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Degradation of Transcriptional Repressor ATF4 During Late-Phase Long-Term Potentiation

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Degradation of Transcriptional Repressor ATF4 During Late-Phase Long-Term Potentiation

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History

Memories are one of the most personal and intimate aspects of the human personality. As such, the formation and storage of memories has been one of the most intriguing topics of psychology and cognitive neuroscience for many years. The earliest speculation regarding the underlying components of memory formation can be traced back to the writings of the ancient philosophers Aristotle and Cicero. However, it was not until the 19th century when neuroscientists began to outline the structural, cellular, and molecular underpinnings of memory formation. In 1890, William James was the first to differentiate between short-term memory (STM) and long-term memory (LTM), or primary and secondary memory, respectively, as he described it in his book *Principles of Psychology*. At that time, the cellular basis for LTM formation remained undefined. However, Semon (1904) and Cajal (1912) individually postulated the initial hypotheses for the cellular mechanism of memory formation in the early 20th century, suggesting that the processing of information requires neural plasticity at the synaptic level, simply referred to as synaptic plasticity. Years later, Donald Hebb (1949) notably and accurately proposed a theory, which stated that synaptic strengthening would occur if two neurons rapidly fired together within a short period of time. This Hebbian theory became commonly summarized as "neurons that fire together, wire together." At the time, these novel hypotheses of synaptic plasticity were initially viewed with skepticism by many neuroscientists due to the widely-accepted theory proposing that individual synapses had an inherently determined and static magnitude of neurotransmission from birth. Since then, however, many studies have shown that long-term synaptic plasticity requires the activity-dependent molecular and structural alterations of individual synapses, which can elicit

the strengthening or weakening of a synapse, hereafter referred to as long-term potentiation (LTP) and long-term depression (LTD), respectively. Currently, both of these mechanisms are known shown to play a vital role in LTM formation.

Long-term potentiation

The strengthening of synapses occurs through the cellular mechanism known as LTP. As mentioned earlier, Semon, Cajal, and Hebb collectively proposed the first correct hypothesis of this mechanism; however, it took several more decades for this process to be experimentally documented in neurons. Electrophysiological experiments by Bliss and Lomo (1973) first demonstrated LTP in the perforant neural circuit of the rat hippocampus. It was shown that the repetitive high-frequency electrical stimulation of the presynaptic perfomant neuron resulted in a long-lasting increase in response in the postsynaptic dentate gyrus (DG) neuron. This type of strengthening of synapses have been shown by a plethora of studies in a wide range of animal models. Accumulated evidence over the years has established LTP to be the cellular basis for memory formation (Bliss and Collingridge 1993; Moser et al. 1994). Since then, LTP has been one of the most well studied areas of neuroscience throughout the past four decades.

A number of relatively recent studies have described the molecular changes associated with LTP. Specifically, the dependence of LTP on protein modifications, mRNA synthesis, and protein synthesis have been well documented (Frey et al. 1988; Kelleher et al. 2004). These molecular changes appear to be the differentiating factor between the individual phases of LTP. In general, LTP can be divided into two broad phases – the early-phase (E-LTP) and the late-phase (L-LTP). E-LTP is a transient process (1-2 hours) induced by a single, high-frequency stimulation and is dependent on the posttranslational modification of preexisting proteins throughout the postsynaptic neuron (Lynch 2004). Indeed, the phosphorylation of glutamatergic receptors has

been described as a vital mechanism during this phase (Lee 2006), and the incubation of neurons in a general kinase inhibitor has been shown to inhibit the induction of E-LTP (Malinow et al. 1989). E-LTP is thought to underlie STM formation (Nguyen et al. 1994; Kandel 2001). In contrast, L-LTP is the process responsible for LTM formation. L-LTP is a lengthy process (6-24 hours) induced by repetitive, high-frequency stimulations and is dependent on mRNA and protein synthesis (Kelleher et al. 2004). L-LTP can be further subdivided into the induction/early-phase (Ep-L-LTP) and maintenance/late-phase (late L-LTP/L-LTP maintenance). Similar to the broader phases of LTP, the dependence of these phases on molecular activities appears to be the differentiating factor between them. Ep-L-LTP is a relatively transient process dependent on the translation of preexisting dendritic mRNA (Kelleher et al. 2004; Dong et al. 2008). L-LTP maintenance is a longer process and is dependent on new gene transcription (Nguyen et al. 1994). Previous pharmacological studies have demonstrated this differential molecular dependence by the inhibition of Ep-L-LTP and L-LTP maintenance in cultured murine hippocampal neurons incubated with a translation (anisomycin) or transcription (actinomycin-D) inhibitor (Kelleher et al. 2004; Banko et al. 2005). Taken together, these data demonstrate that LTM formation is dependent on the repetitive high-frequency stimulation of a presynaptic neuron and the subsequent activation of transcription and translation machinery.

Long-term depression

Two decades after the discovery of LTP, the opposite cellular mechanism – LTD – was discovered in cerebellar neurons by Ito and colleagues (1982). LTD has been shown to occur when the presynaptic and postsynaptic neurons are simultaneously stimulated at a low-frequency (Blanke and VanDongen 2008). This results in a decrease of the excitatory postsynaptic potential (EPSP) (Bear 1999) and the eventual weakening of the synapse. Although appearing

counterintuitive, LTD has been implicated as an important mediator of LTM formation in the mammalian hippocampus (Dudek and Bear 1992). The vulnerability of a hippocampal synapse to an LTD stimulus depends on the experience of the individual synapse. That is, a synapse that has been hyperstimulated by LTP stimuli is more susceptible to an LTD stimulus. In this manner, LTD influences LTM formation by acting as a negative feedback regulatory mechanism on individual synapses, such that each synapse never reaches the maximal level of LTP stimulation. As such, LTD is the cellular mechanism underlying the theoretical potential for unlimited LTM formation and learning capacity in that synapses are kept below stimulation saturation (Ito 1989).

Animal models of long-term memory formation

Although LTM formation is dependent on both forms of long-term synaptic plasticity, LTP functions as the primary cellular mechanism, while LTD, as previously outlined, plays a role in maintenance. As such, LTP has consumed much of the research efforts regarding LTM formation throughout the recent decades. LTP has been studied *in vivo* and *in vitro* in numerous animal models, such as *Drosophila*, honey bees, *Aplysia*, nematodes, and rodents. The molecular underpinnings of long-term synaptic plasticity have been best outlined in the sensorimotor synapse of *Aplysia* in the laboratory of the 2000 Nobel Prize in Physiology or Medicine recipient, Eric Kandel, at Columbia University. The sensorimotor synapse of *Aplysia* controls the defensive gill reflex and exhibits the capacity for long-term facilitation (LTF), a basal form of LTP and simple model of LTM formation (Abrams 1985). Subsequently, these studies, along with the identification of the hippocampus as an important mediator of memory, established the groundwork for later mammalian studies. Likewise, the evolutionary conservation of many molecular mechanisms associated with LTP has permitted the application of results documented in more basal animal

models to that of higher order animal models. Indeed, this strategy has been, and continues to be, used to further elucidate the underpinning molecular mechanisms associated with LTP.

Mammalian hippocampal neurons

The hippocampus is a bilateral brain structure located in the medial temporal lobe and is a component of the limbic system. The limbic system also includes the amygdala and some midbrain regions that collectively function to regulate emotion, memory, and motivation. The role of the hippocampus in memory formation was initially described by Scoville and Milner (1957) who performed experimental bilateral temporal lobe lesions in the brain of a patient exhibiting chronic epilepsy. Despite aiding much of the seizure complications, the surgery consequently resulted in permanent anterograde amnesia (Mishkin 1978; Zola-Morgan et al. 1982). This patient later became famously known as "patient H.M." Since then, the role of the hippocampus in learning and memory has been well described, and it has been implicated in the pathology of memory-related disorders, such as Alzheimer's disease in humans (Firsoni et al. 2008).

Hippocampal neurons are unique in that they have the capacity to undergo robust LTP relative to neurons of other brain regions (Racine et al. 1983). The trisynaptic neural circuitry of the hippocampus was first outlined by Andersen and colleagues (1971), who described three neural pathways: the perforant pathway, the mossy fiber pathway, and the Schaffer collateral pathway. The perforant pathway sends afferent projections to the dentate gyrus (DG), while the mossy fiber pathway projects from the DG to the cornu ammonis (CA) area 3 (CA3) region. The Schaffer collateral pathway, the most well understood of the hippocampal pathways, connects the CA3 neurons with the CA1 pyramidal neurons via CA3 afferents (Figure 1). Each of these pathways are important in distinctive forms of memory formation; however, the relative simplistic structure of the circuit and ease of experimental manipulation make the Schaffer collateral pathway an ideal

region to study LTP. Behavioral analyses in rodent models using contextual fear conditioning (Blanchard and Blanchard 1972), the Morris water maze (Teng and Squire 1999), and the T-maze task (Bannerman et al. 2003) have demonstrated the importance of these neurons in mediating LTM formation. Many structural changes have also been described at the cellular level in these neurons during LTP, such as increased spine number (Desmond and Levy 1990), altered synaptic vesicle concentration (Applegate et al. 1987), and structural alterations of synapses (Desmond and Levy 1990). The molecular changes in the Schaffer collateral pathway during LTP have also been described. During Ep-L-LTP, repetitive high-frequency electrical stimulation of the presynaptic (CA3) neuron has shown to facilitate the release of glutamate onto the postsynaptic (CA1) *N*-methyl-D-aspartate (NMDA) receptors (NMDARs). The dependence on NMDAR stimulation during LTP in these neurons was demonstrated by incubating neurons in an NMDAR antagonist, which significantly attenuated the postsynaptic response (Morris et al. 1986). NMDAR stimulation facilitates a robust postsynaptic calcium influx (Lynch et al. 1983; Harris et al. 1984; Malenka et al. 1988) that positively regulates cAMP activity, and, consequently, the hyperactivation of numerous intracellular kinases, such as PKA and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) (Haung and Kandel 1994; Michael et al. 1998; Roberson et al. 1999). Lastly, these kinases mediate the transcription of memory-dependent immediate early genes, which are necessary for L-LTP maintenance (Silva et al. 1998) and will be elaborated further below.

Chemical long-term potentiation

Modeling LTM formation in the murine Schaffer collateral neural circuit *in vitro* has historically been performed by inducing L-LTP with the presynaptic electrical stimulation protocols of either high-frequency and theta burst stimulation. Both of these protocols have been

shown to mimic the electrophysiological postsynaptic response of presynaptic stimulation *in vivo* (Wilson 1978; Ylinen et al. 1995). However, Dong and colleagues (2008) noted that these protocols only induce L-LTP in a small subset of neurons, thereby hindering the ability to examine the molecular changes in the neurons of a whole brain region. Alternatively, a chemical LTP (cLTP) protocol has been shown to induce an electrophysiological response comparable with that resulting from the electrical stimulation protocols in the majority of CA1 murine neurons *in vitro* (Dong et al. 2008). This protocol was previously outlined by Otmakhov and colleagues (2004), and require a series of chemical incubation steps that induce LTP in hippocampal slices. Thus, the cLTP protocol provides a suitable alternative to electrically-stimulated LTP and can be used to better examine the molecular changes associated with LTM formation because inducing LTP globally improves the signal-to-noise ratio.

THE UBIQUITIN-PROTEASOME PATHWAY

Covalent attachment of ubiquitin, a small 76 amino acid protein, to a target protein (ubiquitination), is a well-studied, highly conserved posttranslational protein modification mechanism (Ciechanover et al. 1980; Hershko et al. 1980; Khoury et al. 2011). Ubiquitination can occur either through the attachment of a single ubiquitin molecule (monoubiquitination) or a chain of ubiquitin molecules (polyubiquitination) to the target protein. Monoubiquitination usually marks a protein for endocytosis or lysosomal degradation (Polo 2012) while polyubiquitination marks a target protein for proteolysis by the proteasome through a process known as the ubiquitin-proteasome pathway (UPP) (Hershko and Ciechanover 1998). This molecular mechanism has been identified as an important mediator of a wide range of cellular processes.

Enzymes of ubiquitin conjugation

The polyubiquitination mechanism (Figure 2) is a complex process requiring the action of three primary ubiquitin-conjugating enzymes that attach a series (chain) of ubiquitin molecules to a target protein. The ubiquitin-activating enzyme (E1) activates ubiquitin through an adenosine triphosphate (ATP)-dependent process and transfers it to the ubiquitin-carrying enzyme (E2). The ubiquitin-containing E2 binds to a ubiquitin ligase (E3) to form the E2-E3 complex. The E2-E3 complex binds the target protein and performs the ubiquitination process. The initial ubiquitin molecule is attached to a lysine (Lys) residue of the target protein, and subsequent ubiquitin molecules are attached to an internal Lys residue in ubiquitin (Hegde and DiAntonio 2002). E3s are the most diverse of the ubiquitination enzymes and provide the necessary specificity for target proteins. E3s can be broadly divided into homologous to E6-AP carboxyl terminus (HECT) domain and really interesting new gene (RING) finger ligases. Further, the latter category can be subdivided into Skp1/Cul-1/F-box (SCF) and anaphase-promoting complex (APC) E3s (Hegde and DiAntonio 2002; Hegde 2010; Hegde 2017). SCF E3s have an additional specificity element through the variable F-box protein subunit (Skowyra et al. 1997) which acts as the target protein receptor element (Patton et al. 1998; Craig and Tyers 1999). The UPP can be regulated by the posttranslational modification of the ubiquitin enzymes and the target protein or the sequestration of the ubiquitination machinery to a particular cellular region (Stacey et al. 2012; Hegde et al. 2014; Ohtake et al. 2015).

The proteasome

The 26S proteasome is a highly conserved (Hegde and DiAntonio 2002) cytoplasmic and nuclear proteolytic structure that recognizes and degrades polyubiquitinated proteins. The 26S proteasome is a multi-subunit complex consisting of two 19S regulatory particles (19S RPs) that cap each end of the single 20S catalytic cylinder (Hegde and Upadhya 2006). The 19S RP function

to identify the polyubiquitin chain and use it to guide the target protein to the catalytic chamber (Fu et al. 2001). The 20S proteasome degrades the polyubiquitinated target protein into small peptides or amino acids (Hegde and DiAntonio 2002). The polyubiquitin chain is not degraded, but rather disassembled by a deubiquitinating enzyme (DUB), and the free, inactive ubiquitin molecules are subsequently recycled (Tanaka and Chiba 1998).

THE UBIQUITIN-PROTEASOME PATHWAY AND LONG-TERM POTENTIATION

The UPP has been identified as a key mechanism in several aspects of neural activity, such as axonal outgrowth (Muralidhar and Thomas 1993; Oh et al. 1994; Campbell and Holt 2001) and synaptogenesis (DiAntonio et al. 2001). Most recently, however, the UPP has been identified as one of the molecular mechanisms involved in LTM formation. Early studies in *Aplysia* were the first to identify the activity of the UPP during LTF (Hegde et al. 1993; Zhao et al. 2003). Since then, studies have further outlined the role of the UPP during LTP in mammalian models (Dong et al. 2008; Whalley 2012; Dong et al. 2014). .

Protein kinase A regulatory subunit proteolysis

At the sensorimotor synapse of *Aplysia*, presynaptic 5-hydroxytryptamine (5-HT; i.e. serotonin) stimulation facilitates intracellular elevations in cAMP during LTF, which subsequently increases PKA activity and its phosphorylation of downstream targets. PKA is a heterotetramer that contains two regulatory (R) subunits and two catalytic (C) subunits (Bauman and Scott 2002). Considering this structure, it was hypothesized that the PKA R subunit could be degraded or downregulated, as either scenario would elicit an increase in the activity of the PKA C subunit. However, a previous study in this model demonstrated homeostatic PKA R subunit gene expression during LTF (Bergold et al. 1992), suggesting that PKA R subunit degradation might

account for augmented PKA C subunit activity. Hegde and colleagues (1993) later described the PKA R subunit as a novel substrate of the UPP through a series of biochemical experiments. For example, Western blot analyses demonstrated that the PKA R subunit was polyubiquitinated and subsequently degraded by the proteasome in this study. This was later corroborated in pharmacological proteasome inhibition experiments (Chain et al. 1999). Moreover, the microinjection of PKA C subunits into the presynaptic sensory neuron was shown to facilitate LTF at the sensorimotor synapse of *Aplysia* (Chain et al. 1999), demonstrating the importance of PKA C subunit activity during LTM formation. Abel and colleagues (1997) documented the behavioral implications of the PKA R subunit as it relates to memory formation in mammals. A significant impairment in Morris water maze performance was documented in transgenic mice with a dominant-negative PKA R subunit, such that the PKA C subunit was irreversibly inhibited by the PKA R subunit, suggesting that decreased PKA activity impairs spatial memory (Abel et al. 1997).

Regulation of protein synthesis

Recall that the induction of L-LTP (Ep-L-LTP) is dependent on the translation of preexisting dendritic mRNA. Recently, Dong and colleagues (2008; 2014) have shown that the UPP might play an important role in the regulation of protein synthesis and, by extension, the capacity for LTM formation. The initial evidence of this was shown by the enhancement of Ep-L-LTP and the inhibition of L-LTP maintenance in murine hippocampal neurons incubated in a proteasome inhibitor (β-lactone) and then stimulated by a repetitive high-frequency stimulus (100 Hz x 4). To determine if translation was involved in the augmentation of Ep-L-LTP, the neurons were first incubated in anisomycin, followed by β-lactone and the induction of LTP by repetitive high-frequency stimulation. Indeed, anisomycin preincubation was found to block the enhancement of Ep-L-LTP of neurons incubated in β-lactone. Moreover, a novel experiment, such

that the dendrite was isolated from the soma, was used to evaluate the cellular region of protein synthesis in β-lactone-mediated Ep-L-LTP enhancement. Electrophysiological data demonstrated that the enhancement of Ep-L-LTP in disconnected dendrites was the same as intact neurons. Taken together, these data suggest that the UPP regulates local dendritic translation effectors to establish the stimulus threshold for Ep-L-LTP. Although L-LTP maintenance is specifically dependent on new gene transcription, the role of translation must also be considered to account for the inhibition of this stage that was previously described in β-lactone-incubated neurons. To evaluate this, Dong and colleagues (2008) first incubated neurons in β-lactone, followed by anisomycin and the induction of LTP by repetitive high-frequency stimulation. This incubation sequence was used, in contrast to the former experiments, to exclusively evaluate the role of translation during L-LTP maintenance in β-lactone-incubated neurons. It was shown that this incubation sequence resulted in the recovery of L-LTP maintenance. This experiment suggests that the UPP regulates L-LTP maintenance by modulating translation effectors. Taken together, these experiments demonstrated that the UPP differentially modulates the stimulus threshold of Ep-L-LTP and the induction of L-LTP maintenance by regulating the concentration of translation effectors.

A previous study by Steward and Schuman (2003) outlined the signaling cascade responsible for the translation of preexisting mRNAs in hippocampal neurons. It was shown that the mammalian target of rapamycin (mTOR) pathway regulated this process. Could the differential increase in mTOR positive translation effectors and decrease in negative translation effectors during Ep-L-LTP and L-LTP maintenance, respectively, underlie the differential effect of proteasome inhibition on LTP? Dong and colleagues (2014) evaluated this by incubating hippocampal neurons in an mTOR inhibitor (rapamycin) followed by β-lactone, which resulted in

an attenuation of the Ep-L-LTP enhancement and facilitation of inhibited L-LTP maintenance associated with proteasome inhibition. Thus, it was hypothesized that both positive and negative translation effectors may be downstream components of the mTOR pathway, and may elicit the differential effect of LTP in neurons incubated in β-lactone. Indeed, it has been shown that the translation activators (positive effector), eukaryotic initiation factor 4E (eIF4E) and eukaryotic elongation factor 2a (eEF2a), and translation repressor (negative effector), eukaryotic initiation factor 4E binding protein 2 (4E-BP2) are downstream targets of mTOR signaling pathway. Indeed, confocal microscopy experiments of cultured murine hippocampal neurons preincubated in β-lactone and subsequently electrically stimulated showed that the fluorescence of eIF4E and eEF2a peaked during Ep-L-LTP, and fluorescence of 4E-BP2 peaked during L-LTP maintenance (Dong et al. 2014). Taken together, these data demonstrate that the UPP functions to establish the stimulus threshold for L-LTP induction (Ep-L-LTP) by regulating the concentration of mTOR-dependent translation activators, and to mediate L-LTP maintenance induction by regulating mTOR-dependent translation repressor concentrations.

cAMP-responsive element-binding protein repressor: Regulation by proteolysis

The formation of LTMs requires memory consolidation, which is a process dependent on the transition from Ep-L-LTP to L-LTP maintenance. Recall that the defining molecular mechanism associated with L-LTP maintenance depends on transcription. It has been well established that these genes are modulated by the cAMP-responsive element-binding protein (CREB), a highly conserved transcription factor that regulates genes required for formation of LTM. The dependence of LTM formation on CREB activity has been shown in pharmacological inhibition and genetic inactivation studies in *Aplysia* (Dash et al. 1990; Bourtchuladze et al. 1994; Martin et al. 1997; Casadio et al. 1999), *Drosophila* (Silva et al. 1998), and murine hippocampal

neurons (Silva et al. 1992; Chen et al. 2003; Dong et al. 2008). Similar to most transcription factors, CREB activity is phosphorylation-dependent. At the induction of L-LTP maintenance, PKA has been shown to translocate to the nucleus and phosphorylate CREB (pCREB) (Bacskai et al. 1993), allowing for the transcription of immediate-early genes (Michael et al. 1998). This was demonstrated by the nuclear microinjection of pCREB into the presynaptic sensory neuron of the *Aplysia* sensorimotor synapse, which positively regulated LTF (Bartsch et al. 1998). Moreover, a mammalian behavioral study by Gooney and colleagues (2002) supported this by documenting an increase in pCREB in hippocampal neurons of rats trained in the Morris water maze. Together, these studies demonstrate the importance of the phosphorylation-dependent expression of CREB-mediated memory-dependent genes during L-LTP.

The activity of CREB, like most other transcription factors, is modulated by other transcription factors. CREB belongs to a group of transcription factors that contain a basic leucine zipper (bZIP) domain, which have the capacity to dimerize with other factors in this group. Specifically, CREB has been shown to be repressed by such dimerization. The repression of CREB activity was first demonstrated by Bartsch and colleagues (1995) in *Aplysia*, and was later found to be the result of its heterodimerization with ApCREB2 (Bartsch et al. 2000). The pharmacological inhibition of this repressor element was found to decrease the induction threshold necessary for LTF of the presynaptic sensory neuron at the *Aplysia* sensorimotor synapse (Bartsch et al. 1995). This was subsequently corroborated by other studies (Abel et al. 1997) and has since been shown in mammalian hippocampal neurons (Chen et al. 2003; Liu et al. 2014). The mammalian ApCREB2 homolog was later identified as activating transcription factor 4 (ATF4) (Karpinski et al. 1992; Lee et al. 2003). A Western blot analysis has shown the polyubiquitination and degradation of ATF4 in murine hippocampal neurons during LTP. Moreover, the same study

found that the incubation in β-lactone elicits the nuclear accumulation of ATF4-ubiquitin conjugates and reduction in expression of *Brain-derived neurotrophic factor* (*Bdnf*) (Dong et al. 2008), which is an immediate-early gene critical for LTM formation (Impey et al. 1996; Barco et al. 2002). The nuclear translocation of ATF4 during LTD in murine hippocampal neurons has also been shown (Liu et al. 2008), providing further evidence of CREB repression by ATF4. Taken together, these reports demonstrate that the transcription of CREB-mediated immediate-early genes is negatively modulated by ATF4, and the degradation of ATF4 by the UPP is necessary for LTP and LTM formation.

Potential mechanism of Activating Transcription Factor 4 proteolysis

To date, the ubiquitination mechanism of ATF4 remains undefined in hippocampal neurons. However, considering the high evolutionary conservation of CREB-mediated genes, ATF4 activity, and the UPP, it is reasonable to hypothesize ATF4 degradation as a potential mechanism based on prior studies in non-neuronal cells and the limited existing studies in neuronal cells. As such, previous studies outlining this mechanism may provide some details regarding the mechanistic activity of ATF4 proteolysis by the UPP.

The initial steps in elucidating the mechanisms of ATF4 degradation require investigation of the enzymes that conjugate ubiquitin to ATF4. Through a series of *in vivo* molecular probing experiments, Lassot and colleagues (2001) first documented ATF4 as a novel substrate for the SCF E3 containing the F-box protein beta-transducing repeat-containing protein (βTrCP) in HeLa cells. This was later corroborated in a siRNA-mediated knockdown of βTrCP in NIH3T3 mouse cell culture (Yang and Karsenty 2004). The ATF4-βTrCP interaction is demonstrated in Figure 4. Of the known βTrCP substrates, the highest binding affinity has been shown for ATF4, and this interaction has been shown to be cAMP-dependent (Pons et al. 2011). Furthermore, the ATF4-

βTrCP interaction has been shown to be phosphorylation-dependent. The βTrCP binding motif of ATF4 is located between amino acid 218 and 224 (Lassot et al. 2001; Lassot et al. 2005; Koditz et al. 2007). It was shown that ATF4 was stabilized in mutation experiments of serine (Ser) residue 219 (218 in the mouse) of ATF4 (S219-ATF4) to alanine (Ala) (Lassot et al. 2001). This suggests that the phosphorylation of S219-ATF4 (pS219-ATF4) is necessary for the ATF4-βTrCP interaction. However, some studies have provided evidence for other posttranslational modifications that may influence this interaction. For example, pS224-ATF4 (223 in the mouse) has been suggested as a phosphorylation site by numerous studies (Pons et al. 2007; Pons et al. 2008; Wang et al. 2011). Moreover, Frank and colleagues (2010) showed the proline-directed phosphorylation of five other sites – four Ser residues and one threonine (Thr) residue – outside the βTrCP binding motif that might play a role in the binding affinity between the two proteins. Indeed, the mutation of all of these residues to Ala resulted in the stabilization of ATF4 and inhibited its ubiquitination. Lastly, it has also been shown that the acetylation of ATF4 by p300 histone acetyltransferase also results in its stabilization (Lassot et al. 2005). Together, these studies provide strong evidence for the pS219-ATF4-dependent interaction between ATF4 and βTrCP, but the influence of posttranslational modifications at other sites remains inconclusive and should be considered in future studies.

Taken together, studies in murine and human non-neuronal cells and, in a limited capacity, neurons suggest some potential mechanisms underlying ATF4 degradation by the UPP during LTP. However, no definitive details of the mechanism could be drawn from these reports due to inconsistencies and lack of replication; although the broader points could be considered as a guide for neural-based inquires. First, these studies indicate that ATF4 proteolysis by the UPP may be dependent on the phosphorylation-dependent interaction with βTrCP. Previous studies have shown

that the phosphorylation-dependent interaction between a target protein and βTrCP is not unprecedented in hippocampal neurons. Indeed, βTrCP has been implicated in the degradation of the repressor element-1 silencing transcription factor (REST) (Kaneko et al. 2014) and spine-associated Rap GTPase activating protein (SPAR) (Ang et al. 2008), which modulate neural differentiation and dendritic spine morphology, respectively. Considering ATF4, however, the pS219-ATF4 is most likely required, but the specific kinase responsible for this and the cellular localization of the subsequent proteolysis remain ambiguous. Despite this, the nuclear translocation of MAPK during L-LTP maintenance and its potential ability to elicit pS219-ATF4 during LTP suggests a promising hypothesis for this mechanism. Although these data in non-neuronal cells and hippocampal neurons is supportive of ATF4 degradation by βTrCP, further studies are necessary to elucidate this mechanism during LTP and determine how it relates to LTM formation.

Given the above information regarding the molecular underpinning of long-term memory formation and the regulatory role that the UPP plays during these processes, it is not unreasonable to continue elucidating the details of these mechanisms. Specifically, the potential for the UPP to regulate transcription by the proteasome-mediated de-repression of CREB during long-term synaptic plasticity is a valid avenue to explore. As such, the focus of this thesis and the experiments within are focused on this question.

MATERIALS AND METHODS

Animals

All experiments were done with C57BL/6 male mice between the ages of 6-12 weeks old (Charles River, Wilmington, MA). The vast majority of studies within this field of neuroscience

have solely used male subjects. However, it is well documented that a hippocampal-pituitary axis exist and future studies should consider potential sexually dimorphic characteristics on both the cellular and molecular level. Animal experiments were approved by the Institutional Animal Care and Use Committee of Georgia College & State University. Transverse hippocampal slices (400 μm) were made using a standard mechanical tissue chopper and allowed to recover in oxygenated (95% O_2/5% CO_2) ACSF at 32°C for 1 h. cLTP was induced using 200 nM N-Methyl-D-aspartate (NMDA; Cayman Chemical, Ann Arbor, MI) in 0 Mg^{2+} artificial cerebral spinal fluid (ACSF) followed by 0.1 μM rolipram + 50 μM forskolin (Cayman Chemical, Ann Arbor, MI) in 0 Mg^{2+} ACSF. ACSF lacking Mg^{2+} was used as NMDA receptors are normally blocked by such ions.

Proteasome, kinase, and neddylation inhibitors

After 1 h of recovery, cLTP slices were incubated in ACSF with 25 μM β-lactone for 30 min or the following specific kinases inhibitors for 1 h; 20 μM U0126 (Cayman Chemical, Ann Arbor, MI), 5 μM KT5720 (Cayman Chemical, Ann Arbor, MI), or 5 μM KT5823 (Cayman Chemical, Ann Arbor, MI), or the neddylation inhibitor MLN4924 (2 μM) (Cayman Chemical, Ann Arbor, MI) followed by cLTP. Slices were then collected at 0, 5, 10, 15, 20, 25, and 30 min and processed for immunofluorescent labeling.

Confocal microscopy

After being subjected to chemically-induced LTP with or without preincubation with proteasome, kinase, or neddylation inhibitors, cLTP and time-matched control slices were collected and fixed in 4% paraformaldehyde for 1 h followed by five 30 min washes with PBS at room temperature. After washing, slices were blocked in a solution containing 4% normal goat

serum (Vector Laboratories), 0.4% Triton-X-100, and 0.05% sodium azide in PBS at 4°C for 6 h. Slices were then incubated in blocking solution containing polyclonal antibody against pSer219-ATF4 (1:50; MyBioSource, San Diego, CA) at 4°C overnight. Following primary antibody incubation, slices underwent three 20 min washes in PBS containing 0.2% Triton-X-100 and were then incubated in Alexa 488-conjugated goat anti-rabbit secondary antibody (1:300; Invitrogen) and To-Pro-3 (1:500; Invitrogen) at 4°C for 6 h. Following secondary antibody incubation, slices underwent four 30 min washes in 0.2% Triton-X-100 in PBS and one 30 min wash in PBS. Slices were mounted with Prolong Gold antifade reagent (Invitrogen). Images were taken with an Olympus FV3000 confocal microscope and analyzed using ImageJ (National Institutes of Health (NIH), Bethesda, MD).

Statistical analysis

Data are expressed as mean ± standard error of the mean. The sample size (*n*) reflects the number of animals used for each experiment, not the number of slices. This accounts for the inevitable biological diversity. Immunoreactivity was measured in five slices for each experimental group (20 cells/slice). Data were analyzed by one-way ANOVA and Tukey post-hoc test.

RESULTS

Degradation of phosphorylated ATF4 during cLTP

It has previously been shown that after both electrically- and chemically-induced LTP that the quantity of ATF4 is diminished 30 min post-stimulation and the previous experiments evidence indicated that ATF4 is degraded by the UPP (Dong et al. 2008). In addition, the available evidence

in the literature suggests that ubiquitin-proteasome-mediated degradation of ATF4 is likely to be controlled by its phosphorylation on Serine-219. Therefore, we investigated the regulation of phosphorylated ATF4 during cLTP around this time period. We collected slices that were subjected to cLTP-inducing treatments and time-matched controls every 5 min during the first 30 min time period and carried out immunohistochemical experiments using an antibody raised against ATF4 phosphorylated on Serine-2019 (anti-pSer219-ATF4). We then quantified phosphorylated ATF4 immunofluorescence (Figure 1A and 1B) and found that ATF4 phosphorylation remains relatively low during the early part of this period (0-10 min) in both controls and cLTP slices, but increases at 15 min in cLTP slices (cLTP: 173.9% ± 5.9%, control: 99.2% ± 7.6%; n=6, $p < 0.01$, one-way ANOVA and Tukey post hoc test), with a peak at 20 min (cLTP: 313.2% ± 10.8%, control: 98.5% ± 3.9%; n=6, $p < 0.01$, one-way ANOVA and Tukey post hoc test). Phosphorylated ATF4 levels begin to fall at 25 min and reach levels comparable to controls by 30 min (cLTP: 105.6% ± 10.0%, control: 99.3% ± 9.0%; n=6, p = ns, one-way ANOVA and Tukey post hoc test).

Phosphorylated ATF4 levels are stabilized by proteasome inhibition during cLTP

The attenuation of phosphorylated ATF4 during cLTP could likely be explained by its degradation by the UPP. To examine this hypothesis, we incubated slices in β-lactone, a highly selective proteasome inhibitor. We chose to examine the effect of proteasome inhibition at the 25 min mark, as this is the timepoint between the peak and decline to normal levels of phosphorylated ATF4 (Figure 2A and 2B). As expected, ATF4 immunoreactivity was increased in cLTP slices relative to control slices. Furthermore, β-lactone treatment significantly increased ATF4 levels in cLTP slices (β-lactone+cLTP: 550.3% ± 20.0%, cLTP: 220.0% ± 15.5%, control: 98.9% ± 10.9%; n=6, $p < 0.01$, one-way ANOVA and Tukey post hoc test). These data indicate that the

decline of phosphorylated ATF4 levels associated with LTP stimulation is because of proteasome-mediated degradation.

ATF4 phosphorylation is mediated by PKA during cLTP

Together, these data demonstrate that the proteasome-mediated degradation of ATF4 is dependent on the phosphorylation of Serine reside 219 (pS219-ATF4). To further elucidate the mechanism of ATF4 degradation and the signaling cascade that may underlie its phosphorylation, we performed a series of experiments by incubating the slices with different protein kinase inhibitors prior to cLTP induction. We specifically investigated the role of three kinases: PKA, PKG, and ERK. Prior incubation in KT5720 (PKA inhibitor) was found to significantly reduce ATF4 phosphorylation 20 min after cLTP induction (KT5720+cLTP: 109.3% ± 18.3%, cLTP: 234.9% ± 16.7%, control: 100.3% ± 7.9%; n=6 for all groups, $p < 0.01$ for KT5720+cLTP vs. cLTP and control vs. cLTP, p = ns for KT5720+cLTP vs. control, one-way ANOVA and Tukey post hoc test). However, prior incubation in the ERK inhibitor U0126 (U0126+cLTP: 240.2% ± 12.4%, cLTP: 234.9% ± 16.7%, control: 100.3% ± 7.9%; n=6 for all groups, $p < 0.01$ for U0126+cLTP vs. control and control vs. cLTP, p = ns for U0126+cLTP vs. cLTP, one-way ANOVA and Tukey post hoc test) and the PKG inhibitor KT5823 (KT5823+cLTP: 251.2% ± 4.3%, cLTP: 234.9% ± 16.7%, control: 100.3% ± 7.9%; n=6 for all groups, $p < 0.01$ for KT5823+cLTP vs. control and control vs. cLTP, p = ns for KT5823+cLTP vs. cLTP, one-way ANOVA and Tukey post hoc test) did not have a significant impact on phosphorylated ATF4 levels. These data demonstrate that PKA is the likely kinase responsible for the phosphorylation of ATF4 during LTP.

ATF4 degradation during cLTP requires neddylation of E3 ligases

Collectively, these data indicate that the proteasome-mediated degradation of ATF4 is necessary for long-term synaptic plasticity and is likely to be regulated by its PKA-mediated phosphorylation Polyubiquitination of ATF4 is necessary for targeting it for degradation by the proteasome. The enzyme known to be responsible for polyubiquitination of ATF4 is an E3 ligase called $SCF^{\beta TrCP}$ (Lassot et al. 2001). The activation of SCF-type ligases is dependent on the neddylation of the cullin subunit. Thus, it is possible to determine whether $SCF^{\beta TrCP}$ is responsible for the polyubiquitination of ATF4 by inhibiting this process, thereby preventing the activation of the ligase itself. We used the small molecule inhibitor MLN4924 to test this hypothesis and observedthat incubation in MLN4924 prior to cLTP induction resulted in a significant reduction in ATF4 phosphorylation (MLN4924+cLTP: 350.7% $\pm$ 8.1%, cLTP: 225.3% $\pm$ 4.5%, control: 100.6% $\pm$ 6.2%; n=6 for all groups, $p < 0.01$ for MLN4924+cLTP vs. cLTP and control vs. cLTP, p = ns for KT5720+cLTP vs. control, one-way ANOVA and Tukey post hoc test). These results suggest that the degradation of ATF4 is likely dependent on the activation of the $SCF^{\beta TrCP}$ E3 ligase and provides a novel mechanism in the regulation of ATF4 during long-term potentiation.

DISCUSSION

Long-term synaptic plasticity is the primary mechanism underlying formation of memory that lasts for long periods of time. This process is dependent on the synthesis of proteins from pre-existing mRNAs as well as from mRNAs transcribed from a new set of genes. In a widely studied model of long-term synaptic plasticity L-LTP, these processes can be clearly distinguished in its two phases. The induction phase is dependent on the translation of pre-existing local mRNAs residing mainly in dendrites and the maintenance phase is dependent on the transcription of new genes (Hegde 2010). With respect to the investigations on the transcription occurring during the maintenance phase, much of the attention throughout the decades has been devoted to CREB and

the genes that it regulates. However, it should be noted that a number of other transcription factors have since been identified in the recent past (Hegde and Smith 2019). Although the regulation of transcription and translation during long-term synaptic plasticity is mediated by numerous mechanisms, it has been shown that the UPP plays a part.

To understand how transcription is regulated during L-LTP, it is ideal to study CREB-mediated gene synthesis during the maintenance phase. CREB is a transcription factor belonging to the basic leucine zipper category of transcription factors (Alberini 2009). These transcription factors have the ability to form dimers with other transcription factors in this category. Not surprisingly, dimerization can significantly affect their ability to regulate new gene transcription. One such transcription factor is ATF4, and it has been shown (despite its name) to inhibit CREB-mediated gene transcription during long-term synaptic plasticity in both murine and *Aplysia* neurons. Moreover, it has been shown that the de-repression of CREB by ATF4 is necessary for the maintenance phase of L-LTP to occur (Chen et al. 2003; Liu et al. 2014).Therefore, the focus of this study was to elucidate the mechanism by which removal of repression by ATF4 occurs during LTP within the mouse hippocampus.

To better understand the decrease of ATF4 during L-LTP maintenance we used chemically induced LTP (cLTP). We have previously shown that cLTP elicits the same neuronal response as that of electrically-induced LTP (Dong et al. 2008), which has traditionally been used in similar studies. We designed a time-course experiment based on the previous finding that the CREB-mediated *Bdnf* gene synthesis is upregulated 30 min after LTP induction. Our results revealed that phosphorylated ATF4 levels peak at 25 min post-cLTP induction and begin to return to levels comparable to controls at the 30 min timepoint. Because ATF4 represses CREB, this finding is not surprising. However, the mechanism by which ATF4 levels decrease during L-LTP maintenance

was still not fully understood. A logical approach in elucidating ATF4 regulation is to determine if this was due to its degradation by the UPP. For this purpose, we used a highly specific proteasome inhibitor β-lactone and quantified phosphorylated ATF4 levels at 25 min. Indeed, we found that phosphorylated ATF4 levels were significantly increased by inhibition of the proteasome relative to both time-matched controls and cLTP slices. Together, these data suggest that the proteasome-mediated degradation of phosphorylated ATF4 is a necessary mechanism during LTP maintenance.

The next question we wanted to address was determining the signaling cascade that underlies the phosphorylation of ATF4 during LTP. It has been previously shown that the phosphorylation of the Serine-219 residue is necessary for ATF4 degradation (Lassot et al. 2001). We chose to evaluate specific inhibitors of three different protein kinases that could be responsible for ATF4 phosphorylation. When protein kinase A (PKA) was inhibited during LTP, the phosphorylation of ATF4 was significantly reduced compared to time-matched control and cLTP slices. Inhibition of ERK and PKG did not have an effect on ATF4 phosphorylation. These results suggest that, during L-LTP, PKA functions both to activate CREB and to signal the proteolytic removal of ATF4 through the UPP.

Considering that we have shown that the proteasome-mediated degradation of ATF4 occurs during cLTP, it is important to understand the specific details of this process, such as the enzymes responsible for ATF4 ubiquitination. ATF4 has previously been identified as a substrate for the $SCF^{\beta TRCP}$ E3 ligase (Lassot et al. 2001), which belongs to the cullin-RING ubiquitin ligases (CRLs) class of E3 ligases. To activate CRLs, the cullin protein subunit must undergo neddylation, which facilitates the completion of the protein complex. Neddylation of CRLs is driven by the NEDD8-activating enzyme (NAE) (El-Mesery et al. 2019). To evaluate the prospect of $SCF^{\beta TRCP}$

as the possible mediator of phosphorylated ATF4 degradation, the inhibition of neddylation is a logical approach to use. The indirect inhibition of CRLs has been achieved in many model systems by selectively inhibiting NAE using a small molecule called MLN4924 (aka Pevonedistat), which selectively targets NAE. MLN4924 has only recently been used in neuronal studies, primarily in cell culture. Although limited data exist, it appears that MLN4924 significantly impairs synaptic plasticity as revealed by cellular and molecular studies (Scudder and Patrick 2015; Vogl et al. 2015; Bayraktar and Kreutz 2018). We observed that the incubation of hippocampal slices with MLN4924 prior to the induction of cLTP significantly inhibited ATF4 degradation during LTP. Thus, these data provide initial evidence that the SCF ubiquitin ligase that attaches polyubiquitin to ATF4 is likely to be $SCF^{\beta TRCP}$. However, MLN4924 might inhibit neddylation of cullins that are part of other SCF ligases and therefore future studies should consider devising methods to specifically inhibit the ubiquitin ligase that acts on ATF4.

The molecular underpinnings of long-term synaptic plasticity have been the focus of much research over the past decades although many mechanistic questions remain unanswered. The de-repression of CREB by ATF4 degradation during L-LTP can be included in that category. The present study is the initial attempt to elucidate the molecular processes responsible for proteolytic removal of ATF4. Our results suggest that degradation of ATF4 is likely to be necessary during LTP maintenance and that phosphorylation of ATF4 is mediated by PKA.

Furthermore, our data suggest that the polyubiquitination of ATF4 might be regulated by the $SCF^{\beta TRCP}$ E3 ligase. However, additional questions need to be addressed to fully elucidate the degradation of ATF4 by the UPP during cLTP. For example, it is not known whether Serine-219 is the only site phosphorylated in the ATF4 protein. In addition, it is not clear whether the $SCF^{\beta TRCP}$ E3 ligase is subject to regulation by signaling molecules activated during cLTP (Hegde 2004).

Future studies will build on these data to continue the elucidation of ATF4 regulation through UPP-mediated proteolysis during long-term synaptic plasticity.

FIGURES

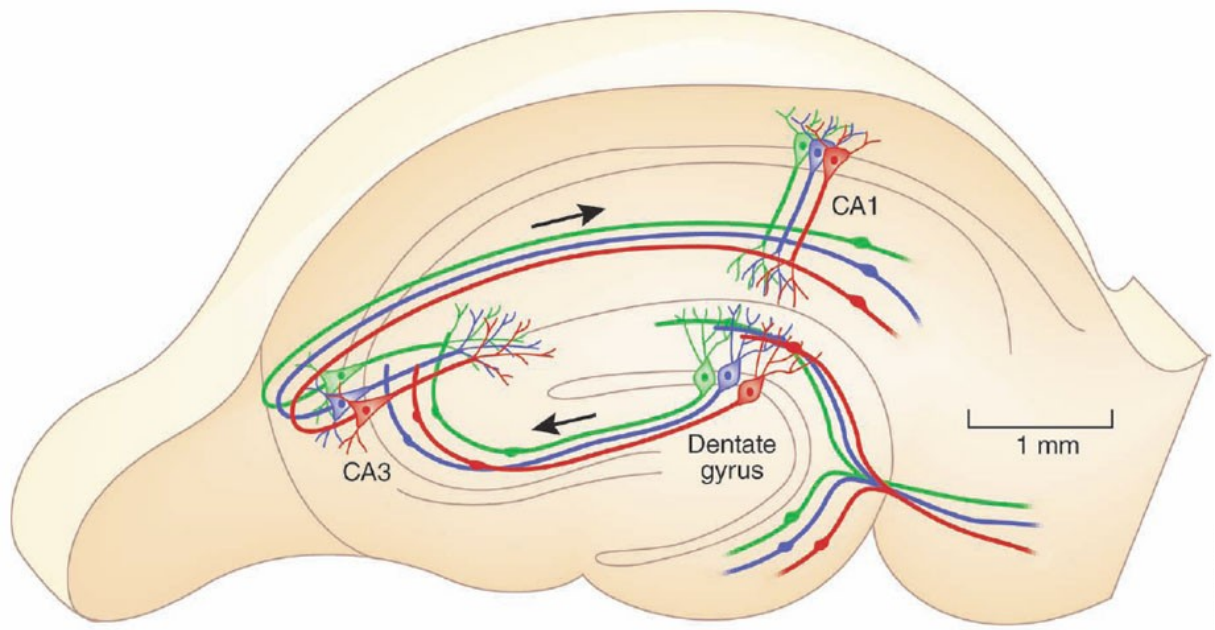


Figure 1. The tri-synaptic neuronal orientation of the mammalian hippocampus. The perforant pathway sends afferent neuron projections to the dentate gyrus (DG). The mossy fiber pathway projects from the DG to the cornu ammonis (CA) area 3 region. Similarly, the Schaffer collateral pathway connects the CA3 neurons to the CA1 pyramidal neurons via CA3 afferents. [Figure adapted from (Moser 2011)].

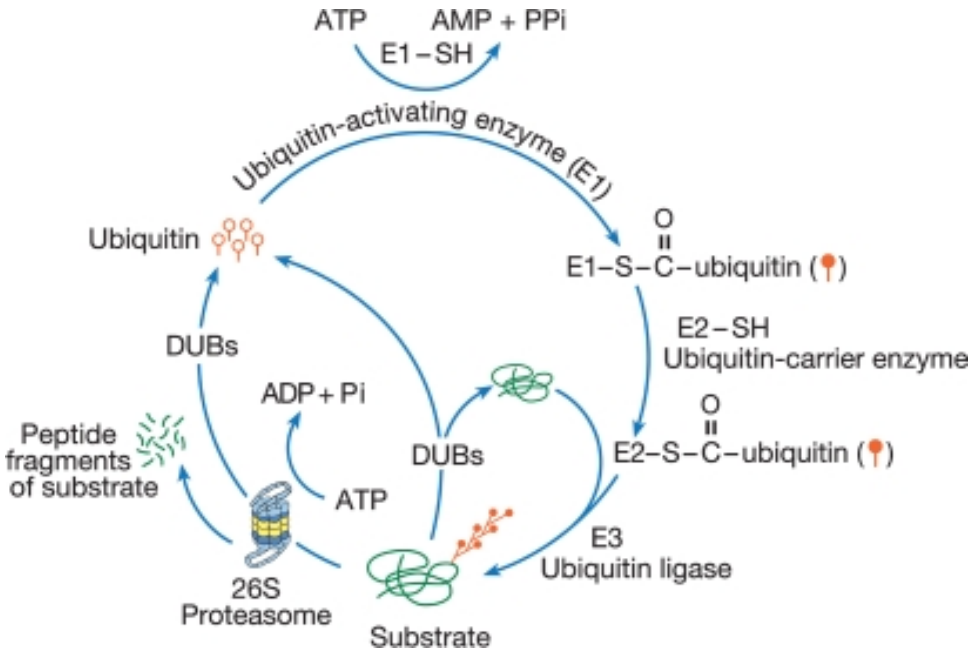


Figure 2. The ubiquitin-proteasome pathway (UPP). First, the ubiquitin activating enzyme (E1) activates ubiquitin through an ATP-dependent process. Next, the activated enzyme is transferred to the ubiquitin conjugating enzyme (E2), which further transfers the activated ubiquitin (red circle with stem) to the ubiquitin ligase (E3). The E3 ligates the initial ubiquitin onto the target protein, and then successively attaches subsequent ubiquitin molecules to form the polyubiquitin tail. The polyubiquitin tail is the cellular signal for proteolysis by the 26S proteasome. The proteasome degrades the target protein into polypeptide fragments or amino acids in an ATP-dependent manner. The polyubiquitin tail is digested back to individual, inactive ubiquitin molecules by a deubiquitinating enzymes (DUB). [Figure adapted from (Upadhya et al. 2004)].

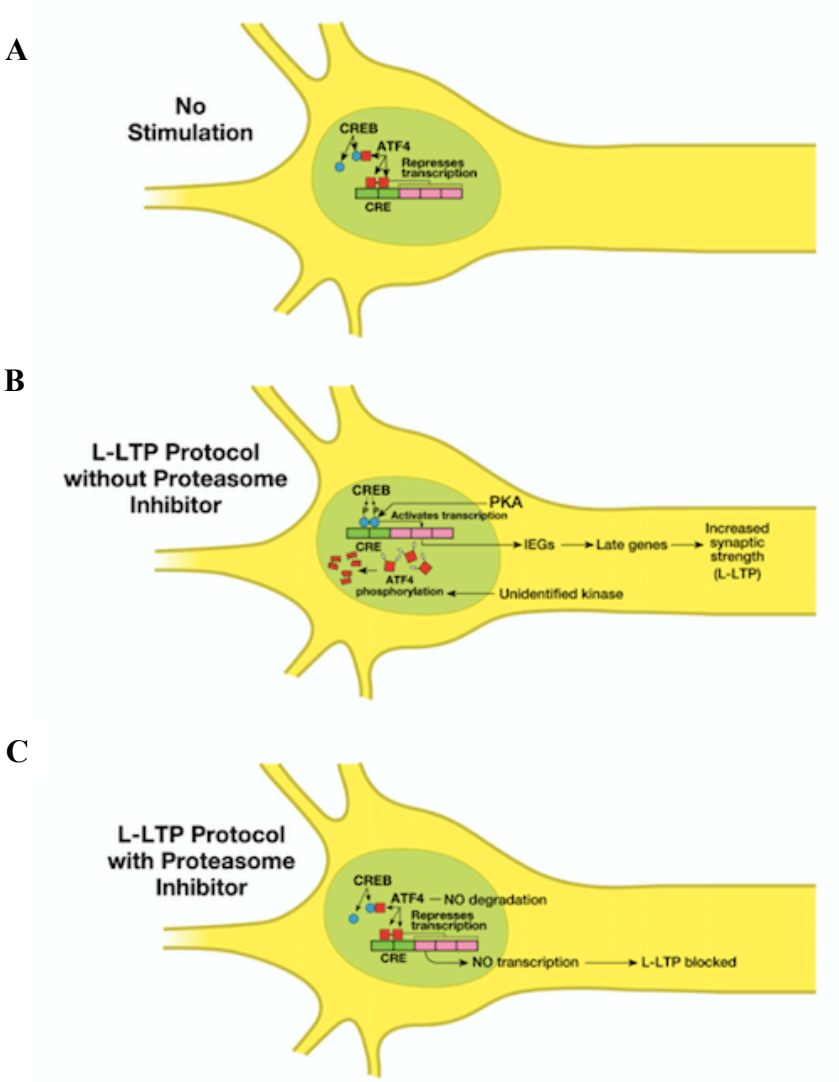


Figure 3. Summary of the role of ATF4 during long-term potentiation and its regulation by the proteasome. A) Under basal conditions, ATF4 dimerizes can either form a heterodimer with CREB or bind directly to the CRE consensus sequences to repress transcription. B) During synaptic stimulation, ATF4 is phosphorylated on amino acid residue 219, and PKA is thought to underlie this post-translational modification. This indirectly facilitates its degradation by the UPP, and subsequently, the transcription of immediate early genes (IEGs). C) When the proteasome is inhibited, ATF4 continues to be phosphorylated (not shown), but these levels are stabilized due to the inability for proteasomal degradation.

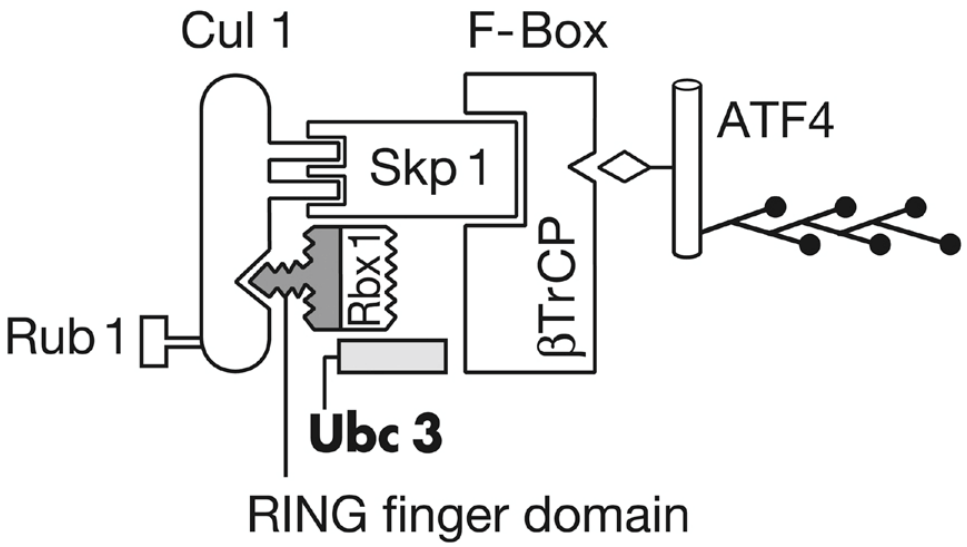
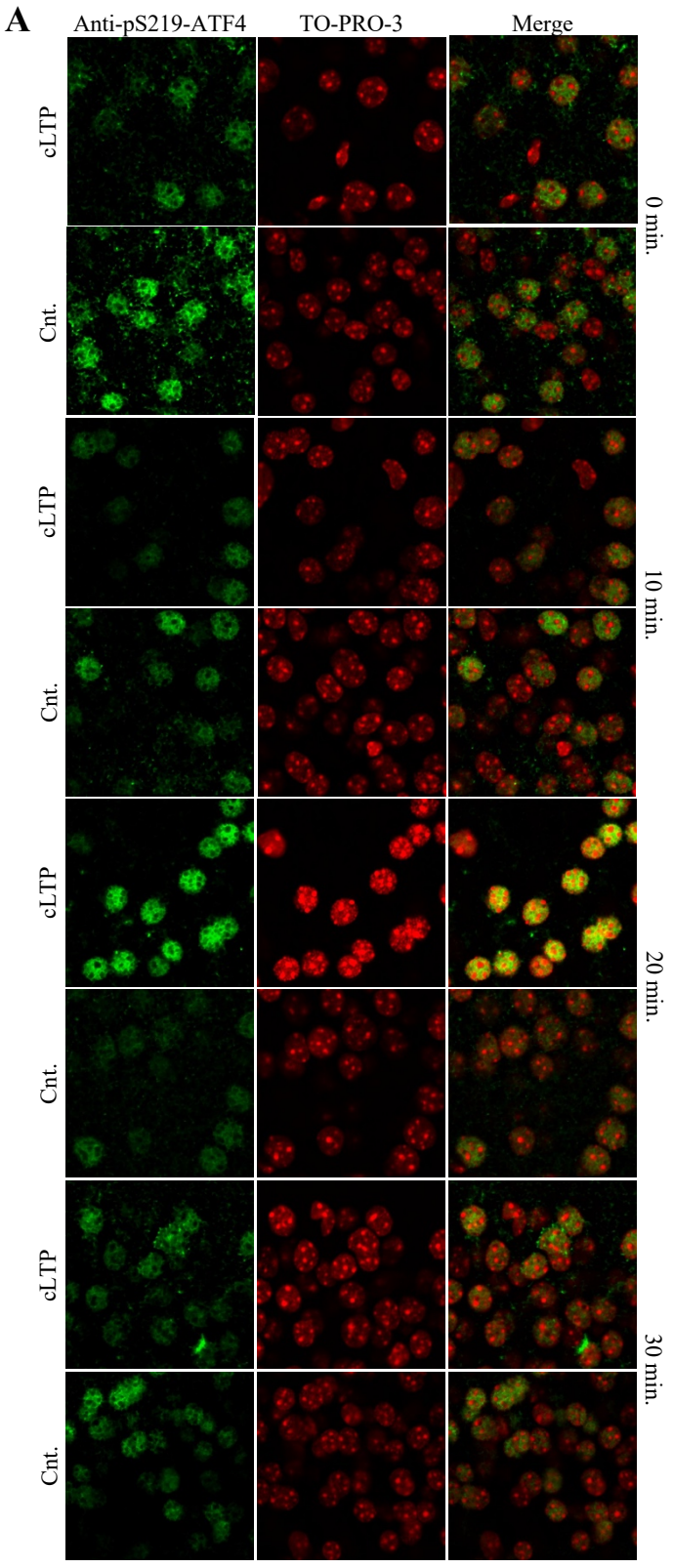


Figure 4. The interaction of ATF4 and SCF$^{\beta TrCP}$ E3 Ligase. The F-box protein βTrCP acts as the target protein receptor subunit of the SCF, and binds pS219-ATF4 in the nucleus. The βTrCP F-box protein containing ATF4 then binds to the other adaptor protein subunits of the SCF E3. Ubc3 is the E2 that carries the activated ubiquitin to the E3. Rbx1 contains the RING finger domain. Skp1 connects the F-box protein βTrCP to Cul-1, which is bound by Rub1 that augments the ligating activity of the E3. [Figure from (Hegde 2004)].


A
Anti-pS219-ATF4
TO-PRO-3
Merge
cLTP
Cnt.
0 min.
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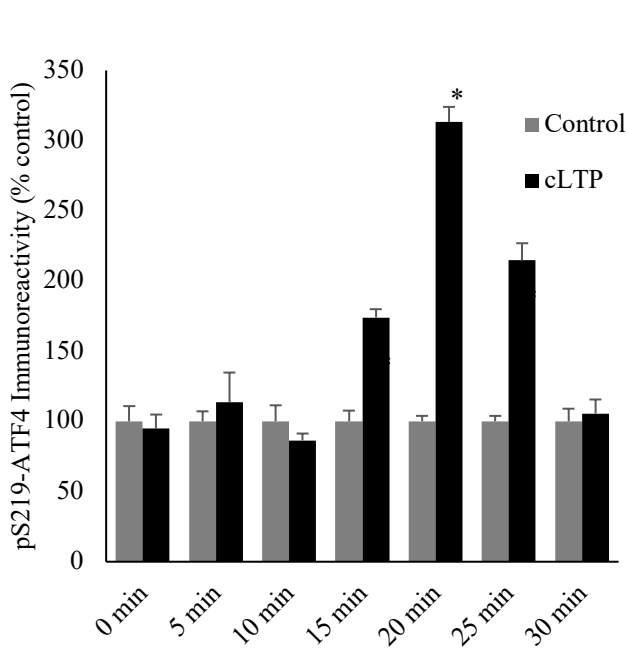
B
pS219-ATF4 Immunoreactivity (% control)
350
300
250
200
150
100
50
0
*
Control
cLTP
0 min
5 min
10 min
15 min
20 min
25 min
30 min

Figure 5. Phosphorylated ATF4 is degraded during chemically induced long-term potentiation. (A) Representative confocal microscopy images of pS219-ATF4 immunofluorescence and the nuclear counterstain TO-PRO-3. pS219-ATF4 levels remain basal during the early timepoints after cLTP but peak at 20 min, eventually falling to levels comparable to time-matched controls by the end of the 30 min time window. (B) Quantification of pS219-ATF4 immunoreactivity every 5 min during the 30 min time course showing a peak at the 20 min time point. * $p < 0.01$ between cLTP and time-matched controls, # $p < 0.01$ between treatment groups, $n = 6$, one-way ANOVA and Tukey post hoc test.

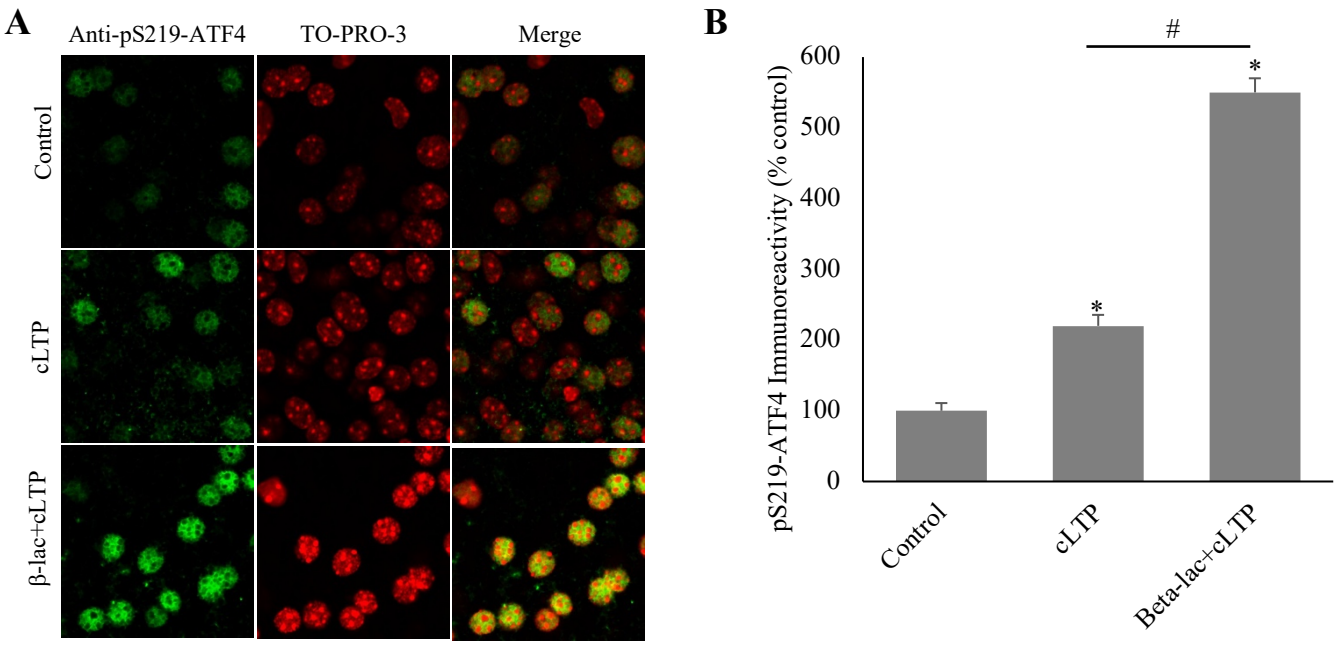

Figure 6. Phosphorylated ATF4 is stabilized by the proteasome inhibitor β-lactone during chemically induced long-term potentiation. (A) Representative confocal microscopy images of pS219-ATF4 immunoreactivity and the nuclear counterstain TO-PRO-3 at 25 min after cLTP induction with treatments as indicated. pS219-ATF4 levels are slightly higher in cLTP slices relative to time-matched controls, and markedly increased by the pretreatment with the proteasome inhibitor β-lactone. (B) Quantification of pS219-ATF4 immunoreactivity showing the significant increase caused by the inhibition of the proteasome during cLTP. * $p < 0.01$ between cLTP and time-matched controls, # $p < 0.01$ between treatment groups, $n = 6$, one-way ANOVA and Tukey post hoc test.

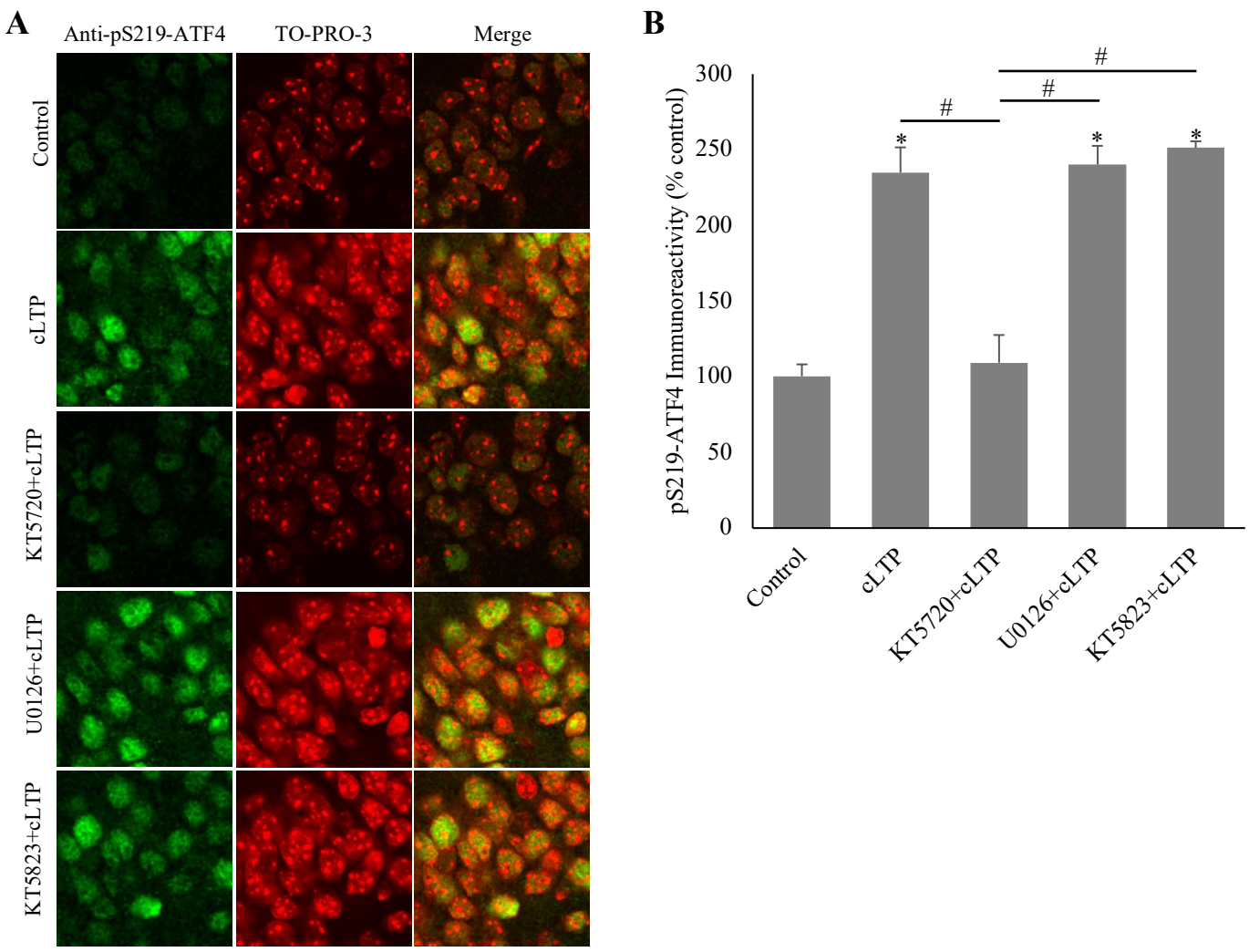

Figure 7. ATF4 is phosphorylated by protein kinase A during chemically induced long-term potentiation. (A) Representative confocal microscopy images of pS219-ATF4 and the nuclear counterstain TO-PRO-3 20 min after cLTP induction, time-matched controls, and slices pretreated with specific kinase inhibitors for either PKA, ERK, or PKG before cLTP induction. pS219-ATF4 levels are increased in cLTP slices relative to time-matched controls and this is significantly attenuated in slices pretreated in the PKA inhibitor KT5720, but not the ERK inhibitor U0126 or the PKG inhibitor KT5823. (B) Quantification of pS219-ATF4 immunoreactivity showing PKA inhibition prevents the phosphorylation of serine 219-ATF4 caused by cLTP at 20 min post-

induction. * $p < 0.01$ between cLTP and time-matched controls, # $p < 0.01$ between treatment groups, $n = 6$, one-way ANOVA and Tukey post hoc test.

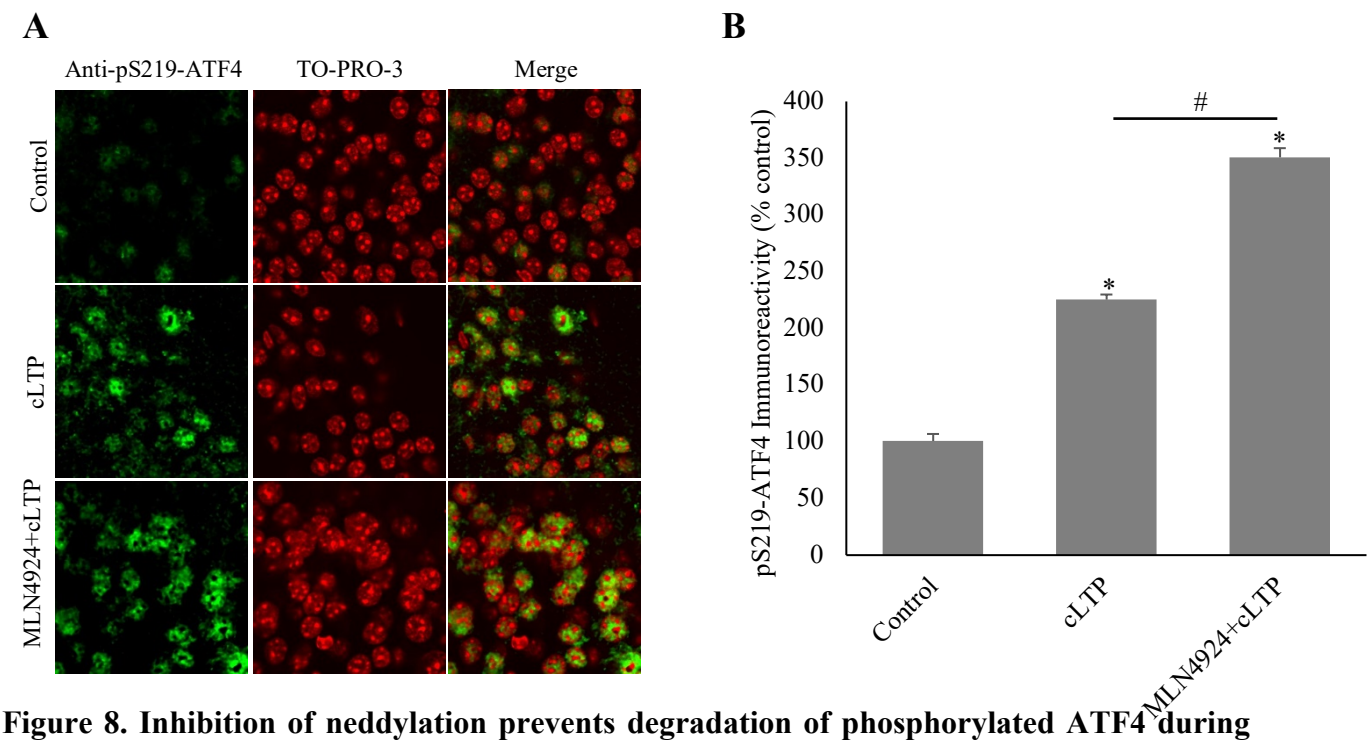

Figure 8. Inhibition of neddylation prevents degradation of phosphorylated ATF4 during chemically induced long-term potentiation. (A) Confocal microscopy images of pS219-ATF4 and the nuclear counterstain TO-PRO-3 25 min after cLTP induction, time-matched controls, and slices pretreated with the small molecule neddylation inhibitor MLN4924 before cLTP induction. pS219-ATF4 levels are markedly increased relative to time-matched controls and the inhibition of neddylation significantly increases pS219-ATF4 levels after cLTP. (B) Quantification of pS219-ATF4 immunoreactivity shows that the neddylation inhibitor MLN4924 causes an accumulation of phosphorylated ATF4 after cLTP induction. * $p < 0.01$ between cLTP and time-matched controls, # $p < 0.01$ between treatment groups, $n = 6$, one-way ANOVA and Tukey post hoc test.

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