Intracellular Signalling Targets Downstream of GT 1061, a Novel Nitric Oxide Mimetic

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Abstract

Nitric oxide (NO) is a gaseous intracellular messenger that mediates a wide range of physiological, behavioural, and cognitive events. A novel nitric oxide mimetic, GT 1061 was developed as an Alzheimer's Disease therapy, and has previously been shown to improve performance in rodent learning paradigms. The aim of the present studies was to determine if GT 1061 confers protection against neurotoxicity, and activates via phosphorylation, survival and learning-related intracellular targets. Cell viability assays showed that GT 1061 (30-300 μM) was not able to protect neurons against hydrogen peroxide toxicity or serum deprivation. Primary rat hippocampal and cortical cultures treated with GT 1061 showed some elevation in phosphorylated CREB (cyclic-AMP response element binding protein) and MAPK (mitogen-activated protein kinase) between concentrations of 10-300 μM, though without reaching statistical significance. No change in phosphorylated Akt (protein kinase B) or PKC (protein kinase C) was observed. Intraperioneal injection of GT 1061 (1 and 5 mg/kg) had no effect on the levels of phosphorylated CREB, MAPK, or PKC in the CNS, but phosphorylated Akt showed a tendency to increase with GT 1061. Taken together, these data suggest that these intracellular signalling pathways are not strongly stimulated by GT 1061 in our experimental models.

Résumé

Le monoxyde d'azote (NO) est un messager intracellulaire gazeux impliqué dans une série de fonctions physiologiques, comportementales et cognitives. Le GT 1061, un nouveau composé mimant les effets du NO et développé pour le traitement de la maladie d'Alzheimer, améliore l'apprentissage chez le rongeur. L'objectif de notre étude est de déterminer si le GT 1061 possède des propriétés neuroprotectrices, et active des cibles intracellulaires liées à l'apprentissage et la survie cellulaire. La mesure de la viabilité cellulaire a montré que le GT 1061 (30-300 μM) ne protège pas les neurones de la toxicité induite par le péroxyde d'hydrogène ou la privation de sérum. Une augmentation (statistiquement non significative) de la protéine CREB (cyclic-AMP response element binding protein) et de la MAPK (mitogen-activated protein kinase) phosphorylés est observée dans des cultures primaires de cortex et d'hippocampe de rat traitées au GT 1061 (10-300 μM). En revanche, l'Akt (protéine kinase B) ou la PKC (protéine kinase C) sous leur forme phosphorylée ne sont pas modifiées. L'injection intrapéritonéale de GT 1061 (1 et 5 mg/kg) ne modifie pas les taux de CREB, de MAPK ou de PKC phosphorylés mais semble augmenter l'Akt phosphorylée dans le cerveau. Ces résultats suggèrent que les voies de signalisation intracellulaire etudiées ici ne sont pas fortement modulées par le GT 1061 dans nos modèles expérimentaux.

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Abbreviations

Beta-amyloid	Aβ
Adenylyl cyclase	AC
Alzheimer's disease	AD
Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid	AMPA
Acetylcholine	ACh
Acetylcholinesterase inhibitor	AChEI
Protein kinase B	Akt
Amyloid precursor protein	APP
Apolipoprotein E	ApoE
Brain-derived neurotrophic factor	BDNF
Cornu ammonis	CA
Calcium-calmodulin	CaMP
Adenosine 3',5'-cyclic monophosphate	cAMP
Catalase	CAT
CREB binding protein	CBP
Guanosine 3',5'-cyclic monophosphate	cGMP
Choline acetyltransferase	ChAT
Central nervous system	CNS
Cyclic-AMP response element binding protein	CREB
Enhanced chemiluminescence	ECL
Extracellular-signal related kinase	ERK
Food and Drug Administration (United States of America)	FDA
Gamma-aminobutyric acid	GABA
Glycogen synthase kinase 3	GSK3
Glyceryl trinitrate	GTN
Guanosine triphosphate	GTP
Hydrogen peroxide	H_2O_2
Intracerebroventricular	ICV
Intraperitoneal	i.p.
c-Jun N-terminal kinase	JNK
Long-term potentiation	LTP
Muscarinic receptor 1	M_1
Mitogen activated protein kinase	MAPK
Metabotropic glutamate receptor	mGluR
Magnetic resonance imaging	MRI
3-4(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide	MTT
Nerve growth factor	NGF
N-methyl-D-aspartate	NMDA
Nitric oxide	NO
Nitric oxide synthase	NOS
Neutral red (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride)	NR

NMDA receptor 2B subunit	NR2B
Platelet-derived growth factor promoter transgenic mice expressing human APP	PDAPP
Positron emission tomography	PET
Paired helical filament	PHF
Phosphorylated	phospho-
Phosphatidylinositol-3-kinase	PI3-K
Protein kinase A	PKA
Protein kinase C	PKC
Protein kinase G	PKG
Presenilin	PS
Reactive nitrogen species	RNS
Reactive oxygen species	ROS
Soluble guanylyl cyclase	SGC
Sodium nitroprusside	SNP
Superoxide disumutase	SOD
Transgenic	Tg
3-(5-hydroxymethyl-2-furyl)-1-benzyl-indazole	YC-1

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1. INTRODUCTION

Nitric oxide (NO) is a gaseous intracellular messenger that mediates a wide range of physiological functions, including neurotransmission, vasodilation, neuroplasticity, and free-radical scavenging, and is implicated in a variety of behavioural and cognitive events. High levels of NO can be neurotoxic, primarily through the generation of free radical reactive oxygen species (ROS). However, low concentrations of NO are not only essential for normal neuronal function, but may be neuroprotective against cellular insults. Accordingly, the NO system is disrupted in many neuropathological conditions, including Alzheimer's Disease, Parkinson's Disease, ischemia, and neuroinflammation. A novel nitric oxide mimetic, GT 1061, has previously been shown in rats to improve performance in learning paradigms with few adverse side effects. This suggests that GT 1061 may be able to exploit beneficial NO-activated pathways. The aim of the present experiment is to determine whether GT 1061 is able to phosphorylate, and thus stimulate, known neuroprotective and learning-related intracellular targets, including protein kinase B (Akt), mitogen-activated protein kinase (MAPK), cyclic-AMP response element binding protein (CREB), and protein kinase C (PKC). Western blot analyses were performed on cortical and hippocampal cell extracts taken from both an *in vitro* rat primary neuronal cell culture model and from rat brain tissue after systemic injection of GT 1061. The results underscore the intricate complexity of the NO system, and the importance of evaluating such interactions in the context of both neuropathological and normal neuronal events.

1. 1. Alzheimer's Disease

1. 1. 1. Etiology and Clinical Presentation

Dementia refers to a collection of symptoms related to loss of cognitive function, including disruption of memory, judgment, reasoning, language abilities, and orientation, and is associated with neuropathologies such as Alzheimer's Disease (AD), Parkinson's Disease, Huntington's Disease, and vascular dementia. Presently, AD is the leading cause of dementia (Rahkonen et al., 2003; Stevens et al., 2002). Alois Alzheimer first characterized a distinct form of dementia in a patient, Auguste D., instituted at a German asylum at the beginning of the 20th century. During the post-mortem examination, Alzheimer identified the presence of amyloid plaques, neurofibrillary tangles, and distinct cortical atrophy, which he reported in 1906, characterizing the disease that now bears his name.

Primary symptoms of AD include progressive decline in cognitive functioning, and loss of memory and awareness. This is accompanied by difficulties with language, orientation, judgment, problem-solving, and motivation. The patient may become subdued and withdrawn or experience changes in mood and personality. There is progressive decline in ability to perform daily living tasks, and in later stages, general motor ability is impaired. There is also high incidence of comorbid depression, anxiety, and psychosis in AD patients (Boland, 2000; Piccininni et al., 2005).

AD affects more than 15% of North Americans over the age of 65 and accrues costs of more than 100 billion dollars per year in health care expenses (www.alz.org). At present, there is no cure for AD. On average, patients live for 8-10 years after they are diagnosed. However, some have estimated that a delay in symptom onset by 10 years

would reduce the number of AD cases in one generation by as much as 75%. As a result, most research efforts are currently focused on halting disease progression in its early stages.

To date, there has not been a singular cause of AD identified. Familial or inherited-type AD account for only about 10% of all AD cases, and involve autosomal dominant mutations of one or several genes that confer increased susceptibility and earlier disease onset.

The first gene to be linked with heritable AD was the amyloid precursor protein (APP). This gene is located on chromosome 21 and encodes a 770 amino acid transmembrane protein. Several mutations have been identified in related populations of AD, the most common of which is a substitution at codon 717 (Chartier-Harlin et al., 1991). These mutations result in overproduction of toxic beta-amyloid deposits. Presenilin 1 (PS1) and presenilin 2 (PS2), are related genes located on chromosome 14 and chromosome 1, respectively. There have been over forty PS mutations identified and associated with early onset AD (Campion et al., 1995). The role of these proteins is still unclear, but they have been shown to interact with γ-secretase, an enzyme that processes APP (Kimberly et al., 2000; Wolfe et al., 1999; Xia et al., 1997). Apolipoprotein E (ApoE) is the major apolipoprotein in the central nervous system, and facilitates triglyceride metabolism and cholesterol regulation. ApoE is expressed as three polymorphisms; ApoE2, which confers protection against AD, ApoE3, which is the most common variant, and ApoE4, which is a risk factor for sporadic and late-onset AD (Nalbantoglu et al., 1994; Raber et al., 2004; Poirier et al., 1995; Poirier et al., 1993).

However, the ApoE4 allele is considered only to be a risk factor and not a true genetic determinant of AD.

The large majority of AD cases are largely sporadic or non-inherited. Age, obesity, and previous head trauma all correlated positively with increased risk of AD (Gustafson et al., 2003; Mortimer et al., 1991), while education and physical exercise correlate negatively with AD (Evans et al., 1997; Lindsay et al., 2002). Women are thought to be more likely to develop AD than men (Andersen et al., 1999; Launer et al., 1999).

1. 1. 2. Affected Brain Structures

Two areas of the brain that are critical for cognitive function, learning, and memory are the cerebral cortex and hippocampus.

The cerebral cortex is the outer surface of the cerebrum and is composed of 6 distinct cellular layers. Functionally, the cortex is divided into 4 lobes; the temporal, frontal, parietal, and occipital lobes. Sensory and motor information is relayed from the thalamus to primary sensorimotor areas of the cortex to be processed. The frontal and temporal cortices are largely responsible for higher cognitive functions including language, consciousness, attention, and memory. The cortex is also capable of immense plasticity, and it has been suggested that memory storage occurs here (Maviel et al., 2004; Squire & Zola-Morgan, 1991). Cortical volume changes very little with age in healthy adults, but shows significant loss in patients with AD (Ohnishi et al., 1995), as detected through imaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET).

Likewise, hippocampal volume also decreases very little with age alone, but drops substantially in patients with AD even in early stages of the disease (Ohnishi et al., 1995). The hippocampus is a bilateral subcortical structure that forms part of the limbic system. Other regions of the limbic system include the amygdala, which is involved in emotion and aggression, the cingulate gyrus, the archicortex, and the hypothalamus, which regulates the autonomic nervous system. The hippocampal structure is divided into 4 regions - the dentate gyrus, Cornu ammonis region 1 (CA1), CA2, and CA3. Information is conveyed through the hippocampus in a tri-synaptic loop. The entorhinal cortex of the medial temporal lobe sends inputs to the granule cells of the dentate gyrus by the perforant path. The mossy fibre pathway connects the granule cells to the pyramidal cells of the CA3, which project through the Schaeffer collateral to the CA1. Most of the major central nervous system (CNS) neurotransmitters are represented in the hippocampus including glutamate, acetylcholine, norepinephrine, serotonin, gamma-aminobutyric acid (GABA), and dopamine. The hippocampus, which is particularly susceptible to anoxia and oxidative stress, is the site of continuous neurogenesis and neuroplasticity, and contains place cells, which form a spatial map representative of the surrounding physical environment. Acquisition and consolidation of spatial and episodic memory are thought to involve the hippocampus. Studies by Brenda Milner (Scoville & Milner, 1957) and many others have shown that disruption of this area can cause anterograde amnesia (inability to form new memories).

1. 1. 3. Affected Neurotransmitter Systems

Neurons that release or receive signals via acetylcholine (cholinergic system) are especially vulnerable in AD. The basal forebrain, which sends cholinergic projections to the hippocampus, olfactory bulb, amygdala, and cerebral cortex, is particularly impaired in AD (Auld et al., 2002; Vogels et al., 1990; Whitehouse et al., 1982; Whitehouse et al., 1981). There is an early and progressive loss of cholinergic markers including choline acetyltransferase (ChAT), and muscarinic and nicotinic acetylcholine receptor binding (Araujo et al., 1988; Aubert et al., 1992; Bartus et al., 1982; Lyness et al., 2003; Rodriguez-Puertas et al., 1997; Ruberg et al., 1990; Svensson et al., 1997). Many attempts have been made to exploit the M_1-type muscarinic receptor, a postsynaptic metabotropic receptor, for treatment of AD. Spatial memory has been shown to be disrupted by M_1 receptor antagonists, and rescued by M_1 receptor agonists in rodent models of memory impairment (Brandeis et al., 1990; Brandeis et al., 1995; Fisher et al., 1998; Ohno et al., 1994). However, past clinical trials of M_1 receptor agonists have revealed low bioavailability, lack of efficacy, or significant side effects (Adamus et al., 1995; Rajeswaran et al., 2001; Wienrich et al., 2001). Much of this has been attributed to lack of M_1 receptor subtype specificity. Further identification of M_1-specific targets should lead to safer and more effective agonists.

Neurons that signal via glutamate (glutamatergic system) are also affected in AD. This is likely due in large part to the significant role the glutamatergic system plays in neurotransmission, excitotoxicity, and hippocampal plasticity. Hippocampal CA1 neurons and pyramidal neurons of the neocortex are lost in AD, and these areas are particularly susceptible to neurofibrillary tangle accumulation (Kowall & Beal, 1991).

Glutamate binds to three types of ionotropic receptors: the AMPA receptor, kainate receptor, and NMDA (N-methyl-D-aspartate) receptor. NMDA receptors play a fundamental role in Hebbian learning and memory, requiring binding of both glutamate and glycine, in addition to membrane depolarization, in order to influx Ca^{2+} and other ions. Levels of NMDA receptor density and innervation in the dentate gyrus and cortex are generally thought to be decreased in AD (Hardy et al., 1987; Ikonomovic et al., 1999), though there are some conflicting reports (Wakabayashi et al., 1999). The decrease in NMDA receptor levels in AD may suggest that glutamatergic neurons are particularly susceptible to cell death. Glutamatergic neurons that do not succumb to cell death may undergo changes in composition or distribution in AD. The NR2B subunit of the receptor, which is strongly associated with synaptic plasticity underlying formation of new memories (Tang et al., 1999), is decreased in AD hippocampus and elsewhere in the brain (Hynd et al., 2004; Mishizen-Eberz et al., 2004). Recently, it has been shown that Aβ induces dephosphorylation of the NR2B subunit in addition to endocytosis of NMDA at synaptic surfaces (Snyder et al., 2005). Thus, synaptic transmission may be impaired by dysfunctional glutamate neurotransmission as a result of increased levels of Aβ.

There is growing interest in the role of metabotropic glutamate receptors (mGluRs) in AD. Lee et al. (2004) found mGluR2 to be increased in hippocampal AD neurons, in populations showing high levels of hyperphosphorylated tau. Activation of mGluRs is thought to shift APP processing in favour of the less toxic, non-amyloidic pathway (Lee et al., 1995; Ulus & Wurtman, 1997). mGluRs are also mostly noted to be protective against excitotoxicity (Kingston et al., 1999; Koh et al., 1991), thus, it is

possible that upregulation of mGluR2 in AD may be a compensatory reaction against cell injury (Lee et al., 2004).

Taken all together, the hippocampus and surrounding circuitry is one of the earliest and most severely affected areas in AD, which is not surprising given that memory dysfunction is the most prominent clinical symptom.

1. 1. 4. Neuropathology

Long before a clinical manifestation of AD is detected, the brain begins to undergo distinct morphological changes. Neuroimaging such as MRI, and neuropsychological assessments such as the Mini-Mental State Examination (MMSE) are tools being used evaluate cases of dementia. Unfortunately, these tools are currently only able to give a probable diagnosis of AD, which is confirmed upon postmortem examinations of brain tissue.

1. 1. 4. 1. Beta Amyloid Plaques

The beta-amyloid hypothesis of AD posits that amyloid accumulation, either due to over-expression or lack of clearance from the brain, is the primary cause of AD. APP is processed via two different pathways. In a non-amyloidic pathway, cleavage of APP by the α secretase at residue 687 yields a soluble ectodomain and a transmembrane fragment. Cleavage of the transmembrane fragment by γ secretase at residues 711 or 713 releases a non-amyloid peptide, p3. Amyloidic processing of APP by β secretase at residue 671 releases a comparatively smaller soluble ectodomain and longer transmembrane fragment. When the transmembrane fragment is subsequently cleaved by

γ secretase, β-amyloid (Aβ) is released. Depending on the γ secretase cleavage site, the resulting Aβ peptide is either 40 or 42 amino acid residues. These fibrillar amyloid peptides can seed insoluble extracellular deposits (amyloid plaques), which are cytotoxic (Jarrett et al., 1993; Maruyama et al., 1995). Beginning in early stages of AD, plaques can be found in most neocortical areas and subcortical areas (Price et al., 1991). Aβ can trigger apoptotic cell pathways (Harada & Sugimoto, 1999; Ivins et al., 1999a; Lakshmana et al., 2005), necrosis (Floden et al., 2005; Suzuki, 1997), and endocytosis of glutamate receptors (Snyder et al., 2005). Though plaques may be observed in normal aging or mild cognitive deficit, they are significantly elevated in AD postmortem tissue (Masliah et al., 1993; Wang et al., 1999). Using APP as a therapeutic target in AD is difficult due to APP's physiological functions. APP modulates neurite outgrowth, synaptic plasticity, synaptogenesis, and even protects neurons from excitotoxcity (Allinquant et al., 1995; Goodman & Mattson, 1994; Morimoto et al., 1998). Selectively targeting Aβ instead of the precursor protein or gene may hold more therapeutic potential. Initial studies have shown both promise and potential complications. On-going studies have shown that Aβ immunization is able to clear amyloid burden and prevent synaptic degeneration in rodents, and reverse cognitive deficits in both mice and humans (Buttini et al., 2005; Hock et al., 2003; Kotilinek et al., 2002; Sigurdsson et al., 2001). Unfortunately, clinical trials in humans were halted after several patients developed subacute meningoencephalitis (Orgogozo et al., 2003).

1. 1. 4. 2. Neurofibrillary Tangles

AD is also considered to be a tauopathy (a disorder related to dysfunction of tau proteins). Tau proteins are microtubule-associated proteins involved with tubule assembly and stability, and are essential for intracellular transport and establishment of cell polarity (Mandelkow & Mandelkow, 1998). Tau contains multiple Ser/Thr phosphorylation sites (Lund et al., 2001), though they are not all normally occupied. Hyperphosphorylated tau is no longer able to bind microtubules, and adopts a paired helical filament (PHF) conformation (Biernat et al., 1993; Crowther et al., 1994; Bramblett et al., 1993). Abnormal buildups of PHF and other microtubule proteins result in neurofibrillary tangles, causing disruptions in protein trafficking, metabolism, and axonal structure (Mandelkow et al., 2003). Neurofibrillary tangle and PHF formation occur increasingly with normal aging (Price et al., 1991; Uboga & Price, 2000; Yang et al., 2005). However, AD patients show abnormally high densities of neurofibrillary tangles compared with non-demented age-matched controls, particularly in the anterior olfactory nucleus, entorhinal cortex, and CA1 subfield (Giannakopoulos et al., 1993; Price et al., 1991; Wilcock & Esiri, 1982). Tangles are also observed in the amygdala, neocortical areas, and basal forebrain in moderate to late stages of AD.

Interestingly, the realms of Aβ processing and neurofibrillary tangle formation are ever-more overlapping. There is accumulating evidence that fibrillar Aβ peptides may be able to phosphorylate tau protein in septum, hippocampus, and cortex, leading to a specific loss of cholinergic neurons (Busciglio et al., 1995; Greenberg & Kosik, 1995; Zheng et al., 2002a). This may occur through the stimulation of glycogen synthase

kinase 3β (GSK3β) and possibly MAPK, both of which are involved in tau phosphorylation (Takashima et al., 1998a; Zheng et al., 2002a). GSK3β not only has the ability to phosphorylate tau, but conversely, can also phosphorylate the C-terminal of APP, at least in vitro (Aplin et al., 1996), suggesting that both plaque and tangle processing pathways may converge on a common effector. Another protein that may play a role in both neuropathologies is presenilin 1. PS1 mutations are known to increase Aβ production, but the PS1 protein also has binding sites for both tau protein and GSK3β, raising the possibility that it may serve to facilitate tau phosphorylation (Takashima et al., 1998b). However, the interaction between Aβ and neurofibrillary tangles is far from clear, as it appears that plaques and tangles can also be distributed in different areas of the brain, and develop at different stages of the disease (Price et al., 1991).

1. 1. 4. 3. Oxidative Stress

Free radicals, or species with a single unpaired electron, inflict damage to cells by pulling electrons away from other molecules, thereby propagating a chain reaction of radical formation and reduction. It has been purported to be the earliest event leading to AD, preceding both Aβ deposition and emergence of neurofibrillary tangles (Nunomura et al., 2001). Like other AD neuropathologies, oxidative stress is also a major event in normal cellular aging; however, damage in AD is thought to be much higher. Aβ induces oxidative stress indirectly through microglial activation, but it can also directly contribute to oxidative stress by generating the free radical hydrogen peroxide through a Cu^{2+}-dependent reaction (Huang et al., 1999a; Huang et al., 1999b; Milton, 2004; Opazo et al., 2002; Tabner et al., 2002). Oxidative stress can cause damage to lipids, DNA, and

proteins, and lead to cell death. Levels of lipid peroxidation are significantly increased in AD tissue, specifically in the temporal and, to some degree, the frontal cortices (Karelson et al., 2001). Early-stage AD tissue shows evidence of oxidized RNA-derived nucleosides, particularly in the parietal and temporal lobes, and primarily in mitochondrial DNA (Nunomura et al., 2001; Wang et al., 2005), and several enzymes and proteins that are essential for neuronal function, such as glutamine synthetase, are abnormally oxidized in cortical AD tissue (Pamplona et al., 2005; Smith et al., 1991). The total capacity of the protective antioxidative system is diminished, and specific antioxidative enzymes such as catalase (CAT) and superoxide dismutase (SOD), show decreased activity in the temporal cortex (Karelson et al., 2001; Marcus et al., 1998). SOD, along with nitric oxide, catalyzes the conversion of the free radical, superoxide, to hydrogen peroxide, and CAT reduces hydrogen peroxide to water. Diminished activity of SOD and CAT in the temporal cortex suggests an accumulation of reactive oxygen species in an area that is highly damaged in AD.

1. 1. 4. 4. Atrophy and Cell Death

Patients suffering from Alzheimer's, Parkinson's and other dementias show high levels of brain atrophy, which advance with time. There is an enlargement of the ventricles, and total brain volume is decreased in AD, with significant changes to the hippocampus, and frontal and temporal lobes (Forstl et al., 1995). Though atrophy occurs during the course of normal aging, AD-associated changes are defined by specificity of affected areas and severity of loss (Ohnishi et al., 2001). AD atrophy is linearly correlated with neuronal cell death, suggesting a causative relationship (Kril et

al., 2004). The entorhinal cortex shows profound loss of neurons, even in very early stages of cognitive decline. Layer II entorhinal neurons are decreased by 60% by the first clinically-detectable stage of AD, and drop further to 90% by severe stages of dementia (Gomez-Isla et al., 1996). Similarly, layer IV neurons decrease by 40% and 70% through the first through last stages of AD, respectively (Gomez-Isla et al., 1996). Neurons from the CA1 are also lost as a function of disease duration (Kril et al., 2004).

Both apoptotic and necrotic mechanisms have been implicated in genetic and sporadic AD. Cultured neurons exposed to Aβ show characteristic signs of apoptosis, such as condensation of chromatin, fragmentation of DNA, and formation of membrane blebs (Loo et al., 1993; Pike et al., 1991; Watt et al., 1994). Oxidative stress is a strong initiator of apoptosis. Mitochondria, as high producers of oxidants, are particularly sensitive to oxidative damage. If free radical production is left unchecked, mitochondria will release cytochrome c, leading to the activation of intrinsic apoptotic pathways via caspase-9 activation. Aβ-mediated apoptosis can also be triggered by extrinsic pathways involving death receptors such as Fas and Fas ligand (Su et al., 2003), and there is also evidence that death receptor TR3 may be upregulated in AD neurons (Newman et al., 2000). Hence, both the extrinsic death receptor mediated pathway and the intrinsic mitochondrial pathway can be activated by Aβ, either through direct stimulation of pro-apoptotic factors or indirectly through Aβ-related oxidative stress (Ivins et al., 1999b; Rohn et al., 2002; Rohn et al., 2001). Both apoptotic pathways converge on caspase-3, which cleaves cellular proteins, including tau (Rametti et al., 2004). Tau in the cleaved form then promotes formation of tangles (Cotman et al., 2005; Rissman et al., 2004).

Consequently, caspase activation may be yet another mechanism through which Aβ accelerates neurofibrillary tangle pathology.

Not all AD-related cell death is of apoptotic nature. Programmed cell death involves characteristic parceling and degradation of cellular contents, stimulating several signaling cascades, which can be identified by positive apoptotic markers. Necrotic cell death is much more reactive and explosive, with fewer regulated intracellular signaling events. Morphological and immunohistological evidence using in situ end labeling show that a large proportion of cell death in AD occurs in the absence of apoptotic markers (Lucassen et al., 1997; Stadelmann et al., 1998). Furthermore, there is a significant inflammatory component in some AD cell death, indicative of necrotic, not apoptotic, cell death. This includes expression of microglial-induced proinflammatory cell surface molecules, cytokines, and other associated proteins (Kalaria, 1999). Microglia can be activated by Aβ, and accrue at sites of amyloid plaque. Initially, inflammation may be a host response in an attempt to clear amyloid, but the sustained, low-grade inflammatory response eventually becomes harmful to the cell (Akiyama et al., 2000). Likewise, Aβ can stimulate microglial NADPH oxidase to elicit the release of reactive oxygen species through a respiratory burst in an attempt to clear pathogens (Bianca et al., 1999; McDonald et al., 1997). However, this strategy is also double-edged, causing oxidative damage to the host cell as well as cells involved in pathogenic processes.

1. 1. 5. Clinical and Neuropathological Correlates

There is some debate over which neuropathological marker best correlates with disease progression. Much of this disparity arises from comparisons of different

cognitive rating scales, of probands of different ages or disease stages (early cognitive impairment versus severely cognitively impaired), and of different methodologies in measurement of plaques and tangles. But the greatest source of variation arises from trying to identify the borders separating and defining normal aging-related cognitive decline, mild cognitive decline, and dementia. Groups of age-matched controls may inadvertently include subjects that are in mild pre-clinical stages of dementia. Traditionally, neurofibrillary tangles are thought to correlate most closely with severity of clinical symptoms (Giannakopoulos et al., 2003; Guillozet et al., 2003). However, recent studies by McKeel et al. (2004) and others have argued that amyloid plaques are more indicative of disease progression.

Neuronal loss rate has, too, been suggested to be the strongest indication of disease progression (Niikura et al., 2002). Rusinek and colleagues (2003) recently measured changes of the medial temporal lobe using MRI and predicted in advance, with nearly 90% accuracy, the likelihood of future AD development. This is particularly significant clinically, because unlike postmortem studies of neurofibrillary tangles and amyloid plaques, imaging can be performed on living humans even before the emergence of symptoms. This opens the possibility of early detection and treatment of AD.

Can strategies that decrease cellular atrophy halt or reverse cognitive decline? Drapeau et al. (2003) showed that superior performance in the Morris Water Maze can predict a corresponding high level of hippocampal neurogenesis in aged rats, suggesting that those with more new brain cells might be protected from aging-related cognitive decline. Peterson and colleagues (1999) demonstrated that mice carrying a mutation in the neurotrophin receptor p75 gene had impaired performance in Morris Water Maze and

avoidance memory tasks, and showed a loss of neurons in the basal forebrain, compared with controls. This suggests that neurotrophic factors may be involved in mediating cognitive tasks, and that loss of survival factors can directly disrupt cognitive performance. However, it is not clear if and how neurogenesis and increased cell survival directly improve learning and memory. In addition, it is unknown if promoting the survival of older existing neurons will benefit cognitive function in the same manner as the generation of new neurons.

1. 1. 6. Alzheimer's Disease Therapies

Currently, only two classes of pharmacological therapies have been approved by the U.S. Food and Drug Administration (FDA) for treatment of AD. Both target neurotransmission processes. The first class of AD drugs has been developed to target the negative association between disease progression and acetylcholine (ACh) levels. Decreased ACh levels are thought to result from cell death in the basal forebrain, hippocampus and cortex, leading to memory loss and cognitive deficit (Geula et al., 1994). The acetylcholinesterase inhibitor (AChEI) tacrine (Cognex®, which is now no longer prescribed due to hepatotoxic side effects) was first marketed in 1993, and since then, donepezil (Aricept®), rivastigmine (Exelon®), and galantamine (Reminyl® or Razadyne ®) have been approved for clinical use. This class of drugs increases the level of functional ACh by inhibiting the enzyme acetylcholinesterase, which degrades ACh in the synaptic cleft. AChEIs are most effective in the early to moderate stages of the disease, but cannot reverse existing damage or completely halt disease progression.

A second type of drug, memantine (Namenda®), was recently approved by the FDA in 2003 for effective use in moderate to late stages of AD. Memantine is a weak NMDA receptor antagonist, reducing but not completely inhibiting the opening of the ion channel in response to glutamate signals. Excessive activation of glutamate receptors can lead to an excessive influx of Ca^{2+}, resulting in excitotoxicity and cell death. While complete blockade of NMDA receptors would result in disruption of normal neurotransmission throughout the brain, memantine is effective in slowing AD progression by muffling (but not preventing) NMDA activation through an uncompetitive mechanism (Reisberg et al., 2003).

None of these drugs are efficacious in halting disease progression (Courtney et al., 2004; Doody, 2003; Lleo et al., 2005). The therapies affect symptomology, but do not modify the underlying pathology and disease progression is inevitable. Neither are these drugs effective for all AD patients (Trinh et al., 2003), but they are still widely used, for lack of alternatives. Thus, today, AD is a terminal diagnosis. In the search for more effective therapies for AD, current research efforts have widened to include cellular and molecular signaling pathways that are thought to play a role in cell survival or cognitive functioning. In this way, researchers hope to be able to identify specific therapeutic targets that can modulate endogenous cellular activity.

1. 2. Signalling Pathways Associated with Cognition

1. 2. 1. Signal Transduction

Cellular function and survival is largely dependent on cellular responses to external signals. The vast list of neurotransmitters, neurotrophic factors, and signaling

pathways associated with cognitive function is continually expanding. Rapid cellular communication is particularly important in the nervous system, which regulates, in addition to cognitive processes, physiological autonomic functions, sensory information, and motor commands. Ionic species can elicit very rapid cellular responses, largely due to tightly regulated voltage-sensitive mechanisms. A number of extracellular signals are able to bind and open ionic channels, allowing the influx or efflux of ions such as calcium (Ca^{2+}), potassium (K^{+}), and chloride (Cl^{-}). In some instances, membrane depolarization or mechanosensitive triggers also activate ionic channels. Ca^{2+} plays a role in many types of signal transduction events, including the regulation of synaptic transmission. Influx of Ca^{2+} is particularly important for docking and fusion of vesicles with the presynaptic membrane, and the subsequent exocytotic release of neurotransmitter (Catterall, 1998; Heidelberger et al., 1994). Acetylcholine, glutamate, dopamine, serotonin, gamma-aminobutyric acid (Elmqvist & Feldman, 1965), and glutamate are some of the neurotransmitters that have shown a Ca^{2+}-dependent component in transmitter release (Burke et al., 1993; Carvalho et al., 1986; Elmqvist et al., 1965; Henderson et al., 1983; Turner et al., 1993; Turner et al., 1992).

Other signalling mechanisms involve activation of intracellular cascades via second messenger systems or a series of kinase phosphorylation events, which lead to transcriptional regulation. The resulting protein expression serves to change the cell in some way – for example, by increasing the cell's resistance to stress or conversely, rendering the cell more susceptible to insult. The signaling pathways do not frequently act in isolation; rather, there are countless interactions, or "crosstalk" between pathways.

In addition, each extracellular signal and kinase is often simultaneously induced in multiple pathways.

Amidst the myriad of signals, there are some targets upon which many different pathways converge. The redundancy in activation suggests that these common effectors likely play critical roles in mediating cellular function.

1. 2. 2. Akt/Protein Kinase B

The serine-threonine kinase Akt (also known as protein kinase B) is the main effector downstream of phosphatidylinositol 3-kinase (PI3-K). The PI3-K pathway is a cell survival pathway and is activated by several neurotrophic factors, including insulin-like growth factor-1 (IGF-1), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (Liot et al., 2004; Zheng & Quirion, 2004), and vascular endothelial growth factor (Wick et al., 2002). Once phosphorylated at Thr-308 and Ser-473 residues, active Akt promotes cell survival in several different ways.

Akt is able to phosphorylate and subsequently inactivate pro-apoptotic proteins such as caspase-9 (Cardone et al., 1998), Bad (del Peso et al., 1997), and members of the winged-helix family of transcription factors, including Foxo3 (FKHRL1) (Brunet et al., 1999; Zheng et al., 2000), Foxo 1 (FKHR) (Tang et al., 1999; Zheng et al., 2002b), and Foxo4 (AFX) (Kops et al., 1999; Zheng et al., 2002b).

Akt is also able to regulate transcription through activation of transcription factors. The nuclear factor κB (NF- κB) transcription factor is dependent on the degradation of an inhibitor of NF- κB, IκB, and stimulation of the IκB kinase, both of which involve PI3-K and Akt activity. NF-κB induces the transcription of pro-survival

genes, including gadd45β (a c-jun amino-terminal kinase cascade inhibitor), bcl-xL, X-linked inhibitor of apoptosis, and inhibitors of caspases and c-myc (Cavin et al., 2005; De Smaele et al., 2001; Khoshnan et al., 2000). Akt can also repress the pro-apoptotic transcription factor p53 by activation of murine double minute 2 (Mdm2) E3 ubiquitin ligase (Gottlieb et al., 2002), and induce CREB transcription of pro-survival genes (Du & Montminy, 1998).

Importantly, particularly within the context of AD, Akt is also able to inactivate glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995). GSK-3β activity has been positively correlated with neurofibrillary pathology (Hanger et al., 1992; Lovestone et al., 1994), and GSK-3α is a necessary component of amyloid precursor processing (Phiel et al., 2003). Thus, it might be suspected that tissue from AD patients would show lower than normal levels of active Akt, and in fact, Akt activity is decreased in familial AD presenilin 1 and 2 lymphoblast cell lines, a cellular model of AD (Ryder et al., 2004). However, immunohistological data of human tissue suggests that Akt levels are actually increased in AD patients (Pei et al., 2003). It has been postulated that Akt activity is elevated as a compensatory mechanism against neurofibrillary pathology (Pei et al., 2003). Other studies have shown that tau proteins are phosphorylated by Akt at Ser214, which unlike phosphorylation of Thr212, confers neuroprotection rather than degeneration (Ksiezak-Reding et al., 2003). So conceivably, increased levels of Akt seen in AD patients are not a contributing factor, but a reaction against, cell death.

1. 2. 3. Mitogen Activated Protein Kinase

Mitogen activated protein kinases (MAPKs) are a broad family of protein kinases implicated in many different signaling pathways primarily related to cell growth, differentiation and survival, synaptic plasticity, and transcriptional regulation (English & Sweatt, 1996; English & Sweatt, 1997; Impey et al., 1998). The extracellular signal regulated kinase 1/2 (ERK 1/2) family member, otherwise known as MAP kinase p44/42, phosphorylates Ser or Thr residues that precede a proline residue, on transcription factors, cytoskeletal proteins, and other signaling molecules (Pearson et al., 2001). MAPK p44 and 42 are distinct but closely related kinases activated via phosphorylation by MAP kinase kinase 1/2 (Pearson et al., 2001).

MAPK's role in neuroprotection against cellular injury is well-documented (Hetman & Gozdz, 2004). MAPK has been implicated in a variety of cell survival pathways, including those activated by glutamate receptors (Lu et al., 2002), cyclic AMP (Troadec et al., 2002), estrogen (via ERα) (Acconcia et al., 2005), and brain-derived neurotrophic factor (Almeida et al., 2005), amongst others. Downstream of MAPK are several transcription factors, including elk-1, myc-c and CREB (Chuang & Ng, 1994; Sato et al., 1997), which induce transcription of a host of survival genes (Riccio et al., 1999).

MAPK has dual anti-apoptotic action. In addition to stimulating transcription of survival genes via CREB, MAPK also inhibits, by phosphorylation, pro-apoptotic proteins such as Bad (Bonni et al., 1999). In fact, after deprivation of survival factors, inhibition of pro-apoptotic proteins occurs earlier than stimulation of survival gene transcription (Bonni et al., 1999). MAPK also inhibits GSK3β (a pro-apoptotic kinase)

(Hetman et al., 2002) and Bim40 (a pro-apoptotic factor that mediates NGF-withdrawal cell death) in PC12 cells (Biswas et al., 2002).

In addition to cell survival, MAPK activity is also important for cognitive function. Sweatt's laboratory first demonstrated that MAPK activation is required for expression of long term fear conditioning (Atkins et al., 1998), and later showed that other forms of memory, such as spatial memory are also MAPK-dependent (Selcher et al., 1999). MAPK has been shown to play an essential role in the induction of long-lasting Long-Term Potentiation (LTP), a cellular model of learning and memory. English and Sweatt (1997) demonstrated that pharmacological inhibition of MAPK prevented the formation of LTP in rat hippocampal slices, and Kelleher and colleagues (2004) used knockout mice to show that MAPK is necessary for long-lasting forms of synaptic plasticity and to mediate certain spatial and contextual memory behaviours.

1. 2. 4. Cyclic AMP Response Element Binding Protein

In 1987, Montminy and Bilezikjian first described a phosphorylated CRE-binding protein, CREB (cAMP response element binding protein), which appeared to be necessary for transcription of the somatostatin gene (Montminy & Bilezikjian, 1987). Since then, CREB has been widely implicated within many signalling pathways, particularly those involved with neuronal survival and function, including Akt and MAPK –related pathways. A member of the basic-region leucine zipper transcription factor superfamily, CREB is composed of several functional domains, which vary slightly from one spliced variant to another. Of the three major variants, $CREB_{\alpha}$ and $CREB_{\Delta}$ are found in greater abundance than $CREB_{\beta}$. In the central nervous system,

CREB is a transcriptional regulator for genes related to development, differentiation, growth, and survival of neurons. Disruption of CREB causes apoptosis, neurodegeneration, and axonal growth deficits (Lonze et al., 2002; Mantamadiotis et al., 2002). Analyses of rat and human genomes reveal that CREB-regulated loci number in the thousands, and possibly even ten thousands (Impey et al., 2004; Zhang et al., 2005).

There are many pathways through which CREB phosphorylation is induced. Cyclic AMP-dependent protein kinase (PKA) is one such mechanism (Hagiwara et al., 1993). PKA activity is modulated by cAMP produced by adenylyl cyclase (AC). ACs are associated with G-protein coupled receptors on the cell surface and as such, establish a mechanism through which CREB phosphorylation is responsive to extracellular signalling cues such as neurotransmitters and neuropeptides. CREB can also be stimulated by an increase in Ca^{2+} concentration. During membrane depolarization, voltage-gated calcium channels and glutamate receptors influx Ca^{2+}. Through interaction with calmodulin, several calcium/calmodulin-dependent kinases including CaMKI, CaMKII, and CaMKIV can be activated and phosphorylate CREB (Blanquet et al, 2003; Matthews et al., 1994; Sheng et al., 1991; Wu et al., 2001). CREB activation involves the binding of a coactivator, CREB Binding Protein (CBP), to the phosphorylated Ser-133 residue of CREB's kinase inducible domain region. Phosphorylation of Ser-133 is stimulus-dependent, and occurs in response to a variety of external cues such as growth factors, synaptic activity and depolarization of the cell, hypoxia, stress, and other signalling mechanisms (Johannessen et al., 2004; Lonze & Ginty, 2002).

The landmark study showing that CREB plays a role in learning and memory was the first evidence that gene transcription is necessary for long-term memory formation

(Yin et al., 1994). Since then, many observations have been made in many different vertebrate and invertebrate models showing that both protein synthesis and CREB are necessary for learning and long-term retention. Early studies showed that *Creb*$^{\alpha/\delta}$ knockout mice were significantly impaired in spatial memory ability (Bourtchuladze et al., 1994). Likewise, mice with disruption of CREB protein production by antisense oligodeoxynucleotides were shown to have impaired LTP and learning (Guzowski & McGaugh, 1997). However, Balschun et al. (2003) demonstrated that not all forms of memory or LTP are disrupted in animals devoid of all isoforms of CREB.

1. 2. 5. Protein Kinase C

Protein kinase C (PKC) is a family of over 12 Ca^{2+}/phospholipid-dependent isoenzymes, which are involved in many different intracellular signaling pathways. In survival pathways, PKC can protect neurons from NGF deprivation-induced apoptosis in a calcium-independent manner (Pierchala et al., 2004; Tanaka & Koike, 2001). Interestingly, this appears to be mediated by activation of the PI3-K/Akt pathway. PKC also inhibits apoptosis by up-regulating anti-apoptotic factors Bcl-xL, Bcl-w, and BDNF, and down-regulating proapoptotic factors Bad and Bax (Weinreb et al., 2004). The delta isoform of PKC has shown to have both pro- and anti- apoptotic properties (Bharti et al., 1998; Li et al., 1998; Mecklenbrauker et al., 2004), suggesting that it may function as a gate-keeper between proliferation and negative cell-cycle progression (Jackson & Foster, 2004).

Amongst other normal functions, activation of PKC has been shown to be necessary for memory consolidation (Wallenstein et al., 2002). During classical

conditioning training, PKC undergoes a redistribution from the cytosol to the cellular membrane in hippocampal neurons, suggesting that PKC is amenable to learning-induced long-lasting changes (Bank et al., 1988). PKC may also help mediate the temporal specificity of memory consolidation. Along with PKA, PKC must be active during a critical time window of approximately 1-2 hours following memory acquisition in order for the memory to remain intact (Wallenstein et al., 2002).

1. 3. Nitric Oxide

1. 3. 1. Nitric Oxide as a Biomolecule

Nitric oxide (NO) is a small, clear, gaseous species that is found throughout the nervous system. It was first recognized as a biological signalling molecule in 1987 when Ignarro and colleagues (Ignarro et al., 1987; Ignarro et al., 1987), and Moncada and colleagues (Palmer et al., 1987) identified nitric oxide as the potent vasodilator and smooth-muscle relaxant previously termed 'endothelial-derived relaxant factor' (Furchgott & Zawadzki, 1980). In 1998, the Nobel Prize in physiology and medicine was awarded to Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad for their contributions to discovering the biophysical properties of NO. Today, it is known to contribute to normal functioning of many different physiological events.

NO is produced by the enzyme nitric oxide synthase (NOS) during the conversion of L-arginine to L-citrulline (Bredt & Snyder, 1990). In mammals, there are 3 identified isoforms of NOS, each encoded on a unique gene (Bredt et al., 1991; Lowenstein et al., 1992; Marsden et al., 1993). All NOS enzymes are composed of an oxygenase domain and a reductase domain. The L-argining binding site, as well as the heme catalytic site, is

located on the oxygenase domain. As the names suggest, neuronal NOS (nNOS or NOS1) is localized in neuronal cells throughout the central nervous system. nNOS is a cytosolic enzyme containing a PDZ domain, which localizes it to nerve synapses (Alderton et al., 2001). Endothelial NOS (eNOS or NOS3) is predominantly localized in endothelial cells of the cerebral vasculature. eNOS contains hydrophobic branches that anchor the enzyme to the plasma membrane (Venema et al., 1995). Inducible NOS (iNOS or NOS2) is found mostly in glia and some neuronal cells. nNOS and eNOS isoforms constitutively and calcium-calmodulin-dependently produce low levels of NO. In contrast, inducible NOS (iNOS) produces high levels of NO as an inflammatory or immunological response, and independently of calcium levels. It is a cytoplasmic enzyme, not usually expressed under normal conditions, but once NO production is activated, it is sustained until iNOS is degraded (MacMicking et al., 1997; Small et al., 2004).

NO is completely soluble in aqueous fluids, diffusing easily across cellular membranes (including the blood-brain barrier), and has a half-life of approximately 5 seconds (Ignarro, 1989). NO can be both beneficial and harmful to a cell, depending upon the concentration. High levels of NO can cause oxidative stress and free radical production, leading to cell injury and death. Paradoxically, low physiological levels of NO or NO-donors can be both beneficial and necessary for cell survival (Chiueh, 1999; Ciani et al., 2002; Contestabile & Ciani, 2004).

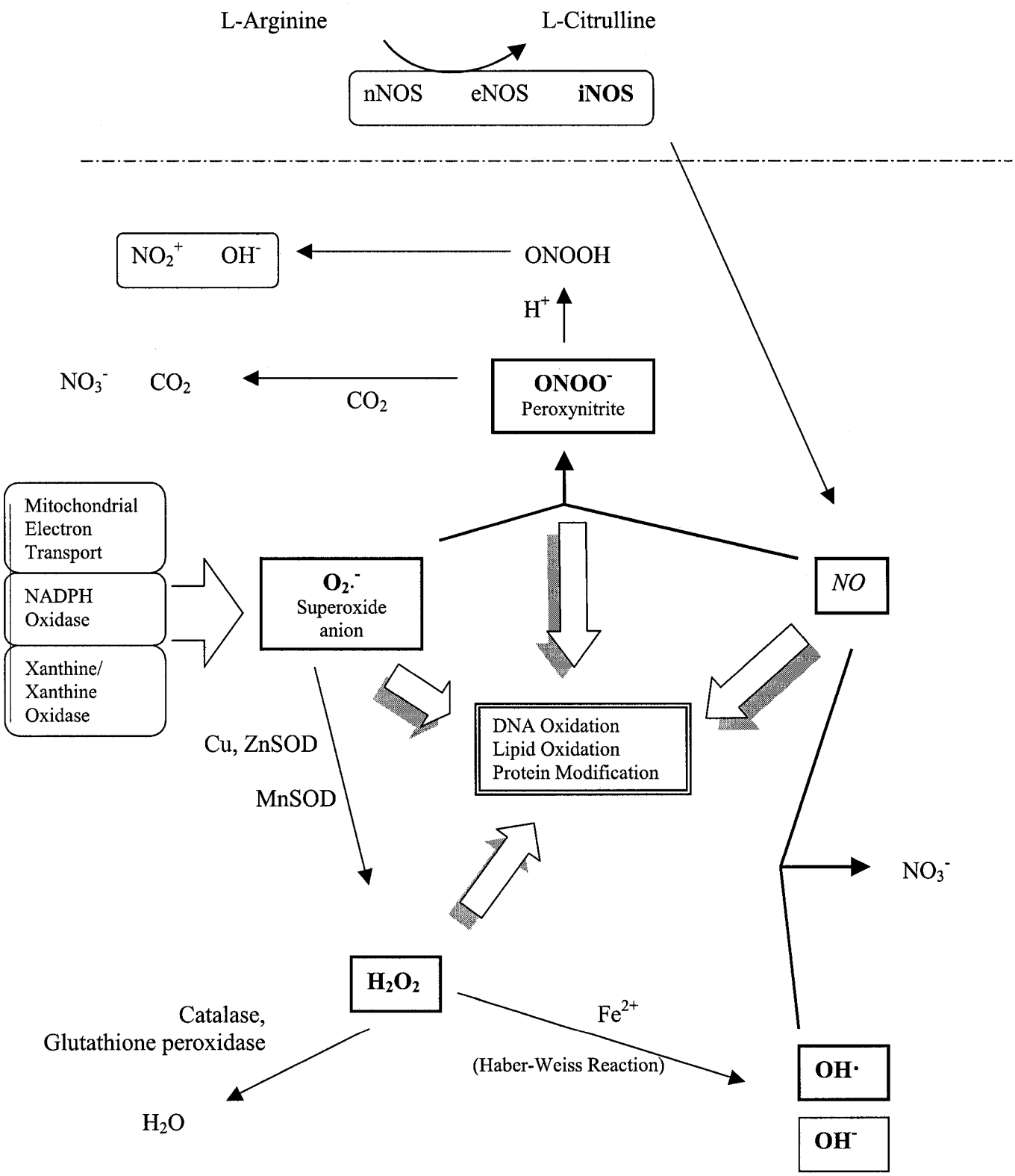
1. 3. 2. Free Radical Producer and Scavenger

NO is an uncharged lipophilic molecule and contains an unpaired electron, and thus, functions both as an electron donor (oxidant) and an electron acceptor (antioxidant)

Free radicals are species with reactive unpaired electrons. They are produced during electron-transport reduction-oxidation reactions, and can cause cell death through DNA damage and activation of harmful enzymatic pathways, as previously described (see Sect. 1. 1. 3. 3.; Chien et al., 2004; Lee et al., 2005). Destructive effects occur when the unpaired electron draws another electron away from a neighbouring molecule, which becomes a free radical itself, and initiates a chain reaction of electron loss and gain. Reactive oxygen species (ROS) are oxygen-based free radicals and other molecules containing a reactive oxygen group. Included in this group are hydrogen peroxide (H_2O_2), singlet oxygen (O_2), nitric oxide ($NO\cdot$), peroxynitrite ($ONOO^-$), and superoxide anion ($O_2^{\cdot-}$) (Bruckdorfer, 2005). Likewise, Reactive Nitrogen Species (RNS) are molecules containing a reactive nitrogen group, including nitric oxide, peroxynitrite, nitrate (NO_3^-), nitrite (NO_2^-), and 3-nitrotyrosine (Aslan & Ozben, 2004; Ischiropoulos & Beckman, 2003).

NO is a ROS and RNS, but it is also a free radical scavenger, particularly of superoxide. Superoxide anion is continually produced in mitochondria by incomplete reduction of water, NADPH oxidase, and by other enzymatic reactions (Bruckdorfer, 2005). Figure 1 summarizes the formation of major ROS species. Superoxide is harmful to the body, so there are several defense mechanisms through which it is neutralized to more benign species.

Figure 1. Formation of Reactive Oxygen Species and Their Products



Superoxide Dismutase (SOD), which is found in the cytosol as Cu, ZnSOD and in mitochondria as MnSOD, converts superoxide to hydrogen peroxide (de Haan et al., 1995; Li et al., 1995). Hydrogen peroxide itself is a free radical, but it is less reactive than superoxide and can be converted to water by catalase or glutathione peroxidase (Aebi, 1984; Hothersall et al., 1981; Milton, 2004). However, in the presence of ferrous iron, hydrogen peroxide can undergo an enzyme-independent Haber-Weiss reaction to produce toxic hydroxyl radicals ($OH\cdot$), which are very reactive. Superoxide also reacts with NO to form peroxynitrite ($ONOO^-$). Superoxide anion's affinity for NO is actually greater than for SOD (Huie & Padmaja, 1993), suggesting that in the presence of NO, peroxynitrite is the major product. In this way, NO serves as a free radical scavenger. Though not a free radical, peroxynitrite itself can be highly reactive. Together with NO, peroxynitrite can induce apoptosis by directly releasing mitochondrial cytochrome c (Chae et al., 2004), inducing p53 expression (Yamaguchi et al., 2001), generation of ceramide (Takeda et al., 1999), and activation of c-Jun N-terminal kinase (JNK) (Li et al., 2004), all of which lead to activation of proapoptotic caspases. However, peroxynitrite can be converted by carbon dioxides to less toxic nitrates, which are easily eliminated from brain tissue (Chiueh, 1999; Squadrito & Pryor, 1998). NO can also scavenge hydroxyl radicals to form nitrates (Chiueh, 1999). Thus, the antioxidant properties of endogenous NO are very real. In addition, NO can also directly act on NADPH oxidase to prevent superoxide production, and regulate intracellular iron homeostasis to inhibit the formation of hydroxyl radicals from hydrogen peroxide (Clancy et al., 1992; Fernandez-Tome et al., 1999; Matsunaga et al., 2004; Rauhala et al., 2005).

1. 3. 3. Nitric Oxide and Cognition

Disruption of the nitric oxide system interferes with some cognitive-related behaviour in animal models. Pharmacological inhibition of NOS has been shown to impair spatial memory (Bohme et al., 1993; Koylu et al., 2005; Qiang et al., 1997), olfactory memory (Kendrick et al., 1997), and fear conditioning (Schafe et al., 2005), whereas augmentation of nitric oxide activity has been shown to enhance passive and active avoidance memory (Chien et al., 2005) and spatial memory (Prusky et al., 2004).

Disruption of NO also interferes with learning and memory processes at a cellular level. It has been postulated that LTP involves the strengthening of synaptic connection in response to repeated stimulation. A potentiated presynaptic neuron will release more neurotransmitter when stimulated, relative to its unpotentiated state. Key studies have indicated an essential role of NO in this synaptic modulation, and that disruption of NO activity inhibits the establishment of LTP (Bohme et al., 1991; Bon et al., 1992; Haley et al., 1992).

How might nitric oxide mediate learning? NO's unique permeability and short half-life make it well suited to function as a retrograde messenger. Unlike conventional neurotransmitters, gaseous NO is not stored in vesicles, but is released directly after production and diffuses to neighbouring cells in a range of 0.3-0.4 mm (Lancaster, Jr., 1997). The post-synaptic cell produces NO in response to Ca^{2+}-influx following conventional neurotransmission by classical neurotransmitters. A positive feed-back loop is formed as NO diffuses in the reverse direction, back to pre-synaptic neurons. The end result of this signalling by NO is an enhancement of neurotransmitter release in

subsequent cell firing events (Grassi & Pettorossi, 2000; O'Dell et al., 1991; Schuman & Madison, 1991).

1. 3. 4. Nitric Oxide Signalling

NO is diffusible and does not require a cell surface receptor. Once in the cytosol, NO binds to soluble guanylyl cyclase (sGC). sGC are heterodimers composed of two subunits. The large subunit is expressed in two isoforms (α1 and α2), and the small subunit is also expressed in two isoforms (β1 and β2). All isoforms are expressed in the rat brain except β2. sGC is distributed in multiple regions throughout the brain including the hippocampus and throughout all cerebral cortical layers of the neocortex (Ding et al., 2004). sGC contains a prosthetic ferrous-heme centre. When NO binds to the ferrous group, a conformational change occurs and sGC is activated. sGC converts GTP to cGMP. As a second messenger, cGMP can activate cyclic nucleotide-gated channels, cyclic nucleotide phosphodiesterase, and protein kinase G (PKG), stimulating different signal transduction cascades and other downstream kinases. cGMP is degraded by a family of enzymes called phosphodiesterases.

1. 3. 5. Nitric Oxide and Alzheimer's Disease

Several aberrations in NOS and NO concentration suggest that NO may be implicated in AD (Law et al., 2001a). In analysis of human brains, tissue from AD patients showed differential distribution of NOS across the cortex and abnormally in pyramidal cell populations (Fernandez-Vizarra et al., 2004). Aβ has been shown to elevate NO release, possibly mediating the increase in oxidative stress seen in AD (Law

et al., 2002b; Smith et al., 1997). Correspondingly, aged rats with cognitive impairments showed significantly higher levels of inflammatory iNOS expression and lower levels of constitutive nNOS expression than that of young or aged rats without cognitive impairment. However, general NOS enzymatic activity was increased in young animals compared with aged animals. This suggests that the nitric oxide system may be disrupted during neurodegeneration, though it is still unclear how the balance of NO production and NOS expression is modulated in AD (Law et al., 2002a).

1. 3. 6. Possibility of Nitrate-based Neuropharmacotherapy?

Nitrate-based drugs have existed since 1878 when glyceryl trinitrate (GTN) was formulated for treatment of angina. Since then, nitrate pharmacotherapies have primarily targeted the cardiovascular system. Along with compounds that induce vasodilation and hypotension, current on-going drug developments include a nitrate-based ATP-sensitive K^+ channel activator (Minamino et al., 2004), and a class of NO-donating nonsteroidal anti-inflammatory drugs/cyclooxygenase inhibitory NO donors (Burgaud, Ongini, & Del Soldato, 2002).

Mechanisms that modulate the NO/cGMP/PKG pathway are also being investigated in the context of cognitive enhancement and neuroprotection. YC-1 [3-(5-hydroxymethyl-2-furyl)-1-benzyl-indazole] is a compound that increases sGC activation by 100-1000 fold via enhanced NO binding to sGC. YC-1 has been shown to facilitate both LTP and behavioural performance in memory tasks (Chien et al., 2003; Chien et al., 2005). The ras/MAPK pathway and CREB phosphorylation are suggested to be downstream of this pathway.

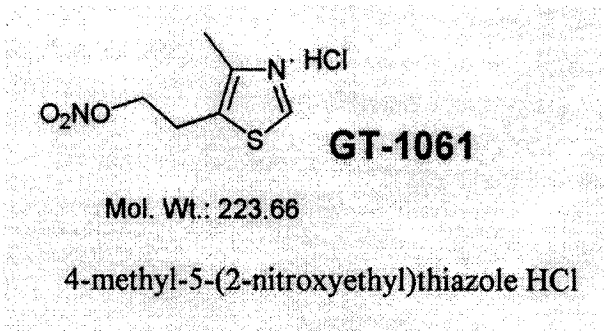
Several cell culture models have shown neuroprotective properties of NO. In particular, the NO survival pathway appears to be able to promote cell survival through a pathway that operates in parallel with NGF mechanisms, and is activated during serum deprivation (Akassoglou, 2005; Farinelli et al., 1996; Figueroa et al., 2005). Neonatal dorsal root ganglia neurons deprived of NGF undergo apoptosis if cultured alone, but in the presence of NO, there is no evidence of apoptosis. In fact, the neurons display extensive neurite outgrowth (Thippeswamy et al., 2005). Culmsee et al. (2005) demonstrated that in the absence of NGF, NO-donors enhanced the phosphorylation of the NGF receptor, TrkA, as well as PI3-K and MAPK (ERK 1/2), thereby preventing apoptosis.

1. 3. 7. GT 1061, a novel nitric oxide mimetic

In the search for alternative AD therapies, there has been recent interest in targeting sGC activation through NO-like compounds. To this purpose, a novel class of NO mimetics based on nitrate ester chemistry has been developed (Smith, Dringenberg, Bennett, Thatcher, & Reynolds, 2000; Thatcher, Bennett, Dringenberg, & Reynolds, 2004). In initial screening in comparison with GTN, this group of disulfanyl *S*-nitrate compounds, the GT 715 family, was more effective in activating hippocampal sGC, and showed a much smaller vasodilatory effect. (Reynolds et al., 2002). Thus, GT 715 appears to selectively target the central nervous system without inducing significant peripheral effects, rendering it much more suitable as a neuropharmacological agent than traditional NO donors.

GT 1061 [4-methyl-5-(2-nitroxyethyl)thiazole HCl] (Figure 2) is a compound of this class that has been shown to rescue learning behaviour of 192-IgG saporin-lesioned rats in a visual matching to sample test of memory, and also in the Morris Water Maze spatial memory task after scopolamine-induced amnesia (Thatcher et al., 2004). Preliminary testing in rodent models has indicated that GT 1061 is absorbed rapidly into the circulation, is able to cross the blood brain barrier, and has high selectivity for CNS. In keeping with its nitrate chemistry, it shows low levels of systemic toxicity, and is associated with tolerable cardiovascular side effects. Consequently, there is high potential for clinical development of GT 1061 as cognitive enhancement therapy in AD.

Figure 2. Structure of GT 1061



1.4. Objective, Hypothesis, and Rationale

The objective of this thesis was to identify key intracellular signalling pathways activated by GT 1061, a novel nitric oxide mimetic, and determine its capacity to protect cultured cells against neurotoxicity.

We hypothesize that molecular targets known to be involved in cell survival and cognitive function will be stimulated following exposure to GT 1061. We choose to study Akt, MAPK, CREB, and PKC because they have all been demonstrated to mediate learning and memory behaviours, and are effectors upon which many cell survival pathways converge. We also hypothesize that GT 1061 will be able to protect neurons from toxic insult. We chose to use hydrogen peroxide toxicity both as a general model of ROS oxidative stress, which is observed to be elevated in AD, and as a specific model of AD neuropathology, as hydrogen peroxide specifically is generated directly from Aβ, and has been implicated in mediating AD neuropathology.

2. MATERIALS AND METHODS

2. 1. Primary Neuronal Cultures

Cell culturing allows for the study of specific cellular events in a controlled environment (Vierck et al., 2000). By isolating specific populations of cells, it is possible to eliminate many of the multiple interactions that occur between different regions of the brain, and also between different physiological systems. Primary cell cultures keep dissociated tissue alive without subculturing. Tissue is taken directly from an organism, triturated, and allowed to adhere to a substrate in the presence of a growth medium. Embryonic tissue yields more viable cells than does adult tissue, and is usually the preferred model. Neurons, even those from embryos, generally do not spontaneously proliferate in vitro (thus, the lack of subculturing), but will extend neuritic outgrowth on the appropriate substrate. Embryonic neurons are viable in primary culture for 1-4 weeks, depending on the growth conditions.

Hippocampal and cortical tissue were dissected from embryonic day 19-20 Sprague-Dawley rat pups (Charles River Canada, Montreal, QC, Canada). Animal care followed the protocols and guidelines of McGill University Animal Care Committee and the Canadian Council for Animal Care. Tissues were rinsed four times with Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution (Gibco/Invitrogen, Burlington, ON, Canada) supplemented with15mM HEPES buffer, 10 U/mL penicillin (Invitrogen), and 10 μg/mL streptomycin (Invitrogen), and digested with 0.25% trypsin (Sigma, St. Louis, MO, USA) at 37°C for 10 minutes. Fetal bovine serum (FBS) was added to halt the digestion reaction, and tissues were rinsed in HBSS an additional three times to remove serum. The tissue was then triturated 30 times using a sterile Pasteur pipette, followed by a

single aspiration through an 18.5 gauge needle. Samples were centrifuged at 1000xg for 10 minutes and supernatant removed. Cells were filtered and resuspended in serum-free Neurobasal culture medium (Invitrogen) supplemented by 2% B27 (Invitrogen), 20 μM L-glutamine, 15 mM HEPES (Invitrogen), sodium pyruvate (Sigma), potassium chloride, 10 U/mL penicillin, and 10 μg/mL streptomycin. Neurobasal has been developed as an optimal growth medium for embryonic hippocampal neuronal cells (Brewer, 1995). Cells were plated at a density of 1 x 10^6 cells/mL in 6 or 96 –well plates coated with 10 μg/mL poly-D-lysine. Poly-D-lysine promotes the adhesion and survival of neurons, and permits neuritic outgrowth (Riopelle & Cameron, 1984). Cells were incubated in a humidified incubator at 37°C with 5% CO_2. Culture medium was replaced with fresh B27 supplemented Neurobasal medium without L-glutamine on days 1 and 3 after plating.

2. 2. Neutral Red Cell Viability Assay

Neutral red (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride, NR) was used to evaluate cell viability of primary cultured neurons. NR is a weak cationic dye that easily permeates cellular membranes. It accumulates in lysosomal compartments of living cells, where it binds to the anionic sites in the lysosomal matrix. After solubilization, NR is detected by measurement of optical density by spectrophotometry. Congo red dye directly binds to amyloid-like peptides (Klunk et al., 1989), and NR is thought to also interact with amyloid in the same manner. Unfortunately, this precludes the use of NR assays in models of Aβ toxcitiy.

Hippocampal and cortical primary neurons were cultured for one week as described. To study possible dose-dependent neuroprotective effects of GT 1061, on day 7 after plating, cells were incubated in fresh Neurobasal medium with 100 μM hydrogen peroxide and either 0, 30, 100, or 300 μM GT 1061 for 24 hours. To study the possible time-dependent effects of GT 1061, cells were incubated in 100 μM GT 1061 and 100 μM H_2O_2 for 24 hours, or 60 or 15 minutes. All treatments were performed in triplicate. Treatment medium was then removed and replaced with Neurobasal containing 25 μg/mL NR. After 3 hours of incubation at 37°C, cells were washed with PBS to remove excess NR. Remaining NR was solubilized with 50% aqueous ethanol with 1% acetic acid. Cell viability was determined by optical density measurement at 540 nm using a microplate reader (Bio-Tek Instruments ®, Ville St. Laurent, QC, Canada).

2. 3. Treatments

Serum deprivation of 24 hours or longer is often used in primary neuronal culture as an experimental paradigm to induce apoptosis. Here, cells are serum deprived for 2 hours prior to addition of agents to reduce basal levels of phosphorylation activity. This makes it easier to distinguish drug effects from endogeneous cell activity in studying phosphorylation-related mechanisms.

All drugs were freshly prepared on the day of experimentation. On day 7 after plating, culture medium was removed and replaced with fresh Neurobasal medium without B27 supplement for 2 hours. GT 1061 (GB Therapeutics/Cita Pharmaceuticals, Mississauga, ON, Canada) was dissolved in sterile water and diluted in Neurobasal to give final concentrations of 0.01, 0.03, 0.1, 0.3, 1, and 3 mM. To study the short-term

effects of GT 1061, the compound was added to the culture medium for 15 minutes and incubated at 37°C. Cells were then washed in ice-cold PBS and frozen at -80° until Western blot analysis.

On the day of protein analysis, plated cells were manually dissociated in RIPA lysis buffer containing detergents and proteases (to prevent degradation of proteins) (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM EDTA, 1% Igepal CA-630, 0.1% SDS, 50 mM NaF, 1 mM $NaVO_3$, 5 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin (Sigma), and 50 μg/mL aprotinin (Sigma)). Samples were further incubated in lysis buffer for 20-30 minutes, then centrifuged to remove cellular debris.

2.4. In Vivo Administration of GT 1061

Three groups of 8 male Sprague-Dawley rats (Charles River), each of 300-350 grams, were acclimatized to housing and handling for one week prior to experimental procedures. Animal procedures were carried out according to protocols and guidelines of McGill University Animal Care Committee and the Canadian Council for Animal Care. 1 or 5 mg/kg GT 1061 in sterile saline or saline vehicle were administered via intraperitoneal injection 15 minutes prior to sacrifice. Following decapitation, brains were rapidly removed into ice-cold PBS, and hippocampal regions were dissected and frozen in 2-isopentane. Collected tissues were stored at -80°C until analysis. On the day of analysis, samples were thawed and homogenized in RIPA buffer by sonication, and centrifuged 1000xg for 1 hour at 4°C to remove cellular debris.

2. 5. Western Blot

Western blot analysis measures levels of specific proteins within a mixture of proteins and molecules. Samples are separated by size and incubated with antibodies to tag a particular target. These antibodies conjugated with a substrate that can be detected and recorded. Most chemicals were obtained from Sigma-Aldrich (ON, Canada)

Western blots were performed on both in vitro cultured cells and in vivo brain tissue homogenates. After tissue lysis and extraction, sample protein levels were measured using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA) and added to 6X sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 1% glycerol, 50 mM dithiothreitol, and 0.1% (w/v) bromophenol blue). Equal amounts of protein (roughly 20 μg of cultured extracts or 100 μg of fresh brain tissue homogenates) were separated by electrophoresis on a 4-20% Novex ® Tris-glycine polyacrylamide gel (Invitrogen), and transeferred onto a Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech, ON, Canada). Membranes were blocked in 6% skim milk powder in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.2% Tween 20) for 1 hour at room temperature before incubation with primary antibody (1:1000 or 1:500) overnight at 4°C with gentle shaking. Primary antibodies used include phospho-Akt (Ser 473), phospho-MAPK p44-42, phospho-CREB (Ser-133), and phospho-PKC(δ) (Cell Signaling Technologies, Beverly, MA, USA). Membranes were rinsed 3 times in TBST and probed with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour at room temperature. Secondary antibodies used included anti-rabbit, anti-goat, and anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Following 4 additional rinses in TBST for 10 minutes each, bands were visualized by

enhanced chemiluminescence detection (Perkin Elmer, Boston, MA, USA) exposed on x-ray film (Perkin Elmer).

Detection of the bound antibodies can occur through several mechanisms. The most common method is detection by enhanced chemiluminescence (ECL). The horseradish peroxidase conjugate reduces hydrogen peroxide in the presence of other reagent substrates, causing an enhancement of the oxidation of luminol to produce a visible wavelength. This signal is detected by x-ray film. This method is one of the more sensitive methods for signal detection. However, it is also sensitive to background signals. Alternatively, horseradish peroxidase can undergo a colourimetric reaction with tetramethylbenzidine or 4-chloro-1-naphthol substrates to give a blue precipitate. Other colourimetric reactions utilize the enzyme alkaline phosphatase, which reacts with a toluidine salt and nitro-blue tetrazolium chloride products to give an insoluble dark purple precipitate. These precipitates can be measured by relative optical densities, using spectrophotometry (www.promega.com). Alkaline phosphatase can also give fluorescent products, whereby phosphate substrates are cleaved to a phosphate and an alcohol. The alcohol product emits excitation wavelengths that are detectable by photosensors. This is considered to be one of the most sensitive detection methods. Radioactive antibodies can give signals detectable by x-ray film. This method bypasses enzymatic reactions, but is not common any longer, due to high expenses and safer alternatives.

Blots were stripped with stripping buffer (62.5 mM Tris (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol) at 50°C for 30 minutes, and rinsed well in TBST for a total of 1 hour. After re-blocking, membranes were incubated with native form antibodies, Akt, ERK 1/2, CREB, PKC(δ), (Santa Cruz Biotechnology) or β-actin (Sigma), and Western

blot analysis repeated. Semi-quantification was performed with MCID image analyzer (Image Research Inc., ON, Canada). Optical densities were estimated for each band and expressed as a ratio of phosphorylated to native proteins.

The limitation of this method is that it is only estimation and not an absolute value. While immunoblotting has been optimized for common use, it is neither precise, nor sensitive to very small changes in protein levels. Semi-quantification is vulnerable to experimenter discrepancy, and levels of variation in semi-quantification can be quite high.

2.6. Statistical Analyses

One and two-way between group analyses of variance (ANOVA), for Western blot and cell viability assays respectively, were performed using GraphPad Prism ® software (GraphPad Software Inc., San Diego, CA, USA), with the level of significance set at $p < 0.05$. Where variance was large, a data transformation of $\sqrt{(\chi+1)}$ was performed.

3. RESULTS

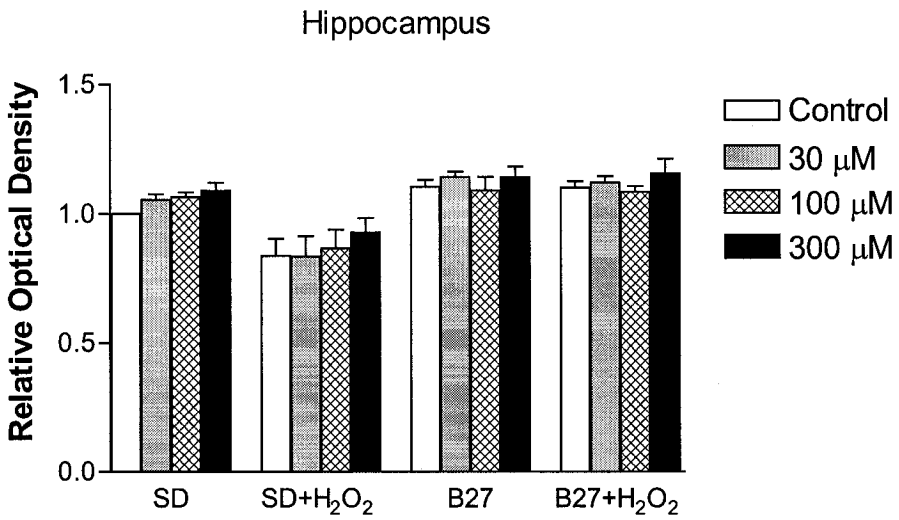
3. 1. Neuroprotection against toxicity

GT 1061 was added to hippocampal and cortical cultures to determine the extent to which it can protect neurons against hydrogen peroxide toxicity. Each culture condition was replicated 3-5 times.

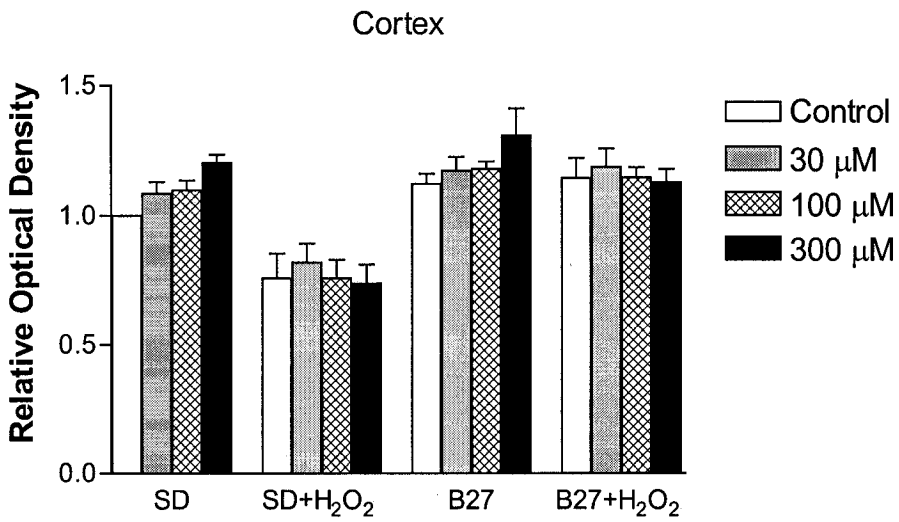
GT 1061 could not protect cortical ($F(9, 48)=0.848$, $p>0.05$) or hippocampal ($F(9, 48)=0.271$, $p>0.05$) neurons against hydrogen peroxide, as measured by Neutral Red cell viability assays. As predicted, B27 was able to protect cells against 100 μM hydrogen peroxide. There was a non-significant trend towards increased cell viability with increasing concentration of 30, 100, 300 μM of GT 1061 (Figure 3). Across both serum deprived and B27 supplemented media, there was also trend towards increased cell viability with short exposure to 100 μM GT 1061 (15 minutes) compared with longer exposures (1 and 24 hours) in cortical cultures, though analysis of variance did not reveal any significant differences (Figure 4).

Figure 3. Neuroprotective effects of increasing GT 1061 concentration on hippocampal (A) and cortical (B) cultures exposed to hydrogen peroxide.

A.



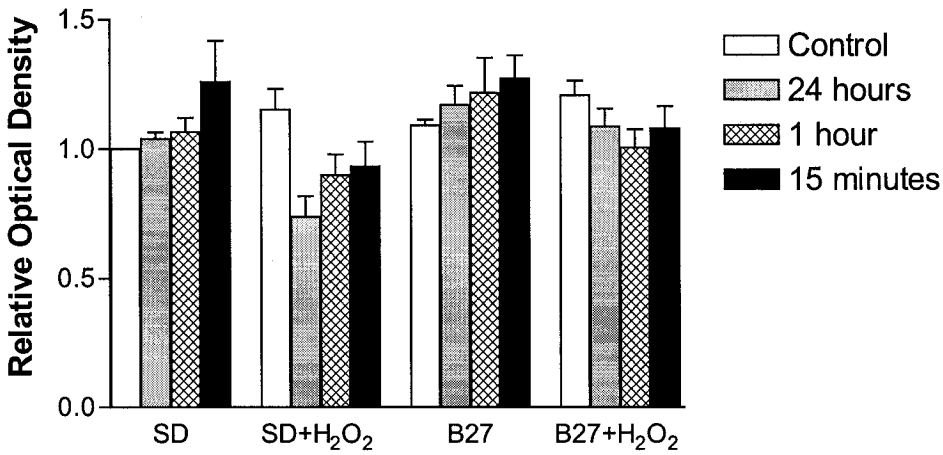
B.



Hippocampal and cortical neurons were grown in either serum deprived (SD) medium or B27 supplemented medium (B27). Half of each type of culture was exposed to 100 μM hydrogen peroxide (H_2O_2). Values are expressed as ratio relative to serum deprived control cultures (far left-hand bar), and represent mean ± standard error of the mean

(SEM) of 3-5 replicates. Hydrogen peroxide elicits neurotoxic response in serum deprived cells, but not in B27 supplemented cells. There are non-significant trends showing increased cell survival with increasing GT 1061 concentration.

Figure 4. Neuroprotective effects of GT 1061 across different exposure durations in cortical cultures exposed to hydrogen peroxide.



Cortical neurons were grown in either serum deprived (SD) medium or B27 supplemented medium (B27). Half of each type of cultures was exposed to 100 μM hydrogen peroxide (H_2O_2). Values are expressed as ratio relative to serum deprived control cultures (far left-hand bar), and represent mean ± standard error of the mean (SEM) of 3-5 replicates. Non-significant trends suggest a time-dependent increase in cell survival with shorter GT 1061 treatment duration.

3. 2. In vitro phosphorylation of downstream targets

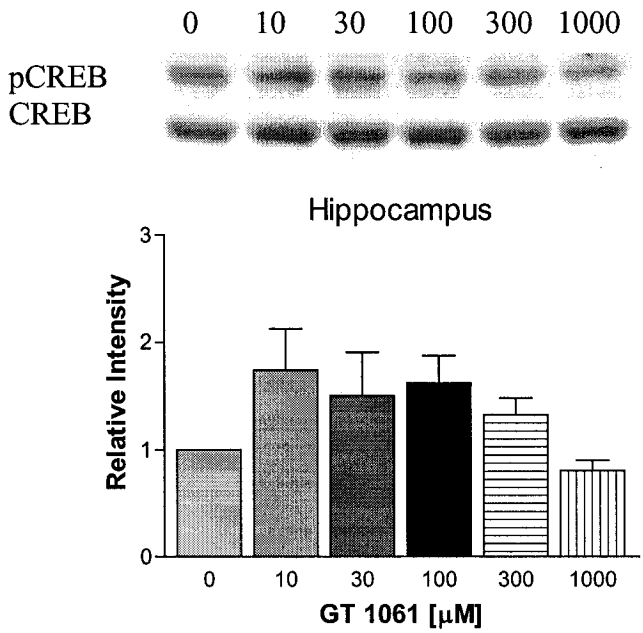
Levels of phosphorylated Akt, MAPK, CREB, and PKC(δ) were measured by immunoblotting of cultured hippocampal and cortical neurons after treatment with different concentrations of GT 1061. Each Western blot was repeated 3-5 times, and semi-quantification was expressed relative to control culture conditions. There were no significant differences between any treatment groups, as evaluated by between group analysis of variance [pAkt: $F(5, 17)= 1.02$, $p>0.05$; pMAPK: $F(5, 17)=1.84$; pCREB: $F(5, 17)=1.97$, $p>0.05$; pPKC(δ): $F(5, 17)=1.04$, $p>0.05$].

The mean level of phospho-CREB in hippocampal cultures was increased by roughly 50% at 10, 30, and 100 μM concentrations of GT 1061 compared with control cultures (Figure 5A). However, significance was not reached, partly owing to large sample variation, and this was not altered by a data transformation [$F(5, 17)=2.15$), $p>0.05$]. Phospho-CREB levels were also increased slightly in cortical cultures at 30 μM GT 1061, though there was no increase at other concentrations (Figure 5B).

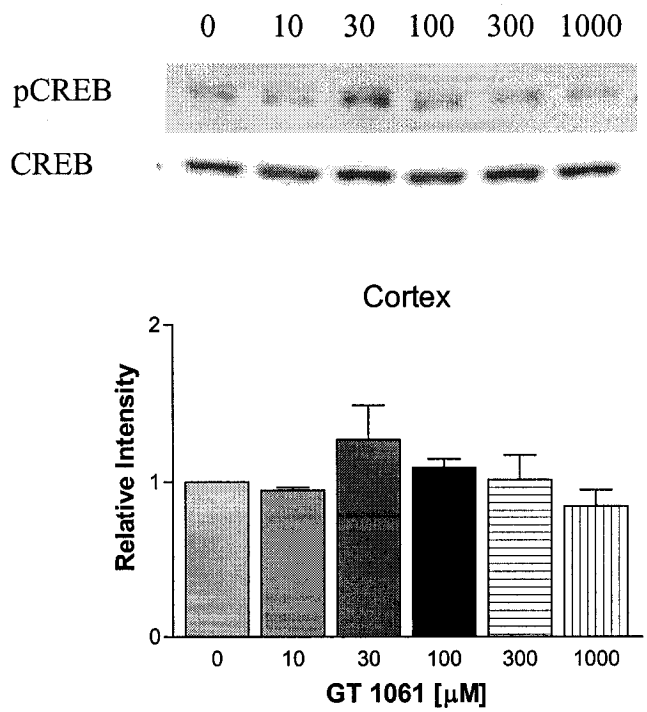
There also appeared to be a small increase in phospho-MAPK levels at 30 μM in hippocampal cultures (Figure 6A), but not in cortical cultures (Figure 6B). There was very little change in levels of phosphorylated Akt (Figure 7) and PKC(δ) (Figure 8) independent of GT 1061 concentration in either hippocampal or cortical cultures.

Figure 5. Phosphorylated CREB levels from hippocampal (A) and cortical (B) cultured neurons

A. Hippocampus



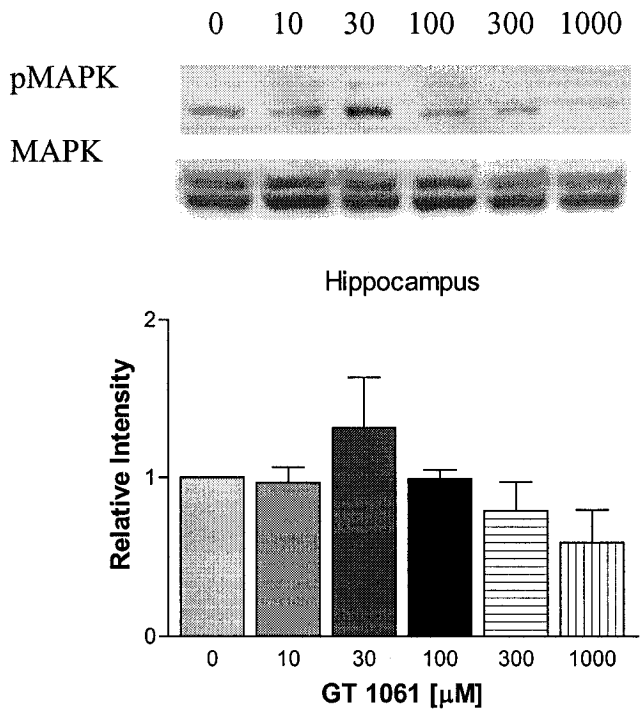
B. Cortex



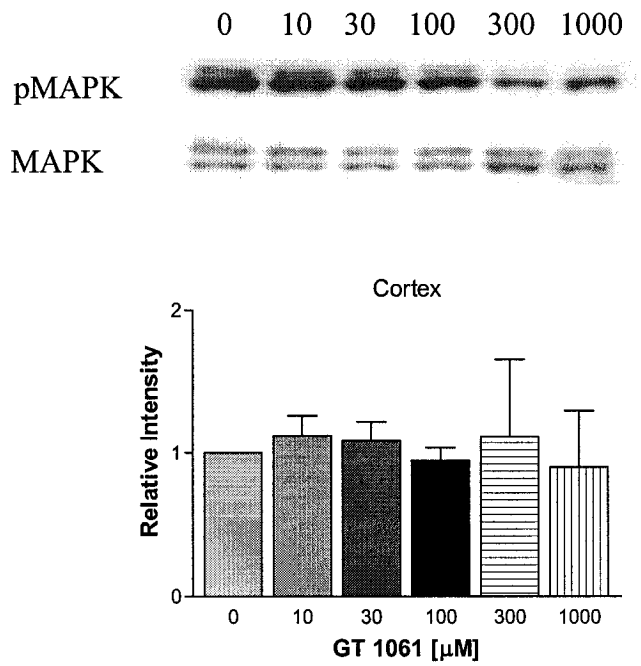
Representative Western blots of phospho-CREB from A) hippocampal and B) cortical cells. Histograms show means $\pm$ SEM of replicated (3-5 times) Western blots, expressed as ratio relative to control cultures (0 μM GT 1061). An increase in hippocampal pCREB signal occurred at 10, 30, 100, and 300 μM GT 1061 in hippocampal cells failed to reach statistical significance, possibly due to high variability. Note the larger scale of the intensity axis in (A). In cortical cultures, an increase in phospho-CREB was detected at 30 μM, but did not reach statistical significance.

Figure 6. Phosphorylated MAPK p44/42 levels from hippocampal (A) and cortical (B) cultured neurons

A. Hippocampus



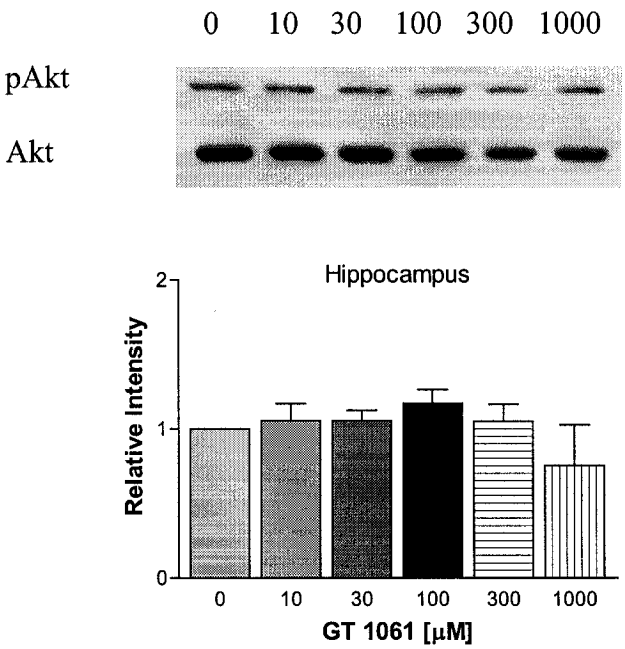
B. Cortex



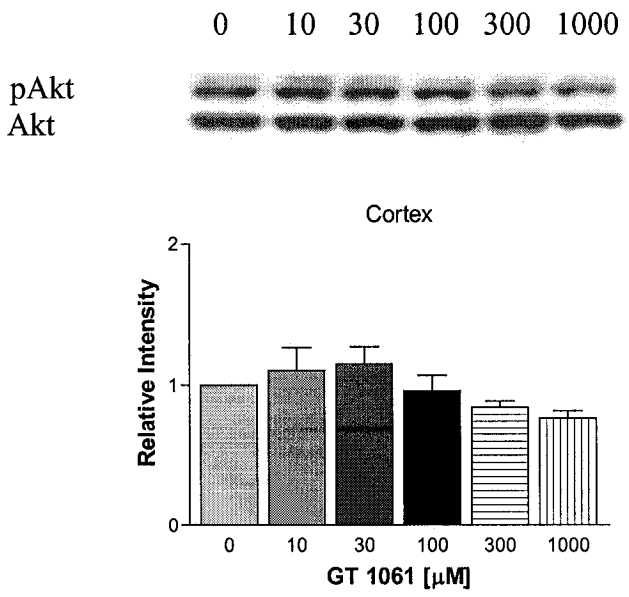
Representative Western blots of phospho-MAPK p44/42 from A) hippocampal and B) cortical cells. Histograms show means $\pm$ SEM of replicated (3-5 times) Western blots, expressed as ratio relative to control cultures (0 μM GT 1061). An increase in phospho-MAPK p44/42 signal was present at 30 μM GT 1061 in hippocampal cells but did not reach statistical significance. There was no discernable change in cortical phospho-MAPK p44/42 levels.

Figure 7. Phosphorylated Akt levels from hippocampal (A) and cortical (B) cultured neurons

A. Hippocampus



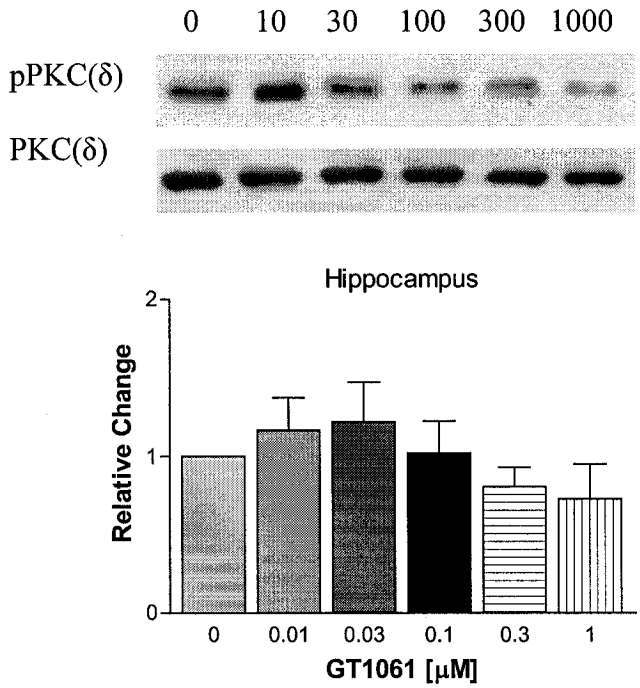
B. Cortex



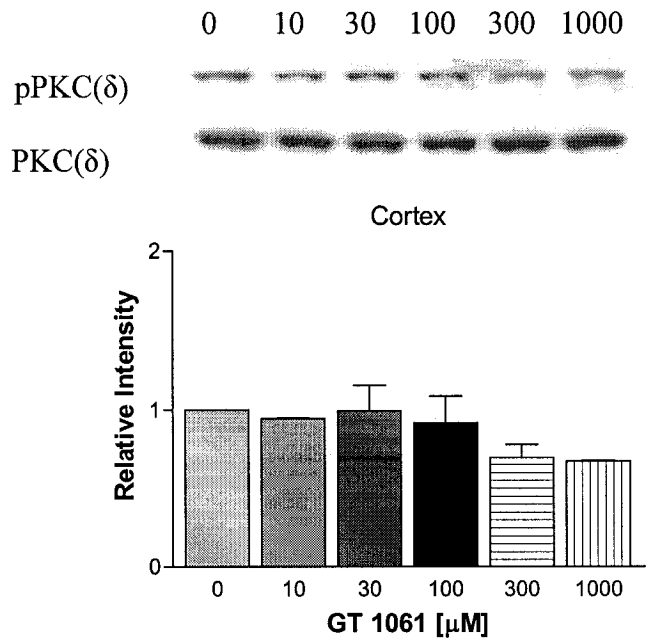
Representative Western blots of phospho-Akt from A) hippocampal and B) cortical cells. Histograms show means $\pm$ SEM of replicated (3-5 times) Western blots, expressed as ratio to control cultures (0 μM GT 1061). No qualitative or semi-quantitative differences between different GT 1061 concentrations were observed.

Figure 8. Phosphorylated PKC(δ) levels from hippocampal (A) and cortical (B) cultured neurons

A. Hippocampus



B. Cortex



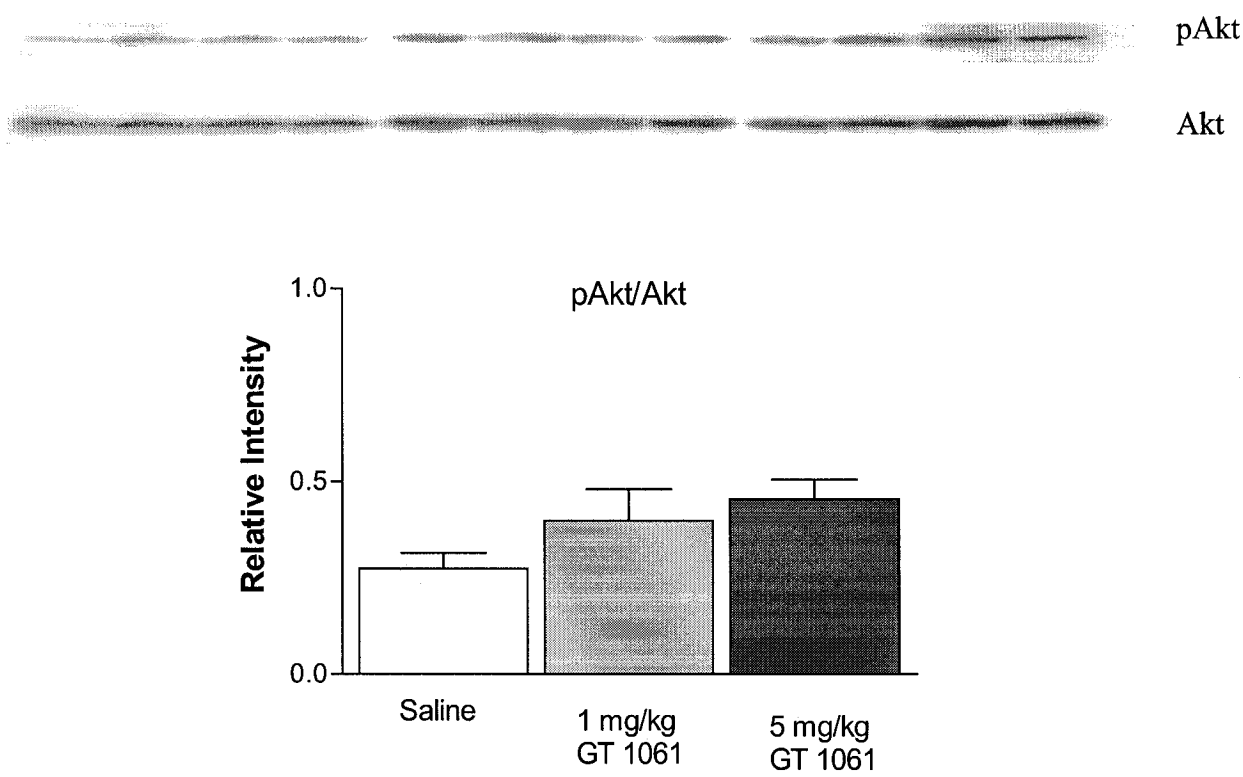
Representative Western blots of phospho-PKC(δ) from A) hippocampal and B) cortical cells. Histograms show means ± SEM of replicated (3-5 times) Western blots, expressed as ratio to control cultures (0 μM GT 1061). No qualitative or semi-quantitative differences between different GT 1061 concentrations were observed.

3. 3. In vivo phosphorylation of downstream targets

GT 1061 was administered to groups (n=8) of adult male Sprague-Dawley rats through intraperitoneal (i.p.) injection, and Western blot analyses of in vivo brain extracts were performed. Levels of phosphorylated Akt, MAPK, CREB, and PKC(δ) were semi-quantified, and expressed as a ratio of phosphorylated protein to native species.

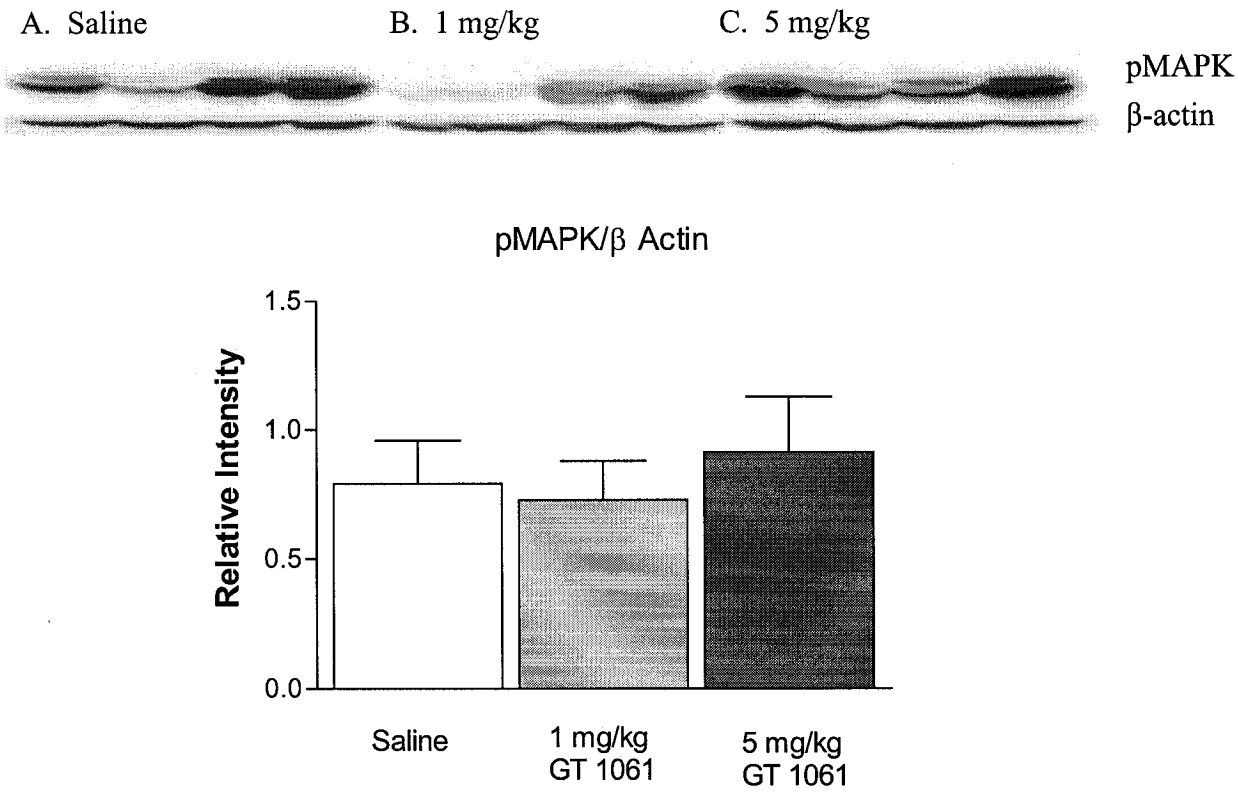
Analysis of variance did not reveal any statistically significant differences between groups [pAkt: $F(2, 23)=2.31$, $p>0.05$; pMAPK: $F(2, 23)=0.276$, $p>0.05$; pCREB $F(2, 23)=0.791$, $p>0.05$; pPKC(δ): $F(2, 23)=0.828$, $p>0.05$], even after data transformation [pAkt: $F(2, 23)=2.43$, $p>0.05$; pMAPK: $F(2, 23)=0.228$, $p>0.05$; pCREB $F(2, 23)=0.770$, $p>0.05$; pPKC(δ): $F(2, 23)=0.686$, $p>0.05$]. However, there was a minor trend towards increased phospho-Akt with increasing concentration of GT 1061 (Figure 9). Despite extensive acclimatization procedures prior to experimental manipulation, there was wide variability between animals in levels of phospho-MAPK (Figure 10). However, when grouped together, levels of phospho-MAPK did not change significantly with GT 1061, nor did phospho-CREB (Figure 11). Finally, phospho-PKC(δ) also did not change considerably either at 1 mg/kg or 5 mg/kg concentrations, compared with saline-injected controls (Figure 12).

Figure 9. Phosphorylated Akt levels in hippocampal tissue after intraperitoneal injection of GT 1061



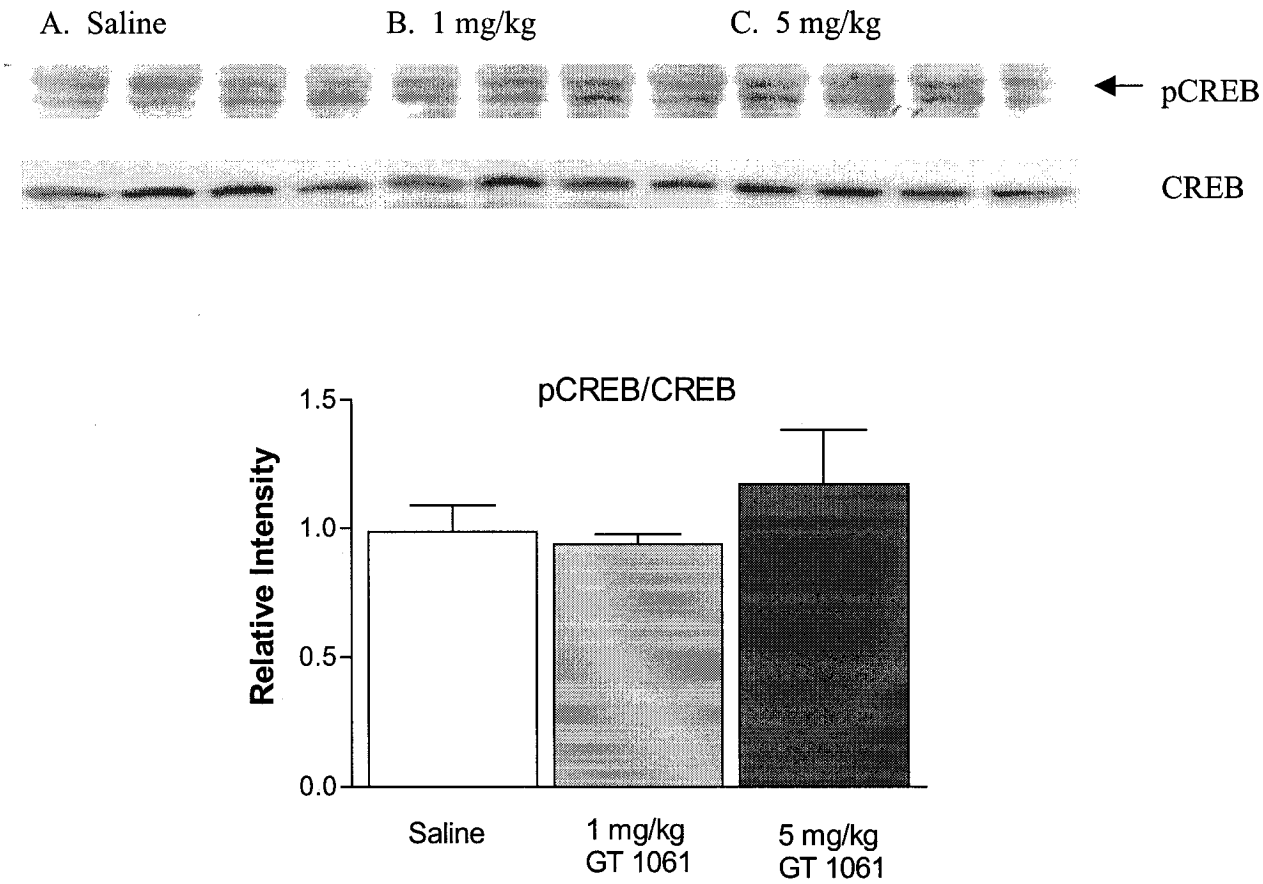
Each band represents protein from a single rat. Representative Western blots for rats given i.p. injections of (A) saline, (B) 1 mg/kg GT 1061, and (C) 5 mg/kg GT 1061. Histograms express the ratio of phosphorylated versus native Akt signal intensity, means from each group ± SEM, showing slight dose-dependent increases in phospho-Akt levels.

Figure 10. Phosphorylated MAPK p44/42 levels in hippocampal tissue after intraperitoneal injection of GT 1061



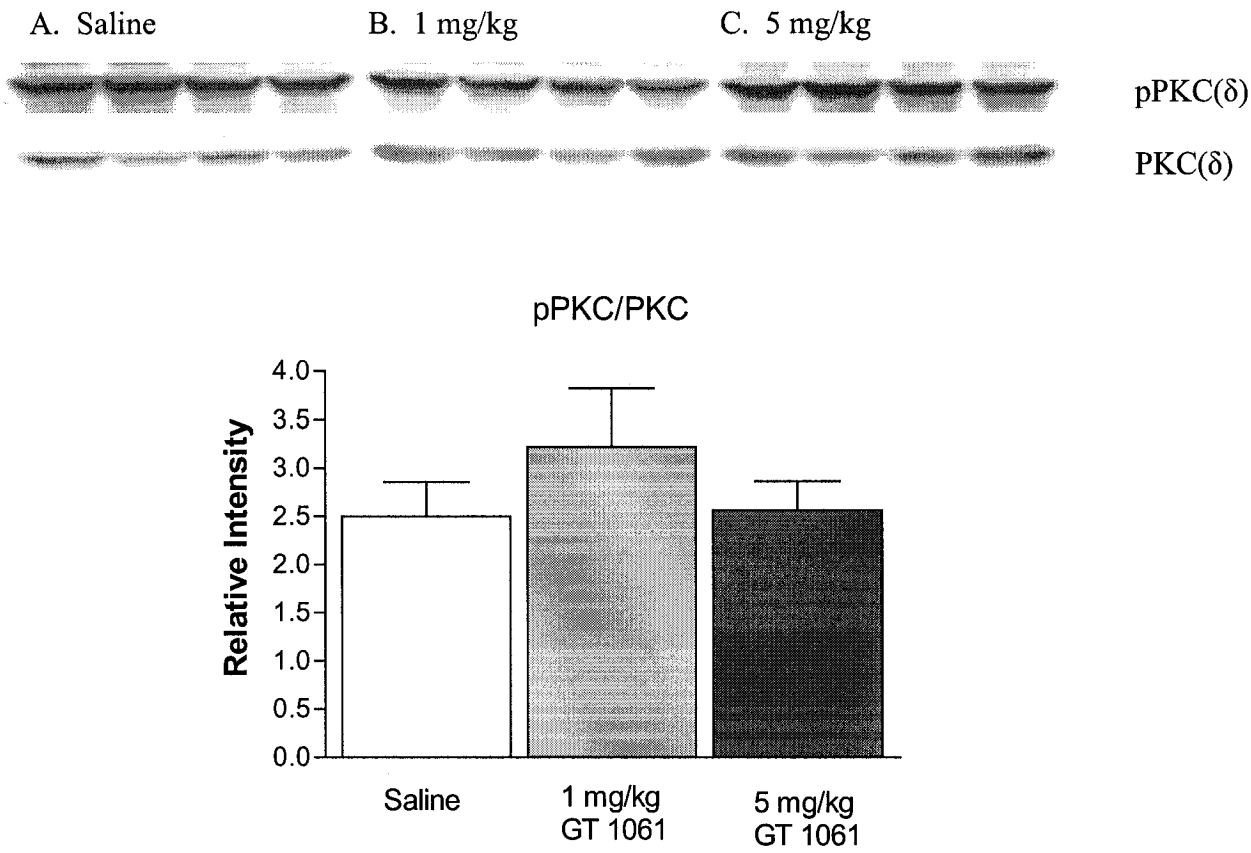
Each band represents protein from a single rat. Representative Western blots of rats given i.p. injections of (A) saline, (B) 1 mg/kg GT 1061, and (C) 5 mg/kg GT 1061. Histograms express the ratio of phosphorylated MAPK p44/42 versus β-actin signal intensity, means from each group ± SEM, showing no discernable change in levels of phospho-MAPK p44/42 with GT 1061.

Figure 11. Phosphorylated CREB levels in hippocampal tissue after intraperitoneal injection of GT 1061



Each band represents protein from a single rat. Representative Western blots of rats given i.p. injections of (A) saline, (B) 1 mg/kg GT 1061, and (C) 5 mg/kg GT 1061. Histograms express the ratio of phosphorylated versus native CREB signal intensity, means from each group ± SEM, showing no discernable change in levels of phospho-CREB with GT 1061.

Figure 12. Phosphorylated PKC(δ) levels in hippocampal tissue after intraperitoneal injection of GT 1061



Each band represents protein from a single rat. Representative Western blots of rats given i.p. injections of (A) saline, (B) 1 mg/kg GT 1061, (C) 5 mg/kg GT 1061. Histograms express the ratio of phosphorylated versus native PKC(δ) signal intensity, means from each group ± SEM, showing no discernable change in levels of phospho-PKC(δ) with GT 1061.

4. DISCUSSION

This was the first series of studies to investigate the molecular effects of GT 1061, a novel nitric oxide mimetic. Neuronal loss is a central pathology of AD, and NO and NO-related signalling have been shown to modulate, and possibly mediate directly, cell survival. Therefore, we evaluated the capacity of GT 1061 to protect neurons from hydrogen peroxide toxicity. We also investigated the possible activation of established cell survival and memory-related signalling pathways using both in vitro and in vivo models.

4. 1. GT 1061 does not confer protection against hydrogen peroxide toxicity in cultured cells

GT 1061 did not significantly increase cell viability in cultures with or without hydrogen peroxide (modeling oxidative stress), and with or without B27 growth supplement (modeling serum deprivation). These results were rather unexpected.

It is possible that the effects of GT 1061 are dependent on the model of insults. Our cell viability assays showed that GT 1061 is not neuroprotective against hydrogen peroxide oxidative toxicity, and with serum deprivation, only showed slight trend for protection in cortical cells. It is possible that other cytotoxic insults might have been able to trigger greater protective effects. As a nitric oxide mimetic it may have been better able to protect cells against superoxide, which is endogenously neutralized by NO binding. Previously, GT 1061 was shown to rescue cognitive ability in models of cholinergic system disruption (Thatcher et al., 2004). Perhaps GT 1061 is particularly effective against agents that disrupt cholinergic function.

Another functionally relevant model of cytotoxicity that could be used is beta-amyloid. We initially studied the effects of GT 1061 (30-300 μM) on $A\beta_{25-35}$ toxicity (25 μM) of cultured neurons using MTT cell viability assays. Our preliminary data showed that GT 1061 afforded very little neuroprotection against Aβ toxicity (data not shown). However, concerns arose regarding the chemical compatibility of the nitrate-based drug with MTT (personal communication, J. Reynolds et al.). Additionally, NR is thought to bind to amyloid regardless of whether or not cells are alive, thus, rendering NR cell viability assays incompatible with this model. Other strategies such as fluorescent labelling should be used to accurately assess GT 1061's effects against Aβ toxicity.

Does the lack of neuroprotective capacity render GT 1061 unsuitable as an AD therapy? Not necessarily. Currently-marketed cholinesterase inhibitors are first-line defenses in AD therapy, and yet each have different neuroprotective capacities and different mechanisms by which they may protect cells. In a study by Arias and colleagues (2005), cultured neurons exposed to $A\beta_{25-35}$ and okadaic acid were most protected by donepezil, followed by galantamine and rivastigmine, while tacrine showed no cytoprotection. Akasofu and colleagues (2003) also showed that donepezil, but not tacrine, rivastigmine, or galantamine, had neuroprotective effects against oxygen-glucose deprivation of cortical cell cultures. This suggests that neuroprotection of currently used AD drugs may be distinct from clinical efficacy and dependent on injury model.

4. 2. GT 1061 only modestly stimulates CREB phosphorylation in vitro

Western blot analyses were used to measure relative protein levels in hippocampal and cortical cultures. No significant increases were observed following treatment with

GT 1061. Comparisons of drug-treated and control cells suggest that CREB may possibly be a weak target for GT 1061 in cultured cells. There was also a slight increase in phospho-MAPK levels in hippocampal culture. The effect of GT 1061 on phospho-CREB is somewhat greater on hippocampal cultured cells than on cortical cells, suggesting that GT 1061 might be more effective in the hippocampus than the cortex, although this will have to be confirmed by additional experiments.

Though not strongly stimulated here, MAPK is one of the most common and important kinases upstream of CREB, and the ras/MAPK/CREB cascade has been implicated in learning and memory (Adams & Sweatt, 2002) and in the induction of LTP (Ying et al., 2002). Several mental retardation syndromes have been linked to dysregulation of MAPK, CREB, and associated pathways, including neurofibromatosis (Ingram et al., 2001) and Rubinstein-Taybi Syndrome (Alarcon et al., 2004).

In addition to MAPK, PKA and calcium-calmodulins, other pathways that lead to phosphorylation of CREB at Ser-133 include PI3K/Akt (Du et al., 1998), and PKC (Johannessen et al., 2004). However, our results do not show concomitant increases in either Akt or PKC(δ), suggesting that these last two pathways do not contribute to the small effect of GT 1061.

4. 3. GT 1061 does not stimulate CREB phosphorylation in vivo

Causal effects are relatively easy to observe in isolated vitro models. In comparison, intact in vivo models involve many different interacting systems. Therefore, it is important not to generalize results from one type of model to another. In our experiments, the slight increases in phospho-CREB and phospho-MAPK levels seen in

vitro were not reproduced in the in vivo model. No significant changes in phosphorylation levels were observed, though there was a trend towards phospho-Akt stimulation in vivo, which was not seen in vitro.

Phospho-Akt has been shown to play an active role in learning and memory. Robles et al., (2003) demonstrated that Akt is also upregulated after training in a hole-board spatial discrimination task. Using the radial arm maze, Mizuno and colleagues (2003) showed that maze training induced significant phosphorylation of hippocampal PI3-K and Akt, and infusion of BDNF antisense inhibited both memory and memory-induced phosphorylation of PI3-K. This may suggest that phosphorylation of the Akt pathway is activity dependent. In comparison, phospho-MAPK was not increased after spatial learning, and decreased from basal levels after BDNF antisense infusion, suggesting that MAPK phosphorylation may be less activity-dependent and more neurotrophin-dependent. In our experiment with GT 1061, we observed a trend towards increased phospho-Akt levels with GT 1061 while MAPK was not affected. This might suggest that GT 1061 could play a role in enhancing activity-dependent cognitive function. It would thus be of interest to establish if spatial learning and injection of GT 1061 could together induce a significant increase in the phosphorylation and activation of Akt.

Some precautions must be taken in the interpretation of our results due to limitations of the experimental model. Large variations are observed between samples from different animals (for example, levels of phospho-MAPK). This can create difficulties in qualitative and semi-quantitative interpretation. Another limitation is related to the temporal and spatial range of the drug's activity. We studied the effects on

the hippocampus because this structure is involved in almost every working or spatial memory-related event, and there is a high probability of observing memory-related changes in this area. Other areas that should have been investigated are the pre-frontal or temporal cortices, as both are relevant to memory (Kessels et al., 2000; Markowitsch et al., 1993; Rosen et al., 2005; Rowe et al., 2000; Squire et al., 1991).

In addition to these limitations, our results must be interpreted in the context of several variables. Previous characterization of this compound showed that GT 1061 reaches peak absorption into the bloodstream by approximately 3 minutes, and plasma levels of GT 1061 are not detectable by about 60 minutes (personal communication, J. Reynolds et al.). Initial behavioural testing showing cognitive enhancement was first performed after 20 minutes after the administration of the drug. Therefore, we decided to investigate the short-term effects of GT 1061. However, if GT 1061's effects are primarily involved with memory consolidation or retention, rather than acquisition, the drug's optimal action on the brain may possibly occur at a later time point. Moreover, events further downstream in signalling cascades may require longer to be fully activated. It is also possible that the availability of the drug varies with the route of administration. Intravenous administration results in a much larger and quicker peak in GT 1061's plasma concentrations, while i.p. injection and oral dosing result in much smaller peaks (personal communication, J. Reynolds et al.). However, plasma concentrations do not necessarily predict effective concentrations to specific brain regions, and drug availability to specific brain regions has not been previously measured. Thus, comparative in vitro and in vivo concentrations of the drug have yet to be determined, making it rather difficult to compare results obtained in our two different model systems.

Previous experiment using sodium nitroprusside (SNP), an NO donor, as a positive control, have indicated that NO downstream signalling pathways appear to be intact in embryonic primary cell culture (data not shown). However, a direct measurement of sGC activation by SNP or GT 1061 has yet to be taken.

GT 1061 has been shown to ameliorate memory deficits caused by amnesic agents, but behavioural performance did not improve above normal in unchallenged animals (Thatcher et al., 2004). Certain cellular events, (for example, some forms of MAPK phosphorylation, as mentioned previously), are activated only as reaction to injury or insult. Hence, differential levels of phosphorylated proteins may be dependent on the environment. In our in vitro model, we observed increases in CREB and MAPK phosphorylation levels in cells that had been serum deprivation for 2 hours prior to treatment, but in our in vivo studies, we investigated intact, young adult rats with no challenge. It is possible that effects of GT 1061 are more pronounced in models of injury or impairment, rather than at normal, non-challenged conditions. It would thus be of interest to investigate the effects of GT 1061 in aged and memory impared rats, for example.

4. 4. Possible Neuromodulatory Targets

It is difficult to conclusively elucidate the mechanism of GT 1061 with this data. Many different signalling pathways converge on Akt, MAPK, CREB, and PKC phosphorylation, and collectively, they are extremely broad-reaching signalling targets. All of these targets have been implicated in mediating neuroprotection, synaptic

remodeling, or modulation of synaptic transmission, so it is somewhat surprising that we failed to observe more pronounced modulatory effects of GT 1061 on these markers.

One possibility is that GT 1061's actions are not expressed simply by an increase in phosphorylation levels of these targets, but by more specific changes. For example, CREB exists in two isoforms: CREB1 is a transcriptional activator, while CREB2 is a transcriptional repressor (Karpinski et al., 1992). In addition, aged, memory-impaired rats show decreases in CREB1, but not CREB2 (Brightwell et al., 2004). Could GT 1061 act by shifting the balance of phosphorylated CREB in favour of the CREB1 isoform? MAP kinases and PKC are also members of large families of isoforms and related kinases. GT 1061 may act specifically on a single subset of these kinases.

4. 5. Studying Non-Human Models of Alzheimer's Disease

Some behavioural data suggests that GT 1061 is effective at ameliorating cognitive deficits, shortly after administration (Smith et al., 2000; Thatcher et al., 2004). In future studies on GT 1061, it will be important to consider other models of abnormal cognitive behaviours such as AD.

One of the difficulties in studying AD is finding an appropriate animal model. Like many other neurological and psychiatric disorders, AD's multifactorial neuropathology coupled with cognitive symptomology, makes modeling of any sort, a very difficult task. In vitro cell culture approach is a simple and precise method of studying Aβ toxicity (Busciglio et al., 1992), oxidative stress, and excitotoxicity. Inducing tau hyperphosphorylation and related pathophysiology is possible (Vincent et

al., 1994), but more difficult (Delobel et al., 2003), and reproducing the complexity of AD in culture models is nearly impossible.

Some inference to cognitive processes can be made from in vivo models, but only if the disease or dimension is authentically represented in the animal. There has yet to be an animal model developed that successfully encompasses all clinical and pathological criteria of AD.

An animal's cognitive ability can be extrapolated through measurements of behaviour. Dementia-like behaviour is often correlated to performance on learning and memory tasks. For rodents, common spatial memory tests include the Morris Water Maze, radial arm maze, and Y- or T- mazes. These tests measure accuracy, search strategy (as assessed by types of errors made and search paths taken), relative speed, and perseverance of learned behaviour. Other forms of memory tested in models of dementia include avoidance behaviour, object recognition and novel-stimulus memory, and working memory-specific tasks, such as delayed-visual match-to-sample tasks. Rodents are often tested on these behavioural tasks after they have been challenged with pharmacological or environmental manipulations known to impair performance. Novel therapeutics can then be assessed based on their ability to rescue learning behaviours.

GT 1061 has been reported to reverse some scopolamine –induced memory deficits in the Morris Water Maze task (Thatcher et al., 2004), and preliminary studies have suggested that it is also beneficial against 192 IgG-saporin –induced deficits in delayed visual matching to sample task (Prusky et al., 2004). Authors also described (though without explicit experimental details) that GT 1061 was effective in reversing scopolamine and 192 IgG-saporin –induced cognitive deficits in step through passive

avoidance tests and 192 IgG-saporin –induced Morris Water Maze deficits (Thatcher et al., 2004).

A variety of transgenic mice lines have been developed as potential animal models of Aβ expression (Kobayashi & Chen, 2005). Mice expressing the 717 (V→F) mutated form of human APP driven by platelet-derived growth factor promoter (PDAPP) show amyloid deposits by 6-9 months, which increase with age (Games et al., 1995). The Tg2576 and the APP23 transgenic mice express the K670N/M671L mutation of human APP driven by the hamster prion protein and murine Thy-1 promoters, respectively. These mice also develop amyloid plaques by 6-9 months (Hsiao et al., 1996; Sturchler-Pierrat et al., 1997). As observed in AD, plaques in APP transgenic mice develop initially in the cortex and hippocampus. Plaques in the septum and thalamus develop later and to a lesser extent, and plaques in the cerebellum are virtually absent. Evidence of hyperphosphorylated tau has been reported in these three transgenic models (Masliah et al., 2001; Sturchler-Pierrat et al., 1997; Tomidokoro et al., 2001a; Tomidokoro et al., 2001b). However, the formation of PHF in APP transgenic mice has yet to be confirmed. PDAPP mice show age-dependent spatial and working memory impairments beginning as early as 3 months, and this may be correlated to Aβ burden (Chen et al., 2000; Dodart et al., 2000; Dodart et al., 1999; Huitron-Resendiz et al., 2002). There is some evidence for alterations in synaptic transmission, which remains to be fully characterized (Larson et al., 1999). Tg2576 transgenic mice also show impairments in spatial memory (Chapman et al., 1999) though there is contradictory evidence over synaptic plasticity dysfunction (Chapman et al., 1999; Fitzjohn et al., 2001).

PS proteins, which are mutated in one form of famililal AD, regulate the γ-secretase enzyme that cleaves Aβ peptides from APP. AD patients with PS mutations show high levels of insoluble Aβ linked with early onset of disease. PS1-null mice are not viable, indicating a critical role for PS1 in development (Shen et al., 1997). PS1 transgenic mice show increased $A\beta_{42}$ (Borchelt et al., 1996; Duff et al., 1996); however mice carrying conditional knockout, overexpression, and knockin mutations of PS1 showed more modest behavioural and morphological abnormalities than human-APP transgenic animals (Janus et al., 2000; Shen et al., 1997; Yu et al., 2001). PS2-null mice also have a mild phenotype, but PS2 transgenic animals show spatial memory deficits in the Morris Water Maze (Herreman et al., 1999; Hwang et al., 2002). Notably, most APP and PS transgenic strains of mice do not show neurodegeneration in the hippocampus, cortex, or anywhere else in the brain (Irizarry et al., 1997; Irizarry et al., 1997; Kohler et al., 2001; Stein & Johnson, 2002).

Recent advances have allowed for the generation of double and triple mutant mice. Combinations of APP, PS, and tau mutations can be manifested simultaneously in a single animal. The TgCRND8 mice with two mutant forms of APP develop amyloid plaques by 90 days of age, and by 1 month with an additional PS1 mutation transgene (Chishti et al., 2001). Plaque deposition spreads from the frontal cortex to the hippocampus, olfactory bulb, and striatum with time. The cerebellum and brainstem are affected last. This temporal pattern of plaque development in the TgCRND8 mouse closely resembles that seen in AD. No atrophy of the dorsal hippocampus or surrounding region was observed (Chishti et al., 2001). These mice showed significantly impaired

spatial and reference memory when assessed in the Morris Water Maze by 11 weeks of age.

The 3xTg-AD triple transgenic mice developed by LaFerla and colleagues incorporate transgene mutations of PS1, APP, and tau (Oddo et al., 2003). Aβ deposits become apparent in the frontal cortex by 6 months, and are evident in the hippocampus and other cortical regions by 12 months. Memory deficits emerge around the same time as Aβ pathology (Billings et al., 2005). Tau pathology is first apparent in the hippocampus, and then progresses to cortical areas (Oddo et al., 2003). By 6 months, synaptic transmission is dysfunctional, and LTP is impaired. Various other multiple transgenic strains have shown phenotypes that include progressive loss of hippocampal synapses and neurons, cytoskeletal abnormalities, gliosis, microglial activation, and hyperphosphorylated tau (Boutajangout et al., 2004; Nalbantoglu et al., 1997; Rutten et al., 2005; Schmitz et al., 2004). Although AD neuropathologies can be modeled using genetic modification, none have been successful in inducing an authentic model of AD itself. Regardless, each genetic modification reflects slightly different facets of AD pathology, and employing these types of models could provide valuable information on the effects of GT 1061 on progressive cognitive loss, as seen in AD.

Drug-induced AD –like pathology has also been modelled in rodents by intracerebroventricular (icv) infusion of Aβ peptides. While this technique is more physically invasive for the animal effects of Aβ on disease pathology can be studied without interfering with early development and maturation, unlike the generation of traditional genetic mutants. Studies have shown that infusion of human Aβ 1-40 in rats disrupts cortical and hippocampal ACh release, striatal dopamine release, Morris Water

Maze and passive avoidance task acquisition, LTP (Itoh et al., 1999; Itoh et al., 1996; Nitta et al., 1997; Nitta et al., 1994), and decreases levels of endogenous antioxidants in the brain (Kim et al., 2003). Thatcher et al. (2004) also reported that GT 1061 was effective in reversing some deficits in Morris Water Maze performance after icv infusion of Aβ1-40, though these results are difficult to interpret given a lack of reported experimental details and controls. It would thus be highly relevant to explore the effects of GT 1061 in aged rodents with memory deficits and in various transgenic mouse models.

4. 6. Other Possible Effects of GT 1061

Classical nitrates such as GTN have been used for many years to treat angina (Warren & Francis, 1978). However, GTN is a potent hypotensive agent, and its use as a CNS therapy has been limited due to risk of cardiovascular side effects. Given its chemistry, it is possible that GT 1061 may also have some vasodilatory actions, but apparently to a much smaller extent. GT 715, another S-nitrate in the same class as GT 1061, has been shown to have remarkably fewer effects on mean arterial blood pressure compared with GTN in anaesthetized rats (Reynolds et al., 2002). In addition, GT 715 was less potent, by one order of magnitude, in relaxing rat aortic muscle compared with GTN (Reynolds et al., 2002). To our knowledge, there have not yet been any published studies on the vasodilatory effects of GT 1061 on brain vasculature.

Impaired regional cerebral blood flow in hypertensive individuals is associated with impaired performance in verbal memory tasks (Jennings et al., 2005). AD patients have been shown to have relatively lower regional cerebral blood flow to prefrontal,

parietal, and possibly temporal regions, and the extent of hypoperfusion has been demonstrated to correlate with Mini-Mental State Examination scores (Montaldi et al., 1990; Muller et al., 1999; Trollor et al., 2005). If GT 1061 is able to effectively improve cerebral blood flow at concentrations low enough to avoid significant peripheral cardiovascular effects, there could be vast therapeutic potential, not only for the treatment of AD, but also in vascular dementia and ischemia. In fact, subcutaneous injection of GT 715, 2-4 hours post-ischemia (temporary middle cerebral artery occlusion) has been shown to diminished brain infarct volume (Reynolds et al., 2002). Similar studies on GT 1061 have yet to be published, but similar chemical properties suggest that this molecule could be similarly effective.

5. CONCLUSION

The existing therapies for AD and other cognitive dysfunctions have focused on neurotransmission and the modulation of neurotransmitter levels. However, it is becoming evident that solely treating neurotransmission dysfunction is not enough to halt or reverse AD progression. There is growing interest in targeting intracellular survival pathways not only in order to prevent cell death, but also for directly enhancing cognitive functioning.

As studies on the role of endogenous NO in the nervous system continue, it is increasingly apparent that NO is a versatile molecule involved in many neurophysiological functions, in addition to pathological conditions. The focus is now shifting toward isolating and promoting the cell survival properties of NO whilst bypassing cell death mechanisms. The stimulation of CREB, a crucial mechanism in both memory formation and cell survival, may be a useful therapeutic target in the treatment of AD. However, in our primary cell culture and in vivo models, GT 1061 failed to strongly stimulate the activity of intracellular CREB or related kinases, and was not able to protect neurons against H_2O_2 toxicity. Hence, the molecular mechanism of GT 1061 may involve other signalling pathways, or may be dependent upon particular situation and disease conditions. Further studies will be required to clearly establish the possible use of GT 1061-like molecules in the clinics.

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Appendix A

Ethics Certificates (2)

Appendix B

Publication

Material presented in this thesis has appeared previously as an abstract.

A. M. Li, W. H. Zheng, M. De Somer, A. J. Giovinazzo, & R. Quirion. GT1061, a novel nitric oxide mimetic compound, stimulates the phosphorylation of CREB and protein kinase C in cortical neurons. Program No. 219.8, *2004 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience, 2004. Online.