
Electronic Thesis and Dissertation Repository

10-27-2015 12:00 AM

Mechanisms of neuroprotection against ischemic insult by stress-inducible phosphoprotein-1

Jason Xu, *The University of Western Ontario*

Supervisor: Dr. Marco Prado, *The University of Western Ontario*

Joint Supervisor: Dr. Vania Prado, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology

© Jason Xu 2015

Follow this and additional works at: <https://ir.lib.uwo.ca/etd>



Part of the [Cell Biology Commons](#), and the [Molecular and Cellular Neuroscience Commons](#)

Recommended Citation

Xu, Jason, "Mechanisms of neuroprotection against ischemic insult by stress-inducible phosphoprotein-1" (2015). *Electronic Thesis and Dissertation Repository*. 3463.
<https://ir.lib.uwo.ca/etd/3463>

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.

MECHANISMS OF NEUROPROTECTION AGAINST
ISCHEMIC INSULT BY STRESS-INDUCIBLE
PHOSPHOPROTEIN-1

(Thesis format: Monograph)

by

Jason Xu

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Jason Xu 2015

Abstract

Stress-inducible phosphoprotein-1 (STI1) levels are increased in the brain following ischemia. STI1 is a co-chaperone for Hsp70/Hsp90 modulating protein folding. STI1 can also be secreted by a number of cells and function to activate extracellular signalling by the prion protein (PrP^C) and type-I bone morphogenetic protein receptor ALK2. However, the mechanisms by which STI1 can protect neurons against ischemia are currently unknown. A caspase-3 reporter mouse line was used to evaluate the consequences of increased extracellular STI1 levels. Neurons were treated with recombinant STI1 and specific agonists/antagonists for PrP^C, α 7nAChR, and ALK2 prior to oxygen-glucose deprivation (OGD). STI1 treatment significantly decreased apoptosis and cell death in neurons submitted to OGD in a manner dependent on PrP^C, α 7nAChR, and ALK2. Activation of both the α 7nAChR and ALK2 receptor were effective at decreasing neuronal death in response to ischemia, while BMP-4 treatment post-OGD also decreased neuronal death, suggesting that α 7nAChR and ALK2 receptor may be novel targets for preventing death of neurons after ischemia.

Keywords:

Ischemia, stroke, OGD, STI1, prion protein, α 7nAChR, ALK2

Co-Authorship Statement

No co-authorship in this thesis.

Acknowledgments

I would like to thank Dr. Marco Prado and Dr. Vania Prado for the opportunity to complete my masters degree in their laboratory. I am extremely grateful for all the resources and mentorship I have received in the past two years. I would also like to acknowledge my advisory committee members Dr. Patrick Lajoie, Dr. David Cechetto, and Dr. Shawn Whitehead for their time and feedback during my committee meetings. Together, your guidance has been extremely valuable to me as I progressed through my research.

My project would not be possible without the generous help I have received from Flavio, Val, Andrew, Ash, and Momo, who have both kick started my project and taught me almost all I know about the methods employed in my two years of work. I am forever grateful for the countless hours they have dedicated in the microscope room and the cell culture room.

I would also like to thank Jue and Sanda, who have supported me throughout my time in the lab. From genotyping to data filing, you have laid the foundation for all my work in the past two years.

Table of Contents

MECHANISMS OF NEUROPROTECTION AGAINST ISCHEMIC INSULT BY STRESS-INDUCIBLE PHOSPHOPROTEIN-1	i
Abstract	ii
Co-Authorship Statement.....	iii
Acknowledgments.....	iv
Table of Contents	v
List of Tables	1
List of Figures	2
List of Abbreviation, Symbols, Nomenclature	4
1 Introduction and Literature Review	5
1.1 Stroke	5
1.1.1 Types of Stroke.....	6
1.1.2 Risk Factors and Current Treatments for Ischemic Stroke.....	7
1.1.3 Neurotoxicity during Stroke	7
1.1.4 Recent Pharmacological Approaches	10
1.2 Prion Protein	11
1.2.1 Roles of the Prion Protein in the Nervous System.....	11
1.2.2 Diseases Involving the Prion Protein	14
1.2.3 The Role of the Prion Protein in Ischemic Conditions	15
1.2.4 Prion Protein Ligands and Targets.....	16
1.3 Stress-inducible phosphoprotein-1.....	18
1.3.1 STI1 structure.....	18
1.3.2 Intracellular roles of STI1	18
1.3.3 Secretion and binding of STI1 to Prion Protein.....	22
1.3.4 STI1 during Ischemia.....	24

1.4	Alpha-7 nicotinic acetylcholine receptor	26
1.5	ALK2 receptor	29
1.5.1	Activin receptors	29
1.5.2	Activin signalling.....	29
1.5.3	BMP-4 in the brain.....	30
1.5.4	STI1 signalling via ALK2.....	31
1.5.5	The role of activin receptors during ischemia.....	32
1.6	Rationale, Study Aim, and Hypothesis	33
2	Materials and Methods	35
2.1	Animals	35
2.2	Ethics Statement.....	35
2.3	Reagents	37
2.4	Primary cell culture.....	38
2.5	Oxygen Glucose Deprivation.....	42
2.6	Immunofluorescence.....	42
2.7	Caspase-3 reporter mouse	43
2.8	LIVE/DEAD assay.....	45
2.9	Statistical Analysis.....	46
3	Results	48
3.1	STI1 decreases apoptosis and neuronal death induced by OGD	48
3.2	STI1 engages $\alpha 7$ nAChR for neuroprotection	52
3.3	Activation of the $\alpha 7$ nAChR protects neurons against OGD.....	56
3.4	STI1 activates ALK2 receptors.....	58
3.5	Treatment of neurons with BMP-4 during reperfusion rescues neurons	62
4	Discussion	69
4.1	STI1 as a neuroprotective factor	69

4.2	Activin receptor family member ALK2 offers unique protection against ischemia	71
4.3	Treatment during reperfusion	73
4.4	Limitations and Future Directions	75
4.4.1	<i>In vivo</i> experiments	75
4.4.2	Protection beyond the hippocampus	77
4.4.3	Recombinant vs. Endogenous STI1	77
4.4.4	Calcium Signalling.....	78
4.4.5	STI1 signalling via the ALK2 receptor in neuroprotection	78
4.5	Conclusions.....	79
	Bibliography	82
	Appendices.....	100
	Curriculum Vitae	101

List of Tables

Table 1: Overview of the expression of PrP ^C in mice and humans.....	12
Table 2: Cellular roles of STII	21
Table 3: Mouse lines used in this thesis.....	36

List of Figures

Figure 1: Dissection of hippocampus and cortex from embryonic E16.5 brains.....	39
Figure 2: Schematic illustrating the mating, culturing, and experiment of hippocampal neurons.....	44
Figure 3: Schematic illustrating the caspase-3 reporter (Apo) mouse line.....	41
Figure 4: Time course of caspase-3 activity and cell death in neurons treated with STI1 and OGD.....	49
Figure 5: Treatment of wild-type and <i>Prnp</i> ^{-/-} hippocampal neurons with STI1 and OGD.....	51
Figure 6: Neuroprotection by STI1 against OGD is attenuated in wild-type hippocampal neurons treated with α 7nAChR inhibitor MLA.....	54
Figure 7: Neuroprotection against OGD by STI1 is prevented in alpha-7 nicotinic acetylcholine receptor (α 7nAChR) knock-out hippocampal neurons.....	55
Figure 8: Treatment of wild-type hippocampal neurons with α 7nAChR agonist PNU 282,987 significantly reduced cell death due to OGD.....	57
Figure 9: Expression of ALK2 in primary cultured wild-type hippocampal neurons.....	59
Figure 10: Inhibition of the ALK2 receptor prevented the neuroprotective effects of STI1 against OGD.....	60
Figure 11: Treatment of wild-type hippocampal neurons with BMP-4 significantly decreased cell death due to OGD treatment.	61
Figure 12 PNU 282987 and STI1 only protected neurons if treated prior to OGD, but not during reperfusion.....	63

Figure 13: Treatment neurons with BMP-4 during reperfusion significantly decreased OGD-induced cell death..	65
Figure 14: Neuroprotection against OGD by PNU 282,987 and BMP4 was independent of the PrP ^C	68
Figure 15: BMP-4 protection against OGD was dependent on the $\alpha 7$ nAChR.	67
Figure 16: Schematic of the proposed model of the $\alpha 7$ nAChR, PrPC, ALK2, and their interaction with STI1..	81

List of Abbreviation, Symbols, Nomenclature

$\alpha 7nAChR$: Alpha-7 nicotinic acetylcholine receptor

ALK2: Activin A receptor, type 1

BMDC: Bone marrow-derived stem cell

BMP: Bone morphogenetic proteins

CFP: Cerulean fluorescent protein

EPSP: excitatory postsynaptic potentials

ERK: Extracellular signal-regulated kinase

GSK-3 β : glycogen synthase kinase-3 β

Hop: Heat-shock organising protein

Hsp: Heat-shock protein

OGD: Oxygen-glucose deprivation

NMDA: N-methyl-D-aspartate

NSC: Neural stem cell

PI3K: phosphoinositide 3-kinase

PKA: Protein kinase A

PrP^C: Prion protein

ROS: Reactive oxygen species

R-SMADs: recruits receptor-regulated SMADs

SARA: SMAD anchor for receptor activation

STI1: Stress-inducible phosphoprotein-1

TGF- β : Transforming growth factor-beta

TNF: Tumour necrosis factor

TPR: Tetratricopeptide repeat

1 Introduction and Literature Review

1.1 Stroke

Stroke is currently the third leading cause of death in Canada. Fifty thousand Canadians suffer from stroke each year, resulting in one stroke every ten minutes. With 315,000 Canadians currently living with the effects of stroke, its societal burden is estimated to be \$3.6 billion annually (Statistics Canada, 2012).

Stroke occurs when blood flow towards the brain is disturbed. When blood flow is obstructed or reduced within a blood vessel that supplies the brain, the brain tissue downstream of the occlusion becomes deprived of oxygen and glucose. Deprivation of these essential nutrients gives rise to a rapidly expanding region of irreversible necrotic death. Within the tissue surrounding the perimeter of the area of necrosis, known as the penumbra, resides cells that receives limited blood flow. Unlike the necrotic center, the penumbra is an area in which damage due to ischemia is not entirely irreversible, but the cells are pushed towards apoptosis due to an array of insults such as rising levels of reactive oxygen species, depletion of cellular ATP, glutamate toxicity, and increased intracellular calcium (Lau et al., 2010, Lakhan et al., 2009, MacDonald et al., 2006). Neurons are quickly lost during stroke and permanent neurological damage, or even death, can occur within just minutes of oxygen deprivation. Studies have shown that for every minute of delay in treating stroke patients, the average brain loses 1.9 million neurons, 13.8 billion synapses, and 12 km of axonal fibers (Saver, 2006). To put this into perspective, for every 60 minutes of untreated stroke progression, the patient loses as many neurons in their brain as they normally would in nearly four years of aging (Saver

et al., 2006). This further emphasizes the importance of a rapid treatment response for minimizing neurological damage due to ischemia.

1.1.1 Types of Stroke

There are two types of stroke: ischemic stroke and haemorrhagic stroke. In ischemic stroke, a clot forms within the arteries that supply blood to the brain (Andersen et al., 2009). When formed elsewhere in the body, the clot may break off and travel towards the brain, arrest in a narrow artery, and block any downstream blood flow. Treatment for ischemic stroke typically involves the intravenous administration of tissue plasminogen activators that break down blood clots in order to re-introduce blood flow downstream of the affected areas (Lansberg et al., 2012). Intra-arterial thrombolysis and mechanical clot removal have also been used. This involves using a catheter entering from the upper thigh, extending into the arteries of the brain, which allows a more direct delivery of antithrombolytics (Andersen et al., 2009). When tissue plasminogen activators are not readily available, an antiplatelet medicine such as aspirin can be used to slow or stop platelets from aggregating and forming blood clots (Andersen et al., 2009). Lastly, anticoagulants can also be prescribed to decrease blood clot formation (Lansberg et al., 2012).

In contrast, haemorrhagic stroke involves a weakened vessel that ruptures and damages the surrounding areas due to both blood loss and increased pressure (Andersen et al., 2009). Unlike ischemic stroke, the use of antithrombolytics would exacerbate haemorrhagic stroke damage, by making the bleeding worse. At times, surgical procedures must be administered to locate and stop the source of blood loss using procedures such as aneurysm clipping, coil embolization, and arteriovenous

malformation repair (Andersen et al., 2009, NHLBI, 2014). Although accounting for only about 15% of all strokes, intra-cerebral haemorrhage has the highest morbidity and mortality rates amongst all stroke subtypes (Broderick et al., 2007).

1.1.2 Risk Factors and Current Treatments for Ischemic Stroke

Risk factors for stroke include diabetes, high blood pressure, and old age (Bousser, 2012). After the age of 55, the risk of stroke doubles every 10 years (Hinkle et al., 2007). Women have been found to have poorer outcomes from stroke than men, due to their greater longevity (Statistics Canada, 2012). Despite stroke and stroke treatments being studied extensively over the past two decades, patients are still limited to a single approved treatment: intra-venous thrombolytic therapy (Lansberg et al., 2012). To make matters worse, recent studies have shown that intra-venous thrombolytic therapy is only effective within 4 hours of the stroke (6 hours if given intra-arterially), and after this time its efficacy declines significantly (Lansberg et al., 2012). The limited therapeutic window, coupled by the lack of treatment options, has called attention to the need for novel therapies that aim to reduce cell death or promote survival following ischemia.

1.1.3 Neurotoxicity during Stroke

There are many factors that contribute to neurotoxicity following ischemic stroke. During ischemia, disturbances in cellular homeostasis such as ion shifts, excessive calcium influx, dysregulation of glutamate receptors, and ATP-depletion have been extensively studied (Lau et al., 2010, MacDonald et al., 2006).

In neurons, calcium is a key regulator in numerous signalling and regulatory processes. Dysregulation of calcium can occur as a result of increased calcium influx and

excess intracellular calcium (Berridge, 1998). Thus, both intracellular and extracellular sources of calcium are tightly regulated. In basal conditions, the osmotic gradient (ratio of extracellular to intracellular calcium is 10,000:1) and the electrical gradient (negative on the intracellular side) creates a great potential for calcium influx (Brini et al., 2014). This electrochemical gradient is upheld by the active $\text{Ca}^{2+}\text{-}2\text{H}^{+}$ ion transporter and $3\text{Na}^{+}\text{-}\text{Ca}^{2+}$ exchanger on the cell membrane (Brini et al., 2014). Normally, signal transduction begins with presynaptic glutamate release, activation of AMPA and NMDA receptors, sodium influx, postsynaptic depolarization, and calcium influx via calcium-permeable channels (Brini et al., 2014). Termination of the glutamate/calcium transients occurs via glutamate reuptake, conversion of glutamate to glutamine/lactate via glial cells, or activation of potassium channels that result in hyperpolarization of the membrane (Attwell et al., 1994, Magistretti et al., 1996, Kennedy, 1989). The endoplasmic reticulum (ER) also stores calcium concentrations equal to the extracellular environment, serving as a source of calcium that is activated by the phospholipase-C- IP_3 signalling pathway (Irvin, 1986). Although the extracellular environment has 10,000 times higher calcium concentrations than intracellular environments, almost all of the intracellular calcium is bound to calcium binding proteins or stored in the ER and mitochondria (Kristian et al., 1998). This intracellular source of calcium is also believed to be a contributor to calcium-related cell damage during ischemia excitotoxicity (Paschen, 1996)

During ischemia, dysregulation of ion channels such as voltage-gated calcium channels and NMDA receptors leads to excess calcium influx resulting in neuronal excitotoxicity (Choi, 1992, Kristian et al., 1998). Within a minute, concentration of extracellular calcium drops by 90%, indicating that almost all extracellular calcium is

now intracellular (Kristian et al., 1994). This effect is worse in hippocampal neurons as CA1 cells have a higher density of NMDA receptors compared to the rest of cortex (Silver et al., 1992). Calcium from intracellular organelles can also contribute to excitotoxicity (Kristian et al., 1998). Excess accumulation of intracellular calcium leads to glutamate toxicity, increase in reactive oxygen species (ROS; such as $\cdot\text{O}_2^-$, H_2O_2 , and $\cdot\text{OH}$), peroxynitrate generation, and degradation of cytoskeletal proteins (Choi et al., 1988, Tymianski et al., 1996, Siesjo, 1981, Eimerl et al., 1994.). Following excitotoxicity, many protein kinases and phosphatases are activated or inactivated aberrantly, and fragmentation of DNA ultimately results in cell death (Hossmann, 1993, Wieloch et al., 1996).

The mitochondria have been shown to modulate calcium homeostasis by serving as a sink for transient increases in intracellular calcium (Nicholls, 1985). However, during glutamate toxicity, the mitochondria increase the production of ROS that deteriorates the inner mitochondrial membrane (Dykens et al., 1994, Dugan et al., 1995). Briefly, the electron transport chain in mitochondria relies on a large electrochemical gradient of H^+ established across the inner mitochondrial membrane. In basal conditions, the membrane is impermeable to H^+ . However, studies have shown that exposure of mitochondria to excess calcium and oxidative stress leads to osmotic swelling of the organelle and release of intramitochondrial components (Gunter et al., 1990, 1994). As a result, ATP production is stopped due to the breakdown of the electron transport chain. Dysfunction of the mitochondria as a result of glutamate toxicity and mitochondrial membrane depolarization is the primary event that leads to cell death (Schinder et al., 1996, Richter, 1993).

One of the key regulators of calcium homeostasis is calcium binding proteins. Interestingly, studies have shown that the expression of calcium binding proteins in neurons is correlated to resistance against excitotoxicity (Rami et al., 1992, Freund et al., 1990). In fact, the level of calcium binding proteins decreases during aging, as well as in Alzheimer's disease and Parkinson's disease, which may contribute to cognitive decline (Thibault et al., 2007, Iacopino et al., 1990). Since the major risk factor for stroke is aging, the decline in calcium binding protein expression may be a contributing factor as well.

1.1.4 Recent Pharmacological Approaches

Recent cellular and molecular studies have reported treatments that offer neuroprotection in rodent models (Tymianski et al., 2010). However, these candidate neuroprotectants have not been successfully translated into the clinic as treatments for patients. One significant barrier that needs to be overcome at the clinical trials phase of candidate drugs development is lack of tools and biomarkers to assess the relationship between brain damage due to ischemia and clinical outcome (Tymianski et al. 2010). Unlike myocardial infarctions, which have a straightforward relationship between myocardial infarct size and myocardial function, it is much more difficult to establish and evaluate the same relationship in stroke (Cerqueira et al., 1991).

The majority of studies conducted for stroke treatments have been focused on the signalling mechanisms of regulating neuronal cell death during ischemia and how to decrease/prevent activation of these pathways in order to increase cell viability (Tymianski et al. 2010). A recent study found that treatment of hippocampal neurons with apoaequorin, a calcium binding protein isolated from jellyfish, protects against

oxygen-glucose deprivation (OGD), which models ischemia, via up-regulation of the anti-inflammatory cytokine interleukin-10 (Detert et al., 2013). Another study found that treatment of hippocampal neurons *in vitro* with melatonin also protected against OGD in manner dependent on the alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) (Parada et al., 2014). Our laboratory has discovered a potential mechanism involving the prion protein (PrP^C) that specifically capitalizes on an endogenous homeostatic response that plays a critical role during neuronal injury (Beraldo et al., 2013). We anticipate that understanding the brain's defense mechanisms during ischemia could help to reveal new ways to interfere with neuronal death and improve outcomes of stroke patients in the future.

1.2 Prion Protein

1.2.1 Roles of the Prion Protein in the Nervous System

The prion protein PrP^C is a glycosylphosphatidylinositol (GPI)-anchored protein found abundantly throughout the brain (Sales et al., 1998) and to a lesser degree in select non-neuronal tissues such as lymphoid tissues, cardiac muscle, and gastrointestinal mesenteric ganglion cells (Brown et al., 2000, see Table 1). PrP^C was first identified from scrapie-infected hamster brain tissue (Bolton et al., 1984). Coded by the *Pnrp* gene, the C-terminal of the PrP^C hosts a globular domain (consisting of three alpha-helices and antiparallel beta-pleated sheets) and the N-terminal hosts a random coil sequence, both of approximately 100 amino acids in length (Zahn et al., 2000).

Table 1: Overview of the expression of PrP^C in mice and humans

Species	Tissue	Cell type/location	Reference
Mouse	Brain	Neurons – Intracellular	Piccardo et al., 1990
		Neuronal processes	Ford et al., 2002b
	Spinal cord	Neurons	Ford et al., 2002a
	Intestine	Submucosa	Manson et al., 1992
	Bone marrow	Hematopoietic stem cells	Kubosaki et al., 2001
Human	Brain	Hippocampus – Presynaptic	Fournier et al., 2000
		Cerebellum	Laine et al., 2001
	Blood	Lymphocytes, monocytes, T-cells, NK cells, B cells	Durig et al., 2000
Both	Brain	Neurons - Cortex	Piccardo et al., 1990
	Muscle	Subsynaptic sarcoplasm	Gohel et al., 1999

More than two decades ago, a PrP^C-knockout mouse named *Pnnp*^{0/0} was produced to study the functions of PrP^C (Bueler et al., 1992). Since then, several PrP^C-knockout mouse lines have been generated. Initial studies of *Pnnp*^{0/0} mice showed no observable morphological differences in the brain, skeletal muscles, and visceral organs, nor any behavioural/learning deficiencies (Bueler et al., 1992). However, later studies have found that mice without PrP^C expression demonstrate increased locomotor activity when placed in a novel environment and reduced levels of anxiety during stress (Roesler et al., 1999, Nico et al., 2005), suggesting that PrP^C may play a role in behavioural responses during stress.

PrP^C has also been suggested to play a role in learning. PrP^C-null mice demonstrate normal short-term and long-term memory at 3 months of age, but display memory impairments at 9 months (Coitinho et al., 2003). Furthermore, pharmacological antagonism of PrP^C leads to impairments of memory in old rats as well (Coitinho et al., 2003). One of the ligands of PrP^C, which will be discussed below in greater detail, is stress-inducible phosphoprotein-1 (STI1). Interestingly, binding of STI1 to PrP^C increases short-term memory retention and long-term memory consolidation by acting on the CA1 region of the hippocampus (Coitinho et al., 2007), suggesting that the interaction between STI1 and PrP^C may play an endogenous role in learning and memory. The functional roles of STI1 and PrP^C will be discussed in greater detail in the next section.

1.2.2 Diseases Involving the Prion Protein

Prion diseases, such as Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy (mad cow disease) in other mammals, are identified by protein aggregation and cellular degeneration in the brain (Prusiner et al., 1998). In prion diseases, PrP^C undergoes a conformational change of its protein structure into PrP^{SC}, resulting in a fatal and incurable neuronal disease in mammals that leads to death within few months following diagnosis (Prusiner et al., 1998). The misfolded prions cause transmissible spongiform encephalopathies by acting as self-replicating infectious agents (Knight et al., 2004). Both PrP^C and PrP^{SC} share the same amino acid sequences coded by the *Prnp* gene, suggesting that the different functions between the two are due to post-translational modifications (Basler et al., 1996). The conformational change from alpha-helix to beta-sheets also explains the ability of PrP^{SC} to form and accumulate as compact aggregates in the brain (Baldwin et al., 1994). Interestingly, studies have shown that when PrP^{SC}-infected tissue is grafted into a PrP^C-knockout mouse, the host tissue is not affected by PrP^{SC}, suggesting that the development of the prion disease requires misfolding of the endogenous protein (Brandner et al., 1996).

The progression of prion diseases may also be related to the loss of the neuroprotective functions of the endogenous PrP^C (Linden et al., 2008). Studies have suggested that the loss of resistance to oxidative stress due to the lack of PrP^C plays a vital role in the pathogenesis of prion diseases (Giese et al., 2001). Although the pathology of prion disease has been extensively studied, less is known about the endogenous roles of the PrP^C.

PrP^C has also been identified to play a role in the progression of other neurodegenerative diseases such as Alzheimer's disease. The accumulation of soluble oligomers of the amyloid-beta peptide has been found to contribute to the neuronal dysfunction of this disease in a PrP^C-dependent way (Lauren et al., 2009). The interaction of the amyloid-beta oligomer and PrP^C leads to a variety of toxic events such as glutamate excitotoxicity, synaptic dysfunction, and neuronal death, although the specific mechanisms are still to be determined (Paula-Lima et al., 2013, Quenfurth et al., 2010). It is interesting to note that both Alzheimer's disease and prion diseases involve aggregation of misfolded proteins. Moreover, studies have demonstrated that brains of scrapie-infected mice showed higher beta-secretase activity compared to their control counterparts (Parkin et al., 2007), suggesting that PrP^C has an endogenous role in beta-secretase modulation. As mentioned earlier, STII is one of the ligands of PrP^C. Recent studies have shown that STII can protect neurons against amyloid-beta oligomer toxicity by binding to the PrP^C and also activating the $\alpha 7$ nAChR (Ostapchenko et al., 2013a). The role of STII as a ligand for PrP^C will be discussed in greater detail in the following sections. This recent finding regarding the neuroprotective role of the prion protein and STII against Alzheimer's disease raises questions regarding what other potential neurological insults PrP^C-STII may protect against. Oxidative stress is often implicated in numerous human neurodegenerative diseases such as Alzheimer's disease and prion disease (Beal et al., 1995, Smith et al., 2000).

1.2.3 The Role of the Prion Protein in Ischemic Conditions

In vitro studies using PC-12 rat adrenal Pheochromocytoma cells showed that the level of prion protein expression correlated with resistance to oxidative stress, a

component of ischemic damage (Brown et al., 1997). Furthermore, studies have shown that during ischemic conditions, PrP^C mRNA expression is increased in both human and rodent tissues (McLennan et al., 2004). In a different study involving multicellular prostate tumour spheroids, the authors found that the generation of reactive oxygen species, a hallmark of ischemic damage, also led to an increase in PrP^C expression (Sauer et al., 1999). Injured neurons may increase the production of proteins vital for survival while decreasing the synthesis of non-relevant molecules (Papadopoulos et al., 2000). Thus, the observation that PrP^C is increased in brain tissues during ischemia supports the proposed protective role of PrP^C during hypoxia. In fact, studies involving PrP^C-null mice found increased infarct volume as well as oxidative stress markers following ischemia compared to their wild-type counterparts (McLennan et al., 2004, Wong et al., 2011, Weise et al., 2006). In contrast, mice showing overexpression of PrP^C by adenovirus-mediated gene targeting demonstrated decreased injury and behavioural dysfunction compared to control animals (Shyu et al., 2011, Weise et al., 2008). These studies involving genetically modified mice with a knockout or overexpression of PrP^C suggests a potential role for PrP^C during ischemia.

1.2.4 Prion Protein Ligands and Targets

The prion protein serves as a receptor for the extracellular matrix proteins laminin and vitronectin in modulation of neuronal processes such as neuritogenesis and axonal growth, respectively (Graner et al., 2000, Hajj et al., 2007). The binding of neural cell adhesion molecules to PrP^C has been found to be involved in the differentiation of neuronal stem cells (Schmitt-Ulms et al., 2001, Prodromidou et al., 2014). PrP^C has also been found to play a role in the homeostasis of serotonergic neuronal cells by modulation

of serotonergic G protein-coupled receptors (Mouillet-Richard et al., 2005). The laminin $\gamma 1$ chain has been found to contain the laminin binding site for PrP^C, and laminin-PrP^C interaction leads to neuritogenesis and intracellular calcium mobilization modulated by metabotropic glutamate receptors (Beraldo et al., 2011).

Glutamate is the major excitatory neurotransmitter in the central nervous system (Mayer, 2005). Glutamate acts upon both ionotropic (ion channels) and metabotropic (G protein-coupled receptors) glutamate receptors. AMPA, kainate, and NMDA receptors belong to the ionotropic glutamate receptor family (Mayer, 2005). During depolarization, the magnesium ion block of the channel pore is removed and activation of NMDA receptors by co-agonists glutamate and glycine or D-serine mediates the influx of sodium and calcium ions that results in excitatory postsynaptic potentials (EPSPs) (Mayer, 2005). However, during excitotoxicity, excess glutamate causes aberrant NMDA activity and subsequent uncontrolled calcium influx (Lau et al., 2010). The prion protein has been shown to attenuate excitotoxicity in hippocampal neurons by inhibiting NMDA receptors (Houman et al., 2008). Furthermore, up-regulation of NMDA receptors containing NR2D subunits and the resulting prolonged NMDA-evoked current was found in PrP^C-knockout mouse hippocampal neurons, an effect that was reversed by treatment with exogenous PrP^C (Houman et al., 2008). Taken together, these results suggest that PrP^C might be important for the modulation of NMDA receptors during excitotoxicity.

Based on the multi-faceted cellular mechanisms that PrP^C is involved in, this GPI-anchored protein has been suggested to play the role of a scaffolding protein, serving as a receptor for a wide range of neuronal functions (Linden et al., 2008). The major characteristics of prion diseases are similar between mice and humans, as PrP^C is a

conserved protein between the two species. Coupled with the similarities in PrP^C expression following hypoxia, this suggests that the use of mouse models for studying the role of PrP^C is appropriate. Understanding the underlying mechanisms of PrP^C and its neuroprotective roles may help uncover novel mechanisms that could be applied to treatment of an array of neurodegenerative diseases.

1.3 Stress-inducible phosphoprotein-1

1.3.1 STI1 structure

Stress-inducible phosphoprotein-1 (STI1), also known as the Hsp70/Hsp90 organising protein (Hop), has recently received attention in the literature for its wide range of roles in both healthy and diseased cells. The structure of STI1 is composed of two types of domains: the tetratricopeptide repeat (TPR) motif domain and the aspartate-protein (DP) motif domain. There are three TPR domains and two DP domains (Scheufler et al., 2000, Brinker et al., 2002). The TPR domains, responsible for protein-protein interactions, are involved in many cellular processes such as transcription and protein degradation (Allan and Ratajczak, 2011).

1.3.2 Intracellular roles of STI1

In eukaryotic cells, the final stages of translation often require heat-shock protein chaperones that assist in the folding of the final protein structure. Within the cell, heat shock proteins are expressed abundantly in both cytoplasm and the nucleus (Lassle et al., 1997). Without the actions of chaperones, proteins will begin to aggregate due to exposed hydrophobic residues (Martin 2004). Hsp90, a 90kDa chaperone, is important for the final folding steps of many regulatory client proteins (Lee et al., 2012). The binding of

client proteins to Hsp90 requires prior interactions between Hsp70 and STI1. STI1 acts as a co-chaperone that helps the transfer of client proteins from Hsp70 to Hsp90 by binding onto both simultaneously via its TPR domains (Lassle et al., 1997, Schmid et al., 2012). Studies have shown that from the three TPR domains, the TPR1 and TPR2B domains are responsible for Hsp70 binding, while the TPR2AB domain is responsible for Hsp90 binding (Odunuga et al., 2003, Rohl et al., 2015). Formation of this protein complex plays a role in many cellular functions in addition to protein folding, such as viral replication, cell division, and signal transduction (Hu et al., 2002). Without the co-chaperone activity of STI1, the level of client proteins of Hsp90, including kinases, transcription factors, and steroid hormone receptors responsible for proliferation, apoptosis, and tumorigenesis, are significantly decreased (Caplan et al., 2007, Lanneau et al., 2008, Miyata et al., 2013, Walsh et al., 2011, Beraldo et al., 2013).

The role of STI1 also extends to cancerous cells such as breast cancer and colon cancer cells (Willmer et al., 2013, Kubota et al., 2010). In breast cancer cells, STI1 has been found to be expressed in isolated pseudopodia fractions and localized with actin in lamellipodia, suggesting that STI1 may regulate cell migration (Willmer et al., 2013). STI1 expression has also been found to be increased in colon cancer cells, and the level of STI1 expression has been correlated with Hsp90 expression in tumour tissues (Kubota et al., 2010). However, the exact involvement of STI1 is not fully understood. Overall, studies have shown that inhibition of STI1 is toxic to cancer cells, as many of the client proteins of Hsp90 are involved oncogenic activity (Horibe et al., 2011). As a result, STI1 has been recently studied as a potential therapeutic target for cancer cells (Walsh et al., 2011, Willmer et al., 2013).

STI1 has also been identified as a regulator of the cellular stress response in *C. elegans* models (Song et al., 2009). In these models, both STI1 mRNA and protein expression is increased following heat stress, and STI1-knockout mutants have shortened life spans, thus suggesting that STI1 is involved in stress responses and longevity. The authors further suggest that the two roles are related, as stress events can induce increased expression of pro-survival genes, and in turn increasing longevity of the organism. In fact, in *C. elegans*, the genes that regulate lifespan including those in the insulin-signalling pathway also regulate thermo-tolerance (Ogg et al., 1997).

As previously mentioned, deletion of the prion protein gene *Pnrp* is nonessential for viability (Büeler et al., 1992). In contrast, STI1 appears to play a critical role during embryonic development. Studies involving genetically modified mice with deletion of the STI1 gene (*Stip1*) have led to the finding that mice homozygous for the STI1 knockout died at E9.5-E10.5 (Beraldo et al., 2013). More specifically, in these mutant mice, the client proteins of Hsp90 have been found to be significantly reduced, indicating that the cochaperone activity of STI1 for Hsp90 is important during early development. Interestingly, deletion of *Stip1* is not lethal in *C. elegans* and yeast (Chang et al., 1997, Song et al., 2009), suggesting that despite the conservation of STI1 across a variety of species, there is evidence of evolution and further functions of STI1 in higher organisms.

STI1 has also been found to be secreted by several different types of cells (Santos et al., 2011, Lima et al., 2007, Arantes et al., 2009, Erlich et al., 2007, Tsai et al., 2012). Secreted STI1 appears to function as a cytokine, inducing a wide range of signalling pathways (Hajj et al., 2013, Beraldo et al., 2010, Caetano et al., 2008, Erlich et al., 2007, Tsai et al., 2012) (See Table 2 for a summary of the roles of STI1).

Table 2: Cellular roles of STI1

Cell type/Model	Targets	Function	Reference
Intracellular function	Hsp70/Hsp90	Cochaperone	Lassle et al., 1997
Retinal/Hippocampal Neurons	PrP ^C	Neuroprotection Neuritogenesis	Beraldo et al., 2010
Hippocampal neurons	PrP ^C / α 7nAChR	Protection against soluble A β O in Alzheimer`s disease	Ostapchenko et al., 2013a
Ovarian Cancer Cells	ALK2-SMAD signalling	Cell proliferation	Tsai et al., 2012
Neurons	PrP ^C + PKA and ERK1/2	Neuroprotection Neuritogenesis	Lopes et al., 2005
Ischemic brain <i>in vivo</i>	Bone Marrow Derived Cells	Proliferation Migration	Lee at al., 2013
Neurons	PrP ^C - PI3K -mTOR	Protein Synthesis	Roffe et al., 2010
Embryonic mouse model		Embryonic development	Beraldo et al., 2013
<i>C. elegans</i>	Hsp	Stress-response Fertility/Lifespan	Song et al., 2009
Breast cancer cells	Actin	Cell migration	Willmer et al., 2013
Colon cancer	Hsp70/90	Upregulation of Hsp complex formation	Kubota et al., 2010

1.3.3 Secretion and binding of STII to Prion Protein

STII has been found to be a ligand of PrP^C and STII-PrP^C binding leads to a plethora of signalling cascades responsible for neuronal events such as neuritogenesis, neuronal protein synthesis, neuroprotection against staurosporine and ischemia (Zanata et al., 2002, Chiarini et al., 2002, Lopes et al., 2005, Roffe et al., 2010, Caetano et al., 2008, Beraldo et al., 2010, Beraldo et al., 2013). The secretion mechanism of STII is rather unconventional, as STII lacks a signalling peptide. STII is secreted by astrocytes and other cells, likely via a diverse population of extracellular vesicles derived from multi-vesicular bodies (Hajj et al., 2013). Extracellular vesicles released by astrocytes have been shown to contain STII on the outer leaflet, and the association of STII with these extracellular vesicles greatly increased STII activity on PrP^C (Hajj et al., 2013). The binding sites of STII to PrP^C have been identified as amino acids 113-128 on the prion protein, and 230-245 on STII (Zanata et al., 2002). The interaction between the two proteins is specific and shows high affinity, with a K_d of 10^{-7} M (Zanata et al., 2002, Ostapchenko et al., 2013).

Endocytosis of the PrP^C following STII binding has been found to be an important step in PrP^C-STII signalling pathways. By using both STII and STII $_{\Delta 230-245}$ (deletion of PrP^C-binding site), it was determined that STII treatment yielded in dynamin-dependent PrP^C-internalization, an effect that is not observed with STII $_{\Delta 230-245}$ (Caetano et al., 2008). Endocytosis of PrP^C has been found to be a necessary step for STII-induced ERK1/2 activation, which in turn has been shown to promote neuronal differentiation and synaptic plasticity (Lopes et al., 2005, Coitinho et al., 2007). Furthermore, STII itself was found to be internalized in a manner independent of the

PrP^C (Caetano et al., 2008). These results indicate that although STI1-PrP^C engagement on the cell surface results in endocytosis, the interaction between the two proteins is only transient once internalized.

STI1 has been shown to convey neuroprotection of both retinal and hippocampal neurons against staurosporine-induced programmed cell death by the activation of protein kinase A (PKA) (Chiarini et al., 2002, Zanata et al., 2002, Lopes 2005, Beraldo et al., 2010). Unlike STI1-induced ERK1/2 activation, PKA activation does not require endocytosis of PrP^C (Caetano et al., 2008). Furthermore, the interaction between STI1 and PrP^C was found to induce both differentiation and protein synthesis in hippocampal neurons through the extracellular signal-regulated kinase 1 and 2 (ERK1/2) and phosphoinositide 3-kinase (PI3K)-Akt mTOR activation, respectively (Lopes et al., 2005, Roffe et al., 2010). Both neuroprotection and neuritogenesis mediated by the interaction of PrP^C with STI1 in hippocampal neurons depends on the α 7nACh receptor, and inhibition of the latter with the selective α 7nACh receptor antagonist α -bungarotoxin blocks these effects (Beraldo et al., 2010).

Although many extracellular functions of STI1 depend on PrP^C, few PrP^C-independent roles have been identified. Recently, expression and secretion of STI1 has been identified in both ovarian cancer cells and glioblastomas and has been shown to induce intracellular signalling pathways that leads to cell proliferation (Tsai et al., 2012, Erlich et al., 2007). However, the proliferative effect of extracellular STI1 on glioma has been suspected to have both PrP^C-dependent and -independent mechanisms (Erlich et al., 2007). Taken together, it appears that the role of STI1 expands beyond just being a ligand

for PrP^C. However, these studies have not directly provided evidence that the PrP^C is not involved, thus it is still possible that PrP^C also play a role in these mechanisms.

1.3.4 STI1 during Ischemia

Recently, studies have shown that STI1 protein secretion from astrocytes is significantly increased following oxygen glucose deprivation (OGD) (Beraldo et al., 2013). In fact, 9 hours following OGD, there is a 3-fold increase in extracellular STI1 levels (Beraldo et al., 2013). This increased STI1 expression during hypoxic conditions has been found to result from direct binding of the hypoxia inducible factor-1-alpha onto the promoter of STI1 gene (Lee et al., 2013).

The recruitment of bone marrow-derived endothelial cells has been described as an endogenous recovery mechanism in the brain following stroke (Hess et al., 2002). Recently, STI1 has also been shown to recruit bone marrow-derived stem cells (BMDCs) into ischemic brains *in vivo* (Lee et al., 2013). Furthermore, STI1 signalling promoted the proliferation and migration of BMDCs *in vitro* (Lee et al., 2013), suggesting that the recruitment of BMDCs into hypoxic areas of the brain by STI1 may be an endogenous response to facilitate recovery following ischemia.

Apoptosis and necrosis are two forms of cell death with distinct morphological features. While necrosis is a passive, irreversible process, apoptosis is much more active and requires the action of numerous apoptotic regulatory proteins (Walker et al., 1988). There are two types of apoptosis. The first type is intrinsic and involves the mitochondria. The second type depends on the action of membrane death receptors, and is thus extrinsic (Walker et al., 1988). In intrinsic apoptosis, the balance of both pro-apoptotic proteins

(such as BIM and Bax) and anti-apoptotic proteins (such as Bcl-2 and Bcl_{XL}) regulate the release of cytochrome c from the mitochondria. In both forms of apoptosis, caspase enzymes are activated and damages occur to the DNA, chromatin, and the plasma membrane (Walker et al., 1988). Cyclic AMP (cAMP) is one of the most well studied second messengers, especially in regulation of apoptosis. In neuronal cell types, cAMP has been found to have an anti-apoptotic effect (Insel et al., 2012). Activation of PKA, an effector of cAMP, has been found to protect cells against TNF-alpha-induced apoptosis (Kragsted et al., 2004).

Anisomycin induces apoptosis by inhibition of protein synthesis (Croons et al., 2009). In early studies, activation of PrP^C using a PrP^C-binding peptide modeling STI1 was found to transduce neuroprotective signals against anisomycin-induced apoptosis in retinal cells (Chiarini et al., 2002). While activation of PrP^C has been found to activate both cAMP/PKA and ERK pathways, inhibition of PKA activity blocked the protective effect of PrP^C (Chiarini et al., 2002). Treatment of hippocampal neurons with STI1 led to a PrP^C-dependent increase of PKA activity to a level similar to that observed with forskolin, a potent activator of PKA (Lopes et al., 2005). Furthermore, PrP^C has been documented to modulate calcium signalling (Whatley et al., 1995). In fact, the interaction between STI1 and PrP^C triggers calcium influx via physical interaction with the α 7nAChR, leading to protection against staurosporine-induced apoptosis (Beraldo et al., 2010). Unlike the massive influx of calcium during excitotoxicity, this controlled increment of calcium influx via α 7nAChR is upstream of PKA activation and is subsequently protective against staurosporine (Cooper et al., 1995, Beraldo et al., 2010).

1.4 Alpha-7 nicotinic acetylcholine receptor

In intra-cerebral haemorrhage, the development of brain edema induces apoptosis in surrounding neurons, extending the damage beyond the initial site of ischemia (Matsushika et al., 2000). Secondary injury mechanisms, such as the inflammatory cascade, are significant contributors to neuronal ischemic damage (Keep et al., 2012, Lakhan et al., 2009). Excessive inflammation and the production of tumour necrosis factor (TNF) leads to tissue injury and cell death (Tracey et al., 1986). The $\alpha 7$ nAChR is a member of the nicotinic acetylcholine receptor family and has been shown to have an important role in anti-inflammatory pathways (Rosas-Ballina et al., 2009). These receptors are ligand-gated pentameric ion channels that transmit signals for acetylcholine at neuromuscular junctions in both the central and peripheral nervous systems (Leonard and Bertrand, 2001, Marubio and Changeux, 2000). In human macrophages, acetylcholine has been found to target nicotinic receptors and inhibit the release of inflammatory cytokines such as TNF (Borovikova et al., 2000). Acetylcholine agonists such as nicotine have anti-inflammatory effects by inhibition of cytokine synthesis in numerous cell types (Sadis et al., 2007). However, the role of the $\alpha 7$ nAChR during ischemia in the central nervous system is not completely understood.

In rats, stimulation of the $\alpha 7$ nAChR has been shown to attenuate apoptosis induced by subarachnoid haemorrhage by down-regulation of caspase-3 activity via increased Akt phosphorylation (Duris et al., 2011). More specifically, in neurons, activation of Akt leads to inhibition of glycogen synthase kinase-3 β (GSK-3 β) (Grimes and Jope, 2001), an enzyme that shows increased expression following oxygen-glucose deprivation in ischemic stroke (Valerio et al., 2011). During ischemia, phosphorylation of

GSK-3 β in specific residues involved in its activity is increased, and the activated GSK-3 β induces caspase-3 activation and subsequent apoptosis (King et al., 2001). In addition, inhibition of GSK-3 β also increases the expression of β -catenin, an important component of the blood brain barrier (Nelson and Nusse, 2004). Hence, increased phosphorylation of Akt by α 7nAChR activation leads to decreased GSK-3 β activity, thereby attenuating apoptosis and blood brain barrier degradation following ischemia.

Furthermore, studies using murine models of intra-cerebral haemorrhage have shown that activation of the α 7nAChR via PHA-543613 (an agonist of the α 7nAChR) leads to attenuation of behavioural deficits such as sensorimotor function, reflexive motor ability, and spatial exploratory and cognitive performance (Krafft et al., 2012). In contrast, treatment with MLA (a selective α 7nAChR antagonist) leads to exacerbation of these behavioural deficits (Krafft et al., 2012). The authors suggested that the activation of the α 7nAChR lead to both anti-apoptotic and anti-inflammatory effects via the inhibition of pro-inflammatory cytokines. More recently, studies have shown that PNU 282,987, a selective α 7nAChR agonist, protects against hepatic ischemia-reperfusion injury (Li et al., 2013). It was found that treatment with PNU 282,987 attenuated liver injury following ischemia-reperfusion by suppression of cytokine and NF- κ B activation. These findings further suggest that activation of the α 7nAChR leads to attenuation of apoptosis and inflammation.

Interestingly, a recent study found that hippocampal cultures treated with melatonin during reperfusion protected against OGD (Parada et al., 2014). The protective effect of melatonin was prevented when co-treated with α -bungarotoxin, a selective α 7nAChR antagonist, indicating that the α 7nAChR plays a critical role in the protective

effect of melatonin against OGD (Parada et al., 2014). The authors suggest that the activation of the $\alpha 7$ nAChR and subsequent overexpression of heme oxygenase-1, a key enzyme in the protection against oxidative and inflammatory stress (Kim et al., 2005), greatly contributes to the neuroprotective effect of melatonin (Parada et al., 2014). *In vivo* studies demonstrate that melatonin administration following stroke results in reduced infarct size and attenuated behaviour deficits, while administration of MLA partially blocks the protective effect of melatonin (Parada et al., 2014).

Recently the $\alpha 7$ nAChR has been identified in signalling pathways of STI1 and PrP^C in hippocampal neurons (Beraldo et al., 2010, Ostapchenko et al., 2013a). STI1 binding to PrP^C leads to a physical interaction with the $\alpha 7$ nAChR, inducing calcium influx required for protection against staurosporine-induced apoptosis via PKA activity (Beraldo et al., 2010). Since calcium influx induced by STI1 is abolished in both PrP^C-null neurons or with $\alpha 7$ nAChR inhibition, STI1-PrP^C interaction is suggested to be upstream of $\alpha 7$ nAChR-induced calcium influx (Beraldo et al., 2010). One possible mechanism could be that STI1-PrP^C modulates the responsiveness of the $\alpha 7$ nAChR to choline present in the extracellular milieu, since the concentration of choline is too low to activate the $\alpha 7$ nAChR under basal conditions (Beraldo et al., 2010). The signalling pathways activated by STI1 and $\alpha 7$ nAChR overlap, including ERK1/2 inducing neuritogenesis and PKA inducing neuroprotection (Caetano et al., 2008, Beraldo et al., 2010). However, the role of the $\alpha 7$ nAChR in OGD-induced apoptosis is not fully understood.

1.5 ALK2 receptor

1.5.1 Activin receptors

ALK2 receptors are members of the activin receptor family, a group of growth and differentiation factors that belong to the transforming growth factor-beta (TGF- β) superfamily (Shi et al., 2003). These receptors are involved in various cell functions including cell proliferation and migration (Kitisin et al., 2007). Activin receptors are involved in many biological processes during early development, particularly in the differentiation of stem cells during organogenesis (Pauklin et al., 2015). In embryonic stem cells, activin signalling has been documented to be both necessary and sufficient in the maintenance of pluripotency of the epiblast (Vallier et al., 2004). On the other hand, activin signalling has been found to be an important step for the differentiation of adult stem cells (Kadaja et al., 2014). In the brain, activin signalling has been found to induce the differentiation of neural stem cells into cortical interneurons (Cambray et al., 2012). However, reliable detection of activin receptors has been difficult, and their roles in adult tissues are yet to be fully understood (Pauklin et al., 2015). Interestingly, it has recently been shown that decreased activity of the Activin A-Smad pathway leads to increased sensitivity to OGD as evidenced by higher apoptosis rates, suggesting that this pathway plays a neuroprotective role against OGD (Wang et al., 2013).

1.5.2 Activin signalling

Activin receptors have a cysteine-rich extracellular domain, a trans-membrane domain, and a cytoplasmic serine/threonine-rich domain (Kitisin et al., 2007). There are two types of activin receptors, categorized as type-I or type-II. Seven type-I receptors and five type-II receptors have been identified in the human genome (Manning et al., 2002).

All activin receptors transduce signals in a similar manner that involves one of each receptor dimers (Shi et al., 2003). Activation of type II receptor dimers (which are typically responsible for ligand-binding specificity) via ligand binding recruits the corresponding type I receptor dimer to form a hetero-tetrameric complex (Hardwick et al., 2008). Type II receptors, which are serine/threonine kinase receptors, phosphorylate serine residues on type I receptors (Kitisin et al., 2007). Generally, type-I receptors determine the downstream signalling pathway to be activated (Shi et al., 2003). Once type I receptors are phosphorylated, SMAD anchor for receptor activation (SARA) recruits receptor-regulated SMADs (R-SMADs) and orients the serine residue on the C-terminus of the R-SMAD for phosphorylation by the type I receptor (Kitisin et al., 2007). The phosphorylated R-SMADs dissociate from SARA, bind onto a co-SMADs, and form a complex that migrate to the nucleus and binds transcription factors to induce changes in gene expression (Kitisin et al., 2007).

1.5.3 BMP-4 in the brain

Bone morphogenetic proteins (BMPs) were first identified in protein extracts that were able to induce cartilage formation and *de novo* bone growth (Wozney et al., 1988). Since then, more than 20 BMPs have been identified and comprise the largest subgroup of the TGF-beta superfamily, with a wide range of cellular roles in addition to differentiation including proliferation, morphogenesis, and apoptosis (Hogan, 1996). Despite their wide range of cellular mechanisms, all BMPs signal in a similar fashion compared to other TGF-beta superfamily members, which is through activin receptors (Shi et al., 2003). BMP-4 has been found to play a critical role in the differentiation of neural stem cells (NSCs) into neurons, oligodendrocytes, and astrocytes during periods of

spatiotemporal development (Abematsu et al., 2006). Recent studies have shown that BMP-2 and BMP4 are highly expressed in astrocytes, thus identifying astrocytes as a major source of BMPs during development and differentiation of NSCs (Hu et al., 2012).

Outside of the central nervous system, BMP signalling has also been identified in the mammalian neuromuscular system (Chou et al., 2013). In neuromuscular junctions, BMP-4 has been identified to be expressed by Schwann cells and skeletal muscle fibers (Chou et al., 2013). During embryonic development, peripherally derived BMP-4 was found to play a role in the survival of motor neurons by protecting the cells from glutamate toxicity (Chou et al., 2013). However, whether or not BMP-4 can protect CNS neurons from other types of toxicity during ischemia is not well understood.

1.5.4 STI1 signalling via ALK2

Recent work has shown that ovarian cancer cells release large amounts of STI1 into the blood (Tsai et al. 2013). As a result, STI1 concentrations in the blood stream can be used as a potential diagnostic biomarker for patients with suspected ovarian cancer (Wang et al., 2010, Kim et al., 2010). Additionally it has been shown that in cancer cells, STI1 acts as a cytokine to stimulate cell proliferation (Wang et al., 2010). More recently, it has been shown that ALK2 receptors (Activin A receptor, type 1 or ACVR1) can be directly activated by STI1 in ovarian cancer cells (Tsai et al., 2012). Secreted STI1, acting in an autocrine and paracrine fashion, binds and activates ALK2, which results in cell proliferation via SMAD-regulated transcription of target genes such as inhibitor of DNA binding (IDs) and increased protein synthesis (Tsai et al., 2012). However, whether this STI1 signalling pathway is present in neurons is unknown.

1.5.5 The role of activin receptors during ischemia

A recent study focused on the role of SARA during ischemia showed that expression of SARA was significantly increased following OGD in PC12 cells (Wang et al., 2013). Furthermore, down-regulation of SARA by siRNA lead to decreased mRNA expression of Smad2, 3, and 4, as well as decreased phosphorylation of the Smad2 protein (Wang et al., 2013). As a result of decreased SARA activity and ultimately the Activin A-Smads pathway, there was an increased sensitivity in PC12 cells to OGD as evidenced by higher apoptosis rates. Taken together, the authors suggested that SARA, along with the ActA/Smad pathway, may play a protective role against OGD (Wang et al., 2013).

1.6 Rationale, Study Aim, and Hypothesis

Current treatment for ischemic stroke is limited to intra-venous thrombolytic therapy. Recent studies have shown that the therapeutic window of thrombolytic therapy is within 4 hours following ischemic onset (Lansberg et al., 2012). To develop novel therapies for stroke, the molecular mechanisms by which the brain protects itself against ischemia must be fully understood. One of these mechanisms activated during ischemia involves PrP^C and STI1. Studies have shown that the expression of both PrP^C and STI1 are increased during stroke, and that the level of PrP^C expression directly correlates with resistance against oxidative stress (McLennan et al., 2004, Lee et al., 2013, Brown et al., 1997). STI1, the ligand for PrP^C, has recently been shown to protect neurons from OGD-induced cell death (Beraldo et al., 2013). However, the exact mechanisms by which STI1-PrP^C protects neurons against ischemia is not fully understood. **The aim of this thesis is to investigate the role of the alpha-7 nicotinic acetylcholine receptor and the ALK2 receptor in STI1-PrP^C-mediated protection against ischemia.**

One of the receptors modulated by STI1-PrP^C is the $\alpha 7$ nAChR. The binding of STI1 to PrP^C leads to calcium influx via the $\alpha 7$ nAChR (Beraldo et al., 2010). Furthermore, the protective effect of STI1 against staurosporine has been previously shown to depend on the $\alpha 7$ nAChR (Beraldo et al., 2010). On the other hand, STI1 has also been shown to induce cell proliferation in ovarian cancer cells via the ALK2 receptor of the activin receptor family (Tsai et al., 2012). Recent studies have shown that inhibition of the downstream effectors of activin receptors exacerbates damage induced by OGD (Wang et al., 2013). However, the involvement of the $\alpha 7$ nAChR and the ALK2 receptor in STI1-PrP^C-mediated neuroprotection against ischemia is unknown. *We*

hypothesize that the $\alpha 7nAChR$ is required for STII-PrP^C-mediated neuroprotection against OGD.

2 Materials and Methods

2.1 Animals

The caspase 3 reporter mouse line (ApoMouse, Nicholls et al., submitted) provides a way to identify living neurons that will undergo caspase-3-mediated apoptosis in response to insults. This mouse line is genetically modified to express cerulean fluorescent protein (CFP). Activated caspase can be identified thanks to using bimolecular complementation technique (ApoMouse, Nicholls et al., submitted). The prion protein-knockout mouse line ($Prnp^{0/0}$), descendants of the *Zrch1* line, originally generated by Dr. Charles Weissman (Scripps Florida) was provided by Dr. Frank Jirik (University of Calgary), and had been backcrossed to C57BL/j for 10 generations (Khosrayani et al., 2008). The $\alpha 7$ nAChR-knockout mouse line (B6.129S7-*Chrna7*^{tm1Bay/J}) was purchased from Jax labs. The mouse line was originated on a mixed C57BL/6 and 129/SveV background and posteriorly backcrossed to C56BL/6 for more than 8 generations. The mouse was developed by the deletion of the last three exons of the *Chrna7* gene. Table 3 provides a summary of mice used in this thesis.

2.2 Ethics Statement

The animals used in this research were maintained by the University of Western Ontario Animal Care and Veterinarian Services. All procedures were done in accordance with approved animal use protocols at the University of Western Ontario (2008-127) (Appendix 1) following the guidelines of the Canadian Council of Animal Care (CCAC).

Table 3: Mouse lines used in this thesis

Strain	Background	Origin	Modification	Use
Prnp ^{0/0} (Zurich)	C57BL/6j	C. Weissmann but strain donated by Frank Jirik (U. Calgary)	Prnp (PrP ^C) knockout	To determine if neuroprotection by STI1 against OGD <i>in vitro</i> is dependent on the PrP ^C
B6.129S7 - <i>Chrna7</i> ^{tml} <i>Bay/J</i>	C57BL/6	Jax labs	<i>Chrna7</i> (alpha7 nicotinic receptor)	To determine the role of $\alpha 7$ nAChR in the STI1-PrP ^C – mediated neuroprotection <i>in</i> <i>vitro</i> against OGD
Apo mice	C57BL/6	Generated by Marc Caron at Duke University Medical Centre	Bimolecular fluorescence complementation technique of the Cerulean protein	To determine the caspase-3 activity of neurons following OGD

2.3 Reagents

Mouse recombinant STI1 (His₆-STI1) was generated and purified as described previously (Zanata et al., 2002). Staurosporine (Invitrogen Burlington ON, Canada), a protein kinase inhibitor that triggers apoptosis, was used as a positive control in apoptosis experiments (Chae et al., 2000). Staurosporine was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25mg/mL and stored at -20°C. The concentrations used for staurosporine were chosen based on their effectiveness at inducing apoptosis in neuronal cultures as determined by our previous studies (Beraldo et al., 2010). The competitive inhibitor of α 7nAChR, methyllycaconitine citrate (MLA, Abcam Cambridge MA, USA), was used to determine if the α 7nAChR is involved in the effects of STI1 (Bertrand et al., 1997). MLA was dissolved in saline at a concentration of 100mM and stored at -20°C. To activate α 7nAChR, its agonist PNU282987 [(N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride) Abcam Cambridge MA, USA] was used (Hajós et al., 2005). PNU 282987 was dissolved in water at a concentration of 100mM and stored at -20°C. Effective final concentrations used for MLA and PNU 282987 in neuronal cultures were determined by previous studies at 20nM and 0.5 μ M, respectively (Bodnar et al., 2005, Hu et al., 2007, Cheng et al., 2014). To inhibit the ALK2 receptor, LDN193189 (Abcam Cambridge MA, USA), a small molecule inhibitor of bone morphogenetic protein type 1 receptors, was used (Cuny et al., 2008). LDN 193189 was dissolved in water at a concentration of 10mM and stored at -20°C. Final LDN 193189 concentrations used were determined by previous experiments describing its effect as a highly selective antagonist of ALK2 at 5nM (Yu et al., 2008). BMP-4 (Sigma-Aldrich St. Louis MO, USA), a polypeptide from the TGF-beta superfamily of proteins, was used as an agonist

to ALK2 receptors (Chaikuad et al., 2012). BMP-4 was dissolved in 10mM citric acid at a concentration of 1mg/mL and stored at -20°C. BMP-4 (EC50 ranging from 5 – 10 ng/mL) used in our LIVE/DEAD experiments (final concentration of 10ng/mL - 100ng/mL) was determined by previous experiments that found 100ng/mL to be effective *in vitro* (Vrijens et al., 2013, La Rosa et al., 2011).

2.4 Primary cell culture

Male and female mice were paired and kept in the same cage for 3 days. After 3 days, mice were separated into individual cages. This provided a 3-day window of estimation for the age of the embryos. On day 15, successful mating was determined by observing the abdomen of the female mouse for visual confirmation of pregnancy. Pregnant females were euthanized by decapitation at embryonic day 17 and embryos were collected and kept on glass plates over ice until dissection. Embryonic brains were dissected under the microscope for removal of the cortex and the hippocampus. In brief, the mouse brain was separated into halves along the sagittal section (Figure 1A,B). With the superficial surface facing down, the meninges were removed from the forebrain by gently grasping the olfactory bulb using forceps (Figure 1C,D). Then, the midbrain was separated from the forebrain. Once again with the superficial surface of the forebrain facing down, the hippocampus was observed and removed from the cortex using forceps (Figure 1E,F). Once separated, the fimbriae of the hippocampus were removed by careful clipping using forceps (Figure 1H). Tissues were kept on ice in Eppendorf tubes with Hank's Balance Salt Solution (HBSS) until tissue dissociation.

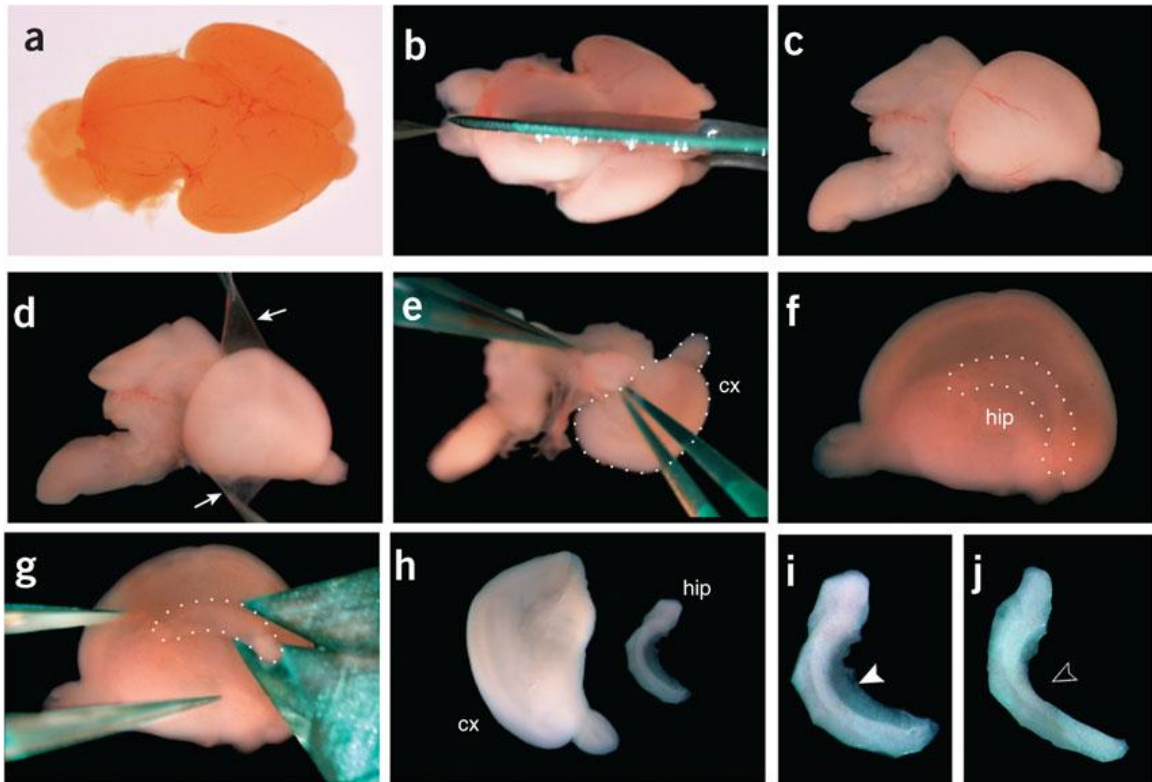


Figure 1: Dissection of hippocampus and cortex from embryonic E16.5 brains. (a)

The E16.5 mouse brain is (b) cut into halves via the midline sagittal section. (c) The halves are placed such that the cortex is facing up. (d) Meninges (arrows) are removed from the hemispheres with Dumont no. 5 forceps by gently pulling. (e) While holding the brainstem, the cortex (cx) is flipped outward and clipped off the midbrain. (f) The hippocampus (hip) is visible on the inner surface of the forebrain. (g) The hippocampus is removed with a microscissor. (h) Cortex (cx) and hippocampus (hip) are separated. (i,j) The fimbriae (arrowhead) on the concave side of the hippocampus are removed (open arrowhead). Image from Fath et al., 2008.

HBSS was then replaced with 37 °C trypsin-EDTA (0.25%, phenol red, (Life Technologies Burlington ON, Canada) and tubes were placed for 20 minutes in a 37°C water bath for tissue dissociation. Cells were then washed with sterile plating media (Minimum essential media with GlutaMAX, 20% Fetal Bovine Serum, 1% penicillin & streptomycin, 1% sodium pyruvate, 1.75% glucose, (Life Technologies) and incubated for 1 minute in plating media supplied with 0.2mg/mL DNase (Roche). Cells were then mechanically dissociated and homogenized by careful pipetting up and down. Cell concentration was determined by counting dissociated cells with a hemocytometer. Number of cells plated varied according to each experiment and plate sizes. Apo mouse neurons were plated in P35 (5×10^4 cells) glass-bottom dishes. Neurons for the LIVE/DEAD assay were plated in 4-well P15 (8×10^4 cells) dishes. Neurons for immunofluorescence were plated in 24-well P15 (5×10^4 cells) dishes.

Neurons were plated in dishes previously coated with poly-L-lysine under UV light overnight and washed with sterile HBSS prior to plating (Sigma Aldrich Canada). After 4 hours, the plating medium was removed and replaced with the maintenance medium (Neurobasal medium supplied with 1% penicillin/streptomycin mix, 0.5mM L-glutamate, and B27 supplement, Life Technologies). Cells were maintained at 37°C and 5% carbon dioxide for 7 days before experimentation. Half of the medium was replaced with fresh maintenance medium every two days. See Figure 2 for general experimental timelines.

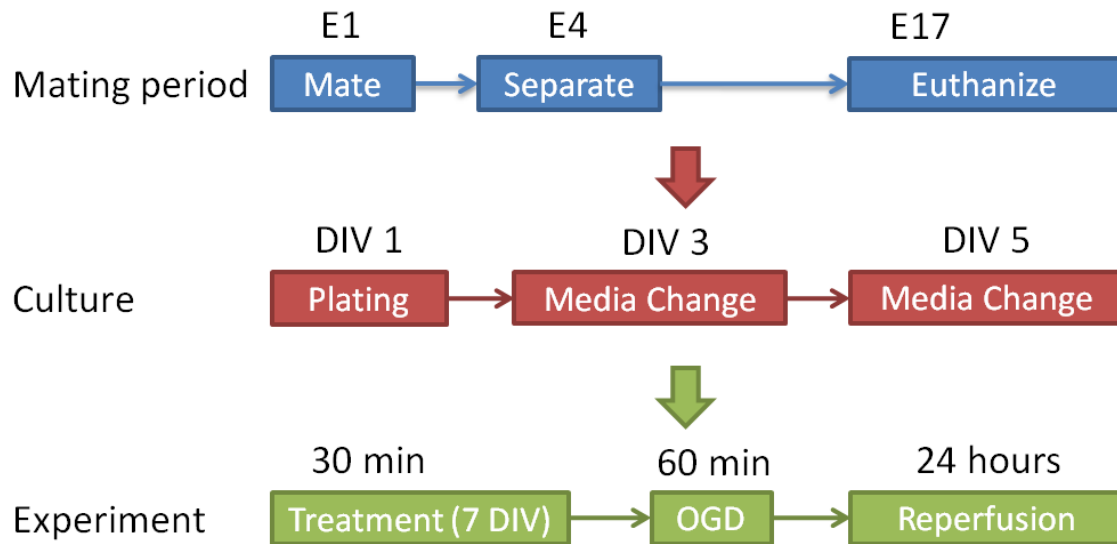


Figure 2: Schematic illustrating the mating, culturing, and experiment of hippocampal neurons.

2.5 Oxygen Glucose Deprivation

For oxygen glucose deprivation (OGD) experiments, the original media of the cells was replaced with a glucose-free maintenance media as described previously (Beraldo et al., 2013). Cells were then placed in an incubator (AutoFlow NU-4950 Oxygen and Humidity Control Water Jacket CO₂ Incubator, NuAire, Plymouth MN, USA) with 0% oxygen (95% nitrogen/5% carbon dioxide) for 1 hour, beginning when oxygen was completely depleted from the chamber. Upon completion of OGD, cells were removed from the incubator, glucose-free maintenance medium was replaced with the original maintenance medium, and the cells were returned to normal conditions at 37°C and 5% carbon dioxide for 3, 12, or 24 hours for reperfusion (Beraldo et al., 2013).

2.6 Immunofluorescence

After 7 days of culture *in vitro*, hippocampal neurons were isolated from E17 wild-type mice were fixed using 2% paraformaldehyde (Millipore) and permeabilized using 0.5% Triton X-100 (Millipore). Cells were then blocked using 2% BSA (Sigma) before applying the primary antibodies against NeuN (mouse, 1:200 dilution, Abcam) and ALK2 (rabbit, 1:200 dilution, Abcam) overnight at 4°C, followed by secondary antibodies anti-mouse Alexa Fluor-488 (1:1000 dilution, Invitrogen) and anti-rabbit Alexa Fluor-633 (1:1000 dilution, Life Technologies) for 1 hour at room temperature. Cells were also stained using Hoechst reagent (1µg/mL) to visualize the nucleus. Fifteen images were taken per embryo per treatment using a LSM 510 META confocal microscope (Zeiss Jena, Germany) equipped with 63x/1.4NA objective and analyzed using LSM 5 software (Zeiss Jena, Germany) and ImageJ (NIH).

2.7 Caspase-3 reporter mouse

We used a caspase 3 reporter mouse line to identify living neurons that are undergoing caspase-3-mediated apoptosis. In a healthy cell, expression of Apo transgenes produces two halves of CFP, tagged with recombinant sequences (inteins) and signal peptides attached to the protein via caspase 3 cleavage site. These signal peptides contain PEST protein degradation sequences and target CFP halves to different cellular compartments, which prevents formation of full fluorescent CFP. In apoptotic cells, caspase-3 activation removes the signal peptides and the degradation sequences, allowing the two halves to bind using complementary inteins and reconstitute the fully functional monomeric CFP (Figure 3). Fluorescence was quantified by using a confocal microscope (LSM 510 META, 10x, 485nm excitation, 530nm emission) and fluorescence per cell was normalized to the control cells that were not subjected to OGD. Eight images were taken per treatment per embryo dish, and four embryos were cultured per experiment.

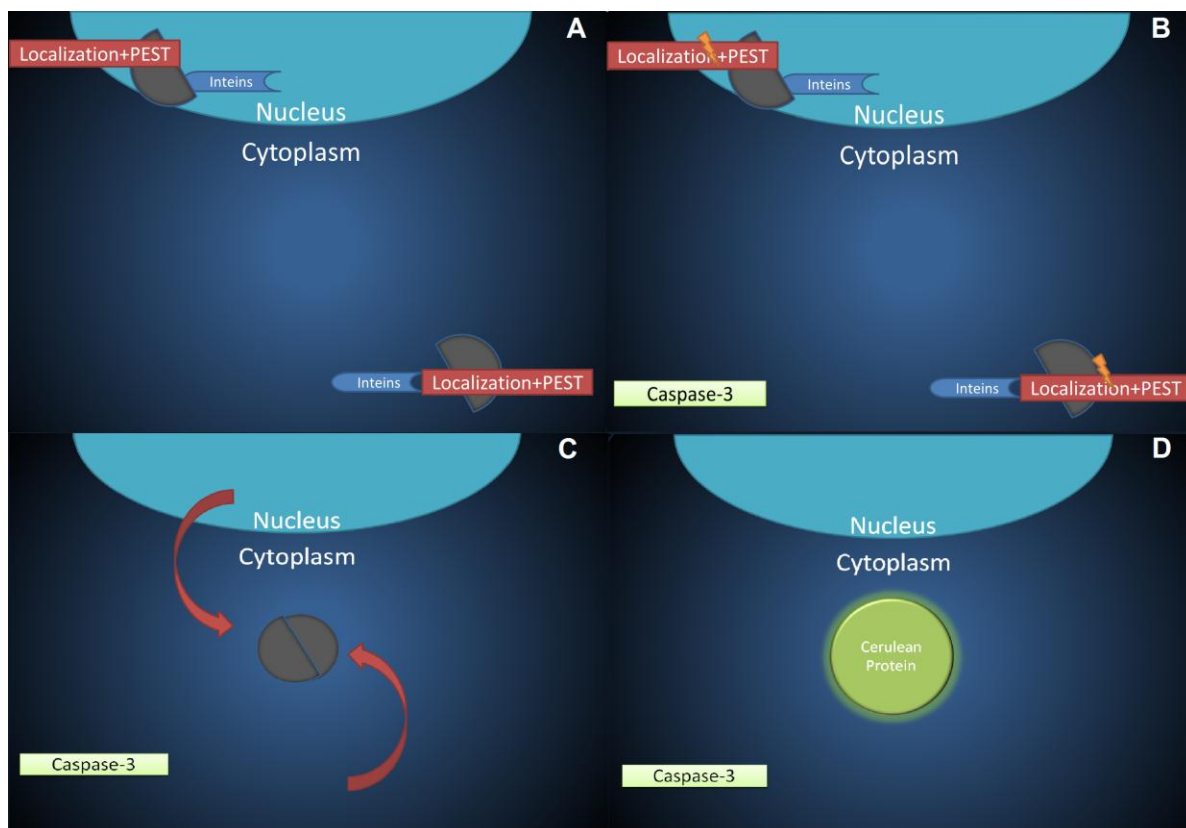


Figure 3: Schematic illustrating the caspase-3 reporter (Apo) mouse line. (A) Complementary inteins, subcellular localization sequences, and the PEST protein degradation sequences were added and the two halves were separated under basal conditions. (B) Caspase-3 activation led to cleavage of the subcellular localization and PEST protein degradation sequence. (C) Two halves freely diffuse throughout the cell and bind through complementary inteins. (D) Reconstitution resulted in the fully functional and fluorescent Cerulean protein.

2.8 LIVE/DEAD assay

The LIVE/DEAD mammalian cell viability assay (Invitrogen, Burlington ON, Canada) was used to analyze cell viability following OGD or staurosporine-treatment. Calcein-AM, a membrane permeable dye that expresses green fluorescence when cleaved by ubiquitous intracellular esterases, was used to determine the amount of live cells. Ethidium homodimer-1, a membrane impermeable dye that expresses red fluorescence upon binding to DNA and indicates loss of plasma membrane integrity, was used to determine the amount of dead cells. For the Ethidium homodimer-1 dye, 15 μ L of the 2mM stock solution [dissolved in DMSO/H₂O 1:4 (v/v)] was added per 10mL of maintenance solution for a working concentration of 3 μ M. For the Calcein-AM, 2.5 μ L of the 4mM stock solution (dissolved in anhydrous DMSO) was added per 10mL of maintenance solution for a working concentration of 1 μ M. Following the experiment, cells were incubated with the dye in the original maintenance medium for 30 minutes at 37°C at 5% carbon dioxide and then washed three times with Krebs-Ringers Henseleit (KRH) solution (in mM: 125 NaCl, 5 KCl, 5 HEPES, 2.6 MgSO₄, and 10 glucose, pH 7.2). After washing, the cells were imaged using an LSM 510 META confocal microscope equipped with 10x/0.4NA objective. Calcein-AM was excited using a fluorescein optical filter (485nm) and the Ethidium homodimer-1 was excited with a rhodamine optical filter (530nm). Fluorescent emissions were captured at 530nm and 645nm for the Calcein-AM and the Ethidium homodimer-1, respectively. Six random images were taken per embryo (dish) per treatment. Live (green) and dead (red) cells were counted using the Image-based Tool for Counting Nuclei (ITCN, Center for Bio-image Informatics at UC Santa Barbara) with a diameter of 16 pixels for live cells and 8

for dead cells. Threshold to distinguish two adjacent cells as individual cells was set at 1 pixel. Images were analyzed using ImageJ software (NIH) and cell death was calculated as the ratio of dead cells to the total number of cells.

2.9 Statistical Analysis

Data was obtained in each condition from cultures with at least 4 independent embryos of either sex. Caspase-3 activity from Apo mice was quantified in arbitrary units as fluorescence per cell normalized to the control. Cell death from the live/dead mammalian viability assay was quantified as percentage of total cells that are dead. All data were expressed as mean +/- standard error and analyzed using one-way or two-way ANOVA (When comparing multiple mouse lines) and Tukey's post-hoc test performed on GraphPad Prism (Version 5.0 for Windows, La Jolla California USA). In all cases, $P < 0.05$ was considered statistically significant.

3 Results

3.1 STI1 decreases apoptosis and neuronal death induced by OGD

To investigate the mechanisms involved with the actions of STI1 against OGD-induced cell death, hippocampal neurons obtained from a reporter mouse for caspase-3 activation were subjected to 1 hour OGD treatment and then reperfusion in the presence or absence of STI1. Caspase-3 activity and cell death were measured in the same fields using fluorescence from CFP and Ethidium homodimer-1 dye, respectively. Cells were imaged at 3, 12, and 24 hours of reperfusion after OGD. As a positive control, neurons were treated with 200nM of staurosporine for the same period of time as OGD treatment. Increased CFP fluorescence was observed in neurons after exposure to 1 hour OGD or in neurons treated with staurosporine as early as 3 hours after treatment (Figure 4A,B; $P < 0.05$). Interestingly, the time course of apoptosis fluorescence is distinct from the cell death fluorescence. Cerulean fluorescence was higher at 3 hours of reperfusion and decreased with time, whereas labeling of dead cells increased progressively until 24 hours (Figure 4A,B). Treatment of neurons with STI1 significantly decreased caspase-induced fluorescence compared to non-treated cells (Figure 4A,B; $P < 0.05$). Furthermore, cell death in neurons treated with STI1 was also significantly lower than non-treated neurons in all 3 durations of reperfusion (Figure 4A,C; $P < 0.05$).

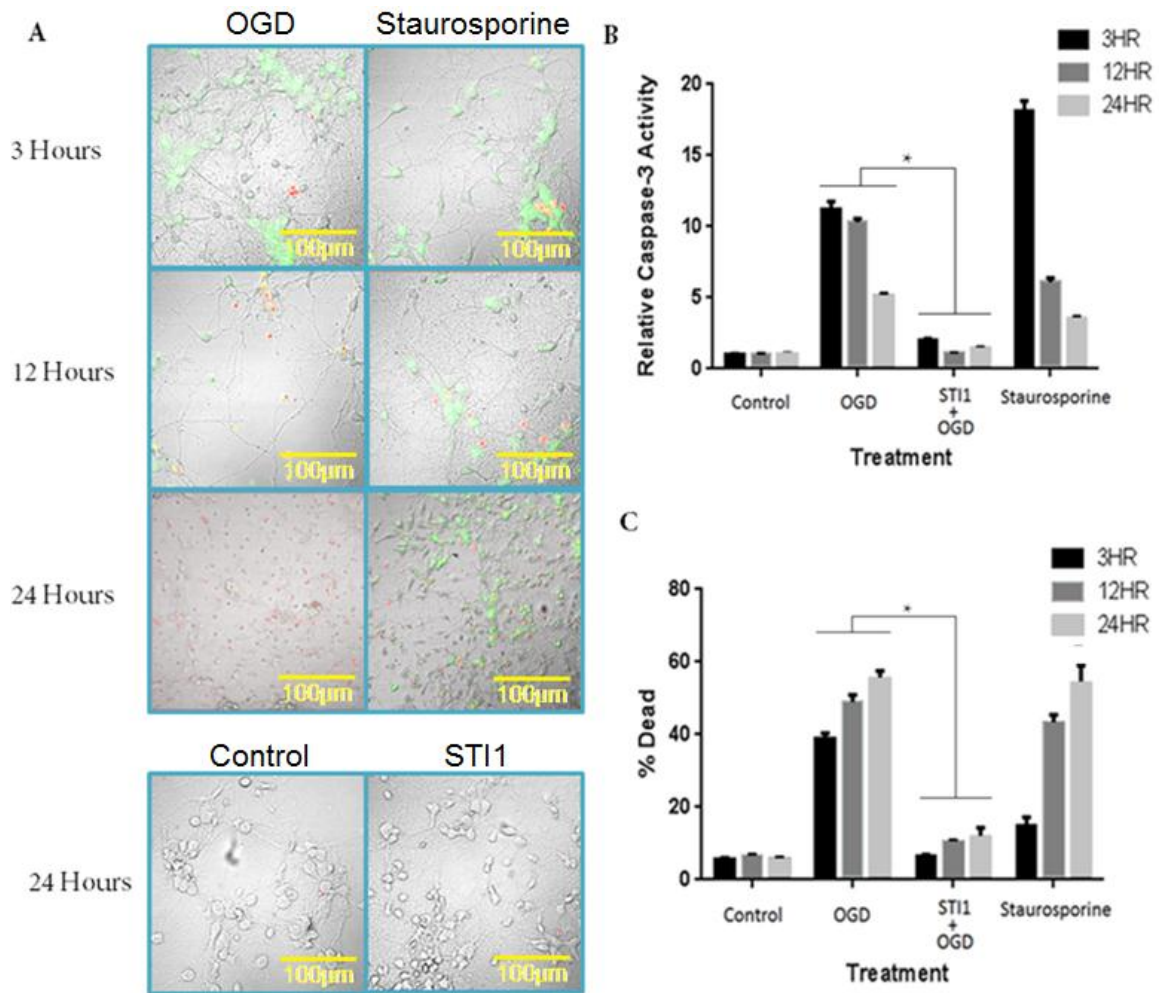


Figure 4: Time course of caspase-3 activity and cell death in neurons treated with STI1 and OGD. Apo mouse hippocampal neurons were treated with 1 μ M of recombinant STI1 (1 μ M, 30 minutes) and then subjected to 1 hour OGD. Cells were imaged at 3, 12, and 24 hours of reperfusion. (A) Representative images of cells per treatment at different time points as indicated on the left. Green fluorescence indicates activated caspase-3 and red fluorescence (Ethidium Homodimer-1 staining) indicates dead cells. Neurons treated with staurosporine for 3, 12, and 24 hours were also imaged using the same methods. (B,C) Caspase-3 activity was quantified in arbitrary units as fluorescence per cell normalized to the control. Cell death was quantified as percentage of total cells that were dead. Treatment of neurons with STI1 significantly decreased

caspace-3 fluorescence in all 3 durations of reperfusion. The number of dead cells in neurons treated with STI1 remained significantly lower than on neurons that were not treated with STI1 (OGD)- or staurosporine-treated cells. * $P < 0.05$. Results are presented as mean \pm SEM; data were analyzed and compared by two-way ANOVA and Tukey's post hoc test. (n=4).

Previous experiments have suggested that STI1-mediated neuroprotection against staurosporine or OGD-induced cell death is PrP^C-dependent (Lopes et al., 2005, Caetano et al., 2008, Beraldo et al., 2010, Beraldo et al., 2013). To confirm whether STI1 prevents neuronal death in a PrP^C-dependent way, wild-type and *Prnp*^{0/0} hippocampal neurons were treated with STI1 (1 μ M) and then submitted to 1 hour OGD and 24 hour reperfusion prior to live/dead imaging. Treatment with STI1 significantly decreased cell death induced by OGD in wild-type neurons (Figure 5; $P < 0.05$). However, treatment with STI1 was ineffective to decrease cell death in *Prnp*^{0/0} hippocampal neurons (Figure 5).

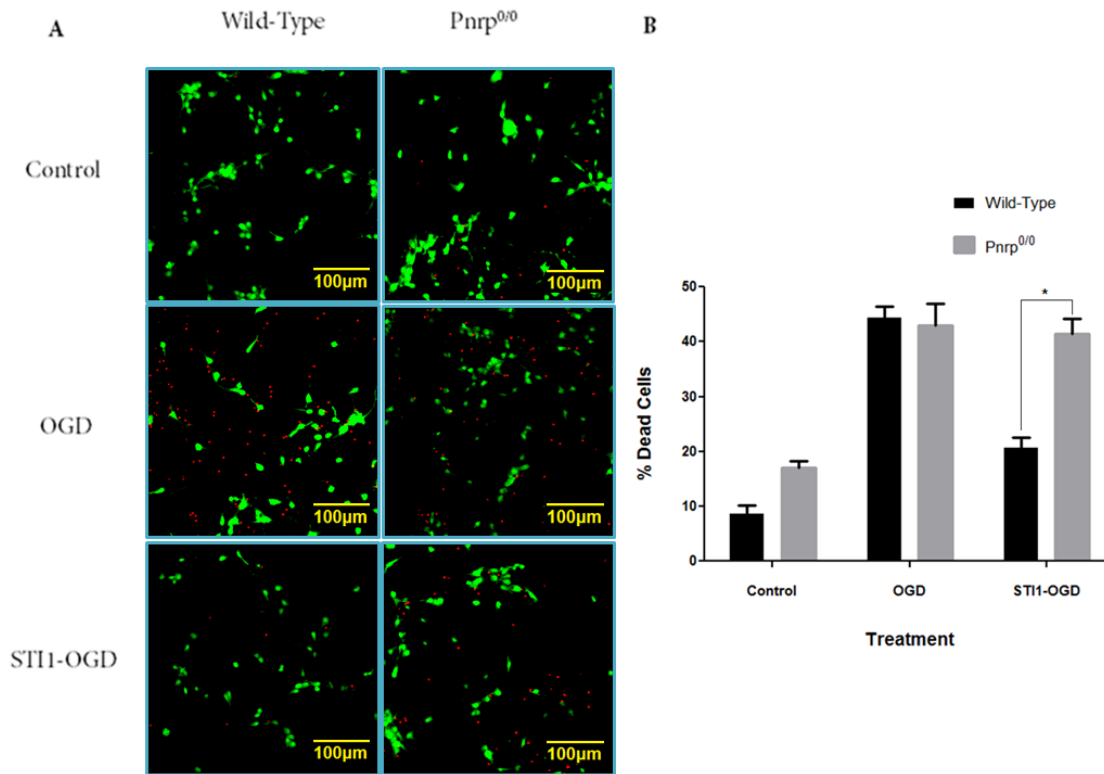


Figure 5: Treatment of wild-type and *Pnnp*^{0/0} hippocampal neurons with STI1 and OGD. Wild-type (n=4) and *Pnnp*^{0/0} (n=4) hippocampal neurons were treated with recombinant STI1 (1 μM, 30 minutes) and then subjected to 1 hour OGD. (A) Representative images obtained by confocal microscopy. (B) Cell death was quantified as percentage of total cells that were dead using Calcein-AM (Live, green) and Ethidium homodimer-1 (dead, red) dyes. Treatment of neurons with STI1 decreased OGD-induced cell death in wild-type cells, but not in *Pnnp*^{0/0} cells. Results are presented as mean +/- SEM; data were analyzed and compared by two-way ANOVA and Tukey's post hoc test. (n=4). *P<0.05.

3.2 STI1 engages $\alpha 7$ nAChR for neuroprotection

Previous experiments suggested that STI1 can engage a complex containing PrP^C and $\alpha 7$ nAChRs in hippocampal neurons for neuronal signalling and protection against staurosporine-induced neuronal cell death (Beraldo et al., 2010). However, in other neuronal types and cancer cells, STI1 can activate signalling independent of PrP^C, $\alpha 7$ nAChR or both (Arruda-Carvalho et al., 2007, Tsai et al., 2012, Santos et al., 2013). To determine whether $\alpha 7$ nAChRs are important for STI1-mediated neuroprotection against ischemic insults, wild-type hippocampal neurons were treated with several concentrations of the $\alpha 7$ nAChR-selective antagonist MLA for 30 minutes prior to treatments with 1 μ M recombinant STI1 and 1 hour OGD plus reperfusion. Inhibition of $\alpha 7$ nAChRs by MLA attenuated the neuroprotective effect of STI1 against OGD (Figure 6; $P < 0.05$). Interestingly, even neurons treated with the highest dose of MLA and STI1 presented better survival than untreated neurons, where treatment with MLA was not able to completely abrogate the neuroprotective effect of STI1 against OGD (Figure 6A,B). To further investigate the role of $\alpha 7$ nAChR in STI1-mediated neuroprotection against ischemic insults, we then used cultured hippocampal neurons obtained from $\alpha 7$ nAChR-knockout mice. Hippocampal neurons were treated with 1 μ M of recombinant STI1 and exposed to OGD for 1 hour and reperused for 24 hours. Treatment of wild-type neurons with STI1 significantly decreased cell death compared to neurons that were not treated with STI1 (Figure 7; $P < 0.05$), whereas this effect is lost in $\alpha 7$ nAChR-knockout mouse hippocampal neurons (Figure 7). In contrast, treatment of neurons with staurosporine induced levels of cell death not significantly different between wild-type and $\alpha 7$ nAChR-knockout mouse neurons (Figure 7). These results further support the finding that STI1

selectively engages PrP^C/α7nAChRs to protect neurons against OGD/reperfusion-mediated cell death.

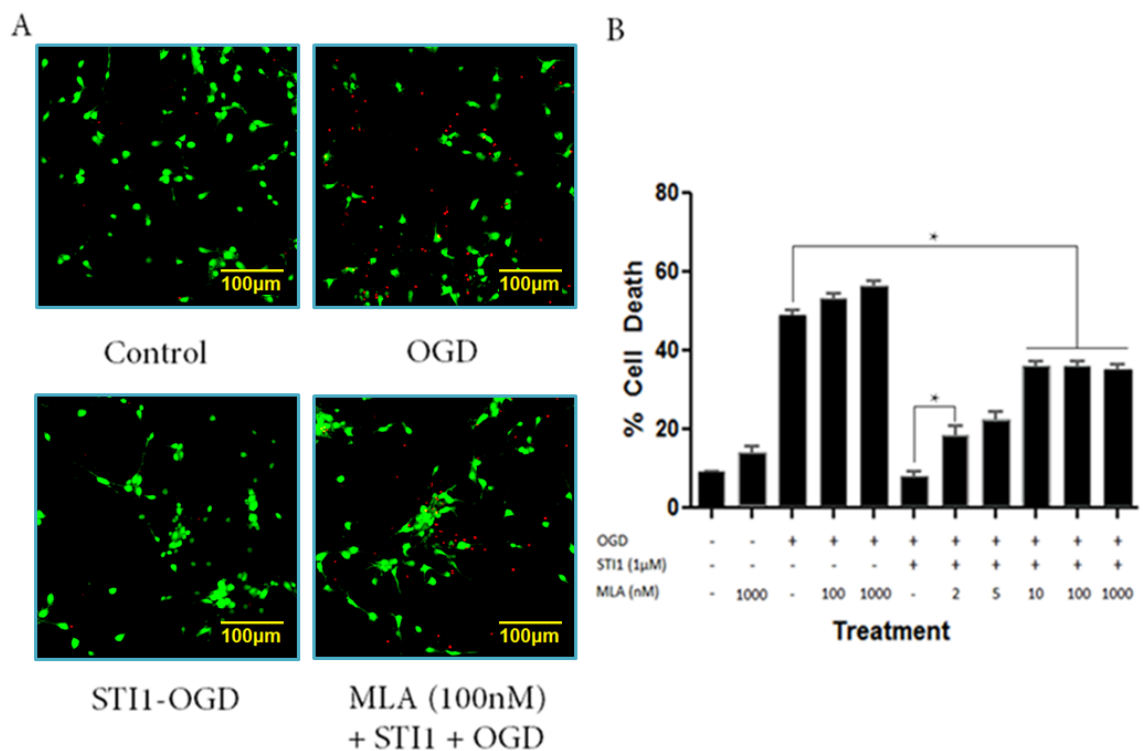


Figure 6: Neuroprotection by STI1 against OGD is attenuated in wild-type hippocampal neurons treated with $\alpha 7$ nAChR inhibitor MLA. Wild-type hippocampal neurons were treated with a MLA (2-1000nM) and recombinant STI1 (1µM, 30 minutes) and then subjected to 1 hour OGD. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using Calcein-AM (Live, green) and Ethidium homodimer-1 (dead, red) dyes. While treatment of neurons with STI1 decreased OGD-induced cell death, neurons were no longer protected when treated with the $\alpha 7$ nAChR inhibitor MLA. Results are presented as mean \pm SEM; data were analyzed and compared by one-way ANOVA and Tukey's post hoc test. (n=4). *P<0.05.

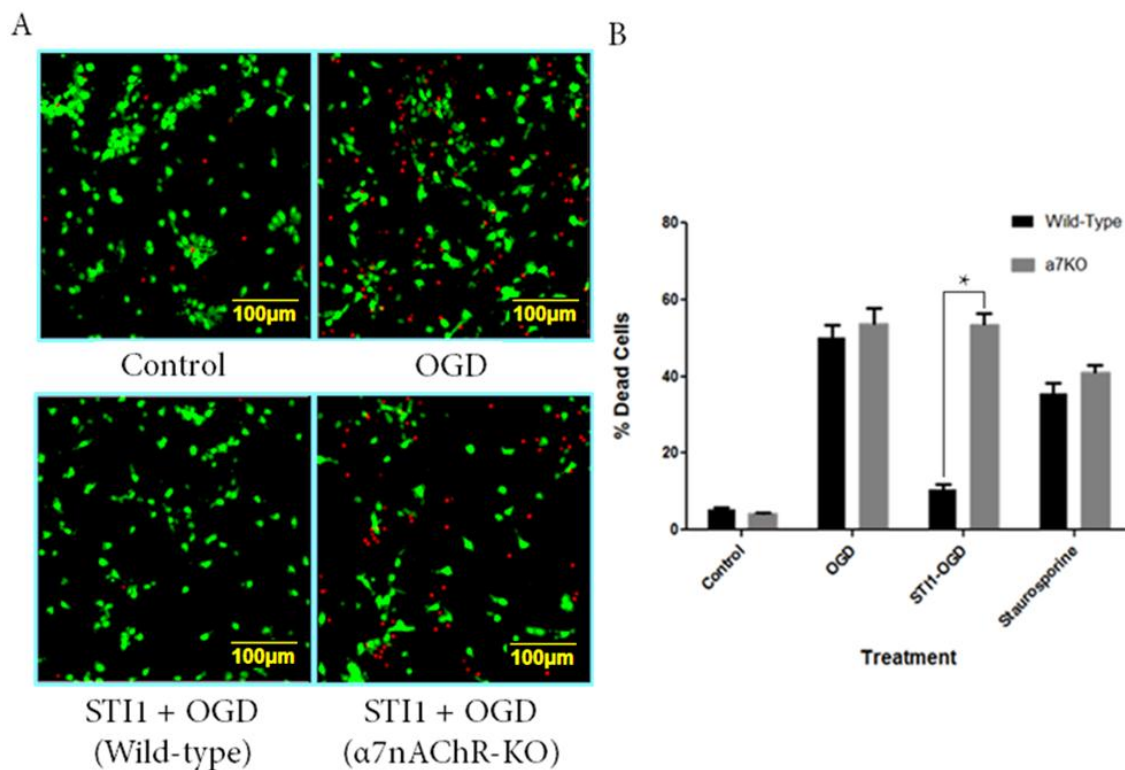


Figure 7: Neuroprotection against OGD by STII is prevented in hippocampal neurons from alpha-7 nicotinic acetylcholine receptor ($\alpha 7$ nAChR) knock-out mice. Wild-type and $\alpha 7$ nAChR-KO hippocampal neurons were treated with recombinant STII (1 μ M, 30 minutes) and then subjected to 1 hour OGD. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using Calcein-AM (live, green) and Ethidium homodimer-1 (dead, red) dyes. Treatment of wild-type neurons with STII significantly decreased OGD-induced cell death, an effect not observed in $\alpha 7$ nAChR-KO neurons. Results are presented as mean \pm SEM; data were analyzed and compared by two-way ANOVA and Tukey's post hoc test. (n=4). *P < 0.05.

3.3 Activation of the $\alpha 7$ nAChR protects neurons against OGD

Previous experiments have suggested that nicotinic receptors may provide a new pathway to interfere with outcomes in hemorrhagic stroke (Krafft et al., 2012) and activation of nicotinic receptors can protect neurons against OGD (Li et al., 2013). To further investigate the potential for activation of $\alpha 7$ nAChR to protect neurons against OGD-induced cell death, we used PNU 282,987, a selective $\alpha 7$ nAChR agonist. Wild-type mouse hippocampal neurons were treated with variable concentrations of PNU 282,987 for 30 minutes prior to exposure to OGD. Activation of $\alpha 7$ nAChR with PNU 282,987 impaired neuronal death induced by OGD in a dose-dependent manner (Figure 8A,B; $P < 0.05$). Furthermore, we tested whether this effect of PNU 282,987 was selective to $\alpha 7$ nAChRs. Similar to the effects of STI1, PNU 282,987 was unable to prevent OGD-induced neuronal death of $\alpha 7$ nAChR-knockout neurons (Figure 8A,C).

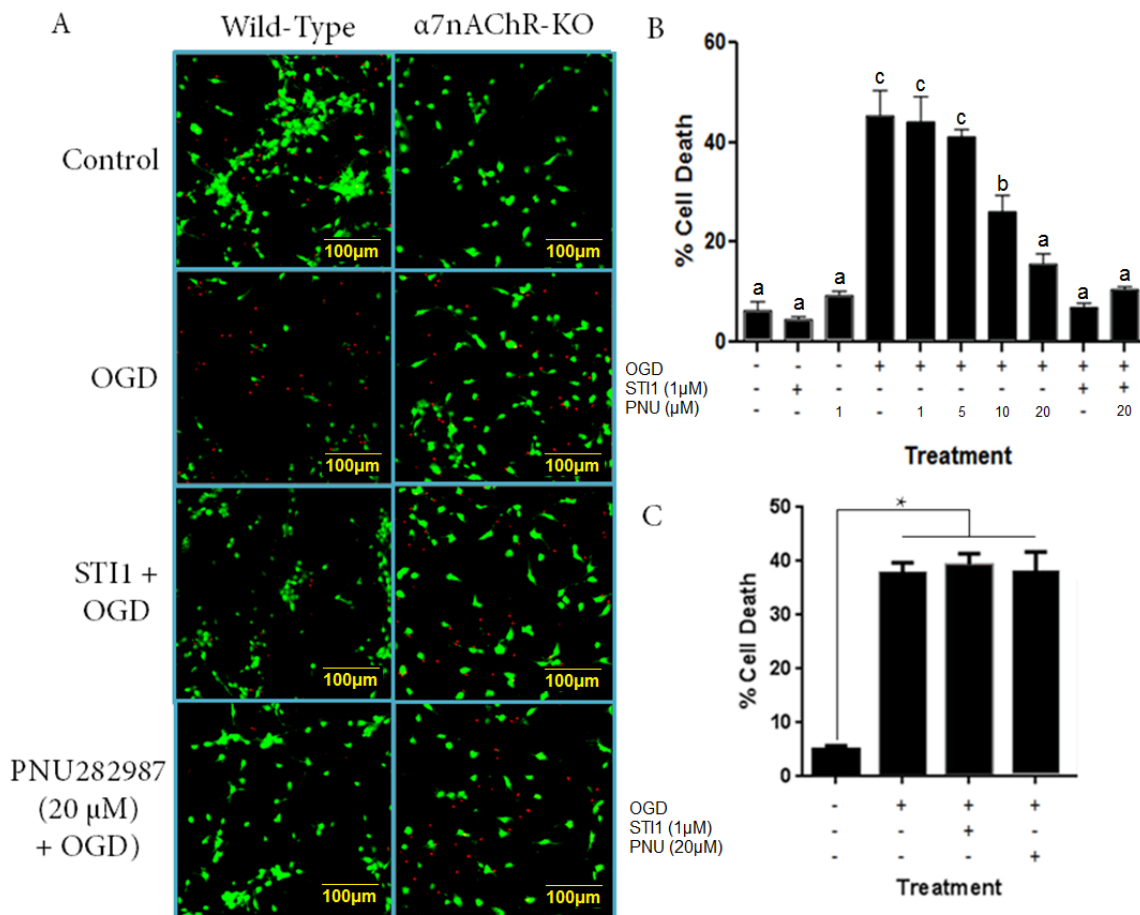


Figure 8: Treatment of wild-type hippocampal neurons with the $\alpha 7$ nAChR agonist PNU 282,987 significantly reduced cell death due to OGD. Wild-type and $\alpha 7$ nAChR-KO hippocampal neurons were treated with recombinant STI1 (1µM) and/or PNU 282,987 (1-20 µM, 30 minutes) and then subjected to 1 hour OGD. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B,C) Cell death was quantified as percentage of total cells that were dead using Calcein-AM (live, green) and Ethidium homodimer-1 (dead, red) dyes. Treatment of neurons with 20µM of PNU 282,987 significantly decreased OGD-induced cell death (B). However, this protection was not observed in $\alpha 7$ nAChR-KO neurons (C). Results are presented as mean \pm SEM; data were analyzed and compared by one-way ANOVA and Tukey's post hoc test. (n=4). * or different letters above the bars indicate significant difference ($P < 0.05$), whereas bars denoted with the same letter are not significantly different ($P > 0.05$).

3.4 STI1 activates ALK2 receptors

Previous experiments have shown that STI1 can also modulate signalling pathways independent of $\alpha 7$ nAChRs in astrocytes (Arantes et al., 2009), peripheral neurons (Santos et al., 2013), and cancer cells (Tsai et al., 2012). To further test whether other signalling pathways can be activated by STI1 in hippocampal neurons, we focused on ALK2, a poorly studied receptor in the brain. Expression data obtained from the Allen Brain Atlas indicate selective expression of ALK2 receptors in the hippocampus (Figure 9A). Staining of mouse hippocampal neurons for the ALK2 receptor shows membrane expression in NeuN labeled neurons (Figure 9B).

To determine the potential involvement of the ALK2 receptor in STI1-mediated neuronal protection against OGD, wild-type mouse hippocampal neurons were treated with variable concentrations of the ALK2 antagonist LDN 193198 and 1 μ M of recombinant STI1 prior to exposure to OGD for 1 hour followed by 24 hours of reperfusion. Our results show that inhibition of the ALK2 receptor prevented the effect of STI1 against OGD (Figure 10; $P < 0.05$), suggesting the potential involvement of ALK2 receptors.

To further test the possibility that activation of ALK2 receptors can modulate cell death in response to ischemia-reperfusion we used the ALK2 agonist BMP-4. Treatment of neurons with variable concentrations of BMP-4 significantly decreased cell death induced by ischemia-reperfusion (Figure 11; $P < 0.05$).

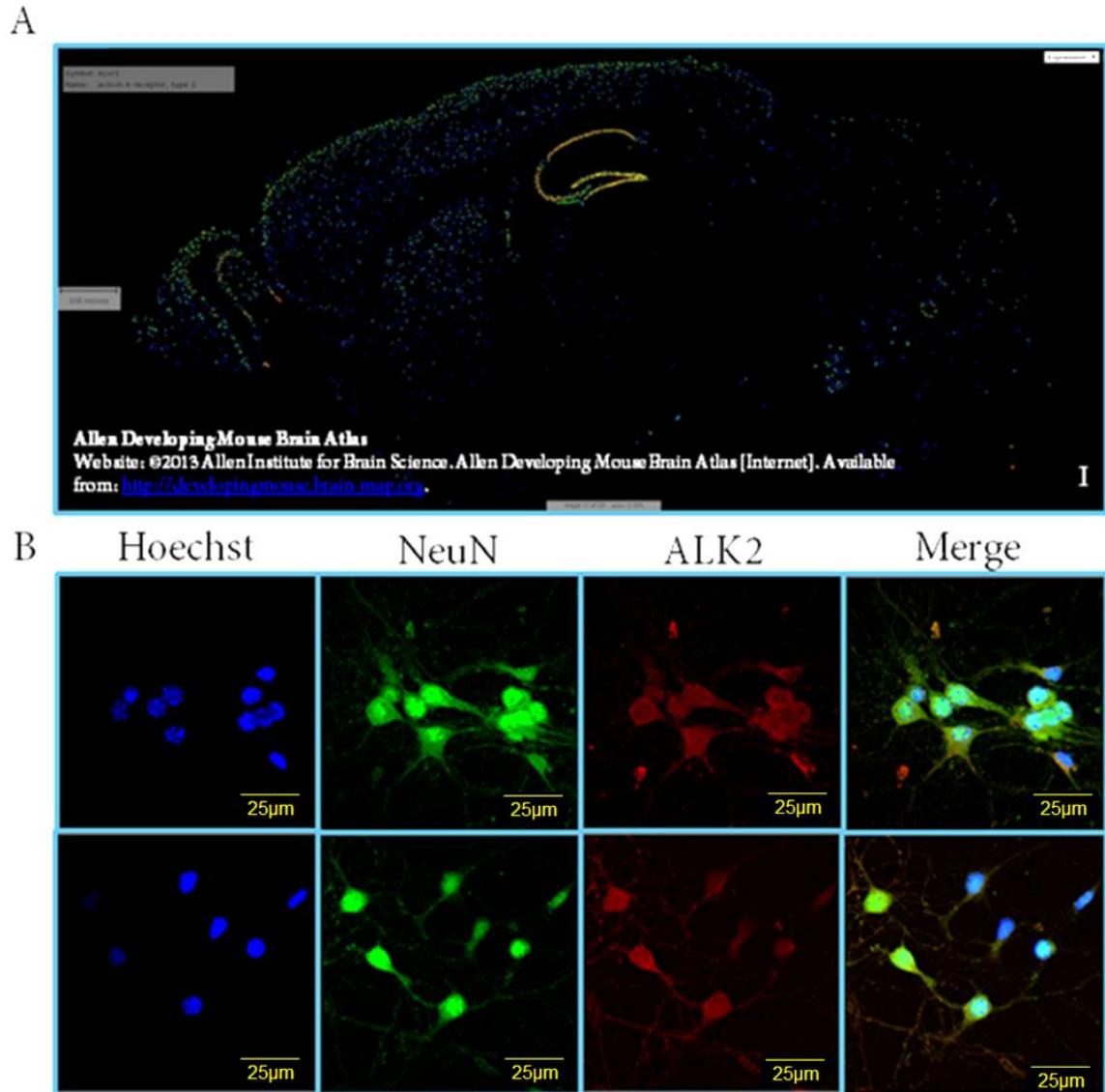


Figure 9: Expression of ALK2 in primary cultured wild-type hippocampal neurons.

(A) The Allen Developing Mouse Brain Atlas image showing *in situ* hybridization for the *acvr1* gene (ALK2) in a mouse brain sagittal section. Warmer colours indicate higher gene expression. (B) Wild-type mouse E17 primary hippocampal neurons after 7 days in culture were stained with nuclear marker Hoechst (blue), mature neuronal marker NeuN (green), and ALK2 (red). Composite image shows ALK2 and NeuN colocalization. ALK2 is enriched in the cell membrane.

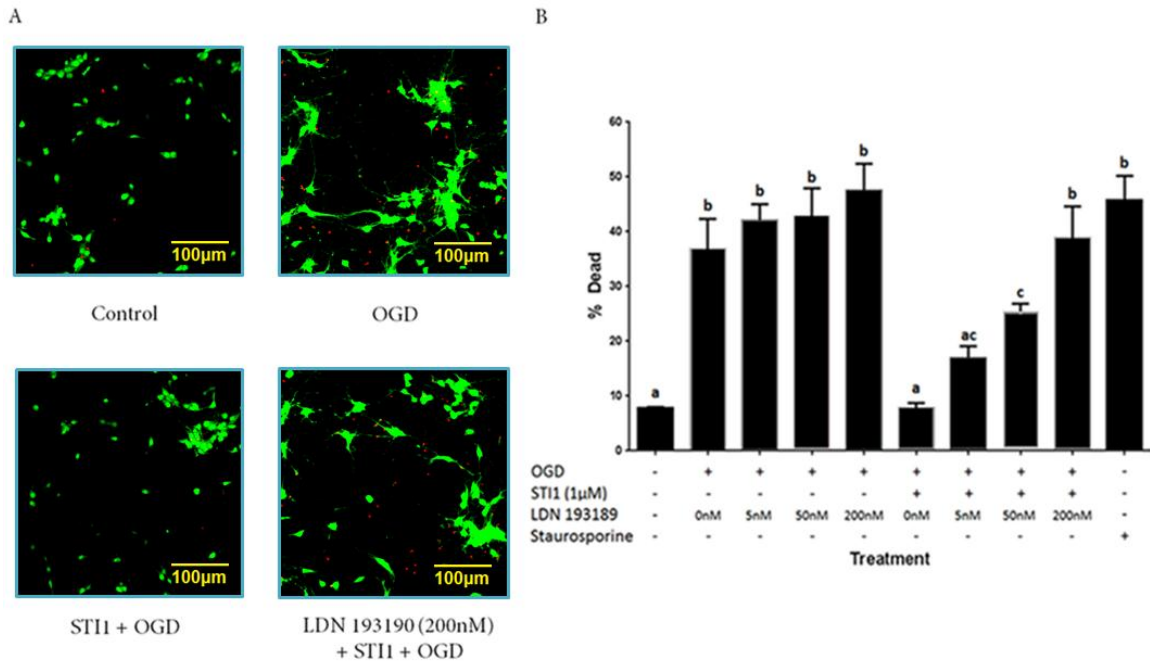


Figure 10: Inhibition of the ALK2 receptor prevented the effects of STI against OGD. Wild-type hippocampal neurons were treated with LDN 193189 (5-200nM) and/or recombinant STI1 (1µM, 30 minutes) and then subjected to 1 hour OGD. Staurosporine (200nM) was also used as a positive control. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using Calcein-AM (live, green) and Ethidium homodimer-1 (dead, red) dyes. Treatment of neurons with STI1 significantly decreased cell death by OGD, an effect not observed when neurons were treated with LDN 193189 in which cell death increased in a dose-dependent manner ($P < 0.05$). Results are presented as mean \pm SEM; data were analyzed and compared by one-way ANOVA and Tukey's post hoc test. ($n=8$). Different letters above the bars indicate significant differences ($P < 0.05$), whereas bars denoted with the same letter are not significantly different ($P > 0.05$).

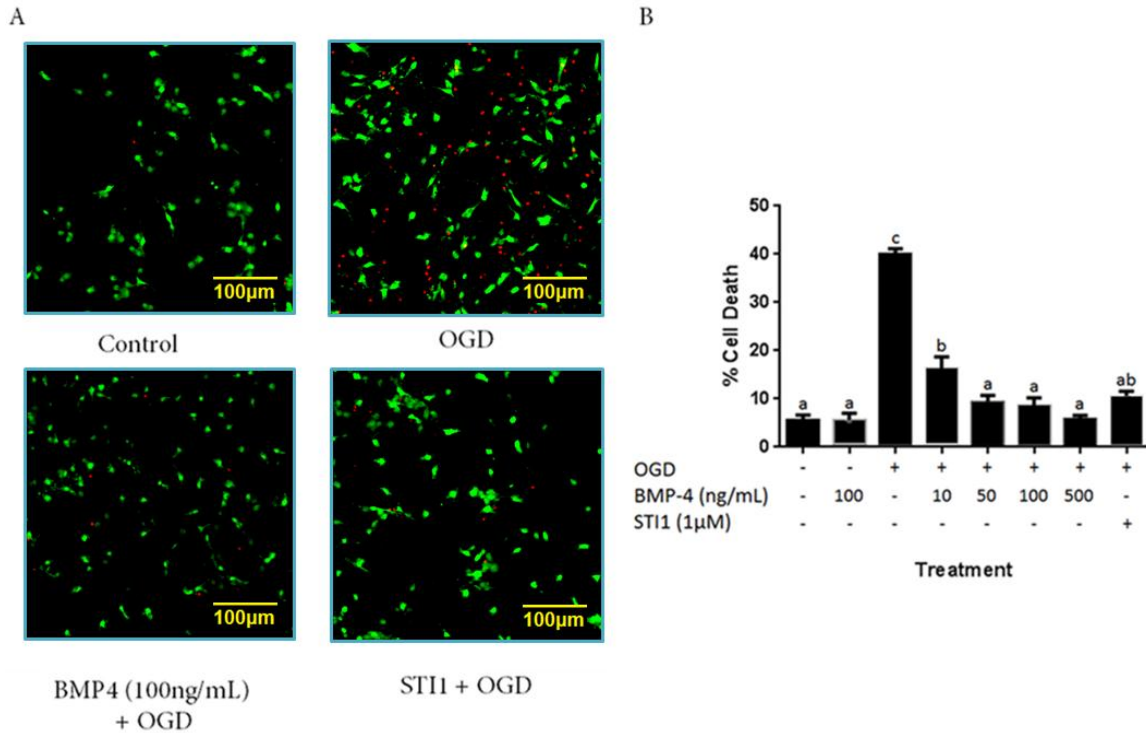


Figure 11: Treatment of wild-type hippocampal neurons with BMP-4 significantly decreased cell death due to OGD treatment. Wild-type hippocampal neurons were treated with recombinant STII (1µM) and BMP-4 (10-500ng/mL, 30 minutes) and then subjected to 1 hour OGD. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using calcein-AM (Live, green) and ethidium homodimer-1 (dead, red) dyes. Treatment of neurons with 10ng/mL of BMP-4 for 30 minutes prior to 1 hour OGD significantly decreased cell death compared to neurons that were not treated with BMP-4. At 50ng/mL of BMP-4, cell death following OGD was not significantly different from cells treated with STII. Results are presented as mean \pm SEM; data were analyzed and compared by one-way ANOVA and Tukey's post hoc test. (n=4). Different letters above the bars indicate significant difference ($P < 0.05$), whereas bars denoted with the same letter are not significantly different ($P > 0.05$).

3.5 Treatment of neurons with BMP-4 during reperfusion rescues neurons

The manipulations reported so far using STI1 are known to activate several distinct and overlapping signalling pathways (Caetano et al., 2008, Zanata et al., 2002, Tsai et al., 2012) prior to submitting neurons to ischemia-reperfusion. It would be relevant for future pharmacological manipulations to understand whether activation of these signalling pathways by STI1 after the onset of ischemia can also prevent neuronal death. To answer that question, neurons were treated with STI1, PNU 282987 or BMP-4 prior to induce ischemia or just after the OGD treatment (during reperfusion). Figure 12 reveals that, as expected, activation of PrP^C/α7nAChRs by STI1 (1μM) or α7nAChRs by PNU 282987 (20μM) prevents neuronal death if neurons are treated prior to the OGD exposure. In contrast, these two treatments were ineffective if the neurons were exposed to them after the period of ischemia (Figure 12).

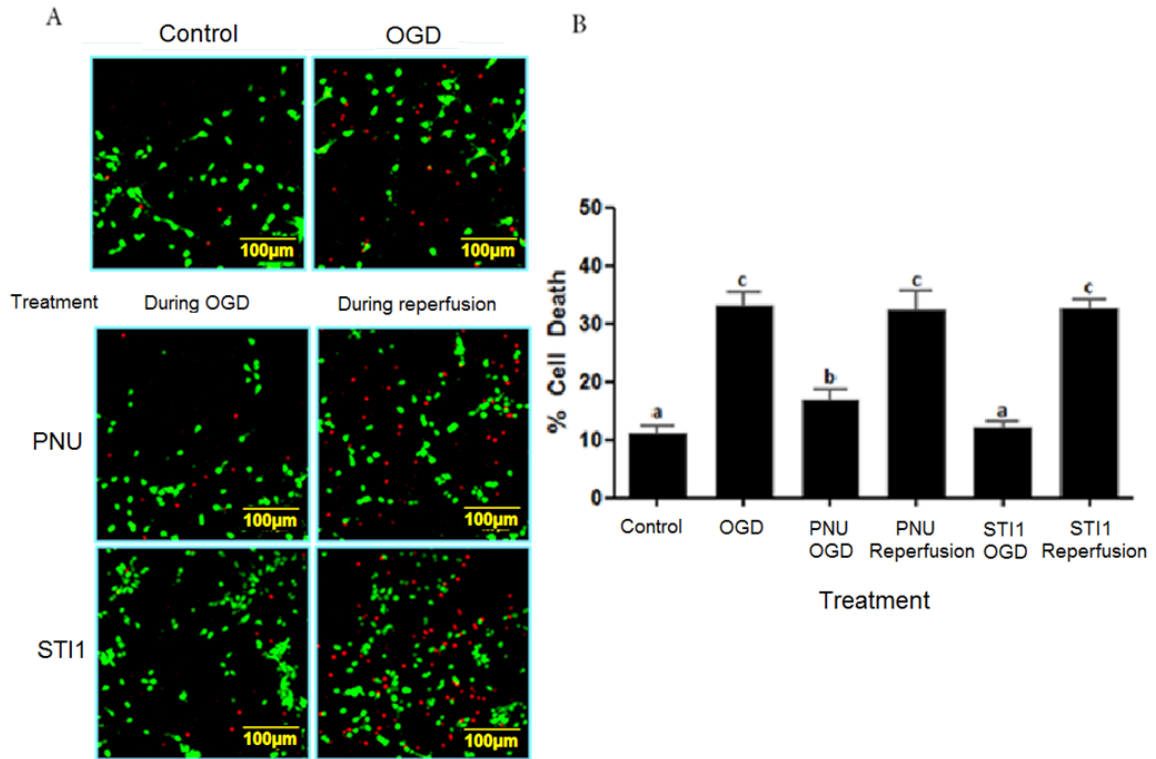


Figure 12: PNU 282987 and STI1 only protected neurons if treated prior to OGD, but not during reperfusion. Wild-type hippocampal neurons were treated with recombinant STI1 (1µM) or PNU 282,987 (1-20 µM, 30 minutes) and then subjected to 1 hour OGD or treated during reperfusion. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using calcein-AM (live, green) and ethidium homodimer-1 (dead, red) dyes. Treatment of neurons with STI1 or PNU 282987 decreased OGD-induced cell death, an effect not observed when these drugs were treated during the reperfusion period (B). Results are presented as mean \pm SEM; data were analyzed and compared by one-way ANOVA and Tukey's post hoc test. (n=4). Different letters above the bars indicate significant difference ($P < 0.05$), whereas bars denoted with the same letter are not significantly different ($P > 0.05$).

Interestingly, BMP-4 (100ng/mL) was effective at protecting neurons against OGD-induced cell death if it was used prior or after the OGD treatment (Figure 13). In addition, prevention of neuronal death by BMP-4 was attenuated by the ALK2 selective antagonist LDN 193189 (200nM) (Figure 13). The concentrations used in these experiments were established as the minimum concentration for the maximum effect on cell death following OGD-reperfusion observed in the previous experiments. That is, we found that the level of cell death observed in STI1-treated neurons that received 200nM of LDN 193189 was similar to level of cell death observed in neurons submitted to OGD that did not receive STI1 (Figure 10B). For BMP-4, we found that with treatment of neurons with 100ng/mL was able to decrease cell death following OGD to a level not significantly different to cell death observed in neurons not submitted to OGD, or STI1 treated neurons submitted to OGD (Figure 11). Thus, we used these concentrations in our experiments with treatments during reperfusion.

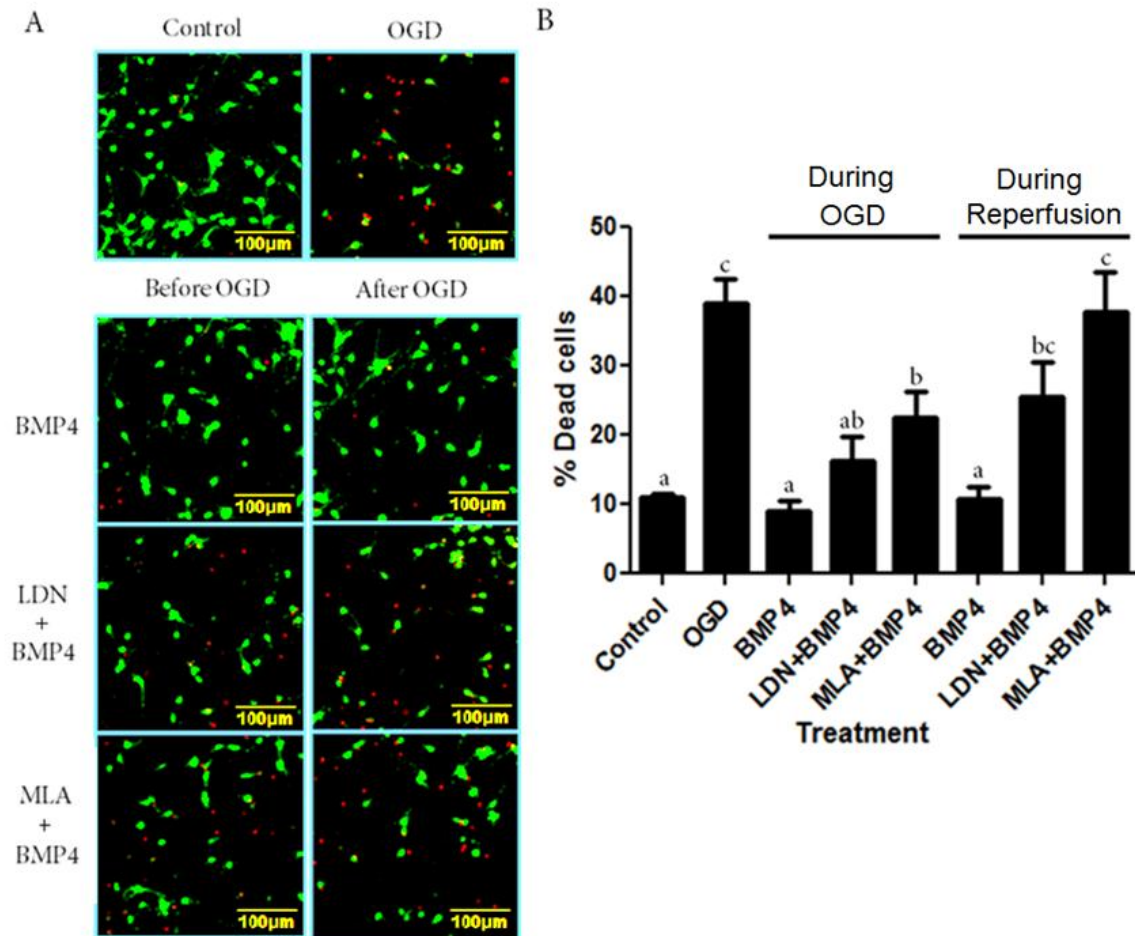


Figure 13: Treatment neurons with BMP-4 during reperfusion significantly decreased OGD-induced cell death. Wild-type hippocampal neurons were treated with MLA (10nM), LDN 193189 (200nM), and/or BMP-4 (100ng/mL, 30minutes) and then subjected to 1 hour OGD. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using calcein-AM (Live, green) and ethidium homodimer-1 (dead, red) dyes. Treatment of BMP-4 (100ng/mL) either before OGD or during reperfusion significantly reduced cell death. Treatment of either LDN 193189 or MLA prior to OGD decreased protection. Results are presented as mean \pm SEM; data were analyzed and compared by one-way ANOVA and Tukey's post hoc test. (n=4). Different letters above the bars indicate significant difference ($P < 0.05$), whereas bars denoted with the same letter are not significantly different ($P > 0.05$).

We have shown that neuroprotection by STI1 depends on PrP^C (Figure 5). We then tested whether neuroprotection by BMP-4 and PNU 282,987 bypasses the PrP^C-dependent step activated by STI1 or if it also requires PrP^C for preventing neuronal death induced by OGD. We treated wild-type and *Prnp*^{0/0} neurons with BMP-4 and PNU 282,987 and compared cell death between the two after OGD and reperfusion. In the absence of PrP^C, both BMP-4 and PNU 282,987 were still able to prevent OGD-induced neuronal death (Figure 14). Furthermore, treatment of BMP-4 during reperfusion still protected the neurons against OGD even in the absence of PrP^C. Interestingly, in line with a potential role of α 7nAChRs after BMP-4 treatment, MLA or absence of α 7nAChRs completely abolished the rescue from cell death in BMP-4-treated neurons (Figure 15).

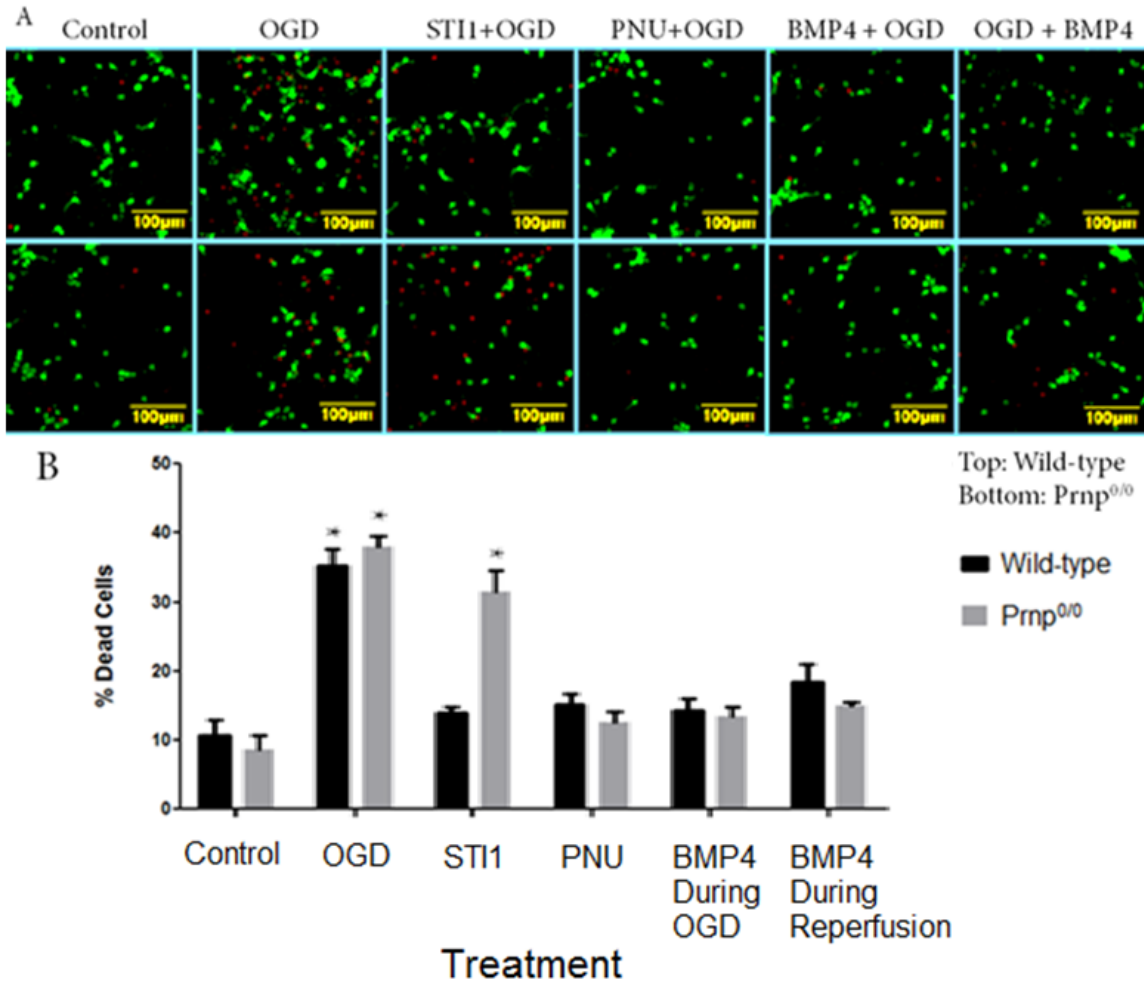


Figure 14: Neuroprotection against OGD by PNU 282,987 and BMP4 was independent of the PrPC. Wild-type and *Prnp*^{0/0} hippocampal neurons were treated with recombinant STI1 (1 μM), PNU 282,987 (20 μM), or BMP-4 (100ng/mL, 30minutes) and then subjected to 1 hour OGD. BMP-4 was also treated during reperfusion in a separate experiment group. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using Calcein-AM (live, green) and Ethidium homodimer-1 (dead, red) dyes. Cell death following OGD in *Prnp*^{0/0} neurons treated with STI1 was significantly higher compared to wild-type counterparts. No significant differences were found between wild-type and *Prnp*^{0/0} neurons when treated with PNU 282987 or BMP-4 prior to OGD or during reperfusion. Results are presented as mean +/- SEM; data were analyzed and compared by two-way ANOVA and Tukey's post hoc test. (n=4) *P<0.05.

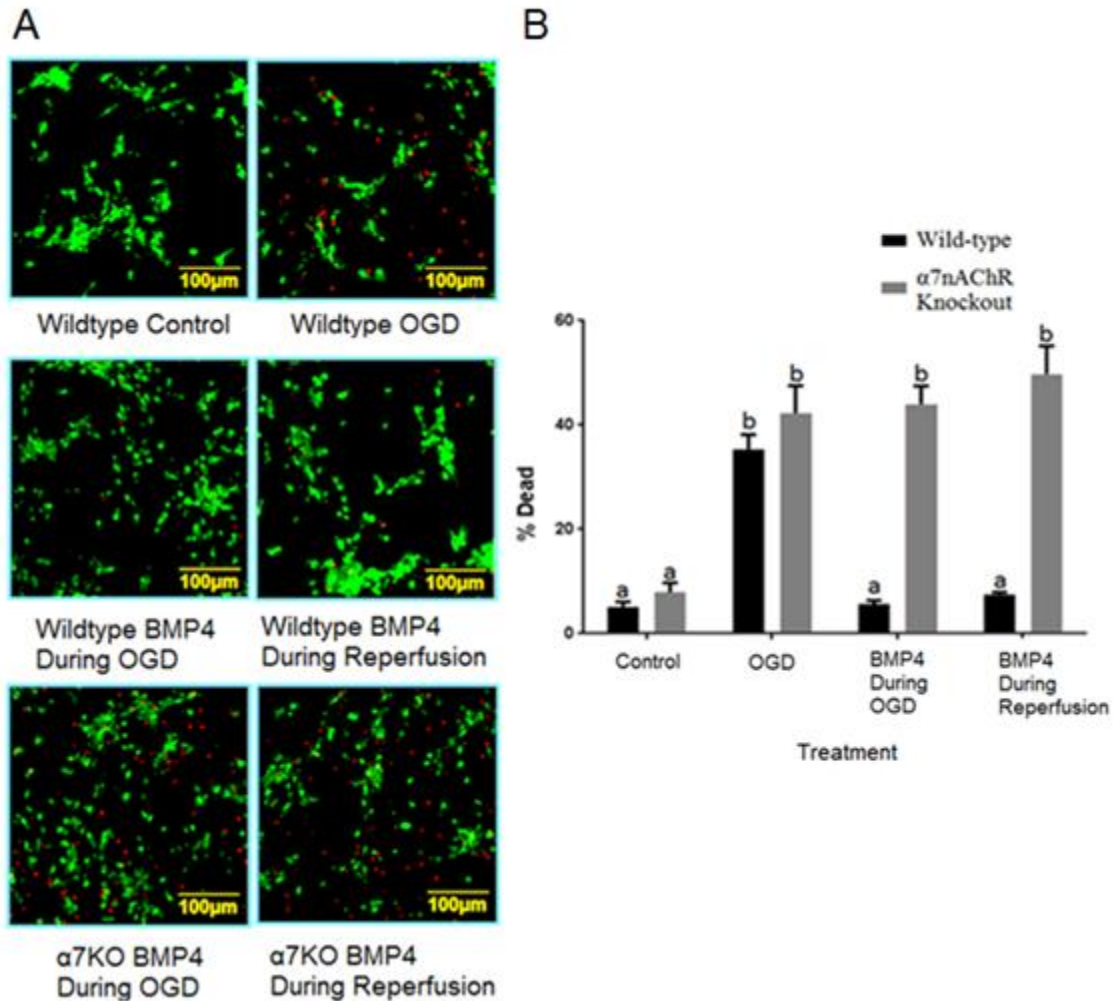


Figure 15: BMP-4 protection against OGD was dependent on the $\alpha 7$ nAChR. Wild-type and $\alpha 7$ nAChR-KO hippocampal neurons were treated with BMP-4 (100ng/mL, 30 minutes) and then subjected to 1 hour OGD or during reperfusion. (A) Representative images obtained by confocal microscopy after 24 hours of reperfusion. (B) Cell death was quantified as percentage of total cells that were dead using Calcein-AM (live, green) and Ethidium homodimer-1 (dead, red) dyes. Treatment of BMP-4 (100ng/mL) either before OGD or during reperfusion significantly reduced cell death, an effect not observed in $\alpha 7$ nAChR-null neurons. Results are presented as mean \pm SEM; data were analyzed and compared by two-way ANOVA and Tukey's post hoc test. (n=4). Different letters above the bars indicate significant difference ($P < 0.05$), whereas bars denoted with the same letter are not significantly different ($P > 0.05$).

4 Discussion

Current treatments for ischemic stroke are limited in efficacy, thus calling for the development of novel therapeutics. Recent studies have identified both STI1 and PrP^C as potential targets for stroke treatment. However, the exact mechanism by which STI1-PrP^C protects the brain during ischemia is poorly understood. Here we demonstrated that the STI1-PrP^C neuroprotective mechanism is dependent on the α 7nAChR and ALK2 receptor. Activation of the α 7nAChR and ALK2 receptor by PNU 282,987 and BMP-4, respectively, also protected neurons against OGD in a manner independent of PrP^C. Interestingly, BMP-4 protected neurons even if treated during reperfusion. The neuroprotective signalling mechanisms of these receptors, limitations of this study, and future directions will be discussed in the following sections.

4.1 STI1 as a neuroprotective factor

Caspase-3 is a critical mediator of apoptosis and is often activated during programmed cell death as a protease in the cleavage of vital cellular proteins (Porter et al., 1999). Using our Apo mouse, we were able to quantify caspase-3 activity via the CFP technique and demonstrate that STI1 plays a vital role in cellular survival following OGD. One of the mechanisms suggested to be responsible for neuroprotection is activation of PKA. Previous studies have shown that activation of cAMP and its effector PKA protects cells against TNF- α -induced apoptosis (Insel et al., 2012, Kragsted et al., 2004). In fact, PKA activation is one of the mechanisms by which STI1-PrP^C protects against staurosporine-induced cell death (Caetano et al., 2008, Beraldo et al., 2010). Moreover, studies have shown that inhibition of PKA activity abolished the protective effect of PrP^C activation (Lopes et al., 2005). However, unlike ERK1/2 activation by

STI1, PKA activation does not require PrP^C endocytosis (Caetano et al., 2008). The STI1-PrP^C complex may remain near the cell surface and physically interacts with the α 7nAChR to induce calcium influx and subsequent activation of PKA, thereby inducing protection against elevated pro-apoptotic enzymes during ischemia. Here we showed that the α 7nAChR is required in neuroprotection by STI1 against OGD, supporting the model in which α 7nAChR activation by STI1-PrP^C interaction leads to PKA activation and subsequent neuroprotection against OGD. To further examine the role of PKA in protection against OGD-induced apoptosis, future studies should determine if the endocytosis of PrP^C is required for the anti-apoptotic effects observed in this study.

Interestingly, the protective effect of STI1 was observed as early as 3 hours following OGD. Previous studies investigating signalling pathways of STI1 have shown that STI1 induces PrP^C internalization as early as 45 minutes following treatment (Caetano et al., 2008), with the internalization of PrP^C by STI1 being a necessary step in STI1-mediated neuritogenesis via ERK1/2 signalling. However, STI1-PrP^C has also been shown to enhance protein synthesis in neurons via PI3K-mTOR signalling (Roffe et al., 2010). The increase in protein synthesis as evidenced by [³⁵S]-methionine incorporation, which occurred after only 30 minutes of incubation with STI1 (Roffe et al., 2010). The authors suggested that the increase in protein synthesis via the interaction between PrP^C and STI1 is critical for both neuritogenesis and neuroprotection against staurosporine-induced cell death (Roffe et al., 2010). Together, the timing of STI1-mediated signalling demonstrated by these studies align with our observations that STI1 decreases both caspase-3 induced fluorescence and cell death as early as 3 hours following OGD. Future studies using the Apo mouse should examine the caspase-3 activity and cell death as

early as one or two hours following OGD to determine how quickly STI1 acts on the cell following or even during injury. This will help identify the timing in which signalling pathways are active when neurons are protected by STI1, and how quickly these cellular changes can translate into protection against OGD.

STI1 has also been suggested to have cell signalling pathways independent of the prion protein. For example, in ovarian cancer cells, secreted STI1 acts as a ligand for ALK2 receptors, leading to cancer cell proliferation (Tsai et al., 2012). Furthermore, STI1 has also been shown to regulate retinal proliferation independent of the prion protein (Arruda-Carvalho et al., 2007). However, in hippocampal neurons, neuroprotection by STI1 against OGD was determined to be dependent on the prion protein. Our results confirm findings in previous studies in which neuroprotection by STI1 is dependent on the activation of the PrP^C-regulated signalling pathways (Lopes et al., 2005, Lima et al., 2007, Caetano et al., 2008, Roffe et al., 2010).

4.2 Activin receptor family member ALK2 offers unique protection against ischemia

Activin receptors have been found to play a role in many cellular functions including facilitation of neuronal survival following ischemia (Mukerji et al., 2007). Studies have shown that endogenous activin can protect neurons *in vitro* from hydrogen peroxide free radical stress (Mukerji et al., 2007). Here we showed that ALK2 receptors were expressed in hippocampal neurons and played a role in STI1-PrP^C neuroprotection against OGD. In ovarian cancer cells, STI1 activates ALK2, which triggers SMAD signalling and induces cell proliferation. Interestingly, this pathway was also observed to be up-regulated in neurons adjacent to areas of ischemia *in vivo* (Mukerji et al., 2007).

Furthermore, in a separate study, SARA was found to be up-regulated following OGD in PC12 cells (Wang et al., 2013). As described earlier, recruitment and orientation of SMAD proteins for phosphorylation by ALK2 receptors is dependent on SARA activity (Kitisin et al., 2007). The authors found that down-regulation of SMAD signalling by inhibition of SARA exacerbated neuronal death during OGD. It is possible that, in addition to PrP^C, STI1 also targets ALK2 receptors and subsequent SMAD signalling in neurons to induce neuroprotection against OGD.

However, the two signalling pathways activated by STI1 may not be mutually exclusive, as we observed that inhibition of the ALK2 receptor completely abolished the neuroprotective effect of STI1 against OGD. PrP^C has been described as a multi-faceted scaffolding protein and modulates a plethora of cell surface receptors. One of the receptors modulated by the PrP^C is the α 7nAChR (Beraldo et al., 2010). Interestingly, our study demonstrates that the α 7nAChR is required for BMP-4 neuroprotection against OGD. Since BMP-4 activates ALK2, and PrP^C modulates the α 7nAChR, it is possible that there is cross-talk between PrP^C and ALK2. The mechanisms by which PrP^C protects neurons is by activation of PKA, attenuation of excitotoxicity by modulation of NMDA receptors, and its interactions with the α 7nAChR (Chiarini et al., 2002, Zanata et al., 2002, Lopes 2005, Houman et al., 2008, Beraldo et al., 2010). However, in experiments with *Pnrp*^{0/0} neurons, while neuroprotection by STI1 is dependent on the PrP^C, BMP-4 does not require the PrP^C for neuroprotection. While it may be possible that BMP-4 is able to activate some or all of those pathways independently of the PrP^C, it is also possible that BMP-4 may activate an entirely different set of signal cascades downstream of PrP^C. Furthermore, neuroprotection against OGD by BMP-4 was not observed in

neurons from the $\alpha 7$ nAChR-KO mouse line, suggesting an interaction between BMP-4 and the $\alpha 7$ nAChR. Activation of the $\alpha 7$ nAChR has been documented to play a role in attenuating inflammation-induced damage following ischemia by inducing Akt phosphorylation and inhibition of GSK-3 β activity (Duris et al., 2011, Grimes and Jope, 2001). Neuroprotection by PKA activation from STI1-PrP^C is also dependent on the $\alpha 7$ nAChR (Chiarini et al., 2002, Beraldo et al., 2010). It is possible that BMP-4 may also use these pathways in protection against OGD. While there is no direct evidence of interactions between BMP-4, ALK2, and the $\alpha 7$ nAChR, future studies should examine both Akt/GSK-3 β phosphorylation and PKA activity following treatment with BMP-4. Furthermore, future studies should also focus on the potential mechanism in which BMP-4 possibly interacts with the $\alpha 7$ nAChR to elicit a calcium influx in manner similar to STI1-PrP^C interaction.

4.3 Treatment during reperfusion

We have investigated the potential of the $\alpha 7$ nAChR, ALK2 receptor, and PrP^C in protecting neurons from OGD. In the experiments described above, manipulation of these receptors occurred before the 1 hour exposure to OGD. However, in practical situations, treatments administered following ischemic insult are much more prevalent than those administered prior to insult. In this study we showed that neither STI1 nor PNU 282,987 were able to protect the neurons if treated after OGD. Our findings with PNU 282,987 were in contrast to those of a study in which continual post-intracerebral haemorrhage treatment with PNU 282,987 *in vivo* for durations ranging from 3 hours to 3 days led to an increased number of surviving neurons when observed three days post-insult (Hijioka et al., 2012). Although the study focused on haemorrhagic stroke, the authors suggested

that the $\alpha 7$ nAChR agonist protected the brain in a manner that was independent of its anti-inflammatory properties. This was evidenced by the lack of effect of PNU 282,987 on the expansion of the hematoma as well as the edema formation (Hijioka et al., 2012). One explanation was that, *in vivo*, $\alpha 7$ nAChRs are also expressed by microglia/macrophages (Suzuki et al., 2006), which may play a role in the survival of neurons following intracerebral haemorrhage, a model that is absent in our *in vitro* experiments. Another explanation for PNU 282,987 not protecting neurons when treated during reperfusion is that compared to *in vivo* models, cells *in vitro* have been shown to be much more vulnerable to anoxic and excitotoxic transients (Kristian et al., 1998). To further evaluate this, a milder insult such as a 30-minute exposure to OGD should be conducted to see if PNU 282,987 can protect neurons if treated during reperfusion. Furthermore, since the penumbra is the primary area of interest for pharmaceutical intervention following stroke, a milder model of OGD using 2% oxygen and 10% of the original concentration of glucose should be used.

However, although the lack of surrounding cellular architecture and increased vulnerability may explain why PNU 282,987 did not protect our neurons *in vitro*, our results show that BMP-4 was able to decrease cell death even if used as a treatment during reperfusion. Although we have shown that BMP-4 neuroprotection is dependent on the $\alpha 7$ nAChR, unlike PNU 282,987, BMP-4 may interact with other receptors and signalling pathways in addition to the $\alpha 7$ nAChR. *In vivo* studies have shown that BMP-4 has been found to play a critical role in the differentiation of NSCs into neurons, oligodendrocytes, and astrocytes during periods of spatiotemporal development (Abematsu et al., 2006). Recent studies have also shown that BMP-4 is highly expressed

in astrocytes, which can be a major source of BMPs (Hu et al., 2012). In addition, STII has been shown to recruit BMDCs into the area of ischemia (Lee et al., 2013). The recruitment of BMDCs, which have been suggested to play a vital role in recovery following ischemia, may be further influenced by BMP-4 to induce differentiation into neurons, oligodendrocytes, and astrocytes. However, this *in vivo* model of recovery following ischemia does not explain how BMP-4 protected neurons *in vitro* when treated during reperfusion. Regardless, future experiments should focus on treatment of BMP-4 in *in vivo* models of stroke, and see if it can still protect if administered post-ischemia.

4.4 Limitations and Future Directions

4.4.1 *In vivo* experiments

There were several limitations within our model of stroke. During stroke, many cytotoxic effects are present in addition to the lack of oxygen and glucose. In an *in vivo* model, cytotoxic effects such as ATP depletion, increase in ROS, excessive intracellular calcium, and ion shifts (Lau et al., 2010, MacDonald et al., 2006) may be attenuated by the glial, astrocyte, and macrophages within the architecture surrounding the neurons (Suzuki et al., 2006, Kristian et al., 1998). However, due to time limitations and the need to first obtain experimental data supporting the rationale for further *in vivo* experiments, all experiments in this study were carried out *in vitro*. It would be interesting to see if the findings from this study can be recapitulated *in vivo*. For example, BMP-4 can be administered to mice following various durations of middle cerebral artery occlusion. Neurological damage can be assessed using the Bederson scale (Bederson et al., 1986) or measurement of infarct size. Behavioural and neuromuscular deficits can be measured using tests such as grip strength (described in Lee et al., 2013), elevated body swing test

(Shyu et al., 2004), and adhesive removal (described in Beraldo et al., 2013). Evaluation of the drugs used in our study *in vivo* will be further characterized in future studies for their efficacies in treatment of stroke.

Many recent advances in stroke treatment involve thrombolytics and therapeutic strategies that target the survival of the cells directly. However, secondary injury mechanisms involving the inflammatory cascade can be equally damaging as the initial cytotoxicity (Aronowski et al., 2005). Cellular damage following ischemic onset is contributed to by the failure of membrane sodium/potassium ATPase pumps and intracellular calcium accumulation (Emsley et al., 2008). However, while cytotoxic edema develops within minutes of ischemia onset, damage due to inflammation can occur from hours to days, spreading from the ischemic core into the surrounding ischemic penumbra (Baron 2001). This suggests that treatments targeting the inflammatory response can have greater success in neuroprotection relative to treatments that combat initial cytotoxicity. Since agonists of the $\alpha 7nAChR$ have been identified to have a potent anti-inflammatory effect, the $\alpha 7nAChR$ has been discussed as a potential target for neuroprotection against haemorrhagic stroke (Duris et al., 2011, Krafft et al., 2012, Li et al., 2013). In addition, puerarin, a major isoflavonoid derived from *Gegen*, has been found to attenuate cerebral ischemia by counteracting the inflammatory response via the $\alpha 7nAChR$ (Liu et al., 2013). Pro-apoptotic inflammatory enzymes such as nuclear factor kappa ($NF-\kappa B$), interleukin-6 (IL6), and tumour necrosis factor- α (TNF- α) are increased following ischemia (Liu et al., 2013, Barnes et al., 1997). Future studies *in vivo* should further examine the suppression of the anti-inflammatory cascade in the STII-PrP^C-

$\alpha 7$ nAChR mechanism of neuroprotection by measuring the levels of these inflammatory markers.

4.4.2 Protection beyond the hippocampus

The most common site of stroke occurs in the middle cerebral artery (MCA) that supplies blood to the cerebrum, resulting in deficits in both motor and cognitive functions. On the other hand, damage to the hippocampus, which is often accompanied by amnesia, results from infarctions in the posterior cerebral artery (PCA) (Szabo et al., 2009). Since MCA occlusions occur more often than PCA occlusions, future studies investigating STI1-PrP^C neuroprotection should also be conducted in neurons from the cortex and the striatum, which are more commonly damaged during ischemic stroke.

In our experiments, we primarily used hippocampal neurons. Unlike PrP^C and STI1, the expression of ALK2 is relatively scarce, and more focused in the hippocampus. Future experiments should explore if our observations in hippocampal neurons are reflective of the rest of the brain by repeating these experiments in neurons from different areas of the cortex, the midbrain and the hindbrain. Immunohistochemistry experiments should also be conducted to further examine the expression of the $\alpha 7$ nAChR and ALK2 receptors.

4.4.3 Recombinant vs. Endogenous STI1

The difference between recombinant and endogenous astrocyte-derived STI1 has been studied previously (Caetano et al., 2008). In conditioned media from astrocytes, the amount of endogenous STI1 was estimated to be approximately 0.5nM, and 5nM of STI1 was required to induce ERK1/2 activation. In contrast, 100 times more recombinant STI1

(0.5 μ M) was required to induce the same effect. The authors proposed that the difference in the efficacy may be attributed to improper folding of recombinant STI1, lack of post-translational modifications, or absence of potential co-activators in the media (Caetano et al., 2008). In our experiments, we used recombinant STI1 concentration of 1 μ M to induce neuroprotection. It would be of interest in future experiments to use astrocyte-derived endogenous STI1 and determine if there is a difference in neuroprotection against OGD, or perhaps a protective effect of endogenous STI1 if treated during ischemia similar to BMP-4.

4.4.4 Calcium Signalling

Previous studies have shown that STI1 induces calcium influx via the α 7nAChR (Beraldo et al., 2010). Here we have demonstrated that the neuroprotective effect of BMP-4 against OGD is dependent on the α 7nAChR, as its effects were abolished by treatment with MLA or in α 7nAChR-null neurons. Early preliminary results conducted showed a transient increase in calcium influx when hippocampal neurons (7 DIV) were treated with 100ng/mL of BMP-4 (data not shown). Future experiments should fully characterize the interaction of BMP-4 and calcium influx, and more specifically its involvement with the α 7nAChR by using both pharmacological and genetic models.

4.4.5 STI1 signalling via the ALK2 receptor in neuroprotection

In ovarian cancer cells, the secreted STI1 binds to ALK2 receptors, leading to the recruitment and phosphorylation of SMAD 1/5, subsequent translocation to the nucleus, up-regulation of ID3 genes, and ultimately resulting in increased cell proliferation (Tsai et al., 2012). Here we showed that ALK2 receptors are not only expressed in hippocampal neurons, but are also necessary for neuroprotection by STI1 against OGD. It

would be interesting to see if the STI1-ALK2 signalling in neurons also acts through the same or a similar signalling cascade compared to ovarian cancer cells. Future studies should conduct Western blot analysis to see if the treatment of STI1 or BMP-4 (as a positive control) leads to an increase in Phospho-SMAD1/5 relative to total SMAD1/5. To further elucidate the signalling mechanism of STI1 and ALK2, a recombinant STI1 deleted of amino acids 230-245, which is involved in PrP^C binding, should be used. This will allow us to determine if SMAD signalling induced by STI1 is independent from PrP^C. The *Prnp*^{0/0} mouse line can also be used to confirm these results. In ovarian cancer cells, STI1 was also observed to interact with clathrin, and the inhibition of clathrin-dependent endocytosis resulted in compromised STI1-ALK2 interaction, and ultimately decreased SMAD1/5 phosphorylation and cell proliferation (Tsai et al., 2012). In comparison, the endocytosis of STI1-PrP^C was also found to be necessary for ERK1/2 signalling (Caetano et al., 2008). It would be of interest to see if clathrin-dependent endocytosis also plays a role in STI1/ALK2-mediated neuroprotection. Future studies should use immunocytochemistry to determine if treatment of neurons with STI1 increases cytoplasmic ALK2 staining. Furthermore, treatment of neurons with Dynasore, the inhibitor of dynamin-dependent endocytosis, before OGD could further elucidate the ALK2 pathway in neuroprotection (Macia et al., 2006).

4.5 Conclusions

Both STI1 and PrP^C have been suggested to play a role in endogenous neuroprotective mechanisms during stroke. With our findings, we suggest that the α 7nAChR and the ALK2 receptor are also potential targets for stroke treatments. Future studies should evaluate these receptors *in vivo*. Overall, our findings, summarized in

Figure 16, further elucidate the molecular mechanisms and the receptors involved in STII-PrP^C neuroprotection, which is an important step towards the development of novel treatments for stroke.

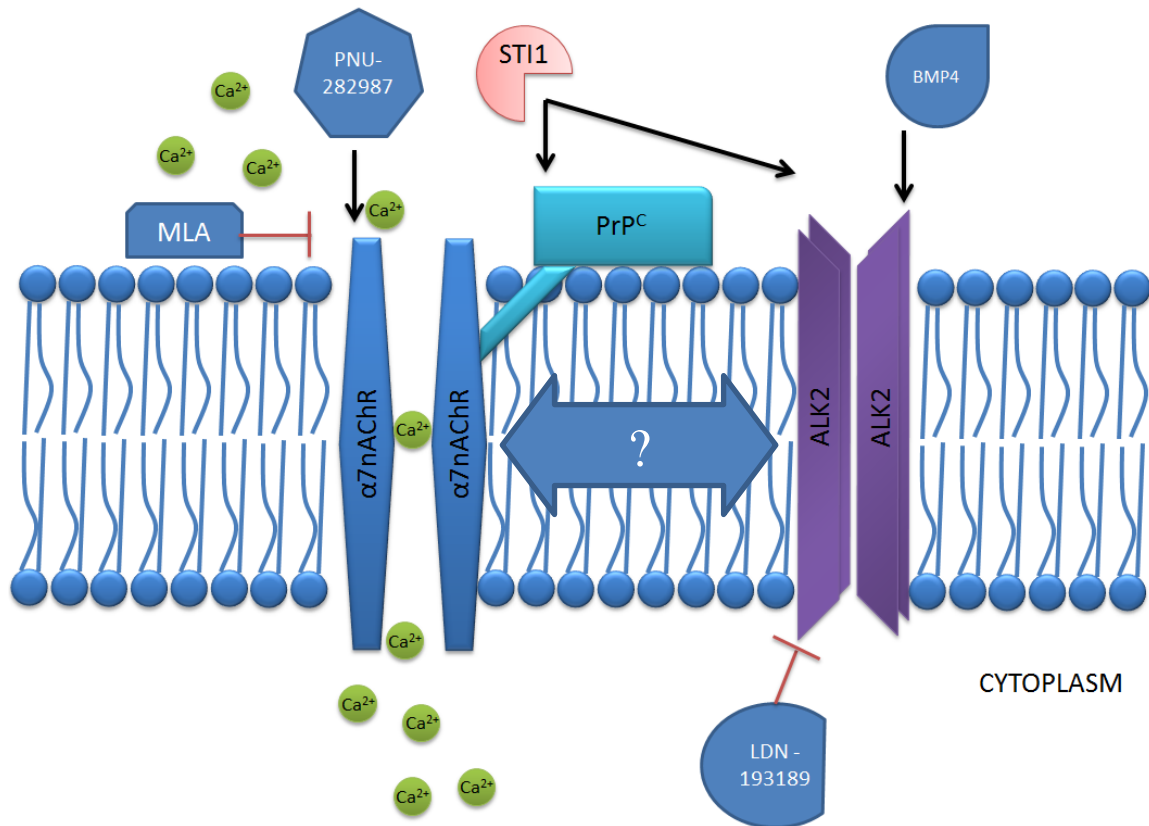


Figure 16: Schematic of the proposed model of the $\alpha 7$ nAChR, PrP^C, ALK2, and their interaction with STI1. Extracellular STI1 has been shown to act as a ligand for the GPI-anchored PrP^C leading to calcium influx via the $\alpha 7$ nAChR. Here we propose the role of the ALK2 receptor in hippocampal neurons as a receptor for the STI1 leading to neuroprotection in conjunction to PrP^C. Arrow heads indicate activation, while blunt heads indicate inhibition. Future studies should aim to elucidate the interaction between ALK2 and $\alpha 7$ nAChR.

Bibliography

- 1) Allan RK, Ratajczak T. Versatile TPR domains accommodate different modes of target protein recognition and function. *Cell Stress Chaperones* 16:353–367(2011)
- 2) Andersen KK, Olsen TS, Dehlendorff C, Kammergaard LP. Hemorrhagic and ischemic strokes compared: stroke severity, mortality, and risk factors. *Stroke*. 40(6):2068-72 (2009)
- 3) Arantes C, Nomizo R, Lopes MH, Hajj GN, Lima FR, Martins VR. Prion protein and its ligand stress inducible protein 1 regulate astrocyte development. *Glia*. 57(13):1439-49 (2009)
- 4) Aronowski J, Hall CE. New horizons for primary intracerebral hemorrhage treatment: experience from preclinical studies. *Neurol Res*. 27(3):268-79 (2005)
- 5) Arruda-Carvalho M, Njaine B, Silverira MS et al. HOP/STI1 modulates retinal proliferation and cell death independent of PrPC. *Biochem Biophys Res Commun*. 361:474-480 (2007)
- 6) Attwell D, Mobbs P. Neurotransmitter transporters. *Curr Opin Neurobiol*. 4:353–359 (1994)
- 7) Baldwin MA, Pan KM, Nguyen J, Huang Z, Groth D, Serban A, Gasset M, Mehlhorn I, Fletterick RJ, Cohen FE, et al. Spectroscopic characterization of conformational differences between PrPC and PrPSc: an alpha-helix to beta-sheet transition. *Philos Trans R Soc Lond B Biol Sci*. 343(1306):435-41 (1994)
- 8) Barnes PJ, Karin M. Nuclear factor-kb: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med*. 336:1066-71 (1997)
- 9) Baron JC. Perfusion thresholds in human cerebral ischemia: historical perspective and therapeutic implications. *Cerebrovasc Dis*. 11 Suppl 1:2-8. (2001)

- 10) Basler K, Oesch B, Scott M, Westaway D, Wälchli M, Groth DF, McKinley MP, Prusiner SB, Weissmann C. Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. *Cell*. 46(3):417-28 (1986)
- 11) Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* 17:472–476 (1986)
- 12) Beraldo FH, Arantes CP, Santos TG, Queiroz NG, Young K, Rylett RJ, Markus RP, Prado MA, Martins VR. Role of alpha7 nicotinic acetylcholine receptor in calcium signaling induced by prion protein interaction with stress-inducible protein 1. *J Biol Chem*. 285(47):36542-50 (2010)
- 13) Beraldo FH, Soares IN, Goncalves DF, Fan J, Thomas AA, Santos TG, Mohammad AH, Roffé M, Calder MD, Nikolova S, Hajj GN, Guimaraes AL, Massensini AR, Welch I, Betts DH, Gros R, Drangova M, Watson AJ, Bartha R, Prado VF, Martins VR, Prado MA. Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein. *FASEB J*. (9):3594-607 (2013)
- 14) Berridge MJ. Neuronal Calcium Signalling. *Neuron*. 21(1):13-26 (1998)
- 15) Bertrand S, Devillers-Thiéry A, Palma E, Buisson B, Edelstein SJ, Corringer PJ, Changeux JP, Bertrand D. Paradoxical allosteric effects of competitive inhibitors on neuronal alpha7 nicotinic receptor mutants. *Neuroreport*. 8(16):3591-6 (1997)
- 16) Bodnar AL, Cortes-Burgos LA, Cook KK, Dinh DM, Groppi VE, Hajos M, Higdon NR, Hoffmann WE, Hurst RS, Myers JK, Rogers BN, Wall TM, Wolfe ML, Wong E. Discovery and structure-activity relationship of quinuclidine benzamides as agonists of alpha7 nicotinic acetylcholine receptors. *J Med Chem*. 24;48(4):905-8 (2005)
- 17) Bolton DC, McKinley MP, Prusiner SB. Molecular characteristics of the major scrapie prion protein. *Biochemistry*. 23(25),5898-5906 (1984)

- 18) Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature*. 405(6785):458-62 (2000)
- 19) Bousser MG. Stroke prevention: an update. *Front Med*. 6,22-34(2012)
- 20) Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S, Weissmann C, Aguzzi A. Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature*. 379(6563):339-43 (1996)
- 21) Broderick JP, Diringer MN, Hill MD, Brun NC, Mayer SA, Steiner T, Skolnick BE, Davis SM; Recombinant Activated Factor VII Intracerebral Hemorrhage Trial Investigators. Determinants of intracerebral hemorrhage growth: an exploratory analysis. *Stroke*. 38(3):1072-5 (2007)
- 22) Brini M, Calì T, Ottolini D, Carafoli E. Neuronal calcium signaling: function and dysfunction. *Cell Mol Life Sci*. 71(15):2787-814 (2014)
- 23) Brinker A, Scheufler C, Von Der Mülbe F et al. Ligand discrimination by TPR domains. Relevance and selectivity of EEVD-recognition in Hsp70•Hop•Hsp90 complexes. *J Biol Chem* 277:19265–19275(2002)
- 24) Brown DR. PrPSc-like prion protein peptide inhibits the function of cellular prion protein. *Biochem J*. 352 Pt 2:511-8 (2000)
- 25) Büeler H1, Fischer M, Lang Y, Bluethmann H, Lipp HP, DeArmond SJ, Prusiner SB, Aguet M, Weissmann C. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature*. 356(6370):577-82 (1992)
- 26) Caetano FA, Lopes MH, Hajj GN, Machado CF, Pinto Arantes C, Magalhães AC, Vieira Mde P, Américo TA, Massensini AR, Priola SA, Vorberg I, Gomez MV, Linden R, Prado VF, Martins VR, Prado MA. Endocytosis of prion protein is required for ERK1/2 signaling induced by stress-inducible protein 1. *J Neurosci*. 6691-702 (2008)
- 27) Cambray S, Arber C, Little G, Dougalis AG, de Paola V, Ungless MA, Li M, Rodríguez TA. Activin induces cortical interneuron identity and differentiation in

- embryonic stem cell-derived telencephalic neural precursors. *Nat Commun.* 3:841 (2012)
- 28) Caplan AJ, Mandal AK, Theodoraki MA. Molecular chaperones and protein kinase quality control. *Trends Cell Biol* 17:87–92 (2007)
- 29) Cerqueira MD, Maynard C, Ritchie JL. Radionuclide assessment of infarct size and left ventricular function in clinical trials of thrombolysis. *Circulation.* 84(3 Suppl):I100-8. (1991)
- 30) Chae HJ, Kang JS, Byun JO, Han KS, Kim DU, Oh SM, Kim HM, Chae SW, Him HR. Molecular mechanism of staurosporine-induced apoptosis in osteoblasts. *Pharmacol Res.* 373-81 (2000)
- 31) Chaikuad A, Alfano I, Kerr G, Sanvitale CE, Boergermann JH, Triffitt JT, von Delft F, Knapp S, Knaus P, Bullock AN. Structure of the bone morphogenetic protein receptor ALK2 and implications for fibrodysplasia ossificans progressiva. *J Biol Chem.* 26;287(44):36990-8 (2012)
- 32) Chang HC, Nathan DF, Lindquist S. In vivo analysis of the Hsp90 cochaperone Sti1 (p60). *Mol. Cell Biol.* 318-325 (1997).
- 33) Cheng Q, Yakel JL. Presynaptic $\alpha 7$ nicotinic acetylcholine receptors enhance hippocampal mossy fiber glutamatergic transmission via PKA activation. *J Neurosci.* 34(1):124-33 (2014)
- 34) Cheng Z, Li-Sha G, Jing-Lin Z, Wen-Wu Z, Xue-Si C, Xing-Xing C, Yue-Chun L. Protective role of the cholinergic anti-inflammatory pathway in a mouse model of viral myocarditis. *PLoS One.* 9(11):e112719 (2014)
- 35) Chiarini LB, Freitas AR, Zanata SM, Brentani RR, Martins VR, Linden R. Cellular prion protein transduces neuroprotective signals. *EMBO J.* 21(13):3317-26.(2002)
- 36) Choi DW. Excitotoxic cell death. *J Neurobiol.* 23(9):1261-76 (1992)
- 37) Choi DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron.* 1:623–634. (1988)

- 38) Chou HJ, Lai DM, Huang CW, McLennan IS, Wang HD, Wang PY. BMP4 is a peripherally-derived factor for motor neurons and attenuates glutamate-induced excitotoxicity in vitro. *PLoS One*. 8(3):e58441 (2013)
- 39) Coitinho AS, Lopes MH, Hajj GN, Rossato JI, Freitas AR, Castro CC, Cammarota M, Brentani RR, Izquierdo I, Martins VR. Short-term memory formation and long-term memory consolidation are enhanced by cellular prion association to stress-inducible protein 1. *Neurobiol Dis*. 26(1):282-90 (2007)
- 40) Coitinho AS, Roesler R, Martins VR, Brentani RR, Izquierdo I. Cellular prion protein ablation impairs behavior as a function of age. *Neuroreport*. 14(10):1375-9 (2003)
- 41) Cooper DM, Mons N, Karpen JW. Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature*. 374(6521):421-4 (1995)
- 42) Croons V, Martinet W, Herman AG, Timmermans JP, De Meyer GR. The protein synthesis inhibitor anisomycin induces macrophage apoptosis in rabbit atherosclerotic plaques through p38 mitogen-activated protein kinase. *J Pharmacol Exp Ther*. 329(3):856-64 (2009)
- 43) Cuny GD, Yu PB, Laha JK, Xing X, Liu JF, Lai CS, Deng DY, Sachidanandan C, Bloch KD, Peterson RT. Structure-activity relationship study of bone morphogenetic protein (BMP) signaling inhibitors. *Bioorg Med Chem Lett*. 18(15):4388-92 (2008)
- 44) Detert JA, Adams EL, Lescher JD, Lyons JA, Moyer JR Jr. Pretreatment with apoaequorin protects hippocampal CA1 neurons from oxygen-glucose deprivation. *PLoS One*. 8(11) (2013)
- 45) Dugan L, Sensi S, Canzoniero L, Handran S, Rothman S, Lin T, Goldberg M, Choi D. Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci*. 15:6377-6388 (1995)
- 46) Dürig J, Giese A, Schulz-Schaeffer W, Rosenthal C, Schmäcker U, Bieschke J, Dührsen U, Kretzschmar HA. Differential constitutive and activation-dependent expression of prion protein in human peripheral blood leucocytes. *Br J Haematol* 108(3):488-95 (2000)

- 47) Duris K. et al. Alpha7 nicotinic acetylcholine receptor agonist PNU-282987 attenuates early brain injury in a perforation model of subarachnoid hemorrhage in rats. *Stroke*, 3530-3536 (2011).
- 48) Dykens J. Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca²⁺ and Na⁺: implications for neurodegeneration. *J Neurochem*. 63:584–591 (1994)
- 49) Eimerl S, Schramm M. The quantity of calcium that appears to induce neuronal death. *J Neurochem*. 62:1223–1226 (1994)
- 50) Emsley HC, Smith CJ, Tyrrell PJ, Hopkins SJ. Inflammation in acute ischemic stroke and its relevance to stroke critical care. *Neurocrit Care*. 9(1):125-38. (2008)
- 51) Erlich RB, Kahn SA, Lima FRS et al. STI1 promotes glioma proliferation through MAPK and PI3K pathways. *Glia* 55:1690–1698 (2007)
- 52) Fath T, Ke YD, Gunning P, Gotz J, Ittner LM. Primary support cultures of hippocampal and substantia nigra neurons. *Nat Protoc*. 4(1):78-85 (2009)
- 53) Ford MJ, Burton LJ, Morris RJ, Hall SM. Selective expression of prion protein in peripheral tissues of the adult mouse. *Neuroscience*. 113(1):177-92 (2002)
- 54) Fournier JG, Escaig-Haye F, Billette de Villemeur T, Robain O, Lasmézas CI, Deslys JP, Dormont D, Brown P. Distribution and submicroscopic immunogold localization of cellular prion protein (PrP_c) in extracerebral tissues. *Cell Tissue Res*. 292(1):77-84 (1998)
- 55) Freund TF, Buzsáki G, Leon A, Baimbridge KG, Somogyi P. Relationship of neuronal vulnerability and calcium binding protein immunoreactivity in ischemia. *Exp Brain Res*. 83(1):55-66 (1990)
- 56) Grimes CA, Jope RS. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog Neurobiol*. 65(4):391-426 (2001)
- 57) Gohel C, Grigoriev V, Escaig-Haye F, Lasmézas CI, Deslys JP, Langeveld J, Akaaboune M, Hantai D, Fournier JG. Ultrastructural localization of cellular

- prion protein (PrPc) at the neuromuscular junction. *J Neurosci Res.* 55(2):261-7 (1999)
- 58) Gunter T, Pfeiffer D. Mechanisms by which mitochondria transport calcium. *Am J Physiol.* 258:C755–C786 (1990)
- 59) Gunter T, Gunter K, Sheu S, Gavin C. Mitochondrial calcium transport: physiological and pathological relevance. *Am J Physiol Soc* 267:C313–C339 (1994)
- 60) Hardwick JK, Kodach LL, Offerhaus GJ, van den Brink GR. Bone morphogenetic protein signaling in colorectal cancer. *Nat. Rev. Cancer.* 806-812 (2008)
- 61) Hajj GN et al. The unconventional secretion of stress-inducible protein 1 by a heterogeneous population of extracellular vesicles. *Coll Mol Life Sci.* 3211-27 (2013)
- 62) Hajós M, Hurst RS, Hoffmann WE, Krause M, Wall TM, Higdon NR, Groppi VE. The selective alpha7 nicotinic acetylcholine receptor agonist PNU-282987 [N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride] enhances GABAergic synaptic activity in brain slices and restores auditory gating deficits in anesthetized rats. *J Pharmacol Exp Ther.* 312(3):1213-22 (2005)
- 63) Hess DC, Hill WD, Martin-Studdard A, Carroll J, Brailer J, Carothers J. Bone marrow as a source of endothelial cells and NeuN-expressing cells After stroke. *Stroke.* 33(5):1362-8 (2002)
- 64) Hijioka M, Matsushita H, Ishibashi H, Hisatsune A, Isohama Y, Katsuki H. a7 nicotinic acetylcholine receptor agonist attenuates neuropathological changes associated with intracerebral hemorrhage in mice. *Neuroscience* 222:10–19 (2012)
- 65) Hinkle JL, Guanci MM. Acute Ischemic Stroke Review. *J Neurosci Nurs.* 285-293 (2007)
- 66) Hogan BL. Bone morphogenetic proteins in development. *Curr Opin genet Dev.* 6(4):432-8 (1993)

- 67) Horibe T, Kohno M, Haramoto M et al. Designed hybrid TPR peptide targeting Hsp90 as a novel anticancer agent. *J Transl. Med* 9:8 (2011)
- 68) Hossmann KA. Disturbances of cerebral protein synthesis and ischemic cell death. *Neurobiology of Ischemic Brain Damage*. 161–177. (1993)
- 69) Hu CD, Kerppola TK. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat Biotechnol*. 539-45. (2003)
- 70) Hu J, Toft D, Anselmo D, and Wang X. In vitro reconstitution of functional hepadnavirus reverse transcriptase with cellular chaperone proteins. *J. Virol*. 269-279 (2002)
- 71) Hu JG, Zhang YX, Qi Q, Wang R, Shen L, Zhang C, Xi J, Zhou JS, Lu HZ. Expression of BMP-2 and BMP-4 proteins by type-1 and type-2 astrocytes induced from neural stem cells under different differentiation conditions. *Acta Neurobiol Exp (Wars)*. 72(1):95-101 (2012)
- 72) Hu M, Schurdak ME, Puttfarcken PS, El Kouhen R, Gopalakrishnan M, Li J. High content screen microscopy analysis of A beta 1-42-induced neurite outgrowth reduction in rat primary cortical neurons: neuroprotective effects of alpha 7 neuronal nicotinic acetylcholine receptor ligands. *Brain Res*. 1151:227-35 (2007)
- 73) Iacopino A1, Christakos S. Specific reduction of calcium-binding protein (28-kilodalton calbindin-D) gene expression in aging and neurodegenerative diseases. *Proc Natl Acad Sci U S A*. (11):4078-82 (1990)
- 74) Insel PA, Zhang L, Murray F, Yokouchi H, Zambon AC. Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. *Acta Physiol (Oxf)*. 204(2):277-87 (2012)
- 75) Irvin RF. Calcium transients: mobilization of intracellular Ca²⁺. *Br Med Bull*. 42:369–374. (1986)
- 76) Kadaja M, Keyes BE, Lin M, Pasolli HA, Genander M, Polak L, Stokes N, Zheng D, Fuchs E. SOX9: a stem cell transcriptional regulator of secreted niche signalling factors. *Genes Dev*. 28, 328-341 (2014)

- 77) Kennedy M. Regulation of neuronal function by calcium. *Trends Neurosci.* 12:417–424 (1989)
- 78) Khosravani H, Zhang Y, Tsutsui S, Hameed S, Altier C, Hamid J, Chen L, Villemaire M, Ali Z, Jirik FR, Zamponi GW. Prion protein attenuates excitotoxicity by inhibiting NMDA receptors. *J Gen Physiol.* 131(6):i5 (2008)
- 79) Kim YS, Zhuang H, Koehler RC et al. Distinct protective mechanisms of HO-1 and HO-2 against hydroperoxide-induced cytotoxicity. *Free Radic Biol Med* 38:85–92 (2005)
- 80) King TD, Bijur GN, Jope RS. Caspase-3 activation induced by inhibition of mitochondrial complex I is facilitated by glycogen synthase kinase-3beta and attenuated by lithium. *Brain Res.* 919(1):106-14 (2001)
- 81) Kitisin K et al. Tgf-beta signalling in development. *Sci. STKE.* Cm1 (2007)
- 82) Knight RSG, Will RG. Prion Diseases. *J Neurol Neurosurg Psychiatry.* 75:i36-i42 (2004)
- 83) Kristián T, Katsura K, Gidö G, Siesjö BK. The influence of pH on cellular calcium influx during ischemia. *Brain Res.* 641:295–302 (1994)
- 84) Kristián T, Siesjö BK. Calcium in ischemic cell death. *Stroke.* 29(3):705-18 (1998)
- 85) Kubosaki A, Yusa S, Nasu Y, Nishimura T, Nakamura Y, Saeki K, Matsumoto Y, Itohara S, Onodera T. Distribution of cellular isoform of prion protein in T lymphocytes and bone marrow, analyzed by wild-type and prion protein gene-deficient mice. *Biochem Biophys Res Commun* 282(1):103-7 (2001)
- 86) Kubota H, Yamamoto S, Itoh E, Abe Y, Nakamura A, Izumi Y, Okada H, Iida M, Nanjo H, Itoh H, Yamamoto Y. Increased expression of co-chaperone HOP with HSP90 and HSC70 and complex formation in human colonic carcinoma. *Cell Stress Chaperones.* 15(6):1003-11
- 87) La Rosa I, Camargo L, Pereira MM, Fernandez-Martin R, Paz DA, Salamone DF. Effects of bone morphogenic protein 4 (BMP4) and its inhibitor, Noggin, on in

- vitro maturation and culture of bovine preimplantation embryos. *Reprod Biol Endocrinol.* 9:18. doi: 10.1186/1477-7827-9-18 (2011)
- 88) Lainé J, Marc ME, Sy MS, Axelrad H. Cellular and subcellular morphological localization of normal prion protein in rodent cerebellum. *Eur J Neurosci.* 14(1):47-56 (2001)
- 89) Lanneau D, Brunet M, Frisan E et al. Heat shock proteins: essential proteins for apoptosis regulation: apoptosis review series. *J Cell Mol Med* 12:743–761(2008)
- 90) Lansberg MG. Antithrombotic and thrombolytic therapy for ischemic stroke: antithrombotic therapy and prevention of thrombosis, 9th ed: American college of chest physicians evidence-based clinical practice guidelines. *Chest.* 141, e601S-e636S (2012)
- 91) Lakhan SE, Kirchgessner A, Hofer M. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *J Transl Med* 7:97 (2009)
- 92) Lassle M, Blatch G, Kundra V, Takatori T, Zetter BR, Stress-inducible, murine protein mSTI1. Characterization of binding domains for heat shock proteins and in vitro phosphorylation by different kinases. *J. Biol. Chem.* , 1876-1884 (1997).
- 93) Lau A. & Tymianski M. Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch.* 525-542 (2010).
- 94) Lauren J, Gimbel DA, Nygaard H.B, Gilbert JW., & Strittmatter SM. Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* 1128-1132 (2009).
- 95) Lee CT, Graf C, Mayer FJ, Richter SM, Mayer MP. Dynamics of the regulation of Hsp90 by the co-chaperone Sti1. *EMBO* 1518-28 (2012)
- 96) Lee SD, Lai TW, Lin SZ, Lin CH, Hsu YH, Wang HJ, Lee W, Su CY, Yu YI, Shyu WC. Role of stress-inducible protein-1 in recruitment of bone marrow derived cells into the ischemic brains. *EMBO Mol Med.* 1227-46 (2013)
- 97) Leonard S, Bertrand D. Neuronal nicotinic receptors: from structure to function. *Nicotine Tob Res.* 3(3):203-23 (2001)

- 98) Li F1, Chen Z, Pan Q, Fu S, Lin F, Ren H, Han H, Billiar TR, Sun F, Li Q. The protective effect of PNU-282987, a selective $\alpha 7$ nicotinic acetylcholine receptor agonist, on the hepatic ischemia-reperfusion injury is associated with the inhibition of high-mobility group box 1 protein expression and nuclear factor κ B activation in mice. *Shock*. 197-203 (2013)
- 99) Lima FR et al., Cellular prion protein expression in astrocytes modulates neuronal survival and differentiation. *J. Neurochem*. 103, 2164-2176 (2007)
- 100) Linden R. et al. Physiology of the prion protein. *Physiol Rev*. 673-728 (2008).
- 101) Liu X, Mei Z, Qian J, Zeng Y, Wang M. Puerarin partly counteracts the inflammatory response after cerebral ischemia/reperfusion via activating the cholinergic anti-inflammatory pathway. *Neural Regen Res*. 8(34):3203-15 (2013)
- 102) Lopes MH, Hajj GM, Muras AG et al. Interaction of cellular prion and stress-inducible protein 1 promotes neuritogenesis and neuroprotection by distinct signaling pathways. *J Neurosci*. 25:11330–11339(2005)
- 103) MacDonald JF, Xiong ZG., & Jackson MF. Paradox of Ca^{2+} signaling, cell death and stroke. *Trends Neurosci*. 75-81 (2006)
- 104) Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, Kirchhausen T. Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell*. 10(6):839-50. (2006)
- 105) Magistretti PJ, Pellerin L. Cellular mechanisms of brain energy metabolism: relevance to functional brain imaging and to neurodegenerative disorders. *Ann N Y Acad Sci*. 777:380–387 (1996)
- 106) Manning G, Whyte DB, Marintez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 298:1912-34 (2002)
- 107) Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J. The prion protein gene: a role in mouse embryogenesis? *Development*. 115(1):117-22 (1992)
- 108) Martin J. Chaperonin function—effects of crowding and confinement. *J Mol Recognit*. 17:465-472 (2004)

- 109) Marubio LM, Changeux J. Nicotinic acetylcholine receptor knockout mice as animal models for studying receptor function. *Eur J Pharmacol.* 393(1-3):113-21 (2000)
- 110) Mayer ML. Glutamate receptor ion channels. *Curr Opin Neurobiol.* 15(3):282-8 (2005).
- 111) McLennan NF et al. Prion protein accumulation and neuroprotection in hypoxic brain damage. *Am. J. Pathol.* , 227-235 (2004).
- 112) Miyata Y, Nakamoto H, Neckers L. The therapeutic target Hsp90 and cancer hallmarks. *Curr Pharm Des.* 19:347–365 (2013)
- 113) Mukerji SS Katsman EA, Wilber C, Haner NA, Selman WR, Hall AK. Activin is a neuronal survival factor that is rapidly increased after transient cerebral ischemia and hypoxia in mice. *J Cereb Blood Flow Metab* (2007) 27(6):1161–1172
- 114) National Heart, Lung, and Blood Institute. How is a stroke treated? <http://www.nhlbi.nih.gov/health/health-topics/topics/stroke/treatment> (2014)
- 115) Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science.* 303(5663):1483-7 (2004)
- 116) Nico PB, de-Paris F, Vinadé ER, Amaral OB, Rockenbach I, Soares BL, Guarnieri R, Wichert-Ana L, Calvo F, Walz R, Izquierdo I, Sakamoto AC, Brentani R, Martins VR, Bianchin MM. Altered behavioural response to acute stress in mice lacking cellular prion protein. *Behav Brain Res.* 162(2):173-81 (2005)
- 117) Nicholls DG. A role for the mitochondrion in the protection of cells against calcium overload? *Prog Brain Res.* 63:97–106 (1985)
- 118) Odunuga OO, Hornby JA, Bies C et al. Tetratricopeptide repeat motif-mediated Hsc70-mSTI1 interaction. Molecular characterization of the critical contacts for successful binding and specificity. *J Biol Chem* 278:6896–6904 (2003)

- 119) Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, Ruvkun G. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature*. 389(6654):994-9 (1997)
- 120) Ostapchenko VG, Beraldo FH, Mohammad AH, Xie YF, Hirata PH, Magalhaes AC, Lamour G, Li H, Maciejewski A, Belrose JC, Teixeira BL, Fahnestock M, Ferreira ST, Cashman NR, Hajj GN, Jackson MF, Choy WY, MacDonald JF, Martins VR, Prado VF, Prado MA. The prion protein ligand, stress-inducible phosphoprotein 1, regulates amyloid- β oligomer toxicity. *J Neurosci*. 33(42):16552-64 (2013a)
- 121) Ostapchenko VG, Beraldo FH, Guimarães AL, Mishra S, Guzman M, Fan J, Martins VR, Prado VF, Prado MA. Increased prion protein processing and expression of metabotropic glutamate receptor 1 in a mouse model of Alzheimer's disease. *J Neurochem*. 127(3):415-25 (2013b)
- 122) Papadopoulos MC, Giffard RG, Bell BA. An introduction to the changes in gene expression that occur after cerebral ischaemia. *Br J Neurosurg*. 14(4):305-12 (2000)
- 123) Parada E, Buendía I, León R, Negredo P, Romero A, Cuadrado A, López MG, Egea J. Neuroprotective effect of melatonin against ischemia is partially mediated by alpha-7 nicotinic receptor modulation and HO-1 overexpression. *J Pineal Res*. 56(2):204-12 (2014)
- 124) Parkin ET, Watt NT, Hussain I, Eckman EA, Eckman CB, Manson JC, Baybutt HN, Turner AJ, Hooper NM. Cellular prion protein regulates beta-secretase cleavage of the Alzheimer's amyloid precursor protein. *Proc Natl Acad Sci U S A*. 104(26):11062-7 (2007)
- 125) Paschen W. Disturbances of calcium homeostasis within the endoplasmic reticulum may contribute to the development of ischemic-cell damage. *Med Hypotheses*. 47:283-288 (1996)
- 126) Pauklin S, Vallier L. Activin/Nodal signalling in stem cells. *Development*. 142(4):607-19 (2015)

- 127) Paula-Lima AC, Brito-Moreira J, Ferreira ST. Deregulation of excitatory neurotransmission underlying synapse failure in Alzheimer's disease. *J Neurochem* 126:191–202. (2013)
- 128) Piccardo P, Safar J, Ceroni M, Gajdusek DC, Gibbs CJ. Immunohistochemical localization of prion protein in spongiform encephalopathies and normal brain tissue. *Neurology*. 40(3 Pt 1):518-22 (1990)
- 129) Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. *Cell Death Differ*.6(2):99-104 (1999)
- 130) Prodromidou K, Papastefanaki F, Sklaviadis T, Matsas R. Functional cross-talk between the cellular prion protein and the neural cell adhesion molecule is critical for neuronal differentiation of neural stem/precursor cells. *Stem Cells*. 32(6):1674-87 (2014)
- 131) Rami A, Rabié A, Thomasset M, Kriegstein J. Calbindin-D28K and ischemic damage of pyramidal cells in rat hippocampus. *J Neurosci Res*. (1):89-95. (1992)
- 132) Richter C. Pro-oxidants and mitochondrial Ca²⁺: their relationship to apoptosis and oncogenesis. *FEBS Lett*. 325:104–107 (1993)
- 133) Roesler R, Walz R, Quevedo J, de-Paris F, Zanata SM, Graner E, Izquierdo I, Martins VR, Brentani RR. Normal inhibitory avoidance learning and anxiety, but increased locomotor activity in mice devoid of PrP(C). *Brain Res Mol Brain Res*. 71(2):349-53(1999)
- 134) Roffé M, Beraldo FH, Bester R, Nunziante M, Bach C, Mancini G, Gilch S, Vorberg I, Castilho BA, Martins VR, Hajj GN. Prion protein interaction with stress-inducible protein 1 enhances neuronal protein synthesis via mTOR. *Proc Natl Acad Sci U S A*. 107(29):13147-52. (2010)
- 135) Röhl A, Wengler D, Madl T, Lagleder S, Toppel F, Herrmann M, Hendrix J, Richter K, Hack G, Schmid AB, Kessler H, Lamb DC, Buchner J. Hsp90 regulates the dynamics of its cochaperone Sti1 and the transfer of Hsp70 between modules. *Nat Commun*. 6:6655 (2015)

- 136) Rosas-Ballina M, Tracey KJ. Cholinergic control of inflammation. *J Intern Med.* 265(6):663-79 (2009)
- 137) Sadis C, Teske G, Stokman G, Kubjak C, Claessen N, Moore F, Loi P, Diallo B, Barvais L, Goldman M, Florquin S, Le Moine A. Nicotine protects kidney from renal ischemia/reperfusion injury through the cholinergic anti-inflammatory pathway. *PLoS One.* 2(5):e469 (2007)
- 138) Santos TG, Beraldo FH, Hajj GN, Lopes MH, Roffe M, Lupinacci FC, Ostapchenko VG, Prado VF, Prado MA, Martins VR. Laminin- γ 1 chain and stress inducible protein 1 synergistically mediate PrPC-dependent axonal growth via Ca²⁺ mobilization in dorsal root ganglia neurons. *J Neurochem.* 124(2):210-23 (2013)
- 139) Santos TG, Silva IR, Costa-Silva B, Lepique AP, Martins VR, Lopes MH. Enhanced neural progenitor/stem cells self-renewal via the interaction of stress-inducible protein 1 with the prion protein. *Stem Cells.* 29(7): 1126-36 (2011)
- 140) Sauer H, Dagdanova A, Hescheler J, Wartenberg M. Redox-regulation of intrinsic prion expression in multicellular prostate tumor spheroids. *Free Radic Biol Med.* 27(11-12):1276-83. (1999)
- 141) Saver JL. Can acute MRI predict outcome following transient ischemic attack and minor stroke? *Nat Clin pract Neurol.* 2(1):14-5 (2006)
- 142) Scheufler C, Brinker A, Bourenkov G et al. Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. *Cell* 101:199–210 (2000)
- 143) Schinder AF, Olson EC, Spitzer NC, Montal M. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J Neurosci.* 16:6125–6133 (1996)
- 144) Schmid AB, Lagleder S, Gräwert MA, Röhl A, Hagn F, Wandinger SK, Cox MB, Demmer O, Richter K, Groll M, Kessler H, Buchner J. The architecture of functional modules in the Hsp90 co-chaperone Sti1/Hop. *EMBO.* 1506-17 (2012)

- 145) Shi Y, Massague J. Mechanisms of TGF-beta signalling from cell membranes to the nucleus. *Cell*. 113(6):685-700 (2003)
- 146) Shyu WC et al. Overexpression of PrP^C by adenovirus-mediated gene targeting reduces ischemic injury in a stroke rat model. *J. Neurosci.* , 8967-8977 (2005)
- 147) Siesjö BK. Cell damage in the brain: a speculative synthesis. *J Cereb Blood Flow Metab*. 1:155–185. (1981)
- 148) Silver IA, Erecinska M. Ion homeostasis in rat brain in vivo: intra- and extracellular Ca²⁺ and H⁺ in the hippocampus during recovery from short-term, transient ischemia. *J Cereb Blood Flow Metab*. 12:759–772. (1992)
- 149) Statistics Canada. Mortality, summary list of causes. <http://www.statcan.gc.ca/pub/84f0209x/84f0209x2009000-eng.pdf> (2012)
- 150) Song HO *et al.* C. elegans STI-1, the homolog of Stt1/Hop, is involved in aging and stress response. *J. Mol. Biol.* 604-617 (2009).
- 151) Szabo K, Förster A, Jäger T, Kern R, Griebe M, Hennerici MG, Gass A. Hippocampal lesion patterns in acute posterior cerebral artery stroke: clinical and MRI findings. *Stroke*. 40(6):2042-5 (2009)
- 152) Thibault O, Gant JC, Landfield PW. Expansion of the calcium hypothesis of brain aging and Alzheimer's disease: minding the store. *Aging Cell*. 6(3):307-17. (2007)
- 153) Tracey KJ, Beutler B, Lowry SF, Merryweather J, Wolpe S, Milsark IW, Hariri RJ, Fahey TJ 3rd, Zentella A, Albert JD, et al. Shock and tissue injury induced by recombinant human cachectin. *Science* 234(4775):470-4 (1986)
- 154) Tsai CL, Tsai CN, Lin CY, Chen HW, Lee YS, Chao A, Wang TH, Wang HS, Lai CH. Secreted stress-induced phosphoprotein 1 activates the ALK2-SMAD signaling pathways and promotes cell proliferation of ovarian cancer cells. *Cell Rep*. 2(2):283-93. (2012)

- 155) Tymianski M. Can molecular and cellular neuroprotection be translated into therapies for patients? Yes, but not the way we tried it before. *Stroke*. Octo;41 (2010)
- 156) Tymianski M, Tator CH. Normal and abnormal calcium homeostasis in neurons: a basic for the pathophysiology of traumatic and ischemic central nervous system injury. *Neurosurgery*. 38:1176–1195. (1996)
- 157) Valerio A, Bertolotti P, Delbarba A, Perego C, Dossena M, Ragni M, Spano P, Carruba MO, De Simoni MG, Nisoli E. Glycogen synthase kinase-3 inhibition reduces ischemic cerebral damage, restores impaired mitochondrial biogenesis and prevents ROS production. *J Neurochem* 116(6):1148-59 (2011)
- 158) Vallier L, Reynolds D, Pedersen RA. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev Biol*. 275(2):403-21 (2005)
- 159) Vrijens K, Lin W, Cui J, Farmer D, Low J, Pronier E, Zeng FY, Shelat AA, Guy K, Taylor MR, Chen T, Roussel MF. Identification of small molecule activators of BMP signaling. *PLoS One*. 8(3):e59045 (2013)
- 160) Walker NI, Harmon BV, Gobé GC, Kerr JF. Patterns of cell death. *Methods Achiev Exp Pathol*. 13:18-54 (1988)
- 161) Walsh N, Larkin A, Swan N et al. RNAi knockdown of Hop (Hsp70/Hsp90 organising protein) decreases invasion via MMP-2 down regulation. *Cancer Lett*. 306:180–189 (2011)
- 162) Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, Li JH, Wang H, Yang H, Ulloa L, Al-Abed Y, Czura CJ, Tracey KJ. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature*. 421(6921):384-8 (2003)
- 163) Wang HY *et al*. S 24795 limits beta-amyloid-alpha7 nicotinic receptor interaction and reduces Alzheimer's disease-like pathologies. *Biol. Psychiatry* 522-530 (2010)
- 164) Whatley SA, Powell JF, Politopoulou G, Campbell IC, Brammer MJ, Percy NS. Regulation of intracellular free calcium levels by the cellular prion protein. *Neuroreport*.6(17):2333-7 (1995)

- 165) Weise J. *et al.* Overexpression of cellular prion protein alters postischemic Erk1/2 phosphorylation but not Akt phosphorylation and protects against focal cerebral ischemia. *Restor. Neurol. Neurosci.* 57-64 (2008).
- 166) Wieloch T, Hu BR, Kamme F, Kurihara J, Sakata K. Intracellular signal transduction in the postischemic brain. *Adv Neurol.* 71:371–387. (1996)
- 167) Willmer T, Contu L, Blatch GL, Edkins AL. Knockdown of Hop downregulates RhoC expression, and decreases pseudopodia formation and migration in cancer cell lines. *Cancer Lett.* 328(2):252-60 (2013)
- 168) Wong BS, Liu T, Li R, Pan T, Petersen RB, Smith MA, Gambetti P, Perry G, Manson JC, Brown DR, Sy MS. Increased levels of oxidative stress markers detected in the brains of mice devoid of prion protein. *J Neurochem.* 76(2):565-72 (2001)
- 169) Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel regulators of bone formation: molecular clones and activities. *Science.* 242(4885): 1528-34 (1988)
- 170) Yu PB, Deng DY, Lai CS, Hong CC, Cuny GD, Boussein ML, Hong DW, McManus PM, Katagiri T, Sachidanandan C, Kamiya N, Fukuda T, Mishina Y, Peterson RT, Bloch KD. BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nat Med.* 14(12):1363-9 (2008)
- 171) Zahn R, Liu A, Lührs T, Riek R, von Schroetter C, López García F, Billeter M, Calzolari L, Wider G, Wüthrich K. NMR solution structure of the human prion protein. *Proc Natl Acad Sci U S A.* 97(1):145-50 (2000)
- 172) Zanata SM, Lopes MH, Mercadante AF, Hajj GN, Chiraini LB, Nomizo R, Freitas AR, Cabral AL, Lee KS, Juliano MA. Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *EMBO J.* 3307-3316 (2002)

Appendices

Animal Protocol Certificate

eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2008-127::6

2008-127::6:

AUP Number: 2008-127

AUP Title: a)Elucidating the Function acetylcholine in the central and peripheral nervous system; b)Role of the Cellular Prion Protein in physiological and pathological conditions

Yearly Renewal Date: 03/01/2015

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2008-127 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

Curriculum Vitae

Name: Jason Xu

Post-secondary Education and Degrees: University of Western Ontario
London, Ontario, Canada
2009-2013 BMSc

The University of Western Ontario
London, Ontario, Canada
2013-2015 MSc (Candidate)

Honours and Awards: Academic Excellence Scholarship
Mandarin
2008

Western Scholarship of Distinction
Western University
2009-2010

Dean's Honor List
2009-2010, 2010-2011, 2011-2012, 2012-2013, 2013-2014

Ontario Volunteer Service Award
Government of Ontario
2011-2015

Related Work Experience Research Associate
St. Joseph's Health Care London
2012

Teaching Assistant
The University of Western Ontario
2013-2015