pro-survival substrates, such as endothelial nitric oxide synthase (eNOS) (Bell and Yellon, 2003), mouse double minute (MDM2) (Mocanu and Yellon, 2003) and CREB (Papadia et al., 2005), become activated after phosphorylation and hence stimulate cellular processes essential for growth, survival and proliferation.

Besides PI3K/Akt cascade, the neuroprotective effect of NMDARs is also mediated by CREB-regulated gene expression. Generally, $Ca^{2+}$ influx through NMDARs triggers phosphorylation and activation of CREB through two pathways: one is a fast, sensitive, nuclear $Ca^{2+}$/calmodulin-dependent protein kinase (principally CaMKIV) pathway, while the other is a slow, less sensitive, Ras/mitogen-activated protein kinase (MAPK) pathway (Wu et al., 2001). Upon activation, CREB binds to the cAMP response element (CRE) and promotes expression of many pro-survival genes. One of the CREB-regulated targets is brain-derived neurotrophic factor (BDNF), which is a crucial neurotrophin in promoting neuronal survival, growth and differentiation (Shieh et al., 1998). Another candidate of CREB regulation is Bcl-2, which exerts its anti-apoptotic effect through preventing caspase activation (Riccio et al., 1999). Other CREB target genes include Bcl6, a transcriptional repressor implicated in p53 suppression (Zhang et al., 2007b); Btg2, which can inhibit apoptosis induced by trophic factors deprivation (Zhang et al., 2007b); superoxide dismutase 2 (Sod2), an

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important defense against oxidative damages (Papadia and Hardingham, 2007) and so on. Possibly through up-regulation of the above pro-survival genes, the NMDAR-mediated CREB activation is proposed to contribute to establishment of ischemic tolerance and preconditioning (Mabuchi et al., 2001).

The NMDAR-mediated nuclear $Ca^{2+}$/calmodulin signaling can also mediate neuronal survival through triggering nuclear export of the class II HDAC, which is responsible for repression of the transcription factor myocyte-enhancing factor 2 (MEF2) (McKinsey et al., 2000, Chawla et al., 2003). Once exported, the class II HDAC relieves its repression on MEF2. The activated MEF2, in turn, stimulates the expression of pro-survival genes, and hence contributes to neuroprotection (Mao et al., 1999).

***Pro-death signaling pathways downstream of NMDA receptors***

In contrast to the physiological activation of NMDARs, pathological conditions, such as ischemic stroke and traumatic injuries, usually lead to excessive NMDAR activation. The associated massive influx of $Ca^{2+}$ then induces cell death through triggering mitochondrial-dependent apoptosis (Kluck et al., 1997, Reed, 1997), as well as activating various pro-death signals, such as DAPK1 (Tu et al., 2010), nNOS (Aarts et al., 2002, Sun et al., 2008), p38/JNK MAK kinases (Cao et al., 2005, Centeno et al., 2007, Soriano et al., 2008, Lai et al., 2011), calpain (Bi et al., 1996, Bi et al., 1998, Xu et al., 2007), SREBP1 (Taghibiglou et al., 2009) and PTEN (Gary and Mattson, 2002, Ning et al., 2004, Chang et al., 2010, Jurado et al., 2010).

Increase in cytoplasmic $Ca^{2+}$ induces excessive $Ca^{2+}$ uptake by mitochondria through its potential-driven uniporter (Gunter and Pfeiffer, 1990). The $Ca^{2+}$ overload then results in opening of mitochondrial permeability transition (MPT) pore, which is a high conductance inner membrane channel that leads to further imbalance in electrochemical gradients (Siesjo and Siesjo, 1996, Dubinsky and Levi, 1998). The disturbance in ion gradient equilibration not only uncouples the electron transport from ATP synthesis, but also enhances swelling of mitochondria which may eventually cause rupture of the outer mitochondrial membrane and release of pro-apoptotic proteins, particularly cytochrome c, leading to initiation of apoptotic cell death cascade (Kluck et al., 1997, Reed, 1997).

A recent study indentified death-associated protein kinase 1 (DAPK1) as a novel downstream molecule of NMDARs (Tu et al., 2010). Upon ischemic challenges, DAPK1 is recruited into NMDAR complex and directly binds with NR2B subunit C-terminal tail. DAPK1 then phosphorylates NR2B subunit at $Ser^{1303}$ and hence enhances NR1/NR2B receptor channel conductance. Uncoupling DAPK1 from NMDAR complex using an interfering peptide ($NR2B_{CT}$) significantly protects neurons against ischemic insults. Therefore, DAPK1 may represent a novel strategy for stroke treatment.

Neuronal nitric oxide synthase (nNOS), another pro-death protein, is suggested to be coupled to NMDARs through scaffolding protein PSD95 (Sun et al., 2008). Suppressing PSD95 expression using antisense oligodeoxynucleotide attenuates NMDAR-mediated NO production and hence protects neurons against NMDA toxicity. On the other hand, the NMDAR function and nNOS expression are

unaffected by the PSD95 suppression, suggesting that PSD95 merely acts as an intermediary in the NMDAR-mediated excitotoxic signaling cascade. The way that the $Ca^{2+}$ influx through NMDAR is brought into close proximity to specific neurotoxic signaling pathways (e.g. nNOS) through some intermediary proteins (e.g. PSD95) is termed "source-specific $Ca^{2+}$ neurotoxicity" (M. Arundine and M. Tymianski, 2004). This concept also well explains why the $Ca^{2+}$ inflow through NMDARs is more neurotoxic than that through voltage-gated $Ca^{2+}$ channels (Tymianski et al., 1993, Sattler et al., 1998, Cao et al., 2005). Further studies have been carried out to utilize this "source specific $Ca^{2+}$ neurotoxicity" in treating excitotoxic injuries, such as ischemic stroke. Pre-clinical data showed that membrane permeable interfering peptide (TAT-NR2B9c) that disassociates PSD95 from NR2B containing-NMDARs (NR2BRs) can significantly reduce the neuronal damages following ischemic insults (Aarts et al., 2002, Sun et al., 2008). This intervention has two major advantages over the conventional NMDAR blockers. First, it provides a wider therapeutic time window (up to 3 hours post-stroke onset) than that permitted by the universal NMDAR inhibitors (usually within 1 hour of stroke onset). Second, inhibiting the activation of nNOS, a neurotoxic signal, avoids many side effects involved in using NMDAR antagonists that block both pro-survival and pro-death signaling pathways. This interfering peptide is currently tested for treating ischemic monkeys, a closer animal model to human being, by Michael Tymianski group (Canadian Stroke Network Annual Meeting, 2009 Ottawa). The outcome will be extremely interesting, considering the lack of efficient treatment for stroke in the current market.

Following-up studies further proved that p38, the mitogen-activated protein kinase (MAPK), is one of the death signals downstream of NR2BR-PSD95-nNOS (Cao et

al., 2005, Soriano et al., 2008). P38 activation, triggered by $Ca^{2+}$ influx through NMDARs, can be suppressed by either NOS inhibitors or TAT-NR2B9c peptide. However, the therapeutic application of p38 inhibition is restricted, due to the early onset and decay of p38 activation that is completed within the first hour of excitotoxic insult (Cao et al., 2005). It suggests that other signaling molecules, rather than p38, are responsible for the wide therapeutic time window (up to 3 hours) of the TAT-NR2B9c peptide for treating ischemic injuries.

Another MAPK, c-Jun N-terminal kinase (JNK), is also found to be a downstream molecule of NMDARs (Centeno et al., 2007, Lai et al., 2011). Unlike other downstream molecules of NMDARs, JNK remains activated as long as 24 hours post-stroke onset. Inhibition of JNK activation by peptide D-JNKI1 could abolish NMDA-induced excitotoxicity *in vitro* and ischemia-induced neuronal damages *in vivo*. More excitingly, D-JNKI1 provides a very wide post-stroke therapeutic time window, which is up to 12 hours for transient focal ischemia (Centeno et al., 2007, Esneault et al., 2008) and 3 hours for permanent focal ischemia (Hirt et al., 2004).

Besides nNOS and p38/JNK MAPK, another critical downstream signal of NMDARs is calpain, a $Ca^{2+}$-activated neutral cysteine protease. Application of toxic level of glutamate in cultured neurons leads to activation of calpain, which, in turn, truncates AMPARs (Bi et al., 1996), NMDARs (Bi et al., 1998) as well as metabotropic glutamate receptor-1 (mGluR1) (Xu et al., 2007). Among the many substrates of calpain, it is very interesting to note that truncation of NR2A-containing NMDARs results in loss of receptor functions (Guttmann et al., 2002), while cleavage of NR2B-containing NMDARs is somewhat silent (Simpkins et al., 2003). Therefore, it appears

that activation of calpain renders the neurons more vulnerable to pro-death NR2B pathways over pro-survival NR2A cascades in the presence of high dose glutamate. Besides the truncation of NMDARs, activated calpain also indirectly cleaves mGluR1, which plays both neuroprotective and neurotoxic roles under different conditions (Xu et al., 2007). The neuroprotective function of mGluR1 is mediated by activation of PI3K-Akt cascades through formation of an mGluR1-Homer-PIKE-L signaling complex, whereas its neurotoxic effect is due to its effect on cytoplasmic free $Ca^{2+}$ and its stimulation of glutamate release following pathological insult such as ischemia. Truncation of mGluR1 by calpain selectively disrupts the mGluR1-PI3K-Akt signaling cascades, but leaves its ability to increase cytoplasmic free $Ca^{2+}$ preserved (Xu et al., 2007), suggesting that this post-translational modification transforms mGluR1 from a pro-survival receptor to a pro-death receptor. The above findings shed light on the relationship between NMDARs and other glutamate receptors, and imply the existence of a positive feedback loop of NMDA excitotoxicity that is mediated by calpain activation.

Due to the lack of specific calpain inhibitor, the recent discovery of sterol regulatory element binding protein-1 (SREBP-1) as the calpain downstream molecule is of great significance (Taghibiglou et al., 2009). SREBP-1, a transcription factor previously known to be activated following cellular cholesterol deprivation, is here proved to respond to excitotoxic level of NMDA stimulation, too. Under resting condition, the immature SREBP-1 is restricted in the endoplasmic reticulum (ER) by an ER-anchoring protein called insulin-induced gene-1 (Insig-1). Over-activation of NMDARs causes the ubiquitination and degradation of Insig-1, leading to release of immature SREBP-1 from the ER, and subsequent cleavage and activation of

SREBP-1 in the Golgi. The mature SREBP-1 then translocates into the nucleus to initiate the cell-death cascades. To further prove the role of SREBP-1 in NMDAR-mediated neurotoxicity, the authors also demonstrate that suppressing SREBP-1 activation, either by oversupplying cholesterol or by shRNA knockdown, has a strong protective effect against NMDA excitotoxicity in cultured cortical neurons. However, in human stroke patients, it's impractical to oversupply cholesterol or to knockdown SREBP-1. In order to solve the problem, an interfering peptide was designed to resemble the ubiquitination sites on Insig-1 and act as the pseudo-substrate for Insig-1 degradation. As a result, sufficient amount of Insig-1 is preserved to retain the immature SREBP-1 in the ER and prevents the SREBP-1-mediated cell death. In rats subjected to stroke, intravenous injection of the interfering peptide 2 hours post-onset of ischemia not only substantially reduces the brain infarction but also improves the behavioral outcomes, suggesting that agents, which reduce SREBP-1 activation, represent a new class of neuroprotective therapeutics against stroke.

Phosphatase and tensin homolog deleted on chromosome TEN (PTEN), a dual protein and lipid phosphatase, is recently discovered to be another critical downstream death signal of NMDARs (Gary and Mattson, 2002, Ning et al., 2004, Chang et al., 2010, Jurado et al., 2010). The detailed mechanisms through which PTEN mediates NMDA-induced neuronal death will be thoroughly discussed in the following chapter 1.3.5.

**1.2.3 Whether an episode of NMDAR activity is neuroprotective or excitotoxic?**

As mentioned above, NMDARs activate both pro-survival and pro-death downstream

signaling pathways. However, what determines whether an episode of NMDAR activity is neuroprotective or excitotoxic remains unclear. Several popular theories co-exist regarding this question. They are 1) stimulus intensity hypothesis (Hardingham and Bading, 2003), 2) subcellular location hypothesis (Soriano and Hardingham, 2007), and 3) subtype composition hypothesis (Liu et al., 2007b).

The stimulus intensity hypothesis proposes that physiological level of NMDAR activity triggers neuroprotective cascades, while pathological stimulation that is too low or too high promotes cell death. For example, *in vivo* blockade of NMDARs in developing rat brain results in suppression of physiological level of NMDAR activity, and hence causes extensive apoptosis in many brain regions (Gould et al., 1994, Ikonomidou et al., 1999). In contrast, excitotoxic insults, such as ischemic stroke, lead to excessive activation of NMDARs and therefore trigger various pro-death downstream cascades (Arundine and Tymianski, 2004). The underlying mechanism for this stimulus intensity hypothesis is proposed to be that the $Ca^{2+}$ concentration threshold for activating pro-survival signals, such as PI3K, ERK1/2 and CaMKIV, is lower than that necessary for triggering pro-death scenarios, such as calpain activation, mitochondrial $Ca^{2+}$ overload and excessive NO production (Soriano and Hardingham, 2007).

Aside from the stimulus intensity theory, the subcellular location of NMDAR may also have profound influence on the nature of NMDAR signaling. This theory suggests that synaptic NMDARs are primarily coupled to cell survival pathways, while extrasynaptic NMDARs are more likely to activate pro-death cascades (Vanhoutte and Bading, 2003). Specifically, synaptic NMDARs are suggested to activate

extracellular signal–regulated kinase (ERK) signaling cascades, while extrasynaptic NMDARs evoke ERK inactivation (Hardingham et al., 2001, Ivanov et al., 2006). Also, synaptic NMDAR activation strongly triggers CREB-dependent gene expression, while extrasynaptic NMDARs mediate CREB shut-off pathways that cause CREB dephosphorylation of its activator site (Ser[133]) (Hardingham et al., 2002). Moreover, the synaptic NMDARs activate a number of pro-survival genes including BDNF, Btg2 and Bcl6 (Zhang et al., 2007b), while extrasynaptic NMDARs induce pro-death gene expression such as Clca1 (Wahl et al., 2009). One good example of the subcellular location hypothesis is that, during hypoxia/ischemia challenges, the glutamate transporters operate in reverse and hence lead to an uncontrolled release of glutamate in to the extrasynaptic space; therefore, the number of extrasynaptic NMDARs largely determines the ability of neurons to survive those insults (Rossi et al., 2000). Notably, although the subcellular location of NMDARs profoundly affects the nature of NMDA signaling, exclusive localization is not a prerequisite for the functions mentioned above. For example, retinal ganglion cells that express only extrasynaptic NMDARs are resistant to NMDA challenge (Ullian et al., 2004), while synaptic NMDARs can also cause excitotoxicity (Sinor et al., 2000).

In addition to the intensity/location theories, the subtype composition of NMDARs is also suggested to be a major factor that determines whether an episode of NMDAR activity is neuroprotective or excitotoxic. This hypothesis proposes that activation of either synaptic or extrasynaptic NR2A-containing NMDARs (NR2ARs) promotes neuronal survival, whereas activation of either synaptic or extrasynaptic NR2B-containing NMDARs (NR2BRs) increases neuronal death (Liu et al., 2007b). The

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subtype-specific functions of NMDARs are explained by the preferential coupling of NR2ARs to Ras–guanine nucleotide–releasing factor 2 (RasGRF2), and NR2BRs to Ras–guanine nucleotide–releasing factor 1 (RasGRF1) (Li et al., 2006b). RasGRF2, in response to NR2ARs, triggers ERK signaling pathway, and therefore contributes predominantly to the induction of LTP and promotion of cell survival. In contrast, RasGRF1, in response to NR2BRs, activates p38 MAP kinase, and thus contributes mainly to the generation of LTD and enhancement of cell death. In light of the different activities of NR2ARs and NR2BRs, specific agonists/antagonists may be exploited for the development of treatments for excitotoxic neuronal damages, such as ischemic stroke. In fact, one study has successfully proved that selective enhancement of NR2AR activation with glycine can significantly attenuate ischemic brain damages in rats even when given 4.5 hours after stroke onset (Liu et al., 2007b). This may constitute a promising therapy for stroke in human patients.

The above theories emphasize on different aspects of NMDA excitotoxicity but are not contradictory. For example, both the subcellular location hypothesis and the subtype composition hypothesis agree that synaptic NMDARs, which are mostly NR2A-containing receptors, primarily activate cell survival pathways; while extrasynaptic NMDARs, which are mainly NR2B-containing receptors, promote cell death cascades.

## 1.2.4 NMDA excitotoxicity in ischemic stroke

Ischemic stroke is a pathological condition that is due to an interruption of cerebral blood supply. The resultant depletion of oxygen and glucose causes anoxic

depolarization, leading to excessive release and accumulation of extracellular glutamate and other neurotransmitters. In turn, the toxic level of extracellular glutamate over-activates NMDARs in adjacent neurons, triggering pro-death downstream pathways (Rothman, 1983, Rothman, 1984, Simon et al., 1984, Arundine and Tymianski, 2004).

The affected ischemic regions differ considerably not only in the level of glutamate increase but also in the duration of glutamate elevation (Lipton, 1999). In the core area, the extracellular glutamate could reach as high as 50~90μM; and in the penumbra area, it rises to about 30~50μM. In contrast, the glutamate level in the extrapenumbral area increases to about 6μM, only slightly higher than the normal concentration which is about 1~5μM (Shimada et al., 1989, Graham et al., 1994, Baker et al., 1995). The durations of glutamate elevation vary considerably too. The extracellular glutamate remains high in the core region for many hours after prolonged ischemia, while the penumbral and extra-penumbral glutamate returns to baseline at about 1 hour post ischemia onset (Graham et al., 1990, Morimoto et al., 1996).

The efflux pathways of glutamate remain controversial. Some evidences support the classic exocytosis, while others support the reversal of $Na^+$-glutamate transporter or spontaneous vesicular release. An *in vivo* experiment showed that the N-type $Ca^{2+}$ channel blocker reduces the glutamate release by 50%, suggesting the importance of classical exocytosis in mediating glutamate release during ischemic stroke (Takizawa et al., 1995). On the contrary, another experiment using hippocampal slices showed absolutely no inhibition of glutamate release when extracellular $Ca^{2+}$ is

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removed (Pellegrini-Giampietro et al., 1990), excluding the possibility of classical exocytosis. Unlike the contradictory results for classical exocytosis, the evidence for reversed operation of $Na^+$-glutamate transporter is much more consistent. Preloading hippocampal slices with inhibitors of $Na^+$-glutamate transporter reduces the glutamate release by 75%, indicating that the reversal of glutamate transporter may represent a major pathway for glutamate efflux during ischemic stroke (Longuemare and Swanson, 1995). Last, the spontaneous vesicular release hypothesis is supported by the evidence that glutamate release can be blocked by the inhibitor of phospholipase A2, an enzyme that is involved in plasma membrane disruption (Phillis and O'Regan, 1996). More studies are needed to resolve the conflicting results on the different glutamate release mechanisms.

Based on the above observations of excitotoxicity in ischemia, NMDAR has been a longstanding therapeutic target for drug development. In the animal studies, NMDAR antagonists (Park et al., 1988b, Nishikawa et al., 1994, Sauer et al., 1995) and NMDAR subunit knockout/knockdown (Wahlestedt et al., 1993, Morikawa et al., 1998) all show very promising protection for ischemic stroke. They not only reduce the infarct size, but also significantly improve the functional recovery. Several well-controlled studies also proved that the protective effect of NMDAR blockade is not mediated through reducing skull/body temperatures (Takizawa et al., 1991, Frazzini et al., 1994) or increasing cerebral blood flow (Park et al., 1989, Dezsi et al., 1992), but rather through blocking the excessive $Ca^{2+}$ influx through NMDARs (Choi, 1987). However, in contrast to the encouraging results in animal studies, the clinical trials turned out to be quite disappointing. All of NMDAR antagonists are proved to be either ineffective or have adverse CNS effects in human stroke patients (Muir, 2006).

Several explanations for these failures have been proposed by clinicians and scientists, including poor experimental designs, lack of long-term effect evaluation, et al. (Gladstone et al., 2002, Ikonomidou and Turski, 2002, Roesler et al., 2003). But in my current study, only two factors will be emphasized as they may be circumvented by the novel intervention proposed here. First, excessive glutamate release and NMDAR over-activation are relatively early events during ischemic insults. Thus, NMDAR antagonists only allow a short therapeutic time window of about 1 hour post-stroke onset. However, the patient inclusion periods used by most clinical trials are generally more than 6 hours post-ictus, which is way beyond the time window permitted by NMDAR antagonists, hence resulting in lack of efficacy in stroke treatment. Second, NMDAR antagonist, when used at the concentration required to block excitotoxicity, also block the normal physiological functions of NMDAR in facilitating neuronal survival and regeneration, hence leading to intolerable adverse effects (Ikonomidou et al., 1999). Despites of these limits, NMDA excitotoxicity is still a hotly investigated topic as it's one of the most important mechanisms for neuronal loss during ischemic stroke. But instead of aiming at the receptor *per se*, investigations turn to focus on the downstream death signals of NMDARs. Because activation of these downstream death molecules occurs sequentially after activation of NMDARs, interventions targeting these effectors are expected to permit a much wider therapeutic time window; also, because these death effectors don't interfere with NMDAR's pro-survival functions, only minimal side effects should be observed.

## 1.3 PTEN

Phosphatase and tensin homolog deleted on chromosome TEN (*PTEN*), also known as mutated in multiple advanced cancers 1 (*MMAC1*), is a tumor suppressor gene that is mapped to 10q23.3. Ever since its original identification (Steck et al., 1997), deletion or mutations of this gene have been reported in a large fraction of tumors, such as glioblastoma, prostate cancer, melanoma, breast cancer and ovarian cancer (Sansal and Sellers, 2004). In addition, mechanisms including transcriptional repression, epigenetic silencing, posttranslational modification and aberrant localization of PTEN protein all lead to subtle or dramatic loss of PTEN functions in various cancers (Salmena et al., 2008). Besides its well-characterized tumor suppressive functions, PTEN is also highly expressed in the brain and plays a critical role in NMDAR-mediated excitotoxic neuronal injuries (Ning et al., 2004).

PTEN protein contains three major domain structures that are the phosphatase domain (residues 15-186), the C2 domain (residues 186-351) and the C-terminal tail (residues 352-403) (Wang and Jiang, 2008). The phosphatase domain is the center for PTEN's catalytic activity, as mutation on residue 124 (Cysteine) will result in loss of PTEN phosphatase function (Ali et al., 1999). The C2 domain, together with the N-terminal (residues 6-15), is required for PTEN's interaction with membrane (Lee et al., 1999, Campbell et al., 2003). The C-terminal tail of PTEN contains several recognizable motifs that are involved in regulation of PTEN functions. These motifs include a cluster of putative phosphorylation sites, a PDZ binding motif and two PEST motifs (Wu et al., 2000, Tolkacheva et al., 2001).

## 1.3.1 Functions of cytoplasmic PTEN and nuclear PTEN

The protein encoded by *PTEN* gene is a dual lipid and protein phosphatase. It shuttles between cytoplasm and nucleus to exert many distinct physiological and pathological functions, such as dephosphorylating $PI(3,4,5)P_3$ into $PI(4,5)P_2$ and hence antagonizing PI3K/Akt pro-survival pathways (Vivanco and Sawyers, 2002, Deleris et al., 2006), mediating NMDA excitotoxicity (Ning et al., 2004), inducing cell cycle arrest (Chung and Eng, 2005, Chung et al., 2006), reciprocally cooperating with p53 (Stambolic et al., 2001, Li et al., 2006a), maintaining chromosomal integrity (Shen et al., 2007), et al.

### *Functions of cytoplasmic PTEN*

In the cytoplasm, PTEN primarily functions as a lipid phosphatase, which specifically targets the 3-position of the inositol ring on phosphatidylinositol phosphate (PIP) (Maehama and Dixon, 1998). $PI(3,4,5)P_3$ is suggested to be the primary *in vivo* substrate for PTEN, even though PTEN can dephosphorylate PIP(3)P, $PI(3,4)P_2$ and $PI(3,4,5)_3$ *in vitro* (Vivanco and Sawyers, 2002, Deleris et al., 2006). PTEN and phosphatidylinositol-3-kinase (PI3K) have opposing effects on $PI(3,4,5)P_3$ levels, and therefore have opposite influences on cell growth, survival, proliferation (Cully et al., 2006). Briefly, PI3K catalyzes the conversion of $PI(4,5)P_2$ into $PI(3,4,5)P_3$ and hence promotes cell growth, survival and proliferation; in contrast, PTEN dephosphorylates $PI(3,4,5)P_3$ into $PI(4,5)P_2$ and thus suppresses the PI3K-mediated pro-survival signaling pathways. The PI3K signaling cascades usually start with binding of ligand to receptor tyrosine kinases (RTKs), such as insulin-like growth factor receptor 1 and

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epidermal growth factor receptor (Vivanco and Sawyers, 2002). The activated RTKs recruit PI3K to plasma membrane, where PI3K catalyzes the phosphorylation of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ (Liu et al., 2009). Next, $PI(3,4,5)P_3$ binds to Akt's PH (pleckstrin homology) domain and recruits Akt to the plasma membrane, leading to a conformational change in Akt (Freudlsperger et al., 2011). Subsequently, Akt is phosphorylated at three regulatory sites: $Thr^{308}$ in the catalytic domain by PDK1 (Vivanco and Sawyers, 2002, Papadia et al., 2005), $Ser^{473}$ in the COOH-terminal domain by mTOR-RICTOR complex (Sarbassov et al., 2005) and $Ser^{129}$ by casein kinase 2 (CK2) (Di Maira et al., 2005). Once activated, Akt phosphorylates two types of substrates: the pro-survival and the pro-death substrates (Mocanu and Yellon, 2007). The pro-survival substrates, such as eNOS (Bell and Yellon, 2003), mouse double minute (MDM2) (Mocanu and Yellon, 2003) and CREB (Papadia et al., 2005), become activated after phosphorylation and trigger signaling pathways essential for cell survival, proliferation and growth (Du and Montminy, 1998, Mocanu and Yellon, 2007). In contrast, the pro-death substrates, including GSK3β (Cross et al., 1995), FOXO transcription factors (Brunet et al., 1999, Soriano et al., 2006), c-Jun N-terminal kinase (JNK)/p38 activator ASK1 (Kim et al., 2001) and BAD (Jonassen et al., 2001), are inactivated by phosphorylation, and, as a result, fail to promote cell death processes (Soriano and Hardingham, 2007). Therefore, by antagonizing PI3K/Akt signaling pathways, PTEN functions as a suppressor for cell growth, proliferation and survival.

PTEN is also a unique protein phosphatase that can dephosphorylate both phospho-serine/threonine and phospho-tyrosine (Li et al., 1997). The protein phosphatase activity of PTEN is involved in regulating many cell processes. For example, PTEN

disrupts cell spreading and migration by dephosphorylating and inactivating its protein substrate focal adhesion kinase (FAK) (Tamura et al., 1998). More, PTEN can enhance NMDARs surface expression through its protein phosphatase activity (Ning et al., 2004). Evidence showed that overexpression of PTEN protein phosphatase-inactive mutant results in a dramatic reduction in NMDAR current in cultured hippocampal neurons, and siRNA knockdown of PTEN protein *in vivo* significantly reduces the NMDAR-mediated neuronal death in an ischemia model (Ning et al., 2004), suggesting PTEN plays a critical role in mediating NMDA excitotoxicity.

### *Functions of nuclear PTEN*

Although PTEN's tumor suppressor function is previously believed to be solely due to its lipid phosphatase activity at plasma membrane, recent studies revealed that PTEN is also found in the nucleus in a number of different tissues and cultured cells, and plays an important role in regulating cell processes as well (Baker, 2007, Gil et al., 2007, Planchon et al., 2008). More importantly, accumulating evidence indicates that tumors, which retain a strong nuclear PTEN staining, remain of lower grade, while decrease in nuclear PTEN staining is associated with higher tumor stage (Perren et al., 2000, Lian and Di Cristofano, 2005, Trotman et al., 2007), strongly suggesting a more critical role of nuclear PTEN in tumor suppression than cytoplasmic PTEN.

Given that all the major components of PI3K/Akt cascade, such as $PI(4,5)P_2$, $PI(3,4,5)P_3$, PI3K, PDK1, and Akt, are found in the nucleus, it is possible that nuclear

PTEN also functions as a PI(3,4,5)$P_3$ phosphatase and consequently suppresses nuclear PI3K/Akt mediated pro-survival signaling pathways (Deleris et al., 2006). Supporting evidence showed that PTEN purified from vascular smooth muscle cell nuclei can dephosphorylate nuclear PI(3,4,5)$P_3$ (Deleris et al., 2003); also, nuclei from PC12 cells incubated with PTEN show increased apoptotic DNA fragmentation, which could be rescued by PI(3,4,5)$P_3$ oversupplying (Ahn et al., 2004, Ahn et al., 2005). Further, the level of nuclear PTEN is found to be inversely correlated to that of activated nuclear Akt. In the primary follicular thyroid tumor cells, nuclear PTEN level diminishes during the progression from normal tissue to adenoma to carcinoma, whereas the level of nuclear phospho-Akt increases (Vasko et al., 2004). In addition, enforced nuclear PTEN expression can reduce cellular levels of phospho-Akt (Trotman et al., 2007). These results demonstrated that nuclear PTEN plays an essential role in inhibiting cell survival through dephosphorylating nuclear PI(3,4,5)$P_3$ and hence antagonizing nuclear Akt signaling pathways.

Another important function of nuclear PTEN is to induce cell cycle arrest through down-regulation of phospho-MAKP and cyclin D1 levels by its protein phosphatase activity (Radu et al., 2003, Chung and Eng, 2005, Chung et al., 2006). Initially, nuclear localization of PTEN was found to coincide with the G0-G1 phases of the cell cycle (Ginn-Pease and Eng, 2003). Cells expressing wide-type PTEN exhibit a slower growth rate as well as higher G1/S ratio than cells expressing nuclear-localization-defective mutant of PTEN. Further studies then revealed that PTEN capable of nuclear localization results in a decrease in phospho-MAKP and cyclin D1 levels, whereas the nuclear-localization-defective mutant of PTEN has no effect, indicating that nuclear accumulation of PTEN plays an important role in regulating

carcinogenesis (Radu et al., 2003, Chung and Eng, 2005, Chung et al., 2006).

*Functions of nuclear PTEN beyond phosphatase activity*

PTEN and p53 are two of the most important tumor suppressors. Their reciprocal cooperation has been proposed, as PTEN is found to enhance p53 stability and p53 can increase PTEN transcription (Stambolic et al., 2001, Li et al., 2006a). PTEN and p53 have some striking similarities as well as differences. PTEN and p53 are both tightly regulated by ubiquitination. The mechanisms are surprisingly similar only with a small twist. NEDD4-1, the ubiquitin ligase for PTEN, mediates PTEN nuclear import (mono-ubiquitination) and degradation (poly-ubiquitination) (Trotman et al., 2007, Wang et al., 2007), whereas Mdm2, the ubiquitin ligase for p53, catalyzes p53 nuclear export (mono-ubiquitination) and degradation (poly-ubiquitination) (Li et al., 2003). On the other hand, a major difference between the two tumor suppressors is the basic levels and activities. Under normal conditions, PTEN is a stable and readily detectable protein, while its ubiquitin ligase, NEDD4-1, is generally suppressed and only gets activated upon stimulation (Trotman et al., 2003). On the contrary, p53 protein expression is normally maintained at very low level through Mdm2-mediated ubiquitination and proteasomal degradation, and only increases in response to cellular stress signals, such as DNA damage and hypoxia (Brooks and Gu, 2006). Therefore, it appears that PTEN is constantly required as a default gatekeeper for cancer suppression, whereas p53 is only activated under certain conditions (Wang and Jiang, 2008). In cells lacking PTEN, the half-life of p53 is significantly reduced. The mechanisms by which PTEN stabilizes p53 are suggested to be direct protein-protein interaction (Freeman et al., 2003, Tang and Eng, 2006) and maintenance of

p53 acetylation (Li et al., 2006a). Through its C2 domain, PTEN forms a complex with the p53 tumor suppressor. This nuclear complex enhances p53's stability by protecting it from Akt/Mdm2-mediated sequestration, as well as increases p53's DNA binding and transcriptional activity (Freeman et al., 2003, Tang and Eng, 2006). Further, PTEN promotes p53 acetylation through direct association with the histone acetyl transferase p300/CBP in response to DNA damage (Li et al., 2006a). Acetylation enhances p53 tetramerization, which in turn, is required for the PTEN-p53 interaction and subsequent maintenance of high p53 acetylation. On the other hand, p53 can up-regulate *PTEN* transcription through binding to the upstream of *PTEN* gene (Stambolic et al., 2001). In addition to the above reciprocal relationship between PTEN and p53, they are also suggested to from a functional network through which loss of PTEN could trigger a cellular senescence program via activation of p53. In support of this theory, *in vivo* evidence showed that *p53* inactivation cannot induce any tumor phenotype in mice, while *PTEN* inactivation only causes non-lethal invasive prostate cancer after long latency (Chen et al., 2005). In striking contrast, inactivation of both *PTEN* and *p53* results in a lethal prostate cancer phenotype with very early onset (Chen et al., 2005). The reason why *PTEN* inactivation alone cannot induce malignant cancer phenotype is due to activation of p53-dependent senescence, indicating that p53 functions as a fail-safe protein when the function of PTEN is lost (Wang and Jiang, 2008). Taken together, the above results strongly suggest a reciprocal relationship between the two most important tumor suppressors: PTEN and p53.

Nuclear PTEN also plays a critical role in maintaining chromosomal integrity. Shen et al. found that nuclear PTEN can stabilize chromosomes through physically

interaction with centromere-specific binding protein C (CENP-C), as well as promotes double-strand-break repair by upregulation of RAD51 (Shen et al., 2007). In contrast, PTEN mutants that lack association with CENP-C result in extensive centromere breakage and chromosomal translocations. Thus, nuclear PTEN is proposed to be a potential target for anti-cancer therapy development, as many chemotherapeutic drugs act through DNA repair mechanisms (Planchon et al., 2008).

In all, cytoplasmic PTEN and nuclear PTEN have different functions in regulating various cell processes, and the nuclear localization of PTEN is more and more believed to be indispensable for PTEN to execute its tumor suppression and pro-apoptosis activities.

### 1.3.2 Modulations of PTEN stability and activity

Modulations of PTEN can be roughly divided into two categories: transcriptional level and post-translational level. Transcriptional regulations of *PTEN* are mediated by several transcription factors that directly bind to *PTEN* promoter and regulate *PTEN* expression positively or negatively. Post-translational regulations of PTEN include ubiquitination (Trotman et al., 2007, Wang et al., 2007), phosphorylation (Vazquez et al., 2000, Okahara et al., 2004), acetylation (Okumura et al., 2006) and oxidation (Leslie et al., 2003, Kwon et al., 2004).

The transcription factors that positively regulate *PTEN* expression include: peroxisome proliferators-activated receptor γ (PPARγ) (Patel et al., 2001), early growth-regulated transcription factor-1 (EGR-1) (Virolle et al., 2001), p53 (Stambolic

et al., 2001) and human sprout homolog 2 (SPRY2) (Edwin et al., 2006). Activation of PPARγ by its selective ligand, rosiglitazone, leads to directly binding of PPARγ to *PTEN* promoter and upregulates *PTEN* expression in normal and cancer cells. Antisense-mediated disruption of PPARγ expression prevents the upregulation of *PTEN*. Therefore, PPARγ agonist has been proposed to be a potential treatment for cancers in which PTEN is still functional (Patel et al., 2001). Besides PPARγ, *PTEN* mRNA and protein levels are also positively regulated by EGR-1 in response to irradiation treatment, leading to apoptosis. Loss of EGR-1, which often occurs in human cancers, results in deregulation of *PTEN* gene and radiation resistance of some cancer cells (Virolle et al., 2001). In addition to PPARγ and EGR-1, p53 binds to *PTEN* promoter and upregulates *PTEN* transcription, too. Irradiation can increase *PTEN* mRNA level in primary and tumor cell lines with wild-type p53, but not in those with p53 mutant (Stambolic et al., 2001). Last, SPRY2, a modulator of growth factor actions, mediates its anti-proliferative function through increasing *PTEN* mRNA and protein expression, which is accompanied by decrease in Akt activity (Edwin et al., 2006). Together, PPARγ, EGR-1, p53 and SPRY2 positively regulates *PTEN* transcription and therefore its tumor suppressive function.

On the other hand, *PTEN* expression is negatively regulated by many scenarios, including transcription factors, miRNA miR-21, and epigenetic silencing through promoter methylation. MKK4 (Xia et al., 2007) and JNK suppress *PTEN* transcription via direct binding of NFκB to *PTEN* promoter (Xia et al., 2007). Transforming growth factor-beta (TGFβ) is also shown to down-regulate *PTEN* expression in pancreatic cancer cells (Chow et al., 2007) and in mesangial cells (Mahimainathan et al., 2006). Similarly, c-Jun, a component of the AP-1 family

transcription factors, is found to promote cellular survival by suppression of *PTEN* transcription (Hettinger et al., 2007). Besides transcription factor, an alternative way to modulate *PTEN* expression is by microRNA miR-21, a short non-coding RNAs that regulate gene expression negatively (Meng et al., 2007). The miR-21 is one of the most frequently upregulated microRNAs in cancers. Inhibition of miR-21 in cultured cancer cells increases *PTEN* expression, whereas miR-21 enhancement by transfection with precursor miR-21 suppresses *PTEN* expression. Last, *PTEN* expression can also be regulated by epigenetic silencing through promoter methylation. High percentages of *PTEN* methylation and low *PTEN* transcription levels are frequently co-observed in many cancer cell lines, such as melanoma (Mirmohammadsadegh et al., 2006), gastric carcinoma (Kang et al., 2002) and breast cancer (Garcia et al., 2004a). Treating the melanoma cells with the demethylation agent, 5-aza-2'-deoxycytidine, results in a reduction in methylation and an increase in PTEN protein level in an in vitro kinase assay (Mirmohammadsadegh et al., 2006). In all, aberrant regulations by transcription factors, miRNA miR-21 and promoter methylation are the major mechanisms to suppress *PTEN* expression in cancer cells.

In addition to transcription regulation, PTEN is subject to multiple post-translational modification, including ubiquitination (Trotman et al., 2007, Wang et al., 2007), phosphorylation (Vazquez et al., 2000, Okahara et al., 2004), acetylation (Okumura et al., 2006) and oxidation (Leslie et al., 2003, Kwon et al., 2004)

Many studies have suggested that the stability of PTEN is regulated by poly-ubiquitination, whereas the nuclear/cytoplasmic shuttling of PTEN is controlled by

mono-ubiquitination. The major residues for PTEN ubiquitination are reported to be Lys13 and Lys289 (Trotman et al., 2007, Wang et al., 2007, Fouladkou et al., 2008, Song et al., 2008, Van Themsche et al., 2009). Under normal conditions, PTEN is a long-lived and stable protein, so it is surprising to find that its C-terminal tail contains two so-called PEST motifs, which are characteristic of short-lived proteins that are subject to ubiquitin-mediated degradation by the proteasome. More strikingly, deletion of the PEST motifs is reported to enhance PTEN poly-ubiquitination followed by proteasomal degradation (Wang et al., 2008). To explain this inconsistency, Wang et al. proposed that the C-terminal PEST motifs binds intramolecularly with the C2 domain of PTEN and therefore masks the binding sites for its ubiquitin ligase; however, under stimulation, PTEN goes through conformational change and exposes its binding sites that allow easy access for its ubiquitin ligase (Wang and Jiang, 2008). Wang et al. revealed that neural precursor cell expressed, developmentally downregulated-4-1 (NEDD4-1) acts as an ubiquitin ligase for PTEN ubiquitination (Wang et al., 2007). Mono-ubiquitination of PTEN by NEDD4-1 promotes PTEN nuclear translocation, whereas poly-ubiquitination of PTEN by NEDD4-1 leads to PTEN degradation by the proteasome. Moreover, in consistency with PTEN's tumor-suppressive ability, overexpression of NEDD4-1 promotes cellular transformation, while elimination of NEDD4-1 expression inhibits tumor growth in a PTEN-dependent manner (Wang et al., 2007). However, two other groups disagreed about the role of NEDD4-1 as the universal PTEN ubiquitin ligase. Instead, they proposed that X-linked inhibitor of apoptosis protein (XIAP) is another ubiquitin enzyme for PTEN ubiquitination (Fouladkou et al., 2008, Van Themsche et al., 2009). It is likely that PTEN ubiquitination is differentially regulated by the two enzymes, NEDD4-1 and XIAP, under different environmental conditions with

unknown mechanisms.

It is widely accepted that PTEN exists in a predominantly phosphorylated and inactive state in cytoplasm (Vazquez et al., 2000). Once its C-terminal tail is dephosphorylated, PTEN translocates to plasma membrane and acts as an active $PI(3,4,5)P_3$ phosphatase. This modulation is proposed to be due to an intra-protein binding between the phosphorylated C-terminal tail and C2/phosphate domain, thus causing auto-inhibition (Vazquez et al., 2001, Das et al., 2003). Phosphorylation not only regulates PTEN's catalytic activity, but also controls PTEN's stability (Vazquez et al., 2000). Mutation of phospho-acceptor sites in the C-terminal tail of PTEN results in a large increase in catalytic activity and a dramatic reduction in half-life. To explain this modulation, one hypothesis suggests that the phosphorylated C-terminal tail is able to bind intramolecularly with the C2 domain and, as a result, mask the residue K289, which is the major ubiquitination site of NEDD4-1, hence preventing ubiquitination-mediated PTEN degradation (Wang and Jiang, 2008). PTEN's phosphorylation status could be regulated by estradiol and progesterone (Guzeloglu-Kayisli et al., 2003). Estradiol down-regulates PTEN activity by increasing its phosphorylation, whereas progesterone up-regulates PTEN activity by decreasing its phosphorylation. The kinases responsible for PTEN phosphorylation include casein kinase 2 (CK2), glycogen synthase kinase 3 β (GSK3β), glioma tumor suppressor candidate region 2 (GLTSCR2), src kinase, LKB1 and RhoA-associated kinase (ROCK). CK2 phosphorylates PTEN on ser370 and ser385, resulting in reduced PTEN phosphatase activity and increased stability (Torres and Pulido, 2001). PTEN phosphorylation can be increased by CK2 activator, spermine, but decreased by CK2 inhibitors, heparin or 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole. GSK3β is

another kinase that phosphorylates PTEN on Ser362 and Thr366 (Al-Khouri et al., 2005). GSK3β can be inhibited by insulin and other activators of PI3K pathway, indicating a negative feedback loop for PI3K-PTEN signaling cascade. GLTSCR2, also known as PICT-1, is responsible for phosphorylation of ser380, a crucial phosphorylation site necessary for stabilizing PTEN (Okahara et al., 2006). Additionally, it is proposed that the src kinase phosphorylates PTEN on tyrosine residues in pervanadate-treated cells, thus reducing the phosphatase activity and stability of PTEN (Lu et al., 2003). Also, the tumor suppressor kinase, LKB1, is suggested to phosphorylate PTEN on ser385 and adjoining sites (Mehenni et al., 2005). Different from the above C-terminal tail kinases, ROCK targets ser229, thr232, thr319 and thr321 in the C2 domain, and activates, rather than inactivate, PTEN in chemoattractant-stimulated leukocytes (Li et al., 2005, Papakonstanti et al., 2007). Collectively, phosphorylation by various kinases is one of the most important modulations of PTEN's phosphatase activity and stability.

PTEN activity can also be down-regulated by acetylation (Okumura et al., 2006). Interaction of PTEN with nuclear histone acetyltransferase p300/CBP-associated factor (PCAF) can promote PTEN acetylation at Lys125 and Lys128, which then suppresses PTEN's catalytic activity by interfering with the binding between PTEN and its substrate $PI(3,4,5)P_3$.

Oxidation is another post-translational modification of PTEN (Seo et al., 2005). In response to cellular stress or receptor activation, cells generate reactive oxygen species (ROS), which can induce formation of a intracellular disulfide bond between the phosphatase active site Cys124 and Cys71 of PTEN, therefore causing

abrogation of PTEN activity.

Besides the above transcriptional and post-translational modulations of PTEN, several inhibitor compounds are also available. However, those inhibitors are not only non-specific, but also toxic in physiological setting. These inhibitors include vanadium compounds, zinc, bisperoxovanadium and sodium orthovanadate.

All together, the stability and activity of PTEN are both strictly controlled by various transcriptional and post-translational modulations. Aberrance in any of those mechanisms may lead to subtle or dramatic loss of PTEN functions.

### 1.3.3 Regulations of PTEN subcellular localization

*Membrane recruitment*

The ability of binding with membrane lipid is crucial for PTEN's phosphatase activity, which is to convert $PI(3,4,5)P_3$ into $PI(4,5)P_2$ and hence inhibits cell growth, proliferation and survival (Wang and Jiang, 2008). One study using *in vitro* binding assays revealed that clusters of positively charged residues on the surface of phosphatase domain and C2 domain are critical for PTEN binding to anionic lipids, even though its phosphatase activity *per se* is not required for this membrane binding (Vazquez and Devreotes, 2006). The binding between PTEN and plasma membrane is a dynamic process, and is regulated by a complex mix of mechanisms, including C-terminal tail phosphorylation and protein-protein interactions (Tamguney and Stokoe, 2007). The phosphorylated C-terminal tail of PTEN is suggested to

mask the membrane binding domains intramolecularly, thus resulting in a low membrane association rate. Once dephosphorylated, PTEN is in an open status and becomes accessible for membrane binding. Additionally, PTEN can be recruited to plasma membrane by interaction with PDZ domain-containing proteins through its PDZ binding motif. Among these are MAGI1b, 2 and 3, hDlg, NHERF1, NHREF2, MAST205, MAST3 and SAST (Vazquez and Devreotes, 2006).

### Shuttling between cytoplasm and nucleus

Many hypotheses have been proposed about the mechanisms of PTEN cytoplasmic and nuclear shuttling. Among these include mono-ubiquitination-mediated PTEN nuclear translocation (Trotman et al., 2007), Ran-GTPase-dependent import mediated by an N-terminal nuclear localization domain (Gil et al., 2006), active $Ca^{2+}$-dependent transport via non-canonical nuclear localization signals within the C-terminus of PTEN with the help of major vault protein (MVP) (Chung et al., 2005), and passive transport by diffusion through nuclear pores (Liu et al., 2005).

PTEN is among those proteins whose nuclear/cytoplasmic shuttling is regulated by mono-ubiquitination. (Li et al., 2003, Plafker et al., 2004, Massoumi et al., 2006). Two lysine residues, K13 and K289, are proved to be the key sites for ubiquitination by the ubiquitin ligase known as neural precursor cell expressed, developmentally downregulated-4-1 (NEDD4-1) (Trotman et al., 2007, Wang et al., 2007). Knockdown of NEDD4-1 redistributes PTEN to the cytoplasm, whereas over-expression of NEDD4-1 increases nuclear PTEN localization. In addition, wild-type GFP-PTEN fusion protein displays a normal ubiquitination level and is found in both

nuclear and cytoplasmic compartments, whereas the over-expressed mutant proteins, GFP-PTEN$_{K13E}$ and GFP-PTEN$_{K289E}$, exhibit a decreased mono-ubiquitination level and are excluded from the nucleus. Moreover, over-expression of wile-type ubiquitin can rescue the deficient nuclear import of GFP-PTEN$_{K13E}$ and GFP-PTEN$_{K289E}$, while over-expression of a modified ubiquitin capable of only mono-ubiquitination results in an even greater increase in PTEN nuclear import (Trotman et al., 2007, Wang et al., 2007). The above evidence strongly suggests a causative role of mono-ubiquitination in mediating PTEN nuclear accumulation. Therefore, it has been proposed that mono-ubiquitination may function as a tag for PTEN to be recognized by nuclear transportation machinery or it may mediate PTEN nuclear translocation through an independent unknown process. Further, ubiquitination not only regulates PTEN cytoplasmic/nuclear shuttling, but also determines PTEN stability (Trotman et al., 2007). The mono-ubiquitinated PTEN can be further poly-ubiquitinated by NEDD4-1 in the cytoplasm and then degraded by the proteasome. Also, the mono-ubiquitinated PTEN can be de-ubiquitinated in the nucleus and remains nuclear and hence be protected from cytoplasmic degradation. The above findings are further supported by an analysis of PTEN's half-life (Trotman et al., 2007). Wide-type PTEN that has an intact cytoplasmic/nuclear shuttling ability has a half life of roughly 7.5 hours. However, forced nuclear localization increases PTEN half-life to 15 hours, because NEDD4-1 is mainly cytoplasmic. In contrast, forced cytoplasmic accumulation decreases PTEN half-life to about 4.5 hours. All together, ubiquitination by NEDD4-1 not only regulates PTEN cytoplasmic/nuclear shuttling, but also determines PTEN stability.

In addition to the above mechanism, another study suggests that N-terminus of

PTEN contains a nuclear localization signal (NLS), which may mediate PTEN nuclear translocation in a Ran GTPase-dependent manner (Gil et al., 2006). The authors also suggest that there are multiple nuclear exclusion signals (NES) located in PTEN's PTP, C2 and C-terminal domains, whose mutations can trigger strong PTEN nuclear accumulation. Very interestingly, this study also pointed out that the PTEN mutants, which reside primarily in the nucleus, can enhance apoptosis, whereas the apoptosis-inducing agents reciprocally promote PTEN nuclear accumulation, indicating a pro-apoptotic role of nuclear PTEN.

Moreover, Chung et al. identified two putative NLS-like sequences located in the PTP and C2 domains of PTEN, which facilitate PTEN nuclear translocation through interacting with major vault protein (MVP), a previously hypothesized nuclear-cytoplasmic transport protein (Chung et al., 2005). Surprisingly, importin α and importin β interact with wide-type PTEN and NLS-like mutant PTEN equally, indicating that the importin complex may not be required in the nuclear translocation of PTEN. Additionally, the author found that neither the phosphatase activity nor the phosphorylation residues of PTEN are required for its nuclear/cytoplasmic transportation.

Unlike the above models which describe PTEN nuclear transportation as a activate process, Liu et al., on contrary, suggests that PTEN enters the nucleus by passive diffusion through nuclear pores (Liu et al., 2005). Their results showed that PTEN nuclear transportation is independent of Ran GTPase, because nuclear localization of GFP-PTEN is not altered at a non-permissive temperature (39.5°C) for Ran-dependent transportation. Furthermore, large PTEN fusion proteins, such as GFP-

GFP-PTEN, have little or no nuclear localization, whereas PTEN alone (47kDa), which is smaller than the widely-believed upper limit (60kDa) for passive diffusion, has intact nuclear transportation.

To summarize the above hypotheses of PTEN cytoplasmic/nuclear shuttling, a comprehensive model was proposed by Gil et al (Gil et al., 2007). PTEN is suggested to exist in two conformations: the "closed" and the "open" ones. In the "closed" conformation, PTEN's NLS-like domain at N-terminus is masked by multiple NES motifs residing in the C2 core region and the C-terminal tail. The "closed" PTEN resides primarily in cytoplasm, and some nuclear translocation is mediated through passive diffusion or other importin-independent mechanisms. However, upon dephosphorylation of C-terminal tail or alterations of NES motifs, such as binding to regulatory partners or partial proteolysis, a conformational change occurs that exposes the NLS-like domain and triggers PTEN nuclear accumulation. The "open" PTEN is then transported into the nucleus through either Ran GTPase-, MVP-, NLS-like domain-dependent-, or importin-related mechanism. Also, the "open" PTEN could be recruited to plasma membrane/PI(4,5)$P_2$ through its lipid binding motifs at N-terminus, or through its PDZ binding motif at C-terminus that binds to various plasma membrane protein partners.

***Events that trigger PTEN nuclear import***

PTEN normally localizes in both the cytoplasm and the nucleus. Nuclear accumulation of PTEN could be enhanced under certain conditions, such as apoptotic stimulation (Gil et al., 2006), ATP depletion (Lobo et al., 2008),

neurotrophin treatment (Lachyankar et al., 2000) and cell cycle traverse (Ginn-Pease and Eng, 2003).

Gil et al. found that apoptosis-inducing agents, such as TNF-α and sorbitol, promote PTEN nuclear accumulation in cell lines, while PTEN mutants that reside primarily in the nucleus reciprocally enhance apoptosis, thus implying a pro-apoptotic role of nuclear PTEN (Gil et al., 2006). Moreover, Lobo et al. revealed that cells deficient in ATP show an increased level of nuclear PTEN (Lobo et al., 2008). This increase in PTEN can be reversed by supplementation with ATP, ADP, AMP, but not the non-hydrolyzable analogue ATPγS, suggesting that ATP deficiency-induced cell death may be mediated through PTEN nuclear translocation. Further, Lachyankar et al. revealed that treatments of CNS stem cells with NGF or BDNF induce nuclear accumulation of PTEN as well as neuronal differentiation (Lachyankar et al., 2000). Suppression of PTEN expression with antisense oligonucleotides lowers the yield of differentiated neurons, suggesting that nuclear PTEN plays a critical role in regulating neuronal differentiation. Last, Ginn-Pease et al. proposed that nuclear import and export of PTEN are associated with cell cycle traverse (Ginn-Pease and Eng, 2003). Higher nuclear PTEN levels are related to G0-G1 phase, while lower nuclear PTEN levels are related to S phase, implying that nuclear PTEN activity might directly regulate the cell cycle. All together, the above evidence strongly suggests that modulation of PTEN subcellular localization is an important mechanism for regulating PTEN activities.

## 1.3.4 PTEN and ischemic neuronal death

In addition to its well-defined role in tumor suppression, emerging evidence suggests that PTEN is highly expressed in the brain and may play a critical role in ischemia-induced neuronal damages (Gary and Mattson, 2002, Ning et al., 2004, Wu et al., 2006, Zhang et al., 2007a). Many previous studies showed that the process of ischemic injuries involves dynamic changes of phospho-PTEN and phospho-Akt (Noshita et al., 2001, Omori et al., 2002, Lee et al., 2004, Choi et al., 2005, Zhao et al., 2005). Also, the ischemic neuroprotective effects of many procedures and pharmacological agents are mediated through inhibiting PTEN and/or promoting PI3K/Akt signaling cascades (Yoshimoto et al., 2002, Jin et al., 2003, Garcia et al., 2004b, Saito et al., 2004, Kaya et al., 2005, Zhao et al., 2005, Zhao et al., 2006, Lee et al., 2009). Thus, a thorough understanding of these pathways is very important in developing effective therapeutic measures for ischemia treatment.

Despite some conflicting results, the general consensus is that the phospho-PTEN, an inactive form of PTEN, decreases immediately after onset of ischemia, and returns to normal or slightly increases at 24 hours post-ictus. The early decrease of phospho-PTEN precedes cytochrome *c* release, suggesting that the disorder of PI3K/PTEN pathway occurs before apoptosis, while the later increase of phospho-PTEN may represent an intrinsic protective mechanism (Omori et al., 2002, Lee et al., 2004, Choi et al., 2005, Zhao et al., 2005). On the other hand, the change of phospho-Akt follows a different pattern. In the ischemic penumbra, the phospho-Akt (Ser 473), an active form of Akt, usually decreases immediately after ischemia onset, and then increases above normal level from 1 to 9 hour after reperfusion, but

decreases again at 24 h after reperfusion (Zhao et al., 2006). Unlike ischemic penumbra, the phospho-Akt (Ser 473) in the ischemic core decreases throughout all the time points monitored (Noshita et al., 2001). In contrast, regarding phospho-Akt on Thr 308, one study reported its sustained decrease after ischemia onset (Yoshimoto et al., 2001), whereas another study did not detect its significant decrease until 48 hours after ischemic insult (Zhao et al., 2005).

As dramatic changes of phospho-PTEN and phospho-Akt are observed in the process of ischemia, many studies continued to investigate whether inhibition of PTEN is protective against ischemic neuronal injuries. Indeed, the siRNA knockdown and pharmacological inhibition of PTEN are reported to be associated with reduced ischemic neuronal death, further confirming the pro-death role of PTEN during ischemic challenges (Ning et al., 2004, Wu et al., 2006, Zhang et al., 2007a). What's more, many other agents (e.g. growth factor, free radical scavengers, estrogen) and procedures (e.g. preconditioning and hypothermia) are suggested to protect neurons via inhibiting PTEN and/or promoting PI3K/Akt signaling pathways (Yoshimoto et al., 2002, Jin et al., 2003, Garcia et al., 2004b, Saito et al., 2004, Kaya et al., 2005, Zhao et al., 2005, Zhao et al., 2006, Lee et al., 2009). Altogether, inhibition of the PTEN signaling cascades may represent a potential therapeutic target for ischemia treatment.

**1.3.5 PTEN and NMDA receptors**

As discussed in the previous chapters 1.1.3 and 1.2.4, excessive activation of NMDA receptors (NMDARs) is believed to be one of the primary mechanisms leading

ischemia-induced neuronal damages. Given that overwhelming body of evidence has also proved a critical role of PTEN in mediating ischemic injuries (chapter 1.3.4), many studies continued to investigate the potential reciprocal relationship between NMDARs and PTEN.

Indeed, accumulating studies revealed that PTEN is actually a crucial downstream effector of NMDARs. Ning et al. and many groups found that PTEN forms a functional complex with NR2B-containing NMDARs (NR2BRs), but not NR2A-containing NMDARs (NR2ARs) (Ning et al., 2004, Chang et al., 2010, Jurado et al., 2010). In addition, this complex is further enhanced by a direct interaction between PTEN and PSD95 in a manner that is dependent on $Ca^{2+}$ influx through NMDARs. Given that the NR2BR-PSD95 complex is primarily coupled with pro-death signaling pathways (Aarts et al., 2002, Hardingham et al., 2002, Liu et al., 2007b), PTEN may be brought into close proximity with those death signals through its binding with NR2BRs and PSD95, hence functioning as a downstream death effector of NMDARs. As predicted, several *in vitro* and *in vivo* studies found that over-expression of phosphatase-inactive PTEN mutant can block glutamate-induced excitotoxicity in cultured hippocampal neurons (Gary and Mattson, 2002), and siRNA knockdown of PTEN protein also strongly protects against NMDARs-mediated neuronal injuries in a rat model of ischemia (Ning et al., 2004), confirming the role of PTEN as an essential death signal downstream of NMDARs.

All together, previous literature strongly suggests a critical role of PTEN not only in tumor suppression, but also in ischemia/NMDAR-mediated neuronal injuries. Suppression or blocking PTEN signaling pathways may represent a novel target

upon which neuroprotective therapeutics can be developed.

## 1.4 Rationales, hypotheses and specific aims

Ischemic stroke is a leading cause of death and disability in developed countries. It not only causes huge human suffering but also exerts a heavy economic burden on society (Lloyd-Jones et al., 2010). Therefore, a thorough understanding of its pathological mechanisms and development of effective treatments are badly needed. Ischemic stroke occurs when occlusion of blood vessel(s) disturbs the blood supply to the brain, leading to massive neuronal damage. The progression of ischemic injuries likely involves multiple events, although their relative importance is still not fully understood (Lipton, 1999). Increasing evidence suggests that ischemic neuronal death is caused, at least in part, by excessive release and accumulation of extracellular glutamate, which in turn over-activates NMDARs in adjacent neurons and hence initiates pro-death signaling cascades (Lucas and Newhouse, 1957, Olney, 1969, Lipton, 1999, Arundine and Tymianski, 2004). Although blocking NMDARs with antagonists has shown promising neuroprotective effects in treating ischemic animals, its therapeutic outcomes in human clinical trials turn out to be very disappointing  (Muir, 2006). Many explanations for these clinical failures have been proposed by clinicians and scientist, including poor experimental designs, lack of long-term evaluations, et al. (Gladstone et al., 2002, Roesler et al., 2003). But in my current study, only two major factors will be emphasized as they may be circumvented by the novel therapeutics proposed here. First, the patient inclusion periods used by most clinical trials are generally more than 6 hours post-ictus, which

is way beyond the time window permitted by NMDAR antagonists, hence resulting in lack of efficacy in stroke treatment (Gladstone et al., 2002, Ikonomidou and Turski, 2002, Roesler et al., 2003). Second, NMDAR antagonist, when used at the concentration required to block excitotoxicity, also blocks the normal, physiological functions of NMDAR in facilitating neuronal survival and regeneration, hence leading to intolerable adverse effects (Ikonomidou et al., 1999). Therefore, instead of aiming at the NMDAR *per se*, my present research turns to focus on identifying downstream death effectors of NMDARs that may permit wider therapeutic time windows and are solely involved in the pro-death pathways of NMDARs.

Given the pro-death role of NR2B-containning NMDARs (NR2BRs) (Liu et al., 2007b), identifying the interacting partners of NR2BRs has facilitated the discovery of NMDAR's downstream signals that are responsible for neuronal death during ischemic stroke. One of the well-characterized such proteins is PTEN, a dual lipid/protein phosphatase previously known as an important tumor suppressor. Several studies showed that PTEN forms a functional complex with NR2BRs, but not NR2ARs (Ning et al., 2004, Chang et al., 2010), and this complex is further enhanced by PTEN interaction with PSD95 in a $Ca^{2+}$ dependent manner (Jurado et al., 2010). Further, the role of PTEN as a critical pro-death effector of NMDARs is supported by many *in vitro* and *in vivo* studies. Gary et al. found that over-expression of catalytically inactive PTEN mutant almost completely abolishes the glutamate-mediated neurotoxicity in cultured hippocampal cultures (Gary and Mattson, 2002). In addition, Ning et al. and many other groups revealed that conditional knockdown or pharmacological inhibition of PTEN protein can dramatically reduce the NMDAR-mediated neuronal injuries in *in vivo* models of ischemia (Ning et al., 2004, Wu et al.,

2006, Zhang et al., 2007a). Taken together, the above studies have strongly suggested that PTEN plays an essential role in the NMDA-induced neurotoxicity, and thus represents a potential therapeutic target for stroke treatment.

In spite of increasing interest in downregulation of PTEN as a means for ischemic stroke treatment, the experimental progression has been slow. The difficulty in developing blockers for PTEN lies in several aspects. First, with regard to potential pharmacological manipulation of PTEN, highly specific activators or inhibitors are still unavailable. Second, it must be noted that chronic inhibition of PTEN pathways may lead to hypertrophy and malignancy because of PTEN's crucial role in tumor suppression; therefore, it is very difficult to establish the fine balance between the beneficial effects and harmful consequences of PTEN inhibition. Third, besides its pro-apoptotic activities, PTEN is also involved in many cell processes, such as chromosomal integrity maintenance and cell cycle regulation; as a result, completely blocking PTEN activities may interfere with its normal physiological functions and produce adverse effects. Therefore, instead of aiming at developing more potent PTEN inhibitors, my current study turns to investigate the detailed mechanisms through which PTEN mediates ischemic cell death. This will enable me to develop efficient and specific therapy that only interferes with PTEN's pathological functions in mediating ischemic neuronal injuries, and hence may have minimal side effects.

Previous studies showed that PTEN is subjected to strict cellular controls, including transcriptional modulations, post-translational modifications, sub-cellular localization regulations and so on. Aberrance in any of the above regulating mechanisms will lead to subtle or dramatic loss of PTEN functions. Recently, accumulating evidence

revealed a very interesting trend that normal cells have PTEN present in both the cytoplasm and the nucleus, whereas some cancer cell lines express PTEN that predominantly reside in the cytoplasm (Duerr et al., 1998, Perren et al., 2000, Whiteman et al., 2002, Trotman et al., 2007). This discrepancy between normal cells and cancer cells strongly implies that nuclear PTEN, compared to cytoplasmic PTEN, may be more critical in mediating tumor suppression. Moreover, a number of studies provided even more direct evidence about the pro-apoptotic role of nuclear PTEN. Isolated nuclei that are pre-incubated with purified PTEN exhibit increased apoptotic DNA fragmentation (Ahn et al., 2004, Ahn et al., 2005). PTEN mutants that reside primarily in the nucleus enhance cell apoptosis, while apoptosis-inducing agents reciprocally enhance nuclear translocation of PTEN (Gil et al., 2006). Taken together, the above evidence strongly suggests that nuclear accumulation of PTEN is critically important for its pro-death functions.

Therefore, I hypothesize that ***NMDARs may mediate excitotoxic neuronal injuries through enhancing nuclear translocation of PTEN, which may represent a novel therapeutic target for ischemic stroke treatment.***

The main objectives of the present thesis research include the followings:

1) *In vitro*: a. to investigate whether excitotoxic NMDA stimulation can trigger PTEN nuclear translocation in cultured cortical/hippocampal neurons, b. to determine mechanisms by which NMDA stimulation promotes PTEN nuclear translocation, and c. to examine whether inhibiting PTEN nuclear translocation can protect neurons against NMDA excitotoxicity.

2) *In vivo*: to investigate whether ischemic challenges (MCAO in rat) can promote PTEN nuclear translocation in the infarct area of the brain, and whether blocking PTEN nuclear translocation using the interfering peptide can reduce infarct volume as well as rescue neurological deficits caused by the ischemic insults.

I propose that inhibition of PTEN nuclear translocation may have two advantages over other therapeutic approaches. First, it may provide a much wider therapeutic time window, as it targets the downstream effector of NMDARs rather than NMDAR *per se*. Second, it may only cause minimal side effects, as it solely interferes with the downstream pathway that is abnormally altered during excessive NMDAR activation. Thus, this work will provide a novel therapeutic means for ischemic stroke treatment.

**Figure 1: NMDAR-mediated signaling pathways**

NMDARs are suggested to mediate both pro-survival and pro-death signaling pathways. Activation of synaptic NMDARs mainly triggers pro-survival signaling pathways, including PI3K-Akt cascade and CREB-regulated gene expression. On the other hand, extrasynaptic NMDARs are primarily coupled with pro-death signals, such as DAPK1, nNOS, p38/JNK MAK kinases, calpain, SREBP1 and PTEN.
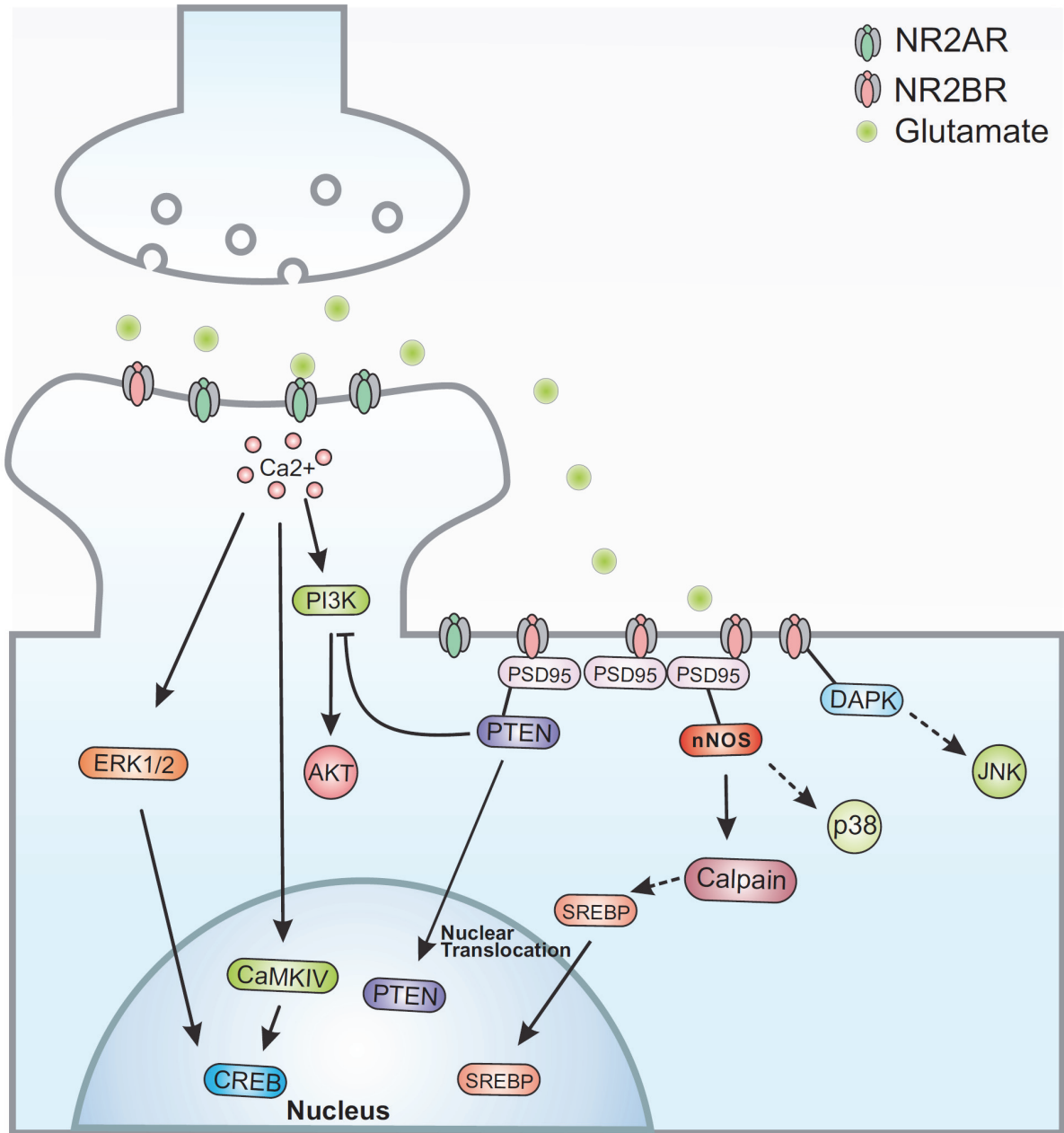
# 2 METHODS

## 2.1 Antibodies and reagents

Mouse anti-PTEN (#sc-7974), goat anti-HDAC1 (#sc-6298) and anti-Insulin Receptor β were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-TATA binding protein (TBP, #ab818) was purchased from Abcam Inc. (Cambridge, MA). Mouse anti-Hsp90 (#610418) was obtained from BD transduction laboratories (San Jose, California). Hoechst 33342 nucleic acid stain, ProLong Gold mounting medium and secondary fluorophore-bound IgGs (Alexa Fluor series) for immunocytochemistry were obtained from Invitrogen-Molecular Probes (Portland, OR). NMDA was purchased from Ascent Scientific (Weston, UK). AP5 was purchased from Tocris (Cookson Bristol, UK). Complete Protease Inhibitor Cocktail Tablets (#04693116001) and Phosphatase Inhibitor Cocktail Tablets (04906845001) were obtained from Roche Applied Science (LSC). Reagents for Protein Concentration Assay (Reagent A #500-0113, reagent B #500-0114) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Cell Toxicity Colorimetric Assay Kit (LDH assay, #TOX7) was purchased from Sigma-Aldrich (St. Louis, MO). Nuclear extraction kit was purchased from Panomics (AY2002, Redwood City, CA). Transfection system (Calcium phosphate) was purchased from Promega (Madison, WI). Tat-fused peptides (Tat-K13, Tat-K13R and Tat-K289) were custom synthesized by Peptide Synthesizing Facility at UBC (Vancouver, BC, Canada).

## 2.2 Buffers

All buffers were sterilized by autoclaving or vacuum filtration (Corning, Polyethersulfone with 0.22 µm pore size). *Phosphate Buffered Saline (PBS)* contains 137mM NaCl, 2.7mM KCl, 8.1mM $Na_2HPO_4$, 1.76mM $KH_2PO_4$, pH to 7.4. *Radioimmunoprecipitation assay (RIPA) buffer* contains 150mM NaCl, 0.3% Deoxycholic Acid Sodium, 50mM Tris, 1mM EDTA, 1.0% Triton-100, pH to 7.4. *Washing Buffer for Immunoprecipitation* contains 500mM NaCl, 50mM Tris-HCl, 1% Triton-100, pH to 7.4. *4 X Sample Buffer (SB)* contains 50% Glycerol, 125mM Tris-HCl (pH6.8), 4% SDS, 0.08% Bromophenol Blue, 5% β-mercaptoethanol (add right before using). *Stripping buffer* contains 2% SDS, 62.5mM Tris-HCl (pH 6.7), 100mM β-mercaptoethanol (add right before using). Poly-D-Lysine Coating Solution contains 10µg/ml poly-D-lysine. *HBSS Dissection Buffer (DB)* contains 5.0g/L glucose, 1.25g/L Sucrose, 0.89g/L Hepes, pH to 7.4, osmolarity to 310-320 mOsm, stored at -20°C. *Neurobasal Plating Media (NP media)* contains 487.75mL Neurobasal Media, 0.5mM GlutaMAX$^{TM}$-I Supplement, 2% B27 supplement, 25µM of glutamic acid. *Neurobasal Feeding Media (NF media)* contains 488.75 mL of Neurobasal Media, 0.5mM GlutaMAX$^{TM}$-I Supplement, 2% B27 supplement. *Buffer A (for nuclei fractionation)* contains 10mM HEPES-KOH, 10mM KCl, 10mM EDTA, 0.4% NP40, 1.5mM $MgCl_2$, 1mM DTT (add right before using), protease inhibitor cocktail (add right before using), pH to 7.9. *Buffer B (for nuclei fractionation)* contains 20mM HEPES-KOH, 400mM NaCl, 1mM EDTA, 10% Glycerol, 1mM DTT (add right before using), protease inhibitor cocktail (add right before using), pH to 7.9. *Protein A beads* were prepared from powder Protein A Sepharose (GE Life Sciences). 100mg of

Protein A Sepharose beads were incubated with 1mL of PBS for 4 hours at room temperature. The swollen beads were then centrifuged down at 500 X $g$ and washed with fresh PBS for three times. After the final wash, the beads were mixed with 500µl lysis buffer made with protease/phosphatase inhibitors (Roche). The slurry was then ready for use. Protein A beads were specifically used for purifying mouse or rat monoclonal antibodies. *Protein G beads* were prepared from pre-swollen Protein G Sepharose™ 4 Fast Flow (GE healthcare). The pre-swollen beads were centrifuged down at 500 X $g$ and washed with fresh PBS for three times as described above. After the final wash, the beads were mixed with 500µl lysis buffer made with protease/phosphatase inhibitors (Roche). The slurry was then ready for use. Protein G beads were specifically used for purifying rabbit polyclonal antibodies.

## 2.3 Plasmids

PTEN plasmids (GFP-PTEN$_{WT}$, GFP-PTEN$_{K13R}$, NLS-GFP-PTEN$_{WT}$, and NLS-GFP-PTEN$_{C124S}$) were kind gifts from Dr. Alonzo H. Ross (University of Massachusetts Medical School, USA). Specifically, the coding sequence of human PTEN was cloned into pcDNA3 plasmid. An enhanced Green Fluorescent Protein (GFP) was inserted into the 5' end of PTEN to facilitate microscopic visualization. In addition, N-terminus of GFP-PTEN was fused with a Nuclear Localization Signal (NLS), which can force the newly synthesized PTEN into the nucleus through nuclear pore complex. The NLS tag used in the PTEN constructs is SPKKKRKV, which is a strong NLS based on a hexamer protein called SV40 Large T-antigen (Liu et al., 2005). GFP-PTEN$_{K289R}$ plasmid was constructed by site-directed mutagenesis using Pfu

DNA polymerase. The PCRs contained the following components: 2μL Pfu Turbo DNA polymerase (5.0U/ μL, Stratagene, La Jolla, CA, USA), 2.5μL DNA template (100ng/μL, GFP-PTEN$_{WT}$), 1μL forward primers (200ng/μL), 1 μL reverse primers (200ng/μL), 1μL dNTP (2mM dNTP), 5 μL 10× Pfu buffer (Stratagene, La Jolla, CA, USA) and 37.5μL sterile water. Standard PCR cycling conditions were used:  95°C for 5min; 30 cycles of 95°C for 1min, 48.5°C for 1min, and 68°C for 13min; and a final extension at 68°C for 13min. The PCRs product was then subjected to 1μL DpnI (20 U/μl, New England Biolab) digestion at 37°C for 1 hour, and then transformed into DH5α competent cells to grow at 37°C for overnight. Last, clones were picked for mini-preparation and subsequent automated DNA sequencing.

## 2.4 Primary culture of cortical/hippocampal neurons

One day prior to primary culture, coverslips or plates were incubated with poly-D-lysine solution and allowed to sit overnight at room temperature. Immediately prior to plating, the coverslips or plates were washed with sterile water for three times, air-dried and ready for use. Pregnant rat (E18) was sacrificed by overdosing with 3.5mL of a 25% Urethane solution. Embryos were transferred to a pre-cooled sterile culture dish. Using a dissecting scope, the cortex or hippocampi were isolated and incubated in ice-cold Dissection Buffer (DB). After dissection was completed, DB was replaced with 2-4mL of pre-warmed 0.25% Trypsin-EDTA and incubated at 37°C for 30min. Then, the Trypsin was inactivated by 8-10mL warm DMEM media (with 10% FBS). The dissociated cells were washed with fresh DMEM for three times, followed by gentle pipetting for several times to ensure a single cell suspension. Next, the

DMEM was replaced by Neurobasal Plating (NP) media by centrifuging at 2,500 RPM for 1 min. The cells were counted using a hemocytometer, and plated onto poly-D-lysine-coated culture dishes at a density of $6.0 \times 10^6$ per plate (100mm), $4.0 \times 10^5$ per well (12 well) or $2.0 \times 10^5$ per well (24 well). The cells were then cultured in a 37°C incubator with 95% $O_2$ and 5% $CO_2$. At day 2 in vitro, 1/3 NP media was replaced by equal volume of Neurobasal Feeding (NF) media. At day 3 in vitro, uridine (10 µM) and 5-Fluor-2'-deoxyuridine (10 µM) were added to the culture medium and maintained for 48 h to inhibit non-neuronal cell proliferation before washout. Media swap was performed every 4 days until cells were used for additional experiments.

## 2.5 NMDA-induced excitotoxicity

Primary cultures of mature cortical/hippocampal neurons (12-14 DIV) were used in this study. Right before NMDA treatment, half of the conditioned medium was taken out and saved in *BD* Falcon™ tube for further use. Neurons were stimulated with 25µM NMDA through bath application, together with other drug or peptide treatments as specified in each individual experiment. After 60 min incubation with NMDA, neurons were washed with fresh neural basal medium for once and then returned to the previously saved NMDA-free conditioned medium. Neurons were allowed to recover for different periods of time, ranging from 0 hour to 24 hours until collection or additional assay.

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## 2.6 Nuclei staining

Apoptotic cell death was determined by measuring the percentage of neurons displaying condensed/fragmented nuclei, which are one of the most typical indicators for cell apoptosis (Hardingham et al., 2002, Wang et al., 2004). Briefly, neurons were incubated with DNA dye Hoechst-33342 (1μg/mL) for 20min at room temperature. Four visual fields were randomly selected from each coverslip and imaged with a Leica DMIRE2 fluorescence microscope. The number of apoptotic nuclei and total nuclei were counted, respectively. The condensed or fragmented nuclei were regarded apoptotic, whereas the large and round nuclei were counted as healthy ones. The death rate was expressed as a ratio (%) between number of apoptotic nuclei and that of total nuclei. This experiment was performed double-blindly.

## 2.7 LDH assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme, which can convert NAD into NADH. LDH is released from cells into culture medium upon compromise of plasma membrane integrity. Therefore, the amount of released LDH represents the degree of cell death. In my current study, the extracellular LDH level was measured using Cell Toxicity Colorimetric Assay Kit obtained from Sigma-Aldrich. The basis of this LDH assay is: 1) LDH reduces NAD into NADH, 2) the resulting NADH is then utilized in the stoichiometric conversion of a tetrazolium dye, and 3) the resulting colored compound is measured by a spectrophotometric microplate reader at a wavelength of 490nm. The cell death rate was expressed as a ratio (%) between the

absorbance of treated group and that of control group.

## 2.8 Nuclei fractionation

***Nuclei fractionation from cultured neurons:*** Nuclear extracts were isolated from cultured neurons (6.0 X $10^6$ cells/100mm dish) by using the Panomics nuclear extraction kit (Panomics, catalog number AY2002) with several modifications. Briefly, Buffer A and Buffer B were freshly prepared as described in the ***Buffer*** section, and kept on ice for later use. Culture neurons were rinsed with cold PBS for one time, and lysed by 1mL cold Buffer A for 30min or until no intact neurons could be observed under microscope. To achieve the best result, this lysis step was performed on ice, along with gentle rocking. Neuronal lysate was then pipetted up and down for several times to break down any cell clumps and collected into 1.5mL centrifuge tube for centrifuging at 14,000 x *g* for 3 min at 4°C. The supernatant was cytoplasmic fraction, which was removed into a new tube and centrifuged again to further remove nuclear debris. On the other hand, the pellets were intact nuclei, which were subjected to lysis by 150 µL Buffer B for another 2 hours. This lysis step was also performed on ice, along with gentle rocking. To ensure complete dissolution of nuclei, the pellets were vortexed briefly for several times during the 2 hours incubation. Last, the nuclear lysate was centrifuged at 14,000 x *g* for 5min at 4°C, and the supernatant (nuclear fraction) was transferred into a new tube for protein concentration assay and western blotting. The purity of different cellular fractions was confirmed by probing each fraction for corresponding subcellular marker proteins (Kim et al., 2008, Song et al., 2008). As shown in Fig. 3A, the cytoplasmic

marker heat shock protein 90 (Hsp90) and the plasma membrane marker insulin receptor β primarily localize in the cytoplasmic fraction, while the nuclear markers, histone deacetylase 1 (HDAC1) and TATA-binding protein (TBP), mainly reside in the nuclear fraction, suggesting this nuclei fractionation is pure enough for my purpose.

***Nuclei fractionation from brain tissue:*** Nuclear extracts were isolated from brain tissue of Sprague-Dawley rat by using the Panomics nuclear extraction kit (Panomics, catalog number AY2002) with several modifications. Briefly, Buffer A and Buffer B were freshly prepared as described in the ***Buffer*** section, and kept on ice for later use. The brain tissues were collected from the area outlined by the dashed box in Fig. 10A, and finely diced into very small pieces with clean scissors on ice. This step is critically important, as any big cell clumps may result in cross-contamination between different subcellular fractions. The brain tissues were then transferred into a pre-chilled, clean Dounce homogenizer, and homogenized with 15-20 strokes. 1mL of Buffer A was added to the brain tissues to facilitate further homogenization with another 15-20 strokes. The homogenate was incubated on ice for 15min, and then transferred to a pre-chilled microcentrifuge tube and centrifuged at 850 x *g* for 10min at 4°C. The supernatant was discarded. At this stage, most cells have not been lysed yet. Again, the homogenate was added a second 1mL of Buffer A and dispersed thoroughly by pipetting up and down for 15-20 times. The homogenate was then incubated on ice for another 15min and centrifuged at 14,000 x *g* for 3min at 4°C. The supernatant was cytoplasmic fraction, which was removed into a new tube and centrifuged again to further remove nuclear debris. On the other hand, the pellets were intact nuclei, which were washed with fresh Buffer A (~1mL) for twice, and then subjected to lysis by 300μL Buffer B for another 2 hours. This

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lysis step was also performed on ice, along with gentle rocking. To ensure complete dissolution of nuclei, the pellets were dispersed by pipetting up and down for several times during the 2 hours incubation. Last, the nuclear lysate was centrifuged at 14,000 x $g$ for 5min at 4°C.  The supernatant (nuclear fraction) was transferred into a new tube for protein concentration assay and western blotting as described above.

## 2.9 Western blotting

Samples were aliquoted into the same amount (40μg) of total protein, and boiled with 4X sample buffer at 95°C for 5min. The samples were then separated on 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. To block non-specific background, the membranes were incubated with 5% fat-free milk for 1 hour at room temperature. The target proteins were immunoblotted with primary antibody (overnight at 4°C) and then corresponding HRP-conjugated secondary antibody (1 hour at room temperature). For sequential blotting, the membranes were stripped with stripping buffer, and probed with another antibody. The blots were developed by enhanced chemiluminescence detection system (Amersham ECL), and imaged by Bio-Rad ChemiDoc™ XRS+ system. The intensities of interested bands (raw data) were quantified using Bio-Rad Quantity One® software. The relative level of target protein is expressed as the percentage between intensity of target protein and that of marker protein, such as nuclear marker HDAC1 and cytoplasmic marker β-actin.

## 2.10 Immunocytochemistry

Mature hippocampal neurons (12-14 DIV) cultured on poly-D-lysine-coated coverslips were fixed with 4% formaldehyde in PBS for 30 min at room temperature. The neurons were then permeabilized by 0.1% Triton X-100 in PBS for 2 min on ice. To block non-specific background, the neurons were incubated with 2% normal goat serum in PBS for 1 hour at room temperature. Next, the endogenous PTEN protein was probed by mouse anti-PTEN primary antibody (2μg/ml) for overnight at 4°C, and subsequently incubated with fluorophore-conjugated secondary antibodies (Alexa Fluor 555 goat anti-mouse IgG) for 1 hour at room temperature. Nuclei were stained with fluorescent dye Hoechst 33342 (1μg/ml) for 20min at room temperature. Last, the coverslips were mounted onto slides with ProLong Gold mounting medium (Invitrogen) and ready for microscopy visualization. Images of PTEN and nuclei were taken with a Leica DMIRE2 fluorescence microscope. The region of nucleus was defined by overlapping each PTEN image with its corresponding nucleus image. Signals of nuclear PTEN and cytoplasmic PTEN (excluding neuritis) were quantified separately by drawing irregular circles around corresponding areas and reading the mean intensities with software *Image J*. The relative nuclear PTEN level of each neuron was expressed as a ratio between the intensity of nuclear PTEN and that of cytoplasmic PTEN from the same cell. Double-blind quantification was used in this experiment.

## 2.11 Primary culture transfection

Mature hippocampal neurons (10–12 DIV) cultured on coverslips in a 12-well plate were subjected to transfection by ProFection® Mammalian Transfection System - Calcium Phosphate (Promega, PRE1200). Briefly, all transfection reagents were thawed and warmed to room temperature. The DNA solution and 2X HEPES-buffered saline (HBS) solution, enough for one whole 12-well plate, were prepared in two separate sterile tubes. In Tube 1, 20µl DNA (1µg/µl) and 418µl water were mixed well first, then joined by 62µl $CaCl_2$ (2M) and mixed well again. Tube 2, which contains 500µl 2X HBS, was gently vortexed while the prepared DNA solution from Tube 1 was slowly added (dropwise) to Tube 2. Fine calcium phosphate-DNA coprecipitates were formed and incubated at room temperature for 30min. Next, the DNA mix was vortexed briefly again just before being added to the neurons (80µl mix/well). Plate was swirled to evenly distribute the calcium phosphate-DNA coprecipitates, and returned to incubator (37°C) for another 2 hours. Last, the neurons were washed with pre-warmed neurobasal medium for three times to remove the calcium phosphate-DNA coprecipitates, and then returned to incubator for another 48 hours until additional assay.

## 2.12 Cell line transfection

Transfection was performed on COS7 (African Green Monkey SV40-transfromed kidney fibroblast cell line) using Lipofectamine™ 2000 transfection reagent (Invitrogen). Briefly, COS7 cells were re-plated (100mm plate) so that they could

reach 90% confluence the next day. The DNA solution and Lipofectamine solution were prepared in separate sterile tubes. In Tube 1, 12µl DNA (1µg/µl) was diluted with 500µl Opti-MEM® I Reduced Serum Medium without serum. In Tube 2, 30µl Lipofectamine™ 2000 was diluted with 500µl Opti-MEM® I Medium. Following 5min incubation at room temperature, the two solutions were combined and mixed gently. The mix was then allowed to sit at room temperature for another 20min before being added to COS7 cells. The plate was rocked gently back and forth to evenly distribute the transfection mix, and returned to incubator (37°C) for another 48 hours until addition assay.

## 2.13 Co-Immunoprecipitation

Cells were washed with cold PBS for one time, and incubated with RIPA buffer containing complete protease and phosphatase inhibitor cocktails (Roche) for 30min on ice. Lysate was collected into microcentrifuge tube and centrifuged at 14,000 RPM for 10min at 4°C. The supernatant was removed into a new tube and subjected to protein concentration assay (Bio-Rad). Each sample was aliquoted into the same amount (1mg) of total protein, and incubated with 4µg primary antibody against the target protein for 2 hours at 4°C. Then, 30µl pre-swollen protein A (for rabbit primary antibody) or protein G (for mouse/rat primary antibody) was added to the lysate and incubated for overnight at 4°C. The beads were washed with washing buffer for two times and PBS for another two times before being boiled with 4X sample buffer at 95°C for 5min. Samples were then subjected to western blotting as described above.

## 2.14 Three-vessel middle cerebral artery occlusion (MCAO)

The MCAO surgery was done in our collaboration lab (Dr. Woei-Cherng Shyu Lab) as previously described (Chen et al., 1986, Shyu et al., 2008). Briefly, adult male Sprague-Dawley rats (weight ~200g) were anesthetized with chloral hydrate (0.4g/kg i.p.). A craniotomy, 2mm in diameter, was drilled 1mm rostal to the anterior junction of the zygoma and the squamosal bone using a surgical microscope. The exposed MCA was ligated with a square knot using a 10-0 nylon suture. Next, the bilateral common carotid arteries (CCAs) were clamped with non-traumatic arterial clips. Successful surgery was confirmed by a marked drop in regional cerebral blood flow, monitored by a laser doppler flowmeter (PF-5010, Periflux system; Perimed AB). Moreover, the core body temperature was monitored with a thermometer probe (Hewlett-Packard Model 21090A probe, Hewlett-Packard Company, Andover, MA), and maintained at $37.0 \pm 0.5°C$ with a heating pad. Both blood pressure and blood gas levels were also monitored during the experiment. After 90min ischemia, the suture and clips were removed to allow instant reperfusion. Experimental rats were subdivided into several groups to receive different treatments of interference peptides (Tat-K13, Tat-K13R or Tat-K289 at dosage of 10mg/kg) or saline via femoral vein injection. The first bolus of Tat-K13 was administered at either 2 hours or 6 hours post-stroke onset, while Tat-K13R, Tat-K289 and saline were all given at 2 hours post-stroke onset. To achieve the optimal outcome, another two doses of peptides were administered on the 2nd day and the 3rd day, respectively. Rats were then allowed to recover for different periods of time until additional experiments.

## 2.15 Magnetic resonance image (MRI)

MRI was performed in our collaboration lab (Dr. Woei-Cherng Shyu Lab) in order to evaluate the brain infarct volume post-ischemia insults (Shyu et al., 2004, Shyu et al., 2008). The 3.0 T General Electric imaging system (R4, GE) was used. Briefly, at Day 7 post-stroke onset, rats were anesthetized with chloral hydrate (0.4g/kg i.p.) and the body temperature was kept at 37.0 ± 0.5 °C with a heating pad during imaging process. The T2-weighted Spin Echo imaging sequence (T2WI) was used with the following parameters: repetition time, 4000ms; echo time, 105ms; 6-8 contiguous coronal slices with each 2mm thick. At this stage of stroke development (7 days post-ischemia), brain infarcts manifest as high signals on the MRI images. The non-infarct areas were drawn manually from slice to slice and the volumes were measured with the analysis software called Voxtool (General Electric). The infarct size was quantified by subtracting the non-infarct volume of the ischemic hemisphere from the total volume of the contralateral hemisphere.

## 2.16 [$^{18}$F]fluoro-2-deoxyglucose positron emission tomography (FDG-PET) examination

In collaboration with Dr. Woei-Cherng Shyu group, the microPET scanning of [$^{18}$F]fluoro-2- deoxyglucose (FDG) was performed to assess the recovery of brain glucose metabolic activities post-ischemia insults (Matsumura et al., 2003, Shyu et al., 2004, Shyu et al., 2008). FDG is an analogue of glucose. The amount of FDG uptake by the brain represents the level of brain glucose metabolism. Therefore, by

tracing the $^{18}$F-FDG signals, we can evaluate the effects of peptide treatments on brain metabolic activities following ischemic injuries. Briefly, $^{18}$F was produced by the $^{18}$O(p, n)$^{18}$F nuclear reaction in a cyclotron, and $^{18}$F-FDG was synthesized with an automated $^{18}$F-FDG synthesis system (Nihon Kokan) at China Medical University and Hospital, Taiwan. The PET scanning was performed by a high-resolution small-animal PET scanner (microPET, Rodent R4, Concorde Microsystems) with the system parameters described previously (Carmichael et al., 2004). At Day 7 post-stroke onset, rats were anesthetized with chloral hydrate (0.4g/kg, ip) and fixed in a customized stereotactic head holder in the microPET scanner. The rats were then given a bolus i.v. injection of $^{18}$F-FDG (200-250 μCi/rat) dissolved in 0.5ml of saline. Immediately following the $^{18}$F-FDG administration, rats were subjected to PET scanning for 60min using a 3-D acquisition protocol (Visnyei et al., 2006, Shyu et al., 2008). Data collected were analyzed by Interactive Data Language (IDL) ver. 5.5 (Research Systems) and ASIPro ver. 3.2 (Concorde Microsystems). Last, the deficit of brain glucose metabolic activity was expressed using the following formula: [1-ipsilateral hemisphere signal/contralateral hemisphere signal] X100%.

## 2.17 Neurological behavioral tests

A battery of behavioral tests was done in our collaboration lab (Dr. Woei-Cherng Shyu Lab) as described previously (Shyu et al., 2004, Shyu et al., 2008). They are elevated body swing test, vertical movement time and grip strength test. Elevated body swing test is a measurement of body asymmetric motor behavior (Chang et al., 2003, Shyu et al., 2008). It was performed by elevating the rat by its tail and counting

the frequency of its swing behavior. Previous studies revealed that rats subjected to ischemia exhibit a significantly biased swing activity with the direction contralateral to the ischemic side, while the native rats have no preference for either side. Therefore, the frequency of the biased swing activity may represent the degree of neurological deficits caused by ischemic stroke. The detailed experimental design is as follows. The rat was lifted to about one inch above the floor by its tail. One swing behavior was counted when the rat moved its head more than 10 degree from the vertical axis to either the contralateral side or ipsilateral side of ischemia. Twenty continuous swings were counted in total, and the percentage of recovery was normalized using the following formula: % recovery = [1 – (No. of contralateral swings – 10) / 10] x 100%. Vertical movement of the rat was monitored using the VersaMax Animal Activity Monitor obtained from Accuscan Instruments (Chang et al., 2003, Shyu et al., 2004, Shyu et al., 2008). The Monitor has 16 horizontal X-Y and 8 vertical Z infrared sensors spaced 87 cm apart. The vertical sensors were situated 10 cm from the floor of the chamber. The rat was placed in the recording chamber at night, and its vertical movement time (seconds) was automatically recorded by computer when the infrared beams were broken by its movement over a 2-hour period. The length of its vertical movement represents the recovery of locomotor circuits injured by ischemic stroke. Grip strength is another index for testing motor functions (Dunnett et al., 1998, Shyu et al., 2004, Shyu et al., 2008). It is recorded by a grip strength meter manufactured by TSE system. Briefly, the rat was mounted onto the grip sensor and pulled backwards gently by its tail with a continuous movement. Naturally, the rat clung to the grip sensor firmly to resist the pull until it could no longer hold on and let it go. The maximum force at which the animal released the grip was recorded by the grip strength meter. 20 pulls were performed for both left forelimb and right forelimb,

respectively, and the readings were averaged to get the mean grip strength for each forelimb. The strength of the impaired forelimb (contralateral to stroke side) was calculated as the percentage of the intact forelimb's performance (ipsilateral to stroke side). Then, the improvement of motor function was expressed as the ratio between the grip strength measured at 28 days post-stroke and that measured at 1 day following MCAO surgery.

## 2.18 Statistical analysis

All measurements in this study were performed blindly. Results were expressed as mean ±Standard Deviation. One Way ANOVA was used for comparison among multiple groups and followed by post-hoc (Tukey) test where appropriate. Data lacking normal distribution were analyzed by nonparametric ANOVA (Kruskal-Wallis test). Student's $t$-tests were used to evaluate mean differences between two groups. Data lacking normal distribution were analyzed by a nonparametric Rank Sum Test (Mann-Whitney). Statistical significance was defined as $*P < 0.05$, $** P < 0.01$.

# 3 RESULTS

## 3.1 NMDA stimulation induces excitotoxic neuronal death in cultured neurons

In order to develop an effective treatment for excitotoxic/ischemic neuronal injuries, first of all, I need to establish a good *in vitro* model, which allows careful dissection of the detailed cellular mechanisms underlying excitotoxic/ischemic cell death. I choose to induce NMDA excitotoxicity by using our lab's standard protocol, which is to challenge 12-14 days old cultured cortical/hippocampal neuronal with NMDA (25µM) for 1 hour, and then allow 0-24 hours' development of cell death before final quantification. It is necessary to point out that my preliminary experiments have showed that both cortical neurons and hippocampal neurons produce very similar results under NMDA stimulation. However, as biochemistry experiments generally require a large number of cells, cortical neurons were used instead of hippocampal neurons to provide sufficient material.

Previous literature suggests that NMDA treatment is able to induce both apoptosis and necrosis, depending on the intensity of NMDA insults (Bonfoco et al., 1995). Mild NMDA stimulation primarily induces apoptosis, whereas intense NMDA challenge mainly causes necrosis. In order to determine the degree of cell death our protocol can produce, two independent methods, nuclei staining and LDH assay, were performed, respectively. Nuclei condensation and fragmentation are widely-accepted as one of the most typical indicators for cell apoptosis (Hardingham et al., 2002,

Wang et al., 2004). Hence, the percentage of neurons exhibiting condensed/fragmented nuclei represents the degree of apoptosis. As described above, cultured hippocampal neurons (12-14 DIV) were treated with NMDA (25µM) for 1 hour, and then returned to conditioned medium (NMDA-free) for recovery. Six hours after treatment, I stained the neuronal nuclei with a fluorescent dye named Hoechst-33342, and then quantified the ratio of condensed/fragmented nuclei to total nuclei by using microscopic visualization. As predicted, the nuclei staining revealed that NMDA treatment results in a dramatic increase in the percentage of neurons displaying condensed or fragmented nuclei, suggesting a significantly enhanced neuronal apoptosis (Fig. 2A, quantification is included in Fig. 9B). To confirm this result, another cell death measurement, LDH assay, was used. LDH is a soluble enzyme located in the cytoplasmic compartment. LDH is released into extracellular medium upon plasma membrane damage, a process that occurs during both apoptosis and necrosis. Therefore, the amount of released LDH represents the degree of total neuronal death (Koh and Choi, 1987, Liu et al., 2007b). Another major advantage of LDH assay is that it is a non-biased and population-based test, and thus provides reliable information about the cell death rate. Briefly, cultured cortical neurons (12-14 DIV) were challenged with the same protocol (25µM NMDA for 1 hour plus 6 hours recovery). Cultured medium was then collected and subjected to LDH colorimetric assay. Similarly, the readout showed a significantly enhanced necrotic neuronal death post-NMDA treatment. To confirm this toxicity is indeed caused by NMDAR overactivation, a specific NMDAR antagonist AP5 (50µM) was used along with NMDA stimulation. The result showed a complete abolition of cell death by AP5, confirming the specificity of the NMDA-induced excitotoxicity (n=7; One Way ANOVA on Ranks, H=15.217, P=0.002; Fig. 2B). All together, the results of

nuclei staining and LDH assay proved that our NMDA protocol is strong enough to trigger significant cell death in cultured neurons, and thus represents a good *in vitro* model for studying NMDAR-mediated neuronal injuries.

## 3.2 NMDA stimulation enhances PTEN nuclear translocation in cultured neurons

Previous studies have showed that NMDARs have dual roles in governing neuronal fates. While NR2A-containing NMDA receptors (NR2ARs) mainly trigger the activation of neuronal survival pathways, the NR2B-containing NMDARs (NR2BRs) are primarily responsible for initiating neuronal death cascades (Aarts et al., 2002, Liu et al., 2007b). The difference between NR2ARs and NR2BRs is suggested to be due to their distinct channel properties and downstream signaling pathways. First, the two receptors mediate different kinetics of $Ca^{2+}$ influx (Choi, 1994, Cull-Candy and Leszkiewicz, 2004). NR2ARs have fast deactivation kinetics and function as the coincidence detectors of synaptic excitation, whereas NR2BRs have longer decay time and are more efficient at excitatory postsynaptic potential (EPSP) summation. Second, the two receptors are coupled to distinct downstream effectors (Lee et al., 2002, Kohr et al., 2003, Liu et al., 2004, Martin and Wang, 2010). NR2ARs are linked with pro-survival signals, such as PI3K/Akt; in contrast, NR2BRs form functional complex with pro-death molecules, such as PTEN. Given that excitotoxicity induced by NMDARs, particularly NR2BRs, plays an essential role in mediating neuronal injuries in ischemic stroke, I propose that identification of the downstream death signals of NR2BRs may provide novel therapeutic targets for ischemia treatment.

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Interventions targeting those death effectors may have two major advantages over other stroke therapies (e.g. NMDAR antagonists). First, they will provide much wider therapeutic time windows, as activation of those death effectors is far downstream of NR2BRs. Second, they will not interfere with the pro-survival functions of NMDARs, because those death signals are only coupled to NR2BRs, but not NR2ARs. Within all the interaction partners of NR2BRs, I am particularly interested in PTEN, as PTEN is a well-characterized protein whose phosphatase activity is previously known to be indispensible for triggering glutamate/ischemia-induced neuronal injuries (Gary and Mattson, 2002, Lee et al., 2004, Ning et al., 2004, Wu et al., 2006, Zhang et al., 2007a, Chang et al., 2010). However, none of the previous studies were able to identify the detailed mechanisms by which PTEN mediates the excitotoxic neuronal death, and hence failed to develop practical intervening measures for stroke treatment. Given that overwhelming body of evidence from cancer studies has revealed that PTEN has to translocate into the nucleus to exert its tumor suppressive functions (Perren et al., 2000, Lian and Di Cristofano, 2005, Liu et al., 2007a, Trotman et al., 2007), I propose to investigate whether NMDA challenge also triggers PTEN nuclear accumulation in neurons, and whether this translocation represents a critical step leading to excitotoxic neuronal damage.

Two approaches were used to examine the change of PTEN's subcellular distribution triggered by NMDA stimulation. The first approach is nuclei fractionation followed by western blotting for PTEN protein. The purity of different cellular fractions was confirmed by probing each fraction for corresponding subcellular marker proteins (Kim et al., 2008, Song et al., 2008).  As shown in Fig. 3A, the cytoplasmic marker heat shock protein 90 (Hsp90) and the plasma membrane marker insulin receptor β

primarily localize in the cytoplasmic fraction, while the nuclear markers, histone deacetylase 1 (HDAC1) and TATA-binding protein (TBP), mainly reside in the nuclear fraction, suggesting that this nuclei fractionation method is pure enough for my purpose. Briefly, cultured cortical neurons (12-14 DIV) were treated with NMDA (25µM) for 1 hour and then allowed to recover for 6 hours, followed by nuclei fractionation and western blotting. As predicted, a significantly stronger nuclear PTEN signal was observed in the NMDA-treated group than the control group, suggesting that NMDA challenge triggers a dramatic nuclear translocation of PTEN. Further, in order to confirm that the PTEN nuclear translocation is genuinely triggered by NMDA stimulation, the specific NMDAR antagonist AP5 (50µM) was also bath applied into culture medium along with NMDA treatment. The result showed that AP5 completely abolished the NMDA-induced nuclear accumulation of PTEN, confirming that this translocation is indeed specifically mediated by NMDAR overactivation (n=4; One Way ANOVA, F=50.318, P<0.001; Fig. 3B). In contrast to the pattern of nuclear PTEN, cytoplasmic PTEN only exhibited a small reduction after NMDA stimulation and this reduction could also be restored by AP5 administration (n=4; One Way ANOVA, F=4.947, P=0.018; Fig. 3C). To further verify the result of nuclei fractionation experiment, another approach, immunochemical staining, was used. Cultured neurons (12-14 DIV) were treated with NMDA (25µM) for 1 hour and then recovered for 6 hours. Next, neurons were fixed, permeabilized, and immunostained with PTEN antibody as well as nuclei dye Hoechst 33342. In consistency with nuclei fractionation experiment, the immunostaining result also showed a significantly enhanced nuclear PTEN signal in the NMDA-treated group compared to control, confirming that NMDA challenge indeed triggers a dramatic increase in PTEN nuclear accumulation, which may causatively mediate NMDA-

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induced neurotoxicity (Fig. 3D).

The next question is which subtype of NMDARs mediates the nuclear translocation of PTEN. Previous paper showed that PTEN forms a functional complex only with NR2B-containing NMDARs (NR2BRs), but not NR2A-containing NMDARs (NR2ARs) (Ning et al., 2004, Chang et al., 2010). Therefore, I predict that it is NR2BRs that trigger this PTEN nuclear accumulation. Cultured neurons (12-14DIV) were pre-treated with either NR2BR-specific antagonist Ro25-6981(0.5µM) or NR2AR-specific antagonist NVP-AAM077 (0.4µM) for 30min, and then subjected to the same NMDA treatment and subsequent nuclei fractionation as described above. Not surprisingly, the result showed very good consistency with the previous literature: only NR2BR antagonist Ro25-6981, but not NR2AR antagonist NVP-AAM077, is able to block the NMDA-induced PTEN nuclear translocation, suggesting that PTEN nuclear translocation is primarily mediated by NR2BRs, but not NR2ARs (Fig. 3E). It's worth noting that the subunit specificities of Ro25-6981 and NVP-AAM077 are hotly debated in the literature (Berberich et al., 2005, Weitlauf et al., 2005). The discrepancy is likely due to large variations in experimental settings among different labs. In fact, under our lab's experimental conditions, my colleague found that the cross-inhibition degrees of NR2BRs by NVP-AAM077 and NR2ARs by Ro25-6981 are actually very small and not statistically significant (Liu et al., 2007b). Thus, I conclude with confidence that the two antagonists have little cross-receptor subtype inhibition under the present experimental conditions and the above results are reliable.

## 3.3 NMDA stimulation enhances PTEN nuclear translocation in a time-dependent manner

Next, in order to investigate whether the NMDA-induced PTEN nuclear translocation permits a wider intervention time window than NMDARs *per se*, a time course study was carried out. After 12 to 14 days' *in vitro* culture, cortical neurons were treated with NMDA (25μM, 1 hour) and allowed to recover for different periods of time, ranging from 0 hour to 24 hours. Neurons were then collected and subjected to nuclei fractionation and subsequent western blotting to probe for PTEN protein. Very excitingly, the result showed a dramatic time-dependent nuclear translocation of PTEN following NMDA challenge. The translocation initiated immediately after NMDA treatment, peaked at around 6-9 hours and dropped back at 24 hours later (Fig. 4A and 4B). Even more interestingly, the time course of cell death, measured by LDH assay, matches perfectly with that of PTEN nuclear translocation. The cell death began to reveal itself at around 3 hours after NMDA stimulation, became significant at 6 hours, and reached a plateau at 9 hours later (Fig. 4C). LDH assay measures the accumulation of LDH released by neurons into the culture medium. Therefore, the change of LDH level from one time point to the next represents the cell death happening within that period of time. As showed in Fig. 4C, the most dramatic increase of cell death occurred between 3 hour and 6 hour, which is exactly when the major PTEN nuclear translocation took place. On the other hand, extracellular LDH level stayed relatively stable between 9 hours to 24 hours, suggesting that very little cell death occurred during that period. This result strongly implies that PTEN nuclear translocation may causatively lead to NMDA-induced excitotoxicity. All

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together, these exciting findings proved that PTEN nuclear translocation (peak at 6-9 hours post-NMDA) permits a sufficient therapeutic time window for stroke patient recruitment (average ~6 hours), and thus PTEN well qualifies as an ideal therapeutic target for developing stroke treatment.

## 3.4 PTEN nuclear translocation following NMDA stimulation is a pro-death process rather than a self-defense mechanism

The above experiments revealed a strong positive correlation between PTEN nuclear accumulation and neuronal death, implying PTEN nuclear translocation may causatively contributes to NMDAR-mediated neurotoxicity. However, it's also possible that PTEN nuclear translocation is a self-defense mechanism rather than a pro-death event. To address this question, NMDA excitotoxicity was induced in cultured neurons that were transfected with either wild-type or phosphatase-inactive mutant of PTEN. Then, cell death assay was performed to test whether loss of phosphatase activity of nuclear PTEN could actually rescue the NMDA-induced toxicity or make the neuronal injuries worse. The phosphatase dead mutant of PTEN used in my experiment is $PTEN_{C124S}$, which was previously proved to have lost both protein and lipid phosphatase activities (Liu et al., 2005). In addition, the $PTEN_{C124S}$ mutant is directed into nucleus upon expression by fusing with the classic nuclear localization signal (NLS) (Liu et al., 2005). Therefore, the $NLS\text{-}PTEN_{C124S}$ could function as a dominant negative competitor for endogenous PTEN, which will also accumulate into the nucleus upon NMDA stimulation. Further, both the wild-type and mutant PTEN are tagged with Green Fluorescent Protein (GFP) to enable

microscopic visualization. The detailed experiment design is as follows. Cultured hippocampal neurons (11-12DIV) were transfected with GFP alone (negative control), NLS-GFP-PTEN$_{WT}$ and NLS-GFP-PTEN$_{C124S}$, respectively; 48 hours after transfection, neurons were treated with NMDA (50μM) for 30min and allowed to recover for another 6 hours, followed by fixation and nuclei staining (Hoechst 33342). Only the neurons with GFP fluorescence (an indicator of successful transfection) were counted for cell death evaluation. Neurons with large and round nuclei are regarded as healthy, while those with condensed or fragmented nuclei are counted as apoptotic ones. The death rate was calculated by dividing apoptotic GFP-expressing neurons by total GFP-expressing neurons. The result showed a significantly reduced cell death rate in the PTEN$_{C124S}$ group than the PTEN$_{WT}$ group, suggesting that the phosphatase activity of nuclear PTEN is critically important for NMDA-induced neurotoxicity (n=12; One Way ANOVA, F=9.483, P<0.001; Fig. 5A and 5B). Therefore, I came to the conclusion that NMDA-induced PTEN nuclear translocation is a neurotoxic event rather than a self-defense mechanism.

It is worth pointing out that the dose of NMDA used on transfected neurons (50μM) is higher than that used in previous experiments (25μM). It is because the transfected neurons are more resistant to NMDA excitotoxicity than the non-transfected ones. A possible explanation is that neurons, which can hold up to the shock of transfection, are usually stronger and healthier than other neurons. Thus it takes more intense NMDA stimulation to initiate their apoptotic response. Also, it's worth mentioning that the NLS-GFP-PTEN$_{WT}$ expressing neurons, unexpectedly, didn't exhibit an increased cell death compared to those expressing GFP-alone (Fig. 5A and 5B). Many previous studies, using cancer cell lines, all showed that forced nuclear

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accumulation of PTEN can enhance apoptotic response (Ahn et al., 2004, Ahn et al., 2005, Gil et al., 2006). Therefore, at the beginning, I predicted that overexpression of NLS-GFP-PTEN$_{WT}$ in neurons should also be able to induce a significant apoptosis response. However, the final result turned out to be unexpected. It showed that NLS-GFP-PTEN$_{WT}$- and GFP- expressing neurons have similar cell death rates. There are several possible explanations for this unexpected result. First, neuronal death may be more complicated than that of cancer cell lines, and other proteins or signaling pathways may be required to trigger activation of PTEN in the nucleus. Thus, PTEN nuclear accumulation alone is necessary but insufficient to induce significant neuronal death. Second, maybe the endogenous PTEN is more than enough to mediate NMDA excitotoxicity. So the overexpressed nuclear PTEN cannot "top up" this already saturated cell death. Third, it's also possible that NLS-GFP-PTEN$_{WT}$ indeed caused significant neuronal death; however, those dying neurons quickly lost their GFP signals after expressing NLS-GFP-PTEN$_{WT}$ and failed to be picked up by fluorescence microscopy. Therefore, NLS-GFP-PTEN$_{WT}$ neurons ended up having the same death rate as the GFP-expressing neurons. Fourth, PTEN exists in a predominantly phosphorylated and inactive state in cytoplasm (Vazquez et al., 2000). It is activated by de-phosphorylation upon certain stimuli, such as ischemia. Here, the over-expressed PTEN might trigger some intrinsic protection mechanisms which inactivate the exogenous PTEN by increasing its phosphorylation. Hence, even though the over-expressed PTEN aggregates into the nucleus, it fails to promote excitotoxic neuronal death as it is in an inactive dephosphorylated status.

## 3.5 Lysine13 (K13), but not Lysine289 (K289), is critical for PTEN nuclear translocation in neurons

In order to support the above result gathered from transfected neurons, another approach was used to investigate whether PTEN nuclear translocation is indeed critically important for NMDA-induced excitotoxicity. First, I seek to develop a specific blocker that targets the process of PTEN nuclear translocation. Then, the inhibition efficacy of this blocker on NMDA-induced PTEN nuclear accumulation will be confirmed by using two methods: immunochemical staining and nuclei fractionation. Last, the effect of the blocker on NMDAR-mediated neurotoxicity will be tested by both nuclei staining and LDH assay.

The mechanisms of PTEN nuclear import have been extensively researched by many previous studies. Of those proposed mechanisms, the mono-ubiquitination-mediated PTEN nuclear accumulation may be the best characterized (Trotman et al., 2007, Wang et al., 2007). Two lysine residues, K13 and K289, are suggested to be the key sites for PTEN ubiquitination. While mono-ubiquitination on K13 and K289 promotes PTEN nuclear translocation, their poly-ubiquitination enhances proteasome-mediated degradation of PTEN. However, whether neuronal PTEN shares the same nuclear import mechanism with cell line PTEN remains elusive. Thus, I propose to investigate the relative importance of K13 and K289 in mediating neuronal PTEN subcellular localization by using single-point mutants on either site K13 or site K289 (Fig. 6A). Cultured neurons (11-12 DIV) were transfected with wide-type GFP-PTEN, GFP-PTEN$_{K13R}$ and GFP-PTEN$_{K289R}$, respectively. Forty-eight

hours after transfection, neurons were fixed and stained with nucleus dye Hoechst 33342. The relative amount of nuclear PTEN was then calculated as a ratio between fluorescent intensity of nuclear GFP-PTEN and that of cytoplasmic GFP-PTEN from the same cell. The result revealed that the over-expressed GFP-PTEN$_{K13R}$ mutant displayed a strong deficit in nuclear translocation, whereas the GFP-PTEN$_{K289R}$ mutant and the GFP-PTEN$_{WT}$ showed an equal distribution between nuclear and cytoplasmic compartments (n=33; One Way ANOVA, F=155.076, P<0.001; Fig. 6B and 6C), strongly suggesting that, unlike cancer cell lines, only the residue K13 (but not the residue K289) is critically important for PTEN nuclear translocation in neurons.

## 3.6 Interfering peptide Tat-K13 inhibits mono-ubiquitination of PTEN

Next, two interfering peptides were developed based on the above finding that mono-ubiquitination site K13, but not K289, is required for PTEN nuclear import in neurons. The peptide-K13 is designed to mimic the PTEN sequence that flanks K13 residue (KEIVSRN**K$_{13}$**RRYQED). It would function as a competitive inhibitor for PTEN mono-ubiquitination and hence block PTEN nuclear translocation. In contrast, the peptide-K289 flanks the K289 site (GPEETSE**K$_{289}$**VENGS) and may function as an inactive control (Fig. 7A). Both peptides were rendered membrane permeable ability by fusing with cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) Tat protein (GRKKRRQRRR) (Schwarze et al., 1999). I have a lot of confidence in the Tat fusion peptide strategy, because our lab

and many other groups have previously successfully used it for *in vitro* delivery of peptides into cultured cells, as well as *in vivo* delivery of peptides into neurons in the brain (Aarts et al., 2002, Borsello et al., 2003, Liu et al., 2007b, Taghibiglou et al., 2009). Following *in vitro* bath application to culture medium, the Tat-fused peptide can be detected in neurons as short as 10 min post-administration, reaches peak levels within 30 min, and remains detectable for several hours after washing peptide from the bath (Aarts et al., 2002). Similar to the *in vitro* application, the Tat-fused peptide can cross the blood-brain-barrier and becomes detectable in the brain within 30 min of peripheral intravenous (i.v.) injection, peaks in the next 1 hour or so, and remains at high levels for many hours post-injection (Aarts et al., 2002, Borsello et al., 2003, Liu et al., 2007b, Taghibiglou et al., 2009). Therefore, by fusing with Tat-sequence, peptides used in my current study could be efficiently delivered into neurons in both *in vitro* and *in vivo* systems, and function as a specific blocker for PTEN nuclear translocation.

The following step is to validate the rationale behind the ubiquitination-blocking peptide. First of all, I tested the effect of Tat-K13 on PTEN ubiquitination in COS-7 cell line system. Two plasmids, HA tagged-ubiquitin and GFP tagged-PTEN, were co-transfected into COS-7 cells, along with Tat-K13 (10μM) or Tat-K289 (10μM) treatment. At 36 hours post-transfection, two membrane permeable proteasome inhibitors, MG132 (25μM) and epoxomicin (1μM), were added to culture medium to inhibit degradation of ubiquitinated proteins. At 48 hours post-transfection, cells were collected with RIPA buffer containing protease inhibitor cocktail, proteasome inhibitors (25μM MG132 and 1μM epoxomicin), and de-ubiquitination inhibitor (0.5μM ubiquitin aldehyde). The lysates were then subjected to immunoprecipitation

(IP) with HA (Ubiquitin) antibody and subsequent western blotting with GFP (PTEN) antibody. The bands detected are ubiquitinated-PTEN, with the smallest one at around 90kD (82kD GFP-PTEN plus 8kD ubiquitin) as the mono-ubiquitinated-PTEN, and larger ones (>90kD) as the poly-ubiquitinated PTEN. The Tat-K13-treated group showed a significant decrease in mono-ubiquitinated-PTEN, whereas the Tat-K289-treated group displayed no difference from control condition (n=4; One Way ANOVA, F=6.196, P=0.024; Fig. 7B and 7C). This result further confirmed that the K13 residue is more crucial than the K289 residue in mediating PTEN mono-ubiquitination, and the Tat-K13 is an efficient and specific intervention to block mono-ubiquitination of PTEN. Next, I continued to test the effect of Tat-K13 on endogenous PTEN mono-ubiquitination in cultured neurons. Similarly, cortical neurons were treated with proteasome inhibitors, MG132 (25µM) and epoxomicin (1µM), 12 hours before collection. The lysate was then subjected to immunoprecipitation with ubiquitin antibody and subsequent western blotting with PTEN antibody. However, unfortunately, the signals of bands were so weak that they were barely detectable (Data not shown). Therefore, I failed to collect reliable data to demonstrate the effect of Tat-K13 on endogenous PTEN mono-ubiquitination. The possible reason could be due to inefficient ubiquitin antibody for immunoprecipitation, because re-probing membrane with the same ubiquitin antibody also revealed very weak IP signals. Hence, further experiment may be carried out when an efficient and reliable antibody for ubiquitin immunoprecipitation is found.

## 3.7 Interfering peptide Tat-K13 inhibits NMDA-induced PTEN nuclear translocation

The next step to validate the rationale underlying Tat-K13 peptide is to test its blocking efficiency for NMDA-induced PTEN nuclear translocation. To address this question, two approaches were used. The first approach is nuclei fractionation from peptide/NMDA-treated neurons. Cultured cortical neurons were first incubated with either Tat-K13 (10μM) or Tat-K289 (10μM) for 30 min, and then subjected to NMDA challenge (25μM, 1 hour) and recovery (6 hours). Next, the neuronal nuclei were isolated according to the protocol described before (Fig. 3A), followed by western blotting for PTEN antibody. As predicted, the NMDA-triggered nuclear accumulation of PTEN could be completely inhibited by pre-treating neurons with Tat-K13, but not Tat-K289 (n=4; One Way ANOVA, F=23.809, P<0.001; Fig. 8A and 8B). More, the cytoplasmic PTEN exhibited an obvious, though not significant, reduction after NMDA treatment, and this reduction can be largely restored by Tat-K13, but not Tat-K289 (n=4; One Way ANOVA, F=5.299, P=0.004; Fig. 8C). These results suggest that mono-ubiquitination on K13 residue is critically important for NMDA-induced nuclear import of PTEN, and Tat-K13 functions as an efficient and specific blocker for PTEN nuclear accumulation. To support the nuclei fractionation results, another approach, endogenous PTEN immunostaining, was used. First, cultured neurons (12-14 DIV) were pre-treated with either Tat-K13 (10μM) or Tat-K289 (10μM) for 30min. Next, neurons were challenged with 25μM NMDA for 1hour and returned to original medium for recovery for another 6 hours. Last, neurons were fixed and subjected to immunostaining by PTEN antibody as well as nuclei staining by Hoechst

33342. The relative nuclear PTEN level was expressed as a ratio between fluorescent intensity of nuclear PTEN and that of cytoplasmic PTEN. In consistence with the result of nuclei fractionation, pre-treating neurons with Tat-K13 successfully abolished the NMDA-induced PTEN nuclear translocation, while Tat-K289 had no effect (n=7 cover slips per group, 20 neurons per cover slip; One Way ANOVA, F=6.581, P<0.001; Fig. 8D and 8E). All together, the above results strongly suggest that mono-ubiquitination of residue K13 is a critical step leading to PTEN nuclear accumulation following NMDA challenge, and Tat-K13 is an effective intervention strategy for studying the role of PTEN nuclear import in NMDAR-mediated neurotoxicity.

## 3.8 Interfering peptide Tat-K13 inhibits NMDA-induced neuronal death

With the effective blocker (Tat-K13) for PTEN nuclear translocation, I am able to continue to investigate whether PTEN nuclear accumulation plays a causative role in NMDA-induced excitotoxicity. Two methods, nuclei staining and LDH assay, were used for this purpose. The nuclei staining experiment was performed in a similar setting as described above. Following 30 min pre-treatment with peptide (10μM), cultured hippocampal neurons were subjected to 1 hour NMDA challenge (25μM) and 6 hours recovery. Then, nuclei were stained with fluorescent dye Hoechst 33342 for cell death assay (Hardingham et al., 2002, Wang et al., 2004, Liu et al., 2007b). The cell death is expressed as a ratio between number of condensed/fragmented nuclei and that of total nuclei. The result revealed that NMDA treatment results in a

significant increase in the percentage of neurons exhibiting condense/fragmented nuclei, and this enhanced cell death can be completely abolished by pre-treating neurons with Tat-K13, but not Tat-K289 (n=8; One Way ANOVA, F=14.254, P<0.001; Fig. 9A and 9B). This result suggests a critical role of PTEN nuclear import in NMDAR-mediated excitotoxicity, as blocking PTEN nuclear translocation with interfering peptide Tat-K13 also blocks NMDA-induced neuronal death. Next, LDH assay was carried out to support the result of nuclei staining. LDH assay measures the amount of LDH released into culture medium by neurons. It represents the plasma membrane damage and thus the cell death degree. Briefly, cultured cortical neurons were subjected to a similar peptide/NMDA treatment as described above: 30min peptide pre-treatment (10µM) followed by 1 hour NMDA stimulation (25µM) and 24 hours recovery. The culture medium was then collected for LDH assay. The result showed that NMDA-induced neuronal death is almost completely blocked by Tat-K13 pre-treatment, whereas the control peptide Tat-K289 has no protective effect (n=28; One Way ANOVA on Ranks; H=130.923, P=<0.001; Fig. 9C). It proved that Tat-K13, by preventing PTEN nuclear translocation, protects neurons against NMDA-induced excitotoxicity. All together, the results of nuclei staining and LDH assay confirmed that PTEN nuclear translocation is a critical step leading to NMDA-induced neurotoxicity, and its specific blocker (Tat-K13) has a strong protective effect against NMDAR-mediated neuronal damages.

## 3.9 Ischemia promotes time-dependent PTEN nuclear translocation in an *in vivo* animal model

The above *in vitro* experiments strongly suggest that PTEN nuclear translocation plays a causative role in NMDA-induced neuronal damages, and its specific blocker, Tat-K13, represents a novel therapeutic intervention that can protect neurons against excitotoxic insults. As excessive activation of NMDARs is regarded as one of the major mechanisms for neuronal injuries during ischemic stroke (Lucas and Newhouse, 1957, Olney, 1969, Lipton, 1999, Arundine and Tymianski, 2004), I continued to investigate whether PTEN nuclear translocation also causatively leads to ischemic neuronal death, and whether its specific blocker (Tat-K13) can also prevent neuronal injuries following ischemic challenges in an *in vivo* animal model.

First of all, it is very important to select the appropriate *in vivo* animal model to test my hypothesis. As my current research aims to investigate the underlying mechanisms for human ischemic stroke, a type of stroke that accounts for about 87% of all stroke cases (Green and Shuaib, 2006), I therefore chose the transient model of middle cerebral artery occlusion (MCAO) as my *in vivo* model. The reason to choose MCA occlusion is because the majority cases of human ischemic stroke result from an occlusion of this artery (Garcia, 1984). Another reason to choose the transient type of MCAO is because most human strokes involve both occlusion of MCAO and spontaneous reperfusion (Ringelstein et al., 1992, Carmichael, 2005). Thus, the transient MCAO model, the most widely-used ischemia model in current stroke research, fits my purpose very well.

The next question is to decide the occlusion part of MCA: proximal or distal (Garcia, 1984). Proximal MCAO is achieved by inserting a suture into the internal carotid artery of rats and then advancing the suture cranially to block the MCA at its junction with Willis' Circle (Traystman, 2003, Carmichael, 2005). Proximal MCAO does not require craniotomy and can be performed at high throughput manner. However, Proximal MCAO generates relatively large ischemic infarcts which include striatum, overlying cortex and thalamus/hypothalamus. Ischemia in hypothalamus produces a hyperthermic response in rats, which is rarely seen in human stroke. Thus, it may not be the best model to mimic the type of human stroke I am interested in. In contrast, there are two distal MCAO models. Compared to the proximal model, both distal models produce much smaller ischemic infarcts, which include an ischemic core in the frontal and parietal cortex, and a slowly-evolving ischemic penumbra in adjacent temporal, frontal and cingulated cortex and a small region of dorsolateral striatum (Traystman, 2003, Carmichael, 2005). As no damage is done to the hypothalamus, hyperthermic response is avoided in both distal models. The first distal MCAO model was originally developed by Tamura et al., in which the MCA is occluded after it gives off lencticulostriate branches at the basal surface of the lateral part of the cerebral hemisphere (Tamura et al., 1981, Bederson et al., 1986). The second distal MCAO model is also called three-vessel occlusion model because it involves occlusion of the MCA on the surface of the brain above the rhinal fissure as well as bilateral common carotid arteries (Robinson et al., 1975, Chen et al., 1986). In general, the distal models, particularly the three-vessel occlusion model, are more favorable in most labs than the proximal model, because they generate a smaller and more reproducible infarct volume (Carmichael, 2005). Thus, the distal model will be

chosen in my current study. To occlude the distal MCA, several methods have been developed, including photochemical irradiation, intravenous injection of blood clots, or suture ligation. Photochemical irradiation involves irradiating several branches of MCA with laser beams following intravenous administration of the photosensitive dye Rose Bengal (Traystman, 2003). The advantage of photochemical irradiation is that it only requires a small craniotomy and keeps dura intact. However, the disadvantage of the model is that the photochemical reaction can result in microvascular injury and reperfusion is not an option. Also, occlusion of MCA could be achieved by directly injecting homologous blood clot fragments into MCA (Beech et al., 2001). Although this model closely resembles human ischemic stroke, its main disadvantage is that it produces infarcts of variable size and location, which makes the analysis of experimental outcome difficult. Last, MCA could be ligated with a square knot using nylone suture. This suture ligation is easy to perform, and, at the same time, avoids the above disadvantages of photochemical irradiation and blood clots injections (Chen et al., 1986). Therefore, after careful and thorough evaluation of all the MCAO models and my purposes, I choose to use the transient distal three-vessel MCAO achieved by suture ligation, because it involves a simpler surgical technique, produces consistent infarcts, and closely mimics human stroke.

The third step is to select the appropriate animal model to do the MCAO surgery on. Generally speaking, animals used in stroke research include large animals, such as dog, cat and monkey, as well as small animals, like rat and mouse (Traystman, 2003, Carmichael, 2005). Large animals have gyrencephalic brains, which are structurally and functionally closer to those of humans. However, experiments with large animals are very costly, labor intensive and involve public animal welfare concerns. Instead,

the small animals, even though their brains are lissencephalic, are much more affordable, and thus represent the most popular models for stroke studies. The most widely-used small animals are rat and mouse. They share many characteristics in modeling human stroke. However, unlike rat, mouse is extremely sensitive to ischemia duration, and generates infarct size of substantial variations. Previous studies showed that mouse MCAO could produce measures of infarct volume that range over a fivefold difference, even when the same strain, the same duration of ischemia, and the same survival period were used (Carmichael, 2005). Therefore, in my current study, the rat model is chosen as it is much more reproducible than the mouse model.

Following choosing the transient distal three-vessel MCAO on rat as my in vivo model, I continue to test my hypothesis that PTEN nuclear translocation may also represent a critical step leading to ischemic neuronal injuries.

First of all, I need to test whether ischemia insults can also trigger PTEN nuclear translocation in my current *in vivo* model. To address this question, a time course study was carried out. Adult male Sprague-Dawley (SD) rat were subjected to MCAO surgery, along with a careful monitoring of core body temperature and cerebral blood flow. Following a 90 min ischemia, the occlusion of MCA was released, and reperfusion was restored instantly. Rats were then allowed to recover for various time periods, ranging from 2 hours to 24 hours post-ictus. At the end of recovery, the brain tissues were collected from the area outlined by the dashed box, which includes both ischemic core and penumbra (Fig. 10A). Nuclei fractionation was then performed, followed by western blotting for nuclear PTEN. Very excitingly, the result

showed that ischemia insult triggers a time-dependent PTEN nuclear translocation, with the peak nuclear PTEN level reached at 12 hours post-stroke onset and baseline level restored at 24 hours (n=2, Fig. 10B). Given that the average hospital recruitment time for stroke patients is about 6 hours post-stroke onset, this time course study strongly suggests that PTEN nuclear import well qualifies as a potent target for stroke treatment development, as it permits a very wide therapeutic time window (up to 12 hours).

## 3.10 Interfering peptide Tat-K13 inhibits ischemia-induced PTEN nuclear translocation in a dose-dependent manner

In the previous *in vitro* experiments, Tat-K13 was proved to be a specific blocker for NMDA-induced PTEN nuclear import and neuronal damages. Therefore, I proposed to test whether Tat-K13 also functions as an efficient inhibitor for PTEN nuclear translocation in the current *in vivo* animal model. In order to find out the most effective dose of Tat-K13, A dose-response study was designed. Based on our lab's previous experience with other peptides (Liu et al., 2007b, Taghibiglou et al., 2009), three doses were selected: 0.1mg/kg, 1mg/kg, 10mg/kg, with the 10mg/kg expected to be the optimal concentration along with an acceptable toxicity. More, a post-treatment of Tat-K13 was used in order to mimic the real clinical situation, as most stroke patients arrive at hospital several hours (6 hours average) after the first symptom. Given that PTEN nuclear import peaks around 12 hours post-ictus and Tat-peptide injected through femoral vain can quickly reach a high concentration in the brain within 1 hour (Aarts et al., 2002, Liu et al., 2007b, Taghibiglou et al., 2009),

a post-treatment of 6 hours should be sufficient to achieve the optimal inhibition effect for PTEN nuclear translocation. Last, in the *in vivo* experiments, another control peptide Tat-K13R was introduced to further confirm the specificity of the working peptide Tat-K13. In Tat-K13R, the active lysine residue (K13) was switched to an arginine (R13). Therefore, Tat-K13R losses its ability to competitively inhibit PTEN mono-ubiquitination on the K13 site, and hence functions as an inactive control peptide for Tat-K13. In brief, adult SD rats were challenged with a 90 min ischemia, followed by femoral vain injection of Tat-K13 or Tat-K13R at 6 hours post-stroke onset. The rats were then sacrificed at 12 hours post-ictus for brain tissue collection and subsequent nuclei fractionation/western blotting. As predicted, Tat-K13 significantly suppressed the PTEN nuclear translocation in a dose-dependent manner, with 10mg/kg as the most effective dosage. On the other hand, the control peptide Tat-K13R failed to block PTEN nuclear accumulation even when administered at a high concentration of 10mg/kg (n=6; One Way ANOVA, F=21.128, P<0.001; Fig. 11A and 11B). It's also worth mentioning that Tat-K13 didn't evoke any obvious dose-limiting toxicity in the rats subjected to ischemia. It further proved that PTEN nuclear translocation is a pro-death event, and blocking this translocation does not interfere with the pro-survival activities of NMDARs.

## 3.11 Post-treatment of interfering peptide Tat-K13 reduces infarct volume of ischemic rat brain

After confirming that Tat-K13, when administered at a dose of 10mg/kg, can significantly suppress the ischemia-induced PTEN nuclear accumulation, I continue

to test whether Tat-K13 can protect neurons against ischemic injuries. In order to learn about the dynamic course of neuronal recovery following ischemia, I propose to monitor the morphological and functional change of brain by using magnetic resonance image (MRI), $[^{18}F]$ fluoro-2-deoxyglucose positron emission tomography (FDG-PET) and a battery of behavioral tests (Visnyei et al., 2006, Shyu et al., 2008). The reason to choose MRI and PET scanning is because they are both non-invasive techniques and allow us to keep monitoring the same animals during the entire course of their recovery.

In brief, adult SD rats were subjected to a 90 min ischemia insult, and then subdivided into five groups to receive different interfering peptides. The first bolus of Tat-K13 (10mg/kg) was administered at either 2 hours or 6 hours post-stroke onset, while all the controls, Tat-K13R (10mg/kg), Tat-K289 (10mg/kg) and saline, were given at 2 hours post-stroke onset. To achieve the optimal outcome, another two doses of peptides were administered on the 2nd day and the 3rd day, respectively (Fig. 12A). Next, the morphological change of brain infarction was assessed by MRI scanning at 7 days post-stroke onset. At this stage of stroke development, brain infarcts manifest as high intensity signals on the MRI images, and are quantified by subtracting the non-infarct volume of the ischemic hemisphere from the total volume of the contralateral hemisphere (Shyu et al., 2008). The MRI result showed that rats treated with Tat-K13, no matter at 2 hours or 6 hours post-ictus, can dramatically reduce brain infarct volumes, compared to saline- or control peptides-treated rats (n=10; One Way ANOVA on Ranks, H=38.639, P=<0.001; Fig. 12B and 12C). It suggests that Tat-K13, by blocking PTEN nuclear translocation, strongly protects neurons against ischemic injuries.

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## 3.12 Post-treatment of interfering peptide Tat-K13 reduces metabolism deficit of ischemic rat brain

To assess the effect of Tat-K13 on the brain glucose metabolic activities, FDG-PET scanning was also performed on the same groups of rats at Day 7 post-stroke onset (Fig. 13A). Rats were first given a bolus i.v. injection of $^{18}$F-FDG (an analogue of glucose), and then subjected to PET acquisition for tracer signals (Visnyei et al., 2006, Shyu et al., 2008). High PET signal means high regional FDG uptake, or, in other words, high regional brain activities. The percentage of deficit is expressed as 1-[ipsilateral hemisphere signal/contralateral hemisphere signal] X 100%. From the PET images, a striking recovery in FDG uptake over the ischemic hemisphere of the Tat-K13-treated rats was observed, while the control peptides-treated rats exhibited similar brain activities as the saline-treated rats (n=10; One Way ANOVA, F=246.269, P<0.001; Fig. 13B and 13C). The PET acquisition, together with the MRI result, strongly suggests that Tat-K13, by inhibiting PTEN nuclear accumulation, can rescue the neuronal damages caused by ischemic stroke, and the Tat-K13 may represent a novel stroke treatment with a particularly wide therapeutic time window of 6 hour post-stroke onset.

## 3.13 Post-treatment of interfering peptide Tat-K13 promotes the neurological recovery of rats subjected to ischemia

The above experiments proved that Tat-K13 has a strong neuroprotective effect over

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morphological recovery of brain infarction. However, the infarct volume does not always faithfully represent the functional recovery of neural circuits. Therefore, the following experiments were designed to investigate the effect of Tat-K13 on neurological recovery following ischemic insults. Sensorimotor circuit is often the major network that is impaired by ischemic stroke. Hence, a battery of behavioral assessments was used to test whether Tat-K13 could improve the sensorimotor performance of rats subjected to ischemia. Another concern is that previous studies showed that some neuroprotective interventions simply delay the ischemic injury process rather than provide a sustained neuroprotection (Valtysson et al., 1994, Corbett and Nurse, 1998, Gladstone et al., 2002). Therefore, in order to achieve high clinic feasibility, the neurological recovery was assessed over a relatively long-term period. Specifically, the behavioral tests were performed weekly up to 4 weeks to examine whether Tat-K13 actually offers a long-lasting neurological protection (Fig. 14A). Three typical sensorimotor deficit assessments were chosen for this purpose. They are elevated body swing test, vertical movement time and grip strength test (Dunnett et al., 1998, Chang et al., 2003, Shyu et al., 2004).

Elevated body swing test is a measurement of body asymmetric motor behavior (Chang et al., 2003, Shyu et al., 2008). It is performed by elevating the rat by its tail and counting the frequency of its swing behavior. Previous studies revealed that rats subjected to ischemia exhibit a significantly biased swing activity with the direction contralateral to the ischemic side. Therefore, the frequency of the biased swing activity may represent the degree of neurological deficits caused by ischemic stroke. Indeed, on the very first day following stroke surgery, all five groups of rats showed a significantly biased swing behavior toward the contralateral side of MCA occlusion.

However, in the following 4 weeks, rats treated with Tat-K13 (both 2 hours and 6 hours groups) exhibited a much faster recovery in the body swing behavior than those treated with saline and control peptides (n=10; One Way ANOVA; Fig. 14B). It suggests that Tat-K13 can not only reduce the brain infarcts but also repair the neural circuits damaged by stroke insult. Next, vertical movement of the rats was also monitored to evaluate recovery of the locomotor circuits injured by ischemic stroke (Chang et al., 2003, Shyu et al., 2008). Rats were placed in a computerized recording chamber at night, and their vertical movement time was automatically recorded over a 2-hour period. The result showed that Tat-K13-treated rats (both 2 hours and 6 hours groups) displayed a dramatic improvement in locomotor activities over the 4-week recovering period following MCAO surgery, while the saline- and control peptides- treated rats only showed a very slow recovery (n=10; One Way ANOVA; Fig. 14C). This result further proved that the locomotor circuits can be largely repaired by the Tat-K13 treatment, no matter given at 2 hours or 6 hours post-stroke onset. Last, another widely-used measurement, grip strength test, was also performed to further evaluate the recovery of motor function following MCAO surgery (Dunnett et al., 1998, Shyu et al., 2008). With the help of Grip-Strength Meter, the grip strength of each forelimb was measured separately, and the strength of the impaired forelimb (contralateral to stroke side) was calculated as a percentage of the intact forelimb's performance (ipsilateral to stroke side). Then, the improvement of motor function was expressed as a ratio between the grip strength measured at 28 days post-stroke and that measured at 1 day following surgery. The result showed that Tat-K13 treatment, but not the saline and control peptides treatments, significantly improved the grip strength performance of the impaired forelimb after the 4 weeks' recovery, suggesting a long-lasting therapeutic effect of Tat-K13 peptide

over the motor functions (n=10; One Way ANOVA on Ranks, H = 35.840, P=<0.001; Fig. 14D). All together, the above experiments strongly indicate that Tat-K13 can not only reduce brain infarct size but also repair the neurological circuits that are damaged by ischemic insults.

In all, my current study proposed a previously unknown death signaling cascade, PTEN nuclear translocation, which can be triggered by both NMDAR overactivation and ischemia insults. Its specific blocker, Tat-K13, exhibited a strong neuroprotective effect against the excitotoxic/ischemic injuries, along with many advantages over conventional stroke treatments. First, Tat-K13 provides a much wider therapeutic time window (6 hours) than the only clinically available anti-stroke drug tPA (3 hours); second, Tat-K13 did not evoke any obvious dose-limiting toxicity in rats subjected to ischemia; third, Tat-K13 can not only significantly reduce the brain infarcts but also repair the neurological deficits; fourth, Tat-K13 offers a long-lasting neuroprotection (4 weeks) rather than simply delays the ischemia progress. Thus, Tat-K13 presents a novel therapeutic intervention for ischemic stroke with exclusive advantages over other previous stroke therapies.

**Figure 2: NMDA stimulation induces excitotoxic neuronal death**

(A) NMDA stimulation (bath application of 25µM NMDA to cultured neurons for 1 hour followed by 6 hours recovery) triggers a dramatic increase in the percentage of neurons displaying condensed/fragmented nuclei. Scale bar represents 20µm. (B) The same NMDA stimulation results in a significantly enhanced LDH release of neurons into culture media, an indicator for plasma membrane disintegration; and this enhanced neuronal death can be completely abolished by the specific NMDAR antagonist AP5 (n=7). Error bars represent Standard Deviation, **$P < 0.01$.
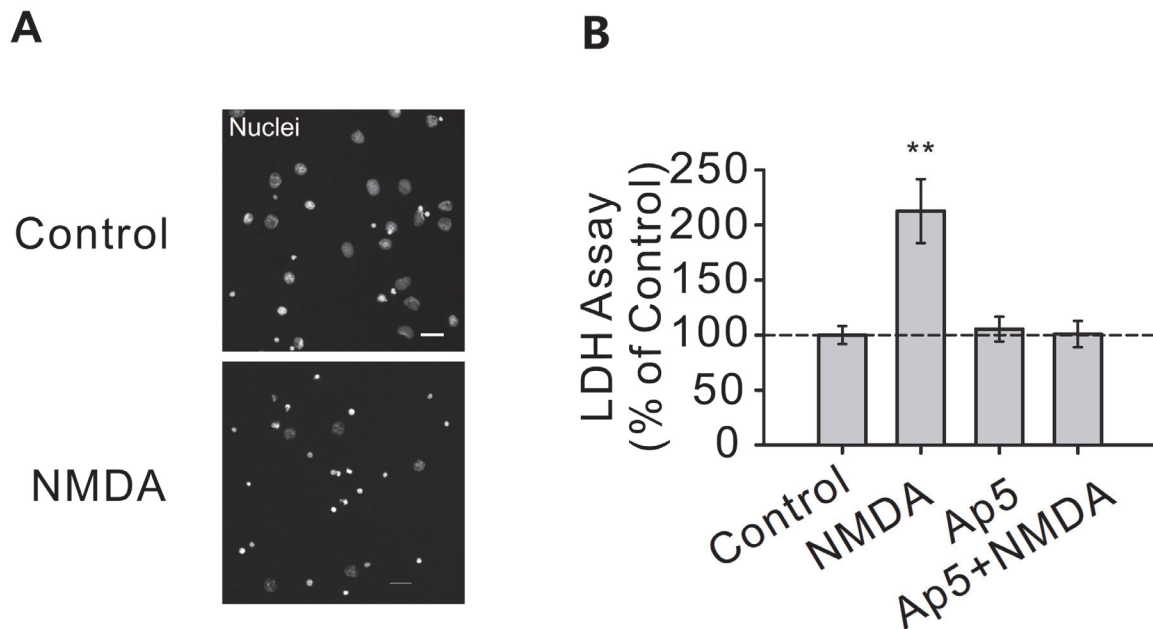
**Figure 3: NMDA stimulation enhances PTEN nuclear translocation in cultured neurons**

(A) Nuclei fractionation method was used to study the change of subcellular localization of PTEN post-NMDA stimulation (25µM for 1 hour followed by 6 hours recovery). The purity of different cellular compartments was confirmed by probing each fraction for corresponding subcellular marker proteins, with insulin receptor β and heat shock protein 90 (Hsp90) as the cytoplasmic markers, and histone deacetylase 1 (HDAC1) and TATA-binding protein (TBP) as the nuclear markers. (B) NMDA stimulation triggers significant PTEN nuclear accumulation, which can be completely abolished by the specific NMDAR antagonist AP5 (n=4). (C) NMDA stimulation leads to a reduction in cytoplasmic PTEN level, which can also be restored by AP5 (n=4). (D) Immunostaining method was also used to study the subcellular localization of PTEN. The result showed a significant increase in nuclear PTEN level following the same NMDA stimulation as described above. Scale bar represents 20µm. (E) Pretreatment of NR2BR-specific antagonist Ro25-6981(0.5µM) can block NMDA-induced PTEN nuclear translocation, while NR2AR-specific antagonist NVP-AAM077 (0.4µM) is ineffective, suggesting PTEN nuclear translocation is mediated by NR2BRs, but not NR2ARs. Error bars represent Standard Deviation, **$P < 0.01$. *Experiment in Fig.3E was performed by my colleague Dr. Changiz Taghibiglou.*

**A**

|  | Nuclear Fraction | Cytosolic Fraction | Lysate |
|---|---|---|---|
| Ap5 | − − + + | − − + + | |
| NMDA | − + − + | − + − + | |
| PTEN | | | |
| Insulin Rβ | | | |
| Hsp90 | | | |
| HDAC1 | | | |
| TBP | | | |

**B**
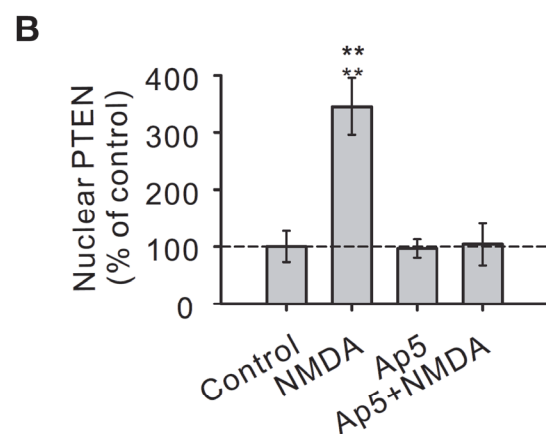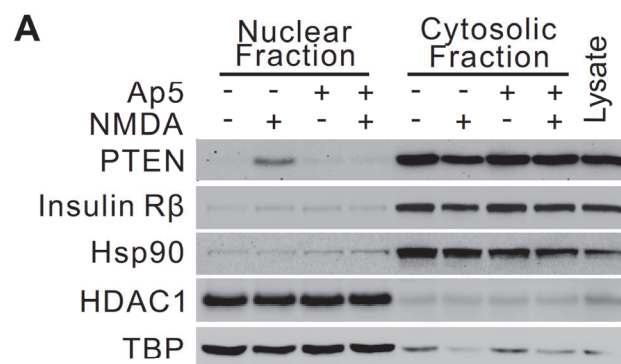
**C**

**D**  Control    NMDA

**E**
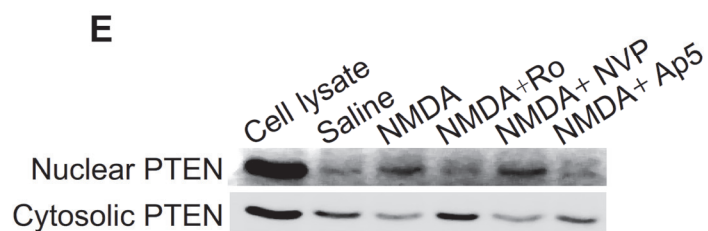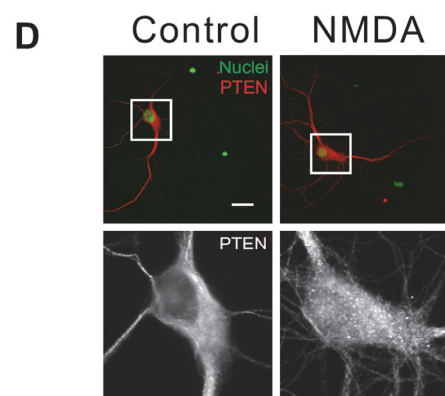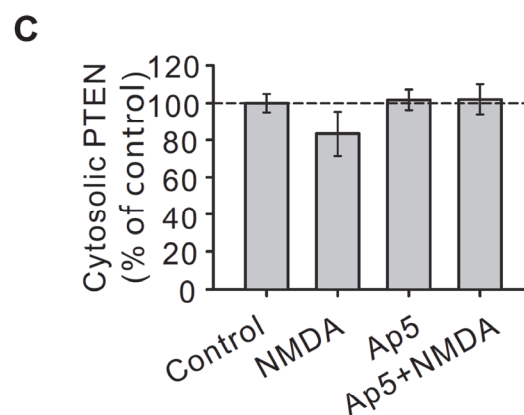
**Figure 4: NMDA stimulation enhances PTEN nuclear translocation in a time-dependent manner**

(A, B) Following NMDA stimulation, PTEN translocates into the nucleus in a time-dependent manner. It initiates immediately after NMDA treatment, peaks at around 6-9 hours post-ictus and drops back at 24 hours later (n=2). (C) PTEN nuclear translocation is coupled with a time-dependent increase of LDH release, an indicator for plasma membrane disintegration (n=2). Error bars represent Standard Deviation.
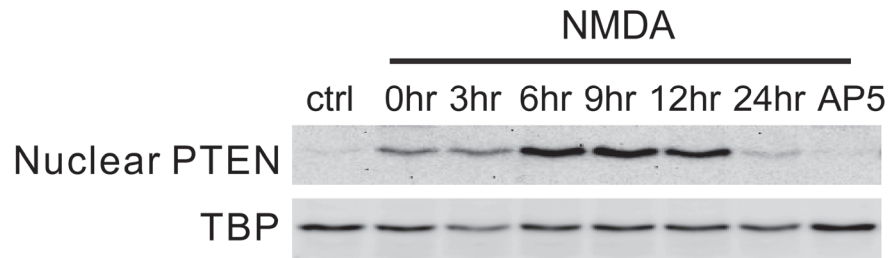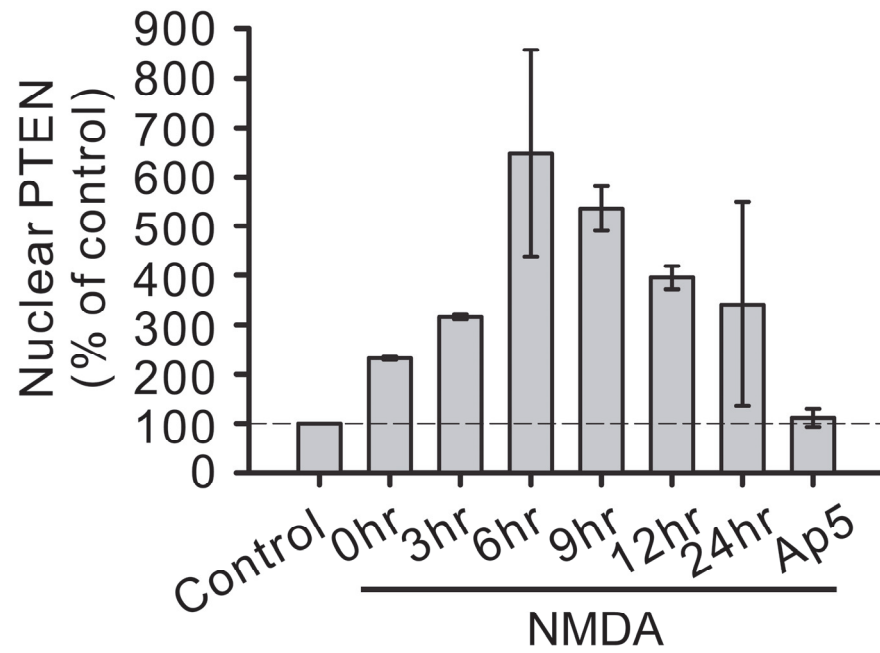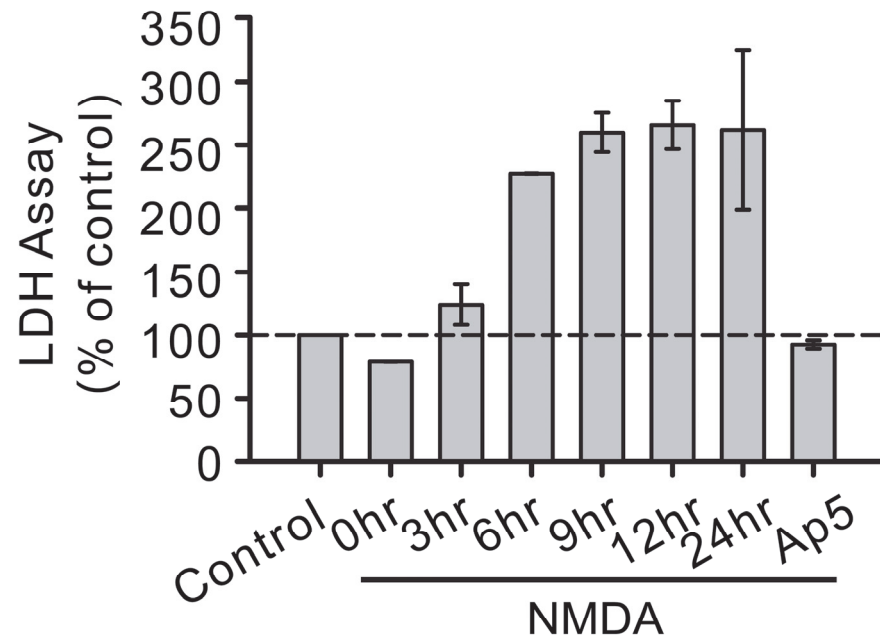
**Figure 5: PTEN nuclear translocation following NMDA stimulation is a pro-death process rather than a self-defense mechanism**

(A, B) Overexpression of phosphatase inactive mutant $PTEN_{C124S}$ rescues, rather than enhances, the NMDA-induced neuronal death, suggesting that PTEN nuclear translocation is a pro-death process but not a self-defense mechanism (n=12). Scale bar represents 20μm. Error bars represent Standard Deviation, **$P < 0.01$.
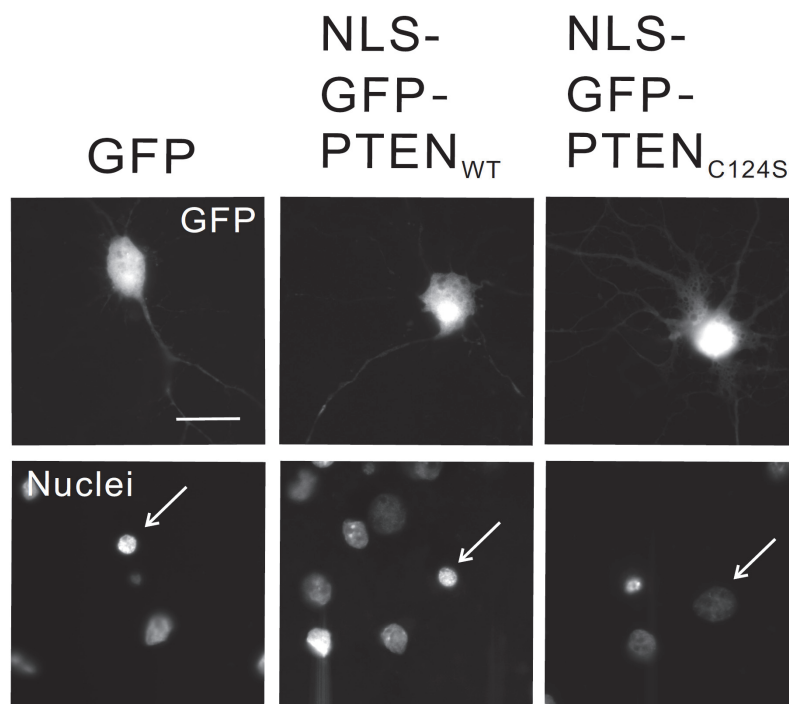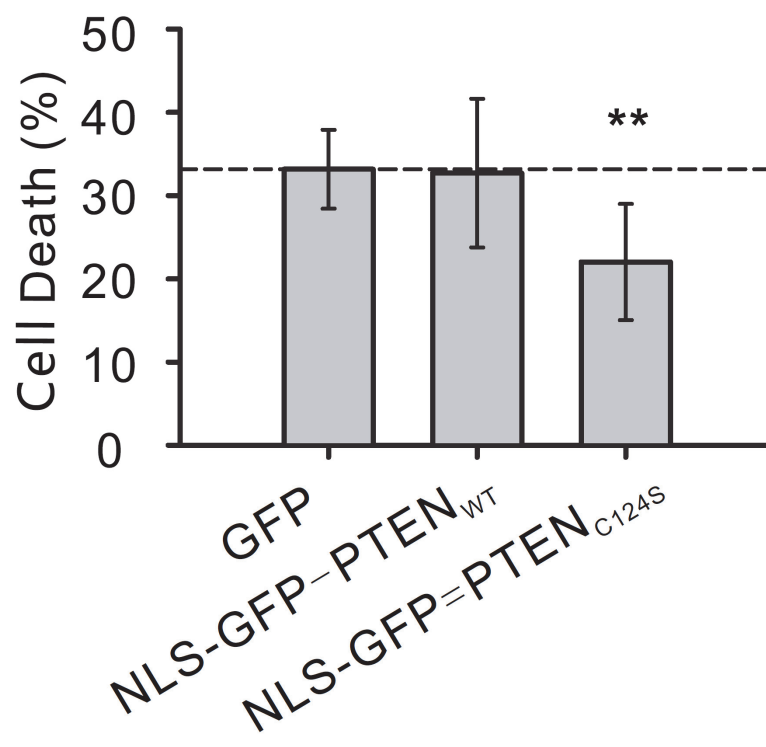
**A**

GFP | NLS-GFP-PTEN_{WT} | NLS-GFP-PTEN_{C124S}

GFP

Nuclei

**B**



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**Figure 6: Lysine13 (K13), but not Lysine289 (K289), is critical for PTEN nuclear translocation in neurons**

(A) Previous cancer studies revealed that nuclear translocation of PTEN depends on mono-ubiquitination on two lysine residues: K13 and K289. Mutation on either residue can result in abolishment of PTEN nuclear translocation ability and its tumor suppressive functions. (B, C) Unlike the mechanism in cancer cell lines, only PTEN$_{K13R}$ mutant (but not PTEN$_{K289R}$ mutant) shows strong deficit in nuclear translocation ability in neurons (n=33). Scale bar represents 20µm. Error bars represent Standard Deviation, **$P < 0.01$.
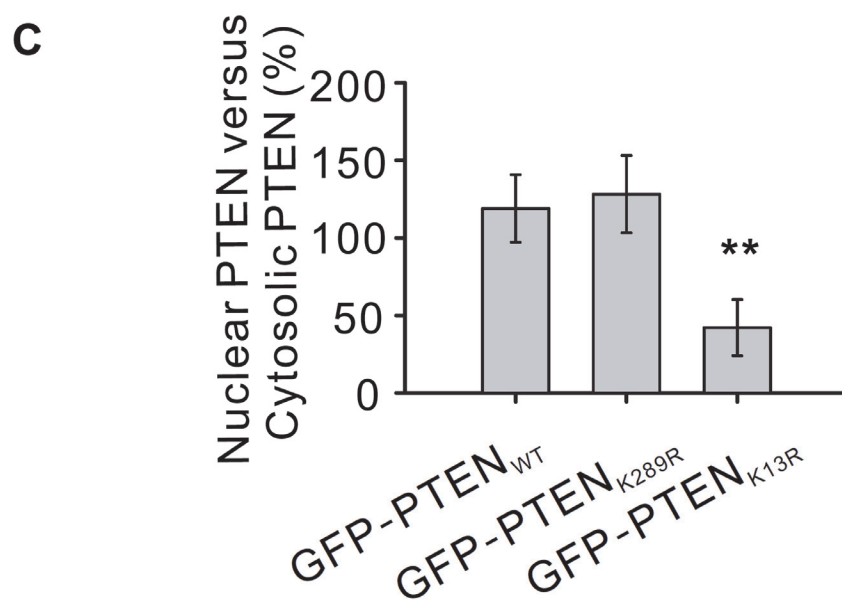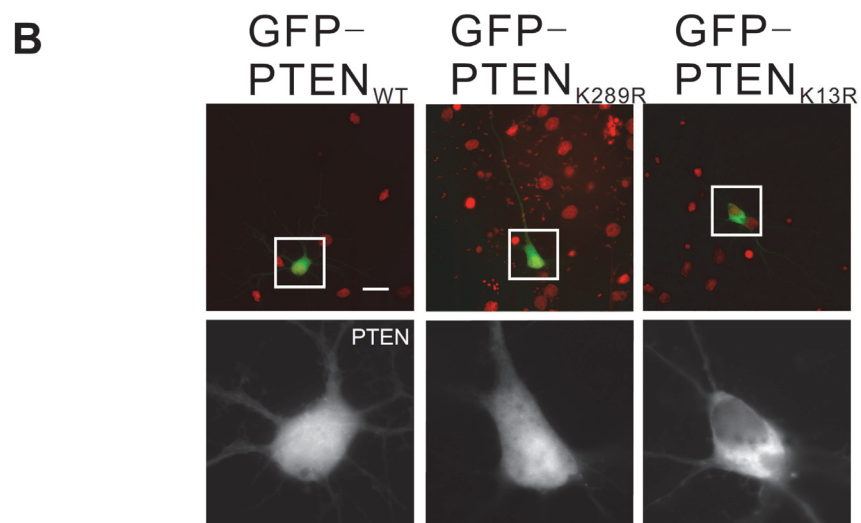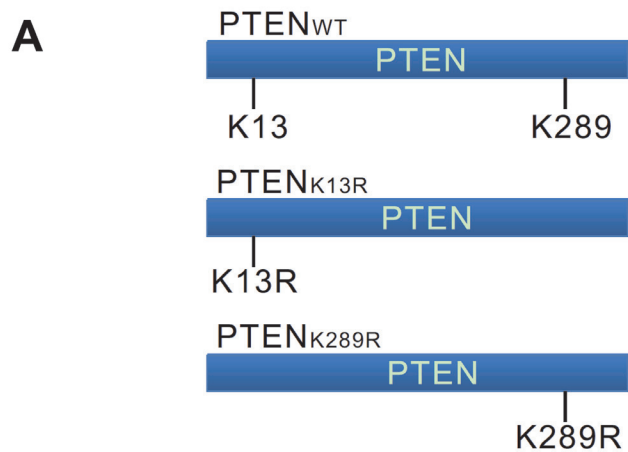
**A**

PTEN_WT

PTEN

K13    K289

PTEN_K13R

PTEN

K13R

PTEN_K289R

PTEN

K289R

**B**

GFP–PTEN_WT    GFP–PTEN_K289R    GFP–PTEN_K13R

PTEN

**C**

**Figure 7: Interfering peptide Tat-K13 inhibits mono-ubiquitination of PTEN**

(A) Interfering peptide-K13 is designed to mimic the PTEN sequence that flanks K13 residue (KEIVSRN**K₁₃**RRYQED), functioning as a competitive inhibitor for PTEN mono-ubiquitination. In contrast, interfering peptide-K289 flanks the K289 residue (GPEETSEK$_{289}$VENGS) and functions as an inactive control. Both peptides were rendered membrane permeable ability by fusing with cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) Tat protein (GRKKRRQRRR). (B, C) Pretreatment of interfering peptide Tat-K13 (10µM) leads to a significant reduction in mono-ubiquitination of PTEN, while the control peptide Tat-K289 (10µM) is ineffective (n=4). Error bars represent Standard Deviation, *$P < 0.05$.
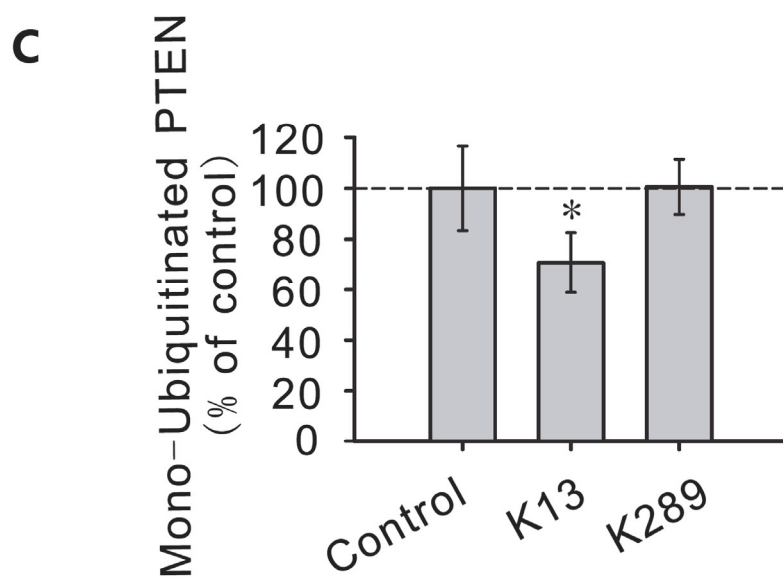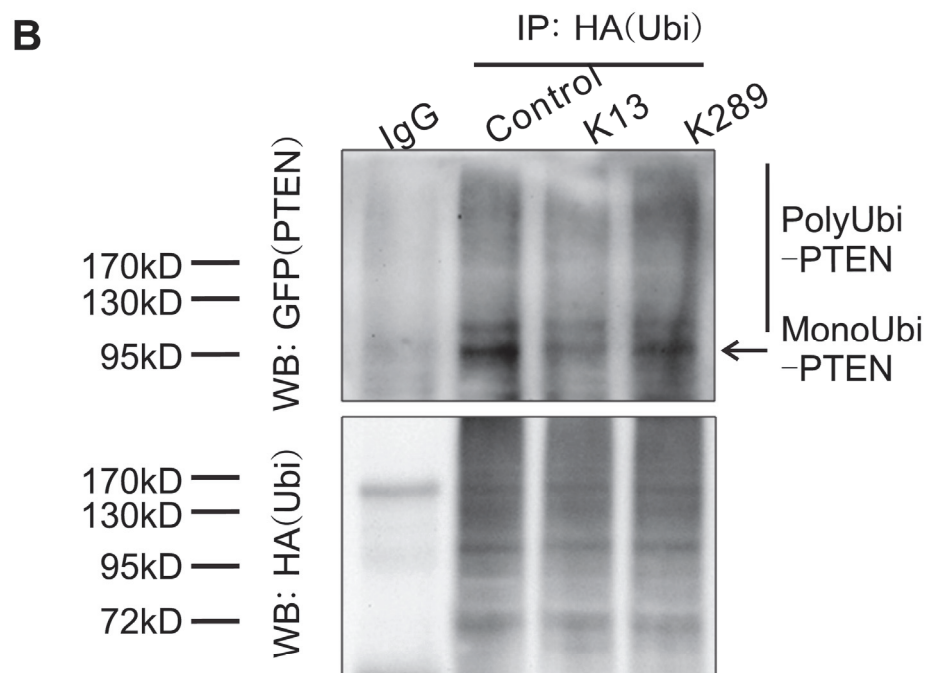
**A**

K13

PTEN

KEIVSRN**K13**RRYQED-TAT

K289

PTEN

TAT-GPEETSE**K289**VENGS

**B**

IP: HA(Ubi)

IgG    Control    K13    K289

WB: GFP(PTEN)

170kD —
130kD —

95kD —

PolyUbi
-PTEN

MonoUbi
-PTEN

WB: HA(Ubi)

170kD —
130kD —
95kD —
72kD —

**C**

Mono−Ubiquitinated PTEN
(% of control)

120
100
80
60
40
20
0

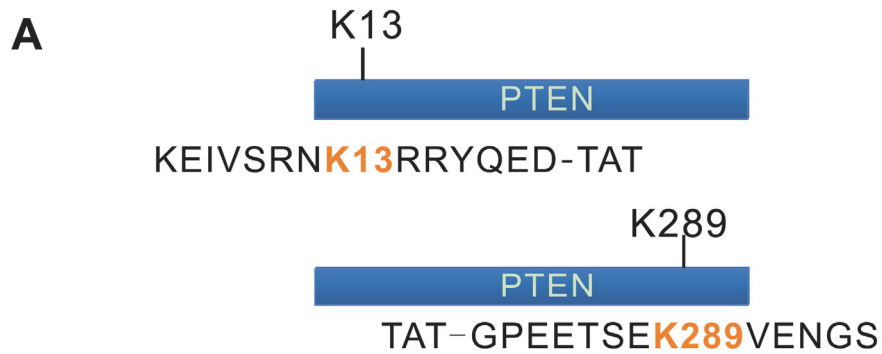Control    K13    K289

*

120

**Figure 8: Interfering peptide Tat-K13 inhibits NMDA-induced PTEN nuclear translocation**

(A, B) Result of nuclei fractionation revealed that pretreatment of interfering peptide Tat-K13 (10µM) abolishes NMDA-induced PTEN nuclear translocation, while the control peptide Tat-K289 (10µM) has no effect (n=4), suggesting Tat-K13 is an effective and specific inhibitor for PTEN nuclear translocation. (C) On the other hand, the NMDA-induced reduction in cytoplasmic PTEN level could be largely restored by pretreatment with Tat-K13, but not Tat-K289 (n=4). (D, E) Result of immunostaining also confirmed that pretreatment of Tat-K13 completely inhibits NMDA-induced PTEN nuclear translocation, while Tat-K289 fails to do so (n=7), confirming Tat-K13 as an effective and specific intervention strategy for PTEN nuclear translocation. Scale bar represents 20µm. Error bars represent Standard Deviation, *$P < 0.05$, **$P < 0.01$.
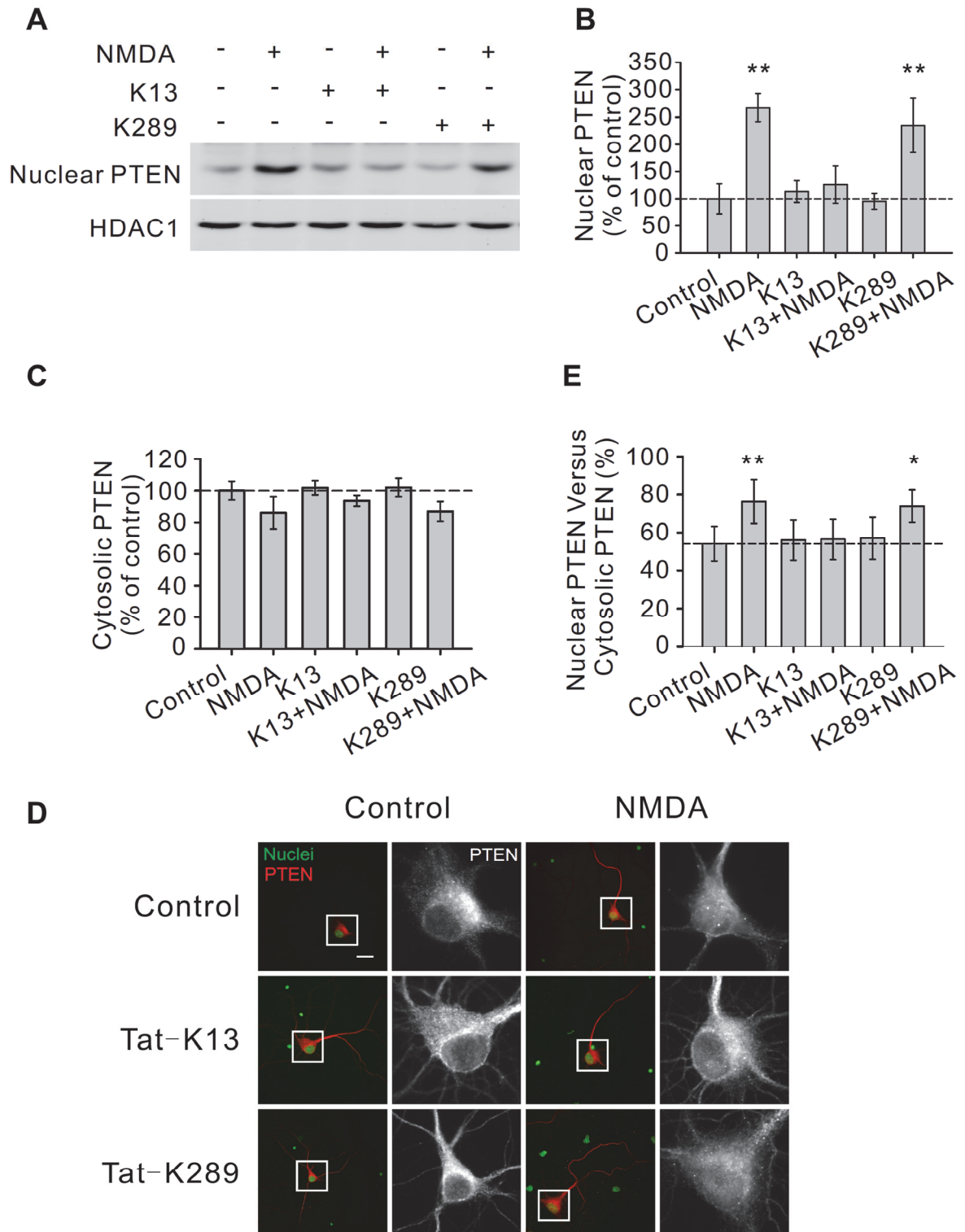
**Figure 9: Interfering peptide Tat-K13 inhibits NMDA-induced neuronal death**

(A, B) The NMDA-enhanced nuclei condensation/fragmentation can be completely blocked by pretreatment of interfering peptide Tat-K13 (10μM), but not the control peptide Tat-K289 (10μM), suggesting PTEN nuclear translocation is causatively contributive to NMDA-induced excitotoxicity in cultured neurons (n=8). Scale bar represents 20μm. (C) In addition, the increased LDH release triggered by NMDA stimulation can be abolished by pretreatment of Tat-K13, but not Tat-K289, further confirming the causative role of PTEN nuclear translocation in NMDAR-mediated neuronal injuries (n=28). Error bars represent Standard Deviation, **$P < 0.01$.
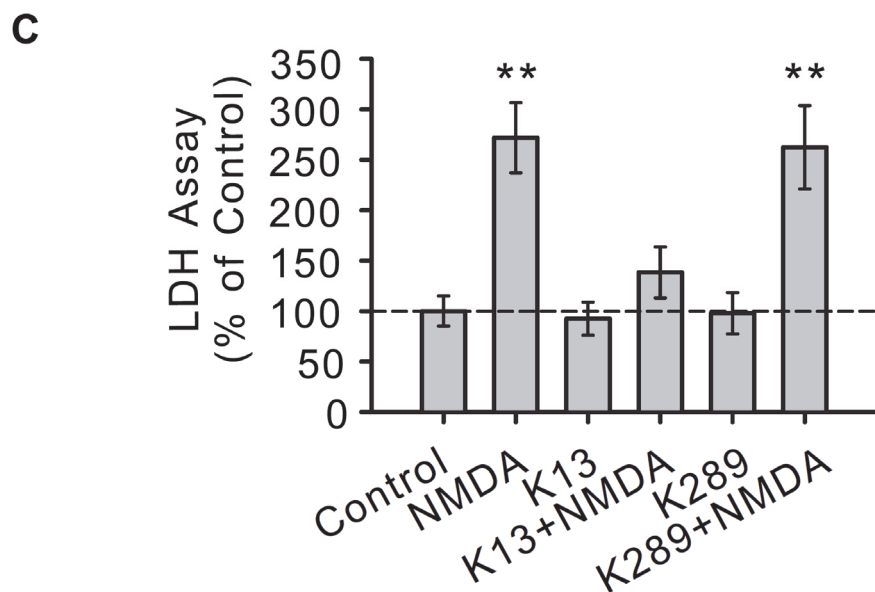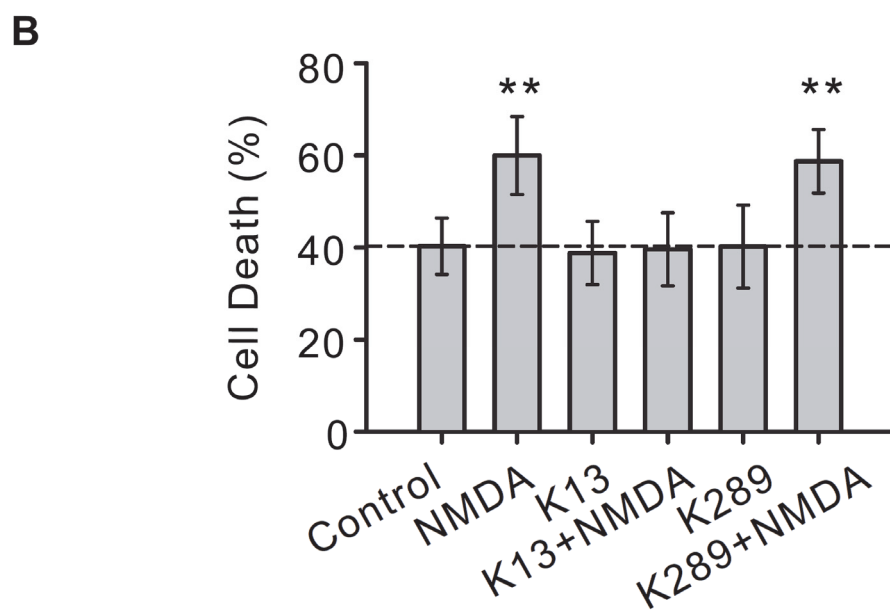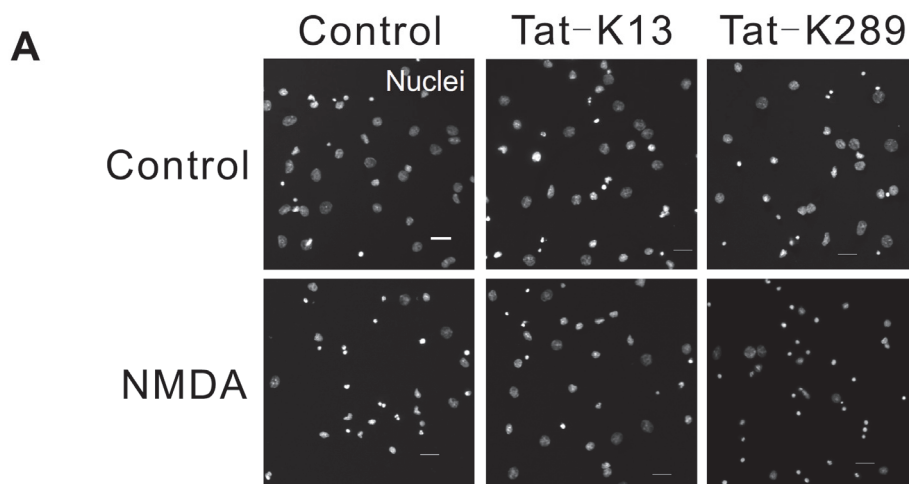
**A**

Control  Tat−K13  Tat−K289

Control

Nuclei

NMDA

**B**

Cell Death (%)

80

60  **

40

20

0

Control  NMDA  K13  K13+NMDA  K289  K289+NMDA

**C**

LDH Assay
(% of Control)

350
300
250  **
200
150
100
50
0

Control  NMDA  K13  K13+NMDA  K289  K289+NMDA

124

**Figure 10: Ischemia promotes time-dependent PTEN nuclear translocation in an *in vivo* animal model**

(A) Rats were subjected to 90min MCAO surgery and allowed to recover for different periods of time, ranging from 2 hours to 24 hours post-stroke onset. Next, brain tissues were collected from the area outlined by the dashed box, which includes both ischemic core and penumbra (MRI image taken at Day 7 post ischemia). Nuclei fractionation was then performed, followed by western blotting for nuclear PTEN. (B) The result showed a time-dependent PTEN nuclear translocation, which peaks around 12 hours and returns to baseline level 24 hours post-stroke onset (n=2), suggesting a therapeutic time window that may be much wider than that of the only clinically available anti-stroke drug tPA (3 hour post-ictus). *The MCAO surgery was performed by our collaboration lab (Woei-Cherng Shyu Lab), while the nuclear fractionation and western blotting were done by me.*

**A**

**B**

Cytosolic

Nuclear fractions
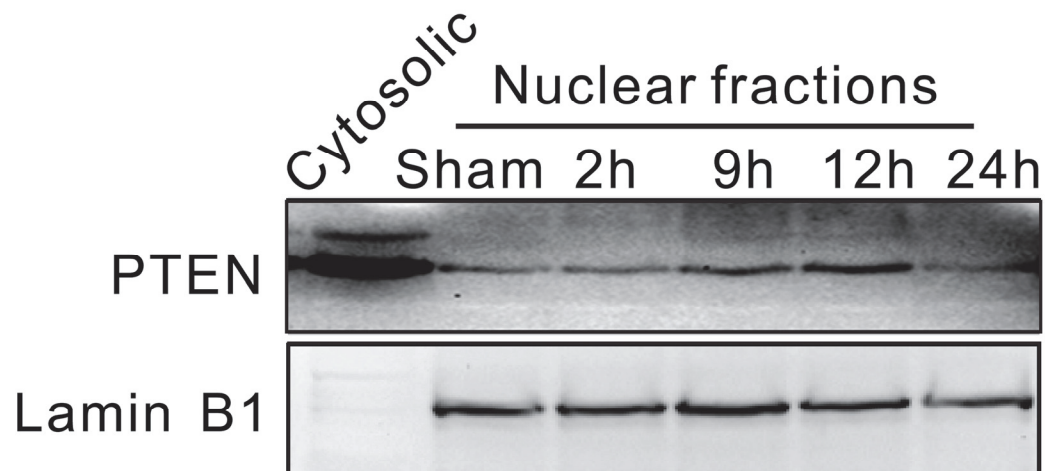
Sham 2h 9h 12h 24h

PTEN

Lamin B1
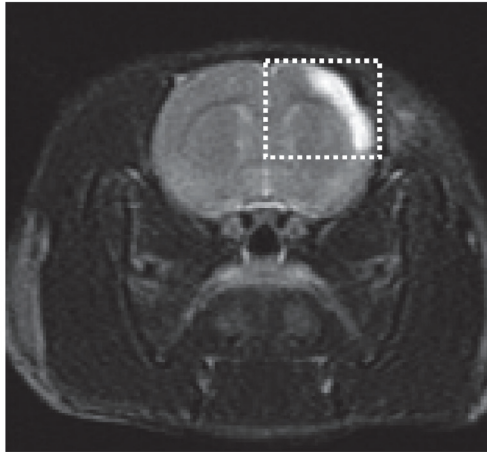
**Figure 11: Interfering peptide Tat-K13 inhibits ischemia-induced PTEN nuclear translocation in a dose-dependent manner**

(A, B)  Rats were subjected to 90min MCAO, and administered with Tat-K13, the single point mutant Tat-K13R or saline at 6 hours post-stroke onset. At 12 hour post-ictus, brain tissues were collected for nuclei fractionation and western blotting for nuclear PTEN. The result showed that interfering peptide Tat-K13 inhibits the ischemia-induced PTEN nuclear translocation in a dose-dependent manner, with 10mg/kg as the most effective dose, whereas the single point mutant Tat-K13R (10mg/kg) have no effect on PTEN nuclear translocation (n=6). Error bars represent Standard Deviation, $**P < 0.01$. *The MCAO surgery and peptide injection were performed by our collaboration lab (Woei-Cherng Shyu Lab), while the nuclear fractionation and western blotting were done by me*.

**A**

Tat-K13 (mg/kg)

| 10 | 1 | 0.1 | Tat-K13R (10mg/kg) | Saline Control |

Nuclear PTEN
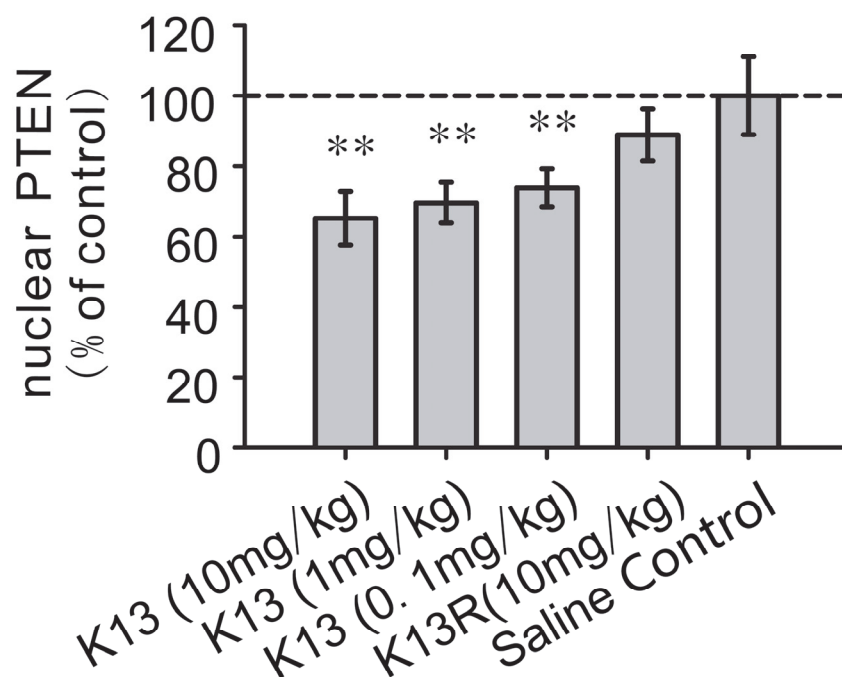
Lamin B1

Nuclear fractions
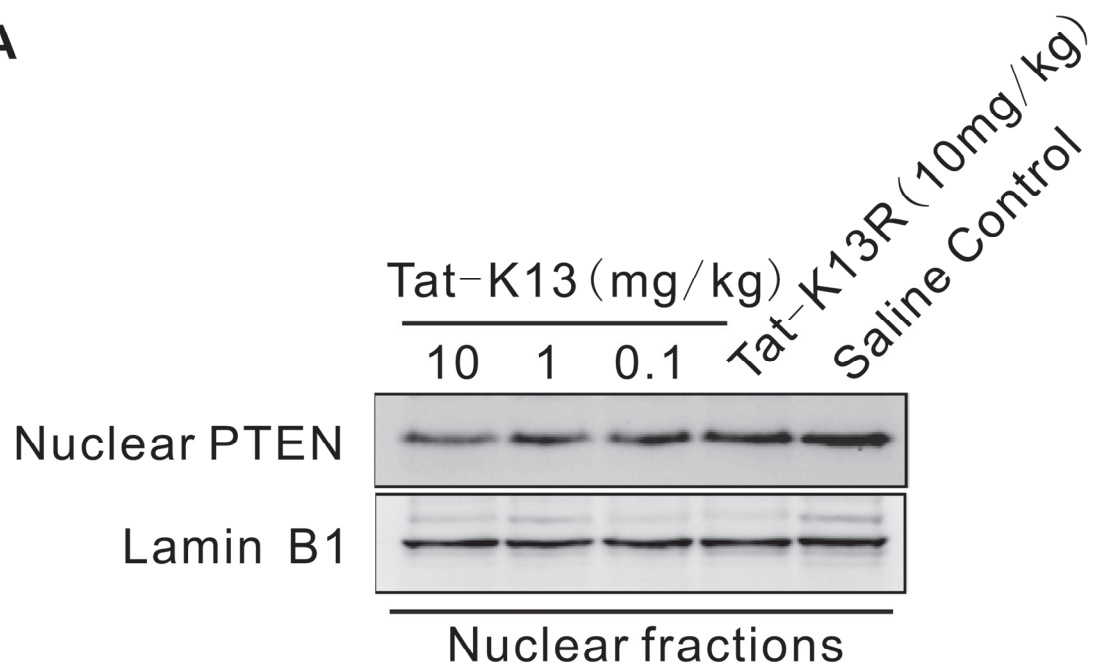
**B**

**Figure 12: Post-treatment of interfering peptide Tat-K13 reduces infarct volume of ischemic rat brain**

(A) Rats were subjected to a 90 min MCAO insult, and then subdivided into five groups to receive different interfering peptides. The first bolus of Tat-K13 (10mg/kg) was administered at either 2 hours or 6 hours post-stroke onset, while all the controls, Tat-K289 (10mg/kg), Tat-K13R (10mg/kg) and saline, were given at 2 hours post-stroke onset. To achieve the optimal outcome, rats received another two doses of peptide injection on the second and the third day, respectively. (B, C) At Day 7 post-stroke onset, brain infarction was measured by MRI scanning. The result showed that post-treatment of interfering peptide Tat-K13, no matter at 2 hours or 6 hours post-ictus, dramatically reduces the brain infarct volume, compared to the control peptides- and saline- treated groups (n=10). Error bars represent Standard Deviation, **$P < 0.01$. *Data from this figure were generated by our collaboration lab (Woei-Cherng Shyu Lab).*

**A**

| Day 1 | Day 1-3 | Day 7 |
| MCAO | Peptide 2hr or 6hr post stroke onset | MRI |

**B**

Tat-K13 (2 hrs)  Tat-K13 (6 hrs)  Tat-K13R (2 hrs)  Tat-K289 (2 hrs)  Saline (2 hrs)
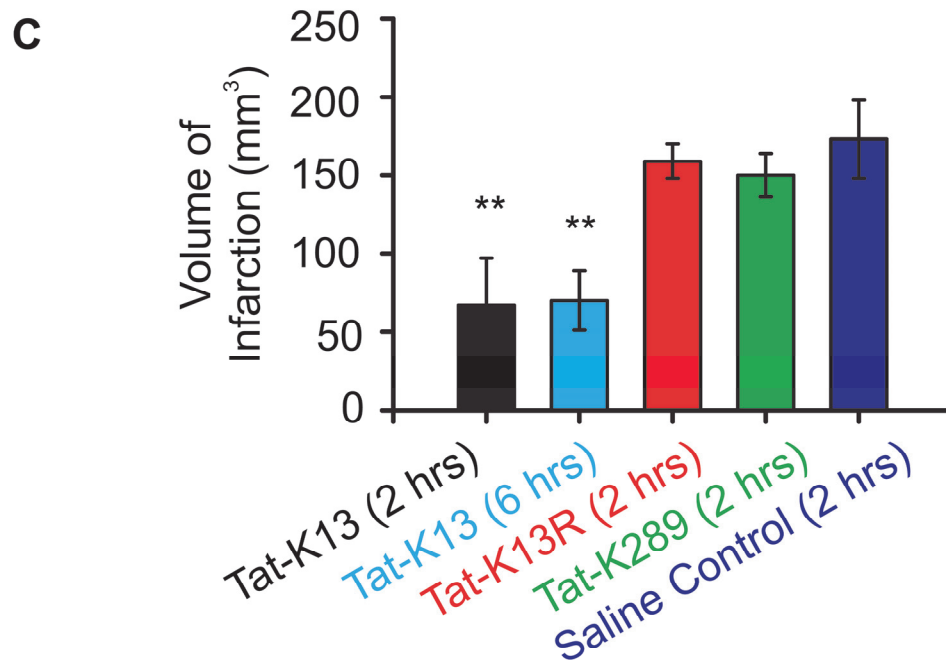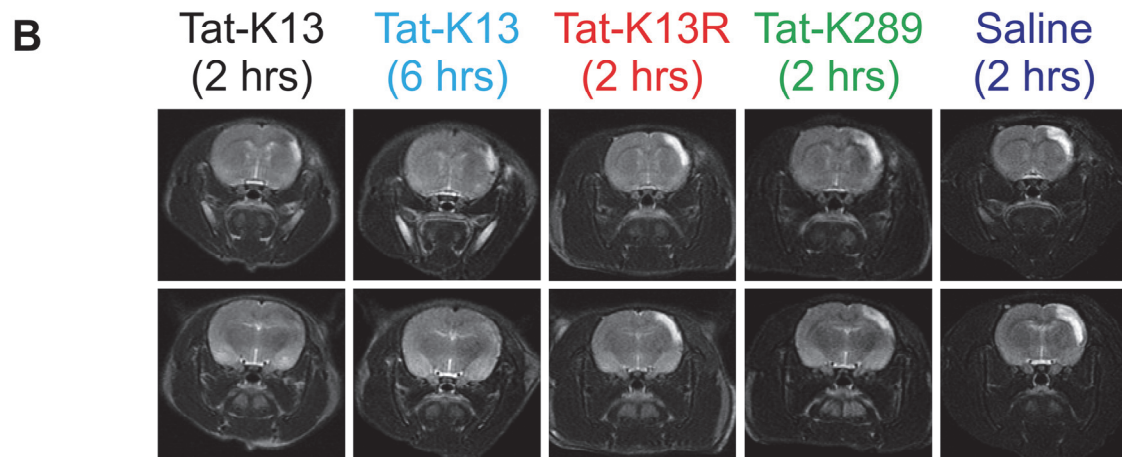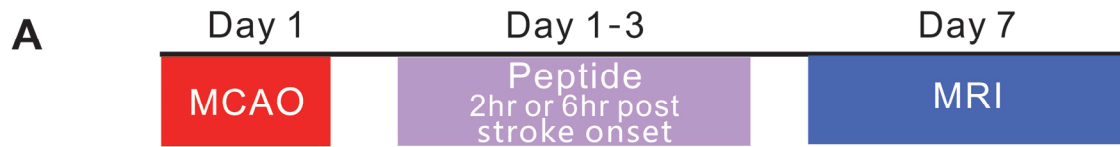
**C**

**Figure 13: Post-treatment of interfering peptide Tat-K13 reduces metabolism deficit of ischemic rat brain**

(A) Also at Day 7 post-stroke onset, the same groups of rats from the above experiment were given a bolus i.v. injection of $^{18}$F-FDG (an analogue of glucose), and then subjected to PET acquisition for tracer signals in the brain. High $^{18}$F-FDG uptake represents high regional glucose metabolic activities. (B, C) The result showed that post-treatment of Tat-K13 (both 2 hours and 6 hours groups) can largely rescue the brain glucose metabolism deficit caused by ischemic insults, whereas the control peptides, Tat-K289 and Tat-K13R, are as ineffective as the saline treatment (n=10). The color scale represents relative levels of glucose metabolism, from high (red) to low (blue). Error bars represent Standard Deviation, **$P < 0.01$. *Data from this figure were generated by our collaboration lab (Woei-Cherng Shyu Lab).*
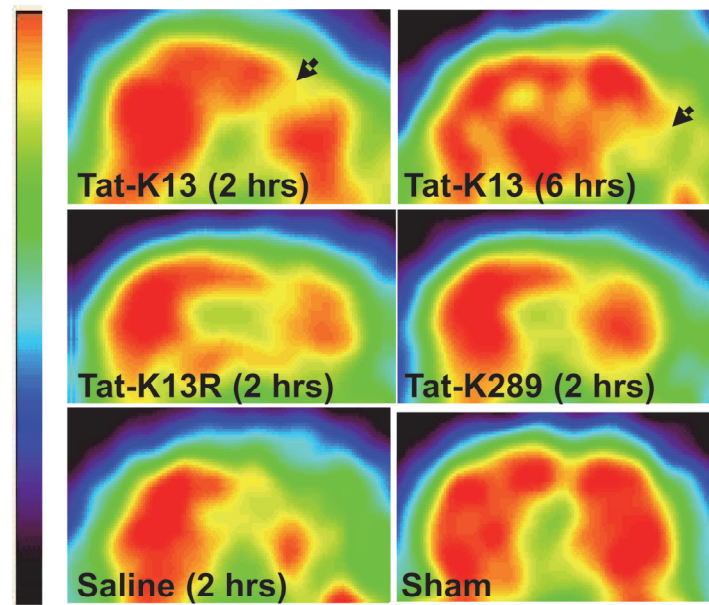
**A**
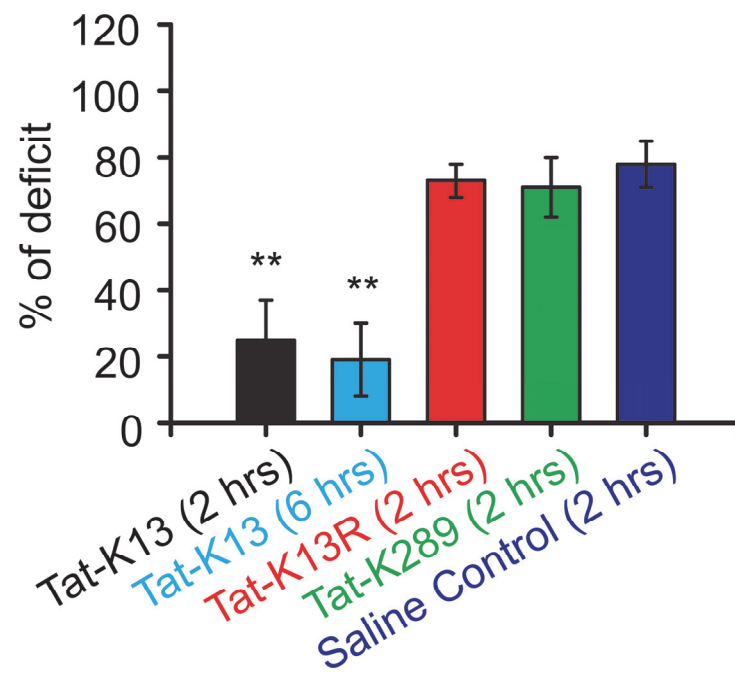
| Day 1 | Day 1-3 | Day 7 |
|:---:|:---:|:---:|
| MCAO | Peptide<br>2hr or 6hr post<br>stroke onset | FDG-PET |

**B**



**C**

**Figure 14: Post-treatment of interfering peptide Tat-K13 promotes the neurological recovery of rats subjected to ischemia**

(A) The same groups of rats from the above experiments were further subjected to a battery of behavioral tests to assess their neurological recoveries over a 4-week period of time following ischemic insults. (B) Rats treated with Tat-K13, either at 2 hours or 6 hours post-stroke onset, exhibited a significantly enhanced improvement in elevated body swing test, whereas rats treated with control peptides and saline only showed a very slow recovery (n=10). (C) Similarly, the Tat-K13-treated rats (both 2 hours and 6 hours groups) showed a much faster recovery in vertical movement test compared to the control peptides- and saline-treated rats (n=10). (D) Again, the grip strength test showed that post-treatment of Tat-K13, but not the control peptides or saline, can significantly improve the grip strength performance of the impaired forelimb following the 4 week recovery period (n=10). Error bars represent Standard Deviation, *$P < 0.05$, **$P < 0.01$. *Data from this figure were generated by our collaboration lab (Woei-Cherng Shyu Lab).*
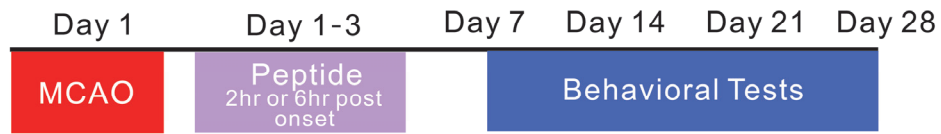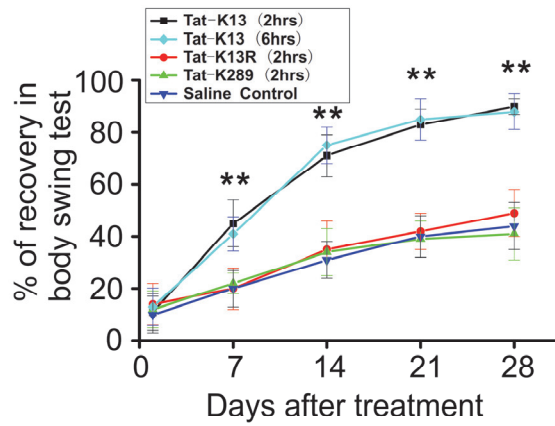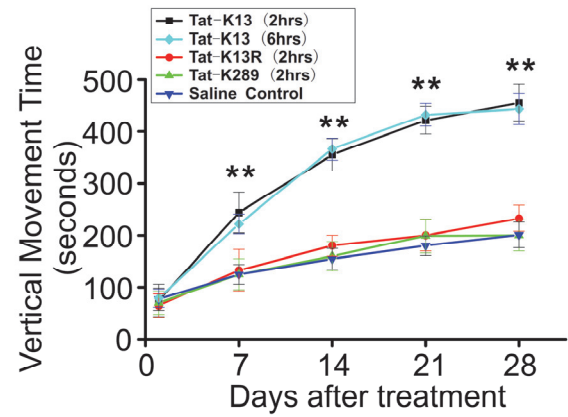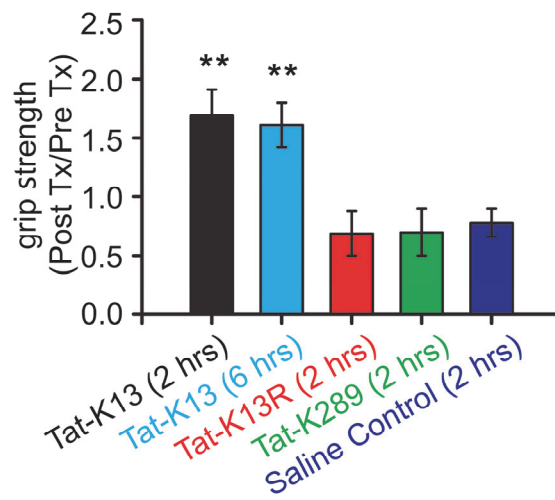
# 4 CONCLUDING REMARKS

## 4.1 Research summary

My current study revealed a previously unknown death signaling cascade, PTEN nuclear translocation, which is causatively contributive to NMDAR/ischemia-mediated neuronal injuries. Through this study, I developed a specific PTEN nuclear translocation blocker (Tat-K13) and demonstrated that it represents a novel effective stroke treatment, as it not only dramatically reduces brain infarction but also significantly improves the functional recovery of sensorimotor circuits in rats subjected to ischemia. Most excitingly, this intervention remains effective 6 hours after stroke onset, the longest time window tested in the present study. This is notably longer than that of tPA (~3 hours), the only clinically available drug for treating ischemic stroke. Moreover, this novel intervention causes no obvious side effects, as it only specifically interferes with excitotoxic NMDA and ischemia-induced nuclear translocation of PTEN, but leaving the NMDAR's pro-survival pathways unaltered.

## 4.2 Too much and too little nuclear PTEN are both harmful

My present research revealed that excessive PTEN nuclear translocation caused by NMDAR over-activation or ischemic insults causatively leads to neuronal death in both *in vitro* and *in vivo* models. On the other hand, previous cancer studies proved

that lack of PTEN nuclear accumulation, due to mutations on either K13 residue or K289 residue, results in uncontrolled growth and proliferation of cancer cells. Together, my current finding and the previous literature strongly suggest that a physiological level of nuclear PTEN is critically important for maintaining a well-balanced survival, growth and proliferation of cells.

**4.2.1 Too much nuclear PTEN leads to excitotoxic/ischemic neuronal death**

Under normal conditions, nuclear PTEN is maintained at a moderate amount in neurons. However, during NMDA stimulation or ischemic insults, PTEN excessively accumulates in the nucleus and triggers signaling cascades that lead to neuronal death.

This observation of enhanced PTEN nuclear translocation as the key step in NMDA/ischemia-induced neurotoxicity is in a good agreement with previous literature. Gary et al. revealed that over-expression of phosphatase-dead PTEN mutant abolishes the glutamate-induced toxicity in cultured hippocampal neurons (Gary and Mattson, 2002). Ning et al. and many other groups found that conditional knockdown or pharmacological inhibition of PTEN protein can significantly reduce the excitotoxic neuronal injuries in the *in vivo* models of ischemia (Ning et al., 2004, Wu et al., 2006, Zhang et al., 2007a). In spite of increasing interest in down-regulation of PTEN as a means for ischemic stroke treatment, the experimental progression has been slow. It is not only because specific and potent inhibitors for PTEN are still lacking, but also because complete blockade of PTEN activities significantly increases the risk of hypertrophy and malignancy. Hence, instead of

aiming at PTEN *per se*, my current study turns to focus on identifying the detailed mechanisms by which PTEN mediates the excitotoxic/ischemic neuronal death, and aims to develop intervention that specifically targets this abnormally-altered PTEN signaling cascade. Excitingly, my study revealed that NMDAR/ischemia actually mediates neuronal death through enhancing PTEN nuclear translocation, and blocking this excessive nuclear accumulation of PTEN with interfering peptide Tat-K13 strongly protects against excitotoxic/ischemic neuronal damages in both *in vitro* and *in vivo* models. Hence, I reached the conclusion that too much nuclear PTEN causatively leads to excitotoxic/ischemic neuronal injuries.

## 4.2.2 Too little nuclear PTEN leads to uncontrolled growth and proliferation of cancer cells

In contrast to the neuronal death caused by excessive PTEN nuclear accumulation during excitotoxic/ischemic insults, overwhelming body of evidence from cancer studies suggests that too little nuclear PTEN leads to uncontrolled growth and proliferation of cancer cells.

Early studies claimed that PTEN was exclusively cytoplasmically localized. However, recent findings revealed that PTEN exists in both the cytoplasm and the nucleus (Planchon et al., 2008). This discrepancy is likely due to that early studies primarily focused on tumor cell lines and tissues (Furnari et al., 1997, Li and Sun, 1997, Whang et al., 1998), whereas the recent studies mainly used normal cells, such as primary neurons and endothelial cells (Sano et al., 1999), follicular thyroid cells (Gimm et al., 2000) and myoepithelial cells of breast ducts (Perren et al., 1999). It is

not difficult to find that the above evidence implies an inverse relationship between the amount of nuclear PTEN and tumor progressive stage. In normal cell, PTEN localizes in both the cytoplasm and the nucleus, but in tumor cells, cytoplasmic PTEN predominates. Indeed, further experiments proved this proposition to be true. For example, nuclear PTEN immunostaining is apparent in normal islet cells of pancreatic tissue, whereas PTEN localizes primarily in the cytoplasm in the sporadic endocrine pancreatic tumors (Perren et al., 2000). Even more strikingly, in the thyroid, normal follicular cells are characterized by a stronger staining in the nucleus than in the cytoplasm, whereas the nuclear staining intensity progressively diminishes during progression from normal to follicular adenoma to carcinoma (Gimm et al., 2000). Similar trends have been observed in a wide variety of tumor tissues (Depowski et al., 2001, Brenner et al., 2002, Whiteman et al., 2002, Dreher et al., 2004), indicating that nuclear localization is indispensible for PTEN's tumor suppressive activity, and failure in this nuclear accumulation leads to loss of PTEN's anti-tumor function.

All together, both my current finding and the previous literature suggest that the amount of nuclear PTEN is strictly regulated and maintained at a moderate level in normal cells. Too much nuclear PTEN, triggered by excitotoxic/ischemic insults, and too little nuclear PTEN, caused by *PTEN* mutations, are both harmful.

## 4.3 Clinical relevance of the present thesis research

Ischemic stroke is the third leading cause of death and the leading cause of disability

in the world (Lloyd-Jones et al., 2010). It occurs when cerebral artery becomes occluded, resulting in disturbance of blood supply to the brain. In turn, the mismatch between blood supply and high energy demand of the brain triggers cascades of events leading to progressive neuronal death and neurological deficits characteristic of a stroke. Current treatments for ischemic stroke include two major approaches. One is vascular-based therapeutics that aims to relieve the vascular occlusion after stroke occurs. The other is neuroprotection-based therapeutics that aims to inhibit the signaling cascades leading to ischemic neuronal death.

At present, the only clinically available treatment for ischemic stroke is a vascular-based therapeutics known as tissue plasminogen activator (tPA) (NINDS, 1995, Wardlaw et al., 1997). It is a serine protease that catalyzes the conversion of plasminogen to plasmin, a major enzyme responsible for dissolving blood clot. When administered to stroke patients within 3 hours post stroke onset, tPA can significantly improve the stroke outcome (NINDS, 1995, Hacke et al., 1998). However, this therapeutics comes with several limitations. First, there is a risk that tPA may transform ischemic stroke into a more severe and often fatal hemorrhagic stroke (Wardlaw et al., 1997), possibly through increasing the level of matrix metalloproteinase-9 (MMP-9) and hence enhancing the hemorrhage rate (Lapchak et al., 2000). Second, tPA treatment is almost always fatal for hemorrhagic stroke patients, who may present indistinguishable clinical symptoms from ischemic stroke patients (Green and Shuaib, 2006). Therefore, when stroke patients are admitted to a hospital, they need to be further diagnosed by x-ray computed tomography (CT) scan to rule out any signs of hemorrhage, and then finally given tPA for ischemic stroke treatment. All together, the above limitations have significantly restricted the

timely treatment for stroke patients, and resulted in tPA administration in less than 5% of clinical stroke cases (Green and Shuaib, 2006).

In contrast, the neuroprotection-based therapeutics aims to inhibit pro-death signaling pathways that lead to ischemic injuries. This approach has two important advantages over the conventional vascular-based therapies. The first one is that it can be given to both ischemic and hemorrhagic stroke patients; as a result, no CT scanning is required for this type of treatment (Green and Shuaib, 2006). The second advantage is that interventions targeting this process are expected to have a much wider therapeutic time window than vascular-based therapeutics, because the progression of ischemic infarct usually takes several hours to several days to fully mature (Hossmann, 2006). As discussed before, NMDAR-mediated excitotoxicity is believed to one of the primary causes for ischemic neuronal death. Indeed, a large number of NMDAR antagonists-based therapies have been proved to be neuroprotective in various ischemic animal models (Park et al., 1988b, Bullock et al., 1990). However, to everyone's dismay, all of those antagonists failed in the human clinical trials, either due to low efficacy or adverse effects (Muir, 2006). Besides the problems of poor experimental design, such as lack of proper blinding and randomization, another two major reasons for these clinical failures have been proposed (Gladstone et al., 2002, Ikonomidou and Turski, 2002, Roesler et al., 2003, Muir, 2006). First, the patient inclusion periods (usually >6 hours) used by most clinical trials are beyond the therapeutic time window (within 1 hour post-ictus) of NMDAR antagonists. Once the downstream cascades of NMDARs are activated, blocking the receptor *per se* is no longer effective. Second, in addition to their pathological activities, NMDARs also have a wide variety of physiological functions,

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such as regulating synaptic plasticity and mediating neuronal survival/recovery after injuries (O'Dell et al., 1991, Lu et al., 1998, Ikonomidou et al., 2000, Liu et al., 2004). Blocking NMDAR at the receptor level completely abolishes its downstream signaling cascades, both pro-death and pro-survival ones. This explains why most compounds tested in human clinical trials produced adverse effects.

In my current study, the above limitations of NMDAR antagonists are circumvented by targeting NMDAR's downstream death cascade - PTEN nuclear translocation.

First, unlike blocking NMDAR *per se*, targeting the process of PTEN nuclear translocation allows a much wider therapeutic time window. In the *in vivo* experiments, PTEN nuclear translocation is found to reach a peak at around 12 hours post-ischemia onset, and its blocker Tat-K13 has a strong neuroprotective effect even when administered at 6 hours post-ictus. Given that the average hospital recruitment time for stroke patients is about 6 hours, this time window would be sufficient in most clinical cases.

Second, unlike conventional NMDAR antagonists that block both pro-death and pro-survival downstream pathways of NMDARs, Tat-K13 only interferes with the pathologically-enhanced PTEN nuclear translocation, and thus evokes no obvious side effects.

Third, Tat-K13 has many advantages over other conventional inhibitors of PTEN. My *in vitro* and *in vivo* results showed that Tat-K13 can specifically and efficiently suppress mono-ubiquitination of PTEN and thus block PTEN nuclear translocation.

In contrast, the commercially available PTEN inhibitors, such as vanadium compounds, are usually not only non-specific but also toxic in physiological settings. What's more, Tat-K13 just has a half-life of 3 hours, and only transiently intervenes with PTEN signaling pathways. Therefore, PTEN's tumor suppressive functions shouldn't be jeopardized by this temporary inhibition.

Fourth, I propose to further investigate the potential protective effect of Tat-K13 for hemorrhagic stroke. Unlike vascular-based therapeutics (e.g. tPA), Tat-K13 belongs to the neuroprotection-based therapeutics, which in theory should have no adverse effects on hemorrhagic stroke, but protect against its neurotoxicity. Therefore, Tat-K13 may be given to stroke patients even in the ambulance without a necessity for CT scanning, and hence may save a lot of time for prompt treatment. In contrast, the only clinically available stroke medicine tPA has adverse effects on hemorrhagic stroke and thus requires CT scanning before administration. This limitation significantly delays tPA treatment and results in tPA administration in less than 5% of total stroke cases (Green and Shuaib, 2006).

Fifth, unlike many early studies, my current experiments were carried out with high quality control standards, such as double-blinding, randomization, cerebral blood flow monitoring, et al. Also, unlike many anti-stroke drugs that simply delay the ischemic injury process (Valtysson et al., 1994, Corbett and Nurse, 1998, Gladstone et al., 2002), my Tat-K13 has been proved to provide a sustained neuroprotection that lasts up to 4 weeks post-stroke onset, the longest time window tested in the present study.

All together, Tat-K13 circumvents many of the limitations of other stroke treatments, such as narrow therapeutic time window, dose-limiting toxicity, lack of long-term benefits, et al. To date, the pharmaceutical company, GlaxoSmithKline Inc., is collaborating with us in developing Tat-K13 as a novel post-stroke therapy.

## 4.4 Future directions

### 4.4.1 Upstream signaling pathways of PTEN

My study revealed a novel death signaling cascade for excitotoxic neuronal injuries during ischemic stroke. However, the detailed mechanism by which NMDAR triggers PTEN mono-ubiquitination and hence promotes PTEN nuclear translocation remains elusive. Previously studies showed that NEDD4-1 is the major ubiquitin ligase (E3) for PTEN ubiquitination in both cancer cell lines (Wang et al., 2007) and neurons (Kwak et al., 2010). Moreover, Nedd4-1 is found to be activated by an increase in intracellular $Ca^{2+}$ concentration. The elevated $Ca^{2+}$ level releases the auto-inhibition of NEDD4-1, promotes NEDD4-1 membrane translocation, and hence leads to activation of NEDD4-1's ubiquitin ligase activity (Plant et al., 1997, Wang et al., 2010). All together, NEDD4-1's direct interaction with PTEN and its calcium-dependent activation make it a likely candidate ubiquitin ligase for NMDA-induced PTEN mono-ubiquitination. Very excitingly, my co-immunoprecipitation experiment confirmed that NEDD4-1 is indeed constitutively present in the NR2BR complex in cultured cortical neurons. Moreover, this NEDD4-NR2BR association could be further enhanced by NMDA stimulation (25µM, 1 hour) in a time-dependent manner.

The peak interaction is detected at 3 hours post-NMDA treatment, while the basal level interaction is restored at 6 hours post-ictus (n=6; One Way ANOVA, F=3.739, P=0.028; Fig. 15A and 15B). The time course of NEDD4-NR2BR well fits into that of PTEN nuclear translocation (*in vitro*), which peaks around 6 hours post-NMDA treatment. Therefore, I predict that NEDD4-1 may be recruited into NR2BR complex by the $Ca^{2+}$ influx through NMDARs; as PTEN is also an interacting partner of NR2BR (Ning et al., 2004), NEDD4-1 is then able to mono-ubiquitinate PTEN within close proximity and hence promotes PTEN nuclear translocation. More experiments will be needed to further validate this time-dependent interaction between NEDD4-1 and NR2BRs in the *in vivo* ischemic system. In addition, manipulating the NEDD4-NR2BR interaction using interfering peptide or other approaches may also allow us to obtain direct evidence regarding the role of this interaction on PTEN mono-ubiquitination and nuclear translocation.

**4.4.2 Downstream signaling pathways of PTEN**

My current study revealed that PTEN nuclear translocation is causatively contributive to NMDA/ischemia-induced neuronal death. However, the detailed mechanisms by which nuclear PTEN initiates the pro-death cascades are still unclear.

Given that all the major components of PI3K/Akt cascade, such as PI(4,5)P2, PI(3,4,5)P3, PI3K, PDK1, and Akt, are all found in the nucleus, it is possible that nuclear PTEN functions as a $PI(3,4,5)P_3$ phosphatase and consequently suppresses nuclear PI3K/Akt-mediated pro-survival signaling pathways (Deleris et al., 2003, Ahn et al., 2004, Vasko et al., 2004). To validate this hypothesis, further experiments will

be needed to evaluate whether excitotoxic/ischemic insults can trigger a reduction in nuclear level of PI(3,4,5)P$_3$ and phospho-Akt, and whether interfering peptide Tat-K13, by inhibiting PTEN nuclear translocation, can rescue this reduction.

Also, it's possible that PTEN translocates into the nucleus to form a functional complex with its downstream effectors, hence initiating the signaling cascades that lead to neuronal death. In order to identify the binding partners of nuclear PTEN following ischemic insults, a pull-down assay was performed. Adult SD rats were first subjected to a 90 min ischemia (MCAO) and sacrificed at 9 hours post-stroke onset. Brain tissues from the infarct area on the ischemic side and the corresponding area on the contralateral side were both collected, and then subjected to nuclei fractionation, respectively. The nuclear lysates from each hemisphere were co-immunoprecipitated with PTEN antibody, and subsequently separated by SDS-PAGE gel, followed by mass-spectrometry assay. The result showed that a few proteins are specifically recruited into nuclear PTEN complex following the ischemic insult (data not shown). I predict that this ischemia-induced recruitment may lead to activation of the pro-death signals or inactivation of the pro-survival molecules. As this recruitment occurs downstream of PTEN nuclear translocation, interventions targeting this process may provide an even wider therapeutic time window for stroke treatment. This hypothesis will be validated by future experiments.

### 4.4.3 Tat-K13 may have a broader clinic application

My current study demonstrated a very promising neuroprotective effect of Tat-K13 in

treating ischemic injuries when administered up to 6 hours post-stroke onset. Given that my *in vivo* experiment showed that PTEN nuclear translocation peaks around 12 hours following stroke onset, I propose that Tat-K13 may provide an even wider therapeutic time window that's up to 12 hours post-ictus. What's more, the clinical application range of Tat-K13 might be further broadened by co-application with tPA, as combination of neuroprotection-based and vascular-based therapeutics could offer many synergistic advantages in extending therapeutic time window, decreasing adverse effects, improving clinical outcomes, et al. Moreover, a small portion of human stroke cases displays a late onset of spontaneous reperfusion (Carmichael, 2005). Therefore, investigating the potential protective effect of Tat-K13 or Tat-K13/tPA combination in a permanent stroke model will further increase the chance of clinical success. Last, NMDAR overactivation-induced excitotoxicity is believed to be a common pathological mechanism for neuronal damages in many brain disorders, including acute brain insults like ischemic stroke, as well as chronic neurodegenerative diseases such as Huntington's disease (Taghibiglou et al., 2009). Therefore, Tat-K13 may have a broad clinic application in treating diseases besides ischemic stroke, as investigated here, highlighting the need for future studies.

**Figure 15: NR2B-containing NMDARs interact with NEDD4-1 in a time-dependent manner**

(A, B) Cultured cortical neurons were challenged with NMDA (25μM) for 1 hour and allowed to recover for different periods of time, ranging from 0 hour to 6 hours post-ictus. Neurons were then collected for co-immunoprecipitation with NR2BR antibody, and subsequently subjected to western blotting with NEDD4-1 antibody. The result showed a constitutive interaction between NEDD4-1 and NR2BRs, which could be further enhanced by NMDA stimulation in a time-dependent manner. The peak interaction is detected around 3 hours post-NMDA, while the basal level interaction is restored at 6 hours post-ictus (n=6). Error bars represent Standard Deviation, *$P < 0.05$. *The basal level interaction between NR2B and NEDD4-1 was first discovered by Dr. Yuan Ge (Dr. Yu Tian Wang Lab), while the time-course study was performed by me.*
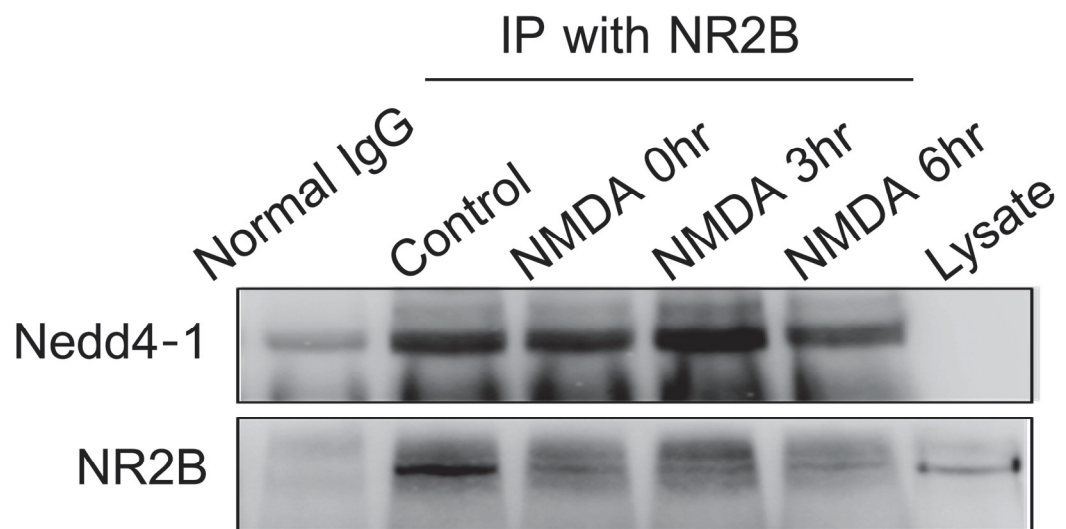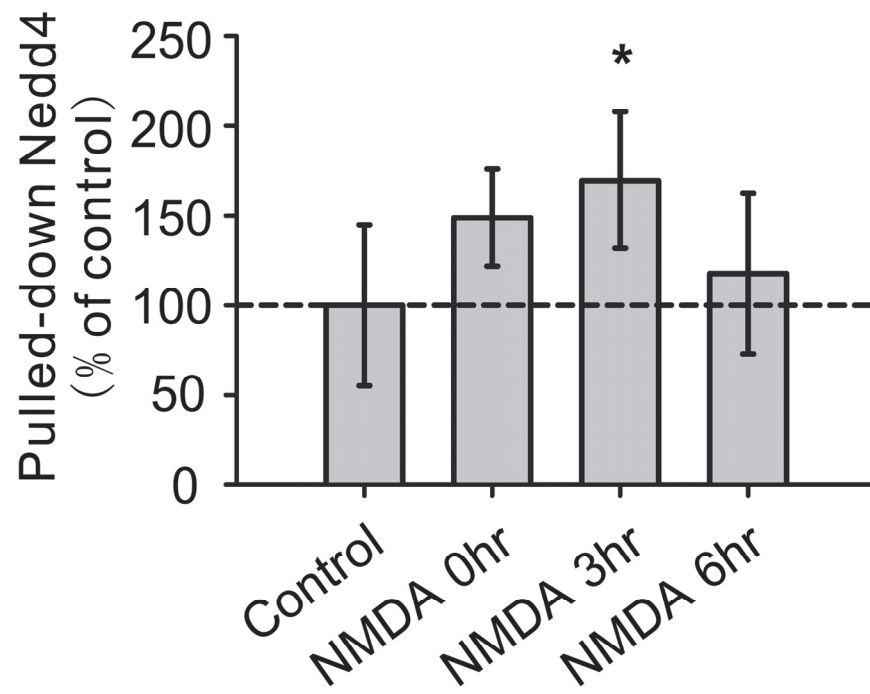
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