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The involvement of atypical PKC and the ubiquitin-proteasome system  
in memory and addiction

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of  
Philosophy

in

Psychology

by

Kristin K. Howell

Committee in charge:

Professor Stephan Anagnostaras, Chair  
Professor Robert Clark  
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Professor John Wixted

2015

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University of California, San Diego

2015

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Chapter 2, in full, is currently being prepared for submission to *Journal of Neuroscience*. Howell, KK\*; Gonzales, FR\*; Dozier, LE; Anagnostaras, SG; Patrick, GN. (\*authors contributed equally). The dissertation author was the primary author of this paper.



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## ABSTRACT OF THE DISSERTATION

The involvement of atypical PKC and the ubiquitin-proteasome system  
in memory and addiction

by

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Doctor of Philosophy in Psychology

University of California, San Diego, 2015

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Addiction results in persistent neural and behavioral modifications characterized by compulsive drug use, compulsive drug seeking, and repeated relapse. The neural substrates associated with drug-use and addiction are believed to overlap with those involved in memory. Many protein kinases, transcription factors, and receptors are implicated in both processes; however, drug related and traditional associative memory may produce different neural modifications. One such addiction related memory is sensitization, a persistent increase in the response to a drug following repeated administration. The purpose of my dissertation is to examine whether certain post-translational mechanisms are similarly involved in associative

forms of memory and sensitization. In my first experiment, I investigated the role of atypical protein kinase C (PKC) in the development and expression of locomotor sensitization. I used zeta inhibitory peptide (ZIP) to block atypical PKC activity. We found that the induction, but not maintenance, of locomotor sensitization was impaired when ZIP was administered both continuously and with a single infusion. In the second and third series of experiments, I examined the effects of disrupting the ubiquitin-proteasome system (UPS), a pathway that mediates protein degradation, on learning, memory, and addiction using two lines of novel knockin mice. Both mice had a mutation at a single amino acid residue, serine 120, a site on Rpt6, part of the proteasome's regulatory component. This site is important as it is phosphorylated by CaMKII in an activity-dependent manner. In one line of mice the serine was switched to an aspartic acid to create a phospho-mimetic mutation (S120D), whereas in the second line of mice serine was switched to an alanine to create a phospho-dead (S120A) mutation. We found this mechanism to be a critical regulator of the UPS during addiction-related memory, as sensitization was completely blocked in phospho-mimetic mice. In contrast, both lines of knockin mice displayed intact associative memory, as assessed using Pavlovian fear conditioning and context discrimination. Therefore, while atypical PKCs and the UPS may be involved in both traditional, associative memory and addiction-related memory, the functional role of these mechanisms seems to differ between the two processes.

## INTRODUCTION

Addiction is a disorder characterized by long-lasting behavioral and neural adaptations that contribute to compulsive drug use, compulsive drug seeking, and repeated relapse even long after withdrawal from the drug (Hyman et al 2006, Josselyn et al 2001, Lüscher & Malenka 2011, Robbins & Everitt 1999, Robinson & Kolb 1997, Russo et al 2010, Volkow et al 2003). The enduring modifications and chronic relapse associated with addiction suggest the involvement of memory. It has been proposed that the mechanisms of learning and memory and addiction overlap implying learning and memory or memory-like neuronal remodeling may underlie addiction (Carmack et al 2013, Kauer & Malenka 2007, Kelley 2004, Robinson & Berridge 2008, Torregrossa et al 2011). Formalizing a definition for learning is difficult, as learning can be defined as generally as ‘a modification to behavior based on previous experience’. By this definition, both associative and non-associative processes are types of learning that underlie memory. Associative learning and memory is assessed using canonical tasks such as Pavlovian fear conditioning and the Morris watermaze, while nonassociative learning encompasses habituation and sensitization. For the purposes of this dissertation, ‘learning’ or ‘memory’ refers to associative forms of learning and memory unless otherwise clarified.

In discussing the overlap between learning and memory and addiction, it is important to consider how these two processes are related. Current theories on the intersection between memory and addiction can be categorized into four closely related models. (1) In the strongest version, it is hypothesized that during addiction drugs of abuse hijack the circuitry underlying beneficial forms of reward or habit learning and pathological learning ensues (Berke & Hyman 2000, Hyman 2005, Hyman & Malenka

2001). (2) Another model proposes that addiction and memory share neural substrates (Kelley 2004). (3) A third variation posits that the molecular mechanisms involved in memory and addiction overlap (Nestler 2001, Nestler 2002). (4) Finally, the fourth model proposes that there is some interaction between the two distinct processes of memory and addiction (Volkow et al 2002, Volkow et al 1999).

*Hijacking/pathological learning model of addiction and memory*

The hijacking/pathological learning model proposes that during addiction the neural mechanisms that subservise 'normal' learning and memory are usurped (Hyman 2005). Under typical circumstances, the neural substrates that underlie an organism's pursuit of rewards, as well as cues that come to predict these rewards, serve an important role for the organism's survival. However, during addiction a pathological hijacking of the circuitry that is often harmful and difficult to overcome occurs (Berke & Hyman 2000, Hyman 2005, Hyman & Malenka 2001). For example, resources (e.g. food, shelter) and mating are key for survival, and therefore serve as sources of positive reinforcement. Internal cues such as hunger, thirst and sexual arousal and environmental cues such as the smell of food or a female in estrous motivate animals to obtain the respective rewards. Drugs of abuse produce similar behavioral patterns in an addict, but these behaviors are now directed toward obtaining drugs of abuse (Berke & Hyman 2000, Hyman 2005, Hyman & Malenka 2001). The addicted individual is highly motivated to obtain drugs of abuse; in this case the drugs of abuse serve as a source of positive reinforcement despite the consequences associated with use. In this pathological form of learning, internal states such as drug craving and environmental cues such as people or places associated with drug use, drive the addict to seek out, hoard, and consume drugs of abuse (Hyman 2005, Hyman & Malenka 2001, Robinson & Berridge 2003).

The mesolimbic dopamine pathway is important for the experience of reward both from natural stimuli (food, mating) as well drugs of abuse (Di Chiara 1998, Everitt et al 1999, Koob & Bloom 1988, Wise & Bozarth 1987). In this pathway, dopamine (DA) neurons in the ventral tegmental area (VTA) synapse with cells in the nucleus accumbens (NAc; Wise and Bozarth, 1987; Di Chiara, 1998; Koob and Bloom, 1988). VTA DA neurons project to other areas in the forebrain such as the prefrontal cortex (PFC) and amygdala as well (Everitt et al 1999). DA is released and VTA/NAc/forebrain circuitry is engaged. Thus, it is thought that addictive drugs mimic naturally rewarding stimuli. However, addictive drugs produce greater levels of dopamine release and stimulate the neural circuitry described above for longer periods of time. This could contribute to a drug of abuse's ability to supersede all other rewarding stimuli (Hyman 2005).

Experimental evidence for this theory is supported by a series of studies conducted by Schultz and colleagues (Hollerman & Schultz 1998, Schultz 1998, Schultz et al 1993, Schultz et al 1997). These studies demonstrate how DA neuron firing patterns can function as a learning signal. Schultz and colleagues recorded from midbrain dopamine neurons in awake monkeys either anticipating or consuming a juice reward. Monkeys learned to predict a reward under certain circumstances (i.e. when certain predictive cues were presented). Subsequently, changes in the firing pattern of DA neurons were observed. At baseline, DA neurons fire tonically (consistently), with the delivery of unexpected rewards producing a transient increase in firing (phasic firing). Importantly, the increase in firing following a reward no longer occurs if the monkey has learned a certain cue is predictive of the reward. Instead, the neurons displayed phasic activity in response to the predictive cue (reviewed in Schultz 1998). If a stimulus that typically predicted a reward was presented, and no reward was delivered, a suppression in the firing of DA neurons at the predicted time of reward was observed (Hollerman & Schultz 1998, Schultz 1998, Schultz et al 1993, Schultz et al 1997). Thus, based on these three scenarios, it is

hypothesized that the firing patterns of DA neurons function as a learning signal. While the studies described above primarily focus on neurons in the midbrain, learned associations regarding the predictive value of a stimulus likely involve the interaction of neurons and neurotransmitters in a distributed system including the NAc, striatum, PFC, hippocampus, and amygdala (Hyman & Malenka 2001). Together, the results of these studies demonstrate that there are distinct changes to neural circuitry and firing patterns in response to learned reward-related associations. While similar experiments to those carried out by Schultz and colleagues (Hollerman & Schultz 1998, Schultz 1998, Schultz et al 1993, Schultz et al 1997) have yet to be conducted with addictive drugs, the hijacking/pathological learning model would predict similar results if drugs of abuse were used as the rewarding stimulus.

*Shared neural substrate model of addiction and memory*

This model argues that the process of addiction shares certain commonalities with learning and memory for natural rewards. It is hypothesized that the circuitry involving dopamine and glutamate, necessary for motivation, learning, and memory is similarly involved in addiction (Kelley 2004). The learning induced by exposure to drugs of abuse produces long-lasting changes to reward-related circuitry. In the strongest version of this model, the mesolimbic and mesocortical dopamine systems involved in addiction are argued to be the same as those involved in habit learning (Kelley 2004).

Much evidence for this model of addiction comes from the interaction between dopamine (specifically D1 receptors) and glutamate, in the cortex, limbic system, and basal ganglia. The glutamatergic receptors for *N*-methyl-D-aspartate (NMDA) and  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) play a critical role in learning and memory by mediating transcriptional and translational changes that subsequently influence plasticity and behavior. Typically, these mechanisms contribute to the survival of an organism; however, during

addiction drugs of abuse alter this circuitry resulting in enduring maladaptive behaviors (Berke & Hyman 2000, Hyman & Malenka 2001, Kelley 2004, Kelley & Berridge 2002, Koob & Le Moal 1997).

It is theorized that cells in the striatum and cortex, which receive both dopaminergic and glutamatergic signals, act as coincidence detectors important for the formation of associations during learning (Berke & Hyman 2000, Horvitz 2002, Kelley 2004, Sutton & Beninger 1999). Some evidence for this idea comes from studies examining long-term potentiation (LTP) at hippocampal-PFC synapses. LTP is an increase in synaptic strength between two neurons, and is theorized to be a key cellular mechanism in learning and memory (Bliss & Collingridge 1993). LTP at hippocampal-PFC synapses requires the co-activation of D1 and NMDA receptors (Gurden et al 2000). Similar dopaminergic and glutamatergic interaction is hypothesized to be involved in addiction. For example, at a cellular level, potentiation of AMPA currents in dopamine cells was induced by a single exposure to cocaine or amphetamine (Ungless et al 2001). At a behavioral level, glutamate and dopamine are both involved in locomotor sensitization. Sensitization is an increase in the response to a particular drug following repeated administration (discussed in detail below). Glutamate levels in the NAc increase following repeated administration of cocaine as well as in response to cues associated with cocaine (Hotsenpiller et al 2001, Pierce et al 1996). The studies described above support the theory that there are shared neural substrates between learning, memory, and addiction, specifically the interactions between dopaminergic and glutamatergic neurotransmitter systems.

#### *Shared molecular mechanisms model of addiction and memory*

According to this model, many of the molecular and cellular changes induced by drugs of abuse and addiction are also implicated in traditional forms of memory. Further, drug-induced neural changes can be regarded as types of molecular or cellular memory (Nestler 2013).



Evidence in support of this model comes from studies demonstrating that both processes are mediated by the same neurotrophic factors, cellular signaling pathways, and transcription factors (specifically cAMP response element binding protein; CREB). Through these mechanisms long-term alterations in cell morphology, plasticity, and behavior are produced (Nestler 2002, Nestler 2004, Nestler 2013, Nestler & Aghajanian 1997).

In the past, it was believed that there was overlap between the molecular and cellular mechanisms involved in learning, memory, and addiction, but the neural circuits mediating these processes were distinct. There had been much evidence implicating the mesolimbic dopamine system as a substrate highly involved in addiction whereas, the hippocampus, amygdala, and PFC were the neural structures thought to mediate learning and memory (Hyman & Malenka 2001, McGaugh 2000, Nestler). In recent years, a more integrative view has emerged. It is now recognized that circuits between the hippocampus, PFC, amygdala, NAc, and striatum all interact. Despite differences in neural circuitry proposed in early iterations of this model, many of the same molecular and cellular events were being investigated in both processes. For example, learning, memory and addiction all result in changes in the expression levels of AMPA and NMDA receptors (discussed in detail below; Luscher et al., 1999; Nestler, 2002). Further, LTP- and LTD-like plasticity in the NAc and VTA have been shown to be modified by cocaine (Nicola et al 2000). Finally, strong support for this model has come from studies investigating the role of the transcription factor, CREB.

Many upstream signaling pathways converge through the activation of CREB. This transcription factor has been examined in multiple model systems (*Aplysia*, *Drosophila*, mice) and has been demonstrated to be important for behavioral forms of learning and memory (Elgersma & Silva 1999, Josselyn et al 2001, Kida et al 2002, Yin & Tully 1996). Specifically, CREB in the hippocampus and amygdala has been implicated in a multitude of associative

learning and memory tasks such as an olfactory memory task and fear conditioning (Silva et al 1998). CREB has also been shown to play an equally critical role in addiction. Drugs of abuse activate CREB expressed in the NAc and amygdala (Berke & Hyman 2000, Josselyn et al 2001, Nestler 2002). While it is clear that CREB is involved in these processes, CREB activation alone cannot necessarily explain the endurance of learning, memory, and addiction. It is currently hypothesized that the ability of a memory (whether traditional or addiction-related) to persist may be the result of morphological changes in the hippocampus or NAc and that these changes could result from alterations in the expression of molecular substrates such as CREB (Nestler 2002, Robinson & Berridge 2001). Another mechanism through which CREB may influence these processes is by mediating protein synthesis. The specific role of protein synthesis in learning, memory and addiction is discussed in detail below. However, evidence of protein synthesis involvement does not necessarily support this theory specifically. Other theories would concur that long-lasting, persistent changes are due, at least in part, to new protein synthesis.

#### *Addiction and memory interact*

According to this model, there is some interaction between addiction and memory. Anagnostaras and colleagues (2002) proposed that there are both associative and nonassociative processes involved in addiction. An associative component of addiction, such as cue-elicited craving, may share similar neural adaptations to those involved in traditional forms of associative memory. However, there may also be some processes unique to addiction, as can be seen in sensitization (Anagnostaras and Robinson, 2002; discussed in detail below). Behavioral changes reflecting the nonassociative component of addiction (e.g. an increase in the unconditioned response to the drug) may be driven by changes to cellular plasticity resulting from repeated drug administration (Anagnostaras et al 2002, Kuczenski et al 1982, Robinson & Becker 1986). Thus,

nonassociative and associative learning are dissociable, and the involvement of each of these processes in addiction suggests that addiction and memory interact.

Additional support for this model comes from studies investigating the role of dopamine in addiction and memory. Both processes are critically mediated by dopamine (Castellano et al 1991, Setlow & McGaugh 1999). Dopamine cells exhibit similar patterns of firing to hippocampal cells characteristic of learning, when exposed to rewards or conditioned stimuli (Waelti et al., 2001). For this reason, dopamine neurons are thought to be involved in associative learning. Associative learning of drug-related stimuli has been shown to occur in the NAc (Di Chiara 1999). Since drugs of abuse increase levels of dopamine, especially in the NAc, it is thus hypothesized that dopamine may help consolidate the drug-taking experience (Volkow et al 2002).

Memory systems likely influence the drug intoxication and craving involved in addiction (Volkow et al 2002). Drug intoxication is altered by previous experience, as previous experience with the drug sets expectations for the drug's effects and influences the user's response (Volkow et al 2002). Drug craving occurs when the user has formed associations between the drug, drug-taking environment, and experience of pleasure (or overpowering sensation). Subsequently, drug-related cues such as particular contexts or people can trigger memories related to the drug, leading to craving and potentially relapse.

Evidence implicating memory-related neural structures in craving, comes from studies designed to induce craving through exposure to a drug, video or by recalling an experience with the drug. During the experience of craving, imaging studies revealed activation in the amygdala, hippocampus, and striatum (Childress et al 1999, Kilts et al 2001, Volkow et al 1999). Further, the mesocortical dopamine system encompasses areas of the frontal cortex such as the prefrontal cortex, orbitofrontal cortex, and cingulate gyrus. These structures are likely involved in memory-

related aspects of addiction (Volkow et al., 2002; Kilts et al., 2001). According to this model, interactions between the circuitry connecting these regions, contribute to the memory system's influence on addiction by strengthening the reinforcing value of the drug and increasing the salience of the drug during craving. Further, dopamine released during drug-taking may help consolidate and strengthen the drug-related memory (Volkow et al., 2002).

The four models described above can be conceptualized as various ways to frame the relationship between memory and addiction. Each model has its distinct characteristics, yet, the role of neurotransmitters such as dopamine and glutamate, protein kinases, and types of synaptic plasticity (LTP, LTD) overlap. With the accumulation of more evidence from both the learning and memory and addiction fields, there is a move toward a perspective supporting an interaction between the circuitry and molecular and cellular mechanisms implicated in each of these processes.

#### *Incentive sensitization theory of addiction*

In line with the fourth model described above that proposes memory and addiction interact, is the incentive sensitization theory of addiction. The four core principles of the incentive sensitization theory are (1) drugs of abuse have the ability to induce persistent changes in neuronal circuitry, (2) the neural substrates affected by drugs of abuse are typically involved in incentive motivation and reward, (3) the neural changes produced by addiction leave the involved circuitry in a sensitized state, and (4) the neural circuitry that becomes sensitized is involved in 'drug wanting' (attributing salience to the drug) and is distinct from the circuitry involved in drug 'liking' (pleasurable effects), which does not undergo sensitization (Robinson & Berridge 1993, Robinson & Berridge 2000, Robinson & Berridge 2008).

According to this theory, repeated exposure to drugs of abuse can produce persistent changes in the cells and circuits involved in ascribing salience to particular stimuli. Typically this

circuitry is important for motivating behavior, but in the addicted brain these circuits become sensitized (hypersensitive) and drugs of abuse and drug-related cues become overly salient (Robinson & Berridge 1993, Robinson & Berridge 2000, Robinson & Berridge 2008). Sensitization refers to the increase in a drug's effects resulting from repeated administration. According to this theory, after taking a drug repeatedly, the appeal of drug taking and drug-associated stimuli increases. The persistence of sensitization may help explain the pathological wanting (craving) of drugs of abuse for years even after the discontinuation of use, often resulting in relapse (Robinson & Berridge 1993, Robinson & Berridge 2000, Robinson & Berridge 2008).

This theory states that associative learning and memory contribute to some, but not all aspects of sensitization. Associative learning may mediate the expression of sensitization under certain circumstances or in particular contexts (Anagnostaras & Robinson 1996, Robinson & Berridge 1993, Robinson & Berridge 2000, Robinson & Berridge 2008, Stewart & Vezina 1991). However, learning cannot fully account for the pathological urge to take drugs. Parallels can be drawn between the contribution of learning to other non-associative processes such as pain or stress. Most likely, learning modulates or interacts with the expression of sensitization (Robinson & Berridge 1993, Robinson & Berridge 2000, Robinson & Berridge 2008).

#### *Behavioral assessment of addiction-related memory*

Two prominent behaviors that model aspects of addiction-related memory are psychomotor sensitization and conditioned place preference (CPP). Psychomotor sensitization has both non-associative and associative components, while place preference is an associative form of memory.

#### *Sensitization*

Sensitization is induced following multiple administrations of a drug and is characterized by increased psychomotor activation (locomotor sensitization), increased dopamine

neurotransmission (neural sensitization), and hypersensitivity to a drug's rewarding value (incentive sensitization). Sensitization models the transition from casual to compulsive drug use (Anagnostaras & Robinson 1996, Anagnostaras et al 2002, Robinson & Berridge 1993, Robinson & Berridge 2000, Shuman et al 2012, Steketee & Kalivas 2011). While locomotor activity (horizontal distance traveled) is most often used as a measure to assess sensitization, other behaviors such as stereotypy, vertical activity, or rotational behavior can be used to detect sensitization as well (Anagnostaras & Robinson 1996, Carmack et al 2013). A sensitized animal will exhibit progressively increasing activity to the same drug dose until a ceiling is reached. At a neural level, sensitization produces an upregulation in dopamine transmitter, transporters, and receptors (Paulson & Robinson, 1995).

#### *Context-dependency in sensitization*

As mentioned above, under some circumstances context can affect the extent that sensitization is expressed. Sensitization has been shown to be entirely context dependent in some studies (Anagnostaras & Robinson 1996, Anagnostaras et al 2002, Stewart & Vezina 1991). In rats with a lesioned nigrostriatal dopamine system (6-OHDA lesions), amphetamine-induced sensitization was only expressed in a context previously paired with the drug, but not in an unpaired environment (Anagnostaras & Robinson 1996). However, in the same study, context-independent sensitization was also demonstrated. This was achieved by using multiple contexts to create a "truly random" contextual group (Anagnostaras & Robinson 1996, Rescorla 1967). Additionally, this experiment distinguished between a conditioned response and true sensitization. A conditioned response refers to a response similar to that the drug would produce (e.g. elevated locomotor activity), but in the absence of the drug. The conditioned response is driven by cues associated with the drug such as the context previously paired with the drug. In this experiment rats exhibited sensitization in a novel context, but to a lesser extent than in the paired context

(Anagnostaras & Robinson 1996). As expected, there was no conditioned response observed in an unpaired environment. In a follow-up study, Anagnostaras and colleagues (2002) elaborated on the conditions that induce context specificity during sensitization to amphetamine in 6-OHDA lesioned rats. Rats given a sensitization challenge test in an environment previously paired with amphetamine exhibited robust sensitization, but not rats given a challenge test in an unpaired environment (Anagnostaras et al 2002). Rats given saline in the paired context demonstrated a conditioned response. Electroconvulsive shock was given to certain treatment groups to induce amnesia for the reactivated memory. ECS did not affect sensitization in paired contexts, nor did it impair the conditioned response (Anagnostaras et al 2002). However, when ECS was given to rats in the unpaired context, they exhibited robust sensitization (Anagnostaras et al 2002). ECS seems to have lifted an inhibitory constraint that blocks the expression of sensitization in an unpaired environment under certain circumstances. Together, these studies demonstrate that sensitization can be expressed in both paired and unpaired contexts, which supports the theory that there are both associative and non-associative components to sensitization.

#### *Conditioned Place Preference*

Conditioned place preference is an associative addiction-related memory that is characterized by an animal's preference for a context previously paired with a drug (Bardo & Bevins 2000, Rowlett et al 1994, Seymour & Wagner 2008). Place preference is used as a model for drug seeking (Bardo & Bevins 2000, Rowlett et al 1994, Seymour & Wagner 2008). Typically, place preference is induced by pairing a chamber with distinct contextual cues (odor, tactile, visual) with a drug, and pairing a second chamber with different cues with saline. The experimenter is then able to assess preference and associative, addiction-related memory, by measuring the time spent in each of the chambers. These experiments are carried out when the animal has not been administered a drug. Animals with a drug-elicited preference will spend more

time in the drug-paired chamber. Both place preference and sensitization serve as useful behavioral assays to assess different aspects of addiction.

In describing the four models used to link memory and addiction as well as the incentive sensitization theory of addiction, I have touched upon some of the relevant molecular mechanisms and cellular events involved in memory and addiction. Here, I will provide further detail implicating the glutamate receptor AMPA, as well as the protein kinase CaMKII $\alpha$  in learning, memory, and addiction. Finally, I will discuss current evidence demonstrating the importance of protein consolidation and degradation in memory and addiction. These molecular substrates and cellular processes will be the focus as they are most relevant to subsequent studies presented in this dissertation.

#### *Role of AMPA in memory and addiction*

AMPA trafficking has been theorized to underlie forms of synaptic plasticity involved in learning and memory including LTP and long-term depression (LTD). Exocytosis (trafficking to the surface of the membrane) of AMPARs is enhanced during LTP, while during LTD, endocytosis (internalization) of AMPARs increases (Anggono & Huganir 2012, Kessels & Malinow 2009, Lüscher et al 1999, Shepherd & Huganir 2007). There have multiple behavioral studies demonstrating a role for AMPARs in fear learning (Humeau et al 2007, Rumpel et al 2005). Mice lacking GluR1-containing AMPARs were unable to acquire cued or contextual Pavlovian fear memory and lacked LTP at thalamo-amygdala and cortico-amygdala synapses (Humeau et al 2007). Furthermore, expression of GluR1-containing AMPARs increased following the associative memory tasks, fear conditioning and one-trial inhibitory avoidance (Whitlock et al 2006, Yeh et al 2006). Endocytosis of AMPARs in the amygdala also facilitated extinction-induced erasure of a fear memory (Clem & Huganir 2010). Together these studies



suggest that AMPARs, specifically GluR1 containing receptors, are recruited or incorporated into synapses during learning.

Direct evidence supporting a role for AMPA in addiction-related behavior has been shown in several studies. Infusion of an AMPA antagonist into the dorsal striatum decreased cue-controlled drug seeking behavior (Vanderschuren et al 2005). Delivery of an AMPA antagonist into the NAc attenuated cocaine seeking modeled through Pavlovian conditioned-approach and place preference (Di Ciano et al 2001, Di Ciano & Everitt 2004, Kaddis et al 1995). AMPA has also been implicated in behavioral sensitization. In rats pretreated with cocaine, injection of an AMPA antagonist into the NAc blocked sensitization, whereas an injection of AMPA increased locomotor activity (Pierce et al 1996). Surface expression of AMPARs in the NAc increased in cocaine-sensitized rats sensitized. This increase correlated with the degree to which rats exhibited behavioral sensitization (Boudreau & Wolf 2005). AMPA has also been theorized to play a role in relapse; cocaine-induced reinstatement of drug seeking has been shown to be dependent on the activation of AMPARs in the NAc (Cornish & Kalivas 2000). An infusion of AMPA into the NAc induced reinstatement while an infusion of an AMPA antagonist blocked reinstatement (Cornish & Kalivas 2000).

The involvement of AMPARs in learning, memory, and addiction is quite complex and thus, a specific role in each of these processes is difficult to discern. The specific function of AMPA in each of these processes seems to vary based on the receptor subtype as well as the site of expression. Interestingly, the role of AMPA in learning, memory, and addiction has converged in studies investigating atypical PKC isoforms, specifically, protein kinase M  $\zeta$  (PKM $\zeta$ ). There is evidence demonstrating PKM $\zeta$  maintains LTP and behavioral forms of associative and spatial memory (Ling et al 2002, Parsons & Davis 2011, Pastalkova et al 2006, Sacktor et al 1993, Serrano et al 2005). Similarly, PKM $\zeta$  has been shown to be important for addiction-related

behavior and plasticity (Crespo et al 2012, Ho et al 2012, Howell et al 2014, Li et al 2011). Downstream, AMPAR trafficking is theorized to mediate the actions of PKM $\zeta$  (Migues et al 2010, Shema et al 2009). An increase in AMPAR trafficking to postsynaptic sites accompanies the activation of PKM $\zeta$  and inhibition of AMPAR endocytosis prevents the memory impairment typically produced by PKM $\zeta$  inhibition (Migues et al 2010, Shema et al 2009). Similar results have been obtained upon investigation of addiction-related behavior. Mice co-treated with cocaine and an atypical PKC inhibitor produced a reduction in global AMPAR expression (Howell et al 2014). Thus, the effects of AMPAR trafficking in mediating the actions of PKM $\zeta$  provide a link between the role of AMPARs in memory and addiction.

#### *Role of CaMKII $\alpha$ in memory and addiction*

Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII), is a protein kinase activated by the entry of Ca<sup>2+</sup> into neurons and has been implicated in learning, memory and related plasticity (Lisman et al 2002). Multiple studies have demonstrated that inhibition of CaMKII prevents the induction of LTP. However, CaMKII inhibition does not disrupt LTP once it is already established (Malenka et al 1989, Malinow et al 1989, Otmakhov et al 1997). CaMKII remains active for at least one hour post-LTP induction, even when Ca<sup>2+</sup> is no longer present. Thus, it has been theorized to help maintain memory (Fukunaga et al 1993). Results using pharmacological inhibitors parallel those found using CaMKII mutant mice. These mice also exhibit impaired LTP (Giese et al 1998, Hinds et al 1998, Silva et al 1992).

Our understanding of the importance of CaMKII in behavioral forms of learning, memory, and addiction largely comes from two lines of knockin in mice, T286A and T286D mice (Giese et al 1998, Mayford et al 1996, Mayford et al 1995). Typically autophosphorylation of CaMKII occurs at the site threonine 286. In T286A mice, the threonine was replaced by alanine creating a mutant with no autophosphorylation. In addition to impaired LTP, these mice had

severe spatial memory deficits suggesting the autophosphorylation of CaMKII is necessary for learning spatial tasks (Cho et al 1998, Giese et al 1998). In T286D mice the threonine was switched with aspartic acid, mimicking constitutive phosphorylation (Mayford et al 1996, Mayford et al 1995). T286D mice had significantly enhanced levels of Ca<sup>2+</sup>-independent activity in vitro (Fong et al 1989). However, similar to the T286A mice, T286D transgenic mice had impaired spatial memory (Bach et al 1995, Mayford et al 1996). Later, a version of this mouse was created with spatial and temporal control of the mutation using a tetracycline-dependent transactivator system (Mayford et al 1996). This allowed mice to be raised with the mutation ‘turned off’, but allowed for the mutation to be ‘turned on’ during or after training. Like the non-inducible version of the T286D mice, these mice exhibited impaired spatial memory (Bejar et al 2002, Mayford et al 1996).

An alternate version of the inducible T286D mouse was created that restricted expression of the mutation to the NAc/striatum. This mouse was created to examine the role of CaMKII activity in reward- and addiction-related behaviors (Kourrich et al 2012, Wiltgen et al 2007). A single injection of cocaine induced elevated locomotor activity in T286D mice relative to wild-type mice (Kourrich et al 2012). Conditioned-place preference was also more easily induced in T286D mice; a low dose of cocaine produced stronger place preference in T286D mice relative to controls (Kourrich et al 2012). Interestingly, in T286D mice, motivation toward goal-directed actions elicited by cues associated with reward was impaired (Wiltgen et al 2007). T286D mice were examined in a Pavlovian instrumental transfer test. Initially, mice were trained to press two levers, one delivered food, the other a sucrose solution. Mice were then conditioned to two stimuli; they learned one predicted food delivery and the other predicted sucrose delivery (typically preferred over food). Mice were able to learn to lever press to obtain the reward and learned the association between the stimuli paired with each reward. However, the cues did not

motivate T286D mice to lever press for reward (Wiltgen et al 2007). Thus, the motivational effects of reward cues seem to be dependent on normal levels of CaMKII activity in the striatum. Taken together, these findings highlight the importance of CaMKII in learning, memory, and reward-related behavior. Disruption in CaMKII seems to impair spatial memory and aspects of reward related memory, while facilitating the addiction-related memories place preference and sensitization.

#### *Role of protein synthesis and degradation in memory and addiction*

Newly acquired information is converted into a stable, long-term memory through consolidation and is dependent on *de novo* protein synthesis (Bahar et al 2003 1986, Davis & Squire 1984, Frey et al 1988, Gold 2008, Jarome & Helmstetter 2014, Schafe & LeDoux 2000, Schafe et al 1999). Reconsolidation, also protein synthesis-dependent, is the process by which a memory is restabilized if returned to an active state during recall (Alberini & Ledoux 2013, Nader et al 2000). Protein synthesis has been shown to be critical for consolidation and reconsolidation on a wide variety of behavioral memory tasks. Infusion of anisomycin, the pharmacological inhibitor used in the majority of studies, into the amygdala disrupted both the consolidation and reconsolidation of auditory and contextual fear memory, conditioned taste aversion, and inhibitory avoidance (Bahar et al 2003, Jarome et al 2011, Milekic et al 2007, Nader et al 2000, Rodriguez-Ortiz et al 2012, Schafe & LeDoux 2000). Anisomycin infused into the hippocampus similarly impaired the consolidation and reconsolidation of contextual fear conditioning, Morris water maze, and object recognition memory (Artinian et al 2008, Bourchouladze et al 1998, Debiec et al 2002, Rossato et al 2007).

The importance of protein synthesis during consolidation and reconsolidation has also been investigated in the context of reward-related memories and addiction. Infusions of anisomycin into the NAc disrupted the consolidation of an appetitive instrumental learning task

(lever-pressing for food; Hernandez et al., 2002). Once the task was learned, anisomycin did not produce impairment. Thus, protein synthesis was shown to be critical for the consolidation, but not reconsolidation of appetitive instrumental learning (Hernandez et al 2002). However, overall the necessity of protein synthesis during addiction-related memories is less clear. While it seems *de novo* protein synthesis is critical for the consolidation of place preference under some conditions, there have been other findings that suggest anisomycin does not disrupt this behavior (Robinson and Franklin, 2007; Yim et al., 2006 ). In the context of addiction, drug-related memories are reactivated after each drug-use, and are theorized to be reconsolidated after reactivation (Milekic et al 2006, Sanchez et al 2010, Sorg 2012, Tronson & Taylor 2007). Thus, understanding the reconsolidation of addiction-related memories is especially important. However, whether protein synthesis is necessary for the reconsolidation of place preference and sensitization as it is with traditional forms of memory is not yet clear (Milekic et al., 2006; Valjent et al., 2006; Bernardi et al., 2007; Robinson and Franklin, 2007).

Recently, the view has emerged that long-term memory formation depends not only on *de novo* protein synthesis, but on protein degradation as well (Artinian et al., 2008; Jarome et al., 2011; Lopez-Salon et al., 2001; Rodriguez-Ortiz et al., 2010; Fioravante and Byrne, 2010; Cajigas et al., 2010; Chain et al., 1999). Protein degradation is important for the remodeling of synapses and release of inhibitory constraints on protein expression, which influence plasticity and memory (Bingol & Sheng 2011, Chain et al 1999, Fioravante & Byrne 2010, Patrick 2006). Protein expression at the synapse is likely regulated through the dynamic interplay between protein synthesis and degradation. The majority of studies conducted have used the inhibitors lactacystin or clasto-lactacystin  $\beta$ -lactone ( $\beta$ -lac; Jarome et al., 2011; Rodriguez-Ortiz et al., 2011; Lee et al., 2008; Artinian et al., 2008, reviewed in Jarome and Helmstetter, 2014; Fioravante et al., 2008). Infusion of these inhibitors into the amygdala or hippocampus have been

shown to impair the consolidation and reconsolidation of both auditory and contextual fear conditioning, conditioned taste aversion, trace fear conditioning and the Morris water maze (Artinian et al 2008 2014, Jarome et al 2011, Lee et al 2008, Rodriguez-Ortiz et al 2011). These studies indicate that protein degradation, like protein synthesis is important for a variety of tasks assessing learning and memory. However, a mechanistic understanding of the role of protein degradation in learning and memory remains to be determined.

Recently, a few studies have begun to investigate whether or not protein degradation is similarly involved in addiction. Massaly and colleagues (2013) examined the effects of inhibiting protein degradation during the development and expression of opiate-induced addiction related behaviors. Inhibition of protein degradation impaired the development, but not expression of conditioned place preference. Protein degradation seems to be similarly important for the development of sensitization to morphine (Massaly et al., 2013). As discussed above, inhibition of protein synthesis impairs conditioned place preference. However, administration of a protein degradation inhibitor into the NAc blocks the impairment to reconsolidation produced by anisomycin (Ren et al., 2013). Together, these studies suggest that protein degradation may mediate both the consolidation and reconsolidation of stimulant and opiate-induced place preference (Massaly et al, 2013; Ren et al., 2013). The investigation of protein degradation in the context of addiction is still quite a new field. These early studies do suggest, that similar to its role in memory, protein degradation is important for at least some aspects of addiction. Many questions have yet to be answered including: at what synapses is protein degradation especially important, what is the mechanism by which protein degradation occurs in addiction, and what are the most important targets. More work will be required to understand the functional role of protein degradation during addiction.

*Overview of studies*

### *Inhibition of PKC disrupts addiction-related memory*

Recently, the persistently active, atypical PKC isoforms, PKM $\zeta$  and PKC $\lambda$  were demonstrated to be essential for many forms of long-term associative and spatial memories as well as late long-term potentiation (L-LTP; Kwapis et al., 2012; Kwapis et al., 2009; Parsons and Davis, 2011; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2009). However, the effects of these atypical PKCs in addiction-related memory was not well understood. Previous studies have typically infused a single, region specific application of the pseudosubstrate inhibitor, zeta inhibitory peptide (ZIP). In this series of experiments (Chapter 1), I investigated the effects of continuous and acute ZIP on the non-associative, addiction-related memory locomotor sensitization. Specifically, these studies address: (1) the effects of continuous ZIP administration on locomotor sensitization, (2) the effects of continuous chelerythrine (a more general PKC inhibitor) administration on locomotor sensitization, (3) the effects of acute, pre-induction ZIP on locomotor sensitization, (4) the effects of acute, post-induction ZIP on locomotor sensitization and (5) the effects of ZIP on AMPAR density (Howell et al 2014). Taken together these studies (Chapter 1) demonstrated that aPKCs are critically involved in the development of locomotor sensitization, but inhibition fails to disrupt sensitization once it has been established, despite reducing the density of membrane-bound AMPARs.

### *Proteasome phosphorylation regulates cocaine-induced sensitization*

As discussed above, long-term memory formation is known to involve de novo protein synthesis. Recent investigation into the effects of protein degradation is beginning to shape the view that memory formation requires a complex balance between protein synthesis and degradation (Artinian et al., 2008; Jarome et al., 2011; Lopez-Salon et al., 2001; Rodriguez-Ortiz et al., 2011; Fioravante and Byrne, 2010; Cajigas et al., 2010; Chain et al., 1999). Together, these processes influence plasticity and memory by regulating the proteins expressed at synapses

(Bingol & Sheng 2011, Cajigas et al 2010, Chain et al 1999, Ciechanover 2005, Fioravante & Byrne 2010, Patrick 2006). In eukaryotic cells, protein degradation largely occurs through the ubiquitin proteasome system (UPS; Bingol and Sheng, 2011; Ciechanover, 2005; Patrick, 2006). In this pathway, a ubiquitin chain tags a target protein, which is then delivered to the 26S proteasome where it is degraded (Ciechanover 2005, Glickman & Raveh 2005, Nagy & Dikic 2010). The 26S proteasome is a large complex with two components, the 20S core particle and 19S regulatory cap. Several ATPases, which are responsible for opening the pore of the 20S core particle, unfolding the targeted proteins and transferring them into the 20S core, make up the 19S caps (Bingol & Schuman 2005, Ciechanover 2005, Glickman & Raveh 2005, Nagy & Dikic 2010, Patrick 2006). Recently, Djakovic and colleagues demonstrated that CaMKII $\alpha$  phosphorylates the ATPase Rpt6 at serine 120 (S120) in an activity-dependent manner (Djakovic et al 2012, Djakovic et al 2009).

In this series of experiments (Chapter 2), I investigate the role of protein degradation on cocaine-induced locomotor sensitization through the use of a novel knock-in mouse. This line of mice was created based on the findings of Djokovic and colleagues (2009, 2012) and is phospho-mimetic (ser120 to aspartic acid; AGC  $\rightarrow$  GAC; S120D). These mice are the first discrete model of proteasomal function. Locomotor sensitization to cocaine was assessed in four groups of mice; S120D mice administered cocaine and saline and WT mice administered cocaine and saline. Proteasomal activity was also assessed in the nucleus accumbens and prefrontal cortex. There were two main findings. (1) In tissue collected from the NAc and PFC of S120D mice, proteasomal activity did not increase following the application of cocaine. This is in contrast to findings obtained from WT tissue in which cocaine induced a robust increase in proteasomal activity. (2) Behavioral sensitization to cocaine was completely blocked in S120D mice. Together,



these findings demonstrate an essential role for the precise regulation of protein degradation via Rpt6 phosphorylation at serine 120.

*Regulation of the UPS through Rpt6 phosphorylation at S120 is not required for associative fear memory*

Along with the S120D mice described above, a second line of knock-in mice was generated. These mice were created to have a phospho-dead mutation at serine 120 (ser120 to alanine; AGC → GCC; S120A). In S120A mice, phosphorylation at serine 120 remains in a ‘locked-off’ state. I examined S120D and S120A mice on two tasks that assess associative learning and memory (1) cued and contextual fear conditioning and (2) context discrimination, as well as a third task, (3) elevated plus maze, to assess baseline anxiety levels (Chapter 3). Surprisingly, there were no impairments on any of the tasks in either line of mice. Thus, bi-directional regulation of the proteasome at serine 120 on Rpt6 does not seem to be the mechanism underlying the effects of protein degradation during associative fear memory.

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## **CHAPTER 1: Inhibition of PKC disrupts addiction-related memory**

### **Abstract**

The atypical PKC isoforms, PKM $\zeta$  and PKC have been proposed as integral substrates of long-term memory. Inhibition of these isoforms has recently been demonstrated to be sufficient for impairing the expression and maintenance of long-term potentiation. Additionally, the pseudosubstrate inhibitor, zeta inhibitory peptide (ZIP), which effectively blocks PKM $\zeta$  and PKC, has previously been shown to disrupt associative memory; very little is known about its effects on pathological nonassociative forms of memory related to addiction. The neural and molecular substrates of memory and addiction have recently been argued to overlap. Here, we used ZIP to disrupt PKM $\zeta$  and PKC activity to examine their role in cocaine sensitization, a nonassociative, addiction-related memory argued to underlie the transition from casual to pathological drug use. We examined the effects of both continuous and acute administration of ZIP. Even a single application of ZIP blocked the development of sensitization; sustained inhibition using osmotic pumps produced an almost complete blockade of sensitization. Further, a single application of ZIP was shown to reduce membrane-bound AMPAR expression. These results demonstrate a novel, critical role for the atypical PKC isoforms in nonassociative memory and cocaine addiction.

## Introduction

Addiction involves long-lasting behavioral and neural changes thought to render the addict chronically susceptible to relapse (Hyman, Malenka, & Nestler, 2006; Koob & Volkow, 2010; Lüscher & Malenka, 2011; Nestler, 2001; Robbins & Everitt, 1999; Robinson & Kolb, 1997; Russo et al., 2010). Recently, it has been proposed that the mechanisms of learning and memory, and addiction overlap and that memory or memory-like neuronal remodeling subserve addiction (Carmack et al., 2013; Kauer & Malenka, 2007; Kelley, 2004; Robinson & Berridge, 2008; Russo et al., 2010; Torregrossa, Corlett, & Taylor, 2011).

In both processes, these changes involve the activation of multiple protein kinases including CaMKII, PKA, and PKC (Lee & Messing, 2008; Kandel, 2012; Lisman et al., 2012; Mayford, 2007). Recently, there has been growing evidence specifically implicating atypical isoforms of PKC (aPKCs) in LTP and memory (Pastalkova et al., 2006; Shema et al., 2007; Serrano et al., 2008; Sacktor, 2008; Ren et al., 2013). One isoform that has received much attention is protein kinase M $\zeta$  (PKM $\zeta$ ). PKM $\zeta$  is persistently active and lacks the regulatory domain present on most protein kinases, giving rise to the idea that PKM $\zeta$  may be essential for long-term memory (LTM) and late long-term potentiation (L-LTP). An abundance of studies implicating PKM $\zeta$  in LTM and L-LTP currently exists (Kwapis, Jarome, Gilmartin, & Helmstetter, 2012; Kwapis, Jarome, Lonergan, & Helmstetter, 2009; Parsons & Davis, 2011; Pastalkova et al., 2006; Serrano et al., 2008; Shema, Sacktor, & Dudai, 2007). However, recent studies using PKC $\zeta$ /PKM $\zeta$  knockout mice have questioned the idea that PKM $\zeta$  is necessary and sufficient for L-LTP and LTM and proposed that a second atypical PKC isoform, PKC, is involved, especially in early stages (Volk et al., 2013; Lee et al., 2013; Ren et al., 2013; Frankland & Josselyn, 2013; Matt & Hell, 2013).

Evidence implicating PKM $\zeta$  in LTM and L-LTP comes from studies using a single application of zeta-inhibitory peptide (ZIP) or the more general PKC inhibitor chelerythrine (Herbert, Augereau, Gleye, & Maffrand, 1990; Yao et al., 2013). A few studies have used the dominant negative form of PKM $\zeta$  to inhibit PKM $\zeta$  and subsequently disrupt LTP (Ling et al.,

2002) and established memory (Shema et al., 2002). Once believed to be selective to PKM $\zeta$ , ZIP was recently shown to also inhibit PKC (Ren et al., 2013). ZIP is derived from the autoinhibitory pseudosubstrate segment of PKC $\zeta$ , which is the same as that of PKC (Standaert et al., 2001; Bosch et al., 2004). Thus, the effects of ZIP may result from PKM $\zeta$  and/or PKC inhibition. For the current studies, it is only important that plasticity and associative memory are disrupted by ZIP.

While many prior studies have demonstrated the efficacy of ZIP in impairing both associative memory and L-LTP (Barry et al., 2012; Kwapis et al., 2009; Parsons & Davis, 2011; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2007), the role of aPKCs in addiction-related memory is unclear. We examined if ZIP disrupts the nonassociative, addiction-related memory, psychomotor sensitization to cocaine. Sensitization is an enhanced sensitivity to a drug characterized by increased psychomotor activation (locomotor sensitization), increased dopamine release (neural sensitization), and hypersensitivity to the drug's rewarding value (incentive sensitization) and is used to model the transition from casual to compulsive drug use (Anagnostaras & Robinson, 1996; Anagnostaras, Schallert, & Robinson, 2002; Robinson & Berridge, 1993; Shuman, Cai, Sage, & Anagnostaras, 2012; Steketee & Kalivas, 2011). Thus, this memory is thought to reflect pathological and compulsive behavior rather than ordinary associative learning.

Cocaine has been shown to change properties associated with excitatory synaptic transmission. Both in slice and in vivo, alterations in AMPAR/NMDAR ratios and increases in AMPAR rectification have been demonstrated following cocaine treatment (Kauer & Malenka, 2007; Kessels & Malinow, 2009). PKM $\zeta$  and PKC may exert their effects through AMPAR trafficking (Ling, Benardo, & Sacktor, 2006; Miguez et al., 2010; Ren et al., 2013; Yao et al., 2008; Sacktor, 2011). Perfusion of PKM $\zeta$  into cells doubled the AMPA mediated EPSC and inhibition of PKM $\zeta$  decreased postsynaptic GluR2 (Miguez et al., 2010; Sacktor, 2008). Similarly, PKC inhibition blocked the enhancement of GluA1/GluA2 typically induced by LTP suggesting the elevation of postsynaptic AMPARs is dependent on PKC activity (Ren et al., 2013). Thus, the

aPKCs, PKM $\zeta$  and PKC may also mediate modifications in AMPARs during addiction-related memory and plasticity.

We examined the effects of disruption of aPKCs at multiple time points during the induction of sensitization using continuous or acute intracerebroventricular (ICV) application of ZIP or chelerythrine. Finally, we examined whether acute disruption of PKM $\zeta$  reduced membrane-bound AMPAR density.

## **Materials and Methods**

### **Animals**

Ninety-six hybrid C57BL/6Jx129T2SvEms/J (129B6, Jackson Labs) adult mice were used. Mice were group housed in a vivarium on a 14:10 light:dark schedule. Testing was performed during the light phase. All procedures were approved by the UCSD IACUC and compliant with the NRC Guide.

### **Drugs**

The myristolated PKC Zeta pseudosubstrate inhibitory peptide (AnaSpec) was dissolved in phosphate-buffered saline (PBS, Ricca) to a dose of 10 nmol. In experiment 1, 10 nmol of ZIP was administered across 3 days at a rate of 0.25  $\mu$ L/hr whereas in experiments 3 and 4, the 10 nmol dose of ZIP was given in a single 1 $\mu$ L infusion. Chelerythrine Cl (Enzo) was dissolved in PBS to a concentration of 10 nmol/ $\mu$ L. Buprenorphine HCl (0.05 mg/kg, s.c.) was given for post-operative pain (Reckitt-Benckiser). Cocaine HCl (Sigma) was dissolved in physiological saline (salt weight, 15 mg/kg, 10 ml/kg, i.p.).

### **Surgery**

For all experiments mice were anesthetized with isoflurane dispensed from a precision vaporizer and mounted in a stereotaxic apparatus (myNeuroLab.com). A single hole was drilled in the skull for infusion into the third ventricle (AP: -0.5mm; ML: 0 mm, DV: -3 mm, Franklin & Paxinos, 2007). Following surgery, all animals were given an injection of buprenorphine. For experiments examining continuous inhibition of aPKCs (experiments 1, 2), osmotic pumps

(Alzet-Durect model 1002) and PE60 tubing were implanted subcutaneously and connected to an infusion headstage attached to the skull (Alzet, Brain infusion kit 3).

*Experiments 1 and 2.* In experiment 1, 16h prior to surgery, pumps were filled with aCSF (100  $\mu$ L; ion concentrations in mM: Na 150, K 3.0, Ca 1.4, Mg 0.8, P 1.0, Cl 155; Harvard) and connected to tubing containing ZIP and/or aCSF. A “leader” and “trailer” of aCSF was placed before and after the ZIP in the tubing (separated with mineral oil) timed such that ZIP administration began 8.5 h prior to the beginning of cocaine administration, and ended 23 h after the sixth cocaine administration session (**Figure 1.1A**). In experiment 2, pumps and tubing were filled with chelerythrine or aCSF. Animals recovered for 3d.

*Experiments 3 and 4.* In experiment 3, mice were given one microinfusion of ZIP prior to the induction of sensitization. Mice were implanted with 20-ga guide cannulae (PlasticsOne) 1mm above the target. A dummy was placed inside the guide to prevent clogging. Animals recovered for 3 days. Prior to ZIP infusion, mice were briefly anaesthetized with isoflurane. Dummies were removed and a 24-ga injection cannula that extended 1 mm below the guide was attached. ZIP was infused at a rate of 1  $\mu$ L/min using a syringe pump (Kd Scientific) and injection cannulas were left in place for 3 min following the infusion. Animals recovered for 2h. In experiment 4, after the induction of sensitization, mice were given an infusion of ZIP or aCSF. A 29-ga stainless steel needle connected to a syringe and pump infused ZIP at a rate of 1  $\mu$ L/min. The needle remained in place for 3 additional min. Animals recovered for 24 h.

### **Behavioral Assessment**

Mice were tested in individual chambers housed in a windowless room as described previously (Carmack et al., 2013; Shuman et al., 2012). The apparatus consisted of a two-sided, 44 cm. x 44 cm. x 31 cm chamber, bisected by an opaque wall with a removable insert (Med-Associates). Sides assigned for drug and saline pairings were counterbalanced. Activity monitor software (Med-Associates) tracked the distance traveled. Two 100-watt bulbs lit the room and white noise (65 dBA) played continuously. All animals were handled for 5 d prior to the



experiments. Prior to behavioral assessment, animals were habituated to the chambers during two, 1h sessions (30 min each side).

*Experiment 1.* Mice were divided into 4 groups: Veh/Coc, ZIP/Coc, Veh/Veh, ZIP/Veh (**Figure 1.1A**). Sensitization was induced during 6 sessions of cocaine administration (2 sessions/d for 3 d). Animals received an injection of saline (10 ml/kg) and were immediately placed in the saline-paired side of the chamber. Animals were restricted to this side for 15 min. Mice were then removed, given an injection of cocaine (Veh/Veh and ZIP/Veh mice received a second injection of saline) and restricted to the drug-paired side of the chamber. After 15 min, mice were removed from the chambers and returned to their home cages. ZIP was infused continuously throughout the 6 cocaine administration sessions. 48 h following the 6th cocaine administration session, animals underwent a conditioned place preference test. All animals were off-ZIP and off-drug. The insert bisecting the two sides of the chamber was removed and animals were allowed to freely explore both sides of the chamber for 15 min. Place preference was measured as the difference in percent time spent on the drug-paired side and saline-paired side. A final, off-ZIP sensitization challenge test was conducted 24 h later. All animals were given an injection of cocaine (15 mg/kg) and were immediately placed on the drug-paired side of the chamber. Animals were restricted to the drug-paired side and remained in the chambers for 15 min. Sensitization was measured as the increase in locomotor activity following repeated drug-context pairings.

*Experiment 2.* Chelerythrine was delivered continuously throughout the entire experiment. Procedures were the same as those described above; however, in this experiment, animals were given 5 cocaine administration sessions across 5 d, followed by a place preference test 24 h later and the sensitization challenge test another 24 h later (session 6).

*Experiment 3.* Mice were infused with ZIP (described above) 2 h prior to the first cocaine administration session (session 1). 24 h later mice were given an off-drug place preference test. Another 24 h later, mice were given a sensitization challenge test (session 2).

*Experiment 4.* Animals were given 4 cocaine administration sessions across 4 d. 24 h after the final session, animals underwent surgery and were infused with ZIP or vehicle (described above). Following recovery, animals were given an off-drug place preference test followed 24 h later by a sensitization challenge test (session 5).

### **Histology**

In experiments 1 and 2 mice were anaesthetized and perfused with 1 x PBS and 4% paraformaldehyde. Tissue was sliced into 1mm coronal sections using an acrylic matrix (Braintree). For experiments 3 and 4, animals were anesthetized and then decapitated for fresh tissue collection. Brains were extracted, frozen in 2-methylbutene and stored at -80°C. At -20°C brains were cut in 20 µm coronal sections at 200 µm intervals using a cryostat (Microm HM550, Fisher). Cannula placement was verified by visual inspection. Brain morphology remained grossly intact following the application of ZIP and chelerythrine. No animals were excluded.

### **Radioligand incubation and liquid scintillation**

Optimal binding procedures for the [<sup>3</sup>H]AMPA radioligand are adapted from previous literature (Monk et al., 2012; Olsen et al., 1987; Jang et al., 2000). Sections were pre-incubated with 50 mM Tris-HCl buffer for 20 min, then incubated for 30 min with 15nM [<sup>3</sup>H]AMPA (Sigma) in the same buffer at 25°C. After incubation, the sections were rinsed in the Tris-HCl buffer, then washed in distilled water. Sections from each slide were transferred to vials containing a liquid scintillation cocktail (EcoLume Liquid Scintillation Fluid, MPBiomedicals) to assess global AMPA receptor expression density using automated liquid scintillation (Tricarb 2900TR, PerkinElmer).

### **Data Analysis**

Data were entered into a multivariate analysis of variance (MANOVA; PASW18). The level of significance was set at  $p \leq .05$ . Following a significant omnibus comparison, or group x time interaction, post-hoc comparisons were made using univariate ANOVAs or Fisher's protected least significant difference (PLSD). In order to simplify data presentation, univariate

ANOVAs are reported for group differences, followed by Fisher's PLSDs for interesting comparisons.

## Results

### Experiment 1: Effects of continuous ZIP administration on locomotor sensitization

We first examined the effects of continuous aPKC inhibition on psychomotor sensitization to cocaine. Mice were implanted with osmotic pumps that delivered continuous, ICV ZIP or aCSF (10 nmol, 0.25  $\mu$ L/h) throughout 6 cocaine (15 mg/kg, i.p.) or saline administration sessions (**Figure 1.1A, 1.1B**). ZIP administration was timed such that it began prior to the first cocaine administration session and ended after the sixth session. Mice were divided into four groups (n=7-11 per group): (1) Veh/Coc mice received vehicle, ICV, in the pumps and i.p. cocaine injections, (2) ZIP/Coc mice were administered ZIP ICV through pumps and received i.p. cocaine injections, (3) Veh/Veh mice received vehicle both ICV and i.p., (4) ZIP/Veh mice received ICV ZIP through the pumps, but received i.p. injections of vehicle. We found an initial elevated locomotor response in groups receiving cocaine compared to those receiving vehicle, (**Figure 1.1C**; ANOVA, [F(3,32)=4.6, p=.009]), but no difference in the acute response to cocaine between ZIP/Coc mice and Veh/Coc mice (Fisher's PLSD, p=.78). Across the 6 sessions of cocaine administration, differences between ZIP/Coc and Veh/Coc mice emerged (**Figure 1.1B**; [F(3,32)=18.3, p<.001]). ZIP/Coc mice demonstrated a dramatic reduction in locomotor activity compared to Veh/Coc mice (p<.05). When paired with saline, ZIP did not produce any locomotor attenuating effects (ZIP/Veh vs. Veh/Veh, p=.93). We then measured sensitization as the difference between the acute (session 1) and sensitized (session 6) response (Carmack et al., 2013; Shuman et al., 2012). There were significant group differences [F(3,32)=6.9, p=.001; **Figure 1.1E**]; Veh/Coc mice exhibited robust sensitization, showing a greater response than all other groups (p-values<0.02). Sensitization was blocked in ZIP/Coc mice, as they did not differ from control groups (p-values>0.1; **Figure 1.1E**). We conducted a final sensitization challenge test, during which all animals were off-ZIP and all groups received cocaine (15 mg/kg,i.p.;

**Figure 1.1F).** Groups that had previously received cocaine (ZIP/Coc, Veh/Coc) showed greater activity compared to groups that had previously received vehicle (ZIP/Veh, Veh/Veh; [F(3,32)=3.2,  $p < .05$ ]; **Figure 1.1F**), but ZIP/Coc mice showed attenuated sensitization relative to Veh/Coc mice (first 5 min, main effect [F(3,32)=10.4,  $p < .001$ ], ZIP/Coc vs Veh/Coc,  $p < .05$ ; **Figure 1.1F**).

### **Experiment 2: Effects of continuous chelerythrine administration on locomotor sensitization**

As we used a novel, chronic procedure to inhibit aPKCs, in experiment 2, we investigated whether continuous chelerythrine administration would affect sensitization similarly to ZIP. Chelerythrine more generally blocks PKCs by competitively inhibiting the catalytic domain and effectively inhibits PKM isoforms (Herbert et al., 1990; Serrano et al., 2008; Yao et al., 2013). This experiment was conducted to validate the effectiveness of using osmotic minipumps and continuous delivery to inhibit aPKCs. Mice were implanted with osmotic pumps which delivered chelerythrine or vehicle throughout the experiment at a dose established by others (10nmol/ $\mu$ L, 0.25 $\mu$ L/h, Serrano et al., 2008; Yao et al., 2013). Mice were divided into two groups (n=8-9 per group): (1) received chelerythrine (10 nmol/ $\mu$ L) ICV as well as i.p. cocaine injections and (2) vehicle mice received aCSF ICV and i.p. injections of cocaine. All animals underwent 6 cocaine administration sessions (15mg/kg,i.p.; **Figure 1.2A**). As with ZIP, chelerythrine did not affect the acute response to cocaine during session 1 [F(1,15)=0.1,  $p=.76$ ]. After the final cocaine administration session, sensitization was assessed as the difference between the acute (session 1) and sensitized response (session 6). Sensitization was dramatically attenuated in mice previously treated with chelerythrine relative to mice that had received vehicle (**Figure 1.2B, 1.2C**; [F(1,15)=11.1,  $p < .01$ ]).

### **Experiment 3: Effects of acute, pre-induction ZIP on locomotor sensitization**

As most previous studies have given a single infusion of ZIP to assess the effects on memory, we examined if a single infusion could disrupt sensitization (Kwapis et al., 2009;

Parsons & Davis, 2011; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2007). In this experiment, we used two groups of mice (n=13 per group): (1) received a single pre-induction application of ZIP (10nmol/ $\mu$ L, 1 $\mu$ L) 2h prior to the first cocaine administration session, while (2) received a 1 $\mu$ L infusion of aCSF prior to cocaine administration (**Figure 1.3A**). During this initial session (15mg/kg,i.p.), ZIP did not affect the response to cocaine (**Figure 1.3A**; [F(1,24)=22, p=.65]). In contrast, when challenged with cocaine while off-ZIP, 48 h later, mice that had previously received ZIP showed substantial impairment in sensitization (**Figure 1.3A**; [F(1,24)=5.8, p<.05]). Further, ZIP also impaired sensitization when assessed as the difference in activity between the two sessions (**Figure 1.3B**; [F(1,24)=5.7, p<.05]).

#### **Experiment 4: Effects of acute, post-induction ZIP on locomotor sensitization**

We then examined whether a single, post-induction application of ZIP could disrupt locomotor sensitization. Two groups of mice were used (n=8-9 per group); both groups received i.p. injections of cocaine (15mg/kg, i.p.), but one group received an ICV infusion of ZIP while the other received an ICV infusion of aCSF. Four cocaine administration sessions produced robust sensitization that did not differ across groups (**Figure 1.3C**; [F(1,15)=.29, p=.59]). After the fourth session, mice were given a single microinfusion of ZIP (10 nmol/ $\mu$ L, 1 $\mu$ L) or a comparable infusion of aCSF. Forty-eight hours later, we conducted an off-ZIP sensitization test. Post-induction ZIP failed to affect sensitization (**Figure 1.3C**; [F(1,15)=.23, p=.63]).

#### **Experiment 5: Effects of ZIP on AMPAR density**

Finally, we examined whether a single application of ZIP was sufficient to reduce AMPAR density in sensitized brain tissue. As it has been argued that both PKM $\zeta$  and PKC exert their effects through AMPAR trafficking (Ling et al., 2006; Miguez et al., 2010; Yao et al., 2008; Ren et al., 2013), we used a radioligand binding procedure to detect membrane-bound [ $^3$ H]AMPA. We found that membrane-bound AMPARs were significantly reduced in tissue previously exposed to ZIP (**Figure 1.3D**; [F(1,34)=6.2, p < .02]).

## Discussion

In the present study, we examined the effects of both continuous and acute inhibition of the  $\alpha$ PKC isoforms, PKM $\zeta$  and PKC, on the nonassociative, addiction-related memory, locomotor sensitization. There were two main findings. First,  $\alpha$ PKCs are critically involved in the development of locomotor sensitization; ZIP was highly effective at disrupting sensitization if infused prior to cocaine administration. Second, infusion of ZIP after sensitization had been established failed to produce impairment, despite reducing the density of membrane-bound AMPARs. The current findings extend the existing evidence regarding which forms of memory are susceptible to disruption by ZIP. The novel method for ZIP administration reveals nonassociative memory may have different requirements for memory maintenance than traditional forms of memory, as pre-induction ZIP was required to produce impairment.

### ZIP administration disrupts the development of sensitization

Growing evidence supports the view that ZIP disrupts not only PKM $\zeta$ , but also a second atypical PKC isoform, PKC (Ren et al., 2013; Volk et al., 2013; Lee et al., 2013). The majority of studies have used a single application to disrupt associative or spatial forms of memory such as conditioned taste aversion, Pavlovian fear conditioning, fear potentiated startle, and the Morris water maze (Kwapis et al., 2009; Parsons & Davis, 2011; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2007). ZIP is derived from the pseudosubstrate sequence of PKC $\zeta$ , which is identical to that of PKC. At higher concentrations, ZIP inhibits both PKM $\zeta$  and PKC (Ren et al., 2013; Standaert et al., 2001; Bosch et al., 2004). Here, we expand current findings to include a role for PKM $\zeta$  and PKC in the nonassociative, addiction-related memory, locomotor sensitization. Administration of either ZIP or chelerythrine prior to induction impaired the development of sensitization. ZIP dramatically impaired sensitization regardless of whether it was given continuously or in a single infusion, but the effects were largest when given continuously (**Figure 1.1 B,E**). Still, a single application of ZIP disrupted sensitization 48h after administration (**Figure 1.3 A,B**), a time point when ZIP would have been fully degraded (Kwapis et al., 2012). These

results demonstrate ZIP persistently effects sensitization if administration occurs prior to acquisition.

There are a few previous reports demonstrating ZIP's ability to disrupt certain forms of addiction-related plasticity and memory including cocaine-induced spontaneous synaptic transmission, the cocaine-induced enhancement in AMPA/NMDA ratio and conditioned place preference (Ho et al., 2012; Li et al., 2011). However, these studies focused on associative forms of addiction related memory. The present study is the first to demonstrate its ability to disrupt nonassociative addiction related memory. Nonassociative aspects of addiction are important to consider as they model key pathological components of what drive addiction.

### **ZIP does not impair the maintenance of sensitization**

Studies that have examined the application of post-training ZIP have found that it often produces amnesia (Kwapis et al., 2009; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2007; Gámiz & Gallo, 2011). However, Parsons and Davis (2011) suggested the effects of ZIP were dependent on the timing between training and administration. While memory and addiction have been argued to share overlapping neural substrates (Kauer & Malenka, 2007; Kelley, 2004; Lee & Messing, 2008; Robinson & Kolb, 1997), findings from the current study suggest the role of PKM $\zeta$  and PKC in sensitization differs somewhat from their role in associative memory. Once sensitization had been established, ZIP administration was unable to produce subsequent impairment (**Figure 1.3C**). Moreover, sustained inhibition was required to fully prevent sensitization.

One possible reason that may account for these differences is the region-specificity of the infusion. Most prior studies have infused ZIP into a particular region (e.g. amygdala, insular cortex, hippocampus), however we administered ZIP ICV. It is possible that the concentration of ZIP required to produce an effect after sensitization had been established was not achieved. Previous work has established that a certain concentration of ZIP is required to block PKM $\zeta$  and PKC and impair plasticity (Ren et al., 2013; Sacktor & Fenton, 2012; Serrano, Yao, & Sacktor, 2005). A similar explanation could potentially account for differences in the effects of ZIP on

conditioned place preference found in this study compared to other studies that have shown the apparent erasure of CPP memory following the administration of PKM $\zeta$  inhibitors. (Lee et al., 2013; Shabashov et al., 2012; Li et al., 2011; He et al., 2011). It is also possible that by infusing ZIP ICV the peptide did not reach regions critical for the behavior, such as the amygdala (He et al., 2011; Hsu et al., 2002; Everitt et al., 1991). While this explanation may explain the negative result in our place preference experiments, it likely does not account for our sensitization results because the concentration achieved in the current study was sufficient to disrupt sensitization prior to induction and produced a decrease in AMPAR density when given post-induction.

An alternate reason we did not find an effect of ZIP on sensitization when given after induction is that the mechanism of ZIP may be different when given pre-training compared to post-training. It is possible there is a shift to the right in the dose-effect curve for ZIP given post- vs. pre-training. While we used the standard dose of ZIP in the current study, in future studies, it would be interesting to examine the effects of a higher dose of ZIP on sensitization when given after induction; however, it is possible there would be non-specific effects at higher doses. In the future it would be interesting to compare the effects of a single post-induction ZIP infusion and continuous ZIP infusion on AMPAR density. It is possible, in our experiments, that continuous ZIP infusion reduced postsynaptic AMPAR density below a critical threshold necessary to sustain memory, while the single, post-induction infusion did not (despite using the same total dose of ZIP). Similarly, it is also possible that inhibiting aPKCs prior to training impairs AMPAR insertion or that the newly inserted AMPARs are more vulnerable to the effects of ZIP, potentially because of a difference in sub-unit composition. A more detailed analysis of the type of AMPARs affected by pre- vs. post training infusions could help to tease apart these explanations. Another alternative is that the neural adaptations produced by a nonassociative, drug-related memory may be more enduring than those in associative memory or the mechanisms may only partially overlap (Carmack et al., 2013; Robinson & Kolb, 1997). A recent study conducted by Carmack and colleagues (2013), using the NMDA receptor antagonist CPP, found that NMDARs were not essential for the induction of sensitization, whereas NMDARs were essential for the formation of



place preference. A study conducted by Cai and colleagues (2011) was one of the few studies to examine the effects of aPKC inhibition on non-associative memory. In this study, both ZIP and chelerythrine were found to disrupt long-term sensitization of the gill-withdrawal reflex in *Aplysia*, even when given 7 days after training. While both the current study and the Cai et al. study examine the effects of inhibition of aPKCs on sensitization, the mechanisms underlying each of these forms of sensitization is quite different.

AMPA trafficking is believed to mediate the downstream effects of PKM $\zeta$  and PKC (Ling et al., 2006; Miguez et al., 2010; Yao et al., 2008). PKM $\zeta$  has been reported to enhance AMPA-mediated mEPSCs and application of the synthetic peptide GluR2<sub>3Y</sub> effectively prevented the endocytosis of GluR2 AMPAR subunits and prevented the deficit in fear memory typically produced by PKM $\zeta$  inhibition (Ling et al., 2006; Miguez et al., 2010). Similarly, PKC also affects AMPAR trafficking. Inhibition of PKC blocked the LTP-induced enhancement of post-synaptic responses of GluA1 and GluA2 and postsynaptic AMPARs, mEPSCs, and EPSC magnitude are reduced by application of ZIP or PKC knockdown (Ren et al., 2013). Our data support and extend previous findings, which suggest the effects of PKM $\zeta$  and PKC are mediated by AMPARs, to cocaine-induced sensitization. In future studies, an interesting comparison would be to examine the effects of ZIP on both sensitized and non-sensitized brain tissue, but for the purposes of this experiment we were primarily concerned with any differences in AMPAR density in cocaine-sensitized animals exposed to ZIP vs. non-ZIP.

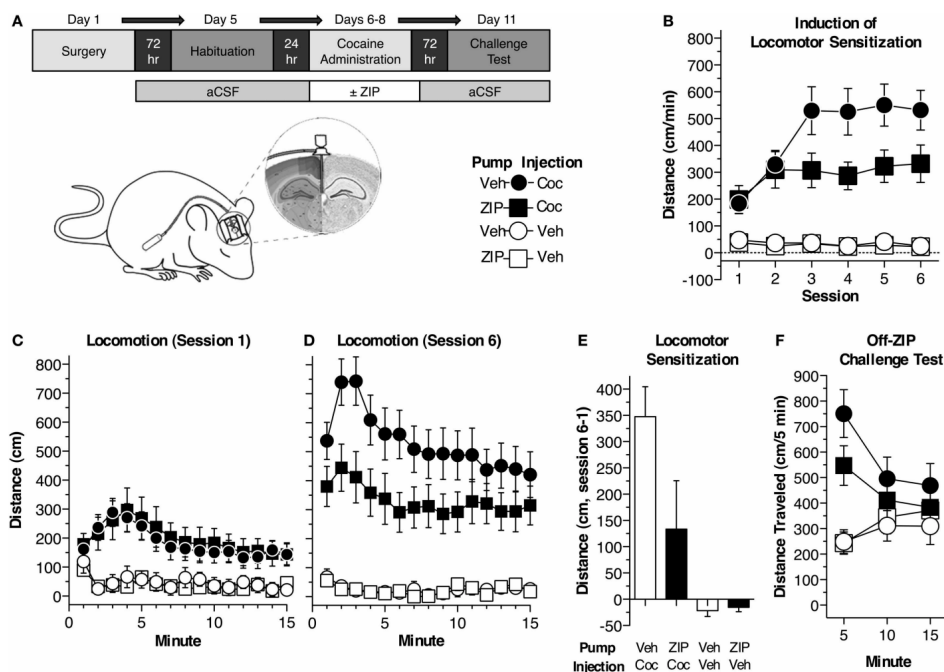
While ZIP was initially believed to exert its effects on plasticity and memory by selectively inhibiting PKM $\zeta$ , emerging evidence suggests at concentrations of at least 2M, the peptide acts on PKC as well (Ren et al., 2013); this likely accounts for the controversial findings obtained from mice with a deletion of the *Prkcz* gene (Volk et al., 2013; Lee et al., 2013). ZIP still effectively reversed LTP and cocaine-induced place preference in these mice despite the absence of PKM $\zeta$  (Volk et al., 2013; Lee et al., 2013). Both lines of PKC $\zeta$ /PKM $\zeta$  knockout mice exhibit levels of PKC that do not differ from controls (Volk et al., 2013; Lee et al., 2013). We found that ZIP effectively impaired nonassociative addiction-related memory and membrane-bound AMPAR

expression, but future work will be needed to directly assess the extent to which ZIP exerts its effects on PKM $\zeta$ , PKC or both atypical PKCs. Additional future work will be needed to mitigate the discrepancy between the post-training effects of ZIP on AMPAR density and behavior. As mentioned above, it is possible that a higher dose of ZIP is needed to disrupt AMPAR expression enough to disrupt established sensitization. In the current study we examined global AMPAR density, while future work will examine AMPAR density in a region specific manner.

### **General Conclusion**

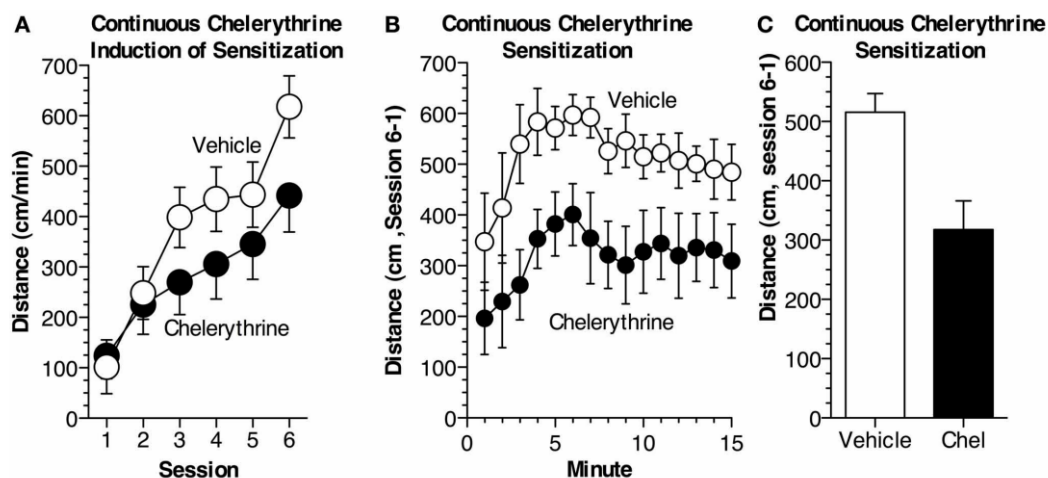
In summary, we found that atypical PKC isoforms play a critical role in cocaine-induced locomotor sensitization and addiction. Future work should further explore the differences between traditional forms of associative memory and nonassociative addiction related memory. These differences may elucidate how certain forms of memory may become pathological. Taken together, these findings support a critical role for the atypical PKCs, PKM $\zeta$  and PKC in cocaine-induced sensitization and therefore in mediating the transition from casual to pathological drug use.

Chapter 1, in full, is a reprint of the material as it appears in Inhibition of PKC disrupts addiction related memory 2014. Howell, KK; Monk, BR; Carmack, SA; Mrowczynski, OD, Clark, RE, Anagnostars, SG in *Frontiers in Behavioral Neuroscience*. The dissertation author was the primary author of this paper.

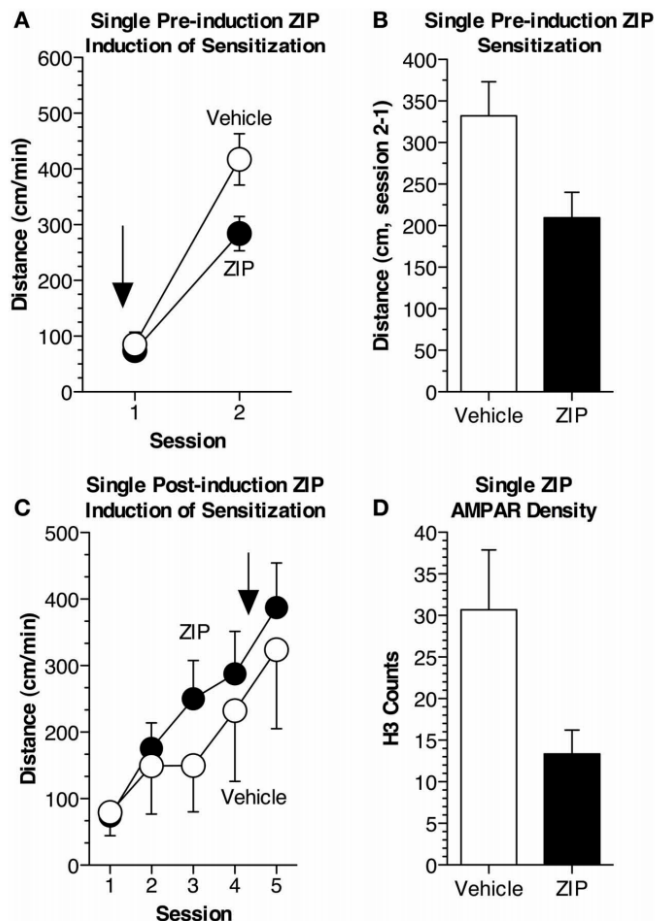


**Figure 1.1** Continuous ZIP administration blocks cocaine-induced locomotor sensitization.

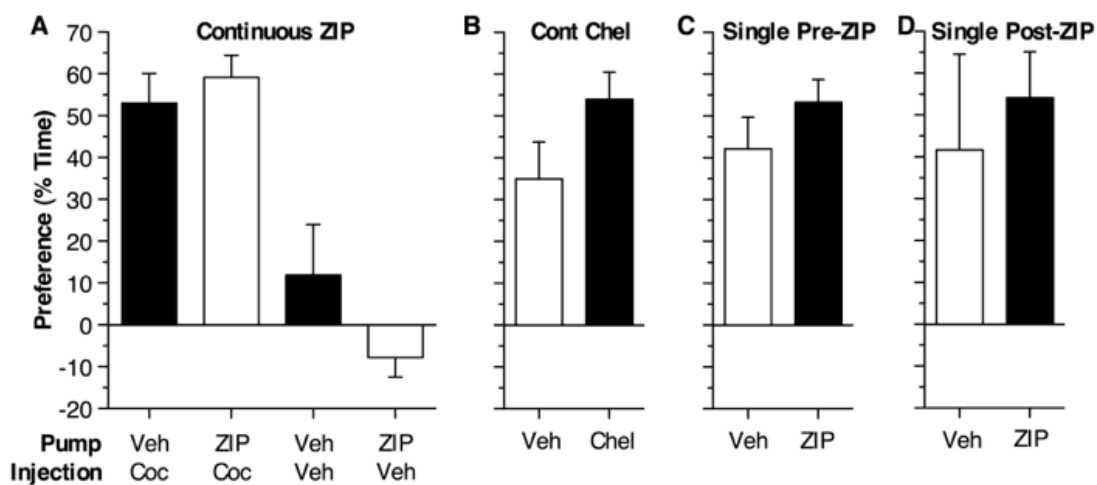
(A) Depiction of the procedure used in experiment 1 ( $n=10$  Veh/Coc,  $n=11$  ZIP/Coc,  $n=7$  Veh/Veh,  $n=8$  ZIP/Veh). Mice were implanted with osmotic pumps, filled such that ZIP would be delivered ICV just prior to the first cocaine administration session and terminate 24 h following the sixth session (10 nmol, 0.25  $\mu$ L/h). Animals underwent 6 cocaine administration sessions (2 sessions per day) during which time the development and expression of sensitization was assessed. (B) ZIP impaired the development of sensitization. The data represent the average distance traveled for each session ( $\pm$  SEM). The distance traveled by mice receiving ZIP/Coc was reduced compared to mice treated with Veh/Coc across the 6 cocaine administration sessions (15 mg/kg, i.p.; MANOVA,  $F(3,32)=18.3$ ,  $p<.001$ ; Fisher's PLSD  $p < .05$ ). ZIP alone did not produce any locomotor attenuating effects (ZIP/Veh, Veh/Veh,  $p = .93$ ). (C) ZIP did not alter the acute response to cocaine (session 1). Distance traveled at each minute of the session ( $\pm$  SEM) is shown. Animals receiving cocaine (ZIP/Coc, Veh/Coc) traveled a greater distance compared to mice receiving vehicle (ZIP/Veh, Veh/Veh,  $p$ -values  $< .05$ ), but there was no difference between the cocaine-treated groups ( $p = .78$ ). (D) ZIP/Coc mice showed reduced locomotor sensitization compared to Veh/Coc mice during session 6 (ANOVA,  $F(3,32)=16.7$ ,  $p < .001$ ; ZIP/Coc, Veh/Coc Fisher's PLSD  $p < .05$ ). Distance traveled at each minute of the session ( $\pm$  SEM) is shown. ZIP alone did not produce any effects on locomotor activity. (E) Sensitization, measured as the difference between the sensitized (session 6) and acute (session 1) responses, was blocked in ZIP/Coc mice. The average difference in distance traveled ( $\pm$  SEM) is shown for each group. Sensitization in ZIP/Coc mice did not differ from mice treated with ZIP/Veh or Veh/Veh ( $p$ -values  $> 0.1$ ). (F) Sensitization was also assessed while all animals were off-ZIP. ZIP/Coc and Veh/Coc groups showed greater activity than mice that previously received vehicle (ZIP/Veh, Veh/Veh; MANOVA,  $F(3,32)=3.2$ ,  $p < .05$ ). Animals in the ZIP/Coc group showed reduced sensitization compared to animals that had previously received Veh/Coc during the first 5 min of the test (ANOVA [ $F(3,32)=10.373$ ,  $p < .001$ ], Fisher's PLSD, ZIP/Coc vs Veh/Coc,  $p < .05$ ). There were no differences in the acute response to cocaine between animals that had not previously received cocaine (Fisher's PLSD, Veh/Veh, ZIP/Veh,  $p = .96$ ).



**Figure 1.2** Continuous chelerythrine reduces locomotor sensitization. **(A)** Mice were implanted with osmotic pumps containing chelerythrine or vehicle. Chelerythrine (n=9 Chel, n=8 Veh) was delivered continuously, ICV (10 nmol/ $\mu$ L, 0.25  $\mu$ L/h), across 6 sessions of cocaine administration (15 mg/kg, i.p.). Average distance traveled ( $\pm$  SEM) during each session is depicted. Chelerythrine did not alter the acute response to cocaine (ANOVA,  $F(1,15)=.097$ ,  $p=.76$ ) but did reduce the development of sensitization across the 6 sessions. **(B)** Sensitization, measured as the difference in distance traveled between the acute (session 1) and sensitized (session 6) response was significantly impaired in mice receiving chelerythrine across 15 min (MANOVA,  $F(1,15)=11.1$ ,  $p < .01$ ). The difference in distance traveled ( $\pm$  SEM) is shown for each minute. **(C)** Average sensitization ( $\pm$  SEM) measured as the difference in distance traveled is shown for each group.



**Figure 1.3** Single pre- but not post-induction application of ZIP reduces locomotor sensitization. **(A)** Mice received a single pre-induction infusion of ZIP (10 nmol/ $\mu$ L, 1  $\mu$ L). Sensitization was induced during 2 sessions of cocaine administration (15 mg/kg, i.p.). ZIP was administered 2 h prior to the first cocaine administration session (n=13 ZIP, n=13 aCSF), indicated by the arrow. The average distance traveled ( $\pm$  SEM) for each session is shown. While on-ZIP, the acute response to cocaine was not altered (ANOVA,  $F(1,24)=.215$ ,  $p = .65$ ). Cocaine was given a second time, 48 h later, during session 2. When measured off-ZIP, distance traveled was significantly reduced in animals that had previously received ZIP (ANOVA,  $F(1,24)=5.8$ ,  $p < .05$ ). **(B)** Sensitization was significantly reduced in animals given ZIP prior to cocaine administration (ANOVA,  $F(1,24)=5.7$ ,  $p < .05$ ). Sensitization is represented as the average difference in distance traveled ( $\pm$  SEM) between the two sessions. **(C)** Mice received a single post-induction infusion of ZIP (10 nmol/ $\mu$ L, 1  $\mu$ L). Sensitization was induced across 4 sessions of cocaine administration (15 mg/kg, i.p.), after which mice were given a single infusion of ZIP, represented by the arrow. Post-induction ZIP did not produce impairment when sensitization was assessed 72 h later off-ZIP (ZIP n=9, Veh n=8). **(D)** A single infusion of ZIP reduces AMPAR density following sensitization. H3 counts ( $\pm$  SEM) for each group are depicted.



**Figure 1.S1 Supplementary Figure** Conditioned place preference assessment.

(A) Place preference was examined for all experiments. In experiment 1 mice that had previously received cocaine (Veh/Coc, ZIP/Coc) showed place preference whereas mice that had received vehicle (Veh/Veh, ZIP/Veh) did not [ $F(3,32)=15.048$ ,  $p < .01$ ]. Continuous ZIP did not affect place preference (Veh/Coc, ZIP/Coc,  $p = .45$ ). (B) Experiment 2. Chelerythrine did not disrupt place preference [ $F(1,15)=3.121$ ,  $p = .10$ ]. (C) An infusion of ZIP given prior to any cocaine administration did not impair place preference [ $F(1,15)=1.440$ ,  $p = .24$ ]. (D) Place preference remained intact when ZIP was given after the induction of sensitization [ $F(1,15)=0.023$ ,  $p = .88$ ].

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## **CHAPTER 2: Proteasome phosphorylation regulates cocaine-induced sensitization**

### **Abstract**

Addiction is characterized by compulsive drug use, drug seeking, and repeated relapse despite long periods of withdrawal from the drug. Exposure to addictive drugs produces structural and functional modifications in neurons and synapses that entail the dynamic regulation of protein synthesis and degradation (Djakovic et al., 2009, Djakovic et al., 2012, Hamilton et al., 2012). The ubiquitin proteasome system (UPS) is a major pathway responsible for the breakdown of proteins in eukaryotic cells. We were interested in the effects disrupting the UPS would have on addiction-related behavior. To address this, we generated a novel line of knockin mice with a mutation in the ATPase 19S regulatory particle subunit of the proteasome, Rpt6, at serine 120 (S120). This is the first mouse model with a mutation on the proteasome. We found that increases in Rpt6 phosphorylation correlated with enhanced proteasome activity. Next, we assessed the effects of this mutation on cocaine-induced sensitization. In nucleus accumbens and prefrontal cortex tissue, a phospho-mimetic mutation at S120 prevented the increase in proteasomal activity following repeated cocaine treatment that was observed in wild-type mice in both the nucleus accumbens and prefrontal cortex. Similarly, behavioral sensitization completely failed to develop in knockin mice. Together, these findings highlight that Rpt6 phosphorylation and proteasome activity critically regulate cocaine-induced sensitization at both a molecular and behavioral level.

## Introduction

The persistent neural and behavioral adaptations characteristic of addiction can render an addict permanently susceptible to relapse even years after cessation of drug use (Robinson, 1997, Volkow, 2003, Hyman, 2006, Luscher, 2011). The enduring nature of these modifications suggests the involvement of memory or memory-like neuronal remodeling (Hyman, 2001, Kelley, 2004, Kauer, 2007, Robinson, 2008). Sensitization is an increase in the sensitivity to a drug following repeated administration, characterized by enhanced locomotor activity, dramatically increased dopamine release, and hypersensitivity to the rewarding value of the drug (Paulson, 1995, Robinson, 2008). Sensitization is argued to mediate the transition from ordinary goal-seeking behavior to compulsive behavior as the drug comes to elicit a much stronger dopamine response than natural reinforcers. The involvement of protein synthesis in traditional forms of memory, as well as addiction-related memory is well demonstrated (Davis, 1984, Schafe, 2000, Hernandez, 2002, Gold, 2008, Jarome, 2014). Indeed sensitization is accompanied by large scale remodeling of the dopamine system, including an increase in the number of synapses and dendritic spines (Robinson, 1997). More recently however, the importance of protein degradation has begun to be investigated in memory and addiction-related plasticity and behavior.

It is now clear that regulated proteolysis via the ubiquitin proteasome system (UPS) plays a major role in the development, maintenance and remodeling of synaptic connections in the brain (Patrick, 2006, Mabb and Ehlers, 2010, Hamilton and Zito, 2013). The UPS is one of the major cellular pathways controlling protein turnover in eukaryotic cells. The selective degradation of proteins via the UPS involves three steps: recognition of the target protein via specific signals, marking of the target protein with ubiquitin chains, and delivery of the target protein to the 26S proteasome, a multi-subunit protein complex that degrades the ubiquitinated proteins. The 26S proteasome is a large energy-dependent protease formed by the co-assembly of a 20S proteasome (the catalytic component) and 19S cap (the regulatory component). The 20S proteasome is a self-compartmentalizing protein assembly with a barrel shape. The 20S consists of 28 subunits ( $\alpha$  and

$\beta$ ), six of which are catalytic, while the 19S consists of Rpt (ATPase) and Rpn (non-ATPase) regulatory subunits (Hershko and Ciechanover, 1998, Pickart, 2004). The 19S subunits contain several ATPases that are involved in opening the pore of the 20S proteasome, binding and unfolding the target proteins as they enter the proteasome and translocating the proteins into the interior of the 20S proteasome core (Voges et al., 1999).

We and others recently found a novel form of regulation for the 26S proteasome in neurons involving the plasticity kinase  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases II alpha (CaMKII $\alpha$ ). CaMKII $\alpha$  phosphorylates the ATPase 19S regulatory particle (RP) subunit of the 26S proteasome, Rpt6, at serine 120 (S120) in an activity-dependent fashion to control the activity and distribution proteasomes in neurons (Djakovic et al., 2009, Bingol et al., 2010, Djakovic et al., 2012). We predicted that the functional relevance of Rpt6 phosphorylation on proteasome-dependent synaptic remodeling to be high as we have shown it to regulate synaptic strength and activity-dependent generation of new spines (Djakovic et al., 2012, Hamilton et al., 2012). Historically, proteasome inhibitors have been used to understand the role of protein degradation in synaptic plasticity and behavior. However, they are quite toxic to cells and therefore have a limited utility. We have recently generated Rpt6 S120D phospho-mimetic knock-in mice ser120 to aspartic acid; AGC  $\rightarrow$  GAC). Here, we examined the effects of sensitization to cocaine on proteasomal activity in the nucleus accumbens (NAc) and prefrontal cortex (PFC) in wild type and Rpt6 S120D knockin (KI) mice. We also assessed behavioral sensitization to cocaine. While repeated cocaine administration elevated proteasomal activity in both the NAc and PFC in control mice, the Rpt6 S120D KI mutation disrupted cocaine-induced proteasomal activity. Furthermore, cocaine produced a dramatic sensitization of locomotor activity in wild-type mice, whereas sensitization was completely blocked in S120D KI mice. Together, our findings indicate regulation of proteasome by Rpt6 phosphorylation at serine 120 is critical for maintaining cocaine-induced sensitization at both a molecular and behavioral level.

## Materials and Methods

*Generation of Rpt6 Ser120Asp (S120D) phospho-mimetic Knockin (KI) mutant mice.* We generated Rpt6 phospho-mimetic (ser120 to aspartic acid; S120D) KI mice (iTL; www.genetargeting.com). The strategy for generating the KI mice is described in Figure 2. Ten micrograms of the targeting vector was linearized by NotI and then transfected by electroporation of BA1 (C57Bl/6 x 129/SvEv) (Hybrid) embryonic stem cells. After selection with G418 antibiotic, surviving clones were expanded for southern blot analysis to identify recombinant ES clones (data not shown). Targeted iTL BA1 (C57BL/6N x 129/SvEv) hybrid embryonic stem cells were microinjected into C57BL/6 blastocysts. After germline transmission, the Neo cassette was removed by mating to C57BL/6 FLP mice. Tail DNA was analyzed by PCR to identify heterozygous mice and verify deletion of the Neo cassette. Mutant heterozygous mice were backcrossed to C57BL/6. By visual inspection, Rpt6 S120D homozygous mutants (confirmed by PCR and sequencing) obtained by crossing heterozygous mutants, have normal body size, feeding, and mating. The intercross of heterozygotes resulted in production of wild-type, heterozygous, and homozygous offspring at the expected 1:2:1 Mendelian ratio. All procedures relating to animal care and treatment conformed to the institutional and NIH guidelines.

*Antibodies and Reagents.* Mouse mAb Rpt6, mAb 20S was purchased from Enzo Lifesciences, mAb CaMKII $\alpha$  and mAb phospho-CaMKII $\alpha$  Abcam and custom rabbit pAb phospho-specific Rpt6 S120 (Djakovic et al., 2012) antibodies were used. *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) substrate was purchased from Enzo Lifesciences. Cocaine HCl (from Sigma) used during behavioral assessment was dissolved in physiological saline to a dose of 15 mg/kg (salt weight) and administered i.p. (10 ml/kg).

*Neuronal cultures.* High Density Rat dissociated cortical neurons from postnatal day 1 pups of either sex were plated onto poly D-lysine-coated 6-well plastic dishes at ~500,000 cells per well (cortical cultures) and were maintained in B27 supplemented Neurobasal media (Invitrogen) until  $\geq 14$  d *in vitro* (DIV), as previously described (Djakovic et al., 2009).

*Proteasome activity assays.* Proteasome activity was measured as previously described with slight modifications (Kisselev and Goldberg, 2005, Djakovic et al., 2009). Briefly, cultured neurons were incubated for 24 h in either plain media (control), or media containing Cocaine (1  $\mu$ m, 5  $\mu$ m, 10  $\mu$ m). Neurons were then lysed in Affinity Purification Buffer (APB) (25mM HEPES-KOH pH=7.4, 5mM MgCl<sub>2</sub>, 10% Glycerol, 1mM DTT, 2mM ATP). Lysates were cleared by centrifugation at 14,000 rpm for 20 min. Whole brain and tissue punches were lysed in APB using a dounce homogenizer, and cleared by centrifugation at 14,000 rpm for 20 min. Protein concentration was measured using a coomassie blue colorimetric protein assay (Bio-Rad) and equal amounts of protein were used as inputs for the peptidase assay. The chymotrypsin-like activity of 26S proteasomes in lysates was monitored over time using *N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) substrate. Experiments were run in triplicate at 37<sup>o</sup>C using a microplate fluorimeter (HTS7000 Plus, Perkin-Elmer) with excitation and emission filters of 360nm and 465nm, respectively. Kinetics data was taken every 60 sec for 2 h using 96-well microplates (Costar). The data was averaged and plotted to find the kinetic rates of the chymotrypsin-like activity.

*Western blot analysis.* Total protein lysates from either cultured neurons, whole brain extracts or tissue punch extracts were generated by lysing in APB (25mM HEPES-KOH pH=7.4, 5mM MgCl<sub>2</sub>, 10% Glycerol, 1mM DTT, 2mM ATP) then clearing by centrifugation at 14,000 rpm at 4<sup>o</sup>C. Protein concentration was determined by colorimetric protein assay (Bio-Rad), and equal amounts of protein were loaded on SDS-PAGE. Samples were boiled with sample buffer, resolved on SDS PAGE, and probed with primary mouse mAb Rpt6, mAb 20S, mAb CaMKIIa, mAb phospho-CaMKII $\alpha$  and custom rabbit pAb phospho-specific Rpt6 S120 (Djakovic et al., 2012) antibodies. Resulting blots were digitized and band intensities quantitated using NIH ImageJ. For quantification of total phospho-Rpt6 S120 levels, band intensities in each condition were normalized to total Rpt6 band mean intensity from the same sample. Experimenters were blinded to condition during data collection and analysis.

*Behavioral Sensitization.* 40 adult Rpt6 S120D and WT mice were used for behavioral experiments. All animals were group housed. The vivarium was maintained on a 14:10 light:dark schedule. Testing was performed during the light phase. Mice had *ad libitum* access to food and water. All procedures were approved by the UCSD IACUC and compliant with the NRC Guide. All mice were handled for five days prior to behavioral assessment. Mice were then habituated to testing chambers during two, 1h sessions (30 min per side). Testing was conducted in individual chambers housed in a windowless room (described previously Howell et al., 2014; Carmack et al., 2013). Two-sided chambers were 44 cm. x 44 cm. x 31 cm, bisected by an opaque wall (Med-Associates). Sides paired with saline and drug were counterbalanced. Activity monitor software (Med-Associates) was used to track locomotor activity. The testing room was lit by two 100-watt bulbs and white noise (65 dBA) played continuously. Mice were divided into four groups: S120D-Cocaine, WT-Cocaine, S120D-Saline, and WT-Saline. Sensitization was induced during four sessions of cocaine administration. Sessions one through three were separated by 24h, while 48h separated sessions three and four. Initially, all animals received an injection of saline (10 ml/kg) and were placed in the saline side of the chamber, where they remained for 15 min. Mice were then removed, given an injection of either cocaine (S120D-Cocaine, WT-Cocaine groups) or a second injection of saline (S120D-Saline, WT-Saline groups). Immediately following injections, animals were placed in the drug-paired side of the chamber for 15 minutes.

*Histology.* Twenty-four hours following the final behavioral testing session, mice were given a final injection of cocaine (S120D-Cocaine, WT-Cocaine groups) or saline (S120D-Saline, WT-Saline groups) in their home cage. Approximately 30 minutes later, mice were anesthetized using isoflurane dispensed from a precision vaporizer and then decapitated for fresh tissue collection. For whole brain biochemical experiments, brains were removed, flash frozen in liquid nitrogen and stored at -80°C. For experiments where tissue was obtained from the nucleus accumbens (NAcc) and prefrontal cortex (PFC), brains were removed and frozen on dry ice. 1mm coronal sections were obtained using an acrylic matrix (A/P: 2.10-1.10 Braintree Scientific). 1mm



punches (Miltex disposable biopsy punch) were then taken from the NAcc (M/L:  $\pm 0.5$ , D/V: 2.0) and PFC (M/L:  $\pm 1.25$ , D/V: 4.25), flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

*Data Analysis.* For western blot experiments statistical significance was determined using unpaired Student *t* tests (Prism, GraphPad). Behavioral data were entered into a multivariate ANOVA (MANOVA; SPSS Statistics Desktop, V22.0). If a significant omnibus comparison or group x time/session interaction was achieved, post-hoc comparisons were made using Fisher's protected least significant difference (PLSD). Group differences are reported using univariate ANOVAs, followed by Fisher's PLSD for interesting comparisons. For all experiments, significance was set at a level of  $p \leq .05$ .

## Results

### Cocaine increases Rpt6 S120 phosphorylation and proteasome activity.

It is known that cocaine activates CaMKII $\alpha$  through T286 phosphorylation (Anderson, 2008). CaMKII $\alpha$  is also capable of phosphorylating S120 of the proteasome regulatory particle Rpt6, modulating the proteasome's chymotrypsin-like peptidase activity (Djakovic et al., 2009) would lead to alterations in Rpt6 phosphorylation and proteasome activity. We examined Rpt6 phosphorylation and proteasome activity in lysates from cultured neurons treated with increasing amounts of cocaine. We found that treatment with 5 $\mu\text{M}$  cocaine significantly increased proteasome activity as monitored by the rate of Suc-LLVY-AMC cleavage (Figure 1A). Moreover, we observed a significant increase in Rpt6 S120 phosphorylation relative to control treated neurons (Figure 1A;  $p < 0.001$ ). As predicted, we found that CaMKII $\alpha$  T286 phosphorylation was also increased in cocaine-treated cultures (Figure 1B). We additionally evaluated whether cocaine would produce similar effects on Rpt6 S120 and proteasome activity when administered to mice (i.p.). We used lysates from whole brains of WT mice that were treated with either cocaine or saline for 6 days thereby inducing cocaine sensitization (Figure 1C). We then performed western blot analysis as well as peptidase assays. Rpt6 S120 phosphorylation significantly increased in WT whole brains treated with cocaine compared to saline (Figure 1D,E). We also find that this increase in phosphorylation is correlated with a slight increase in the

rate of proteasome chymotrypsin-like activity (Figure 1D,E). However, this increase did not reach statistical significance ( $p = 0.068$ ). Taken together, we show that cocaine concomitantly increases Rpt6 phosphorylation and proteasome activity.

#### **Generation of *Rpt6* S120D Knockin (KI) mice.**

To begin to assess the biological relevance of *Rpt6* S120 phosphorylation, we generated genetically modified mice by homologous recombination with a targeting vector carrying a *gacAGctac* to *gacGActac* mutation (coding nucleotides 358–359 of exon 5 of *Rpt6*) encoding a serine 120 to aspartic acid phospho-mimetic mutant of the Rpt6 protein (Figure 2A). The resulting modified *Rpt6* gene can be distinguished from the wild type *Rpt6* gene by PCR-based genotyping (Figure 2B and C). Rpt6 S120D homozygous KI mice expressed similar levels of Rpt6 protein as compared to wild-type (+ / +) mice. We found that our custom pS120 phospho-specific antibody, which does not recognize Rpt6 S120A mutant protein to slightly cross react with the Rpt6 S120D which is likely due to the mimetic nature of the negative charge (Figure 2D; Djakovic, 2012). Gross brain anatomy in Rpt6 S120D homozygous mutant animals was comparable to wild-type (Figure 2E).

#### **Rpt6 S120 phosphorylation and peptidase activity is increased in nucleus accumbens and pre-frontal cortex in cocaine treated wild type mouse brains, but not in S120D mutant mice.**

Major biochemical and structural changes in both the nucleus accumbens (NAc) as well as the pre-frontal cortex (PFC) are associated with cocaine sensitization (Robinson, 1997). Mice were administered five cocaine (15 mg/kg, i.p.) or saline treatments across 6 days (Figure 3A). We then extracted lysates from tissue punched specifically from the NAc and PFC of both wild type and Rpt6 phospho-mimetic S120D mutated mice (Figure 3B). Western blot analysis and peptidase assays were performed on tissue from these regions. In WT mice, we found that cocaine administration increases Rpt6 phosphorylation in the NAc in comparison to treatment with saline (Figure 3E). This increase is also correlated with a significant enhancement in proteasome activity in the NAc ( $p = 0.031$ ,  $n=4$ ; Figure 3C). Interestingly, however, cocaine-induced increases in proteasome activity was not observed in the NAc of S120D mice (Figure 3D;  $p =$

0.847,  $n=4$ ). A similar trend was observed in PFC. Cocaine increases Rpt6 S120 phosphorylation in PFC in cocaine-treated WT mice (Figure 3H). Again, this correlated with increased peptidase activity measured in the PFC (Figure 3F;  $p = 0.016$ ,  $n=4$ ). As in the NAc, this increase was occluded in the PFC of S120D mutated mice, as no difference was observed between mice administered cocaine compared with saline controls (Figure 3F;  $p = 0.934$ ,  $n=4$ ).

### **Behavioral sensitization is completely blocked in Rpt6 S120D mutant mice**

We were interested in examining the effects of constitutive Rpt6 phosphorylation at serine 120 on addiction-related behavior. We assessed locomotor sensitization to cocaine (15 mg/kg i.p.) in four groups of mice; S120D mice administered cocaine and saline (S120D-Cocaine ( $n=11$ ) and S120D-Saline ( $n=5$ ), respectively) and WT littermates administered cocaine and saline (WT-Cocaine ( $n=18$ ) and WT-Saline ( $n=6$ ), respectively). Baseline activity, examined prior to the administration of any i.p. injections, (habituation (H), Figure 4A) did not differ between groups [ $F(3,36) = .037$ ,  $p=0.99$ ]. However, across subsequent days of behavioral assessment, differences between groups emerged [Figure 4A,  $F(3,36)=5.79$ ,  $p<.005$ ]. While WT-Cocaine mice demonstrated increasing locomotor activity with each cocaine injection, the activity levels of S120D-Cocaine mice remained constant across days (Figure 4A, Fisher's PLSD,  $p=0.007$ ). Further, locomotor activity did not differ between S120D-Cocaine mice and groups administered saline (S120-Cocaine/S120D-Saline,  $p=0.59$ ; S120D-Cocaine/ WT-Saline,  $p=0.36$ ). Examining minute by minute activity during the first session of cocaine (or saline) administration revealed no differences between groups [Figure 4B,  $F(3,36)=.778$ ,  $p=0.51$ ]. Importantly, there was no difference in the acute response to cocaine between S120D and WT mice ( $p=0.76$ ). During session four, after mice in the cocaine groups had received repeated administrations, activity was elevated in WT-Cocaine mice in comparison to all other groups [Figure 4C,  $F(3,36)= 4.57$ ,  $p<0.01$ ; S120D-Cocaine/ WT-Cocaine  $p= 0.01$ ]. Despite receiving multiple injections of cocaine, locomotor activity of S120D-Cocaine mice did not differ from either saline group (S120D-Cocaine/S120D-Saline,  $p=0.64$ ; S120D-Cocaine/WT-Saline,  $p=0.67$ ). We then assessed sensitization as the difference between the acute (session 1) and sensitized (session 4) response

(Figure 4D; Howell et al., 2014; Carmack et al., 2013). There were significant group differences [ $F(3,36)= 3.70, p<0.05$ ]. S120D-Cocaine mice did not exhibit sensitization, whereas WT-Cocaine mice demonstrated robust sensitization (Figure 4D; S120D-Cocaine/WT-Cocaine,  $p=0.008$ ). Again, S120D-Cocaine mice did not differ from either saline control group (S120D-Cocaine/S120D-Saline,  $p=0.87$ ; S120D-Cocaine/WT-Saline,  $p=0.79$ ).

## Discussion

In order to assess the importance of proteasome-dependent protein degradation in addiction-related behavior we generated a line of novel mutant mice. Specifically, we utilized mice with a single point mutation in a single subunit of the 26S proteasome. The phosphorylation of the 19S ATPase subunit Rpt6 at S120 has been shown to be regulated by CaMKII $\alpha$  in an activity-dependent manner (Djakovic et al., 2012). We found that increases in Rpt6 S120 phosphorylation correlated with increased proteasome activity (Figure 1 and 3; Djakovic, 2012 #27). We suggest the regulation of Rpt6 phosphorylation is important as Rpt6 phosphorylation has been shown to regulate synaptic strength and activity-dependent new spine generation (Djakovic et al., 2012, Hamilton et al., 2012). Here we demonstrate that Rpt6 phosphorylation at serine 120 is critical for cocaine-induced sensitization, a prominent addiction-related behavior that models the transition from casual to compulsive drug use. Specifically, we show that behavioral sensitization to cocaine is completely absent in Rpt6 S120D mutants. This indicates that altering the dynamics of Rpt6 S120 phosphorylation by locking it in the phospho-mimetic state (S120D) alters cocaine-induced sensitization.

These mice, when treated with multiple administrations of cocaine, did not display locomotor activity different from mice receiving saline. It is important to note though, that while sensitization was blocked in these mice, the acute response to cocaine did not differ from WT animals (Figure 4B). Thus, we are able to conclude that there is a disruption in nonassociative memory rather than simply an impaired response to cocaine.

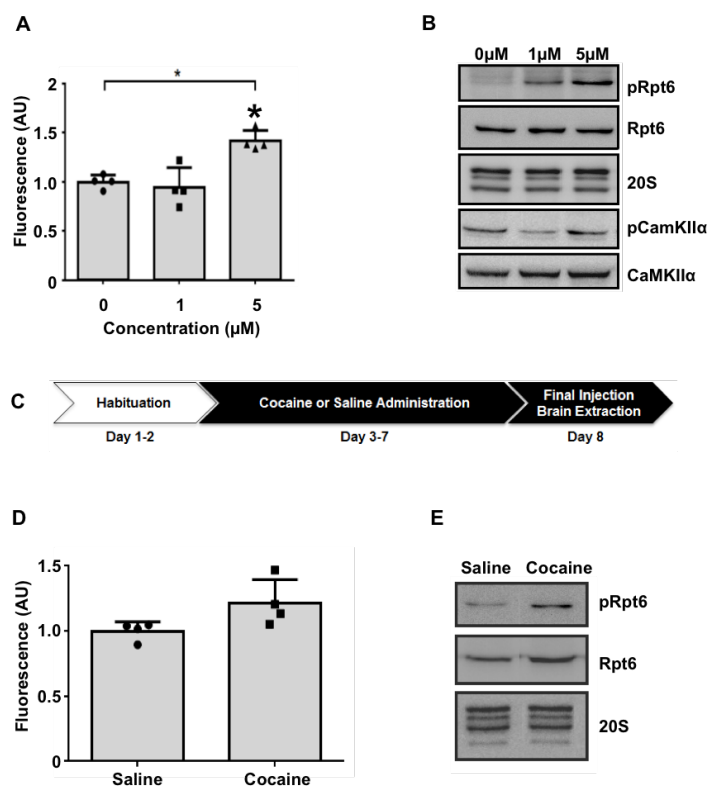
Our results are in line with the interactionist view of memory and addiction (Volkow et al 2002, Volkow et al 1999). According to this model there are distinguishable associative and nonassociative components of addiction, and the molecular mechanisms and neural substrates underlying these processes may overlap with those involved in canonical forms of memory (Leith, 1982, Anagnostaras, 2002). Here, we demonstrate that behavioral changes reflecting the nonassociative component of addiction, e.g. sensitization, that result from repeated drug administration are affected by changes to cellular plasticity mediated by the ubiquitin proteasome system.

Other recent studies have also begun to investigate the importance of the ubiquitin proteasome system during addiction. Most recently, Werner and colleagues (2015) examined drug-related memory retrieval after self-administration or at various times during withdrawal from cocaine. In groups retrieving cocaine-related cues, there was an increase in proteasome activity in the NAc one day into withdrawal, but not 50-60 days into withdrawal (Werner, 2015). These findings demonstrate that the activation of the UPS during memory retrieval may be influenced by the time between training and testing. Earlier studies examined the effects of protein degradation inhibition during the development and expression of conditioned place preference (a task that models drug-seeking) and sensitization (Massaly, 2013, Ren, 2013). Development, but not expression of morphine-induced CPP was impaired by administration of UPS inhibitor (Massaly, 2013). Similarly, treatment with a proteasome inhibitor during the induction of sensitization to morphine produced impairment (Massaly, 2013). In a second study, Ren and colleagues (2013) investigated the effects of co-administration of a protein synthesis inhibitor and inhibitor of the UPS on cocaine-induced conditioned place preference. Co-treatment with a UPS inhibitor reversed the memory impairments typically produced by the administration of the protein synthesis inhibitor alone (Ren, 2013). Understanding the role of protein degradation during addiction is a young area of investigation and many questions remain. The present study advances our current understanding by utilizing the first discrete mouse-model of altered

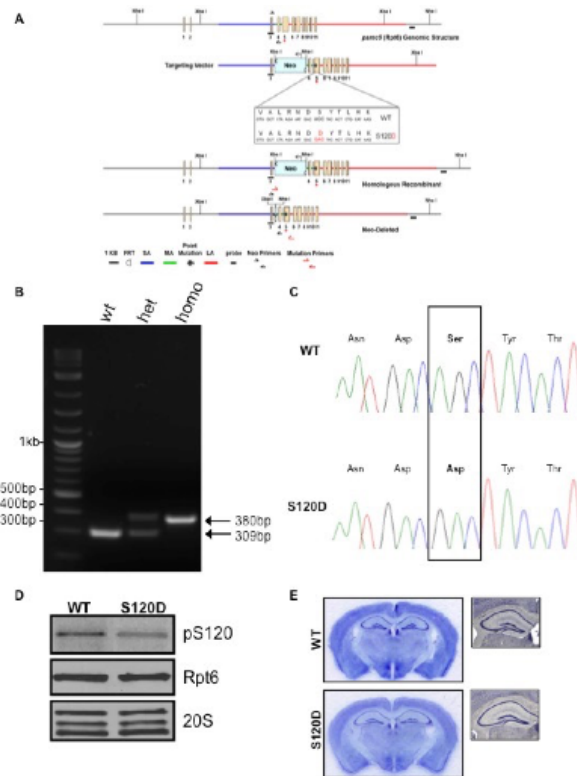
proteasome function. This is important, as many of the previous studies have relied upon the use of inhibitors, which have been shown to be toxic to cells (Reaney, 2006, Jantas, 2011).

We also found that the increase in proteasomal activity in the PFC and NAc typically induced by cocaine administration was absent in S120D KI mice. The PFC and NAc have previously been implicated in behavioral sensitization (Robinson, 1997, Thomas, 2001, Steketee, 2003). Enduring morphological alterations were found in NAc and PFC neurons following amphetamine-induced sensitization including an increase in the length of dendrites, density of spines, and number of branched spines (Robinson, 1997). These results highlight the long-lasting adaptations to synaptic connectivity that result from repeated experience with drugs of abuse. Specifically how these morphological changes contribute to addiction is unknown. Interestingly, the UPS has previously been shown to be implicated in spine stability and recently Rpt6 phosphorylation and proteasome function have been shown to regulate the formation of new dendritic spines (Patrick, 2006, Mabb and Ehlers, 2010, Hamilton et al., 2012). One intriguing possibility that may account for the present findings is that activity-dependent Rpt6 phosphorylation at serine 120 may contribute to the structural changes that occur following repeated psychostimulant administration. Potentially, constitutively active phosphorylation at this site may interfere with the stimulant-induced morphological changes, thus preventing sensitization.

Chapter 2, in full, is currently being prepared for submission to *Journal of Neuroscience*. Howell, KK\*; Gonzales, FR\*; Dozier, LE; Anagnostaras, SG; Patrick, GN. Proteasome phosphorylation regulates cocaine-induced sensitization (\*authors contributed equally). The dissertation author was the primary author of this paper.

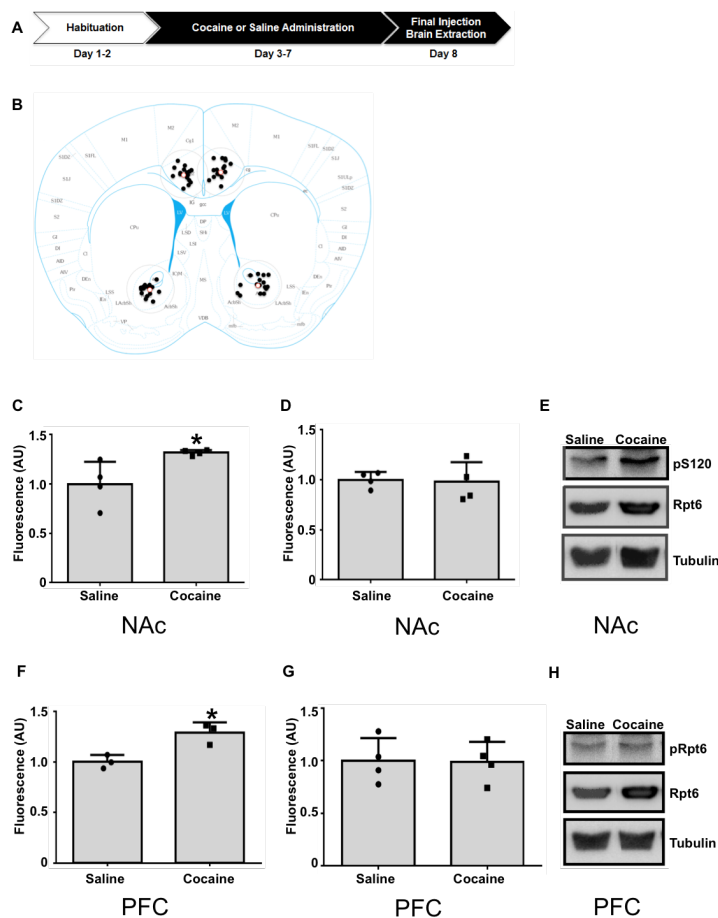


**Figure 2.1.** Cocaine increases Rpt6 S120 phosphorylation and proteasome activity. **A**, Dissociated cortical neurons (DIV >17) were treated with vehicle or cocaine (1 and 5 $\mu\text{M}$ , 24h) and chymotrypsin-like proteasome activity was measured in resulting lysates with the fluorogenic substrate Suc-LLVY-AMC. Graph depicts rate of Suc-LLVY-AMC cleavage (mean  $\pm$  S.E. fluorescence) normalized to control-treated neurons. As depicted, cocaine (5 $\mu\text{M}$ , 24h) significantly increases proteasome activity (\*,  $p < 0.05$ , unpaired Student's  $t$  test;  $n = 4$  independent experiments). **B**, Representative western blot of lysates in (A) probed with phospho Rpt6 pS120, total Rpt6, phospho-CaMKII $\alpha$  T286, total CaMKII $\alpha$ , and 20S core antibodies. Cocaine increases Rpt6 S120 phosphorylation in a dose dependent-manner. **C**, Schematic of drug administration protocol. After a habituation period on day 1 and 2, cocaine (15 mg/kg, i.p) or saline was administered on days 3 thru 7 and a final injection delivered on day 8 immediately prior to brain extraction. Whole brain lysates were prepared and proteasome activity and Rpt6 phosphorylation examined as above. **D**, Proteasome activity was increased in brains of cocaine-injected animals relative to saline (albeit statistical significance was not reached due to variability in cocaine-treated animals; ( $p = 0.068$ , unpaired Student's  $t$  test;  $n = 4$  independent experiments). **E**, Representative western blot of lysates in (D) probed with phospho Rpt6 pS120, total Rpt6 and 20S core antibodies shows that a concomitant increase in Rpt6 S120 phosphorylation occurs in cocaine-treated animals when compared to saline.

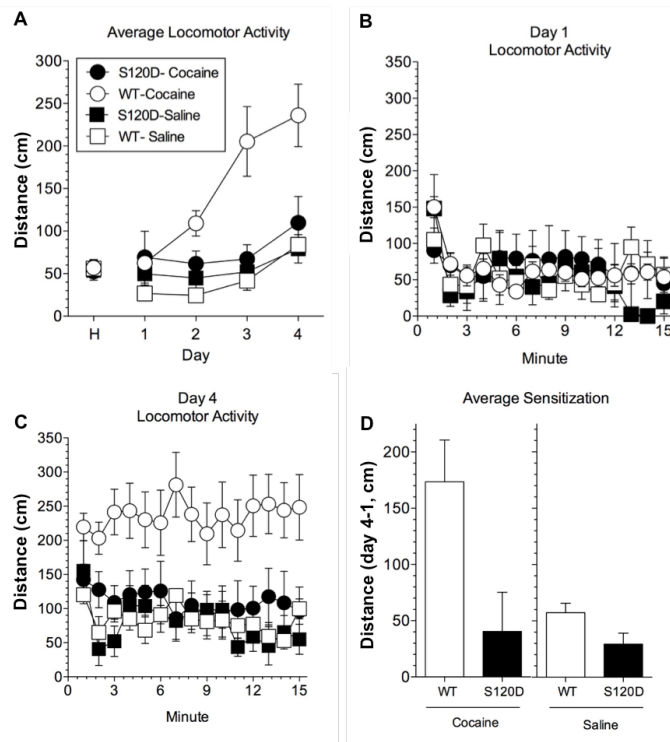


**Figure 2.2.** Generation of *Rpt6* S120D Knockin (KI) mice. *A*, Schematic of targeting strategy. Genomic DNA structure of *psmc5* (*Rpt6*) region is shown. The targeting vector was designed such that the long homology arm (LA) extends 5.98kb 3' to the first point mutation (asterisk; AG GA, S120D) in exon 5. The FRT flanked Neo resistance cassette was inserted 463 bp 5' to the point mutation. The short homology arm (SA) extends 2.79 kb 5' to the FRT flanked Neo cassette. *B*, Tail genomic DNA was analyzed by PCR screening for genotyping and to verify deletion of the Neo cassette. *C*, Electropherograms confirming the presence of the mutation in homozygous *Rpt6* S120D mutant male mice. *D*, Western blot analysis of affinity purified 26S proteasomes from *Rpt6* S120D KI mice and wt littermates and probed with phospho *Rpt6* pS120, total *Rpt6* and 20S core antibodies. The pS120 antibody cross-reacts slightly with 26S proteasomes purified from S120D mutants due to the acidic charge of aspartic acid. *E*, Representative images of Nissl stained fixed whole brain coronal sections of 60 day old mice and cropped region of hippocampal formation (right).





**Figure 2.3.** Cocaine increases Rpt6 phosphorylation and proteasome activity in NAc and PFC in wildtype but not *Rpt6 S120D* KI mutant mice. **A**, Schematic of drug administration protocol. After a habituation period on days 1 and 2, cocaine (15 mg/kg, i.p) or saline was administered on days 3 thru 7 and a final injection delivered on day 8 immediately prior to brain extraction. **B**, 1mm coronal sections were obtained using an acrylic matrix and tissue punches were taken from NAc and PFC. Depicted are the centers of individual punches (black dots), the arithmetic mean of the punch location (red dot), and average extent of all punches (dashed line). Lysates were prepared and chymotrypsin-like proteasome activity and Rpt6 phosphorylation was examined. **C thru E**, Cocaine increases proteasome activity in NAc of wildtype (**C**) but not *Rpt6 S120D* KI mutant mice (**D**). Graph depicts rate of Suc-LLVY-AMC cleavage (mean  $\pm$  S.E. fluorescence) normalized to saline-treated animals (\*,  $p < 0.05$ , unpaired Student's *t* test;  $n = 4$  independent experiments;  $p = 0.031$  and  $0.847$  for wildtype and *Rpt6 S120D* cocaine to saline-treatments, respectively). **E**, Representative western blot of lysates in (WT NAc) probed with phospho Rpt6 pS120, total Rpt6, and tubulin antibodies shows a dramatic increase in the NAc in cocaine-treated animals compared to saline-treated controls. **F thru H**, Cocaine increases proteasome activity in the PFC of wildtype (**F**) but not *Rpt6 S120D* KI mutant mice (**G**). Graph depicts rate of Suc-LLVY-AMC cleavage (mean  $\pm$  S.E. fluorescence) normalized to saline-treated animals (\*,  $p < 0.05$ , unpaired Student's *t* test;  $n = 3$  independent experiments;  $p = 0.015$  and  $0.933$  for wildtype and *Rpt6 S120D* cocaine to saline-treatments, respectively). **H**, Representative western blot of lysates in (F, wt PFC) probed with phospho Rpt6 pS120, total Rpt6, and tubulin antibodies shows a increase in PFC in cocaine-treated animals compared to saline-treated controls.



**Figure 2.4.** Complete disruption of locomotor sensitization in S120D mice. **A**, S120D mice did not sensitize across 4 days of cocaine administration (n=18 WT-Cocaine, n=11 S120D-Cocaine, n=6 WT-Saline, n=5 S120D-Saline). The average distance traveled for each session ( $\pm$  SEM) is depicted. All mice showed a similar level of activity during habituation and day 1. However, during days 2-4 locomotor activity of WT-Cocaine mice was elevated compared to S120D-Cocaine mice and mice receiving saline (15 mg/kg, i.p;  $p < 0.005$ ). **B**, Locomotor activity during day 1. The data represent the distance traveled per minute of the session ( $\pm$  SEM). There were no differences between the four groups ( $p = 0.51$ ). **C**, Locomotor activity during day 4. Distance traveled per minute of the session ( $\pm$  SEM) is depicted. While the response to cocaine was elevated in WT-Cocaine mice, S120D-Cocaine mice did not exhibit locomotor activity different from saline groups (WT-Cocaine, S120D-Cocaine Fisher's PLSD  $p = 0.01$ ; S120D-Cocaine, WT-Saline, S120D-Saline Fisher's PLSD  $p > 0.5$ ). **D**, Sensitization was measured as the difference in locomotor activity between day 4 (sensitized response) and day 1 (acute response). The average difference in distance traveled ( $\pm$  SEM) per group is shown. S120D-Cocaine mice did not develop sensitization and did not differ from mice that received saline (S120D-Cocaine/S120D-Saline, S120D-Cocaine/WT-Saline,  $p > 0.7$  WT-Cocaine mice displayed sensitized, elevated locomotor activity that differed from S120D-Cocaine mice ( $p < 0.01$ )).

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**CHAPTER 3:** Regulation of the UPS through Rpt6 phosphorylation at S120 is not required for associative fear memory

**Abstract**

It has been well established that protein synthesis is required for the formation of new memories. Less well understood is the role of protein degradation during learning and memory. The ubiquitin-proteasome system (UPS) is the major pathway by which proteins are degraded in eukaryotic cells. Recently, inhibitors that target the UPS have been used to demonstrate the importance of protein degradation during learning and memory related plasticity and behavioral tasks. However, use of these inhibitors can be toxic to cells and their use does not provide a mechanism by which protein degradation may be mediating these processes. To address these gaps, we generated two lines of novel knockin mice with specific mutations within the UPS. We manipulated the ability for phosphorylation of Rpt6 (part of the regulatory subunit of the proteasome) to occur at serine 120 in a bi-directional manner, such that in one line of mice phosphorylation was blocked while in the other phosphorylation remained constitutively active. Behavior was examined on two associative memory tasks, Pavlovian fear conditioning and a context discrimination task. Neither line of mice were impaired on these tasks suggesting that while protein degradation may play a key role, Rpt6 phosphorylation at serine 120 does not seem to be the mechanism involved, during associative learning and memory.

## Introduction

Experiences such as learning and memory produce synaptic modifications resulting in functional and structural changes. These changes entail the dynamic synthesis of new proteins, as well as the degradation of existing proteins (Bingol & Sheng, 2011). It has been well established that de novo protein synthesis is an integral part of long-term memory formation (Jarome & Helmstetter, 2014; Bahar et al., 2003, Goelet et al., 1986; Schafe et al., 1999; Gold, 2008; Davis & Squire, 1984; Schafe & LeDoux, 2000; Frey et al., 1988). Less well known though, is the role of protein degradation in learning and memory. It is theorized that protein degradation releases inhibitory constraints by breaking down proteins that need to be degraded in order for plasticity to occur or that it functions as an inhibitory constraint itself, thereby influencing synaptic strength, plasticity and memory (Fioravante and Byrne, 2010; Cajigas et al., 2010; Patrick, 2006; Bingol and Sheng, 2011; Ciechanover, 2006; Artinian et al., 2008; Chain et al., 1999). One of the major pathways involved in protein degradation is the ubiquitin proteasome system (UPS; Bingol and Sheng, 2011; Ciechanover, 2006; Patrick, 2006; Chain et al., 1999).

In the ubiquitin proteasome pathway, a ubiquitin chain, created by transferring ubiquitin molecules from three distinct types of enzymes (E1s, E2s, E3s), is attached to target protein marked for degradation (Patrick, 2006; Hershko & Ciechanover, 1998; Bingol & Schuman, 2005). Ubiquitin molecules are first activated by the ubiquitin-activating enzyme (E1). Activated ubiquitin is then transferred to E2 ubiquitin-conjugation enzymes and finally to E3 ubiquitin ligases that target the proteins for degradation (Figure 3.1; Patrick, 2006; Hershko & Ciechanover, 1998; Bingol & Schuman, 2005). The process of ubiquitination can be reversed via deubiquitinating enzymes (DUBs). Once the protein has been targeted by ubiquitin it is sent to the 26S proteasome for degradation. The 26S proteasome is made up of two distinct components, the 20S core particle and 19S regulatory cap. The 19S caps are comprised of several ATPases which are responsible for opening the pore of the 20S core particle, unfolding the targeted proteins and transferring them into the 20S core (Patrick, 2006; Ciechanover, 2006; Nagy and Dikic, 2010;

Glickman and Raveh, 2005; Bingol & Schuman, 2005; Voges, 1999). Through this complex and tightly regulated system, the UPS is able to modulate protein dynamics at the synapse.

Previous studies have shown that administration of pharmacological inhibitors that target the UPS impact long-term potentiation (LTP), long-term depression (LTD), and behavioral learning and memory tasks. However, the exact role of protein degradation is not yet clear. Inhibition of the UPS has been shown to impair the maintenance of late LTP, while enhancing the induction of early phases of LTP (Hegde, 2010; Fonseca et al., 2006). Protein degradation is also implicated in LTD; inhibition of the UPS blocks LTD in *Aplysia* (Fioravante et al., 2008). Whether protein degradation is necessary for behavioral tasks that assess learning and memory in rodents is a question that has recently begun to garner attention. The infusion of UPS inhibitors into the amygdala or hippocampus has been shown to impair the consolidation and reconsolidation of both auditory and contextual fear conditioning, conditioned taste aversion, trace fear conditioning and the Morris water maze (Jarome et al., 2011; Rodriguez-Ortiz et al., 2011; Lee et al., 2008; Artinian et al., 2008, reviewed in Jarome and Helmstetter, 2014). In contrast, protein degradation does not seem to be required for short-term memory (STM; Artinian et al., 2008). These studies have primarily used the UPS inhibitors lactacystin (lac) or clasto-lactacystin  $\beta$ -lactone ( $\beta$ -lac), which inhibits the 20S component of the proteasome (Jarome et al., 2011; Rodriguez-Ortiz et al., 2011; Lee et al., 2008; Artinian et al., 2008, reviewed in Jarome and Helmstetter, 2014; Fioravante et al., 2008). However, these studies are correlational and do not provide a mechanism for the regulation of protein degradation by the UPS.

Djakovic and colleagues (2009) demonstrated that  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII), a protein kinase implicated in plasticity and memory, stimulated proteasome activity. Further, CaMKII $\alpha$  was shown to directly phosphorylate Rpt6, an ATPase subunit part of the 19S regulatory particle, *in vitro* (Djakovic et al., 2009). In later experiments, it was discovered that the phosphorylation of Rpt6 occurs at serine 120 (S120) in an activity-dependent manner (Djakovic et al., 2012). Expression of a phospho-dead mutation (serine to alanine; S120A) or phospho-mimetic mutation (serine to aspartic acid; S120D) at the site of phosphorylation



produced opposite effects on synaptic strength (Djakovic et al., 2012). These findings suggest that CaMKII-regulated phosphorylation of Rpt6 at S120 may be an important mechanism underlying the involvement of the UPS in synaptic regulation and plasticity.

To examine the role of this mechanism in behavioral forms of learning and memory, we developed two lines of knock-in mice (Rpt6 S120A and S120D), with mutations that regulate the proteasome in a bi-directional manner. These novel knockin mice are highly specific and are the first discrete models of proteasomal function. Initially, we examined behavior using the Pavlovian conditioned freezing paradigm, as it has become a leading model for the assessment of learning and memory in rodents (Anagnostaras et al., 2010). Next, we assessed performance on a context discrimination task in which mice must learn to discriminate between two similar contexts (shock context vs. no-shock context Hyde et al., 2001; Frankland et al., 1998). This task has been shown to be a more sensitive measure of hippocampal dysfunction than Pavlovian contextual conditioning (Frankland et al., 1998). Performance was not affected by either mutation on either task. Thus, it seems that CaMKII-dependent Rpt6 phosphorylation at S120 may not be the mechanism underlying protein degradation involved in associative fear memory.

## **Materials and Methods**

### **Subjects**

#### *Generation of S120A and S120D Knock-In Mice*

Two lines of novel knock-in mice were generated. Rpt6 phospho-dead (ser120 to alanine; AGC → GCC; S120A) and phospho-mimetic (ser120 to aspartic acid; AGC→GAC; S120D) KI mice (ingenious targeting laboratory). The S120D phospho-mimetic mouse line has been described previously (insert citation of addiction paper). The gene targeting strategy used to generate the line of S120A phospho-dead mice is depicted in Figure 3.2A and B. There were no gross structural abnormalities in the brain of either mutant (Figure 3.2C). Proteasomes purified from S120A mice do not react to an antibody against Rpt6 S120 (pAB; Figure 3.2D).

80 adult group-housed mice were used for behavioral testing. The vivarium was kept on a 14:10h light:dark schedule. Testing occurred during the light phase and mice were handled for 5 days prior to behavioral assessment. All procedures were approved by the UCSD IACUC and were compliant with the NRC Guide.

## **Behavioral Assessment**

### *Fear Conditioning*

*Apparatus.* Mice were tested in individual conditioning chambers. The VideoFreeze system (Med Associates) was used to assess behavior as described previously (Anagnostaras et al., 2010; Carmack et al., 2014).

*Training.* Training consisted of one, 10 min session. Mice were placed in chambers and baseline activity was assessed for 2 min. Mice then received 3 tone-shock pairings beginning at minutes 2, 3, and 4. Tone-shock pairings consisted of a 30-sec tone (2.8 kHz, 90 dB) that co-terminated with a 2-sec scrambled, AC food shock (0.75 mA, RMS). Immediate memory was assessed during the last 5 min of training. Freezing behavior and locomotor activity were recorded (Anagnostaras et al., 2010; Carmack et al., 2014). Freezing was defined as the absence of all movement except respiration (Anagnostaras et al., 2010; Carmack et al., 2014).

*Context Test.* Twenty-four hours post-training, mice were returned to the training context. Once mice were placed in the chambers, freezing was measured for 5 minutes to assess fear to the context.

*Tone Test.* Twenty-four hours after the context test a 5 min tone test was conducted to assess cued memory. The context was altered on multiple dimensions. White acrylic sheets were placed over the grid floors, a black plastic triangular insert was used to alter wall shape, and chambers were cleaned and scented with a 5% vinegar solution. Near-infrared light was used, in the absence of white light, to create a dark environment. The test consisted of a 2 min baseline period, followed by the presentation of 3-30 sec tones (2.8 kHz, 90 dBA) at minutes 2, 3, and 4. The tones matched those used during training.

### *Context Discrimination*

Context discrimination was carried out in the same fear conditioning chambers as described above. Testing consisted of 16, 6-minute sessions in alternating contexts, A+ and A-; 8 days in context A+ starting on day 1 and 8 days in context A- starting on day 2. Both contexts consisted of grid floors and were scented with a 7% isopropyl solution. Contexts differed on one dimension. In context A+ white light was turned on, whereas in context A- only near-infrared light was used, producing a dark environment. In context A+ mice were given one, 2-sec scrambled, AC food shock (0.75 mA, RMS) shock at minute 5. Freezing behavior and locomotor activity were recorded during the 6 min session.

#### *Elevated Plus Maze*

Mice were individually tested on an elevated plus maze (Med Associates). The maze was placed in a dimly lit room and had two open and two enclosed arms (6.5 cm x 36 cm each) joined by a center section (6.5 cm x 6.5 cm). The floor of the maze was lit with a near infrared backlight. This light was invisible to the mice but provided a high contrast for video tracking. A camera and video tracking software (Panlab Smart 3.0, Harvard Apparatus) were used to track time spent and distance traveled. Mice remained on the maze for 5 min and distance traveled and time spent in each arm was measured.

#### **Data Analysis**

Behavioral data were analyzed using multivariate or univariate analyses of variance (ANOVAs). Significance was set at a level of  $p \leq 0.05$  (SPSS Statistics Desktop, V22.0).

### **Results**

#### **Fear Conditioning**

The effects of mimicking and blocking Rpt6 phosphorylation at serine 120 were examined using Pavlovian fear conditioning to assess immediate and long-term associative memory (Fig. 3.3, 3.4). Baseline activity, measured during the first 2 min of training, did not differ between S120D (n=21) mice and WT (n=13) controls [Fig. 3.3A,  $F(1,32) = 1.25$ ,  $p = 0.27$ ]. However, shock reactivity in S120D mice was significantly dampened [Fig. 3.3A,  $F(1,32) =$

10.48,  $p < 0.005$ ]. Freezing measured during the last 5 min of training served as an indicator of immediate memory. There were no immediate memory differences between S120D and WT mice [Fig. 3.3B,  $F(1,32) = 0.004$ ,  $p = 0.95$ ]. When assessed 24 h later, we did not find any differences in contextual memory [Fig. 3.3C,  $F(1,32) = 0.087$ ,  $p = 0.77$ ]. Similarly, cued memory, measured as average freezing during tone presentation, did not differ between groups [Fig. 3.3D,  $F(1,32) = 1.20$ ,  $p = 0.28$ ].

S120A ( $n = 16$ ) and WT ( $n = 8$ ) mice displayed similar activity levels at baseline [Fig. 3.4A,  $F(1,22) = 0.25$ ,  $p = 0.62$ ], but as with S120D mice, shock reactivity was reduced in S120A mice compared to WT littermates [Fig. 3.4A,  $F(1,22) = 4.33$ ,  $p = 0.049$ ]. There were no immediate memory deficits in S120A mice [Fig. 3.4B,  $F(1,22) = 1.29$ ,  $p = 0.27$ ]. Context memory and cued memory were also normal in S120A mice when compared to WT controls [Fig. 3.4C,  $F(1,22) = 1.645$ ,  $p = 0.21$ ; Fig. 3.4D,  $F(1,22) = 0.119$ ,  $p = 0.73$ ].

### **Context Discrimination**

In order to further examine whether a mutation at serine 120 would produce any perturbations in hippocampal-dependent memory, we examined both S120D and S120A groups in a context discrimination task (Fig. 3.5, 3.6). Average shock reactivity was assessed across all eight days in context A+. We did not find any statistically significant differences between either S120D mice ( $n = 4$ ) and WTs ( $n = 6$ ) or S120A mice ( $n = 3$ ) and WTs ( $n = 7$ ) [Fig. 3.5A,  $F(1,8) = 2.58$ ,  $p = 0.15$ ; Fig. 3.6A,  $F(1,8) = 0.91$ ,  $p = 0.37$ ]. Each day mice were placed in the chambers, we examined baseline freezing during the first four minutes. There were no baseline freezing differences found between S120D [Fig. 3.5A,  $F(1,8) = 0.11$ ,  $p = 0.75$ ] mice or S120A [Fig. 3.6A,  $F(1,8) = 0.79$ ,  $p = 0.79$ ] mice and their respective WT littermates. In order to assess whether the mice were able to discriminate between the A+ and A- contexts, we took a difference score by subtracting the amount of freezing in A- from that in A+ on consecutive days. Day 1 was excluded since it was the first exposure to the context and there was no freezing as mice had not yet received a shock. Across sessions, all groups of mice were able to learn to distinguish the contexts and no significant differences were found (Fig. 3.5C, 3.6C) between either line of

knockins and their respective control groups [S120D  $F(1,8)=0.13$ ,  $p=0.73$ ; S120A  $F(1,8)=4.48$ ,  $p=0.067$ ].

### **Elevated Plus Maze**

In an attempt to explain differences in shock reactivity found during fear conditioning training, we were interested in examining baseline anxiety differences between S120D knockins and WT littermates. We investigated behavior on the elevated plus maze (Figure 3.7). We examined percent time in each arm (Fig. 3.7A), the total distance traveled (Fig. 3.7B), and the distance traveled in each arm (Fig. 3.7C). Both S120D and WT groups spent more time in the closed arms (Fig. 3.7A). There were no between group differences in time spent in either open [Fig. 3.7A,  $F(1,19)=2.96$ ,  $p=0.10$ ] or closed [Fig. 3.7B,  $F(1,19)=2.16$ ,  $p=0.16$ ] arms. We also examined total distance traveled during the 5 min test. Again, there were no between group differences [Fig. 3.7B,  $F(1,19)=0.025$ ,  $p=.87$ ]. Finally, we assessed the distance traveled in each of the arms (Fig. 3.7C). While both S120D and WT mice traveled a greater distance in the closed arms, there were no differences between groups in the distance traveled in the closed [Fig. 3.7C,  $F(1,19)=0.83$ ,  $p=.37$ ] or open [Fig. 3.7C,  $F(1,19)=0.93$ ,  $p=.35$ ] arms.

### **Discussion**

In the current study, we examined the effects of a phospho-mimetic and phospho-dead mutation at serine 120 (S120D, S120A respectively), the site on Rpt6 phosphorylated by CaMKII, on learning and memory and anxiety-related behaviors. Surprisingly, we did not find differences in contextual or cued fear conditioning, context discrimination, or on the elevated plus maze. These studies are the first to examine learning, memory, and anxiety using a discrete model of proteasomal function. Findings suggest that the mechanism investigated in this study is not implicated in associative fear memory.

Shock reactivity did differ between both lines of knockins and WT littermates (Fig. 3.3A, 3.4A). Activity measured during the 2 sec shock was dampened in both S120A and S120D mice. Baseline activity did not differ however, and long-term memory for both cued and contextual fear was not impaired. In order to investigate whether the difference in shock reactivity could be

attributed to a change in the baseline level of anxiety in the knockin mice, we examined behavior in S120D mice on the elevated plus maze and did not observe any differences. Thus, altered anxiety levels in the knockin mice likely does not account for the disparity in shock reactivity. Despite an impairment in the perception of the shock (unconditioned stimulus), both groups of knock-in mice displayed normal cued and contextual freezing. Further, there were no differences in shock reactivity between knockin mice and WT controls during the context discrimination task (Fig 3.5A, 3.6A). Since there were no meaningful differences on any of the measures assessed, we did not examine S120A mice on the elevated plus maze.

Results from previous studies investigating the role of protein degradation in learning and memory have obtained different findings depending on the type of manipulation, task, and neural structures targeted (Jarome et al., 2011, Pick et al., 2013, Lee et al., 2008). Jarome and colleagues (2011) found that infusions of  $\beta$ -lac into the amygdala impair the consolidation of both cued and contextual fear conditioning. In contrast, when Lee and colleagues (2008) infused  $\beta$ -lac into the hippocampus, they did not find any effect on contextual fear memory. The same dose of  $\beta$ -lac was used in these studies, and therefore cannot account for differences in the results (Lee et al., 2008; Jarome et al., 2011). More recently, Pick and colleagues (2013) examined the effects of a conditional knock-out (cKO) of Cdh1, which regulates an E3 ligase, in the forebrain, on cued and contextual fear memory. Both cued and contextual fear were impaired in these mice (Pick et al., 2013). However, performance on hippocampal-dependent water maze was normal in these mice (Pick et al., 2013). Based on these previous findings, it is somewhat surprising that we did not observe any differences in either S120A or S120D mice on cued and contextual fear or context discrimination tasks as the mutation was expressed throughout the forebrain using the CaMKII $\alpha$  promotor. Though, the mutation used in this study is quite different than the cKOs examined by Pick and colleagues (2013).  $\beta$ -lac exerts its effects on the UPS by inhibiting the proteolytic activity of the 20S core particle. This mechanism is different and more general than the manipulation used in this study in which we targeted a single site on the 19S regulatory particle.

It should also be pointed out that while  $\beta$ -lac seems to be most selective for the proteasome, some non-specific effects have been observed as well (Adams, 2002; Kozlowski et al., 2001).

While our results seemed to differ from previous behavioral findings, they did parallel the electrophysiological results we obtained (data not published, Scudder and Patrick). Hippocampal baseline physiology, E-LTP, and L-LTP were assessed in S120A and S120D mice (unpublished data, Scudder and Patrick). Baseline physiology, measured through paired pulse facilitation and I/O currents appeared normal in both S120A and S120D lines and E-LTP and L-LTP in both lines of knockin mice did not differ from controls (unpublished data, Scudder and Patrick). Thus, there was consistency across our behavioral and electrophysiological results in that neither plasticity nor memory was impaired.

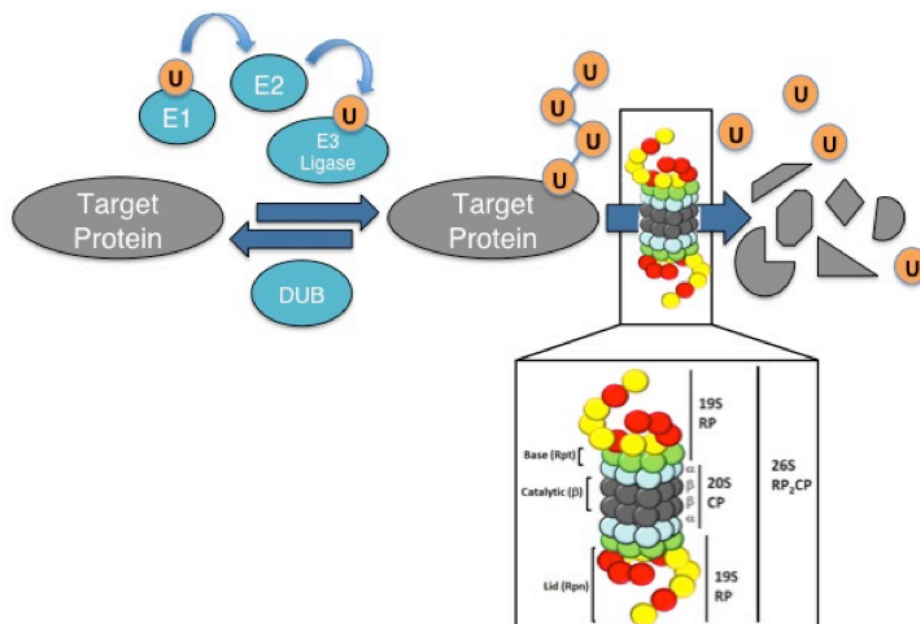
A similar approach to examining the importance of a particular phosphorylation site was taken in research investigating the role of CaMKII (Giese et al., 1998; Mayford et al., 1995; Mayford et al., 1996; Elgersma et al., 2004). A single site, threonine 286, important for the autophosphorylation of CaMKII, was altered (Giese et al., 1998; Mayford et al., 1995; Mayford et al., 1996). Similar to the current study, two lines of mice were created; T286 phosphorylation was blocked in T286A mice so there was no autonomous CaMKII activity (Giese et al., 1998). T286 phosphorylation was mimicked in a second line of T286D mice (Mayford et al., 1995; Mayford et al., 1996). T286A mice exhibited impaired LTP as well as deficits on the Morris water maze task (Giese et al., 1998). LTP was normal in T286D mice, however these mice were impaired on the hippocampal-dependent Barnes maze (Mayford et al., 1995; Mayford et al., 1996). In light of these findings, it is surprising there were no plasticity or hippocampal-dependent memory deficits in the S120A and S120D mice examined in our study.

It is possible that other types of memory, not examined here, would be impaired in S120D and S120A mice. For example, while associative memory appears to be normal in both knockin lines, working memory, which places a high cognitive demand on the animal could potentially be impaired. Mice with a targeted mutation in ubiquitin C-terminal hydrolase L3, which enhances proteasome activity, have significantly impaired working memory, despite

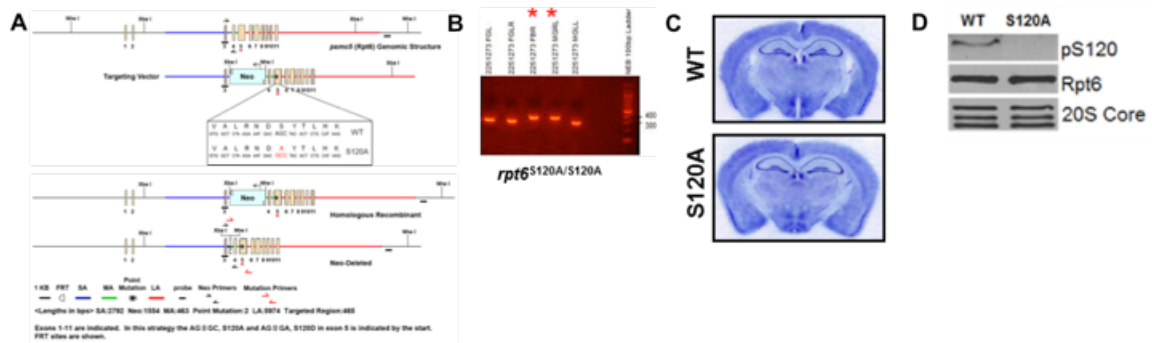
exhibiting intact hippocampal LTP (Wood et al., 2005; Chain et al. 1995; Hedge et al., 1997). It is possible a similar result would be obtained for Rpt6 S120A and S120D mice.

The role of protein degradation via the UPS in learning and memory has begun to garner further investigation. Previously, the UPS has been implicated in various neurodegenerative and neurodevelopmental diseases such as Alzheimers disease and Angelman Syndrome (Dickey et al., 2006; Bedford et al., 2008; Kishino et al., 1997). While it does not seem to be phosphorylation of Rpt6 at serine 120, further investigation into the mechanism underlying activity of the UPS during associative memory is warranted as it may help further our understanding of these disorders.

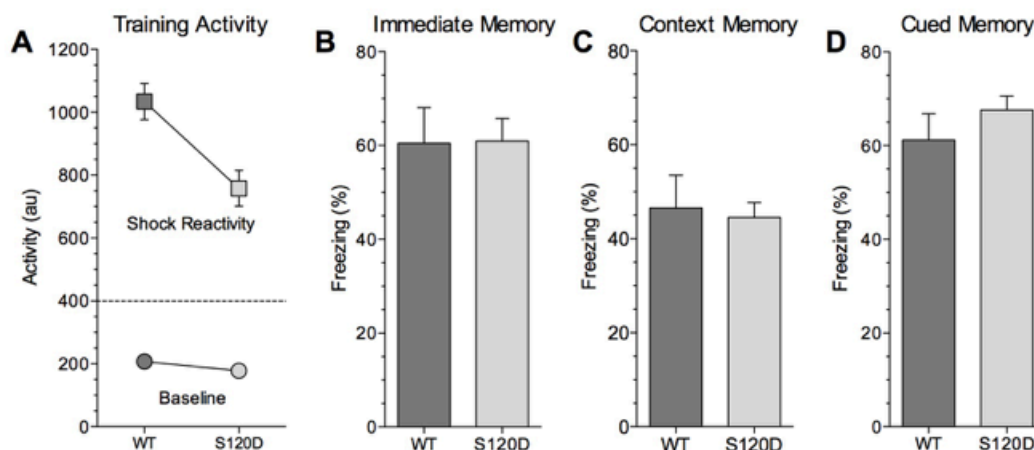




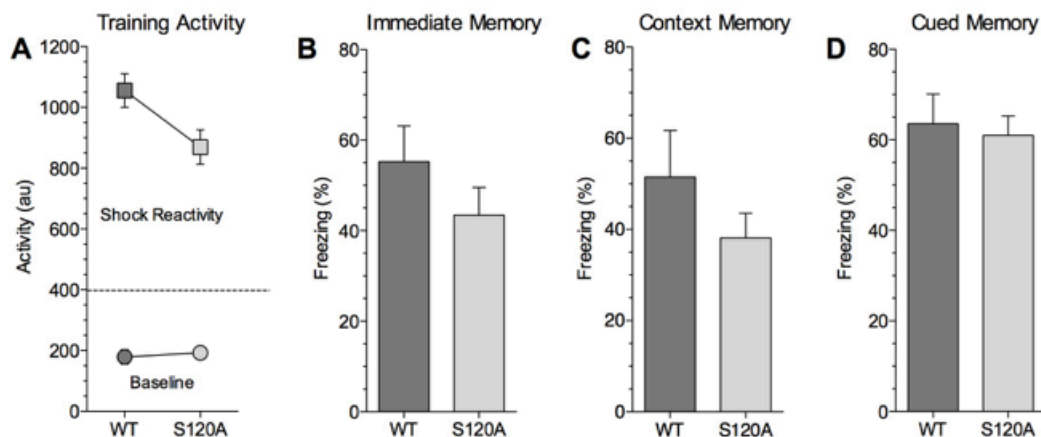
**Figure 3.1** Depiction of protein degradation via the ubiquitin-proteasome system (UPS). Three classes of enzymes (E1s, E2s, E3s) transfer ubiquitin molecules to create a chain that marks the protein for degradation. The target protein is then transferred to the 26S proteasome, which is composed of two parts: the 20S core particle and 19S regulatory caps (inset). Several ATPases make up the 19S caps (Patrick, 2006; Ciechanover, 2006; Nagy and Dikic, 2010; Glickman and Raveh, 2005; Bingol & Schuman, 2005). Upon delivery to the proteasome, the target protein is degraded. (Image adapted from Sullivan et al., 2003).



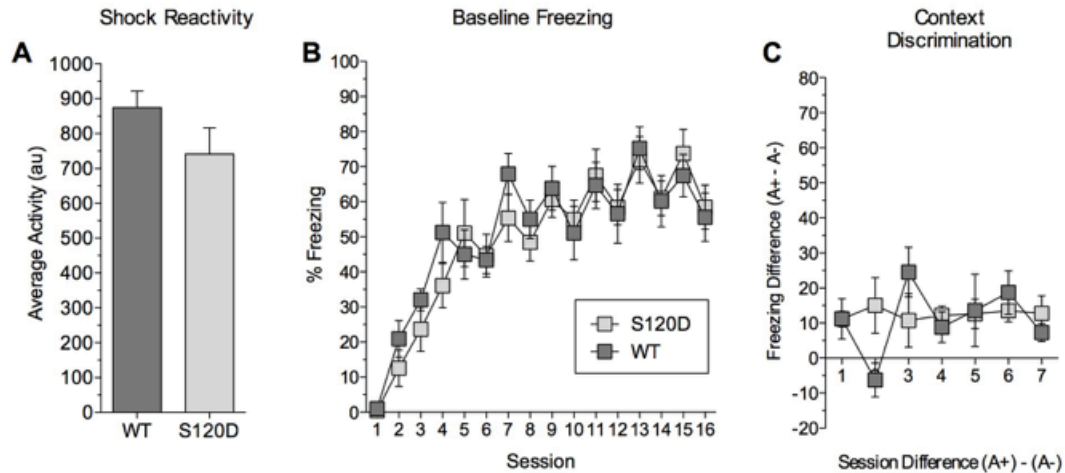
**Figure 3.2** Generation of S120A Knock-in mice. **A** Genetic targeting strategy for the creation of Rpt6 S120A mice. **B** PCR screening verifying the genotype of mice. WT, 309 bp; heterozygous S120A mice, 309 bp and 380 bp; homozygous S120A mice (red asterisks), 380 bp. **C** There were no gross neuroanatomical differences observed between S120A and WT mice. Images are of coronal slices taken from 60-day-old mice. Whole brains were fixed and Nissl stained. **D** Proteasomes purified from S120A mice do not react to an antibody against Rpt6 S120. A western blot analysis of affinity purified 26S proteasomes from Rpt6 WT and S120A brain homogenates probed with phospho-specific Rpt6 pS120, total Rpt6, and 20S core antibodies is depicted.



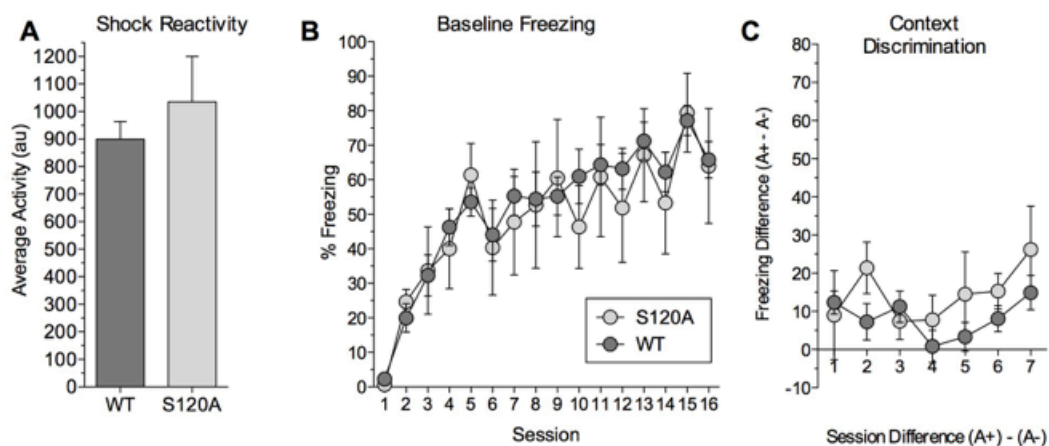
**Figure 3.3** Contextual and cued fear memory is not impaired in S120D mice. **A** Shock reactivity was significantly lower in S120D mice. The data depict average activity for S120D (n=21) and WT (n=13) groups during training for both the 2 min baseline and during the 2s shock. Baseline activity did not differ between S120D and WT mice ( $p=0.27$ ) but S120D mice exhibited significantly lower shock reactivity (ANOVA,  $F(1,34)=10.48$ ,  $p<0.005$ ). **B** Immediate memory was not impaired in S120D mice. Immediate memory was measured as the average percent time freezing during the last 5 min of training. Percent time freezing during the immediate memory test is depicted. There were no differences between S120D mice and WT mice ( $p=0.95$ ). **C** S120D mice had normal contextual fear memory. 24 h post-training mice were placed back into the training context and freezing was assessed across the 5 min test. There were no significant differences between S120D and WT mice ( $p=0.77$ ). **D** Cued fear memory did not differ between S120D and WT mice. 24 h after the context test, mice were placed in a novel context and three tones were presented. Average percent time spent freezing during the three tone presentations is depicted. There were no between group differences ( $p=0.28$ ).



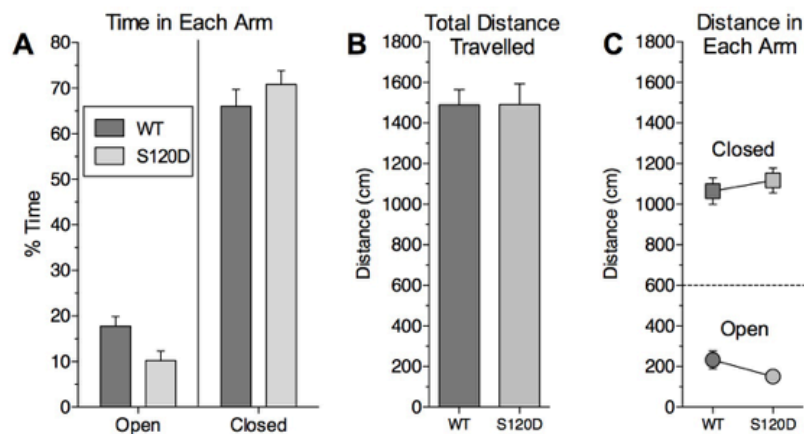
**Figure 3.4** Contextual and cued fear memory is not impaired in S120A mice. **A** Shock reactivity was attenuated in S120A mice. Average activity during the initial 2 min baseline and average activity during the 2s shock is depicted for S120A (n=16) and WT (n=8) mice. Baseline activity did not differ between groups ( $p=0.62$ ) but shock reactivity was significantly lower in S120A mice (ANOVA,  $F(1,22)=4.33$ ,  $p<0.05$ ). **B** Immediate memory did not differ between groups. Immediate memory was measured as the average percent time freezing during the last 5 min of training. S120A mice did not exhibit impaired immediate memory ( $p=0.27$ ). **C** Contextual fear memory was not disrupted in S120A mice. Contextual fear memory was assessed 24 h post training, in the same context as training. Average percent time spent freezing during the 5 min test is depicted. Freezing did not differ between S120A and WT mice ( $p=0.73$ ). **D** Cued fear memory did not differ between S120A and WT mice. 24 h after the assessment of contextual memory, mice were placed in a novel context and three tones were played. Depicted, is average percent time spent freezing during the three tone presentations. No differences were observed between S120A and WT mice ( $p=0.21$ ).



**Figure 3.5** Context discrimination was not impaired in S120D mice. **A** Average shock reactivity did not differ. The data depict average activity for S120D (n=4) and WT (n=6) mice during the presentation of the 2s shock across days in the A+ context. No between group differences were observed ( $p=0.15$ ) **B** Baseline freezing did not differ between S120D and WT mice. Average percent time spent freezing during the first 4 min in the chambers is depicted. There were no between group differences ( $p=0.75$ ). **C** Context discrimination, measured as a difference score, did not differ between groups. The data depict the difference in percent time freezing in Context A+ (shock context) and Context A- (no shock context) on consecutive days. Freezing on Day 1 was not included. S120D mice did not exhibit differences relative to WT controls ( $p=0.73$ ).



**Figure 3.6** Context discrimination was not impaired in S120A mice. *A* S120A mice exhibited normal shock reactivity. The data depict activity for S120A (n=3) and WT (n=7) mice during the presentation of the 2s shock on days when mice were placed in context A+. There were no significant between group differences ( $p=0.37$ ). *B* Baseline freezing did not differ between S120A and WT mice. Freezing was assessed during the initial 4 min in the chambers each day. There were no between group differences ( $p=0.77$ ). *C* Context discrimination was not impaired in S120A mice. Context discrimination was assessed using a difference score. The difference in percent time freezing in Context A+ (shock context) and Context A- (no shock context) on consecutive days is depicted. No differences were observed ( $p=0.07$ ).



**Figure 3.7** Performance on the elevated plus maze was not impaired in S120D mice. **A** Time spent in each arm of the elevated plus maze did not differ between S120D (n=14) and WT (n=7) mice. Percent time spent in the open and closed arms is depicted for each group. There were no differences observed ( $p>0.10$ ). **B** Total distance traveled was assessed. There were no differences between S120D and WT mice ( $p=0.87$ ). **C** Distance traveled was calculated for the open and closed arms separately. There were no between group differences for distance travelled in either open or closed arms ( $p>0.30$ ).

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## GENERAL DISCUSSION

Three studies were conducted to assess the involvement of memory in addiction. In the first study I demonstrated that the administration of zeta inhibitory peptide (ZIP), an inhibitor of atypical PKC isoforms, blocks the development of cocaine-induced sensitization (Chapter 1). However, once sensitization has been established, ZIP administration no longer disrupts the addiction-related memory, yet reduces AMPAR expression. In Chapters 2 and 3, I use two novel lines of knock-in mice that are the first discrete models of proteasomal function. First, I show that tight regulation of the ubiquitin-proteasome system (UPS) is necessary for cocaine-induced sensitization (Chapter 2). Knock-in mice with a phospho-mimetic mutation on the ATPase, Rpt6 (part of 19S cap), at serine 120 (ser120 to aspartic acid; AGC → GAC; S120D) were generated. Sensitization was completely blocked in these mice, indicating that a precise regulation of the UPS, and protein degradation, is required for addiction-related memory. Finally, the third series of experiments shows that unlike sensitization, Rpt6 phosphorylation at serine 120 is not required for associative fear memory (Chapter 3). In addition to the S120D mice, these studies utilized a second line of novel knock-in mice, with a phospho-dead mutation (ser120 to alanine; ACG → GCC S120A). Here, I examined behavior in cued and contextual fear conditioning as well as a context discrimination task. Memory was not impaired on these tasks, indicating this mechanism does not regulate the involvement of the UPS in associative fear memory. Together, these studies extend findings suggesting that addiction and memory share overlapping molecular substrates. However, the functional role of these substrates does not seem to be identical. Thus, the results discussed in this dissertation support the theory that memory and addiction interact.

### *Atypical PKC inhibition impairs addiction and memory*

Our examination of the effects of atypical PKC inhibition revealed that these protein kinases are involved in the development of locomotor sensitization, but the maintenance of this behavior may have different molecular requirements than traditional forms of memory (Chapter 1). Prior to these experiments most studies used a single, post-training application of ZIP to

disrupt associative or spatial forms of memory. Results from these studies suggested atypical PKCs are most important for the maintenance of these forms of memory (Kwapis et al., 2009; Parsons & Davis, 2011; Pastalkova et al., 2006; Serrano et al., 2008; Shema et al., 2009). However, in our study we found that the application of ZIP prevented the induction of sensitization when given continuously or with a single infusion, suggesting atypical PKCs might be more involved in the development of nonassociative addiction-related memory. Interestingly, there have been a few previous reports demonstrating that atypical PKCs may be required for maintaining associative forms of addiction-related memories. These studies examined the effects of ZIP on cocaine-induced spontaneous synaptic transmission, cocaine-induced enhancement in AMPA/NMDA ratio and conditioned place preference (Ho et al., 2012; Li et al., 2011). Unfortunately, our study examining conditioned place preference did not provide conclusive results. Thus, the difference between the role for atypical PKCs our study compared to previous studies may be due to the nonassociative component of sensitization.

As mentioned in the introduction to this dissertation, AMPARs are implicated in both memory and addiction. Despite the fact that sensitization was not disrupted when ZIP was administered post-training, we did observe a reduction in AMPA receptor expression. Previous reports have also demonstrated that the downstream effects of PKM $\zeta$  and PKC $\lambda$  were mediated by AMPAR trafficking (Ling et al., 2006; Miguez et al., 2010; Yao et al., 2008). The deficits in fear memory typically produced by PKM $\zeta$  inhibition, were not observed when the endocytosis of AMPARs was prevented (Ling et al., 2006; Miguez et al., 2010). In addition, application of ZIP blocked the LTP-induced enhancement of post-synaptic AMPARs (Ren et al 2013). Thus, while the specific effects of ZIP and atypical PKC inhibition may be different between associative and non-associative forms of memory, the mechanism proposed to regulate these effects does seem to be conserved.

#### *Disruption of the ubiquitin-proteasome system in addiction and memory*

I examined the effects of disrupting an activity-dependent mechanism that regulates the UPS in addiction-related memory and more traditional, amygdala- and hippocampal-dependent

memory (Chapters 2, 3). Two lines of mice, in which proteasomal activity is regulated in a bi-directional manner were used; one in which Rpt6 phosphorylation at serine 120 remains in a constitutively active state (i.e. ‘locked on’; S120D), the other in a permanently ‘locked-off’ state (S120A). In S120D mice, locomotor sensitization was completely blocked, whereas robust sensitization developed in wild-type mice. Further, proteasomal activity in the NAc and PFC did not increase following cocaine administration in S120D mice, as it did in wild-type controls. These results suggest that precise control of the UPS through this mechanism is necessary for the development of nonassociative, addiction-related memory. In contrast, neither S120A nor S120D mice were impaired in cued or contextual fear memory or the hippocampal-dependent context discrimination task. These results are surprising for two main reasons: (1) expression of a phospho-dead mutation (S120A) or phospho-mimetic mutation (S120D) in hippocampal slices (in vitro) produced opposite effects on synaptic strength (Djakovic et al 2012) and (2) Administration of the pharmacological agents lactacystin or clasto-lactacystin  $\beta$ -lactone ( $\beta$ -lac) into the amygdala or hippocampus disrupted many forms of associative memory including cued and contextual fear conditioning, conditioned taste aversion, trace fear conditioning and the Morris water maze (Artinian et al 2008, Jarome & Helmstetter 2014, Jarome et al 2011, Lee et al 2008, Rodriguez-Ortiz et al 2011). However, these studies using lactacystin or  $\beta$ -lac were correlational and did not provide a mechanism by which the UPS is regulated during these types of memories. The current studies cannot rule out that the UPS is involved in associative fear memory; it may just be that CaMKII $\alpha$  dependent Rpt6 phosphorylation at serine 120 is not the mechanism involved.

As discussed earlier, a similar genetic strategy was used to create two lines of mice that targeted autophosphorylation of CaMKII, a phospho-dead T286A line (threonine to alanine), and a phospho-mimetic T286D line (threonine to aspartic acid; Giese et al., 1998; Mayford et al., 1995; Mayford et al., 1996). Both T286A and T286D mice exhibited impaired spatial memory, but associative memory for cued and contextual fear conditioning were spared (Bach et al 1995, Cho et al 1998, Giese et al 1998, Mayford et al 1996). Thus it seems that a discrete mutation that changes a single amino acid residue, may only produce selective memory deficits. It is possible,

that in addition to behavioral sensitization, other types of memory, such as working memory, would be disrupted in the knock-in lines we generated. Taken together results from the current studies do suggest that the UPS may be differentially regulated during non-associative addiction related memory and associative fear memories. One explanation may be that the downstream targets or processes affected by the UPS differ depending on the type of memory.

#### *Differences in associative and nonassociative memories*

The above studies suggest the functional role of molecular and cellular changes that underlie nonassociative addiction-related memory may be somewhat different than those that underlie associative memory. Similar findings have been published previously on the role of NMDARs in nonassociative and associative memory. Carmack and colleagues found that administration of a competitive NMDAR antagonist blocked conditioned place preference and cued and contextual fear conditioning (both associative memories). However, this treatment did not impair behavioral sensitization (Carmack et al 2013). NMDARs have been shown to be an integral aspect of the induction of associative memory, but may not be required for nonassociative forms of memory.

While these studies point to differences between associative and nonassociative memories, they do not provide an account of what might be responsible for these differences. One hypothesis is that the structural changes that result from associative vs. nonassociative learning may not be the same. Both processes can induce persistent structural changes (Robinson and Kolb, 2004; Robinson and Kolb, 1997; Lamprecht and LeDoux, 2004; Weiler et al., 1995; Nikonenko et al., 2002; Muller et al., 2002). Sensitization to stimulants produces robust, long-lasting structural modifications in the NAc and PFC (Robinson & Kolb 1997). Robinson and Kolb showed that chronic (>30 days) exposure to amphetamine produces an increase the length of dendrites, the density of dendritic spines, and in the number of branched spines on major outputs of the cells (Robinson & Kolb 1997). Further, there have been reports demonstrating a correlation between increases in spine density and locomotor sensitization to cocaine (Li et al., 2004; Wheeler et al., 2013). A recent study that examined whole-brain volumetric changes in

response to cocaine exposure in mice found a decrease in the volume of the NAc and areas of the PFC; however, there were also increases in the volume of the substantia nigra and other areas of the PFC (Wheeler et al., 2013). While interesting, the specific impact of these volumetric changes is not known, but worth further exploration.

Similarly, the induction of traditional forms of associative memory and LTP result in changes to the number and shape of spines (Lamprecht and LeDoux, 2004; Weiler et al., 1995; Nikonenko et al., 2002; Muller et al., 2002). Specifically, associative memory has been shown to induce increases spine density in the hippocampus (Leuner et al., 2003). It is worth pointing out however, that there have not been studies directly comparing the ability of these changes to endure, or how widespread they are, between addiction and memory. Further, addiction studies, specifically those examining modifications resulting from sensitization have focused on medium spiny neurons in the NAc (Robinson and Kolb, 1997, 1999). Associative memory studies have primarily examined changes to pyramidal cells in the hippocampus (Robinson and Kolb, 1997; 1999; Lamprecht and LeDoux, 2004; Leuner et al., 2003; Sorra and Harris 2000). Technological advances to imaging techniques will facilitate our ability to more accurately and more easily answer these questions.

### *General Conclusions*

Taken together, these studies extend findings in support of the theory that there are shared molecular mechanisms between the processes of addiction and memory. However, these studies do not address whether there are shared neural substrates between the two processes. Since the specific involvement of atypical PKCs and the UPS does diverge between nonassociative addiction-related memories and associative memories, it seems a view in which the two processes interact is more likely true than one in which the same circuitry is usurped from one process to the other. In summation, three points can be made: (1) while involved in both processes, the functional role of atypical PKCs and the UPS is different between addiction-related memories and traditional forms of memory (2) this may be due to the fact that there are associative and nonassociative components of sensitization and the nonassociative components seem to be



differentially affected and (3) together, these results support the theory that memory and addiction interact.

Currently, relapse is the biggest problem in treating addiction. It is estimated that 40-60% of addicts will relapse (NIDA, 2008). Continuing to investigate the overlapping molecular mechanisms and neural substrates may elucidate key therapeutic targets in treating addiction. As discussed earlier, according to the incentive sensitization theory of addiction, associative learning only partially accounts for the neural changes involved in sensitization. Addiction likely involves both associative and nonassociative components and while both need to be studied, it is important to consider differences between these forms of memory, as the nonassociative aspects of addiction are thought to model key pathological components of what drive addiction.

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