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A role for cell cycle protein E2F1 in HIV-induced neurotoxicity

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A role for cell cycle protein E2F1 in HIV-induced neurotoxicity

Abstract
HIV-associated neurocognitive disorders (HAND) are a spectrum of HIV-related conditions affecting the central nervous system that range from mild memory impairments to severe dementia. HAND results from the release of inflammatory factors and excitotoxins by HIV-infected macrophages in the brain. These factors alter the extracellular environment and provoke a neuronal response, ultimately causing dendritic damage, synaptic loss, and neuronal death. Our previous data indicate that components of the cell cycle regulatory machinery are elevated in neurons from post-mortem brain tissue of HAND patients. One of these upregulated proteins, the transcription factor E2F1, is known to activate gene targets required for G1-to-S phase progression as well as for apoptosis. Despite its increased neuronal expression, E2F1 target genes are unchanged by HIV-induced neuronal damage in vitro. Furthermore, E2F1 displays a predominantly cytoplasmic localization, a site inconsistent with its role as a transcription factor.

Utilizing an in vitro model of HIV-induced neurotoxicity, we assessed the role of E2F1 in HIV-mediated neuronal damage. To begin, we evaluated the contributions of two death pathways - calpain-mediated and caspase-mediated cell death - in cortical neurons treated with H2O2 and NMDA as they mature in culture. Although both calpain-activated and caspase-activated death were detected in cortical neurons at 1 week of age, only calpain-mediated neuronal death was observed at 3 weeks of age, suggesting that calpain is the dominant death pathway. We then tested the effect of E2F1 gene disruption on HIV-induced neuronal loss. Neuronal damage and death was significantly attenuated in neurons expressing mutant E2F1 protein compared to wildtype cultures. Furthermore, we identified E2F1 as a novel calpain substrate and showed that E2F1 cleavage by calpain produces an E2F1 fragment that stably accumulates in neurons during HIV-mediated neuronal damage, suggesting a neurotoxic role for this E2F1 isoform. Finally, preliminary studies to investigate a putative RNA-binding role for E2F1 in the neuronal cytosol are outlined. Together, these data implicate E2F1 in a calpain-mediated pathway of HIV-induced neurotoxicity. Future work to understand the role of E2F1 in neurons and the consequences of calpain cleavage of E2F1 could provide important insights into disease progression and therapeutic strategies.

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A ROLE FOR CELL CYCLE PROTEIN E2F1 IN HIV-INDUCED NEUROTOXICITY

Jacob Zyskind

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A ROLE FOR CELL CYCLE PROTEIN E2F1 IN HIV-INDUCED NEUROTOXICITY

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Jacob Zyskind
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ABSTRACT

A ROLE FOR CELL CYCLE PROTEIN E2F1 IN HIV-INDUCED NEUROTOXICITY

Jacob Zyskind
Kelly L. Jordan-Sciutto

HIV-associated neurocognitive disorders (HAND) are a spectrum of HIV-related conditions affecting the central nervous system that range from mild memory impairments to severe dementia. HAND results from the release of inflammatory factors and excitotoxins by HIV-infected macrophages in the brain. These factors alter the extracellular environment and provoke a neuronal response, ultimately causing dendritic damage, synaptic loss, and neuronal death. Our previous data indicate that components of the cell cycle regulatory machinery are elevated in neurons from post-mortem brain tissue of HAND patients. One of these upregulated proteins, the transcription factor E2F1, is known to activate gene targets required for G1-to-S phase progression as well as for apoptosis. Despite its increased neuronal expression, E2F1 target genes are unchanged by HIV-induced neuronal damage in vitro. Furthermore, E2F1 displays a predominantly cytoplasmic localization, a site inconsistent with its role as a transcription factor.
Utilizing an *in vitro* model of HIV-induced neurotoxicity, we assessed the role of E2F1 in HIV-mediated neuronal damage. To begin, we evaluated the contributions of two death pathways – calpain-mediated and caspase-mediated cell death – in cortical neurons treated with H₂O₂ and NMDA as they mature in culture. Although both calpain-activated and caspase-activated death were detected in cortical neurons at 1 week of age, only calpain-mediated neuronal death was observed at 3 weeks of age, suggesting that calpain is the dominant death pathway. We then tested the effect of E2F1 gene disruption on HIV-induced neuronal loss. Neuronal damage and death was significantly attenuated in neurons expressing mutant E2F1 protein compared to wildtype cultures. Furthermore, we identified E2F1 as a novel calpain substrate and showed that E2F1 cleavage by calpain produces an E2F1 fragment that stably accumulates in neurons during HIV-mediated neuronal damage, suggesting a neurotoxic role for this E2F1 isoform. Finally, preliminary studies to investigate a putative RNA-binding role for E2F1 in the neuronal cytosol are outlined. Together, these data implicate E2F1 in a calpain-mediated pathway of HIV-induced neurotoxicity. Future work to understand the role of E2F1 in neurons and the consequences of calpain cleavage of E2F1 could provide important insights into disease progression and therapeutic strategies.
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CHAPTER 1 - INTRODUCTION

1.1 HIV-ASSOCIATED NEUROCOGNITIVE DISORDERS

According to the most recent epidemiological data from the World Health Organization (WHO), over 35 million people are currently infected with the Human Immunodeficiency Virus (HIV). Although effective anti-viral therapy has been developed to slow disease progression, HIV remains a global health concern. In 2013 alone, WHO reported 2.1 million people newly infected with HIV and 1.5 million HIV-related deaths. HIV is a retrovirus that can infect host cells expressing the surface glycoprotein CD4 and either the CCR5 or CXCR4 chemokine receptors. These proteins serve as surface receptors for the viral coat protein gp120 and mediate fusion of viral particles with the host cell membrane, allowing entry of the viral capsid and incorporation of the viral genome into the host cell DNA (Stevenson, 2003). After an initial latency period, viral replication increases and ultimately leads to host cell death and release of viral particles that spread the infection to remaining CD4-positive T-cells. The perpetuation of the infection ultimately depletes this population of T-cells, leaving the host susceptible to opportunistic infection (Stevenson, 2003). This final stage of the infection is termed autoimmune deficiency syndrome (AIDS).

In addition to the immunosuppressive effects for which it is best known, HIV also invades the central nervous system (CNS) and can cause neurological deficits characterized by motor, cognitive and behavioral abnormalities. These complications, collectively referred to as HIV-associated neurocognitive disorder (HAND), are
estimated to affect up to 30% of HIV-positive individuals (Sacktor et al., 2002, McArthur, 2004, Antinori et al., 2007). Symptoms range from mild impairments such as forgetfulness and agitation to a clinically demented state known as HIV-associated dementia (HAD) (Gonzalez-Scarano and Martin-Garcia, 2005). The neuropathological hallmarks underlying HAND consist of astrocytosis, syncytia formation, dendritic damage, synaptic loss and neuronal loss, which typically manifest in the cerebral cortex, hippocampus and basal ganglia (Gonzalez-Scarano and Martin-Garcia, 2005, Kaul et al., 2005). While no genetic factors have been shown to increase the risk of HAND, susceptibility does correlate with high viral levels in the plasma (Childs et al., 1999).

Following the widespread administration of anti-retroviral therapy (ART) and its effective suppression of viral replication, the incidence of HAND among HIV patients has decreased (Sacktor et al., 2002). However, the prevalence is increasing due to the longer life expectancy afforded by ART (Dore et al., 1999, McArthur, 2004, Gonzalez-Scarano and Martin-Garcia, 2005).

HIV entry into the CNS occurs soon after systemic infection (Davis et al., 1992, An et al., 1999). Since the virus is unable to cross the blood brain barrier (BBB) itself, infiltration of the CNS occurs through a ‘Trojan Horse’ mechanism (Haase, 1986, Gonzalez-Scarano and Martin-Garcia, 2005). This process involves HIV-infected monocytes, some of which cross the BBB to replenish the population of resident macrophages and microglia that carry out immune surveillance in the brain (Gonzalez-Scarano and Martin-Garcia, 2005, Kaul et al., 2005). Once inside the brain, these infected cells serve as viral reservoirs, releasing viral particles, viral proteins, and a host of soluble
factors such as chemokines, cytokines, trophic factors, reactive oxygen species, and excitotoxins into the extracellular milieu (Gonzalez-Scarano and Martin-Garcia, 2005, Kaul et al., 2005). These secreted factors activate and spread infection to nearby microglia and macrophages that in turn, release signaling molecules that further alter the extracellular environment (Gonzalez-Scarano and Martin-Garcia, 2005, Kaul et al., 2005).

Although neuronal loss is observed in HAND, HIV does not infect neurons due to their lack of CD4 receptor expression (Adle-Biassette et al., 1995, Petito and Roberts, 1995). Neuronal death is instead mediated by HIV indirectly through the collection of factors released by the infected/activated macrophages and microglia in the brain (Gonzalez-Scarano and Martin-Garcia, 2005, Kaul et al., 2005). Efforts to determine the neurotoxic contributions of individual factors have produced two potential models: the ‘direct’ model which argues that secreted viral proteins are the major contributors to neurotoxicity (Hesselgesser et al., 1998, Kaul and Lipton, 1999, Ohagen et al., 1999), and the ‘indirect’ model which identifies the inflammatory factors released from activated macrophages and microglia as the key effectors of neuronal demise (Lipton et al., 1991, Achim et al., 1993, Bukrinsky et al., 1995). Although there is debate over the precise identity of the damaging factors, the altered extra-cellular environment triggers HIV-mediated neuronal degeneration and death through neuronal pathways that respond to excitotoxicity and neuroinflammation (Kaul and Lipton, 2004). These pathways involve stimulation of glutamate receptors, protease activation, and upregulation of components of the cell cycle machinery (Jordan-Sciutto et al., 2002a, Jordan-Sciutto et al., 2002b,
Garden et al., 2004, Kaul and Lipton, 2006, O'Donnell et al., 2006, Akay et al., 2011).
The subsequent sections will focus on some of these mechanisms as they relate to this body of work.

1.2 CALPAIN STRUCTURE, REGULATION AND FUNCTION IN NEURONS

Calpains are a family of cytoplasmic cysteine proteases that are activated by Ca$^{+2}$ (Liu et al., 2008). They function as a major proteolytic system within the cell alongside the proteasome and autophagic lysosomal proteases. However, unlike these proteolytic systems, calpains do not degrade their substrates but instead proteolytically process them, leaving a truncated product that is either inactivated or functionally altered. There are currently 15 known Calpain isoforms in the human genome. These isoforms are broadly classified into two groups based on their domain structure: “classical” and “non-classical” calpains. Classical calpains contain four domain regions, (1) an N-terminal anchor helix region, (2) a catalytic CysPc protease domain that consists of two protease core domains PC1 and PC2, (3) a C2 domain-like domain termed C2L that helps stabilize the protease core, and (4) a penta-EF-hand that binds Ca$^{2+}$ at the C-terminus (Figure 1). Non-classical calpains lack the C2L domain and/or the penta-EF-hand domain.
Figure 1.1. Schematic of domain structure for calpain-1 and calpain-2. The domain organization of the calpain-1 and calpain-2 heterodimers are laid out. The classical calpain domain structure is exemplified by the large catalytic subunit schematic. An N-terminal helix segment, the protease core domain divided into subdomains PC1 and PC2; the C2-like domain C2L; and the penta-EF-hand domains of the large and small subunits PEF(L) and PEF(S) respectively. The small regulatory subunit also contains a glycine-rich (GR) domain at its N-terminus. Domain boundaries are indicated by residue numbers flanking each domain. Adapted from Campbell and Davies, 2012.
The best characterized calpain isoforms are calpain-1 and calpain-2, two classical calpains that are ubiquitously expressed and heterodimerize with a small regulatory subunit encoded by CAPN1/CAPN4 to form functional proteases. Both isoforms share the same domain architecture, contain ~60% sequence homology, and exhibit virtually identical substrate specificity \textit{in vitro}. However, they display different \textit{in vitro} requirements for Ca\textsuperscript{2+} in order to become activated. Half-maximal activity of calpain-1 has been shown with \textit{in vitro} Ca\textsuperscript{2+} concentrations of 3-50 uM, while calpain-2 achieves half-maximal activity within a [Ca\textsuperscript{2+}] range of 0.4-0.8 mM (Cong et al., 1989, Kapprell and Goll, 1989). Hence, calpain-1 and calpain-2 are often referred to as μ-calpain (for μM [Ca\textsuperscript{2+}]) and m-calpain (for mM [Ca\textsuperscript{2+}]), respectively. Although the penta-EF hand domains of both calpain-1 and calpain-2 bind Ca\textsuperscript{2+} ions, their primary function is to serve as the heterodimerization interface for the small regulatory subunit (Kretsinger, 1997, Maki et al., 2002). In fact, the Ca\textsuperscript{2+} binding of this domain does not trigger structural changes within the protease domain to turn the protease “on” or “off,” but rather provides structural support for the protease domain when it adopts an active conformation (Hanna et al., 2008, Moldoveanu et al., 2008). Instead, cooperative Ca\textsuperscript{2+} binding at two sites within the catalytic CysPc protease domain – one site each in the PC1 and PC2 core domains – is responsible for protease activation (Dainese et al., 2002, Moldoveanu et al., 2002, Moldoveanu et al., 2004). Ca\textsuperscript{2+} binding at these sites causes a conformational change within CysPc, bringing PC1 and PC2 closer together and positioning the catalytic cysteine, histidine and asparagine residues of the active site within the necessary proximity for proteolytic cleavage of the substrate protein (Moldoveanu et al., 2004).
The *in vitro* Ca\(^{2+}\) requirements for calpain-1 and especially calpain-2 activity are orders of magnitude higher than the nanomolar Ca\(^{2+}\) concentration inside a resting cell. Given this barrier to activation, several mechanisms have been proposed to lower the [Ca\(^{2+}\)] requirement and allow calpain activation *in vivo*. One mechanism that has been investigated is post-translational modification of calpain. Calpain-2 has been shown to be activated as a result of serine phosphorylation by ERK/MAPK. This activation was reported in cultured murine fibroblasts during epidermal growth factor (EGF)-induced cell motility and de-adhesion in the absence of intracellular Ca\(^{2+}\) changes (Glading et al., 2004). Similarly, calpain-2 activation in neurons was also achieved by MAPK phosphorylation in response to both brain-derived neurotrophic factor (BDNF) and EGF signaling (Zadran et al., 2010). Another possible activation mechanism involves calpain binding to phospholipids along the plasma membrane. Several studies have highlighted the ability of calpain-1 and calpain-2 to bind polyphosphoinositides such as phosphoinositide biphosphate (PIP\(_2\)) via their C2L domains (Saido et al., 1992, Tompa et al., 2001, Shao et al., 2006). This interaction was shown to significantly lower the calcium requirement for protease activation (Saido et al., 1992). This mechanism is particularly attractive given the convenient location of phospholipids to plasma membrane ionotropic receptors, and Ca\(^{2+}\) channels on the ER and mitochondrial surface that facilitate rapid spikes of cytosolic [Ca\(^{2+}\)]. By ensuring that calpain localizes near the center of these spikes, the cell can achieve rapid, brief periods of calpain activity in a spatially restricted fashion.
To date, the only endogenous specific inhibitor of calpains that has been identified is a protein called calpastatin. One calpastatin molecule contains four inhibitory units that can each bind and inhibit one calpain molecule (Emori et al., 1987, Maki et al., 1987). Despite relatively poor calpastatin sequence conservation across species, the inhibitory effect on calpain is highly specific. Co-crystallization of calpastatin and calpain-2 has shown that calpastatin binds tightly to Ca\(^{2+}\)-bound calpain while looping out several amino acids from the catalytic cleft, protecting itself from calpain cleavage (Moldoveanu et al., 2008). Given that physiological activation of calpain is brief and that calpastatin knockout mice lack a phenotype under basal conditions, it seems likely that the inhibitory function of calpastatin is primarily involved during periods of stress to prevent prolonged over-activation of calpain (Takano et al., 2005).

When transiently activated, calpains function in a diverse set of processes including cell adhesion and cell migration (Chan et al., Wiemer et al., Franco et al., 2004), muscle contraction (Whidden et al., Verburg et al., 2009), and cytoskeletal dynamics (Franco et al., 2004, Wang et al., 2005). Calpain-1 and calpain-2 are ubiquitously expressed, and their deficiency through targeted deletion of the small regulatory subunit CAPN4/CAPNS1 causes embryonic lethality in mice (Arthur et al., 2000, Zimmerman et al., 2000). Both calpain isoforms are particularly abundant in the CNS and show expression in both neurons and glia (Goll et al., 2003). Given the Ca\(^{2+}\) influx in postsynaptic compartments mediated by NMDA receptors during synaptic transmission, a role for calpain in synaptic plasticity was hypothesized and has been studied for several decades. These studies have shown that induction of long-term
potentiation (LTP) by high frequency burst stimulation is blocked by application of calpain inhibitors such as leupeptin in both acute hippocampal slices and in vivo (Staubli et al., 1988, Oliver et al., 1989, Denny et al., 1990). Furthermore, rats carrying a genetic deficiency in calpastatin show enhanced induction of hippocampal LTP following high frequency stimulation, suggesting that synaptic strength can be increased by promoting calpain activity (Muller et al., 1995).

While the mechanism for this effect on synaptic potentiation is not fully elucidated, calpains have been shown to cleave numerous synaptic and cytoskeletal proteins that help establish LTP. Among them is the first identified calpain substrate αII-spectrin, a neuronal cytoskeleton adaptor that is anchored to the plasma membrane and binds actin, calmodulin and microtubules (Siman et al., 1984). Cleavage of αII-spectrin has been shown to alter membrane domain organization and influence membrane trafficking events (Dosemeci and Reese, 1995). Calpains also proteolytically process membrane receptors responsible for synaptic transmission and plasticity such as AMPA receptors (AMPARs) and NMDA receptors (NMDARs) (Bi et al., 1997, Wu et al., 2005, Dong et al., 2006), synaptic scaffolding proteins PSD95 and SAP97 that are involved in trafficking and anchoring of AMPARs (Lu et al., 2000, Jourdi et al., 2005), and postsynaptic kinases such as CamKII and Protein Kinase C that influence AMPAR and NMDAR conductance through receptor phosphorylation (Hrabetova and Sacktor, 1996, Hajimohammadreza et al., 1997). Finally, conditional disruption of calpain-1 and calpain-2 in the mouse CNS via CAPNS1 deletion revealed reduced dendritic branching complexity, lower spine density and deficits in LTP and memory, providing further
evidence of calpain involvement in regulating neurotransmission and synaptic plasticity (Amini et al., 2013).

In addition to these physiological effects, calpains can have a detrimental impact on cell viability when over-activated. While physiological activation of calpain is brief, sustained dysregulation of calcium homeostasis during many neurodegenerative conditions – specifically those involving excitotoxicity – leads to prolonged calpain activation and cleavage of a wide range of substrates (Vosler et al., 2008). These cleaved substrates are either rendered inactive or display an altered function that contributes to disease pathogenesis. A well-studied example of these calpain-mediated functional alterations is the processing of neuronal kinase cyclin-dependent kinase 5 (CDK5), a protein involved in neuronal maturation and migration that is required for proper development of the mammalian cortex (Dhavan and Tsai, 2001, Tanaka et al., 2001). CDK5 activity is regulated through its interaction with p35, a small protein that serves as a regulatory subunit for the kinase (Tsai et al., 1994). During pathological calpain activation, p35 is proteolytically cleaved to a smaller isoform p25, which aberrantly activates CDK5 and causes off-target phosphorylation of substrates such as NMDARs, the peroxidase Prx2, the cell cycle regulator Retinblastoma protein (Rb), and other disease-related proteins such as the ubiquitin ligase Parkin and the cytoskeletal protein Tau (Lopes and Agostinho, 2011).

Sustained calpain activation has been reported in a number of chronic neurodegenerative states such as Alzheimer Disease (AD) (Saito et al., 1993, Kelly et al., 2005), Parkinson’s Disease (PD)(Esteves et al., 2009), and Huntington’s Disease
(HD)(Gafni and Ellerby, 2002, Gafni et al., 2004) as well as acute conditions such as cerebral ischemia (Gascon et al., 2008). In AD, calpain activity has been a major focus of investigation. The protein Tau is best known for its involvement in AD neuropathology, producing intraneuronal aggregates termed ‘neurofibrillary tangles.’ Tau was first identified as a calpain substrate through cell-free studies, and later shown to be proteolytically processed by calpain-1 into a 17-kiloDalton fragment in cultured hippocampal neurons following treatment with Aβ oligomers (Yang and Ksiezak-Reding, 1995, Park and Ferreira, 2005). Although initially identified as neurotoxic, a follow-up report suggests that the 17-kiloDalton fragment itself does not affect cell viability (Park and Ferreira, 2005, Garg et al., 2011). Nonetheless, calpain appears to play a key role in disease progression. Immunohistochemical staining of AD post-mortem tissue revealed activated calpain-2 associated with neurofibrillary tangles and amyloid plaques, two of the major pathological features of the disease (Grynspan et al., 1997, Higuchi et al., 2012). Furthermore, manipulations of the calpain system both in cell culture and animal models of AD have been shown to alter disease pathology and neuronal impairments. For instance, mice lacking calpastatin show enhanced Aβ accumulation and Tau hyperphosphorylation (Higuchi et al., 2012). Pharmacological inhibition of calpains in a transgenic mouse model of AD attenuated synaptic dysfunction and cognitive impairment while reducing Aβ plaques and Tau hyperphosphorylation (Sinjoanu et al., 2008). These pathological alterations appeared to result in part from down-regulation of β-secretase 1 leading to reduced production of Aβ40 and Aβ42 and from reduced activation of pathological Cdk5 activity, a kinase known to phosphorylate Tau (Sinjoanu et al., 2008). More recent studies have demonstrated that AD patients exhibit elevated calpain activity
in cerebrospinal fluid, suggesting that calpain activity may have diagnostic value in multi-variate analyses (Laske et al., 2014).

Calpain activation has also been reported in PD, HD and HAND suggesting a widespread involvement in neurodegenerative conditions. The pathological protein aggregates called Lewy Bodies, which are a hallmark of PD, are primarily comprised of fibrillar $\alpha$-synuclein protein (Jadhav et al., 2013). $\alpha$-synuclein serves as a calpain substrate and has been shown to be cleaved by calpain both in its soluble form and as a fibrillized structure \textit{in vitro} and that cleavage of fibrillized $\alpha$-synuclein produces fragments that retain their fibril form and promote co-assembly of soluble $\alpha$-synuclein (Mishizen-Eberz et al., 2003, Mishizen-Eberz et al., 2005). Additionally, site-specific antibodies to calpain-cleaved $\alpha$-synuclein fragments immunolabeled cortical tissue in aged PD transgenic mice and Lewy Bodies in the substantia nigra of PD patients post-mortem (Dufty et al., 2007). Consistent with these findings, $\alpha$-synuclein aggregation was reduced by calpastatin overexpression in $\alpha$-syn transgenic mice, leading to reduced $\alpha$-syn truncation and aggregation and improved maintenance of synaptic integrity (Diepenbroek et al., 2014).

In the genetic neurodegenerative disease HD, a mutant form of huntingtin (mHtt) protein containing poly-glyutamine (polyQ) repeat expansion shows a propensity to form intraneuronal inclusions in the striatum. These inclusions are comprised at least in part of N-terminal fragments of mHtt that have been proteolytically cleaved (Martindale et al., 1998). Several reports have identified calpains as proteases responsible for this cleavage (Gafni and Ellerby, 2002, Gafni et al., 2004). Calpain-resistant forms of mHtt were
shown to attenuate toxicity produced by calpain activation in Htt-overexpressing cells (Gafni et al., 2004). In addition, post-mortem tissue analysis of HD patients revealed elevated calpain activity in the caudate nucleus, pointing to a role for calpain in HD pathology (Gafni and Ellerby, 2002).

Finally, a number of studies have implicated calpains in HAND. Using an *in vitro* model of HIV-induced neurotoxicity consisting of rat primary neuronal cultures treated with conditioned media from HIV-infected macrophages, we and others have shown that HIV-mediated neuronal damage is prevented by inhibiting NMDAR stimulation and calpain activation (O'Donnell et al., 2006, Wang et al., 2007, White et al., 2011). Investigation of the effect of HIV infection on CDK5 activation in cultured neurons revealed increased levels of the calpain-cleaved CDK5 activator p25 and higher CDK5 activity following addition of HIV conditioned media (Wang et al., 2007). Similarly, examination of midfrontal cortex autopsy tissue from patients with HAND revealed elevated p25 levels, further suggesting that calpain activation in neurons is increased following HIV infection (Wang et al., 2007). In this same *in vitro* model, the pro-survival protein MDMx was shown to be degraded by calpain in neurons treated with HIV-macrophage supernatants, causing neurotoxicity that was partially rescued by MDMx overexpression (Colacurcio et al., 2013). Finally, in a separate model of HIV-mediated neuronal damage, the HIV protein TAT was shown to activate calpain via ryanodine receptors, causing neuronal death that was attenuated using pharmacological inhibitors to ryanodine receptors and calpain (Perry et al., 2010). Together, these data provide a
compelling argument for the involvement of calpain activation in the neuronal damage of HAND.

Given this widespread involvement in neuronal damage, understanding the pathological activities of calpain and identifying its pathological substrates could provide insights into neuronal loss that are applicable to many diseases. Toward that end, several studies have attempted to uncover a preferred sequence for calpain cleavage (Tompa et al., 2004, Cuerrier et al., 2005). However, results have been inconclusive, and it now seems likely that calpain cleavage involves recognition of secondary or tertiary features, making substrate and cleavage site predictions more difficult. In spite of this challenge, understanding the various roles of calpain substrates and the functional implications of their proteolytic cleavage will provide critical insights into disease mechanisms and potential therapeutic strategies.

1.3 CASPASES: ROLES IN NEURONAL PHYSIOLOGY AND NEURONAL DEATH

Caspases are a family of cysteine proteases best known for their role in mediating a type of programmed cell death called apoptosis that is characterized by DNA fragmentation and membrane blebbing. Caspases cleave their target substrates at tetrapeptide motifs where aspartic acid is in the P1 position. As with calpains, caspases are post-translationally regulated to allow for their rapid activation. They are translated as inactive zymogens called procaspases and contain a large subunit, a small subunit, and an N-terminal prodomain that must be proteolytically cleaved for enzyme activation. While
some caspases are primarily involved in immune regulation and cytokine processing, those that are implicated in apoptosis are typically classified into two groups – initiator caspases and executioner caspases. Initiator caspases (caspase-2, -8, -9, -10) are the first line of caspases to be activated following a death stimulus. They contain long N-terminal prodomains that facilitate interactions with specific adaptor proteins. These adaptors recruit and complex with the initiator caspases, allowing initiator caspase to aggregate and auto-activate through homodimerization and self-cleavage. Once activated, initiator caspases cleave and activate a second line of caspases termed executioner caspases (caspase-3, -6, -7). These executioner caspases then go on to cleave a host of protein substrates that promote cell death.

The above cascade can be triggered in response to a variety of pro-death stimuli, and the initial stimulus determines the identity of both the adaptor proteins and initiator caspases that participate. The influence of the initial stimulus can best be illustrated by comparing the two major apoptosis pathways that utilize the caspase cascade: the extrinsic pathway and intrinsic pathway (Figure 2). The extrinsic pathway is initiated by binding of ligands such as Fas ligand or tumor necrosis factor alpha (TNFα) to ‘death receptors’ on the cell surface (Lavrik et al., 2005). Death receptor binding causes receptor clustering followed by recruitment of several adaptors such as Fas-associated protein with Death Domain (FADD), TNF receptor associated protein 2 (TRAF2), and cellular inhibitor of apoptosis proteins (cIAP1 and cIAP2) to form the death-inducing signaling complex (DISC). DISC formation, in turn, promotes binding and activation of initiator caspase-8 and caspase-10, leading to activation of the executioner caspases.
In the intrinsic (also called mitochondrial) pathway of apoptosis, cellular stresses such as growth factor deprivation, DNA damage, or developmental death signals cause mitochondrial membrane permeabilization and subsequent release of mitochondrial cytochrome c into the cytosol. This release promotes assembly of a protein complex called the ‘apoptosome, which consists of cytochrome c, apoptosis peptidase activating factor 1 (Apaf-1), and dATP. The apoptosome recruits and activates initiator caspase-9, leading to downstream activation of executioner caspases such as caspase-3 and ultimately cell death.
Figure 1.2. Schematic of Apoptotic Pathways. During intrinsic pathway activation, cellular stresses induce mitochondrial membrane permeabilization and cytochrome c release. Together with Apaf-1, cytochrome c forms the multimeric ‘apoptosome’ that recruits and activates caspase-7, which in turn activates executioner caspase-3 to carry out cell death. In the extrinsic pathway, a death receptor ligand binds to the death receptor, triggers formation of a membrane-bound Death-Inducing Signaling Complex (DISC) involving the death receptor, adaptor proteins such as FADD, and recruitment of procaspase-8. Activation of caspase-8 leads to caspase-3 activation and apoptosis. In some cell types, caspase-8 can also cleave pro-apoptotic protein Bid to tBid (dashed arrow), which interacts with Bax/Bak to trigger mitochondrial membrane permeabilization and induction of the intrinsic pathway.
Caspase-mediated cell death plays an important role in the developing nervous system. Mice deficient in either caspase-3, caspase-9, or Apaf-1 display embryonic or perinatal lethality marked by exencephaly (Cecconi et al., 1998, Kuida et al., 1998, Pompeiano et al., 2000). This phenotype is due to reduced apoptosis in immature neurons and in neural progenitors, leading to their excessive amplification. Cell culture studies have also implicated caspase-dependent pathways in neuronal death following trophic factor deprivation. In cultured sympathetic neurons, nerve growth factor deprivation leads to apoptosis that can be blocked by caspase inhibitors (Deshmukh et al., 1996, McCarthy et al., 1997). Programmed cell death has also been identified in regulating adult neurogenesis, the production of new postmitotic neurons in the adult brain. For instance, adult mice with genetic deficiency of BAX, a pro-apoptotic protein involved in mitochondrial release of cytochrome c, show an increased number of new neurons in the dentate gyrus and subcallosal zone, resulting from a reduced rate of programmed cell death among adult-generated neurons (Sun et al., 2004, Kim et al., 2011).

Aside from their traditional roles in cell death, caspases have been implicated in a number of non-apoptotic cellular processes including cell differentiation, proliferation and inflammation (Yi et al., 2009). In the nervous system, it is now appreciated that caspases influence neuronal connectivity through several mechanisms including axon guidance, synaptic plasticity and dendritic pruning (Hyman and Yuan, 2012). For instance, Casp9-/− mice exhibit aberrant olfactory sensory neuron projections to the olfactory bulb, possibly due to disruption of semaphorin 7A cleavage by caspase-9 at the axon surface (Ohsawa et al., 2009, Ohsawa et al., 2010). In hippocampal CA1 neurons from caspase-3 -/- mice, experimentally induced NMDAR-dependent long-term
depression (LTD) is impaired, suggesting that caspase activation participates in synapse elimination and dendritic spine shrinkage (Li, Jia, Jo, 2010). Additionally, pharmacological inhibitors of caspases also block LTD and were shown to reduce AMPA receptor internalization, providing a potential mechanism for this caspase-mediated effect on synaptic transmission (Li et al., 2010). Caspase activation has also been demonstrated to contribute to dendritic pruning. Through an elegant optogenetic approach that activates the intrinsic apoptotic pathway in dendrites, localized activation of caspase-9 and caspase-3 led to dendritic retraction and spine shrinkage while sparing the neurons from cell death (Erturk et al., 2014). These findings highlight the importance of caspases in neuronal physiological function and provide an attractive mechanism of localized caspase activation to carry out non-apoptotic functions in the CNS.

Given the slow progression and chronic nature of most neurodegenerative conditions, it is thought to be unlikely that the typically rapid cell death brought on by classical apoptotic cascades could contribute to disease pathogenesis. However, evidence of caspase activation in a number of neurodegenerative disorders has been clearly documented. Caspase activity has been shown both in tissue from Alzheimer Disease (AD) patients and in cell culture and animal models of the disease. For instance, caspase-cleaved Tau fragments have been detected in neurofibrillary tangles and dystrophic neurites of hippocampal tissue from AD patients (Gamblin et al., 2003, Rissman et al., 2004). Furthermore, cortical neuronal cultures treated with fibrillar Aβ42 display caspase activation and the same Tau fragments observed in vivo, and these caspase-cleaved Tau products accelerate Tau filament assembly in vitro (Gamblin et al., 2003). In transgenic mice overexpressing Tau, caspase activation was shown to precede neurofibrillary tangle
formation, again suggesting that caspases play a role in catalyzing AD pathology (de Calignon et al., 2010).

Reports of caspase involvement in neurodegeneration are not restricted to AD studies, but extend to other diseases as well. In Huntington’s disease (HD), striatal neurodegeneration is accompanied by intraneuronal aggregation of toxic N-terminal fragments of mutant huntingtin (mHtt) protein. Caspase-3 and Caspase-6 have both been shown to cleave mHtt and produce N-terminal fragments found in HD post-mortem brain tissue (Kim et al., 2001, Warby et al., 2008). Furthermore, caspase inhibition ameliorates mHtt toxicity in several cell models of mHtt toxicity (Wellington et al., 2000, Leyva et al., 2010). Caspase processing is also thought to play a role in regulating the DNA- and RNA-binding protein TDP-43, a nuclear protein that redistributes to the neuronal cytosol and forms inclusions in a number of neurodegenerative diseases including ALS, Traumatic Brain Injury, and Frontotemporal dementia (Blennow et al., 2012, Baralle et al., 2013). Although a mechanism for TDP-43-mediated neurotoxicity is still not fully understood, several studies have identified caspase truncation of TDP-43 as enhancing aggregation and cellular toxicity, providing a possible link between TDP-43 pathology and neuronal damage in these diseases (Zhang et al., 2009, Yang et al., 2010, Yang et al., 2014). Despite the growing number of studies implicating caspase activity in neurodegenerative states, further studies are warranted to determine whether caspases in mature neurons function within a classical apoptotic pathway or if local, transient activation of caspases are enacted for non-canonical processes.
1.4 E2F1: STRUCTURE, REGULATION AND FUNCTION

E2F1 is a member of the E2F family, a group of nine transcription factors best known for coordinating a transcription program that underlies cell cycle. The E2Fs are commonly divided into two subclasses based on their transcriptional regulatory properties: (1) “activator” E2Fs (E2F1-3a) which function as transcriptional activators and (2) “repressor” E2Fs (E2F3b-8) which repress transcription of target genes (DeGregori and Johnson, 2006). While all E2Fs share a highly homologous ‘winged helix’ DNA binding domain (Zheng et al., 1999), they display differences in their regulatory binding partners, functional capabilities and other structural features.

Human E2F1 protein is 437 amino acids in length. Located within the first 200 amino acids is the ‘winged helix’ DNA binding domain (Zheng et al., 1999) (Figure 3). Next to the DNA binding domain is a dimerization domain, which allows E2F1 to heterodimerize with its DNA binding partner DP1. This interaction augments both DNA binding of E2F1 and transcriptional activation at target promoters (Helin et al., 1993, Bandara et al., 1994). At the C-terminus, E2F1 contains a transactivation domain, which promotes target gene transcription by recruiting the basal transcription subunit TFIID and several co-activators including acetylase p300/CBP to target promoters (Trouche et al., 1996, Ross et al., 1999, Lang et al., 2001). p300/CBP boosts E2F1-mediated transcription both by increasing chromatin accessibility via histone acetylation and by acetylating E2F1 itself, leading to enhanced DNA binding and E2F1 stability (Martinez-Balbas et al., 2000). Embedded within the transactivation domain is the pRb binding motif. pRb binding to this motif during quiescence or early in the G1 phase masks the E2F1
transactivation domain, effectively repressing E2F1 activity (Helin et al., 1993). When pRb is phosphorylated by cyclin D:cdk4/6 just prior to S phase, pRb-E2F1 dissociates and E2F1 repression is relieved, allowing transcriptional upregulation of target genes. Finally, E2F1 also contains a cyclin A binding domain near its N-terminus (Xu et al., 1994). Following S phase entry, this domain is bound by the cyclin A:cdk2 complex, inhibiting E2F1 binding to DNA (Krek et al., 1994, Krek et al., 1995). As the cell completes DNA replication, cyclin A:cdk2-bound E2F1 is ubiquitinated by the Skp1/Cul1/Skp2 ubiquitin ligase and subsequently degraded by the proteasome to allow entry to G2 (Marti et al., 1999). Finally, E2F1 contains nuclear localization signals at the N- and C-terminus that specify E2F1 nuclear targeting (Ivanova et al., 2007).
Figure 1.3. E2F1 domain organization. Pictured are the domains of E2F1. Cyclin A binding domain which facilitates cyclinA:cdk2 binding to E2F1, inhibiting E2F1 DNA binding and targeting E2F1 for proteasomal degradation, (aa 67-108), DNA binding (aa 119-191) which contacts the nucleotides and the DNA backbone directly, Dimerization/Heptad Repeat (aa 201-243) which binds to the transcriptional co-factor DP1 to promote DNA binding, Marked Box (aa 245-317) domain which enhances E2F1-DP1 dimerization, Nuclear localization signals (aa 85-91, 423-428) which retain E2F1 in the nucleus, Transactivation domain(aa 368-437) which facilitates interactions with basal transcription machinery or pRB binding through a pocket binding motif (aa 409-426). The blue horizontal bar maps the epitope that the KH95 E2F1 antibody detects (aa 342-386).
The eukaryotic model of cell division involves the temporal regulation of a host of cell cycle proteins. For the cell cycle to proceed normally, cyclins and cyclin-dependent kinases (CDKs) must coordinate their activity in a periodic and ordered fashion. The transcription factor E2F1 also takes part in this sequential regulation along with its repressor Retinoblastoma protein (pRb). In quiescence and in the early stages of the cell cycle, E2F1 is bound and repressed by pRb, preventing transactivation of target genes. During the G1 phase, pRb is phosphorylated by the cyclin D:cdk4/6 and cyclin E:cdk2 complexes, leading to dissociation of the repressive pRb-E2F1 complex (Giacinti and Giordano, 2006). E2F1 can then activate its targets, prompting S phase entry and DNA replication.

In mitotic cells, E2F1 expression is normally triggered by the presence of growth-promoting mitogens that stimulate cell cycle entry (DeGregori and Johnson, 2006). However, maximal expression of E2F1 occurs late in G1 when E2F1 must transactivate target genes involved in DNA replication such as Pol-α, DHFR, and PCNA (DeGregori and Johnson, 2006). Because an E2F consensus site is located within its promoter, E2F1 is also able to upregulate its own mRNA levels, creating a positive feedback loop for its expression (Iaquinta and Lees, 2007). Translational control of E2F1 occurs as well, most notably through miR 17-5p and 20a, which inhibit E2F1 protein synthesis during early G1 (Pickering et al., 2009).

Aside from promoting cell proliferation, E2F1 functions as a potent and versatile inducer of apoptosis when DNA is irreparably damaged and in other contexts (DeGregori and Johnson, 2006). In such cases, E2F1 is stabilized by phosphorylation and protected
from degradation through an interaction with the phospho-serine binding protein 14-3-3τ (Wang et al., 2004, Iaquinta and Lees, 2007). As E2F1 accumulates, it can initiate apoptosis through direct transcriptional activation of apoptotic targets such as APAF-1, BAX, Puma, Bim, caspase 3, 7-9, and p14ARF. E2F1-mediated transcription can also trigger apoptosis through stabilization of the pro-apoptotic transcription factor p53 (Bates et al., 1998). p53 is typically maintained at low levels in the cell by the E3 ubiquitin ligase Mdm2, which shuttles p53 to the cytoplasm where it is degraded by the proteasome. Following E2F1 transactivation of p14ARF, p14ARF sequesters Mdm2, causing p53 to accumulate and activate apoptotic targets (Bates et al., 1998, Putzer, 2007). E2F1 can also promote cell death independently of p53 by transactivation of the p53 homolog, p73 (Irwin et al., 2000, Stiewe and Putzer, 2000). Alternatively, E2F1 can trigger cell death through several transcription-independent mechanisms: (1) directly binding p53 through the cyclin A binding domain (Hsieh et al., 2002) (2) reducing levels of an inhibitor of death receptor-mediated apoptosis TRAF2, possibly via reduction of the anti-apoptotic NF-κB (Phillips et al., 1999), and (3) activating the calcium-sensitive cysteine protease calpain, which leads to proteolytic degradation of the Mdm2 homolog and fellow p53 inhibitor, MDMx (Strachan et al., 2005).

1.5 E2F1 IN NEURONS

E2F1 regulation and function as described in the previous section has been studied almost exclusively in cycling cells. Neurons are considered to be permanently postmitotic. Given this designation, neuronal expression of cell cycle proteins might appear unlikely. However, E2F1 is indeed present in nervous tissue, beginning early in brain development.
and increasing in expression through maturation (Kusek et al., 2001). This pattern of upregulation is reproduced in differentiated neural cell lines and primary neuronal cultures (Kusek et al., 2001). In stark contrast to the nuclear localization of E2F1 in proliferating cells, neuronal E2F1 is predominantly cytoplasmic (Wang et al., 2010). Furthermore, neuronal E2F1 exhibits active shuttling between the nucleus and cytosol in a Crm1a-dependent manner (unpublished). We have previously reported that immunostaining of E2F1 in cultured neurons reveals a punctate pattern and that biochemical fractionation of brain tissue reveals enrichment of E2F1 levels in synaptic fractions (Ting et al., 2014). These observations suggest E2F1 may have a novel function in postmitotic neurons. Interestingly, the ‘winged helix’ superfamily of DNA-binding proteins to which E2F1 belongs contains several members that physiologically bind RNA in the cytosol (Intine et al., 2003, Yoshizawa et al., 2005). Furthermore, several studies have demonstrated an ability of E2F1 to bind synthetic RNA species (Ishizaki et al., 1996, Darbinian et al., 2006), suggesting a potential role for E2F1 as an RNA-binding protein in the neuronal cytosol. Despite this intriguing possibility, E2F1 in neurons does retain some of the properties it exhibits in undifferentiated cells. Chief among them is its transcriptional regulation of apoptosis.

E2F1-dependent apoptosis has been studied extensively in neuronal culture preparations (Verdaguer et al., 2007). These studies have demonstrated that E2F1 overexpression induces apoptosis in cultured cerebellar granule neurons, while E2F1 deletion attenuates neuronal death caused by potassium deprivation, kainic acid treatment, and Aβ peptide application (Giovanetti et al., 2000, O'Hare et al., 2000, Smith et
Furthermore, E2F1 induces neuronal apoptosis in a dopamine-evoked toxicity model through a transcription-independent reduction of TRAF2 (Hou et al., 2001).

There are a number of in vivo examples demonstrating that neurons aberrantly upregulate E2F1 and other cell cycle proteins during neurodegenerative disease (Herrup and Yang, 2007). Post-mortem analysis has revealed increased E2F1 expression in the substantia nigra of patients with PD, midfrontal cortex of AD patients, motor neurons of Amyotrophic Lateral Sclerosis patients, and brain tissue from Huntington’s Disease (HD) patients (Jordan-Sciutto et al., 2001, Ranganathan and Bowser, 2003, Hoglinger et al., 2007, Pelegri et al., 2008). Upregulation of phosphorylated pRb has similarly been observed in some of these post-mortem samples (Jordan-Sciutto et al., 2001, Ranganathan and Bowser, 2003). In human brain samples with HIV neuropathology and in simian models of HIV-related neurodegeneration, neuronal E2F1 levels are significantly increased in affected regions in comparison to those of infected patients lacking neuropathology (Jordan-Sciutto et al., 2000, Jordan-Sciutto et al., 2002b). Although neurons have never been observed to undergo complete cell divisions, neuronal DNA replication has been reported to coincide with upregulation of cell cycle proteins in AD and PD (Yang et al., 2001, Hoglinger et al., 2007). The above examples and other reports establish a clear link between the activation of cell cycle machinery and neurodegenerative states. Studying E2F1 and other cell cycle proteins in the context of neurodegeneration could advance our understanding of the neurotoxic mechanisms underlying these diseases.
1.6 RATIONALE AND OBJECTIVES

As a result of its unique transcriptional regulation of the cell cycle and cell death, understanding E2F1-mediated transcriptional regulation has been a major point of emphasis. Initial studies of E2F1 in neuronal culture have focused on its ability to induce caspase-mediated neuronal apoptosis. Investigations into neurodegenerative mechanisms have revealed upregulation of E2F1 in CNS neurons of patients with a variety of neurodegenerative disorders. These observations have provided a theory whereby E2F1 and other cell cycle regulators are aberrantly activated in neurons during neurodegenerative disease. This activation causes post-mitotic neurons to attempt to re-enter the cell cycle and induces caspase-mediated neuronal death, both of which rely on E2F1 participation as a transcription factor. Interestingly, we have previously observed E2F1 neuronal expression to be predominantly cytoplasmic both in vitro and in vivo (Jordan-Sciutto et al., 2002b, Wang et al., 2010). Furthermore, we found that expression of canonical E2F target genes is unaffected in an in vitro model of HIV-induced neurotoxicity (Wang et al., 2010). Our past findings suggest that neuronal E2F1 may fulfill an alternate role to its traditional actions as a transcription factor.

To explore this possibility, we first wanted to understand the availability of different death pathways to neurons as they mature (Chapter 2). We examined the relative contribution of two common death pathways: caspase-dependent apoptosis and calpain-mediated cell death. We found that while both caspase- and calpain-mediated death pathways were activated in neurons at an early age in culture, only calpain-mediated death was observed as neuronal cultures matured. This developmental shift in death
pathway availability was associated with an upregulation of calpain-1 (μ-calpain) protein levels and a downregulation of executioner caspase-3 protein expression.

Next, we sought to examine the role of E2F1 in the neuronal damage of HAND (Chapter 3). Utilizing an in vitro model of HIV-induced neurotoxicity, we assessed the effect of E2F1 gene disruption on HIV-induced neuronal loss. We found that neuronal damage and death was significantly attenuated in neurons expressing mutant E2F1 protein compared to wildtype cultures. Furthermore, we identify E2F1 as a novel calpain substrate that is cleaved into a stable fragment in primary cortical cultures following treatment with HIV-macrophage conditioned media, suggesting that calpain-cleaved E2F1 may serve a neurotoxic role. Additionally, we demonstrate cell type-dependent processing of E2F1 by calpain, suggesting a novel mechanism of calpain regulation.

In Chapter 4, we aimed to explore the possibility that E2F1 functions as an RNA-binding protein in neurons. DNA binding domain To investigate putative RNA binding functions of E2F1 in the cytosol, we outline preliminary studies examining the RNA-binding capability of E2F1 by attempting to define the key residues within the DNA binding domain that govern the E2F1-RNA interaction.

Finally in Chapter 5, we provide a summary of our findings, an interpretation of their significance within the context of previous research, and their implications for future studies. We highlight potential areas for further examination, technical considerations for future experiments, and a discussion of clinical utility.
1.7 REFERENCES


CHAPTER 2

Differential roles for caspase- and calpain-mediated cell death in one-week-old and three-week-old rat cortical cultures

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2.1 ABSTRACT

Necrosis and apoptosis are well established as two primary cell death pathways. Mixed neuroglial cultures are commonly used to study cell death mechanisms in neural cells. However, the ages of these cultures vary across studies and little regard is given to how cell death processes may change as the cultures mature. To clarify whether neuroglial culture age affects cell death mechanisms, we treated 1-week-old and 3-week-old neuroglial cultures with either the excitotoxic stimulus, N-methyl-D-Aspartate (NMDA), or with the oxidative stressor, hydrogen peroxide (H₂O₂). While NMDA is known to be toxic only in cultures that are at least 2 weeks old, H₂O₂ is toxic in cultures of all ages. Here we confirm that, in 1-week-old neuroglial cultures, NMDA does not induce toxicity, while H₂O₂ induces both calpain-mediated and caspase-mediated neuronal death. In 3-week-old cultures, NMDA and H₂O₂ both trigger calpain-mediated, but not caspase-mediated, neuronal death. Further, we observed a decline in caspase-3 levels and an increase in calpain levels in untreated neuroglial cultures as they aged. Our findings presented here show that neuronal cell death mechanisms vary with culture age and highlight the necessity of considering culture age when interpreting neural cell culture data.
2.2 INTRODUCTION

Neurons undergo cell death during both development and disease. Classically, cell death falls under two major categories. Apoptosis, considered a programmed cell death, is classified by specific cellular morphological changes such as membrane blebbing and nuclear fragmentation (Edinger and Thompson, 2004). The second major form of cell-death is necrosis, classically defined as a passive and unregulated event (Edinger and Thompson, 2004). However, some reports have suggested that necrosis can also occur as part of a coordinated cell death program comparable to apoptosis and is not an uncontrolled event (Edinger and Thompson, 2004, Festjens et al., 2006).

Caspases, a family of aspartate-specific cysteine proteases, are activated via proteolytic cleavage and are well-characterized for their role in apoptosis (Wyllie, 1997). Caspases fall into one of two groups: initiator caspases (8, 9, and 10) and their downstream targets, executioner caspases (3, 6, and 7). Activation of the initiator caspases serves to initiate apoptosis and can be triggered either extracellularly via stimulation of the Fas receptor and tumor necrosis factor receptor or intracellularly via the release of cytochrome c from mitochondria (Wyllie, 1997). The initiator caspases function to activate caspase-3 or another executioner caspase, which then executes apoptosis (Wyllie, 1997).

Calpain is a ubiquitously expressed Ca\(^{2+}\)-dependent neutral protease. While apoptosis is classically considered to be under the purview of the caspase family, calpain is now believed to play a role in apoptosis under certain conditions (O'Donovan et al., 2001). Calpain is also activated in neurological diseases in instances of both necrotic and apoptotic cell death (Heron et al., 1993, Bonfoco et al., 1995). Calpain is maintained as
an inactive proenzyme until a spike in cytosolic free Ca^{2+} concentration triggers its activation. Cleavage targets of activated calpain comprise many enzymatic, signaling, and cytoskeletal proteins, including the neurofilament proteins, tau and tubulin (Ray and Banik, 2003). Importantly, calpain activity contributes to neuronal death in multiple neuropathological conditions, such as spinal cord injury (Ray and Banik, 2003), cerebral ischemia (Rami, 2003), and Alzheimer disease (Nixon et al., 1994).

Attempts at uncovering the relative roles of caspases and calpains in neuronal death have been complicated by the post-mitotic nature of neurons. Unlike cycling cells, cultured neurons differentiate and mature as they age. Indeed, it is well-documented expression of certain proteins change as neuronal cultures age in vitro. For example, NMDA glutamate receptor expression is absent until approximately 14 days in vitro (DIV) and its subunit composition changes between 14 DIV and 21 DIV (O'Donnell et al., 2006). In previous studies of neuronal death, culture age has varied widely and the contributions of these age variations were not considered. Here, we examine the involvement of caspases and calpains in 1-week-old and 3-week-old neuroglial cultures responding to two different toxicities: NMDA, a toxicity known to depend on culture age, and hydrogen peroxide, which is toxic to neuronal cultures regardless of age (Nakamichi et al., 2005).

2.3 MATERIALS AND METHODS

*Preparation of primary neuronal cultures.* Primary rat neuroglial cortical cultures were prepared from embryonic day 17 Sprague–Dawley rat fetuses as described previously (Brewer, 1995). Cells were plated in dishes pre-coated with poly-L-lysine (Peptides
International, Louisville, KY, USA) at a density of $6 \times 10^6$ cells per 100mm dish or $4 \times 10^4$ cells per well in 96-well plates and were maintained at 37°C, 5% CO$_2$ in neurobasal media (Invitrogen) with B27 supplement (Invitrogen). Neurons make up 90% of the total cells under these culture conditions [13].

**MAP2 cell-based ELISA assay.** Primary rat cortical neuroglial cultures were plated in 96-well plates. Neuronal death and damage were assessed using a MAP2 cell-based ELISA assay, as described previously (White et al.). The reliability of the ELISA as an indicator of cell death was verified by traditional hand counting of MAP2-positive cells (White et al.).

**Western blotting of primary cell cultures.** Whole cell extracts were prepared from primary rat cortical cultures and were subjected to Western blotting. Antibody dilutions are as follows: calpain-cleaved spectrin, 1:15,000 (gift from David Lynch, University of Pennsylvania); u-calpain, 1:500 (#2556, Cell Signal, MA), caspase-3, 1:500 (#06735, Millipore, MA); caspase-9, 1:500 (#04443, Millipore, MA); caspase-8, 1:250 (AB1879, Millipore, MA); cleaved caspase-3, 1:250 (#9661, Cell Signal, MA).

**Statistical Analysis.** Differences between test groups were examined by one-way analysis of variance, with a threshold for significance of $p<0.05$. 

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2.4 RESULTS

**Calpain, not caspase, mediates NMDA-induced neurotoxicity in 3-week-old cultures**

To elucidate age-related differences in neuronal death processes, we treated 1- and 3-week-old mixed rat cortical cultures with varying doses of NMDA, the chemical agonist of the glutamate NMDA receptor. First, we examined cultures for neuronal damage and death using a cell-based (CB)-ELISA to detect the neuronal marker protein, microtubule-associated protein 2 (MAP2). We found that, as expected, 1-week-old cultures showed no loss of MAP2 fluorescence when treated with NMDA, while 3-week-old cultures exhibited a significant decrease in MAP2 levels at all NMDA dosage levels (Figure 2.1A). This decrease in MAP2 fluorescence was rescued by pretreatment with the calpain inhibitor MDL28170 (MDL), but not pan-caspase inhibitor qVD-OPH (OPH). As a positive control for protection, we also pretreated control cultures with the NMDA receptor antagonist, MK801, which completely blocks NMDA-induced toxicity. The result was confirmed by counting the number of MAP2-positive cells (Figure 2.1B).

To verify our pharmacological inhibitor data, we examined levels of calpain-cleaved spectrin and executioner caspase-3 in our treated cultures to assess activation of each protease. As expected, we saw no increase in cleaved caspase-3 levels or in calpain-cleaved spectrin levels in 1-week-old cultures following treatments with 3, 10, 30, or 100µM NMDA (Figure 2.1C). In 3-week-old cultures, NMDA treatment induced an increase in calpain-cleaved spectrin at all treatment doses. However, there was no increase in cleaved caspase-3 levels at any dose of NMDA treatment. Taken together, these data suggest that calpain, rather than caspase-3, mediates NMDA-induced neuronal death in 3-week-old cultures.
Caspase and calpain activity both contribute to H$_2$O$_2$-induced toxicity in 1-week-old cultures, while toxicity in 3-week old cultures is mediated via calpain alone

We next treated 1- and 3-week-old cultures with H$_2$O$_2$ to examine neuronal death processes following an insult known to induce toxicity in neuronal cultures regardless of age (Nakamichi et al., 2005). Specifically, we treated cortical neuroglial cultures with 30, 100, or 300µM H$_2$O$_2$ and examined neuronal death and damage using the MAP2 CB-ELISA. In 1-week-old cultures, we observed significant MAP2 loss following treatment with either 100 or 300µM H$_2$O$_2$; in 3-week-old cultures, all three doses of H$_2$O$_2$ induced significant reduction of MAP2 (Figure 2.2A). The decreased MAP2 fluorescence was rescued by pretreatment with MDL and OPH in 1-week cultures, but only by MDL in 3-week cultures. The result is confirmed by counting the number of MAP2-positive cells (Figure 2.2B).

When we immunoblotted lysates from H$_2$O$_2$-treated cultures for markers of protease activation, we found high levels of calpain-cleaved spectrin in both 1- and 3-week-old cultures treated with 100 and 300µM H$_2$O$_2$ and moderate levels of calpain-cleaved spectrin in 3-week-old cultures treated with 30µM H$_2$O$_2$ (Figure 2.2C). In support of our pharmacological inhibitor data, we observed increased levels of cleaved caspase-3 in 1-week-old cultures treated with 100µM H$_2$O$_2$, but saw no increases in cleaved caspase-3 in 3-week-old cultures subjected to any doses of H$_2$O$_2$ (Figure 2.2C). Interestingly, we saw increased levels of cleaved caspase-3 in 1-week-old cultures treated with 30µM H$_2$O$_2$, even though we had not observed MAP2-loss in these cultures. Further, we observed no increases in cleaved caspase-3 in 1-week-old cultures treated
with 300μM H₂O₂, even though OPH protected against MAP2-loss induced at this dose. These findings suggest that in 1-week-old mixed cortical cultures, caspase-3 plays a role in H₂O₂-induced toxicity, particularly at lower doses, but does not contribute to H₂O₂-mediated cell death in 3-week-old cultures. In contrast, calpain appears to mediate H₂O₂-induced cell death in both 1-week-old and 3-week-old cultures.

**Calpain expression increases while caspase-3 expression decreases as neuroglial cultures age**

We next sought to determine whether 1- and 3-week-old cultures showed any difference in expression levels of calpains and caspases to determine whether our observed differences in protease responses to toxicities might occur solely at the cleavage/activation level, or whether differences in expression levels might also contribute. Thus, via immunoblot, we examined levels of μ-calpain, caspase-3, and of the initiator caspases, caspase-8 and -9 in 2-, 7-, 14-, and 21-day-old mixed cortical cultures. We found that levels of calpain increased with age and that full-length pro-caspase-3 decreased (Figure 2.3). We saw no change in levels of full-length caspase-8 or -9.

2.5 DISCUSSION

In the present study, we highlight how the age of primary neuronal cultures influences the respective contributions of calpain and caspase to neuronal death. We investigated the role of calpain and caspase activation in two toxicity models, NMDA and H₂O₂, using rat
primary cortical neurons aged for 1 week and 3 weeks in culture. We found that 1-week-old cultures treated with NMDA showed no significant calpain- or caspase-mediated toxicity, whereas NMDA treatment of 3-week-old neuronal cultures triggered calpain-dependent neurotoxicity. H₂O₂ treatment of 1-week-old cultures produced both calpain and caspase-3 activation as well as calpain- and caspase-mediated neurotoxicity. In contrast, H₂O₂ treatment of neuronal cultures at 3 weeks of age primarily produced calpain-mediated toxicity. To investigate possible mechanisms for these age-dependent responses, we measured the expression of μ-calpain and caspase-3, -8 and -9 in primary neuronal cultures over time. We found that μ-calpain is expressed at higher levels as cultures age. In contrast, caspase-3 expression shows a decrease in protein levels as the cultures age. Together, our findings suggest that caspase-triggered death may play a major role in younger cultures, but that a calpain-mediated death pathway predominates as cultured neurons mature.

Caspase activation has been reported to play an instrumental role in neurotoxicity caused by Aβ peptides, glutamate, and 6-hydroxydopamine, as well as that caused by serum withdrawal (Du et al., 1997, Marks et al., 1998, Giovanni et al., 2000, Hou et al., 2001). However, the primary neuronal cultures used in each of these studies have been aged for a week or less in vitro. The presence of caspase-dependent neuronal death in 1-week-old cultures and its absence in 3-week-old cultures suggests that caspase involvement in neuronal death is greatest when the cells are immature and highlights the importance of culture age in studies examining death mechanisms in neurons, such as those mentioned above.
Further, although studies had previously suggested that excitotoxic challenge can trigger caspase-mediated apoptosis in cultured neurons (Bonfoco et al., 1995), a recent growing body of evidence has indicated that calpain pathways and not caspase pathways are predominantly responsible for excitotoxic death (Nimmrich et al., White et al., O'Donnell et al., 2006). Our observation that NMDA-mediated calpain activation and excitotoxic death only manifest in older neuronal cultures is similarly supported by several studies highlighting the developmental regulation of NMDA receptor expression in culture. These studies demonstrate (1) that NMDA-induced toxicity occurs selectively in mature neuronal cultures and that it can be blocked by application of the NMDA receptor antagonist MK-801, which blocks open channels on the cell surface, and (2) that excitotoxicity-sensitive NR2A and NR2B subunits are highly expressed between 14 DIV and 21 DIV, but are nearly undetectable at 7 DIV (Eugenin et al., O'Donnell et al., 2006). Consequently, the presence of NR2-containing NMDA receptor subtypes leads to an increased excitotoxic death in older cultures.

The increase in $\mu$-calpain protein expression along with the decrease in caspase-3 expression as our cultures age may partially explain the absence of caspase-dependent neurotoxicity following toxic treatments of our 3-week-old primary neuronal cultures. Interestingly, these converse patterns of calpain and caspase-3 protein expression were previously reported by others using primary rat cortical cultures aged between 5 days and 20 days in vitro (Kim et al., 2007). A number of in vivo studies have also examined temporal expression of these proteases during rat brain development. They similarly found that pro-caspase-3 protein and mRNA levels fall off dramatically in the cortex and in brain homogenates during central nervous system maturation (Hu et al., 2000,
Yakovlev et al., 2001, Li et al., 2009). These reports are accompanied by functional studies demonstrating a progressively reduced ability to activate caspases in neurons as they mature in culture and in vivo (Hu et al., 2000, Yakovlev et al., 2001). In contrast, upregulation of calpain mRNA and protein levels has been repeatedly observed as brains age and undergo neurodegeneration, with abnormally high levels observed in the neocortex of Alzheimer’s Disease patients and substantia nigra and locus coeruleus of Parkinson’s Disease patients (Nixon et al., 1994, Mouatt-Prigent et al., 1996, Li et al., 2009).

While 1-week-old cultures treated with H₂O₂ showed an accumulation of cleaved caspase-3, 3-week-old cultures treated with either NMDA or H₂O₂ showed no caspase-3 activation. Procaspase-3 levels were nonetheless detectable in our 3-week cultures and were noticeably reduced by higher concentrations of NMDA and H₂O₂. Interestingly, this reduction was typically accompanied by calpain activation observed by increased calpain-cleaved spectrin, suggesting that 3-week-old neuronal cultures may utilize calpain-mediated proteolysis or degradation of caspase-3 as a mechanism to silence caspase signaling. Indeed, crosstalk between the calpain and caspase pathways has been reported in several instances, with calpain typically acting upstream of caspases, cleaving caspase-3 as well as caspase-7, -8, and -9 at non-canonical sites rendering them inactive (Chua et al., 2000).

Our results strongly suggest that the caspase-dependent death pathway is active in developing neuronal cultures but is replaced by calpain as the predominant death pathway when cultured neurons mature. We suggest two potential mechanisms that underlie this
change: (1) calpain-mediated inactivation of caspases (Fig. 1 & 2) and (2) downregulation of procaspase expression as neurons age (Fig. 3). Both mechanisms may be at play as neurons age. Given our findings, in vitro studies of caspase involvement in neuronal death should be interpreted with careful consideration given to the age of culture.

2.6 ACKNOWLEDGEMENTS

We would like to thank Dr. David Lynch for generously providing us with the calpain-cleaved spectrin antibody (AB38). We would also like to thank Dr. Marc A. Dichter and Margaret A. Maronski for providing us with primary cultures.
Figure 2.1 Calpain mediates NMDA-induced neurotoxicity in 3-week-old cultures. A, MAP2-cell based ELISA was performed in one or three-week old rat cortical cultures in response to 3, 10, and 30μM NMDA. NMDA has no effect on the MAP2 fluorescence signal strength in 1-week old rat cortical cultures. In 3-week old rat cortical cultures NMDA induced a dose-dependent decrease of the fluorescence signal strength in MAP2-cell based ELISA, and the decrease is blocked by the calpain inhibitor MDL28170, and by the NMDA receptor antagonist, MK801, but is not affected by the caspase inhibitor, OPH (** p<0.001, compared with veh; ###p<0.001, compared with cells treated with 30μM NMDA). B, Neuronal survival as determined by counts of MAP2-positive cells in one or three-week old rat cortical cultures in response to 3, 10, and 30μM NMDA. Data represents the average ±SD counts of multiple wells (n=6; *** p<0.001, compared with veh; ###p<0.001, compared with cells treated with 30μM NMDA). C, One and three-week old rat cortical cultures were treated with 3, 10, 30, or 100μM NMDA, and the expression levels of calpain-cleaved spectrin, caspase-3, and cleaved caspase-3 were detected (n=3) by western blot. The fold change in protein expression compared to that of the control (1.0) is specified beneath the respective bands in each blot. Each sample was subjected to SDS-PAGE and stained with Coomassie blue to demonstrate equal loading of samples.
Figure 2.2 Caspase and calpain activity both contribute to H2O2-induced toxicity in 1-week-old cultures, while toxicity in 3-week old cultures is mediated via calpain. A, MAP2-cell based ELISA was performed in one or three-week old rat cortical cultures in response to hydrogen peroxide. In 1-week-old rat cortical cultures, hydrogen peroxide induced a dose-dependent decrease of the fluorescence signal strength in MAP2-cell based ELISA, and the decrease is blocked by calpain inhibitor, MDL28170, and caspase inhibitor, OPH. In 3-week-old rat cortical cultures hydrogen peroxide induced a dose-dependent decrease of the fluorescence signal strength in MAP2-cell based ELISA, and the decrease is blocked by MDL28170, but not by OPH. B, Neuronal survival as determined by counts of MAP2-positive cells in one or three-week old rat cortical cultures in response to hydrogen peroxide. Data represents the average ±SD counts of multiple wells (n=6; *** p<0.001, compared with veh; #p<0.05, ### p<0.001, compared with cells treated with 300μM hydrogen peroxide). C, One or 3-week-old rat cortical cultures were treated with different concentrations of hydrogen peroxide and expression levels of calpain-cleaved spectrin, caspase-3, and cleaved caspase-3 were detected (n=3). The numerical values specified beneath the respective bands of western blots represent the fold change in protein expression as compared with that of the control (1.0). Each sample was subjected to SDS-PAGE and stained with Coomassie blue to demonstrate equal loading of samples.
Figure 2.3 Representative blot showing age-dependent expressions of calpain and of full-length caspases: 3, 8, and 9. Expression levels of calpain, caspase-3, caspase-8, caspase-9 were detected in d2, d7, d14, and d21 rat cortical cultures (n=3). The numerical values specified beneath the respective bands of western blots represent the fold change in protein expression as compared with that of the control (1.0). Each sample was subjected to SDS-PAGE and stained with Coomassie blue to demonstrate equal loading of samples.
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CHAPTER 3

E2F1 in neurons is cleaved by calpain in an NMDA receptor-dependent manner in a model of HIV-induced neurotoxicity

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3.1 ABSTRACT

The transcription factor E2F1 activates gene targets required for G₁-S phase progression and for apoptosis, and exhibits increased expression levels in neurons in several CNS diseases including HIV encephalitis, Alzheimer disease, and Parkinson Disease. While E2F1 is known to regulate cell viability through activation of caspases, here we present evidence supporting the involvement of E2F1 in NMDA receptor-dependent, HIV-induced neuronal death mediated by calpains. Using an in vitro model of HIV-induced neurotoxicity that is dependent on NMDA receptor and calpain activation, we have shown that cortical neurons lacking functional E2F1 are less susceptible to neuronal death. Additionally, we report that neuronal E2F1 is cleaved by calpain to a stable 55-kiloDalton fragment following NR2B-dependent NMDA receptor stimulation. This cleavage of E2F1 is protein conformation-dependent and involves at least two cleavage events, one at each terminus of the protein. Intriguingly, the stabilized E2F1 cleavage product is produced in postmitotic neurons of all ages, but fails to be stabilized in cycling cells. Finally, we show that a matching E2F1 cleavage product is produced in human fetal neurons, suggesting that calpain cleavage of E2F1 may be produced in human cortical tissue. These results suggest neuronal E2F1 is processed in a novel manner in response to NMDA receptor-mediated toxicity, a mechanism implicated in HAND pathogenesis as well as several other diseases of the CNS.
3.2 INTRODUCTION

E2F1 is a highly conserved member of the E2F family, a group of nine transcription factors that coordinate a transcription program underlying cell cycle progression. E2F1-directed transcription is mediated by coordinated activity of the ‘winged helix’ DNA binding domain and C-terminal transactivation domain, which recruit basal transcription machinery to target gene promoters (Trouche et al., 1996, Ross et al., 1999, Lang et al., 2001). Embedded within the transactivation domain is the pocket binding motif for tumor suppressor Retinoblastoma protein (pRb), which binds E2F1 during quiescence and G1 to repress E2F1 activity (Helin et al., 1993, Lees et al., 1993). Hyperphosphorylation of pRb prior to S phase leads to dissociation of the pRb-E2F1 repressive complex allowing E2F1 to transactivate target genes involved in DNA replication such as DNA polymerase-α, dihydrofloate reductase, and proliferating cell nuclear antigen (DeGregori and Johnson, 2006).

Aside from promoting cell proliferation, E2F1 functions as a potent inducer of apoptosis both in response to cytotoxic events such as irreparable DNA damage and during normal physiologic processes like T-cell maturation (DeGregori and Johnson, 2006). In these cases, E2F1 is stabilized by phosphorylation and protected from degradation through an interaction with the protein 14-3-3τ (Wang et al., 2004, Iaquinta and Lees, 2007). As E2F1 accumulates, it can initiate apoptosis through transactivation of apoptotic targets such as Bax, caspase 3, 7-9, and p14ARF. Alternatively, E2F1 can trigger cell death through multiple transcription-independent mechanisms, by inhibiting
activation of anti-apoptotic signals such as NFκappa-B, reducing levels of TRAF2, and inducing activation of calcium-sensitive cysteine protease calpain, which leads to proteolytic degradation of the Mdm2 homolog and p53 inhibitor, MDMx (Phillips et al., 1999, Hou et al., 2001, Strachan et al., 2005).

E2F1 regulation and function has been studied primarily in cycling cells. Little is known about the role of E2F1 in post-mitotic neurons. E2F1 is present in neurons during brain development and increases in expression through neuronal maturation (Kusek et al., 2001). This upregulation is also observed in differentiation of neural cell lines and cultured primary neurons (Kusek et al., 2001, Ting et al., 2014). In contrast to the nuclear localization of E2F1 in proliferating cells, neuronal E2F1 is predominantly cytoplasmic (Wang et al., 2010). Although neuronal E2F1 does retain some properties observed in mitotic cells, including its ability to induce apoptosis, these observations suggest E2F1 may have a novel function in neurons.

E2F1 deletion in vitro attenuates neuronal death in a number of toxicity models including potassium deprivation and Aβ peptide toxicity, while upregulation of E2F1 has been observed in post-mortem brain tissue of patients with Alzheimer Disease (AD), Parkinson Disease (PD) and Amyotrophic Lateral Sclerosis (ALS), supporting a link between E2F1, neuronal viability and neurodegeneration (Giovanni et al., 2000, O'Hare et al., 2000, Jordan-Sciutto et al., 2001, Ranganathan and Bowser, 2003, Hoglinger et al., 2007). HIV-associated neurocognitive disorder (HAND) is a neurologic syndrome consisting of a spectrum of cognitive, motor and behavioral deficits. Although neurons themselves are not directly infected by HIV, neuropathological hallmarks of the disease
include dendritic damage, synaptic loss and neuronal loss (Masliah et al., 1992, Masliah et al., 1997). Infiltration of HIV-infected macrophages into the central nervous system precedes neuronal damage. Such macrophages and subsequently activated resident microglia secrete inflammatory factors that alter the extracellular environment (Giulian et al., 1996, Gonzalez-Scarano and Martin-Garcia, 2005). The neuronal response to the altered environment involves aberrant activation of the cell cycle regulatory machinery, including upregulation of E2F1 (Wang et al., 2007, Akay et al., 2011). Post-mortem tissue from patients with HIV encephalitis (HIVE), the pathological correlate of advanced disease, shows elevated levels of E2F1 in neurons from basal ganglia, hippocampus and prefrontal cortex, the brain regions most affected in HAND (Jordan-Sciutto et al., 2002), although prototypical E2F1 target genes remain unchanged (Wang et al., 2010). Interestingly, the E2F1 observed in tissue from patients with HIVE is also primarily cytoplasmic. Similar results were observed in cortical samples from SIV-infected encephalitic macaques (Jordan-Sciutto et al., 2000). These findings suggest that E2F1 correlates with HAND but likely fulfills a different function from its classical role as a nuclear transcription factor.

Our lab has previously shown that E2F1 is processed by calpain in dividing cells and overexpression of a cytoplasmic and transcriptionally-inactive E2F1 protein in dividing cells leads to calpain activation and calpain-dependent toxicity (Strachan et al., 2005). Although calpain is ubiquitously expressed, it is particularly abundant in the CNS. Calpain has also been implicated in synaptic potentiation (Khoutorsky and Spira, 2009, Zadran et al., 2009) transcriptional regulation (Abe and Takeichi, 2007, Lynch and Gleichman, 2007, Khoutorsky and Spira, 2009, Zadran et al., 2009) and in both acute and
chronic neurodegeneration (Saito et al., 1993, Gafni and Ellerby, 2002, Gafni et al., 2004, Kelly et al., 2005, Gascon et al., 2008, Esteves et al., 2009). Calpain has two prototypical isoforms, μ-calpain and m-calpain, that differ in their in vitro requirements for Ca$^{+2}$ (μM for μ-calpain, mM for m-calpain). Although they display nearly identical substrate specificity, μ-calpain activation is associated with neuroprotective processes and m-calpain activation may contribute to pathologic states (Wang et al., 2013). Sustained dysregulation of calcium homeostasis during many neurodegenerative conditions, specifically those involving excitotoxicity, leads to calpain cleavage of a wide range of substrates (Vosler et al., 2008). These cleaved substrates are either rendered inactive or display an altered function that may contribute to disease pathogenesis. Given these findings, we assessed the interplay of E2F1 and calpain in a calpain-dependent model of neurotoxicity utilizing an in vitro model of HIV-mediated neuronal damage.

### 3.3 MATERIALS AND METHODS

**Animals:** E2F1$^{tm1}$ transgenic mutant and wild-type mice are C57BL6/SV129 hybrids obtained from Jackson Labs (Bar Harbor, ME). Mice were housed under a 12-hr light/dark cycle and were allowed access to food and water *ad libitum*. Experiments were approved by the Institutional Animal Use and Care Committee and in accordance with the ARRIVE guidelines.

**Chemicals and reagents:** Antibodies used include: Actin (A2066), MAT (M6693) [Sigma], Akt1 (#2938), phospho-Akt1 (#4060), Erk1/2 (#4695), phospho-Erk1/2
(#9101), FLAG (#2368), Lamin A/C (#2032) [Cell Signaling], E2F1 (KH95), GAPDH (6C5) [Santa Cruz], MAP2 (SMI52) [Covance]. Calpain-cleaved aII-spectrin (A38) described previously (Roberts-Lewis et al., 1994) was a generous gift from Dr. Robert Siman (University of Pennsylvania). Other chemicals include N-methyl-d-Aspartate (NMDA), NMDA inhibitor MK801 (Tocris), calpain inhibitor MDL28170 (Tocris), recombinant calpain-1 (208712, Calbiochem), Nicotinamide (Sigma), Trichostatin A (Sigma), Lambda protein phosphatase (New England Biolabs), Conantokin G (Bachem), and Ifenprodil (Tocris), Goat anti-mouse-beta-lactamase TEM-1-conjugated secondary antibody and Fluorocillin™ Green reagent were from Invitrogen.

**Preparation of primary neuronal cultures:** Primary cortical cultures were prepared from E18 Sprague Dawley rat embryos or E16 murine embryos as described previously (Wang et al., 2007). Cells were plated on poly-L-lysine pre-coated 60mm dishes at a density of 2 x 10⁶ cells per dish and maintained in neurobasal media with B27 supplement (Invitrogen) at 37°C, 5% CO₂. Cultures were utilized at 21 days *in vitro* (DIV) unless stated otherwise.

**Monocyte derived Macrophage (MDM) culture:** HIVMDM supernatants were derived from monocyte-derived macrophage cultures infected with HIV-1 as described previously (Chen et al., 2002). HIV infection of macrophages was confirmed by serial quantification of p24 antigen levels in the culture supernatants over time and only
HIVMDM supernatants exhibiting productive infection (p24 levels greater than 100 pg/ml of supernatant) were used to treat neuronal cultures. Supernatants from HIVMDM and non-infected macrophages (mock-infected MDM) were collected at selected time points after infection and stored at -80°C.

**Western blotting:** Whole cell extracts were prepared from primary rat cortical cultures. Cells were lysed for 20 min with ice-cold lysis buffer consisting of 50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 0.1% IGEPAL, 0.5% sodium deoxycholate, and a protease inhibitor cocktail (Sigma). Lysates were centrifuged at 16,100 g, at 4°C for 10 minutes and supernatants were collected. Protein concentrations were determined by Bradford assay, 30 μg of protein was loaded in each lane of a 4-12% Bis-Tris gradient gel for protein separation and subsequently transferred onto PVDF membranes. Routine immunoblotting and autoradiography procedures were followed as described (Akay et al., 2011).

**Subcellular fractionation:** Cytoplasmic and nuclear protein extractions were performed using Panomics’ Nuclear Extraction Kit (Panomics Inc.) following the manufacturer’s protocol. Briefly, cells were lysed with ice cold lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 10 mM EDTA, 4% IGEPAL, 10 mM DTT, protease inhibitor cocktail). Lysates were centrifuged at 16,100 g for 3 min and the supernatant (cytoplasmic fraction) was collected. The nuclear pellet was resuspended in buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 10 mM DTT, protease inhibitor cocktail) and
incubated on a rocking platform at 4°C for 2 hours. Following centrifugation at 16,100 g for 5 min, the supernatant (nuclear extract) was collected. Protein (30μg) was resolved on a 4-12% gradient TBE gel and probed with antibodies against E2F1.

**In vitro calpain cleavage assay:** Primary rat cortical cultures were harvested using lysis buffer (137mM NaCl, 2mM EDTA, 10% Glycerol, 20mM Tris-HCl pH=8, 1% protease inhibitor cocktail). Resulting lysates were incubated with 2mM CaCl₂ at 4°C for 6 hours in the presence or absence of 500nM of purified calpain-1 (EMD Millipore). The reactions were then terminated by SDS-PAGE sample buffer resolved on a 4-12% gradient Bis-Tris gel, followed by western blotting as described above. Calpain cleavage of affinity-purified FLAG/MAT-tagged E2F1 was performed using the above protocol with the following modifications: FLAG-transfected E2F1 protein was affinity-purified as indicated by manufacturer (Sigma). Prior to elution, FLAg-E2F1 was incubated at 4°C with 100ug neuronal protein lysate for 30 minutes followed by addition of calpain and CaCl₂ as indicated above.

**Neurotoxicity assessment:** Neurotoxicity was quantified by a MAP2 cell-based ELISA as described previously (Wang et al., 2007). In parallel cultures, blinded-hand counts of Propidium Iodide-excluded MAP2-positive cells were performed to assess neuronal death as described (Akay et al., 2011).
**Statistical analysis:** Values are expressed as mean ± SEM. Paired results were analyzed by Student’s t-test. Data with multiple categories were analyzed by one-way ANOVA followed by the Newman–Keuls post-hoc test using Prism software (GraphPad Software). Values of p < 0.05 were considered significant.

### 3.4 RESULTS

**HIVMDM-induced neurotoxicity is attenuated in cortical neurons from E2F1 mutant mice**

Increased E2F1 protein levels have previously been observed in post-mortem analysis from patients with HIV (Jordan-Sciutto et al., 2002). We investigated the link between E2F1 and HIV-mediated neurodegeneration, employing an *in vitro* model of HIV-induced neurotoxicity in which primary rat cortical neuroglial cultures are treated at DIV 20 with conditioned media from HIV-infected human monocyte-derived macrophages (HIVMDM) (O'Donnell et al., 2006). In this model, neuronal death is dependent on NMDA receptor stimulation and calpain activation (O'Donnell et al., 2006). To assess the role of E2F1 in HIV-mediated neurotoxicity, we treated cortical cultures from E16 mouse embryos lacking functional E2F1 and from wildtype controls with either HIVMDM or Mock-infected macrophage supernatants (MOCK). Neuronal toxicity was assessed by cell-based ELISA for the dendritic marker MAP2 (*Figure 3.1A*) and confirmed through blinded hand-counts of MAP2-positive cells (*Figure 3.1B*). While HIVMDM supernatants cause dose-dependent neurotoxicity in wildtype cultures, E2F1 mutant cultures displayed lower levels of neurotoxicity relative to wildtype control cultures at all
but the most concentrated HIVMDM dose (Figure 3.1A, 3.1B). The reduced toxicity in neurons lacking functional E2F1 suggests that loss of E2F1 confers partial protection against HIV-induced neuronal damage.

**HIVMDM-treated rat cortical cultures display lower molecular weight E2F1**

We have previously shown that E2F1 expression is predominantly cytoplasmic in post-mitotic neurons and that mRNA levels of E2F1 transcriptional targets remain unchanged in neurons following HIVMDM treatment (Wang et al., 2010). To explore the role of E2F1 in HIV-mediated neurotoxicity, we treated primary neuroglial cultures from rat cortex at DIV21 with HIVMDM supernatants and assessed E2F1 levels in subcellular fractions via immunoblot. E2F1 was primarily in the GAPDH-positive cytosolic fraction of both untreated and HIVMDM-treated neuronal cultures and largely absent from the Lamin A/C-containing nuclear fractions (Figure 3.2B). While the subcellular distribution of E2F1 seemed unchanged following HIVMDM treatment, the cytosolic fraction from HIVMDM-treated cultures contained a lower molecular weight E2F1 immunoreactive band in place of the full length E2F1 found in untreated and mock-treated neuronal cultures (Figure 3.2B). This lower molecular weight E2F1 (LMW-E2F1) band migrates at an apparent molecular weight 5 kDa below the full sized band and displays a dose-dependent accumulation during HIVMDM treatment, showing increased production with decreasing dilutions of HIVMDM supernatant treatment (Figure 3.2C). Furthermore, the LMW-E2F1 is detectable as early as 6 hours following initial treatment and accumulates over time (Figure 3.2D). These results suggest that neuronal E2F1 is cytoplasmic and changes in size in response to HIV-associated neurotoxins.
Production of LMW-E2F1 is mediated by NR2A/2B-containing NMDA Receptor stimulation

Since NMDA receptor subunit expression is developmentally regulated, HIVMDM does not induce toxicity in neuronal cultures until DIV 14-21 when NR2B-containing receptor maturation occurs (Wang et al., 2007). Interestingly, production of LMW-E2F1 by HIVMDM treatment is not detectable until after neuronal cultures reach 2 weeks of age (Figure 3.2A) suggesting that LMW-E2F1 may be dependent on NMDA receptor (NMDAR) stimulation. To investigate this possibility, we pretreated cortical cultures at DIV 21 with the NMDAR antagonist MK801 for 30 minutes prior to the addition of HIVMDM supernatant. Blocking NMDAR activation with MK801 eliminated the production of LMW-E2F1 following HIVMDM treatment (Figure 3.3A). Treatment of neurons with synthetic NMDAR agonist NMDA also led to the generation of LMW-E2F1, confirming that LMW-E2F1 production is NMDAR-dependent (Figure 3.4C). To determine whether LMW-E2F1 production was similarly mediated by NR2B containing receptors, we pretreated neuronal cultures with antagonists to different NMDA receptor subtypes prior to incubation with HIVMDM supernatants. While Zinc (NR1/2A) and Ifenprodil (NR1/2B) failed to block HIVMDM-induced formation of LMW-E2F1, Conantokin-G (all NR2B-containing) inhibited LMW-E2F1 production (Figure 3.3C). These results demonstrate that LMW-E2F1 production is selectively dependent on NR2A/NR2B-containing NMDAR stimulation.

LMW-E2F1 is a calpain cleavage product
Our *in vitro* model of HIV-induced neurotoxicity depends on NMDAR stimulation and also on activation of calpains, the calcium-sensitive cysteine proteases subsequently activated by NMDAR-dependent Ca\(^{2+}\) influx (O'Donnell et al., 2006, Wang et al., 2007, White et al., 2011). Calpain was activated in the present experiments as revealed by increased levels of calpain-cleaved αII-spectrin in HIVMDM-treated neurons (*Figure 3.4A*). We therefore hypothesized that NMDAR-dependent production of LMW-E2F1 results from proteolytic cleavage of E2F1 by calpain. To test the involvement of calpain, we pretreated neuronal cultures with the cell-permeable pharmacologic inhibitor MDL28170 (Calpain inhibitor III) prior to HIVMDM incubation and measured resulting LMW-E2F1 production by immunoblot. Pharmacologic inhibition of calpains prevented the production of LMW-E2F1 while preserving the full length form of E2F1 observed in untreated neuronal cultures, suggesting that LMW-E2F1 is dependent on calpain activation (*Figure 3.4B*). To validate E2F1 as a bona fide substrate of calpain, we performed an *in vitro* calpain cleavage assay on untreated neuronal protein lysates. Incubation of the lysates with calcium and calpain reproduced the LMW-E2F1 product observed in HIVMDM- and NMDA-treated neurons, (*Figure 3.4C*). To definitively show that E2F1 is cleaved directly by calpain rather than through an event downstream of calpain activity, we performed an *in vitro* cleavage assay on neuronal lysates as before, then inhibited calpain activity by adding MDL28170 to the cleavage reaction and subsequently incubated the reaction with recombinant FLAG-E2F1 protein. While endogenous E2F1 was cleaved by initial incubation with calpain, calpain inhibition blocked cleavage of the subsequently added FLAG-E2F1, again suggesting that the effect of calpain on E2F1 is through direct cleavage (*Figure 3.4D*). Finally, we incubated
recombinant $^{35}$S-labeled E2F1 with $\mu$-calpain or m-calpain in the presence of calcium. E2F1 was cleaved by both calpains in a similar time course (Figure S1). Together, these results demonstrate that E2F1 is cleaved by calpain to a smaller stable isoform following HIVMDM treatment and NMDAR stimulation.

**Stabilized calpain-cleaved E2F1 is generated preferentially in neurons**

To determine whether calpain cleavage may play a role in other cell or tissue types, we performed *in vitro* calpain cleavage assays on lysates from several different types of cultured cells and determined how efficiently the stabilized E2F1 cleavage product accumulated. Interestingly, the E2F1 cleavage product was produced in a number of different neuronal cell types including primary rat cortical neurons, primary rat hippocampal neurons, and retinoic acid-differentiated SHSY5y neuroblastoma cells, but did not stably accumulate in non-neuronal cells such as HEK293 cells and undifferentiated SHSY5y cells (Figure 3.5A). We found similar results in mouse tissue homogenates, where cleaved E2F1 was stably produced in brain samples but not those of other organs (data not shown). These findings suggest that calpain cleavage of E2F1 into a stable isoform occurs preferentially in neurons. Given that undifferentiated and differentiated SHSY5y cells exhibited different capacities for generating cleaved E2F1 and that cleaved E2F1 production was only detected in HIVMDM-treated neuronal cultures at DIV 14 or later, we suspected that formation of the stabilized cleavage product may be influenced by neuronal maturation. To test this possibility, we performed *in vitro* calpain cleavage assays on protein lysates from rat primary cortical neurons at different ages in culture. Incubation of protein lysates with activated calpain produced the
stabilized E2F1 cleavage product at all culture ages tested (Figure 3.5B), suggesting that both immature and mature neurons have the capacity to generate the calpain cleavage product. Together, these results suggest that production and stabilization of calpain-cleaved E2F1 occurs preferentially in cells of neuronal lineage.

**Stabilization of cleaved E2F1 is protein conformation-dependent**

To determine what factors may be responsible for stabilizing cleaved E2F1 in neurons, we first tested whether heat denaturation of the neuronal protein lysate affected the ability of activated calpain to produce the stabilized product. *In vitro* calpain cleavage assays were performed on boiled and unboiled neuronal protein lysates and assessed by immunoblot for presence of the stabilized E2F1 cleavage product. We found that cleaved E2F1 production was disrupted by heat denaturation of the lysate, even when purified, natively folded E2F1 was added to the boiled lysate prior to calpain incubation (Figure 3.6A). This result suggests that native conformation of neuronal proteins is necessary for stabilizing the cleaved product, providing a potential explanation for how calpain-cleaved E2F1 is preferentially stabilized in neurons.

A growing number of studies have demonstrated that the post-translational modifications, particularly phosphorylation, of calpain substrates or their binding partners can influence their ability to be cleaved by calpain (Huang et al., 1997, Bi et al., 2000, Nicolas et al., 2002, Qin et al., 2010). As E2F1 is both acetylated and phosphorylated in mitotic cells, we disrupted phosphorylation and acetylation of the neuronal protein lysates to determine whether these modifications might alter calpain processing of E2F1. To dephosphorylate neuronal proteins, lysates were incubated with lambda protein
phosphatase, a pan phosphatase with activity toward phosphorylated serine, threonine, and tyrosine residues, prior to incubation with activated calpain. While dephosphorylation activity was confirmed through a decrease in phospho-Akt levels, stabilized calpain-cleaved E2F1 was still produced in our in vitro calpain cleavage assay following phosphatase treatment (Figure 3.6B). We similarly disrupted global acetylation levels in the neuronal lysates through inhibition of histone deacetylases (HDACs) with Trichostatin A, which inhibits class I and II HDACs. Despite observing decreased acetylated alpha-tubulin from Trichostatin A treatment, production of calpain-cleaved E2F1 appeared unaffected (Figure 3.6C). We also inhibited class III HDACs (sirtuins) using Nicotinamide treatment. While Nicotinamide has effects on other targets, Nicotinamide-mediated sirtuin inhibition has frequently been confirmed through decreased Erk1/2 signaling (Li et al., 2008, Lee et al., 2011, Zhao et al., 2012). We did observe decreased levels of phospho-Erk1/2 following Nicotinamide treatment; however, as with trichostatin A treatment, we observed no significant effect on the stable production of cleaved E2F1 (Figure 3.6D). Similar results were also observed following deglycosylation of the neuronal lysates (data not shown). Together, these results suggest that post-translational modifications of the surrounding neuronal proteins may not be important for stabilizing the calpain-cleaved E2F1 product.

Calpain cleaves E2F1 at the N- and C-terminus

To better understand the kinetics of calpain cleavage of E2F1, we assessed E2F1 products produced by addition of calpain over time. By performing a time course of our in vitro calpain cleavage assay, we were able to identify an intermediate E2F1 cleavage product
that was produced early on during calpain incubation (Figure 3.7A). This intermediate product was further processed to the final stabilized cleavage product observed in neurons following HIVMDM treatment, suggesting that calpain cleavage of E2F1 involves at least two distinct cleavage events. Arresting calpain activity during this time course prevented further conversion of full-length E2F1 to the intermediate product and blocked production of the stabilized final product (Figure 3.7A’), further demonstrating that both the intermediate and final E2F1 products are directly produced by calpain cleavage. To identify where the E2F1 protein is cleaved by calpain, we made use of a construct encoding a dually epitope-tagged E2F1 protein with the FLAG-tag at the N-terminus and a metal affinity (MAT)-tag at the C-terminus. We affinity-purified the E2F1 protein from transfected HEK293 cells and with the protein still bound by the affinity resin, we then incubated the purified protein with neuronal protein lysate to reproduce the environment in which production of stabilized calpain-cleaved E2F1 is observed. Following incubation with activated calpain, we assessed which epitope tags were lost and retained in the eluted fraction by immunoblot analysis. We found that both FLAG and MAT tags were lost following calpain incubation (Figure 3.7B) supporting the concept of two distinct cleavage events at each terminus of E2F1.

**Presence of LMW-E2F1 in human fetal neurons**

The data from our *in vitro* model of HIV-mediated neurotoxicity firmly establishes that a calpain-cleaved E2F1 fragment accumulates in primary rodent neuronal cultures following HIVMDM treatment. To determine whether E2F1 in human neurons is similarly processed by calpain, we cultured human fetal neurons and performed an *in
vitrō calpain cleavage assay on the protein lysates obtained from these cells. As observed with primary rat neuronal cultures, activated calpain processed E2F1 from human neurons to a smaller, stable product (Figure 3.8A). This product matched the size of the calpain-cleaved E2F1 isoform from primary rat neurons, suggesting that E2F1 in human neurons displays the same capability to be processed to the smaller product. A smaller E2F1 isoform was also observed in protein lysates from human cortical autopsy tissue by immunoblot. Interestingly, several of the samples from HIV+ patients with minor cognitive motor disorder (MCMD) and with HIV-associated dementia (HAD) contained a lower molecular weight E2F1 band that was not found in a sample from an HIV+ neurocognitively normal patient (Figure S2). Production of this lower band did not correlate with post-mortem interval (Pearson’s Correlation: r=-0.103, p=0.684) or diagnosis (Fisher’s Exact: p=0.571 for HAD, p=0.545 for MCM, p=0.576 for HAD+MCM). Together, these results provide evidence for the production of a calpain-cleaved E2F1 isoform in human neurons.

3.5 DISCUSSION

Unlike other proteolytic systems such as lysosomes and the ubiquitin-proteasome, calpains do not simply breakdown their substrates. In many cases, calpain proteolytic processing leads to modulatory effects on substrate structure and function. Here, we present evidence supporting the involvement of E2F1 in HIV-induced neuronal death mediated by calpains. Using an in vitro model of HIV-induced neurotoxicity that is dependent on calpain activation, we have shown that cortical neurons lacking functional
E2F1 are less susceptible to neuronal death. Additionally, we report that neuronal E2F1 is cleaved by calpain to a stable 55 kiloDalton fragment following NR2B-dependent NMDA receptor stimulation. This cleavage of E2F1 is protein conformation-dependent and involves at least two cleavage events, one at each terminus of the protein. Intriguingly, the stabilized E2F1 cleavage product is produced in postmitotic neurons of all ages, but fails to be stabilized in cycling cells. We have previously reported that neuronal E2F1 displays the ability to shuttle between the cytosol and nucleus, a result also observed in differentiated keratinocytes (Strachan et al., 2005, Ivanova et al., 2007). Despite its nuclear-cytoplasmic shuttling capability, our results and previously published studies indicate that E2F1 is predominantly cytoplasmic in postmitotic neurons, similar to the localization recently reported for E2F1 in fully differentiated oligodendrocytes (Figure 3.2A, 3.2B, 3.3A) (Wang et al., 2010, Magri et al., 2014). Furthermore, neuronal E2F1 appears to be retained in the cytosol of HIVMDM-treated neurons following cleavage by calpain (Figure 3.2A, 3.3A), suggesting that the functional implications of E2F1 cleavage are distinct from those of Beta-catenin or Cdk5. This observation is further supported by the reported cytosolic E2F1 staining in neurons from post-mortem brain tissue of patients with HIVE, AD, ALS and PD (Jordan-Sciutto et al., 2001, Ranganathan et al., 2001, Hoglinger et al., 2007). Finally, our previous studies using this in vitro model have demonstrated that transcription of several E2F target genes remains unchanged in neurons following HIVMDM treatment (Wang et al., 2010). These observations all argue against a functional effect of E2F1 cleavage on its activity as a transcription factor. Future studies to define the E2F1 protein sequence that is preserved...
following cleavage may shed light on the role of this calpain-cleaved transcription factor when it is retained in the cytosol.

Determining cleavage sites within calpain substrates is often challenging given the poor understanding of how substrate specificity is achieved. While several studies have reported amino acid preferences for calpain activity based on analysis of published substrate cleavage sites, these preferences are often insufficient to correctly predict cleavage sites in novel calpain substrates (Tompa et al., 2004, DuVerle et al., 2011). Furthermore, calpain substrates are often cleaved in multiple places, compounding the difficulty of accurately defining the resulting fragment. For example, the microtubule-associated protein Tau is cleaved by calpain at a minimum of three internal sites, resulting in a stable 17-kilodalton fragment that has been reported to play a role in neurotoxicity (Park and Ferreira, 2005, Liu et al., 2011). Similarly, voltage-gated sodium channel Na\textsubscript{V}1.2 is cleaved by calpain in two regions of the alpha-subunit, producing channel fragments that display altered channel properties (von Reyn et al., 2009, von Reyn et al., 2012). Our data suggests a similar pattern of calpain processing for E2F1 in neurons. At least two cleavage events, one at the N-terminus and one at the C-terminus, are responsible for producing the stabilized E2F1 cleavage product (Figure 3.7). E2F1 has a Cyclin A binding domain that begins nearly 70 amino acids into its N-terminus. In dividing cells, E2F1-Cyclin A binding through this domain helps regulate E2F1-DNA binding. However, given the cytosolic localization of E2F1 in mature neurons, it is unlikely that the domain serves the same function in this cellular context. Similarly, the C-terminus of E2F1 contains the transactivation domain along with the pRB pocket binding domain, which has been studied exclusively in the context of E2F1-mediated
transcription. While these sequences have well-characterized roles in cycling cells, it is unclear how they may function in the neuronal cytosol. To what extent either of these domains is affected by cleavage is also unknown. Based on our results, the E2F1 cleavage product displays a 5-10 kDa reduction in apparent molecular weight suggesting a loss of 45-90 amino acids. Future efforts to define the calpain cleavage sites are necessary to determine the consequence of disrupting the N- and C-terminal domains.

Despite a growing list of calpain substrates that have recently been identified, the rules surrounding substrate recognition and sequence cleavage are yet to be fully elucidated. In this study, we identified a stabilized E2F1 isoform produced by calpain processing. Intriguingly, we were unable to observe this stabilized product in cells other than neurons (Figure 3.4). To our knowledge, this is the first example of a calpain substrate exhibiting distinct processing patterns in different cell types. To understand how this could be achieved, we first tested whether the stabilized cleavage product was dependent on protein conformation (Figure 3.6A). We found that protein conformation was indeed critical for the stabilized accumulation of cleaved E2F1, suggesting that neuronal E2F1 may contain unique binding partners that protect regions of the protein from further degradation. Interestingly, the E2F1 protein sequence has a number of P-X-X-P motifs known to bind Src homology 3 (SH3) domains (Alexandropoulos et al., 1995). We have previously reported that neuronal E2F1 is enriched in synaptosomes and co-localizes with the synaptic scaffolding protein PSD-95, which contains a C-terminal SH3 domain (Ting et al., 2014). Exploring whether PSD-95 or other synaptic proteins may bind E2F1 through SH3 binding domains may provide further clarity on the dependence of calpain-cleaved E2F1 on protein conformation. In addition to examining
native protein structure, we also tested whether post-translational modifications such as phosphorylation and acetylation may play a role in modifying calpain activity toward E2F1 in different cellular environments (Figure 3.6B-6D). There are multiple examples showing calpain preferentially cleaving substrates based on their phosphorylation state. Notably, the tumor suppressor p53 is preferentially cleaved by calpain when phosphorylated at one of its serine residues in developing neurons (Qin et al., 2010). A separate study focusing on calpain-mediated cleavage of NMDA receptors found that tyrosine phosphorylation of NR2 subunits protected their C-terminal domains from calpain processing (Bi et al., 2000). Finally, αII-spectrin, one of the prototypical calpain substrates, also shows decreased sensitivity to calpain cleavage when phosphorylated at one of its tyrosine residues (Nicolas et al., 2002). While post-translational modifications to E2F1 have not been explored in neurons, we thought they may provide a basis for calpain to differentially process neuronal E2F1. Given the numerous instances when E2F1 is phosphorylated and acetylated in dividing cells, we attempted to pharmacologically disrupt both of these modification systems in neuronal lysates to determine whether calpain cleavage of E2F1 is altered. However, neither phosphatase treatment nor inhibition of HDACs or Sirtuins significantly affected the formation of the stabilized E2F1 calpain cleavage product (Figure 3.6). Whether other post-translational modifications are involved in directing calpain processing of E2F1 is still a possibility. A comprehensive understanding of neuronal E2F1 post-translational modifications will likely be necessary to fully explore this issue.

Our findings suggest that calpain cleavage of E2F1 occurs during a neurotoxic event and contributes to this process as we have observed reduced neurotoxicity in
HIVMDM-treated cortical neurons lacking functional E2F1 (Figure 3.1). Interestingly, we were able to completely block calpain cleavage of E2F1 using the NR2B subunit inhibitor Conantokin G, suggesting that cleaved E2F1 requires NR2B-NMDAR stimulation (Figure 3.3B). A recent study detailed how synaptic NMDAR stimulation leads to μ-Calpain activation and subsequent neuroprotection, while extrasynaptic NMDAR stimulation leads to m-Calpain activation that promotes neurotoxicity (Wang et al., 2013). A study that deleted both μ-Calpain and m-Calpain in the CNS of mice define roles for these proteases in both excitotoxic death and synaptic plasticity (Amini et al., 2013). Given that NR2B subunits are typically found at extrasynaptic locations, the NR2B-dependent nature of E2F1 processing by calpain suggests that it requires extrasynaptic NMDAR activity and subsequent neurotoxic activation of m-calpain. Although the NR2B-dependent nature of E2F1 processing suggests that it is part of a neurotoxic pathway, it is important to note that both m-calpain and μ-calpain are able to proteolytically cleave E2F1 (Figure S1), and that calpain-cleaved E2F1 may fulfill a neuroprotective function either in our model of HIV-induced neurotoxicity or possibly in other neuronal contexts such as development. Indeed, we have shown that calpain-cleaved E2F1 can be produced in immature neurons that have not yet expressed NR2B subunits (Figure 3.5B), suggesting the possibility of a role in synaptic development. It should also be noted that the attenuated HIVMDM-induced neurotoxicity observed in E2F1 mutant neurons could be due to a loss of physiologic E2F1 function rather than absence of a gain-of-toxic function in wildtype cultures. While it seems unlikely that E2F1 in the cytosol could promote neurotoxicity by activating transcription of apoptotic genes, it is quite possible that it could influence calcium homeostasis. Previous work...
from our lab has shown that overexpression of a transcriptionally inactive N-terminally truncated E2F1 protein in HEK293 cells causes calpain-dependent cell death (Strachan et al., 2005). Interestingly, this truncated E2F1 product is cytoplasmic much like the neuronal E2F1 protein that we detect in primary neuronal cultures. While these results were obtained from cell lines, they provide an intriguing comparator to calpain-cleaved neuronal E2F1 and suggest the possibility that the E2F1 cleavage product may serve to amplify calpain activation. In fact, a number of calpain substrates have been reported to perform this function, promoting further cytosolic calcium increases upon cleavage. For instance, the Type 1 IP3 receptor, which functions as an ER calcium channel, disrupts intracellular Ca\(^{2+}\) buffering when cleaved by calpain (Kopil et al., 2012). Similarly, L-type Calcium channels in cultured neurons are cleaved by calpain, leading to C-terminal truncation of their \(\alpha_1\) pore-forming subunit and enhanced Ca\(^{2+}\) influx from the L-type channels (Klockner et al., 1995, Hell et al., 1996). Cleavage of these substrates and others help create a positive feed-forward pathway that further increases intracellular calcium levels, thereby amplifying calpain activation. Our results showing the presence of a lower molecular weight E2F1 band in a subset of subcortical tissue samples from patients with HAND may similarly be due to intracellular calcium dysregulation (Figure S2) and additional studies with greater statistical power are warranted. Interestingly, neuronal E2F1 has been reported to bind Necdin, a factor involved in maintaining the terminally differentiated identity of postmitotic neurons (Kuwako et al., 2004). Further, Necdin has been linked to intracellular calcium regulation through its interaction with NEFA, a calcium-binding protein located on the ER cisternae which regulates ER calcium release (Taniguchi et al., 2000). As Necdin interacts with the C-terminal domain of E2F1, further
work to determine if calpain-cleaved E2F1 could participate in calcium regulation through changing its interaction with Necdin and NEFA would provide further insight into this pathway.

Our findings indicate that E2F1 exhibits novel regulation by calpain cleavage in neurons. Further, cleavage of E2F1 by calpain is independent of known phosphorylation and acetylation events and appears to be dependent on an interacting protein partner. Finally, an isoform of E2F1 with similar size to calpain-cleaved E2F1 is apparent in cortex from HIV-infected patients with cognitive deficits while it is absent in cognitively normal patients. Understanding the role of this stabilized calpain cleavage project will provide novel therapeutic implications for treating patients with cognitive decline linked to excitotoxic injury including HAND, AD, ALS and PD.
3.6 ACKNOWLEDGMENT: We thank Dr. Harry Ischiropoulos, Dr. Judy Grinspan, Dr. Robert Neumaur, and Dr. Cagla Akay for assistance with experimental design and preparation of the manuscript. We would also like to thank Dr. David Park and Dr. Olimpia Meuicci for providing reagents and E2F1 mutant mice. We also thank the CNAC Tissue processing Core at Temple University, particularly Dr. Dianne Langford, for providing primary human neurons, the National NeuroAIDS Tissue Consortium for providing tissue from HIV-positive patients and Margaret Maronski for her help in preparing primary rat neuronal cultures. This work was supported by the NIH. The authors declare no conflicts of interest.
Figure 3.1. HIVMDM-induced neurotoxicity is attenuated in cortical neurons from E2F1 mutant mice. **a.** Cortical neurons from wild type (black bars) and E2F1 mutant mice (white bars) at DIV 20 were treated with HIVMDM supernatants or Mock-MDM supernatants at indicated dilutions for 20 hours. Resulting neuronal damage was measured by a cell-based MAP2 ELISA (** denotes p<0.01) (White et al., 2011). **b.** Blinded hand counts of MAP2-positive, propidium iodide-negative cells from wildtype (square) and E2F1 mutant (triangle) cortical cultures treated with indicated dilutions of HIVMDM (black) and mock (white) supernatants for 20 hours (*denotes p<0.05).
Figure 3.2. HIVMDM-treated rat cortical cultures display lower molecular weight E2F1 (LMW-E2F1). **a,b.** Immunoblot analysis of LaminA/C-positive nuclear fraction and GAPDH-positive cytoplasmic fraction from rat cortical cultures at DIV14 (a) and DIV21 (b) following 20-hour HIVMDM or Mock treatment. **c.** Immunoblot analysis of E2F1 from GAPDH-positive, LaminA/C-negative cytosolic fractions of rat cortical cultures (DIV21) treated with indicated dilutions of HIVMDM for 20 hours. Coomassie gel stain used for loading control (a, b, c). **d.** E2F1 immunoblot analysis of rat cortical cultures at DIV 21 following a time-course of HIVMDM or Mock treatment (Actin: loading control).
Figure 3.3. Production of LMW-E2F1 is mediated by NR2A/2B-containing NMDAR stimulation. a. Immunoblot of subcellular fractions from rat cortical cultures (DIV21) treated for 20 hours with HIVMDM or mock supernatants. For indicated samples, NMDAR antagonist MK801 (10µM) was added to cultures 30 minutes prior to supernatant treatment. Coomassie-stained gel serves as a loading control. b,c. Rat cortical cultures at DIV21 were pre-treated for 1 hour with different NMDAR subunit inhibitors (Table, c) followed by HIVMDM treatment. Conantokin G (CoG, 10µM), which blocks NR2A/2B-containing NMDARs prevented the production of LMW-E2F1, while Ifenprodil (Ifn, 10µM), which blocks NR2B/2B-containing NMDARs, and Zinc (Zn, 500nM) which blocks NR2A-containing NMDARs, had no effect on HIVMDM-induced formation of LMW-E2F1. Coomassie-stained gel serves as a loading control.
Figure 3.4. LMW-E2F1 is a calpain cleavage product. a. Samples from primary rat cortical cultures at DIV21 were immunoblotted for calpain-cleaved aII-spectrin to measure calpain activation following HIVMDM treatment. Coomassie gel stain serves as a loading control. b. To block calpain activation, cortical cultures were pretreated with calpain inhibitor MDL28170 (20uM) for 30 minutes prior to HIVMDM. Coomassie gel stain serves as a loading control. c. In vitro calpain cleavage assay of neuronal lysates
from untreated cultures at DIV 21 with calpain-1 (10μg/mL) and 2mM CaCl₂. Reaction mixture was incubated at 4°C for 6 hours. Untreated lysate from untreated and NMDA-treated cultures serve as controls for full length and LMW-E2F1 (top and bottom arrowheads, respectively). d. In vitro calpain cleavage assay to test direct cleavage of E2F1 by calpain. Addition of MDL28170 to neuronal lysates prior to incubation with calpain-1 and CaCl₂ blocked formation of LMW-E2F1 (pre-MDL). Following 4 hours of the 6-hour incubation of neuronal lysate with calpain-1, MDL28170 was added along with affinity-purified FLAG-E2F1 to the reaction (post-MDL and post-FLAG-E2F1, respectively) to determine whether exogenous E2F1 is protected by calpain inhibition following cleavage of endogenous targets.
Figure 3.5. Stabilized calpain-cleaved E2F1 is generated preferentially in neurons. 

In vitro calpain cleavage assays were performed on protein lysates from indicated cell types to assess the influence of cell type on production of stabilized calpain-cleaved E2F1. Lysates were collected from primary rat cortical and hippocampal cultures at DIV21, and from differentiated SHSY5y neuroblastoma cells following 10-day retinoic acid treatment. b. In vitro calpain cleavage assay of lysates from primary rat cortical neurons at indicated ages in culture.
Figure 3.6. Stabilization of cleaved E2F1 is protein conformation-dependent. a. In vitro calpain cleavage assays were performed on rat cortical neuronal lysates from cultures at DIV21 that were boiled or unboiled. Purified FLAG-E2F1 protein that was added to the boiled lysate failed to show stable accumulation of cleaved E2F1 when incubated with calpain-1. FLAG-MAPK served as a control to demonstrate calpain substrate specificity in the assay. b. To determine the effect of protein phosphorylation on the stabilization of calpain-cleaved E2F1, neuronal lysate (0.5 μg/μL) was treated with λ protein phosphatase (λPP, 600 units) for 1 hour at 25°C prior to incubation with calpain-1 and CaCl₂ at 4°C. Phospho-Akt levels normalized to total Akt levels were measured to confirm effect of λPP treatment. c,d. Rat cortical cultures were treated for 24 hours with
either 1μM Trichostatin A (TSA) to inhibit class I and II HDACs (c) or Nicotinamide (low=1μM, high=25μM) to inhibit class III HDACs (d). Cultures were harvested and total protein lysates were subjected to in vitro calpain cleavage assay. HDAC inhibition was confirmed by increased levels of acetylated α-tubulin over total α-tubulin and decreased levels of phospho-Erk1/2 over total ERK.
a

Ca\textsuperscript{2+} + Calpain

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E2F1

Calpain-cleaved Spectrin

a'

Ca\textsuperscript{2+} + Calpain

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E2F1

Calpain-cleaved Spectrin

b

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| Calpain: | - | - | - | - | + | - | - | + |

IP: FLAG

IB: FLAG

| Lysate: | - | - | + | + | + |
| Calpain: | - | - | - | - | + |

IP: MAT

IB: MAT
Figure 3.7. Calpain cleaves E2F1 at N- and C-terminus. a. Time course of in vitro calpain cleavage assay was performed on lysates from rat cortical cultures for indicated times. Samples were run on a 10% Bis-Tris polyacrylamide gel to better resolve molecular weight differences of E2F1 bands due to calpain processing. Samples from early time points show generation of an intermediate product (middle arrow) before complete conversion to the stabilized calpain-cleaved E2F1 product (bottom arrow). Lysate from NMDA-treated cultures served as a positive control for calpain-cleaved E2F1. Calpain-cleaved αII-spectrin shows progressive calpain activity over time course. a’. Time course of calpain cleavage assay was performed as in (a) but with addition of MDL28170 (20uM) beginning at t=20 minutes to inhibit calpain activity at all subsequent time points. b. Differentially epitope-tagged FLAG-E2F1-MAT overexpressed in HEK293 cells was affinity purified with either FLAG antibody or Nickel resin. Purified protein was incubated with neuronal lysate (50ug) for 30 minutes while still resin-bound prior to performing an in vitro calpain cleavage assay and subsequent elution. Eluted protein was assessed for presence of N-terminal FLAG tag and C-terminal MAT tag by immunoblot analysis. FLAG-MAPK-MAT served as a control to demonstrate E2F1-specific calpain processing and as a positive control along with FLAG-BAP for successful purification.
Figure 3.8. Lower molecular weight E2F1 is produced in human fetal neurons.

Protein lysates from cultured human fetal neurons (HFN) at DIV 17 were subjected to an in vitro calpain cleavage assay as described above. Immunoblot analysis of resulting samples assessed levels of E2F1 and calpain-cleaved αII-spectrin to measure calpain activation. Lysate from NMDA-treated rat cortical cultures (RCN) served as a positive control for calpain-cleaved E2F1.
3.7 SUPPLEMENTAL DATA

E2F1 in neurons is cleaved by calpain in an NMDA receptor-dependent manner in a model of HIV-induced neurotoxicity.

**Figure S1. Proteolytic processing of E2F1 by Calpain-1 and Calpain-2.** *In vitro* translated full-length E2F1 (2 μg) was incubated with recombinant μ- or m-calpain for indicated times in the presence of 0.1mM or 1mM CaCl$_2$, respectively. The reaction mixtures were then resolved on a 4-12% gradient gel. The cleavage of E2F1 was visualized by SDS-PAGE on a 4-12% gradient gel using Coomassie Blue staining. Arrows denote transiently produced cleavage products.
Figure S2. Lower molecular weight E2F1 is observed in prefrontal cortex of HIV+ patients. E2F1 expression in autopsy brain tissue from HIV-positive patients was measured by immunoblot analysis. Whole cell protein extracts from mid-frontal cortex show a lower molecular weight E2F1 band (arrow) similar to the stabilized calpain-cleaved E2F1 product observed in HIVMDM-treated neuronal cultures. Actin levels serve as a gel loading control.
**Table S1.** Summary of case data for autopsy tissue obtained from the NNTC

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**Table S1.** Data on the HIV status, gender, age in years, post-mortem interval (PMI) in hours, neurocognitive diagnosis (NeuroCog), and neuropathologic diagnosis (Neuropath Dx) are summarized. Abbreviations: F, female; HAD, HIV-associated dementia; HIVE, human immunodeficiency virus encephalitis; M, Male; MCMD, minor cognitive motor disorder; ND, not determined; NNTC, National NeuroAIDS Tissue Consortium; PMI, postmortem interval.
METHODS

In vitro translation of E2F1

In vitro translated $^{35}$S-labeled full-length E2F1 (2μg) was incubated with recombinant μ- or m-calpain, (Calbiochem), in a reaction buffer containing 50mM HEPES buffer (pH 7.4), 150mM NaCl, 1mM EDTA, and 1% Triton X-100. Calpain was activated with 0.1 or 1mM CaCl$_2$, and reactions were incubated at 4°C for indicated times and then terminated by SDS-PAGE sample buffer. The reaction mixtures were then resolved on a 4-12% gradient gel. The cleavage of E2F1 was visualized using Coomassie Blue staining of the gel.

Western blotting of human brain tissue

Frozen tissue samples from midfrontal cortex of HAD (n = 6), MCMD (n = 7), and control (n = 5) human autopsy cases were obtained from the tissue banks of National NeuroAIDS Tissue Consortium (Morgello et al. 2001). Due to limited availability, cortical tissue from only one HIV-infected, cognitively normal case was obtained. Donor demographics are listed in Table 1. For protein extraction, tissue was homogenized and prepared for immunoblot as described (Akay et al. 2012). 100 μg of protein was loaded per lane on a 10% Bis-Tris gel, followed by standard immunoblotting techniques described under Western blotting in Materials and Methods.
3.8 REFERENCES


CHAPTER 4
Investigating the RNA binding domain of E2F1: Preliminary Studies for cytosolic E2F1 functions

Unpublished data
4.1 ABSTRACT

In addition to its well-known role as a transcription factor in cell proliferation, E2F1 has been linked to neuronal death in neurodegenerative diseases such as Alzheimer Disease, Parkinson’s Disease, and HIV Encephalitis. Interestingly, E2F1 is localized predominantly in the cytosol of post-mitotic neurons, a striking finding given its nuclear localization in cycling cells. Previous work has shown that E2F1 binds to synthetic RNA species containing a specific stem loop secondary structure and that E2F1 can bind RNA from cortex, a capability that requires its DNA-binding domain. These findings led us to hypothesize that E2F1 may play a role in neuronal survival during neurodegenerative disease by regulating RNA through direct binding. By using site-directed mutagenesis, electrophoretic mobility shift assays (EMSA), and immunoprecipitation, we conducted a preliminary investigation of the requirement of specific amino acids in the DNA binding domain of E2F1 for RNA binding. Our analysis identifies amino acid R125 and N152 as sites of interest for distinguishing RNA binding and DNA binding capabilities. More work is needed to define the E2F1 RNA binding domain.
4.2 INTRODUCTION

E2F1 is one of 9 members of the E2F family of transcription factors. Traditionally, increased transcriptional activity of the E2F proteins leads to the expression of gene products necessary for DNA synthesis and progression to S-phase. E2F1 is considered an E2F ‘activator,’ which refers to its ability to transcriptionally upregulate transcription of its gene targets. E2F1 is predominantly regulated by interactions with transcription factor DP1 and tumor suppressor Retinoblastoma protein (pRB). Heterodimerization of E2F1 with DP1 and hyperphosphorylation of pRB (ppRB) both lead to enhancement of E2F1 nuclear activity (Bandara et al., 1993, Nicolay and Dyson, 2013).

In addition to its role in cell division, E2F1 has been implicated in cell death through its transcriptional activation of a number of genes in the apoptotic cascade. Multiple studies have demonstrated that activation of E2F1 by phosphorylation of pRb is necessary and sufficient for neuronal cell death in vitro (Giovanni et al., 1999, Park et al., 2000). E2F1 levels as well as ppRb levels increase in neurons during neurodegenerative diseases such as Alzheimer’s Disease, Parkinson’s disease and HIV encephalitis (Jordan-Sciutto et al., 2002a, Jordan-Sciutto et al., 2002b, Hoglinger et al., 2007). Interestingly, it has been observed that E2F1 is predominantly cytoplasmic in post-mitotic neurons both in culture and in post-mortem brain tissue from patients with Alzheimers Disease and HIV Encephalitis (Jordan-Sciutto et al., 2002b, Strachan et al., 2005), suggesting that E2F1 may carry out a neuronal function independent of its role as a transcription factor.
E2F1 belongs to a superfamily of DNA binding proteins that contain winged-helix DNA binding domains (Zheng et al., 1999). A number of proteins in this superfamily, including the Lupus La protein and SelB, have shown the ability to bind RNA in addition to DNA (Dong et al., 2004, Yoshizawa et al., 2005). Furthermore, a previous study reported that E2F1 could bind several synthetic RNA species in vitro. One of these species contains a stem loop secondary structure (Figure 4.1) that was shown to be critical for E2F1 binding (Ishizaki et al., 1996). Work by others in our lab has shown that E2F1 can bind RNA from tissue. To determine if E2F1 can bind endogenous RNA, GST-tagged E2F1 protein was incubated with RNA isolated from human cortex and then pulled down using a Glutathione Sepharose affinity column. RNA bound to affinity-purified E2F1 containing the full length sequence but not to E2F1 lacking the DNA binding domain, suggesting that E2F1 can bind cellular RNA from neurons and that this interaction requires the DNA binding domain (unpublished, A. Barnstable). Finally, RNA isolated from cultured SHSY5y neuroblastoma cell lines were similarly shown to bind E2F1 using a formaldehyde crosslinking assay (not shown).

Figure 4.1 E1 ligand secondary structure. Stem loop secondary structure of E1 RNA, ligand that has been shown to bind efficiently to E2F1 (Ishizaki et al., 1996). The C1 stem loop is denoted in the box.
Given the above observations that E2F1 is predominantly cytoplasmic in post-mototic neurons and capable of binding RNA, we hypothesized that neuronal E2F1 may serve to regulate RNA transcripts through direct binding of cytosolic RNA species. To investigate the RNA binding motif of E2F1, we introduced point mutations to a number of different amino acid residues in the DNA binding domain of E2F1 that have previously been shown to contact DNA in an E2F:DP:DNA complex (Zheng et al., 1999). The wildtype and point mutation-containing constructs were then cloned into FLAG-tagging vectors with the goal of overexpressing these E2F1 variants in cell lines and assessing their relative affinity for DNA and RNA using Electrophoretic Mobility Shift Assays (EMSA). In experiments using cell extracts, supershifts using FLAG antibody were performed to verify the identity of the DNA-bound protein. Furthermore, purified DP1 was prepared and added to the EMSA samples during the incubation step to make the comparison of E2F1:DNA and RNA binding assays physiologically relevant.

4.3 METHODS

Site-Directed Mutagenesis

To introduce point mutations to strategic residues in the DNA binding domain of E2F1, we used Stratagene's QuikChange™ XL Site-Directed Mutagenesis Kit. Mutagenesis primer pairs for each intended point mutations were designed by following the primer design guideline in the protocol prior to performing mutagenesis reactions. Mutagenesis reactions were performed according to the manufacturer’s instructions with commercially-ordered DNA primers purified by standard desalting. For each mutagenesis reaction 10ng of template DNA (FLAG-rE2F1 – a plasmid encoding rat E2F1 with an N-terminal FLAG-tag and C-terminal metal affinity tag)
Following the PCR mutagenesis reaction, methylated and hemimethylated DNA was selectively digest by incubating the reaction with Dpn I to remove any remnants of the parental DNA and to enhance selection for point mutant clones. Bacterial transformations were performed with XL10-Gold Ultracompetent Bacterial cells as indicated in the instructions. Selection on LB-kanamycin agar plates was then performed and DNA was purified from resulting transformants and sequenced to confirm successful mutagenesis.

3' End DNA Biotinylation

By using Thermo Scientific's Biotin 3’ End DNA Labeling Kit, we were able to effectively create 3’ end biotin-labeled double-stranded DHFR DNA, which has been shown to bind E2F1. 25 μl of ddH2O, 5 μl of 5x Tdt reaction buffer, 5 μl of unlabeled oligo (control or forward/reverse strand of DHFR, 100nM final concentration), 5 μl of Biotin-11-UTP (0.5 μM final concentration), and 10 U of diluted Tdt were mixed and incubated for 30 minutes at 37°C. Then, 2.5 μl of 0.2M EDTA and 50 μl chloroform:isoamyl alcohol were added to terminate each reaction and to extract excess Tdt. The remaining samples were vortexed and centrifuged at high speed to separate out phases, conserving the resulting top aqueous layers. Finally, the forward and reverse strands of DFHR were annealed to form double stranded DNA by combining the two samples and incubating at room temperature for 1 hour.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA experiments in this study were conducted using an EMSA Assay Kit from Signosis. First, cell extracts of neurons (magnet-assisted transfection) and HEK 293 (Lipofectamine transfection) were collected in whole cell lysis buffer. DNA/RNA
competition and supershift reactions were then prepared by combining 5 μg of protein extract, 1 μl of poly d(I-C), 2.0 μl of 5x Binding buffer, and ddH2O to create 10 μl total volume reaction samples. The samples were incubated on ice for 5 minutes. Then, 1.0 μl of either cold probe of varying concentrations (10ng, 100ng and 1000ng for DNA cold probe, and 10ng, 50ng, and 200ng for RNA cold probe) for competition assays, or FLAB antibody for supershift assays, was added to the samples. Additional incubation on ice for 20 minutes was made. Finally, 2 μl of biotin labeled DHFR hot probe was added to each sample, and further incubated for 1 hour at room temperature.

6.5% non-denaturing polyacrylamide gels were cast and used to run the EMSA samples. Gels were run at 100V for 90 minutes at 4°C following a 30 minute pre-run, and transferred on to a nylon membrane at 40V for 150 minutes at 4°C. UV crosslinking was performed to capture protein-nucleic acid complexes on the membrane. The membrane was then blocked, incubated with Streptavidin-HRP, and washed before visualizing nucleic acid probes by chemiluminescence.

**Immunoprecipitation**

As mentioned above, overexpressed FLAG-DP1 proteins in HEK 293 cells were purified using FLAG Immunoprecipitation Kit produced by Sigma-Aldrich. 40 μl of ANTI-FLAG M2 affinity gel resin was aliquoted after thorough suspension. This was briefly centrifuged at 6,000 x g and resulting supernatant was removed. The remaining beads were wash with 1x FLAG Wash Buffer 3 times. Then, cleared HEK 293 lysate containing overexpressed FLAG-DP1 proteins was added and incubated at 4°C for 12 hours. After the incubation, the sample was centrifuged and aspirated to remove supernatant, leaving only the FLAG-DP1 bound beads. FLAG-DP1 proteins
were eluted from the beads by incubating in 115 μl of 3x FLAG Elution Buffer (15 μl of 3x FLAG peptide in 100 μl of 1x Wash Buffer). FLAG-BAP was similarly immunoprecipitated as a positive control.

4.4 RESULTS AND DISCUSSION

Previous work in the lab has shown that E2F1 is capable of binding cellular RNA from Rat cortex and that this ability is dependent on the E2F1 DNA binding domain. Additionally, E2F1 localization in neurons has been demonstrated to be predominantly cytoplasmic. These observations suggest that E2F1 may serve as an RNA binding protein in neurons. To better understand the RNA binding capability of E2F1, we sought to define its RNA binding domain. Since the DNA binding domain of E2F1 was necessary for binding cellular RNA, we reasoned that the RNA binding domain likely overlaps with the DNA binding domain. We therefore utilized an approach relying on site-directed mutagenesis of amino acid residues in the E2F1 DNA binding domain known to play a role in DNA binding in order to assess the effect of the mutations on the relative binding affinity for DNA and RNA using EMSA competition assays. E2F1 amino acids R163, R164, and Y166 directly contact cysteine and guanine residues during DNA binding, and are likely necessary for direct binding to RNA as well. Therefore, conservative point mutations changing each of these residues to alanine were made to test their effects on E2F1:DNA and E2F1:RNA interactions. In addition, N152 in E2F1 corresponds to a lysine (K) in the DNA binding domain of E2F4, which is exclusively nuclear in neurons. To test whether this amino acid confers E2F1 with a selective ability to bind RNA, N152 was mutated to lysine, the residue that E2F4 has at that position. N170, a neutral amino acid that binds to the
backbone of DNA, was mutated to A170 to see if it affects E2F1 RNA binding. Amino acids K180 and N184 have been shown to be in direct contact with DNA in a E2F1:DNA complex; therefore, point mutations changing both these amino acids to alanine(K180A and N184A) were made. Finally, R125, which contacts thymidine during DNA binding, was mutated to A to test the effect of this residue on the preference of E2F1 for DNA or RNA. Point mutations were performed using site-directed mutagenesis primers and successful mutants were confirmed by sequencing of the DNA of resulting clones. We then analyzed expression of the cloned mutant constructs by transfecting HEK293 cells and measuring overexpression by Western Blot analysis (Figure 4.2).

Prior to assessing the effect of each of these point mutants on DNA and RNA binding affinity, we set out to determine whether E2F1 displays different relative affinities for DNA and RNA binding depending on the cell type in which E2F1 is expressed. Since E2F1 localization is mainly cytoplasmic in neurons and nuclear in dividing cells, it is possible that these different subcellular localizations reflect different E2F1 RNA binding and DNA binding affinities. To begin testing this possibility, we overexpressed a truncated form of E2F1 missing the last 40 amino acids at the C-terminus in cultured rat cortical neurons. Protein lysates from transfected cultures were then used for EMSA assays using a biotin-labeled DNA probe from the Dhfr gene to detect E2F1-DNA complex formation. Increasing concentrations of unlabeled DNA probe and unlabeled E1 RNA ligand were used in a competition assay similar to those previously described to assess relative DNA and RNA binding (Ryder et al., 2008; Jordan-Sciutto et al., 1999).

The EMSA analysis from these neuronal protein lysates demonstrates that both DNA and RNA show ability to compete for E2F1 binding (Figure 4.3). However,
densitometry analysis of the EMSA image suggests that E2F1 has a higher affinity for RNA than for DNA, a result consistent with previously reported observations in cell-free systems (Ishizaki et al., 1996). In the densitometry plot, the intensity of chemiluminescence was plotted against the amount of unlabeled competition species (DNA and RNA). Resulting slope of each linear regression trend line shows a steeper slope for the E1 RNA competitive inhibitor and therefore a higher affinity for RNA binding than DNA binding in neurons. It is also worth noting that a robust gel shift can be detected in untransfected neuronal lysates, suggesting that the observed gel shift is partially attributed to endogenous protein and that E2F1 may not be optimally overexpressed to obtain reliable data for cross-lane comparisons (lane 2 of Figure 4.3a).

Although one of our ultimate goals is to compare E2F1-nucleic acid binding affinities in neurons and dividing cells, analysis of both the point mutant and wildtype E2F1 proteins would be more reliable in dividing cells where E2F1 overexpression is more robust. Various point mutation-containing E2F1 and wildtype constructs were overexpressed in HEK 293 cells and used in EMSA competition assays to assess their basal DNA binding level and the effectiveness of the unlabeled DNA probe to compete away this binding. Specifically, wildtype, R125A, L130A, N152K, and N170A constructs were tested in this experiment. Interestingly, R125A, which alters the thymidine binding residue, and N152K, which mirrors the E2F4 sequence at this residue, both show an inability to bind DNA regardless of the presence of cold competitor. The inability of R125A to bind DNA is consistent with the previous finding that human E2F1 R127S (the corresponding amino acid position in the human protein) showed an inability to bind DNA (Jordan-Sciutto et al. 1994), and suggests that this residue is critical for DNA binding (Figure 4.4). The inability of N152K to
produce a gel shift is surprising given that E2F4 contains this point mutation in an otherwise highly conserved E2F DNA binding domain and is capable of binding DNA. On the other hand, wildtype E2F1 displayed DNA binding that was clearly detectable by EMSA and efficiently competed away by cold DNA competitor (Figure 4.4). E2F1 N170A similarly showed an ability to bind DNA despite the point mutation, suggesting that altering this residue is not sufficient to significantly disrupt E2F1:DNA binding.

In order to increase sensitivity of E2F1:DNA binding in our EMSA assay and ensure that the lack of gel shifts observed is indeed due to a lack of DNA binding, we decided to introduce increased amounts of the E2F1 DNA binding cofactor DP1 in the EMSA reactions. Since E2F1 affinity for DNA is dramatically enhanced when heterodimerized with DP1, we hypothesized that increased presence of DP1 would enhance E2F1 affinity for DNA. To do so, HEK 293 lysates were transfected with FLAG-DP1-myc transfection with and without FLAG-rE2F1 co-transfection and overexpression was confirmed using Western Blot analysis (Figure 4.5, Top left panel). Successful overexpression was demonstrated using antibodies to DP1, FLAG and myc. Although DP1 overexpression was observed while co-transfecting HEK293 cells with FLAG-E2F1, it was not as robust as when DP1 was overexpressed on its own. To maximize the amount of DP1 added to the reaction, we decided to purify DP1 protein from the single transfection HEK lysates by FLAG affinity purification (Figure 4.5, Top right panel). Following affinity purification, DP1 was added to the EMSA reactions to determine whether it could amplify the gel shift signal. In addition, EMSA reaction samples were tested for the effect of total reaction volume on the binding efficiency of E2F1 species to DNA hot probe, a consideration based on the suspicion that excessively large reaction volume may decrease the binding
efficiency due to dilution. Similar to the EMSA result using neuronal lysates, our experimental results from HEK293 cells also shows a clear sign of a gel shift in the untransfected lysate lane (lane 2, Figure 3 and 4).

The resulting EMSA data was inconclusive regarding the effect of DP1 on E2F1:DNA binding, while suggesting that a larger reaction volume may enhance the binding efficiency (Figure 4.5, bottom panel). DP1 positive lanes do not show enhanced band shift intensity in comparison to DP1 negative lanes. Reaction volume, however, may have an effect since the band intensity of 15 μl reactions appears stronger than that of 10 μl reactions. However, neither variable produced a dramatic increase in E2F1-DNA-bining signal.

Overall, our data so far suggests that E2F1 is able to bind both DNA and RNA, but it may have a stronger affinity for RNA. Also, certain amino acid sites, such as R125A and N152K, within the DNA binding domain of E2F1 could potentially display differential roles in DNA and RNA binding. However, these results are preliminary, and we are still in the process of optimizing the EMSA protocol. As a future direction of this project, we are preparing to test for the effect of ionic concentrations on E2F1:DP:DNA and E2F1:DP:RNA complex formation by changing the ionic make-up of our binding buffer used during EMSA (Jordan et al., 1994). We may also to perform EMSA reactions with affinity purified FLAG-E2F1 or GST-E2F1 in order to increase the concentration of E2F1 bait and to eliminate concerns of endogenous protein in the cell lysates contributing to the observed probe shifts. Finally, switching from a biotin-based EMSA protocol to a more sensitive detection method such as radioactivity may be a necessary change to enhance baseline E2F1-DNA binding signal.
Figure 4.2. Generation of E2F1 point mutants. Top panel, a schematic showing the minimal sequence of E2F1 containing functional DNA binding (amino acids 117-191), in red along with the relevant amino acid sequence in which the mutated residues are located. Residues in red denote amino acids that contact DNA, asterisks refer to the residues that contact the DNA bases as opposed to the phosphodiester backbone. Western Blot showing successful overexpression of point mutant E2F1 constructs in HEK293 cells. Wildtype FLAG-rE2F1 E2F1 (1-397) which lacks the final 40 C-terminal amino acids, serve as positive controls for overexpression.
Figure 4.3. EMSA competition assay in neuronal lysates. Top panel. EMSA competition assay from rat primary neuronal lysates overexpressing C-terminal truncated wildtype rat E2F1 (1-397) with biotin-labeled DHFR DNA probe and either increasing concentrations of unlabeled DHFR DNA probe or unlabeled E1 C1 RNA probe used as cold competitor. The last lane represents a supershift attempt. Bottom panel. Densitometry analysis of the EMSA result from top panel. Relative signal intensities of the bands from DNA and RNA competition assays were plotted against the amount (ng) of competition inhibitor added.
Figure 4.4. EMSA of select E2F1 point mutants. EMSA of E2F1 constructs containing point mutations R125A, L130A, N152K, and N170A overexpressed in HEK 293 cells. E1 RNA ligand was used as a cold competitor.
Figure 4.5. EMSA results with affinity-purified DP1. **Top left panel.** Flag-Dp1-myc plasmid was used to transfect HEK293 cells with and without FLAG-E2F1 co-transfection in HEK 293 cell line. Samples were run on gel in triplicate. Identical thirds of the transferred membrane were probed with FLAG, DP1, and Myc antibodies, respectively. All three antibodies detected DP1 over-expression. Interestingly, DP1 expression level was markedly reduced by co-transfection with E2F1. **Top right panel.** Purification of Dp1 using FLAG affinity column. Dp1 eluate (lane 3) shows DP1 enrichment relative to unbound DP1 supernatant ‘flow-through’ following incubation with affinity column (Dp1 flow). **Bottom Panel.** EMSA testing for the effects of Dp1 and reaction volume on E2F1:DNA binding.
4.5 REFERENCES


CHAPTER 5 - DISCUSSION

5.1 OVERVIEW

One of the pressing needs facing the scientific and medical community is the development of effective therapies to manage neurodegenerative disease progression. In the case of HIV-associated neurocognitive disorders (HAND), the introduction of anti-retroviral therapy (ART) to suppress viral replication has dramatically improved prognosis of HIV-positive patients by both preventing the immune-compromised final state of HIV disease known as AIDS and significantly reducing the incidence of HIV-associated dementia, the most severe form of HAND (Childs et al., 1999, Sacktor et al., 2001). However, the prevalence of more minor cognitive impairments has grown and current estimates suggest that 30-50% of HIV-infected patients suffer from some form of neurocognitive dysfunction (Sacktor et al., 2001, Heaton et al., 2010) (McArthur et al., 2010). This shift in disease landscape is believed to be due to the increase in average lifespan of HIV-positive individuals on ART as well as potentially neurotoxic effects of ART drugs themselves (Sacktor et al., 2003, Sacktor et al., 2006, Sacktor et al., 2009). Recent evidence in both nonhuman primates with SIV-infection and in cultured primary cortical neurons has revealed significant neuronal damage resulting from ART (Akay et al., 2014). In fact, studies have suggested that discontinuation of ART treatment may improve neurocognitive status of HIV-positive individuals suffering from HAND (Robertson et al., 2010). However, given the universal benefit that ART provides to patients by preserving immunity and lifespan, a major emphasis should be placed on developing adjunct therapies to use alongside ART in treating the neurological impairments in HAND.
Toward that end, we have sought to define the neuronal pathways that promote neuronal damage and degeneration during HIV infection with the goal of identifying targets for therapeutic intervention. Two common death pathways that have been implicated in neuronal death are classical apoptotic signaling cascades involving the caspase family of cysteine proteases and necrotic pathways that utilize cysteine proteases called calpains. In advancing our understanding of HIV-induced neurodegeneration, one of our goals was to determine the relative contribution of caspases and calpains to cellular death processes in mature post-mitotic neurons. In Chapter 2, we assessed the effect of neuronal culture age on the capacity of neurons to induce calpain-mediated and caspase-mediated death. We report that primary cortical neuronal cultures exhibited both caspase- and calpain-mediated neuronal death at 1 week of age. However at 3 weeks of age, a stage at which neurons have fully developed synaptic innervations and express a complete repertoire of NMDAR subunits, we observed that cultured cortical neurons maintain their ability to induce death through calpain pathways but no longer induce caspase-dependent death. This shift in death pathway capabilities is accompanied by an upregulation of calpain-1 and a downregulation of executioner procaspase-3 protein levels.

In Chapter 3, we employed an in vitro model of HIV-induced neurotoxicity to identify a role for cell cycle protein E2F1 in calpain-mediated neuronal death. Our results revealed that cortical neuronal cultures from mice lacking functional E2F1 show reduced susceptibility to neuronal damage induced by conditioned media from HIV-infected monocyte-derived macrophage cultures (HIV-MDM). Furthermore, we identified neuronal E2F1 as a novel calpain substrate that is proteolytically cleaved to generate a
small, stable fragment following treatment with HIV-MDM supernatants. This fragment is produced specifically in differentiated neurons and requires native conditions to prevent further calpain degradation, suggesting that calpain-mediated proteolysis of E2F1 is cell-type specific and dependent on the native conformation of E2F1 and possibly other neuronal binding partners.

Given the ability of E2F1 to bind RNA through its DNA binding domain, we hypothesized that the cytosolic localization of neuronal E2F1 may allow it to regulate local protein translation as an RNA-binding protein (Ishizaki et al., 1996, Darbinian et al., 2006); unpublished data). In Chapter 4, we outline preliminary studies examining the RNA-binding capability of E2F1 by attempting to define the key residues within the DNA binding domain that govern the E2F1-RNA interaction.

Taken together, these data: 1) identify calpain activation as a dominant pathway in causing death and damage of fully differentiated neurons, 2) implicate neuronal E2F1 in a calpain-mediated death pathway during HIV infection of the CNS, and 3) highlight a need for understanding both the physiological role of full-length E2F1 in neurons and the function of the cleaved product during pathological conditions.

5.2 CALPAINS AND CASPASES: CELL DEATH IN MATURE NEURONS

Caspase-mediated signaling cascades are widely used by mammalian cells as a form of genetically programmed cellular suicide known as apoptosis. Originally identified in C. elegans, caspases were shown to enact a cell death mechanism that rapidly eliminates excess cells during development. The cells that activate this caspase death program are
predetermined based on cell lineage and activate cell death at predictable times (Lette and Hengartner, 2006). Similarly, the proper development of the mammalian CNS requires activation of caspase-mediated apoptosis, providing a wave of cell death among proliferating neuroblasts and immature neurons that unsuccessfully compete for synaptic innervations and trophic factor support (Gagliardini et al., 1994, Yuan and Yankner, 2000). Disrupting this process through genetic deletion of caspase-3 and caspase-9 in mice causes exencephaly, hyperplasia and perinatal lethality (Kuida et al., 1998, Pompeiano et al., 2000). Caspases have similarly been implicated in activating apoptosis in individual cells of various tissue types as a response to cellular stresses such as DNA damage, oxidative stress and hypoxia (Elmore, 2007). The rapid elimination of these damaged cells serves to protect the surrounding cells of the organism and in the case of DNA damage, to ensure that the defective cells do not proliferate and produce a genetically altered cell population.

However, the mature mammalian CNS primarily contains neurons that are postmitotic and that have established a complex network of synaptic connections with surrounding cells. Preserving these synaptic connections is critical for facilitating cell-cell communication and maintaining intact neural circuitry. Given the dependence of the CNS on this synaptic network, a rapid elimination of a mature neuron would affect each of the surrounding cells with which it shares synaptic connections. Thus, the detrimental effects of rapid elimination of stressed or damaged neurons in the adult brain could outweigh any potential benefits, suggesting that caspase-directed apoptosis may be less likely when the CNS is fully developed. In fact, our results in cultured rat cortical neurons show a clear developmental effect on the induction of caspase-mediated neuronal
death. While H$_2$O$_2$ treatment was able to induce caspase-dependent neuronal death at an early culture age (1 week), caspase-mediated death was undetectable in cultures treated with H$_2$O$_2$ at 3 weeks of age, a time when synaptic surface receptor expression has matured (O'Donnell et al., 2006, Eugenin et al., 2011). Protein expression data from these cultures revealed a reduction in procaspase-3 protein levels in cultures at 3 weeks of age compared to those at 1 week, further supporting a decreased capacity to induce caspase-mediated death as neuronal cultures mature. This finding was mirrored by several studies both in vitro and in animal models demonstrating that procaspase-3 levels decrease during neuronal maturation and that caspase induction in response to stressors such as hypoxia and ischemia was dramatically attenuated with age (Hu et al., 2000, Yakovlev et al., 2001, Li et al., 2009).

Unlike caspase-mediated death that can take on the order of hours to minutes to commit a cell to death, calpain-mediated neuronal death can occur as part of a chronic, slow progressing disease state. Current evidence suggests that calpain dysregulation in the CNS becomes more likely with age in part due to increased expression of calpains and gradual depletion of its endogenous inhibitor calpastatin (Nixon et al., 1994, Mouatt-Prigent et al., 1996, Rao et al., 2008). Our results in cortical culture support those in vivo observations by showing that as caspase-3 expression and caspase-mediated neuronal death are downregulated in culture, calpain-1 expression increases and calpain-activated death becomes more prevalent. Using a similar cortical culture system to model age-related neurodegeneration, Kim and colleagues observed this same increase in expression for both calpain-1 and calpain-2 isoforms as their cultures aged in conjunction with decreased procaspase-3 levels (Kim et al., 2007). However, numerous in vitro studies that
aim to model acute neuronal loss and chronic neurodegenerative states in the mature brain identify caspase involvement in neuronal death (Giovanni et al., 2000, Park et al., 2000a, Kuwako et al., 2002, Benosman et al., 2007, Shiwany et al., 2009, Feng et al., 2014). These studies often utilize neuronal cell lines or cultured primary neurons at an early age. Our findings underscore the importance of carefully considering culture age when interpreting data from in vitro studies. Indeed, our in vitro model of HIV-mediated neurotoxicity illustrates the critical role that culture age can play as HIVMDM treatments fail to elicit neuronal damage and loss at 7 days in vitro, but show significant neuronal loss at DIV 14 and DIV 21 (O'Donnell et al., 2006). Furthermore, our results and those of others suggest that calpain-mediated neuronal death predominates following neuronal maturation and that selecting older neuronal cultures for in vitro studies may be more physiologically relevant for modeling CNS diseases in the adult brain.

5.3 A TRANSCRIPTION-INDEPENDENT FUNCTION FOR NEURONAL E2F1

Traditionally, E2F1 research was aimed at understanding its function in proliferating cells. Studies of E2F1 have uncovered its role as a critical regulator of the G1-S phase transition during cell division as well as an inducer of cell death through its activation of transcriptional programs for DNA replication and classical apoptotic cascades, respectively (DeGregori and Johnson, 2006, Iaquinta and Lees, 2007). However, E2F1 has also received considerable attention in the context of neuronal death and neurodegeneration. In vitro studies have implicated E2F1 transcriptional activation of apoptosis in cultured neurons and neuronal cell lines in response to a variety of cellular
stresses, including DNA damage, potassium deprivation, and Aβ toxicity (Park et al., 1997, Giovanni et al., 2000, O'Hare et al., 2000, Park et al., 2000a, Park et al., 2000b). Furthermore, a number of cell cycle proteins including E2F1 and its transcriptional repressor Rb, show aberrant expression patterns in CNS neurons of patients with neurodegenerative diseases (Ranganathan et al., 2001, Herrup and Arendt, 2002, Jordan-Sciutto et al., 2002a, Jordan-Sciutto et al., 2002b, Jordan-Sciutto et al., 2003, Akay et al., 2011, Folch et al., 2012)(Akay, Lindl, 2011; Jordan-sciutto, Dorsey, 2003; Ranganathan, Scudiere, 2001; Herrup and Arendt, 2002; Jordan-Sciutto, Malaiyandi, 2002; Jordan-Sciutto, Wang, 2002; Folch, Junyent, 2011). These unexpected observations along with discoveries of aneuploidy and DNA replication in postmitotic CNS neurons provided the hypothesis that the observed aberrant activation of cell cycle regulators represented an attempt at cell cycle re-entry that caused neuronal death and contributed to neurodegeneration (Mosch et al., 2007, Arendt, 2012).

However, data from our lab suggests that E2F1 in neurons may have an alternate role to its traditional actions as a transcription factor in cell division and apoptosis. We have observed a predominantly cytoplasmic localization of E2F1 in neurons both in vivo and in vitro (Jordan-Sciutto et al., 2002b, Wang et al., 2010, Ting et al., 2014). Despite reported E2F1 upregulation in neurons of patients with HIV encephalitis, studies from our in vitro model of HIV-induced neurotoxicity show no evidence of transcriptional activation of canonical E2F gene targets (Wang et al., 2010). Furthermore, several studies have noted an ability of E2F1 to induce cell death through transcription-independent mechanisms. For instance, we have shown that overexpression of transcriptionally-inactive E2F1 protein can induce calpain-mediated cell death in
HEK293 cells (Strachan et al., 2005). In cerebellar granular neuronal cultures, Hou et al. reported that overexpression of E2F1 lacking the transactivation domain induces apoptosis through modulation of NFκB protein levels (Hou et al., 2001). Our current studies confirmed the cytosolic localization of neuronal E2F1 and reveal a role for E2F1 in HIV-induced neurotoxicity through a calpain-dependent pathway. Additionally, we identified the stable accumulation of a calpain-cleaved E2F1 fragment in neurons and suggest that production of this cleavage product may serve as a neurotoxic event either through E2F1 physiological loss of function or through a toxic gain of function.

5.4 IDENTIFYING THE SITES OF E2F1 CLEAVAGE

Understanding the functional consequences of neuronal E2F1 cleavage will require the identification of the precise proteolytic sites within the E2F1 protein. This effort has been complicated by several biological and technical challenges. First, unlike proteases such as caspases and trypsins that have well-established cleavage site motifs, the rules governing substrate cleavage by calpains are poorly understood. Given the widespread involvement of calpain activity in neuronal physiology and neuronal damage, there have been numerous attempts to define a consensus sequence or preferred motif for calpain cleavage of its many substrates (Tompa et al., 2004, Cuerrier et al., 2005, DuVerle et al., 2011). Despite these efforts, no definitive method exists to predict cleavage sites and it is now generally appreciated that calpain cleavage site recognition involves secondary and tertiary structure considerations, making site predictions based on primary sequence difficult.
In the absence of reliable prediction tools, calpain cleavage sites are often identified by recapitulating substrate cleavage *in vitro* through incubation of recombinant substrate protein with activated calpain and then identifying cleavage sites through sequencing analyses. Interestingly, we found that incubation of recombinant E2F1 with activated calpain-1 in a cell-free system failed to produce the stabilized fragment observed in neurons. Additionally, calpain cleavage of E2F1 in overexpressing cell lines also failed to generate the stabilized fragment, suggesting that this calpain-cleaved isoform is uniquely produced in neurons. The current technical limitations of both commercially available and our custom-designed E2F1 antibodies have so far prevented immunoprecipitation of the E2F1 cleavage product from neuronal protein lysates. Our attempts to overexpress E2F1 in neurons in order to increase the potential input of cleaved E2F1 have similarly been unsuccessful, likely due to the presence of E2F consensus sites within the plasmid CMV promoter that allow E2F1 to shut off promoter-driven expression when complexed with Rb or other binding proteins (Trouche et al., 1997, Choi et al., 2005). Thus, future attempts to isolate the calpain-cleaved E2F1 product from neurons will focus on: 1) increasing input of E2F1 protein for future immunoprecipitations either by using an *in vitro* transcription/translation system or by identifying a plasmid promoter lacking E2F consensus sites that will support robust E2F1 overexpression in neurons, and 2) performing a two-step chromatographic purification method consisting of either size-exclusion chromatography or ammonium sulfate precipitation followed by a DNA binding affinity column.
5.5 CALPAIN-CLEAVED E2F1: A NEUROTOXIC ISOFORM

We have been able to demonstrate through a calpain cleavage time course and via an epitope tagging approach that neuronal E2F1 is cleaved by calpain at least twice, once at the N-terminus and once at the C-terminus. Identifying the precise cleavage sites would be beneficial not only by clarifying the extent to which the terminal domains are disrupted within E2F1, but also by allowing overexpression studies of the cleavage product to conclusively assess its toxicity. Despite the current limitations that prevent this study, there are several pieces of evidence suggesting that calpain-cleaved E2F1 acts in a neurotoxic fashion. First, pharmacological inhibition of different NMDAR subunits revealed that E2F1 cleavage is dependent on stimulation of NR2B-containing receptor subtypes. NR2B-containing NMDARs are widely reported as extrasynaptic and viewed as the receptors mediating excitotoxicity (Zhou and Baudry, 2006, Liu et al., 2007). Moreover, the regions of the brain with higher NR2B expression such as the prefrontal cortex, hippocampus and basal ganglia are the same regions that are most susceptible to excitototoxicity and HIV-mediated neurodegeneration (Kim et al., 1998, Jordan-Sciutto et al., 2002b). Second, our model of HIVMDM-induced neurotoxicity promotes neuronal damage in a NR2B receptor-dependent and calpain-dependent manner, the same manner in which E2F1 is cleaved (O'Donnell et al., 2006, White et al., 2011). Finally, we have previously shown that overexpressing an N-terminal truncation of E2F1 in HEK293 cells causes calpain-dependent cell death, suggesting that E2F1 truncation can promote toxicity via a calpain pathway (Strachan et al., 2005). Taken together, these data support the view of calpain cleavage of E2F1 as a neurotoxic event (Figure 5.1).
Nonetheless, it is worth considering the possibility that E2F1 cleavage serves a neuroprotective function. In fact, calpain activation has been observed to have neuroprotective roles in certain toxicity models. For instance, calpain activation can attenuate neuronal death in hippocampal and mesencephalic slice cultures treated with the neurotoxin MPP+ through BDNF-dependent signaling (Jourdi et al., 2009). Given the disparity of Ca\(^{2+}\) required in vitro by calpain-1 and calpain-2, there has been a widely held belief that calpain-1 fulfills physiological roles while calpain-2 is activated under conditions of sustained calcium dysregulation. The recent development of isoform-specific pharmacological inhibitors of calpains has helped test this theory. While recent findings present conflicting results, at least one study has reported results that support a neuroprotective role for calpain-1 and a neurotoxic role for calpain-2 (Lee et al., 2013, Wang et al., 2013). Using these isoform-specific calpain inhibitors along with isoform-specific knockdown approaches could help identify which calpain isoform is responsible for processing E2F1 in our HIVMDM-treated cultures and shed light on the role of calpain-cleaved E2F1 in neuronal viability.

5.6 E2F1 IN SYNAPTIC PHYSIOLOGY

The functional implications of a potential E2F1 loss-of-function are difficult to appreciate without understanding the role of E2F1 in neuronal physiology. Our current understanding of neuronal E2F1 function is hampered by the absence of a true E2F1 knockout mouse model. Future development of a conditional ‘Cre-LoxP’ E2F1 knockout animal model will help address E2F1 physiological functions through studies of the
effects of neuron-specific and brain region-specific E2F1 deletion. In spite of this limitation, current evidence supports several possible roles for neuronal E2F1.

In cultured hippocampal neurons, E2F1 expression is punctate and co-localizes with the post-synaptic marker PSD-95 (Ting et al., 2014). Biochemical fractionation similarly reveals enrichment of E2F1 in synaptic fractions (Ting et al., 2014). Furthermore, mice lacking functional E2F1 exhibit age-dependent depletion of critical synaptic proteins such as PSD-95, NMDAR subunits NR1 and NR2A, and Synaptic Ras GTPase activating protein (SynGAP) as well as memory and olfactory deficits with mirroring age-dependent onset (Ting et al., 2014). These findings suggest that E2F1 may contribute to maintenance of synaptic structure or physiology in the adult brain.

Interestingly, the E2F1 amino acid sequence contains multiple P-X-X-P motifs known to bind SH3 domains (Alexandropoulos et al., 1995). A number of synaptic proteins contain SH3 domains, including the scaffolding proteins PSD-95 and SAP97, as well as amphiphysin I (Vandanapu et al., 2009, Neuvonen et al., 2011). These proteins are involved in a variety of synaptic processes including ion channel/receptor clustering, membrane trafficking, and synaptic vesicle recycling. Identifying potential binding interactions between E2F1 and the SH3 domains of these proteins or with other synaptic proteins will be instrumental in clarifying the significance of E2F1 synaptic localization. Additionally, these interactions may be disrupted during HAND following calpain cleavage of E2F1, contributing to synaptic dysfunction and HIV-associated neurotoxicity.

Interestingly, we found that protein conformation was critical for production of the stabilized calpain-cleaved E2F1 isoform, suggesting that unique binding partners in neurons may underlie the cell type-dependent nature of its production by protecting
regions of the E2F1 protein from further degradation. Thus, identifying the synaptic binding interactions with E2F1 that remain preserved in our *in vitro* model of HAND may have important implications for understanding the novel calpain processing of E2F1 in neurons.

Given the important role of calpain in synaptic plasticity and specifically in the induction of long-term potentiation (LTP), it is possible that the calpain-cleaved E2F1 isoform may also participate in this process (Wu and Lynch, 2006). In fact, recent findings regarding CDK5 regulation in neurons show that its activator p35 is cleaved by calpain to p25 not only during pathological conditions, but also following physiological neuronal activity. Transient, NR2B-dependent production of p25 by calpain was shown to enhance LTD in hippocampal slice culture and aid in memory extinction in mice expressing a noncleavable p35 protein (Seo et al., 2014). Development of a calpain-resistant E2F1 knock-in mouse model would similarly allow us to assess whether inhibiting E2F1 cleavage influences synaptic remodeling and ultimately, the potential implications for learning and memory.

### 5.7 E2F1 AND CALCIUM REGULATION

It is well-established that intracellular Ca\(^{2+}\) levels govern calpain activation. Interestingly, a number of calpain substrates are involved in regulating calcium homeostasis. Under conditions of elevated intracellular Ca\(^{2+}\) concentrations, cleavage of these substrates often exacerbates calcium dysregulation and creates a positive-feedback pathway to amplify calpain activity. For instance, the Type 1 IP3 receptor, which functions as an ER calcium
channel, disrupts intracellular Ca\(^{2+}\) buffering when cleaved by calpain (Kopil et al., 2012). L-type Ca\(^{2+}\) channels in cultured neurons are cleaved by calpain, leading to C-terminal truncation of their \(\alpha_1\) subunit and enhanced Ca\(^{2+}\) influx (Klockner et al., 1995, Hell et al., 1996). Similarly, the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) has been shown to be cleaved by calpain during excitotoxic stress, inhibiting its Ca\(^{2+}\) extrusion capabilities (Bano et al., 2005). Interestingly, neuronal E2F1 has been reported to bind Necdin, a factor involved in maintaining the terminally differentiated identity of postmitotic neurons (Kuwako et al., 2004). Further, Necdin has been linked to intracellular calcium regulation through its interaction with NEFA, a calcium-binding protein located on the ER cisternae which regulates ER calcium release (Taniguchi et al., 2000). We have previously found that transcriptionally-inactive N-terminally truncated E2F1 causes cell death when overexpressed in HEK293 cells (Strachan et al., 2005). This truncated E2F1 product localizes to the cytoplasm much like calpain-cleaved E2F1 and induces death through a calpain-dependent mechanism. Together, these findings suggest that E2F1 may play a role in calcium homeostasis and that calpain-cleaved E2F1 could promote calpain amplification by enhancing intracellular calcium elevation. Future work will determine whether an E2F1-Necdin binding interaction might be involved in calcium regulation and whether calpain cleavage of E2F1 during HIV-mediated neurotoxicity disrupts this interaction and this putative function. In addition to this candidate approach, a system-wide effort to identify neuronal binding proteins should be carried out to identify the neuronal interactome of E2F1. This unbiased strategy could confirm an E2F1-Necdin interaction while identifying binding factors involved in other potential processes.
5.8 NEURONAL E2F1: A PUTATIVE RNA-BINDING PROTEIN

We and others have observed that E2F1 can bind synthetic RNA ligands in vitro (Ishizaki et al., 1996, Darbinian et al., 2006). We have also observed that E2F1 interacts with cellular RNA from rat cortex and that this interaction is dependent on the ‘winged helix’ DNA binding domain (unpublished observation). Considering that localization of neuronal E2F1 is predominantly cytoplasmic, E2F1 participation in RNA binding and local translational regulation provides an appealing mechanism for its function in neurons and a target of disruption during HIV-induced neuronal damage. Interestingly, several DNA-binding proteins in the ‘winged helix’ superfamily carry out RNA-binding functions including the Lupus La protein and SelB, both of which function as translation factors in the cytosol (Intine et al., 2003, Yoshizawa et al., 2005). In our current studies, we attempted to determine the identity of the RNA binding domain of E2F1 through a combined approach utilizing site-directed mutagenesis and EMSA. As we continue these studies and identify E2F1 point mutants with disrupted RNA binding ability, we aim to use these point mutants in overexpression studies to assess their influence on neuronal viability.

Further work to investigate E2F1 RNA-binding in neurons will be greatly aided by identification of the RNA species that E2F1 binds. So far, BLAST searches have been difficult to carry out given the short sequence and secondary structure of the synthetic E1 RNA ligand with which E2F1 interacts. A more promising unbiased approach will rely on RNA immunoprecipitation methodologies in conjunction with real-time PCR techniques to isolate E2F1-associated RNA and identify the individual transcripts. Once they are identified, studies to explore their translational regulation during basal and
neurotoxic conditions could show whether E2F1-RNA binding and translational repression is disrupted during HIV-associated neurodegeneration while pinpointing the individual transcripts that are affected.

Putative RNA-binding functions of E2F1 could help regulate local translation in several neuronal contexts. One such context is synaptic plasticity, which depend on local protein synthesis to facilitate rapid changes in synaptic membrane organization and surface receptor distribution (Martin and Zukin, 2006, Derkach et al., 2007). Protein synthesis inhibitors have been shown to block induction of LTP and a host of RNAs have been identified in dendrites along with protein synthesis machinery (Bradshaw et al., 2003, Martin and Zukin, 2006, Pfeiffer and Huber, 2006). Local protein synthesis also plays an important role in axon guidance, where the growth cone must continually respond to chemotactic signals in the surrounding environment (Yoon et al., 2009). For instance, local translation of β-actin is required during Ca$^{2+}$-mediated growth cone turning (Yao et al., 2006). Intriguingly, calpain activation has also been documented both in synaptic potentiation and in axon pathfinding, suggesting a possible regulatory link between calpain processing of E2F1 and E2F1-RNA binding, wherein E2F1 cleavage triggers release of bound, translationally repressed transcripts (Qin et al., 2010, Baudry et al., 2013). Given our results that calpain-cleaved E2F1 is capable of being produced at all culture ages, it is quite possible that calpain-cleaved E2F1 is transiently generated during neurite outgrowth. Thus, performing parallel RNA immunoprecipitation reactions with full length and calpain-cleaved E2F1 could identify which E2F1-bound transcripts are released following calpain cleavage of E2F1 either in physiological or pathophysiological settings.
Although we do not observe detectable levels of E2F1 in nuclear fractions of cultured cortical neurons and fail to detect expression of canonical E2F gene targets in our model of HIV-induced neurotoxicity, it is still possible that neuronal E2F1 retains some role as a transcription factor. In fact, we have previously observed that E2F1 shuttles between the neuronal cytosol and nucleus, and that inhibiting the nuclear export receptor Crm1 causes nuclear accumulation of E2F1. Furthermore, calpain cleavage of certain substrates has been shown to induce nuclear translocation and influence transcriptional programs. For example, in cultured hippocampal neurons treated with glutamate or NMDA, calpain has been reported to cleave the transcription factor beta-catenin at its N-terminus, producing stable truncation products that subsequently translocate to the nucleus and activate gene transcription (Abe and Takeichi, 2007). Following calpain processing of p35 to p25, Cdk5 translocates to the nucleus where it has been shown to inhibit MEF2D, a transcription factor that regulates neuroprotective genes (O'Hare et al., 2005, Smith et al., 2006). To aid in the investigation of E2F1 RNA-binding function, we are in the process of screening for nucleotide analogs that bind E2F1 and selectively disrupt E2F1:RNA binding while leaving E2F1:DNA complexes unaffected. This tool will help identify RNA binding effects of E2F1 that are unrelated to E2F1 transcription factor activity.

5.9 CLINICAL UTILITY OF CALPAIN-CLEAVED E2F1

Calpain activation has been implicated in a host of neurodegenerative conditions (Camins et al., 2006). Despite a well-documented role in neuronal damage, calpains are
ubiquitously expressed and are critical for a variety of physiological processes in different cell types. With the physiological functions of calpains in mind, it is currently difficult to formulate a therapeutic strategy involving calpain inhibition. If a strategy is to be devised to selectively address detrimental calpain activities in a particular disease, it will be critical to understand the role of each calpain substrate, the functional implication of its cleavage, and the disease in which it is processed. By identifying a subset of these substrates whose cleavage significantly contributes to disease pathology, it may be possible to therapeutically target calpain in a substrate-specific manner that eliminates unwanted proteolytic events while sparing those that are beneficial.

Recent work has employed just such an approach with the calpain substrate mGluR1α that is cleaved during excitotoxicity, leading to enhanced calcium dysregulation (Wei et al., 2008). To block this cleavage event, a decoy peptide was designed containing the mGluR1α sequence spanning its calpain cleavage site fused to a cell permeable sequence from the HIV TAT protein. This decoy peptide successfully prevented mGluR1α cleavage by calpain and provided neuroprotection when administered to NMDA-treated cortical cultures and when injected systemically into mice with ischemic injury (Wei et al., 2008). Given this promising result, it may be worthwhile to pursue substrate-specific strategies for attenuating certain calpain cleavage events.

We have identified E2F1 as a novel calpain substrate and provided evidence suggesting that calpain-cleaved E2F1 contributes to HIV-mediated neuronal damage. While there is a clear need for adjunctive therapies to treat patients with HAND, the potential of calpain-cleaved E2F1 as a therapeutic target will depend in part on the nature of neuronal E2F1 physiologic function and the functional consequences of its proteolytic
processing. Furthermore, a specific strategy targeting E2F1 cleavage like the one above for mGluR1α will necessitate identification of the precise cleavage sites within the E2F1 sequence. Thus, future studies are warranted to explore neuronal E2F1 function and to define the sequence of calpain-cleaved E2F1 and its role in neurons.

Aside from the potential therapeutic value of calpain-cleaved E2F1, calpain-cleaved E2F1 may hold promise as a diagnostic marker. Our studies identified production of calpain-cleaved E2F1 in human fetal neurons and observed a low molecular weight E2F1 isoform in a subset of midfrontal cortex autopsy samples from patients with HAND. A larger tissue sample size will need to be evaluated to determine whether there is a significant correlation between lower molecular weight E2F1 presence and disease status. If a correlation does exist, generating site-directed antibodies specific for calpain-cleaved E2F1 may have utility in helping diagnose HAND. A recent study found that measuring levels of activated calpain in the CSF of AD patients improved diagnostic accuracy as part of a multivariate analysis (Laske et al., 2014). Additionally, the diagnostic value of calpain-cleaved αII-spectrin in serum was recently demonstrated in predicting recovery of athletes following concussions, suggesting that calpain substrates may serve as diagnostic marker for mild traumatic brain injury in blood samples (Siman et al., 2013, Siman et al., 2014). Experiments to detect calpain-cleaved E2F1 levels in CSF and serum will help determine whether there is similar utility for this E2F1 isoform in diseases such as HAND and potentially other CNS conditions. Ultimately, detecting calpain-cleaved E2F1 as part of a diagnostic signature may maximize diagnostic accuracy and disease specificity. Future work is needed to demonstrate whether calpain-cleaved E2F1 or other calpain substrates are suitable for this type of application.
Figure 5.1. Proposed mechanism for neuronal E2F1 in HIV-induced neurotoxicity.

HIV-infected monocytes cross the blood-brain barrier and differentiate into macrophages in the brain parenchyma. There, they release excitotoxins and inflammatory factors such as glutamate into the extracellular environment (1). These factors bind and activate NMDA receptors on CNS neurons (2), causing calcium influx (3) and subsequent activation of the cysteine protease calpain (4). Activated calpain cleaves multiple substrates including E2F1, producing a stabilized protein fragment with truncations at the N- and C-terminus (5). Calpain-cleaved E2F1 may contribute to calpain-mediated neuronal damage observed in NMDA receptor-mediated neurotoxicity (6).
5.10 REFERENCES


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