DYSFUNCTION OF THE CREB SIGNALING PATHWAY DURING 6-HYDROXYDOPAMINE NEUROTOXICITY

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Disruption in important cell survival signaling pathways may represent a central mechanism in neurodegenerative disease processes. 6-Hydroxydopamine (6-OHDA) is an oxidative neurotoxin that is commonly used to injure catecholaminergic cells of the central and peripheral nervous systems, and has been used extensively to model Parkinson's Disease. Although it has been documented that 6-OHDA elicits phosphorylation of several kinases, downstream transcriptional effects that influence neuronal cell death are not well defined. The cAMP response element (CRE) is present in the promoter sequences of several important neuronal survival factors. Treatment of catecholaminergic neuronal cell lines (B65 and SH-SY5Y) with 6-OHDA resulted in repression of basal CRE transactivation, and message levels of CRE-mediated genes such as brain derived neurotrophic factor and the survival factor Bcl-2 were decreased in 6-OHDA-treated cells. Message levels of genes lacking CRE sequences were not affected. Interestingly, repression of CRE could be reversed by delayed treatment with cAMP several hours after initiation of 6-OHDA injury. Furthermore, this restoration of CRE-driven transcription, even up to 2 hours post 6-OHDA treatment, was associated with significant neuroprotection. In contrast to observations in other model systems, the mechanism of CRE repression did not involve decreased phosphorylation of its binding protein CREB. Instead, increased phospho-CREB was observed in 6-hydroxydopamine-treated cells, as both total CREB and phospho-CREB were markedly increased in the cytoplasm after treatment. Nuclear expression of p-CREB showed a

different pattern, and was decreased in the nucleus of 6-hydroxydopamine-treated cells. 6-OHDA also decreased nuclear phospho-CREB in dopaminergic neurons of primary mouse midbrain cultures. Co-treatment with cAMP promoted/restored nuclear localization of phospho-CREB in both immortalized and primary culture systems, a trend that was associated with protection in both B65 and SY5Y cell lines. Additionally, when human Parkinson's/Lewy body brain tissue was examined, an intense clumped or granular distribution of cytoplasmic phospho-CREB was observed in degenerating substantia nigra neurons, with little to no cytoplasmic staining seen in age-matched controls. Overall, these studies suggest a common theme of impaired nuclear-cytoplasmic trafficking during oxidative neuronal injury processes, with disruption in CREB sub-cellular localization being a recurring trend. It is interesting to note that cytoplasmic accumulation of upstream CREB kinases has previously been observed in both 6-hydroxydopamine-treated cells and in degenerating Parkinson's disease neurons, further supporting a potential role for impaired nuclear import of phosphorylated signaling proteins in neuronal injury processes. These studies present important insight into oxidant-mediated modulation of survival signaling pathways in neuronal cells, may offer potential relevance to the pathogenic mechanisms underlying the progression of neurodegenerative disease.

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ABBREVIATIONS

ROS: Reactive Oxygen Species

SOD 1,2: Superoxide Dismutases

O2.- : Superoxide

H2O2: Hydrogen peroxide

CNS: Central Nervous System

PD: Parkinson's Disease

DA: Dopamine

6-OHDA: 6-Hydroxydopamine

DAT: Dopamine Transporter

MAPK: Mitogen Activated Protein Kinase

ERK: Extracellular Regulated Kinase

pERK: Phosphorylated ERK

RSK: p90rsk Kinase

pRSK: Phosphorylated RSK

CREB: cAMP Response Element Binding Protein

pCREB: Phosphorylated CREB

PKC: Protein Kinase C

CaMK: Calmodulin-Dependent Kinase

cAMP: cyclic Adenosine Monophosphate

PKA: Protein Kinase A

CRE: CREB Response Element

NLS: Nuclear Localization Signal

CBP: CREB Binding Protein

HD: Huntington's Disease

BDNF: Brain Derived Neurotrophic Factor

Bcl2: B-Cell leukemia/lymphoma 2

LDH: Lactate Dehydrogenase

Db-cAMP: dibutyrate cAMP

DAPI: 4,6-diamidoino-2-phenylindole Dihydrochloride

EMSA: Electrophoretic mobility shift assay

TH: Tyrosine Hydroxyl

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Well, this day is finally here, and I can hardly believe it. I came to Pittsburgh all those years ago with two goals, and for two reasons... One was for a degree. The other was for a life. And- a PhD, a wedding, a dog and two babies later, I have somehow managed to accomplish both. But of course, none of this would be possible without a little help from my friends. Accordingly, I want to thank the following…..

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1.0 INTRODUCTION

1.1 OXIDATIVE STRESS IN NEURODEGENERATIVE DISORDERS

1.1.1 Oxidative Stress

Oxidative stress and the damage that it can inflict on neurons has been implicated in the pathogenesis of many neurodegenerative diseases (Beal 1995) (Souza et al. 2000) (Tyurin et al. 2000). Oxidative stress – induced injury is believed to result from an imbalance between the production of reactive cellular oxidants and the ability of a cell's endogenous antioxidant defenses to control them. Cellular oxidants are generated normally throughout the life of a cell by standard metabolic processes, with a major source of reactive oxygen species (ROS) coming from the mitochondrial electron transport chain (Lass et al. 1997). Additional oxidants can be generated, however, by non-metabolic and aberrant processes within the cell through exposure to cytotoxic chemicals, ionizing radiation, and/or certain drugs (Galan et al. 2001). Once generated, and if not neutralized, these reactive oxidants can inflict damage on proteins, DNA and lipids because of their ability to oxidize these important cellular components and alter them both structurally and functionally. Indeed, markers of oxidative stress like lipid peroxidation and protein nitration are found in postmortem examination of brain tissues from patients with a variety of neurodegenerative disorders (Sayre et al. 2001).

In the normal, healthy cell, antioxidant defense systems exist to counteract and remove harmful oxidants. For example, the highly reactive superoxide ($O_2^{\cdot-}$), which is generated in large amounts by the electron transport chain, is quickly and efficiently converted within the cell to the less toxic H_2O_2 by the superoxide dismutases, SOD1 and SOD2 (Melov et al. 1998). Since H_2O_2 itself can further react to produce the even more damaging hydroxyl radical ($^{\cdot}OH$), enzymes such as catalase and glutathione peroxidase also exist within cells to detoxify H_2O_2 by converting it to O_2 and H_2O, and therefore limit its availability to do damage the cell. It is ultimately when ROS levels within a cell exceed the cell's antioxidant capacity that the condition of oxidative stress occurs.

Neurons within the CNS seem to be particularly vulnerable to oxidative stress due to a variety of reasons. For example, neurons exhibit a relatively high level of metabolic activity along with low levels of some antioxidant enzymes like catalase and glutathione peroxidase, and neurons possess an inherently high amount of easily oxidized substrates, like membrane lipids (Reviewed in (Langley and Ratan 2004)). Additionally, specific neuronal subtypes can have added sources of ROS generation. For example, dopaminergic neurons may be further vulnerable to oxidative stress because dopamine itself can undergo spontaneous or enzyme-catalyzed oxidation to generate its own source of reactive oxidants (Reviewed in (Olanow 1990)). Understanding how the homeostatic balance between oxidants and antioxidant defenses is maintained in healthy cells, as well as how this balance gets tipped in favor of oxidants during neuronal death processes is critical to understanding the pathogenesis of neurodegenerative diseases such as Parkinson's Disease, Alzheimer's Disease and Amyotrophic Lateral Sclerosis (ALS). Given the progressive nature of these diseases, a better understanding of mechanisms that could disrupt antioxidant responses as well as impair a cell's

adaptive responses to injury is critically important and could lead to new strategies in the development of therapeutic agents for neurodegenerative disorders.

1.1.2 Parkinson's Disease

Parkinson's disease (PD) is a common neurodegenerative disorder, affecting approximately 1-2% of people over the age of 65. It is pathologically characterized by a selective loss of dopaminergic (DA) neurons within the mesencephalon (specifically the substantia nigra pars compacta) (Lang and Lozano 1998), and a concurrent degeneration and dysfunction of the nigrostriatal pathway. With the progressive loss of the nigrostriatal dopaminergic neurons, there is a corresponding decrease of dopamine content in both the substantia nigra and striatum. It is the loss of these dopamine-producing projections that is thought to account for the majority of physical and motor deficits seen in PD.

Clinically, Parkinsonian symptoms are characterized and defined by the triad of bradykinesia (slowness and decreased amplitude of movement), tremor at rest, and muscle rigidity, and these symptoms as well as the underlying neuropathological hallmarks have been well-characterized. However, the molecular mechanisms that lead to the progressing neurodegeneration still remain elusive. While PD can affect some individuals with specific genetic susceptibilities, the genetic burden of actual mutations accounts for only 5-10% of the overall PD population. (Gandhi and Wood 2005). The majority of cases occur sporadically in the aging population and lack a clear etiology. However, the remarkably consistent phenotype across all PD cases, whether genetic or sporadic, has lead to the conclusion that there may be common molecular mechanisms that underlie all cases of the disease.

As previously stated, it is widely believed that oxidative mechanisms are a causative factor in the progression of Parkinson's Disease, and evidence has long existed for the presence of increased oxidative stressors in PD brain (Beal 1995) (Souza et al. 2000) (Tyurin et al. 2000). For example, elevated levels of lipid peroxidation markers (4-hydroxynonenal and malonialdehyde) as well as evidence for protein nitration have been found in the substantia nigra of PD patients (Andersen 2004), and reduced levels of the antioxidant glutathione can been seen at early stages of nigral cell loss ((Sian et al. 2004)). Additional support for a role of oxidative stress in PD progression has come from studies of the rarer genetic forms of the disease. For example, mutations in *parkin*, a ubiquitin protein ligase that is mutated in some autosomal recessive forms of PD, have been shown to elevate levels of oxidative stress upon expression and make cells more sensitive to cell death processes. (Hyun et al. 2002). Additionally, overexpression of wild type DJ-1, another gene found mutated in some autosomal recessive forms of PD, appears to protect cells from oxidative stress induced by H_2O_2 (Canet-Aviles et al. 2004). While the exact function of DJ-1 is still unclear, evidence suggests it plays an important role in protecting neurons from mitochondrial-induced oxidative stress (Gandhi, 2005). Taken together, these independent lines of evidence strongly suggest that oxidative mechanisms are playing a role in the neuronal dysfunction and death that occurs in Parkinson's Disease. Defining components of oxidant-induced death pathways in neurons is a valuable pursuit and can potentially lead to the development of novel therapeutic targets and/or agents for fighting this disease. Since the majority of patients afflicted with PD retain otherwise normal cognitive function, the development of strategies to slow or halt the damage occurring in dopaminergic neurons could significantly improve their quality of life.

1.1.3 6-OHDA Model

All commonly accepted models of Parkinson's disease, like the actual disease itself, are thought to involve oxidative processes at the heart of dopaminergic injury. Examples of such models include neuronal death induced by exposure to the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP) (Cadet and Brannock 1998), the stimulant methamphetamine(O'Dell et al. 1991), and the dopamine analog 6-OHDA, which we have chosen to utilize in these experiments. Like dopamine, 6-OHDA can oxidize to generate a wide spectrum of oxygen radicals (Cohen and Heikkila 1974), and targeted intrastriatal injections of either compound produce selective dopaminergic injury in a pattern that is almost identical to the loss and degeneration seen in PD brain (Przedborski et al. 1995) (Zigmond and K.A 1997) and (Hastings et al. 1996). Additionally, 6-OHDA has been isolated from PD patients (Andrew et al. 1993), making this neurotoxin a potentially valuable tool to further investigate the role of oxidative stress in PD.

The *in vitro* system utilized for studies included in this dissertation involves the use of two neuronal cell lines. The first is the CNS-derived B65 cell line, which expresses tyrosine hydroxylase (TH), a precursor enzyme involved in dopamine production, and has electrically excitable membranes that can generate action potentials (Schubert et al. 1974). The second is the human dopaminergic neuroblastoma SH-SY5Y cell line, which expresses dopamine hydroxylase and the dopamine transporter (DAT) (Decker et al. 1993). Cultures of B65 and/or SH-SY5Y cells offer a homogeneous population of neuronal cells to study signaling mechanisms that may be occurring in response to oxidative stress in dopaminergic neurons.

Of course, there are limitations to this model. For example, a transformed cell line cannot be considered completely analogous to mature neurons, and typical neuronal responses

may be lacking in these cells. Therefore, when possible, results obtained were confirmed using primary neuronal cultures obtained from mouse midbrain. Additionally, since we are in the unique position to have access to banked human Parkinson's disease tissue, we were able to use this tissue to further confirm the validity of our observed results as they relate to the human disease itself.

1.2 THE ERK-RSK-CREB NEURONAL SURVIVAL SIGNALING PATHWAY.

1.2.1 ERK Signaling Pathway

Although increasing evidence points to a role for the process of oxidative stress in neuronal degeneration, to date little is known about the molecular players responsible for controlling this avenue of cellular fate. One of the most notable and commonly activated signal transduction pathways within a cell is the Mitogen Activated Protein Kinase (MAP Kinase) signaling cascade. MAP kinases are serine/threonine kinases that play important roles in transducing extracellular signals from the membrane to the nucleus. They are activated by a wide variety of stimuli, ranging from neurosupportive signals like neurotrophic factors, to cell stressors like reactive oxygen species (Reviewed in (Chang and Karin 2001), (Grewal et al. 1999)and (English et al. 1999). The ERK branch of the MAPK pathway includes ERK1 and ERK2 (also known as p44 and p42 MAP kinases), which are activated upon phosphorylation of specific tyrosine and threonine residues by the MAPK kinase, MEK. MEK is in turn activated via phopsphorylation by the MAPK kinase kinase, Raf, which itself can be activated by various mechanisms.

Historically, ERK has been associated with favorable cellular functions, like proliferation, differentiation and survival (Xia et al. 1995) (Segal and Greenberg 1996) and (Ballif and Blenis 2001), and proper ERK function is essential to the normal development and functional plasticity of the CNS (reviewed in (Chu et al. 2004)). Recently however, in our laboratory and in others, the ERK branch of the MAPK family has also been implicated in the death of neuronal cells (see references below). Interestingly, the majority of the studies described to date, using models of neurodegeneration, cerebral ischemia and brain trauma, have involved death induced by oxidative mechanisms, suggesting that ERK may be specifically involved in this mode of neuronal damage. For example, in two laboratories that utilized a glutamate-induced model of oxidative stress, data show that not only does glutamate activate ERK, but inhibition of the upstream ERK activator, MEK, leads to protection from the oxidative toxicity (Stanciu et al. 2000) and (Satoh et al. 2000). This result was evident in a hippocampal cell line (HT22 cells) and primary neuronal cultures. Additionally, it has also been shown that inhibition of ERK phosphorylation (again via MEK inhibition) can protect neurons from damage induced by focal cerebral ischemia in rodents (Alessandrini et al. 1999), as there is increased neuronal survival when ERK activation was blocked. Data from our own laboratory show that in response to the oxidative neurotoxin 6-OHDA, neuronal cells exhibit an abnormal, sustained pattern of ERK phosphorylation, which could be inhibited by MAP kinase kinase (MEK) inhibitors (Kulich and Chu 2001). Since the inhibition of abnormal ERK activation also offered significant protection from the cytotoxic effects of 6-OHDA, these data suggest also that ERK phosphorylation directly contributes to the dopaminergic neuronal death mechanisms that are involved in oxidant-induced neuronal cell death (Kulich and Chu 2001). Given the vastly different and contradictory functions of ERK within the CNS and in various models of death, a

further and more detailed examination of the molecular events that occur downstream of the sustained ERK activation in 6-hydroxydopamine-induced neurotoxicity is essential.

Once activated, ERK can phosphorylate target proteins within the cytoplasm, or can translocate to the nucleus where it can modulate the activity of various transcription factors. One well-known cytoplasmic target of ERK is the serine/threonine kinase, p90rsk. When RSK becomes active, it can similarly target proteins in the cytoplasm, or move to the nucleus (usually co-translocating with ERK) to affect nuclear targets (reviewed in (Grewal et al. 1999)). Members of the RSK family, consisting of RSK1, 2 and 3, are thought to be activated in response to mitogenic stimuli, but the regulation of their activation is complex and not well understood (Alessandrini et al. 1999). Susequent work utilized novel RSK constructs to examine the hypothesis that RSK was a downstream effector of MAP kinase–induced cell survival (Shimamura et al. 2000). These authors found that a kinase-dead mutant of RSK1 inhibited the MEK-dependent survival signal in HEK cells, while a constitutively active RSK1 restored the survival effect observed with activation of MEK. This confirmed that RSK does indeed play a downstream role in this important survival pathway. Additionally, a separate study by Bonni et al (1999) (Bonni et al. 1999) used neuronal primary cultures and showed similar results with RSK2, and went a bit further to suggest that one of the mechanisms by which the MAPK-RSK pathway promotes cell survival is via phosphorylation of RSK's downstream nuclear target, CREB.

1.2.2 CREB.

cAMP-response element binding protein, CREB, belongs to a family of transcription factors that have been implicated in many important neuronal functions (Walton and Dragunow 2000), (Finkbeiner 2000), and (Shimamura et al. 2000). For example, CREB-dependent gene

expression has been reported to play a role in such diverse processes as cell survival, plasticity, growth and development, and most recently, cell death. In neurons and other cells, CREB and its family members function as effector molecules that bring about changes within a cell in response to a wide range of signals. Diverse extracellular stimuli such as growth factors, hormones, membrane depolarization and Ca2+ influx can all cause activation of CREB, and the multiple different signaling cascades all converge to phosphorylate a critical CREB residue-Serine 133 (Reviewed in (Mayr and Montminy 2001)). Since CREB is phosphorylated in response to such diverse signals, as well as through such diverse intracellular pathways (Figure 1), just how specificity is achieved has been the subject of much debate. Unfortunately, these complex mechanisms are still not totally clear.

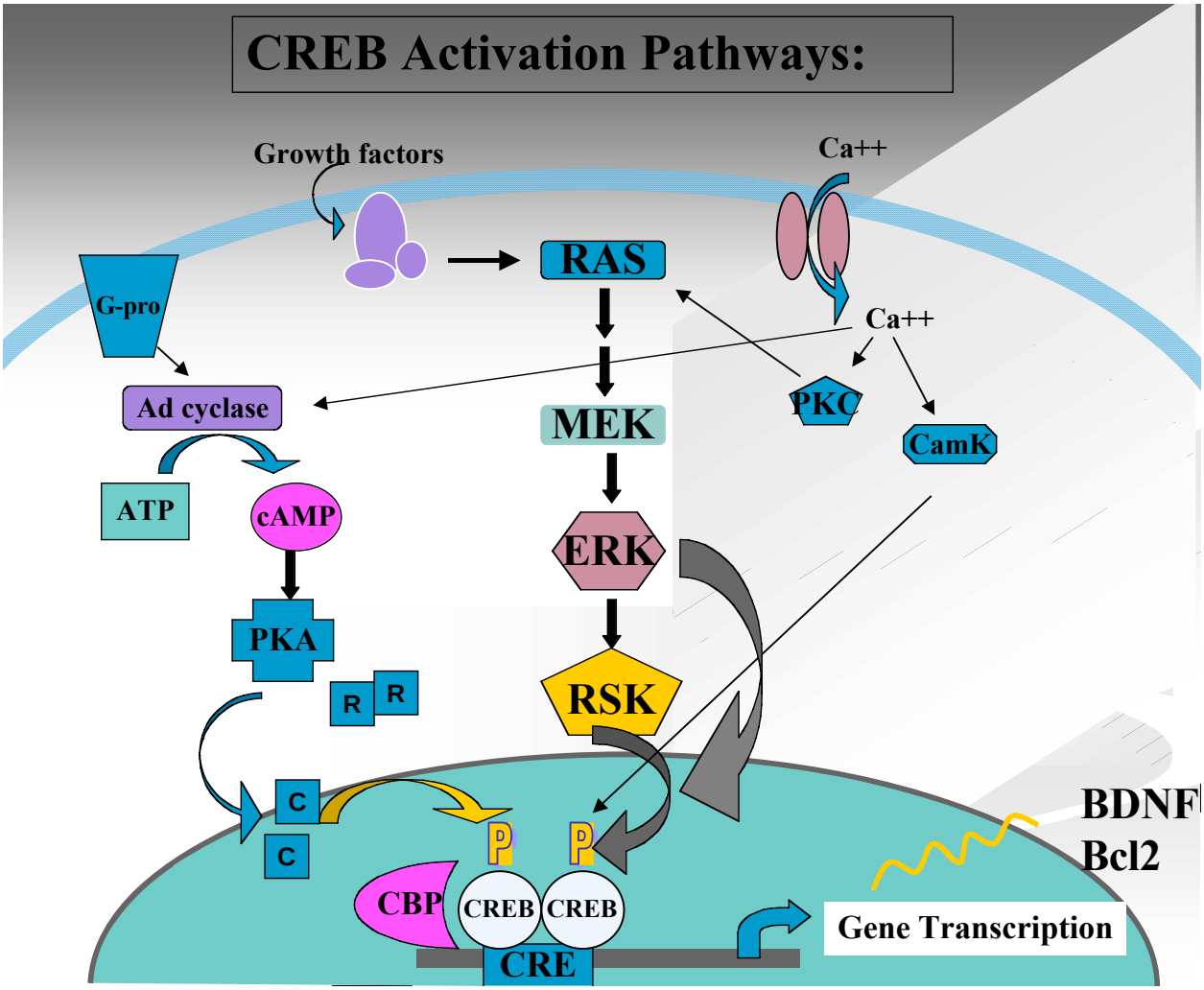


Figure 1. CREB Activation Pathways.
CREB can be activated (phosphorylated) by a variery of signaling pathways. The most common and best elucidated is the cAMP-PKA pathway. Extraceullular signals (ex: Hormones and neurotransmitters) activate heterotrimeric G-proteins, that directly stimulate adenyl cyclase, which can then catalyze the production of cAMP. cAMP then leads to the activation of PKA, which dissociates into active catalytic subunits which can diffuse into the nucleus and phosphorylate CREB. Additionally, growth factors can stimulate their respective receptors, which can lead to stimulation of Raf and the downstream kinases RAS, MEK and ERK. Activated ERK can them stimulate RSK to translocate to the nucleus to phosphorylate CREB. Furthermore, intracellular Ca++ can stimulate the PKA pathway (through calmodulin) or activate members of the Calmodulin-dependent kinase family (CcamK) which can phosphorylate CREB directly. Once activated, pCREB controls the transcription of various important genes.

As its name suggests, CREB is activated in response cAMP, and this mechanism of activation is well-characterized. (CREB is also activated by the MAP Kinase pathway (via

RSK), by PKC, and Ca2+ and calmodulin dependent kinase (CaMKIV), see Figure 1). cAMP accumulates in the cytoplasm in response to stimulation of membrane (G)-protein coupled receptors and stimulates the dissociation of the protein kinase A (PKA) heterotetramer, which consists of a pair of regulatory (R) and a pair of catalytic (C) subunits. Once liberated, the catalytic subunits are free to enter the nucleus by passive diffusion where they phosphorylate CREB on its Ser-133 residue, and gene expression can then be induced. Phosphorylation on this serine is critical to transcriptional activation, as mutagenesis studies (substitution of Ser-133 with Ala) show that Ser-133 mutation causes a disruption in stimulus-induced CREB activity. (Gonzalez and Montminy 1989). Furthermore, gain-of-function mutations that mimic Ser-133 phosphorylation confer on CREB the ability to activate CRE reporter gene transcription in the absence of stimulation (Du et al. 2000).

1.2.2.1 Kinetics of CREB activation.

Once cAMP levels are increased in the cytoplasm, stimulating the dissociation of PKA, the released catalytic subunits are free to diffuse about the cell and phosphorylate target proteins. As mentioned, the subunits can also passively diffuse into the nucleus, where they can activate their nuclear substrate, CREB. Transcription of CREB-mediated genes usually peaks at about 15-30 minutes post-stimulus, which corresponds well with the time needed for PKA's catalytic subunits to become maximal in the nucleus (Hagiwara et al. 1993). Most cAMP-regulated genes studied to date have been found to be induced transiently (Hagiwara et al. 1992). Even in the presence of constant stimulation, CREB activity typically attenuates and declines to baseline over the next 2-4 hours with dephosphorylation of CREB Ser-133 paralleling the decline in transcriptional activity (Hagiwara et al. 1992). Dephosphorylation is achieved by two okadaic acid-sensitive, serine/threonine phosphatases, protein phosphatase-1 (PP-1) and protein

phosphotase PP-2A (Hagiwara et al. 1992) and (Wadzinski et al. 1993). As this is the typical pattern of CREB activation, it has become clear that this is certainly not the only pattern. Even within specific brain regions, responses to the same stimulus can vary neuron to neuron. For example, in some subtypes of striatal neurons, stimulation with dopamine (which activates cAMP) causes CREB phosphorylation that is sustained for more than 30 minutes. Yet in other striatal neuron subtypes, cAMP signals induce a much shorter CREB activation response, such that CREB is dephosphorylated within 15 minutes of the initial stimulus (Liu and Graybiel 1996). Interestingly, these subtle differences result in the differential expression of downstream genes (Liu and Graybiel 1996). Clearly, mechanisms of controlling CREB activation are intricate and complex, and consist of many levels of checks and balances.

1.2.2.2 Binding of CREB to CRE.

One point of control by which specificity in CREB signaling can be achieved within a cell exists at the DNA binding level. Typically, CREB binds as a dimer to a conserved cAMP Response Element (CRE), an 8 base pair sequence of TGACGTCA (Comb et al. 1986). These CRE sites have been found to exist in more than 100 gene promoters, with about half containing this full CRE palindrome, and half containing the half site, CGTCA (Mayr and Montminy 2001). To date, whether CREB binding to CRE occurs before or after phosphorylation remains controversial, as evidence seems to exist for both views. For example, it has also been shown that CREB dimerization is strongly promoted in the presence of CRE DNA, and this dimerization occurs independent of phospohrylation status (Metallo and Schepartz 1997), and (Wu et al. 1998). Additionally, chromatin does not seem to impose a physical barrier on CREB binding to CRE (Mayall et al. 1997). However, for some gene promoters, the CRE site seems to be constitutively bound, while in others, cAMP will actively stimulate CREB to bind to and

occupy CRE (reviewed in (Mayr and Montminy 2001)). It has been suggested that occupancy of CRE by CREB can be regulated by DNA methylation, but studies indicate that additional, unknown levels of regulation also likely contribute. It is reasonable that different levels of CRE occupancy can account for some measure of the cells ability to mediate specificity in CREB signaling.

1.2.2.3 Regulation of CREB transcriptional activity.

Once CREB is bound to CRE and phosphorylated, whether or not gene expression is induced is further regulated, and varies greatly depending on both the stimulus as well as the gene promoter. For example, there are documented differences in CREB activity in cells treated with cAMP versus stress or mitogen signals. When looking at the promoter level, a single CRE site can be sufficient for gene activation through CREB after treatment with cAMP (Matthews et al. 1994). However, in response to growth factors, phosphorylation of CREB may not be enough, as additional promoter-bound factors are needed in order to achieve CREB-mediated gene expression (Bonni et al. 1999). Additionally, to add another level of complexity, in other cell types (Jurkat cells), active CREB phosphorylation can be achieved without any subsequent activation of a CRE reporter gene (Brindle et al. 1995). Thus, various stimuli are able to discriminate between gene targets, and/or gene promoters are able to discriminate between the different signals. How is a cell capable of mediating specificity in CREB signaling, allowing a gene promoter to respond to one signal but not another, despite similar levels of phosphorylation? The answer likely reflects not only different levels of occupancy of CREB over CRE, but more importantly, the ability of a cell to recruit different cofactors to the transcriptional apparatus.

1.2.2.4 CREB localization

CREB is a ubiquitously and constitutively expressed transcription factor that is generally assumed to be targeted to the nucleus immediately after synthesis in the cytoplasm. Indeed, a nuclear localization signal (NLS) has been mapped to the CREB gene (Waeber and Habener 1991). This widely held view, however, has been questioned on occasion, as multiple reports describe the presence of CREB immunoreactivity outside of the nuclear envelope. For example, CREB has been reported in developing dendrites of cultured neurons (Crino et al. 1998), in the postsynaptic densities of rat brain ((Suzuki et al. 1998)), as well as in cytosolic fractions from mouse hippocampus (Azuma et al. 1999). Additionally, CREB immunoreactivity as well as CRE binding activity has been described in mitochondrial fractions from rat brain (Cammarota et al. 1999). However, despite these reports, which all examined neuronal systems, the idea that CREB could function or even exist outside of nuclei in neurons is still widely ignored. In a more recent report, (Kuramoto et al. 2005) found that immunoreactive CREB was detectable in both nuclear and cytosolic fractions of individual mouse brain structures. Interestingly, suppression of new protein synthesis by cycloheximide in vivo resulted in a significant decrease in nuclear CREB and a corresponding increase in cytoplasmic CREB levels in mouse hippocampus. This effect was restricted to specific brain regions, raising the possibility that regulation of nuclear import/export of CREB may vary from brain region to brain region through a previously unidentified mechanism other than an NLS. This additional mechanism could act as a supplementary level of control of a cell's responsiveness to the variety of extracellular signals that CREB must discriminate between. More specifically, this mechanism may represent a cell's response to harmful or toxic stimuli.

1.2.3 CREB Binding Protein.

CBP (CREB-binding Protein) was first identified when a cDNA expression library was screened with 32P-labeled CREB to search for factors that associate with CREB and therefore may contribute to transcriptional control (Chrivia et al. 1993). CBP is a 265 kDa nuclear protein that associates with phosphorylated CREB, and acts as a critical transcriptional co-activator. This idea is supported by multiple lines of evidence. For example, anti-CBP neutralizing antibodies cause inhibition of cAMP-mediated expression of a CRE reporter vector (Arias et al. 1994), and the same amino acids that are most critical for CREB transcription are also those that are required for interaction of CREB with CBP. It is likely that CBP acts as a transcriptional adaptor – serving as a bridge to allow phosphorylated CREB to recruit and interact with the RNA polymerase complex at the TATA box. Indeed, both CBP and RNA polymerase co-immunoprecipitate with Ser-133 pCREB (Kee et al. 1996). Since RNA helicase was also found to immunoprecipitate, and co-expression of CBP with this RNA helicase increased CREB's ability to activate transcription (Nakajima et al. 1997), it is likely that both are involved in linking CREB to the transcriptional machinery.

After CBP is recruited to the gene promoter, it is proposed to mediate activation of target genes not only through its association with RNA polymerase, but also through its intrinsic histone acetyltransferase activity (Ogryzko et al. 1996; Yang et al. 1996). By encouraging the acetylation of amino acids on histones, chromatin structure is altered in such a way as to make it more accessible to the entire transcriptional apparatus. As would be expected, CBP function is critical in the wide range of CREB-mediated cellular functions, and disruption in CBP function can have devastating effects on normal development. For example, mutations in humans causing loss of function of one CBP allele results in the Rubinstein-Taybi syndrome (reviewed in

(Rouaux et al. 2004)), which is characterized by physical abnormalities as well as mental retardation. Additionally, disruptions in CBP function have also been implicated in the pathology of the neurodegenerative disorder, Huntington's Disease, an effect that will be discussed in greater detail below.

1.3 DISRUPTIONS IN ERK-RSK-CREB SIGNALING IN NEURONAL DEATH.

1.3.1 CREB is important in neuronal survival.

While CREB function has been implicated in development, learning and memory, and plasticity, arguably one of the most important roles of CREB is in the promotion and regulation of neuronal survival. The first reports to document CREB-dependent mechanisms of neuronal survival showed that neurotrophic factors could inhibit cell death processes, in large part by working through CREB (Bonni et al. 1999; Riccio et al. 1999). For example, Bonni et al (1999) investigated the role of MAPK in the BDNF-induced survival of cerebellar neurons, and determined that activation of transcription factor CREB was necessary for this survival. Interestingly, they also discovered that if they inhibited the activation of CREB, (by using a dominant-interfering mutant or one in which the crucial ser 133 phosphorylation site was mutated) they could trigger apoptosis in these cells. A separate study using similar mutant constructs, again showed that genetically inactive CREB could cause death of cerebellar granular neurons, while an active CREB mutant reduced cell death in response to oxidative stress (See and Loeffler 2001). Additionally, See and Loeffler (2001) showed that exposure to H_2O_2 blocked CREB phosphorylation, and if CREB phosphorylation is restored, neurons can be

protected from oxidant-induced cell death. Taken together, these results indicate that proper and intact CREB function is critical to neuronal survival, not only during development, but also during cell responses to injury.

CREB may control neuronal survival, in part, by controlling transcription of neuroprotective genes. For example, the promoter regions for both Brain Derived Neurotrophic Factor (BDNF) and the anti-apoptotic protein, Bcl2, each contain CRE sites (Mayr and Montminy 2001) and both of these gene products have been shown to play an important role in neuronal survival. Additionally, transgenic mice that overexpress Bcl2 are protected from naturally occuring neuronal loss as well as experimentally-induced ischemia (Martinou et al. 1994). Additionally, treatment of neuronal cells with oxidants causes a decrease in Bcl2 at both the protein and mRNA levels, as well as a decrease in the CRE-mediated Bcl2 promoter activity (Pugazhenthi et al. 2003). BDNF is also known to affect neuronal survival, as this neurotrophic factor can protect nigrostriatal dopaminergic neurons from neurotoxins in rodent and monkey models of Parkinson's disease (PD) (Sun et al. 2005). Interestingly, BDNF has also been shown to be able to stimulate proliferation of neuronal precursors and the possible generation of new dopaminergic neurons in the striatum and substantia nigra in the unilateral 6-OHDA lesion rat model of Parkinson’s disease (Mohapel et al. 2005). Taken together, these results confirm that CREB, together with its downstream gene products, play an important role in the regulation of neuronal survival throughout the life of a neuron. Activation during development, as well as during times of stress is critical for determining neuronal fate, opening up the possibility that disruption of this important signaling pathway would have detrimental consequences.

1.3.2 CREB is disrupted in neuronal degeneration and death.

Many lines of evidence now exist for the role of CREB function in neuronal survival, and it is likely that this important transcription factor plays a key part in the adaptive response of neurons to insult and/or injury. Accordingly, CREB has been shown to be activated in response to stressful and toxic stimuli. It is possible that the activation of CREB-dependent signaling in response to harmful stimuli may represent a defense mechanism in neuronal cells. Indeed, studies implicate CREB activation in the selective vulnerability of hippocampal neurons in response to injury, and this activation correlates well with neuronal survival. For example, after hypoxic injury, there is an increase in activated CREB within apoptosis-resistant neurons of the dentate granule-cell layer, whereas both CREB and pCREB are markedly decreased in the dying CA1 pyramidal cells. (Reviewed in (Walton and Dragunow 2000)). This activation of CREB within the dentate region and corresponding loss in the CA1 neurons (which precedes the onset of cell death) suggest that neuronal survival is intimately linked to the ability of a cell to activate CREB. This hypothesis is further substantiated by later work showing that injection of CRE decoy oligos during toxic stimuli is associated with increased cell death (Mabuchi et al. 2001), indicating that neuroprotective responses require CREB-dependent gene expression.

In agreement with this idea, several human conditions exist where CREB function is disrupted. For example, in Coffin-Lowrey syndrome, the gene that encodes RSK-2 (an important CREB kinase described earlier) is mutated (Abidi et al. 1999), resulting in severe physical and mental abnormalities in humans. And as previously described, a heterozygous mutation in CBP results in Rubinstein-Taybi syndrome, which is also characterized by mental and skeletal deficits. Additionally, CREB dysfunction is thought to play a role in the neurodegenerative disorder Huntington's Disease.

Huntington's Disease is a member of the CAG repeat family of neurodegenerative diseases and is characterized by the presence of an expanded polyglutamine (polyQ) repeat in the huntingtin (htt) protein. The important CREB co-activator, CBP, has been implicated as being central to the pathology of this disease, as nuclear inclusions that contain mutant huntingtin recruit and sequester CBP, presumably disrupting its ability to influence CREB-mediated gene transcription (Nucifora et al. 2001). Overexpression of CBP can protect cells from toxicity associated with expression of mutant huntingtin, providing further evidence that loss of CBP function contributes to neuronal death. Additionally, in more recent study, Shimohata et al (2005) (Shimohata et al. 2005) showed that expanded polyglutamate stretches can strongly suppress CREB phosphorylation as well as induction of CRE-mediated transcriptional activation. Further, the suppression of CREB dependent transcription was reversibly rescued by increasing concentrations of cAMP, which also protected cells from the neurotoxic effects of expanded polyglutamine. Thus, the ability of CREB to become transcriptionally active, whether through phosphorylation, RSK activation, CBP association, or cAMP, appears to be required for neuronal survival in many circumstances, and may have particular relevance to human diseases.

1.4 SUMMARY

CREB is a nuclear cellular transcription factor that is critical to many important cellular functions. Since CREB is activated in response to various different signals, and controls the expression of more than 100 different genes, just how CREB signaling is differentially regulated within the cell has been the subject of much debate. To date, research has shown that CREB signaling can be modulated at many levels. For example, the mechanisms of CREB

phosphorylation, CREB occupancy of CRE, and the binding of CREB cofactors at the transcriptional apparatus have all been shown to play a role in achieving specificity, and it is likely that additional measures of regulation also exist, but are undetermined as of yet. It is not difficult to surmise that disruption at the level of any of these control mechanisms could impair a cell's ability to respond appropriately to cell signals, and could have detrimental effects on cell function.

One of CREB's most important roles within a neuron is the control of cell survival. Indeed, proper CREB function is needed for neuronal survival not only during development, but also during neuronal response to harmful or toxic stimuli. One would predict that the inability of a cell to activate proper adaptive CREB-mediated survival responses would have devastating effects. In this study, we examined adaptive responses to oxidative stress in neurons, and in particular, examined the impact of the neurotoxin 6-hydroxydopamine on CREB-mediated survival signaling. Defining the mechanism(s) behind oxidant-induced cell death can potentially lead to the development of novel therapeutic strategies for fighting neurodegenerative diseases.

2.0 RATIONALE AND HYPOTHESIS

Oxidative stress has been implicated in a variety of pathological processes, ranging from ischemic cell death and aging to neoplastic transformation and autoimmune disorders. In particular, oxidative mechanisms have been strongly linked to the pathogenesis of age-related neurodegenerative diseases, including Parkinson's Disease, Alzheimer's Disease, and Huntington's Disease (Barnham et al. 2004). The central nervous system may be particularly susceptible to oxidants due to neurons' high levels of metabolic activity and relatively low levels of endogenous antioxidants. Indeed, markers of oxidative stress, including lipid peroxidation and protein nitration, are increased in affected brain areas in Alzheimer's, Parkinson's and Amyotrophic Lateral Sclerosis (Beal 1995) (Souza et al. 2000) (Tyurin et al. 2000) and antioxidants have been shown to provide protection in their respective animal and culture models (Wu et al. 2003) (Cassarino et al. 1997) (Callio et al. 2005) (Wu et al. 2003). While the mechanism(s) by which reactive oxygen species influence cell survival-death decisions still remain incompletely defined (Ellerby and Bredesen 2000) (Barlow et al. 2005), there is little doubt that oxidative processes play an important role in the propagation of neuronal injury. Since the neurons that degenerate in these diseases show a regional vulnerability that is specific to each disorder, oxidative processes may be a factor that links the unique death pathways in neurodegenerative diseases. Given the progressive nature of neurodegenerative diseases, a better

understanding of mechanisms that could impair adaptive responses to injury is particularly important.

6-Hydroxydopamine (6-OHDA) is an oxidative neurotoxin that has been used extensively to lesion the nigrostriatal system that degenerates in Parkinson's and related diseases. In such studies, targeted intrastriatal injections of 6-OHDA in mice and rats result in a pattern of selective dopaminergic injury that is almost identical to neuronal loss and degeneration seen in PD brain (Przedborski et al. 1995) (Zigmond and K.A 1997). Neuronal injury in response to 6-OHDA may result from its ability to spontaneously autoxidize, resulting in the generation of a wide spectrum of reactive oxygen species (ROS) including hydrogen peroxide, superoxide and hydroxyl radical(Cohen and Heikkila 1974). While both *in vivo* and *in vitro* studies implicate this intracellular oxidative stress in 6-OHDA neurotoxicity (Callio et al. 2005) (Asanuma et al. 1998) the exact mechanism of neuronal cell death is still unclear. It has recently been shown that 6-OHDA treatment elicits activation of several kinases including extracellular signal regulated protein kinases (ERK) (Kulich and Chu 2001) (Horbinski and Chu 2005), however the downstream transcriptional mechanisms by which 6-OHDA modulates cell survival-death decisions still remain incompletely defined.

CREB (cyclicAMP Response Element Binding protein) is a transcription factor that plays an important role in neuronal survival, in part by controlling the transcription of neuroprotective genes such as Brain Derived Neurotrophic factor (BDNF) and the pro-survival protein Bcl-2 (Finkbeiner 2000) (Mayr and Montminy 2001). Studies show that cAMP, a potent activator of CREB, acts as a trophic or protective signal for several populations of catecholaminergic neurons (Reviewed in (Goldberg and Barres 2000)), and can enhance the protective effects of noradrenaline (Troadec et al. 2002)and glial cell line derived neurotrophic factor (Engele and

Franke 1996). Interestingly, related studies show that oxidative stressors can disrupt CREB function, for example by interfering with CREB phosphorylation and/or by reducing CRE binding activity (Ito et al. 1999) (See and Loeffler 2001). An additional study examined the impact of oxidative stressors on the downstream CRE-mediated survival gene Bcl2 (Pugazhenthi et al. 2003) and reported that oxidative stress causes a down-regulation of the Bcl2 promoter, resulting in a decrease in Bcl2 protein as well as mRNA levels. Taken together, these results suggest that a loss of CREB signaling could contribute to oxidant – induced neuronal dysfunction, and may be particularly important under conditions of oxidative stress. Specifically, since CREB can be activated by neurons in response to insult, and this CREB activation is typically associated with increased survival, any interruption in this adaptive survival response may hinder a neuron's ability to protect itself.

The potential role of the CREB pathway in neuronal cell responses to 6-hydroxydopamine was investigated in this study. Since previous data has implicated CREB functioning in neuronal death processes, we questioned whether disruption of CREB signaling contributes to the cell death process in Parkinsonian models, to better clarify the mechanisms involved in 6-OHDA-mediated toxicity. Since previous data in our laboratory demonstrated alterations in two upstream CREB kinases, ERK and RSK, with both showing abnormal cytoplasmic accumulation in 6-OHDA-treated cells and degenerating Parkinson's disease neurons (Zhu et al. 2002), we sought to examine downstream effects on transcription that may influence neuronal death. ***We hypothesized that CREB signaling will be altered in response to 6-OHDA, with downstream CREB-mediated transcription disrupted, resulting in a decrease in expression of important survival gene products.*** A better understanding of neurodegenerative mechanisms, with elucidation of a transcriptional mechanism for oxidative stress-induced

neurotoxicity would offer important insights into the pathogenic mechanisms underlying the progression of neurodegenerative diseases.

3.0 MATERIALS AND METHODS

3.1 CELLS AND TISSUES

3.1.1 Cell Culture

B65 cells (ECACC 85042305) were the gift of Dr. David Schubert of the Salk Institute, La Jolla, CA (Schubert et al. 1974). SH-SY5Y cells were purchased from ATCC (Manassas, VA). Both cell lines were maintained in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, 2mM L-Glutamine and 10mM HEPES. Low-serum media consisted of DMEM supplemented with 0.5% heat-inactivated fetal calf serum, 2mM L-glutamine and 10mM HEPES. Cell culture reagents were purchased from Biowhittaker (Walkerville, MD) unless otherwise indicated. In a typical experiment, B65 and SH-SY5Y cells were plated at a density of $3x10^5$ cells/well for 6-well plates, $1x10^4$ cells/well in 96 well plates, or $1x10^6$ cells/well in 100 mm dishes. For all experiments, cells were cultured in a humidified incubator with 5% CO_2 at 37^oC.

3.1.2 Primary midbrain cultures

Primary midbrain neuronal cultures were derived from 15-day C57BL/6 mouse embryos as previously described (Chu et al. 2005). Briefly, the striatum and ventral midbrain were dissected

from the embryos (Hilltop Laboratory Animals, Inc., Scottdale, PA, USA), and incubated with 100 units papain (Worthington Biochem. Corp. Lakewood, NJ, USA) in Hank's Balanced salt solution for 30 min at 37°C. Cells were then mechanically dissociated using a flame-polished Pasteur pipette in Dulbecco's modified Eagle's medium/Ham's F12 1 : 1 (v/v; GIBCO/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine seum (Cambrex Biological Science, Walkersville, MD, USA), 5 mg/L insulin (Invitro Life Technologies) and 30 mM D-glucose (Sigma, St Louis, MO, USA). Cells were collected by centrifugation at 200 *g* for 5 min, resuspended in culture medium and plated in poly-L-lysine coated 16-well chamber slides (Nunc Laboratory-Tek, Fisher Scientific, Agawam, MA, USA) at a density of 2×10^5 cells/cm^2. or seeded into 12 or 96 well plates. Cultures were maintained at 37°C in 5% CO_2. After 3 days, fresh medium containing 2 μM cytosine arabinoside was applied for 72 h to inhibit the proliferation of glia. Cultures were treated at between 7-9 days *in vitro* with 50 μM of 6-OHDA, a dose selected based on published death curves (Guo et al. 2001), and/or 100uM db-CAMP (Kim et al. 2005) as indicated in figure legends. After 3 hours, the chamber slides or cell culture plates were washed in PBS (20mM potassium phosphate, 150mM potassium chloride), fixed in 3% paraformaldehyde for 15 minutes, and then processed for immunofluorescence as described in detail in the Methods below.

3.1.3 Human Tissues

Paraffin-embedded midbrain sections were obtained from the Joseph and Kathleen Price Bryan Brain Bank and the University of Pittsburgh Brain Bank. All banked patients have undergone extensive standardized pre-mortem neurological and post-mortem neuropathological assessments. Available PD, DLB, and incidental Lewy Body disease cases were requested,

along with a set of normal control cases matched for age and postmortem intervals. Additionally, sections of breast cancer tissue were requested to serve as a positive control for CREB antibodies. The study design was approved by the University of Pittsburgh Institutional Review Board.

3.2 TRANSCRIPTION, VIABILITY AND CELL DEATH ASSAYS

3.2.1 Transcription assays

The CRE reporter vector from the Mercury Pathway Profiling System (Clontech, PaloAlto, CA) was cloned and purified using endotoxin-free Maxi-Prep kits (Qiagen, Valencia, CA). The empty, backbone vector (pTAL) was used as a control. Both pTAL and pCRE contain a TATA-like promoter region from the Herpes simplex virus thymidine kinase (HSV-TK) promoter (to provide optimal induction of reporter, while giving low background), and the firefly luciferase coding sequence (from *Photinus pyralis*), which is followed by the SV40 late polyadenylation signal to ensure proper, efficient processing of the luciferase transcript in eurkaryotic cells (See Figure 2). Additionally, there is a transcription blocker, which is composed of adjacent polyadenylation and transcription pause sites for reducing background transcription, as well as an ampicillin resistance gene, to allow for propagation and selection in E.coli. The pCRE vector contains the above components as well as three copies of the CRE-binding sequence fused to the TATA-like promoter region of the HSV-TK promoter. After transcription factors bind CRE, transcription is induced and the reporter gene is activated and ultimately translated into functional enzyme.

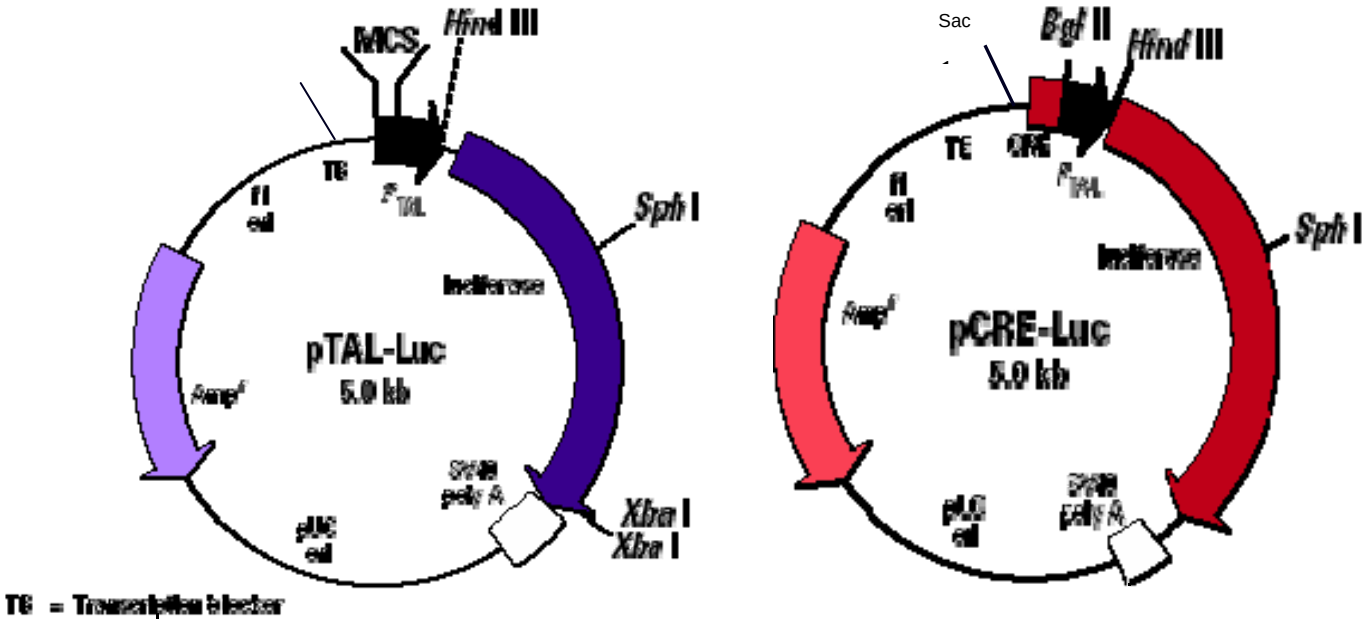


Figure 2. pTAL and pCRE plasmid maps.
The structure of both pCRE and control pTAL plasmids are shown. Note that vectors are identical except for the tandom repeats of CRE found on the pCRE plasmid. Both plasmids contain a TATA-like promoter region (TAL), the firefly luciferase coding sequence, SV40 late polyadenylation signal, a transcription blocker (TB), and an ampicillin resistance gene (Amp^r).

For each CRE transcription assay, cells were plated in 6-well plates, and grown overnight. For each well, 1 µg plasmid DNA was prepared in 500µl Opti-MEM media, combined with 10µL Lipofectamine 2000 (Invitrogen, Carlsbad,CA) in 500µL Opti-MEM, and incubated for 20 min before being applied to plated cells. After 8 hours, the cells were switched to low-serum media for 24 hours prior to the experiment. For calcium phosphate transfections using the CalPhos Mammalian Transfection kit (Clontech, PaloAlto, CA), 1 µg of plasmid DNA was combined with water and Calcium Phosphate reagent, allowed to incubate 20 minutes at room temperature, and then added drop-wise to cells. Transfections were allowed to proceed overnight before switching to low-serum media for 24 hours prior to use. Parallel experiments

were performed using either Lipofectamine 2000 or calcium phosphate, with similar results. However, since the Calcium phosphate method resulted in less injury to the cells, this was the method most often employed.

3.2.2 Luciferase Assay

Cells were assayed for CRE reporter activity using a Luciferase Reporter Assay Kit (Clontech, PaloAlto, CA). Briefly, cells were washed with PBS (without calcium and magnesium) and then lysed using 1X Reporter Assay Kit cell lysis buffer using gentle agitation for 20 minutes. Cell lysates were then collected with cell scrapers, anc centrifuged briefly (500*g*, 2 minutes) to remove cellular debris. After centrifugation, supernatants were assayed for luciferase activity within 20 minutes or frozen at -70^{o}C and assayed within 3 weeks. Equal volumes of lysate, buffer and luciferin substrate were combined (200 μl final volume), followed by a 20 second measurement of emitted light using a Lumat LB 9507 tube luminometer (Berthold Technologies USA, Oak Ridge, TN). Luciferase, expressed in response to CRE transactivation, catalyzes the oxidation of luciferin, resulting in photon production. Light output is directly proportional to amount of luciferase, and thus transcriptional activity driven by CRE in the cells. Relative light units were normalized to the untreated control for all experiments. Additionally, as a further control, for some experiments protein concentrations of cell lysates was determines, and used to normalize activity across wells.

3.2.3 Toxicity Assays

For each assay, 6-OHDA (Sigma, St. Louis, MO) was prepared in ice cold 0.5% (wt/vol) ascorbate or sterile water immediately before use. Since equivalent results were obtained with either vehicle system, sterile water was used preferentially. Dibutyryl-cyclic-adenosine monophosphate (db-cAMP) (Sigma, St. Louis, MO) was prepared in sterile water, and was also prepared fresh immediately prior to each experiment. To determine protection, reagents (catalase (30U/ml) or db-cAMP (250 μM-500μM)) were dissolved in cell culture media, and added to cells at times and concentration indicated in figure legends. Toxicity was measured using a Lactate dehydrogenase (LDH) release assay (Sigma 340-UV LDH detection system). Briefly, cells were grown in 96-well plate format and exposed to a dose curve of concentration of 6-OHDA (0-1400 μM for B65 cells; 0-300 μM for SH-SY5Y cells) or to vehicle for 18-20 hours. Following visual inspection of cell morphology, cells were centrifuged (5 minutes, 500*g*) and aliquots of supernatant were obtained. An equal volume of 2% (wt/vol) Triton X-100 was added to the wells and cells were lysed by gentle pipetting followed by a 30 minute incubation at 37^{o}C. The amount of LDH activity in the supernatant ("released" LDH) and lysate ("total" LDH) were determined by measuring the decrease in absorbance at 340nM associated with the reduction of pyruvate in a linear velocity reaction. The level of LDH release into the culture medium was expressed as percent of total LDH in the wells.

To determine if any of the toxicity treatments elicited caspase activation, a Caspase 3 activity assay was used. Briefly, B65 and/or SH-SY5Y cells were grown and treated as described. To separate nuclear and cytoplasmic protein fractions, the NePUR Kit (Pierce, Rockford,IL) was used as directed by the manufacturer, and cytoplasmic fractions only were

analyzed. Prior to use, the lysates were then centrifuged for 30 min at 4°C, 16 000 *g*, and after centrifugation, protein concentrations of the supernatants were ascertained using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Samples containing 100 µg protein were mixed with 20 µM of the fluorogenic substrate Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluoromethyl-coumarin (Chemicon International, Temecula, CA, USA), followed by a 2-h incubation at 37°C. The changes in fluorescence were quantified every 20 min using a luminescence spectrometer (Winlab; Perkin Elmer, Shelton, CT, USA) (excitation 400 nm, emission 505 nm).

3.2.4 Cell Viability Assays

To assay cell viability, the metabolic dye alamarBlue (Biosource, Camarillo, CA) was utilized. As a redox indicator, alamarBlue is reduced by reactions innate to cellular metabolism and, therefore, provides an indirect measure of viable cell number. Reduction was determined by measuring fluorescence as a function of time. Fluorescence measurements were made by exciting at 530-560 nm and measuring emission at 590 nm. In reporting alamarBlue reduction, data are expressed as fluorescence emission intensity units as a function of time of incubation. Briefly, for each assay, cells were plated in 96-well plates and treated with db-cAMP and 6-OHDA as indicated above and in figure legends. After 18-24 hours, alamarBlue (1:10 dilution) was added to the plates, and fluorescence at time zero was measured to obtain a baseline. Fluorescence was measured again after a 2-hour incubation at 37^{o}. After baseline subtraction, cellular fluorescence is an indicator of cell survival.

3.3 PROTEIN AND RNA PROCEDURES

3.3.1 Protein Isolation

Total cell lysates were obtained at various time points using a lysis buffer containing Triton X-100, and protease and phosphatase inhibitors (Sodium Orthovanidate (1mM final concentration), PMSF (1.25mM), Sodium Pyrophosphate, (2mM), and E-64 (100uM)). Briefly, after treatments cells were washed in 1X scraping buffer (25mM Hepes, 50mM NaCl, 5mM EDTA, pH 7.5) to remove traces of remaining media, and then collected in appropriate volume of scraping buffer and centrifuged (1000 RPM) for 5 minutes. All steps were performed on ice or at 4^{o}C. After centrifugation and removal of supernatent, cell pellets were resuspended in 40-100uL Lysis buffer (25mM Hepes, 150mM NaCl, 5mM EDTA, 100mM Triton-X 100, pH 7.5) and pipetted vigorously to disrupt cell membranes. After a 30 minute incubation on ice, lysates were centrifuged briefly to pellet debris and stored at –80^{o}C until use.

To separate nuclear and cytoplasmic protein fractions, the NePUR Kit (Pierce, Rockford,IL) was used as directed by the manufacturer. Protein concentration was determined using the Coomassie Plus Protein Assay (Pierce, Rockford,IL), and the purity of the fractions was assessed by immunoblotting for cytoplasmic and nuclear markers (Beta-Actin and Lamin, respectively).

3.3.2 Immunoblotting.

For both total cell protein and nuclear/cytoplasmic fractions, equal amounts of protein were electrophoresed through 5-15% SDS-PAGE gels under reducing conditions, transferred to

Immobilon-P membranes (Millipore, Bedford,MA), and blocked in 5% non-fat dry milk in PBST (20mM potassium phosphate, 150mM potassium chloride, and 3% (w/v) Tween 20, pH 7.4), for 1-2 hours at room temperature. Blots were probed overnight at 4^{o}C with the following antibodies and dilutions:

Table 1. Information for antibodies used in Western blots.

AB Target	***Vendor***	***Source Species***	***Dilution***	***Secondary AB***
Phospho-CREB	**Cell Signaling**	**mouse**	**1:1000**	**Anti-mouse 1:5000**
Total CREB	**Cell Signaling**	**rabbit**	**1:1000**	**Anti-rabbit 1:5000**
B-Actin	**Sigma**	**mouse**	**1:10,000**	**Anti-mouse 1:10,000**
Lamin	**Cell Signaling**	**rabbit**	**1:1000**	**Anti-rabbit 1:5000**
CBP	**Cell Signaling**	**rabbit**	**1:1000**	**Anti rabbit 1:5000**

CREB control extracts (Cell Signaling) were used as positive control. After washing, blots were probed with a HRP-conjugated IgG secondary antibody, and antibody detection was carried out using a chemiluminescence detection kit (ECL, Amersham, Piscataway,NJ). Blots were stripped in stripping buffer (50mM sodium dodecyl sulfate (SDS), 25mM glycine, pH 2) for 20-30-minutes, washed in PBS and then re-probed with new antibodies as indicated in the figure legends. After use, blots were stained with Coomassie Blue to confirm equal protein loading and efficient transfer.

3.3.3 Isolation of RNA and RT-PCR

Total RNA was isolated from treated B65 cells using Qiagen RNeasy Kits (Qiagen, Valencia, CA). RNA was quantified by spectrophotometry and 1 μg RNA was used for each PCR reaction. Primers were designed using the on-line Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), unless otherwise stated. The sequences of forward and reverse primers were as follows (Table 2):

Table 2. List of Oligos used for RT-PCR.

Gene	*Forward Primer (5'-3')*	*Reverse Primer (5'-3')*
Bcl-2		
BDNF	GTGACAGTATTAGCGAGTGGG	GGGTAGTTCGGCATTGC
CREB	TCAGCCGGGTACTACCATTC	CCTCTCTCTTCGTGCTGCT
B-Actin	TGTTTGAGACCTTCAACACC	TAGGAGCCAGGGCAGTAATC
GAPDH	GGGTGGTGCCAAAAGGGTC	GGAGTTGCTGTTGAAGTCACA
MKP 3	GGCAAAAACTGTGGTGTCCT	CCAGGGTCCTTTCAAAGTCA

Samples were amplified in a PTC-100, Programmable Thermal Controller (MJ Research, Inc. (BIORAD) Waltham, MA), using the GeneAmp EZ rTth RNA PCR Kit (Perkin Elmer/ Roche, Branchburgh, NJ) following manufacturers instructions. Reaction products were electrophoresed through 1% agarose gels and stained with ethidium bromide. To determine density of visualized bands, gels were scanned in and photographed using a on Kodak Gel Logic 2200, and Kodak Imaging Software 4.0.4 was used to perform densitometry on observed bands.

Typically, date is expressed as a ratio between the densitry of the b-actin band and the band of interest.

Quantitative RT-PCR was performed using a LightCycler Instrument (Roche Molecular Biochemicals, Indianapolis, IN), and the LightCycler-RNA Amplification Kit SYBR Green I (Roche Molecular Biochemicals, Indianapolis, IN) to measure RNA in a one step RT-PCR reaction in real time, following manufacturers instructions. Melting curves and gel electrophoresis of the products were used to insure specificity of amplification products.

3.3.4 Electrophoretic mobility shift assay

EMSA was performed as described (Oury et al. 2002) on double stranded oligonucleotides by hybridizing complementary denatured single stranded oligonucleotides with10X annealing buffer (200 mM Tris-HCl pH8.0, 100 mM $MgCl_2$, 500 mM NaCl). An established palindromic CRE probe sequence (5'-AGAGATTGCC**TGACGTCA**GAGAGC-3') (Chandran et al. 1996) was used. This CRE consensus sequence, taken from the rat somatostatin gene promoter, has been used extensively in the literature (Zhang et al. 2004)and (Hershko et al. 2003) and is also commercially available (Promega, Madison, WI). The CRE probe (^{32}P end labeled; 20,000 cpm per lane) was incubated with 30 μg of nuclear protein extract in 5X binding buffer (50 mM Tris-HCl pH8.0, 62.5% glycerol, 2.5 mM EDTA, 750 mM KCl) in a 30 μl total reaction volume for 15-20 minutes at 25°C. Specificity was assessed by competition with unlabeled CRE probe. Poly-dIdC (15 ng) was used as a nonspecific competitor. For supershift assays, a mixture of antibodies to CREB and Ser133 phosphorylated CREB (pCREB) (3uL each) was added 15 minutes prior to the addition of labeled CRE probe. Protein-DNA complexes were resolved on a 1X TBE/ 10% PAGE, and detected by autoradiography.

3.4 MICROSCOPY STUDIES

3.4.1 Immunocytochemistry

B65 and SH-SY5Y cells were plated on glass coverslips in 12-well plates at a density of $1.5x10^5$ cells/well, treated with 6-OHDA or cAMP as indicated in figure legends, then washed with phosphate-buffered saline (PBS) and fixed in ice-cold 4% paraformaldehyde for 15 minutes (Chu et al. 2005). Cells were permeabilized with 0.1% Triton X-100/PBS, and then blocked in 5% normal donkey serum. To visualize phosphorylated CREB, a pCREB monoclonal antibody (1:1000, overnight at 4°C, Cell Signaling) was used. Coverslips were then washed with PBS and incubated with Cy3-conjugated secondary antibodies (1:200, Jackson ImmunoResearch) for 1 hour at room temperature. Nuclei were counterstained with DAPI (Molecular Probes). For additional experiments, the mitochondrial voltage-dependent ion channel marker, anti-ANT (Adenine nuclerotide transporter) (1:200, Santa Cruz Biotechnilogy, INC, Santa Cruz, CA) was used in a triple label. Coverslips were mounted in gelvatol, and cells visualized and photographed using an Olympus Provis fluorescence microscope (Olympus America Inc., Melville, NY, USA) equipped with three filter cubes: FITC (excitation 490/emission 520), TRITC (excitation 541/emission 572 nM) and DAPI (excitation 350/emission 470 nM).

Primary cell cultures were obtained as described above. For immunofluorescence studies, cells were treated with 6-OHDA and/or db-cAMP for 3 hours as indicated, and then immediately fixed in 4% paraformaldehyde for 15 minutes. After rinsing with PBST, cells were blocked in 5% Donkey Serum for 1 hour at room temperature, then probed with indicated antibodies. For striatal cultures, cells were co-labeled with antibodies against pCREB (1:1000) and the neuronal marker neurofilament (polyclonal, 1:500, Sigma, St. Louis, MO). For midbrain

cultures, cells were co-labeled with antibodies against pCREB, and TH to label dopaminergic neurons (polyclonal, 1:2000, Chemicon, Temecula, CA). After washing in PBST, cells were incubated in appropriate secondary antibodies, anti-mouse Cy3 and anti-rabbit Alexa 488, at 1:500 in Link Diluent (BioGenex, San Ramon, CA). For all cultures, the cells were counterstained with the nuclear marker DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (Molecular Probes). The slides were observed using an Olympus Provis fluorescence microscope (Olympus America Inc., Melville, NY, USA) and/or a Nikon Eclipse II microscope.

3.4.2 Immunohistochemistry

Paraffin-embedded human midbrain sections, from a previously characterized subset of 3 Parkinsonian and 2 control brains (Callio et al. 2005) (Zhang et al. 2004), were stained for pCREB using previously described protocol (Jordan-Sciutto et al. 1999). Briefly, sections were deparaffinized using a series of zylene and ethanol washes, and treated with 3% H_2O_2 for 30 minutes to quench endogenous peroxidases. Sections were then heated in target retrieval solution (DAKO Target Retrieval Solution, Carpinteria, CA) at 95^{o}C for an hour, allowed to cool, and then treated with Immunon protein blocking agent (Shandon, Pittsburgh, PA) and incubated at 4^{o}C overnight with appropriate antibody in antibody diluent (DAKO Antibody Diluent with Background Reducing Components, Carpinteria, CA). This was followed by incubation with biotinylaterd (anti-rabbit or anti-mouse secondary where appropriate) IgG (1:500, Jackson ImmunoResearch, West Grove, PA) at room temperature for one hour and then streptavidin-horseradish peroxidase (1:500). For tyramine amplification, biotinyl tyraminde (1:100, TSA; Perkin-Elmer, Emeryville, CA) was applied for 30 minutes at room temperature, followed by streptavidin-horseradish peroxidase (1:500), as described by the manufacturer. The

peroxidase reaction was visualized using 3-amino-9-ethyl-carbazole (AEC) substrate (BioGenex, San Ramon, CA) and sections were then counterstained with Mayer's hematoxylin. Equivalent results were obtained using avidin-biotin or tyramide amplification. Slides were visualized and photographed, and examined for positive staining.

3.5 STATISTICAL ANALYSIS

All results are expressed as mean ± SEM unless otherwise indicated. Two-group comparisons were performed using Student's *t*-test. Multiple-group comparisons were performed using ANOVA. *Post hoc* testing used the Student's *t*-test with Bonferroni correction, and $p < 0.05$ was accepted as statistically significant.

4.0 RESULTS

4.1 CRE/CREB FUNCTION

4.1.1 6-OHDA Represses the CRE Promoter

The luciferase reporter transcription assay was used to monitor induction of the CRE element in B65 cells treated with media, db-cAMP, 6-OHDA or vehicle. Luciferase is expressed in response to CRE transactivation, and will catalyzes the oxidation of the substrate luciferin (added during the assay), resulting in photon production. Light output is directly proportional to amount of luciferase present in each sample, and thus is a measure of CRE transcriptional activity in the cells. Luciferase activity from 6 independent experiments were normalized to the untreated control of each experiment, and averaged (Figure 3). Results show treatment with db-cAMP (a cell permeable cAMP analog, and potent activator of CRE) resulted in a 2.5 fold induction of the CRE promoter, while treatment with 6-OHDA caused a significant repression in the basal activity of the CRE promoter. Control cells stimulated with vehicle alone did not show significantly different responses from untreated cells.

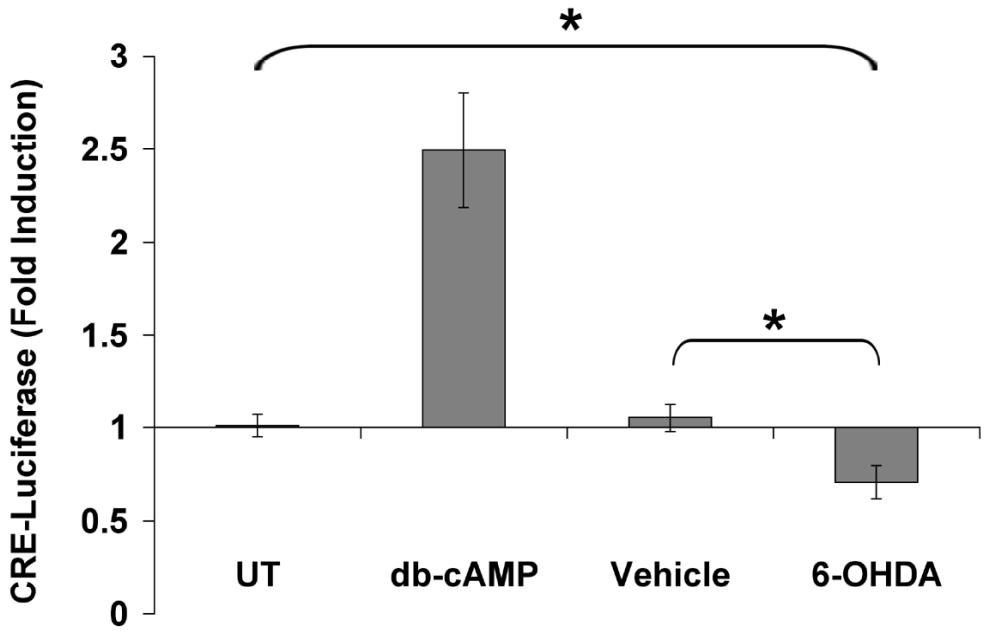


Figure 3. 6-OHDA treatment causes repression of the CRE promoter.
The CRE-luciferase reporter vector was used to monitor induction of the CRE element. Cells were transfected with pCRE-luc as described in Methods, then stimulated with media alone (UT), cAMP, 6-OHDA or ascorbate vehicle for 3 h and assayed for luciferase activity as described in Methods. Data is expressed as the fold induction of luciferase activity normalized to the untreated control group, and represents the mean of 6 independent experiments, +/- standard deviation. *p < 0.05 (ANOVA followed by Student's t-test with Bonferroni correction).

Next, to verify that the observed repression was not due to cell death, several measures of cell viability and the transcription of control luciferase constructs lacking CRE elements (Figure 4) were examined. At the time points used for transcriptional assays (30 min to 3 hours of 6-OHDA exposure) no morphological changes of cell death are apparent (Figure 4B). Cells treated with 6-OHDA for 3 hours looked as robust and healthy as untreated cells. Moreover, there is no evidence of LDH release at 4 hours of 6-OHDA exposure (Figure 4C), indicating that cells have not begun to lyse at this time point. LDH release is not apparent until 8 h after 6-OHDA initiation indicating that cell rupture and death begins somewhere between 4-8 hours of 6-OHDA treatment in B65 cells. Additional control experiments were conducted using the pTAL plasmid,

which lacks CRE sequences, but is otherwise identical to the TATA-like promoter-containing CRE reporter vector (See Figure 2 in Materials and Methods). 6-OHDA treatment had no effect on pTAL-driven luciferase expression (Figure 4A), indicating that 6-OHDA is not causing a general disruption in transcriptional responses. Therefore it is likely that the observed transcriptional repression is due to disrupted CRE transactivation specifically, and not to general defects in transcription or cell viability.

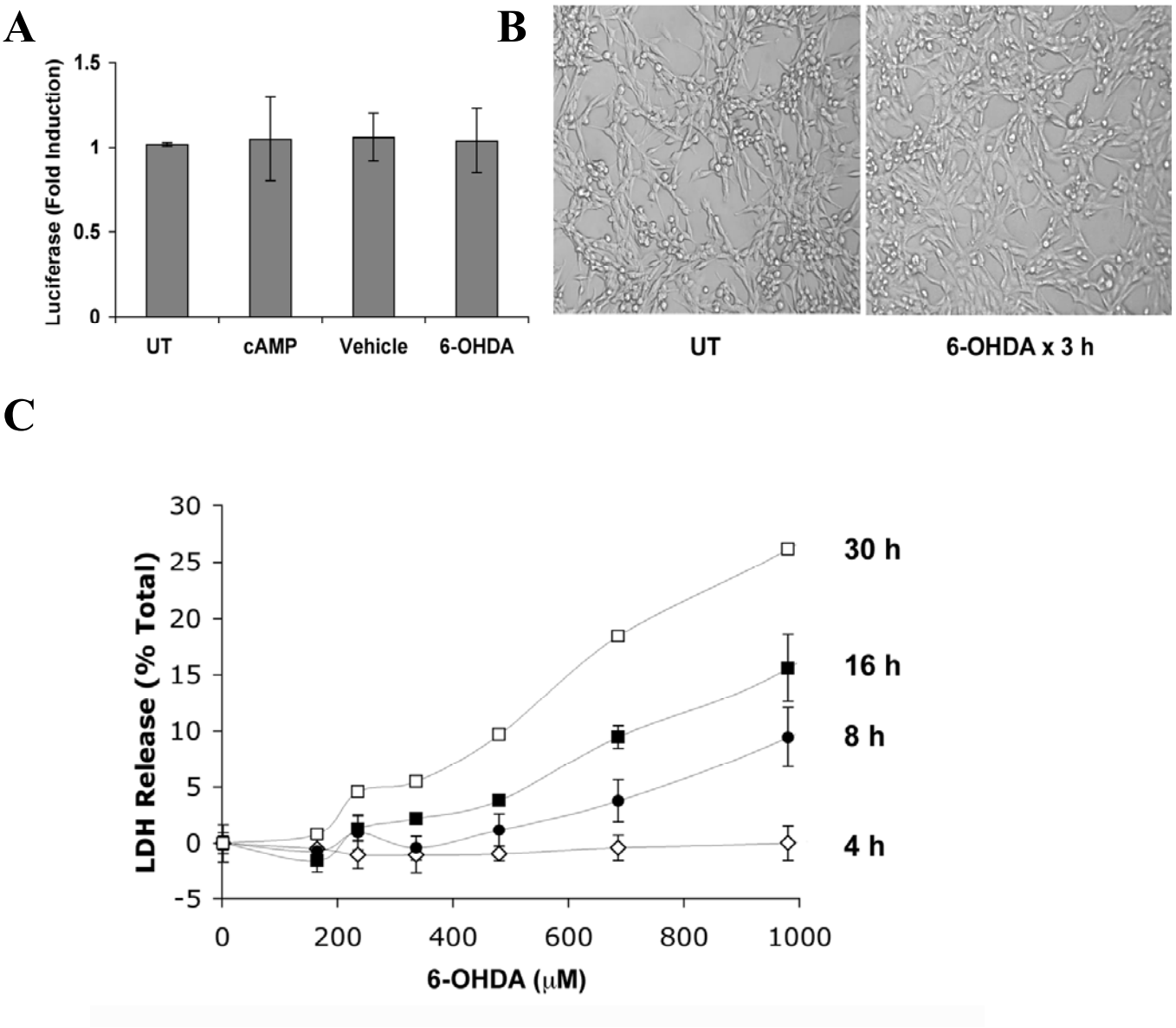


Figure 4. 6-OHDA-induced CRE repression is not due to decreased cell viability

A. Cells were transfected with a control luciferase construct lacking CRE enhancer elements as described in Methods, treated as above, and assayed for luciferase activity. There were no significant effects of any treatment condition on basal levels of cellular transcription. B. Representative phase contrast images of the cell cultures obtained at 3 h, immediately prior to cell lysis for luciferase assays. No morphologic evidence of cell injury or death was observed at this time point. C. Cells were treated with a range of 6-OHDA concentrations for different amounts of time. Cell injury was assayed using the LDH release assay. Data represent the average of triplicate wells +/- standard deviation. There is no evidence of cell death until 8 h after 6-OHDA exposure.

4.1.2 Disruption in CREB function contributes to 6-OHDA induced cell death.

Next, the potential ability of cAMP treatment to reverse the repressive effects of 6-OHDA on the CRE promoter was examined. Cells were treated with a cell permeable form of cAMP 10 min prior to exposure to 6-OHDA. The results demonstrate that cAMP pre-treatment prevented the 6-OHDA-induced repression of CRE activity (Figure 5). Treatment of B65 cells with cAMP in the presence of 6-OHDA not only restored CRE activity, but resulted in CRE activity levels that were higher than those elicited by treatment with the same concentration of cAMP alone.

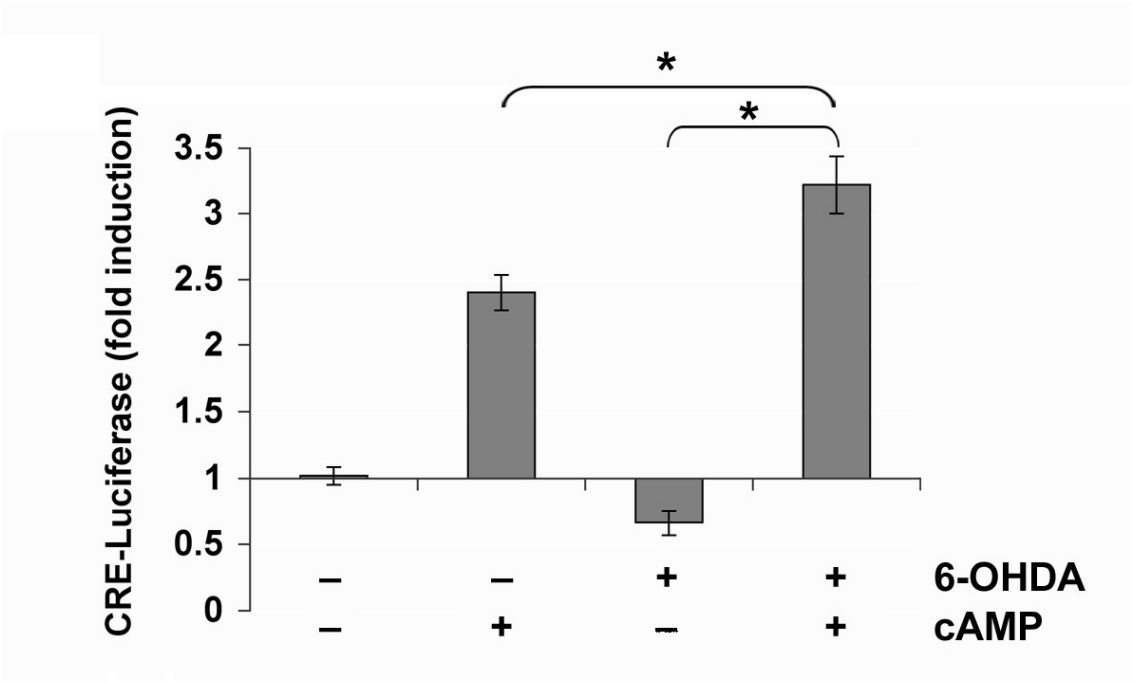
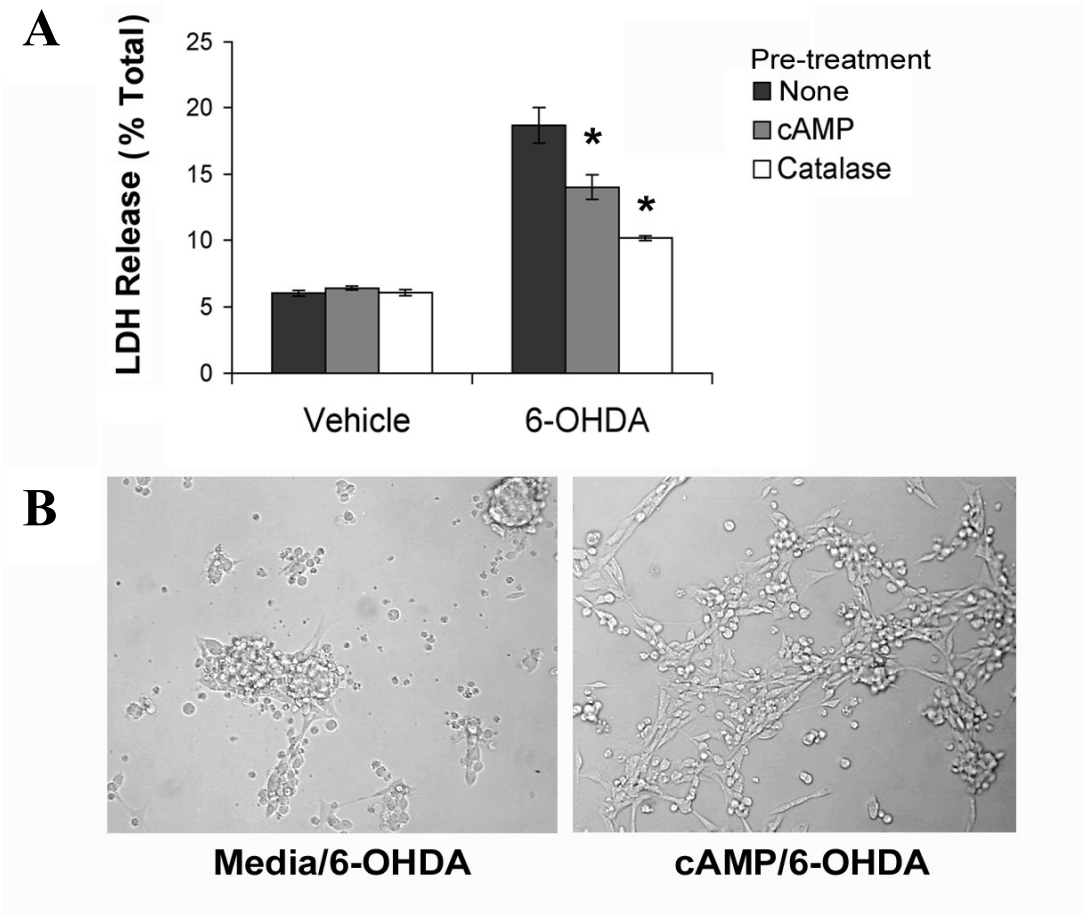


Figure 5. cAMP pre-treatment reverses 6-OHDA-induced repression of CRE.
Cells were transfected with pCRE-luc plasmid, then stimulated with vehicle, cAMP, 6-OHDA or 6-OHDA following a 10 min pre-treatment with cAMP. After 3 h, cells were lysed and luciferase activity measured. Data is the mean +/- standard deviation, and is representative of 5 independent experiments. *p < 0.05 by ANOVA followed by Student's t-test with Bonferroni correction.

Since cAMP reverses the 6-OHDA-induced CRE repression, experiments were conducted to determine if cAMP treatment during 6-OHDA exposure would also confer protection against 6-OHDA-mediated toxicity. Since the antioxidant catalase has been previously shown to protect cells from 6-OHDA – induced injury, it was used here as a positive control (Kulich and Chu 2001). Indeed, a 10 minute pre-treatment with cAMP resulted in significantly decreased cell injury at 18 h by both LDH release assay and morphological examination (Figure 6A & B). This cAMP-induced protection was apparent at a range of 6-OHDA concentrations, determined by LDH Assay (Figure 6C), and morpholoical examination (Figure 7). Since addition of cAMP reversed the 6-OHDA-induced repression while conferring protection, these data suggest that disruption in CRE function contributes to 6-OHDA-induced cell death.



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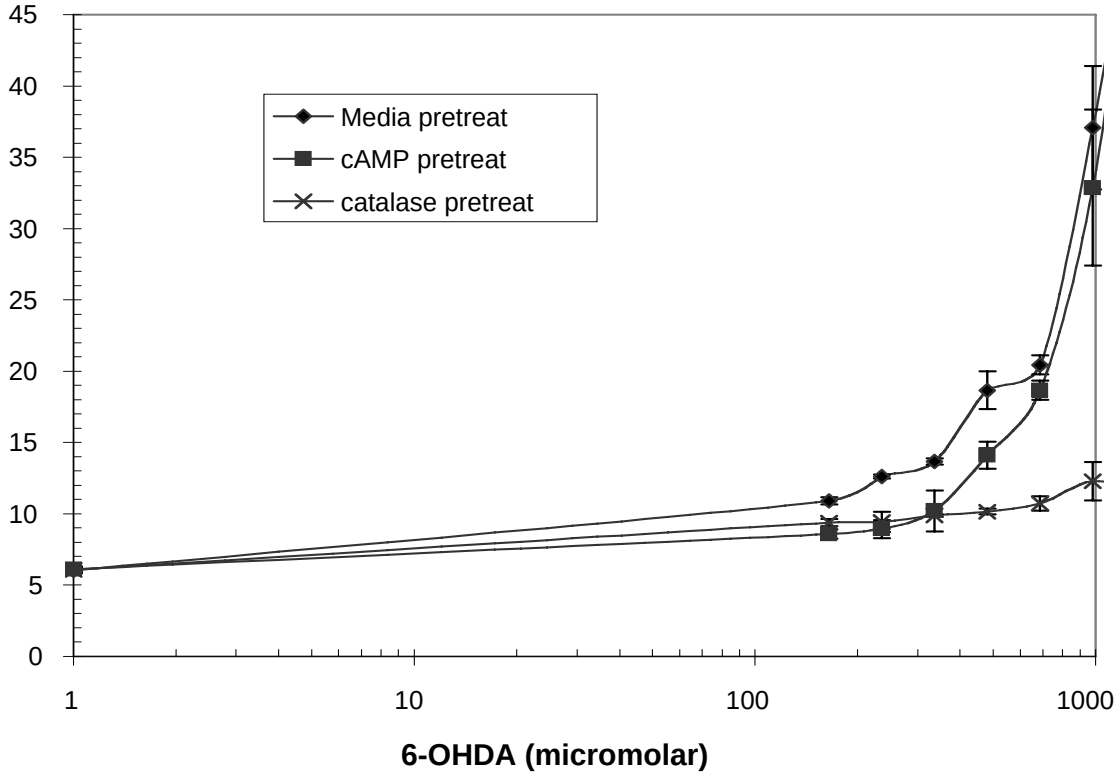

Figure 6. cAMP protects against 6-OHDA- toxicity.

A. B65 cells were treated for 10 min with media (None), cAMP (250uM)or catalase (30U/ml) before exposure to vehicle or 6-OHDA for 18 hours. Cell injury was assayed using the LDH release assay, and are representative of at least 3 independent experiments. *p < 0.05 vs. 6-OHDA with no pre-treatment (Student's t-test). B. Cells were photographed immediately prior to performing the LDH assay, showing that cAMP pre-treatment results in greater numbers of viable neuronal cells (right) compared to the shrunken, dead morphology observed with 6-OHDA alone (left). C. Cells were exposed to a dose curve of 6-OHDA treatment and LDH release was examined as described above. Data is representative of 4 independent experiments.

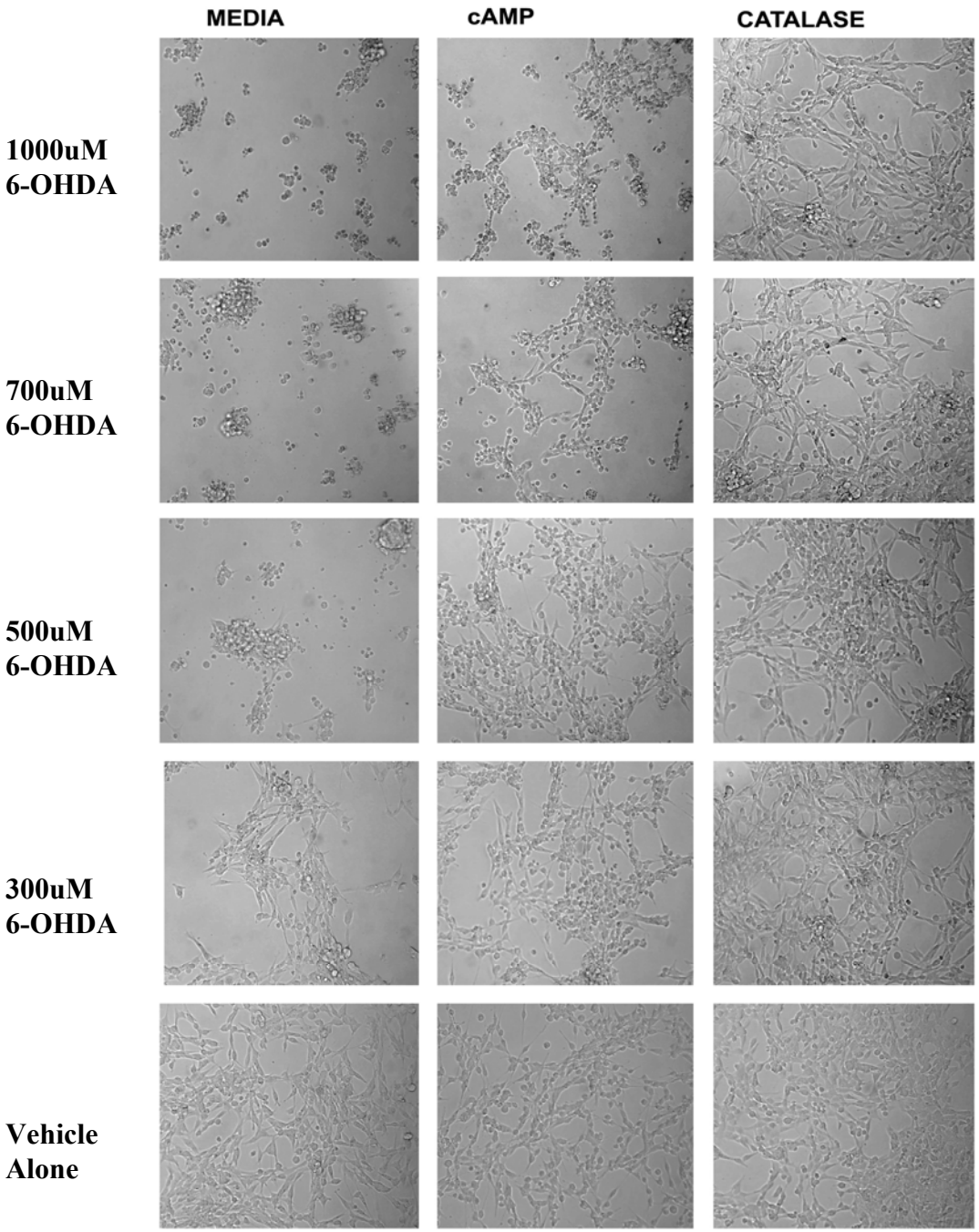

Figure 7. Morphological Evidence of cAMP and Catalase protection from 6-OHDA-induced Toxicity at a Range of Concentrations.

B65 cells were treated for 10 min with media (None), cAMP (250uM) or catalase (30U/ml) before exposure to vehicle or 6-OHDA for 18 hours. Cell injury was assessed my morhological examination. It is clear that cAMP-treated cells appear more healthy, and have less evidence of injury than the cells treated with media alone. The anti-oxidant catalase was used as a positive control for protection.

4.1.3 Delayed administration of cAMP reverses CRE repression and confers protection.

This effect was further characterized by determining whether delayed treatment with cAMP following initiation of 6-OHDA toxicity would still effectively reverse 6-OHDA-mediated CRE repression. We exposed the CRE-transfected B65 cells to cAMP at different intervals of time after 6-OHDA exposure. Results showed that delayed addition of cAMP indeed caused a reversal of 6-OHDA-induced repression (Figure 8A), an effect that was obvious up to one hour after toxin treatment. Moreover, delayed cAMP treatments also resulted in significant protection against toxicity even when administered up to 4 hours after initiation of 6-OHDA treatment (Figure 8B), further supporting the interpretation that perturbations to the CREB signaling pathway contributes to 6-OHDA toxicity.

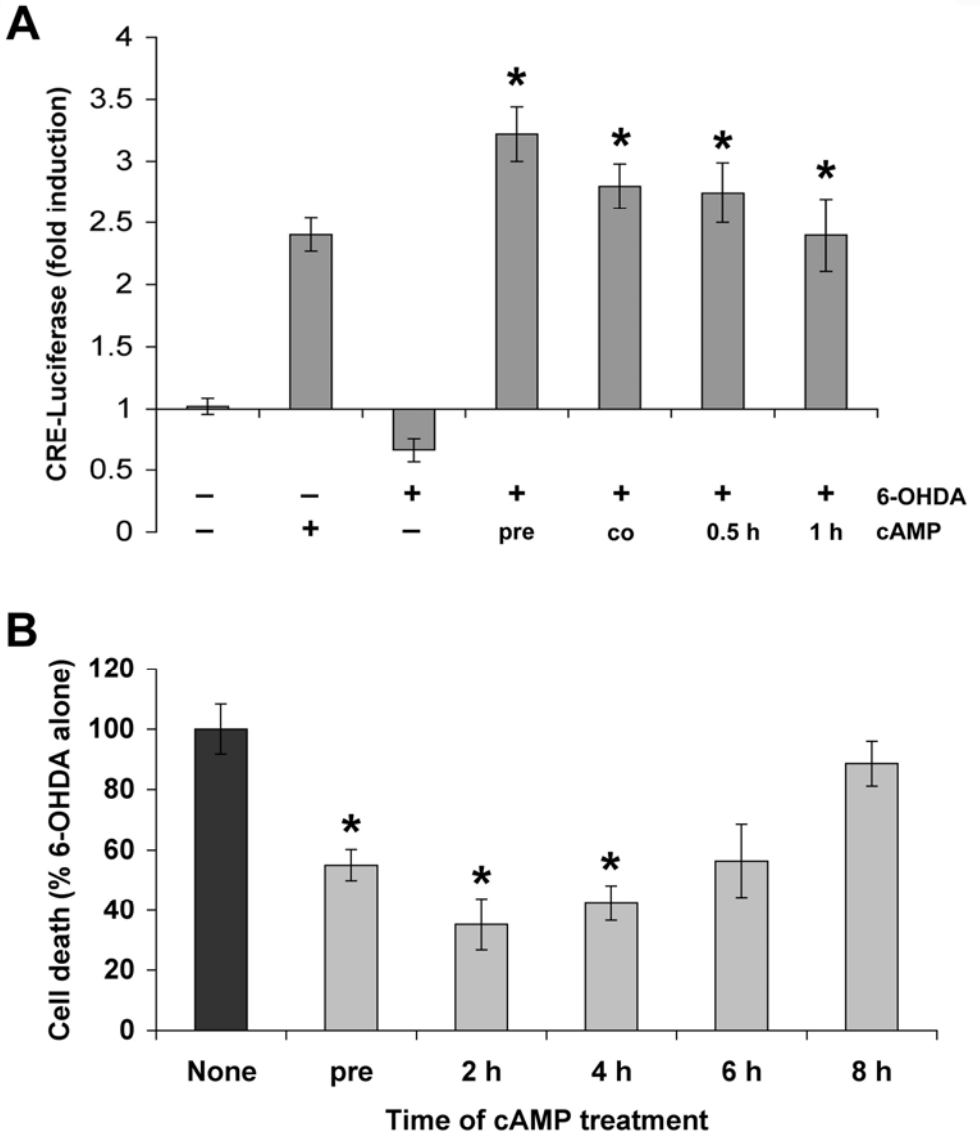

Figure 8. Delayed cAMP administration following 6-OHDA treatment reversed CRE repression and rescues cells from toxicity.

A. Cells were transfected with pCRE-luc plasmid and treated with 6-OHDA and/or cAMP as indicated. The cAMP was added 10 min prior to 6-OHDA (pre), simultaneously with 6-OHDA (co), or at different intervals after initiation of 6-OHDA injury. After 3 h, cells were lysed and luciferase activity measured. Data is the mean +/- standard deviation, and is representative of at least 3 independent experiments. *$p < 0.05$ vs. 6-OHDA alone (ANOVA followed by Student's t-test with Bonferroni correction). B. Cells were pre-treated with cAMP 10 minutes prior to 6-OHDA, or at different intervals after initiation of 6-OHDA injury. After 18 h, cell death was measured using the LDH release assay. Data represents the mean +/- standard deviation from at least three independent experiments. *$p < 0.05$ vs. 6-OHDA alone (ANOVA followed by Student's t-test with Bonferroni correction).

We next looked to determine if administration of BDNF would protect B65 cells from 6-OHDA**.** Since BDNF is a CRE-mediated gene and is well known for its role in neuronal survival (Mayr and Montminy 2001), and CRE-mediated transcription is repressed in response to 6-OHDA, we hypothesized that pre-treating cells with BDNF would diminish the cytotoxic effects of 6-OHDA. However, this was not the case, as BDNF treatment had no effect on 6-OHDA-induced cell death (Figure 9).

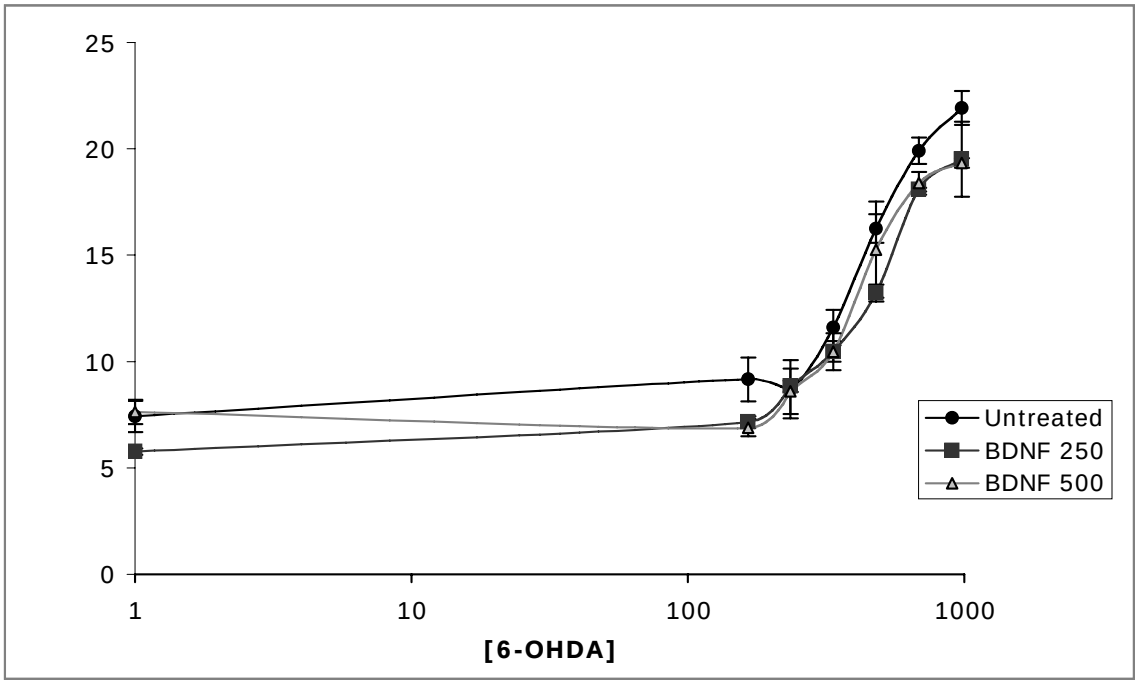

Figure 9. BDNF does not confer protection from 6-OHDA-induced toxicity.
B65 cells were treated for 10 min with media (None) or BDNF (250 or 500nM) before exposure to vehicle or 6-OHDA for 18 hours. Cell injury was assayed using the LDH release assay, and are representative of at least 3 independent experiments. These results are representative of 2 independent experiments.

4.1.4 6-OHDA reduces expression of CRE-regulated genes:

To confirm that 6-OHDA-induced repression of CRE transactivation was associated with functional effects on endogenous genes, we examined the expression of downstream CRE-regulated genes (Mayr and Montminy 2001). BDNF and Bcl-2 were selected due to their well-documented roles in neuronal growth and survival as well as prior studies showing that both CREB-induced genes can protect from 6-OHDA toxicity (Jordan et al. 2004) (Klein et al. 1999). Semi-quantitative and quantitative RT-PCR showed decreased *BCL2* and *BDNF* mRNA in response to 6-OHDA treatment (Figure 10). No changes were observed in the mRNA levels of MAPK phosphatase-3 (MKP-3), which lacks CRE sequences in its promoter region, or in β-actin mRNA levels.

In addition, stimulation of CRE by cAMP prevented the 6-OHDA-induced reduction in mRNA expression of both Bcl2 and BDNF (Figure 10, see lanes labeled "Both"), with cAMP and 6-OHDA co-treatment resulting in BDNF and Bcl2 gene expression that was even higher than baseline, or with cAMP stimulation alone. Since previous results showed cAMP pre-treatment also conferred significant protection (see Figures 6,7), these data indicate that 6-OHDA-mediated repression of CRE transactivation results in decreased expression of CRE-controlled survival genes during 6-OHDA-induced neuronal cell death, an effect that may be contributing to the cell death process.

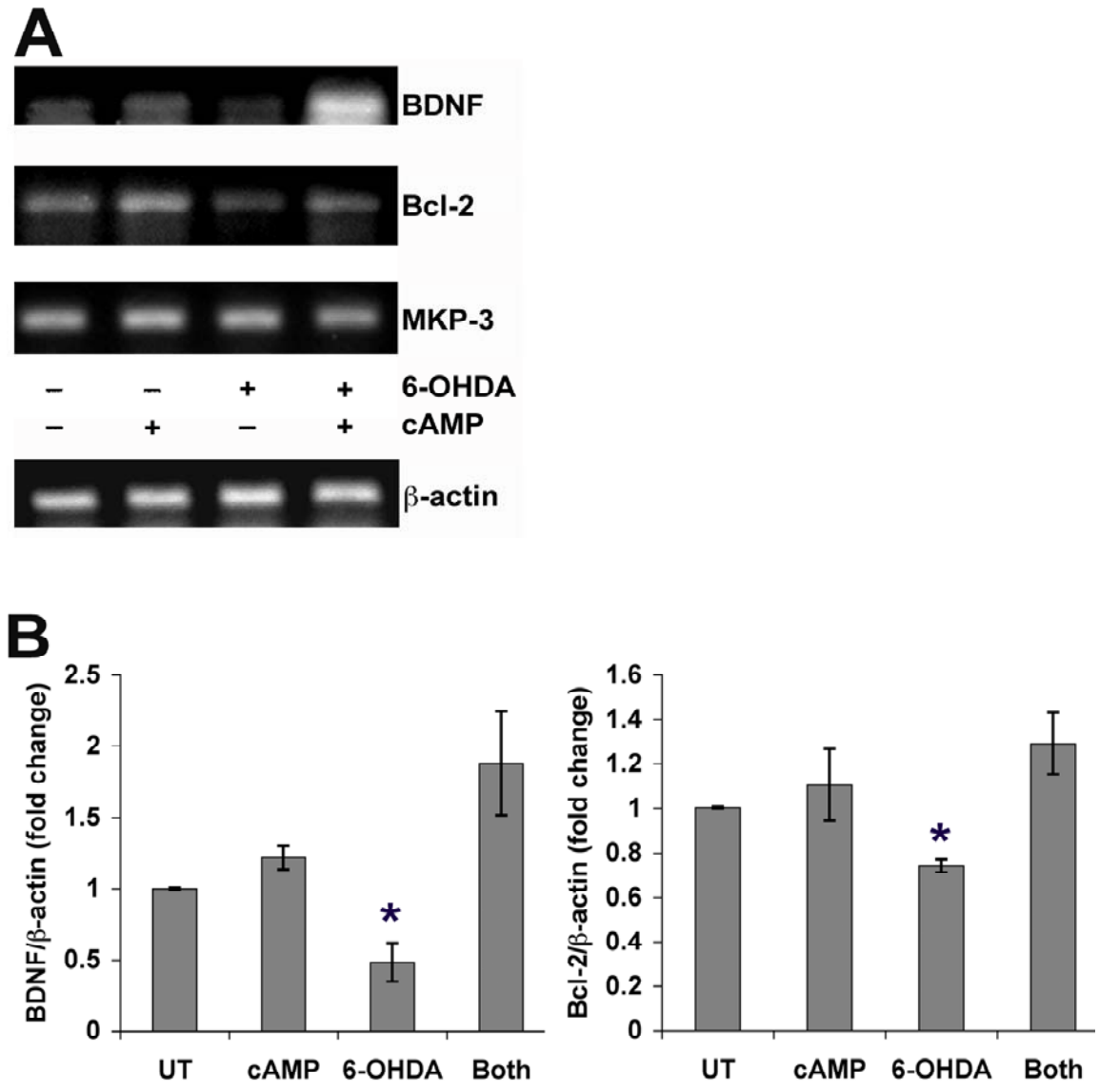

Figure 10. 6-OHDA results in decreased BDNF and Bcl-2 mRNA expression, which is reversed by cAMP.

A. B65 cells were treated with combinations of 6-OHDA and cAMP as indicated and subjected to RT-PCR analysis as described in Methods. Note the decreased levels of BDNF and Bcl-2 mRNA in 6-OHDA treated cells, which is reversed by addition of cAMP. In contrast, there are no changes in the mRNA levels of MKP-3 or □-actin, two genes that lack CRE elements in their promoter regions. Gels are representative of three independent experiments. B. For quantitative RT-PCR, cells were treated as above and analyzed by real time RT-PCR using the Roche LightCycler SYBR Green I system. Data was analyzed with relation to □actin crossing points using the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). Each graph reflects the average of three independent experiments. *p < 0.05 vs. UT/Both (ANOVA & Student's t-test with Bonferroni correction).

Interestingly- the promoter of the CREB gene itself also contains a CRE site, and CREB activation (via phosphorylation) allows CREB to positively regulate its own expression (Mayr and Montminy 2001). Therefore, message level for CREB were also examined. As hypothesized, qualitative RT-PCR showed that levels of CREB mRNA were decreased in response to 6-OHDA (Figure 11). This decrease was not as strong as seen with other CRE-mediated genes, Bcl2 and BDNF, however, the minor decrease was reversed by cAMP co-treatment.

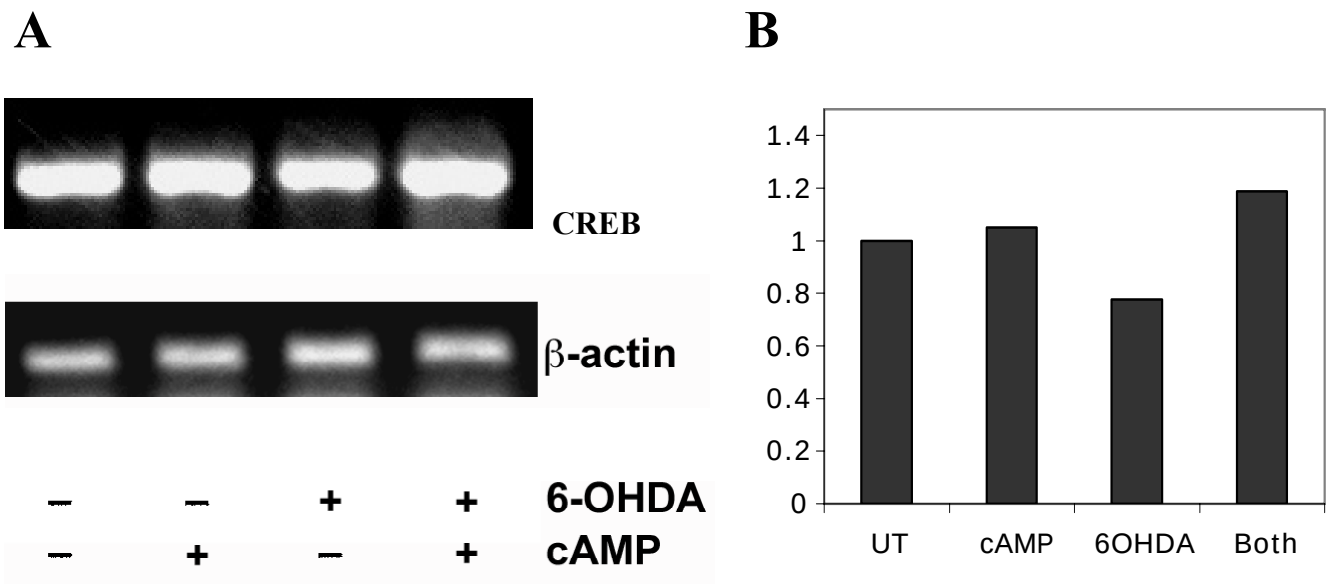

Figure 11. 6-OHDA treatment resulted in a decrease in CREB mRNA expression.

A. B65 cells were treated with combinations of 6-OHDA and cAMP as indicated and subjected to RT-PCR analysis as described in Methods. B. The graph on the right shows densitomitry analysis of the observed bands. Note the decreased levels of CREB mRNA in 6-OHDA treated cells, which is reversed by addition of cAMP. In contrast, there are no changes in the mRNA levels of □-actin, which lacks a CRE element in its promoter regions. Data are reprecsentative of two independent experiments.

4.1.5 Electromobility shift assay demonstrates decreased CRE binding activity in 6-OHDA treated cells.

Next, to determine the effect of 6-OHDA on CRE DNA binding activity, nuclear extracts from B65 cells treated with 6-OHDA and/or cAMP for 3 h were incubated with radiolabeled DNA containing a palindromic CRE sequence. This consensus CRE sequence has been used extensively in the literature and is also commercially available (See Materials and Methods). Use of specific (unlabeled probe) and nonspecific (poly-dIdC) competitors revealed a specific protein-CRE doublet. Treatment with 6-OHDA resulted in *decreased* CRE binding activity of both bands compared to control and cAMP treated cells (Fig. 12A). Supershift assay using a mixture of CREB antibodies confirmed the presence of CREB/pCREB in the complex (Fig. 12B). Interestingly, cAMP co-treatment did not reverse the decrease in CRE binding activity observed in 6-OHDA treated cells, despite its ability to reverse CRE-transcriptional repression, as indicated by CRE-luciferase and quantitative RT-PCR data shown above. It is possible that the kinetics of cAMP-mediated changes do not correspond with the time point analyzed. A more likely explanation is that complex multifactorial interactions (co-activators and repressors) underlie the ability of cAMP to reverse 6-OHDA mediated transcriptional repression.

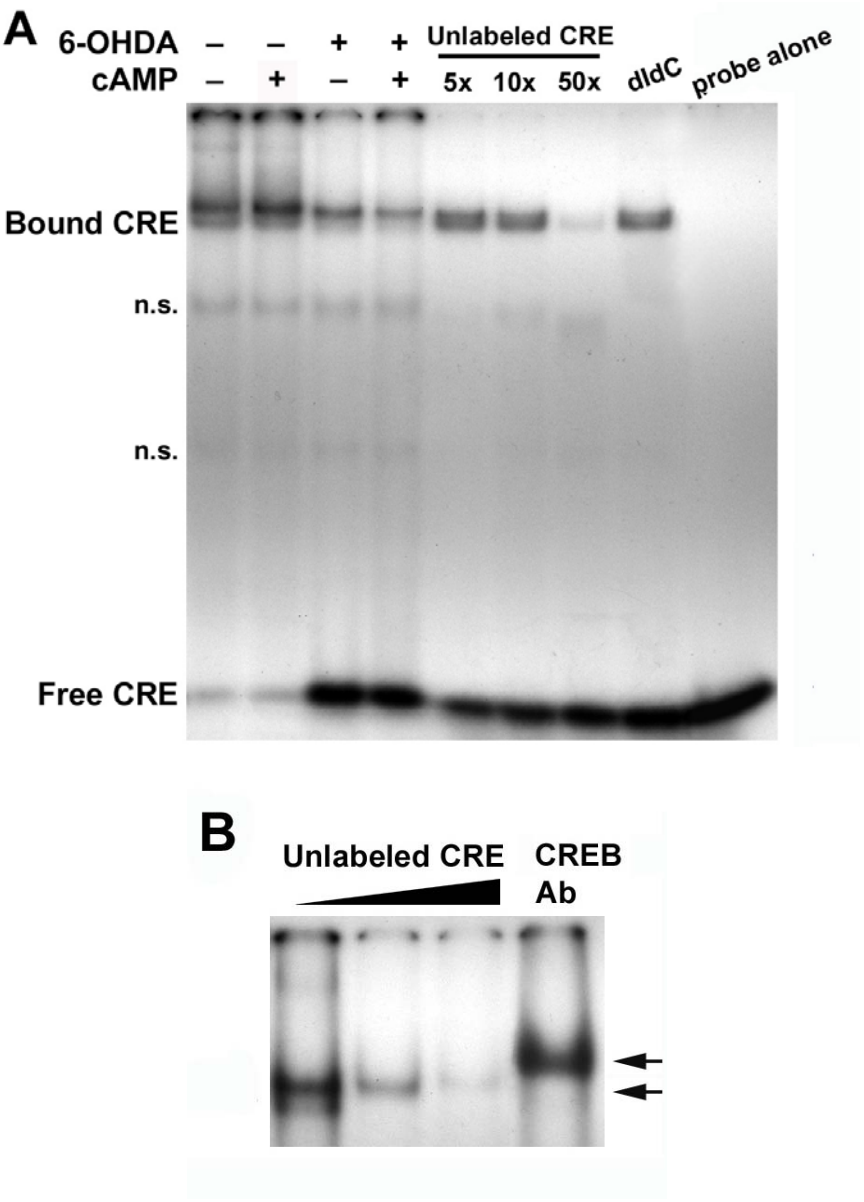

Figure 12. Electrophoretic mobility shift assay of CRE-binding activity in B65 cells.

A. Nuclear extracts prepared from B65 cells that were untreated or treated with dibutyryl-cAMP alone/or prior to 6-OHDA for 3 h were incubated with a ^{32}P-labeled CRE probe for 15-20 minutes at room temperature. Where indicated, incubations were performed in the presence of a 5-fold, 10-fold or 50-fold molar excess of unlabeled CRE probe to inhibit the specific band (panel A), in the presence of poly-dIdC to inhibit the nonspecific bands (n.s., panel A), or in the presence of CREB antibodies to supershift the protein-CRE complex (panel B). The migration of protein-bound CRE probe is retarded relative to that of the free CRE probe. B. To determine whether the protein-CRE complexes included CREB, antibodies were used to supershift the complexes. Arrows indicate the relative migration of protein-bound CRE with and without the CREB antibodies.

4.1.6 The Protective effects of cAMP work though PKA.

To determine whether the effects of cAMP were mediated through activation of protein kinase A, cells were treated in the presence of a protein kinase A inhibitor H89 (10mM, (Sanchez et al. 2001)). The presence of H89 suppressed cAMP-mediated increases in CRE-luciferase activity to baseline levels in cells co-treated with cAMP and 6-OHDA (Figure 13). These results suggest that the transcriptional effects of cAMP are mediated through protein kinase A, although additional mechanisms affecting 6-OHDA-mediated repression cannot be excluded.

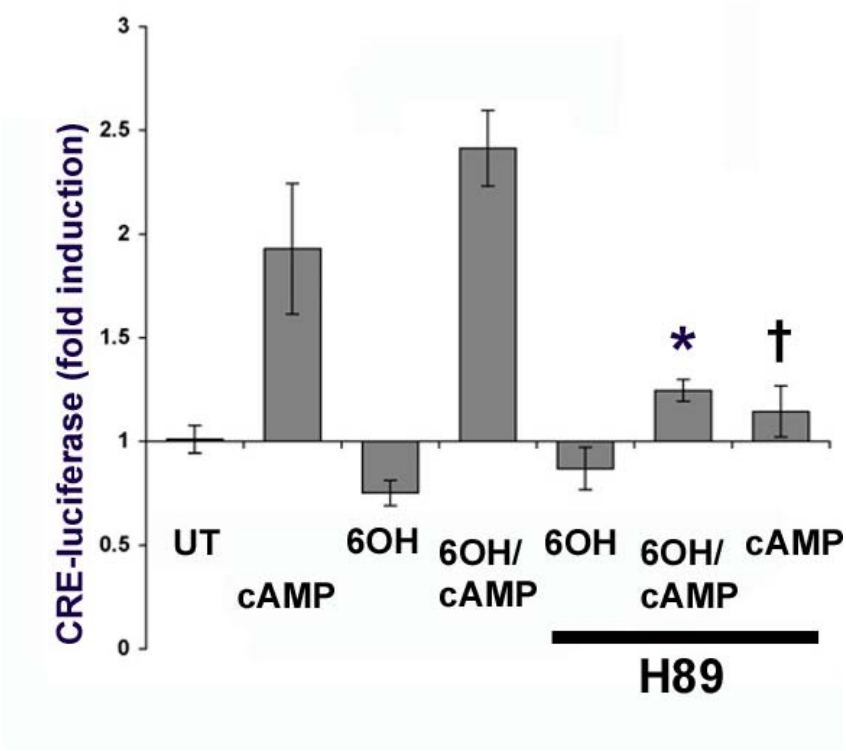

Figure 13. H89 supresses cAMP-mediated increases in CRE-luciferase activity.

B65 cells transfected with CRE-luciferase were treated with cAMP, 6-OHDA (6OH), or both cAMP and 6-OHDA, in the absence or presence of the PKA inhibitor H89 (10 □M). After 3 h, lysates were collected for luciferase assay, and the results normalized to untreated control cultures. 6-OHDA caused significant repression of CRE-luciferase expression, which was not reversed by H89. H89 co-treatment significantly inhibited effects of cAMP alone and the effects of cAMP on cells treated with 6-OHDA. * $p < 0.01$ versus 6-OHDA/cAMP. † $p < 0.01$ versus cAMP.

Additionally, the effects of inhibitors of both the PKA pathway (H89) and of the ERK pathway (PD098059 and OU126) on the basal levels of CRE activity in B65 cells were examined. Since CRE-mediated can be activated by multiple pathways in neuronal cells, we sought to determine the relative contribution of these two main CREB-activating pathways to baseline CRE stimulation in growing neurons. Interestingly, MEK inhibitors repressed basal levels of CRE activation (Figure 14 A,B), while the PKA inhibitor had no effect (Figure 14 C). These results suggest that cellular processes activated during neuronal growth in culture function primarily through the ERK pathway, with the PKA path making only a very minor, if any, contribution.

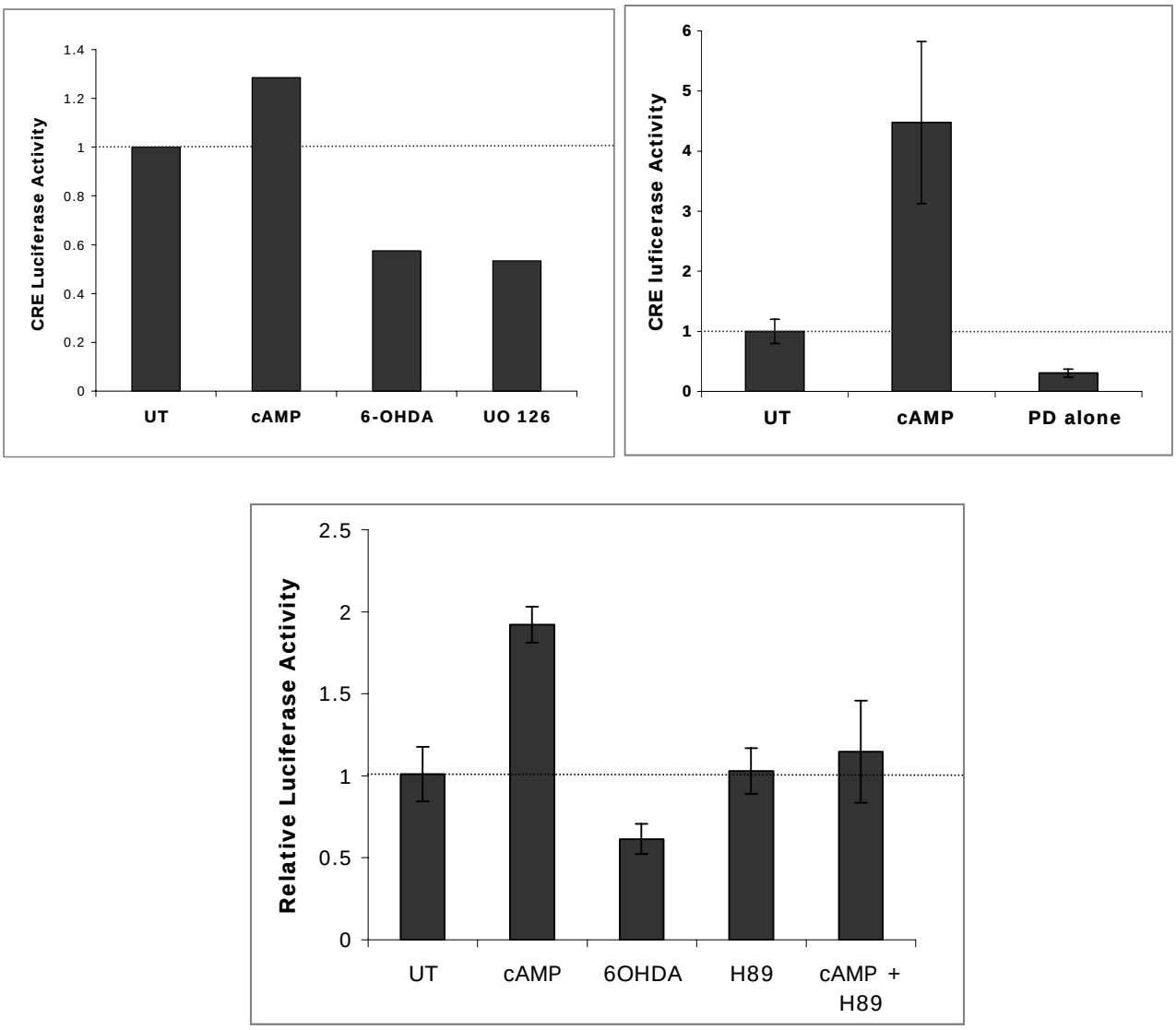

Figure 14. The MEK inhibitors, UO126 and PD98, represses CRE transactivation, but the PKA inhibitor, H89, does not.

B65 cells were transfected with CRE-luciferase and treated with cAMP. 6-OHDA, UO126, PD098059 or PKA , depending on experiment, and as indicated in figure legends. After 3 hours, lysates were collected for luciferase assay and results were normalized to the luciferase activity in the untreated control cultures for each experiment. Both PD and UO126 repressed CRE luciferase activity, and H89 had no effect.

4.1.7 CONCLUSIONS

The oxidative neurotoxin, 6-OHDA, is toxic to the B65 neuronal cell line. We have determined that 6-OHDA represses the CRE promoter, and reversal of that repression with cAMP treatment protects cells from toxicity. This protection was evident even with delayed cAMP administration, up to 4 hours following 6-OHDA treatment. The observed repression had a functional effect, as downstream CRE-mediated genes, BDNF, Bcl2, and CREB were all decreased in response to the toxin. Restoration of gene expression occurred with cAMP stimulation, an effect that was associated with protection from death. When DNA binding was examined using EMSA, we see that 6-OHDA decreases in CRE binding activity. Interestingly, the PKA inhibitor, H89, did not affect basal CRE activity. However, inhibition of the ERK pathway (via MEK inhibitors, PD098059 and OU126) did results in CRE repression, indicating that basal CRE activity is working, at least in part, through the ERK and not PKA. Additionally, since camp-mediated reversal of 6-OHDA induced CRE repression could be blocked with H89, this indicates that the protective effects of camp observed in this cell line occur through PKA. In the next set of experiments, potential mechanisms for these observed results were examined.

4.2 CREB LOCALIZATION

4.2.1 Effects of 6-OHDA treatment on CREB phosphorylation and localization.

To further investigate potential mechanism(s) underlying CRE repression, we examined the expression levels and subcellular localization patterns of CREB and pCREB in B65 cells. In

contrast to studies in other systems that showed decreased CREB phosphorylation as the major mechanism of signaling pathway repression (See and Loeffler 2001) (Ito et al. 1999), 6-OHDA treated cells exhibited a clear increase in pCREB (Figure 15A), using whole cell lysates.

Since CREB can be activated in the cytoplasm by phosphorylation of Ser-133 before translocating to the nucleus to control gene transcription (Stevenson et al. 2001), we performed subcellular fractionation studies. Time course studies revealed that 6-OHDA caused progressive accumulation of pCREB in the cytoplasm (Figure 15C), with a mild, but persistent decrease in nuclear fractions of 6-OHDA treated cells (Figure 15B). A similar pattern was observed using an antibody that recognizes CREB irrespective of its phosphorylation state (FIGURE 16). The altered subcellular distribution of pCREB in 6-OHDA treated cells likely contributes to the functional repression of CRE transactivation, despite increased total levels of pCREB. Interestingly, the addition of protective doses of cAMP to 6-OHDA treated cells ameliorated the decrease in nuclear pCREB, but had little effect on pCREB accumulation in the cytoplasm (Figure 15B & C, last lane).

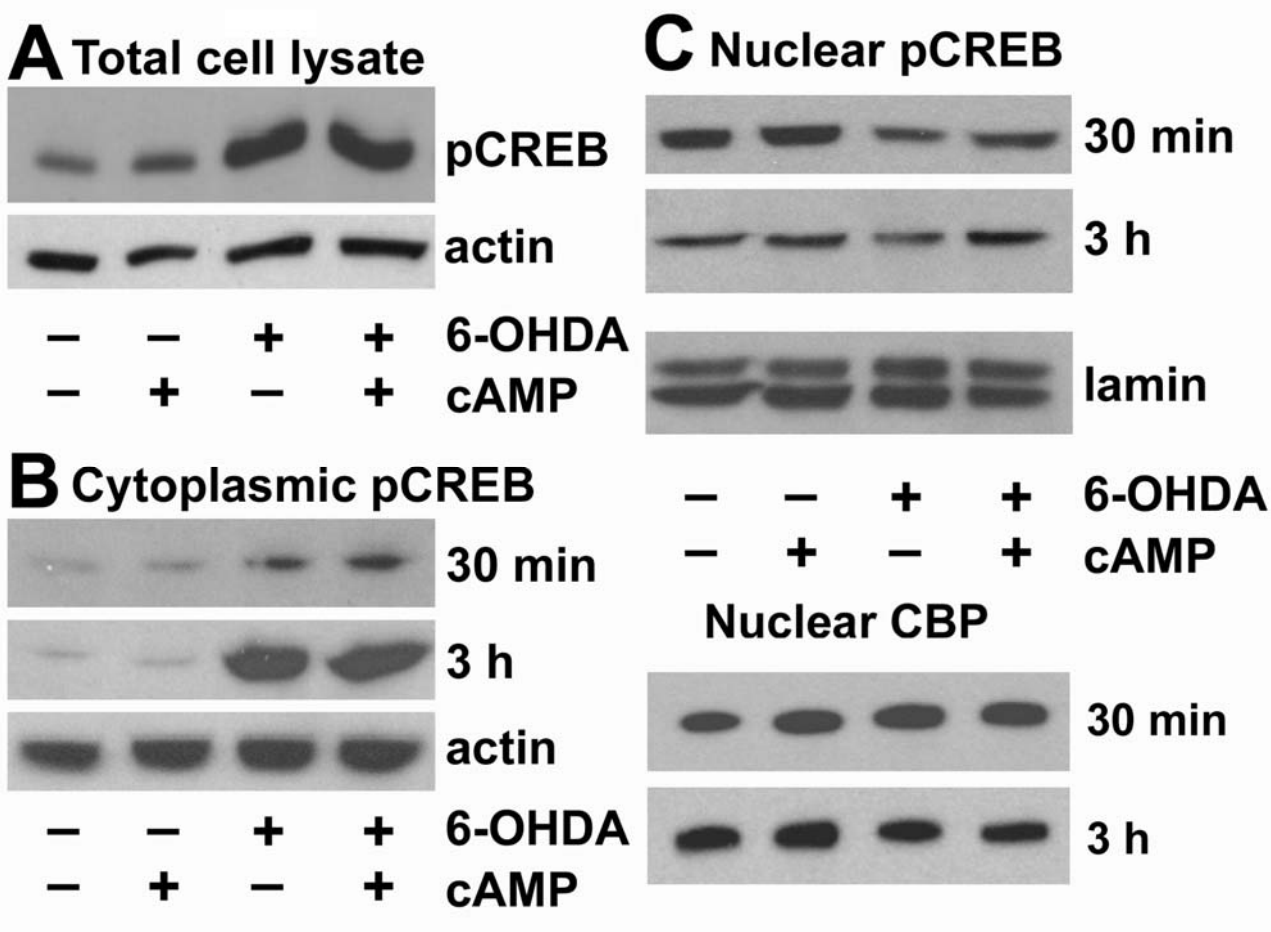

Figure 15. Effects of 6-OHDA treatment on subcellular distribution of pCREB.

A. Total Triton-X100 cell lysates were prepared following treatment with the indicated combinations of cAMP and 6-OHDA, and subjected to western blot analysis for pCREB. B. Nuclear fractions were prepared from cells treated with cAMP and 6-OHDA for the indicated time points, and analyzed for pCREB, then stripped and reprobed for the nuclear marker lamin A. C. Cytoplasmic fractions were prepared from cells treated with cAMP and 6-OHDA for the indicated time points, and analyzed for pCREB, then stripped and reprobed for b-actin as a loading control. Data is representative of at least 3 independent experiments.

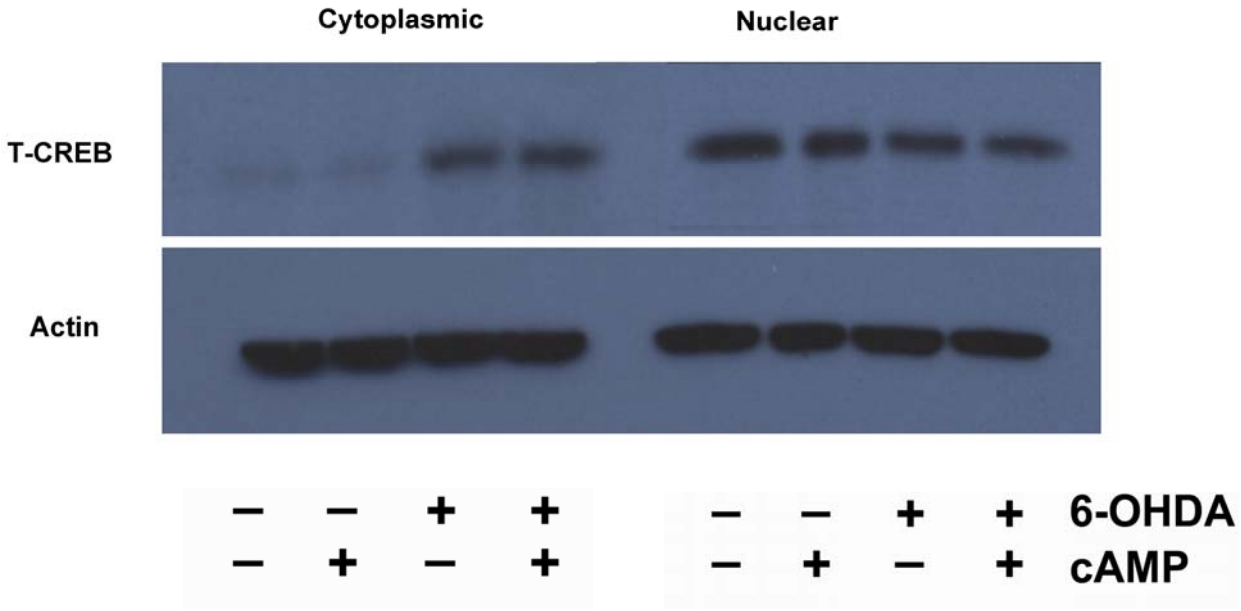

Figure 16. Effects of 6-OHDA on Total-CREB.

Cytoplasmic and nuclear fractions were prepared from cells treated with cAMP and 6-OHDA for the indicated time points, and analyzed for total CREB. Blots were then stripped and re-probed for b-actin as a loading control. Data is representative of at least 3 independent experiments.

Since binding of co-factors is often critical for stimulus-induced activation of CREB in various important cell processes, we next looked at the expression levels of CREB-binding protein (CBP) in B65 cells in response to 6-OHDA. CBP is a 265 kDa nuclear protein that associates with phosphorylated CREB (reviewed in (Goldberg and Barres 2000))and functions as a CREB co-activator. CBP sequestration (and subsequent disruption of function) has been shown to be a contributing factor in the pathogenesis of various polyglutamine-mediated diseases (Reviewed in (Rouaux et al. 2004). To examine if disruption of CBP played a role in our observed decrease in CRE function, we looked at expression levels in nuclear and cytoplasmic fractions. Our data indicate that there was no change in the levels of CREB binding protein

(CBP), which was present only in the nuclear fractions (Figure 17). Thus, the altered nuclear-cytoplasmic distribution of CREB is not due to generalized disruption of all nuclear transcription related proteins, nor is a loss of this important CREB co-activator contributing to CRE disruption in our model.

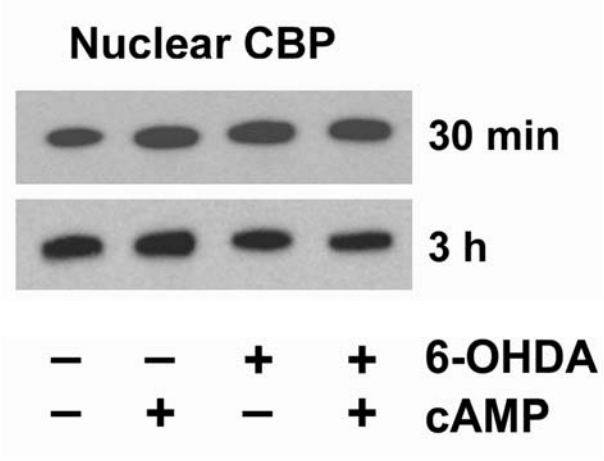

Figure 17. CBP Expression in nuclear fractions of 6-OHDA-treated B65 cells.

Nuclear and cytoplasmic fractions were prepared from B65 cells treated with cAMP and/or 6-OHDA for the indicated time points, and analyzed for CBP as described in Methods. Note that CBP expression in response to 6-OHDA is not changed from the untreated. CBP was not detected in cytoplasmic fractions.

4.2.2 Immunofluorescence localization of pCREB in 6-OHDA treated cells.

The distribution of pCREB was further characterized using immunofluorescence. In control cells, there is a low diffuse background level of pCREB staining, which is predominantly cytoplasmic in distribution (Figure 18A). The addition of cAMP to otherwise untreated cells had no effect on the intensity of pCREB staining in the cells, but increased the numbers of cells showing nuclear localization of pCREB (Figure 18B). In contrast, treatment with 6-OHDA for three hours resulted in markedly increased cytoplasmic pCREB staining intensity (Figure 18C). Co-treatment of 6-OHDA with cAMP promoted nuclear localization of pCREB in a subset of cells (Figure 18D). These *in situ* techniques support the results obtained from the subcellular fractionation studies, and demonstrate that 6-OHDA elicits accumulation of pCREB in the cytoplasm. Despite this increase in cytoplasmic pCREB levels, nuclear expression of pCREB and transactivation of CRE-containing genes are suppressed during 6-OHDA toxicity.

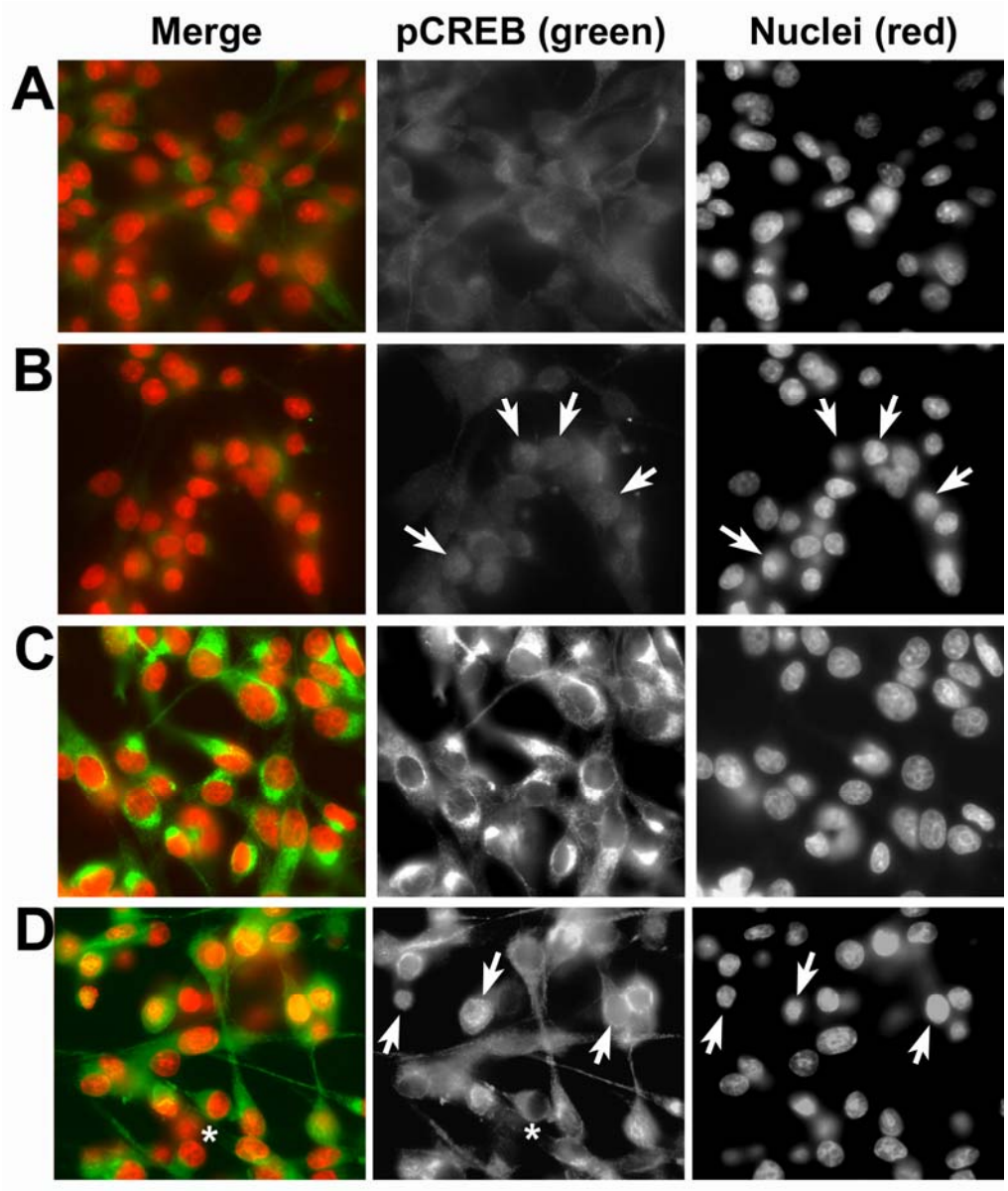

Figure 18. 6-OHDA elicits increased cytoplasmic and perinuclear staining for pCREB.

Cells were treated as indicated below. After 3 h, coverslips were fixed and double labeled for pCREB and the nuclear marker DAPI as described in Methods. A. A light, predominantly cytoplasmic, staining pattern is observed for pCREB in control cultures. B. Addition of cAMP alone promotes nuclear staining for pCREB (arrows, note that pCREB staining is diffuse with no evidence of nuclear exclusion in many cells). C. 6-OHDA treated cells display increased cytoplasmic staining. D. 6-OHDA/cAMP co-treated cells display both increased cytoplasmic staining with exclusion of the nucleus (asterisks) in some cells and diffuse nuclear or nuclear-cytoplasmic staining (arrows) in other cells. Data is representative of 3 independent experiments.

4.2.3 Conclusions

To examine the potential mechanism underlying 6-OHDA-induced CRE repression, a series of immunoblot experiments were preformed. Data indicate that 6-OHDA causes a diversion of CREB/pCREB out into the cytoplasm, and protection from the toxin was associated with restoration of nuclear expression. Expression patters of the important CREB co-activator, CBP, were not altered in response to 6-OHDA. These data suggest that cytoplasmic diversion of the important nuclear transcription factor CREB may be a mechanism that underlies the cellular processes that are activated during 6-OHDA-induced neuronal death.

4.3 SH-SY5Y CELLS, PRIMARY NEURONS AND HUMAN TISSUE

4.3.1 6-OHDA induces CRE repression and decreased nuclear CREB that is reversed by cAMP in SH-SY5Y cells.

The effects of 6-OHDA on CREB signaling was confirmed using a different neuronal cell line. SH-SY5Y is a human neuroblastoma cell line that produces dopamine and expresses dopamine transporters (Decker et al. 1993). SH-SY5Y cells have been extensively used to model dopaminergic neuron injury and death. In this cell line 6-OHDA caused a 50% repression in basal CRE-luciferase activity in SH-SY5Y cells (Fig. 19), an effect that concurs with the 6-OHDA-induced repression seen in B65 cells (See Figure 1). While co-treatment with cAMP completely reversed the 6-OHDA mediated transcriptional repression, co-treatment did not result in the degree of increased CRE transactivation observed in B65 cells.

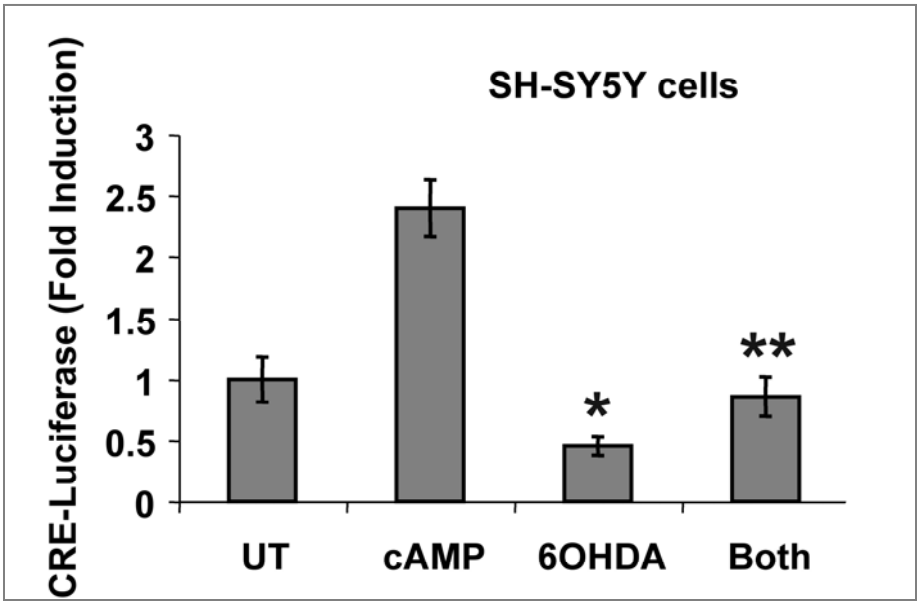

Figure 19. 6-OHDA represses the basal activity of the CRE promoter in SH-SY5Y cells.

A human neuroblastoma-derived dopaminergic cell line was treated with LD_{50} doses of 6-OHDA (150 uM) for 3 h. A. In these experiments, cells were transfected with CRE-luciferase reporter 24 h earlier, and lysates were analyzed in quadruplicate wells for luciferase activity. Data is expressed as the fold induction of luciferase activity normalized to the untreated control group +/- standard deviation.

We next examined whether co-treatment with cAMP could confer protection against 6-OHDA-mediated toxicity. Indeed, a 10 minute pre-treatment with cAMP prior to 6-OHDA initiation resulted in significantly decreased cell injury at 18 hours, as shown by LDH assay (Figure 20A). Additionally, a separate cell viability assay (alamarBlue) showed increased cell number with cAMP co-treatment, again indicating that reversing 6-OHDA-induced repression protected SY5Y cells from cell death. (Figure 20B). Evidence of protection was observed by morphological observation as well (Figure 21). Taken together, these data further support the hypothesis that disruption in CRE function contributes to 6-OHDA-induced death in neuronal cells.

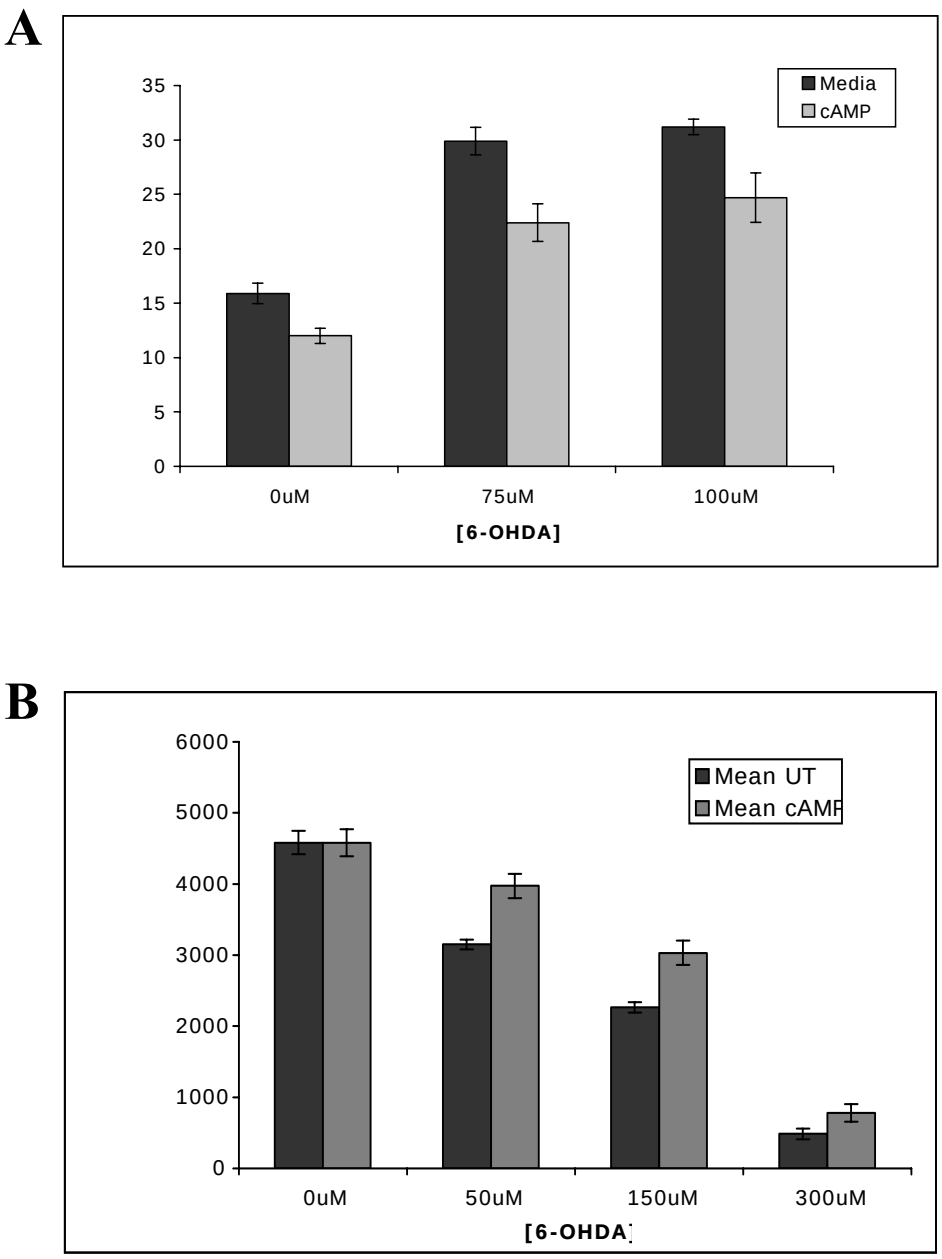

Figure 20. cAMP Protects SH-SY5Y Cells from 6-OHDA toxicity, as shown by two cell death assay (LDH Release), cell viability assay (alamarBlue).

B65 cells were treated for 10 minutes with media or camp (500uM) before exposure to vehicle or 6-OHDA (at indicated concentrations) for 18 hours. A. Cell injury was assessed using the LDH release assay, and results shown are representative of at least 2 independent experiments. B. Cell viability was assessed using the AlimarBlue assay system. Increased fluorescence is indicative of increased cell viability.

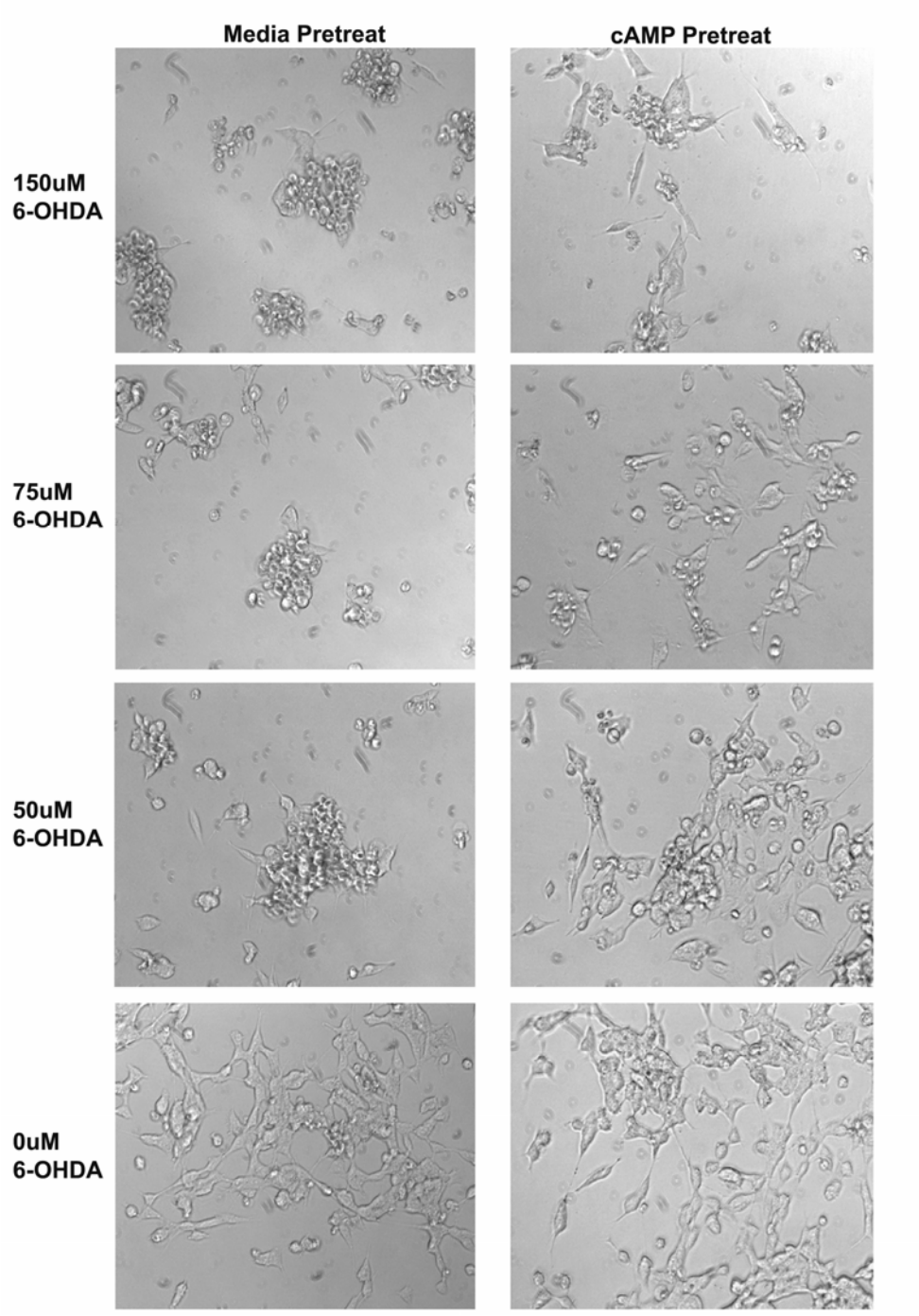

Figure 21. Morphological Evidence of cAMP Protection of SH-SY5Y Cells from 6-OHDA toxicity.

SH-SY5Y cells were treated for 10 minutes with media alone or camp (500uM), before exposure to vehicle (0uM 6-OHDA) or 6-OHDA at indicated concentrations for 18 hours. Cells were photographed and morphology was examined, indicating that cAMP pretreatment resulted in a greater number of viable cells.

4.3.2 Altered subcellular localization of CREB in SY5Y cells.

We next examined sub-cellular expression patterns of CREB and pCREB in SY5Y cells. As in B65 cells, Western blot indicates that repression of basal CRE transactivation was accompanied by increased cytoplasmic CREB/pCREB and by decreased nuclear CREB/pCREB in SH-SY5Y cells (Fig. 22A). Restoration of CRE transcription in cells co-treated with cAMP and 6-OHDA was accompanied by increased nuclear pCREB, although levels of total CREB were not appreciably altered relative to 6-OHDA alone. Immunofluorescence studies also confirm an increase in cytoplasmic pCREB staining in 6-OHDA-treated SH-SY5Y cells (Fig. 22B). Interestingly, there was also a change in the cytoplasmic distribution of pCREB. Control cells show a light diffuse, finely stippled staining pattern. The addition of 6-OHDA resulted in the appearance of a clumped perinuclear staining pattern.

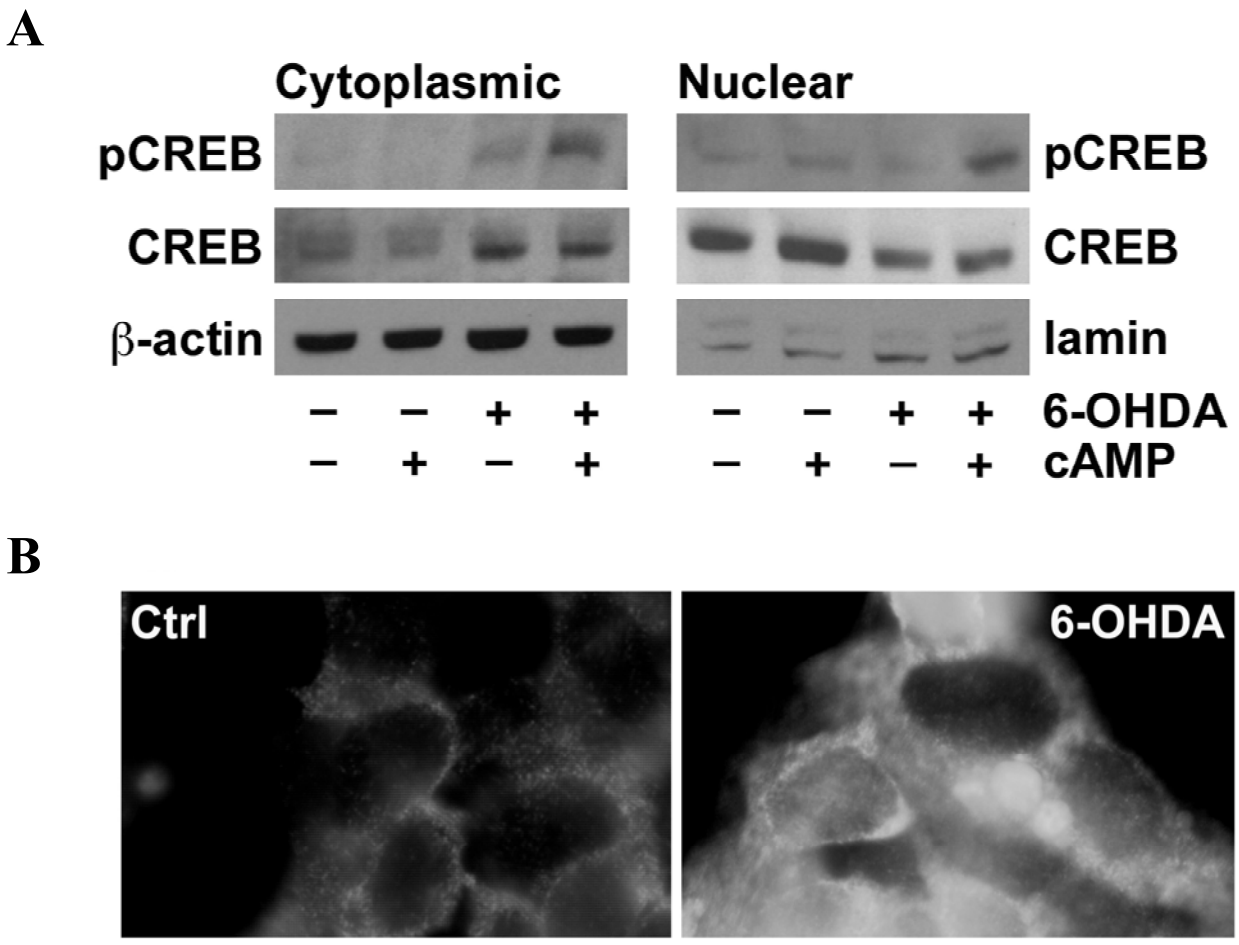

Figure 22. 6-OHDA treatment results in increased cytoplasmic CREB/pCREB and decreased nuclear CREB/pCREB in SH-SY5Y cells.

A human neuroblastoma-derived dopaminergic cell line was treated with LD_{50} doses of 6-OHDA (150 □M) for 3 h. A. **Equal amounts of protein were loaded from cytoplasmic and nuclear fractions of lysates from cells treated as indicated. Immunoblot analysis for pCREB, total CREB and loading controls were performed.** B. **SH-SY5Y cells were treated with vehicle or 6-OHDA x 3 h and subjected to immunofluorescence for pCREB. Note that 6-OHDA treated cells display increased cytoplasmic pCREB staining which shows a clumped distribution in contrast to the diffuse light pCREB staining in control cells**

Consistent with previously published studies, 6-OHDA toxicity was accompanied by caspase 3 activation in SH-SY5Y cells (Fig. 23A), which is indicative of activation of an apoptotic pathway. This increase in caspase 3 was significantly decreased by cAMP co-treatment. Note that 6-OHDA treatment did not elicit caspase 3 activation in B65 cells after 3 hours of 6-OHDA exposure- Figure 23B).

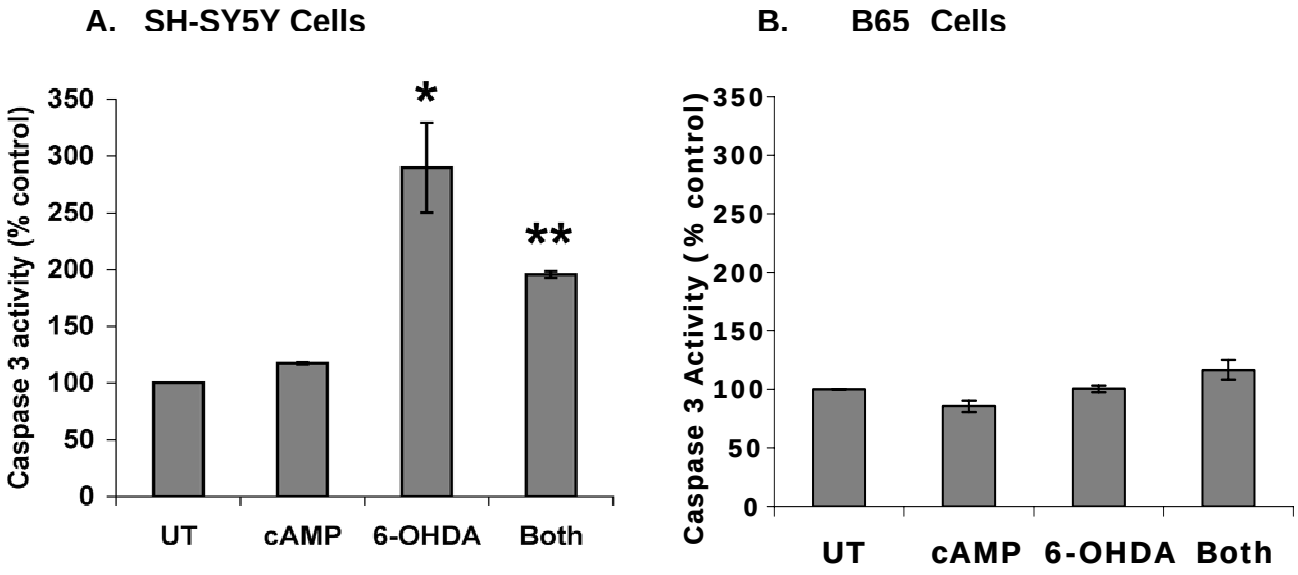

Figure 23. 6-OHDA elicits caspase 3 activity in SH-SY5Y cells, but not B65 cells.

6-OHDA elicits increased caspase 3 activity in SH-SY5Y cells that is significantly attenuated by co-treatment with cAMP. Cells were treated with LD_{50} doses of 6-OHDA (150 uM for SY5Y and 500 uM for B65 cell lines) for 3 h. Equal amounts of protein were analyzed from cytoplasmic fractions of lysates as indicated in the Methods section.
***p < 0.05 vs. UT; **p < 0.05 vs. 6-OHDA alone (ANOVA followed by Student's t-test with Bonferroni correction).**

4.3.3 6-OHDA injury to primary dopaminergic neurons is accompanied by loss of nuclear pCREB.

To determine the effects of 6-OHDA toxicity on primary dopaminergic neurons, midbrain cultures were derived from embryonic mice as described in Methods. Because the neuronal population of interest accounts for 10% of cells in these cultures, assays involving cell disruption are difficult to interpret. 6-OHDA treated cultures were triple labeled for pCREB, tyrosine hydroxylase to identify dopaminergic neurons, and DAPI as the nuclear marker. In contrast to the immortalized neuronal cells, primary neurons exhibit a higher basal expression of nuclear pCREB, which was enhanced by cAMP treatment (Fig. 24AB). In contrast, 6-OHDA exposure resulted in two staining patterns, both characterized by absence of nuclear pCREB. The more common pattern involved granular or clumped cytoplasmic staining for pCREB with exclusion from the nuclear outline (Fig. 24C). Other TH neurons exhibited a complete absence of pCREB staining all together (Fig. 24C asterisk). Interestingly, non-TH neurons in the cultures, which are more resistant to 6-OHDA injury as they lack an active transport mechanisms for the toxin, retained robust nuclear pCREB expression (Fig. 24D). Quantitative analysis of nuclear staining in TH+ neurons was performed using image analysis protocols described previously to measure nuclear versus cytoplasmic staining for another death regulator (Chu et al. 2005). These results indicate significant loss of nuclear pCREB in 6-OHDA cells, which was reversed by cAMP treatment (Fig. 24E).

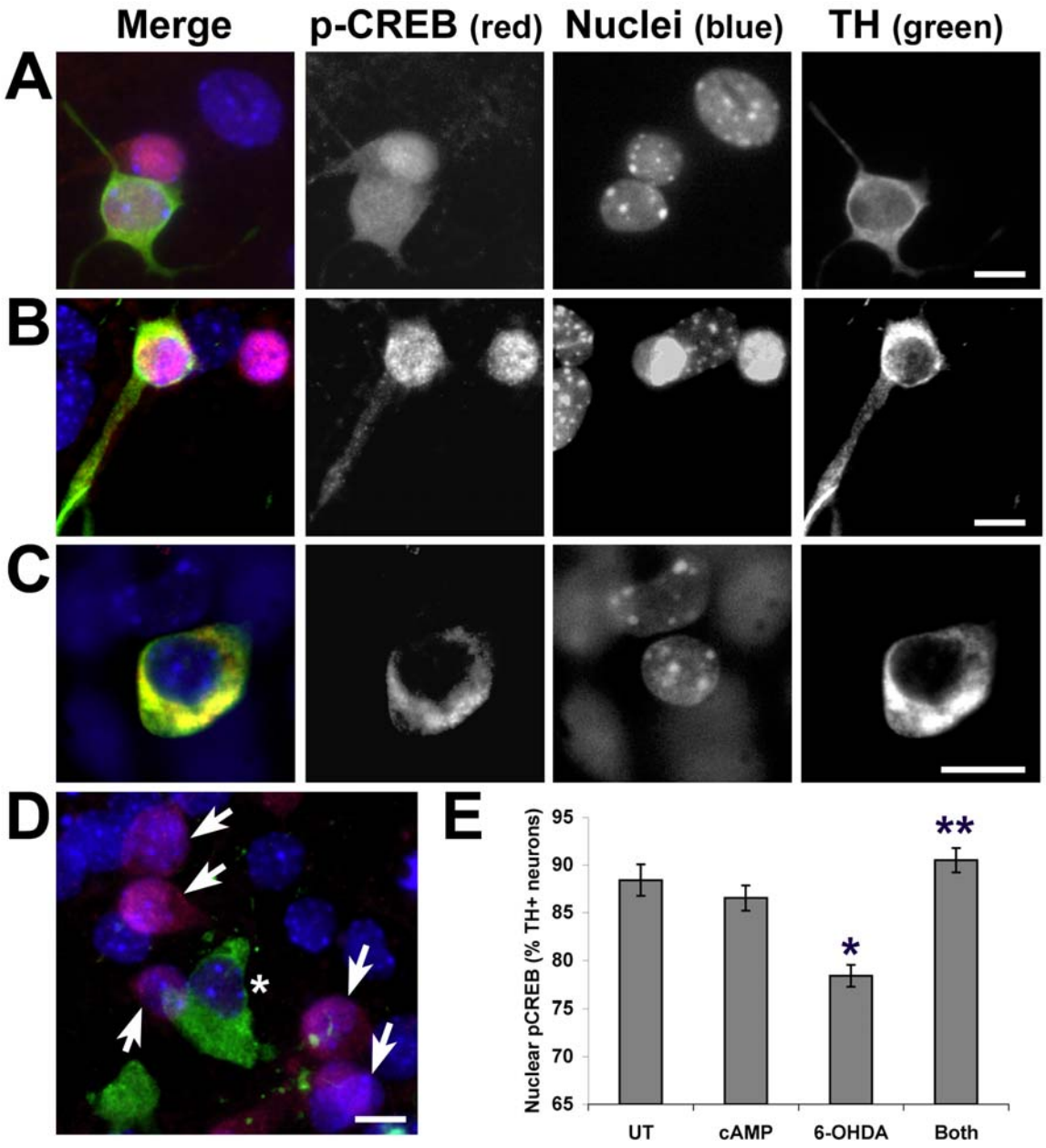

Figure 24. Decreased nuclear pCREB is observed specifically in 6-OHDA treated TH+ neurons, which is reversed by cAMP co-treatment.

Primary midbrain cultures from E15 mice were plated on chamber slides and treated with 50 □M 6-OHDA in the presence or absence of cAMP. Nuclear pCREB appears purple due to co-localization of the red pCREB signal and blue nuclear counterstain. A. In control cultures, both TH+ and non-TH neurons show diffuse nuclear and cytoplasmic staining for pCREB. B. Treatment with cAMP results in accentuated nuclear staining for pCREB in both TH+ and non-TH neurons. C. 6-OHDA, which is internalized by high affinity dopamine transporters on TH+ neurons, selectively decreases nuclear pCREB staining in many TH neurons. Cytoplasmic pCREB is preserved, sometimes developing a clumped

appearance similar to that observed in SH-SY5Y cells (yellow due to colocalization of red pCREB and green TH fluorescence). D. In other TH+ neurons, there is absence of nuclear and cytoplasmic pCREB staining (asterisk), but non-TH neurons in the same culture show nuclear pCREB (arrows). E. TH+ neurons from four independent chambers per treatment condition were scored as either showing nuclear pCREB staining or loss of nuclear pCREB staining. Note that 6-OHDA caused a significant decrease in nuclear pCREB at this early time point, which is reversed by co-treatment with cAMP. *p < 0.05 vs. UT; **p < 0.05 vs. 6-OHDA alone (ANOVA followed by Student's t-test with Bonferroni correction).

4.3.4 Cytoplasmic accumulation of pCREB is observed in dopaminergic neurons of human Parkinson's/Lewy body disease patients.

We previously found that kinases that functioning upstream of CREB and are capable of activating CREB were involved in granular or clumped cytoplasmic aggregates in Parkinson's disease and the closely related diffuse Lewy body dementia, and that this alteration is an early change observed in presymptomatic patients (Zhu et al. 2002). Accordingly, we next sought to look downstream of those kinases and examine the expression patterns of pCREB in human disease tissue, to determine the relevance of our results in an actual human neurodegenerative diseased brain. Immunohistochemical study of pCREB was performed on a subset of cases from this series for which tissue sections were available. In normal brains, pCREB staining is limited to glial nuclei (Fig. 25AB) and nuclei of endothelial cells (not shown). In the diseased human neurons, there was increased expression of granular or clumped pCREB immunoreactivity (Fig. 25, panels C-G) in both pigmented and depigmented (pale body) regions of cytoplasm. (Fig. 25FG). This clumped pattern of pCREB expression was reminiscent of the pattern observed with pERK in similar diseased tissue (Zhu et al. 2002), and suggest there may be a common disruption in nuclear/cytoplasmic trafficking occurring in neurodegenerative disease processes.

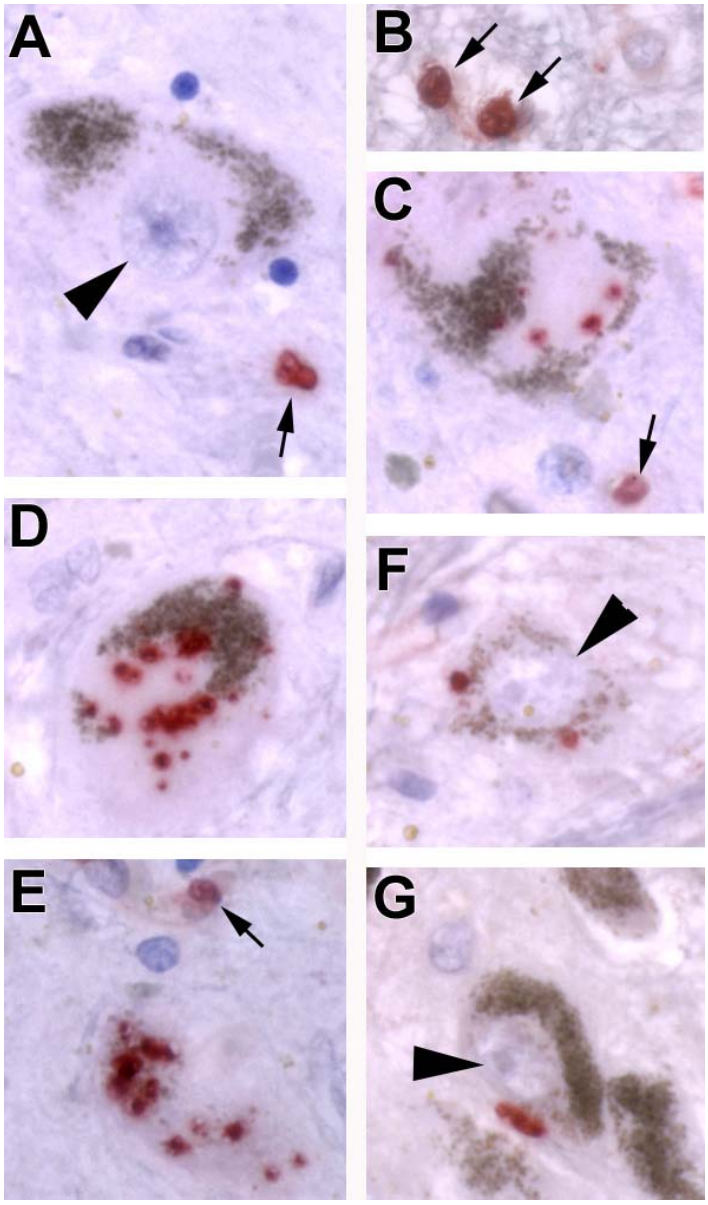

Figure 25. Clumped or granular pCREB is increased in the cytoplasm of dopaminergic neurons in the substantia nigra of parkinsonian patient brains.

A & B. In elderly control subjects, there is normally no pCREB staining (red) in SNc neurons (arrowhead), but staining of glial nuclei (arrow) is seen. Note the brown endogenous pigment that characterizes dopaminergic neurons in this nucleus, which is readily distinguished from the red chromogen used for the pCREB stain. C, D, E. In different Parkinson's/Lewy body disease patient brains, there was increased granular or clumped pCREB staining, often in association with regions of depigmentation (pale bodies). Positive staining is also observed in glial nuclei (arrows). F & G. The increase in pCREB staining was restricted to cytoplasm of SNc neurons, and was not observed in neuronal nuclei (arrowheads). Scale: 10 um.

4.3.5 Conclusions.

The effects of 6-OHDA on the B65 neuronal cell line were confirmed in three separate cell systems: the Sh-SY5Y dopaminergic neuronal cell line, primary midbrain neurons, and post-mortem Parkinson's Disease brain tissue. These data show that in all model systems, 6-OHDA disrupts CREB signaling. In SY5Y cells, 6-OHDA causes repression of CRE transactivation, and reversal of this effect with cAMP offers protection. Additionally, 6-OHDA treatment resulted in increases in cytoplasmic CREB/pCREB expression, and decreases in nuclear immunoreactivity. Similar alterations in CREB localization were seen in primary midbrain neurons, as well as in Parkinson's/Lewy Body Disease brain tissue, as both exhibited abnormal cytoplasmic accumulations of pCREB in injured neurons. Since previous results in our lab showed similar cytoplasmic diversion of important cell signaling proteins, these data describe a common deficit in nuclear/cytoplasmic trafficking that is occurring during neuronal death processes.

5.0 DISCUSSION

5.1 SUMMARY OF RESULTS.

Oxidative stress is increased in both aging and in age-related diseases of almost every major organ system, including the nervous system. In particular, cells of the central nervous system are believed to be particularly susceptible to the damage of oxidants, and continual exposure to oxidative stress over the years may wear down neurons' antioxidant responses (Cassarino and Bennett Jr. 1999) However, mechanisms that contribute to impaired cellular adaptation to oxidative neuronal injuries remain to be fully defined. Using two CNS-derived neuronal cell lines, we have determined that the oxidative neurotoxin 6-OHDA causes a decrease in transactivation of the CRE promotor, resulting in reduced expression of downstream CREB-regulated genes, including two well-characterized survival mediators, Bcl2 and BDNF. CRE transcriptional repression is accompanied by altered subcellular localization of CREB/pCREB, with increases of this important transcription factor being seen in the cytoplasm, and a corresponding decrease seen in nuclei. Similar alterations are observed in dopaminergic neurons of primary midbrain cultures and human parkinsonian post-mortem tissue, as both show abnormal expressions pattern for CREB.

We also have determined that 6-OHDA-induced repression of CRE could be reversed with cAMP treatment, an effect that was associated with significant protection from toxicity. Interestingly, since this repression could be reversed by delayed cAMP treatment several hours after initiation of 6-OHDA induced injury, and this delayed cAMP administration could also confer protection even when applied up to four hours after injury, it is likely that these effects are occurring *early* in the injury process, prior to cell death commitment and lysis. These data indicate that inhibition of the CREB signaling pathway plays a direct role in oxidative stress–induced toxicity in neuronal cells. Since a similar disruption in CREB was seen in primary neurons as well as in human brain tissue, it is likely that the effects seen in our cell lines are relevant to neuronal cell death processes that occur in neurodegenerative diseases.

5.2 CREB DYSFUNCTION IS DUE TO SUB-CELLULAR DIVERSION, NOT INHIBITION OF PHOSPHORYLATION.

While previously published papers have implicated disruptions in CREB signaling in neuronal death, these current studies provide a *novel* mechanism of CREB disruption, with potential relevance to Parkinsonian neurodegeneration. For example, in other model systems, it has been shown that oxidative stressors can disrupt CREB function by interfering with CREB phosphorylation and/or by reducing CRE binding activity (Ito et al. 1999) (See and Loeffler 2001). More specifically, exposure of neuronal cells to H_2O_2 significantly decreased CREB phosphorylation, and if this phosphorylation is restored, neurons are protected from oxidant-induced death (See and Loeffler 2001). While our own studies similarly show that a disruption in CREB pathway contributes to neuronal death, the mechanism by which CREB function is

altered appears to be different. Their observation of decreased CREB phosphorylation was not seen in our studies, as we have determined that CREB phosphorylation is *increased* in response to 6-OHDA, with most of the CREB immunoreactivity retained in the cytoplasm (Figures 15, 18, 22, 15). The accumulation of CREB and pCREB in the cytoplasmic compartment indicates that 6-OHDA treatment may interfere with nuclear translocation of phosphorylated CREB. Whether nuclear import of CREB (after its synthesis in the cytoplasm) is being inhibited or nuclear export is being activated is still unclear, but each is an exciting possibility that warrants further study.

One possible mechanism responsible for inhibition of nuclear translocation is modification of the nuclear localization signal within CREB (located within the basic leucine-zipper domain(Waeber and Habener 1991). While there are numerous studies on the nuclear import of transcription factors from the cytoplasm through NLS (Kallio et al. 1998) and (Fenarli et al. 2004), relatively little attention has been paid to the *export* of different transcription factors from the nucleus, and even less so regarding CREB. However, one study that did examine CREB subcellular localization determined that loss of CREB can be induced in smooth muscle cells (SMC's) by chronic exposure to hypoxia or platelet-derived growth factor BB, and treatment with the nuclear export inhibitor leptomycin B *prevented* this depletion of nuclear SMC CREB (Garat et al. 2006). This work indicates active export of CREB as a mechanism for transcriptional control. It would be worthwhile to examine the effects of the nuclear export inhibitors in our model system, to further substantiate the hypothesis that active nuclear export is contributing to cytoplasmic CREB accumulation.

Additionally, it is also possible that the accumulation of CREB in the cytoplasm may further suggest the possiblity that dephosphorylation of pCREB is interrupted in response to oxidants, or that degradative systems are disrupted (Zhu et al. 2003) (Keller et al. 2004) (Chu

2006). Both of these options would contribute to the cytoplasmic accumulation of signaling proteins within cells. Regardless of the contribution, these results suggest that subcellular diversion of CREB proteins, rather than inhibition of phosphorylation, is responsible for the loss of neuroprotective CRE transcriptional activity.

5.2.1 Nuclear/cytoplasmic trafficking of transciption factors.

The regulation of nuclear/cytoplasmic trafficking of transcription factors is a central mechanism to controlling gene expression. It is widely believed that nuclear import and export proceed through nuclear pore complexes and can occur by a variety of distinct mechanisms (Reviewed in (Nakielny and Dreyfuss 1999). For example, a major nuclear-cytoplasmic trafficking pathway is believed to consist of cytosolic receptor molecules of the importin superfamily, which recognize and dock the NLS-containing proteins at the nuclear pore. The affinity of the importin-targeting sequence interaction is a crucial factor in determining the efficiency of translocation, and can be influenced by phosphorylation. Phosphorylation can result in the masking of targeting sequence or the increased affinity of the interaction with importins (Jans et al. 2000). For example, phosphorylation of the yeast transcription factor, PHO4, blocks its interaction with nuclear import proteins, keeping it retained in the cytoplasm (O'Neill et al. 1996). Subsequent dephosphorylation allows binding with its import receptor, permitting nuclear translocation, DNA binding and initiation of transcription to occur. In another example, phosphorylation of the cytoplasmic retention factor, kB, disrupts its interaction with the transcription factor NFkB, allowing the NLS of NFkB to be recognized by import receptors (reviewed in (Nakielny and Dreyfuss 1999). Although phosphorylation seems to be the predominant mechanism in controlling transciption, it might not be the only posttranslational modification involved. For

example, methylation of arginine residues has also been found to have an effect on the export of some yeast proteins (see review above).

It is possible that phosphorylation or oxidation of the NLS in CREB may be responsible for the altered subcellular localization described in our studies. While CREB is generally thought to be constitutively expressed in the nucleus, this widely held belief is beginning to be challenged. For example, studies have shown that CREB can be localized to the neuronal dendrites (Crino et al. 1998) as well as other cellular compartments including the cytoplasm (Kuramoto et al. 2005) It is possible that under some circumstances, cell stimulation could prompt CREB to translocate into or out of the nucleus. Alterations in nuclear translocation in neurons during injury may represent a previously unexplored control mechanism, allowing the cell an additional level of control over CREB signaling in neuronal cells, or may represent an abnormal response to oxidant exposure. Further examination of CREB NLS is necessary.

5.2.2 Nuclear translocation of CREB.

Interestingly, it has been shown in other cell types that inhibitors of nuclear import can elicit altered subcellular localization patterns of CREB as well as disruptions in CREB-mediated transcriptional responses. For example, in smooth muscle cells, Stevenson et al (Stevenson et al. 2001) described a new and potentially important mechanism for the differentiation of CREB signals. Blockage of nuclear transport led to the accumulation of phosphorylated CREB in the cytoplasm and reduced pCREB activity. However, this reduced activity was restricted to specific stimuli. For example, CRE transcriptional responses to platelet derived growth factor receptor (PDGF) or membrane depolarization were inhibited when nuclear import was blocked, which contributed to cytoplasmic accumulation of pCREB. But inhibition of nuclear import had

no effect on the subcellular distribution of pCREB or CRE-mediated transcription when muscle cells were stimulated by forskolin (Stevenson et al. 2001). Interestingly, PDGF signals to CREB via the MAP Kinase pathway, while forskolin activates the cAMP/PKA pathway. Because the catalytic subunit of PKA is small and can passively diffuse into the nucleus through nuclear pores, active nuclear import is not required. However, both PDGF and depolarization stimuli regulate CREB phosphorylation via large cytoplasmic kinases such as ERK and RSK, which need to translocate into the nucleus to take effect, and therefore would be sensitive to disruption in nuclear trafficking. Cellular regulation of nuclear import adds another level of control to CREB signaling, and may represent an additional mechanism that mediates specificity. Additionally, this regulation may become altered, or abnormally activated in response to oxidant treatment.

5.2.3 Summary.

Taken together, the current and previously published data suggest that 6-OHDA treatment results in impaired nuclear import of pCREB, which accumulates in the cytoplasm. The addition of cAMP is able to bypass the signaling blockage through mechanisms that likely involve PKA, resulting in restoration of neuroprotective CRE transcriptional responses (Figure 11). Indeed, our H89 experiments that were performed implicate involvement of PKA in cAMP effects in our system (Figure 13), strengthening this hypothesis. Interestingly, we also determined that treatment of neuronal cells with MEK inhibitors, which ultimately inhibit the activation of ERK, caused a repression in basal levels of CRE activation (Figure 14). Since the PKA inhibitor H89 did not affect basal CRE activity at all, it is likely the basal levels of CREB activity in these neuronal cells in culture work predominantly through the MAP Kinase pathway, and not through

PKA. 6-OHDA caused inhibition of this ERK-CREB pathway, which resulted in cell death, while stimulation of the alternate cAMP/PKA pathway conferred protection. Since a disruption of nuclear translocation would affect only the MAP Kinase pathway, leaving PKA untouched and free to diffuse and activate any nuclear CREB, these results strengthen our hypothesis that inhibition of CREB signaling occurs through alterations in nuclear/cytoplasmic trafficking. It is necessary and important to extend these studies by examining any alterations in the CREB nuclear localization signal (phosphorylation, methylation, etc) as well as examining the effects of inhibitors of nuclear import/export to determine the potential mechanism responsible for altered CREB trafficking in our model system.

5.3 A COMMON DEFICIT IN NUCLEAR TRANSLOCATION IN NEURODEGENERATION

5.3.1 ERK signaling is disrupted in neuronal death.

Previous data from our laboratory and others demonstrated that spatial and temporal alterations in the ERK signaling pathway, a well-known upstream CREB activator, may be contributing to neurodegenerative disease pathologies. For example, pERK is aberrantly expressed in the brains of patients with Alzheimer's Disease (Zhu et al. 2002), and is increased in the vulnerable penumbra in the brains of patients following acute ischemic stroke (Slevin et al. 2000). pERK has also been implicated in Parkinson's pathogenesis and 6-OHDA-mediated neuronal death (Zhu et al. 2002) (Zhu et al. 2003), where it was demonstrated that granular cytoplasmic accumulations of pERK are present in both a 6-OHDA treated neuronal cell lines as well as in

degenerating neurons of human Parkinson's disease brain (Zhu et al. 2002) (Zhu et al. 2003). In human PD tissue, the cytoplasmic aggregates were localized to the substantia nigra, and were largely absent in control cases. Interestingly, examination of pre-clinical cases indicate that these pERK alterations may be occurring relatively early in the disease process, a finding that may have important clinical implications, and correlates well with our current findings. It was also demonstrated that neuroprotective anti-oxidant treatments blocked cytoplasmic accumulation in the neuronal cell line (Zhu et al. 2002), supporting a role for oxidants eliciting neurotoxic patterns of ERK activation.

In these human tissue studies, when the subcellular localization of phospho-ERK immunoreactive granules was examined using double label confocal microscopy and immuno-electron microscopy, it was revealed that p-ERK labeling was targeted to mitochondria, and heavily labeled mitochondria were observed within autophagosomes of damaged neurons (Zhu et al. 2003). This suggests that a potential interaction between dysfunctional mitochondria, autophagy, and ERK signaling pathways exists in neuronal pathologies. Additonally, since separate, independent studies have suggested the possibility that CREB can accumulate in mitochondria under pathological conditions (Cammarota et al. 1999), (Lee et al. 2005), it is possible that the increased expression of pCREB in the cytoplasm shown in our studies in this report similarly represents an increase in mitochondrial accumulation. The extraction protocol used in these studies to isolate nuclear and cytoplasmic fractions (NePER, Pierce Biotechnology) concentrates mitochondria in the cytoplasmic/cytosolic fraction. This fraction could be further processed for mitochondrial isolation, with subsequent separation of mitochondria by centrifugation and percoll gradient (See (Zippin et al. 2003) for technique). This was not attempted in these studies. However, a preliminary examination using immunofluorescence studies of 6-OHDA-treated B65 cells showed that pCREB did *not* co-localize with mitochondrial markers (data not shown). Additional studies are needed

to determine if cytoplasmic pCREB, like pERK, is being targeted to mitochondria, which may be subsequently targeted to lysosomes. While these preliminary results in the one cell line indicate that pCREB is not accumulating in mitochondria, further clarification of this issue should be pursued in SH-SY5Y cells to more concretely define subcellular localization patterns.

Taken together, these data lend further support to our hypothesis that diversion of important signaling components may directly contribute to cell death. However, while abnormal activation of ERK seems to be a common theme in the neuronal death process, its exact mechanism remains unknown. Since studies have suggested that ERK may be accumulating in mitochondria, some of which are damaged and being autophagocytosed (Zhu et al. 2003), it is unclear to date whether CREB shares a similar fate. Additional studies to determine precise cytoplasmic location of CREB are needed.

5.3.2 RSK is disrupted in neuronal death.

As previously described, one important target of pERK is the ribosomal S6 kinase (RSK), which, once activated, can phosphorylate targets in both the cytoplasm and nucleus. Further examination of this ERK-RSK-CREB pathway by our laboratory showed that phosphorylated-RSK is also increased within the cytoplasm in neuronal cells in response to 6-OHDA, in an aggregated pattern similar to the abnormal ERK distribution induced by 6-OHDA (Zhu et al. 2002). Increases in cytoplasmic pRSK were similarly seen in the substantia nigra neurons in PD brain tissue, again in the aggregated cytoplasmic pattern. Interestingly, this effect was specific for RSK, as a separate ERK target, the transcription factor Elk-1, was not altered in substantia nigra neurons (Zhu et al. 2002). Since RSK typically translocates to the nucleus after stimulation by ERK where it can phosphorylate CREB, these results further suggest that nuclear/cytoplasmic

trafficking is altered in neuronal death pathways. With RSK unable to translocate to the nucleus, alterations in CREB activation would be not be unexpected. Additionally, with p-RSK accumulating in the cytoplasm, it raises the possibility that any accumulated cytoplasmic CREB might, in theory, be phosphorylated locally by pRSK. However, while cytoplasmic pERK has been determined to be active in this model, the enzymatic activity of pRSK was not tested in these studies. Further examination of this important kinase is warranted.

5.3.3 Evidence of disruption in other important signaling proteins.

Additional evidence of cytoplasmic diversion of important nuclear signaling proteins has been described in other models of neuronal death. For example, aberrant cellular localization of E2F1 and pRb, two key regulators of the cell cycle, have been described in damaged neurons in the simian immunodeficiency virus encephalitis (SIVE) model. Compared to non-infected and non-encephalitic controls, expression of both of these nuclear factors was abnormally increased in the cytoplasm of neurons (Jordan-Sciutto et al. 2000). Additionally, in Parkinson's Disease brain tissue, immunoreactivity for pRB was also shown to be abnormally increased in neuronal cytoplasm of the substantia nigra neurons (Jordan-Sciutto et al. 2003). In fact, pRB exhibited a staining pattern that was strikingly similar to our observed results with pCREB. These combined results indicate that cytoplasmic diversion of important signaling components is likely a common factor in damaged and dying neurons in human disease. Whether this result is due to a disruption in nuclear import, an abnormal activation of nuclear export, and/or alterations in dephosphorylation mechanisms still remains unclear, and awaits further study.

5.3.4 Summary

The current sub-cellular fractionation and immunofluorescence data showing accumulation of pCREB in the cytoplasm and loss of CRE-mediated transcriptional activity in 6-OHDA treated cells along with the abnormal expression patterns of ERK and RSK suggest the possibility of a common deficit in nuclear translocation. Since the phosphorylated forms of ERK, RSK, and CREB are all increased in granular or clumped cytoplasmic distribution in degenerating human substantia nigra neurons, it is likely that altered nuclear trafficking may underlie impaired survival signaling in human neurodegenerative diseases. Additionally, as the Parkinson's disease related protein α-synuclein is also known to accumulate in the cytoplasm of affected neurons, and shares homology with 14-3-3 like properties (Ostrerova et al. 1999), the potential involvement of α-synuclein aggregation in altering the distribution of these signaling proteins deserve further study.

5.4 DOWNSTREAM CREB-MEDIATED GENES ARE DISRUPTED IN RESPONSE TO 6-OHDA.

A role for altered expression of BDNF and Bcl2 in parkinsonian neurodegeneration has been suggested by several studies of diseased human tissues and model systems. For example, BDNF expression levels are decreased in the substantia nigra of Parkinson's disease patients (Mogi et al. 1999; Howells et al. 2000), and BDNF treatment reverses the motor deficits induced by 6-OHDA *in vivo* (Klein et al. 1999). Additionally, over-expression of Bcl-2 in rat brain prior to 6-OHDA administration results in increased survival of neurons within substantia nigra (Yamada et al.

1999; Natsume et al. 2001), and Bcl-2 family members can inhibit 6-OHDA-induced death in SH-SY5Y cells (Jordan et al. 2004). A further study reported that oxidative stress causes a down-regulation of the Bcl2 promoter, resulting in a decrease in Bcl2 protein as well as mRNA levels (Pugazhenthi et al. 2003) It is clear that proper expression of both of these survival mediators plays a critical role in neuronal survival.

5.4.1 Functional relevance of decreased BDNF and Bcl2 message.

Our findings that BDNF and Bcl2 mRNA levels are decreased with 6-OHDA treatment (Figure 10) indicate a loss of CRE-controlled gene expression, and provide a functional consequence for decreased CRE transcription. These and previous data support the concept that impaired survival signaling contributes to dopaminergic cell death as well as parkinsonian pathogenesis. However, whether the loss of either of these two gene products has a direct role in neuronal death mechanisms in our model is not completely clear, and requires further investigation. The fact that stimulation of B65 cells with BDNF alone was not able to protect them from 6-OHDA-induced toxicity (Figure 9), despite the fact that these cells express TrkB receptors and respond to BDNF treatment by increasing CREB activity (data not shown), may indicate that restoration of BDNF alone is not enough to compensate for all of the 6-OHDA induced changes. Since BDNF signals through neurotrophin receptors that typically stimulate the ERK pathway, if nuclear trafficking is disrupted in response to 6-OHDA as hypothesized, then one would predict that BDNF would not be able to successfully activate survival mechanisms. In our model, the ERK pathway is disrupted in such a way that important components are sequestered in the cytoplasm and unable to enter the nucleus, and would result in disrupted BDNF signaling. In

short, BDNF would not be able to bypass the 6-OHDA-induced nuclear translocation blockade of the ERK-RSK-CREB pathway.

As 6-OHDA also represses the CRE-related gene Bcl-2 and co-treatment with cAMP restores Bcl-2 mRNA, one would predict reciprocal effects on caspase 3 activity. Indeed, we found that 6-OHDA elicits increased caspase 3 activity, and co-treatment with cAMP significantly attenuates this response in SH-SY5Y cells (Figure 23) which undergo well-described apoptosis in response to several stimuli (Yuste et al. 2002; Kalivendi et al. 2003; Lee et al. 2005). In contrast, B65 cells appear to undergo an alternative form of ERK dependent cell death that lacks classic apoptotic features (CT Chu, unpublished data), and accordingly, caspase 3 was not activated in response to 6-OHDA. However, it is possible that at the time point examined (3 hours) apoptosis has simply not yet begun in B65 cells, and apoptotic changes occur after longer exposures to toxin. Regardless, the similarities between the two cell lines in terms of early transcriptional alterations further suggest that CREB-related changes are upstream to and independent of the specific execution mechanism. Since Bcl-2 can protect against both apoptotic and alternative death pathways (Yuan et al. 2003) and CREB regulated transcription is neuroprotective in ischemic and excitotoxic neuronal death (Mabuchi et al. 2001), these results along with our delayed protection experiments (Figure 8) indicate that the CREB alterations are likely involved in early cell fate decisions in injured neurons prior to cell death commitment. A loss of both BDNF and Bcl2 mRNA is occurring in both cell lines in response to 6-OHDA, and impaired survival signaling is likely contributing to dopaminergic cell death.

5.4.2 CREB controlled genes are not upregulated.

Previous studies of transcriptional responses to 6-OHDA focused upon upregulation of genes potentially involved in death pathways (Holtz and O'Malley 2003; Ryu et al. 2005). Both the unfolded protein/endoplasmic reticulum stress response and c-jun induction were implicated. Of interest is the data indicating that c-jun/ATF/CREB heterodimerization may determine whether increased protein-CRE binding activity results in suppression rather than induction of CRE-transcription (Zhang et al. 2004). While genes whose expression was downregulated by 6-OHDA were not analyzed in these reports, the fact that CRE-regulated genes, such as Bcl-2, were not upregulated along with other genes involved in bioenergetics and death regulation are consistent with our results showing decreased CRE-regulated transcription in 6-OHDA treated cells.

5.4.3 Summary

In summary, it is clear that the downstream CREB-mediated gene products, BDNF and Bcl2, are disrupted in response to 6-OHDA. Since disruptions in both of these survival mediators have been implicated in neuronal death, it is possible that the loss of one or both of them contribute to death in our model system. Further study examining the impact of decreased BDNF and Bcl2 expression in response to oxidative toxins is warranted.

5.5 PROTECTION OF NEURONAL CELLS FROM OXIDATIVE STRESS

5.5.1 Protection from 6-OHDA toxicity may require unidentified cofactors

While our data indicate that cytoplasmic diversion/retention of P-CREB contributes in part to 6-OHDA repression of CRE transcription, cAMP may act through additional mechanisms other than simple reversal of 6-OHDA induced decreases in nuclear CRE binding activity in order to induce protection. It is not clear why cAMP-induced restoration of nuclear pCREB observed in B65 and SY5Y cells by western blot and immunoflourescence (Figures 15, 22) is not reflected by increased CRE probe binding in EMSA (Figure 12). Although it is accepted that cAMP enhances the transactivation potential of CREB, this is often not correlated with increased DNA binding affinity (Mayr and Montminy 2001). As discussed earlier, basal levels of CRE site occupancy vary from promoter to promoter, as for example, CREB occupancy of CRE seems to be constitutive for promoters such as Cyclin-D and cfos but CREB binding to CRE in the CRF promoter is heavily stimulated by cAMP agonists (Sibinga et al. 1999) (Dey et al. 1991). The fact that the altered regulation of CRE transcription observed in our system seems to show a greater magnitude than the alterations in nuclear pCREB levels detected by western blot or EMSA suggests that a heterogeneity in the protein composition of the complexes bound to CRE may be responsible.

Additionally, it is widely believed that differences in CREB activity in response to various stimuli can be attributed to the differential binding of specific cofactors, as recruitment of various complexes to the different gene promoters allow a particular CREB target gene to respond to one signal but not the other. For example, phosphorylation of CREB alone is typically not sufficient for growth factor stimulation of CRE gene expression, as additional

promoter-bound factors are necessary. In our model system, it is possible that cAMP increases the transcriptional efficiency of bound CREB through increased binding of co-activators or perhaps through changes of the stoichiometry of CREB heterodimers to favor transcription. One possible candidate is CREB binding protein (CBP), as sequestration of CBP is believed to be a pathogenic mechanism on other systems (Nucifora et al. 2001). However, our immunoblots of CBP show no differences in nuclear levels of this protein, and no alteration in cytoplasmic fractions (Figure 17). It would be worthy to examine other known CREB co-activators and repressors in the future, to determine specific alterations in CRE binding components. Additionally, since CREB can be phosphorylated as a consequence of calcium signaling, through activation of ERK regulated kinases, or by cAMP-activated protein kinase A (PKA), it is also possible that the integration of these pathways may be necessary for full stimulation of CREB-dependent transcription.

5.5.2 A role for antioxidants in 6-OHDA toxicity.

While 6-OHDA toxicity can be ameliorated by a spectrum of antioxidants (Kulich and Chu 2001; Zhu et al. 2002; Kulich and Chu 2003), the roles of specific oxidative mediators and their compartmentalization are much less clear. Previous studies of the ERK signaling pathway indicate that LD_{50} doses of hydrogen peroxide do not have the same signaling effects as LD_{50} doses of 6-OHDA (Kulich and Chu 2001). Additional studies further suggest that while antioxidants may inhibit 6-OHDA-mediated CRE repression, hydrogen peroxide does not repress CRE transcription in B65 or SH-SY5Y cells (Figure 26). Moreover, hydrogen peroxide increases, rather than decreases CREB DNA binding in primary cortical neurons (J Caltagorone & R Bowser, unpublished data) which is in contrast to our observations with 6-OHDA. Thus,

while antioxidants inhibit 6-OHDA-induced toxicity and signaling alterations in vitro (Kulich and Chu 2001; Zhu et al. 2002; Kulich and Chu 2003), and in vivo (Callio et al. 2005), the signaling alterations likely result from specific mechanisms relating to the nature of the toxin itself, or to specific interactions within dopaminergic cells.

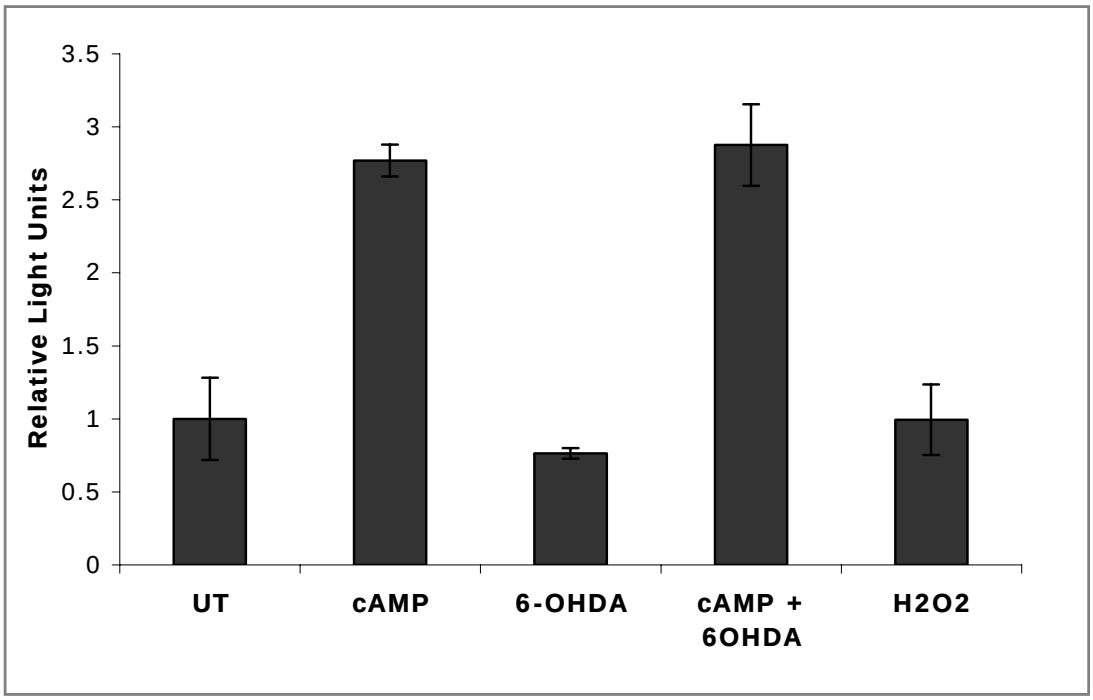

Figure 26. H2O2 does not repress the CRE promoter.

B65 cells were transfected with CRE-luciferase plasmid, and treated with media (UT), cAMP (500uM), 6-OHDA (500uM) or H_2O_2 (500mM) for 3 hours. Lysates were collected and analyzed for luciferase activity as described in Methods. Data is expressed as fold induction over untreated control. 6-OHDA causes a repression of CRE, but H_2O_2 does not.

Preliminary experiments done in our lab examining the effect of antioxidants on CRE activity and CREB localization provided some interesting results. For example, pre-treatment of neuronal cells with antioxidants (Catalase and TBAP) prior to 6-OHDA exposure resulted in

decreased CRE repression (Figure 27 below). Additionally, antioxidant treatment also decreased the 6-OHDA-induced cytoplasmic accumulation of pCREB (data not shown). These results are preliminary and need to be repeated, but suggest that antioxidants can partially alleviate CRE repression and cytoplasmic buildup of CREB, effects that may play a role in antioxidant-induced protection from 6-OHDA toxicity.

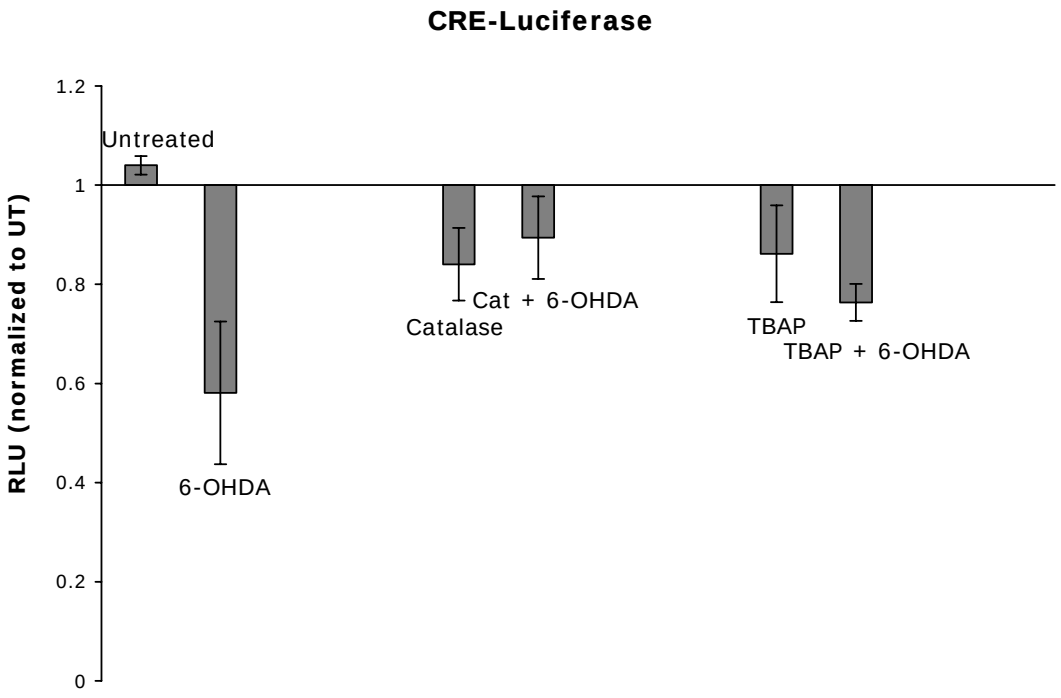

Figure 27. Antioxidants affect 6-OHDA-mediated effects in B65 cells.

Cells were transfected with CRE luciferase and treated with either media, catalase (30 U/ml), or MnTBAP (100 uM) 10 minutes prior to treatment with either vehicle or 500 uM 6-OHDA. Luciferase activity was normalized to cells treated with media alone (untreated).

5.5.3 6-OHDA toxicity is specific to dopaminergic neurons.

Similar observations in two catecholaminergic cell lines and in primary dopaminergic neurons indicate that altered CREB regulation is a common response of catecholaminergic cells to this toxin. As 6-OHDA is targeted to cells that express the high affinity dopamine transporter, it can be difficult to separate cell type specific effects from toxin specific effects. Interestingly, while *loss of* nuclear pCREB is observed in 6-OHDA injured TH+ neurons in primary culture, the non-TH neurons retain robust nuclear pCREB expression (Fig. 24D). These non-TH neurons are largely spared during Parkinson's Disease. This supports the hypothesis that failure to successfully upregulate neuroprotective CREB dependent transcription may underlie lethal effects of 6-OHDA in catecholaminergic cells.

5.5.4 A window for protection

Pulse-chase experiments indicated that 6-OHDA treated B65 cells are committed to die in a delayed fashion following transient exposures to 6-OHDA (Kulich and Chu 2001). The current data indicate that there is no significant LDH release until 8 h after 6-OHDA exposure (Figure 4C). It is interesting to note that cAMP is able to confer significant protection even when applied 4 h after injury, a time occurring after commitment to death (in the absence of protective interventions) and prior to initiation of cell lysis. In contrast, antioxidants can prevent cell death only when added within the first 15-60 min of injury (Kulich & Chu, unpublished data). This indicates that downstream signaling alterations induced by oxidative neurotoxins may prove amenable to therapeutic manipulation after the window of time when antioxidants are able to prevent injury.

5.6 SUMMARY AND CONCLUSIONS

5.6.1 Clinical Relevance.

These results indicate that a disruption of the CRE signaling pathway is involved in 6-OHDA-mediated neuronal death. As CREB dysfunction has also been implicated in many human pathologies, it is likely that these results may have important clinical implications. Adding strength to this concept is the recent data showing CREB imvolvement in levodopa- (L-dopa) induced improvement in motor responses in Parkinson's Disease models (Oh et al. 2003). In PD patients, treatment with the dopamine precurser, L-dopa, usually confers significant clinical benefit, providing drastic improvement in patients' motor function. Since dopamine receptors (the D1 subtype) stimulate PKA-mediated phosphorylation of CREB (Surmeier et al. 1995), it was hypothesized that L-dopa treatment stimulates striatal CREB phosphorylation in the brain and contributes to the maintenance of motor response changes. Indeed, a study by Oh et al (2003) using 6-OHDA lesioned rats, showed that treatment with L-dopa resulted in a dramatic increase in Ser-133 phosphorylated CREB in the striatum and concurrently improved motor function. In addition, the time course of alterations in CREB phosphorylation correlated with the changes in motor function after L-dopa treatment was stopped, and inhibitors of PKA resulted in decreased striatal pCREB as well as a loss of motor improvement. These results further support the idea that disruption in phosphorylated CREB is contributing to the progression of PD symptoms, and that stimulation of CREB, via PKA, may improve neuronal function and survival. Since our current results show sequestration of pCREB in the cytoplasm of affected neurons in PD brain tissue, it is possible that L-dopa treatment causes stimulation of any remaining nuclear CREB (via the dissociation and diffusion of PKA) and can buy neurons more

time. In the future, additional therapies designed to target PKA and/or CREB directly may offer further benefit to Parkinson's patients.

Sequestration of CRE activator proteins (other than CREB itself) has also been suggested to play a causal role in the progression of additional neurodegenerative diseases, such as Huntington's disease (Nucifora et al. 2001). In these studies, intra-nuclear aggregation of CREB binding protein (CBP) occurs, preventing access to its CREB target and resulting in decreased gene transcription and subsequent disease pathology. Additionally, persistent ERK activation in liver cells results in increased binding of CBP to RSK within the nucleus, which resulted in decreased phosphorylation of CREB and attenuation of CRE-mediated gene expression (Wang et al. 2003), which was associated with cell growth inhibition. While these differ from our study in showing nuclear sequestration and reduced levels of CREB phosphorylation, impaired ability to mount protective CRE transcriptional responses may represent a common mechanism in cellular damage and in neurodegenerative diseases. CREB activators, as well as CREB targets may potentially offer new avenues of therapy for treatments of neurodegenerative diseases.

5.6.2 Conclusions

In conclusion, this data taken together with previously published results suggest that 6-OHDA treatment results in impaired nuclear import of ERK, RSK and CREB, all of which accumulate in the cytoplasm. The addition of cAMP, likely working through PKA, is able to bypass the signaling blockage, resulting in restoration of neuroprotective CRE transcriptional activity (Figure 28). Given the increasing reports of signaling proteins and transcription factors accumulating in the cytoplasm of degenerating neurons, strategies to bypass potential

translocation deficits may aid in the development of new strategies for neurodegenerative disease treatment.

This cytoplasmic sequestration of signaling proteins or transcription factors may represent a novel mechanism contributing to failed compensation during oxidative neuronal injuries. The discovery of impaired signaling mechanisms in injury models indicates that trophic factor supplementation therapies may need to consider strategies for bypassing or reversing potential downstream blockages elicited by disease or age-associated oxidative stressors. While the specific mechanisms leading to impairment of protective CRE transactivation in the 6-OHDA model differs from those described previously for other oxidative or neurodegeneration models, the overall effect of disruption in CREB function is a consistent theme. The ability of cAMP to mobilize potentially active pools of pCREB and/or to provide compensatory stimulation of nuclear CREB to override the deficit in CRE-dependent transcription may prove beneficial to neurodegenerative therapies.

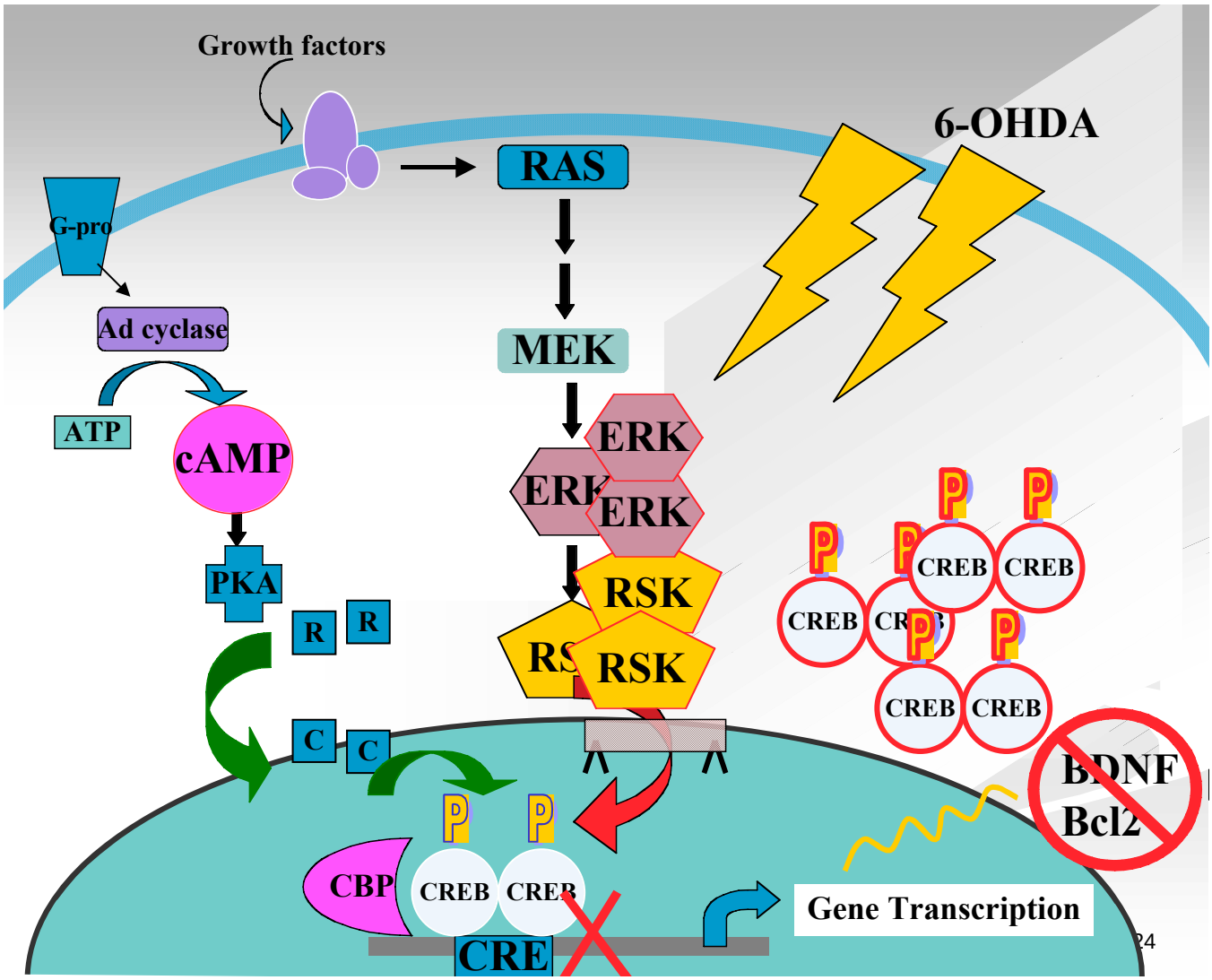

Figure 28. Possible disruption in the CREB Pathway in response to 6-OHDA.

In our model, 6-OHDA exposure results in cytoplasmic sequestration of ERK, RSK and CREB, which is accompanied by the repression of CRE-mediated transcription (X) and subsequent decreases in downstream BDNF and Bcl2 gene expression (∅). It is possible that a blockage of nuclear translocation (red arrow) is occurring in response to 6-OHDA. Administration of cAMP bypasses this blockade (green arrows) and restores CRE transcriptional activity, and conferring significant protection if administered prior to the initiation of cell lysis. Given the increasing reports of signaling proteins and transcription factors accumulating in the cytoplasm of degenerating neurons, strategies to bypass potential translocation deficits may prove promising.

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