Tissue plasminogen activator (tPA) promotes postnatal cortical neuron survival *in vitro* via JAK2- and mTOR-dependent mechanisms

by

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Abstract

Tissue plasminogen activator (tPA) is the only approved drug for ischemic stroke in Canada but is limited in its clinical efficacy due to its short therapeutic window. This study sought to determine the effect of tPA on postnatal primary cortical neuron viability and aimed to identify the relevant cellular signalling mechanisms underlying the tPA-mediated effects *in vitro*. The data revealed that tPA significantly increased the propensity for cell survival within a time latency window of up to 3 hours. tPA-induced neuroprotective effects were significantly dependent upon the mTOR and JAK/STAT signalling pathways, while the MEK and PKA signalling pathways were found to play a less critical role. Immunocytochemical staining showed a marked increase in p-S6 expression following treatment with tPA, substantiating the vital role of mTOR activation in tPA-mediated neuroprotection. These results suggest the possibility of targeting the defined mechanisms to expand the therapeutic window of tPA in stroke recovery.

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***β*-Tubulin** – beta-III-Tubulin

ALR – autophagic lysosome reformation

ANOVA – analysis of variance

Apaf-1 – apoptotic protease activating factor 1

ATF – activating transcription factor

ATP – adenosine triphosphate

BBB – blood brain barrier

Bcl – B-cell lymphoma

BDNF – brain-derived neurotrophic factor

BR-C – Broad Complex

cAMP – cyclic adenosine monophosphate

CBF – cerebral blood flow

CNS – central nervous system

CNTF – ciliary neurotrophic factor

CREB – cAMP response element-binding protein

CREM – cAMP response element modulator

DAPI – 4'6-diamidino-2-phenylindole

DAPk – death-associated protein kinase

ddH2O – double-distilled water

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DPBS – Dulbecco's phosphate-buffered saline solution

DR6 – death receptor 6

DRG – dorsal root ganglion

DRP-1 – DAPk-related protein-1

EBSS – Earle's balanced salt solution

EGF – epidermal growth factor

ER – endoplasmic reticulum

ERK – extracellular signal-regulated kinase

FADD – Fas-associated death domain

FDA – Food and Drug Administration

FGF – fibroblast growth factor

Gab-1 – Grb2-associated binder-1

GDNF – glial cell-derived neurotrophic factor

GM-CSF - granulocyte-macrophage colony-stimulating factor

IEX-1 – immediate early response gene X-1

IGFI – insulin-like growth factor 1

IKK – I_KB kinase

iNOS – inducible nitric oxide synthase

IRS – insulin-receptor substrate

JAK – Janus kinase

JNK – c-Jun N-terminal kinase

LDL – low-density lipoprotein

LRP – lipoprotein receptor-related protein

MAPK – mitogen-activated protein kinase

MCAO – middle cerebral artery occlusion

Mcl-1 – Myeloid cell leukemia-1

MKK1 – mitogen-activated protein kinase kinase 1

MMP-9 – matrix metallopeptidase 9

mTOR – mammalian target of rapamycin

mTORC1 – mTOR complex 1

mTORC2 – mTOR complex 2

NCCD – Nomenclature Committee on Cell Death

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NGF – nerve growth factor

NMDA – N-methyl-D-aspartate receptor

NVU – neurovascular unit

OGD – oxygen-glucose deprivation

p-S6 - phospho-S6 ribosomal protein

p-STAT3 – phospho-signal transducer and activator of transcription 3

PACAP – pituitary adenylate cyclase-activating polypeptide

PAI-1 – plasminogen activator inhibitor-1

PBS – phosphate-buffered saline

PDGF-CC – platelet-derived growth factor receptor CC

PDL – poly-D-lysine

PFA – paraformaldehyde

PI3K – phosphoinositide 3-kinase

PKA – protein kinase A

PNS – peripheral nervous system

PRAS40 – proline-rich Akt substrate of 40 kDa

PROTOR – protein observed with RICTOR

PTEN – phosphatase and tensin homologue

RAPTOR – regulatory-associated protein of mTOR

RasGAP – Ras GTPase activating protein

RGC – retinal ganglion cell

RICTOR – rapamycin-insensitive companion of mammalian target of rapamycin

RIP – receptor-interacting protein

S6K1 – S6 kinase 1

SEM – standard error of the mean

SOCS3 – suppressor of cytokine signaling 3

STAT – signal transducer and activator of transcription

TNF – tumour necrosis factor

TNFR – tumour necrosis factor receptor

tPA – tissue plasminogen activator

TRAIL – TNF-related apoptosis-inducing ligand

TrK - tropomyosin receptor kinase

TSC1 – Tuberous Sclerosis 1

TUNEL – terminal deoxynucleotidyl transferase

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Introduction

Development of the nervous system

During early development, neurons within the central nervous system (CNS) show a broad permissiveness for growth and survival. In addition to the rapid genesis of neuronal cell bodies, dendrites attached to the cell bodies expand, glial cells proliferate, blood vessels arborize, and the extracellular matrix becomes fully developed due to secretions from neighbouring cells (Sanes, Reh, & Harris, 2011). The fate of a neuron during this time period, however, is mediated by several survival factors that are vital to its viability. Survival factors may be target-derived, by receiving signals from the cells that they innervate, afferent-derived, by receiving information through the synaptic inputs, paracrine, by receiving information from neighbouring neuron cell bodies, blood-born, by receiving information from the circulatory system, as well as glia-derived, having information communicated via homeostatic, supportive non-neuronal cells (Sanes et al., 2011). A class of naturally occurring proteins called neurotrophins play a dynamic role in determining whether or not a neuron survives, such that they are critical for stimulating neuron growth, proliferation, differentiation, and repair (Huang & Reichardt, 2001). The mammalian nervous system as a complex cellular, heterogeneous system is purported to be made up of a relatively small number of growth factors, inclusive of neurotrophins, and one of the final frontiers of developmental neuroscience is uncovering how these growth factors act to direct the development of the nervous system and sustain cell viability in the living organism (Landreth, 1999). While some growth factors are present solely during specific times of neural development and play very precise roles, others function

throughout the lifespan and help to sustain neurons by stimulating factors to promote cell survival (Landreth, 1999). The goal of the present study was to examine the impact of tissue plasminogen activator (tPA), the only approved drug for ischemic stroke in Canada, on postnatal cortical neuron viability. Since the developing brain shows vastly different molecular properties compared to the adult brain and that these differences are thought to correlate closely with the limited reparative capacity of the adult brain following injuries, including stroke, insight into the impact of tPA on postnatal cortical neuron survival and its mechanistic effects in these neurons will provide clarity surrounding its precise role in mediating cell viability. This information can then be later translated to the mature brain to potentially allow individuals who have experienced cerebral insults, such as stroke, to achieve a better and more functional recovery.

Growth and survival factors within the developing nervous system

Growth factors have the ability to regulate cell growth, proliferation, maintenance, and sustain cell survival. One growth factor, and in fact the first to ever be discovered, is nerve growth factor (NGF; Levi-Montalcini & Angeletti, 1968). NGF is only present in the nervous system, hence is a neurotrophin, and is required for sympathetic neuron survival not only during neural development but also throughout the length of the lifespan (Landreth, 1999). NGF is unique in that instead of supporting the proliferation and growth of all neuronal precursor cells, it acts to regulate cell survival by selectively protecting neurons that are unlikely to undergo programmed cell death (Landreth, 1999). NGF also plays a nurturing role by promoting a permissive environment for differentiation processes in neurons to occur as well as axon outgrowth, and has been shown to spearhead phenotype specification in neurons (Lewin, 1996). A later descendant of NGF, brain-derived growth factor (BDNF), also serves as a neurotrophin within the developing nervous system. Within the CNS specifically, BDNF supports the survival

and outgrowth of several types of neurons including basal forebrain cholinergic neurons, striatal dopaminergic neurons, and retinal ganglion cells (Landreth, 1999). BDNF plays an important role in cell survival as evidenced by knockout studies revealing that when the BDNF gene is deleted in specific types of neurons (i.e. vestibulocochlear neurons) in mice, mice lose their ability to maintain respective functioning (i.e. gait and balance; Bianchi et al., 1996). The pro-survival effects of BDNF are further substantiated with the evidence that BDNF knockout mice tend to die within 1 to 2 weeks of birth (Landreth, 1999). Other growth factors known to play important roles in the development of the nervous system, and specifically within the CNS, are neurotrophin-3 and neurotrophin 4/5, which work on the tropomyosin receptor kinase (TrK) and p75 receptors to sustain neuronal survival (Landreth, 1999). In addition, some cytokines have been found to promote cell survival, including ciliary neurotrophic factor (CNTF), a cytokine that has been found to support the survival of autonomic, dorsal root ganglion, hippocampal, and motor neurons (Oppenheim, Prevette, Yin, Collins, & MacDonald, 1991; Pun, Santos, Saxena, Xu, & Caroni, 2006), and glial cell-derived neurotrophic factor (GDNF), a cytokine identified as preventing naturally occurring neuron death (Li et al., 1995; Oppenheim et al., 1995; Pascual, Hidalgo-Figueroa, Gómez-Díaz, & López-Barneo, 2011; Wu et al., 2003). Fibroblast growth factors (FGFs) have also been shown to play a variety of fundamental roles across the continuum of neuronal development (Vaccarino et al., 1999), and have also been identified as essential mediators of neurogenesis, axonal growth, neuroprotection, cell maintenance, lesion repair, as well as learning and memory in the CNS (Caldwell et al., 2001; Morrison, Sharma, De Vellis, & Bradshaw, 1986; Paek, Gutin, & Hébert, 2009; Perrone-Capano & Di Porzio, 2000; Reuss & und Halbach, 2003). It could be that tPA acts as a growth or survival factor within postnatal

development by promoting neuron survival; a spectulation that has not precisely been examined before and an area that will be investigated by the current study.

Naturally occurring cell death

During the development of the nervous system, while there is exists an ongoing, vast neurogenesis of cells, including the growth and proliferation of neurons, glia, and neural progenitor cells due to factors such as FGFs and neurotrophins, a prominent feature of neural development is the pervasive process of naturally occurring cell death (Ford-Perriss, Abud, & Murphy, 2001; Nijhawan, Honarpour, & Wang, 2000). It has been estimated that as many as 20 to 80%, depending on the brain region, of the original differentiated cell population becomes eliminated during this period due to death-initiating signalling mechanisms (Oppenheim, 1981; Oppenheim, 1991; Oppenheim & Johnson, 2003), and that this substantial cell death occurs in the majority of developing tissues part of living multicellular organisms (Buss, Sun, & Oppenheim, 2006). The role of this period of naturally occurring cell death could be regarded as a means for optimizing synaptic connections, removing unnecessary neurons, and forming neural patterns (Burek & Oppenheim, 1999). Naturally occurring cell death occurs at a time when axons and dendrites are innervating their targets and is thus suggested as a process representing the pruning of excess neurons (Ford-Perriss et al., 2001). This indicates that in the developing nervous system, neurons are initially overproduced and then compete for connections to a postsynaptic target in order to receive target-derived neurotropic factor support (Cowan, Fawcett, O'Leary, & Stanfield, 1984). The forms of death listed below describe the various processes by which cells can age and die, both programmed and unprogrammed, and illustrate the possible routes to naturally occurring cell death that can lead to premature aging and disease.

Apoptosis and its mechanisms of cell death. Neuron survival is mediated by pro- and

anti-apoptotic mechanisms. When a survival factor binds to its receptor, anti-apoptotic proteins become activated via phosphorylation and further multiply to enhance survival, whereas when a survival factor is eliminated, pro-apoptotic proteins become activated via phosphorylation and become further expressed to ultimately limit survival (Sanes et al., 2011). To date, literature has identified two primary apoptotic pathways: the extrinsic pathway, mediated by 'death receptors'; and the intrinsic pathway, mediated by mitochondrial processes. Recent research has provided evidence that these two pathways are linked and can influence one another to determine neuronal survival (Igney & Krammer, 2002). Downstream, this interlinked pathway becomes initiated by the cleavage of caspases, specifically caspase-3, which begins a cycle of deoxyribonucleic acid (DNA) fragmentation, cytoskeletal and nuclear protein degradation, protein cross-linking, apoptotic cell body formation, phagocytic cell receptor ligand expression, and ultimately phagocytic cell uptake (Hengartner, 2000). There is evidence that the pathway interacts with an additional pathway, T-cell in nature, that is responsible for inducing cytotoxicity and cell death via granzyme A or granzyme B interference (Elmore, 2007). The granzyme A pathway works in parallel with the interlinked intrinsic and extrinsic pathways and activates caspase-independent cell death via single stranded DNA damage (Martinvalet, Zhu, & Lieberman, 2005). In addition to the cell death-targeted activity of these pathways, the caspase part can also activate other pro-caspases, allowing for the initiation of a protease cascade to further amplify the apoptotic signalling pathway and produce additional rapid cell death (Elmore, 2007) (see Figure 1).

In experiments involving *C. elegans*, it has been shown that blocking engulfment genes, the gene-type whose sole purpose is to remove dead cells, enhances cell survival when the cells are subjected to weak pro-apoptotic signals (Hoeppner, Hengartner, & Schnabel, 2001). Additionally, mutations that cause a "partial loss of functioning" (Elmore, 2007) of these genes

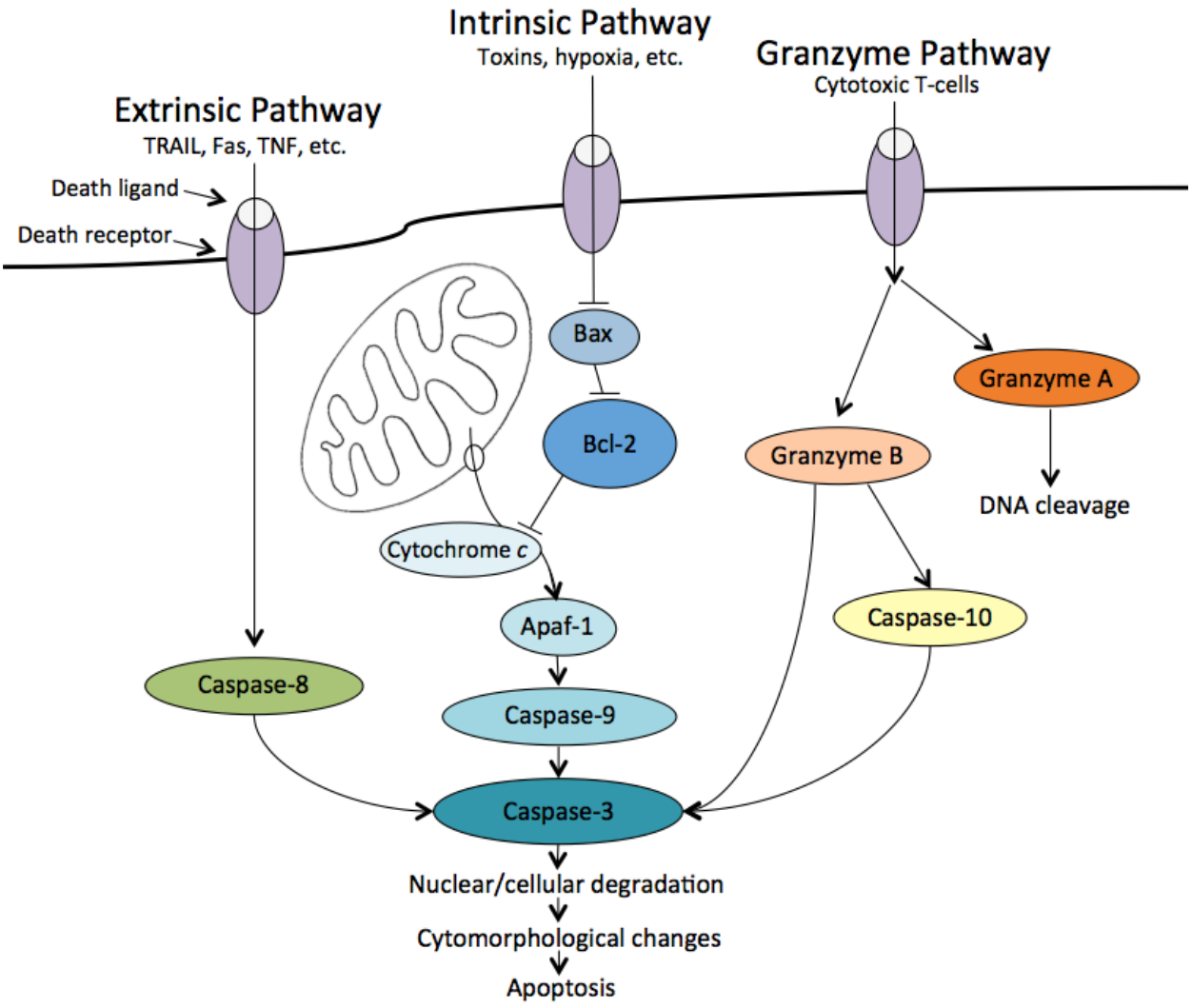

Figure 1. The apoptotic signalling cascade. The extrinsic pathway involves the activity of transmembrane death receptors, and the activation of TNF, Fas, or TRAIL can cause an auto-catalytic activation of caspase-8 and then caspase-3 for induction of apoptosis. The intrinsic pathway, activated by the infiltration of toxins, radiation, hypoxia, and other harmful stimuli, is influenced by the activity of Bcl-2 members: Bax, a pro-apoptotic protein, can inhibit anti-apoptotic proteins such as Bcl-2 to promote apoptosis, and anti-apoptotic proteins like Bcl-2 can help to inhibit the formation of a caspase-dependent mitochondrial cascade. If initiated, the mitochondria will release cytochrome *c*, which will activate Apaf-1, caspase-9, and ultimately caspase-3 for induction of apoptosis. The granzyme pathway, caused by the infiltration of cytotoxic T-cells, activates either granzyme A for initiating DNA damage or granzyme B which activates caspase-10 and/or caspase-3 for induction of apoptosis. Activation of caspase-3 induces nuclear and cellular degradation, including DNA fragmentation, followed by cytomorphological changes, including chromatin condensation, and ultimately apoptosis and phagocytic cell uptake.

have been shown to permit cells originally programmed to die via apoptosis to bypass the death process and survive instead, and mutations to the engulfment genes themselves have helped to improve the observed frequency of this survival (Reddien, Cameron, & Horvitz, 2001). Mutations to the genes alone have also allowed for the survival and differentiation of neuronal cells that were originally destined for programmed cell death (Reddien et al., 2001). These findings illustrate that engulfing cells do not only participate in dead cell removal, but they also ensure that cells labeled for apoptosis do, indeed, go on to die rather than recover in the beginning stages of the dying process (Elmore, 2007).

The extrinsic pathway of cell death works through transmembrane receptor-mediated interactions in order to initiate apoptosis. One of the key players of this pathway is tumor necrosis factor (TNF) and its receptors. They play a critical role in the transmittance of the death signal between the cell surface and the intercellular signalling pathways and have been shown to do this via activation of Fas, TNF-related apoptosis-inducing ligand (TRAIL), and death receptor 6 (DR6) receptors and their respective ligands and an auto-catalytic activation of procaspase-8 (Kischkel et al., 1995; Morrison et al., 2002). TNFs have also shown to be involved in a number of physiological processes including neuronal cell death during development and after injury (Morrison et al., 2002).

The intrinsic pathway of cell death works through non-receptor-mediated stimuli that create intracellular signals that act in either a positive or negative way on targets within each cell (Elmore, 2007). The events are mitochondrial-initiated and signals can lead to the failure of the suppression of death programs and ultimately trigger apoptosis if the signal being communicated involves the absence of growth factors, hormones, or cytokines (Elmore, 2007). Conversely, the death signals can communicate in the opposite way as well, stimulating processes for induction

of cell damage and apoptosis through hypoxia, toxins, viral infection, free radicals, and others (Elmore, 2007). These stimuli work to induce apoptosis by increasing the permeability of the mitochondrial transmembrane and allowing pro-apoptotic proteins into the cytosol (Elmore, 2007). This ultimately facilitates the initiation of a caspase-dependent mitochondrial pathway cascade where the proteins form an "apoptosome", consisting of the proteins cytochrome *c*, apoptotic protease activating factor 1 (Apaf-1), and procaspase-9 (Chinnaiyan, 1999; Hill, Adrain, Duriez, Creagh, & Martin, 2004).

The balance between pro- and anti-apoptotic signals is mediated in large by a family of genes called B-cell lymphoma (Bcl) -2. The proteins associated with these genes are important for determining the fate of a neuron, such that they determine whether a cell will undergo apoptosis or bypass the death process (Elmore, 2007). Bcl-2 and Bcl-xL, being the two primary anti-apoptotic members of the family (Cory & Adams, 2002), inhibit apoptotic death by controlling the activation of caspase proteases (Newmeyer et al., 2000). However, when Bad, a type of pro-apoptotic Bcl-2 protein, is introduced into an environment enriched with Bcl-2 and Bcl-xL, it has been shown to neutralize their neuroprotective effects and promote cell death (Yang et al., 1995), while deletion of these pro-apoptotic proteins, including the Bcl-2 protein Bax, have proven to increase neuron survival (Deckwerth et al., 1996; Vekrellis et al., 1997). In terms of axon growth, it has been shown that neurons upregulate Bcl-2 after injury and regenerate axons in order to facilitate re-organized connections (Cristino, Pica, Della Corte, & Bentivoglio, 2000). cAMP response element-binding protein (CREB) has also been shown to play a pivotal role in increasing the expression of anti-apoptotic proteins, including Bcl-2, once it is activated and present within the cell nucleus (Sanes et al., 2011). However, in the developing CNS, Bcl-2 expression declines as neurons lose their ability to create new axons. In

the peripheral nervous system (PNS), conversely, Bcl-2 is maintained at high levels and allows for axon regeneration throughout the lifespan (Merry & Korsmeyer, 1997).

Alternative forms of naturally occurring cell death. Although neurons die as a normal physiological process throughout neural development and apoptosis is the best understood mechanism of this neuronal cell death, literature suggests that apoptosis may not be the only cellular mechanism that regulates death in the neuronal cell. Evidence shows that alternative forms of cell death exist and that these alternative forms of cell death may exhibit morphological features different from that of canonical apoptosis, including autophagy and necrosis (Yuan, Lipinski, & Degterev, 2003). According to the Nomenclature Committee on Cell Death (NCCD), cell death can be classified according to its morphological appearance (i.e. apoptotic, autophagic, or necrotic), enzymological criteria (i.e. involvement of nucleases or proteases including caspases, calpains, cathepsins or transglutaminases), functional aspects (i.e. programmed or accidental; physiological or pathological), and immunological characteristics (i.e. immunogenicity; Kroemer et al., 2008). In addition to autophagy and necrosis, the NCCD identifies cornification as another type of neuronal cell death, and also lists eight other types of atypical cell death modalities, including mitotic catastrophe, anoikis, excitotoxicity, Wallerian degeneration, paraptosis, pyroptosis, pyronecrosis, and entosis. Necrosis and autophagy, the two most investigated forms of alternative cell death, are described below.

Necrosis, an alternative form of cell death. An alternative to apoptotic cell death is necrosis, a form of cell injury that works via autolysis to induce premature death in cells of living tissue (Proskuryakov, Konoplyannikov, & Gabai, 2003). In contrast to apoptosis which involves a naturally occurring programmed and targeted cause of cell death, necrosis is thought to be caused by factors external to the cell itself, such as infection or trauma, that provide a

degradative, energy-independent mode of death (Elmore, 2007). The process of necrosis is initiated by oncosis, a process characterized by cell swelling, and is followed by karyolysis, the degradation of chromatin in the cell nucleus, whereas the process of apoptosis is accompanied by cell shrinkage, pyknosis, the condensation of the nucleus and its chromatin, karyorrhexis, the rupture and fragmentation of the nucleus, and consequent irregular distribution of chromatin throughout the cell cytoplasm (Elmore, 2007).

Necrosis has historically been referred to as an accidental and unregulated pathological form of cell death; however, accumulating research has suggested that it is, in some cases, a regulated cellular mechanism controlled by a set of signal transduction pathways and catabolic mechanisms (Festjens, Vanden Berghe, Vandenabeele, 2006; Golstein & Kroemer, 2007). In apoptosis, the Fas and TNF family of receptors regulate the apoptotic pathway and induce the recruitment of various proteins and caspases, including Fas-associated death domain (FADD), and caspase-8, which downstream activate further caspases including caspase-3 (Cryns & Yuan, 1998). It has been shown that when these caspases and their associates are inhibited, Fas and TNF induce cell death with features characteristic of necrosis including oncosis, intracellular vacuolization, and dilation of the nuclear membrane (Matsumura et al., 2000; Vercammen et al., 1998). This process is accompanied by a loss of transmembrane potential, but not by the release of cytochrome *c* as seen with apoptosis, indicating that the mitochondrial damage caused by the necrosis is apoptotic-pathway-independent and that the cellular mechanism of necrosis is physiologically different from that of apoptosis (Yuan et al., 2003). The exact mechanism by which necrosis occurs remains to be determined; however, it has been suggested that both the adaptor protein FADD, and receptor-interacting protein (RIP), a Ser/Thr kinase, are required in the necrotic mechanism (Festjens, Vanden Berghe, Cornelis, & Vandenabeele, 2007; Holler et

al., 2000). The kinase activity of RIP has been shown to be required for the activation of the necrosis cascade (Holler et al., 2000), but not for the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$_K$B) activation in apoptosis (Ting, Pimentel-Muinos, & Seed, 1996) that RIP has been shown to mediate through TNF-α-induced NF-$_K$B-activation via recruitment of the I_KB kinase (IKK) complex to tumour necrosis factor receptor (TNFR; Yuan et al., 2003). Although the exact role of RIP in neuronal cell death remains unclear, upregulation of the Fas receptor death-inducing signaling complex, including RIP, after traumatic brain injury has been observed (Qiu et al., 2002), substantiating its role in necrosis.

One of the most distinctive features of necrosis is an early loss of adenosine triphosphate (ATP) synthesis. This has been suggested as a contributing factor to the failure of the homeostatic mechanism by which neurons survive (Yuan et al., 2003). It has been proposed that since necrosis is characterized by oncosis in the intracellular organelles, the loss of ATP synthesis is a prime contributor to the mitochondrial swelling seen throughout this process (Yuan et al., 2003).

It has been postulated that loss-of-function mutations in calreticulin, a calcium ion binding chaperone in the endoplasmic reticulum (ER), and calnexin, a ER membrane protein, can suppress necrotic degeneration (Xu, Tavernarakis, & Driscoll, 2001). The same effect can be seen with mutations to *unc*-68 and *itr*-1, which block calcium ion release from the ER channels (Xu et al., 2001). This research suggests that an increase in the concentration of cytoplasmic calcium ions, due to modulation by the ER, may impact downstream targets promoting necrotic cell death and induce abnormal functioning of cytoplasmic membrane sodium ion channels to cause neuronal degeneration including oncosis (Yuan et al., 2003). A selection of calpains and aspartyl proteases have been labeled as essential inducers of necrotic cell death due to their stark

responsiveness to excess intracellular calcium ion levels (Syntichaki, Xu, Driscoll, & Tavernarakis, 2002), and their activation is thought contribute to downstream processes leading to necrosis (Yuan et al., 2003). While an elevated calcium ion level in the cytoplasm has been proposed to induce neuronal cell death, its significance and mechanism of action has yet to be fully elucidated and this, in part, is due to the complex roles of calcium ions in the regulation of cellular processes (Kroemer et al., 2008). In addition, while calpains and cathepsins have been proposed to influence neuronal cell death mechanisms under neurotoxic conditions including ischemia (i.e., the "calpain-cathepsin hypothesis", see Yamashima, 2000), and how injury-induced N-methyl-D-aspartate receptor (NMDA) activation leads to extreme intracellular calcium ion surplus, activating calpain and then lysosomal cathepsin to ultimately induce necrosis, although these results provide compelling evidence for the possible underlying mechanistic processes of necrosis, they are still speculative and need to be further investigated (Yuan et al., 2003).

Although apoptosis and necrosis are mediated through different, distinct pathways, the pathways have been shown to reciprocate and overlap and the same insult could actually lead to either apoptosis or necrosis depending on its intensity, the neuronal specimen involved, and the type of biological organism at play (Yuan et al., 2003). The decision to undergo a caspase-dependent apoptotic death or a caspase-independent necrotic death is a complex process and it has been shown that some cells may actually undergo both concurrently (Yuan et al., 2003). For example, ischemic brain damage has been shown to induce a mix of necrosis and apoptosis amongst cells in the area of insult (Yuan et al., 2003). In a study utilizing caspase inhibitors in a model of ischemia, it was shown that caspase inhibitors significantly protected against apoptotic neuronal cell death; however, the majority of the injured neurons later exhibited features

associated with necrotic cell death (Moskowitz & Lo, 2003). Futhermore, significant increases in the intracellular levels of calcium ions, due to ischemia or severe ER stress, may release calpain in amounts great enough to reach the threshold for initiation of necrotic cell death through the activation of caspases and the aspartyl protease pathways, but potentially not enough to initiate an apoptotic death cascade, or vise versa (Nakagawa & Yuan, 2000). A study by Martinon and colleagues (2000) showed that Fas-induced apoptosis caused rapid cleavage of RIP, indicating that apoptotic-cleaved RIP may effectively and prematurely block the activation of the necrosis cell death pathway, as it has been identified as a critical mediator of necrosis. Therefore, due to the cross talk between the apoptotic and necrotic pathways, even though they could be regarded as entirely separate pathways, successful execution of an appropriate death program, or a mixture of the two, will likely become activated depending on the physiological or pathological situation.

Autophagy and its role in cell viability. Autophagy is an evolutionarily conserved mechanism characterized by autophagic vacuolization of the cellular components of the cytoplasm in the absence of chromatin condensation (Kroemer et al., 2008). Unlike apoptosis, which includes the involvement of engulfment genes and degeneration, cells that undergo autophagic cell death do not encounter phagocytic activity. During autophagy, lysosomes in the cell form an autolysosome and the cytoplasmic components of the cell become degraded due to lysosomal hydrolases (Rong et al., 2011). Autophagy has traditionally been understood to serve as a cell survival mechanism in starving cells; however, recent studies have indicated that autophagy also plays an important role in cell death (Baehrecke, 2005). For example, in a study by Boya and colleagues (2005), the inhibition of autophagy was shown to trigger apoptosis, providing a compelling case that autophagy positively contributes to sustaining cell survival.

However, an alternate perspective was provided by Lee, Cooksey, and Baehrecke (2002), after they found that the same steroid signal that activates the transcription of caspases for cell death actually activates *Atg* genes for promoting cell survival just a few hours before the death of salivary glands in *Drosophila melanogaster,* indicating the putative, pleiotropic role of autophagy in the regulation of cell viability. In specific cases, autophagy has shown to participate in the destruction of cells, due to atrophy of the cytoplasm (Berry & Baehrecke, 2007; Scott, Juhász, & Neufeld, 2007), while it has also shown to contribute to a pro-survival pathway as demonstrated in an *Atg* gene knockout/knockdown study that revealed that cell death does not become inhibited when the *Atg* gene is deleted but instead promotes cell death at an accelerated pace (Galluzzi et al., 2008).

A possible mechanism for autophagy in regulating cell survival and cell death may involve coordination of signals that suppress apoptosis and inhibit protein synthesis. The phosphoinositide 3-kinase (PI3K) signalling pathway has been postulated as playing a role in this process (Baehrecke, 2005). In the context of cell death, a possible hypothesis could be that autophagy kills cells by rapidly consuming the organelles in the cytoplasm, including the mitochondria, while concomitantly maintaining protein synthesis and other energy-dependent processes (Baehrecke, 2005). Lee and colleagues (2002) proposed that the transcription regulators Broad Complex (BR-C), E74A and E93 are required for autophagic cell death in *Drosophila melanogaster* salivary glands. Death-associated protein kinase (DAPk) and its protein, DAPk-related protein 1 (DRP-1), have also shown to play mechanistic roles in the cell death process, regulating membrane blebbing during apoptosis and vacuole formation in autophagy (Inbal, Bialik, Sabanay, Shani & Kimchi, 2002). In starvation-induced autophagy, lysosome homeostasis becomes restored via autophagic lysosome reformation (ALR), and this

lysosome reformation has been shown to require mammalian target of rapamycin (mTOR) kinase activation (Rong et al., 2011; Nakano et al., 2001). Spinster, a novel membrane protein, has also been shown to interfere with programmed cell death and cause neural degeneration in *Drosophila melanogaster* (Nakano et al., 2001) and defects in spinster have been shown to lead to the accumulation of enlarged autolysosomes (Rong et al., 2011). In brief, autophagy may play an active role in programmed cell death, assisting with the death process when apoptosis is lacking or inhibited, and it also may play a pro-survival role as evidenced by its activity with *Atg* genes in dying cells.

Promotion of cell survival and the vital role of cellular signalling pathways

Neuron viability is maintained through complex, interacting networks of signalling pathways that work together to regulate cell survival, metabolism, and proliferation. In the presence of cellular stress, whether due to injury, toxicity, or the like, the fate of a neuron is largely determined by the input communicated by various signal transduction pathways regulating cell survival. Dysregulated signals within these signalling pathways are often the result of abnormal genetic modifications to the critical components of the pathways and their upstream activators, and can influence signals to induce neuronal cell death (Steelman et al., 2011). However, these pathways can also engage in pro-survival signalling cascades and promote unrestricted cell proliferation and decreased sensitivity to apoptotic-inducing signals in the context of naturally occurring cell death, thereby boosting apoptotic-signal resistance (Steelman et al., 2011). Several cellular signalling pathways have been identified as regulating cell survival through the transmission of proliferative signals from membrane bound receptors to other proteins and ultimately to the nucleus to control gene expression (Steelman et al., 2011). Some of the most important pathways for regulating cell growth and survival include the

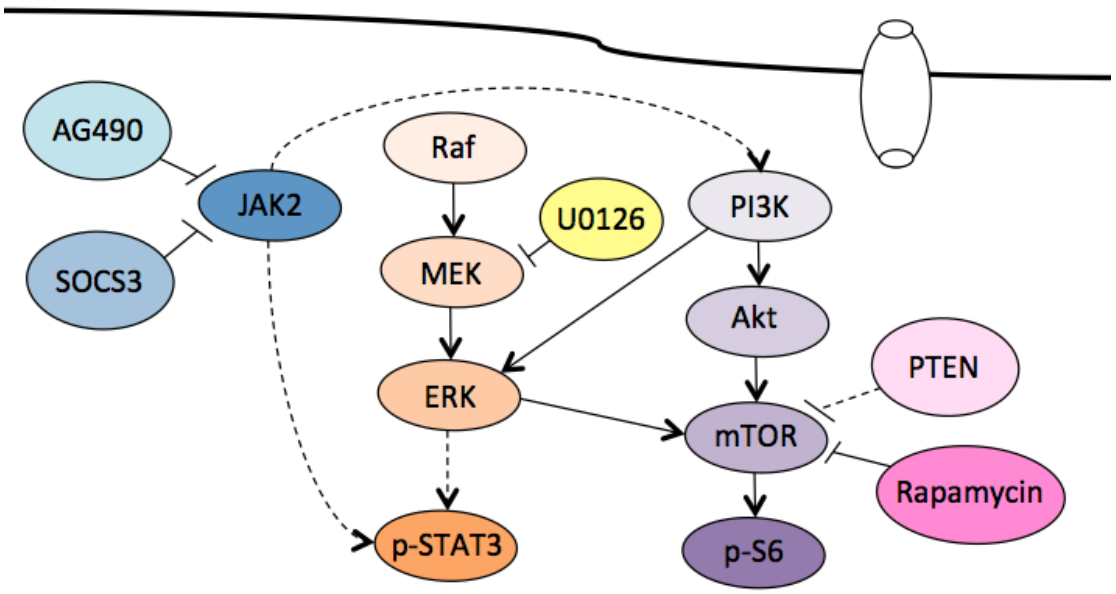

Figure 2. The JAK/STAT, Raf/MEK/ERK, and PI3K/PTEN/Akt/mTOR pathway cascades. When stimulated, each individual pathway invokes responses from the molecules downstream within its cascade, as well as engages in signalling crosstalk with neighbouring pathways. In the JAK/STAT pathway, JAK2, which can be inhibited by AG490 and/or SOCS3, can phosphorylate STAT3 through downstream signalling, and so can the Raf/MEK/ERK pathway, which can be inhibited by U0126, to ultimately upregulate gene transcription. The Raf/MEK/ERK pathway can also interact with the PI3K/Akt/mTOR pathway to phosphorylate S6. The PI3K/Akt/mTOR pathway, which can be inhibited by PTEN and/or rapamycin, can directly trigger an up-regulation of p-S6 for protein translation.

JAK/STAT pathway, ERK pathway, PKA pathway, and mTOR pathway (see Figure 2).

JAK/STAT Pathway. The JAK/STAT pathway is composed of a variety of cellular cascades that transduce signals for development and homeostasis in living organisms (Rawlings, Rosier, & Harrison, 2004). Cytoplasmic Janus kinase (JAK)s have been shown to be crucial components of diverse cellular signalling pathways that, when activated, govern cell survival and stimulate cell proliferation, differentiation, migration, apoptosis (Rane & Reddy, 2000), and most recently, synaptic plasticity (Nicolas et al., 2012). Recent evidence suggests that JAKs integrate with components of a number of diverse signalling cascades, not just internally, but also externally (Rane & Reddy, 2000). The phosphorylation of cytokines and growth factor receptors has been implicated as the primary mode of action that JAKs work through to

transduce their pro-survival and pro-growth effects (Rane & Reddy, 2000). Once a ligand binds to its receptor, intracellular activation occurs, inducing a multimerization of the receptor subunits. JAKs are composed of two domains located at the C-terminus: one for non-catalytic regulation; and the other for tyrosine kinase activity (Rawlings et al., 2004). The JAKs become activated via ligand-mediated receptor multimerization where two JAKs undergo trans-phosphorylation due to their inherent close proximity (Rawlings et al., 2004). The JAKs then go onto phosphorylate further targets, including the receptors and substrates of signal transducer and activator of transcription (STAT)s, factors part of the cytoplasm that are responsible for regulating gene transcription (Rawlings et al., 2004). This cascade, involving the activation of JAKs and STATs, provides a direct pathway for generating transcriptional responses from signals derived from outside of the cell (Rawlings et al., 2004).

The JAK/STAT pathway has been shown to integrate internal signals with that of specialized components of neighbouring signalling pathways to produce anti-apoptotic responses via signalling 'cross talk'. Some of the most substantiated interactions of the JAK/STAT pathway involve interaction with the Ras, PI3K, and mitogen-activated protein kinase (MAPK) pathways, all of which may contribute to proliferative processes (Rane & Reddy, 2000). Although the JAK/STAT pathway and alternative pathways including that of Ras, PI3K, and MAPK pathways mediate distinct signals and cascades for the promotion of cell survival, it is suggested that an interdependence between the different pathways likely exists (Rane & Reddy, 2000). Phosphorylation of other receptors, due to JAK activity, can create binding sites for adaptor molecules including Shc, the p85 subunit of PI3K, STAT proteins, and other kinases, to subsequently phosphorylate multiple downstream targets to further propagate JAK/STAT and other proliferative stimuli to encourage cell growth and survival (Rane & Reddy, 2000).

The activation of the JAK/STAT pathway has been widely substantiated as integrating several of the constitutive elements of the Ras pathway (Alam, Pazdrak, Stafford, & Forsythe, 1995; Bates, Bertics, & Busse, 1996; Kumar, Gupta, Wang, & Nel, 1994). A study by Mizuguchi and colleagues (2000) showed that Ras and STAT are essential and sufficient downstream components of JAK in cell proliferation. JAK/STAT signalling has also been shown to indirectly promote Ras signalling via transcription of suppressor of cytokine signalling 3 (SOCS3). SOCS3 reduces the activity of Ras GTPase activating protein (RasGAP), a negative regulator of the Ras signalling pathway, consequently promoting activation of the Ras pathway for cell growth (Alexander, 2002; Rawlings et al., 2004). The activation of Ras, PI3K and STAT proteins have also been substantiated as increasing transcription via factors such as a *c-jun*. Goodman, Niehoff, and Uckun (1998) showed that when cells were treated using ionizing radiation, the induction of *c-jun* was due to the exclusive activation of JAK3 (Goodman et al., 1998). In addition, the JAK/STAT pathway has been shown to modulate PI3K function. In a study by Al-Shami and Naccache (1999), neurotrophils stimulated by granulocyte-macrophage colony-stimulating factor (GM-CSF) was shown to trigger JAK2, STAT3, STAT5B, and PI3K activation. When treated with AG490, a JAK2 inhibitor, a decrease in phosphorylation of the p85 subunit of PI3K was observed, likely in response to the GM-CSF stimulation (Al-Shami & Naccache, 1999). In a different study by Yamauchi and colleagues (1998), insulin-receptor substrate (IRS) proteins in cells either JAK2 deficient or JAK2 dominant-negative became phosphorylated by JAK2 and upon phosphorylation, the IRS proteins were shown to actually provide a platform for attachment and subsequent activation of PI3K (Yamauchi et al., 1998). In addition, JAKs have been implicated as modulating cell survival and apoptosis via Bcl-2 regulation, as evidenced by multiple studies (Packham et al., 1998; Quelle et al., 1998; Sakai &

Kraft, 1997). Hence, as a mediator to the cellular responses of many cytokines and growth factors, the JAK/STAT pathway indeed serves as an important player in cell viability.

Raf/MEK/ERK Pathway. The extracellular signal-regulated kinase (ERK) pathway, along with its intermediaries, is an evolutionarily conserved MAPK pathway and was originally found to be important for the regulation of neuron survival in the absence of neurotrophin support (Xia, Dickens, Raingeaud, Davis, & Greenberg, 1995). More recent literature has demonstrated that the ERK pathway also plays a pivotal role in neuronal plasticity and memory formation (Impey, Obrietan, & Storm, 1999; Sweatt, 2004). Although some studies identify the ERK pathway as being harmful to neuronal cells and contributing to cell death (Murray, Alessandrini, Cole, Yee, & Furshpan, 1998; Stanciu & DeFranco, 2001), the ERK pathway works primarily to promote cell survival and protect against injury-induced apoptosis.

The mechanism by which the ERK pathway becomes activated is through trophic signalling cascades that involve the engagement of the membrane receptor on the surface of the neuron and activation of p21 GTPases, thereby activating Raf, and igniting a process of phosphorylation of MAPK kinase 1/2 and ultimately ERK (Morrison et al., 2002). Intracellular calcium ion levels, protein kinase A (PKA), diacylglycerol, and cyclic adenosine monophosphate (cAMP) are also known to stimulate and modulate the ERK pathway (Morrison et al., 2002).

The activation of ERK 1 and ERK2 (ERK1/2) has been substantiated as inhibiting apoptosis in a variety of cellular situations including hypoxia (Buckley et al., 1999), growth factor withdrawal (Erhardt, Schremser, & Cooper, 1999), hydrogen peroxide (Wang, Martindale, Liu, & Holbrook, 1998), matrix detachment (Le Gall, 2000), radiation (Kumar et al., 2007), TRAIL (Tran, Holmstrom, Ahonen, Kahari, & Eriksson, 2001), and chemotherapeutic agents

including cytosine arabinoside (Anderson & Tolkovsky, 1999). Additionally, the ERK pathway is also one of the primary mechanisms that molecules such as BDNF work through to provide neuroprotection, such as protecting the neonatal brain from hypoxic-ischemic injury (Han & Holtzman, 2000). ERK1/2 activity has been hypothesized to undergo either transient or prolonged activation during these situations and thus provide an anti-apoptotic effect (Lu & Xu, 2006). The anti-apoptotic effect of ERK1/2 can be diminished with its inhibition as well as following prolonged stimulation due to its downregulation caused by dephosphorylation or degradation (Lu & Xu, 2006). Hence, in order for ERK1/2 to promote cell survival, prevention of its downregulation is vital (Lu & Xu, 2006).

The ERK pathway has been shown to interact with p53, a critical tumour suppressor gene that encodes a transcription factor often mutated in cancer and is also widely apparent in cellular aging (Demidenko, Korotchkina, Gudkov, & Blagosklonny, 2010; Liu et al., 2009; Steelman et al., 2011; Wang, Ziao, Ko, & Ren, 2010). p53 regulates a variety of critical processes and is activated in response to DNA damage (Cheng & Chen, 2010), apoptosis (O'Prey et al., 2010), senescence (Campisi, 2005; Kelley et al., 2010; Maier et al., 2004), metastasis (Ho & Alman, 2010), autophagy (Galluzzi, Morselli, Kepp, Maiuri, & Kroemer, 2010), and aging (Demidenko et al., 2010; Jung-Hynes & Ahmad, 2009; Steelman et al., 2011). The ERK pathway can regulate the activity of p53 and, conversely, p53 is able to induce the activity of the key components of the ERK pathway (McCubrey et al., 2007). The Akt pathway has also been shown to 'cross talk' with the ERK pathway and contribute to its regulation, and thus, Akt has been implicated as playing critical roles in the regulation of cell cycle progression, including responses to aging and disease, as well (Brauer & Tyner, 2009; Yang, Wu, Wu, & Lin, 2010).

Literature has suggested that the ERK pathway plays an important role in protecting

neurons after injury. ERK pathway activation has been shown to be essential for BDNF-mediated protection from DNA damage in cortical neurons (Hetman, Kanning, Cavanaugh & Xia, 1999) as well as glutamate-induced excitotoxicity (Almeida et al., 2005) and hypoxic preconditioning (Hausenloy, Tsang, Mocanu, & Yellon, 2005). A mitogen-activated protein kinase kinase 1 (MKK1) inhibitor has also been shown to protect against damage due to focal cerebral ischemia (Alessandrini, Namura, Moskowitz & Bonventre, 1999), indicating that ERK regulation could be controlled independently of MKK1 mechanisms (Namura et al., 2001). In addition, the ERK pathway has been identified as promoting cell survival by enhancing the activity of anti-apoptotic molecules. Myeloid cell leukemia-1 (Mcl-1) is an anti-apoptotic member of the Bcl-2 family and becomes phosphorylated by ERK1/2 to increase its anti-apoptotic activity (Domina, Vrana, Gregory, Hann & Craig, 2004). Furthermore, the inhibition of ERK1/2 activity has been shown to result in a downregulation of Mcl-1 and Bcl-xL (Boucher et al., 2000; Jost, Huggett, Kari, Boise, & Rodeck, 2001). Immediate early response gene X-1 (IEX-1) is an early response factor to NF-κB and becomes phosphorylated by ERK1/2 to prevent the release of cytochrome *c* from the mitochondria, as mentioned before, to inhibit cell death due to cytokine deprivation (Garcia et al., 2002). In the same vein, IEX-1 demonstrates regulatory processes of ERK1/2 by enhancing its activation in response to growth factors (Garcia et al., 2002). ERK1/2 also regulates caspase-9, sufficiently blocking its activity as well as the activation of caspase-3, illustrating the ERK-induced inhibited activation of caspases downstream of cytochrome *c* (Allan et al., 2003; Means, Muro, & Clem, 2005). It can also phosphorylate Bad and prevent it from inducing apoptosis in neuronal cells and has been postulated as activating CREB for cell survival (Bonni et al., 1999). In conclusion, it is clear that although ERK signalling may function in a pro-apoptotic manner under certain circumstances,

its regulation of cell proliferation, differentiation and survival is considerately more apparent. However, because the exact mechanisms by which the ERK pathway mediates its neuroprotective effects are still widely unknown, further investigation is warranted.

cAMP/PKA/CREB Pathway. CREB becomes activated in response to a wide variety of stimuli and, when activated, has been shown to initiate gene transcription and an array of other cellular responses (Lonze & Ginty, 2002). The degree to which CREB is expressed and utilized at the molecular level is both diverse and specific, and its role within cellular signalling cascades has been proposed as vital for supporting the development of the nervous system, promoting neuroprotection and providing defense against disease, especially within stressful and toxic settings (Lonze & Ginty, 2002; Walton & Dragunow, 2000).

CREB is a transcription factor that is part of a family of structurally-related transcription factors, including activating transcription factor (ATF)s 1, 2, 3, and 4, that act to control the transcriptional processes of a variety of genes that are expressed when cytoplasmic cAMP and calcium ion levels are elevated (Walton & Dragunow, 2000). The activation of CREB can be achieved by the phosphorylation of Ser133 by PKA (Gonzalez & Montminy, 1989), ribosomal S6 kinase 2, calcium ion-activated calmodulin kinases, or MAPK2 (Beitner-Johnson & Millhorn, 1998; Walton & Dragunow, 2000). It has been proposed that Ser133 phosphorylation activates CREB by converting it from an inactive to an active configuration (Brindle, Linke, & Montminy, 1993; Gonzalez, Menzel, Leonard, Fischer, & Montminy, 1991). One study found that Ser133-induced phosphorylation of CREB was induced by Akt overproduction in serum-stimulated cells (Du & Montminy, 1998). In its active form, studies have demonstrated CREB to be a vital regulator of several neuronal processes, one of particular note being the promotion of neuronal survival (Walton & Dragunow, 2000).

CREB and its related family members have been shown to play important roles in promoting survival by mediating gene expression in a variety of cell types, and this has been evidenced in several *in vitro* neuronal studies that have utilized inhibitors of CREB (Bonni et al., 1999; Riccio, Ahn, Davenport, Blendy, & Ginty, 1999; Walton et al., 1999; Lonze & Ginty, 2002). Specifically, cell survival has been shown to require CREB activation in dorsal root ganglion (DRG) sensory and sympathetic neurons (Lonze, Riccio, Cohen & Ginty, 2002) in experiments using CREB knockout mice (Rudolph et al., 1998; Lonze & Ginty, 2002). Within the CNS, a less elucidated role of CREB has been established; however, similar findings to date have been established. Specifically, a burgeoning body of literature now supports the important role of the CREB family member cAMP response element modulator (CREM) in the survival process, especially during neuronal development. One study showed that the elimination of CREB alone did not impact neuronal survival nor did it compromise CNS integrity including that of glial cell survival (Mantamadiotis et al., 2002; Lonze & Ginty, 2002). This is in contrast to the finding that the complete knockout of CREB in mice results in lethality (Rudolph at al., 1998). However, when CREB is concurrently eliminated with CREM, a robust decrease in neuronal survival has been observed, and this decrease is suggested to be due to an increase in apoptotic signalling activity beginning from mid-gestation and onwards throughout early neuronal development (Mantamadiotis et al., 2002; Lonze & Ginty, 2002). Furthermore, within the postnatal age group, robust degeneration of the CNS, especially within the regions of the cortex, hippocampus, and striatum, have been observed when CREB is removed within a CREM knockout setting, illustrating the independent and dependent roles of the CREB and CREM transcriptional factors for sustaining neuronal survival, especially within the later developmental stages (Mantamadiotis et al., 2002; Lonze & Ginty, 2002). Furthermore, it has been shown

within an *in vitro* setting that inhibiting CREB decreases the outgrowth of cortical neuron dendrites, illustrating that CREB could also be important for assisting with precursor proliferation within neuronal development (Redmond, Kashani, & Ghosh, 2002; Lonze & Ginty, 2002).

The upstream effectors of the CREB signalling pathway remain to be fully elucidated; however, findings related to how the activation of CREB affects programmed cell survival are becoming more established. For example, BDNF was proposed as being an important mediator of the CREB cascade following the publication of a study using a hypoxic ischemia model that demonstrated that BDNF was co-localized with phosphorylated CREB in dentate granule cells (Walton et al., 1999; Walton & Dragunow, 2000). Moreover, CREB has also been shown to directly regulate the transcription of the BDNF gene, suggesting a possible reciprocal relationship between BDNF and CREB for promoting cell survival (Shieh, Hu, Bobb, Timmusk, & Ghosh, 1998; Tao et al., 1998; Walton & Dragunow, 2000). Other survival factors have been proposed to control transcription and promote neuronal survival through a CREB-mediated pathway as well, including that of estrogen (Watters & Dorsa, 1998) and pituitary adenylate cyclase-activating polypeptide (PACAP; Campard et al., 1997). PACAP activation, in particular, has been shown to promote neuronal survival via an complex CREB-dependent cascade involving cAMP, PKA, and MAPK constituents (Campard et al., 1997; Tanaka et al., 1997; Villalba, Bockaert, & Journot, 1997). A variety of neurotransmitters and modulators have also been shown to converge on CREB in order to regulate neuronal survival, including a wide range of kinase pathways (Walton & Druganow, 2000), and many of them ultimately phosphorylate CREB via second messenger circuitry to activate protein kinase C and/or PKA (Roberson et al., 1999). The activation of the ERK and MAPK pathways for ultimately phosphorylating CREB

has also been well characterized (Deak, Clifton, Lucocq, & Alessi, 1998; Xing, Ginty, & Greenberg, 1996), and research also suggests that the PI3K/Akt pathway can play an important role in activating CREB and its effectors, as well (Du & Montminy, 1998; Perkinton, Ip, Wood, Crossthwaite, & Williams, 2002; Pugazhenthi et al., 2000).

Previous research has examined the efficacy of the CREB pathway and its effectors in blocking cellular apoptosis, and it has been suggested that CREB induces a potent survival response not only in the presence of pro-survival stimuli but also in response to pro-apoptotic signals (Deak et al., 1998; Iordanov et al., 1997; Lonze & Ginty, 2002). Akt-mediated phosphorylation of the Bcl-2 pro-apoptotic member, Bad (Datta et al., 1997; del Peso, Gonzalez-Garcia, Page, Herrera, & Nunez, 1997), and the protease, caspase 9 (Cardone et al., 1998), could be targets that the CREB pathway suppresses to enhance survival and moderate the effects of pro-apoptotic signalling on cells (Lonze & Ginty, 2002). Additionally, following hypoxia, heightened expression of the CREB-mediated anti-apoptotic gene Bcl-2 has been shown to occur (Freeland, Boxer, & Latchman, 2001), and, in separate study, upregulated CREB expression in neurons was observed in response to harmful stimuli *in vivo* and *in vitro* (Mabuchi et al., 2001). It has been hypothesized that given the nature of CREB and its variety of responses, two streams of signals initiate in response to a cerebral insult: one that recognizes and determines the nature of the injury; and a second that activates the CREB cascade to promote the survival of a cell (Lonze & Ginty, 2002).

Taken together, research investigating the effects of the CREB pathway, including that of its effectors, has supported the notion that CREB activation provides protection to neurons both intrinsically, by encouraging neuronal survival, and also extrinsically in response to pro-apoptotic signalling, by providing resistance to incoming threats such as signals initiated during

cellular stress (Walton & Dragunow, 2000). Some well-characterized anti-apoptotic proteins have shown to converge on CREB in order to promote their neuroprotective effects as well, substantiating CREB and the members of its cascade as key players in the pro-survival response (Walton & Dragunow, 2000). Because of the demonstrated effects of this molecular cascade in providing a neuroprotective effect, the cAMP/PKA/CREB pathway was targeted in the current study and experiments incorporating an inhibitor specific to this pathway were implemented to determine whether a cAMP/PKA/CREB-mediated response underlies the neuroprotective effect induced by tPA.

PI3K/PTEN/Akt/mTOR Pathway. The mTOR pathway, and its upstream and downstream constituents PI3K, phosphatase and tensin homologue (PTEN), and Akt, are important regulators of cell growth and proliferation and have been shown to integrate signals from growth factors, nutrients, and energy status to regulate various processes including autophagy, ribosome biogenesis and cell metabolism (Sarbassov, Ali, & Sabatini, 2005). Because of the pathway's proliferative and anti-apoptotic effects, recent evidence has suggested that its deregulation could actually be associated with physiological diseases such as cancer, diabetes, and aging (Sarbassov et al., 2005; Zoncu, Efeyan, & Sabatini, 2010).

The PI3K/Akt pathway has been identified as a key mediator of cell survival in both peripheral (Crowder & Freeman, 1998) and central nervous system cultured neurons (Ghosh & Greenberg, 1995). Neurotrophic factors including NGF, BDNF, GDNF, and insulin-like growth factor 1 (IGFI) have been shown to activate the PI3K/Akt signalling pathway and initiate a signalling cascade via receptor kinases including TrKs (Morrison et al., 2002; Segal & Greenberg, 1996). The binding of the ligand to its respective receptor allows p85 to connect with phosphorylated tyrosine residues on the receptor via a Src-homology 2 domain (Steelman et al.,

2011). Following receptor dimerization, PI3K relocates to the plasma membrane where its subunit activates PIP_2 and PIP_3, phosphoinositide phosphatases, which act as lipid second messengers and communicate downstream to ultimately attract kinases to the plasma membrane, activate Akt, and initiate further downstream signalling cascades (Martelli, Evangelisti, Chiarini, & McCubrey, 2010; Morrison et al., 2002, Steelman et al., 2011; Zhao & Vogt, 2010). PI3K can also be stimulated via the TrK receptors due to Ras G-protein, IRS signalling, and Grb2-associated binder-1 (Gab-1) activation (Holgado-Madruga, Moscatello, Emlet, Dieterich, & Wong, 1997; Kauffmann-Zeh et al., 1997).

After Akt becomes activated, it is able to translocate to the nucleus and modulate a variety of transcriptional regulators (Martelli et al., 2010; Steelman et al., 2011): CREB (Du & Montminy, 1998); E2F (Brennan et al., 1997); NF-$_K$B through IKK inhibition (Kane, Shapiro, Stokoe, & Weiss, 1999); and forkhead transcription factors via phosphorylation and inactivation, thereby inhibiting the induction of death gene expression (Brunet et al., 1999; Buitenhuis & Coffer, 2009). In addition to transcription factors, Akt is able to target various other molecules to encourage a pro-survival response. Akt can directly phosphorylate and inactivate Bad, preventing it from communicating with its anti-apoptotic Bcl-2 relatives Bcl-2 and Bcl-xL (del Peso et al., 1997). It can also inhibit cell death by preventing the release of cytochrome *c* from the mitochondria (Kennedy, Kandel, Cross, & Hay, 1999) and effectively phosphorylate and inhibit caspase-9 downstream (Cardone et al., 1998). Foxo-3, a protein capable of upregulating Fas-L and Bim, two potent inducers of apoptosis, has been shown to become localized to only the cytosol where it is unable to induce expression of its death genes upon inactivation by Akt (Buitenhuis & Coffer, 2009; Dijkers, Lammers, Koenderman, & Coffer, 2000; Steelman et al., 2011). Moreover, Akt has the capability to phosphorylate and inactivate Bim, allowing for a

complete suppression of its pro-apoptotic activity (Qi, Wildey, & Howe, 2006). As described above, it is now fairly well established that Akt in concert with PI3K and its downstream targets play vital roles in regulating cell viability via up-regulating anti-apoptotic and down-regulating anti-apoptotic signalling molecules.

PTEN is a known buffer of PI3K signalling and negatively regulates the pathway. PTEN is commonly mutated in its phosphatase domain (Eng, 2003) and is therefore an attractive target for activation since its inactivation is observed in pathological states including cancer (Steelman et al., 2011). For example, in glioblastoma, mutations that impair the functioning and localization of PTEN have been shown to result in deficient tumor-suppressive activity (Song, Salmena, & Pandolfi, 2012; Walker, Leslie, Perera, Batty, & Downes, 2004) and thus elevate Akt activity and abnormal growth regulation (Chalhoub & Baker, 2009; McCubrey et al., 2011).

Another key component of this pathway is the mTOR constituent. mTOR, similar to the other kinases of this pathway, is a conserved regulator of cell growth, proliferation, survival and recovery (Hay & Sonenberg, 2004; Hung, Garcia-Haro, Sparks, & Guertin, 2012; Shi et al., 2011). While the exact mechanism involved in coordinating the processes of cell growth still remains unknown, mTOR and its effectors have recently emerged as central regulators of both cell growth and division. This insight came as a result of the inhibition of mTOR by rapamycin in mammalian cells (Fingar, Salama, Tsou, Harlow, & Blenis, 2002) and the knockout mutation of *Drosophila* TOR, both leading to decreased cell size (Leevers & Hafen, 2004; Ruvinsky & Meyuhas, 2006). The mTOR kinase is composed of two distinct complexes: mTOR complex 1 (mTORC1); and mTOR complex 2 (mTORC2). mTORC1 contains regulatory-associated protein of mTOR (RAPTOR) and proline-rich Akt substrate of 40 kDa (PRAS40) subunits whereas mTORC2 contains rapamycin-insensitive companion of mammalian target of rapamycin

(RICTOR) and protein observed with RICTOR (PROTOR) subunits. mTORC1 has been implicated as controlling cell growth via several methods: regulating autophagy via phosphorylation of ULK1 (Chan, 2009; Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009); regulating lipid metabolism by phosphorylating Lipin1 (Peterson et al., 2011); modulating insulin signalling through Grb10 (Hsu et al., 2011; Hung et al., 2012); and directly phosphorylating S6 kinase 1 (S6K1) and 4EBP1, regulators of protein translation (Fingar et al., 2004). Research has substantiated S6K1 as being an important regulator of cell size, cell proliferation, and glucose homeostasis and that it is exclusively managed by mTOR and its downstream effectors (Ruvinsky & Meyuhas, 2006). S6K1 has also been shown to rescue cells from the apoptotic effects of Bad (Harada, Andersen, Mann, Terada, & Korsmeyer, 2001) and may also decrease the induction of apoptosis through an increase in anti-apoptotic Bcl-2 and Bcl-xL expression (Pastor et al., 2009).

mTORC1 is responsible for sensing nutrient availability in the cell as well as scouting out nutrient levels based on surrounding growth factor signalling pathways (Hung et al., 2012). mTORC2, on the other hand, has functions that are widely less known; however, appears to only identify proximal growth factors within its environment (Hung et al., 2012). mTORC1 has been shown to mediate growth factor signalling through the PI3K/Akt cascade, and the widespread role of Akt in regulating cell survival has been suggested to be influenced, or even fully controlled, by mTORC2 activity (Hung et al., 2012). Akt has been shown to have the ability to directly activate mTORC1 via phosphorylating PRAS40 and relieve its inhibitory effect on the complex (Sancak et al., 2007; Vander Haar, Lee, Bandhakavi, Griffin, & Kim, 2007). In conclusion, while the PI3K/PTEN/Akt/mTOR pathway and its effects on neurons have yet to be fully elucidated, its role in maintaining cell viability, through a variety of signalling cascades

that contribute to the up-regulation of anti-apoptotic signals and down-regulation of pro-apoptotic signals, is well recognized. A major goal of this study is to elucidate a possible interaction between the mTOR signalling pathway and the activity of tPA in neurons.

Stroke

Stroke is a neurological event whereby there is a significant reduction in the cerebral blood flow (CBF) of an affected part of the brain due to a sudden or gradual obstruction within a cerebral artery (Hossmann, 2006). There are two main types of stroke: hemorrhagic and ischemic. Hemorrhage stroke can be caused by aneurysms, arteriovenous malformations, and/or elevated hypertension levels, and is caused by the rupture of a weakened blood vessel (American Heart Association, 2012). Conversely, when an obstruction within a blood vessel supplying blood to the brain occurs, this constitutes what is called an ischemic stroke, and accounts for approximately 87 percent of all stroke cases (American Heart Association, 2012). Within ischemic stroke, plaque buildup causes atherosclerosis, which serves as a main contributor to the development of an ischemic stroke (Heart and Stroke Foundation, 2014). Ischemic stroke can be dichotomized into two subtypes: thrombotic and embolic; the former being caused by a blood clot that forms in an artery directly supplying blood to the brain, and the latter being caused by a clot developing somewhere in the body and travelling through the blood steam and ultimately reaching the brain (Heart and Stroke Foundation, 2014). Following the onset of ischemia, the functional activity of the part of the brain affected by the obstruction becomes impaired, and, as the degree of hypoxia becomes more severe, metabolic activity becomes suppressed as well (Hossmann, 2006). These conditions are known to lead to the initiation of apoptotic and necrotic cellular signalling cascades within neurons of the affected area and contribute to the physiological and functional disturbances that individuals may experience when suffering from

an ischemic attack (see Chan, 2004). The region adjacent to the directly affected area is termed the ischemic penumbra, a crescent-shaped region contiguous to both the necrotic tissue and normal, healthy tissue, and has come to be known an as an area "of constained blood supply in which energy metabolism is preserved" (Hossmann, 2006; Hossmann, 1994). The ischemic penumbra has historically been a key area of interest within the ischemic brain because it is an area of the brain that may be salvageable and eligible for therapeutic reversal of the neuropathological symptomatology arising as a consequence of the ischemic attack (Hossmann, 2006; Ginsberg, 2003; Fisher, 2004). Although the consequences of ischemic stroke can be very serious, if not lethal, the availability of effective clinical treatments for ischemic stroke has remained grim. There is currently one approved drug for the treatment of acute ischemic stroke in Canada, tPA, and it involves its use as an intravenous thrombolytic to promote reperfusion within the area of the brain that is affected by the obstruction. tPA is the agent targeted by the current study and is described in greater detail below.

Tissue plasminogen activator (tPA)

tPA is a serine protease involved in the breakdown of blood clots. It acts as an enzyme that catalyzes the conversion of plasminogen to plasmin and works in conjunction with the clotting system to promote clot breakdown, fibrinolysis homeostasis, and matrix regulation (Hu et al., 2008; Mars, Zarnegar, & Michalopoulos, 1993; Fredriksson, Li, Fieber, Li, & Eriksson, 2004). Because of its thrombolytic properties, tPA is used to treat embolic- and thrombotic-type stroke but is contraindicated for the treatment of hemorrhagic stroke or head trauma (Pfeilschifter, Kashefiolasl, Lauer, Steinmetz, & Foerch, 2013; Yepes, Roussel, Ali, & Vivien, 2009). Although proven to be a life saving and powerful 'clot buster' for individuals experiencing ischemia, tPA is limited in that it has a very short therapeutic window for stroke

treatment: approximately 3 hours after symptom onset (Lees et al., 2010; Ringleb, Schellinger, Schranz, & Hacke, 2002). When administered outside of this prescribed timeframe, evidence suggests that tPA becomes neurotoxic to the surrounding brain tissue (ischemic penumbra), causing further growth of the ischemic infarct due to activation of several signalling processes associated with cell death, degradation of the extracellular matrix, and increase in the permeability of the neurovascular unit (NVU; Yepes et al., 2009). Using tPA for the treatment of ischemia has proven to significantly increase the number of patients with a 'good outcome' and reduce the proportion of patients severely disabled or dead three months following ischemia; however, it has also been shown to produce deleterious effects in patients including a 10-fold increase in the incidence of intracerebral hemorrhage (The N.I.N.D.S. Stroke Study Group, 1995). Evidently, tPA remains a controversial agent for the treatment of ischemia and with so many acute stroke patients receiving tPA as a treatment for their condition in the modern age, it is important to determine the underlying mechanisms mediating its harmful effects on the brain.

tPA's pleiotropic role in neuronal viability. At the molecular level, tPA is a glycoprotein consisting of 527 or 520 amino acids (Lin & Hu, 2014). tPA is a single chain enzyme that is cleaved by plasmin into a two chain form composed of a heavy chain and a light chain (Lin & Hu, 2014). The single chain is composed of four conserved domains: (1) a finger domain; (2) an epidermal growth factor (EGF) domain; (3) two kringle domains; and (4) a protease domain (Lin & Hu, 2014). The heavy chain of the two-chain form of tPA is composed of finger, EGF, and kringle domains and the light chain is composed of a single protease domain. tPA has an active site consisting of Histidine 322, Asparagine 371, and Serine 478 (Vivien, Gauberti, Montagne, Defer, & Touze, 2011). Serine 478 mutations have been shown to inactivate tPA and play vital roles in the protease-independent effects of tPA (Lin & Hu, 2014;

Olson et al., 2001). Each of the unique domains of tPA are differentially involved in the pleiotropic function of the molecule (Yepes et al., 2009). The finger domain has been shown to be involved in annexin-II and microglial activation (Nicole et al., 2001), have an affinity for fibrin (Thorsen, Glas-Greenwalt, & Astrup, 1972), and interact with plasminogen activator inhibitor-1 (PAI-1; Kaneko, Sakata, Matsuda, & Mimuro, 1992). The EGF domain interacts with platelets and contributes to the stabilization of the catalytic active site (Yepes et al., 2009). The kringle domains allow the molecule to bind to membranes, proteins, and phospholipids, regulate catalytic activity, have affinity to fibrin and plasminogen, interact with platelet-derived growth factor receptor CC (PDGF-CC; Fredriksson et al., 2004), and also is involved in the cleavage of the NR1 subunit of the NMDA receptor (Matys & Strickland, 2003; Yepes et al., 2009). Lastly, the protease domain is responsible for interacting with target sequences within substrates of other molecules and also for performing proteolysis (Yepes et al., 2009). Although tPA is considered a potent protease, evidence suggests that tPA also possesses a variety of other functions that are separate from its proteolytic activity (Benarroch, 2007; Fernandez-Monreal et al., 2004). tPA can act as a serine protease as well as a cytokine, executing multiple actions to trigger intracellular signalling cascades (Hu et al., 2006; Hu, Wu, Mars, & Liu, 2007; Lin et al., 2010), as well as induce a variety of responses in the face of pathological and diseased states, including ischemia, that can serve to either protect or harm neuronal cells, depending on the time and location.

While tPA has been substantiated as a reasonably effective treatment for acute ischemic stroke if administered within the prescribed time frame and remains the only drug approved by the Food and Drug Administration (FDA) for the treatment of the condition (The N.I.N.D.S. Stroke Study Group, 1995), it still remains a highly controversial agent due to its pleiotropic

effects on the brain. Recent evidence has identified that when tPA interacts with the NMDA receptor, lipoprotein receptor-related protein (LRP), and annexin-II in neuronal and glial cells, cerebral edema and cell death occur (Nicole et al., 2001; Siao & Tsirka, 2002; Yepes et al., 2003). Conversely, in the intravascular compartment, tPA has been identified as providing a substrate of inactive zymogen plasminogen to help promote thrombolysis during ischemic attacks (Yepes et al., 2009). It is currently speculated that tPA provides a plasminogen-dependent, beneficial effect in the intravascular space and a plasminogen-dependent and -independent, deleterious effect in the area of cerebral insult; however, these hypotheses require much more rigorous testing. An overview of the putative neurotoxic and neuroprotective effects of tPA are provided below.

tPA-induced neurotoxicity. To date, tPA has been shown to contribute to neurotoxicity via several distinct ways. One of the most recognized ways by which tPA induces neurotoxicity is through its effects on the neurovascular unit (NVU), or the blood brain barrier (BBB). The NVU is composed of endothelial cells, a basement membrane, perivascular astrocytes, and neurons (Abbott, Ronnback & Hansson, 2006), and serves to maintain a bidirectional passage of molecules between the brain and the intravascular space (Yepes et al., 2009). It has been shown that the components of the NVU begin to degrade, including its tight-junction proteins and basement membrane, in response to cerebral insults like ischemia (del Zoppo & Mabuchi, 2003; Fukuda et al., 2004), thereby increasing the permeability of the NVU and invoking the development of cerebral edema (Yang & Rosenberg, 2011). Studies have revealed that this process is predominately due to the effects of tPA; specifically, its ability to increase the expression and activity of matrix metallopeptidase (MMP-9), an enzyme involved in the degradation of the extracellular matrix (Aoki, Sumii, Mori, Wang, & Lo, 2002; Lee et al., 2007).

tPA's ability to increase the permeability of the NVU has been substantiated in an experimental model of ischemia called middle cerebral artery occlusion (MCAO; Yepes et al., 2003). Additionally, activation of PDGF-CC and its receptors by tPA (Su et al., 2008), as well as tPA's interaction with LRP under ischemic conditions have shown to induce a signalling cascade that increases the permeability of the NVU as well (Polavarapu, An, Zhang, & Yepes, 2008). The interaction of tPA with LRP induces the shredding of the LRP ectoderm into the vascular basement membrane and this consequently detaches the astrocytic end-feet into the basal lamina to ultimately produce increased NVU permeability (Polavarapu et al., 2007; Polavarapu et al., 2008). The interaction of LRP and tPA has also been shown to induce the phosphorylation and translocation of p65, an indicator of NF-$_K$B pathway activation (Zhang, Polavarapu, She, Mao, & Yepes, 2007), which, in the central nervous system, is known to lead to an augmentation of MMP-9 and inducible nitric oxide synthase (iNOS) expression and activity in cells which ultimately increases the permeability of the NVU (Yepes et al., 2009). Furthermore, activation of NF-$_K$B and its targets have been shown to be activated in various models of cerebral ischemia (Scheider et al., 1999; Stephenson et al., 2000) and its activation by tPA has actually been shown to be an effect mediated by LRP (Zhang et al., 2007) and MMP-9 (Wang et al., 2003). As a consequence of increased NVU permeability, tPA may be able to cross from the intravascular compartment into the BBB and cause cerebral edema and hemorrhage transformation under ischemic conditions, as suggested by several studies (Benchenane et al., 2005; Polavarapu et al., 2007).

The neurotoxic effect of tPA in the brain is further substantiated by the fact that a genetic deficiency in tPA (Wang et al., 1998), a deficiency in plasminogen activator inhibitor-1 (PAI-1; Nagai, De Moi, Lijnen, Carmeliet, & Collen, 1999), or treatment with neuroserpin (Cinelli et al.,

2001; Yepes et al., 2000) all independently demonstrate neuroprotective effects in animal models of ischemia. It has been suggested that tPA's catalysis of plasmin generation could contribute to the degradation the components of the extracellular matrix (Chen & Strickland, 1997), the activation of microglia via annexin-II (Yepes et al., 2003), and MCP-1 induction (Sheehan et al., 2007), responses seen in animals that have undergone experimentally-induced cerebral ischemia. Regulated intramembranous proteolysis of LRP via interaction with tPA has been shown to mediate ischemic cell death as well (Polavarapu et al., 2008).

The interaction of tPA with NMDA receptors has also been a field of intense study since it was discovered that the injection of kainic acid into the CA1 region of the hippocampus was associated with neuronal damage in wild-type but not tPA-deficient or plasminogen-deficient mice (Chen & Strickland, 1997; Indyk, Chen, Tsirka, & Strickland, 2003; Tsirka, Gualandris, Amaral, & Strickland, 1995). tPA has been shown to interact with NMDA receptors and play an important role in the initiation of ethanol withdrawal seizures (Pawlak, Melchor, Matys, Skrzypiec, & Strickland, 2005). The need for further clarification of tPA's proteolytic cascade and elucidation of the detailed mechanisms mediating its detrimental impact on neurons highlight the heightened complexity and diverse effects of tPA. Although tPA continues to be used to treat neurological ailments including ischemia, its stark side effects, as evidenced by the above studies, could ultimately lead to unfavorable neurological outcomes in some circumstances.

tPA-induced neuroprotection. While tPA has been shown to induce deleterious effects on neurons by promoting excitotoxicity and increasing the permeability of the NVU under ischemic conditions, a growing body of evidence suggests that tPA may be therapeutic and provide neuroprotection in a number of pathological situations. In the context of ischemia, in

addition to providing potent clot busting activity, tPA administration has been touted as being ten times more likely to help than to harm eligible patients with acute ischemic stroke (Demaerschalk, 2007).

At the molecular level, tPA has been shown to have a plasminogen-dependent beneficial effect in the intravascular space where it acts as a thrombolytic enzyme (Yepes et al., 2009). It is here where tPA is regulated by PAI-1 and is released by endothelial cells in the presence of a thrombotic event to preserve the patency of the blood vessel (Zlokovic et al., 1995). tPA has also demonstrated the ability to convert neurotrophins, including BDNF and NGF, into their active forms to provide anti-apoptotic neuroprotection in neurons (Bruno & Cuello, 2006; Pang et al., 2004). In addition, it has been established that tPA inhibits apoptotic cell death in neuronal culture in a dose-dependent manner from serum deprivation-induced apoptosis via up-regulating the PI3K pathway (Liot et al., 2006).

As further evidence of its anti-apoptotic effects, tPA has been shown to play a critical role in promoting neurite outgrowth (Krystosek & Seeds, 1981), synaptic plasticity, learning, and memory (Baranes et al., 1998; Seeds, Williams, & Bickford, 1995). Specifically, Calabresi and colleagues (2000) identified tPA as a regulator of multiple forms of synaptic plasticity and memory, with tPA knockout mice expressing more learning and memory-related behavioural abnormalities including deficits in habituation and reactivity to spatial change compared to wild-type controls (Calabresi et al., 2000). These effects have been shown to exist independently of tPA's proteolytic activity or interaction with LRP and likely work through interactions with annexin-II (Lee, Hwang, Im, Koh, & Kim, 2007).

Recent research investigating the effects of tPA in both *in vivo* and *in vitro* studies has demonstrated robust tPA-dependent neuroprotection in neurons. In a study by Echeverry and

colleagues (2010), sublethal hypoxia, a type of ischemic preconditioning, was found to induce a rapid release of neuronal tPA, the first step towards a neuroprotective response leading to neuronal survival, and the treatment of tPA in hippocampal neurons subjected to a lethal dose of hypoxia, applied either immediately or 24 hours after ischemic preconditioning, provided a tPA-dependent tolerance against the insult. The study also found that the ischemic preconditioning increased tPA activity within the CA1 region of the hippocampus (Echeverry et al., 2010). The neuroprotection provided by tPA in this study was found to be independent of plasminogen or plasmin and was shown to require the interaction of tPA with low-density lipoprotein (LDL) and an NMDA-receptor-dependent phosphorylation of Akt (Echeverry et al., 2010). Activation of Akt signalling pathway as well as the ERK and c-Jun N-terminal kinase (JNK) pathways by tPA has now also been discovered in a study elucidating the underlying molecular mechanisms responsible for tPA-mediated microglial activation (Pineda et al., 2012). Haile and associates (2011) proved that tPA serves as a neuroprotectant, devoid of neurotoxic effects, in neurons exposed to lethal hypoxia and ischemia and found that tPA rendered the neurons robustly resistant to the harmful stimuli. The study revealed that both endogenous and recombinant tPA induces the expression of TNF-α and that the neuroprotective effect by tPA is mediated by a plasmin and NMDA-receptor interaction, which leads to increased expression of p21 and ultimately the development of hypoxic and ischemic tolerance (Haile et al., 2011). Wu and colleagues (2012) exposed neurons to hypoxic conditions and discovered, through liquid chromatography and tandem mass spectrometry, that the endogenous release of neuronal tPA or neuronal treatment with recombinant tPA promotes cell survival via activation of the mTOR pathway. The study also showed that neuronal tPA induces the uptake of glucose under ischemic conditions and that this response improves neurological outcome following the induction of

ischemic stroke due to the decrease in lesion volume (Wu et al., 2012). In a separate study, Wu and colleagues (2013) investigated the effect of tPA on excitotoxin-induced neuronal death, and found that either the genetic overexpression of neuronal tPA or neuronal treatment with recombinant tPA rendered neurons resistant to the harmful effects induced by the excitotoxicity. An ERK1/2-mediated activation of CREB was found to underlie the neuroprotective effects observed in this study (Wu et al., 2013). In light of these findings, An, Haile, Wu, Torre, and Yepes (2014) conducted a study that sought to determine the role of the interaction between neurons, astrocytes, and endothelial cells in the regulation of energy supply in times of neuronal risk. Results revealed that oxygen and glucose deprivation, a condition that induces metabolic stress, induced a rapid release of tPA from cerebral cortical neurons but not from astrocytes, and that the tPA-induced glucose uptake via AMPK activation in astrocytes and endothelial cells was followed by the synthesis and release of lactic acid by the astrocytes that ultimately contributed to the promotion of neuronal survival (An et al., 2014).

Evidence presented by the above studies make a compelling case for tPA as a positive mediator of neuronal viability. tPA exhibits the ability to initiate anti-apoptotic signalling cascades for the promotion of neuroprotection in cells subjected to harmful or toxic stimuli. Although tPA shows potent neuroprotective effects in neurons in a variety of neurotoxic circumstances including ischemic preconditioning, hypoxia, excitotoxin-induced cell death, and animal models of ischemia, further investigation of its underlying mechanisms is necessary for substantiating its role in neuronal viability. Additional research in this area will help to improve the current understanding of the function of tPA and its plasminogen system and this information could be utilized to identify novel therapies for slowing the pathogenesis of brain-related diseases and improving the treatment outcomes for individuals with neurological conditions such

as ischemia.

Research Objectives

The following are the objectives of the study: (1) to determine the effect of tPA on CNS neuronal survival by testing its effects on cortical neurons in primary neuronal cell culture; and (2) to determine the cell signalling mechanisms underlying the tPA-mediated effects on cortical neurons in primary neuronal cell culture. The results from this study will help to garner a better understanding of the mechanisms underlying tPA activity through investigating a new, untested paradigm of cell death. Insight into tPA's impact on CNS neurons will provide clarity surrounding the mechanisms mediating tPA-induced responses that can later be translated to the treatment of ailments in the brain for a better and more functional recovery following cerebral insults such as ischemic stroke.

Major Hypotheses

The following are the hypotheses of the study: (1) If tPA does promote cell survival, it is hypothesized that the tPA-treated cultures will have significantly greater numbers of neurons compared to the vehicle-treated cultures, *in vitro*; and (2) the tPA-induced responses will be due, at least in part, to the activation of the JAK/STAT and/or mTOR signalling pathways via an up-regulation of phospho-S6 ribosomal protein (p-S6) and/or phospho-signal transducer and activator of transcription 3 (p-STAT3).

Methods

Animals

Experiments were performed using C57BL/6 mice from Charles River Laboratories (Montreal, Quebec, Canada). Animal experimentation was performed according to the

guidelines of the Canadian Council on Animal Care and was carried out with the approval of the Carleton University Animal Care Committee.

Tissue isolation and primary neuronal cell culture

The cerebral cortices of C57BL/6 post-natal day 2 (P2) mice were dissected and the neuronal tissue was prepared for primary neuronal cell culture. Tissue was placed in Dulbecco's phosphate-buffered saline solution (DPBS) 1X (GIBCO, Invitrogen Laboratories) until the beginning of the isolation procedure. Tissue from n = 2 to 3 P2 mice were pooled into one sample for each culture experiment. One pooled biological sample was used to represent n = 1. This study had n = 6 pooled biological samples per treatment group. Tissue was placed in papain solution (Papain Dissociation System; Worthington Biochemical Inc., Columbus, OH) and incubated at 37°C with frequent agitation for approximately 45 minutes. The incubator used throughout the experiments was CO_2-controlled. The mixture was triturated and any pieces of undissociated tissue remaining after trituration were allowed to settle at the bottom of the tube. The cloudy cell suspension was removed and centrifuged at 300g for 5 minutes at room temperature. The supernatant was discarded and the cell pellet was immediately resuspended in DNase dilute albumin-inhibitor solution, containing 2.7 mL Earle's balanced salt solution (EBSS), 300 μL reconstituted albumin-ovomucoid inhibitor and 150 μL of DNase solution. A discontinuous density gradient was prepared by adding 5.0 mL of albumin-inhibitor solution to the resuspended DNase dilute albumin-inhibitor solution and then centrifuged at 70g for 6 minutes at room temperature. The supernatant was discarded and the pelleted cells were immediately resuspended in culture medium before plating. The culture medium used was neurobasal-A medium (Invitrogen), supplemented with 2% B-27 serum-free supplement

(Invitrogen), 0.3% L-glutamine (Invitrogen), and 0.5% penicillin streptomycin (Invitrogen). All components were warmed to 37°C in the incubator before use.

Plating

Prior to plating, cell counts were verified using a hemacytometer to ensure equal cell yields. Plates were coated with 20 μg/mL poly-D-lysine (PDL; Sigma-Aldrich) prepped 24 hours before the experiment at 37°C and 10 μg/mL laminin (Corning) substrate prepped 1 hour before experiment at 37°C before cells were added. All cells were distributed across wells in 24-well plates (Fisher Scientific) with each well containing a total of 500 μL solution and cells were fed 2 days after plating. The duration of time between first dissection of the cortices to time of first plating for each individual experiment was approximately 3 hours.

tPA time course and inhibitor treatments

For the time course of tPA administration experiments, 5 nM tPA (EMD Millipore) was administered to the experimental dissociated neuronal cells in medium at 0 minutes, 15 minutes, 30 minutes, 60 minutes, and 180 minutes after plating. The vehicle, double-distilled water (ddH_2O), was administered to the control dissociated neuronal cells in medium at time of plating. For the inhibitor experiments, cells were treated with either vehicle or 5 nM tPA. The inhibitors administered included 50 μM AG490 (Sigma-Aldrich), an inhibitor of JAK2 signalling; 10 μM U0126 (Promega), an inhibitor of MEK signalling; 100 nM rapamycin (Sigma-Aldrich), an inhibitor of mTOR signalling; and/or 1 μM KT5720 (Calbiochem), an inhibitor of PKA signalling. All inhibitors were diluted in culture medium at the time of plating. Cells were cultured for 3 DIV and then fixed with 4% paraformaldehyde (PFA) before undergoing immunocytochemical staining and analysis.

Immunocytochemistry

Cortical neuronal samples were incubated with a primary mouse or rabbit antibody containing 1:1000 concentration of beta-III-Tubulin (β-Tubulin; Sigma-Aldrich). β-Tubulin is a neuron-specific marker that is widely utilized within the field of developmental neurobiology that identifies the microtubules found solely in neurons (Katsetos, Legido, Perentes, & Mörk, 2003). The antibody was diluted in a solution of 0.01 M phosphate-buffered saline (PBS) containing 0.5% Triton X-100. Cells were washed 3 times with 0.01 M PBS before the primary antibody was applied. After primary incubation overnight in a dark 4°C refrigerator, cells were washed 3 times with 0.01 M PBS and incubated with a secondary antibody containing a 1:200 concentration of Alexa 488 donkey anti-rabbit IgG or Alexa 555 anti-mouse IgG in a dark room at room temperature. After secondary antibody incubation for 1 hour, cells were washed 3 times with 0.01 M PBS and stained with 4'6-diamidino-2-phenylindole (DAPI) in 0.01 M PBS containing 0.5% Triton X-100 for an incubation period of 10 minutes in a dark room at room temperature. DAPI is a fluorescent stain used to mark nuclear DNA in both living and fixed cells (Tarnowski, Spinale, & Nicholson, 1991). Cells were washed once with 0.01 M PBS and each well was left incubated in 500 μL 0.01 M PBS for microscopic analysis. Cells were visualized using a Zeiss Axiovert inverted microscope equipped with a camera prior to analysis with INFINITY Analyze, Release 5.0, Revision 5.0.3 (Lumenera Corporation) imaging software.

Cell quantification

Six frames were taken randomly across each well using a Zeiss Axiovert inverted microscope fitted with a X 10 objective. The number of cells in each frame was counted manually. For each frame, two images were taken (one for each fluorescence channel): one for the β-Tubulin staining and the other for the DAPI staining. Cells were only considered neuronal

and included in the quantification and final analysis if there was precise colocalization of the β-Tubulin and DAPI fluorescent staining for each individual cell in each individual frame.

Statistical analysis

Statistical analyses were conducted using SPSS Statistical Package Version 22.0 (IBM Corporation). Statistical analysis was carried out using one-way analysis of variance (ANOVA) for the time course of tPA administration and inhibitor experiments. Significant one-way ANOVA results were followed by pair-wise Bonferroni post-hoc tests. A p-value $\leq .05$ was considered statistically significant. All data are presented as mean percentage ± the standard error of the mean (SEM).

Results

tPA increases postnatal cortical neuron survival *in vitro*

To assess the effect of tPA administration on cell survival, primary cortical neurons were isolated from early postnatal mice and treated with 5 nM tPA at different time points (0, 15, 30, 60, and 180 minutes) and compared to a vehicle-treated group. In comparison to the vehicle-treated group, the tPA-treated groups, regardless of the time point at which the tPA was administered, showed a marked increase in cell survival (Figure 3, $F(5, 30) = 4.1$, $p < .01$). A high magnification image of a postnatal cortical neuron is provided in Figure 4. Specifically, post hoc comparisons revealed that cell survival in the vehicle-treated group significantly differed from that of the tPA-treated groups at the 0 ($p < .05$), 30 ($p < .05$), 60 ($p < .01$), and 180 ($p < .05$) minute time points (Figure 5). The 15 minute tPA-treated time point group did not significantly differ from that of the vehicle-treated group; however, cell survival in the tPA-treated group at this time point showed a marked increase compared to the vehicle-treated group. These findings indicate that treatment of 5 nM tPA to postnatal primary cortical neurons

significantly increases their propensity for cell survival within a time window of up to 3 hours.

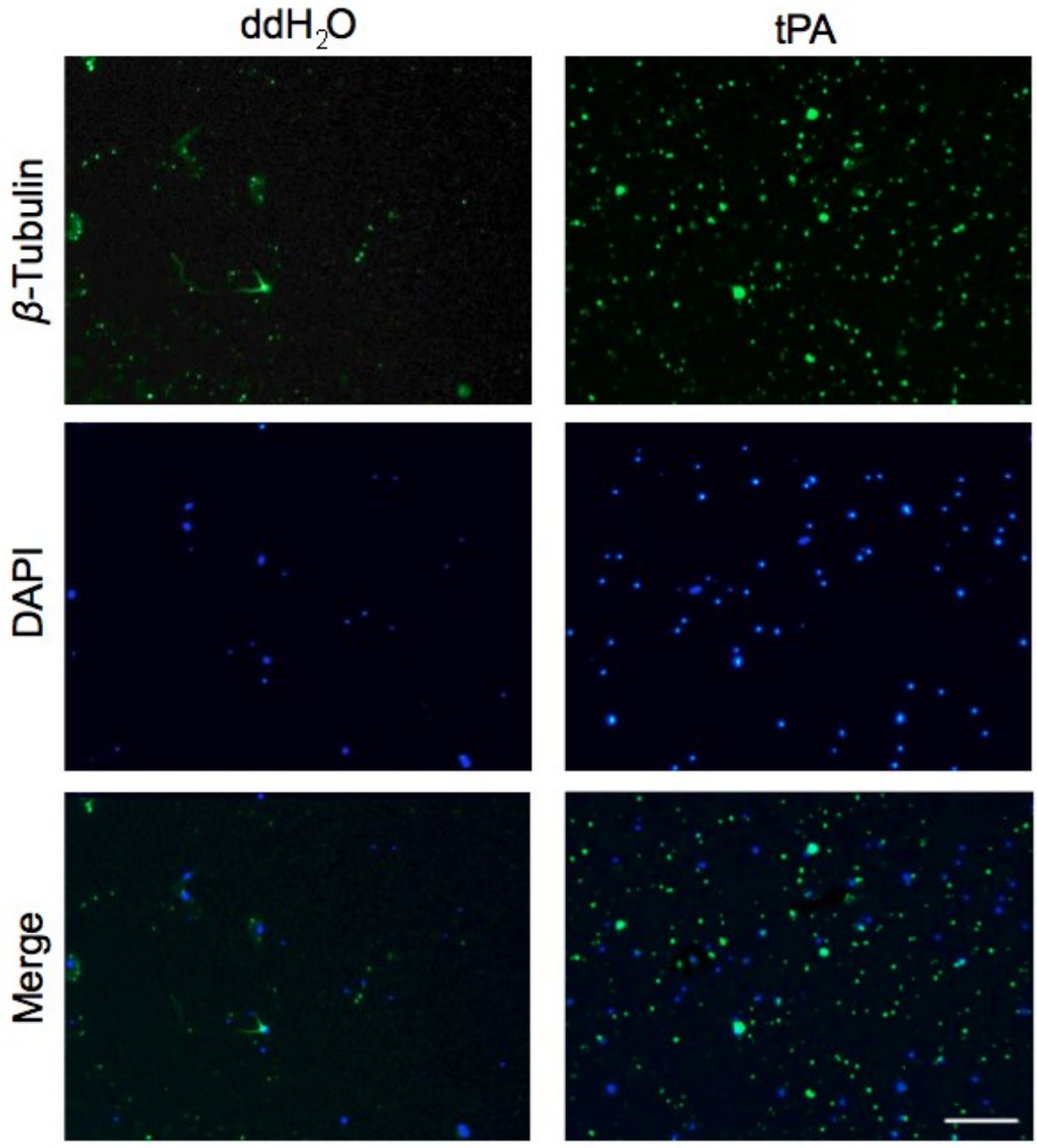

Figure 3. Protective effect of tPA on postnatal cortical neuron viability. Representative fluorescence micrographs at 0 minute time latency illustrating the expression of *β*-Tubulin (green), DAPI (blue) and merge of *β*-Tubulin and DAPI (green + blue) in postnatal primary cortical neurons following treatment with ddH2O (vehicle) or 5 nM tPA. Magnification 10X, scale bar 100 μm.

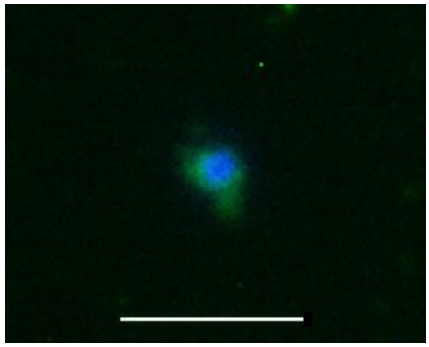

Figure 4. Representative micrograph showing *β*-Tubulin staining and colocalization of DAPI in an immunostained postnatal cortical neuron. Colocalization of positively stained *β*-Tubulin (green) and DAPI (blue), with the DAPI centralized in the nucleus and the *β*-Tubulin localized to the microtubules. Only cells that showed clear colocalization of *β*-Tubulin and DAPI were quantified as surviving cells in all experiments. Magnification 40X, scale bar 25 μm.

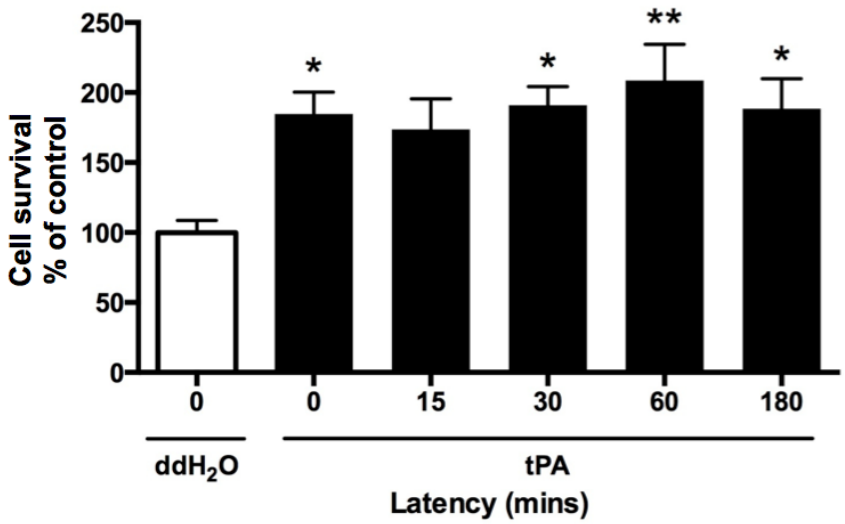

Figure 5. tPA promotes postnatal cortical neuron survival within a 3-hour time interval. Percent cell survival (positively-stained *β*-Tubulin neurons) of neuronal cells treated with tPA during time course experiment. Data are presented as the mean percentage of the control ± SEM. n=6 pooled biological samples per group. *$p < 0.05$; **$p < 0.01$ denotes a statistically significant Bonferroni post-hoc between the ddH_2O-treated control group and the tPA-treated groups.

tPA promotes postnatal cortical neuron survival via mTOR and JAK/STAT pathway activation

Due to the observed beneficial effect of tPA on postnatal primary cortical neuron survival, inhibitor experiments were carried out in order to elucidate the relevant mechanisms underlying the tPA-induced neuroprotection. The established signalling pathways, mTOR, MEK, JAK/STAT, and PKA, were targeted to assess whether the tPA-mediated effects on cell survival were dependent upon these cascades. Accordingly, postnatal primary cortical neuron cultures were treated with known inhibitors of these pathways, including rapamycin, U0126, AG490, and KT5720, respectively.

The vehicle-treated postnatal primary cortical neuron cultures did not significantly differ between the control, vehicle-treated, 10% dimethyl sulfoxide (DMSO)-treated, or rapamycin-treated groups (Figure 6A). However, a significant difference in cell survival was observed between the vehicle-treated group and the tPA-treated groups (Figure 6B, $F(3, 20) = 12.1$, $p < .001$). The tPA-treated rapamycin-treated group showed a marked decrease in cell survival compared to the tPA-treated vehicle-treated group ($p < .01$) and the tPA-treated 10% DMSO-treated group ($p < .01$). Specifically, cell survival within the tPA-treated, rapamycin-treated group diminished to levels similar to that of the vehicle-treated vehicle group, as the two groups did not significantly differ from each other (Figure 6B). These data suggest that tPA protects postnatal cortical neurons from cell death through an mTOR dependent mechanism because the neuroprotective effect of tPA is diminished to vehicle-treated levels in the presence of rapamycin for the tPA-treated group, and also because this decrease in neuroprotection is not seen within cultures treated solely with vehicle.

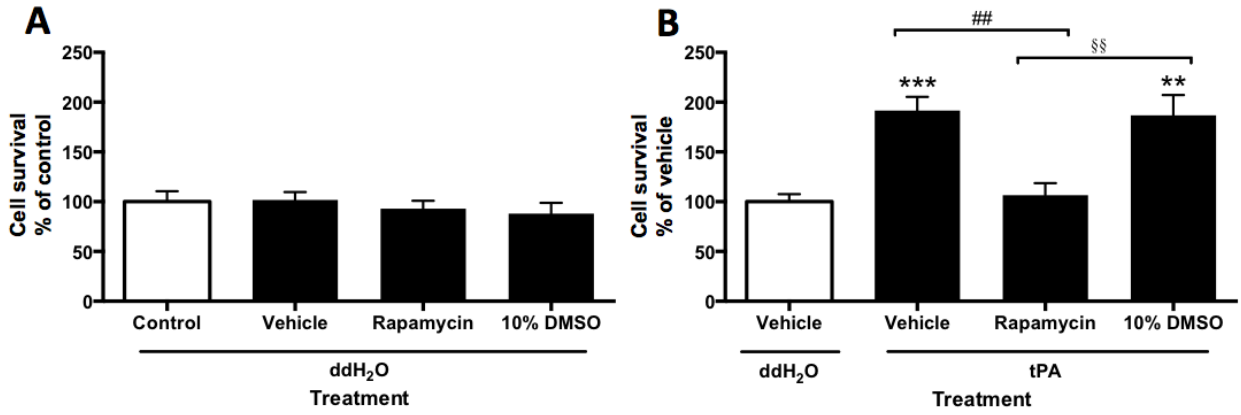

Figure 6. tPA promotes survival of postnatal cortical neurons via activation of the mTOR pathway. Graphical representation of positively-stained *β*-Tubulin cultured primary cortical neurons following treatment with ddH2O (vehicle) or 5 nM tPA, and ddH_2O, 10% DMSO, or 100 nM rapamycin. Data are presented as the mean ± SEM percentage of the control. n=6 pooled biological samples per group. **p* < .05; ***p* < .01 denotes a statistically significant Bonferroni post-hoc between the ddH_2O-treated vehicle and tPA-treated groups. #*p* < .05; ##*p* < .01 denotes a statistically significant difference between the tPA-treated vehicle and tPA-treated inhibitor group. §*p* < .05; §§*p* < .01 denotes a statistically significant Bonferroni post-hoc between the tPA-treated 10% DMSO and the tPA-treated inhibitor group.

To investigate whether tPA promoted its beneficial effects on cell survival via the MEK pathway, postnatal primary cortical neuron cultures were treated with U0126. The vehicle-treated postnatal primary cortical neuron cultures did not significantly differ between the control, vehicle-treated, 10% DMSO-treated, or U0126-treated groups (Figure 7A). A significant difference in cell survival was observed between the vehicle-treated group and the tPA-treated groups (Figure 7B, $F(3, 20) = 9.7$, $p < .001$). The tPA-treated group did not show a discernable decrease in cell survival when treated with U0126 when compared to the tPA-treated vehicle-treated group or the tPA-treated 10% DMSO-treated group. Cell survival within the tPA-treated U0126-treated group remained at a higher level than the vehicle-treated vehicle group; however, this effect was only marginal (Figure 7B, $p = .072$). Results from these data suggest that tPA-induced neuroprotection does not significantly rely on a MEK-dependent mechanism, as treatment with U0126 did not significantly reduce cell survival within the tPA-treated group.

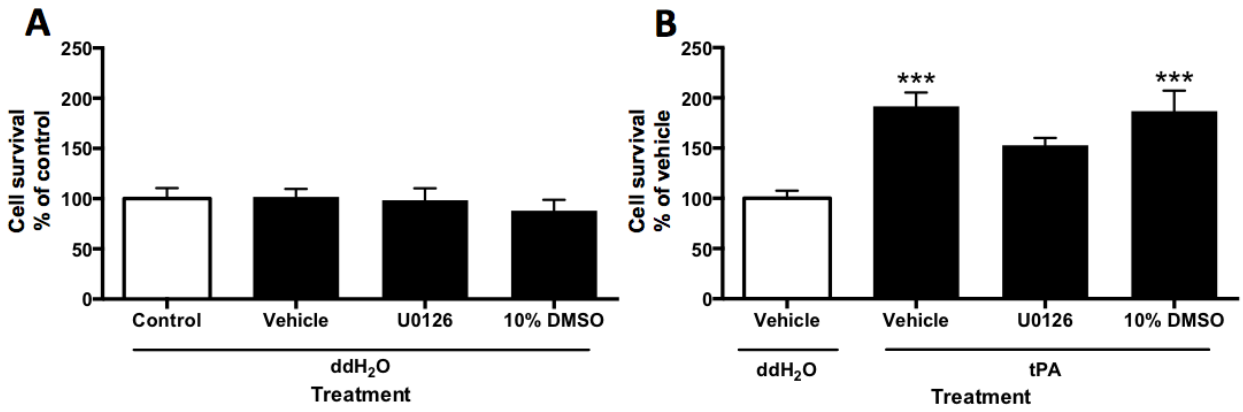

Figure 7. tPA does not significantly rely on the Raf/MEK/ERK pathway to promote postnatal cortical neuron survival. Graphical representation of positively-stained *β*-Tubulin cultured primary cortical neurons following treatment with ddH2O (vehicle) or 5 nM tPA, and ddH2O, 10% DMSO, or 10 μM U0126. Data are presented as the mean ± SEM percentage of the control. n=6 pooled biological samples per group. **p* < .05; ***p* < .01 denotes a statistically significant Bonferroni post-hoc between the ddH2O-treated vehicle and tPA-treated groups.

To determine if tPA-induced promotion of cell survival was dependent on the JAK/STAT pathway, AG490 was administered to cultures. The vehicle-treated postnatal primary cortical neuron cultures did not significantly differ between the control, vehicle-treated, 10% DMSO-treated, or AG490-treated groups (Figure 8A). However, a significant difference in cell survival was observed between the vehicle-treated group and the tPA-treated groups (Figure 8B, $F(3, 20) = 12.2$, $p < .001$). The tPA-treated AG490-treated group showed a marked decrease in cell survival compared to the tPA-treated vehicle-treated group ($p < .01$) and the tPA-treated 10% DMSO-treated group ($p < .01$). Similar to the rapamycin results, cell survival within the tPA-treated AG490-treated group diminished to levels similar to the vehicle-treated vehicle group, as the two groups did not significantly differ from each other (Figure 8B). These data evidence the involvement of the JAK/STAT pathway in tPA-mediated cell survival because the neuroprotective effect seen due to tPA is diminished to vehicle-treated levels in the presence of AG490 for the tPA-treated group, and also because this decrease in cell survival is only seen within cultures that were treated with tPA.

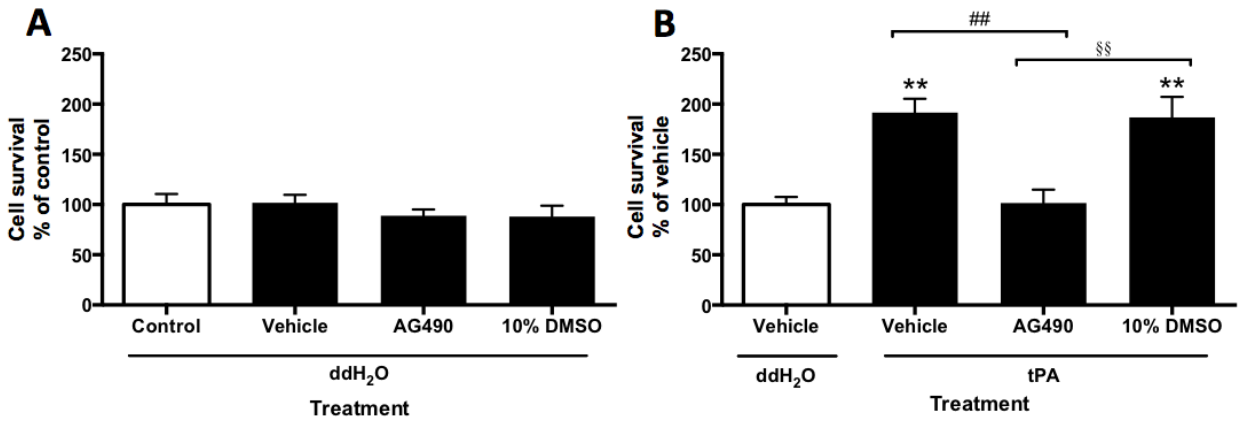

Figure 8. tPA promotes survival of postnatal cortical neurons via activation of the JAK/STAT pathway. Graphical representation of positively-stained *β*-Tubulin cultured primary cortical neurons following treatment with ddH2O (vehicle) or 5 nM tPA, and ddH2O, 10% DMSO, or 50 μM AG490. Data are presented as the mean ± SEM percentage of the control. n=6 pooled biological samples per group. **p* < .05; ***p* < .01 denotes a statistically significant Bonferroni post-hoc between the ddH2O-treated vehicle and tPA-treated groups. #*p* < .05; ##*p* < .01 denotes a statistically significant difference between the tPA-treated vehicle and tPA-treated inhibitor group. §*p* < .05; §§*p* < .01 denotes a statistically significant Bonferroni post-hoc between the tPA-treated 10% DMSO and the tPA-treated inhibitor group.

Lastly, for the vehicle-treated postnatal primary cortical neuron cultures, the control, vehicle-treated, 10% DMSO-treated, and KT5720-treated groups did not significantly differ from one another (Figure 9A). A significant difference in cell survival was observed between the vehicle-treated group and the tPA-treated groups (Figure 9B, $F(3, 20) = 10.8$, $p < .001$). The tPA-treated KT5720-treated neuronal cultures did not significantly differ from the tPA-treated vehicle-treated group (Figure 9B) or the tPA-treated 10% DMSO-treated group, but cell survival within the tPA-treated KT5720-treated group significantly differed from the vehicle-treated vehicle group (Figure 9B, $p \leq .001$). These results indicate that the PKA pathway does not significantly contribute to the tPA-induced neuroprotective effect seen in postnatal primary cortical neurons.

Combinations of inhibitors were employed to elucidate whether interactions between the signalling pathways produced synergistic effects and may have contributed to the heightened

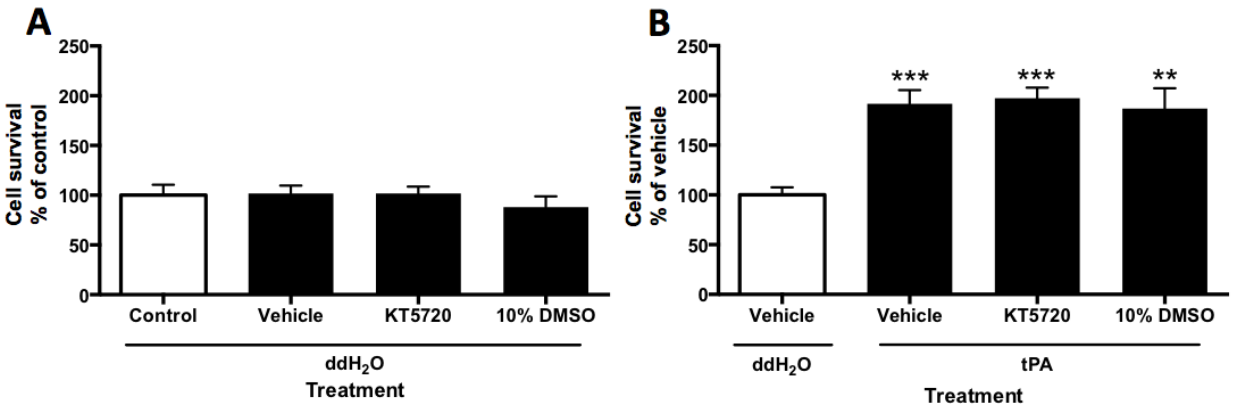

Figure 9. tPA does not significantly rely on the cAMP/PKA/CREB pathway to promote postnatal cortical neuron survival. Graphical representation of positively-stained *β*-Tubulin cultured primary cortical neurons following treatment with ddH2O (vehicle) or 5 nM tPA, and ddH2O, 10% DMSO, or 1 μM KT5720. Data are presented as the mean ± SEM percentage of the control. n=6 pooled biological samples per group. *$p < .05$; **$p < .01$ denotes a statistically significant Bonferroni post-hoc between the ddH2O-treated vehicle and tPA-treated groups.

neuroprotection observed from tPA. None of the vehicle-treated postnatal primary cortical neuron cultures that were also treated with any of the combinations of inhibitors significantly differed from one another (Table 1). In regards to the tPA-treated postnatal primary cortical neuron cultures, results indicate that all combinations of inhibitors had a significant negative effect on cell survival in comparison to both the tPA-treated vehicle- and 10% DMSO-treated groups (Table 1, $p \leq .05$), except for the U0126+KT5720 and Rapamycin+KT5720 groups ($p > .05$). Of note amongst the tPA-treated cultures, the rapamycin+AG490 combination treatment decreased cell survival to levels lower than the vehicle-treated vehicle group, and so did the rapamycin+AG490+U0126 and rapamycin+AG490+U0126+KT5720 treatments, albeit only slightly and non-significantly ($p > .05$). Treatment with rapamycin or AG490 to tPA-treated cultures were both found to be individually capable of diminishing cell survival to levels similar to that of the vehicle-treated groups, as described above; however, when combined together to provide a double inhibitor treatment, minimal further decrease in cell survival was observed

Table 1. Mean raw cell counts per well and percentage of control cell survival levels for the tPA- or ddH2O-treated groups treated with one or more inhibitor solutions.

Treatment	Raw Cell Counts Mean ± SEM	Cell Survival % of Control/Vehicle ± SEM
ddH_2O		
Control	189.6 ± 20.1	100.0 ± 10.6
Vehicle	188.9 ± 15.3	101.8 ± 7.8
10% DMSO	160.7 ± 19.9	87.9 ± 10.9
Rapamycin	171.7 ± 13.7	92.9 ± 8.0
U0126	178.6 ± 15.1	98.5 ± 11.7
AG490	163.1 ± 8.2	88.8 ± 6.4
KT5720	186.5 ± 6.1	101.9 ± 6.8
Rapamycin + U0126	170.5 ± 11.9	94.3 ± 10.3
Rapamycin + AG490	142.9 ± 15.6	81.5 ± 13.8
AG490 + U0126	154.8 ± 11.8	83.9 ± 7.1
Rapamycin + KT5720	175.7 ± 9.3	97.1 ± 10.5
AG490 + KT5720	183.2 ± 4.8	100.3 ± 7.0
U0126 + KT5720	181.2 ± 4.5	100.7 ± 10.4
Rapamycin + AG490 + U0126	142.1 ± 15.6	78.2 ± 9.9
Rapamycin + AG490 + U0126 + KT5720	172.3 ± 7.5	95.5 ± 9.6
ddH_2O		
Vehicle	188.9 ± 15.3	100.0 ± 7.7
tPA		
Vehicle	354.9 ± 25.5	191.5 ± 13.9[a]
10% DMSO	338.3 ± 16.2	186.7 ± 20.6[a]
Rapamycin	194.4 ± 15.1	106.6 ± 11.9[b,c]
U0126	284.1 ± 16.2	152.8 ± 7.4
AG490	182.7 ± 9.1	101.5 ± 13.3[b,c]
KT5720	364.6 ± 14.3	197.1 ± 10.6[a]
Rapamycin + U0126	209.3 ± 4.8	114.8 ± 10.6[b,c]
Rapamycin + AG490	180.5 ± 13.8	98.7 ± 11.0[b,c]
AG490 + U0126	184.8 ± 15.7	101.3 ± 12.7[b,c]
Rapamycin + KT5720	226.0 ± 10.6	122.9 ± 9.4[b]
AG490 + KT5720	206.2 ± 9.5	115.1 ± 15.8[b,c]
U0126 + KT5720	305.6 ± 19.0	170.2 ± 24.4[a]
Rapamycin + AG490 + U0126	147.1 ± 21.6	78.9 ± 10.2[b,c]
Rapamycin + AG490 + U0126 + KT5720	185.2 ± 10.2	99.8 ± 5.6[b,c]

[a] denotes a statistically significant difference between the standardized ddH2O-treated vehicle and the tPA-treated groups as determined by a Bonferroni post-hoc test, $p \leq .05$.

[b] denotes a statistically significant difference between the tPA-treated vehicle and the tPA-treated inhibitor group as determined by a Bonferroni post-hoc test, $p \leq .05$.

[c] denotes a statistically significant difference between the tPA-treated 10% DMSO and tPA-treated inhibitor groups as determined by a Bonferroni post-hoc test, $p \leq .05$.

(Table 1, $p > .05$). This data suggests that both JAK/STAT and mTOR signalling can facilitate tPA-mediated neuroprotection but the two signalling pathways do not significantly synergize to produce a positive impact on cell survival. Hence, this data shows that the tPA-induced beneficial effect on cell survival can be slightly enhanced via interaction of the JAK/STAT and mTOR pathways; however, activation of either the mTOR or JAK/STAT pathways, individually, can produce nearly the same effect. The only significant differences noted between the cell survival levels of the tPA-treated individual, specific inhibitor treatment groups in comparison to the tPA-treated combination inhibitor treatment groups included the following: U0126 showed significantly greater cell survival compared to the Rapamycin+AG490+U0126 group ($p \leq .05$); and KT5720 showed significantly greater cell survival compared to the KT5720+Rapamycin, KT+AG490, and Rapamycin+AG490+U0126+KT5720 groups ($p \leq .05$). Additionally, it was observed that when cultures treated with tPA and with an inhibitor in addition to KT5720, cell survival was greater than when treated individually with an inhibitor (i.e. without KT5720), although not statistically significant. This suggests that KT5720 may be altering the energy state of the neurons and thus allowing them to survive better when under the influence of KT5720.

As a whole, the results from the inhibitor experiments suggest that tPA-induced neuroprotection does not significantly rely on MEK- or PKA-dependent mechanisms but rather acts on the mTOR and JAK/STAT signalling pathways to promote cell survival.

tPA-induced signalling mechanisms at 3 DIV show marked activation of p-S6 expression

Since the data obtained with the inhibitor experiments suggested that the tPA-mediated effects on cell survival could be due to an mTOR- and/or JAK/STAT-dependent mechanism, further experiments were conducted to verify these results. Postnatal primary cortical neuron cultures were double stained with β-Tubulin, and p-S6, a protein involved in mTOR signalling,

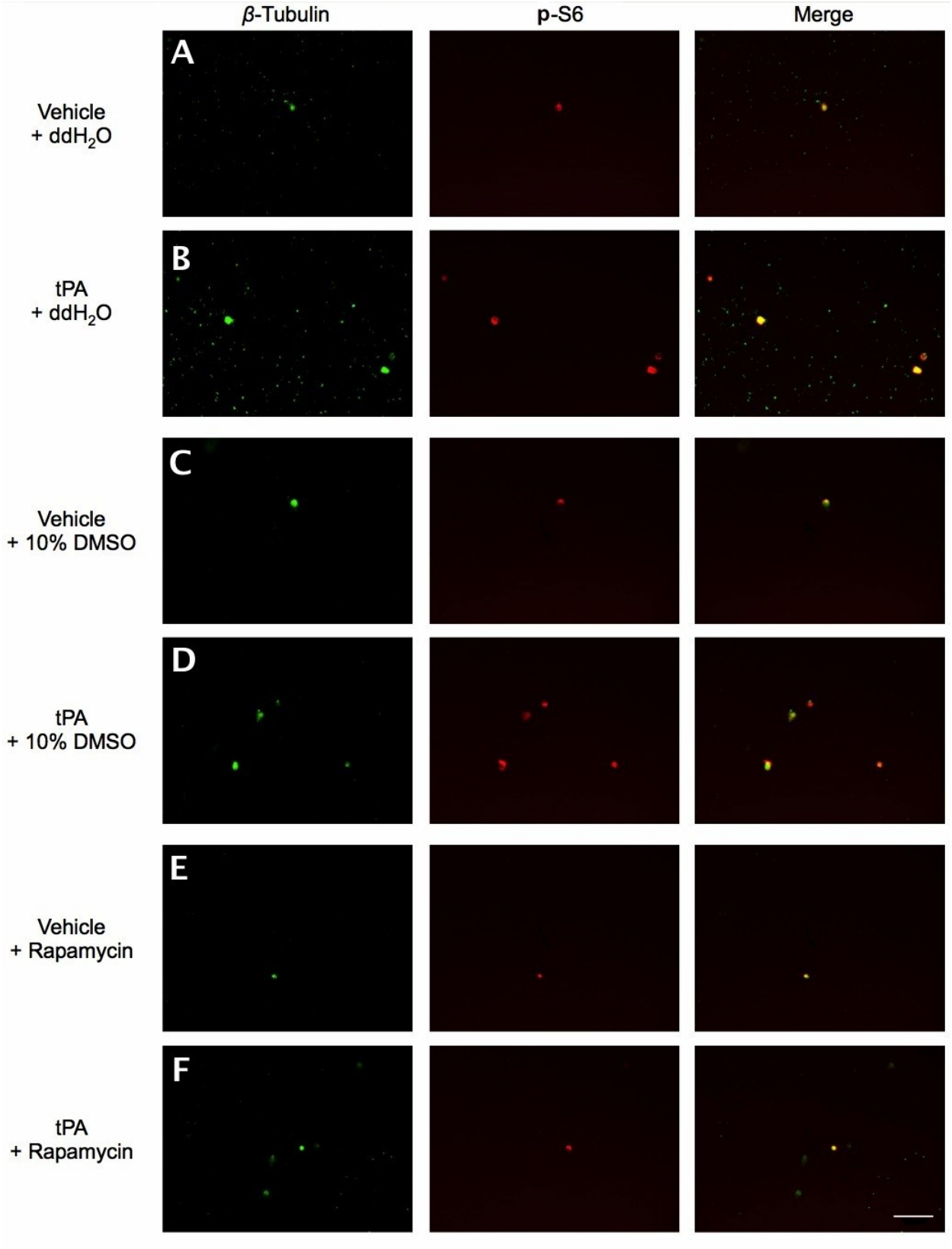

Figure 10. Increased expression of p-S6 in postnatal cortical neurons following treatment

with 5 nM tPA. Representative fluorescence micrographs illustrating the expression of *β*-Tubulin (green), p-S6 (red), and merge of *β*-Tubulin and p-S6 (green + red) in cultured primary cortical neurons following treatment with vehicle (ddH_2O) or 5 nM tPA, and ddH_2O or 10% DMSO or 100 nM rapamycin. Magnification 20X, scale bar 100 μm.

or p-STAT3, a protein involved in JAK/STAT signalling, in order to determine whether expression of either of these proteins was increased following treatment with tPA. Results indicated very minimal p-S6 expression in the vehicle-treated vehicle group compared to the tPA-treated vehicle group, which showed a marked increase in p-S6 expression; representative images are shown in Figures 10A and 10B, respectively. The p-S6 expression in the tPA-treated 10% DMSO group was also compared to that of the vehicle-treated 10% DMSO group and remarkably less p-S6 expression was detected in the vehicle-treated group (Figure 10C) than in the tPA-treated group (Figure 10D). In order to confirm that the expression of p-S6 was mTOR-dependent, the p-S6 expression in the tPA-treated rapamycin group was compared to that of the vehicle-treated rapamycin group. The rapamycin treatment did not considerably affect p-S6 expression within the vehicle-treated group (Figure 10E). However, the rapamycin treatment markedly diminished the tPA-mediated expression of p-S6 (Figure 10F), as the p-S6 expression within the tPA-treated rapamycin group fell to levels similar to that of the vehicle-treated groups. Of note, all p-S6 expression appeared to be in cells also co-labeled with *β*-Tubulin, indicating that the p-S6 expression was neuron-specific. These results suggest that the mTOR pathway plays a significant role in the tPA-mediated beneficial effects on cell survival. While the data revealed that p-S6 expression was remarkably increased in the presence of tPA, no major difference in p-STAT3 expression levels was detected between the vehicle- and tPA-treated groups (data not shown). Because the p-STAT3 staining did not show significant differences between the tPA- and vehicle-treated groups, and because its expression was so minimal, it can be suggested that the involvement of the mTOR pathway may be the most

necessary for producing the tPA-mediated beneficial effect on cell survival in postnatal primary cortical neurons. However, at the same time, both the mTOR and JAK/STAT pathways could be converging and acting on p-S6 as the main downstream effector, highlighting the importance of the involvement of both pathways, reciprocally, in order to produce the neuroprotective effect.

Discussion

tPA is a thrombolytic agent used in the treatment of ischemic stroke but is limited in its clinical efficacy due to its short therapeutic window. Given the widespread use of tPA within the clinical setting and current dearth of research focusing on the impact of this molecule on the brain parenchyma, including its underlying mechanisms of action, this study sought to determine not only the effects of administration of tPA to postnatal cortical neuron viability but also the underlying tPA-mediated effects on postnatal cortical neurons, in culture. Statistical analyses revealed a significant neuroprotective effect of tPA in postnatal cortical neurons *in vitro*. The tPA-induced neuroprotection was found to rely most significantly upon the mTOR and JAK/STAT signalling pathways, and less so on the PKA and MEK signalling pathways. While both the rapamycin and AG490 inhibitors were independently capable of decreasing cell survival levels to amounts similar to those in the vehicle treated vehicle-group, solely the molecular target of the mTOR pathway, p-S6, was able to be detected in cultures at 3 DIV, substantiating the vital role of the mTOR pathway in providing a neuroprotective effect when under the influence of tPA. While the JAK/STAT pathway was also found to significantly underlie the neuroprotective effect seen due to tPA, no discernable differences between the vehicle- and tPA-treated groups could be detected when cultures were stained with p-STAT3, suggesting that the JAK/STAT pathway may be imparting its beneficial effects solely early in the pro-survival signalling cascade, and/or converging onto a pathway that results in an eventual phosphorylation of p-S6.

tPA-induced cell survival

Recent literature has suggested that tPA can provide a protective effect to neurons when they are undergoing various forms of cellular stress, including excitotoxin-induced neuronal death, serum deprivation-induced apoptosis, oxygen-glucose deprivation (OGD), and sublethal and lethal hypoxia (An et al., 2014; Echeverry et al., 2010; Haile et al., 2011; Liot et al., 2006; Wu et al., 2012, Wu et al., 2013). However, a dearth of research evidence exists on the effect of tPA on postnatal neuron viability and its underlying mechanism of neuroprotection. In this regard, this study aimed to investigate the effects of tPA administration on postnatal primary cortical neuron viability, in culture, using a series of time course experiments. The data revealed that a specific time course of tPA administration, up to 3 hours after treatment, resulted in significantly improved cell survival effects. Specifically, the 0, 30, 60, and 180 minute time latency periods that received tPA treatment showed the most dramatic positive effects on cell survival. The 0 minute latency period represented the point at which tPA treatment was administered immediately to the cells. The data from the current study suggests that tPA, like a variety of other pharmacological agents including NGF and BDNF (Nguyen, Lee, Lee, Lee, & Ahn, 2009), could be increasing anti-apoptotic cell signalling cascades and/or suppressing pro-apoptotic signalling mechanisms in order to promote its beneficial effect on cell survival. Findings from the time course of tPA administration experiments shed light on the process of cell death in postnatal primary cortical neurons and reveal how tPA initiates cell signalling mechanisms which could be effective in preventing, or at least delaying, the initiation of a cell death cascade in neurons.

Results from these experiments support previous studies that have investigated the effects of tPA administration in animal models of cerebral ischemia. For example, Wu and

colleagues (2012) found that after embryonic cerebral cortical neurons were exposed to 55 minutes of OGD, the cells treated with tPA showed improved survival up to and including six hours following OGD, and, more specifically, tPA was shown to consistently keep cell survival rates at levels higher than those of the tPA-unexposed groups (Wu et al., 2012). In a separate study, Wu and colleagues (2013) also found that neuronal tPA had a protective effect against excitotoxin-induced cell death within three hours of administration, again using an embryonic tissue culture model. In addition, the study investigated whether treatment with tPA after exposure to an excitotoxic injury, induced by incubation with NMDA, had a protective effect in neurons. It was found that cell survival decreased by about 50 percent in the control group when neurons were exposed to NMDA without subsequent treatment with tPA, while the neurons treated with tPA up to 3 hours following exposure to NMDA showed increased survival compared to the control group across all time points, with reductions in cell survival levels of only approximately 20 to 30 percent depending on the time point (Wu et al., 2013). However, embryonic neurons have been known to respond differently to injury/insult (see Goldberg & Barres, 2000; Moore et al., 2009; Park et al., 2008; Rossi, Gianola, & Corvetti, 2007) thereby justifying the need to assess the impact of tPA on postnatal cortical neurons. Therefore, results presented by the current study support and extend previous findings in the literature that support a role for tPA as a positive mediator of neuronal viability and further exemplify the ability of tPA to protect neurons from undergoing programmed cell death through a time-dependent mechanism, particularly in postnatal neurons, which are known to be more susceptible to damage.

The natural cell death processes *in vitro* following the dissociation of postnatal neurons is a major caveat that generally precludes their analysis (Beaudoin III et al., 2012). However, given

that postnatal neurons behave drastically different than embryonic neurons (Goldberg & Barres, 2000; Goldberg, Klassen, Hua, & Barres, 2002; Shewan, Berry, & Cohen, 1995), it is imperative to assess the cell survival responses in these neurons empirically. The paradigm of cell death that has been investigated in the current study is novel in that the conditions by which neurons were exposed were not intentionally manipulated to induce cell death. In other words, this study captured a rudimentary form of cell death in that the type of apoptosis occurring within cultures was undergoing naturally and not induced by direct experimental manipulation of environmental conditions (i.e. excitotoxin-induced neuronal death, Wu et al., 2013; serum deprivation-induced apoptosis, Liot et al., 2006) or nutrient deficiencies (ie. OGD, An et al., 2014; sublethal hypoxia, Echeverry et al., 2010; lethal hypoxia, Haile et al., 2011; Wu et al., 2012). This is important because the results obtained by this study illustrate the natural sequence of cell death that exists biologically within neurons and thus may have more closely related implications for recovering from brain insults such as ischemic stroke, as well as naturally occurring maturational processes such as aging.

Another aspect of the current study that is novel is that it illustrates the effects of tPA on postnatal cortical neurons, a developmental time point that previous studies have not precisely investigated. Postnatal neurons differ from embryonic neurons in that neurons from later developmental time points (i.e. postnatal neurons) are not able to survive as well in culture as neurons from earlier time points (i.e. embryonic neurons) (Beaudoin III et al., 2012; Chen, Schneider, Martinou, & Tonegawa, 1997; Goldberg et al., 2002; Kole, Annis, & Deshmukh, 2013). For example, Goldberg and colleagues (2002) cultured retinal ganglion cell (RGC)s from rats of different ages and found a stark difference in the intrinsic axon growth ability of the RGCs depending on the age of the animal. Specifically, Goldberg and colleagues (2002)

presented an axon growth curve and revealed that the embryonic stages of development, especially E18 to E21, were the most prone to intrinsic growth ability, while the postnatal stages of development, especially P2 onwards, were very limited in their growth abilities, having about a 90 percent drop in measured axon length by age P2 compared to age E21. Similar results were documented in a different study that illustrated a marked decrease in the number of growing axons at age E18 or older in wild-type animals (Chen et al., 1997). Specifically, Chen and associates (1997) found that the active state of regeneration is highest during the embryonic stages, as evidenced by the vastly extended and numerous neurites seen in culture during this time, which is followed by a downward trend through the postnatal ages, and then decreases significantly following P4, a time point at which the up-regulation of intrinsic growth factors, including Bcl-2, likely stops and regeneration has been shown to become significantly halted (Chen et al., 1997). The current study investigated the protective effects of tPA on postnatal cortical neuron survival by culturing neurons at age P2, and then fixing the neurons at age P5 in culture. Because of the postnatal age under study, it can be proposed that during the experiments the neurons in culture were exhibiting decreases in their capacity for survival due to this documented developmental decrease in survival ability and decrease in pro-survival signalling cascades. In addition to the age of the neurons, cell survival could have been reduced by the cell culture conditions themselves, as culture systems are inherently unable to effectively support cell survival for prolonged periods of time by nature. However, in terms of the cell culture procedure itself, the current study used the Papain Dissociation System to dissociate cells, a method that has been proven to be far more efficient and less destructive to cells than other protease-based systems for cell isolation. In support of this, Kaiser and colleagues (2013) found a higher yield of well distributed neurons, higher branching prevalence, more organized neuronal mesh, and

less debris in cultures that were digested by papain than those that were digested by trypsin. In this regard, because the current study utilized papain for its dissociation procedure, it is likely that less negative effects on cells (i.e. activation of cell death processes) were present compared to if another dissociation system was utilized, like trypsin for example, and this likely ultimately helped to preserve cell viability to a greater capacity in culture. In the current study, results from the inhibitor experiments showed that cell survival levels in the cultures that were treated with vehicle did not significantly decline when incubated with any inhibitor or combination of inhibitors, illustrating that the cells in culture were already undergoing individualized death programs and exhibiting minimal growth. Therefore, results obtained from the study suggest that while the neurons in culture were biologically predisposed to undergo imminent cell death due to their maturational age and culture environment, tPA was able to provide a sufficient, yet temporally-regulated, neuroprotective factor to the cells to ultimately increase their propensity for survival.

Additionally, the rationale behind investigating postnatal cortical neuron survival rather than embryonic cortical neuron survival for this study is evident because neurons from this developmental stage are arguably more representative of neurons that may be exposed to brain pathologies, including acute ischemic attacks, which are more prevalent in postnatal developmental stages. This is important because it has been reported that older adults are more at risk for experiencing ischemia than young adults; nearly a 10-fold difference in stroke prevalence has been observed among adults aged 65 years or older compared to those aged 18 to 44 years (Centers for Disease Control and Prevention, 2012). Consequently, investigating the effects of tPA on neurons at developmental time points that are more mature is warranted. While it is not currently feasible to culture adult cortical neurons due to enhanced cell death

vulnerability, culturing postnatal neurons is indeed a step in the right direction to identify mechanisms that could be manipulated for improving the natural vulnerability to cell death within neurons that are undergoing aging.

tPA has been documented as having the ability to convert some neurotrophins into their active forms. In this regard, it has been shown to convert the precursor proBDNF into the its mature version BDNF for mediating late-phase long-term potentiation for long term memory (Pang et al., 2004), as well as convert proNGF into mature NGF to provide neurotrophic support in a variety of healthy and diseased states including cerebral ischemia (Bruno & Cuello, 2006). It is possible that tPA is promoting its beneficial effects on primary cortical neuronal survival via an upregulation of neurotrophins to provide an anti-apoptotic defense against pro-apoptotic signals. In order to determine the means by which tPA was found to be promoting its beneficial, protective effect on postnatal cortical neurons, a pharmacological approach was undertaken and specific inhibitor experiments were conducted. A discussion of the results from the inhibitor experiments and their implications for potentially expanding the therapeutic window of tPA for stroke recovery is provided below.

Mechanisms underlying tPA-induced neuroprotection

Both the mTOR and JAK/STAT cellular signalling pathways have been established as being important regulators of cell growth, survival, and proliferation within the CNS (Cattaneo, Conti, & De-Fraja, 1999; De-Fraja et al., 2000; Dziennis & Alkaved, 2008; Park et al., 2008; Smith et al., 2009; Sun et al., 2011). In this regard, the second major hypothesis of the current study was that the tPA-induced responses would be due, at least in part, to the activation of the JAK/STAT and/or mTOR signalling pathways via an up-regulation of p-S6 and/or p-STAT3. In order to determine not only whether both the mTOR and JAK/STAT pathways played an

underlying mechanistic role in producing the tPA-induced neuroprotective response, the MEK and PKA pathways were also targeted due to their demonstrated effects in promoting cell survival (Bonni et al., 1999; Ryu, Lee, Impey, Ratan, & Ferrante, 2005). Accordingly, cultures were treated individually with, or in combinations of, specific inhibitors of these pathways: AG490, an inhibitor of JAK2 signalling; and/or U0126, an inhibitor of MEK signalling; and/or rapamycin, an inhibitor of mTOR signalling; and/or KT5720, an inhibitor of PKA signalling. Results revealed clear evidence of the involvement of the mTOR and JAK/STAT pathways in the neuroprotective effect induced by tPA. The MEK pathway, and to a lesser extent, the PKA pathway, showed minimal involvement in the underlying signalling mechanics of the tPA-induced increase in cell survival. Interestingly, inhibiting the PKA pathway increased cell survival levels past those of non-inhibitor treated tPA-treated groups in some cases, albeit not significant statistically.

The mTOR pathway has been implicated in promoting cell growth, proliferation, and survival, and rapamycin consistently acts as a potent and extremely selective inhibitor of mTOR and its direct effectors by binding to a domain separate from its catalytic site to effectively block mTOR and the majority of its functions (Ballou & Lin, 2008). In the current study, when cultures treated with tPA were also treated with rapamycin, significant decreases in cell survival were observed compared to solely cultures treated with tPA. Specifically, cell survival within the tPA-treated cultures treated with rapamycin reduced to nearly vehicle-treated rapamycin cell survival levels. Results from these findings highlight the important role of the mTOR pathway for promoting cell survival when in the presence of tPA. The mTOR pathway itself has two well-established targets, one of them being ribosomal S6K1, which later phosphorylates p-S6 to regulate protein translation (Guertin, & Sabatini, 2007; Luo, Manning, & Cantley, 2003; Park et

al., 2008). In order to verify the involvement of the mTOR pathway in mediating tPA's effect on neurons, antibodies to p-S6 were utilized. Immunocytochemistry revealed a stark increase in the expression of p-S6 following treatment with tPA and minimal expression following treatment with vehicle or tPA with rapamycin. Results from the fluorescence staining substantiate the vital role of p-S6 in promoting neuron survival, specifically when under the influence of tPA.

Of particular importance, it has been established that strong p-S6 signals exist within most embryonic neurons but this signal becomes diminished by about 90 percent once neurons reach a developmentally mature age (Park et al., 2008). This indicates that within adult neurons, mTOR signalling is vastly down-regulated compared to younger neurons and only a small portion of the adult neurons retain any discernable amount of mTOR activity (Park et al., 2008). In this regard, it can be suggested that by increasing p-S6 expression within postnatal neurons undergoing a developmental decline due to age, the robustness of tPA's neuroprotective ability is quite powerful as experiments in the current study were conducted on neurons that were postnatal and thus more prone to imminent cell death. Additionally, results from the current study support findings from other studies that have shown that p-S6 is intrinsically expressed in neurons, as tPA appeared to have increased the expression of p-S6 in neurons specifically (Liu et al., 2010; Park et al., 2008; Smith et al., 2009).

The current study found that the mTOR pathway was independently capable of inducing the neuroprotective effect by tPA. Park and colleagues (2008) investigated the activity of the mTOR pathway in adult RGCs following optic nerve injury. Findings from the study revealed robustly suppressed mTOR activity, as well as p-S6 expression, following axotomy at a variety of time points, which, one could speculate, would ultimately suppress the initiation of protein synthesis and limit axon regeneration and cell survival (Park et al., 2008). The same study also

found that by reactivating the mTOR pathway via deletion of PTEN or Tuberous Sclerosis 1 (TSC1), negative regulators of the mTOR pathway, axon regeneration becomes robustly restored following optic nerve injury, indicating that the maintenance of active protein synthesis via activation of the mTOR cascade is sufficient for producing a cellular program in favour of axon regrowth and survival (Park et al., 2008). Additionally, it has been shown by other studies that mTOR signalling becomes suppressed within cells experiencing other stressors, like hypoxia, as an evolutionary means to conserve energy homeostasis and maintain survival (Brugarolas et al., 2004; Reiling & Hafen, 2004). It has also been noted that the up-regulation of Redd1/2, now a well documented repressor of mTORC1, during hypoxic stress is involved in the inhibition of mTOR (Corradetti, Inoki, & Guan, 2005; Gordon, Steiner, Lang, Jefferson, & Kimball, 2014; Park et al., 2008). As a whole, results garnered from the current study suggest a similar theory in that the mTOR pathway was shown to provide a protective response to harmful stimuli in neurons, and in this case was induced, most predominately, by the presence of tPA.

In addition to the involvement of the mTOR pathway in the tPA-mediated effect on cell survival, results obtained from the study showed that the JAK/STAT pathway could also play an important role in achieving the increased cell survival induced by tPA. When cultures were treated concurrently with tPA and AG490, a highly specific inhibitor of JAK2 (Bright, Du, & Sriram, 1999; Meydan et al., 1996), significant decreases in cell survival were observed compared to cultures treated only with tPA. Similar to the rapamycin findings, cell survival within the cultures treated concurrently with tPA and AG490 reduced to nearly vehicle-treated AG490-treated cell survival levels. These findings cohere with previous studies that have evidenced the role of the JAK/STAT pathway in promoting cell growth, proliferation, and survival. For example, it has been established that the JAK/STAT pathway becomes activated in

injured sensory neurons following sciatic nerve lesion, which increases the expression of interleukin 6 in axotomized sensory neurons and Schwann cells within the lesion site (Cafferty et al., 2001; Cao et al., 2006). Activation of the JAK/STAT pathway has also shown to be correlated with enhanced axonal regenerative ability in DRG neurons (Miao et al., 2006), speaking to the beneficial effects of the JAK/STAT pathway in promoting cell regeneration following injury and maintaining cell survival in the PNS. Within the CNS, a less elucidated paradigm for the JAK/STAT pathway promoting cell growth and survival has been established; however, a study by Smith and associates (2009) found that the deletion of SOCS3, an inhibitor of JAK2 (Babon et al., 2012), in RGCs promotes both neuronal survival and axon regeneration following optic crush injury. Specifically, the study found that further enhancement of axon regeneration in SOCS3 deleted mice could be accomplished via exogenously delivered CNTF, alluding to the possibility that a tight regulatory mechanism between the JAK/STAT pathway and CNTF, as well as other possible cytokines, could be working together to promote the regenerative responses observed (Smith et al., 2009). Although this postulation should be readily amenable to testing, previous studies have also supported this notion by proposing that CNTF-triggered effects on neuronal survival following injury could be due to the involvement of the PI3K/Akt, MAPK/ERK, and/or JAK/STAT pathways, either concurrently or independently of one another (Park, Luo, Hisheh, Harvey, & Cui, 2004). Conversely, Liu and Snider (2001) found that the JAK2 inhibitor, AG490, did not significantly affect axonal growth in embryonic DRG neurons, but did find that AG490 was indeed able to robustly block axonal elongation in adult sensory neurons following a conditioning lesion (Liu & Snider, 2001; Neumann & Woolf, 1999). Furthermore, Liu and Snider (2001) postulated that the axon elongation observed following the conditioning lesion was due to increased phosphorylation of STAT3 in cells by

cytokine release from neighbouring cells, which has previously been shown to occur following peripheral axotomy (Schwaiger et al., 2000). In addition, a phase-specific, rapid phosphorylation of STAT3 has been shown to occur following CNS injury (Bareyre et al., 2011). Taken together, results obtained in the current study resonate with former research that evidence the beneficial role for JAK/STAT signalling in neuronal viability. Administration of the JAK/STAT pathway inhibitor AG490 significantly diminished survival levels in postnatal cortical neurons in the current study, suggesting that activation of the JAK/STAT pathway, and consequent increased expression and phosphorylation of STAT3, could be necessary for achieving the neuroprotective effect of tPA.

In the present study, while a significant decrease in cell survival was observed following administration of AG490, no discernable difference in p-STAT3 expression between the tPA-treated and vehicle-treated groups could be detected. An explanation for this could be that the JAK/STAT pathway may play an initial, upstream role in the anti-apoptotic cascade by producing an early neuroprotective effect via phosphorylation and activation of STAT3, as proposed in a study by Bareyre and colleagues (2011), but the effect is short-lived and/or instead converges onto a pathway whose main molecular target is p-S6. In this regard, because p-S6 has been associated with enhancing neuron growth and survival, it can be suggested that the neuroprotective effects of tPA are dependent upon p-S6-mediated protein translation mechanisms. Additionally, it is possible that the mTOR pathway may be more necessary for tPA-induced neuroprotection than the JAK/STAT pathway; however, at the same time, results obtained by the current study support the hypothesis that both pathways, individually, are capable of mediating tPA-induced neuroprotection, and that when paired together or in conjunction with other cascades, the two pathways can reciprocate and produce modest

synergistic effects. A schematic illustrating the proposed cellular signalling mechanism underlying the tPA-mediated neuroprotective effect is provided in Figure 11.

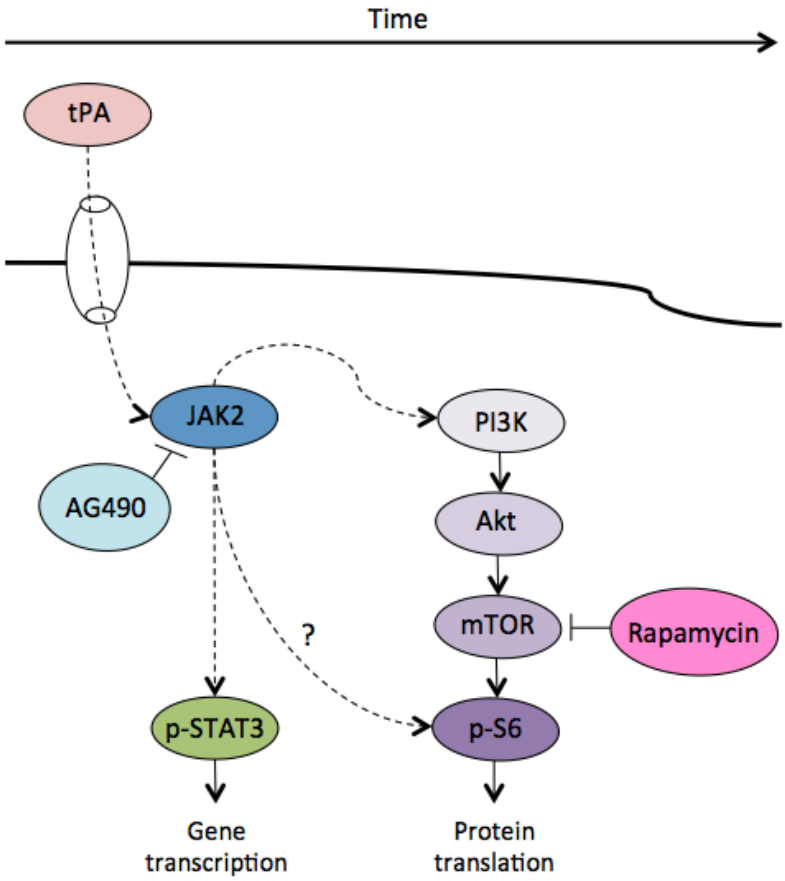

Figure 11. Proposed underlying cellular signalling mechanism of tPA-mediated neuroprotection. tPA enters the cell where it indirectly activates cellular signalling mechanisms including that of JAK2 and mTOR. tPA may work through the JAK2 pathway initially to activate p-STAT3 and promote gene transcription, and/or act on and activate the mTOR cascade for eventual activation of p-S6, and/or converge onto another pathway to activate p-S6. When activated, both the JAK2 and mTOR pathways may converge and consequently act to phosphorylate p-S6, which in turn may contribute to enhanced protein translation and ultimately improved neuronal survival.

Sun and colleagues (2011) proposed the existence of a synergistic relationship between the mTOR and JAK/STAT pathways for producing a regenerative response in axons of RGCs. Although deletion of PTEN or SOCS3 individually was shown to promote significant optic nerve regeneration, the regrowth observed in these studies diminished significantly after about

two weeks following the injury (Park et al., 2008; Smith et al., 2009), prompting the authors to investigate the effects of simultaneous deletion of the two negative regulators. It was found that PTEN and SOCS3 regulate two independent pathways that work together to enable robust and sustained axon regeneration, due to the induction of growth-related genes and sustained expression of p-STAT3 after crush injury (Sun et al., 2011). The same study also found that compared to wild type controls, the mice mutants lacking PTEN, SOCS3, and PTEN and SOCS3 together all showed significantly increased RGC survival following injury (Sun et al., 2011). Notably, this increase in survival was sustained up to 4 weeks following injury, after which the survival rate in the double mutant group prevailed (Sun et al., 2011). Because results garnered from the above mentioned studies verify not only that the mTOR and JAK/STAT pathways are independently capable of producing regenerative effects, but also that concurrent activation of the two pathways produces a more powerful effect for sustaining longer-term regeneration in the adult CNS, the findings of the current study regarding the underlying mechanisms of tPA for neuroprotection over time resonate with this previous literature. Another study by Kretz and colleagues (2005) found that erythropoietin promotes regeneration of adult RGCs via JAK/STAT and PI3K/Akt pathway activation, further evidencing the integrative, tightly knit nature of the two pathways for promoting neuroprotection and regeneration. In the current study, the cell survival in the tPA-treated double inhibitor rapamycin- and AG490-treated group showed a significant decrease compared to the tPA-treated vehicle-treated group, and this decrease in cell survival was greater than that seen in the tPA-treated rapamycin and AG490 inhibitor groups individually. This finding suggests that the mTOR and JAK/STAT pathways could be working synergistically and within an integrative pattern to promote the tPA-induced neuroprotection.

In order to determine whether the MEK pathway was also contributing to the protective effects on neurons due to tPA, U0126, a MEK-specific inhibitor (Favata et al., 1998), was administered to cultures. Surprisingly, while a slight decrease in cell survival counts was observed, the U0126 inhibitor did not significantly decrease cell survival levels in the tPA-treated inhibitor group compared to the tPA-treated vehicle group. This finding goes against previously published literature that has shown that the MEK pathway, specifically that involving ERK, plays a crucial role in mediating cortical neuronal growth and neurogenesis (Hao et al., 2004; Singer, Figueroa-Masot, Batchelor, & Dorsa, 1999). Wu and colleagues (2013) also found that tPA promotes protection from excitotoxin-induced cell death in neurons via the activation of the ERK1/2 signalling pathway. However, Liu and Snider (2001) found that administration of a MEK inhibitor to neuronal cultures had no effect on axon growth induced by a conditioning lesion, and concluded that MEK/ERK signalling is not necessary for producing robust axon extension in adult sensory neurons. In the current study, the minimal decrease in cell survival observed when cultures were incubated with U0126 could likely be due to the underlying mechanics of the tPA-induced effect whereby the cellular signals do not significantly rely on the MEK cascade to provide neuroprotection. It could be hypothesized that following a shorter or longer incubation period, the MEK pathway could contribute more significantly in maintaining postnatal cortical neuron viability.

In order to investigate the existence of interactions between pathways in providing a neuroprotective effect on cells due to tPA, multiple inhibitors were administered concurrently to the neuronal cultures. Of the tPA-treated groups, results showed a noteworthy, significant decrease in cell survival within the group treated with rapamycin and AG490, as explained earlier; however, the maximum decrease in cell survival was obtained in the rapamycin and

AG490 and U0126 treated triple inhibitor group. This result could be due to a form of "cross talk" between the MEK pathway and its neighbouring pathways. For example, it could be that the MEK pathway coalesced with the mTOR pathway, which then interacted with the JAK/STAT pathway due to their close reciprocal relationship, and thus the decrease in cell survival could be due primarily to the robust inhibition of the mTOR cascade and subsequent secondary effects on the MEK and JAK/STAT pathway. Alternatively, it could be postulated that because the mTOR pathway acts on the MEK pathway, albeit very upstream through PI3K signalling, the pro-survival signals originating from the mTOR cascade could be projecting onto the MEK pathway, and promoting a neuroprotective response through this route. Therefore, when U0126 is added as a third inhibitor within the group, further decrease in cell survival is observed because the pro-survival cellular signals being communicated peripherally by the MEK pathway due to mTOR's upstream effectors could also be inhibited. When rapamycin and AG490 were paired, individually, with U0126, synergistic effects were not observed, as the rapamycin and AG490 paired groups with U0126 maintained similar levels of cell survival as their individual inhibitor groups, evidencing the need for concurrent co-communication between the three pathways to produce the neuroprotective response induced by tPA. Of particular note, the AG490 and U0126 double inhibitor group showed a sustained decrease in cell survival, suggesting that the strong inhibitory effect of AG490 dominated the relationship between the two inhibitors and that the U0126 did not significantly assist in further down-regulating cell survival when in the presence of AG490 and tPA.

In order to determine whether the PKA pathway was playing a vital role in the tPA-induced neuroprotection, KT5720, a selective inhibitor of the PKA pathway (Kohda & Gemba, 2001; Pae et al., 2000), was administered to cultures and cell counts were analyzed between

vehicle-, tPA-, and/or PKA-treated groups. Surprisingly, inhibition of the PKA pathway by KT5720 had no significant effect on cell survival in any of the PKA-treated groups, as cultures treated with KT5720 exhibited similar amounts of cells compared to the cultures treated without it. Previous studies have shown that treatment with 1 μM of KT5720 is capable of significantly inhibiting cortical neuron survival (Qiang, Denny, & Ticku, 2007), human neuroblastoma cell survival (Wang & Yang, 2009), attenuating METH-stimulated RyR-1 and -2 expressions in cerebral cortical neurons (Kurokawa et al., 2011), and blocking dopaminergic facilitation of excitatory post-synaptic currents in layer II entorhinal neurons (Glovaci, Caruana, & Chapman, 2014). In the current study, treatment with the same dose did not result in any discernable effect on neuronal survival. In this regard, it can be suggested that the PKA pathway does not significantly mediate the mechanisms underlying the neuroprotective effect of tPA. This finding does not support previous research that has postulated that the cAMP/PKA/CREB pathway is critical to sustaining cell survival and acts on substrates regulated by Akt to phosphorylate negative regulators of cell survival including the pro-apoptotic member of the Bcl-2 family, Bad (Brunet, Datta, & Greenberg, 2001; Harada et al., 1999). Estrogen has also been shown to regulate transcription via the cAMP/PKA/CREB pathway and its direct effectors (Watters & Dorsa, 1998). Additionally, because PKA has been established as one of the major downstream effectors of cAMP, the significance of PKA activity for cAMP-mediated survival due to neurotrophins, including BDNF (Tao et al., 1998), has been investigated. Studies have found that cAMP works to support neurotrophins in their myriad of roles including supporting axon growth and facilitating RGC response to growth factors following injuries such as axotomy (Cai, Shen, De Bellard, Tang, & Filbin, 1999; Shen, Wiemelt, McMorris, & Barres, 1999).

Despite the above evidence that demonstrates that the cAMP/PKA/CREB pathway serves as a vital regulator of programmed neuron survival and could be key to maintaining cell survival (Walton & Dragunow, 2000), results from some studies suggest that the cAMP/PKA/CREB pathway plays only a minor role in enhancing neuron viability. For example, Liu and Snider (2001) found that the PKA pathway did not play a significant role in adult axon morphology nor embryonic sensory axon growth, and, more precisely, administration of its inhibitor KT5720, did not significantly contribute to axon growth length following axotomy. These experiments, however, were carried out in DRG cells, which are sensory neurons that are known to be vastly different in cellular responses compared to cortical neurons. Furthermore, while cAMP has been demonstrated as regulating neuronal survival and mediating BDNF-induced cell survival, Poser, Impey, Xia, and Storm (2003) proposed an opposing paradigm after their research revealed that BDNF protection of cortical neurons from serum withdrawal-induced apoptosis was mediated by PI3K signalling and inhibited by cAMP signalling. Activation of PTEN by PKA, which is responsible for inhibiting PIP_3 signalling (Wu, Senechal, Neshat, Whang, & Sawyers, 1998), could constitute a reason for the inhibition of the PI3K pathway by cAMP, as explained by the authors (Poser et al., 2003). Taken together, findings from the current study correlate with some of the previous research that has evidenced a survival-independent role of the PKA pathway for neuron viability. In the current study, while cultures treated with KT5720 showed cell survival counts similar to that of cultures treated with tPA and vehicle, administration of KT5720 in conjunction with other inhibitors seemed to increase cell survival levels in cultures, albeit only marginally, compared to when cultures were treated with the same combination of inhibitors sans the KT5720. Similar to the suggestion by the above study, increased cell survival when under the influence of KT5720 could be attributed to an underlying complex mechanism

whereby decreased PTEN inhibitory activity due to decreased PKA activity subsequently increases the mTOR activity within the neurons for heightened cell growth, proliferation, and survival capability when under the influence of tPA.

Of particular note, the administration of 10% DMSO to cultures decreased cell survival by approximately 10% across all groups. This finding is similar to previously published studies including one by Yang and colleagues (2014), which revealed a decrease in cell viability of about 12% following administration of DMSO at a similar percentage. While a decrease in cell survival was seen in cultures due to the neurotoxic nature of DMSO (Galvao et al., 2014), the decreases were not selective and all groups responded similarly to the chemical, ultimately causing no significant confounds.

As a whole, the findings from the current study indicate that the relationship between tPA administration and primary cortical neuron survival is certainly a complex process. Combinatorial interactions between pathways mediating transcription and translation factors and engagement in crosstalk between the aforementioned pathways seems to regulate tPA-mediated beneficial functioning in neurons to provide pro-survival effects on cell viability. Results from the current study revealed that tPA not only promotes survival in postnatal primary cortical neurons, but also that its most predominate route for enhancing cell survival is via an mTOR- and JAK2-dependent signalling mechanism.

Limitations and future directions

This study revealed that tPA can act as a neuroprotectant in a temporally-regulated manner and that both JAK/STAT and mTOR signalling effects are critical mediators of this effect. Given that the results indicate that tPA promotes its neuroprotective effect predominantly through the mTOR and JAK/STAT pathways, it is likely that p-S6 is a key molecular target of

the signalling cascade. In addition, because of the predominance of the mTOR pathway in producing the neuroprotective effect observed by tPA, it can be posited that one of the negative regulators of the pathway, PTEN, may also be an important molecular target of the signalling cascade. Although this requires further investigation, the inactivation of PTEN and subsequent over-activation of mTOR to promote increased growth and proliferative effects could be part of the underlying cellular mechanism mediating the beneficial effect of tPA on postnatal cortical neurons. Additionally, because JAK/STAT was also shown to contribute significantly to cell survival under the influence of tPA, it is likely that SOCS3, a negative regulator of the JAK/STAT pathway, could also be a molecular target of tPA. However, this postulation may be less probable given that the molecular target of the JAK/STAT pathway, p-STAT3, could not be detected among the tPA-treated groups upon qualitative analysis.

While results suggest that the mTOR and JAK/STAT pathways are the cascades upon which tPA acts most predominately through to phosphorylate its downstream targets, including that of p-S6, further studies are needed to confirm that other pathways are not also significantly contributing to the neuroprotective effect of tPA. A recent proteomics study found new candidates that could be implicated as key regulators in injury response signalling in mature RGCs, including c-*myc*, NF_KB, and Huntingtin (Belin et al., 2015). Specifically, it was found that co-deletion of PTEN and SOCS3 paired with c-*myc* overexpression resulted in 5 times more regenerating axons compared to PTEN/SOCS3 co-deletion alone at 4 weeks post-injury, and at 8 weeks, many of the regenerating axons were maintained in the mice with the triple treatment. Therefore, examining the role of c-*myc* signalling within the context of tPA-induced neuroprotection could prove particularly beneficial, especially because this study also suggested that c-*myc* could be working in concert with mTOR to produce a pro-survival and regenerative

response in mature neurons (Belin et al., 2015). Additionally, investigating whether tPA promotes its pro-survival effects via activation of anti-apoptotic members of the Bcl-2 family, including Bcl-xL, which has been shown to increase intrinsic growth potential and cell survival in CNS neurons (Jonas, Porter, & Alavian, 2014; Kretz, Kügler, Happold, Bähr, & Isenmann, 2004; Park et al., 2015; Wen et al., 2002), as well as determining whether tPA promotes its pro-survival effects via deactivation of its pro-apoptotic members, including Bad, would be an appropriate aspect for further analysis. Evaluating the effect of PI3K inhibition with LY294002 would also provide more specific information about the underlying mechanism of tPA and details as to where in the PI3K/PTEN/Akt/mTOR cascade the neuroprotective response originates. Analysis of any increase of neurotrophin expression following the administration of tPA, including BDNF, CTNF, and/or NGF would further elucidate the underlying pro-survival architecture of tPA and provide an ecological groundwork for expanding its therapeutic window. In addition, assessment of survival within the cultures treated with or without tPA and conducting a comparison between the groups in terms of death processes would be a noteworthy avenue for future exploration. For example, analysis with trypan blue, a stain used to selectively detect dead cells, could be employed to assess the prevalence of dead cells within cultures before they undergo fixation. Another technique that could be employed to quantify death processes is a terminal deoxynucleotidyl transferase (TUNEL) assay, a method of detecting DNA fragmentation due to apoptotic signalling cascades after cell fixation. Furthermore, assessment of any reductions in oxidative stress by intervention of tPA could also be assessed through immunocytochemistry and provide further information about how tPA induces its neuroprotective effects. Investigation into whether tPA promotes its pro-survival effects through minimizing death processes or by maximizing cell survival capability would be helpful for

determining the exact functions and mechanisms inherent to the molecule and for targeting new strategies to expand the beneficial effects of tPA in therapy.

Another aspect for further investigation could be the analysis of tPA administration on cell types other than primary cortical neurons, including hippocampal, cerebellar, or midbrain neurons, as well as even PNS neurons in addition to CNS neurons to determine if the tPA-induced neuroprotective effect is widespread throughout the nervous system. The effect of tPA on RGC survival and the propensity for axon outgrowth could also be assessed to substantiate its molecular and therapeutic effects within an alternative cellular setting.

It would also be beneficial to experiment with different time frames, age ranges, and incubation periods to determine the exact temporal relationship of tPA-induced neuroprotection. For example, administration of tPA past a 3 hour time latency would be beneficial to determine the effects of tPA outside of its prescribed timeframe for therapy. Specifically, analysis of any changes in tPA's effects after more prolonged and delayed time periods could provide further insight into its underlying characteristics and possibly identify markers related to tPA-induced neurotoxicity, if such an effect was observed. In addition, it would interesting to incubate the cultures for timespans longer than 72 hours, or create a continuum experiment across a range of incubation periods, to determine at which point, if any, tPA-induced neuroprotection diminishes and/or tPA-induced neurotoxicity develops.

Lastly, while the results presented by this study make a compelling case for the mTOR and JAK/STAT pathways being the mediators underlying the neuroprotection induced by tPA, it should be noted that further confirmatory evidence should be sought and additional research studies incorporating the use of alternative techniques are needed in order to corroborate the conclusions reached by this study. For example, utilization of flow cytometry could be used to

verify cell count results, as well as western blotting to assess the amounts of p-S6 and p-STAT3 present following tPA administration. In future studies, AraC, a glial cell proliferation inhibitor should be utilized to limit the amount of glial cells present in cultures, although cultures in the current study were made up of approximately 90 percent neurons and 10 percent glia (see Legacy, Hanea, Theoret, & Smith, 2013). Additionally, it would be advantageous to investigate whether the amount of cells plated altered the effect of tPA on neuronal survival, since a higher yield of cells would boast more neurotrophic support between cells and thus increase cell survival (Banker & Cowan, 1977).

Further understanding of the impact of tPA on neurons could open new doors towards expanding the therapeutic window of tPA and perhaps enhancing the efficacy of this drug clinically. The results from this study help to garner a better understanding of the protective effect of tPA on postnatal primary cortical neurons and the underlying mechanisms mediating its effect, and add to the literature with the goal of fostering a framework for expanding the therapeutic window of tPA in stroke recovery.

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