SIGNALING EVENTS IN ACTIVITY DEPENDENT NEUROPROTECTION, NEURODEGENERATION, AND SYNAPTIC PLASTICITY

DISSERTATION

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By

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CREB-(cAMP response element binding protein) dependent gene transcription within the context of physiological and patho-physiological conditions is a central focus of my dissertation. CREB is a transcription factor that plays a role in many activity-dependent neuronal processes, such as learning and memory, neuroprotection and neurotransmission. Initially, in chapter 1, I examined cellular and molecular signaling events that couple excitotoxic and nontoxic levels of NMDA receptor stimulation to activation of the CREB/cAMP response element (CRE) pathway in cultured cortical neurons. In this study, I report that the temporal regulation of CREB activation is an essential cue that controls the efficacy of NMDA as a regulator of CRE-mediated transcription. Low concentration of NMDA, a non-toxic stimulus, evoked prolonged CREB activation subsequent gene transcription and, as a result cell survival, whereas high, potentially excitotoxic, levels of NMDA, shut off CREB activation leading to cell death. In addition, I found that calcineurin plays a role in regulating the temporal status of CREB activation under different stimulus intensities. Together these data provide a framework to begin to understand how the neuroprotective and excitotoxic effects of NMDA receptor activity function in an antagonistic manner at the level of the CREB/CRE transcriptional pathway. In chapter 2, I examined the role of the CREB/CRE-dependent signaling pathway under pathological conditions elicited by status epilepticus (SE). SE triggers neuronal death, reactive gliosis and remodeling of synaptic circuitry, thus leading to profound pathological alterations in CNS physiology. First, to characterize how seizure activity regulates the activation state of the
CREB/CRE pathway in both glia and neurons of the hippocampus, I utilized the pilocarpine model of SE on a mouse strain transgenic for a CRE-reporter construct (β-galactosidase). I found that SE triggered a rapid (4-8 h post-SE) but transient increase in CRE-mediated gene expression in the neuronal sublayers. In contrast to neurons, SE induced a lasting increase (up to 20 days) in CRE-mediated transcription in both reactive astrocytes and microglia. I also found that CRE-mediated gene expression correlated with expression of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2). Collectively these data show that SE triggers two waves of CREB-mediated gene expression, a transient wave in neurons and a long-lasting wave in reactive glial cells, and that CREB couples SE to COX-2 expression. In chapter 3, I examined the role of the CREB/CRE pathway as a signaling intermediate that couples a BDNF-evoked preconditioning stimulus to protection against SE-induced neuronal death in the striatum. Preconditioning has been reported to attenuate the pathophysiological effects resulting from an array of brain insult, including ischemia and SE. I found that SE evoked cell deaths were dramatically attenuated by BDNF infusion directly into striatum 24 hr prior to SE. Next, I examined the downstream kinases that play a role in BDNF-mediated neuroprotection. To this end, specific kinase inhibitors were infused prior to BDNF infusion. I report that only the MEK inhibitor U0126 attenuated the protective effect of BDNF, indicating a central role for the MAPK cascade in BDNF-mediated neuroprotection. Given that CREB is regulated by MAPK signaling and that CREB plays a role in protective mechanisms, I examined whether CREB-mediated transcription is required for BDNF induced neuroprotection. To this end, I used loss (A-CREB) of CREB function transgenic mouse strains. The protective effect of BDNF was significantly attenuated in A-CREB
expressing striatum, indicating an essential role for CREB in BDNF-mediated neuroprotection. To identify CREB-regulated genes that couple BDNF to neuroprotection, I analyzed the expression of the CREB-target gene, PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1alpha. PGC-1 has recently been shown to regulate the expression of ROS-detoxifying enzymes, including GPx1 and SOD2 (St-Pierre et al., 2006). This led me to examine whether a BDNF/MAPK/CREB signaling cassette confers neuroprotection via the upregulation of PGC-1. Preliminary data in chapter 3 revealed that PGC-1 is upregulated in response to seizure, and that its induction is mediated via a CREB-dependent mechanism. In chapter 4, I redirected my focus on the analysis of signaling pathways that couple excitatory neurotransmission to entrainment of the circadian clock. The mammalian circadian pacemaker located in the suprachiasmatic nuclei (SCN) drives a vast array of biochemical and physiological processes with 24 hr periodicity. This SCN is an ideal model system to examine cellular and molecular signal transduction related to neurotransmission and synaptic plasticity. The phasing of SCN pacemaker activity is tightly regulated by photic input from the retina. Recent work has implicated protein kinase C as a regulator of photic input, although stimulus-induced PKC activity has not been examined. Here I used a combination of biochemical, immunohistochemical and behavioral techniques to examine both the regulation and role of PKC in light-induced clock entrainment. I reported that photic stimulation during the subjective night, but not during the subjective day, stimulates PKC activity within the SCN and that PKC functions as a negative regulator of light entrainment. A combination of cell culture and in vivo experiments revealed that PKC increases the stability of mPERIOD1. Thus, PKC may influence clock entrainment via posttranslational
mechanisms that influence clock protein stability. Together, I employed an array of model systems to examine signaling events that underlie neuroprotection, cell death and neurotransmission.
DEDICATION

My parents, who have dedicated their lives to supporting their children.
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Now, I am about to submit my thesis and prepare for my final defense. I know this could not happen without so many people’s advice and help.

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INTRODUCTION

The CREB/CRE transcription pathway

CREB (cAMP response element-binding protein) is a transcription factor which is regulated by diverse signaling events and regulates the expression of many different kinds of genes which function in array of physiological and pathophysiological cellular events in central nervous system (Carlezon Jr. et al., 2005). CREB was originally identified as an activator responding to the second messenger, cAMP (Goodman et al., 1990). More intense studies revealed that CREB is activated by growth factors and calcium signals (West et al., 2001; Dijkhuizen and Ghosh, 2005). CREB forms homo- or heterodimers with CREB family members and, under most conditions, is constitutively bound to a conserved sequence, the CRE element (cAMP response element, TGACGTCAG) in the promoter of target genes. Its transactivation potential is actuated by phosphorylation of serine 133 within its kinase inducible domain (Lonze and Ginty, 2002). CREB phosphorylation recruits CREB-binding protein (CBP) and other transcriptional machineries such as RNA polymerase (Lonze and Ginty, 2002). The upstream signaling pathways which trigger CREB phosphorylation are diverse. PKA (Protein Kinase A), PKC (Protein Kinase C), PKG (cGMP-dependent protein kinase), CaMKs (calcium calmodulin kinases), MAPks (mitogen activated protein kinases), GSK-3β, Casein kinase II, Dyrk IA and Bruton’s tyrosine kinase have been shown to be Ser 133 CREB kinases in different cell types and different stimulus conditions (Lonze and Ginty, 2002). In addition to Serine 133, several additional phosphorylation sites on CREB protein have been suggested to regulate CREB function (Johannessen et al., 2004). For example, CaMK II phosphorylates CREB at three
different sites; Ser-133, Ser-142 and Ser-143. The phosphorylation of CREB at Ser-142 has been reported to repress CREB-mediated transcription (Parker et al., 1998). CREB transactivation is tightly regulated by a number of phosphatases. The serine-threonine protein phosphatase 2A (PP2A) directly dephosphorylates CREB at Ser-133 in hepatocytes (Wadzinski et al., 1993). However, in NIH 3T3 and PC12 cells, PP1 is the major phosphatase regulating CREB dephosphorylation (Alberts et al., 1994; Hagiwara et al., 1992; Wadzinski et al., 1993). PP2B (calcineurin) also has been suggested to be a CREB phosphatase (Bito et al., 1996). Interestingly, calcineurin potentiates PP1 activity by dephosphorylating the PP1 inhibitor I-1. PTEN (tumor suppressor) has also been shown to function as a CREB phosphatase in cancer cells (Huang et al., 2001). PTP1B (protein tyrosine phosphatase 1B) can be another indirect phosphatase of CREB (Gum et al., 2003). Other post-translational modifications of CREB such as ubiquitination, acetylation and glycosylation have also been suggested to regulate CREB activation (Johannessen et al., 2004). In this study, I am focusing on the role and regulation of CREB in many different experimental paradigms such as neuroprotection, cell toxicity and oxidative stress. Since CREB activation can be regulated by diverse upstream signaling pathways, it is important to appreciate the context-specific effects of CREB. Along these lines, CREB regulates the expression of gene involved in development, learning, memory, plasticity, addiction and neuroprotection (Lonze and Ginty, 2002; Carlezon et al., 2005). As described below, CREB may also contribute to the disease. Thus, I also identified CREB-regulated genes which may underlie unique, context-specific roles for CREB.
The dual role of the NMDA receptor as a regulator of CREB in central nervous system

The NMDA receptor (NMDAR) is an ionotropic glutamate receptor that is specifically activated by the selective agonist, NMDA (\textit{N-methyl d-aspartate}). NMDA receptor activation leads the opening of an ion channel which allows the flow of Na\(^+\) and K\(^+\) and Ca\(^{2+}\) (Yoneda and Oqita, 1991). Calcium influx through NMDARs drives the activation of many downstream signaling molecules, which underlie many physiological processes such as synaptic plasticity, and learning and memory (Coussens and Teyler, 1996; Blair et al., 2001). This idea of a “dual role” for the NMDA receptor (within the context of CREB regulation) was first addressed in Hardingham et al., (2002). This study reported that synaptic NMDA receptor activation triggers a prolonged CREB activation triggering BDNF gene expression and cell survival pathway; on the other hand, extrasynaptic NMDA receptor activation induced CREB shut off resulting in cell death. The relationship between temporal regulation of CREB, cell survival and death mechanisms has also been studied in several other model systems. For example, the activation of L-type calcium channels triggers prolonged activation of CREB resulting in BDNF gene expression. In contrast, glutamate bath application triggered a transient activation of CREB resulting in no BDNF gene expression (Ghosh et al., 1994). It is important to know the signaling mechanisms which control the temporal regulation of CREB activation. Thus, in chapter 1, I characterized which kinases and phosphatases are involved in regulating the duration and in turn transcriptional efficiency of CREB. I also examined whether CREB activation is essential for cell survival.
**Status Epilepticus**

The term epilepsy is derived from the Greek word epilambabein, meaning to seize or to attack. Epilepsy is one of the common disorders of the nervous system. Epilepsy is a neurological condition affecting people of all ages that is characterized by recurring seizures, or brief periods of abnormal electrical activity in the brain. The seizures cause a change in sensation, awareness or behavior. The unpredictability of seizure occurrence may affect patients' ability to drive and can disrupt school days, work responsibilities and social activities. Epilepsy affects 0.5% to 1.0% of the world's population. Infection, neoplasm, trauma, toxic, degenerative, and vascular disease are major etiologies of epilepsy (Engel, 1998). Many research groups are interested in identifying the cellular mechanisms underlying the progression of epilepsy and the development of therapeutic interventions (Loscher, 2002; Schmidt and Rogawski, 2002; Remy and Beck, 2006; Delorenzo et al., 2004; Avanzini and Franceschetti, 2003).

Experimental animal models of epilepsy include induction of epileptic events by acute exposure to convulsants (e.g., penicillin, Ba, Co, Fe, Ni, Cd, fluorothyl), antagonists of GABAergic inhibition (bicuculline, picrotoxin, pentylenetetrazol), neurotoxins (kainic acid, pilocarpine, tetanus toxin), and a variety of lesion-inducing methods (freezing) (White, 2002). Activation of neural pathways by electrical stimulation can drive normal circuitry into hyperexcitable synchronous activity (electroconvulsant shock, or ECS), and chronic periodic application of brief high frequency stimulation induces kindling seizures that progress and ultimately evolve into a state of recurring spontaneous seizures (Goddard et al., 1969). However, from both human and experimental perspectives, the diversity of pathologies and experimental methods that induce seizures implies that epileptogenesis is unlikely to be a unitary process that can characterized in simple terms. In this study, the pilocarpine model of SE (status-epilepticus) was used. This systemic administration of pilocarpine, a potent muscarinic agonist, to rodents
triggers a series of pathophysiological events that parallels the development of temporal lobe epilepsy in humans (Turski et al., 1987; Cavalheiro, 1995).

SE-induced alterations in hippocampal physiology are mediated by a two-stage process. In the initial stage, the release of the excitatory amino acid neurotransmitter glutamate leads to rapid neuronal cell death (Olney et al., 1983; Ingvar et al., 1988; Covolan and Mello, 2000), and in the second phase, glutamate, in combination with trophic factors and cytokines, drives a wave of transcriptional activity that likely confers both a neuroprotective response and contributes to seizure-induced brain pathology. Along these lines, seizure activity has been shown to stimulate the expression of brain derived neurotrophic factor (BDNF; Isackson et al., 1991; Ernfors et al., 1991), which would likely function in a neuroprotective manner (Young et al., 1999; Revuelta et al., 2001), tumor necrosis factor-alpha (TNF-alpha: Plata-Salaman et al, 2000) which would contribute to neuronal cell death (Shinoda et al., 2003) and tissue plasminogen activator (TPA; Qian et al 1993; Tsirka et al 1995), which has been shown to elicit neuronal structural changes (Wu et al, 2000). The expression of many of these genes is regulated by CREB transcription factor. In chapters 2, 3, I examined the role of CREB and the CREB regulated genes in seizure.

**Transgenic mice (Tet inducible system and CRE reporter gene expressing mice)**

To study the role of the CREB/CRE transcription signaling pathway in synaptic reorganization, I used transgenic mice regulated by the CREB-regulatory transgene under control of the tetracycline (tet) Operon system. There are two tetracycline inducible systems; Tet-OFF and Tet-ON. These systems use a chimeric transactivator to regulate transcription of the gene of interest from a silent promoter. The transactivator, either tTA (Tet-OFF) or rtTA (Tet-ON), is expressed from a tissue specific promoter. In the Tet-OFF system, tTA binds to the Tet Response Element (TRE) in the silent promoter and activates transcription in the absence of
tetracycline or doxycycline. In the Tet-ON system, rtTA binds to the TRE and activates transcription in the presence of tetracycline or doxycycline (Zhu et al., 2002; Sprengel and Hasan, 2007). Doxycycline is tetracycline derivative. It has better uptake, less irritation and longer half-life than tetracycline (Kelin and Cunha, 1995). The tet inducible system is a powerful approach to regulate both regional and temporal control of transgene expression. I used CaMK II alpha promoter which is a forebrain specific marker to control the regional expression of CREB mutant genes since there is a research suggesting that dominant negative CREB, A-CREB, expressing mice die minutes after birth, apparently due to respiratory failure from a diminished rib cage circumference (Long et al., 2001). To achieve regulated transgene expression in restricted regions of the forebrain, I utilized a mouse strain developed by Mayford (1996), in which the CaMKII promoter drives the expression of tTA. CaMKII α is Ca2+/calmodulin-dependent protein kinase II and a seine-threonine protein kinase that is restricted to the forebrain (Miller and Kennedy, 1986; Burgin et al., 1990; Hanson et al., 1992; Rothschild et al., 2007). Thus, tet-inducible, tissue-specific, CREB transgenic mice can be used to understand the temporal and regional regulation of CREB/CRE signaling pathway in diverse central nervous systems processes.

In addition to CREB transgenic mice, CRE-reporter transgenic mice were used to study the CREB/CRE dependent gene transcription. This transgenic mouse strain (generated by Dr. Daniel Storm) has 6 CREs elements in the promoter driving the expression of a reporter gene, ß-galactosidase (Impey et al., 1996). By processing tissue for ß-galactosidase expression, one is able to determine the relative level of CRE-dependent transcription. This mouse strain has been used by Dr. Obrietan’s lab to examine how synaptic activity couples to CRE-dependent transcription in a variety of brain regions, including the suprachiasmatic nucleus (the locus of the circadian clock) and the hippocampus (Obrietan and Hoyt, 2004; Obrietan et al., 1998; Obrietan et al., 1999; Dziema et al., 2003). We have also used this mouse to determine whether seizure
stimulates CRE-mediated transcription. By using these transgenic mice, we showed that the pilocarpine-induced seizure activity triggers a robust increase in β-galactosidase expression, indicating that neuronal activity stimulates CRE-dependent transcription. Thus, the CRE-reporter gene mouse strain is an ideal tool to identify neurons which have strong CREB activation and CRE-dependent gene transcription under basal or seizure conditions.

**COX-2 and cell toxicity**

One of the major pathological effects of temporal lobe epilepsy human and animal models is reactive gliosis such as microgliosis and astroglisis. Although glia cells are essential for the neuron viability, the activated form of glia cells can trigger neuronal cell death through the inflammatory response. Several proinflammatory cytokines have been implicated in brain pathophy and neurodegeneration resulting from trauma and (Patel et al., 2003; Yamasaki et al., 1995; Yang et al., 1997). For example, IL-1b (interleukin 1-b) has been shown to increase neuronal cell death induced by experimental trauma, ischemia or excitotoxic brain injury (Patel et al., 2003; Rothwell and Luhehi, 2000; Yamasaki et al., 1995). For the epilepsy model, seizure induces IL1-b and IL1-a protein expression. Furthermore, intracerebral injection of the cytokine dampens the seizure activity suggesting the role of cytokines in pathology of epilepsy.

In addition to these cytokines, prostaglandin E2 (PGE 2) is another key mediator for immune-brain signaling. The PGE 2 level is elevated in the brain during various inflammatory states and evokes fever and the activation of hypothalamo-pituitary-adrenal axis. PGE2 is biosynthesized from membrane phospholipids through three enzymatic steps (phospholipase A2, cyclooxygenase and PGE synthase). Recently, selective inhibitors for cyclooxygenase have been studied as a potential target for many neurodegenerative diseases mediated by inflammatory responses. Two isoforms of COX have been characterized, COX-1 and COX-2. COX-1 is
constitutively expressed in various tissues playing as a house-keeping gene. However, COX-2 is induced strongly by various stimuli, especially by proinflammatory cytokines such as IL1-b and TNF alpha (tumor necrosis factor alpha) (Luan and Xu, 2006; Matsumura and Kobayashi, 2004; Rajakariar et al., 2006, Miller, 2006). The role of COX-2 also has been suggested in pathological brain damage such as Alzheimer’s disease, thus many studies examined the application of COX-2 selective inhibitors for neuroprotective mechanisms. For example, celecoxib and rofecoxib which are specific inhibitors of cyclooxygenase-2 had effects on delaying the onset and progression of Alzheimer’s disease. COX-2 expression is mainly found in degenerating cells in AD patients and inhibition of COX-2 decreases the activation of transcription factors which play a role to induce pro-inflammatory cytokines. (Minghetti, 2004; Gilrov and Colville-Nash, 2000; Hoozemans and O’Banion, 2005). Thus, an understanding of the mechanisms of the gene expression and the spatial and temporal regulation of COX-2 is important in developing therapeutic drugs for many neurodegenerative diseases. In chapter 2, the temporal and spatial regulation of COX-2 in the SE model was examined and CREB/CRE transcription signaling pathway was identified as an upstream regulator SE-induced COX-2 expression.

**BDNF and neuroprotection**

Neurotrophic factors have diverse roles in the growth, development, and plasticity in central nervous system (Alberch et al., 2002). Neurotrophins are also neuroprotective against harmful stimuli such as excitotoxicity, brain injury or ischemia. Recently, transplantation of cells releasing neurotrophic factors and infusion of neurotrophins into striatum was shown to protect neurons from cell toxicity (Alberch et al., 2002). The striatum is a subcortical part of the telencephalon and a major part of the basal ganglia. The striatum is involved in the planning and modulation of movement pathways. It is also suggested to be involved in a variety of other
cognitive processes involving executive function. Functional defects of the striatum are related to many neurodegenerative diseases. Parkinson’s disease results from loss of dopaminergic innervation to the striatum. Huntington disease results, in part from the death of striatal neurons. It has also been suggested that addiction involves plasticity at striatal synapses (Bradberry and Rubino, 2006). Thus, a large number of studies have attempted to address pathogenic mechanisms underlying neurodegenerative diseases related to striatal neuronal physiology. Among the many neurotrophic factors, BDNF has been shown to effectively attenuate striatal cell death. In response to brain insults such as epileptic seizures, cerebral ischemia, glutamate excitotoxicity and traumatic injury, the level of BDNF mRNA increases in the striatum and the exogenous delivery of BDNF into striatum decreases cell death (Canals et al., 2001). These data suggest that BDNF-related pathways can be a target for preventing neuronal degeneration. However, the underlying signaling mechanisms of BDNF induced neuroprotection in the striatum are not well studied. Two major kinases, the phosphatidylinositol 3-kinase (PI3-K) and Ras/mitogen-activated protein kinase (MAPK) pathway, have been suggested as mediators for BDNF neuroprotection (Almeida et al., 2005). However, recent studies suggested that only the extracellular signal-related kinase (ERK). Thus, it is important to note that BDNF regulates downstream signaling pathways in a cell type and context-specific manner. Kinases are not the only downstream regulators which can induce the neuroprotective effects of BDNF. Nuclear factor kappaB (NF-kappaB) and CREB are two major transcription factors function as downstream mediators of BDNF signaling (Marini et al., 2004; Ying et al., 2002; Hsieh et al., 1998). Thus, understanding the combination of kinases and transcription factors is an important first step to begin to understand the role of BDNF in neuroprotection. In chapter 3, I examined which kinase signaling pathway is involved in BDNF mediated neuroprotective effects in striatum and whether CREB is an essential transcription factor which confers protection against excitotoxic conditions.
I then attempted to connect BDNF-mediated signaling to a specific neuroprotective response mechanism. To this end, I focused on oxidative stress. Oxidative stress is a major factor which can induce neuronal cell death resulting from brain insults such as ischemia, trauma, and neurodegenerative disorders (Estevez et al., 1998). Mitochondria are a major source for reactive oxygen radicals. Released reactive oxygen species (ROS) react with molecular oxygen and generate superoxide. In turn, superoxide can react with cellular macromolecules such as DNA, proteins and lipids and impede the function of these macromolecules resulting in pathophysiological conditions which contribute to neurodegenerative diseases (St-Pierre et al., 2006). In addition, superoxide is a major source for generating peroxynitrite (ONOO⁻). ONOO⁻ is formed by the biradical reaction of nitrooxide (NO) and superoxide (O₂⁻) (Huie and Padmaja, 1993). These nitrination products also react with cellular macromolecules and are involved in cell toxicity. 3-nitrotyrosine (3-NT) protein modification is one of the products from this reaction and it can be used as a marker of ONOO⁻ formation in vivo (Beckman, 2002). As a defense system, cells produce antioxidant enzymes to inhibit or scavenge the ROS and RNS (Floyd, 1999). The role and regulatory mechanisms of antioxidant enzymes are studied as potential therapeutic targets against many neurodegenerative diseases. Glutathione peroxidase, glutathione reductase, catalase, thioredoxin reductase, superoxide dismutase, heme oxygenase and biliverdin reductase are the most important antioxidant enzymes and these enzymes provide an important defense against free radicals. Recent studies suggest that exogenous hydrogen peroxide, one potent free radical, induces several antioxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase. Interestingly, the expression of these antioxidant proteins is tightly regulated by PGC-1 alpha (peroxisome proliferator-activated receptor-gamma coactivator-1 alpha), a transcriptional co-regulator that couples changes in cellular activity/stress to the induction of protective antioxidant gene expression (Borniquel et al., 2006; Barger et al., 2001).
Interestingly, CREB has been suggested to regulate the gene expression of PGC-1 alpha (St-Pierre et al., 2006). Thus, in chapter 3, I examined whether CREB transgenic mice show different sensitivities against oxidative stress induced by pilocarpine-induced SE and whether the neuroprotective effects of CREB were associated with the expression of PGC-1.

**Circadian rhythm**

The mammalian circadian clock located in the suprachiasmatic nuclei (SCN) is the major pacemaker that drives physiological processes with ~24 hr periodicity. Extensive studies on the underlying mechanisms which generate circadian cycles have identified a transcriptional-translational negative-feedback loop. Several clock genes are involved in this negative-feedback loop. CLOCK and BMAL1 are two major clock genes which encode for basic helix-loop-helix transcription factor (bHLH/PAS-containing transcription factors) that form a heterodimer. This heterodimer drives the rhythmic expression of three) and two Cryptochrome genes (mCRY1 and mCRY2) in mouse. mPER and mCRY Period genes (mPer1-mPer3form a heterodimer which translocates into nucleus. Translocated mPER and mCRY inhibit the function of CLOCK and/or BMAL1 by direct interaction, and thereby function as negative regulator of their own gene expression (Hastings et al., 1999; Vielhaber et al., 2001). Thus, it is important to understand the signal transduction pathways, which initiate the circadian expression of various genes to maintain and entrain the endogenous time-keeping properties of the SCN. This clock timing process is tightly regulated by photic entrainment cues which are conveyed from the eyes to the SCN via the optic nerve (Dudek et al., 1993). There are several candidate intracellular signaling pathways that couple light core clock transcriptional feedback loop described above. MAPK signaling pathway has been suggested to be involved in clock resetting during subjective night by regulating clock gene expression (Obrietan et al., 1998; Butcher et al., 2003, 2004, 2005).
Ca2+/calmodulin-dependent kinase (CaMK) is another kinase regulating clock resetting triggered by light, external cue (Golombek et al., 2004). Protein kinase A is also reported as a regulator for phase resetting in SCN. PKA inhibitor only affects phase resetting properties only during subjective day not during subjective night (Lee et al., 1999). In addition to these pathways, protein kinase C (PKC) has also been suggested as a potential regulator of photic entrainment (McArthur et al., 1997; Schak and Harrington, 1999; Motzkus et al., 2000; Bult et al., 2001). Protein kinase C (PKC) phosphorylates serine and threonine residues in many substrate proteins. It was first identified in 1977 in bovine cerebellum as a protein kinase that phosphorylated histone and protamine. Since then, its involvement in many biological processes has been demonstrated including development, memory, differentiation, proliferation and carcinogenesis (Dekker and Parker, 1994; Hug and Sarre, 1993). Based on its structure and cofactor regulation, protein kinase C is classified into three groups; conventional (α, two alternatively spliced variants βI and βII, and γ), novel (δ, ε, η and θ) and atypical forms (ζ, ι, and λ). The conventional form of protein kinase C has the pseudosubstrate domain, C1 domain comprising one or two Cys-rich motifs, C2 domain in the regulatory half, and the ATP-binding lobe (C3) and substrate-binding lobe (C4) of the catalytic region. The distinct difference in the primary structure of novel forms is the lack of amino acids involved in binding calcium. Atypical protein kinase Cs does not comprise phorbol ester binding site and one Cys-rich motif (Newton, 1995). For activation, conventional forms need both calcium and diacylglycerol (DAG) as cofactors and novel forms need only DAG. Atypical forms do not require either calcium or DAG (Newton, 1997; Dempsey et al., 2000). The role of protein kinase C is regulated by two important mechanisms. First, the enzyme is activated by a series of phosphorylations that correctly align residues for catalysis and localize protein kinase C to the cytosol. Second, binding of ligands or, in some cases, substrate allows the enzyme activation by releasing the regulatory domain from
the substrate-binding site (Newton, 1995). Its involvement in circadian clock has been suggested in many other researches. PKC isozymes has been found in the SCN and the disruption of its enzymatic function can alter the properties of phase resetting (Cagampang et al., 1998; Bult and Smale, 1999; van der Zee and Bult, 1995; Schak and Harrington, 1999). In chapter 4, I focused on the role of protein kinase C in phase resetting and examined the underlying mechanisms. Here, I suggested its role in posttranslational modification on Period proteins (mPER1 and mPER2).
Excitatory neurotransmission is a critical regulator of both the development and maintenance of normal nervous system physiology. For example, neuronal activity facilitates cell survival, suppresses apoptotic cell death (Catsicas et al., 1992; Sherrard and Bower, 1998; West et al., 2002), functions as a guidance cue during neuronal migration, and refines synaptic circuitry (Katz and Shatz, 1996; Behar et al., 1999; Cohen-Cory, 2002; Cancedda et al., 2003). As the dominant fast excitatory neurotransmitter, glutamate is a key regulator of these processes (Barry and O'Donovan, 1987; Barger and Mattson, 1995). Its capacity to drive synaptic communication is derived in part by the NMDA subtype of ionotropic glutamate receptor. The NMDA receptor functions as a cellular “gate” that allows Ca\(^{2+}\) to enter the cell only under the correct spatial and temporal pattern of synaptic activity (for review, see Nakanishi, 1992; Malenka and Nicoll, 1993; Cull-Candy et al., 2001). In turn, elevated intracellular Ca\(^{2+}\) triggers the expression of new genes, thus converting brief NMDA receptor stimulation into long-term changes in CNS physiology.

NMDA receptor stimulation can protect against cell death resulting from both the withdrawal of trophic factors and excitotoxic insults (Balazs et al., 1988; Chuang et al., 1992;
Rocha et al., 1999; Raval et al., 2003). NMDA receptor-mediated neuroprotection is a transcriptionally dependent process (Marini and Paul, 1992) that appears to be dependent, in part, on the expression of both the anti-apoptotic gene BCL-2 (Mabuchi et al., 2001; Alavez et al., 2003) and the neurotrophin BDNF (Aliaga et al., 1998; Rocha et al., 1999). A number of studies have shown that the cAMP response element-binding protein (CREB)/cAMP response element (CRE) transcriptional pathway regulates the expression of both BCL-2 and BDNF (Wilson et al., 1996; Shieh et al., 1998; Tao et al., 1998; Riccio et al., 1999). With respect to the role of the CREB/CRE transcriptional pathway in neuroprotection, Hara et al. (2003) found that CRE-mediated gene expression is necessary for the induction of ischemic tolerance. Likewise, Mabuchi et al. (2001) showed that the neuroprotective effects of an ischemic preconditioning stimulus involve both NMDA receptor stimulation and CREB-dependent transcription. These observations suggest that a signaling cassette formed by the NMDA receptor and the CREB/CRE transcriptional pathway contributes to activity-dependent neuroprotection.

These neuroprotective effects appear to be mediated specifically by synaptic NMDA receptors; activation of extrasynaptic NMDA receptors has been shown to trigger a transient form of CREB phosphorylation and apoptotic cell death (Hardingham et al., 2002). Although these studies suggest a role for both synaptic activity and CREB-dependent transcription in neuroprotection, issues regarding the signaling events that regulate the induction and duration of NMDA receptor-evoked CREB phosphorylation, as well as how stimulus intensity triggers protection or cell death, have yet to be addressed. The data presented here provide new mechanistic insights into the connections between the NMDA receptor/CREB signaling cassette and the induction of excitotoxic tolerance.
MATERIALS AND METHODS

Tissue culture and transfection. Embryonic day 19-20 Sprague Dawley rat pups were decapitated, their brains were removed, and the cortices were isolated and placed in dissociation media [DM; containing the following (in mM): 90 Na₂SO₄, 30 K₂SO₄, 16 MgCl₂, 0.25 CaCl₂, 32 HEPES, and 0.01% phenol red (Sigma, St. Louis, MO), pH 7.7]. Tissue then was washed three times in DM, finely minced with a razor blade, and digested with a mild protease solution [100 U/ml papain latex (Worthington, Freehold, NJ) and 4.5 mg of cysteine (Sigma) in DM] at 37°C for 30 min. After removal of the digestion solution, the tissue was washed three times in DM and transferred to tissue culture medium [minimal essential medium (MEM); Invitrogen, San Diego, CA] containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Then the tissue was triturated into a single-cell suspension via a 5 ml serological pipette and plated at a density of 2.2 x 10⁵ cells/cm² on poly-L-lysine-coated (>540 kDa; Sigma) glass coverslips or tissue culture dishes. At 2 h after plating, the medium was replaced with MEM containing 2% B-27 (Invitrogen), 1% fetal bovine serum, and 100 U/ml penicillin/streptomycin. Cultures were maintained at 37°C and 5% CO₂ in a Napeco (Winchester, VA) 6100 incubator. Experiments were performed after 10-12 d in vitro (DIV). All animal procedures were in accordance with The Ohio State University animal welfare guidelines.

Fura-2 calcium digital imaging. Time-lapse ratiometric fluorescent digital microscopy was performed as described previously (Obrietan and van den Pol, 1998). Ratiometric single-cell imaging data were collected with MetaFluor software (Universal Imaging, West Chester, PA). Data acquisition was performed every 5 s. Cells were maintained in a HEPES-based buffer containing the following (in mM): 137 NaCl, 25 glucose, 10 HEPES, 5 KCl, 1 MgCl₂, and 3
CaCl₂, pH 7.4. To facilitate NMDA receptor activity during synaptic activity assays and NMDA administration, we added glycine (2 µM) and removed Mg²⁺ from the perfusion medium. Tetrodotoxin (TTX; 1 µM) was added to the perfusion solution to block spontaneous synaptic activity during NMDA administration.

**Western blotting.** Cultured cells were transferred from tissue culture medium to a HEPES perfusion solution containing 1 µM TTX 30 min before stimulation. Cells were stimulated for 10 min and lysed in 200 µl of hot (95°C) 3x SDS sample buffer. Lysates were stored at -80°C until use. Before being loaded, the lysates were heated to 95°C for 10 min, vortexed (10 s), and centrifuged for 7 min at 15,000 x g. Extracts (20 µl/lane) were electrophoresed into a 10% SDS polyacrylamide gel and transblotted onto polyvinylidene fluoride (Immobilon P; Millipore, Bedford, MA). Next the membranes were washed with 5% (w/v) powdered milk dissolved in PBS with 0.1% Triton X-100 (PBST), followed by incubation (4°C overnight) with one of the following antibodies: affinity-purified rabbit polyclonal anti-CREB phosphorylated at Ser¹³³ (1:1000 final dilution; Cell Signaling Technology, Beverly, MA), mouse monoclonal anti-extracellular signal-regulated kinase-1 (ERK-1) and anti-ERK-2 phosphorylated at Thr²⁰² and Tyr²⁰⁴ (1:5000; Sigma), or mouse monoclonal anti-Ca²⁺/calmodulin-dependent protein kinase IV (CaMKIV) phosphorylated at Thr¹⁹⁶ (acquired from Dr. Thomas R. Soderling, Vollum Institute, Portland, OR). Next the membranes were washed and incubated [4 h at room temperature (RT)] with horseradish peroxidase (HRP)-conjugated secondary antibodies directed against the IgG domains of the primary antibodies (1:2000; PerkinElmer Life Sciences, Norwalk, CT). The signal was visualized by using Renaissance chemiluminescent HRP substrate (PerkinElmer Life Sciences). As a control for equal loading of protein across the gel, the membranes were stripped and probed for total ERK expression with a rabbit polyclonal antibody against ERK-1 and ERK-2
ERK expression was revealed with a goat anti-rabbit IgG antibody (1:2000 final dilution; PerkinElmer Life Sciences) conjugated to HRP, followed by visualization as described above. Blots were washed a minimum of six times (5 min per wash) in PBST with 5% milk after each antibody treatment. Quantitation of band intensity was performed with Scion Image analysis software (Frederick, MD). Band intensity was normalized to total ERK-1 for the corresponding lane. Each experiment was repeated a minimum of three times.

**Immunostaining.** After stimulation, the cells were fixed with 4% (w/v) formaldehyde for 15 min, washed five times in PBST, and then blocked (2 h at RT) with 1% normal goat serum and 10% bovine serum albumin in PBST containing 0.02% azide. Cultures were immunolabeled (overnight at 4°C) with an affinity-purified rabbit polyclonal CREB phosphorylation (pCREB) antibody (1:500; Cell Signaling Technology), a monoclonal antibody raised against the neuronal nuclear marker neuronal-specific nuclear protein (NeuN) (1:500; Chemicon, Temecula, CA), or a rabbit polyclonal antibody raised against green fluorescent protein (GFP; 1:5000 final dilution; acquired from Dr. Luc Berthiaume, University of Alberta, Edmonton, Canada). Next the cells were washed and incubated (4 h at RT) with an Alexa 488- or 594-conjugated secondary antibody (1:250 final dilution; Molecular Probes, Eugene, OR). To examine cell health, we also incubated cultures (10 min) with the DNA stain Hoechst 33342 (Hoechst, 1 µg/ml; Molecular Probes) before mounting them with gel mount (Biomedia, Foster City, CA). Photomicrographs were captured by a 16-bit digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ) connected to an inverted epifluorescence microscope [Leica (Nussloch, Germany) DM IRB at 200x magnification]. All tabulated data are expressed as the mean ± SEM. Significance was determined by using the two-tailed Student's t test.
Transfection and reporter gene assays. Primary cultured cortical neurons were transfected after 8 DIV (1.5 µg of DNA per well) by using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. The following constructs were used: pcDNA3 VP16-CREB, pcDNA3 A-CREB, pcDNA3.1 (empty vector), pGL3-CRE-luciferase, and pEGFP-N3. Cells were assayed 48 h after transfection. Luciferase activity was measured by using the Bright-Glo Luciferase Assay System (Promega, Madison, WI). Full-length NF-ATc4 was amplified by PCR from a mouse brain cDNA library and cloned in frame into the pcDNA3.1/CT-GFP expression vector (Invitrogen). Cells were assayed 48 h after transfection. Cells were immunostained for GFP and for the neuronal marker MAP2 (1:2000; Sigma). Fluorescent images were captured with a Zeiss (Oberkochen, Germany) 510 Meta confocal microscope (2-µm-thick optical section). The nuclear/somatic GFP signal was determined for each neuron and expressed as the mean ratio for each condition.

Cell toxicity assay. Cell toxicity was determined by measuring lactate dehydrogenase (LDH) release as described by Koh and Choi (1987). The percentage of LDH release was calculated as LDH in the culture medium divided by total LDH (cellular plus medium LDH). Toxicity also was measured by labeling with the DNA stain Hoechst as described above. For the quantification of apoptotic cell death, the regions were selected randomly as described above, and the viability of all neurons (NeuN-positive) within the region was determined. Neurons with fragmented or condensed nuclei were scored as dead or dying (apoptotic).
**Materials.** Pyruvate, nicotinamide adenine dinucleotide, NMDA, glutamate, nimodipine, bicuculline, 4-aminopyridine (4-AP), TTX, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) disodium salt, and 2-amino-5-phosphonopentanoic acid (APV) were acquired from Sigma. Bisindolylmaleimide, U0126, KN62, KN93, SP600125, and staurosporine were acquired from Calbiochem (La Jolla, CA). H89, FK506, LY294002, PD98059, Rp-cAMPs, and SB203580 were acquired from LC Laboratories (Woburn, MA). Hoechst 33342 and fura-2 were acquired from Molecular Probes.

**RESULTS**

The NMDA receptor couples synaptic activity to CREB phosphorylation

To begin to examine the receptors that mediate synaptically evoked CREB phosphorylation, we prepared cortical neuronal cultures from embryonic day 20 rats and maintained them *in vitro* for 10-12 d. Several groups have shown that 10 DIV cortical neurons form functional synaptic connections and secrete both excitatory and inhibitory amino acid neurotransmitters (Segal and Barker, 1984; Wilcox et al., 1994; Obrietan and van den Pol, 1995; Craig et al., 1996). Fura-2 timelapse digital microscopy was used to monitor neuronal Ca\(^{2+}\) changes mediated by synaptic transmitter release. In the presence of the GABA\(_A\) receptor antagonist bicuculline (50 µM), the withdrawal of the sodium channel blocker TTX (1 µM) from the perfusion solution triggered a series of rapid Ca\(^{2+}\) transients (Figure. 1.1A). The majority of neurons within an imaging region (> 70%) exhibited synchronized Ca\(^{2+}\) transients. The readministration of TTX at the end of the recording returned Ca\(^{2+}\) to basal levels, indicating that the rise in Ca\(^{2+}\) was mediated by the action potential-dependent release of an excitatory neurotransmitter. In parallel with this effect on Ca\(^{2+}\), spontaneous synaptic activity triggered an
increase in the Ser\textsuperscript{133} phosphorylated form of CREB (Fig. 1.1\textit{A}). Next we examined whether signaling via the NMDA receptor was sufficient to drive the synaptic network and trigger CREB phosphorylation. In this experiment, synaptic activity was elicited by the withdrawal of TTX from the perfusion media. To augment network-dependent neuronal activity, we added bicuculline (50 µM) and the potassium channel blocker 4-AP (200 µM) to the perfusion media. Nimodipine (5 µM) was added to the perfusion media to eliminate the potential contribution of L-type voltage-activated Ca\textsuperscript{2+} channels (VACC) to synaptically evoked Ca\textsuperscript{2+} transients and activity-dependent CREB phosphorylation. Under these conditions, network-driven neuronal activity elicited robust Ca\textsuperscript{2+} transients and CREB phosphorylation (Fig. 1.1\textit{B}). When the NMDA receptor antagonist APV was added to the perfusion media, synaptic activity was mostly blocked (we did note a small percentage of cells with increased Ca\textsuperscript{2+} levels even in the presence of NMDA receptor blockade) (data not shown), and CREB phosphorylation was attenuated markedly. The administration of the non-NMDA ionotropic receptor antagonist CNQX did not inhibit the synaptically evoked Ca\textsuperscript{2+} rise, nor was it able to block CREB phosphorylation (Fig. 1.1\textit{B}). Collectively, these data show that a synaptic network can be driven specifically by the NMDA receptor and that NMDA receptor signaling is sufficient to drive CREB phosphorylation.
Figure 1.1. The NMDA receptor couples synaptic activity to the Ser 133 phosphorylated form of CREB. A, Top, Initially, cortical neurons cultured for 10 d were placed in a HEPES-based buffer containing TTX (1 µM), and internal Ca²⁺ levels were monitored by using fura-2 time-lapse digital microscopy. In the presence of bicuculline (50 µM), the withdrawal of the TTX from the buffer elicited rapid and reproducible Ca²⁺ transients. A, Bottom, Relative to cortical cultures maintained in TTX, 10 min of synaptic activity elicited by the removal of TTX triggered a marked increase in pCREB expression. As a protein loading control, this blot was stripped and probed for total ERK expression. Duplicate determinations are shown for each condition. B, Top, Driving the synaptic network via activation of the NMDA receptor. To examine the necessity of L-type Ca²⁺ channels and ionotropic glutamate receptors in synaptic activity-dependent CREB phosphorylation, we placed cortical cultures in HEPES buffer containing bicuculline (50 µM), 4-AP (200 µM), and nimodipine (5 µM). In the majority of neurons, the basal Ca²⁺ levels were maintained by the withdrawal of TTX (1 µM) and the addition of APV (100 µM) to the perfusion medium. Washout of APV and the non-NMDA ionotropic receptor antagonist CNQX (10 µM) triggered a robust and sustained increase in Ca²⁺ levels. Ca²⁺ levels also were increased by the withdrawal of APV, indicating that the synaptic release of glutamate stimulated Ca²⁺ influx via an NMDA receptor-mediated mechanism. B, Bottom, Western blot analysis for pCREB revealed that the NMDA receptor coupled synaptic activity to pCREB expression. C, Synaptic activity attenuates NMDA-induced cell death. Hoechst labeling was used to monitor the effects that synaptic activity has on NMDA-evoked neurotoxicity. A bout (15 min) of synaptic activity was elicited by the application of bicuculline and 4-AP (bic+4AP) in the presence of nimodipine and CNQX at time 0 (t=0). Then 24 h later (t=24), the cultures were exposed to NMDA(50 µM, 15 min); the number of dead and dying cells was scored 8 h later (t=24+8). Arrowheads denote the location of nuclei from healthy cells; arrows indicate apoptotic cells with condensed or fragmented nuclei. D, In the absence of pretreatment with synaptic activity, relatively high numbers (~40%) of apoptotic cells were observed. Pretreatment with synaptic activity significantly (*p<0.05) reduced the apoptotic effects of NMDA. Control data are from cells that were stimulated neither with synaptic activity nor with NMDA. Numbers above each bar indicate the number of cells assayed. E, The neuroprotective effects of synaptic activity also were monitored via LDH release. Cell stimulation was performed as described in C. *Significant difference (p<0.05) from the NMDA treatment condition. NMDA administration was performed in media containing TTX (1 µM).
Synaptic/NMDA receptor stimulation, CREB, and excitotoxic cell death

NMDA receptor stimulation has been shown to be both neuroprotective and excitotoxic. Although the mechanism or mechanisms by which the NMDA receptor exerts this dualistic character are not clear, receptor location, the magnitude of the Ca$^{2+}$ response, and CREB activation may be critical factors. Initially, we tested whether NMDA receptor stimulation mediated by synaptic glutamate release attenuated the apoptotic effects of exogenous NMDA (50 µM) application. For these experiments, NMDA receptor-dependent synaptic activity was elicited by the application of bicuculline and 4-AP in the presence of nimodipine and CNQX for 15 min. Then 24 h later, NMDA (50 µM) was administered for 15 min. Hoechst labeling and LDH release were examined 8 h after NMDA administration. In the absence of a pretreatment with synaptic activity, NMDA elicited a marked increase in the number of dead and dying cells (Fig. 1.1C-E). In contrast, synaptic activity 24 h before NMDA application significantly decreased both the number of apoptotic cells and LDH release. These results indicate that excitatory neurotransmission mediated by NMDA receptor activation protects against NMDA-induced excitotoxic cell death. Upregulation of the neurotrophin BDNF and the anti-apoptotic protein BCL-2 may be a mechanism by which synaptic activity confers neuroprotection against excitotoxic insults. Given that both BDNF and BCL-2 are CREB-regulated genes (Shieh et al., 1998; Tao et al., 1998), these results suggest that neuroprotective (synaptic activity) and toxic (50 µM NMDA) stimuli differentially regulate signaling via the CREB/CRE transcriptional pathway.

To begin to address this issue, we examined the effects that brief stimulations (15 min) with synaptic activity and NMDA have on the activation of CRE-mediated gene expression. For these experiments, the neurons were transfected with a CRE-luciferase reporter construct after 8 DIV and stimulated (48 h later) with either synaptic activity driven specifically by the NMDA receptor (bicuculline, 4-AP, nimodipine, and CNQX) or the exogenous application of NMDA (1, 5, or 50
µM). Luciferase activity measurements revealed that both synaptic activity and low concentrations of NMDA (1 and 5 µM) stimulated significant increases in CRE-mediated gene expression, whereas the application of 50 µM NMDA did not stimulate luciferase expression (Fig. 1.2A). In an attempt to determine why these stimuli have differential effects on CRE-mediated gene expression, we monitored the activation state of CREB. A number of studies have shown that the duration of CREB phosphorylation correlates with transcriptional activation (Bito et al., 1996; Liu and Graybiel, 1996; Impey et al., 1998). The exogenous application of 50 µM NMDA triggered a robust but transient increase in pCREB; levels declined by 15 min after stimulation (25 min time point) and returned to basal levels by 45 min after stimulation (Fig. 1.2D, 45 min time point). A significant increase in NMDA-induced cell death, as assessed by Hoechst labeling (data not shown) and LDH release (Fig. 1.3C, 2 h time point), was not observed during the period of CREB dephosphorylation, indicating that the rapid drop in pCREB levels was not the result of acute cell death. In contrast to the transient pCREB response elicited by 50 µM NMDA, the administration of bicuculline elicited a robust increase in CREB phosphorylation that persisted for ≥90 min after stimulus onset (Fig. 1.2B). Typically, synaptically evoked CREB phosphorylation persisted from 90 to >180 min. Long-lasting CREB phosphorylation was mediated by NMDA receptor activation; in the presence of nimodipine and CNQX, a brief period of synaptic activity triggered long-lasting CREB phosphorylation (Fig. 1.2C, 180 min time point). The transient nature of CREB phosphorylation mediated by exogenous NMDA administration raised the possibility that a process of dephosphorylation also had been activated by the stimulus. Several studies have shown that the phosphatase calcineurin regulates the duration of CREB phosphorylation (Bito et al., 1996; Liu and Graybiel, 1996; Wu et al., 2001). To address the potential role of calcineurin, we incubated cultures with the calcineurin inhibitor FK506 (1 µM). Under this condition, the administration of NMDA (50 µM, 10 min) led to a sustained increase in
CREB phosphorylation that persisted for 180 min (Fig. 1.2E, top), paralleling the long-lasting increase in CREB phosphorylation mediated by synaptic activity. In addition, FK506 significantly attenuated the toxic effects of exogenous NMDA administration (Fig. 1.2E, bottom). These data suggest that strong, potentially neurotoxic levels of NMDA receptor stimulation specifically may activate calcineurin. To assess the relative responsiveness of calcineurin to synaptic activity and NMDA (50 µM), we monitored the subcellular localization of the transcription factor NF-ATc4. Nuclear translocation of NF-ATc4 is elicited by calcineurin-mediated dephosphorylation (Crabtree, 1999; Graef et al., 1999). Thus by monitoring the nuclear/cytoplasmic NF-ATc4 ratio, one may infer the activation state of calcineurin. For these studies, the cortical neurons were transfected with an NF-ATc4-GFP fusion protein and stimulated (10 min) with NMDA (50 µM) or synaptic activity (bicuculline, 4-AP, CNQX, nimodipine); then they were fixed and immunolabeled for GFP and the neuronal marker protein MAP2. NMDA elicited marked NF-ATc4-GFP nuclear translocation, whereas synaptic activity did not increase the nuclear expression of the transcription factor, relative to control conditions (Fig. 1.2F). Pretreatment with FK506 blocked NMDA-induced NF-ATc4 translocation, indicating that calcineurin activity is essential to couple NMDA to NF-ATc4 nuclear accumulation. Interestingly, FK506 reduced NMDA-induced NF-ATc4 nuclear localization to a level that was lower than that observed in unstimulated (control) neurons, indicating that there is tonic calcineurin activity under control conditions. Together these data reveal that NMDA (50 µM) stimulates calcineurin activity but that synaptic activity is not effective on calcineurin activity. These differential response profiles provide mechanistic insight into how the duration of CREB phosphorylation is affected differentially by these two stimuli.

The transient CREB phosphorylation mediated by exogenous NMDA application was in striking contrast to the sustained level of CREB phosphorylation mediated by synaptic NMDA receptor activation. These results raise the possibility that distinct pools of NMDA receptors
Figure 1.2. NMDA, the CREB/CRE pathway, and cell toxicity. A, Neurons transfected with a CRE-luciferase reporter gene construct were stimulated (15 min) with NMDA (1, 5, 50 µM), forskolin (5 µM), or NMDA receptor-dependent synaptic activity (as described in Fig. 1.1C) and assayed 8 h later. *Significant difference (p<0.05) relative to control buffer treatment. Data were averaged from triplicate determinations. B, Synaptic activity triggered a long-lasting form of CREB phosphorylation. In this example, the phosphorylated form of CREB was stimulated after a brief (10 min) administration of bicuculline (bic; 20 µM). Cultures were collected at the poststimulus onset time points indicated. C, Long-lasting CREB phosphorylation was mediated by NMDA receptor activation. In this experiment, synaptic activity was stimulated (10 min) in the presence of CNQX (10 µM) and nimodipine (nim; 5 µM). D, The exogenous application of NMDA (50 µM) triggered a transient form of CREB phosphorylation. Initially, cortical neurons were stimulated for 10 min. Then NMDA was washed from the medium, and the cultures were collected at the poststimulus onset time points indicated. E, Top, Preincubation (120 min) with the calcineurin antagonist FK506 (1 µM) converted the NMDA-evoked (50 µM) transient increase in CREB phosphorylation into a sustained elevation. Basal levels of CREB phosphorylation were not altered by FK506 pretreatment (Con t=0 and t=180 vs FK506 t=0 and t=180). Western blots are representative of at least three independent experiments. E, Bottom, FK506 pretreatment (1 µM, 120 min) attenuated the toxic effects of NMDA (50 µM, 15min stimulation). LDH release was examined 8 h after NMDA administration. For the NMDA administration experiments, all solutions contained TTX. *Significant difference (p<0.05) relative to both control and NMDA administration. F, Neurons were transfected with an NF-ATc4-GFP fusion protein, and the effects of NMDA (50 µM, 10 min stimulation) and synaptic activity (bicuculline plus 4-AP in the presence of nimodipine and CNQX, 10 min stimulation) on NF-ATc4-GFP subcellular localization were examined. F, Top, Representative confocal images of NF-ATc4-GFP expression under the four conditions. Cells were incubated (120 min) with FK506 (1 µM) before NMDA stimulation. Note the marked nuclear translocation after NMDA administration. F, Bottom, Mean nuclear/ cytoplasmic NF-ATc4-GFP ratio under the four treatment conditions. Error bars denote the SEM. Numbers above each bar indicate the number of neurons analyzed. *p<0.005, relative to all other conditions. Con, Control.
(synaptic and nonsynaptic) and/or the intensity of the stimulus may govern pCREB duration. To gain insight into whether pCREB duration is related to NMDA concentration, we monitored the pCREB time course after exogenous stimulation with relatively low concentrations of NMDA. In contrast to the transient increase in pCREB mediated by 50 µM NMDA, brief (10 min) treatment with either 1 or 5 µM NMDA (in the presence of TTX) led to a persistent (~90-180 min) increase in CREB phosphorylation (Fig. 1.3A, B).

To address the issue of whether the duration of CREB phosphorylation corresponds with the toxicity of the treatment, we analyzed LDH release from neurons treated with low and high concentrations of NMDA. Consistent with the idea that the prolonged CREB phosphorylation is associated with nontoxic NMDA stimulation, we found that low concentrations of NMDA (1 and 5 µM) did not trigger significant cell death, whereas 50 µM NMDA stimulated a significant increase in cell death (Fig. 1.3D). In an additional parallel to the results observed by using the synaptic activity paradigm, pretreatment with a low concentration of NMDA (2.5 µM) significantly attenuated excitotoxic effects of 50 µM NMDA (Fig. 1.3D). Collectively, these data indicate that NMDA concentration determines the duration of CREB phosphorylation and determines whether NMDA is protective or toxic.

**Kinase coupling to CREB**

A panel of pharmacological inhibitors was used to identify the signaling events that couple synaptic/NMDA receptor activity to CREB phosphorylation. Initially, cortical neurons were treated with the broad-spectrum CaMK inhibitor KN62 (10 µM), the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor U0126 (10 µM), the PKA inhibitor H89 (5 µM), the inactive cAMP analog Rp-cAMPs (300 µM), or the PKC inhibitor bisindolylmaleimide (1 µM). Western blot analysis and immunofluorescent labeling for pCREB revealed that KN62 and U0126
significantly attenuated the capacity of synaptic activity to stimulate CREB phosphorylation (Fig. 1.4A,B). None of the other inhibitors were effective. As a control experiment, fura-2 Ca\(^{2+}\) imaging was used to examine whether KN62 and U0126 inhibit neurotransmission and thereby nonspecifically affect the ability of synaptic input to regulate CREB phosphorylation. Representative recordings (Fig. 1.4C) revealed that synaptic/NMDA receptor-mediated Ca\(^{2+}\) transients persisted in the presence of the CaMK and mitogen-activated protein kinase (MAPK) inhibitors. Western blot analysis was used to show that ERK phosphorylation at Thr\(^{202}\) and Tyr\(^{204}\) (a marker of ERK activation) was blocked by U0126 and that CaMKIV phosphorylation at Thr\(^{196}\) (a marker of CaMKIV activation) was inhibited by KN62 (Fig. 1.4C). In contrast to the role the MAPK and CaMK pathways play in synaptically evoked CREB activation, neither pathway appeared to contribute to CREB phosphorylation mediated by high levels of NMDA (50 µM) (Fig. 1.5A,C). Control experiments (Fig. 1.5B) validated the capacity of KN62 and U0126 to attenuate NMDA-evoked (50 µM) CaMKIV phosphorylation and MAPK activation, respectively.

We also tested the capacity of a number of other pathways to couple excitotoxic levels of NMDA to CREB phosphorylation. Inhibitors of cAMP/PKA-dependent signaling (5 µM H89; 300 µM Rp-cAMPS) as well as the PKC inhibitor bisindolylmaleimide (1 µM) had no effect on pCREB levels (Fig. 1.5A). Because 50 µM NMDA is neurotoxic, we assessed whether two stress-responsive pathways, the p38 MAPK pathway and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), regulated CREB phosphorylation. Both pathways were activated by NMDA (data not shown); however, neither the disruption of p38 MAPK activity with SB203580 (10-50 µM) nor addition of the JNK/SAPK inhibitor SP600125 (30 µM) had an effect on the NMDA-evoked CREB phosphorylation (Fig. 1.5D,E). In addition, the phosphatidylinositol kinase pathway inhibitor LY294002 (30 µM) and the broad-spectrum protein kinase inhibitor staurosporine (200 nM) had no effect on CREB activation (data not shown). In initial tests of
Figure 1.3. The duration of CREB phosphorylation depends on NMDA concentration. A, In contrast to the transient increase in pCREB elicited by 50 µM NMDA (Fig. 2), brief stimulation (10 min) with low concentrations of NMDA (1 and 5 µM) triggered a sustained (90–180 min) increase in CREB phosphorylation. As a protein-loading control, blots were stripped and probed for total ERK expression. B, Quantitation of the duration of NMDA-evoked (1–50 µM) CREB phosphorylation. pCREB values were normalized to ERK 1 expression, and control conditions were set equal to 100%. Note that low concentrations of NMDA (1 and 5 µM) elicited longer-lasting pCREB than the high (50 µM) concentration of NMDA. *Significant differences (p<0.05) between low (1 and 5 µM) and high (50 µM) NMDA concentrations from the 25–180 min time points. Data are from triplicate determinations. C, The toxic effects of NMDA were correlated inversely with the duration of CREB phosphorylation. LDH assays performed 2 and 8 h after NMDA stimulation (15 min) revealed that low levels of NMDA (1 and 5 µM) are relatively nontoxic, whereas 50 µM NMDA led to a pronounced increase in LDH release at the 8 h time point. As a relative comparison for the toxic effects of NMDA, cortical neurons also were treated with glutamate (1mM, 15 min). Data are from triplicate determinations. *Significant difference (p<0.05) relative to control conditions. D, Pretreatment with a low concentration of NMDA (2.5 µM) significantly attenuated the excitotoxic effects of NMDA (50 µM). In this assay, the cortical neurons were stimulated at time 0 (t=0) with 2.5 µM NMDA for 15 min and then stimulated 24 h later (t=24) with 50 µM NMDA; LDH release was assayed 8 h later. *p<0.05, relative to 50 µM NMDA administration. Con, Control; bic, bicuculline.
Figure 1.4. The MAPK and CaMK pathways couple synaptically evoked NMDA receptor activation to CREB phosphorylation. A, Initially, cortical cultures were pretreated (for 30 min) with U0126 (10 μM), KN62 (10 μM), H89 (5 μM), Rp-cAMPS (300 μM), or bisindolylmaleimide (Bis; 1 μM). Then NMDA receptor-mediated synaptic activity was elicited (10 min) by bicuculline (bic) plus 4-AP in the presence of nimodipine and CNQX. Both U0126 and KN62 attenuated CREB phosphorylation. Mean densitometric band analysis is shown in the bars below. Data are representative of triplicate determinations. *Significant difference (p < 0.05) relative to the bicuculline plus 4-AP treatment condition. Intensity values were normalized to the bicuculline plus 4-AP condition, which was set equal to 100%. Data are representative of triplicate determinations. B, Immunofluorescent staining for pCREB was used to compliment the Western blot analysis. Relative to control cultures, the administration of bicuculline increased pCREB expression; pretreatment with KN62 or U0126 attenuated the synaptic activity-dependent increase in CREB phosphorylation. C, To determine whether inhibition of MAPK and CaMK signaling disrupts Ca²⁺ response characteristics, we loaded cortical cultures with fura-2, pretreated them (10 min) with 10 μM KN6 or 10 μM U0126, and examined the capacity of NMDA receptor-mediated synaptic activity to stimulate Ca²⁺ transients. Representative examples show that robust Ca²⁺ transients were generated in the presence of these inhibitors. As an additional control, Western blot analysis was used to examine the potency of these inhibitors. U0126 (10 μM) attenuated synaptically evoked ERK phosphorylation; KN62 (10 μM) attenuated synaptically evoked CaMKIV phosphorylation. Con, Control.
**Figure 1.5. Coupling excitotoxic levels of NMDA receptor stimulation to CREB phosphorylation.**

A, The small molecule inhibitor panel used in Figure 4A had no effect on CREB phosphorylation mediated by the application of 50 µM NMDA. Intensity values were normalized to the NMDA treatment condition, which was set equal to 100%. Data are representative of triplicate determinations. Bis, Bisindolylmaleimide. B, The capacity of KN62 to disrupt NMDA-evoked CaMKIV activation and of U0126 to disrupt NMDA-evoked MEK-dependent ERK phosphorylation was verified. C, To test for potential redundancy within the MAPK and CaMK pathways, we incubated cells in both 10 µM KN62 and 10 µM U0126 and assayed the effects of NMDA. Treatment with another broad-spectrum CaMK inhibitor, KN93 (10 µM), as well as another MEK inhibitor, PD98059 (50 µM), had no discernible effect on NMDA-evoked CREB phosphorylation. D, E, Inhibition of JNK/SAPK with SP600125 (30 µM) and disruption of p38 MAPK with SB203580 (10–50 µM) did not affect NMDA-evoked CREB phosphorylation. F, Pretreatment with an inhibitor mixture attenuated, but did not block, NMDA-evoked CREB phosphorylation. In this experiment, the neurons were incubated with U0126 (10 µM), KN62 (10 µM), bisindolylmaleimide (1 µM), H89 (5 µM), SB203580 (20 µM), SP600125 (30 µM), LY294002 (30 µM) and staurosporine (200 nM). The number above each lane indicates the pCREB/ERK ratio, which was normalized to the first control condition, which was set equal to 1. Inhibitors were applied 30 min before NMDA stimulation and maintained in the culture media until the cells were lysed. All buffers contained TTX (1 µM). Con, Control.
ERK-5, we found that NMDA (50 µM) did not elicit its phospho-activation (data not shown). Finally, kinase coupling to CREB phosphorylation was tested by using a potent mixture of inhibitors (U0126, KN62, bisindolylmaleimide, H89, SB203580, SP600125, LY294002, and staurosporine). Under this condition, we noted a modest ~40% decrease in CREB phosphorylation (Fig. 1.5F). Together, these data indicate that high, potentially toxic levels of NMDA couple to CREB phosphorylation via an atypical signaling event.

**The MAPK and CaMK pathways do not determine the duration of CREB phosphorylation.**

Next we examined the potential signaling events that govern the duration of CREB phosphorylation. Given the data showing that U0126 and KN62 attenuated synaptic/NMDA receptor-mediated CREB phosphorylation, we focused attention on the MAPK and CaMK cascades. To examine the duration of kinase activation, we collected cultures after 10-60 min of persistent synaptic/NMDA receptor activation and processed them for the phosphorylated form of ERK and for the phosphorylated form of CaMKIV. As expected, synaptic activity led to CREB phosphorylation throughout the stimulus period (Fig. 1.6A). In contrast, CaMKIV activation was transient, peaking 10 min after stimulus onset and returning to near-basal levels before the end of the stimulus period. Given its transient activation profile, these results suggest that CaMK signaling may not contribute to the sustained increase in CREB activation. Indeed, treatment of cortical neurons with KN62 after 10 min of synaptic activity did not decrease the duration of CREB phosphorylation (Fig. 1.6B); in the presence of KN62, elevated pCREB levels were observed 90-180 min after stimulation termination, paralleling the pCREB duration after synaptic stimulation in the absence of inhibitor administration. Persistent synaptic/NMDA receptor activation led to a sustained increase in ERK phosphorylation, paralleling the pCREB activation profile (Fig. 1.6A). Surprisingly, the administration of U0126 (10 µM) after the onset of synaptically mediated CREB phosphorylation also failed to reduce the duration of CREB
activation (Fig. 6B). In control experiments, synaptic activity (10 min) led to a sustained increase in ERK phosphorylation, and the administration of U0126 blocked sustained ERK phosphorylation (Fig. 1.6C). These data suggest that MAPK and CaMK signaling are required for the onset of synaptically evoked CREB phosphorylation but that neither pathway regulates the duration of CREB activation.

**Temporal regulation of CREB "shut-off"**

Hardingham et al. (2002) found that exogenous glutamate applied after 30 min of persistent synaptic activity triggered a rapid decrease in pCREB levels. Under this stimulus paradigm, the extrasynaptic CREB shut-off mechanism determines pCREB duration. In light of our data above showing that the duration of synaptically evoked CREB phosphorylation is independent of persistent kinase activity, we wanted to identify specifically when shut-off occurs: during the induction of synaptically evoked pCREB and/or during the period when persistent CREB phosphorylation has become independent of the MAPK and CaMK pathways. To test whether shut-off occurs during induction, we stimulated synaptic activity while we applied exogenous NMDA (50 µM). After 10 min, the medium was changed to a TTX-containing solution, and the pCREB duration was determined. Under these conditions pCREB levels persisted for up to 90 min, consistent with the pCREB time course elicited by synaptic activity alone (Fig. 1.7A). These data indicate that, when both synaptic and exogenous stimuli are applied simultaneously, synaptic activity dominates and thus determines pCREB duration. In agreement with the work of Hardingham et al. (2002), we found that if synaptically mediated CREB phosphorylation was elicited be These data indicate that the sequence of stimulation determines which pathway exerts control over pCREB duration.
Synaptic activity, CRE-mediated transcription, and neuroprotection

Finally, to form a definitive link between CREB and attenuation of NMDA-mediated toxicity, we transfected cortical neurons with a constitutively active CREB construct (VP16-CREB) and a GFP reporter construct and then, 48 h later, stimulated them with 50 µM NMDA. Fore exogenous NMDA receptor stimulation, pCREB shut-off occurred (Fig. 1.7B). GFP immunofluorescence was used to identify transfected cells, NeuN was used to verify that the cells were neurons, and Hoechst labeling was used to examine cell health. Relative to empty vector-transfected neurons, transfection of VP16-CREB significantly attenuated NMDA-induced cell death (Fig. 1.8A,B). In the absence of NMDA administration, VP16-CREB did not affect cell survival (data not shown). As a control, we show that VP16-CREB stimulated the expression of a CRE-luciferase reporter gene (Fig. 1.8C). A-CREB, a dominant-negative inhibitor of CREB (Ahn et al., 1998), also was used to test the role of CREB in synaptic activity-dependent neuroprotection. In this set of experiments the neurons were transfected with A-CREB and GFP; synaptic activity was elicited 24 h later (at \( t = 0 \)) for 15 min (Fig. 1.8D). At 24 h after synaptic stimulation, the cells were exposed to NMDA (50 µM, 15 min), and the apoptotic effects were assayed 8 h later (Fig. 1.8D). In the absence of NMDA administration, A-CREB did not increase the number of apoptotic cells, relative to empty vector (pcDNA3.1) transfection. However, A-CREB did exacerbate the excitotoxic effects of NMDA and significantly reduce the neuroprotective effects of synaptic activity (Fig. 1.8E). Thus under control conditions (empty vector transfection), synaptic activity reduced the toxic effects of NMDA by 71%, whereas in the presence of A-CREB, the synaptic activity reduced the toxic effects of NMDA by 36%, an approximately two-fold diminution of the neuroprotective effects of synaptic activity. Collectively, these data indicate that the CREB/CRE signaling pathway contributes to the neuroprotective effects of synaptic/NMDA receptor activity.
Figure 1.6. The MAPK pathway and CaMKIV do not regulate the duration of CREB phosphorylation. A, The duration of CREB, CaMKIV, and ERK phosphorylation was monitored during a period of persistent NMDA receptor-mediated synaptic activity. Cultures were lysed 10–60 min after the onset of synaptic stimulation [bicuculline (bic) plus 4-AP containing nimodipine and CNQX]. Control cultures (no stimulation) were collected at the beginning and the end of the stimulus period. Robust increases in both CREB and ERK phosphorylation were observed for the duration of the stimulation. However, CaMKIV phosphorylation was transient, showing maximal activation at the 10 min time point. B, To examine the contribution of CaMK and MAPK signaling to the duration of CREB phosphorylation, we transferred cells to media containing KN62 (10 μM) or U0126 (10 μM) immediately after synaptic stimulation. Samples were collected at the prestimulus and poststimulus time points indicated. Relative to the control synaptic activity condition (no inhibitor), disruption of CaMK signaling and MAPK signaling did not alter the duration of CREB phosphorylation. C, As a control experiment, the duration of ERK phosphorylation was examined. Synaptic activity triggered sustained ERK phosphorylation; the administration of U0126 after stimulation dramatically attenuated ERK activation. Con, Control.
Figure 1.7. Temporal regulation of CREB shut-off. 

A, Cultures were costimulated with NMDA (50 µM) and synaptic activity [bicuculline (bic) plus 4-AP with nimodipine and CNQX] from \( t=0 \) –10 min. After stimulation, the medium was changed to a TTX-containing solution, and the pCREB expression was determined at the time points outlined. Mock stimulation is shown for the \( t=0 \) time point. Under this 10 min stimulus condition, pCREB expression persisted for 90 min.

B, Cultures initially were stimulated (10 min) with synaptic activity (bicuculline plus 4-AP with nimodipine and CNQX from −40 to −30 min) and then placed in a TTX/HEPES buffer (30 min; from −30 to 0 min). After this treatment, NMDA (50 µM) was administered for 10 min (from 0 to 10 min), and the cells were then returned to a TTX/HEPES buffer. pCREB expression was determined at the time points outlined. pCREB levels for both synaptic stimulation (bicuculline plus 4-AP in the presence of nimodipine and CNQX) and mock stimulation [control (Con)] are shown for the \( t=0 \) time point (before NMDA administration). Under this sequential stimulus paradigm, pCREB expression returned to basal levels by 25–45 min after NMDA administration. Data are representative of triplicate determinations.
Figure 1.8. Synaptic activity attenuates NMDA-mediated cell death via a CREB-dependent mechanism. 

A, Neurons were transfected with VP16-CREB or the control vector pcDNA3.1 and stimulated 48 h later with NMDA (50 µM, 15 min). Cell viability was scored 8 h later. Cotransfection with GFP was used to identify transfected cells, NeuN was used to verify that the cells were neurons, and Hoechst labeling was used to assess toxicity. The arrow identifies a representative transfected cell that is dying; arrowheads denote healthy cells. 

B, Quantitation of the percentage of transfected neurons undergoing apoptosis. Relative to vector transfection, the transfection with VP16-CREB attenuated (*p<0.05, significantly different from control vector transfection) the toxic effects of NMDA. Error bars denote SEM. Numbers above the bars indicate the number of neurons assayed. 

C, A CRE-luciferase reporter construct was cotransfected with VP16-CREB or pcDNA3.1 to validate that the constitutively active CREB construct stimulated CRE-dependent transcription (*p<0.05, significantly different from control vector transfection). 

D, A-CREB attenuates the neuroprotective effects of synaptic activity. Neurons were cotransfected with GFP and A-CREB expression vectors. Then 24 h later, neuronal activity was elicited [t=0, bicuculline (bic) plus 4-AP with nimodipine and CNQX, 15 min]. NMDA (50 µM) was administered 24 h after bicuculline stimulation (t=24, 15 min), and cell viability was scored 8 h later. GFP immunofluorescence was used to identify transfected cells, MAP2 was used to verify that the cells were neurons, and Hoechst labeling was used to assess toxicity. Arrows identify transfected cells that were dead or dying; arrowheads denote healthy cells. 

E, Quantitation of transfected neurons undergoing apoptosis. Relative to control expression vector transfection (pcDNA3.1), transfection with A-CREB significantly attenuated (*p<0.05, relative to control vector transfection) the neuroprotective effects of synaptic activity and augmented the neurotoxic effects of NMDA. Numbers above each bar indicate the number of neurons assayed. In the table shown below the bars, the percentage of apoptotic neurons was normalized to control conditions (no stimulation), which was set equal to 1. Error bars denote SEM. Data are the mean of triplicate determinations.
DISCUSSION

Work over the past several years has revealed that the CREB/CRE transcriptional pathway is a complex regulator of a vast array of activity-dependent physiological processes. Of particular prominence have been data examining the role of CREB as a regulator of neuronal health and viability (Walton et al., 1996, 1999; Bonni et al., 1999; Mabuchi et al., 2001; Lonze and Ginty, 2002; Jaworski et al., 2003). In this study, we focused on the interplay between the neuroprotective and cytotoxic effects of NMDA receptor stimulation and on how these extremes in cellular responsiveness affect the activation state of CREB. The data presented here reveal that (1) the magnitude of NMDA receptor stimulation (toxic vs nontoxic) determines the duration of CREB phosphorylation via the selective recruitment of calcineurin activity, (2) permissive and toxic levels of NMDA receptor activation regulate CREB phosphorylation via distinct cellular signaling pathways, (3) the sequence of stimulation with neurotoxic levels of NMDA and neuroprotective synaptic activity determines which pathway exerts control over pCREB duration, and (4) the neuroprotective effects of NMDA receptor activation are mediated, in part, via activation ofCREB-dependent transcription.

We have shown previously that cortical neurons cultured from embryonic day 18 rat pups begin to form functional synaptic connections by 5 DIV and secrete both glutamate and GABA (van den Pol et al., 1995; Obrietan and van den Pol, 1998). To observe robust levels of synaptic activity after 10 DIV, we cultured our neurons under relatively dense conditions. The density of the cultures affects both the rate of synapse formation and the number of functional synapses. For example, van den Pol et al. (1998) found that presynaptic release of glutamate occurred after 3-4 DIV in high-density cultures but was absent in low-density cultures at this time. Furthermore, neurites grow three times faster in high-density cultures than in low-density cultures.
Thus the withdrawal of tetrodotoxin from the perfusion media elicited a rapid rise in neuronal Ca\(^{2+}\) levels. This increase was blocked by the application of APV, indicating that the action potential-dependent release of glutamate drove activation of the network. Synaptic activity triggered a marked increase in the Ser\(^{133}\) phosphorylated form of CREB. This result was expected, given the work showing that synaptic transmission in cultured neurons drives CREB phosphorylation (Bito et al., 1996; Hardingham et al., 2002). However, in the work by Bito et al. (1996) and Hardingham et al. (2002), the receptor subtypes that couple synaptic activity to CREB phosphorylation were not addressed. In an attempt to create a simplified synaptic network that relied specifically on the NMDA receptor, we added antagonists of both non-NMDA ionotropic glutamate receptors and L-type VACC to the bath media. Under this condition, we found that the application of bicuculline and 4-AP stimulated robust Ca\(^{2+}\) transients and a marked increase in CREB phosphorylation, indicating that L-type VACC and the AMPA/kainate subtype of glutamate receptors were dispensable. Many of the effects of synaptic/NMDA receptor activation (persistent CREB phosphorylation, neuroprotection) also were elicited by exogenous application of low concentrations of NMDA (1-5 µM), suggesting that the effects of synaptic activity were mediated by NMDA receptor-dependent signaling. However, it should be noted that synaptic activity also stimulates trophic, hormonal, or metabotropic processes that, in conjunction with NMDA receptor stimulation, might affect CREB phosphorylation. Thus additional work will be required to assess the relative contribution of these other pathways to synaptic/NMDA receptor-mediated CREB phosphorylation and neuroprotection. The cellular signaling events that couple neuronal activity to CREB phosphorylation vary, depending on the developmental state of the cells as well as the source and intensity of the stimulus (Herdegen et al., 1997; Xing et al., 1998; Sala et al., 2000; Hardingham et al., 2002; Obrietan et al., 2002). Two of the best characterized intermediate-signaling pathways are the MAPK pathway and CaMKIV (Enslen et al., 1994; Sun...
et al., 1994; Bito et al., 1996; Xing et al., 1996; Impey et al., 1998). The MAPK pathway is composed of the kinases RAF, MEK, and ERK. The MAPK cascade stimulates the Ser\textsuperscript{133} phosphorylated form of CREB via a kinase functioning downstream of ERK. Several studies have identified RSK (ribosomal S6 kinase) and MSK (mitogen- and stress-activated protein kinase) family members as ERK-activated CREB kinases (Xing et al., 1996; De Cesare et al., 1998; Impey et al., 1998; Arthur and Cohen, 2000). The enzymatic activity of CaMKIV is stimulated by an array of activity-dependent signaling events, including NMDA receptor activation and membrane depolarization (Bito et al., 1996; Kasahara et al., 2000; Soderling, 2000). CaMKIV is enriched in neuronal nuclei (Jensen et al., 1991; Miyano et al., 1992), and its activation is mediated in part by its phosphorylation at Thr\textsuperscript{196} by CaMK (Selbert et al., 1995). Both the MAPK cascade and CaMKIV were activated by neuronal stimulation and found to elicit CREB phosphorylation. Although it is unclear how both pathways contribute to the phosphorylation of CREB at the same site (Ser\textsuperscript{133}), it has been shown that CaMKIV mediates a rapid and transient phosphorylation of CREB, whereas an ERK-activated kinase mediates a subsequent, prolonged phosphorylation of CREB (Impey et al., 1998; Wu et al., 2001).

Although both the MAPK and CaMK pathways stimulate CREB phosphorylation, the duration of CREB phosphorylation is not dependent on the persistent activation of either pathway. These data suggest that other cellular signaling events (or the lack thereof) determine the duration of CREB phosphorylation. Along these lines, Bito et al. (1996) found that calcineurin inhibition with FK506 significantly enhanced the duration of CREB phosphorylation elicited by high-frequency stimulation. In some systems, calcineurin has been shown to function as a Ca\textsuperscript{2+}-activated negative regulator of CREB-dependent transcription by limiting the duration of CREB phosphorylation (Chang and Berg, 2001). To address the activation state of calcineurin, we monitored the subcellular localization of NF-ATc4. The nuclear translocation of NF-ATc4 is
regulated by calcineurin-dependent dephosphorylation (Crabtree, 1999; Graef et al., 1999); thus, by measuring the cytosolic/nuclear ratio of an NF-ATc4-GFP fusion protein, we were able to infer relative levels of calcineurin activity. With this approach, we found that our two stimulus paradigms had strikingly dissimilar effects on calcineurin activity; NMDA (50 µM) elicited a marked increase in calcineurin-dependent NF-ATc4 nuclear accumulation, whereas synaptic activity was relatively ineffective. Furthermore, our data revealed that transient CREB phosphorylation triggered by the exogenous application of a high concentration of NMDA was reversed by FK506 pretreatment, thus supporting the idea that calcineurin is recruited specifically by, and limits the duration of, CREB phosphorylation elicited by excitotoxic levels of NMDA receptor stimulation. Calcineurin activity may account in part for the CREB shut-off (Hardingham et al., 2002) effect elicited by the bath application of a high concentration of NMDA, whereas the lack of synaptically evoked calcineurin activity may contribute to persistent CREB phosphorylation. Interestingly, the induction of CREB shut-off was dependent on the sequence of stimulation. For example, when NMDA (50 µM) was applied and synaptic activity was elicited simultaneously, CREB phosphorylation persisted. Conversely, CREB shut-off occurred after synaptically evoked pCREB had become kinase-independent. Thus when both synaptically evoked kinase activity and NMDA-evoked (50 µM) phosphatase activity are stimulated simultaneously, kinase activity dominates and CREB phosphorylation becomes long-lasting (>90 min). NMDA-mediated toxicity was related inversely to the duration of CREB phosphorylation. Thus a high concentration of NMDA (50 µM) led to transient CREB phosphorylation and significant cell death, whereas low concentrations of NMDA or synaptic activity were nontoxic and triggered long-lasting CREB phosphorylation. Interestingly, long-lasting CREB phosphorylation is observed in cells resistant to toxic insults (Hu et al., 1999; Tanaka et al., 2001; Hara et al., 2003). In our analysis, we did not attempt to address whether neuroprotection with
low concentrations of NMDA was mediated by activation of synaptic NMDA receptors or by a combination of synaptic and extrasynaptic receptors. Additional analysis using receptor subtype-specific antagonists, analogous to the approach used by Lu et al. (2003) and Hardingham et al. (2002), will be required to address this issue. In addition to finding that synaptic activity led to persistent CREB phosphorylation, we observed that synaptic activity triggered a robust increase in CRE-dependent transcription. Extending the duration of CREB phosphorylation may be neuroprotective by increasing the expression of CREB-regulated cell survival genes. Along these lines, a number of studies have shown that the CREB-regulated genes BCL-2 and BDNF are neuroprotective (Martinou et al., 1994; Kitagawa et al., 1996; Lawrence et al., 1996; Bonni et al., 1999; Riccio et al., 1999; Mabuchi et al., 2001; Sugiura et al., 2004). In our model, synaptic activity and low concentrations of NMDA, but not high concentrations of NMDA, stimulated BDNF expression (data not shown). Furthermore, we found that overexpression of constitutively active CREB reduced the toxic effects of high concentrations of NMDA and that A-CREB blocked the neuroprotective effects of synaptic activity. These results raise the possibility that a targeted approach to upregulate CREB-dependent transcription may confer resistance against the neurotoxic effects of excessive excitatory neurotransmission.
CHAPTER 2

CREB MEDIATED GENE EXPRESSION AND COX-2 EXPRESSION IN PILOCARPINE MODEL OF STATUS EPILEPTICUS

INTRODUCTION

Seizures are the result of hypersynchronous electrical activity in the brain. A brain region particularly sensitive to the deleterious effects of status epilepticus (SE) is the hippocampal formation, a structure that is essential for advanced cognitive functions such as learning and memory. Individuals suffering from temporal lobe epilepsy often display pronounced cognitive deficits that dramatically diminish their quality of life (Reviewed by Jokeit and Ebner, 2002; Stefan and Pauli, 2002). A well-established constellation of pathological changes resulting from SE have been characterized in the hippocampus, including neuronal death, reactive gliosis, remodeling of synaptic circuitry and a marked increase in neurogenesis (Reviewed by Morimoto et al., 2004; Parent and Lowenstein, 2002; Jankowsky and Patterson, 2001). These profound alterations in the hippocampus follow a stereotypical pattern, from the acute loss of neurons in the CA1 and CA3 subfields, to the reorganization of the mossy fiber collaterals that occurs several weeks after SE. Many of the SE-induced alterations in hippocampal physiology are mediated by a two-stage process. In the initial stage, the release of the excitatory amino acid neurotransmitter glutamate leads to rapid neuronal cell death (Olney et al., 1983;
Ingvar et al., 1988; Covolan and Mello, 2000), and in the second phase, glutamate, in combination with trophic factors and cytokines, drives a wave of transcriptional activity that likely confers both a neuroprotective response and contributes to seizure-induced brain pathology. Along these lines, seizure activity has been shown to stimulate the expression of brain derived neurotrophic factor (BDNF; Isackson et al., 1991; Ernfors et al., 1991), which would function in a neuroprotective manner (Young et al., 1999; Revuelta et al., 2001), cyclooxygenase-2 (COX-2; Tu and Bazan, 2003; Kawaguchi et al., 2005) which may contribute to neuronal cell death (Dore et al., 2003; Rockwell et al., 2004; Kawaguchi et al., 2005), and tissue plasminogen activator (tPA; Qian et al., 1993; Tsirka et al., 1995), which has been shown to elicit neuronal structural changes (Wu et al., 2000). Because of the role gene expression plays in SE-induced alterations in hippocampal physiology, there is a clear need to begin to identify the cellular signaling and inducible transcription events that are activated by synchronous synaptic discharges. Within this context it is of interest to note studies showing a persistent activation of the transcription factor CREB (cAMP response element binding protein) and expression of CREB-regulated immediate early genes such as EGR-1 and c-fos in epileptic foci (Rakhade et al., 2005; Gass et al., 1997; Kiessling and Gass, 1993; Simonato et al., 1991). These findings suggest a role for CREB in the genetic response to seizure activity. In this study, we examined the regulation of CRE-mediated gene expression in neuronal and non-neuronal cells following pilocarpine-induced status epilepticus (SE). Further, we investigated the potential role of the CREB/CRE transcriptional signaling pathway in hippocampal pathophysiology by assessing its capacity to couple SE to the expression of the pro-inflammatory gene COX-2.
MATERIALS AND METHODS

**Animals.** Adult male C57BL/6 mice (6-10 weeks of age) were initially intraperitoneally (i.p.) injected with atropine methyl nitrate (1 mg/kg), then 30 min later, with pilocarpine hydrochloride (325 mg/kg, i.p.). Status epilepticus (SE) was defined as a continuous motor seizure over 3 hours following several stage 5 seizures (loss of balance, falling or jumping, Racine, 1972). Only mice that developed status epilepticus after pilocarpine administration were used in this study. SE was elicited in approximately 80% of the mice. Control animals were injected with atropine as above, and then injected with saline, instead of pilocarpine. To facilitate recovery from SE, animals received mouse chow soaked in a high sucrose (10%) saline solution for the first four days following SE. All experimental procedures were in accordance with Ohio State University animal care and use guidelines.

**Tissue collection.** Mice were anesthetized with ketamine hydrochloride (91 mg/ml) and xylazine (9 mg/ml) and perfused intracardially with a fixative solution of 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed, post-fixed in 4% PFA for 4 hours and then cryoprotected with 30% sucrose overnight. Thirty-µm thick coronal sections (-1.8 to -2.5 mm from bregma) were cut on a freezing microtome and processed for immunohistochemistry or Fluoro-jade B labeling.

**Immunohistochemistry.** Initially, floating sections were washed (3X) in PBS, incubated (20 min) with 0.3% H2O2, then blocked (60 min) with 10% normal goat serum in PBS. Tissue was then incubated (4º C, overnight) with a rabbit polyclonal antibody against either β-galactosidase (1:10,000 dilution; Cortex Biochem., San Leandro CA, USA) or GFP (1:5000 final dilution,
Molecular Probes, Eugene OR, USA). Next, the tissue was incubated (1 hr at room temperature) in a biotinylated secondary antibody (1:300, Vector Laboratories, Burlingame CA, USA) and then incubated (45 min at room temperature) in an avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories). Nickel intensified diaminobenzidine was used to visualize the signal. Finally, sections were mounted, dehydrated, and coverslipped with Permount (Sigma). Bright-field images were captured using a Leica DMIRB inverted microscope connected to a 16 bit digital camera (Princeton Instruments; Monmouth Junction NJ, USA). Immunofluorescent staining was used for sequential triple labeling experiments. On day one, free-floating sections were blocked with 10% horse serum in PBS, then incubated overnight at 4º C with a goat anti-COX-2 antibody (1:200 final dilution, Santa Cruz Biochem. Santa Cruz CA, USA). On day two, the tissue was labeled (2 hrs at room temperature) with an Alexa 546 conjugated donkey anti-goat IgG antibody (1:250 Molecular Probes, Eugene OR, USA). The tissue was then washed (3X) and blocked with 10% normal goat serum in PBS for 1 hour. Next, the tissue was incubated (4º C, overnight) with a primary antibody against β-galactosidase (1: 2,500 Cortex Biochem.) or GFP (1:1000 Molecular Probes). On day three, the tissue was labeled with an Alexa 488-conjugated goat anti-rabbit IgG antibody (1:250), washed (3X) with PBS and then blocked with 10% normal goat serum in PBS for 1 hour. The last primary antibody against GFAP (1:500, Sigma, St. Louis MO, USA) or CD11b (1:250, Chemicon, Temecula CA, USA) was incubated overnight at 4º C. On day four, the tissue was labeled with an Alexa 633 conjugated goat anti-mouse IgG antibody (1:250). The tissue was then mounted and coverslipped using Gelmount (Biomedia, Foster City CA, USA). For double labeling, the primary antibodies against β-galactosidase and GFAP or CD11b were coincubated and labeled with secondary antibodies, as described above. Double labeling against eGFP (rabbit anti-GFP antibody, 1:1 000 final dilution, Molecular Probes) and COX-2, β-galactosidase (1:2,500, Cortex Biochem.) or JunB (1:2500, Santa Cruz Bio.) was
performed using the methodology described above. Fluorescent images were captured using a Zeiss 510 Meta confocal microscope (Oberkochen, Germany). Data were analyzed using Metamorph image analysis software (Universal Imaging, Downingtown PA, USA).

**Fluoro-jade B histochemistry.** Dead or dying cells were labeled using Fluoro-jade B. Initially, tissue was fixed and thin cut as described above. Next, coronal hippocampal sections were washed in distilled water (3X), and then treated with 0.06% potassium permanganate for 10 min at room temperature with gentle shaking. Sections were then washed in distilled water (3X) and incubated with 0.001% Fluoro-jade B (Histo-Chem Inc., Jefferson, AR, U.S.A.) in 0.1% acetic acid for 20 min with gentle shaking. Sections were then washed in distilled water, dried, dipped in xylene for 2 min and coverslipped with DPX.

**Generation of transgenic mouse lines.** A bitransgenic system was used to generate tetracycline-inducible bicistronic A-CREB-eGFP mice. First, to make the construct that expresses A-CREB and eGFP (enhanced green fluorescence protein), A-CREB was isolated from a pcDNA3 expression vector (provided by Dr. Soren Impey, Oregon Health Sciences University). A-CREB was digested with *HindIII* and *NcoI*, blunted with Klenow and inserted into *Sma I*-digested pIRES2-eGFP (Invitrogen). Primers (Forward: 5’ CGGCAGCCGCGCGACCATGGCT-GACATGACTGGTGACAGC 3’, Reverse: 5’CGGCAGCCGCGCTCTTTGACTTGACAGC-TGTCCATGAC 3’) were used to amplify A-CREB/IRES/eGFP. A Kozak sequence (GCCACCATGG) was added upstream of start codon (bold) of A-CREB to improve expression levels and a *Not I* digestion site (GCCGCG) was added to the 5’ end to clone the construct into the pTRE-Tight vector (Clontech, Mountain View CA, USA). The vector was then digested with *XhoI* to release the TRE promoter, A-CREB/IRES/eGFP and SV 40 polyA sequence. The
fragment was then injected into C57BL/6 embryos at the Ohio State University Transgenic Facility. Founders were screened by PCR. To drive transgene expression, the A-CREB-eGFP mice were crossed with CaMKIIα promoter-tTA transgenic mice (Mayford et al., 1996). Bitransgenic offsprings were identified using PCR, and transgene expression was confirmed using immunohistochemistry against eGFP. Of the 5 A-CREB-eGFP::tTA lines isolated, two had transgene expression within the cortex, hippocampus and striatum. Transgene expression was not repressed with the use doxycycline supplementation at any time during this study. For luciferase reporter gene assays, 293T cells were transfected with a combination of the following vectors: CRE-Luciferase (Lee et al. 2005), TRE-tight A-CREB/IRES/eGFP and pRev-tTA (Clontech, Mountain View, CA). Forty-eight hrs after transfection, cells were stimulated with forskolin (10 µM: 8hrs) and luciferase assays was performed as described (Lee et al., 2005).

**CRE-reporter gene expression.** CRE-β-galactosidase transgenic mice were obtained from Dr. Daniel Storm and genotyping was performed as previously described (Obrietan et al., 2002). As noted in several studies (Impey et al., 1996; Barth et al., 2000; Obrietan and Hoyt, 2004), β-galactosidase expression was not observed in all animals genotyped as CRE positive. In our analysis, we found that ~35% of the CRE-positive animals lacked detectable β-galactosidase expression. Tissue from mice that did not express the transgene were excluded from analysis. To generate A-CREB-eGFP::tTA::CRE-lacZ transgenic mice, the A-CREB-eGFP::tTA mice were crossed with CRE-β-galactosidase mice and tritransgenic offspring were identified using PCR, and transgene expression was confirmed using immunohistochemistry against eGFP and β-galactosidase.
**Western blotting.** Mice were anesthetized as described above and then decapitated. Brains were then removed, placed in chilled oxygenation media and then cut into 500 µm sections with a vibratome. Dorsal hippocampal sections (~ -1.8 mm from bregma) were placed on a microscope slides and frozen with dry ice. Tissue from the hippocampus was isolated and then sonicated in 100 µl of RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM NaF, 25 µM sodium vanadate supplemented with protease inhibitor cocktail: complete mini tablet, Roche Diagnostics), total protein levels were determined, and then 30 µg of protein samples were diluted in 3X sample buffer. Extracts were loaded onto a 10% SDS-PAGE gel, electrophoresed using standard procedures, and protein was transblotted onto polyvinylidene fluoride (PVDF) (Immobilon P; Millipore). Following blocking with 10% (wt/vol) powdered milk, membranes were incubated (4°C overnight) with an affinitypurified rabbit polyclonal antibody against Ser133 phosphorylated CREB (1:1000 dilution, Cell Signaling Technology, Beverly MA, USA). Samples were then incubated with an alkaline phosphatase-conjugated anti-rabbit antibody (1:2,500, Perkin-Elmer, Wellesley, MA USA) and the signal was detected using the Western-CDP star chemiluminescent detection system (Perkin-Elmer). As a protein loading control, membranes were probed with a goat polyclonal antibody against total erk-1 and erk-2 (1:2000, Santa Cruz Biochem.). Samples were then incubated with a horseradish peroxidase-conjugated secondary antibody and the signal was detected using a chemiluminescent HRP substrate (Renaissance chemiluminescent detection system: New England Nuclear).
Quantification and data analysis. To determine the number of COX-2 positive non-neuronal cells expressing β-galactosidase within the dentate gyrus, confocal sections were captured (2-µm-thick optical section, ~ -1.8 mm from bregma) and the total number of positive cells were calculated. Data were averaged from 2 sections (bilateral) per animal, and then averaged from three mice for each time point. For quantitation of COX-2 expression in the dentate gyrus granule cell layer of A-CREB-eGFP::tTA transgenic mice, confocal sections were captured as above and COX-2 and eGFP expression were examined in the dentate granule cell layer. To quantitate COX-2, the perinuclear region of the eGFP-positive cells was digitally outlined using Metamorph and used as a template to quantitate COX-2 expression. To quantitate COX-2 expression in non-transgenic cells, the perinuclear region of cells adjacent to transgenic cells was quantitated. COX-2 expression in control transgenic animals (without pilocarpine) was used as a background subtraction to determine the total inducible signal. There was no significant difference in basal COX-2 expression in transgenic versus non-transgenic cells. For β-galactosidase cell counts, images were captured at 100X magnification and quantitation was performed using MetaMorph software (Universal Imaging). The total number of β-galactosidase positive cells was counted within a digital 200 mm square placed over the GCL, CA3 or CA1. Data were counted from two sections separated by ~ 200 µm (stereotaxic coordinate AP: -1.60 ~ -2.00). Cell counts were analyzed statistically using Student’s t-test, and significance was accepted for P<0.05. All data are expressed as means +/- SEM. All necessary comparisons were carried out using Student’s t-test. A P-value <0.05 was considered as significant.
RESULTS

The muscarinic acetylcholine receptor agonist pilocarpine was used to elicit status epilepticus (SE: Turski et al., 1983 and 1989). Initially, adult mice (C57BL/6) were injected with atropine (1 mg/kg, i.p.), and then injected with pilocarpine (325 mg/kg, i.p.) 30 min later. The seizures were characterized based on Racine’s scale (Racine, 1972). At first, mice typically displayed facial movements (stage 1) and progressed to head nodding (stage 2), forelimb clonus (stage 3), rearing (stage 4), and rearing and falling (stage 5). After the initial stage 5 seizure, a subset of mice developed SE. SE was defined as continuous motor seizures persisting for over 3 hrs. Only mice that developed SE were used. Control animals were injected with atropine, as above, followed by physiological saline (pilocarpine vehicle).

**SE stimulates CRE-mediated gene expression in the hippocampus**

Representative data from brain sections immunolabeled for the CRE-regulated reporter, β-galactosidase, revealed that SE triggered robust CRE-mediated gene expression 8 hrs post SE onset (Fig. 2.1A). Induction was observed throughout the central nervous system. Within the hippocampus, expression was detected in the dentate granule cell layer (GCL) and the CA3 and CA1 subfields. This contrasts with the low, heterogeneous, β-galactosidase expression pattern in control mice not injected with pilocarpine (Fig. 2.1A). By 18 hrs post-seizure onset, β-galactosidase expression had dropped markedly in the excitatory subfields, and by 7 days post-SE, expression had returned to near basal levels. Quantitative data are shown in figure 2.2C. CREB phosphorylation at Ser 133 is a necessary event in the induction of CREmediated gene expression (Gonzalez and Montminy, 1989). Consistent with the effect observed at the level of CRE-mediated transcription, we found that SE triggered a robust increase in the Ser-133
phosphorylated form of CREB in the hippocampus (Fig. 2.1C). For these experiments, the dentate gyrus was isolated from the rest of the hippocampus and the two samples were processed in parallel. Maximum CREB phoshpo-activation was observed at 30 min to 4 hrs post-SE. At 48 hrs post-SE, levels of activated CREB were similar to levels observed in control animals (time point 0). These data reveal that SE triggers CREB transactivation which in turn facilitates the induction of CRE-dependent transcription. Hippocampal neurons are vulnerable to the excitotoxic effects of SE. To assess whether the transient nature of CRE-mediated reporter gene expression within the hippocampal neuronal sublayers correlates with SE-induced cell death, tissue was processed with Fluoro-jade B, a sensitive histochemical marker of dead and dying cells. As expected, at 48 hrs post-SE onset, marked cell death was observed within the CA1 area (Fig. 2.1D) and limited cell death was observed in area CA3 (data not shown). In contrast, cell death was not detected within the GCL, a cell region shown to be highly resistant to the excitotoxic effects of SE (Fricke and Prince, 1984; Lothman et al., 1991, 1992; Schweitzer et al., 1992; Becker et al., 1999). Given that the GCL was refractory to the excitotoxic effects of SE, these data suggest that the short-lived nature of CREB-dependent transcription was not the result of SE-induced cell death. In contrast to the transient increase in CRE-mediated gene expression in hippocampal excitatory neurons, marked β-galactosidase expression was observed outside of the neuronal sublayers 4 days post-SE (Fig. 2.1B). The observation that SE-induced a distinct temporal profile of CRE-mediated gene expression outside of the neuronal sublayers suggests that this transcriptional pathway is differentially regulated within subpopulations of cells. To begin to address this issue, coronal brain sections were double immunolabeled for β-galactosidase and the neuronal cell marker, NeuN (Fig. 2.2A and 2B). Four hours post-seizure onset, β-galactosidase expression was observed in NeuN positive cells in dentate gyrus (Fig. 2.2A and 2B) as well in the CA3 and CA1 (data not
Figure 2.1. Seizure-induced activation of CRE-mediated transcription. (A) Mice transgenic for the CRE-regulated β-galactosidase reporter construct were injected with either pilocarpine (325 mg/kg, i.p.) or vehicle and sacrificed at multiple time points following status epilepticus (SE) onset. Brain sections were immunolabeled for β-galactosidase expression. Small black punctate dots denote cells with a high-level CRE-mediated gene expression. Eight hours after SE, a marked increase in reporter gene expression was observed in the hippocampus excitatory subfields (areas CA1, CA3 and the granule cell layer), as well as in the cortex. This is in contrast to relatively modest staining pattern in control animals sacrificed 8 h post-saline injection. By 18 h post seizure onset, β-galactosidase levels were reduced from the peak level observed at the 8 h time point, and by 7 days, little CRE-mediated gene expression was observed in the hippocampal neuronal sublayers. (B) High magnification images of β-galactosidase staining from animals killed 8 h or 4 days post-SE. Relative to 8 h post-SE, there was a marked increase in CRE-mediated gene expression outside of the neuronal subfields at 4 days post-SE. GCL: granule cell layer, Mol: molecular cell layer, SR: stratum radiatum, SLM: stratum lacunosum moleculare. (C) SE stimulates CREB phosphorylation. Hippocampi from 500 µm-thick coronal brain sections were isolated at multiple time points following SE. At 30 min post-SE, a marked increase in the Ser-133 phosphorylated form of CREB was observed in tissue isolated from the dentate gyrus, as well as non-dentate hippocampus. As a protein loading control, the blots were also probed for total ERK expression. (D) Fluoro-jade B labeling was used to detect SE-induced cell death at 48 h post-SE. Marked cell death was observed in the CA1 and hilus (Hil). DG: dentate gyrus.
shown). Interestingly, by 48 hrs post-SE, the majority of β-galactosidase-expressing cells in the dentate gyrus were outside of the GCL and were not positive for NeuN (Fig. 2.2A and 2B).

**CRE-mediated transcription in reactive astrocytes and microglia.**

Next, we examined whether CREB-dependent transcription was induced in reactive astrocytes and microglia. Astrogliosis and microgliosis occur in response to CNS disease and injury. Initially, to test whether our SE model triggered gliosis, hippocampal brain sections were processed for GFAP (glial fibrillary acidic protein). GFAP is expressed specifically by astrocytes and is upregulated following neurotoxic insults such as lesions, ischemia and seizure activity (Orzi et al., 1990; Steward et al., 1991). At 4 hrs post-SE onset (the earliest time point examined), a modest increase in GFAP immunoreactivity was observed, and by 48 hrs post-SE onset, numerous astrocytes with hypertrophied processes and increased soma size were found throughout the hippocampus (Fig. 2.3A). To assess whether SE stimulated CRE-mediated gene expression in reactive astrocytes, hippocampal sections were double immunolabeled for β-galactosidase and GFAP. At 4 hrs post-SE onset, a modest increase in β-galactosidase expression was detected in astrocytes. This acute induction was coincident with the time period during which robust CRE-mediated gene expression occurred in hippocampal neurons (Fig. 2.3B). This contrasts with control animals injected with atropine, where low levels of β-galactosidase were observed in astrocytes throughout the hippocampus. By 48 hrs post-SE, robust β-galactosidase expression was observed in reactive astrocytes (Fig. 2.3B). These data indicate that SE stimulates a rapid and lasting increase in CRE-mediated gene expression in reactive astrocytes.

Next, we examined whether CRE-mediated gene expression was also upregulated in reactive microglia. To identify microglia, tissue was immunohistochemically processed for the expression of the type 3 complement receptor (CD11b).
Figure 2.2. Seizure-induced activation of CRE-mediated gene expression in neuronal and non-neuronal cells. Animals were sacrificed either 4 or 48 h after pilocarpine-induced SE. The tissue was then fluorescently immunolabeled for β-galactosidase (green) and for the neuronal nucleus-specific antigen NeuN (red). (A) Four hours after SE, β-galactosidase expression was observed in NeuN positive cells of the granule cell layer (GCL) and the hilus. However, by 48 h post-SE onset, β-galactosidase expression was notably absent from the GCL. Furthermore, the remaining β-galactosidase expression did not colocalize with NeuN, indicating that CRE-mediated transcription at the 48 h time point occurred in non-neuronal cells. (B) High magnification of the immunolabeling within the GCL; note the non-overlapping labeling at 48 h post-SE. (C) Quantitation of β-galactosidase expression in excitatory subregions of the hippocampus. Mean±SEM data are from 4–6 animals per time point. *P<0.05 relative to all other data points. #P<0.05 relative to control (0 post-SE) time point.
Figure 2.3. CRE-mediated gene expression in reactive astrocytes. Animals were sacrificed either 4 h or 48 h after pilocarpine-evoked SE. (A) Representative GFAP immunolabeling from the hippocampus reveals that SE triggered a marked upregulation of GFAP labeling relative to control conditions. (B) Tissue was fluorescently immunolabeled for β-galactosidase (green) and GFAP (red). Four hours after SE, β-galactosidase expression was observed in the granule cell layer (GCL). In contrast, limited β-galactosidase expression was detected in GFAP positive cells at this time point. By 48 h post-seizure onset, β-galactosidase expression was observed in astrocytes throughout the dentate gyrus. Arrows denote astrocytes with CRE-mediated reporter gene expression. hil: hilus, mol: molecular layer.
By 4 hrs post SE onset, CD11b expression was observed in the dentate gyrus (Fig. 4A), and by 48 hrs post-SE, marked microgliosis was observed throughout the hippocampus. These findings are consistent with previous studies examining the temporal profile of SE-induced microgliosis (Rizzi et al., 2003; Borges et al., 2003). To determine whether SE stimulates CRE-mediated transcription in microglia, hippocampal brain sections were double labeled for β-galactosidase and CD11b. With our fluorescent immunolabeling technique, it was difficult to detect an increase in CD11b expression 4 hrs post-SE onset. However, by 48 hrs post-SE process-bearing CD11b immunoreactive microglia were detected with colocalized expression of the CRE-regulated reporter gene (Fig. 2.4B). Quantitative analysis revealed that the relative percentage of neuronal and non-neuronal cells that expressed β-galactosidase was dramatically altered from 4 hrs to 48 hrs post-SE. Thus, at 4 hrs post SE, the majority of β-galactosidase-positive cells were neurons whereas, by 48 hrs, only 16% of the β-galactosidase-positive cells were neurons (Fig. 2.4C). It should also be noted that the total number of β-galactosidase-positive cells decreased as a function of time post-SE. These data confirm the transient nature of CRE-mediated gene expression in neurons, and that SE triggers a longer-lasting increase in CREB-dependent transcription in non-neuronal cells.

**Seizure induced-COX-2 expression.**

In response to CNS injury there is a rapid upregulation of pro-inflammatory genes such as cyclooxygenase 2 (COX-2), tumor necrosis factor–α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Taupin et al., 1993; Shohami et al., 1994; Stoll et al., 2002; Turrin and Rivest, 2004). Interestingly, the promoter regions of a number of proinflammatory genes contain CREs, suggesting that they may be regulated by CREB-dependent transcription.
Figure 2.4. CRE-mediated gene expression in microglia. Animals were sacrificed 4 h or 48 h after pilocarpine-evoked SE. The tissue was then immunolabeled with an antibody against CD11b, a marker for reactive microglia. (A) Relative to control animals, SE stimulated robust microglia activation. (B) Double immunofluorescent labeling against β-galactosidase green) and CD11b (red), revealed that seizure activity elicits CREdependent transcription in reactive microglia (arrows). Four hours after status epilepticus, β-galactosidase expression was observed primarily in granule cells (GCL) of the dentate gyrus. However, by 48 h post-SE onset, a marked increase in β-galactosidase expression was observed in CD11bpositive cells along the GCL boarder, as well as in the stratum (St.) radiatum and the molecular cell layer (mol: arrows). (C) Quantitation of β-galactosidase positive cells as a function of both time and cell type. The mean number of β-galactosidase positive neurons (NeuN+) and glia (GFAP +/CD11b+) within the dentate gyrus was determined and expressed as 100% for 3 post-SE time points. Data were collected from three mice per time point. The numbers above bars denote the total number of β-galactosidase positive cells examined at each time point.
A: CD11b

Control  4 hrs post-SE  48 hrs post-SE

B: β-GAL/CD11b

48 hrs post-SE

C

![Graph showing the percentage of GFAP+/CD11b+ and NeuN+ cells over time.](image)
To determine whether there is a correlation between CREB-dependent gene transcription and pro-inflammatory gene expression, we examined the temporal and spatial profile of SE-induced COX-2 expression. Four hours after SE, COX-2 expression was dramatically increased in the GCL and CA3 (Fig. 2.5A). Double labeling experiments revealed a coordinate increase in CRE-mediated gene expression and the induction of COX-2 (Fig. 2.5B). By 48 hours post-SE onset, high levels of COX-2 were observed in CA3 and CA1, whereas expression within the GCL had decreased from the level observed 4 hours post-SE (Fig. 2.5A). This expression pattern paralleled the expression pattern of β-galactosidase. Thus, COX-2 expression was first observed at 4 hrs post-SE and early expression (4-48 hrs) was predominantly located in neuronal sublayers. In contrast, delayed expression (2-14 days post-SE) of COX-2 was detected outside of the neuronal sublayers (Fig. 2.5A: far right panel). Triple immunolabeling experiments revealed that both reactive microglia (CD11b-positive cells) and reactive astrocytes (GFAP-positive cells) that expressed COX-2 also expressed β-galactosidase (2.5C, 5D and 5E). These data raise the possibility that seizures and cell stress stimulate the expression of the pro-inflammatory enzyme COX-2 via a CREB/CRE-dependent mechanism. To provide a definite assessment of the role of the CREB/CRE pathway in the induction of COX-2, we generated a transgenic mouse strain that expresses the CREB repressor A-CREB in a tetracycline-inducible manner within forebrain neurons. A-CREB functions as an inhibitor of CREB-dependent transcription by dimerizing with the basic region of CREB and thus blocking its ability to bind to DNA (Ahn et al., 1998). In addition, the construct was engineered to drive the expression of enhanced green fluorescent protein (eGFP) from an internal ribosomal entry site (IRES), thus allowing for identification of the cells that express the transgene construct. Luciferase reporter gene assays revealed that the construct potently repressed forskolin- (an adenylyl cyclase activation: 10 µM) induced CRE-mediated gene expression Figure 6A.
Figure 2.5. Seizure-induced COX-2 expression. (A) Animals were sacrificed 0, 4, 48 h and 2 weeks after pilocarpine-evoked SE. The tissue was then fluorescently immunolabeled for COX-2 expression. Four hours after SE, COX-2 expression was dramatically increased in the GCL and area CA3. Forty-eight hours after SE, marked COX-2 expression was observed in area CA1. Far right panel shows a high magnification image of COX-2 immunolabeling (boxed region) 2 weeks post-SE. Note that COX-2 expression was detected outside of the GCL. (B) High magnification image of the GCL double labeled for COX-2 and β-galactosidase revealed that CRE-mediated gene expression correlates with the expression of COX-2. Data were collected from animals killed 4 h post-SE onset. (C) Animals were sacrificed 48 h after pilocarpine-evoked SE, and the tissue was immunolabeled for glial markers (GFAP and CD11b), the CRE-regulated reporter (β-galactosidase) and COX-2. Representative images from the molecular layer of the dentate gyrus reveal the colocalized expression of β-galactosidase and COX-2 in both reactive astrocytes (GFAP) and microglia (CD11b). (D) Cell counts denoting the percentage of β-galactosidase positive cells (astrocytes or microglia) that also express COX-2 in the dentate gyrus. Numbers above each bar indicate the total number of positive cells examined. Data were collected from 3 animals per time point. Data were averaged for each animal and then averaged across all animals for each time point. Percentages are expressed as means±SEM.
Immunohistochemical labeling for eGFP detected heterogeneous transgene expression in neurons within the hippocampus, cortex, and striatum (Fig. 2.6B). The efficacy of the construct was initially tested by examining SE-mediated, CREB-dependent gene expression. In A-CREB mice, SE-induced expression of the CREB-regulated gene, JunB, was repressed in cells that express A-CREB-eGFP (Fig. 2.6C). Furthermore, in A19 CREB mice crossed with the CRE-β-galactosidase mice, SE induced β-galactosidase expression was not observed in A-CREB expressing cells (Fig. 2.6D). Together these data reveal that the A-CREB construct functions as a potent repressor of SE-induced, CRE dependent, gene expression. To test the role of CREB in SE-induced COX-2 expression, transgenic mice were killed 8 hrs after SE onset and tissue was double immunolabeled for eGFP and COX-2. A cell-by-cell analysis of the GCL revealed that A-CREB expression significantly attenuated SE-induced COX-2 expression (Fig. 2.7A and 7B). The A-CREB construct is driven by the CaMKII promotor, and as such, is expressed primarily in neuronal cells. In line with this expression pattern, we did not note an effect of transgene expression on SE-induced COX-2 expression in non-neuronal cells (Figure 2.7C). Collectively, these data indicate that SE drives CRE-mediated gene expression in both neuronal and non-neuronal cells of the hippocampus and that CREB couples seizures to the expression of the proinflammatory gene COX-2.
Figure 2.6. A-CREB transgenic mice. (A) 293T cells were transfected with CRE-luciferase, pRev-tTA and either A-CREB/IRES/eGFP or an empty vector (control). Cells were stimulated 48 h later with forskolin (10 µM). Data represent the mean±SEM fold increase in luciferase activity relative to control, untreated cells, which were normalized to a value of 1. (B) Immunohistochemistry for eGFP transgene expression. Expression of the A-CREB-eGFP transgene construct was detected in the cortex, striatum and hippocampus. Hippocampal expression is magnified to the right. As a control, tissue from WT animals was also processed for antigenicity against the GFP antibody. (C) A-CREB blocks SE-induced JunB expression. A-CREB transgenic mice were injected with pilocarpine, and killed 90 min after SE onset. Double fluorescent immunolabeling revealed that JunB expression did not occur in neurons expressing the A-CREB-eGFP transgene construct. Representative data are shown for the striatum and cortex. Note that the red and green signals do not overlap in the merged images. (D) A-CREB blocks SE-induced CRE-mediated gene expression. A-CREB transgenic mice were crossed with CRE-β-galactosidase reporter mice and animals were killed 8 h post-SE onset. Fluorescent immunolabeling of cortex for eGFP (green) and β-galactosidase (red) reveals that reporter gene expression did not occur in neurons expressing the A-CREB construct.
Figure 2.7. A-CREB attenuates SE-induced COX-2 expression. (A) Representative images of the dentate GCL double immunolabeled for eGFP (the transgene marker) and COX-2. eGFP was expressed in a limited number of granule cells. High magnification double labeling (bottom) revealed that COX-2 expression was attenuated in transgenic cells (arrows). (B) Quantitation of SE-induced COX-2 expression in transgenic and non-transgenic granule cells. COX-2 intensity values were determined as described in Materials and methods. Data are expressed as mean fold-induction relative to transgenic cell expression, which was set equal to 1. Error bars denote SEM. Numbers above bars denote the total number of cells examined. *P<0.005. (C) Cortical tissue was triple-labeled for COX-2, A-CREB-eGFP and GFAP. Representative photomicrograph was from a mouse killed 48 h post-SE onset. Robust COX-2 expression was observed in GFAP-positive cells (arrows), but not in eGFP-positive neurons.
DISCUSSION

A principal goal of this study was to examine whether SE stimulates CRE mediated gene expression, and if so to determine both the cell types and temporal regulation of gene induction. The data presented here provide the first detailed information regarding how epileptiform discharges regulate that activation state of the CREB/CRE pathway. Furthermore, the data reveal a divergence in the regulation of CRE-mediated gene expression within neuronal and non-neuronal cells, suggesting that the pathway contributes to distinct cell-type-specific physiological processes. CREB is a member of the basic leucine zipper family of transcription factors that includes CREM and ATF1 (Lonze and Ginty, 2002; Liang et al., 1996). CREB is highly expressed in both neurons and glia (Lonze and Ginty, 2002; Herdegen et al., 1992), and its transactivation potential is regulated in an activity-dependent manner. Thus, within the nervous system, robust neuronal activity has been shown to trigger the phosphorylation of CREB at Serine 133, an event necessary for CREB-dependent transcription. With respect to seizure models, Moore et al. (1996) showed that pentylentetrazol triggers the Serine 133 phosphorylated form of CREB. Although phosphorylation is required for CRE-mediated gene expression, it is only one of several essential events that are required to drive CRE-mediated gene expression (Lonze and Ginty, 2002; Shaywitz and Greenberg, 1999; Thiel et al., 2005; Chen et al., 2003; Impey et al., 1996, 1998; Obrietan et al., 1999, 2004; Lee et al., 2005). Thus, the data presented here are the first to examine the dynamics of seizure-induced CRE-dependent transcription. Beginning 4 hrs post-SE onset, we detected a marked increase in CRE-mediated gene expression within the hippocampal neuronal sublayers. Interestingly, this increase in gene expression was transient. For example, reporter levels in the GCL returned to baseline by ~ 24 hrs post SE onset. This decline in CRE-mediated gene expression within the hippocampal neuronal subfields suggests that there
is an uncoupling of CRE-mediated gene expression from an ostensibly robust extracellular excitatory environment. There are several potential mechanisms that may underlie the transient nature of seizure-induced CRE-mediated gene expression. For example, the duration of CREB phosphorylation appears to be a key event in determining whether CREB effectively couples to the basal transcriptional complex. Work from Hardingham et al., (2002) found that non-excitotoxic levels of NMDA receptor stimulation triggered a lasting form of CREB phosphorylation. Conversely, potentially excitotoxic levels of receptor stimulation led to transient phosphorylation, and abrogation of CRE-mediated gene expression. This effect was found to result from the activation of extrasynaptic NMDA receptors which trigger a discrete set of signaling events that lead to CREB dephosphorylation. In a parallel line of inquiry, we reported that potentially excitotoxic levels of stimulation trigger activation of a phosphatase-dependent shut-off mechanism (Lee et al., 2005). Both of the aforementioned studies were performed with neuronal cultures; additional work will be required to determine the mechanism by which SE triggers transient activation of CRE-mediated gene expression. Thus, within this context, it would appear that the initial bout of neuronal activity elicited by SE was processed as a non-toxic stimulus, and thereby elicits CRE-dependent transcription. This acute induction of SE-induced neuronal activity may be analogous to the level of activity initiated by associative learning or stimuli that elicit long-lasting forms of LTP: two paradigms that have been shown to trigger CRE-mediated gene expression using β-galactosidase transgenic mouse strain (Impey et al., 1996; Impey et al., 1998). Through its capacity to stimulate the expression of neuroprotective genes, the CREB/CRE transcriptional pathway plays a prominent role in cell health (Walton et al., 1996; Bonni et al., 1999; Walton et al., 1999; Mabuchi, 2001; Lonze and Ginty, 2002; Jaworski et al., 2003). Given this context, it was interesting to find that SE-induced CRE-mediated gene expression was not related to sublayer-specific cell death vulnerability. Along these lines,
although both the CA1 area and GCL showed SE induced increases in CRE-mediated gene expression, only CA1 neurons exhibited marked cell death. However, the approach used here did not delineate whether acute CRE-mediated gene expression occurred in neurons that were refractory to the excitotoxic conditions, thus the role of this pathway in cell viability is not clear. Furthermore, in light of its role in neuroprotection, it was also of interest to find that the CRE pathway also regulated the expression of the pro-inflammatory gene COX-2.

The enzymatic activity of COX-2 is the rate-limiting step in prostaglandin synthesis, and its rapid upregulation in response to excitotoxic insults has been suggested to contribute to pathophysiological brain damage (reviewed by Consilvio et al., 2004). Along these lines, a number of studies have shown that blockade of COX-2 attenuates excitotoxic cell death (Ezaki et al., 2005; Kawaguchi et al., 2005). Moreover, transgenic overexpression of COX-2 leads to an increase in cell death resulting from transient focal ischemia (Dore et al., 2003). Conversely, COX-2 and its metabolic products have also been shown to protect neurons against excitotoxic and oxygen/glucose deprivation-induced cell death (Gendron et al., 2005; McCullough et al., 2004; Liu et al., 2005). This dual role of COX-2 (excitotoxic and neuroprotective) is dependent on the model systems and stimulus paradigms used (Gendron et al., 2005). With respect to the cell types and time course of COX-2 induction, our data are consistent with several recent reports using the pilocarpine mode of SE (Turrin and Rivest, 2004; Voutsinos-Porche et al., 2004). The examination of its inducible regulation has revealed that COX-2 has an array of response elements, including a CRE, an NF-kappaB site, a NF-IL6 motif and a binding site for C/EBPβ, (Tanabe and Tohnai, 2002), that would allow for rapid coupling to excitatory stimuli. Interestingly, a number of studies have shown that CREB plays a key role in receptor-dependent coupling to COX-2 (Cho et al., 2004; Yang and Bleich, 2004). Our data are consistent with a role for CREB in COX-2 expression. Along these lines, we show that COX-2 expression parallels the
SE-induced expression pattern of the CRE reporter in both neurons and reactive glia. The causal connection was confirmed using a transgenic mouse strain that overexpresses the CREB inhibitor, A-CREB. Thus, transgenic A-CREB repressed SE-induced COX-2 expression. It is interesting to note that the CREB-regulated transcription factor C/EBPβ has also been implicated in COX-2 expression (Wu et al., 2005; Chen et al., 2004). Given that C/EBPβ is induced by neuronal activity in a CREB-dependent manner (Niehof et al., 1997), it is plausible that CREB might stimulate COX-2 expression via both a direct effect and through the indirect induction of C/EBPβ. Widespread microglial activation and persistent astrogliosis are hallmarks of the pilocarpine model of epilepsy (Borges et al., 2003; Aschner et al., 1999; Hanisch, 2002; Rogove and Tsirka, 1998), and are consistent with the pathological alterations found in human temporal lobe epilepsy. These changes in glial cell physiology are likely mediated by lasting alterations in their transcription profile (Jeon et al., 2004). In this context, it was interesting to find that SE elicited a long-lasting form of CREB-dependent gene expression in reactive astrocytes and reactive microglia. This observation of prolonged CRE-mediated gene expression in glial cells is consistent with pCREB data collected using a number of neuronal injury models. For example, in the hippocampus, increased pCREB levels were observed in reactive glia 7 days following focal stab lesions, and four weeks after kainic acid injection (Carbonell and Mandell, 2003; Ong et al., 2000). Interestingly, in tissue from patients with mesial temporal lobe epilepsy pCREB is also observed in reactive astrocytes (Park et al., 2003). Future experiments will be aimed at addressing the potential role of the CREB/CRE pathway in glial cells as it pertains to SE-induced pathophysiological alterations within the hippocampus. It should be noted that transgenic A-CREB was not expressed in non-neuronal cells, so the connection between CREB and COX-2 expression in glia could not be directly tested. However, given the temporal correlation between β-galactosidase expression and COX-2 expression in reactive glia, it is likely that the
CREB/CRE-pathway also regulates COX-2 expression in non-neuronal cells. In conclusion, this newly identified role of CREB as a positive regulator of SE-induced COX-2 expression is, in some respects, counter to the well established role of CREB as a neuroprotective signaling pathway (Lonze and Ginty, 2002; Tanaka, 2001; Hara et al., 2003). Thus, these data expand the role of CRE-signaling from one that is neuroprotective to one that may contribute to seizure-induced pathological alterations in the CNS.
CHAPTER 3

BDNF INDUCED NEUROPROTECTION THROUGH CREB SIGNALING PATHWAY
IN STRIATUM WITH PILOCARPINE AND CELL TOXICITY MODEL

(ON GOING PROJECT)

INTRODUCTION

BDNF signaling

The neurotrophin brain-derived neurotrophic factor (BDNF) plays diverse roles, regulating neuronal structure, function, and survival during development and into adulthood (Song and Poo, 1999; Bibel and Barde, 2000; Kaplan and Miller, 2000). In addition, recent work indicates that BDNF may have therapeutic value as a treatment for diseases associated with excitotoxicity. For example, Gratacos et al., (2001) showed that BDNF pretreatment protected cultured striatal neurons from kainic acid (KA) induced toxicity in vitro and in vivo, grafting BDNF expressing cells protected striatal neurons from KA-induced excitotoxic damage. The protective efficacy of BDNF has also been shown using global forebrain ischemia model (Larsson et al., 1999; Andsberg et al., 2002). Thus, for a complete picture of how BDNF confers neuroprotection, we must identify the down stream signaling events and genes which are activated by BDNF.

The first step in neurotrophin signaling is the activation of the cognate cell surface
Thus, neurotrophins activated specific receptor tyrosine kinases, Trks, embedded within the plasma membrane (Kaplan and Stephens, 1994; Barbacid, 1995; Kaplan and Miller, 1997). NGF preferentially binds TrkA (Cordon-Cardo et al., 1991), BDNF and neurotrophin (NT) 4/5 bind TrkB (Klein et al., 1991), and NT3 binds TrkC (Lamballe et al, 1991). Binding of ligand to receptor causes receptor dimerization and autophosphorylation (Jing et al., 1992; Clary et al., 1994). On binding to these receptors, neurotrophins activate various intracellular signal transduction pathways, including the Ras/mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3’-kinase/protein kinase B (PI-3-K/PKB) pathway, which appear to interfere with cell survival and cell death mechanisms (Segal and Greenberg, 1996; Nunez and del Peso, 1998). Even though these two major pathways have been shown to regulate BDNF-induced neuroprotection, their precise cell-type specific activation and protective effects within the central nervous system are not known.

**CREB in neuroprotection**

There are a number of studies indicating that CREB is involved in neurotrophin-mediated neuroprotective using several cell toxicity models (Lee et al., 2005; Mabuchi et al., 2001; Glover et al., 2004). CREB function has been implicated in antioxidant or anti-apoptotic gene expression. Along these lines, CREB regulates the expression of several neuroprotective proteins including B-cell CLL/lymphoma 2 (BCL-2) and brain-derived neurotrophic factor (BDNF) (Sugiura et al., 2004). In a recent study, I employed a dominant negative form of CREB to examine neuroprotection and CREB into cultured neuronal model system (Lee et al., 2005). In this study, I transition into an in vivo system, utilizing CREB transgenic mice to address the role of CREB in protection mechanisms against neuronal toxicity induced by pilocarpine, a muscarinic receptor agonist.
PGC-1 in cell toxicity

Reactive oxygen species (ROS) have been implicated as central regulators of cellular damage during neurodegenerative disorders. ROS oxidizes cellular macromolecules resulting in necrosis or apoptosis (Loh et al., 2006). Mitochondrial metabolism is a major source for the majority of ROS production in cells (Balaban et al., 2005). Unpaired electrons escaped from the electron transport chain react with oxygen resulting in generating superoxide. The interaction of superoxide with DNA, proteins and lipids induces many physiological and pathophysiological conditions such as ischemic injury, neurodegenerative diseases and aging (Balaban et al., 2005). A critical cellular defense mechanism against ROS is PPAR\(\gamma\) coactivator 1 (PGC-1). PGC-1 functions as a regulator of mitochondrial biogenesis, fatty acid oxidation, and ROS metabolism through the induction of many ROS-detoxifying enzymes, including GPx1 and SOD2 (St-Pierre et al., 2006; Czubryt et al., 2003). Interestingly, St-Pierre et al., 2006 reported that there are several CRE sites in the promoter of PGC-1 and that its expression is tightly regulated by CREB. These findings reveal a new CREB target gene that plays a role in neuroprotection. Thus, I endeavored to examine whether a signaling cassette formed by BDNF, CREB and PGC-1 play a critical role in neuroprotection against excitotoxic cell death in vivo. Identifying a major regulator of oxidative stress will provide new insights into therapeutic approaches designed to abrogate neurodegenerative disease progression.
MATERIALS AND METHODS

**Animals.** Status epilepticus was induced as described in chapter 2. Briefly, adult C57BL/6 mice (6-10 weeks of age) were first intraperitoneally (i.p.) injected with atropine methyl nitrate (1 mg/kg) 30 min prior to pilocarpine hydrochloride injection (325 mg/kg, i.p.). Animals which developed SE were used in this research. All experimental procedures were in accordance with Ohio State University animal care and use guidelines.

**Cannulation and infusion.** Adult C57/BL6 mice (6-10 weeks of age) were anesthetized with ketamine and xylazine. Cannulation was performed as described in Butcher et al., 2002. The coordinates for striatal injection were: anterior, 0.9mm; lateral midline and dorsoventral, 3.2 mm from dura with head level. After surgery, animals singly housed and allowed to recover at least 10 days. A stainless steel injector needle (30 gauge) extending 500 µm from the tip of the guide cannulae was used to infuse chemicals; kainic acid (100 uM/ul, Sigma), saline, BDNF (50 ng/ul), Me2SO, U0126 (10 uM/µl, Cell Signaling Research), LY294002 (30 mM/ul), K252a (1mM/ul), KN62 (10 uM/µl, Biomol) at a rate of 0.40 µl/min. A total volume of 3 µl was delivered for Kainic acid, Me2SO and inhibitors; 1 µl of saline and BDNF were infused.

**Fluoro-jade B histochemistry.** Fluoro-jade B labels dead and dying cells. The procedure was followed as described in chapter 2. Briefly, the fixed tissue was washed in distilled water (3X), and then treated with 0.06% potassium permaganate for 10 min at room temperature with gentle shaking. Sections were then washed in distilled water (3X) and incubated with 0.001% Fluoro-jade B (Histo-Chem Inc., Jefferson, AR, U.S.A.) in 0.1% acetic acid for 20 min with gentle shaking. Sections were then washed in distilled water, dried, and coverslipped with DPX.
**TUNEL assay.** To identify apoptotic cell death in parvalbumin positive cells, tissue was isolated 3 day after SE onset. Fixed coronal sections were then incubated with anti-mouse parvalbumin antibody (Chemicon international, Temecula, CA) and then the tissue was stained for TUNEL (Chemicon international, Temecula, CA) as described in manufacturer’s instruction. Finally, tissue was processed parvalbumin antibody binding using an anti-mouse secondary antibody conjugated with Alexa 488 (Molecular Probes, Eugene, OR).

**Tissue collection.** Tissue was fixed using the transcardial perfusion method described in chapter 2. Initially, mice were anesthetized with ketamine hydrochloride (91 mg/ml) and xylazine (9 mg/ml) and then perfused intracardially with a fixative solution of 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed, post-fixed in 4% PFA for 4 hours and then cryoprotected with 30% sucrose overnight. Thirty-µm thick coronal sections of striatum were cut on a freezing microtome and processed for immunohistochemistry or Fluoro-jade B labeling.

**Immunohistochemistry.** For diaminobenzidine (DAB) staining, tissue was blocked with 0.3% H2O2 and then incubated overnight with a goat polyclonal antibody against PGC-1 (1:1000; Santa cruz Biotechnology, Santa Cruz, CA). Next, the tissue was incubated in a biotinylated secondary antibody (1:300, Vector Laboratories, Burlingame CA, USA) for 1 hr at room temperature (RT) and then incubated in an avidin-biotin-peroxidase complex (Vectastain ABC kit, Vector Laboratories) for 45 min at RT. Nickel intensified DAB was used to visualize the signal. Finally, sections were mounted, dehydrated, and coverslipped with Permount (Sigma). Bright-field images were captured using a Leica DMIRB inverted microscope connected to a 16 bit
digital camera (Princeton Instruments; Monmouth Junction NJ, USA). For immunofluorescent staining, sections were washed with 1X PBS and then blocked with 10% normal serum in PBS. Then, sections were incubated overnight with one, or a combination of the following primary antibodies: calbindin (1:1000; Sigma), parvalbumin (1:1000; Chemicon international), somatostatin (1:1000, Chemicon international), NeuN (1:500; Chemicon, Temecula, CA), GFP (1:5000; Alberta, Edmonton, Canada), PGC-1 (Santa Cruz Biotechnology), 3-NT (1:500; Calbiochem international) and SOD-1(1:1000; Santa Cruz Biotechnology). The next day, the tissue was labeled with secondary antibodies (Molecular Probes, Eugene OR, USA) for 2 hrs at RT. The images of the mounted sections were captured using a Zeiss 510 Meta confocal microscope (Oberkochen, Germany). Data were analyzed using Metamorph image analysis software (Universal Imaging, Downingtown PA, USA).

**Western blotting.** Striatum tissue was collected as described in chapter 2. Thus, a vibratome was used to cut a 600 µm coronal section. The tissue was then placed on a microscope slides and frozen with dry ice. One-hundred µls of RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM NaF, 25 µM sodium vanadate supplemented with protease inhibitor cocktail: complete mini tablet, Roche Diagnostics) was used to lyse cells and then total protein levels were determined. Thirty µg of protein samples were loaded onto a 10% SDS-PAGE gel, electrophoresed using standard procedures, and the protein was transblotted onto polyvinylidene fluoride (PVDF) (Immobilon P; Millipore). Following blocking with 5 % (wt/vol) powdered milk, membranes were incubated (4°C overnight) with primary antibodies against phospho-ERK1 and ERK2 (1:1000; Cell signaling Technology, Beverly, MA), PGC-1 (Santa Cruz Biotechnology) and 3-NT (Chemicon international). Samples were then incubated with an alkaline phosphatase-conjugated anti-rabbit
antibody (1:2,500, Perkin-Elmer, Wellesley, MA USA) and the signal was detected using the Western-CDP star chemiluminescent detection system (Perkin-Elmer). As a protein loading control, membranes were probed with a goat polyclonal antibody against total erk-1 and erk-2 (1:2000, Santa Cruz Biotechnology). Samples were then incubated with a horseradish peroxidase-conjugated secondary antibody and the signal was detected using a chemiluminescent HRP substrate (Renaissance chemiluminescent detection system: New England Nuclear).

RESULTS (PRELIMINARY DATA)

Pilocarpine-induced cell death in striatum

I used the muscarinic receptor agonist, pilocarpine, to elicit excitotoxic cell death in the striatum. Initially, Fluoro-jade B (FJB) staining was performed to assess the extent of cell death. Three days after status epilepticus (SE) onset, there was a dramatic increase of FJB positive cells in striatum compared to controls animal injected with saline (vehicle) (Figure 3.1). To determine the phenotype of vulnerable cells, I used three different interneuron-subclass-specific markers: calbindin, somatostatin, and parvalbumin. Immunolabeling revealed that SE triggered the loss of all three types of neurons, thus indicating that vulnerability is not specific to distinct subsets of cells (Figure 3.1B). Next, I examined whether SE-induced cell death occurred via an apoptotic mechanism. TUNEL analysis, a marker for apoptotic cell death, detected apoptotic cells in the striatum (Figure 3.1C). However, only a subset of dead/dying cells were TUNEL-positive, suggesting that cell death occurred via both a necrosis and apoptosis.
Figure 3.1. Seizure induced cell death. A. Status epilepticus (SE) was induced with pilocarpine (325 mg/kg) and Fluoro-jade B staining was used to detect dead or dying cells at 3 days post-SE. Relative to animals that exhibited SE, marked cell death was observed in striatum of SE-induced mice. Bright green dots denote FJB-positive cells. B. Three different interneuron markers were used to determine the phenotype of cells vulnerable to SE Brain tissue including striatum region were labeled with calbindin, somatostatin and parvalbumin antibodies at 3 days post-SE. Immunoreactivity against all three types was reduced, indicating that vulnerability is not specific to distinct cell type. To test whether BDNF can protect neurons from cell death, BDNF were infused directly into striatum 24 hours prior to seizure induction. With BDNF pretreatment, parvalbumin positive cells were spared from cell death. C. TUNEL assays were performed to detect apoptotic cell death. Double labeling with parvalbumin (green) and TUNEL (red) reveals colocalization in one of the two parvalbumin-positive cells.
**BDNF induced neuroprotection**

Using SE-induced cell death as a starting point, I examined the potential neuroprotective effects of BDNF infusion into the striatum. To this end BDNF (50 ng/µl) was unilaterally infused 24 hrs prior to SE induction. Representative FJB staining from mice sacrificed 3 days after SE, shows that BDNF (ipsilateral) dramatically decreased the number of FJB positive cells compared to no infusion (contralateral) hemisphere (Figure 3.2). These data show that a single injection of BDNF afforded marked protection from SE-induced cell death. Since pilocarpine induces massive cell death in many brain regions, the toxic effects of SE may have been the resulted from alterations in afferent input to the striatum, rather than a direct excitotoxic effect (e.g. SE-induced glutamate release) in the striatum. Thus, to definitively test whether BDNF affords protection against excitotoxic stimuli within the striatum, 24 hrs after BDNF infusion, I infused kainic acid (3 µl of 100 µM/µl) directly into striatum to elicit excitotoxic cell death. Data collected 3 days after kainic acid infusion revealed that BDNF infusion protected neurons from cell death, indicating that BDNF confers protection against a direct excitotoxic challenged to the striatum (Figure 3.2B). Next, I examined the signaling pathways that confer BDNF-induced protection. A number of studies have shown that BDNF-mediated neuroprotection is through the MAPK and PI3K signaling pathways (Nakazawa et al., 2002; Gottschalk et al., 1999). Here, I examined whether these kinase signaling pathways are involved in BDNF-induced protection against SE in striatum. The possible role of the CaMK pathway was also examined. Cannulated mice were first infused with 3 ul inhibitors of: U0126 (10 µM/µl), LY294002 (30 mM/µl), K252a (1mM/µl) and KN62 (10 µM/µll) or vehicle (Me2SO) 30 min prior to BDNF (50 ng/µl, 1 µl) infusion. Twenty four hour later, SE was elicited with pilocarpine. Striatal tissue was obtained and processed for Fluorojade B staining. Infusion of the MEK inhibitor U0126 attenuated the neuroprotective effects of BDNF, thus implicating MAPK pathway as an effector
Figure 3.2. BDNF protection from SE-induced cell death. A. Cannulated mice were infused with BDNF (1 µl of 50 ng/µl) 24 hours prior to SE induction. Ips-BDNF : ipsilateral side: BDNF infused. Cont-No BDNF : Contralateral site, No BDNF infusion. Compared to contralateral site, BDNF reduced the number of Fluoro-jade B positive cells. B. BDNF protects against kainic acid (3 µl of 100 µM/ µl) induced cell death in the striatum. Three days post injection, marked cell death was observed (ipsilateral). In the BDNF-infused hemisphere (contralateral: right side panel) limited cell death was observed.
of BDNF-induced cell survival (Figure 3.3A). As a confirmation that the MAPK pathway is a relevant effector, I probed striatal extracts of BDNF-induced ERK 1/2 activation. Western analysis revealed that BDNF induced robust ERK phosphorylation. Furthermore, pretreatment with U0126 blocked BDNF-induced MAPK activation (Figure 3.3B). Conversely, neither disruption of PI3K with LY294002, nor abrogation of CaMK signaling with KN62 affected the neuroprotective effect of BDNF (Figure 3.3A). As a final control, the TrkB receptor inhibitor K252a was infused. Disruption of TrkB activity blocked BDNF-mediated neuroprotection (Figure 3.3A), indicating that BDNF is actuating the TrkB receptor to drive MAPK activity and, in turn, neuroprotection.

**CREB is a key regulator of BDNF mediated neuroprotection in the striatum**

Since CREB is a major transcriptional effector of the MAPK pathway, and since CREB has been shown to play a role in protection in central nervous system, I examined the potential role of CREB in BDNF-mediated neuroprotection. To this end, I utilized the A-CREB transgenic mouse strain which was described in chapter 2. As noted, A-CREB is highly expressed in the striatum (Figure 3.4A). Double labeling with NeuN and GFP revealed 100% colocalization, indicating that transgene expression is exclusive to neurons (Figure 3.4B). Since CREB has been implicated in cell survival, I examined transgenic cell viability. Counting the total number of NeuN positive cells in 200 μm squares from three different regions of striatum revealed no difference between transgenic and wild type mice, indicating that, under control conditions, the transgene does not affect cell viability (Figure 3.4C). To determine whether A-CREB affects BDNF-mediated neuroprotection, transgenic mice were infused with BDNF 24 hrs prior to pilocarpine injection; mice were killed 3 days post-SE onset, and processed for FJB. Interestingly, BDNF-evoked neuroprotection was diminished in A-CREB transgenic mice (Figure 3.4D and
Figure 3.3. The MAPK pathway couples BDNF to neuroprotection. A. Cannulated mice were first infused with 3 µl inhibitors of: U0126 (10 µM/µl), LY294002 (30 mM/µl), K252a (1mM/µl) and KN62 (10 µM/µl) or vehicle (Me2SO) 30 min prior to BDNF (50 ng/µl, 1 µl) infusion. Twenty four hour later, SE was elicited with pilocarpine, and 3 days post-SE striatal tissue was processed for Fluorojade B. Values depict the ratio of FJB positive cells in ipsilateral to contralateral striatum. K252a and U0126 infusion significantly attenuated the protective effects of BDNF. *P < 0.05 relative to BDNF. B. Striatum tissue was collected 24 hours after BDNF infusion and probed for ERK phosphorylation via Western analysis. Relative to control tissue, BDNF stimulated ERK activation. Infusion of U0126 30 min prior to BDNF infusion blocked ERK activation. As a protein loading control, the blots were also probed for total ERK expression.
4E). These data indicates that CREB is required to confer the maximal level of BDNF-mediated neuroprotection in the striatum (Figure 3.4D and 4E).

**The role of PGC-1 in BDNF mediated neuroprotection**

PGC-1 is a CREB-inducible gene that has been shown to regulate the level of cytoplasmic reactive oxygen radicals (St-pierre et al., 2006). Since oxidative stress is a major cause of cell death in many models such as ischemic injury and neurodegenerative diseases (Loh et al., 2006; Lang, 2007; Behl, 2005), I examined the potential connection between PGC-1 expression, ROS production and cell death induced by SE. First, I examined whether SE evokes oxidative stress by using a marker of peroxynitrite formation, 3-nitrotyrosine (3NT). At 3 days post-SE, a dramatic increase in the number of nitrotyrosine positive cells was shown in the striatum (Figure 3.5A). As a positive control, mice were injected with kainic acid. Robust 3NT expression was observed following kainic acid induction (Figure 3.5A). Marked double labeling for 3-NT and NeuN was detected, indicating that oxidative stress occurred in neurons (Figure 3.5A). Next, I examined whether PGC-1 expression was induced by SE. At 3 days post-SE, a dramatic increase in PGC-1 was detected in the striatum (Figure 3.5B). To examine whether PGC-1 induction was dependent on CREB signaling, I examined the capacity of SE to stimulate PGC-1 expression in A-CREB mice. Relative to wild-type mice, the SE-induced increase in PGC-1 was attenuated in A-CREB transgenic mice (Figure 3.5C) indicating PGC-1 expression is regulated by CREB transcription factor. As a test of the transactivation potential of PGC-1, I examined the expression of the PGC-1-regulated gene superoxide dismutase 1 (SOD1). SOD1 plays a central role in oxygen radical detoxification (Julka et al., 1992; Li et al., 1999). SOD1 expression was increased following SE, and was colocalized with with PGC-1, suggesting that PGC-1 couples SE to expression of SOD1 (Figure 3.5D).
Figure 3.4. BDNF neuroprotection in A-CREB transgenic mice. A. Immunohistochemistry for GFP. Expression of the A-CREB-eGFP transgene construct was detected in the striatum. As a control, wild type mouse tissue was also processed for GFP immunohistochemistry. B. GFP and NeuN double labeling was performed. Colocalization of the two antigens was detected. C. The total number of NeuN positive cells was counted from wild type and A-CREB transgenic mice. The number above bar indicates the mean value for total number of NeuN positive cells from each striatum. Counting the total number of NeuN positive cells in 200 um squares from three different regions of striatum revealed no difference between transgenic and wild type mice. D. Transgenic and wild-type mice were infused with BDNF 24 hrs prior to pilocarpine injection; mice were killed 3 days post-SE onset, and processed for FJB. BDNF-evoked neuroprotection was diminished in A-CREB transgenic mice. E. FJB positive cells from both ipsilateral (BDNF infused site) and contralateral (No BDNF infused site) were counted and the ratio of FJB positive was obtained.
Figure 3.5. Seizure induced PGC-1 expression and CREB. A. Mice were injected in the striatum with pilocarpine (325 mg/kg) or infused with kainic acid (3 µl of 100 µM/µl) and immunolabeled for 3-nitrotyrosine (3NT). At 3 days post-SE, both pilocarpine and kainic acid induced a dramatic increase in the number of nitrotyrosine positive cells. B. Immunohistochemistry for PGC-1 was performed 3 day after SE onset and in control mice. Relative to control animals, SE induced a dramatic increase in PGC-1 expression. C SE was elicited in wild type (WT) and A-CREB-tTA transgenic mice. Mice were sacrificed 3 days post-SE and double labeled for GFP and PGC-1. PGC-1 expression was lower in A-CREB transgenic cells. D. As a test of the transactivation potential of PGC-1, the expression of the PGC-1-regulated gene superoxide dismutase 1 (SOD1) was analyzed. Double labeling for PGC-1 and SOD1 was revealed that SOD1 expression correlates with the expression of PGC-1.
DISCUSSION AND FUTURE STUDIES

The goal of this study was to understand the underlying mechanisms of BDNF-evoked neuroprotection. To this end, the pilocarpine model of status epilepticus model was used to induce cell death in the striatum. Pilocarpine is a muscarinic receptor agonist which has been shown to damage neurons in diverse brain regions such as cortex, hippocampus and striatum and is used as a tool to induce epileptogenesis in rodent animal models. With this toxicity model, I report here that the MAPK and CREB/CRE transcriptional signaling pathway are principal mediators of BDNF-elicited neuroprotection. Pharmacological inhibition of kinase activity and a transgenic mouse strain which expresses A-CREB were utilized in this study. Finally, my data raise the possibility that the CREB-target gene, PGC-1 is a critical effector of BDNF mediated neuroprotection.

Striatal neurodegeneration is involved in many neurodegenerative disorders such as Huntington’s disease and Parkinson’s disease. Many toxic models have been used to induce striatal neurodegeneration such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Bocharov et al., 2006) and 3-nitropropionic acid (3NP) administration (Lee et al., 2006).

Recently, studies have shown that neurotrophins, such as BDNF can confer protection against excitotoxic stimuli, and that the withdrawal of trophic support can elicit cell death (Yuan and Yanker, 2000 Leeds et al., 2005; Schabitz et al., 1997; Sharma et al., 1998; Almli et al., 2000). Along these lines, it was shown that inhibition of BDNF expression induced significant cell loss in the substantia nigra (Porritt et al., 2005). Even though there are many studies suggesting a role for BDNF in neuroprotection (Mitsumoto et al., 1994; Yan et al., 1992), the underlying neuroprotective mechanisms have not been well studied. First, I confirmed the cell toxicity of pilocarpine mediated SE. Fluorojade B staining revealed a dramatic induction of cell death in
striatum 3 days after SE onset. I was also interested in studying cell-type specific vulnerability among distinct populations of striatal interneurons. Thus, I used three different phenotypic markers; somatostatin, calbindin and parvalbumin. There are several reports which show cell-type specific vulnerability against neurotoxicity (Freund et al., 1990, 1992; Figueredo-Cardenas et al., 1998; Chesselet et al., 1990; Ferrer et al., 1994; Ferrante et al., 1993; Johansen et al., 1990). Calbindin and parvalbumin are calcium-binding proteins. Cells expressing these calcium buffering proteins have been shown to be resistant to excitotoxic insult such as ischemia or seizures (Goodman et al., 1993; Johansen et al., 1990; Nitsch et al., 1989). In contrast, somatostain positive cells have been shown to be relatively sensitive to the excitotoxicity (Figueroed-Cardenas et al., 1998). One interpretation for cell type dependent vulnerability to excitotoxicity is due to excessive entry of Ca2+. Thus, the effect of calcium binding proteins is to buffer cytoplasmic calcium and thus attenuate ischemic or excitotoxic damage (Luiten et al., 1995; Pizzi et al., 1991). My results show that all three types of cells show vulnerability to pilocarpine induced cell death indicating that the level of toxicity induced by pilocarpine is too high to effectively block via calcium buffering. Conversely, cell death may not have been dependent on excessive calcium entry. Next, I examined cell death mechanisms involved in SE-induced toxicity. Apoptosis has been implicated as a SE-activated cell death mechanism (Fujikawa, 2005; Sankar et al., 1998; Sloviter et al., 1996). Here, the data showing colocalization of parvalbumin with TUNEL indicates that in a subset of cells die via an apoptotic-dependent mechanism. However, since TUNEL labeling was not detected in all cells, necrotic mechanisms may also contribute to the cell death process. There are several studies showing apoptosis of select populations of neurons (Filipkowski et al., 1994; Sloviter et al., 1996; Charriaut-Marlangue et al., 1996;). However, in this study, since we did not show the double labeling for TUNEL and FJB, main cell death mechanism is not currently known. In addition to this, many studies argue
that cells which have apoptotic phenotypes, such as DNA laddering and TUNEL positive reactivity, are morphologically necrotic (Fujikawa et al., 2000). Thus, additional studies will be required to determine the pilocarpine-evoked cell death mechanism.

BDNF binds to Trk receptors resulting in receptor activation and kinase activation. Trk receptors have 10 conserved tyrosines in the cytoplasmic domain and three of them exists in the autoregulatory loop of the kinase domain. Phosphorylation of this site is essential for kinase activity. Further, phosphorylation on Trk receptors allows adaptor proteins to couple these receptors to intracellular signaling cascades such as extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol-3-OH kinase (PI3K)/Akt kinase pathway and phospholipase C-r1 (PLC-r1) (Kaplan and Miller, 2000; Pawson and Nash, 2000). Two major signaling pathways have been suggested to couple BDNF to cell protection. One is the p42/p44 mitogen activated protein kinase (MAPK) pathway and the other is phosphatidylinositol 3-kinase (PI3K) pathway (Chen et al., 2006; Ou and Gean, 2006; Leeds et al., 2005; Almeida et al., 2005; Rossler et al., 2004; Nakazawa et al., 2002). Interestingly, with my toxicity model, only the MAPK pathway and Trk B receptor signaling events were essential to elicit BDNF-induced neuroprotection. Many studies suggested that both PI3K and MAPK are essential elements for BDNF evoked neuroprotection (Nakazawa et al., 2002; Gottschalk et al., 1999); however, different studies have shown varied roles for PI3K and MAPK in BDNF-induced trophic responses (Gavalda et al., 2004; Zheng and Quirion, 2004).

CREB is a transcription factor which has diverse functions, controlling cell proliferation and survival (Persengiev and Green, 2003). Gene expression through MAPK and CREB transcription pathway has been implicated in neurotrophin mediated neuroprotection (Arthur et al., 2004; Pizzorusso et al., 2000). Thus, I decided to generate transgenic mice to examine the role of CREB in neurotrophin-mediated neuroprotection. A tetracycline inducible dominant negative
form of CREB, A-CREB, was used to generate a transgenic mouse. Long et al., (2001) have reported that A-CREB expressing mice die minutes after birth, apparently due to respiratory failure from a diminished rib cage circumference. Thus, having a transgenic mouse which can regulate the temporal and regional transgene expression was essential for this research. To that end, we used a forebrain-specific promoter, CaMK II alpha, in combination with the tetracycline transactivator (tTA) developed by Gossen and Bujard (1992). Green fluorescent protein (GFP) was cloned 3’ to an IRES (after A-CREB) to detect transgene expression. Thus, first, I examined whether A-CREB expression changes neuronal viability by counting the number of GFP- and NeuN-positive cells. There was no significant difference in the total number of NeuN positive cells between transgenic and wild type mice, indicating that the basal level of A-CREB expression does not alter cell viability. Since the A-CREB construct does not block CREB activity completely, lower level of CREB activation may be sufficient to maintain cell viability in control condition. However, cell vulnerability is significantly different between transgenic and wild type mice. FJB positive cells were significantly high in A-CREB transgenic mice compared to wild type. Furthermore, the protective effects of BDNF were attenuated in A-CREB transgenic mice indicating that the CREB signaling pathway is essential for BDNF-mediated neuroprotection.

There are a number of well-characterized CREB-regulated neuroprotective genes. For example, liposome-mediated transfer of the human bcl-2 protein, a CREB regulated gene, can protect neurons in a rat model of focal transient ischemia (Cao et al., 2002). Here, I focused on PGC-1 which has been shown to be highly expressed in response to oxidative stress (St-pierre et al., 2006; Venditti and Di Meo, 2006; Suliman et al., 2004). PGC-1 is a cofactor which upregulates antioxidant enzymes to detoxify the oxygen radicals released from mitochondria (St-pierre et al., 2006). In this study, my data reveal that PGC-1 was induced by SE. Next, I examined
whether PGC-1 expression is evoked by SE. PGC-1 immunolabeling revealed a dramatic induction of PGC-1 expression in striatum following SE. Recently, PGC-1 alpha was implicated in ROS metabolism (St-pierre et al., 2006). Since there are numerous studies showing SE-induced ROS generation (Tejada et al., 2007; Layton and Pazdernik, 1999), we examined whether the PGC-1 expression was evoked by oxidative stress mediated by seizure. To detect oxidative stress, I used an antibody against nitrotyrosine. I observed an increase in 3-NT (3-nitrotyrosine) positive cells following SE indicating that SE triggers oxidative stress which triggers cell death. PGC-1 upregulation may represent an inducible neuroprotective response mechanism. This finding led me to hypothesize that the increase in the number of dead or dying cells in A-CREB transgenic mice is due to attenuation of PGC-1 expression. Therefore, I tested whether PGC-1 expression is reduced in GFP expressing cells. The result shows that there is a reduction in PGC-1 expression in GFP positive cells suggesting that CREB regulates the neuroprotective induction of PGC-1. These data also raise the possibility that upregulation of PGC-1 is an essential component of the activity (e.g. BDNF)-mediated neuroprotective response mechanism.

More statistical data and increasing the numbers of samples will be required to finish this project. In addition to this, each finding must be confirmed with at least one distinct assay. For example, the induction of PGC-1 expression will be examined with Western analysis by harvesting and processing striatum tissue from both control and SE-induced animals from both wild type and A-CREB transgenic mice. The big question left is whether PGC-1 expression can be regulated by BDNF and the MAPK signaling pathway. To study the regulation of PGC-1 by BDNF I will employ an in vitro culture model system. This will allow me to examine the signal transduction events that regulate PGC-1 expression. Blunting BDNF-induced PGC-1 expression and challenging cells with oxidative stress is another experiment to examine the role of PGC-1 as an effector of BDNF-mediate neuroprotection. Since PGC-1 is not the only CREB regulated gene
which plays a role in neuroprotection, I also plan to perform microarray analysis to identify the full compliment of SE-induced, CREB regulated, genes. Thus, I have harvested RNA from both wild type and A-CREB transgenic mice. There are three groups. the first group is the control: no SE. The second group was collected 90 min after SE onset: both wild type and A-CREB transgenic mice were collected. The third group was processed 6 hr after SE onset. The 90 min and 6 hr time points will allow early and late response gene profiles to be examined. Identification and characterization of specific genes showing different pattern of expression between wild type and A-CREB transgenic mice will lay the groundwork for future studies related to CREB and neuroprotection.
CHAPTER 4

PROTEIN KINASE C REGULATES THE PHASE-DELAYING EFFECTS OF LIGHT IN THE MAMMALIAN CIRCADIAN CLOCK

INTRODUCTION

The suprachiasmatic nuclei (SCN) located in anterior hypothalamus function as the major biological clock. The inherent pacemaker activity of the SCN is driven by interlocking genetic feedback loops derived from a limited subset of “clock genes” (Sassone-Corsi, 1994; Harms et al., 2004). A number of external stimuli such as light function as potent entrainment cues by regulating the expression of clock genes. Photic information is relayed from the eyes to the SCN via the retinohypothalamic tract (RHT), a branch of the optic nerve. In response to photic stimulation, RHT nerve terminals secrete the excitatory neurotransmitter glutamate (Ebling, 1996; Hannibal, 2002) and the modulatory neurohormone pituitary adenylate cyclase-activating polypeptide (PACAP: Hannibal, 2002; Harrington et al., 1999). These two transmitters work in a coordinated manner to activate a set of second signaling events that couple light to the core clock timing mechanism.

Several intracellular signaling pathways have been implicated in light-induced clock entrainment. For example, the p42/44 mitogen-activated protein kinase (MAPK) pathway is required for both the phase advancing and phase-delaying effects of light (Obrietan et al., 1998;
The MAPK pathway appears to regulate the clock via induction of gene expression; the disruption of MAPK signaling in vivo uncouples light from clock entrainment and inhibits inducible gene expression (Dziema et al., 2003; Akiyama et al., 2003; Coogan & Piggins, 2004). Likewise, both calcium/calmodulin kinases (CaMKs), nitric oxide (NO)/guanosine 3',5' monophosphate (cGMP)/cGMP-dependent protein kinase (PKG) have been implicated in transcriptionally-dependent entrainment of the clock (Golombek & Ralph, 1994; Weber et al., 1995; Mathur et al., 1996; Fukushima et al., 1997; Ding et al., 1998; Yokota et al., 2001; Agostino et al., 2004). In addition to these pathways several studies have identified protein kinase C (PKC) as a potential regulator of photic input (McArthur et al., 1997; Schak & Harrington, 1999; Motzkus et al., 2000; Bult et al., 2001). PKC is a member of the AGC class of Serine/Threonine kinases. PKC comprises a large family (10 at present) of isozymes that fall into three classification groups depending on structure, function and essential cofactors. Conventional PKC isoforms (α, β and γ) require calcium and diacylglycerol (DAG) as cofactors, novel PKC isoforms (δ, ε, η, μ and θ) require DAG, and atypical isoforms (ζ, ι and λ) do not require DAG or calcium for activation (Newton, 1997; Dempsey et al., 2000). In addition to their regulation by cofactors, a series of phosphorylation events are required for catalytic activity, stability and subcellular localization of PKC (Newton, 2001; Newton, 2003).

As a receptor-regulated signaling pathway, PKC has been shown to exhibit diverse, context-specific physiological effects, including transcription activation, alterations in cell morphology and receptor sensitization (Newton, 1995). In the central nervous system, PKC has been implicated in neuronal plasticity-dependent processes, including long-term potentiation and long-term depression (Zhuo & Hawkins, 1995; Hussain & Carpenter, 2005). With respect to the circadian clock, an array of PKC isozymes has been found in functionally discrete regions of the SCN (Cagampang et al., 1998; Bult & Smale, 1999). Furthermore disruption of PKC activity
has been shown to entrain the circadian firing rhythms of the SCN slices in a phase-dependent manner (Schak & Harrington, 1999). Although these data indicate a functional role for PKC in the SCN clock, as yet, an examination of PKC activation by light, and its contribution to clock entrainment have not been performed.

Here we show that photic stimulation during the subjective night triggers PKC activity in the SCN and that the disruption of PKC activity enhances light-induced entrainment of the circadian clock. These effects of PKC do not appear to be mediated at the level of transcription. Rather, these data indicate that PKC regulates the stability of the clock gene product PERIOD1, thus providing a potential post-translational mechanism by which PKC affects clock entrainment.

**MATERIALS AND METHODS**

**Drug infusion and Behavior analysis.** Adult (6 to 10-week-old) C57BL/6 mice were stereotaxically-implanted with a guide cannulae piercing the roof of the third ventricle. The stereotaxic coordinates and surgical procedures are described in (Butcher et al., 2002). After cannulation, mice were individually housed and allowed two weeks to recover. Cannulated mice were then transferred to cages equipped with running wheels (15-cm diameter); wheel rotation was automatically recorded to a PC running Vital View (Minimitter Corp, Bend OR) data acquisition software. For circadian locomotor activity analysis, mice were entrained to a 12hr/12hr light/dark (LD cycle) for 14 days, and then dark-adapted. During the day, luminescence was provided with a fluorescent white light (~ 100 lux at mid-cage level). Animals received a once weekly food and water replenishment, and a change of bedding. Cage maintenance occurred at varying times during the subjective night. During cage maintenance, mice were exposed to a dim red light (< 3 lux). To disrupt PKC activity, dark-adapted mice were
infused under dim red light with the PKC inhibitor (bisindolylmaleimide I: 3 µl, 1 mM, Calbiochem, La Jolla, CA) at CT 14.5. Control animals were infused with drug vehicle (DMSO: 3 µl). Thirty min after infusion, animals were exposed to light (50 lux) for 10 min and then returned to darkness. Control mice not exposed to light were handled in a similar manner as the light-treated animals. After infusion, mice were returned to their home cages and locomotor behavior was monitored. Each mouse was exposed to at least two of the four stimulus paradigms (1: vehicle/light, 2: vehicle/no light, 3: bisindolylmaleimide I/light, 4: bisindolylmaleimide I/no light). A minimum of 10 days separated each stimulus. All animal experiments were performed in accordance with Ohio State University animal welfare guidelines.

To assess the effects of PKC on light entrainment of the circadian clock, we used the linear regression method described in (Daan & Pittendrigh, 1976). To this end, a regression line was drawn through activity onset for the 6 days preceding light treatment. This line was used to project when activity onset should occur following drug infusion and/or light treatment. A second regression line was fitted through the actual activity onset following drug infusion and/or light treatment. Days 3–10 after light treatment were used to generate this line. The difference between the projected and the actual activity onset was the light-induced phase shift. Significance was assessed using the two-tailed Student's t-test and data are expressed as the mean phase shift ± SEM.

**Immunohistochemistry.** For phospho-PKC (pPKC) and PKC phospho-substrate motif immunostaining, mice were dark-adapted for 2 days, exposed to light (100 lux for 15 min) and then immediately killed via cervical dislocation and decapitation. For immediate early gene analysis, mice were killed 90 min after light stimulation. Mice were also sacrificed under a standard 12 hr L/D cycle. Brains were then isolated and immersed in oxygenated physiological
saline, and thick-sectioned (600 µm) with a vibratome. Tissue was then fixed in 4% paraformaldehyde (PFA), incubated in 30% sucrose overnight and then thin cut (40 µm) using a freezing microtome. For DAB staining, sections were initially washed (3X) in phosphate-buffered saline supplemented with 0.1% Triton X-100 (PBST: 10 min), then incubated in 0.3% H2O2 in PBST (15 min). Next, tissue was blocked (1 h) in 5% goat serum/PBS and incubated (overnight, 4°C) in a rabbit phospho-Ser 660 PKC (pPKC) antibody (1:1000 final dilution, Cell Signaling Technology, Beverly, MA), or a rabbit anti-phospho-PKC substrate antibody (1:1000, Cell Signaling Technology). Next, sections were incubated (2 h) at room temperature in biotinylated anti-rabbit IgG (1:300, Vector Laboratories, Burlingame, CA) and then placed in an avidin/biotin HRP complex for 1 h (prepared according to manufacturer’s instruction: Vector Laboratories). The signal was visualized by the addition of DAB-nickel-intensified substrate (Vector Laboratories) and mounted on gelatin-coated slides and coverslipped with Permount media (Fisher Scientific, Houston, TX). For fluorescent immunolabeling against NeuN and pPKC, tissue sections were incubated (overnight, 4°C) with mouse monoclonal NeuN (1:1000, Chemicon, Temecula, CA) and rabbit polyclonal pPKC (1:1000) antibodies. The sections were then washed and incubated (4 h at RT) with an Alexa 488-conjugated goat anti-rabbit IgG antibody (1:500, Molecular Probes, Eugene, OR) and 594-conjugated anti-mouse IgG antibody (1:500, Molecular Probes). Sections were mounted with Gelmount (Biomek, Foster City, CA).

Images of diaminobenzidine- (DAB) labeled sections were captured using a 16-bit digital camera (Micromax YHS 1300; Princeton Instruments, Trenton, NJ) connected to a Leica DM IRB microscope (Nussloch, Germany). For fluorescent immunolabeling, a Zeiss 510 Meta confocal microscope (Oberkochen, Germany) was used to capture 2 µm-thick optical sections.
**Immunohistochemistry Quantiation.** All data were quantified using MetaMorph software (Universal Imaging Corporation, West Chester, PA). For cell counts from DAB-labeled tissue, an intensity threshold filter was initially applied to the SCN image. The filter eliminated nonspecific background labeling from analysis. The threshold value was equal to the mean pixel intensity within a non-immunoreactive region of the lateral hypothalamus. Digital circles were then overlaid on regions with a detectable signal above threshold (defined as positive cells) and counted. Cell counts were averaged over three consecutive central SCN sections for each experimental animal. Average values for each animal were used to generate mean values for each condition. Significance was determined using the two-tailed Student's *t* test and was defined as *p* < 0.05. All data are expressed as the mean ± SEM.

**Immunoprecipitation and Western blotting.** For both immunoprecipitation and Western blotting, SCN-containing coronal brain sections were cut using a vibratome, and then tissue was immediately frozen on dry ice. SCN and piriform cortical tissues were isolated using a razor blade and stored at –80 °C until use. SCN tissue was initially lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium vanadate, 1 mM NaF and 1X protease inhibitor cocktail: Roche). For immunoprecipitation, 500 µg protein lysate samples were precleared with protein-A-agarose and then incubated (60 min) with 2 µg of PKC α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then 30 µl of protein-A-agarose was added to the protein lysates and rotated overnight at 4° C. After centrifugation the agarose-protein complex was washed in RIPA buffer (3X) to remove nonspecific protein binding and then 3X SDS sample buffer was directly added to the agarose-protein complex. Samples were heated (95° C, 5 min) then processed via Western blotting (described below).
For Western blotting, 25 µg of protein lysates from the SCN and piriform cortex were loaded into 8% SDS polyacrylamide gels and trasblotted onto polyvinylidene fluoride (PVDF, Immobilon P, Millipore, Bedford, MA). Next, membranes were washed with PBST (1X), blocked with 5% (w/v) powdered milk dissolved in PBST, then incubated (overnight at 4°C) with primary antibody diluted in PBST supplemented with 5% BSA. The following primary antibodies were used in this study: rabbit polyclonal anti-phospho-Ser 660 PKC (1:1000 final dilution, Cell Signaling Technology), rabbit polyclonal anti-phospho PKC substrate (1:1000, Cell Signaling Technology), rabbit polyclonal anti-phospho PKC ζ/λ (1:1000, Cell Signaling Technology), rabbit polyclonal anti-phospho PKD/PKCµ (1:1000, Cell Signaling Technology), rabbit polyclonal anti-ERK1 and -ERK2 (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-Ras (1:2000, Upstate Biotechnology, Billerica, MA). Next the membranes were washed and incubated with horseradish peroxidase- (HRP) conjugated secondary antibodies directed against the IgG domains of primary antibodies (1:2000, Perkin Elmer Life Sciences, Norwalk, CT). Renaissance chemiluminescent HRP substrate (Perkin Elmer Life Sciences) was used to visualize the signal.

**PERIOD protein stability assay.** Dark adapted cannulated mice were infused with bisindolylmaleimide I (3 µl, 1 mM) or DMSO (3 µl, vehicle) 30 min prior to photic stimulation (100 lux, 10 min) during the early night (CT 15). Mice were then returned to the dark and sacrificed 90 min later (CT 16.5). Tissues were harvested as described above and fixed in 4% PFA. Sections were immunolabeled with polyclonal mPER1 antibody (1:10,000 dilution) provided by Dr. Steven Reppert and polyclonal mPER2 antibody (1:500 dilution, Alpha Diagnostic Intl. Inc., San Antonio, TX). Sections were immunohistochemically processed as described above.
HEK 293T cells were transfected with mperiod1 (mper1)-V5-His or mperiod2 (mper2)-V5-His in pcDNA3.1. The mper expression vectors were a generous gift of Dr. Steven Reppert. Twenty hours after transfection, cells were incubated with cycloheximide (25 µg/ml; Sigma, St. Louis, MO) 60 min prior to phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, 1 µM) stimulation. To block PKC activation, bisindolylmaleimide I (1µM) was added 30 min prior to TPA stimulation. After 90 min of TPA stimulation, cells were harvested with ice-cold RIPA buffer. Lysates were then centrifuged and supernatant protein levels quantitated. Thirtyµg of protein were loaded into 8% polyacrylamide gels and processed via Western analysis as described above. The following primary antibodies were used: mouse monoclonal anti-V5 (1:5000, Invitrogen, Carlsbad, CA), rabbit polyclonal anti-phospho PKC substrate (1:1000, Cell signaling Technology) and rabbit polyclonal anti-ERK1 and ERK2 (1:2000, Santa Cruz Biotechnology).

RESULTS

As a starting point to examine the role of PKC in light-entrainment of the circadian clock, we monitored its activation state following photic stimulation. To this end, mice were dark-adapted for 2 days, and then exposed to light (15 min, 100 lux) during either the subjective night (circadian time 15: CT 15) or the subjective day (CT 6), and the tissue was processed using an antibody that detects the Ser 660 (for the PKCβII isoform) phosphorylated form of PKC. PKC is autophosphorylated at Ser 660, and its phosphorylation is part of a series of sequential phosphorylation events that lead to its activation. Importantly, the Ser 660 motif is conserved in many conventional and novel (but not atypical) PKC isoforms (Keranen et al., 1995), and thus this antibody can be used to examine broadly-reflected changes in PKC activity. Fig. 4.1A reveals that exposure to light during the subjective night led to a marked increase in phospho-
PKC (pPKC) levels. Consistent with the molecular weight of most PKC isozymes, two major bands running from 80 to 85 kDa were detected. To test whether the increase in PKC phosphorylation was consistent with the activation of other signaling events implicated in entrainment, the blot was also probed for the expression of the activated, Thr 202/Tyr 204-phosphorylated, forms of ERK1 and ERK2. As with PKC, photic stimulation during the subjective night triggered an increase in ERK1 and ERK2 phosphorylation. As a protein loading control, the blot was also probed for total ERK expression. The effects of light appear to be specific to the SCN, since photic stimulation did not increase PKC phosphorylation (or ERK activation) in tissue isolated from the piriform cortex (Fig. 4.1A). Consistent with the Western blotting data, immunohistochemical analysis revealed that light triggered an increase in the phospho-PKC in the SCN (Fig. 4.1B). Interestingly, light-induced PKC phosphorylation was robust in the lateral and ventral SCN, two regions that receive limited afferent projections from the RHT (Pickard, 1992; Kita & Oomura, 1982; Johnson et al., 1988). In contrast, the ventral medial “core” region of the SCN, which receives dense projection from the RHT, exhibited a more modest induction pattern. Double immunofluorescent labeling against pPKC and the neuronal nuclear marker NeuN was used to show that light stimulated PKC activation in SCN neurons (Fig. 4.1C). The non-nuclear pPKC expression pattern is consistent with other studies showing a cytoplasmic distribution of activated PKC (Saito et al., 1989; Tanaka & Saito, 1992; Buchner et al., 1999). In contrast to the effects of light during the subjective night, photic stimulation during the subjective day (CT 6) did not lead to an increase in the activated form of PKC relative to control animals not exposed to light (Fig. 4.1A). Interestingly, this result parallels the phase-restricted capacity of light to activate the MAPK pathway. Together, these results reveal that light-induced and phase-restricted PKC activation in the SCN.
Figure 4.1. Light-induced PKC phosphorylation in the SCN. A. Western analysis of SCN tissue from animals exposed to light (15 min, 100 lux) during the subjective night (CT15) and subjective day (CT 6). Light exposure during the subjective night triggered an increase in the Ser 660 phosphorylated form of PKC (pPKC) in the SCN. Light also induced ERK 1 and ERK 2 phosphorylation (pERK). In contrast, light did not induce PKC phosphorylation in the piriform cortex (CTX). As a protein-loading control, blots were stripped and probed for total ERK or Ras expression. B. Immunohistochemical labeling detected a light- (CT 15, 15 min, 100 lux) induced increase in PKC phosphorylation in the SCN. Bar = 125 microns. C. SCN tissue from control (no light) and light-treated animals was immunolabeled with antibodies against pPKC (Green) and NeuN (red). A representative high magnification image of the SCN shows the localized expression of pPKC and NeuN, indicating that light triggers PKC activity in neurons. Bar = 25 microns. D. Light regulation of PKC isozymes. (Boxed) SCN tissue was immunoprecipitated using a PKCα antibody and probed using the phospho-Ser 660 PKC antibody. Light increased PKC α phosphorylation relative to tissue from animals not exposed to light. Light triggered PKC ζ/λ phosphorylation at Thr 410/403, respectively, in the SCN, whereas PKC μ autophosphorylation at Ser 916 was decreased by photic stimulation. As a protein-loading control, blots were stripped and probed for total ERK levels.
To identify specific PKC isotypes which are regulated by light, we probed for PKC activity using isoform-specific antibodies. For PKC\(\alpha\), SCN tissue from control and light-stimulated (100 lux, 15 min, CT 15) animals was immunoprecipitated with a PKC\(\alpha\) antibody, and PKC\(\alpha\) activation was probed using the phospho-Ser 660 antibody. Consistent with the SCN lysate results, a light-induced increase in PKC\(\alpha\) activation was observed (Fig. 4.1D). The activation states of novel and atypical forms of PKC were examined. Along these lines, we found that the phosphorylation of PKC \(\zeta/\lambda\) on Thr 410/413 (activation loop site) was increased after light stimulation. Interestingly, phosphorylation of PKD/PKC \(\mu\) at Ser 916 (autophosphorylation site) was reduced with light stimulation (Fig. 4.1D). These results reveal that photic stimulation triggers complex, and PKC isoform-specific responses within the SCN.

As noted above, PKC is activated by an array of transmitters, peptides and trophic factors. Given that peptide and transmitter content within the SCN is regulated over a 24 hr cycle, we tested whether PKC activity varied as a function of time. pPKC was initially examined under a standard 12 hr L/D cycle; thus animals were sacrificed during the middle of the day (zeitgeber time 6: ZT 6) and the early (ZT 15) and late night (ZT 22). Western analysis revealed an increase in pPKC levels during the late night, and reduced expression during the middle of the day (Fig. 4.2A). Using immunohistochemistry, a similar pattern of PKC activity was detected in dark-adapted mice; minimal activity was observed during the mid-subjective day (CT 6) and maximal expression was detected during the late subjective night (CT 22) (Fig. 4.2B). To determine whether this rhythm in PKC phosphorylation was the result of changing kinase levels, we also probed SCN tissue for total PKC expression. Using both Western blotting and immunohistochemistry, we did not detect marked alteration in total PKC levels as a function of time. Interestingly, PKC antigenicity was most abundant in the dorsal and lateral regions of the SCN (Fig. 4.2B) paralleling the expression pattern of light activated PKC (Fig. 4.1B). Together
Figure 4.2. Rhythmic regulation of phospho-PKC in the SCN. A. SCN tissue was collected during the middle of the day (ZT 6), early night (ZT 15) and late night (ZT 22) and probed via Western analysis for the expression of activated PKC (pPKC). Relatively weak expression was observed during the day, whereas expression during the late night was markedly higher. Variations in total PKC expression were not observed when the blot was probed with a PKC pan antibody. B. Rhythmic PKC activity was also observed in dark-adapted animals. Animals were sacrificed during the subjective day (CT 6), early (CT 15) and late (CT 22) subjective night and SCN tissue was immunohistochemically processed for activated PKC. Clock-regulated PKC activity peaked during the late subjective night (CT 22): minimal expression was observed during the subjective daytime. Immunostaining with a PKC pan antibody did not detect variations in PKC expression over the circadian cycle. Data are representative of three independent experiments.
these data reveal rhythmic PKC activity in the SCN. Next, we addressed the role of PKC in SCN light entrainment. As a starting point for this analysis, we employed a PKC substrate-specific antibody to test for light-induced changes in PKC-dependent phosphorylation patterns in the SCN. As noted by the manufacturer, the PKC substrate antibody detects phosphorylated Serine surrounded by Arg or Lys at the -2 and +2 positions and a hydrophobic residue at the +1 position. This motif is specifically targeted by PKC and its phosphorylation is associated with activation of PKC. For these experiments, dark-adapted animals were exposed to light (100 lux, 5 or 15 min) at CT15 and then immediately sacrificed. SCN lysates were probed via Western blotting for both pPKC and PKC target motif phosphorylation levels. Relative to control animals, photic stimulation triggered an increase in the phosphorylation of a number of unidentified proteins (Fig. 4.3A). Consistent with the pPKC levels, the levels of pPKC substrate labeling were dependent on light duration; thus, relative to a 5 min light stimulus, a 15 min light pulse elicited a higher level of substrate phosphorylation. As a protein loading control, the membrane was also probed for total ERK levels. Next, we used immunohistochemical labeling to detect changes in PKC substrate phosphorylation levels in the SCN. Under control conditions, little immunoreactivity was observed in the SCN. However, following photic stimulation (100 lux, 15 min) at CT 15, an increase in immunoreactivity was observed specifically in the SCN (Fig. 4.3C). To assess the functional role of PKC in the SCN, we devised a ventricular infusion approach to deliver the broad-spectrum PKC inhibitor bisindolylmaleimide I to the SCN. To assess the efficacy of bisindolylmaleimide I infusion, tissue sections were probed for PKC substrate phosphorylation levels. Representative data in figure 4.3B reveal that bisindolylmaleimide I infusion (3 µl, 1 mM, CT 14.5) 30 min prior to sacrifice triggered a reduction in PKC activity along the ventricular border region, which includes the SCN. Importantly, this infusion paradigm potently repressed the light-induced enzymatic activity of
Figure 4.3. Light-induced PKC activity in the SCN. A. SCN tissue from control mice (no light) and animals exposed to light (100 lux) were probed using a PKC phospho-substrate-specific antibody (PKC substrate). Relative to control animals, both 5 and 15 minutes of light triggered an increase in PKC substrate phosphorylation, indicating that light stimulates PKC enzymatic activity in the SCN. Paralleling this effect, levels of phospho-PKC (pPKC) increased with increasing light duration. As a protein loading control, the blot was also probed for total ERK expression. B. Disruption of PKC activity in the SCN. Cannulated mice were infused in the 3rd ventricle with the PKC inhibitor bisindolylmaleimide I at CT14.5. Mice were sacrificed 30 min later and tissue was immunolabeled using the PKC phospho-substrate-specific antibody. This low magnification representative image of the hypothalamus reveals the location of the infusion (arrow). 3V: third ventricle. Bar = 250 microns. C. Cannulated mice were infused with DMSO (vehicle) or bisindolylmaleimide I (Bis) 30 min before light (15 min, 100 lux) exposure at CT 15. Coronal tissue sections were immunolabeled with the PKC phospho-substrate-specific antibody. Light triggered an increase in PKC substrate phosphorylation in animals infused with drug vehicle. In contrast, the infusion of bisindolylmaleimide I effectively blocked PKC enzymatic activity.
PKC (Fig. 4.3C). Thus, bisindolylmaleimide I effectively uncoupled light from PKC activation in the SCN. In a control set of experiments we found that bisindolylmaleimide I led to a decrease in PKC activity that lasted from 90 min to ~ 3 hours post-infusion (data not shown).

Next the potential role of PKC as a regulator of light-induced clock entrainment was analyzed via wheel running behavior. To this end, mice were entrained to an LD cycle for two weeks, and then released into total darkness (DD). After 10 days in DD, animals were either infused with bisindolylmaleimide I (3 µl, 1 mM) or drug vehicle and exposed to light (50 lux, 10 min) 30 min later (CT 15). In vehicle infused mice, photic stimulation led to a significant phase delay (-110 min) in wheel-running activity onset. Interestingly, relative to vehicle infused mice, the disruption of PKC activity led to a significant lengthening (-144 min) of the phase-delaying effect of light (Fig. 4.4). Importantly, in the absence of photic stimulation, bisindolylmaleimide I infusion did not significantly alter clock phase, indicating that light-induced PKC activity modulates the phase-delaying effects of light.

Next, we examined the potential mechanism by which PKC regulates light entrainment of the clock. Given a number of studies showing that PKC stimulates gene transcription, we examined whether disruption of light-induced PKC activation affects immediate early gene expression. For these experiments mice were infused with bisindolylmaleimide I at CT 14.5 and exposed to light (15 min, 100 lux) 30 min later. Animals were killed 90 after light exposure, and the tissue was processed for the expression of JUN-B or EGR-1. As expected, in vehicle-infused mice, photic stimulation led to a dramatic increase in the expression of both immediately early genes (Fig. 5A and 5B). Interestingly, the infusion of bisindolylmaleimide I did not have a significant effect on light-induced JUN-B or EGR-1 expression in the SCN. Furthermore, in control mice (no light) PKC inhibition did not effect basal immediate early gene expression (Fig. 4.5). Together these data indicate that PKC does not significantly contribute to, or influence, the
Figure 4.4. Disruption of light-induced PKC activity augments the phase shifting effect of light. A. Representative double plotted actographs. Initially, mice were maintained on a 12 hr LD cycle, then dark-adapted (DD). After 10 days of free running, mice were infused with either drug vehicle (DMSO, left side) or the PKC inhibitor bisindolylmaleimide I (Bis, right side) 30 min before light (50 lux, 10 min) exposure at CT 15 (asterisk). Relative to the DMSO-infused animal, bisindolylmaleimide I administration enhanced the light-induced phase delay. To determine whether drug infusion altered timing in the absence of photic stimulation, mice were allowed to free run for an additional 14 days, and then infused with DMSO or bisindolylmaleimide I at CT 14.5 (asterisk). Regression lines approximate stimulus-induced phase shift. Please refer to Methods for a description of the regression analysis. Horizontal bars in the activity record denote an “off-line” period when wheel running activity was not recorded. B. Quantitation of the phase-delaying effects of light. Mean ± SEM of the phase-delaying effects of light. *p < 0.05 (significant), **P > 0.1 (not significant): two-tailed Student's t test. Numbers above bars denote sample size for each condition. C. Breakdown of the light-induced phase delays of individual mice infused with either DMSO or bisindolylmaleimide I. Each dot represents a single animal. Open circles denote mean values and error bars denote SEM. A line connecting the two average values was included as a visual aid.
set of signaling events that couple light to rapid transcriptional activation in the SCN. Finally, we examined whether PKC could affect the clock via a post-translational mechanism (Fig. 4.6). For this analysis, mice were infused with bisindolylmaleimide I and the effects on PERIOD 1 (PER 1) and PERIOD 2 (PER 2) protein expression were analyzed. Initially, we found that photic stimulation (100 lux, 10 min) during the early night (CT 15) led to an increase in the number of PER 1 immunoreactive SCN cells (Fig. 4.6A) from mice sacrificed 90 min later (CT 16.5). Given the short interval between photic stimulation and sacrifice (90 min), the increase in PER 1 expression is not likely to result from a transcriptionally-dependent mechanism, but rather, may be the result of an alteration in protein stability. Interestingly, the infusion of bisindolylmaleimide I prior to photic stimulation led to a significant decline in PER 1 expression (Fig. 4.6A), suggesting that PKC influences the stability of PER 1. The effects were specific to PER 1; neither light nor bisindolylmaleimide I had an effect on PER 2 expression. In the absence of photic stimulation, pretreatment (CT 14.5) with bisindolylmaleimide I did not significantly alter PER 1 or PER 2 levels. To test whether PKC alters PER 1 expression, TPA was stimulated and 90 after samples were harvested and western blot was performed. The data shows that TPA led to a marked increase in the expression of mPER1 (Figure. 4.6A). In contrast, the expression of mPER2 was not altered by TPA administration (Figure. 4.6B). Given that translation was blocked with cycloheximide, these results indicate that TPA treatment increased mPER1 stability. To determine whether this effect was mediated by PKC, cells were treated with bisindolylmaleimide I (1 μM) prior to TPA administration. Abrogation of PKC activity blocked the increase in mPER1 expression, indicating that the effects of TPA were mediated by PKC. The efficacy of bisindolylmaleimide I was validated by probing the blot with the PKC phospho-substrate antibody. Thus, TPA treatment triggered an increase in the number of immunoreactive bands and bisindolylmaleimide I pretreatment effectively blocked this increase (Figure. 4.6). Together,
these data indicate that PKC regulates the stability of the clock protein PER1, thus providing a potential mechanism by which light-induced PKC activity influence circadian clock entrainment.

DISCUSSION

The goal of this study was to determine whether PKC plays a role in light-entrainment of the SCN clock. Here we have shown that photic stimulation triggers the phase-specific activation of PKC in the SCN and that the disruption of PKC activity augments the phase-shifting effect of light. Data also suggest that PKC may be affecting the entrainment process by altering the stability of the core clock protein PER1. Together these data identify a physiological role for PKC in light entrainment, and also provide a potential mechanism by which PKC affects the clock.

The activation of PKC is driven by three related processes: subcellular localization, conformation and phosphorylation (Dekker & Parker, 1994; Newton, 1997; Parekh et al., 2000). As with other member of the AGC family of kinases, the primary structure of PKC consists of a C-terminus kinase domain and an N-terminus regulatory domain. The regulatory domain contains a pseudosubstrate motif, which blocks PKC activity by interacting with the catalytic domain, and either a single or tandem C domain. The ligands diacylglycerol and Ca$^{2+}$ target PKC to the membrane by binding to the regulatory domain. Membrane localization disrupts the association of the pseudosubstrate domain with the catalytic domain, thus facilitating PKC activation (Hofmann, 1997; Newton, 1997; Parker & Murray-Rust, 2004). With respect to phosphorylation, three sequential phosphate modifications are required for full PKC catalytic activity. Initially PDK-1-dependent phosphorylation within the catalytic domain “activation
Figure 4.5. PKC does not couple light to immediate early gene expression in the SCN. Drug vehicle (DMSO) or bisindolylmaleimide I (Bis) were infused 30 min before light exposure (10 min, 100 lux) at CT 15. After light exposure, mice were returned to darkness for 90 min and then killed. Immunolabeling revealed that light triggered robust EGR-1 (A) and JUN-B (B) expression in mice infused with either DMSO or bisindolylmaleimide I. 3V: third ventricle. Bar = 125 microns. C and D) Quantitation of light-induced immediate early gene expression. Error bars denote SEM. Three animals were used for each condition. **P > 0.1 (not significant).
Figure 4.6. PKC regulates PER1 protein stability. (A and B) Mice were infused with drug vehicle (DMSO) or bisindolylmaleimide I (Bis) 30 min before light exposure (10 min, 100 lux) at CT 15. After light exposure, mice were returned to darkness for 90 min and then killed. Representative immunolabeling revealed that light triggered an increase in PER1 expression (A) which was attenuated by bisindolylmaleimide I. PER2 expression (B) was not affected by light. Quantitation of PER1 and PER2 immunolabeling is shown below each data set. Error bars denote SEM. Numbers below bars indicate the number of animals examined for each condition. For PER2, no significant difference was observed for any of the conditions (**p > 0.1). HEK293T cells were transfected with mPER1-His-V5 (C) or mPER2-His-V5 (D) constructs. Twenty hours after transfection, cells were pretreated with cycloheximide (25 µg/ml: 60 min) and then stimulated with TPA (1 µM). After 90 min of stimulation, cells were harvested and probed via Western analysis. The V5 antibody was used to detect PER1 or PER2 fusion protein expression, PKC activity was assessed using the PKC substrate motif antibody and protein loading was examined using the total ERK antibody. TPA administration led to an increase in PKC activity and PER1 stability. In contrast TPA did not dramatically alter PER2 stability. Pretreatment (30 min) with bisindolylmaleimide I (1 µM) blocked TPA-induced PKC activity and reversed the stabilization of PER1. Representative samples were run in duplicate and are representative of three independent experiments. These data indicate that PKC regulates PER1, but not PER2, stability.
loop” triggers a conformational change that exposes the substrate binding motif (Le Good et al., 1998). Next, PKC phosphorylates within the “turn motif” of the catalytic subunit. This, in turn, triggers PKC autophosphorylation within the catalytic subunit “hydrophobic motif”. This last phosphorylation event facilitates enzymatic activity, stability and subcellular localization of PKC (Newton, 2001; Newton, 2003). The phospho-specific PKC antibody employed in this study monitored phosphorylation at this hydrophobic motif, and thus could be used to infer the activation state of PKC. It should be noted that atypical PKCs do not contain a phospho-acceptor Ser or Thr residue within the hydrophobic motif, and thus, their activation state was not assessed in this study. Importantly, throughout this study, we employed a PKC motif-specific antibody to confirm that an increase in hydrophobic motif phosphorylation was associated with an increase in PKC enzymatic activity.

Western analysis detected a robust light-induced pPKC signal. This approach was complimented by immunohistochemical and immunoprecipitation assays that also detected a light-induced increase in PKC phosphorylation. Of interest was the finding that photic stimulation during the subjective day did not stimulate PKC activity. This phase-restricted capacity of light to activate PKC parallels the phase-restricted responsiveness of the MAPK pathway to light (Obrietan et al., 1998), thus, raising the possibility that a common phase-regulated upstream event, such as receptor coupling, determines whether these signaling pathways are activated by light.

In addition to its light responsiveness, we detected a diurnal and a circadian variation in the activation state of PKC. Peak activation was detected during the late night, and lower levels of activity were observed during the midday. Interestingly, both PKC mRNA and protein levels have been shown to be rhythmically regulated in the SCN (Cagampang et al., 1998; Jansen et al., 2003). However, we did not detect a marked variation in PKC expression as a function of
circadian time. The absence of detectable PKC rhythm likely reflects the fact that the antibody we employed detects all PKC isoforms, and thus the circadian expression of a subset of isozymes (PKCα, β, and γ; Cagampang et al.,) may have been obscured.

To assess the role of PKC in clock entrainment, we developed an in vivo infusion approach to uncouple light from PKC activation. This method employed the PKC inhibitor bisindolylmaleimide I to block PKC enzymatic activity. The efficacy of the inhibitor was monitored using a phospho-PKC substrate-specific antibody. With this assay we were able to detect inhibition of PKC activity within the periventricular region that includes the SCN. This approach is analogous to the ventricular infusion method used to deliver the MEK inhibitor U0126 and, in turn, implicate the MAPK pathway in transcription regulation and light entrainment of the clock (Butcher et al., 2002).

Interestingly, the disruption of PKC activity significantly enhanced the phase shifting effects of light, indicating that PKC functions as a negative regulator of light entrainment. Importantly, disruption of PKC in the absence of light treatment did not significantly affect the circadian running rhythm or phase shift the clock, indicating that transient disruption of basal PKC activity does not affect inherent pacemaker activity. Rather, it is light-actuated PKC that regulates entrainment. This lack of an effect of PKC inhibition in the absence of photic stimulation is consistent with work showing that PKC inhibition during the early night does not alter the circadian firing properties of SCN slices (Schak & Harrington, 1999). It should be noted though, that PKC inhibition during the late subjective night and early subjective day was found to phase advance clock firing rhythms (Schak & Harrington, 1999). Our data indicating that PKC is a negative regulator of light-induced phase delays are, in some respects, consistent with a study showing that the phase delaying effects of light were inversely correlated with the level of PKCβI in the SCN (Bult et al., 2001). Thus, higher levels of PKCβI were associated with smaller phase
shifts, whereas lower levels of PKCβI were associated with a relatively larger light-induced phase shift.

PKC has been implicated as a signaling intermediate that couples neuropeptide Yergic (NPYergic) input from the thalamic intergeniculate leaflet (IGL) to the circadian clock (Biello et al., 1997). The IGL is a proposed site for the integration of photic and nonphotic inputs to the circadian system (Janik et al., 1995; Moore et al., 2000; Vrang et al., 2003; Thankachan & Rusak, 2005) Interestingly, NPY has been shown to attenuate the phase shifting effects of light (Lall & Biello, 2003a, b; Lall & Biello, 2002; Yannielli et al., 2004). This finding, coupled with the aforementioned study showing that NPY influences the clock via a PKC-dependent mechanism (Biello et al., 1997) provides one potential mechanistic explanation for how disruption of PKC augments light-induced phase shifting. However, it should also be noted that the two principal transmitters of the RHT, glutamate and PACAP, stimulate robust PKC activation (Gillette & Tischkau, 1999; Nowak et al., 2001; Dziema & Obrietan, 2002). Thus, additional work will be required to identify the transmitter and receptor-mediated signaling events that couple light to PKC activation in the SCN.

The role of PKC as a negative regulator of clock entrainment is, in some respects counter to the roles of other light-actuated pathways, which function as positive regulators of clock entrainment. Along these lines, we and several other groups have shown that the MAPK pathway is necessary for robust light-induced phase shifting (Butcher et al., 2002; Coogan & Piggins, 2003). Likewise, disruption of CaMK signaling suppresses light-induced phase shifting, as does inhibition of nitric oxide-dependent signaling (Tischkau et al., 2003; Golombek et al., 2004). The effects of PKC on clock entrainment suggest that it modulates the clock timing process via a mechanism that is distinct from that of the MAPK cascade, CaMKs and nitric oxide/PKG. In line with the supposition, we found that PKC inhibition did not affect light-
induced immediate early gene expression. Again, this contrasts with the aforementioned kinase pathways. For example, disruption of MAPK signaling has been shown to block the induction of Fos, EGR-1 and JunB (Dziema et al., 2003). In addition, the MAPK pathway is a regulator of the cyclic AMP response element, a transcription factor that drives induction of the core-clock gene mper1 (Travnickova-Bendova et al., 2002). Finally, it should also be noted that each light-activated PKC isoform may have a distinct effect on clock entrainment and that our use of a broad-spectrum inhibitor would average these effects, and thus mask potentially unique contributions of each isoform.

Given the lack of an effect on light-induced gene expression, we examined whether PKC regulates the clock via a post-transcriptional mechanism. Initially, we tested whether the in vivo disruption of PKC altered expression of the clock proteins PER1 and PER2. Interestingly, as part of this study, we found that PER1 immunoreactivity was increased 90 min after light stimulation. This increase was in part dependent on PKC; the disruption of PKC activity significantly attenuated light-induced PER1. Both light induction and PKC modulation were restricted to PER1; neither light, nor PKC affected PER2. In some respects, we were surprised to find that light triggered an increase in PER1 immunoreactive 90 min after photic stimulation. If this rapid increase in immunoreactivity were dependent on a transcriptionally-dependent process, Per1 would be considered an immediately early gene. Conversely, most studies have reported that transcriptionally-dependent PER1 expression can be detected 4 hrs or longer post-stimulation (Field et al., 2000; Yan & Silver, 2004; von Gall et al., 2003). Thus, the time course for the effects that we detected is not likely to be mediated by a transcription/translation-dependent mechanism. To our knowledge, this rapid light-induced increase in PER1 has not been previously characterized. It is unclear why this increase in PER1 immunoreactivity has not been documented previously. Possible explanations include differences in stimulus paradigms,
counting criteria and immunolabeling conditions. Along these lines, the ABC-based immunohistochemical labeling technique is extremely sensitive and slight modification in labeling and development conditions can lead to dramatic differences, or, conversely, an absence of a difference, in target protein expression.

To directly test whether PER1 stability is regulated by PKC, we employed a cell culture-based over-expression system. Importantly, to eliminate the potentially complicating effects of inducible PER expression, translation was blocked by pretreatment with cycloheximide. As with photic stimulation PKC activity was required for the stimulus-mediated increase in PER1 levels. This effect was specific to PER1: PKC did not alter the stability of PER2, or PER3 (data not shown). These data raise the possibility that PKC may affect the clock timing process via the regulation of clock protein stability. Hence, PKC appears to counteract an active PER1 degradation pathway. The precise route by which PKC affects PER1 stability is not known. Interestingly, recent work has shown that PKC can abrogate protein degradation via the ubiquitin-proteasome pathway (Leung et al., 2001; Hernandez-Pegeon et al., 2005). This mechanism would counter the effects of casein kinase I, which targets PERs for ubiquitin-mediated degradation (Akashi et al., 2002; Eide et al., 2005). Another possibility is that PKC could stimulate phosphatase activity, which in turn would reduce phosphorylation-targeted degradation. In line with this model, several studies have shown that PKC can stimulate protein phosphatase 1 and protein phosphatase 2A activity (Layne et al., 2001; Li et al., 2006), and that phosphatases play a key role in regulating clock protein stability (Gallego et al., 2006). In conclusion, these data provide a new framework to begin to examine the role of PKC as a regulator the mammalian circadian clock.
CREB signaling is involved in diverse cellular events such as cell survival, development and neuroprotection. In chapter 1, I examined the dual role of the NMDA receptor cell survival and cell death, and the role of CREB as an effector of NMDA receptor-dependent cell viability. I used two different stimuli. One was a non-toxic level of stimulation; synaptic activation and low dose of NMDA application. The other was a toxic level of stimulation; application of a high concentration NMDA. I found that both stimuli evoked CREB activation but that the outcome was different. Thus, I hypothesized that the temporal regulation of CREB might be differently affected by two different stimuli. My data revealed that that a non-toxic level of stimulation evoked prolonged activation of CREB but that a potentially toxic level of stimulation triggered transient activation of CREB. Interestingly, the durational of CREB activation tightly correlated with CREB function. CRE-luciferase assay showed that only prolonged activation of CREB triggered downstream gene expression. The data implicated differential regulation of calcineurin as a principal regulator of phospho-CREB duration, and that calcineurin-regulated CREB phosphorylation determines the transactivation potential of CREB. This was an important study in that it revealed how CREB-mediated neuroprotection can be altered by stimulus intensity, and duration.

In the research project outlined here and in chapter 2, I employed an *in vivo* animal model to understand CREB neuroprotective signaling. As part of this study, I found that SE triggered CREB-dependent gene expression in hippocampus. However, CREB-mediated gene expression
changed as a function of time. At the early time point, 4 hr after SE, CREB mediated gene expression principally in neurons but, by 48 hrs after SE, gene expression was mainly observed in non-neuronal cells: astrocytes and microglia. Given that reactive glia have been implicated in neuronal cell toxicity, I examined a potential CREB target, pro-inflammatory gene COX-2. COX-2 expression is tightly regulated by inflammatory peptides TNF alpha and interleukins, which are released from reactive glia and have been implicated in cell death. A combination of techniques was utilized to support the finding that SE-induced CRE-mediated gene expression drives the expression of COX-2. Thus, in chapter 2, I characterized a potentially pathophysiological role for CREB: the induction of COX-2 gene expression. In chapter 3, I turned to examine the potential role of CREB as a regulator BDNF mediated neuroprotective in the striatum. The preliminary data suggest that the MAPK pathway and CREB transcriptional signaling pathway are involved in BDNF-evoked neuroprotection. In addition to this, PGC-1 expression is a downstream effector of BDNF/CREB and functions a regulator of neuroprotective events controlling seizure-induced cell death via oxidative stress. Further, I pursued the issues of causality by employing a transgenic mouse strain to down-regulate CREB function and, in turn assess BDNF-mediated neuroprotection and PGC-1 expression.

Based on three different sets of experiments described in chapter 1, 2 and 3, the data suggest that CREB can contribute to pathophysiology and neuroprotection. These effects depend upon the cell types in which CRE-mediated gene expression occurs (e.g. microglia or neurons) and the gene(s) expressed (e.g. COX-2 and PGC-1). Furthermore, whether or not CRE-mediated gene expression occurs depends on the stimulus paradigm and the duration of
CREB phosphorylation.

Another line of scientific inquiry was focused on understanding signaling events in clock entrainment. Protein kinase C (PKC) has been suggested to play a role in clock entrainment (McArthur et al., 1997; Schak and Harrington, 1999; Dziema and Obrietan, 2002; Shim et al., 2007), although limited work had been performed using whole-animal models. I examined PKC-regulated SCN physiology in vivo in chapter 4. In this study, phase-restricted PKC activation was shown and more interestingly, it was suggested that specific types of PKC were involved in this process. Via stereotaxic injection, I showed that the delivery of a PKC inhibitor to SCN blunted PKC activation. On a behavioral level, disruption of PKC augmented the phase delaying effects of early night (CT15) light. I examined c-fos and JunB expression as markers of light-induced gene transcription and translation. Interestingly, PKC inhibition did not significantly reduce IEG expression, indicating that PKC does not regulate rapid light-induced transcription or translation. Thus, I hypothesized that PKC affects the clock via a posttranslational mechanism. PER1 and PER2 are major clock proteins playing an important role in clock resetting. Thus, I examined the level of these proteins after light entrainment. The level of PER1 protein was decreased with PKC inhibitors indicating PKC plays a role in PER1 protein stability. Thus, light-induced activation of PKC may affect the clock by regulating the stability of PER1. Alterations in PER1 stability would likely influence clock cycling time and the efficacy of light-entrainment cues.

In conclusion, the seizure model allowed me to understand the role of CREB in cell death and cell survival, while the circadian model allowed me to understand signal transduction related to synaptic activation. Furthermore, the in vitro assay allowed me to study signaling
events in both pathophysiological and physiological conditions. For all these studies, I employed a combination of molecular, cellular, biochemical, pharmacological and behavior techniques.
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