PEPTIDE-MEDIATED NEUROPROTECTION AGAINST EXCITOTOXICITY AND CEREBRAL ISCHEMIA BY REGULATION OF THE JNK ACTIVATION CASCADE

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PEPTIDE-MEDIATED NEUROPROTECTION AGAINST EXCITOTOXICITY AND CEREBRAL ISCHEMIA BY REGULATION OF THE JNK ACTIVATION CASCADE

submitted by Frederike Basedow in partial fulfillment of the requirements for the degree of Master of Science in Neuroscience

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Abstract

Ischemic stroke is one of the leading causes of death and disability worldwide. One of the major pathological processes that causes brain damage after an ischemic insult is excitotoxicity, which initiates inflammation and oxidative stress and eventually leads to apoptotic and necrotic neuronal death. C-Jun-NH_2-Terminal kinases (JNKs) are activated after an ischemic event and contribute to excitotoxicity, inflammation, oxidative stress, apoptosis and necrosis. Targeting JNKs is a promising strategy to mediate the deathly excitotoxic cascade and reduce infarct volume after a stroke.

The aim of this master's thesis was to employ the selective native protein eradication (SNIPER) method to degrade multiple players in the JNK activation pathway as a novel neuroprotective approach. SNIPER peptides consist of a cell-penetrating domain, a protein-binding domain (PBD) derived from a natural binding partner of the protein of interest, and a motif that is recognized by chaperone-mediated autophagy. With these three segments SNIPER peptides can enter cells, bind to the protein of interest and target it to the lysosome for degradation. We designed three peptides with a PBD based on the key JNK-binding site (T1A) of Arrestin-3, which has been shown to bind JNK3 as well as its upstream activators Apoptosis signal-regulated kinase 1 (ASK1) and mitogen-activated protein kinase kinase 7 (MKK7). We hypothesized that these peptides can mediate the JNK activation cascade and act as potent neuroprotectants. We found that a peptide with a protein-binding domain consisting of residues 12 to 25 of T1A, named T1A-3, decreases levels of active JNK and protects cultures of cortical neurons against excitotoxic insult.

Furthermore, T1A-3 dramatically reduces infarct volume in an endothelin-1 model of stroke in rats. The peptide did not reduce protein levels of JNK *in vitro* or in the brains of rats *in vivo*, indicating that T1A-3 likely exerts its protective function by regulating kinases upstream of JNK phosphorylation. Thus, we developed a novel and potent peptide-based tool to inhibit JNK activation and effectively protect against excitotoxicity and ischemic stroke. This tool has great potential to be a more effective and clinically feasible treatment for stroke than previously developed therapies.

Lay Summary

A stroke is characterized by the obstruction of an artery that supplies the brain, which leads to a detrimental series of cellular and molecular events that eventually cause brain cells (neurons) to die. C-Jun-NH_2-Terminal kinases (JNKs) are molecules in the brain that are involved in multiple processes of the stroke-induced death cascade. Targeting these proteins is an effective strategy to prevent neuronal damage. We designed a drug (named T1A-3) that we hypothesized to cause degradation of JNKs as well as of their activators. Our results show that T1A-3 can reduce JNK activity and prevent death of cultured neurons and damage in the brains of rats under experimental stroke conditions. However, it did not reduce levels of JNK, suggesting that T1A-3 acts on activators of JNK rather than on JNK directly. Hence, we have developed a novel and potent drug that mediates JNK activation and protects against stroke-induced neuronal death.

Preface

The project of this thesis was originally suggested by Dr. Javier Marin Prida from the University of Havana, Cuba, when he visited Dr. Yu Tian Wang's lab in 2016. He, together with Dr. Wang, was responsible for the peptide design and initial research plan. All other research steps were jointly conceptualized by Dr. Wang, Dr. Prida and me. Most experiments in this thesis were conducted by me in collaboration with Dr. Prida, except for the peptide synthesis, which was done by Dr. Lidong Liu and Lixia Wang, the isolation of cortical neuronal cultures, which was done by Yuping Li, and the *in vivo* stroke model, which was done by Dr. Prida's group at the University of Havana, Cuba. I was responsible for the data analysis presented in this thesis. The text in this thesis was written by me, except for the description of methods of the *in vivo* stroke model, which was adapted from text written by Dr. Prida. None of the content in this thesis is published at this point in time. All animal experiments done in our lab were approved by the University of British Columbia Animal Care Committee. The following protocols were used:

- Intravenous injections of peptides: A16-0118-001 (Free moving)
- Primary cortical neuron culture: A17-0100 (Slice and Primary Culture Protocol)

I completed the ethics training of the Canadian Council on Animal Care (CCAC) / National Institutional Animal User Training (NIAUT) Program (Certificate # 6611 – 14) and all other relevant Animal Care Services Training courses (Introduction to Working with Rodents in Research, Rodent Restraint and SQ/IP Injections, Introduction to Rodent Anesthesia –

Inhalational and Injectable, Rodent Tail Vein Injection – Conscious and Anesthetized, Introduction to Rodent Aseptic Surgery).

The *in vivo* stroke model was conducted in Cuba and approved by the animal ethics committee of the University of Havana.

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APS	Ammonium persulfate
ASK1	Apoptosis signal-regulated kinase 1
ATF2	Activating transcription factor 2
BSA	Bovine serum albumin
CaMK	Calcium/calmodulin-dependent protein kinase
CMA	Chaperone-mediated autophagy
CPP	Cell-penetrating peptide
CTRL	Control
d.i.v.	Days *in vitro*
DAPK-1	Death-associated protein kinase 1
DMEM	Dulbecco's modified Eagles medium
DMF	*N*,*N*-dimethylformamide
EDT	1,2-Ethandithiol
Elk1	ETS domain-containing protein
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
GluN2BR	GluN2B-subunit-containing N-methyl-D-aspartate receptor
GPCR	G-Protein-coupled receptor
GSTπ	Glutathione S-transferase Pi

H_2O_2	Hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HIV1	Human immunodeficiency virus 1
HOBt	N-Hydroxybenzotriazole –anhydrous
HPCL	high-performance liquid chromatography
HRP	Horseradish-peroxidase
HTBU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
IgG	Immunoglobulin G
IL-1β	Interleukin-1β
IL-6	Interleukin-6
JNK	C-Jun-NH_2-Terminal Kinase
LDH	Lactate dehydrogenase
LTP	Long-term potentiation
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MCAO	Middle-cerebral artery occlusion
NMDA	N-methyl-D-aspartate
NMM	4-Methylmorholine
nNOS	Nitric oxide synthase

NO	Nitric oxide
OD	Optical density
PARP1	Poly(ADP-Ribose)-Polymerase 1
PBD	Protein-binding domain
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide 3-kinases
pJNK	Phosphorylated JNK
PSD-95	Postsynaptic density protein
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PVDF	Polyvinylidene difluoride
RIP	Receptor-interacting protein
RIPA	Radio-immuno precipitation assay
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNIPER	Selective native protein eradication
TAT	Trans-activation transcriptional activator
TBS-T	TRIS-buffered saline
TEMED	Tetramethylethylendiamin
TFA	Trifluoroacetic acid
TFG-β	Transforming growth factor β

TIS	Triisopropylsilane
TNFα	Tumor-necrosis factor α
tPA	Tissue plasminogen activator
TRAF2	Tumor-necrosis factor receptor-associated factor 2
TRIS	Tris(Hydroxymethyl)aminomethane
TTC	2,3,5-triphenyltetrazolium chloride
Wang-resin	p-Alkoxybenzyl alcohol resin

Acknowledgements

I would like to thank my supervisor Dr. Yu Tian Wang for his support, advice and guidance throughout my years as a student in his lab. I furthermore would like to thank my committee members Dr. Ann Marie Craig, Dr. Brian MacVicar and Dr. William Jia for their time, input and support throughout my studies.

A big thank you to Dr. Javier Marin Prida for collaborating with me on this project, for your continuous support and mentorship, your always-positive attitude and your invaluable insights into research and life philosophies.

I would also like to thank all the lab members in Yu Tian's lab who have taught, helped and supported me throughout the last years. Special thanks to Eva So for being an immense help throughout my time in the lab, for sharing all your knowledge, and for teaching me so many things. Thank you also to Yuping Li for all your help with my cell cultures and for making special needs always possible. I would moreover like to acknowledge Dr. Donovan Ashby, Dr. Wu Yang Jin, and Dr. Camille Potey without whom I could not have done any of my animal work. Thank you for going out of your way to teach me. Moreover, I would like to thank Dr. Jie Lu for his continuous support and mentorship throughout my time in the lab.

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1. Introduction

1.1. Overview

Stroke is one of the leading causes of death and disability worldwide. The only currently available treatment for stroke is the anticoagulant tissue plasminogen activator (tPA), the use of which is limited by a narrow therapeutic time window and risk for inducing cerebral hemorrhages (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995; Kuan et al., 2003). The ongoing cascade of molecular and cellular events involved in stroke pathology allows for the interference with late death processes, which is an opportunity to treat brain damage hours to days after a stroke (Lai, Shyu, & Wang, 2011). However, despite decades of research and identification of a large number of mechanisms involved in neuronal damage after a stroke, new treatments that are successful in the clinic are still missing (Neuhaus et al., 2014). Many previous approaches have focused on individual players or specific pathological processes in the deathly stroke cascade, while managing multiple death signals simultaneously may be necessary to achieve better clinical outcomes (Lai, Zhang, & Wang, 2014). In this thesis, I will discuss the pathological events happening in the brain after a stroke and will introduce C-Jun-NH_2-Terminal Kinase (JNK) and its activators as crucial mediators of a number of stroke pathologies. I propose that targeting the JNK activation pathway is a promising way to interfere with a manifold of stroke-induced death processes and may serve as a potent neuroprotective therapy for stroke. To tackle such an intervention, I employed the selective native protein eradication (SNIPER) method (Fan, Jin, Lu, Wang, & Wang, 2014) to develop a peptide that may bind to and degrade JNK as well as upstream kinases that activate it. I

will show that this technology is successful in reducing levels of active JNK and is highly neuroprotective in both *in vitro* and *in vivo* experimental stroke settings. This approach shows great potential to reduce multiple stroke-related death processes and may be a more potent and clinically relevant therapy for stroke than previously developed neuroprotectants.

1.2. Stroke and excitotoxicity

The most common form of stroke is ischemic stroke, which is characterized by the obstruction of an artery that supplies the brain (Benjamin et al., 2017). This leads to a transient or permanent reduction in blood flow and hence lower glucose and oxygen supply to the territory of the blocked artery. Through this reduction in energy supplies, ATP-dependent neuronal functions fail, which triggers a cascade of pathological events that eventually leads to neuronal damage and death (Dirnagl, Iadecola, & Moskowitz, 1999). One of the main processes involved in this death cascade is excitotoxicity. Excitotoxicity is a pathological process that is characterized by excessive stimulation of glutamate receptors and calcium influx into cells, which triggers a series of cytosolic and nuclear events that cause oxidative stress, inflammation, and apoptotic and necrotic death (Dirnagl et al., 1999).

In the healthy brain, neurons actively maintain a potential across their membrane by pumping ions in and out. These processes require sufficient energy supplies and are impaired with reduced oxygen and glucose flow during a stroke. Hence, in an ischemic setting, ion gradients and the membrane potential cannot be maintained and the cells

depolarize (Martin, Lloyd, & Cowan, 1994). This activates voltage-dependent pre-synaptic calcium channels, which triggers the release of neurotransmitters, such as glutamate, into the synaptic cleft (Dirnagl et al., 1999). Furthermore, reuptake of neurotransmitters by energy-dependent transporters is also impaired, which adds to the accumulation of glutamate in the extracellular space (Dirnagl et al., 1999). This overload of glutamate massively activates glutamate receptors on the post-synaptic membrane, which leads to a complex cascade of downstream effects that eventually cause both apoptotic and necrotic death of neurons (Dirnagl et al., 1999).

It has been suggested that particularly the GluN2B-subunit-containing N-methyl-D-aspartate (NMDA) receptor (GluN2BR) mediates neuronal death after excitotoxic insult by recruiting of and signaling through proteins such as postsynaptic density protein 95 (PSD-95), death-associated protein kinase 1 (DAPK-1) and Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Lai et al., 2011). GluN2BRs form a signaling complex with PSD-95 and neuronal nitric oxide synthase (nNOS), which facilitates production of the neurotoxin nitric oxide (NO), causing neuronal damage (Sattler et al., 1999). PTEN and DAPK1 also associate with GluN2BRs and further enhance the receptor's activation, resulting in a positive feedback loop of their association and potentiating GluN2BR's deathly effects (Ning, 2004; Tu et al., 2010; Zhang et al., 2013). Moreover, GluN2BRs promote nuclear translocation of PTEN leading to excitotoxic cell death (Zhang et al., 2013). Additionally, PTEN activation through the GluN2BR inhibits the Phosphoinositide 3-

kinase (PI3K) survival-signaling pathway, which furthermore contributes to GluN2BR-mediated neuronal decease (Lai et al., 2011; Ning, 2004).

Next to direct receptor-mediated mechanisms, the excessive stimulation of postsynaptic glutamate receptors leads to a massive calcium influx into the cell, which additionally initiates death processes (Stanika et al., 2009). Calcium has an important role in regulating neuronal signaling and survival under physiological conditions; however, when critical levels of calcium are exceeded, it causes both apoptotic and necrotic neuronal death (Szydlowska & Tymianski, 2010). High levels of calcium activate proteases, lipases, phophatases, and endonucleases (including calpains, phospholipase A2, caspases, and Calcium/calmodulin-dependent protein kinase (CaMK)), and disturb ion transporters (Dirnagl et al., 1999; Szydlowska & Tymianski, 2010). This results in cytoskeletal and membrane disruption, mitochondrial and endoplasmatic reticulum dysfunction, DNA fragmentation, cell swelling and acidosis (Szydlowska & Tymianski, 2010). Furthermore, it causes oxidative stress through the production of reactive molecules such as hydrogen peroxide (H_2O_2), superoxide radical and NO (Dirnagl et al., 1999; Szydlowska & Tymianski, 2010). Moreover, cytochrome C gets released from damaged mitochondria and furthermore activates pro-apoptotic caspases (Dirnagl et al., 1999; Fujimura, Morita-Fujimura, Murakami, Kawase, & Chan, 1998). All of these events propel apoptotic and necrotic death of neuronal cells (Szydlowska & Tymianski, 2010).

Additionally, excitotoxicity causes inflammation in the brain after a stroke. Injection of NMDA into the brain has been shown to induce inflammatory cytokine expression, such as

interleukin-1 β (IL-1β), interleukin-6 (IL-6), tumor-necrosis factor α (TNFα) and transforming growth factor β (TFG-β) by glial cells (Acarin, González, & Castellano, 2000; Clausen et al., 2008) and blocking NMDA receptors reduces cytokine expression after focal ischemia in the rat brain (Jander, Schroeter, & Stoll, 2000). Moreover, reactive oxygen species stimulate expression of pro-inflammatory genes (Dirnagl et al., 1999) and fuel a pro-inflammatory (or activated) phenotype of microglia (Su et al., 2014). Relieving inflammation by inhibiting IL-1β reduces excitotoxic damage in the ischemic brain (Hara et al., 1997).

Multiple strategies have been employed to target excitotoxicity as a treatment for brain damage after a stroke; yet, none of them have been successful in clinical trials (Neuhaus et al., 2014; Zerna, Hill, & Boltze, 2017). Blocking of glutamate receptors is highly effective in protecting neurons in *in vitro* and *in vivo* models of stroke; however, the therapeutic window is narrow and side effects are significant (Dirnagl et al., 1999; Lai et al., 2011). Targeting excitotoxic mechanisms that happen downstream of glutamate receptor activation can increase the therapeutic window (Lai et al., 2014). Furthermore, mediating specific death-associated molecules may pose fewer side effects than interfering with the broad function of glutamate receptors (Lai et al., 2011). Interfering with multiple excitotoxic players that are activated late in the deathly cascade may be particularly successful in mediating neuronal damage after a stroke (Lai et al., 2014). JNK has been shown to regulate cell damage caused by oxidative stress, inflammation and excitotoxicity and is therefore a promising target to effectively reduce a wide range of pathological mechanisms that are involved in ischemia-induced neuronal death.

1.3. C-Jun NH_2-terminal protein kinases (JNKs) – isoforms and splice variants

JNKs, also known as stress-activated protein kinases, belong to the mitogen-activated protein kinase (MAPK) family of protein kinases. There are three isoforms, JNK1, JNK2 and JNK3, which are derived from three different but closely related genes (Coffey, 2014). For each isoform, a number of different splice variants exists, which are all 46kDa or 54kDa in size and are differentiated as α1, α2 and β1, β2. Splice variants α1 and β1 are the shorter JNK versions, while α2 and β2 are the 54kDa variants consisting of a longer C-terminus, extended by 43 amino acids. All α1, α2 and β1, β2 splice variants have been shown to exist for JNK1 and JNK2, whereas for JNK3 only the α splice variants are well characterized (Coffey, 2014).

1.4. Expression patterns of JNKs

Expression patterns are different for the three JNK genes. JNK1 and JNK2 are present ubiquitously throughout the body, while JNK3 mRNA is mainly expressed in the brain and to a small extent in the testes (Kuan et al., 1999). In the adult brain, JNK3 is the most highly expressed isoform, followed by JNK2 and then JNK1 (Carboni, Carletti, Tacconi, Corti, & Ferraguti, 1998). JNKs are most abundant in the neocortex but are also present in the hippocampus, thalamus, and midbrain. On a cellular level, JNKs exist in both cytoplasm and nucleus and can translocate from one to the other (Coffey, 2014).

1.5. JNK activation

Activation of JNKs is achieved through dual phosphorylation of their Thr-X-Tyr motif within the JNK activation loop by their upstream MAPK kinases (MAP2Ks) MKK4 and MKK7 (Lawler, Fleming, Goedert, & Cohen, 1998). These are activated by upstream MAPK kinase kinases (MAP3Ks) such as Apoptosis signal-regulated kinase 1 (ASK1) (Chang & Karin, 2001). JNK activation requires scaffolding proteins, such as Arrestin, that facilitate proximity of the players in this cascade (Kook et al., 2013; Zhan, Kaoud, Dalby, & Gurevich, 2011). An overview of the JNK-activation pathway is presented in Figure 1.

Arrestins are on average 45kDA-sized proteins that play a crucial role in a wide variety of signaling pathways. They are commonly known for binding and regulating G-Protein-coupled receptors (GPCRs), but they also interact with non-receptor partners and play an important role in MAPK activation. Arrestins act as scaffolds that bring together the different players in the MAPK cascade, facilitating phosphorylation of the more downstream kinases (Gurevich, 2014). All four vertebrate Arrestins have been shown to bind JNK3 and its upstream kinases ASK1 and MKK4, but only Arrestin-3 (also known as β-Arrestin 2) has been shown to promote JNK3 activation (McDonald et al., 2000; Song, Coffa, Fu, & Gurevich, 2009; Zhan et al., 2011). Arrestin-3 can also bind to and activate JNK1 and JNK2 (Kook et al., 2013) and it furthermore interacts with MKK7 (Zhan, Kaoud, Kook, Dalby, & Gurevich, 2013). Recently, the first 25 amino acids on the N-terminal of Arrestin-3 have been identified as the key-binding site for JNK3 (Zhan, Perez, Gimenez, Vishnivetskiy, & Gurevich, 2014). Moreover, this short binding element alone, in the form of a peptide

called T1A, also binds JNK's upstream activators ASK1 and MKK7 and can activate JNK3 (Zhan et al., 2016).

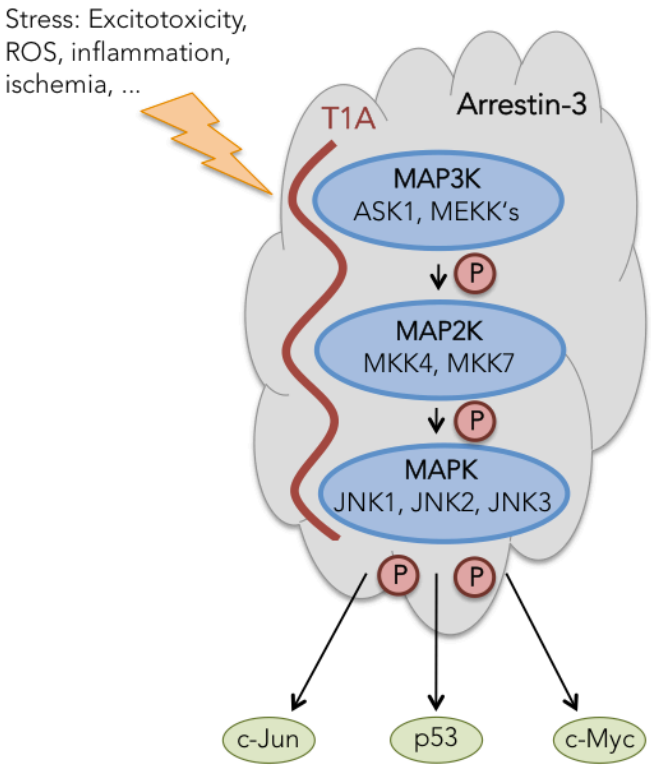

Figure 1 Schematic representation of the JNK activation pathway. JNKs are activated by upstream MAP2Ks, such as MKK4 and MKK7, which are activated by the more-upstream MAP3Ks, including ASK1 and MEKKs (Lawler et al., 1998; Chang & Karin, 2001). Activation of kinases in the MAPK cascade is achieved through phosphorylation and is facilitated by scaffolding proteins, such as Arrestin-3, that bind to, and promote proximity of the different-level kinases (Gurevich, 2014). Arrestin-3, specifically, has been shown to bind to ASK-1, MKK7 and JNK3 via a 25-amino acid-long sequence on it's N-terminal, which, when isolated as a peptide (called T1A), alone is able to promote JNK3 activation (Zhan et al., 2016). JNKs' targets are mainly transcription factors (such as c-Jun, p53 and c-Myc) involved in nervous system development, cell cycle and cell death. The MAPK pathway is known to be induced by various stressors in the central nervous system, including excitotoxicity, reactive oxygen species (ROS), inflammation and ischemia (Coffey, 2014).

1.6. JNK function

JNKs exert a wide variety of functions by phosphorylating downstream targets, which include a variety of transcription factors such as c-Jun, activating transcription factor 2 (ATF2), ETS domain-containing protein (Elk1), c-myc, and p53 that regulate nervous system development, cell cycle and cell death (Coffey, 2014). JNK1, JNK2 and JNK3 are known to have distinct as well as overlapping functions in the brain. JNK1 is largely thought to play a role in developmental processes, as it is highly expressed in the developing brain and mRNA levels decline postnatally. Knockout of JNK1 impairs multipolar transition, bipolar cell movement, and microtubule integrity during development (Chang, Jones, Ellisman, Goldstein, & Karin, 2003; Tararuk et al., 2006; Westerlund et al., 2011). Double knockouts of JNK1 and JNK2 retain an open neural tube and are embryonically lethal (Kuan et al., 1999; Sabapathy et al., 1999). Additionally, apoptosis is dysfunctional during development in these double knockouts (Kuan et al., 1999). JNK1 knockout has also been shown to be protective against kainite-induced neuronal degeneration, suggesting a role of this isoform in death processes in the adult brain (de Lemos et al., 2017). JNK1 furthermore plays a role in synaptic plasticity by phosphorylating PSD-95 and facilitating membrane insertion of the GluR2 α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor subunit following NMDA receptor activation (Kim et al., 2007; Thomas, Lin, Nuriya, & Huganir, 2008).

JNK2 has been shown to be involved in learning and memory mechanisms as well. It plays a role in late-phase long-term potentiation (LTP) and affects learning and LTP in stressed mice (J.-T. Chen et al., 2005; Sherrin et al., 2010). Moreover, it has been implied in the

regulation of pre-synaptic glutamate release (Nisticò et al., 2015). JNK2 has also been implied in neurodegenerative processes in Parkinson's disease. It activates cyclooxygenase 2 and thereby leads to dopaminergic cell death in a mouse model for Parkinson's disease (Hunot et al., 2004).

JNK3 plays a major role in neuronal stress and death processes. It is activated following axonal damage (Cavalli, Kujala, Klumperman, & Goldstein, 2005) and deletion of JNK3 reduces stressed-induced JNK activity as well as excitotoxic neuronal death (Kuan et al., 2003; Yang et al., 1997). Moreover, knocking out JNK3 protects against cell death after kainate-induced seizures (Yang et al., 1997; de Lemos et al., 2017) and results in fewer tonic-clonic seizures after kainate exposure (Brecht et al., 2005). Furthermore, this JNK isoform has been linked to neurodegeneration in Huntington's, Parkinson's and Alzheimer's disease (Hunot et al., 2004; Morfini et al., 2009; Yoon et al., 2012). JNK3 has also been shown to impair GluR1 AMPA receptor trafficking from the Golgi to the membrane under stress conditions (Yang & Cynader, 2014).

1.7. JNKs in stroke

JNKs, in particular JNK3, have been implicated as main players in stroke pathology. Deletion of JNK3, but not of JNK1 or JNK2, protects mice from hypoxia-induced brain injury (Yang et al., 1997; Kuan et al., 2003). JNK, c-Jun and MKK4 activation starts at 3 hours after middle-cerebral artery occlusion (MCAO, a rodent model for stroke) and peaks at 6 hours (Repici et al., 2007). Phosphorylation of JNK, particularly of JNK3, is increased 24

hours after permanent MCAO (Brecht et al., 2005) and after 15-minute transient forebrain ischemia in the rat (Hu, Liu, & Park, 2000). Inhibiting JNK by preventing it from accessing c-Jun or interfering with its interaction with Arrestin 3 reduces infarct volume after MCAO (Borsello et al., 2003; Wei et al., 2018). Furthermore, hindering MKK7 from activating JNK is protective in *in vivo* stroke models (Vercelli et al., 2015). Hence, JNKs play a major role in neuronal death in the brain after a stroke. While JNK3 seems to be the main isoform related to neuronal death, there is also evidence suggesting that only knockdown of all 3 isoforms results in neuroprotection, while individual knockdown is not protective (Björkblom et al., 2008).

1.8. JNKs in excitotoxicity

JNKs are activated by glutamate through NMDA and AMPA receptors (Coffey, 2014). JNK, as well as its upstream kinase MKK7, are activated in cortical neurons as early as one hour after treatment with NMDA (Centeno et al., 2007) and particularly, the death-related GluN2BR has been shown to activate JNK (Lai et al., 2011; Wang et al., 2011). Inhibiting JNK's phosphorylation of c-Jun protects against NMDA-induced cell death (Borsello et al., 2003; Centeno et al., 2007) and knocking out JNK1 or JNK3 reduces excitotoxicity-induced neuronal damage in the hippocampus of mice (de Lemos et al., 2017; Yang et al., 1997). These findings show that JNKs are important players in excitotoxic death processes.

1.9. JNKs in apoptosis and necrosis

JNKs have been shown to be involved in multiple apoptotic mechanisms and inhibiting JNKs is protective against neuronal apoptosis (Yang et al., 1997; Kuan et al., 2003). JNK1 and JNK2 induce cytochrome C release from mitochondria and increase caspase 3 activity (Tournier et al., 2000). Inhibiting JNK's effect on c-Jun prevents caspase-3 activity after excitotoxic insult in cortical neurons (Centeno et al., 2007). JNKs furthermore interact with and phosphorylate the proapoptotic molecule Bim and are involved in Bim- and Bax-mediated apoptosis (Putcha et al., 2003; Okuno, 2004). Activity of JNK's target c-Jun has been correlated to apoptotic cell death and expression of the apoptotic mediator Fas-ligand after ischemia reperfusion injury (Herdegen et al., 1998).

Next to playing a role in apoptosis, JNKs have been shown to mediate necrosis of mouse embryonic fibroblasts after H_2O_2 insult (Shen et al., 2004). Furthermore, JNK1 and JNK3, but not JNK2, have been shown to mediate Poly(ADP-Ribose)-Polymerase 1 (PARP1)-induced necrosis (Muthaiah et al., 2017). Hence, the death processes that JNKs are involved in are of both apoptotic and necrotic nature.

1.10. JNKs in inflammation

Microglia and infiltrating macrophages accumulate at the site of injury and show a pro-inflammatory phenotype after ischemic insult in the brain (Clausen et al., 2008). Microglia with a pro-inflammatory phenotype show increased JNK activation (Hidding et al., 2002; Waetzig et al., 2005). Knock out of JNK1 and JNK3 reduces activity of glia cells after

kainate exposure (de Lemos et al., 2017), indicating a role of these isoforms specifically in excitotoxicity-related inflammation. Additionally, inhibiting JNK by interfering with its effect on c-Jun prevents microglia from being activated 48 hours after oxygen-glucose deprivation of hippocampal slices *in vitro* (Benakis, Bonny, & Hirt, 2010). These findings show that JNK has a significant function in inflammation in the brain after a stroke.

1.11. JNKs and oxidative stress

It has been well established that JNKs are activated by oxidative stress (Shen & Liu, 2006). Reactive oxygen species, such as H_2O_2, activate JNK's upstream MAPK kinase ASK1, but also lead to JNK activation through other pathways involving Src kinase, glutathione S-transferase Pi (GSTπ), or receptor-interacting protein (RIP) and tumor-necrosis factor receptor-associated factor 2 (TRAF2) (Shen & Liu, 2006). Activation of JNK through NO has been shown to depend on MKK4 and may therefore be more specific to the MAPK signaling cascade (Shen & Liu, 2006). Inhibiting JNKs protects cells against oxidative stress-induced apoptosis and necrosis (Shen et al., 2004; Shen & Liu, 2006); hence, JNKs play an important role in oxidative stress-induced cell death.

1.12. JNKs as a target for stroke therapy

From the literature presented above, it becomes clear that JNKs have a significant role in multiple pathological processes involved in ischemic neuronal damage. Interfering with their function is therefore a promising strategy to reduce brain damage after a stroke. Previous attempts, for example with the small peptide JNK inhibitor D-JNKi, have been

successful in reducing excitotoxic neuronal death in vitro and reduce infarct volume in an animal model for stroke (Benakis et al., 2010; Borsello et al., 2003). However, this JNK inhibitor, which prevents JNK from accessing its downstream target c-Jun, was not effective in preventing an inflammatory phenotype of microglia 48 hours after MCAO in rats (Benakis et al., 2010), indicating that inhibiting only one of JNK's functions may not be enough to effectively interfere with all of its pathological actions in a stroke setting. The activation cascade of JNKs and their downstream effects are complex and a more effective approach may be to target multiple players in their activation cascade.

1.13. SNIPER peptides

Our lab has recently developed a peptide-based approach to rapidly and effectively degrade endogenous proteins: the selective native protein eradication (SNIPER) method (Fan et al., 2014; Fan & Wang, 2015). SNIPER peptides consist of three different segments, a cell-penetrating sequence, a protein-binding domain (PBD), and a motif that is recognized by chaperone-mediated autophagy (CMA). With these three segments, SNIPER peptides can enter cells and bind and degrade native proteins in a fast and reversible manner (Fan et al., 2014; Fan & Wang, 2015).

The trans-activating transcriptional activator (TAT) from the human immunodeficiency virus 1 (HIV1) was one of the first cell penetrating peptides (CPP) to be identified (Frankel & Pabo, 1988) and shorter segments of TAT, such as its residues 37-57, 48-60 or 49-57, have been shown to effectively translocate into cells (Green & Loewenstein, 1988; Vivès, Brodin,

& Lebleu, 1997; Park et al., 2002; Ramsey & Flynn, 2015). TAT is commonly used as a delivery tool for peptides in our and other labs (Aarts, 2002; Borsello et al., 2003; Fan et al., 2014).

CMA is an endogenous cellular mechanism for degradation of damaged or redundant molecules by the lysosome. Proteins are targeted to CMA-mediated degradation if they contain the specific pentapeptide motif KFERQ (Dice et al., 1990). SNIPER peptides from our lab that contained this CMA-targeting motif have been shown to efficiently knock down endogenous proteins such as DAPK1 and PSD-95 (Fan et al., 2014).

A crucial step in designing a SNIPER peptide is the selection of a suitable PBD. Protein-binding sequences of SNIPER peptides are usually derived from natural binding partners of the protein of interest. For example, the DAPK1-degrading SNIPER peptide developed in our lab has a PBD that is based on C-terminus residues of the NMDA receptor that is known to interact with DAPK1 (Fan et al., 2014).

SNIPER peptides with TAT as a cell-penetrating domain, KFERQ as a CMA-targeting motif, and a PBD derived from a natural binding partner of the protein of interest are effective tools to degrade endogenous proteins.

1.14. Summary and directions

The development of new therapies for stroke has been a challenge for decades, despite sophisticated research that has led to better understanding of the pathological mechanisms involved. Targeting a wide range of the deathly cascades involved in stroke pathology may

be more successful in the clinic than existing neuroprotective strategies that focus on individual molecules or specific death processes. The JNK signaling pathway plays an important role in several pathological processes that mediate cell death after a stroke, including excitotoxicity, inflammation, oxidative stress as well as apoptosis and necrosis. Targeting several of the kinases in this significant cascade is a promising tactic to mediate a multifold of the deathly mechanisms that lead to brain damage after a stroke. Here, I propose to employ the powerful SNIPER method to degrade multiple players in the deathly JNK cascade in order to target stroke-induced neuronal death. JNK's well-described binding with the T1A domain of Arrestin-3 provides a promising starting point for the development of a PBD that may not only degrade JNK but also additional players in the JNK-activation pathway. Such an approach may be more effective in treating the complex stroke death cascade than previous methods that have only targeted JNK individually or specific JNK functions.

1.15. Hypothesis and aims

For this thesis, I hypothesized that:

A SNIPER peptide, with a PBD derived from the T1A sequence of Arrestin-3 can knock-down multiple players in the JNK-activation cascade and thereby protect against multiple deathly processes that lead to brain damage after a stroke.

To test this hypothesis, I employed the following aims:

1. Develop a series of SNIPER peptide based on the T1A sequence of Arrestin-3,

2. Identify the peptide that is most efficient in reducing levels of active JNK,
3. Determine if this peptide reduces JNK activity via knockdown of target proteins,
4. Test is this peptide is neuroprotective against cell death caused by excitotoxicity and oxidative stress, and to
5. Test if this peptide can reduce brain damage after cerebral ischemia in an animal model for stroke.

By investigating these aims, I may develop a new tool to treat brain damage after a stroke that may be more effective than previously developed methods.

2. Methods

2.1. Primary culture of cortical neurons

Rat cortical neurons were obtained from Sprague Dawley rat embryos (Charles River, Canada), 19 days after fertilization. After sacrificing the mothers with 4mg/kg urethane (Sigma), the embryos were removed from the uterus and their brains were extracted. Cortices were dissected in Hank's Balanced Salt Solution (HBSS, 111mM glucose (Sigma), 146mM sucrose (Fisher), 15mM Hepes (Bioshop), pH=7.4, osmolarity=315mOSM) and cut into three pieces. After removal of the HBSS, they were lysed in pre-warmed 0.25% Trypsin-Ethylenediaminetetraacetic acid (Life Technologies) for 30min at 37°C. Then, 8 to10ml of Dulbecco's modified Eagles medium (DMEM, Sigma) containing 10% fetal bovine serum (ThermoFisher) was added to the cell lysate, gently mixed and transfer into a fresh 50ml Falcon tube (VWR Canada). After letting the cells settle at the bottom of the tube, the media was gently removed and replaced with fresh DMEM 3 times. Next, the cell suspension was gently mixed by pipetting it up and down to allow for single-cell suspension. Next, it was centrifuged for 50s at 2500rpm, after which the DMEM was removed and the cells were resuspended in Neurobasal Plating medium (Neurobasal medium (ThermoFisher) with 2% B27 (ThermoFisher), 0.5mM GlutaMAX (ThermoFisher)). Cell counts were estimated using haemocytometry, the cells were diluted as necessary in Neurobasal plating medium and then plated on poly-D-lysine coated (Sigma-Aldrich) 24-well plates (Sigma) at a density of 0.3×10^6 cells per well. The plates were incubated for 2 days at 37°C with 5% CO_2 before two-thirds of the media was replaced with Neurobasal Feeding media (Neurobasal medium with 2% B27). Subsequently, the cells were kept at

37°C and 5% CO_2 and two-thirds of the media were replaced with fresh Neurobasal Feeding media every 4 days. Experiments were conducted at 14 days *in vitro* (d.i.v.). All animal experiments were performed according to protocols approved by the University of British Columbia Animal Care Committee.

2.2. Peptide synthesis

All peptides were synthesized from the carboxyl to the amide terminal using Fmoc-based solid-phase peptide synthesis technology. The Fmoc-N-protected carboxyl terminal amino acid for each peptide was anchored via its carboxyl group to p-Alkoxybenzyl alcohol resin (Wang-resin). After loading this first amino acid, each peptide was sequenced via repetitive cycles of N-deprotection and amino acid coupling reactions (Howl, 2005): Fmoc protected amino acids (Fmoc-Glu (OtBu)-OH, Fmoc-Met-OH, Fmoc-Leu-OH, Fmoc-Lys (Boc)-OH, Fmoc-Arg (Pbf)-OH, Fmoc-Asn (Trt)-OH, Fmoc-Ser (tBu)-OH, Fmoc-Gln (Trt)-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Pro-OH, Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH, Fmoc-Tyr(tBu)-OH, all from GL Biochem) were dissolved in anhydrous, amide free *N*,*N*-dimethylformamide (DMF, VWR Canada) to 200mM. After loading Fmoc-Arg (Pbf)-Wang-resin (GL Biochem) into the peptide synthesizer (Liberty Blue HT-12 automated microwave peptide synthesizer, CEM, USA), each coupling reaction was initiated with two 2.5-minute repetitions of N-deprotection with 20% piperidine in DMF at 50°C, followed by five 0.5 minute repetitions of DMF wash. The appropriate amino acid solution and the activator cocktail (400mM 4-Methylmorholine (NMM, Alfa Aesar), 200mM 2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, GL Biochem), 200mM N-

Hydroxybenzotriazole –anhydrous (HOBt, GL Biochem) in DMF) were added and the reaction was continued for 10 minutes at 50°C while bubbling in nitrogen. The vessel was washed with DMF for 0.5 minutes and the coupling reaction was repeated to increase coupling efficiency. The vessel was washed 5 times with DMF for 0.5 minutes and the same coupling reaction steps were then completed for each subsequent amino acid. After the last coupling reaction, the peptide was cleaved from the resin and the side-chain protection groups by adding 10ml of TFA cocktail (94% trifluoroacetic acid (TFA, Alfa Aesar), 2% Triisopropylsilane (TIS, Sigma), 2% 1,2-Ethandithiol (EDT, Sigma-Aldrich), 2% ddH_2O) per 100mg of resin to the reaction mixture under nitrogen bubbling for 1 to 2 hours. Then the TFA cocktail was removed and the vessel was washed in DMF at a volume of one third of that used for the TFA cocktail. The resin-cleavage and side-chain protection group-removal steps were repeated 6 times. Then the crude peptide solution was collected and after adding pre-chilled 99% tert-Butyl methyl ether (3:1, Alfa Aesar) it was centrifuged at 1700 x g and 4°C for 10 minutes. The supernatant was removed, the crude peptide was collected, and the same washing was repeated three times. The supernatant was then completely removed and the peptide pellet was air-dried. Next, the peptide was re-solubilized in 1 to 2ml of ddH2O and acetonitrile (high-performance liquid chromatography (HPCL) grade, VWR Canada) was added until the peptide dissolved. The peptide was purified and characterized by a C-18 reverse-phase column with water-acetonitrile gradient (218TP510, Vydac) by HPLC (Agilent 1200 high performance liquid chromatograph) and mass spectrometry (Agilent 6120 mass spectrometer). Finally, the peptide was freeze-dried with a lyophilizer and stored at -80°C until further use.

2.3. *In vitro* peptide testing

Peptides were freshly diluted in ddH_2O prior to each cell culture treatment. Medium was replaced with 0.5ml of fresh Neurobasal Feeding medium and T1A-1, T1A-2, and T1A-3 were added to each well at 5μM, 10μM (all peptides) and 25μM (T1A-3 only). Cell cultures were placed back in the 37°C incubator for different time points. T1A-1 was tested at 4 hours, T1A-2 was tested at 4 hours and 24 hours and T1A-3 was tested after 4 hours, 6 hours, 8 hours, 12 hours or 24 hours. Medium was collected for cytotoxicity detection assay (T1A-3 only) and/or cells were lysed for protein extraction (see below). Experiments done for protein analysis did not include a 25μM dose nor a 6-hour time point. Untreated wells were used as control.

2.4. *In vitro* neuroprotection assay

Peptides were freshly diluted in ddH_2O prior to each cell culture treatment. Medium on all wells was replaced with 0.5ml of fresh Neurobasal Feeding medium and at 5μM of T1A-3 was added to appropriate wells. Cells were incubated for 23 hours at 37°C, after which the medium was replaced with 0.5ml of fresh Neurobasal feeding medium. Again, 5μM of T1A3 were added to the same wells. Separately, 100μM of NMDA (Abcam) and Glutamate (L-Glutamic Acid, Sigma) as well as 10mM of H_2O_2 (Sigma) were each added to at least one well with T1A-3 and one well without T1A-3. After incubation for 1 hour at 37°C, the medium was replaced with 0.5ml of fresh Neurobasal Feeding medium and incubated for 24 hours before medium collection for the cytotoxicity detection assay (see below). Wells

with no treatment (only medium replacement) were used as control. A total of 7 to 11 biological replicates was tested for each treatment condition.

2.5. Cytotoxicity detection assay

Lactate dehydrogenase (LDH) assay kit (Roche) was used to detect cell death in cortical neuronal cultures according to the manufacturer's instructions. In wells of a 96-well microtiter plate (Sigma), 50µl of the reaction mixture were mixed with 50µl of conditioned medium from assay culture plates. The LDH assay plate was shaken at 300rpm and room temperature for 20min and absorbance was measured at 490nm by spectrophotometry (Device: SpectraMax M2 from Molecular Devices, Software: SoftMax Pro 6.5). Background absorbance was measured at 690nm and subtracted from optical densities (ODs) at 490nm. Relative cytotoxicity was calculated by normalizing ODs from the treatment conditions to the ODs from the untreated control. Statistical analyses were conducted on absolute blanked values. Biological replicates varied from 3 to 19 replicates for the different time points and peptide concentrations.

2.6. Peptide testing *in vivo*

Adult male Sprague Dawley rats (180-230g, Charles River, Canada) were anesthetized with 5% isoflurane and held at surgical plane during peptide injection. 50mg/kg of T1A-3 in saline or 1ml/kg of saline was injected into the tail vein after which the rats were immediately recovered. Animals were monitored closely and supplementary care was provided as necessary. 8 hours after injection the animals were euthanized with an

intraperitoneal injection of 4g/kg urethane. The brain was removed, hippocampus and cortex were dissected, and the tissue was stored at -80°C. In total, 4 rats were injected with T1A-3 and 5 rats were injected with saline and used as control.

2.7. Endothelin-1 stroke model

Male Sprague-Dawley rats (250-350 g) (CENPALAB, Havana, Cuba) were maintained under standard laboratory conditions (60% humidity, 22 ± 1 °C, and 12 h light/darkness cycle) with free access to food and water. Endothelin-1 (ET-1)-induced transient focal brain ischemia was induced as previously described (Moyanova et al., 2007). The rats were intraperitoneally anesthetized with 40mg/kg thiopental plus 2.5mg/kg diazepam and placed prone in a stereotaxic frame. ET-1 administration was carried out in the periphery of the middle cerebral artery following the stereotaxic coordinates: 0.2mm anterior, 5.2mm lateral, 8.0mm ventral relative to bregma (Windle et al., 2006; Paxinos & Watson, 2007). ET-1 (Sigma) at 150pmol in 3µL 0.9% saline was injected with a 27G needle at a flow of 1µL/min. Sham animals received an intracerebral injection of 0.9% saline solution instead of ET-1. The rectal temperature was maintained at 37 ± 0.5 °C during the surgery and post-surgical recovery with an incandescent lamp. PBS at 1ml/kg (vehicle) or T1A-3 at 50mg/kg was administered intraperitoneally (i.p.) 30 min before the surgery. 24 hours after the surgery, the rats were anesthetized (40mg/kg thiopental plus 2.5 mg/kg diazepam) and perfused with ice-cold 0.9% saline solution. Their brains were extracted, frozen and sectioned into six 2mm-thickness coronal sections. Brain slices were incubated for 30 min in a 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) solution (in 0.1M PBS, pH 7.4) at 37

°C and fixed by immersion in a 4% paraformaldehyde solution for 48 hours at 4°C. Sections were scanned (Hewlett Packard HP Scanjet G2710) and analyzed using the ImageJ 1.41 software (National Institutes of Health). The scale bar was normalized to 5mm and infarct areas in each slide were marked using the free hand tool, after which the area size was estimated by the software. Infarct areas (in mm^2) of each slide were then added together and multiplied with the slice thickness (2mm) to get infarct volume (mm^3) in each brain. The edema index (ratio of ipsilateral/contralateral hemispheres) was used to correct the infarct measurements for brain swelling. A total of 5 rats were tested for each group. The procedures were approved by the University of Havana's ethics committee in compliance with the Canadian Council on Animal Care (Canadian Council on Animal Care, 1993) and the ARRIVE guidelines for animal experimentation (McGrath, Drummond, McLachlan, Kilkenny, & Wainwright, 2010).

2.8. Protein analysis

2.8.1. Protein extraction

Radio-immuno precipitation assay (RIPA) buffer (150mM sodium chloride, 1% Triton-X-100, 0.5% sodium deoxychloride, 0.1% sodium dodecyl sulfate (SDS), 50mM Tris(Hydroxymethyl)aminomethane (TRIS, Bioshop, pH=8.0) with protease and phosphatase inhibitor cocktail (Bimake) was used for protein collection from *in vitro* and *in vivo* experiments.

For protein collection from cell cultures, the medium was aspirated from all wells of 24-well plates (Sigma), cells were washed in ice-cold phosphate-buffered saline (PBS) and 100µl of RIPA buffer was added per well and pipetted up and down 3 times. To ensure enough protein for subsequent analyses was collected, the RIPA buffer used for lysing cells in one treatment well was transferred to a second well of the same treatment condition and then collected into 1.5ml microcentrifuge tubes (Fisher).

For protein collection from rat brain tissue, frozen cortex and hippocampus samples were thawed and homogenized in 1ml RIPA buffer per 100mg tissue using mortar and pestle. Cell lysates and homogenized tissue in RIPA buffer were incubated on ice for 30min and centrifuged for 15min at 12000rpm and 4°C. The supernatant was then transferred into fresh tubes and either stored at -80°C for later protein concentration determination or it was directly proceeded with the protein assay.

2.8.2. Protein concentration determination and sample preparation for SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The Pierce BCA Protein Assay kit (Thermo Scientific) was used to determine protein concentration of *in vitro* and *in vivo* samples following the manufacturer's instructions. Bovine serum albumin (BSA, Thermo Scientific) at 2mg/ml was 2-fold serially diluted to create a protein standard curve. 10µl of 10x-diluted samples and BSA standard were mixed with 100µl of assay reagent on a 96-well microtiter plate, which was incubated at 37°C for 30min. Absorbance was measured at 562nm using spectrophotometry and protein

concentrations of each sample were determined by relating ODs from samples to the BSA standard curve. The samples were diluted to 0.3µg/µl in ddH_2O and 6x Laemmli sample buffer (10% SDS (Bioshop), 30% glycerol (Fisher), 0.35M TRIS, 0.3% bromophenol-blue (Sigma), 9% 2-Mercaptoethanol (Sigma)) and boiled at 99°C for 5min. Samples were either stored at -80°C or it was directly proceeded with SDS-PAGE.

2.8.3. SDS-PAGE and Western blot analysis

Frozen samples were thawed and reboiled at 99°C for 5min. A total of 10µg of protein from each sample and 5µl of the BLUeye prestained protein ladder (FroggaBio) was loaded on 10-well 1.5mm gels consisting of a 5% stacking (2.82mL ddH_2O, 0.83mL 30% acrylamide solution (Bio-Rad), 1.25mL 0.5M TRIS (pH 6.8), 50µL 10% SDS, 50µL 10% ammonium persulfate (APS, Bioshop), 5µL Tetramethylethylendiamin (TEMED, Bio-Rad)) and a 10% separating gel (4mL ddH2O, 3.3mL 30% acrylamide solution, 2.5mL 1.5M TRIS (pH 8.8), 100µL 10% SDS, 50µL 10% APS, 5µL TEMED). The proteins were run through the stacking gel at 80V and then separated at 120V using the Bio-Rad electrophoresis system. After electrophoresis, proteins were transferred onto a 0.45µm Immobilon-P Polyvinylidene difluoride (PVDF, Millipore) membrane at 110V for 90min. The membranes were blocked in 3% BSA (Fisher) in TRIS-buffered saline with Tween-20 (TBS-T, 50mM Tris-HCl, 150mM NaCl (Bioshop), and 0.1% Tween-20 (Bioshop)) for 1 hour at room temperature. Then the membranes were incubated in 1° antibodies (1:1000 in 3% BSA in TBS-T) overnight at 4°C (see table 2.1 for primary antibody specifications). Membranes were then washed 3 times 5 minutes in TBS-T and incubated in horseradish-peroxidase (HRP)-conjugated 2° antibody

(1:5000 in 3% BSA in TBS-T, see table 2.2 for secondary antibody specifications) for 1 hour at room temperature. After washing the membranes again 3 times 5minutes in TBS-T, blots were developed with Luminata Crescendo Western HRP substrate (Millipore Sigma) using the ImageLab software (Version 5.2.1) and the ChemiDoc MP System (Bio-Rad). White epi illumination and auto exposure for intense bands were used to detect the protein ladder. High sensitivity chemiluminescence and auto exposure for intense bands was used to detect HRP signals.

Next, antibodies were removed from the blots by incubating the membranes in pre-warmed stripping buffer (2% SDS, 62.5mM TRIS, 100mM 2-Mercaptoethanol (Aldrich)) at 50°C for 30min. Membranes were washed 5 times 5min in TBS-T and blocked in 3% BSA in TBS-T for 1 hour at room temperature. The blots were then incubated in anti-β-actin antibody (1:5000 in 3% BSA in TBS-T, Sigma-Aldrich A2228) either overnight at 4°C or at room temperature for 1 hour. After washing 3 times 5 minutes in TBS-T, the membranes were incubated in anti-mouse Immunoglobulin G (IgG, from goat) HRP-labelled (1:5000 in 3% BSA in TBS-T, PerkinElmer, NEF822001EA) for 1 hour at room temperature, washed again 3 times in TBS-T and bands were detected with the same method as for above blots. Band intensity was determined using the band detection function in the ImageLab software (Bio-Rad) and normalized to the band intensity of respective β-actin bands. Normalized band intensities were then furthermore normalized to control band intensities. Statistical analyses were conducted on band intensities normalized to β-actin.

Table 2.1 Primary antibodies

Antibody name	Host species	Company	Catalogue #
Anti - JNK1 + JNK2 + JNK3 (phospho T183+T183+T221)	Rabbit	Abcam	ab124956
Anti-JNK1	Rabbit	Abcam	ab110724
Anti-JNK2	Rabbit	Abcam	ab76125
Anti-JNK3 (55A8)	Rabbit	Cell Signaling Technology	2305S
Anti-β-Actin	Mouse	Sigma-Aldrich	A2228

Table 2.2 Secondary antibodies

Antibody name	Host species	Company	Catalogue #
Anti-Mouse IgG HRP-labeled	Goat	PerkinElmer	NEF822001EA
Anti-Rabbit IgG HRP-labeled	Goat	PerkinElmer	NEF812001EA

2.8.4. Statistical analysis

All data was analyzed and graphed in GraphPad Prism 6.0. The Kruskall-Wallis test with Dunn's multiple comparison test was used for *in vitro* experiments, where multiple treatment conditions were compared to the same control (i.e. peptide effects on pJNK, JNK1, JNK2 and JNK3, cytotoxicity assay, and neuroprotection assay). For *in vivo* experiments, where separate control groups were used, the Mann-Whitney U test was employed to assess statistical significance of differences between untreated and peptide-treated animals. All statistical tests were conducted with a confidence level of 95% and a p-value below 0.05 was considered statistically significant. P-values from Dunn's post test are reported in GraphPad Prism as $p > 0.05$, $p < 0.05$, $p < 0.01$ or $p < 0.001$. Therefore, results from multiple comparison tests are not reported as exact p-values in this thesis. Data is

presented as mean ± standard error of the mean (SEM). In graphs, one asterisk indicates a significant difference from control with a p-value below 0.05, two asterisks indicate $p < 0.01$, three indicate $p < 0.001$, and four asterisks indicate $p < 0.0001$.

3. Results

3.1. Peptide design

The goal of this project was to impair the JNK activation cascade to hinder its action in multiple death processes involved in stroke pathology. In order to develop a successful inhibitor of JNK's deathly functions, we proposed to identify an effective SNIPER peptide based on Arrestin's key JNK-binding site, which also binds JNK's upstream activators ASK1 and MKK7 (Zhan et al., 2016). We suggested that such a peptide, also consisting of the CPP TAT and a CMA-targeting motif, would be able to enter cells, bind to JNK and its upstream kinases and advance their lysosomal degradation. We hypothesized that it thereby interferes with several of the deathly functions of the JNK cascade and may serve as a potent neuroprotective stroke therapy.

The efficacy of a SNIPER peptide depends on the proper binding of its peptide-binding domain to the target protein; hence, finding a suitable binding sequence is a crucial step in designing a SNIPER peptide (Fan & Wang, 2015). The T1A peptide derived from Arrestin-3 has been shown to efficiently bind to JNK3 as well as its upstream kinases ASK1 and MKK7 (Zhan et al., 2016). Hence, this 25 amino acid-long sequence is an excellent candidate for a peptide-binding domain that binds to multiple players in the JNK activation cascade.
We used three shorter segments of the T1A sequence to construct three distinct SNIPER peptides, T1A(1-12), T1A(7-19) and T1A(12-25), and named them T1A-1, T1A-2 and T1A-3, respectively. They were each flanked with TAT(47-57) (YGRKKRRQRRR) at the C-terminal and with the CMA-targeting motif KFERQ at the N-terminal. The full sequence of the three

peptides can be found in table 3.1 and a schematic representation of the peptide design is presented in Figure 3.1.

Table 3.1 Sequence of the T1A peptide and our SNIPER peptides derived from T1A

Peptide name	Sequence
T1A	MGEKPGTRVFKKSSPNCKLTVYLGK
T1A-1 / T1A(1-12)	KFERQ - MGEKPGTRVFKK - YGRKKRRQRRR
T1A-2 / T1A(7-19)	KFERQ - VFKKSSPNCKL - YGRKKRRQRRR
T1A-3 / T1A(13-25)	KFERQ - SSPNCKLTVYLGK - YGRKKRRQRRR

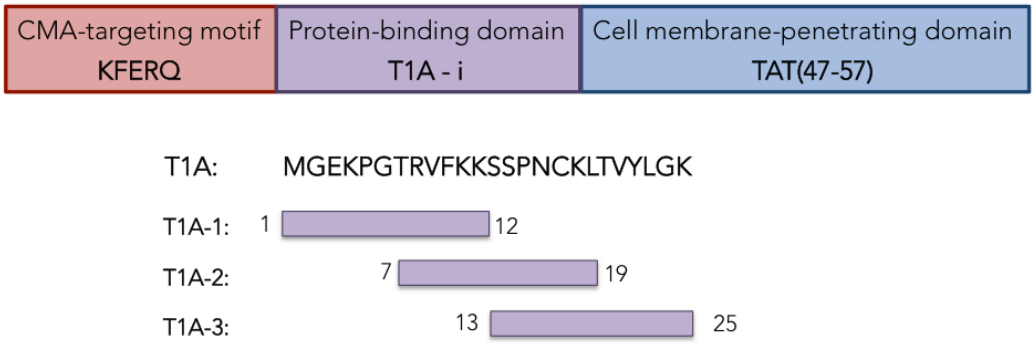

Figure 3.1 Schematic representation of our three SNIPER peptides. Each peptide contains a CMA-targeting motif (KFERQ) on the N-terminal and the cell-penetrating domain TAT(47-57) on the C-terminal of a protein-binding domain. The protein-binding domain of our peptides each consists of a shorter segment of the T1A peptide (Zhan et al., 2016). The binding-domain of T1A-1 contains the first 12 amino acids of T1A, T1A-2 is based on amino acids 7 to 19 of T1A and T1A-3 consists of the last 13 amino acids of T1A.

3.2. T1A-3 is most effective at reducing levels of active JNK

In order to test if our T1A-based SNIPER peptides are effective in impeding the JNK cascade, we first tested whether they reduce levels of JNK phosphorylation, an indicator for JNK activity (Kyriakis & Avruch, 2012). We treated cultures of cortical neurons with 5μM and 10μM of T1A-1, T1A-2 and T1A-3 for different time periods. Protein was collected and analyzed using Western blotting to detect levels of phosphorylated JNK (pJNK). The results are presented in Figure 3.2. Treatment with 10μM of T1A-3 for 4 hours significantly reduced levels of pJNK ($p < 0.05$, $n = 12$). 10μM of T1A-2 also reduced levels of pJNK after 4 hours, however, this effect was not statistically significant ($p > 0.05$, $n = 10$). Levels of pJNK after 5μM of T1A-2 ($n = 9$) and T1A-3 ($n = 11$) treatments were not significantly different from pJNK levels in untreated cells (both: $p > 0.05$). No effect on pJNK levels was seen after treatment with T1A-1 (both doses: $p > 0.05$, $n = 2$).

Next, we tested if T1A-2 and T1A-3, which slightly to significantly reduced levels of pJNK at 4 hours, have an effect on JNK activity with a longer treatment time of 24 hours. In line with the early time point, 24-hour treatments of T1A-2 only slightly but not significantly reduced levels of pJNK with 5μM ($p > 0.05$, $n =6$) and 10μM ($p > 0.05$, $n = 2$). T1A-3, on the other hand, substantially reduced pJNK levels with 10μM ($p < 0.05$, $n = 8$). The lower dose of 5μM also reduced levels of pJNK, but this effect was not significant ($p > 0.05$, $n = 5$; Figure 3.2). Hence, T1A-3 is most effective in reducing levels of active JNK and therefore the most promising candidate of our three peptides to be neuroprotective. This peptide was therefore selected for further investigations.

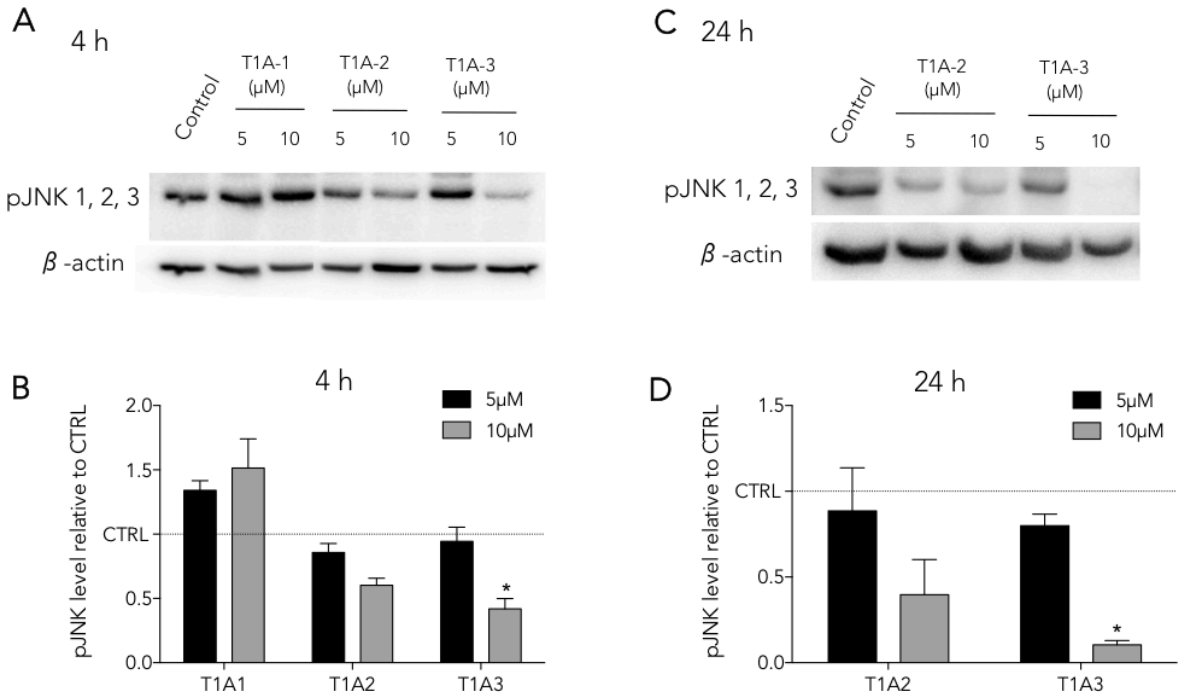

Figure 3.2 Effect of T1A-1, T1A-2 and T1A-3 on levels of pJNK after 4 and 24 hours in cortical neuronal cultures. Representative Western blots showing pJNK 1, 2 and 3 and β-actin levels in untreated cells (CTRL) and cells treated with 5μM and 10μM of T1A-1, T1A-2 and T1A-3 after 4 hours (A) and 24 hours (C). Quantifying graphs show mean pJNK levels normalized to actin + SEM relative to untreated cell (CTRL) after 4 hours of T1A-1, T1A-2 and T1A-3 treatment (B) and after 24 hours of T1A-2 and T1A-3 treatment (D). 10μM of T1A-3 significantly reduced levels of pJNK after 4 hours and 24 hours. No significant effects on pJNK levels were seen with T1A-1 and T1A-2 and with 5μM of T1A-3.

3.3. T1A-3 reduces levels of phosphorylated JNK along a time course of 24 hours

Our initial peptide screening showed that 10μM of T1A-3 can significantly reduce levels of active JNK for 4 hours and 24 hours. To better understand how T1A-3 affects pJNK over time with the two different doses, we next tested additional time points for the effect of the peptide on pJNK levels. The peptide was administered at 5μM and 10μM to cultures of

cortical neurons for 4 hours, 8 hours, 12 hours, and 24 hours, after which protein was collected and tested for pJNK content using Western blot. The results show a significant decrease of pJNK with 10μM of T1A-3 at 4 hours ($p < 0.05$, $n = 12$) and 24 hours ($p < 0.05$, $n = 5$). After 8 hours ($n = 5$) and 12 hours ($n = 4$), 10μM of T1A-3 also reduced pJNK levels, however, this effect was not statistically significant (both time points: $p > 0.05$; Figure 3.3). The lower dose of 5μM of T1A-3 only slightly reduced pJNK levels at 12 hours ($n = 4$) and 24 hours ($n = 8$), but these results were not statistically significant (both: $p > 0.05$). No decrease in pJNK levels was seen after 4 hours ($p > 0.05$, $n = 11$) and 8 hours ($p > 0.05$, $n = 4$) with 5μM of T1A-3. Hence, T1A-3 is most effective in reducing levels of active JNK in cultures of cortical neurons at a dose of 10μM while the lower dose of 5μM only slightly reduces pJNK levels at later time points.

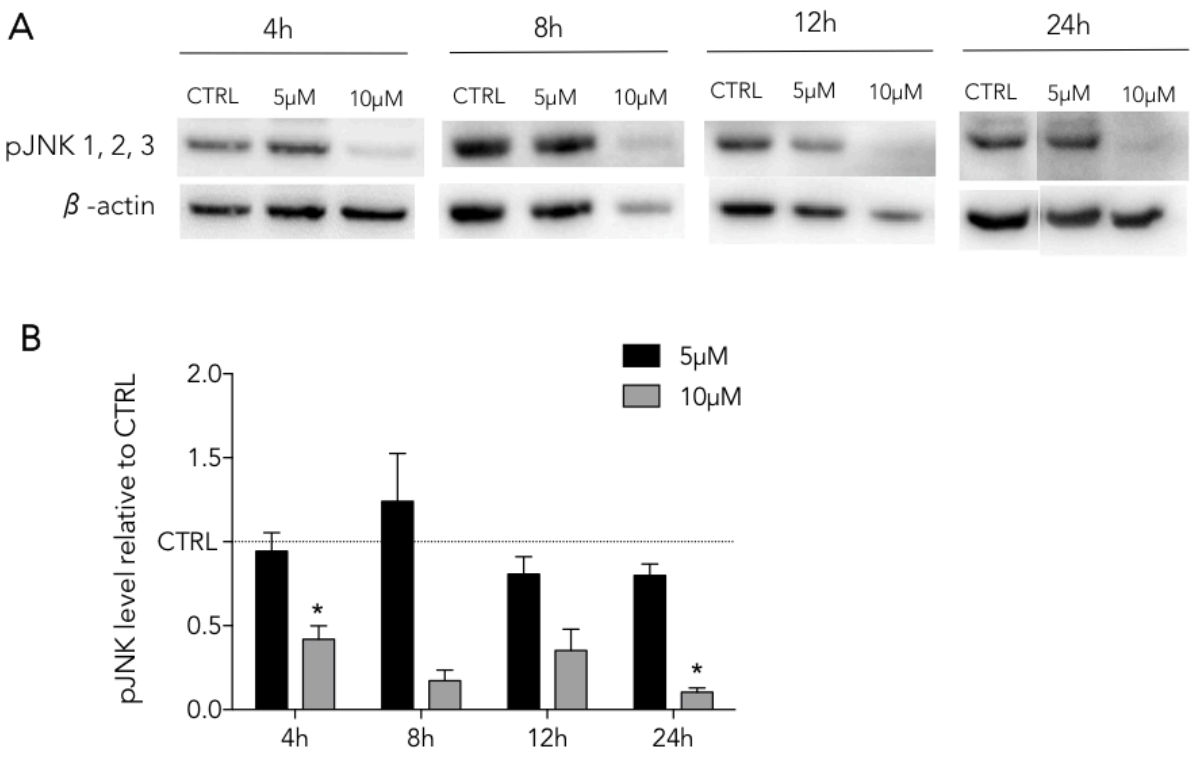

Figure 3.3 Effect of T1A-3 on levels of pJNK after 4 hours, 8 hours, 12 hours, and 24 hours in cultured cortical neurons. A: Representative Western blots showing pJNK 1, 2 and 3 and β-actin levels in untreated cells (CTRL) and cells treated with 5μM and 10μM for 4 hours, 8 hours, 12 hours and 24 hours. The 24 hours blot was cropped for the purpose of this figure (see Appendix A for the full blot). B: Quantification of pJNK levels detected by Western blot after T1A-3 treatment at different concentrations and time points. The graph shows mean pJNK levels normalized to actin + SEM relative to untreated cells (CTRL). There was a significant reduction in pJNK with 10μM of T1A-3 after 4 hours and 24 hours.

3.4. T1A-3 is not toxic at low doses

TAT has been widely used as a delivery tool for peptides; however, whether TAT is toxic to cells remains a controversial topic in the field. Some studies have shown that TAT alone is toxic (Nath et al., 1996; Bonavia et al., 2001), while others report no toxicity (Vivès et al., 1997) or even neuroprotection (Meloni et al., 2014). In order to test if our peptide is safe to

use, we investigated if it is toxic to cortical neuronal cultures when administered at different doses over a time course of 4 to 24 hours. The results can be found in Figure 3.4 and show that 5μM of T1A-3 does not increase LDH release (a measure of cytotoxicity) from cortical neurons at 4 hours ($p > 0.05$, $n = 15$), 6 hours ($p > 0.05$, $n = 9$), 8 hours ($p > 0.05$, $n = 9$), 12 hours ($p > 0.05$, $n = 6$), and 24 hours ($p > 0.05$, $n = 19$). The higher dose of 10μM of T1A-3 elevated LDH release compared to untreated cells; however, this increase was only statistically significant with 12 hours of peptide treatment ($p > 0.05$, $n = 6$). LDH activity with 4 hours ($n = 16$), 6 hours ($n = 9$), 8 hours ($n =9$) and 24 hours ($n = 13$) of 10μM T1A-3 treatment did not significantly differ from that in untreated cells (all time points: $p > 0.05$). A higher dose of 25μM of T1A-3 significantly increased LDH release compared to control after 4 hours ($p > 0.001$, $n = 10$), 6 hours ($p < 0.001$, $n = 9$), 8 hours ($p < 0.0001$, $n = 9$), and 12 hours ($p < 0.001$, $n = 6$) but not after 24 hours ($p > 0.05$, $n = 9$). Hence, T1A-3 is safe to use at a dose of 5μM for the treatment of cultured cortical neurons.

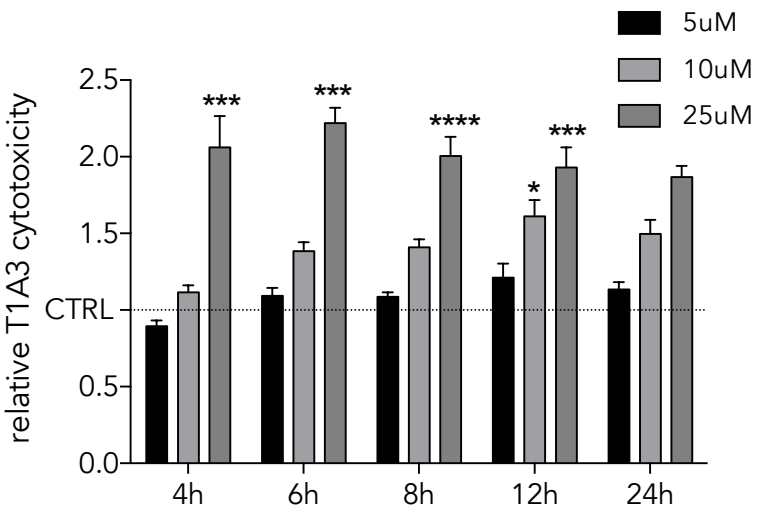

Figure 3.4 Cytotoxicity of 5μM, 10μM, and 25μM of T1A-3 after 4 hours, 6 hours, 8 hours, 12 hours, and 24 hours relative to untreated cells (CTRL) measured by LDH release. T1A-3 is not cytotoxic at 5μM at all time points. LDH is higher with 10μM of T1A-3 compared to CTRL, however, this increase is only statistically significant at 12 hours. 25μM of T1A-3 significantly increase LDH release at all time points, except for at 24 hours.

3.5. T1A-3 reduces protein levels of JNK1 after 24 hours *in vitro*

We hypothesized that through the CMA-targeting motif of T1A-3, the peptide is able to degrade JNK and/or other players of the JNK cascade. To test if T1A-3 reduces JNK protein levels *in vitro*, we treated cortical neuronal cultures with 5μM and 10μM for 4 hours, 8 hours, 12 hours, and 24 hours, harvested the protein and did a Western blot analysis to detect JNK1, JNK2 and JNK3 contents. The results are shown in Figure 3.5. JNK1 was significantly reduced after 24 hours of 10μM of T1A-3 compared to untreated cells ($p < 0.05$, $n = 4$). While there was also a small reduction of JNK1 after 4 hours and a slight decrease of JNK2 and JNK3 after 8 hours of incubation with 10μM of T1A3, there was no

statistically significant effect of T1A-3 on any of the other JNK isoforms or with other time points or doses (all conditions: $p > 0.05$, see Table 3.2. for number of experiments). In summary, T1A-3 reduces JNK1 after 24 hours but not at earlier time points and does not affect levels of the other JNK isoforms.

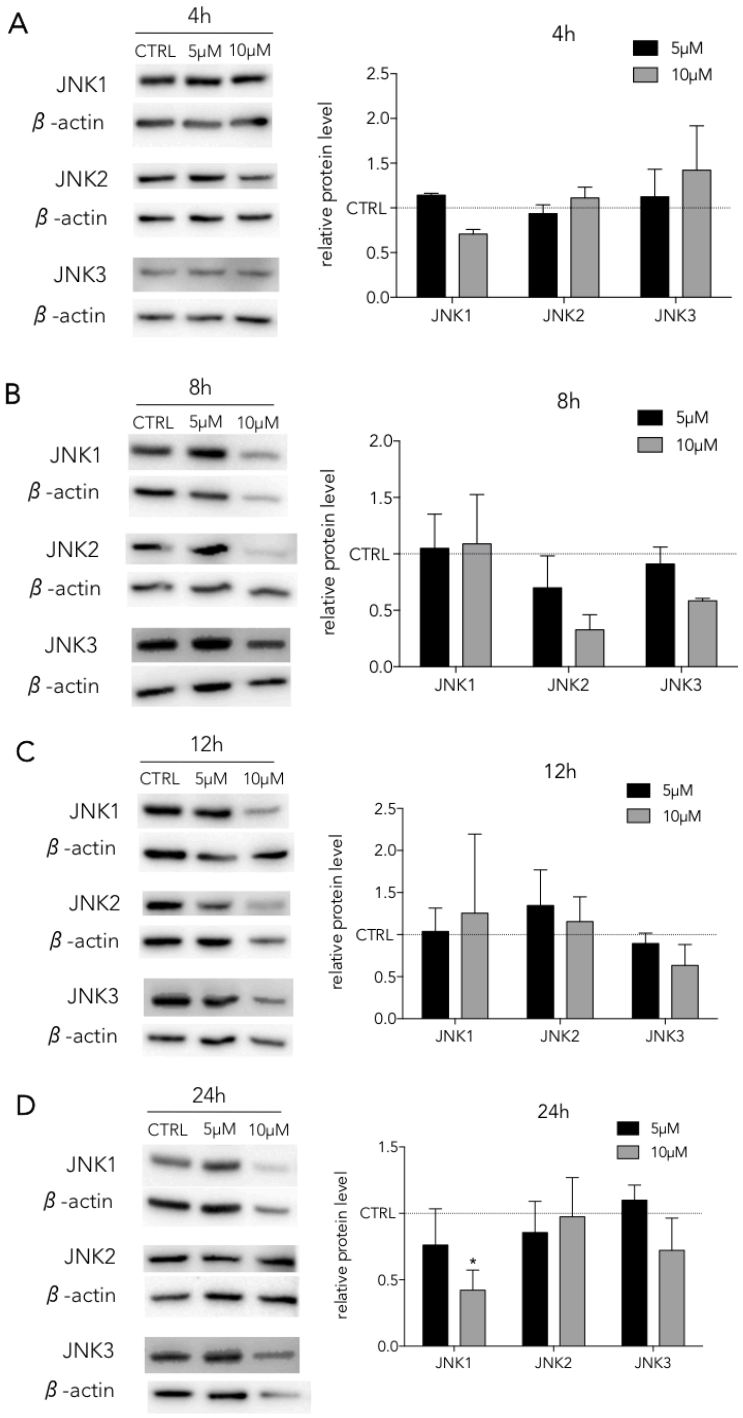

Figure 3.5 Effects of T1A-3 on levels of JNK1, JNK2 and JNK3 after 4 hours (A), 8 hours (B), 12 hours (C) and 24 hours (D) of treatment of cortical neuronal cultures with 5μM and 10μM of T1A-3. Graphs show JNK levels normalized to actin and relative to

untreated cells (CTRL). No statistically significant effect of T1A-3 on levels of JNK1, JNK2, and JNK3 was detected with either dose or at any of the time points.

Table 3.2 Number of experiments (N) conducted for testing JNK1, JNK2 and JNK3 levels in cortical neuronal cultures after treatment with different doses of T1A-3 for different time spans.

Protein	Time treated with T1A-3	Dose of T1A-3	N
JNK1	4 hours	5μM	3
		10μM	3
	8 hours	5μM	3
		10μM	3
	12 hours	5μM	4
		10μM	4
	24 hours	5 μM	4
		10μM	4
JNK2	4 hours	5μM	3
		10μM	3
	8 hours	5μM	3
		10μM	3
	12 hours	5μM	4
		10μM	4
	24 hours	5μM	4
		10μM	4
JNK3	4 hours	5μM	5
		10μM	6
	8 hours	5μM	3
		10μM	3
	12 hours	5μM	3
		10μM	3
	24 hours	5μM	6
		10μM	3

3.6. T1A-3 does not reduce protein levels of JNK *in vivo*

To further investigate the functioning of our peptide, we tested if T1A-3 affects JNK protein levels in living animals. Adult male Sprague Dawley rats were injected with a single dose of 50mg/kg of T1A-3 (n = 4) or 1ml/kg of saline (n =4) intravenously. Eight hours after the injection, the animals were sacrificed, the brains removed, and protein was extracted from the cortex and the hippocampus. Western blot analysis was performed to detect JNK1, JNK2, and JNK3 in the two different brain regions. Interestingly, in contrast to our *in vitro* findings, JNK1 was significantly increased in the cortex ($p = 0.0317$) and hippocampus ($p = 0.0159$) after 8h of T1A-3 treatment *in vivo* (Figure 3.6). Furthermore, JNK2 was also significantly increased in the hippocampus ($p = 0.0159$). There was a small but not statistically significant reduction in levels of JNK2 ($p = 0.1905$) and JNK3 ($p = 0.1111$) in the cortex and no difference in JNK3 levels in the hippocampus ($p = 0.2857$) of T1A-3-treated animals compared to rats treated with saline. Overall, these results suggest that T1A-3 does not reduce levels of JNK in living animals, but rather increases levels of JNK1 and JNK2 in the brain.

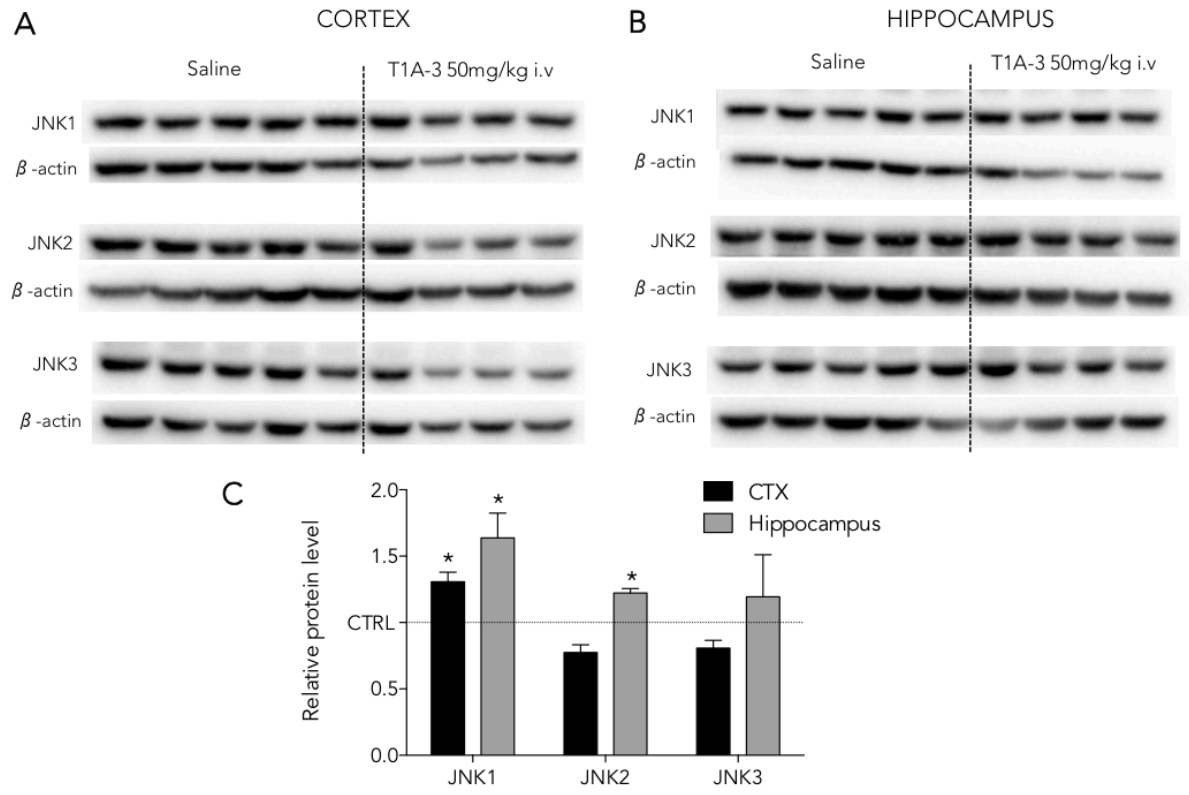

Figure 3.6 Effect of intravenous 50mg/kg T1A-3 compared to 1ml/kg saline on JNK1, JNK2, and JNK3 levels in the cortex (A) and hippocampus (B) of adult male Sprague Dawley rats, 8 hours after injection. The graph (C) shows quantification JNK levels after T1A-3 treatment normalized to β-actin relative to levels in saline-treated animals (CTRL). JNK1 was significantly higher in the cortex and hippocampus and JNK2 was significantly higher in the hippocampus of T1A-3-treated animals compared to saline-treated animals. T1A-3 treatment did not significantly affect levels of JNK2 in the cortex or JNK3 in the cortex and hippocampus.

3.7. T1A-3 is neuroprotective against excitotoxic insult *in vitro*

JNKs are involved in excitotoxicity and oxidative stress (Centeno et al., 2007; Han-Ming Shen & Liu, 2006); two of the main pathological processes that cause brain damage after a

stroke (Dirnagl et al., 1999). We hypothesized that our T1A-3 peptide, by interfering with the JNK activation cascade, is protective against cell death caused by excitotoxicity and oxidative stress. To test this hypothesis, we induced excitotoxic stress in cortical neuronal cultures with 100μM of two potent glutamate receptor activators, NMDA and Glutamate, and oxidative stress with 10mM of the oxidizing agent hydrogen peroxide (H_2O_2) for 1 hour. These toxins significantly increased cell death 24 hours after the 1-hour insult compared to the untreated control (NMDA: $p < 0.001$, $n = 10$; Glutamate: $p < 0.01$, $n = 11$; H2O2: $p < 0.001$, $n = 7$). When neurons were pre-treated with 5μM of T1A-3 for 23 hours before the toxic insult, cytotoxicity (measured by LDH activity) was not significantly different from control with NMDA and Glutamate treatment ($p > 0.05$, $n = 10$ and $p > 0.05$, $n = 11$, respectively; Figure 5). Hence, 5μM of T1A-3 was able to protect cortical neurons against excitotoxic death. T1A-3 furthermore reduced LDH activity after H_2O_2 insult compared to H_2O_2 alone; however, this effect was not statistically significant ($p > 0.05$, $n = 7$) and cytotoxicity with T1A-3 and H_2O_2 was still significantly higher than in untreated cells ($p < 0.05$; Figure 3.7). Thus, a non-toxic dose of 5μM of T1A-3 protects cortical neurons against death from excitotoxicity has a lower effect on death resulting from oxidative stress.

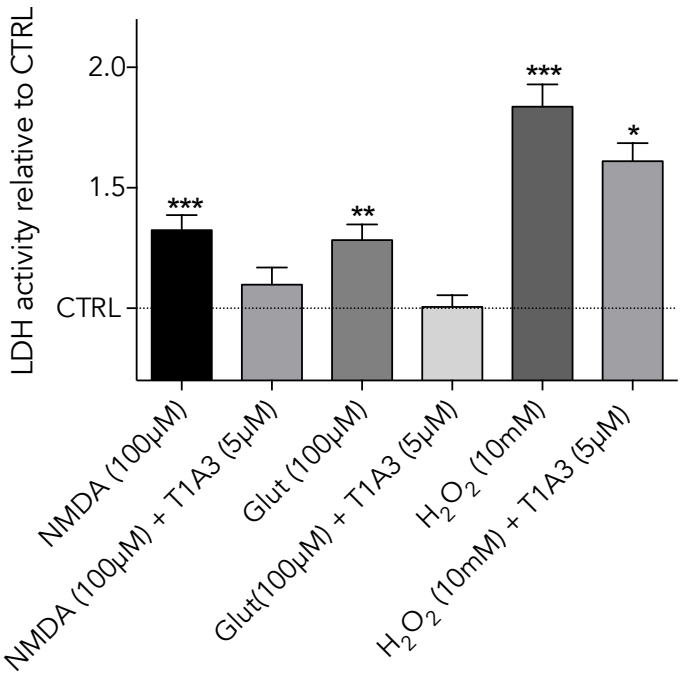

Figure 3.7 Relative cytotoxicity detected by LDH activity in cortical neuronal cultures treated with 100μM of NMDA, 100μM of Glutamate (Glut) and 10mM of H_2O_2 for 1 hour with or without pre-treatment of 5μM of T1A-3. The CTRL represents untreated cells. NMDA, Glutamate and H_2O_2 significantly increased cell death in neuronal cultures, but NMDA and Glutamate did not when 5μM of T1A-3 were also present. No significant difference could be detected between cells treated with H_2O2 with versus without T1A3.

3.8. T1A-3 is protective in against cerebral ischemia in an animal model for stroke

Given that our peptide was highly protective against excitotoxic insult *in vitro*, we next tested if it can protect against cerebral ischemia in an animal model for stroke. Strokes were induced in rats by blocking the middle cerebral artery through cerebral injection of the long-lasting vasoconstrictor ET-1. T1A-3 (n = 5) or PBS (vehicle, n =5) were injected i.p. 30 min before and brains were collected 24 hours after the sterotaxical surgery. TTC staining showed clear infarcts in the brains of vehicle treated animals (mean infarct volume = 149.9

± 23.43mm^3), while animals pre-treated with T1A-3 showed little to no infarct (mean infarct volume = 11.67 ± 8.3mm^3, Figure 3.8). Infarct size was significantly lower in T1A-3-compared to vehicle-treated animals ($p = 0.0079$), suggesting that our peptide is highly protective against stroke-induced brain damage.

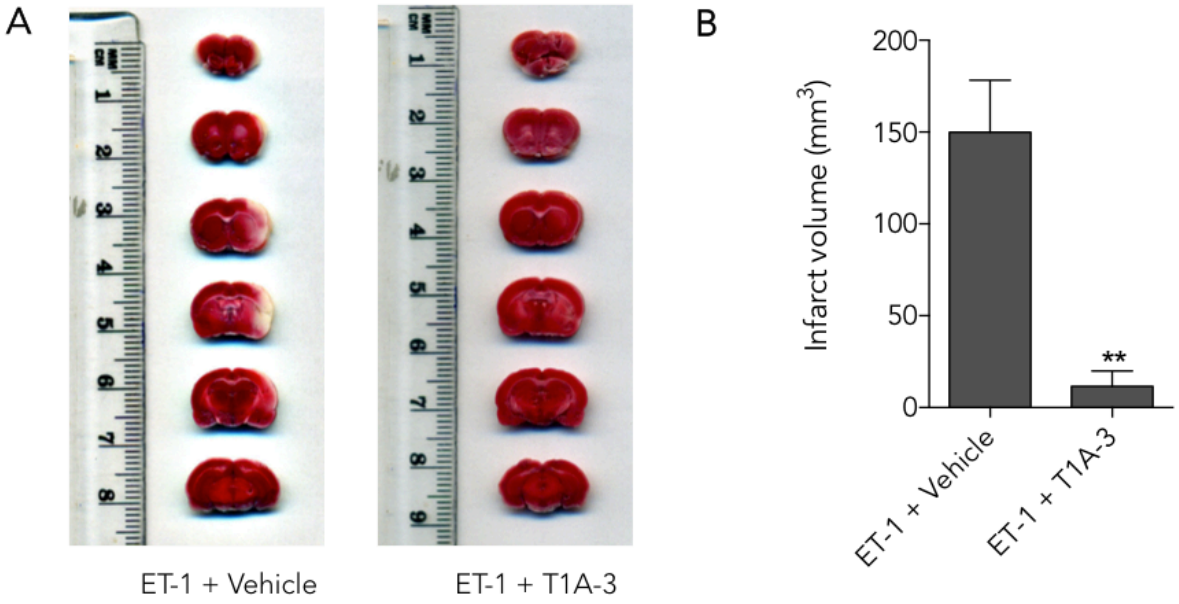

Figure 3.8 Effect of T1A-3 on infarct volume in an ET-1 stroke model in the rat. Representative brain slices from rats treated with 1ml/kg PBS (vehicle) or 50mg/kg T1A-3 i.p. 30min before ET-1 injection (A) and quantification of infarct volume (mm^3) of 5 rats from each treatment (B). Brains from rats treated with T1A-3 had a significantly lower infarct volume than brains from rats treated with vehicle ($p = 0.0079$).

4. Discussion

The aim of this Master's thesis was to develop a peptide that can degrade multiple players in the JNK activation cascade and acts as a neuroprotectant and prospective treatment for stroke. Based on the SNIPER method, we designed three peptides that each contained a PBD based on a shorter segment of the T1A peptide (Zhan et al., 2016), which was flanked with a CMA-targeting motif and a cell-penetrating motif (TAT). We hypothesized that with these three segments, the peptides can enter the cell, bind to JNK and/or upstream kinases and target them to the CMA, thereby degrading the proteins and exerting a protective function. Of the three peptides that we designed, a peptide with the latter 13 amino acids of T1A as a PBD (T1A-3) was most successful in reducing levels of active JNK in cultures of cortical neurons. This peptide was not toxic to neuronal cultures at a dose of 5μM with a treatment time of up to 24 hours. At this non-toxic dose, T1A-3 was able to significantly reduce cell death after excitotoxic insult with NMDA and Glutamate. Moreover, T1A-3 was highly protective against ischemic infarct in an ET-1 stroke model in rats, when administered i.p. 30 minutes prior to the insult. Thus, we have developed a TAT-peptide that can hinder JNK activity, excitotoxic neuronal death and damage in the brain after ischemic stroke. Given that TAT-peptides can penetrate the blood brain barrier following systemic application (Morris, Depollier, Mery, Heitz, & Divita, 2001; Aarts, 2002; Heitz, Morris, & Divita, 2009) and have successfully been used in human clinical trials (Hill et al., 2012) our newly developed peptide may be a novel and clinically applicable neuroprotectant.

4.1. Mechanism of action and neuroprotective function of T1A-3

The SNIPER method employed in this project has previously been shown to efficiently bind to and degrade target proteins (Fan et al., 2014). The design of SNIPER peptides involves the careful selection of a PBD that has a high potential for binding to the protein of interest. In this study we used a segment of the T1A peptide as a PBD for our peptides. T1A is well established to bind JNK3 (Zhan et al., 2014, 2016) and interacts with ASK1 and MKK7 (Zhan et al., 2016), making it an excellent candidate for the PBD of a SNIPER peptide designed to target multiple players in the JNK activation cascade. Yet, while T1A-3 successfully reduced activity of JNK, we did not observe a clear knockdown of JNK protein after treatment with our peptide. JNK1 was significantly reduced after 24 hours of 10μM of T1A-3 but levels of other JNK isoforms did not change significantly *in vitro*. Interestingly, JNK1 was increased in the cortex and hippocampus of adult male Sprague Dawley rats after intravenous injection of 50mg/kg of T1A-3 compared to saline-treated rats. JNK2 was also higher in the hippocampus of rats after T1A-3 injection but there was no difference in levels of JNK3.

Since we did not use the full-length T1A as a PBD, it is possible that T1A-3 does not bind JNK but reduces JNK activity indirectly through mediation of upstream kinases. Given that full-length T1A is able to bind MKK7 and ASK1 (Zhan et al., 2016), our peptide may bind to and degrade one or both of these kinases upstream of JNK and thereby reduce JNK activity and protect against ischemic neuronal death. By degrading more-upstream kinases in the JNK cascade, T1A-3 may moreover affect other death-associated molecules. For

example, ASK1 also phosphorylates p38, another death-related MAPK (Cheon, Kim, Kim, & Koo, 2018). By impairing ASK1's effect on p38, T1A-3 may be neuroprotective in addition to its effect on JNK. An effect of T1A-3 on other players in the MAPK cascade could also explain why JNK1 and JNK2 were upregulated in the brains of rats after T1A3 treatment. There is evidence for compensatory upregulation of JNK with inhibition of other MAPKs, such as extracellular signal-regulated kinase (ERK) and p38 (Jones, Jenney, Joughin, Sorger, & Lauffenburger, 2018; Nutter, Haylor, & Khwaja, 2015; Repici et al., 2009). Hence, by degrading upstream activators of JNK, T1A-3 may affect other targets of these kinases, which may lead to the increase in JNK protein seen in our *in vivo* experiments in a compensatory manner. By affecting additional death-associated molecules, T1A-3 may be particularly potent in protecting against neuronal death.

It is furthermore possible that T1A-3 exerts its effect on JNK activity and neuronal death through interfering with the assembly of JNK in its activation complex with upstream kinases and Arrestin. A small peptide that competes with Arrestin's binding to JNK3 has previously been shown to protect against neuronal damage in a cerebral ischemia reperfusion model in rats (Wei et al., 2018). Since T1A-3's PBD is derived from Arrestin's binding site for JNK3, ASK1, and MKK7, our peptide may compete with the natural binding process of these kinases and thereby inhibit JNK activation and neuronal death without degrading JNK protein.

Another possibility is that T1A-3's neuroprotective effect is mediated by TAT. Previous research has shown that an arginine-rich and positively charged peptide has NMDA

receptor-antagonistic properties and is neuroprotective against excitotoxic insult (McQueen et al., 2017). Considering that T1A-3 also consists of the arginine-rich TAT(47-57) sequence and is positively charged (+10.9 at neutral pH), it is possible that its neuroprotective action is through antagonizing NMDA receptor function. However, since T1A-3 also reduces JNK activity, it is likely that its neuroprotective action originates, at least in part, from its effect on the JNK signaling pathway.

We hypothesized that our peptide can reduce cell death caused by multiple pathological processes including excitotoxicity and oxidative stress. While our results showed that T1A-3 significantly reduces cell death after excitotoxic insult with NMDA and glutamate, it had only a weak, but not significant effect on neuronal death in an oxidative stress setting induced with 10mM of H_2O_2. This may be explained by our treatment protocol, in which the peptide was present before and during the toxic insult, but not after. Previous research has shown that a peptide interfering with the interaction of GluN2BR with PSD-95 is present in cortical neuron cultures for 5 hours after washing out the peptide (Aarts, 2002). Considering that 10mM is a fairly high dose of H_2O_2 and cell death was assessed 24 hours after peptide administration, the transient exposure of T1A-3 may not have been enough to fully protect.

4.2. Peptide toxicity and stability

The findings of our study show that T1A-3 is more effective in reducing levels of active JNK when given at a higher dose. However, at higher doses of our peptide, we also observed more toxicity in neuronal cultures. Previous research has shown that TAT itself can be toxic

to neurons (Nath et al., 1996). Since we used TAT as a cell-penetrating sequence for T1A-3, it is possible that this motif increased our peptide's toxic effects. However, there is also ample contradictory evidence that TAT is not toxic, or even neuroprotective (El-Andaloussi, Järver, Johansson, & Langel, 2007; Meloni et al., 2014; X. Chen, 2017). The toxicity seen with our peptide may rather be explained by TAT's mechanism to get into the cell. TAT induces pores into the membrane as a means to enter cells (Herce et al., 2009) and the increased LDH activity with T1A-3 in our toxicity assay may be due to a leak of LDH through pores formed by TAT, rather than release from dying cells. Thus, the choice of our cytotoxicity assay may have affected our results and T1A-3 may show less toxicity when assessed in other toxicity or viability assays that do not use membrane integrity as a measure.

Interestingly, we did not see a significant increase in LDH release after treatment with a high dose of 25μM of T1A-3 for 24 hours, while the same dose caused significant LDH increases with shorter treatment times. One would expect the effects on LDH release to increase with longer exposure to the peptide. This may be explained by degradation of T1A-3 over time. Tests on peptide stability in our lab have shown that TAT-peptides lose purity with time in solution at room temperature and 4°C (unpublished data). This may be exacerbated by proteases present in culture medium and the increased incubation temperature when applied to neuronal cultures. Furthermore, after entering the cells, the peptide may be subject to degradation by the proteasome and, specifically with its CMA-targeting motif, by the lysosome. These factors may affect the function of our peptides at

later time points and should be considered in future treatment protocols to optimize peptide efficiency.

4.3. Clinical significance

Our findings show substantial neuroprotection with T1A-3 under excitotoxic conditions in neuronal cultures and in an animal model for cerebral ischemia, making it a promising tool to effectively reduce brain damage after a stroke. Our peptide has considerable potential to be successful in the clinic. JNK activation peaks at 6 hours after a stroke and inhibiting JNK has been shown to be protective 6 to 12 hours after MCAO in the rat (Repici et al., 2007). Thus, by targeting JNK, T1A-3 may have a wide therapeutic window, allowing for late application after stroke onset. Moreover, JNK is not only involved in neuronal damage processes after ischemic stroke, but also after hemorrhagic stroke. Inhibiting JNK is protective in this rarer stroke condition as well (Yatsushige, Ostrowski, Tsubokawa, Colohan, & Zhang, 2007; Yin, Huang, Sun, Guo, & Zhang, 2017). While the current stroke therapy tPA worsens hemorrhagic conditions and needs a clear diagnosis of ischemic stroke to be applied (Kuan et al., 2003), T1A-3 would be safe and beneficial to use in both types of stroke without the need for differential diagnostic testing. This allows for safer and faster administration and increases the chances that patients receive treatment in the appropriate time window. TAT-peptides have previously been successfully used in clinical trials, are able to penetrate the blood brain barrier and do not cause any severe side effects (Aarts, 2002; Hill et al., 2012), making a safe and successful use of T1A-3 in the clinic likely. Furthermore, by targeting the stress-activated MAPK pathway, effects on physiological functions and

therefore side effects should be limited. By potentially affecting multiple death-associated players in the JNK activation cascade, T1A-3 may moreover be more potent than previously developed JNK inhibitors that target individual JNK functions. It may affect multiple pathologies involved in stroke-induced death, which eliminates the necessity of treatment cocktails and makes it more likely to be clinically effective than previously developed neuroprotective strategies that focus on individual pathological processes.

Additionally, T1A-3 has a great potential to be applicable beyond stroke. JNKs play a role in pathological processes involved in several neurodegenerative diseases, such as Parkinson's disease (Hunot et al., 2004), Huntington's disease (Morfini et al., 2009) and Alzheimer's disease (Yoon et al., 2012). By mediating excitotoxic death, T1A-3 may be moreover protective in other neurological conditions that involve excitotoxic pathologies, such as epilepsy (de Lemos et al., 2017).

4.4. Summary and Conclusions

In summary, we have found a novel neuroprotective peptide that reduces excitotoxic neuronal death and brain damage after ischemic stroke. This peptide, called T1A-3, is not toxic at low concentrations, even when applied for up to 24 hours, and is able to reduce JNK activity. T1A-3 reduces JNK1 levels after 24hours in neuronal cultures but it does not affect levels of any other JNK isoforms or at any other time points. Moreover, it increases levels of JNK1 and JNK2 in the brains of rats. Therefore, T1A-3 likely exerts its protective function through mediating upstream players in the JNK activation pathway, rather than

through degradation of JNK itself. While its mechanism still needs further investigation, we have found a new effective neuroprotective agent that is promising to be applicable in the clinic and beyond stroke pathologies and that may be safer and more potent than previously developed stroke therapies.

4.5. Future directions

Further research is necessary to determine T1A-3's exact mechanism of action. Performing a binding assay will shed light onto the interaction of T1A-3 with its proposed binding partners JNK, MKK7, and ASK1. Furthermore, testing T1A-3 together with a lysosome inhibitor as well as analyzing the effect of a version of T1A-3 that does not contain the CMA-targeting motif will help us understand if the peptide's action requires lysosomal degradation or rather works through interfering with the assembly of JNK's activation complex. Moreover, it should be assessed how T1A-3 affects levels and activation of other MAPKs such as p38 in order to understand the peptide's full potential. Finally, testing a scrambled peptide and a neutrally-charged version of T1A-3 will be important controls to rule out that its neuroprotective action is mediated by TAT.

Next to understanding T1A-3's mechanism, it will be interesting to explore the peptide's effect on stoke-related pathological processes other than excitotoxicity. Future research should assess if T1A-3 can interfere with microglia activation and pro-inflammatory cytokine expression in experimental stroke settings to test if it impairs inflammation and is thereby more effective in relieving multiple stroke pathologies than previous JNK inhibitors, such as

D-JNKi (Benakis et al., 2010). Furthermore, T1A-3's protective effect in an oxidative stress environment may be further explored by testing if consecutive exposure to T1A-3 after H_2O_2 insult is more efficient in protecting against oxidative stress than the protocol used in our study. To furthermore proof that our peptide exerts a broad effect on multiple stroke pathologies, future research may assess its influence on apoptotic and necrotic cell death by testing apoptotic markers such as caspase activation or Annexin V binding (Cummings & Schnellmann, 2004) or identifying types of cell death using acridine orange and ethidium bromide staining (Mironova, Evstratova, & Antonov, 2007).

Moving forward, it will be particularly important to optimize our peptide's function. Reducing cytotoxicity and increasing stability will be crucial to enhance T1A-3's clinical applicability. For example, incorporation of D-amino acids and retro-inversion of the peptide sequence have been shown to reduce the susceptibility of peptides to proteasomal degradation (Borsello et al., 2003; Fan et al., 2014). While additional tests aside from the LDH assay should be performed to further characterize T1A-3's toxicity, several strategies may be employed to reduce the toxic effects of peptide drugs. The CMA-targeting motif may contribute to toxicity and replacing it with a degron motif that targets its cargo to the proteasome instead of the lysosome may have lower cytotoxic impacts (Chen, 2017). Furthermore, research from our lab has shown that multiple low dose treatments are preferable over higher dose long-term treatment in order to reduce toxic peptide effects (Chen, 2017). Applying multiple lower doses of T1A-3 may more efficiently reduce activity of JNK without the toxic effects seen with higher doses. Increasing the

binding-affinity of peptides may furthermore allow for a lower dose of peptide treatment to get the same effect (Chen, 2017). Other protein-binding sequences, such as an overlapping sequence of T1A-2 and T1A-3 should be tested to determine if they are more effective at reducing pJNK and protecting neurons from ischemic insults than T1A-3 and may therefore be applied at even lower doses.

T1A-3 is protective in an ET-1 stroke model in rats when given 30 minutes prior to stroke onset; however, moving forward it will be important to test if our peptide is also effective when administered after stroke induction and to characterize its therapeutic window. Furthermore, behavioural outcomes are a good indicator of successful translation of new stroke therapies from the pre-clinical to the clinical stage (Neuhaus et al., 2014); thus, behavioural tests will be an important next step to further characterize the effectiveness of T1A-3.

Finally, given JNK's deathly role in Parkinson's, Alzheimers, Huntington's and epilepsy, it will be interesting to test if it is protective in animal models for those diseases as well.

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Appendix

Appendix A Immunoblot for pJNK after 24hours of peptide treatment, of which cropped version is presented in Figure 3.3.-A. T1A-2 lanes were cropped to only present CTRL and T1A-3 results in Figure 3.3.-A.