STRIATAL LEARNING AND MEMORY AFTER METHAMPHETAMINE-INDUCED NEUROTOXICITY AND SUBSEQUENT RESTORATION OF STRIATAL FUNCTION

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Interdepartmental Program in Neuroscience

The University of Utah

May 2014

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ABSTRACT

Methamphetamine (METH) causes partial dopamine (DA) loss in the caudate/putamen and has long-term detrimental effects on cognitive function. We have previously shown that the positive correlation between expression of the immediate-early gene *Arc* in dorsomedial (DM) striatum and learning on a motor response reversal task is lost in rats with METH-induced striatal DA loss, despite normal behavioral performance. This discrepancy suggests that METH-pretreated rats no longer use DM striatum in this task. When function of or *Arc* expression in DM striatum of saline (SAL)-pretreated rats is disrupted, reversal learning and retention of learning, respectively, are impaired. However, METH-pretreated rats are unaffected by either treatment, suggesting that METH-pretreated rats no longer use DM striatum to perform this task. *In situ* hybridization histochemical staining for *Arc* mRNA expression in various brain regions of rats revealed a correlation between *Arc* and response reversal learning in nucleus accumbens (NAc) shell of METH-pretreated rats that did not exist in SAL-pretreated rats. When *Arc* was knocked down in the NAc shell, memory consolidation on the reversal task in METH-pretreated rats was impaired, whereas it was unaffected in SAL-pretreated rats, suggesting that METH-pretreated rats are relying on the NAc shell instead of DM striatum to consolidate reversal memories. Since the above evidence strongly suggests that

METH-induced damage to the striatum forces rats to rely on a different brain region to complete this reversal task, we attempted to restore striatal function in METH-pretreated rats by manipulating extracellular DA levels. METH-pretreated rats are selectively deficient in phasic DA signaling, which generates transient DA changes in response to rewards and their cues. We stimulated the brains of METH- and SAL-pretreated rats in a phasic-like manner and found that the reduced striatal *preprotachykinin* gene expression in METH-pretreated rats was restored to control levels. Furthermore, we found that L-DOPA, the biochemical precursor to DA, restored phasic DA signals in METH-pretreated rats back to the baseline levels in SAL-pretreated rats. These results suggest that METH-induced neurotoxicity results in altered circuitry used in the brain during a reversal learning task, but that restoration of phasic DA signaling may be able to rescue striatal function.

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ACKNOWLEDGEMENTS

The following work was funded by National Institutes of Health National Institute on Drug Abuse grants DA024036 (awarded to Kristen Keefe) and DA032502 (awarded to Elissa Pastuzyn). I would like to thank my friends, family, and mentor for being there with me every step of the way. I could not have done this without you.

CHAPTER 1

INTRODUCTION

Methamphetamine

Methamphetamine (METH) is a highly addictive psychostimulant drug. Thirteen million Americans reported using METH in 2010 (National Survey on Drug Use and Health, SAMHSA). Abuse of METH in humans is associated with decreases in dopamine (DA) transporter (DAT) (Wilson et al., 1996; McCann et al., 1998; Volkow et al., 2001a; Volkow et al., 2001b; McCann et al., 2008) and serotonin (5-HT) transporter (SERT) binding (Sekine et al., 2006; Kish et al., 2009) in the caudate-putamen. Not only are these decreases in DAT and SERT long-lasting, but recent evidence has shown that individuals with a history of hospitalization for METH use also have an increased risk of developing Parkinsonism later in life (Callaghan et al., 2012). Study of the damage that METH abuse induces in the brain is thus important in order to better treat recovering METH addicts.

METH effects in humans. Presently, there is disagreement in the field as to whether METH abuse results in cognitive deficits in humans. On the one hand, two well-designed and controlled studies found no differences in cognitive

functioning between METH abusers and control subjects (Chang et al., 2005; Simon et al., 2010). On the other hand, the reduction in DAT binding has been shown to correlate with cognitive deficits seen in individuals who have abused METH (Volkow et al., 2001). Further, other studies have found cognitive impairments in METH abusers, including impairments in executive function, learning, and memory (for review, see Scott et al., 2007; Marshall and O'Dell, 2012; Dean et al., 2013; but see Hart et al., 2012). For example, neuropsychological studies of METH abusers have discovered deficits in verbal learning and memory (Kalechstein et al., 2003; Gonzalez et al., 2004; Rippeth et al., 2004; Woods et al., 2005; Hoffman et al., 2006), attention (Kalechstein et al., 2003; Salo et al., 2007; Salo et al., 2009), executive control (Kim et al., 2006; King et al., 2010), and social and “real-life” skills, such as prospective memory for events to be done during the week (Henry et al., 2009; Rendell et al., 2009; Henry et al., 2010). Increased impulsivity also has been noted in a delay-discounting test (Hoffman et al., 2006). Thus, despite the controversy, the evidence is compelling that METH abuse is associated with cognitive deficits on certain tasks.

Modeling METH-induced neurotoxicity. It is important to be able to model the consequences of METH exposure in animals in order to study how neurotoxicity to monoamine systems comes about, what the effects of the toxicity are, and ultimately, how cognitive and physiological deficits arising as a result of METH abuse can be treated in order to increase quality of life for recovering addicts. One way to model METH-induced neurotoxicity in rats is via a “binge”

regimen of METH, in which rats are given multiple injections of METH over the course of one day. This regimen causes long-lasting partial depletions of DA and 5-HT levels in the brain, especially in the caudate-putamen/striatum (Seiden et al., 1976; Hotchkiss and Gibb, 1980; Morgan and Gibb, 1980; Ricaurte et al., 1980; Wagner et al., 1980; Ricaurte et al., 1982) comparable to what is seen in human METH abusers (Marshall and O'Dell, 2012).

Similar to observations in humans, METH-induced neurotoxicity in rats is associated with cognitive impairments. Behavioral performance on learning tasks after METH-induced neurotoxicity has been studied more extensively in rats than in humans, due to the tractability of the model organism, allowing researchers to probe in detail the basis of impairments associated with METH, including the extent to which cognitive deficits arise as a consequence of METH-induced neurotoxicity. Rats pretreated with a neurotoxic regimen of METH are impaired on tasks examining motor-sequence learning (Chapman et al., 2001; Daberkow et al., 2005), novel object recognition (Schröder et al., 2003; Belcher et al., 2005; Marshall et al., 2007; Herring et al., 2008; Reichel et al., 2012), visual discrimination and attentional set-shifting (Izquierdo et al., 2010), odor recognition (O'Dell et al., 2011), and spatial learning (Friedman et al., 1998, but see Schröder et al., 2003; Herring et al., 2008). METH-pretreated rats are also more work-averse than normal rats, as demonstrated by choosing a smaller, but easier-to-obtain reward over a larger reward that could only be reached by climbing over a wall (Kosheleff et al., 2012). These experiments have shown that METH-induced partial loss of DA in rodents models cognitive impairments seen

in human METH addicts, suggesting a causal role of METH-induced neurotoxicity in cognitive impairments in humans.

Despite obvious impairments on many behavioral tasks, METH-pretreated rats appear to be normal on other tasks, such as conditioned place aversion (Achat-Mendes et al., 2005), motor response-reversal learning (Daberkow et al., 2008; Pastuzyn et al., 2012; Chapters 2 and 3), and operant conditioning (Son et al., 2011; Son et al., 2013). These tasks can be deceptive, however, as recent research has demonstrated that METH-pretreated rats can appear to be behaving normally, but in reality have subtle impairments on the task or differences from normal rats in the brain regions used to perform the task. For example, METH-pretreated rats have no apparent gross motor impairments and are outwardly indistinguishable from normal rats. However, in the case of instrumental learning tasks, METH-pretreated rats appear to behave normally, but the associations underlying the instrumental behavior are different. Specifically, when those associations are probed by devaluing the reward using conditioned taste aversion, it becomes apparent that METH-pretreated rats never transition from goal-directed responding to habitual, or stimulus-response, responding (Son et al., 2011). METH-pretreated rats also perseverate more during their operant responding than do normal rats (Son et al., 2013). Finally, even though METH-pretreated rats appear to perform the response-reversal learning task as well as normal rats (Daberkow et al., 2008; Pastuzyn et al., 2012), normal rats rely on brain circuitry involving the dorsomedial (DM) striatum (Palencia and Ragozzino, 2004; Pastuzyn et al., 2012; Chapter 2), whereas rats

with METH-induced neurotoxicity rely on circuitry involving the nucleus accumbens (NAc) shell (Pastuzyn and Keefe, in press; Chapter 3). METH-induced neurotoxicity can therefore result in both gross and subtle impairments on cognitive tasks examining basal ganglia-mediated learning and memory processes.

Arc

In order to further explore the nature of METH-induced cognitive and behavioral deficits, we turned our attention to the activity-regulated cytoskeleton-associated gene *Arc*/*Arg 3.1* (Link et al., 1995; Lyford et al., 1995). Almost 20 years of research have cemented Arc's critical role in mediating synaptic plasticity, and it is now known that aberrant *Arc* transcription or translation plays a significant role in many diseases (*e.g.*, Greer et al., 2010; Auerbach et al., 2011; Osterweil et al., 2012). The mechanisms of Arc's role in synapse-level plasticity have been well described in other recent reviews (Bramham et al., 2010; Korb and Finkbeiner, 2011; Shepherd and Bear, 2011). Therefore, this review will focus on the role of Arc in specific behavioral and systems-level learning and memory tasks.

Arc at the synapse. Because the recent findings concerning Arc's role in synaptic plasticity have been reviewed in excellent detail elsewhere (Korb and Finkbeiner, 2011; Shepherd and Bear, 2011), we will only give a brief overview in this review. *Arc* is an immediate-early gene that is transcribed rapidly upon synaptic activity in the absence of new protein synthesis; *e.g.*, within five minutes

of exploration of a novel environment (Guzowski et al., 1999; Vazdarjanova et al., 2002; Barker-Haliski et al., 2012). *Arc*'s rapid induction is due in part to the presence of a negative elongation factor at the *Arc* promoter region that stalls RNA polymerase II at the transcription start site and allows for almost immediate transcription of *Arc* after neuronal activity (Saha et al., 2011). Transcription of *Arc* begins upon calcium influx via L-type voltage-sensitive calcium channels and activation of metabotropic glutamate receptors (Park et al., 2008; Adams et al., 2009); the mRNA is then trafficked to dendrites and Arc protein is translated locally at activated synapses (Steward et al., 1998). The story may be more complicated, however, because recently Arc was shown to play a role in regulating surface GluA1 expression even at inactive synapses (Okuno et al., 2012). Arc is necessary for long-term depression (LTD) (Park et al., 2008; Waung et al., 2008) and homeostatic scaling of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs) at postsynaptic membranes (Rial Verde et al., 2006; Shepherd et al., 2006) by promoting endocytosis of AMPARs (Chowdhury et al., 2006); correspondingly, neurons lacking Arc have increased surface expression of AMPARs. Furthermore, local knockdown of *Arc* mRNA using an antisense oligonucleotide directed against *Arc* prevents maintenance of long-term potentiation (LTP) (Guzowski et al., 2000; Messaoudi et al., 2007). Thus, the evidence points to Arc playing an important role in synaptic plasticity processes (LTD, LTP, and homeostatic scaling) critical for consolidation of learning and memory in the mammalian brain.

The experiments discussed above determining the role of Arc in synaptic plasticity have mostly been carried out in culture or *ex vivo*. An excellent system for studying plasticity *in vivo* is experience-dependent plasticity in visual cortex, since simple monocular deprivation results in a shift in ocular dominance (Wiesel and Hubel, 1963), caused by a strengthening in open eye inputs and corresponding weakening in closed eye inputs (Gordon and Stryker, 1996; Frenkel and Bear, 2004). Because the inputs weaken as a result of AMPAR endocytosis (Allen et al., 2003; Heynen et al., 2003; Crozier et al., 2007; Yoon et al., 2009), two labs examined whether Arc was responsible for ocular dominance shift in monocular deprivation. *Arc* KO mice were found to lack ocular dominance plasticity after monocular deprivation (McCurry et al., 2010) and to have an altered balance of excitatory and inhibitory inputs in visual cortex (Gao et al., 2010), leading to aberrant experience-dependent plasticity. Thus, the *in vitro* and *in vivo* data concur, and point to the significant and essential need for Arc in synaptic plasticity and thus learning and memory.

Arc in spatial learning. Arc knockout (KO) mice have intact short-term memory, but are unable to consolidate long-term memories in a variety of behavioral tasks (Plath et al., 2006). For example, although KOs were able to learn to find the hidden platform in a Morris water maze in a manner similar to that observed in wild-type mice, they were impaired in both remembering the location of a hidden platform in the Morris water maze in a probe test and in "reversal," in which the platform was moved to the opposite quadrant. These latter impairments suggest that Arc is needed for memory consolidation, in

remembering the platform's location, and for relearning, in the case of the new platform location.

Guzowski and colleagues provided similar findings using an *Arc* antisense oligonucleotide to knock down *Arc* expression in the hippocampus (2000). Rats infused with a scrambled oligonucleotide showed a preference for the trained quadrant in a probe test 48 hr later, whereas rats infused with *Arc* antisense either three hours before or immediately after being trained on the Morris water maze showed no preference, suggesting that they had not consolidated the learning during training. Another study by Martínez and colleagues further demonstrates *Arc*'s role in spatial learning, as measured by how much time rats spend exploring a familiar environment (2012). Whereas rats that were infused with a scrambled oligonucleotide showed less exploratory behavior when retested in the open field apparatus 24 hr later, rats that were infused with *Arc* antisense oligonucleotides into dorsal hippocampus three hours before the initial exposure to the open field did not show such a decrease in exploratory behavior when tested in the same context 24 hr later. Rather, they explored the environment as if it were novel. It is thus apparent that either genetic deletion of *Arc* or knockdown of *Arc* in hippocampus results in impaired spatial memory consolidation.

Arc in fear conditioning. Recently, much research has been focused on the role of *Arc* in fear conditioning, mainly with respect to the function it plays in the amygdala, but also regarding its role in the hippocampus in contextual fear behavioral paradigms. Pavlovian fear conditioning is a task in which a tone is

paired with a foot shock, and rats are subsequently tested in a novel environment for freezing when the tone is played. Plath et al. (2006) trained the *Arc* KO mice on this task, and found that when mice were tested for freezing 24 hr later in either the original chamber (contextual test) or in a novel chamber with the tone played (cue test), the KOs demonstrated significantly less freezing than wild-type mice. The role of *Arc* in a specific brain region during fear conditioning was examined by knockdown of *Arc* in the lateral amygdala via an infusion of *Arc* antisense six hours before Pavlovian fear conditioning (Ploski et al., 2008). While short-term memory was intact when rats were tested for freezing to the tone three hours later, long-term memory was impaired when tested 24 hr later. Similar to the spatial memory studies discussed above, *Arc* knockdown-induced disruption of long-term fear memories is further evidence for *Arc*'s specific role in long-term consolidation of memories, rather than in short-term plasticity processes underlying the initial learning and short-term memory.

Arc also appears to be critical for reconsolidation of the memory underlying conditioned fear. For example, in another study by Schafe and colleagues (Maddox and Schafe, 2011), rats underwent Pavlovian fear conditioning, but *Arc* antisense was not infused into the lateral amygdala until 24 hr after training. Ninety minutes later, rats were presented with the tone in the absence of any acute foot shock in a novel chamber to reactivate the fear memory. The rats were then tested for freezing three and 24 hr later to assess short-term and long-term memory, respectively. Knockdown of *Arc* did not impair short-term memory, but did impair long-term memory, as evidenced by less

freezing on the 24-hr test. Importantly, if the tone was not presented before infusion of *Arc* antisense, and thus the fear memory was not reactivated or made labile, knockdown of *Arc* had no impact on long- or short-term memory formation. These findings therefore demonstrate that *Arc* is necessary for consolidation, as well as reconsolidation of an “active”/labile memory.

A fear conditioning paradigm that is similar to the Pavlovian conditioning discussed above is trace fear conditioning, in which 30 s is allowed to elapse between the presentation of the tone and the delivery of the foot shock. Such conditioning has been shown to be dependent on the hippocampus (Yoon and Otto, 2007). Czerniawski and colleagues (2011) infused *Arc* antisense into hippocampus three hours before training rats on the trace fear conditioning paradigm. When rats were tested in a novel environment and the tone was presented 48 hr later, the knockdown of *Arc* was found to have disrupted memory consolidation, as *Arc* antisense-infused rats exhibited less freezing than control rats. Knockdown of *Arc* also impaired memory consolidation in a simpler paradigm in which rats were given a foot shock in a chamber, and then tested for freezing when returned to that same chamber 48 hr later. These studies again demonstrate the essential role of *Arc* in consolidation of fear conditioning memories.

Finally, inhibitory avoidance is another common fear-conditioning task, in which a shock is paired with one side of a chamber, and rats can avoid this shock by spending more time in the other side of the chamber. The role of *Arc* in this task has been tested in two brain regions. In one study, rats were trained on

the task, and then *Arc* antisense was infused into anterior cingulate cortex either immediately after or six or 45 hr later (Holloway and McIntyre, 2011). Long-term memory for the fear conditioning was assessed by testing for latency to enter the shock-paired compartment 48 hr after the initial training. Rats infused with *Arc* antisense either immediately or six hours after training had impaired memory consolidation, measured by shorter latencies than controls to enter the shock-paired chamber, whereas those infused with *Arc* antisense 45 hr posttraining did not differ from controls. Another study showed that infusion of *Arc* antisense three hours before inhibitory avoidance training disrupted memory consolidation, again as demonstrated by the *Arc* antisense-infused rats having shorter latencies to enter the shock-paired chamber (McIntyre et al., 2005; Martínez et al., 2012). In these studies, however, *Arc* antisense was infused into hippocampus, suggesting that multiple brain regions are involved in this kind of fear conditioning, and that *Arc* is a common link across those brain regions that are engaged in the task. *Arc* has thus been shown to be important for long-term memory consolidation of fear memories in three different kinds of fear conditioning tasks and across two brain regions.

Arc in extinction of drug-seeking behavior. Although much research has been performed examining *Arc*'s role in learning and memory in behavioral tasks mediated by the hippocampus and amygdala, comparatively little has been done in another brain region important for learning, the striatum. In particular, with respect to drug addiction, it is apparent that synaptic plasticity underlies the long-lasting changes in circuitry that mediate drug-seeking and cue-invoked relapse

(for review, see Van den Oever et al., 2012); however, despite *Arc*'s well-known role in synaptic plasticity and memory consolidation, just two studies have looked at the causal role of *Arc* in a paradigms relevant to drug abuse and addiction. Hearing and colleagues (2011) trained rats to self-administer cocaine, and then the rats underwent two weeks of abstinence. Three hours before rats were run in a context-induced reinstatement session under extinction conditions (*i.e.*, lever present in chamber, but no cocaine infused), *Arc* antisense was infused into the dorsolateral (DL) striatum. While knockdown of *Arc* did not prevent the context-induced reinstatement, it did impair extinction learning, as evidenced by more lever presses by antisense-infused rats on the second day of reinstatement testing. In the second study, Lv and colleagues (2011) examined the role of *Arc* in the nucleus accumbens (NAc) core and shell in morphine-induced conditioned place preference (CPP). For each test, an *Arc* antisense oligonucleotide was infused into either NAc core or shell three hours prior to testing. *Arc* antisense in NAc core prevented the development of morphine-induced CPP when *Arc* antisense was infused each day, the expression of morphine-induced CPP when *Arc* antisense was infused only on the day of the preference test, and reinstatement of morphine CPP after eight days of extinction. *Arc* antisense in the NAc shell prevented expression of morphine CPP, but had no impact on either development of CPP or its reinstatement. It is clear from these two studies that *Arc* is critical in drug cue-related memories in both DL striatum and NAc.

The findings of Hearing and colleagues detailed above highlight the need to carefully consider task design when assessing the role of *Arc* in a behavior

and in interpreting the results of the *Arc* disruption in terms of the neural circuitry contributing to a particular behavior. In this example, if activation of a memory trace in DL striatum were necessary for the context-induced reinstatement of cocaine seeking, then presumably that memory trace would become labile when the rats were exposed to the context. Based on the evidence from the fear-conditioning studies reviewed above, knockdown of *Arc* would thus have prevented that memory from being reconsolidated, and rats should not have lever-pressed at all the following day when exposed again to the context, as the context-induced cocaine-seeking memory encoded in DL striatum would be gone. In actuality, however, the *Arc* antisense-infused rats pressed more than controls on the second day. These findings suggest that consolidation of new learning occurring on the first reinstatement test day—that is, lever presses now no longer deliver cocaine—was contributing to the behavior observed on the second day and was disrupted by the loss of *Arc* during the first day of context-induced reinstatement. Interestingly, the rats infused with *Arc* antisense, while pressing more than controls on the second day of context-induced reinstatement, did not press as much as they did on the first day, as might be expected if only consolidation of the new extinction learning were blocked. It is thus possible that the intermediate phenotype of the animals infused with *Arc* antisense reflects opponent roles of *Arc* in the consolidation of the new extinction learning and the reconsolidation of the context with the drug-seeking behavior, which was activated and made labile during the first day of context-induced reinstatement testing. Clearly, when interrogating the role of *Arc* in learning and memory

functions, behavioral paradigms and tasks need to be carefully crafted and controlled to isolate the particular process in question.

The meaning of a correlation between Arc mRNA expression in a brain region and behavioral performance. Many studies have used *Arc* mRNA expression as a marker to show activation of neurons or brain regions (*e.g.*, Guthrie et al., 2000; Gusev et al., 2005; Zavala et al., 2008; Robinson et al., 2012; Antoine et al., 2014). However, Guzowski and colleagues (2001) previously argued that it is not simply an increase in *Arc* expression in a brain region that indicates a critical involvement of that brain region in the task being examined. Rather, the work of Guzowski and colleagues, as well as an increasing body of research, suggests that it is the correlation between *Arc* expression and behavioral performance that signifies that task-relevant encoding processes in that brain region are critical for the behavior being observed. For example, as reviewed above, *Arc* is critical for long-term spatial memory formation in the hippocampus (Guzowski et al., 2000; Martínez et al., 2012). Guzowski and colleagues measured *Arc* mRNA expression in dorsal hippocampus of rats sacrificed after training on the spatial version of the Morris water maze and found that rats learning more quickly (*i.e.*, those that found the platform more quickly) had more *Arc* mRNA in the hippocampus than rats that learned more slowly, resulting in a significant correlation between *Arc* mRNA expression in the hippocampus and learning of the spatial location of the platform (2001). Importantly, in rats trained on a cued version of the maze—a task dependent on striatal rather than hippocampal function—*Arc* mRNA expression

was still elevated in the hippocampus, but the degree of expression did not correlate with learning on the cued task. The critical meaning of the correlation between *Arc* expression and behavioral performance has received additional support from studies by Keefe and colleagues, who examined whether a correlation exists between *Arc* mRNA expression in striatum and learning on a basal ganglia-mediated learning and memory task; namely, response-reversal learning on a T-maze, a task known to be critically dependent on the function of the dorsomedial (DM) striatum (Ragozzino et al., 2002). In a study by Daberkow et al. (2007), rats that reached criterion on the reversal task in fewer trials had more *Arc* mRNA expression in DM striatum than rats that took more trials to reach criterion. Importantly, there were significant increases in *Arc* mRNA expression in the DL striatum and the hippocampus of these rats as well, but there were no correlations between expression in those brain areas and trials to criterion on the task. These findings therefore paralleled those of Guzowski and colleagues (2001) in that there was a significant correlation between *Arc* mRNA expression in the critical brain region (DM striatum) and the measure of learning on the task. To further verify that the correlation was indicative of the DM striatum being engaged for this response-reversal learning, *Arc* antisense was infused into DM striatum two hours before rats were trained on the reversal task (Pastuzyn et al., 2012; Chapter 2). Antisense-infused rats had impaired long-term memory consolidation, as shown by their taking more trials to reach criterion than control rats when retested on the reversal direction 24 hr later. Thus, these findings again suggest that it is the correlation between *Arc* expression in a brain

region and the measure of learning, and not simply the induction of *Arc* mRNA in a brain region, that implicates processes occurring in that brain region as being critical to solving the task being completed. Finally, rats that had been pretreated with a binge regimen of methamphetamine (METH) were also trained on the response-reversal learning task. Although METH-pretreated rats performed as well as saline-pretreated controls, reaching criterion on the reversal task in a similar number of trials, METH-pretreated rats lacked the correlation between *Arc* mRNA expression in DM striatum and trials to criterion (Daberkow et al., 2008; Pastuzyn and Keefe in press; Chapter 3). Rather, in the METH-pretreated rats, a significant correlation between *Arc* mRNA expression in NAc shell and trials to criterion on the task was found which did not exist in controls (Pastuzyn and Keefe in press; Chapter 3). Further, knockdown of *Arc* expression via infusion of an *Arc* antisense oligonucleotide into NAc shell of the METH-, but not the saline-, pretreated rats two hours prior to the reversal learning impaired retention of the learned reversal when examined the following day. Taken together, this growing body of evidence strongly suggests that rather than simply *Arc* induction, it is the *correlation* between *Arc* mRNA expression and the measure of learning on the task that is indicative of encoding processes occurring in that brain region being critical for solving the task.

Arc conclusions. The studies reviewed herein have revealed that *Arc* expression is more than just a marker for neuronal activity. Instead, it is critical for memory consolidation and reconsolidation in behavioral tasks across multiple brain regions. Although *Arc* expression is increased in multiple brain regions

when animals are engaged in a learning and memory task, the brain region in which *Arc*-regulated plasticity processes are critical for solving the task depends on the behavioral task. This critical involvement of *A*rc-mediated processes in a brain region in the learning and memory that is occurring appears to be reflected in the correlation between the degree of *Arc* mRNA expression and the measure of learning/memory being examined. It seems plausible to suggest that this correlation could be used in future studies to map, with greater precision than simply examining *Arc* induction, the brain circuitry in which plastic changes underlie the long-term learning taking place.

Phasic Dopamine

The dopaminergic neurons projecting from the ventral tegmental area and the substantia nigra fire in either a phasic or tonic mode (Grace, 2000; Goto and Grace, 2005; Grace et al., 2007; Schultz, 2007). Phasic DA neurotransmission is a bursting pattern of firing of DA neurons and appears to be responsible for mediating reactions to rewards and/or cues. The other mode of DA neuron firing, tonic, is a steady-state firing responsible for the basal levels of DA in the brain (Schultz, 2007). Phasic DA neurotransmission is essential for learning (Zweifel et al., 2009), encoding reward prediction errors (Schultz, 1998, 2007, 2013), and assigning incentive salience to cues (Berridge, 2007). The manner in which DA is released onto the postsynaptic neurons in the striatum modulates the output of basal ganglia components. This review will focus on phasic DA signaling, how it

goes awry as a result of METH-induced neurotoxicity, and what impact this disruption in DA signaling may have on striatal function.

The role of phasic dopamine. There are several hypotheses as to the role phasic DA might be playing in the brain, which are also reviewed elsewhere (Berridge, 2007; Schultz, 2007; Berridge et al., 2009; Schultz, 2013). One idea is that phasic DA acts as a reward prediction error signal (Schultz, 1998, 2007, 2013). In this case, phasic DA release to an unexpected or better-than-expected natural reward creates a positive prediction error and helps strengthen the association between cue and reward. If the reward is completely expected, there is no phasic DA signal, because the animal is not learning anything new. If the reward is expected but does not appear, there is a dip in DA release, creating a negative prediction error theorized to aid in extinction of responding. Studies training rats to work for natural food rewards have demonstrated that phasic DA release initially occurs when the reward is given. This response to the reward diminishes over time and shifts to one occurring in response to the cue as the animal learns that the cue predicts the reward (Day et al., 2007; Brown et al., 2011). Further, a recent paper using optogenetics to artificially cause phasic DA release when there normally would be none resulted in rats forming a cue-reward association that did not occur in controls, providing, for the first time, evidence of phasic DA acting causally as a reward prediction error (Steinberg et al., 2013).

Phasic DA release has also been shown to be involved in behaviors related to drug abuse and addiction. Fast-scan cyclic voltammetry revealed phasic DA release in the nucleus accumbens when rats initiated cocaine-

seeking, as well as in response to a cue, once the rats had learned that the cue predicted cocaine availability (Phillips et al., 2003). Once the rat self-administered cocaine for a period of time, this phasic DA signal to the cue shifts from occurring in nucleus accumbens to dorsolateral striatum (Willuhn et al., 2012), possibly via a dopaminergic "spiral" pathway between nucleus accumbens, substantia nigra, and dorsolateral striatum (Haber et al., 2000; Ikemoto, 2007). Thus, as with natural rewards, phasic DA release to drugs of abuse at first signals the unexpected reward (the drug), and then over time fires to the cue, and not to the primary reinforcer fully predicted by the cue.

Phasic DA release to the cue relates to another hypothesis regarding the role of phasic DA: that of incentive salience, in which the reward-paired cue acquires motivational properties (Berridge, 2007). DA, specifically in the nucleus accumbens core (Saunders and Robinson, 2012), has been shown to be necessary for rats to assign incentive salience to a reward-paired cue (Flagel et al., 2011). Additionally, genetically disrupting phasic DA signaling by deleting NMDA receptors on DA neurons, while leaving tonic DA signaling intact, led to mice being significantly impaired in learning cued behaviors (Zweifel et al., 2009). This evidence suggests that the role of phasic DA is complex and appears to be necessary for learning about both rewards and cues.

Medium spiny neuron gene expression and the effects of METH-induced neurotoxicity. Two populations of GABAergic medium spiny neurons make up ~95 percent of the neurons in the striatum: striatonigral neurons, known as the "direct pathway," which express D1 DA receptors and the neuropeptides

substance P (from *preprotachykinin*, or *ppt*) and dynorphin (from *preprodynorphin*) (Gerfen et al., 1990; Surmeier et al., 1996; Gerfen and Surmeier, 2011); and striatopallidal neurons, known as the "indirect pathway," which express D2 DA receptors and the neuropeptide enkephalin (from *preproenkephalin*, or *ppe*) (Gerfen et al., 1990; Le Moine et al., 1990; Surmeier et al., 1996; Gerfen and Surmeier, 2011). The basal ganglia are hypothesized to act in action selection: the direct pathway, depending on DA levels and signaling type, allows an animal to initiate an action, while the indirect pathway inhibits actions (for review, see Da Cunha et al., 2012). Action selection in each pathway is proposed to occur through the signaling pathways that extend from activation of the D1 DA receptor on direct pathway neurons and the D2 DA receptor on indirect pathway neurons (for review, see Surmeier et al., 2007). Thus, the two main output pathways of the striatum are quite segregated and specialized.

As discussed above, METH-induced neurotoxicity results in long-lasting damage to DA systems in the brain, manifesting in part as a partial loss of DA availability in striatum. Associated with partial DA loss, whether induced by METH (Chapman et al., 2001; Johnson-Davis et al., 2002; Howard et al., 2013a; Chapter 4) or by 6-hydroxydopamine (6-OHDA; Nisenbaum et al., 1996), is a decrease in *ppt* mRNA expression in direct pathway/striatonigral neurons. Conversely, partial DA loss has no effect on *ppe* mRNA expression (Nisenbaum et al., 1996; Chapman et al., 2001; Johnson-Davis et al., 2002; Howard et al., 2013a; Chapter 4). Rather, *ppe* mRNA expression only changes—increases—after extensive (80-90%) DA neuron loss (Gerfen et al., 1991; Nisenbaum et al.,

1996), similar to the extent of loss seen in late-stage Parkinson's disease. This selective effect of METH-induced neurotoxicity on *ppt* expression was the first suggestion that the DA loss associated with METH-induced neurotoxicity might be primarily affecting direct pathway/striatonigral neurons. METH-induced neurotoxicity also affects expression of the immediate-early activity-regulated cytoskeleton-associated (*Arc*) gene. In normal animals, basal levels of *Arc* in striatum are quite low (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012), since *Arc* mRNA transcription is induced by activity (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). However, basal *Arc* in METH-pretreated rats is not only higher than in normal rats, but striatal *Arc* mRNA levels also fail to increase in response to exposure to a novel environment as they do in normal rats (Barker-Haliski et al., 2012). While basal *Arc* transcription is aberrantly regulated in both striatonigral and striatopallidal neurons, the numbers of striatonigral neurons with *Arc* mRNA in the cytoplasm correlates with the degree of METH-induced DA loss, suggesting that METH-induced neurotoxicity significantly disrupts signaling in striatal efferent neurons, particularly striatonigral neurons (Keefe and Horner, 2010; Barker-Haliski et al., 2012).

Impact of METH-induced neurotoxicity on dopamine signaling. Surprisingly, DA released in a phasic pattern appears to signal selectively through DA D1 receptors on striatonigral/direct pathway neurons. For example, stimulation of the medial forebrain bundle (MFB) (through which DA neuron axons project to striatum) in a phasic-like manner results in immediate-early gene (IEG) expression in D1 DA receptor-containing striatonigral neurons (Chergui et

al., 1997; Gonon, 1997; Onn et al., 2000; Howard et al., 2013a; Chapter 4). Studies have also proposed that DA released in a tonic, steady-state firing pattern preferentially affects D2 receptors on striatopallidal/indirect pathway neurons. Computer modeling using values available in the literature suggests that high-affinity D2 receptors are saturated at basal levels of extracellular DA established by DA neurons firing in a tonic manner (Dreyer et al., 2010). When the DA neurons fire phasically, the sudden large increase in DA concentration results in significant increases in DA binding to lower-affinity D1 receptors. Thus, the manner in which DA is released from the presynaptic neuron in striatum controls which receptors are preferentially bound by the DA being released and, therefore, which striatal efferent neurons are preferentially affected.

Because the data reviewed in this section suggest that METH-pretreated rats may have more deficits in functioning of the striatonigral/D1-containing pathway, we hypothesized that the METH-induced partial DA loss might negatively impact phasic DA neurotransmission more than tonic DA transmission. Furthermore, prior work had suggested that partial DA loss is associated with selective impairment of phasic DA signaling (Bergstrom and Garris, 2003). Likewise, microdialysis studies have shown that METH-induced neurotoxicity is not associated with altered tonic/basal DA levels in striatum (Robinson et al., 1990; Cass and Manning, 1999), but is associated with a deficit in phasic DA transmission as assessed using fast-scan cyclic voltammetry (Howard et al., 2011; Loewinger et al., 2012; Howard et al., 2013b; Chapter 5). Specifically, recent work from Garris and colleagues has shown that less DA is

released in the striatum of METH-pretreated rats, relative to saline-pretreated controls, when the MFB is stimulated in a phasic-like manner (Howard et al., 2011). Conversely, the amount of DA released in response to tonic-like stimulation of the MFB is the same in METH-pretreated rats relative to controls. These findings were then replicated in awake, freely-moving rats exploring a novel environment in which spontaneously occurring phasic DA transients were recorded in striatum (Howard et al., 2013b). That is, METH-pretreated rats had smaller amplitude phasic transients than normal rats and the transients were also less frequent. Duration of the transients also increased due to the METH-induced loss of DA transporter-containing terminals which normally clear DA from the extracellular space (Howard et al., 2013b). These experiments suggest that the METH-induced partial loss of DA terminals in striatum does not alter tonic DA signaling, but does impair phasic DA signaling.

Phasic dopamine and medium spiny neuron gene expression. Since phasic DA signaling appears to selectively act at D1 DA receptors, it follows that phasic DA signaling could have effects on striatonigral neurons that are not mirrored in striatopallidal neurons. As noted, Chergui and colleagues (1997) demonstrated that phasic-like stimulation of the MFB increased expression of the IEG *zif268* preferentially in D1-containing medium spiny neurons, whereas tonic-like stimulation of the MFB did not alter striatal *zif268* expression. Recently, we put forth the hypothesis that METH-induced deficits in gene expression and, possibly, behavioral outcomes are due to impairments of phasic DA neurotransmission in these rats (Keefe and Horner, 2010; Barker-Haliski et al.,

2012). Therefore, to further test this hypothesis, Howard et al. (2013a; Chapter 4) determined whether phasic-like stimulation of the MFB could restore the impaired striatal gene expression observed in METH-pretreated rats (as discussed). In these studies, the MFB of anesthetized rats was stimulated in a phasic- or tonic-like manner for 45 min, at which point the rats were sacrificed. *In situ* hybridization histochemical analysis of the IEGs *Arc* and *zif268*, as well as the neuropeptide precursors *ppt* and *ppe*, was then completed. As hypothesized, phasic-like stimulation of the MFB restored *ppt* mRNA expression in METH-pretreated rats back to normal levels, whereas tonic-like stimulation did not change *ppt* mRNA expression. Further confirming the prior findings of Chergui and colleagues (1997), phasic-like stimulation increased *zif268* and *Arc* mRNA expression, whereas tonic-like stimulation did not. Interestingly, in the case of the IEGs, the mRNAs were induced to the same level in both normal and METH-pretreated rats, despite the fact that METH-pretreated rats are lacking 25-50% of their striatal DA terminals, and thus might be expected to be unable to induce mRNA transcription in as many medium spiny neurons as normal rats. Finally, *ppe* mRNA expression was unchanged by either phasic- or tonic-like stimulation. These findings suggest that restoration of phasic DA neurotransmission in the striatum of METH-pretreated rats can rescue impaired striatal gene expression and, by extension, possibly normalize striatal function in these rats.

Pharmacological rescue of METH-induced deficits. The evidence presented above suggests that impairment of phasic DA neurotransmission in METH-pretreated rats may contribute to at least some of the cellular deficits seen

with METH-induced neurotoxicity. Because human METH abusers and animal models of METH exposure also show cognitive deficits (see Section 1.1), it stands to reason that such cognitive/behavioral deficits could also be a result of decreased phasic DA neurotransmission, given that phasic DA transmission has been shown to be critical for certain behaviors (see Section 3.1). Thus, if phasic DA transmission can be restored in a therapeutic fashion, it may be possible to improve cognitive function in individuals with a history of METH abuse and, therefore, improve rehabilitation outcomes.

L-3,4-dihydroxyphenylalanine (L-DOPA), the most common drug used to manage symptoms of Parkinson's disease, is the biochemical precursor of DA. Because the L-aromatic amino acid decarboxylase responsible for converting L-DOPA to DA is not saturated, administration of L-DOPA results in increased DA production in the brain. L-DOPA has been shown to increase vesicular DA content and quantal size of DA release *in vitro* (Pothos et al., 1996; Pothos et al., 1998). Furthermore, L-DOPA increases DA release *in vivo* as measured by fast-scan cyclic voltammetry (Keller Jr. et al., 1988; Wightman et al., 1988; Garris et al., 1994) or amperometry (Rodríguez et al., 2007). These studies used rather large doses of L-DOPA (100-250 mg/kg) and produced significant increases in gross extracellular DA levels. However, there is some intriguing evidence in the literature to suggest that L-DOPA might preferentially affect only phasic DA neurotransmission if the correct dose is used. For example, in intact humans or primates, L-DOPA does not alter DA receptor binding under resting conditions as measured by PET scanning (Antonini et al., 1994; Tedroff et al., 1996b; Tedroff

et al., 1996a; Flöel et al., 2008). However, if the subject is completing a task, administration of L-DOPA increases DA signaling (Flöel et al., 2008). Furthermore, L-DOPA was recently shown to restore reward prediction error in elderly subjects (Chowdhury et al., 2013)—as reviewed here, phasic DA transmission is thought to signal reward prediction error. Thus, we have examined whether striatal functioning in METH-pretreated rats can be restored by specifically rescuing phasic DA transmission via administration of L-DOPA.

In this dissertation (Chapter 5), rats were anesthetized, and the MFB was stimulated in a phasic-like pattern once every five minutes. After establishing baseline levels of phasic-like DA release, rats were given 50 mg/kg L-DOPA. Consistent with our prior observations (Howard et al., 2011), phasic-like DA transmission was decreased in METH-pretreated rats relative to saline-pretreated controls under baseline conditions. However, within 20 min of administration of L-DOPA, phasic-like DA release in the METH-pretreated rats was restored to levels seen under baseline conditions in saline-pretreated controls (Chapter 5). Furthermore, by parsing out the components of the voltammogram using principal component regression (Keithley et al., 2009; Keithley and Wightman, 2011), we determined that 50 mg/kg L-DOPA selectively increased phasic DA neurotransmission in both METH- and saline-pretreated rats without altering basal DA transmission. These findings suggest that just one dose of L-DOPA is sufficient to acutely rescue evoked phasic-like DA release in METH-pretreated rats.

Phasic DA conclusions. The evidence presented in this chapter suggests that METH-pretreated rats have a specific impairment in phasic DA release, which contributes, at least in part, to the deficits in gene expression seen in METH-pretreated rats, and by extension, perhaps to the cognitive deficits observed as well. Since administration of L-DOPA restored phasic-like DA neurotransmission in METH-pretreated rats, this treatment may be useful in helping recovering METH addicts in treatment and in everyday life.

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CHAPTER 2

ALTERED LEARNING AND *ARC*-REGULATED CONSOLIDATION OF LEARNING IN STRIATUM BY METHAMPHETAMINE-INDUCED NEUROTOXICITY

Abstract

Methamphetamine (METH) causes partial depletion of central monoamine systems and cognitive dysfunction in rats and humans. We have previously shown that the positive correlation between expression of the immediate-early gene *Arc* (activity-regulated, cytoskeleton-associated) in dorsomedial (DM) striatum and learning on a response reversal task is lost in rats with METH-induced striatal DA loss, despite normal behavioral performance and unaltered *N*-methyl-D-aspartate (NMDA) receptor-mediated EPSCs, suggesting intact excitatory transmission. This discrepancy suggests that METH-pretreated rats may no longer be using the dorsal striatum to solve the reversal task. To test this hypothesis, male Sprague-Dawley rats were pretreated with a neurotoxic regimen of METH or saline. Guide cannulae were surgically implanted bilaterally into DM striatum. Three weeks after METH, rats were trained on a motor response version of a T-maze task, and then underwent reversal training. Before

reversal training, the NMDA receptor antagonist DL-2-amino-5-phosphonopentanoic acid (AP5) or an *Arc* antisense oligonucleotide was infused into DM striatum. Acute disruption of DM striatal function by infusion of AP5 impaired reversal learning in saline-, but not METH-, pretreated rats. Likewise, acute disruption of *Arc*, which is implicated in consolidation of long-term memory, disrupted retention of reversal learning 24 hr later in saline-, but not METH-, pretreated rats. These results highlight the critical importance of *Arc* in striatum in consolidation of basal ganglia-mediated learning and suggest that long-term toxicity induced by METH alters the cognitive strategies/neural circuits used to solve tasks normally mediated by dorsal striatal function.

Introduction

Methamphetamine (METH) abuse is a significant problem worldwide. METH causes partial loss of dopamine (DA) and serotonin systems in the brain (Seiden et al., 1976; Morgan and Gibb, 1980; Ricaurte et al., 1980; Wagner et al., 1980). In humans, METH-induced neurotoxicity is evident as decreases in DA transporter (DAT) binding in caudate-putamen that can last for up to 11 months (Wilson et al., 1996; McCann et al., 1998; Volkow et al., 2001b; Volkow et al., 2001a). METH-induced toxicity is also evident as decreases in serotonin transporter (SERT) binding across multiple brain regions, including caudate-putamen and frontal cortex (Sekine et al., 2006; Kish et al., 2009), as well as loss of glutamatergic neurons in somatosensory cortex (Pu et al., 1996; Eisch et al., 1998). Cognitive impairments have also been seen in association with METH-

induced neurotoxicity, and include deficits in motor sequence learning (Chapman et al., 2001), object recognition (Schröder et al., 2003; Belcher et al., 2005; Herring et al., 2008), visual discrimination and attentional set-shifting (Izquierdo et al., 2010), and novel odor recognition (O'Dell et al., 2011).

In some tasks, however, behavioral impairments associated with METH-induced neurotoxicity are not apparent. Such tasks include those examining conditioned placed aversion (Achat-Mendes et al., 2005), spatial learning on the Morris water maze (Schröder et al., 2003; Herring et al., 2008), and motor response reversal learning on a T-maze (Daberkow et al., 2008). With regards to the response reversal learning task on the T-maze, expression of *Arc* (activity-regulated cytoskeleton-associated gene), an immediate-early gene important in consolidation of learning, in dorsomedial (DM) striatum is correlated with number of trials to criterion on the reversal learning task in saline-pretreated rats, but not, interestingly, in METH-pretreated rats (Daberkow et al., 2007, 2008). Thus, although METH-pretreated rats behaviorally appear to be normal on this task, the relation between *Arc* expression in striatum and behavior is lost. Guzowski and colleagues (2000; 2001) previously suggested that the correlation between *Arc* expression and learning reflects the involvement of a brain region in a task. Whether *Arc* in DM striatum is necessary for consolidation of response reversal learning has not heretofore been examined; furthermore, whether the loss of correlation in METH-pretreated rats indicates a change in the brain regions engaged during the task is unknown.

Therefore, the goal of the present studies was to test whether *Arc* in DM striatum is critical for consolidation of response reversal learning and whether the loss of the correlation in METH-pretreated rats reflects a loss of dependence of the reversal learning on DM striatal function. We locally infused an *Arc* antisense oligonucleotide (Guzowski et al., 2000; Hearing et al., 2011) or the NMDA receptor antagonist AP5 into DM striatum prior to rats engaging in motor response reversal learning on the T-maze task. The results indicate that *Arc* signaling in DM striatum is necessary for consolidation of the reversal learning and that METH-induced neurotoxicity is associated with a change in the neural substrates mediating such reversal learning.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC; 275-300g) were singly housed in tub cages on a 14:10-hr light cycle. Animal care and experimental procedures followed the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Methamphetamine pretreatment. Rats were treated with a neurotoxic regimen of (±)-METH-HCl (4x10mg/kg free base, s.c.; NIDA, Research Triangle Park, NC) over 6-one day as previously described Daberkow et al., 2008. One hour after the final injection, rats were returned to their home cages and given free access to food and water until behavioral training began (METH-pretreated, *n*=25; saline-pretreated, *n*=29).

Surgery. Two weeks after pretreatment, rats were anesthetized with ketamine/xylazine (90/10mg/kg, i.p.) and placed in a stereotaxic apparatus. A dual, 21-gauge guide cannula (Plastics One, Roanoke, VA) was lowered to end just dorsal to DM striatum (mm from Bregma: AP+0.2; ML±1.9; DV-3.2). The guide was secured with skull screws and dental acrylic, and dummy cannulae inserted. Subsequent infusions were made through 33-gauge infusion cannulae extending 1.8mm beyond the guides. Infusion cannulae remained in place for one min after the infusion before being withdrawn.

Reversal learning task. Response reversal learning on the T-maze was conducted as previously described (Daberkow et al., 2007). Beginning one week after surgery, rats were food restricted and habituated to the food reward and maze. The turn bias of each rat was determined, followed by acquisition training for three days and then reversal learning. During the reversal-learning task, rats had to turn in the opposite direction from acquisition to receive the reward. The criterion for learning on both acquisition and reversal tasks was 9/10 correct turns in a row.

Acute pharmacological manipulations. On the day of reversal training, rats were infused through their cannulae with either AP5 (0.5 µL/2 min, 25 nmol in 0.1M PBS, pH 7.4; Tocris Bioscience, Ellisville, MO) Palencia and Ragozzino, 2004 or an *Arc* antisense oligonucleotide. The *Arc* antisense oligonucleotide was a chimeric phosphorothioate/phosphodiester oligonucleotide against bases 209-228 of the *Arc* gene (Guzowski et al., 2000). The nonsense/control oligonucleotide was comprised of the same bases, but in a scrambled sequence.

One µL of oligonucleotide (1 nmol/µL, 0.1M PBS, pH 7.4) (Guzowski et al., 2000) or PBS vehicle was infused (0.39 µL/min) into each DM striatum. After infusions into DM striata, rats were returned to their home cages for five minutes (AP5) or two hours (*Arc* antisense) prior to reversal training. Five minutes after criterion was reached, rats infused with AP5 and corresponding PBS-infused controls were sacrificed, and the brains removed and frozen in isopentane chilled on dry ice. Rats infused with the *Arc* antisense oligonucleotide and corresponding controls (PBS or *Arc* nonsense oligonucleotide) were returned to their home cages upon reaching criterion. The following day, these rats were tested on reversal retention to determine the number of trials needed to again reach criterion on the reversal direction learned the previous day. Five minutes after reaching criterion, rats were sacrificed and brains removed and frozen.

DAT and SERT autoradiography. Fresh-frozen brains were sectioned (12-µm), thaw-mounted onto Superfrost Plus (VWR, Aurora, CO) slides, and then stored at -20°C. Infusion sites were verified during sectioning (Figure 2.1). DAT levels in striatum were determined with [^{125}I]RTI-55 (PerkinElmer, Waltham, MA) binding, as previously reported (Boja et al., 1992; O'Dell et al., 2011). SERT binding in prefrontal cortex was similarly performed except that fluoxetine was omitted. Prefrontal cortex slides incubated in buffer containing fluoxetine showed no binding (data not shown). Slides were apposed to film (Biomax MR; Eastman Kodak Co., Rochester, NY) for 24 hr and developed. Images were digitized and densitometric analysis accomplished using NIH ImageJ, yielding average, background-subtracted gray values in DM and dorsolateral (DL) striatum and six

prefrontal cortical regions. Two rostral and two middle striatal sections and four prefrontal cortical sections were analyzed per rat. DAT and SERT binding in METH-pretreated rats were then converted to percent of average levels in saline-pretreated rats.

Error analysis. The number of perseverative and regressive errors made during reversal learning by METH- and saline-pretreated rats was calculated as defined by Palencia and Ragozzino (2004), with modification due to the task differences. Wrong turns were counted as perseverative errors if they occurred before a rat made more than three turns in the reversal direction. Incorrect turns occurring after the rat had made more than three turns in the new correct direction were counted as regressive errors.

In situ *hybridization histochemistry.* The left hemisphere from animals used for electrophysiological experiments (see below) was frozen, sectioned (12-µm) and processed for *in situ* hybridization histochemical determination of Grin2a NMDA receptor subunit expression as previously described (Ganguly and Keefe, 2001) using a full-length ribonucleotide probe synthesized from the cDNA (kind gift from Dr. Peter Seeburg) using ^{35}S-UTP and T7 RNA polymerase (Roche, Indianapolis, IN). Slides were hybridized overnight in humid chambers at 55°C, washed, treated with Ribonuclease A (5 µg/mL), washed, dried, and then apposed to X-ray film for one week. Images from films were digitized and densitometric analysis accomplished using ImageJ, yielding average, background-subtracted gray values in DM, DL, and ventromedial striatum.

Determination of striatal DA content. DA content was determined in striatal tissue punches collected during sectioning of frozen brain hemispheres for *in situ* hybridization (Chapman et al., 2001). A blunt-tip, 18-gauge needle was used to collect 1-mm^3 punches from both medial and lateral striatum (+0.3 mm anterior to Bregma). Punches were sonicated in tissue buffer (0.05 M sodium phosphate/0.03 M citric acid buffer, 25% methanol (v/v), pH 2.5) and centrifuged. Twenty µL of supernatant were injected onto a high-pressure liquid chromatography system coupled to an electrochemical detector (EOx = +0.6 V) for separation and quantification of DA levels. Values were expressed per mg of protein. Protein content was determined with the Lowry protein assay.

Striatal slice preparation. Acute brain slices were obtained, as previously described (Chapman et al., 2003), from adult rats (375-460 g) sacrificed three-five weeks after pretreatment with saline or METH. Rats were anesthetized (pentobarbital, 50 mg/kg) and decapitated. Brains were removed and placed into ice cold, oxygenated (95% O_2-5% CO_2) sucrose Ringer solution, pH 7.4, containing (in mM): sucrose (200), KCl (3), NaH_2PO_4 (1.4), $MgSO_4$ (2), $NaHCO_3$ (26), glucose (10), and $CaCl_2$ (2). The brain was divided along the midline, and the right hemisphere was glued caudal-side down to a Vibraslicer chuck (Campden Instruments). Coronal sections (300-350µm) containing striatum were placed in a holding chamber at room temperature containing oxygenated Ringer solution with 126 mM NaCl in place of sucrose, pH 7.37-7.41. Sections remained in the Ringer solution (osmolality 295-305mOsm) for ≥1 hr before recording.

Patch clamp recordings. Slices were transferred into the recording chamber perfused with fresh, oxygenated, Mg^{2+}-free Ringer solution at room temperature (~22°C) via a gravity-feed system (4 mL/min). Whole-cell patch-clamp was used to record from single striatal neurons, using previously described inclusion criteria and data acquisition (Chapman et al., 2003). Borosilicate glass microelectrodes (3-6 MΩ resistance) were pulled using a P-87 micropipette puller (Sutter Instruments). The internal recording solution contained (in mM): K gluconate (130), KCl (10), HEPES (10), EGTA (1), $CaCl_2$ (0.1), ATP (2), GTP (1) and glutathione (1). The external solution was the same as that in the holding chamber.

Excitatory postsynaptic currents (EPSCs) were elicited using local, minimal stimulation to mitigate voltage- and space-clamp errors (Stevens and Wang, 1994; Wilcox et al., 1996). A bipolar stimulating electrode was placed near the recording electrode (<300 μm). The stimulating electrode was used to deliver current pulses (100-μsec duration) of sufficient amplitude to produce the smallest EPSC (25-40 pA) that could be reliably evoked at low frequency (0.1 Hz). To isolate and maximally activate NMDA receptor-mediated EPSCs, the Ringer solution contained 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione, 50 μM picrotoxin, and 10 μM glycine. Data were acquired with an Axopatch ID amplifier and the CLAMPEX8 software package interfaced to a Digidata 1200 acquisition board (Axon Instruments). Signals were filtered at 5 KHz and sampled at 10 KHz.

Only recordings not exhibiting substantial changes in holding current or resistance at the electrode tip were used for analysis. All cells required <100pA

to be clamped to -70mV. Cells with resting membrane potentials above -55mV were omitted from analysis. The following parameters were determined for averaged NMDA receptor-mediated EPSCs: rise times, peak amplitudes, decay time constants, and weighted τ (τ_w). The decay time constants were fit with a double exponential equation: $I(t)=I_f\exp(-t/\tau_f)+I_s\exp(-t/\tau_s)$, where I_f is the amplitude of the fast component, I_s is the amplitude of the slow component, and τ_f and τ_s are the fast and slow time constants, respectively. Weighted time constants were calculated using the equation: $\tau_w=[I_f/(I_f+I_s)]\tau_f+[I_s/(I_f+I_s)]\tau_s$ (Stocca and Vicini, 1998). All data are presented as mean±SEM.

Statistical analysis. Dependent measures from animals used in the behavioral studies were compared across pretreatment and treatment groups using two-way ANOVAs and *post hoc t*-tests (JMP v.9.0). Dependent measures from animals used for electrophysiological studies were analyzed using unpaired *t*-tests for the striatal region of interest.

Results

DAT and SERT autoradiography. Pretreatment of rats used for the behavioral studies with a "binge" regimen of METH resulted in significant decreases in striatal DAT binding. METH-pretreated rats in the AP5 experiment had significantly less DAT binding than saline-pretreated rats in striatum (Fig. 2.2a; DM striatum, mean±SEM, 54.6±6.4% of saline, $F_{(1,22)}=12.8$, $p<0.01$; DL striatum 61.1±6.1%, $F_{(1,22)}=12.2$, $p<0.01$). A similar decrease in DAT binding was seen in METH-pretreated rats in the *Arc* antisense experiment (graph not shown;

DM striatum, 58.4±6.9% of saline, $F_{(1,29)}$=18.4, p<0.001; DL striatum 66.4±6.6%, $F_{(1,29)}$=14.2, p<0.001). METH-pretreated rats also had significantly decreased SERT binding relative to saline-pretreated controls in all prefrontal regions examined (Fig. 2.2b): prelimbic, (57.8±9.5% of saline, $F_{(1,17)}$=14.3, p<0.01), infralimbic (73.6±7.3%, $F_{(1,17)}$=14.5, p<0.01), medial orbitofrontal (58.9±9.9%, $F_{(1,17)}$=13.8, p<0.01), ventral orbitofrontal (47.3±9.2%, $F_{(1,17)}$=25.6, p<0.0001), lateral orbitofrontal (49.4±9.4%, $F_{(1,17)}$=20.0, p<0.001), and cingulate (44.7±10.9%, $F_{(1,17)}$=19.7, p<0.001) cortices.

Effects of acute NMDA receptor blockade in DM striatum. As previously reported by our lab (Daberkow et al., 2008), METH-pretreated rats appear to be behaviorally normal in terms of motor response reversal learning on the T-maze relative to saline-pretreated rats (Fig. 2.3). However, acute disruption of striatal function via bilateral infusion of AP5 into DM striatum revealed differences in DM striatal involvement in this learning. Analysis revealed a significant overall interaction (pretreatment×infusion; $F_{(1,1)}$=4.6, p<0.05), as well as significant main effects of pretreatment ($F_{(1,1)}$=5.2, p<0.05) and infusion ($F_{(1,1)}$=7.6, p<0.05). Saline-pretreated rats that were infused with AP5 (n=5) required significantly more trials to reach criterion than did saline-pretreated, PBS-infused rats (Fig. 2.3; n=8; $t_{(23)}$=-3.4, p<0.01). METH-pretreated rats (n=5), on the other hand, were unaffected by infusion of AP5, and thus were significantly different from saline-pretreated, AP5-infused rats ($t_{(23)}$=2.8, p<0.05), but not METH-pretreated, PBS-infused (n=9; $t_{(23)}$=-0.4, p=0.7) or saline-pretreated, PBS-infused ($t_{(23)}$=-0.3, p=0.7) rats.

Effects of acute Arc *disruption in DM striatum.* Consistent with prior reports making use of *Arc* antisense in different brain regions and in different learning and memory paradigms (Guzowski et al., 2000; Hearing et al., 2011), we observed no effects of *Arc* antisense infusion into DM striatum on initial reversal learning in either saline- or METH-pretreated rats (Fig. 2.4a). Two-way ANOVA on trials to criterion on the reversal learning task revealed no main effects of pretreatment ($F_{(1,30)}$=0.01, p=0.9) or infusion ($F_{(1,30)}$=0.69, p=0.5) and no interaction ($F_{(1,2)}$=0.09, p=0.9). However, again consistent with prior reports (Guzowski et al., 2000; Hearing et al., 2011), analysis of retention of the reversal learning 24 hr after the initial reversal learning task revealed a significant overall interaction ($F_{(1,2)}$=4.07, p<0.05), but no main effects of pretreatment ($F_{(1,30)}$=0.9, p=0.3) or infusion ($F_{(1,30)}$=1.22, p=0.3). *S*aline-pretreated rats infused with *Arc* antisense (n=13) took significantly more trials to reach criterion on the retention test (Fig. 2.4b) than controls (saline-pretreated, *Arc* nonsense-infused: n=4, $t_{(30)}$=-1.8, p<0.05; saline-pretreated, PBS-infused: n=4, $t_{(30)}$=-2.8, p<0.01) and METH-pretreated, *Arc* antisense-infused rats (n=8, $t_{(30)}$=3.7, p<0.001). METH-pretreated, *Arc* antisense-infused rats, however, were not significantly different from control groups (METH-pretreated, *Arc* nonsense-infused: n=4, $t_{(30)}$=1.6, p=0.1; METH-pretreated, PBS-infused: n=3, $t_{(30)}$=0.4, p=0.7). These results indicate that *Arc* in DM striatum is necessary for consolidation of response reversal learning under normal conditions, but not in rats with METH-induced neurotoxicity.

Error analysis. As demonstrated previously (Daberkow et al., 2008) and again in this study (Fig. 2.3), METH-pretreated rats perform as well as normal rats on response reversal learning on the T-maze. We also analyzed the types of errors (Palencia and Ragozzino, 2004) made by METH- or saline-pretreated, PBS-infused rats to determine whether the METH-induced monoamine depletions altered behavioral flexibility. Consistent with the lack of effect on trials to criterion, we found no differences between METH- and saline-pretreated rats in numbers of perseverative (saline-pretreated, 25.5±7.4; METH-pretreated, 28.7±6.5; $t_{(15)}$=-0.3, p=0.8) or regressive (saline-pretreated, 35.9±8.8; METH-pretreated, 45.7±11.0; $t_{(15)}$=-0.7, p=0.5) errors.

In situ *hybridization histochemistry for striatal Grin2a subunit.* The pharmacological properties of NMDA receptors are determined to a large extent by the subunit composition of the receptors, with the NR2 subunits being of critical importance in this regard. In particular, prior work has shown that NR2a subunit incorporation yields NMDA receptors with higher affinity for competitive antagonists such as AP5 (Buller et al., 1994). Therefore, to assess the possibility that the lack of effect of AP5 infusion into DM striatum reflects a change in the pharmacological properties of NMDA receptors in DM striatum in METH-pretreated rats, we examined the expression of the NR2a subunit of the NMDA receptor in striatum of saline- and METH-pretreated rats. In this experiment, the METH-pretreated rats had significant depletions of striatal DA (Table 2.1). These depletions, as determined by HPLC-ECD analysis of tissue DA content in the striatum, are slightly larger than those observed in the cohorts of rats used for the

behavioral experiments described above. Other work in our laboratory (unpublished observations) indicates that the magnitude of the DA depletions estimated by DAT binding is typically less than the magnitude measured via determination of DA tissue content, although the two measures are very highly and significantly correlated (r^2 values of 0.8-0.9). Thus, although the magnitude of the depletions in this cohort of animals used for the determination of NMDA receptor expression and function after METH treatment appears to be greater, we think that they are roughly equivalent degrees of depletion and that any difference simply reflects subtle differences in the actual magnitude of depletion induced in different cohorts of animals treated with METH at different times and by different investigators. Analysis of film autoradiograms for Grin2a mRNA expression in striatal sections (+0.7mm from Bregma) from these saline- and METH-pretreated rats revealed a main effect of region ($F_{(2,39)}$=6.74, p<0.01), but no main effect of pretreatment ($F_{(1,39)}$=0.2, p=0.7) and no significant interaction ($F_{(1,2)}$=0.05, p=0.95) (Fig. 2.5g). *Post hoc* analysis confirmed previous reports (Buller et al., 1994; Standaert et al., 1999; Ganguly and Keefe, 2001) of greater Grin2a mRNA expression in both DM ($t_{(39)}$=-2.1, p<0.05) and DL ($t_{(39)}$=-3.7, p<0.001) striatum relative to VM striatum.

Electrophysiological properties of NMDA receptor-mediated EPSCs. To further assess whether there might be changes in the properties of NMDA receptors in striatal efferent neurons induced by METH exposure and whether this might underlie the differential sensitivity of the METH-pretreated rats to AP5 and *Arc* antisense oligonucleotide infusion, we compared NMDA receptor-

mediated EPSCs from both the DL and VM aspects of the striatum of both saline- and METH-pretreated rats, since there are regional differences in NMDA receptor function in the adult striatum (Chapman et al., 2003). As we have previously reported (Chapman et al., 2003), the kinetics of the NMDA receptor-mediated EPSCs were faster in the DL than the VM striatum; however, prior exposure to a neurotoxic regimen of METH did not change the kinetics (Fig. 2.5, 2.6). That is, the rise times (Fig. 2.6a; main effect of region, $F_{(1,72)}$=9.64, p<0.01), τ_f (Fig. 2.5, 2.6b; main effect of region, $F_{(1,78)}$=11.27, p=0.001), and τ_w (Fig. 2.5, 2.6d; main effect of region, $F_{(1,78)}$=72.66, p<0.0001) were significantly faster in DL striatum, consistent with the greater expression of the Grin2a subunit of the NMDA receptor in that region of striatum. There was also a trend for the τ_s to be faster in DL striatum, although the main effect of region was not statistically significant ($F_{(1,78)}$=1.13, p=0.3). However, for none of these kinetic parameters was there a significant main effect of pretreatment (rise times, $F_{(1,72)}$=0.0001, p=0.99; τ_f, $F_{(1,78)}$=0.03, p=0.9; τ_s, $F_{(1,78)}$=0.02, p=0.9; τ_w, $F_{(1,78)}$=0.2, p=0.7) or a significant interaction (rise times, $F_{(1,72)}$=1.2, p=0.3; τ_f, $F_{(1,78)}$=0.4, p=0.5; τ_s, $F_{(1,78)}$=2.1, p=0.2; τ_w, $F_{(1,78)}$=1.0, p=0.3), indicating that METH-induced neurotoxicity was not associated with changes in the fundamental subunit composition or electrophysiological characteristics of NMDA receptors in dorsal striatum.

Discussion

This study confirms previous observations that DM striatum is involved in motor response reversal learning (Palencia and Ragozzino, 2004) and that METH-pretreated rats appear behaviorally normal on this task (Daberkow et al., 2008). However, the present results extend these prior observations in three important ways. First, the present results establish a critical role for *Arc* in DM striatum in consolidation of reversal learning in normal rats. Second, they provide additional support, in a novel brain area, for the hypothesis put forth by Guzowski and colleagues (2001) that the correlation between *Arc* mRNA in a brain region and behavioral performance reflects task-relevant encoding processes occurring in that brain area. Finally, the present results provide the first direct evidence that METH-induced neurotoxicity is associated with a change in the neural substrates engaged to solve a behavioral task normally dependent on DM striatum. These results therefore highlight the critical importance of striatal *Arc* for consolidation of basal ganglia-mediated learning and suggest that long-term toxicity induced by METH alters neural circuits and/or cognitive strategies used to solve tasks normally mediated by dorsal striatum.

The present data provide the first direct evidence that *Arc* is a critical mediator of consolidation of reversal learning mediated by DM striatum. This brain region has previously been implicated in cognitive flexibility, including that required for motor response reversal learning. In particular, Ragozzino and colleagues have previously established that acute blockade of cholinergic muscarinic or glutamatergic NMDA receptors in DM striatum impairs response

reversal learning (Ragozzino et al., 2002; Palencia and Ragozzino, 2004). Additionally, depletion of DA, but not serotonin, in DM striatum impairs reversal learning (O'Neill and Brown, 2007; Clarke et al., 2011). Furthermore, we have previously demonstrated that, in normal animals, there is a correlation between *Arc* mRNA in DM, but not DL, striatum and trials to criterion on a response reversal learning task (Daberkow et al., 2007). Guzowski and colleagues (2001) initially reported such a correlation between *Arc* expression in hippocampus and spatial learning on the Morris water maze, leading them to speculate that such correlations reflect the involvement of encoding processes in that particular brain region in the consolidation of spatial learning. Therefore, we proposed (Daberkow et al., 2007) that the correlation between *Arc* mRNA in DM striatum and trials to criterion on the reversal learning task reflected the fact that this reversal is normally dependent on DM striatal function, and that *Arc* must be a critical mediator of plasticity in DM striatum underlying consolidation of reversal learning. The present results support this hypothesis, as infusion of an *Arc* antisense oligonucleotide, but not a scrambled oligonucleotide or vehicle, into DM striatum impaired performance in *normal* rats on a reversal retention test given 24 hr later. Taken together with prior results showing that *Arc* antisense oligonucleotide infusions into DL striatum disrupt consolidation of extinction learning occurring during context-induced reinstatement of cocaine-seeking behavior (Hearing et al., 2011), the data strongly implicate *Arc* as a general, critical mediator of encoding processes underlying striatally-based learning and memory functions.

Our previous studies on rats with METH-induced neurotoxicity have shown that although these rats appear to be behaviorally normal with respect to response reversal learning, *Arc* induction in DM striatum is attenuated and no longer correlates with trials to criterion, leading us to hypothesize that METH-induced neurotoxicity promotes a shift in the neural substrates mediating this behavior (Daberkow et al., 2008). The present findings support this hypothesis: in rats with METH-induced neurotoxicity, acute disruption of DM striatal function by infusion of the NMDA receptor antagonist AP5 or an *Arc* antisense oligonucleotide fails to alter response reversal learning or its retention. Thus, although rats with METH-induced neurotoxicity appear to be normal on the surface, the neural substrates mediating the behavior have changed. These findings are similar to those reported, for example, in Parkinson's disease patients, in which behavior appears unimpaired relative to controls, but functional imaging reveals a change in brain regions engaged during the task (Moody et al., 2004). These findings highlight the need for studies assessing the impact of neurotoxicity on learning and memory to examine not simply behavioral measures of the learning, but also the processes and brain regions mediating the behavior, before concluding that there is a lack of effect of such toxicity on a particular behavior.

It is conceivable that the lack of effect of acute disruption of NMDA receptor and *Arc* function in DM striatum on reversal learning and its consolidation reflects a decrease in sensitivity of DM striatum to these manipulations, rather than a reorganization of the neural circuitry mediating the

behavior. However, we think that these former possibilities are unlikely, as *in situ* hybridization histochemical analysis of *Grin2a* mRNA expression and electrophysiological determination of the biophysical properties of striatal NMDA receptors failed to reveal any METH-induced changes in these NMDA receptor subunits or properties. The pharmacology of NMDA receptors is heavily influenced by Grin2 subunit incorporation into the receptor (Buller et al., 1994; Traynelis et al., 2010), as are the rise-time and decay kinetics of the NMDA receptor-mediated current, with Grin2a-containing receptors showing the fastest kinetics (Dingledine et al., 1999). Striatal efferent neurons, which are the striatal neurons in which *Arc* is expressed (Vazdarjanova et al., 2006), express the Grin2a and Grin2B subunits (Standaert et al., 1999). The present results confirm our prior observations and those of others that there is greater expression of Grin2a subunits in DL than VM striatum (Buller et al., 1994; Standaert et al., 1999; Ganguly and Keefe, 2001), and that the rise times and decay kinetics of these currents are correspondingly faster in DL than in VM striatum (Chapman et al., 2003). These results illustrate our ability to detect differences in subunit composition of the NMDA receptor using this electrophysiological approach. Importantly, METH-induced neurotoxicity was not associated with changes in Grin2a subunit mRNA expression or in the biophysical properties of the NMDA receptors in the dorsal striatum, strongly suggesting that METH-induced neurotoxicity is not associated with changes in the subunit composition, and thus the pharmacology, of striatal NMDA receptors. It therefore seems unlikely that a change in the sensitivity of NMDA receptors in METH-pretreated rats to AP5 or

endogenous glutamate underlies the lack of efficacy of acute AP5 infusion or *Arc* antisense infusion in those animals in the present studies. Rather, the data suggest that the lack of effect of these agents more likely reflects a change in the neural circuitry engaged in the reversal learning task.

The consequences of METH exposure that lead to this apparent shift in behavioral control are currently unknown; however, the METH-induced partial loss of DA in DM striatum may be the basis. As is typical (Chapman et al., 2001; Hanson et al., 2009), the binge regimen of METH resulted in an approximately 40% loss of DA tissue content, as measured by DAT levels, in DM striatum at the end of the behavioral training. Although METH also induces a loss of serotonin in DM striatum (Haughey et al., 1999), as noted, DA, not serotonin, neurotransmission in DM striatum appears to mediate reversal learning (O'Neill and Brown, 2007; Clarke et al., 2011; Darvas and Palmiter, 2011). Thus, one strong possibility is that it is the partial loss of DA in DM striatum that results in the change in sensitivity of response reversal learning to acute manipulations of DM striatal function in METH-pretreated rats.

An alternative possibility is that METH-induced damage to extrastriatal serotonin systems disrupts the function of afferents to DM striatum or other neural substrates necessary for reversal learning, thereby altering the circuitry engaged during the reversal learning. The neurotoxic regimen of METH used in the present study also induces a loss of serotonin in prefrontal cortex (Hotchkiss and Gibb, 1980; Ricaurte et al., 1980), including an approximately 50% loss of SERT binding in orbitofrontal cortex (OFC) reported herein. Serotonin function in

OFC is known to be critical for reversal learning (Clarke et al., 2005; Clarke et al., 2007; Robbins and Arnsten, 2009). Thus, changes in the function of OFC as a consequence of METH-induced neurotoxicity to that region may contribute to the changes in reversal learning observed in the present study. However, OFC tends to provide afferent innervation to the central and lateral aspects of dorsal striatum, as well as nucleus accumbens, and largely does not provide afferents to DM striatum (Schilman et al., 2008). On the other hand, prelimbic cortex does project strongly to DM striatum (Lévesque and Parent, 1998; Vertes, 2006). As presented herein, a neurotoxic regimen of METH results in about a 40% loss of SERT in prelimbic cortex. Furthermore, the prelimbic cortex plays a role in reversal learning, although the role is more in controlling complex, higher-order set-shifting tasks, rather than simple one-dimensional reversal learning such as the T-maze task used in this study (Ragozzino et al., 1999; Birrell and Brown, 2000; Ragozzino, 2003). Finally, the centromedian and paracentral nuclei of the thalamus provide excitatory innervation to the DM striatum (Van der Werf et al., 2002). These thalamic nuclei receive relatively dense serotonergic innervation (Vertes et al., 2010), and data obtained from abstinent human METH abusers suggest decreased SERT binding in the thalamus (Sekine et al., 2006). Thus, METH-induced alterations in the function of excitatory afferents from intralaminar cell groups to DM striatum might also play a role in the disruption of DM striatal control over reversal learning observed in the present studies. However, the extent to which neurotoxic regimens of METH damage the intralaminar nuclei of the thalamus in rodents has not heretofore been reported. Clearly, further studies

examining the effects of selective DA depletions induced by substituted amphetamines vs. the effects of combined DA/serotonin depletions will be necessary to conclusively rule out a contribution of serotonin loss to the changes in behavioral control observed in the METH-pretreated rats.

An interesting aspect of the present findings is that METH-pretreated rats appear to be behaviorally normal, both in terms of trials to criterion and in the types of errors made during reversal learning. The neural substrates capable of supporting apparently normal reversal learning despite altered DM striatal function remain to be determined. One possibility for an alternate neural substrate is the nucleus accumbens core, which has been implicated in behavioral flexibility (Goto and Grace, 2005; Haluk and Floresco, 2009; Darvas and Palmiter, 2011). The “binge” regimen of METH exposure often does not induce as much monoamine loss in nucleus accumbens as in dorsal striatum (Eisch et al., 1992; Haughey et al., 1999), and DA signaling in accumbens plays a role in simple reversal learning (Haluk and Floresco, 2009; Darvas and Palmiter, 2011). Future studies thus will be necessary to determine the role of nucleus accumbens in reversal learning in METH-pretreated rats, the circumstances under which DM striatal vs. nucleus accumbens DA signaling normally supports behavioral flexibility, and the cognitive cost associated with a loss of DM striatal control over behavioral flexibility.

In summary, the present study provides the first evidence that *Arc* in DM striatum is a critical mediator underlying consolidation of motor response reversal learning, thereby further validating its importance as a molecular substrate of

learning and memory function. Furthermore, the present results are the first to show that METH-induced neurotoxicity is associated with a change in neural substrates underlying basal ganglia-mediated learning and memory, despite the fact that behavioral indices of that learning appear to be normal. These findings suggest that METH-induced neurotoxicity may have important ramifications for the ability of individuals with a history of METH abuse to engage in cognitive behavioral therapies for management of drug addiction, as well as the extent to which they can function optimally in tasks related to their employment and personal lives. Further studies are therefore needed to fully understand the molecular, cellular, and systems level substrates mediating learning and memory processes in corticostriatal circuits that are compromised by METH-induced monoamine loss and to design approaches to mitigate such effects.

The authors declare no conflicts of interest.

This work was supported by DA024036 and NS35579 (KAK), DC008553 (EDP), and DA14859 (DEC).

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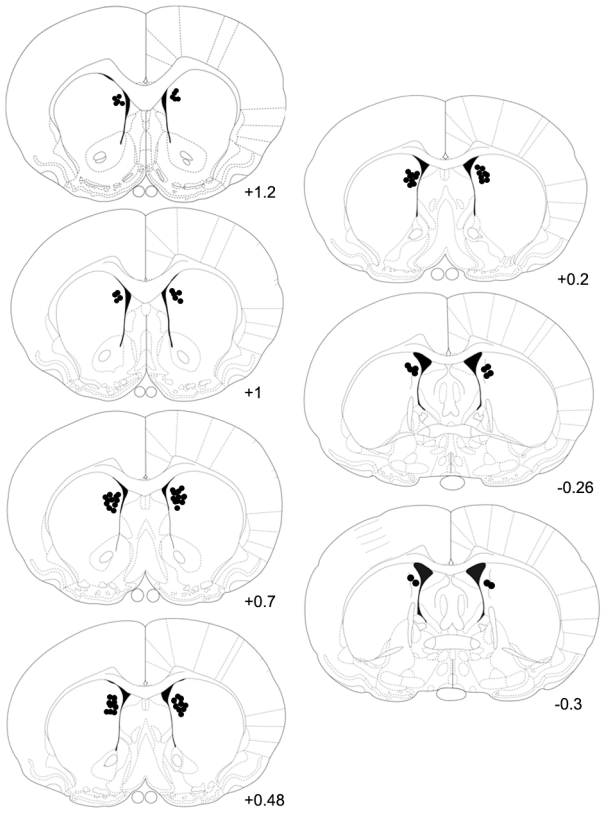


Figure 2.1. Infusion sites in dorsomedial striatum. Black dots indicate placement of infusion sites in DM striatum of rats in AP5 and *Arc* experiments. Numbers indicate mm from Bregma (modified from Paxinos and Watson, 1998).

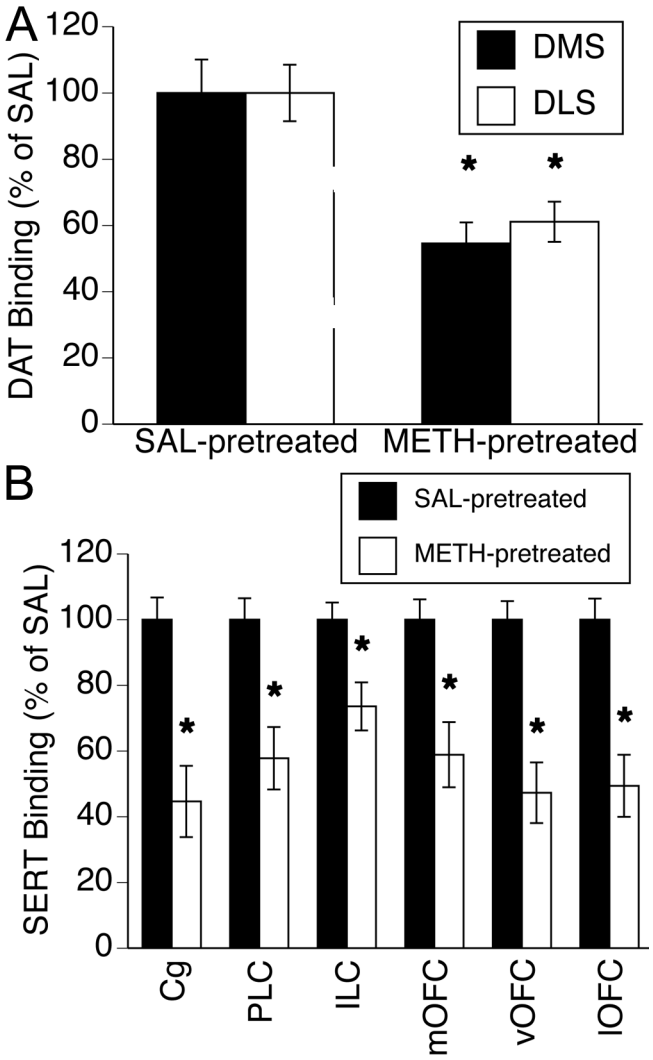


Figure 2.2. DAT and SERT binding. (*A*) DAT decreases (mean±SEM), expressed as percent of average values in saline-pretreated controls, in rats pretreated with (±)-METH (4x10 mg/kg, two-hour intervals; *n*=25) or saline (SAL; *n*=29) approximately seven weeks prior to sacrifice. (*B*), SERT decreases (mean±SEM), expressed as percent of average values in saline-pretreated controls, in rats pretreated with METH (*n*=12) or saline (*n*=11) approximately seven weeks after METH pretreatment. *Significantly different from SAL-pretreated values for same brain region, $p<0.01$. Cg, cingulate cortex; PLC, prelimbic cortex; ILC, infralimbic cortex; mOFC, medial orbitofrontal cortex; vOFC, ventral OFC; lOFC, lateral OFC.

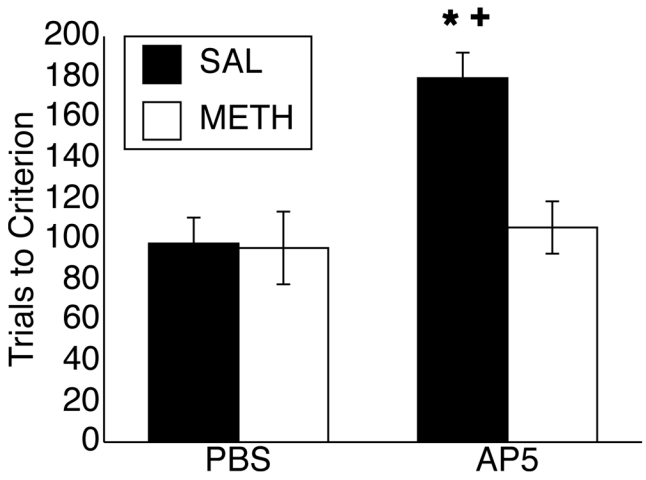


Figure 2.3. Effects of acute NMDA receptor blockade in DM striatum. Mean trials to criterion (9/10 correct consecutive trials; ±SEM) on a motor response-reversal task. Rats were given bilateral infusions of AP5 or PBS 5 min prior to the beginning reversal learning. *Significantly different from SAL-pretreated, PBS-infused rats (p<0.01). $^{+}$Significantly different from METH-pretreated, AP5-infused rats (p<0.05).

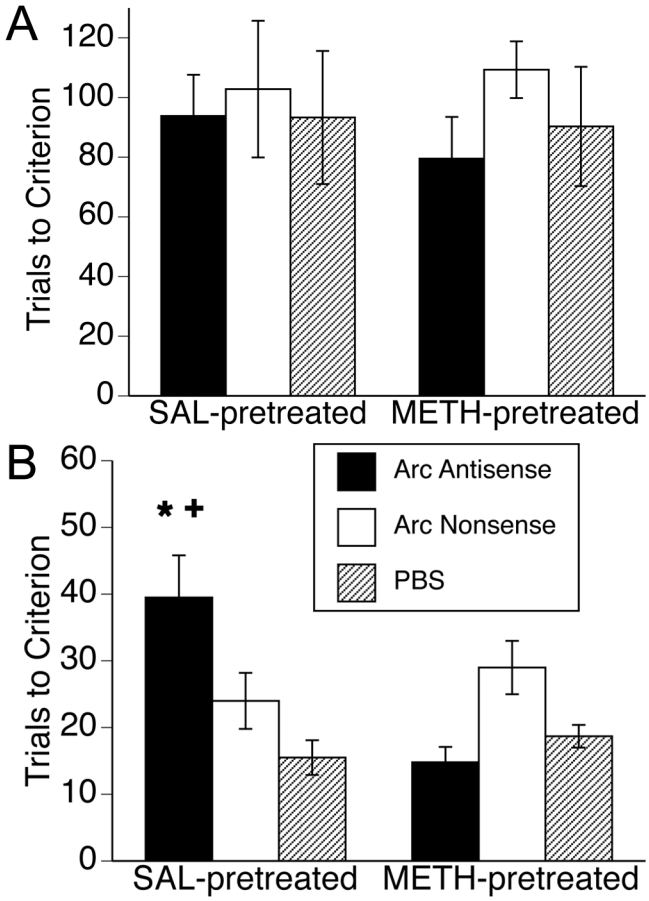
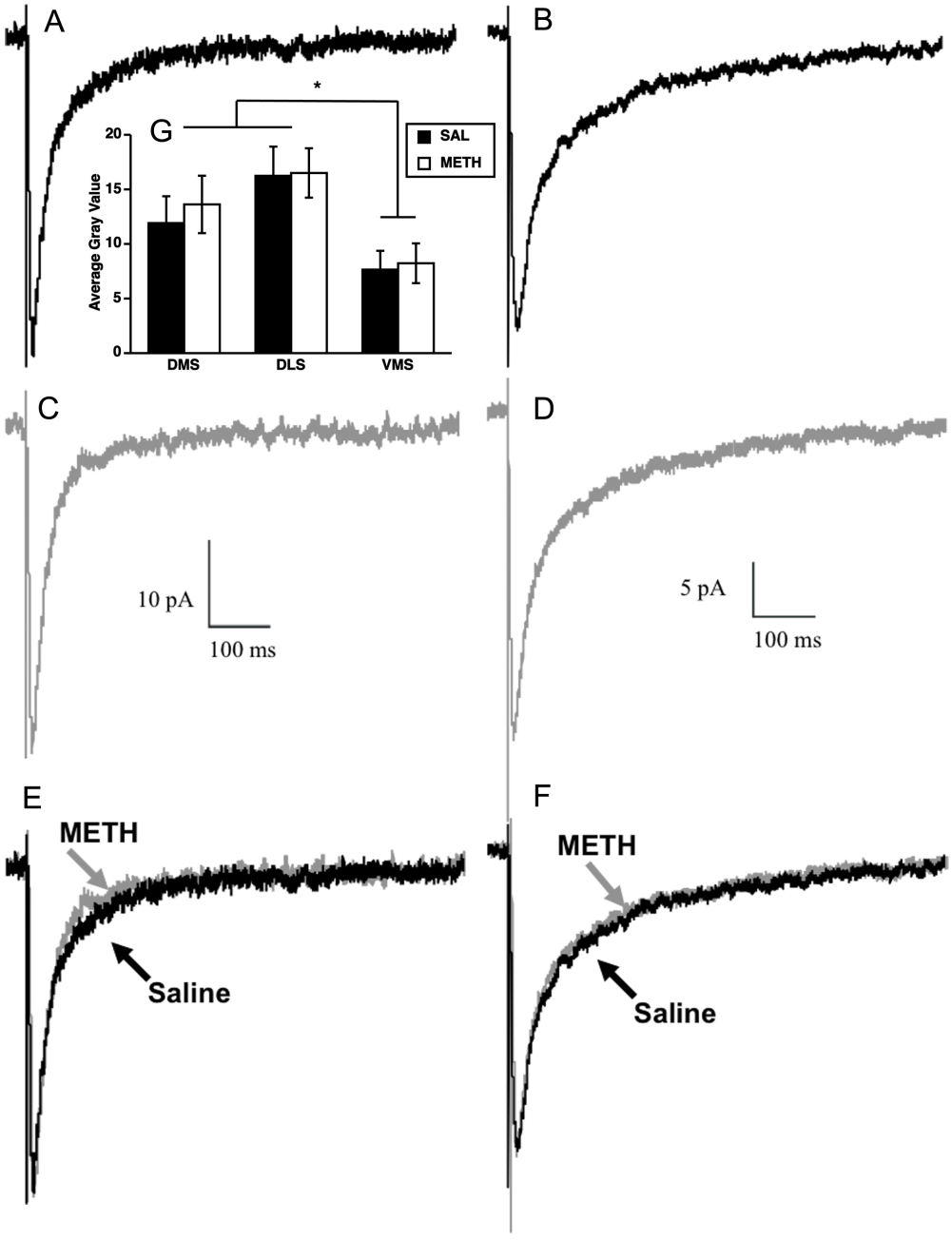


Figure 2.4. Effects of acute *Arc* disruption in DM striatum. Mean trials to criterion (±SEM) on the motor response-reversal task (A) and the reversal-retention tasks (B). (A), Rats were given bilateral infusions of an *Arc* antisense oligonucleotide, *Arc* nonsense oligonucleotide, or PBS two hours prior to beginning reversal training. No significant interactions or main effects of pretreatment or infusion were found. (B), Rats were tested on retention of the previous day's reversal learning. No further infusions were made. *Significantly different from SAL-pretreated, *Arc* nonsense oligonucleotide and PBS controls ($p<0.05$). $^{+}$Significantly different from METH-pretreated, *Arc* antisense-infused rats ($p<0.05$).

Figure 2.5. Grin2a mRNA expression and decay kinetics of NMDA receptor-mediated EPSCs. (A-F), Local, minimal stimulation of striatum in proximity (<300 μm) to the recorded cell elicits a long-lasting, NMDA receptor-mediated EPSC in striatum. The average of 35 EPSCs evoked at 0.1 Hz are shown. Representative traces showing the decay-time kinetics of NMDA receptor-mediated EPSCs in dorsolateral (A, C, E) and ventromedial (B, D, F) striatum of saline- (A, B) and METH- (C, D) pretreated rats are shown, as are normalized, superimposed traces from DL (E) and VM (F). (G), Grin2a mRNA expression in DL, DM, and VM striatum from the hemisphere opposite to that used for electrophysiological recordings. Data are average gray values (±SEM) from densitometric analysis of film autoradiograms. *Both DM and DL striatum are significantly different from VM striatum (p<0.01).


A
B
G
*
SAL
METH
Average Gray Value
20
15
10
5
0
DMS
DLS
VMS
C
D
10 pA
100 ms
5 pA
100 ms
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F
METH
Saline
METH
Saline

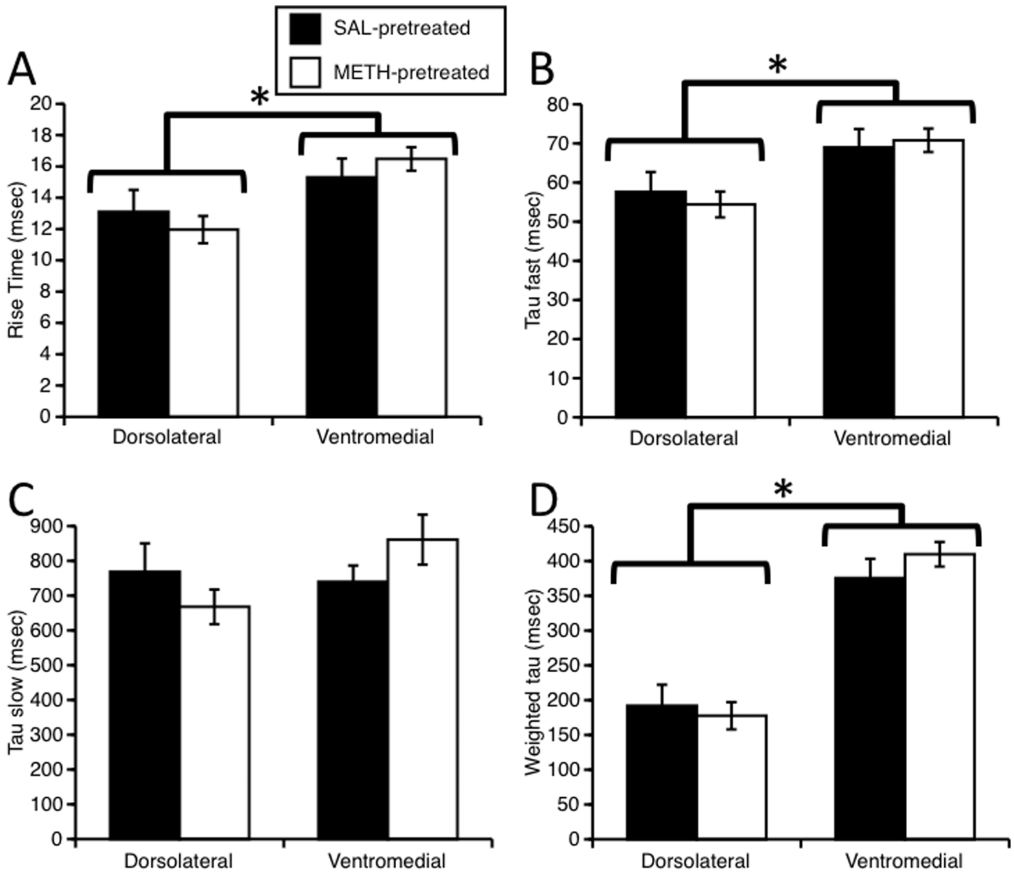

Figure 2.6. Kinetic properties of striatal NMDA receptor-mediated EPSCs in saline- and methamphetamine-pretreated rats. Values are average kinetic parameters (±SEM) calculated from whole-cell, patch-clamp recordings of NMDA receptor-mediated EPSCs in the dorsolateral and ventromedial striata of rats pretreated with saline (SAL-pretreated; *n*=10 for DL, *n*=12 for VM) or a neurotoxic regimen of METH (METH-pretreated; *n*=25 for DL, *n*=19 for VM). (*A*), 10-90% rise time. The decay of the EPSCs were fit with a double exponential equation, $I(t)=I_f\exp(-t/\tau_f)+I_s\exp(-t/\tau_s)$, yielding fast (B; τ fast) and slow (C; τ slow) time constants. Weighted time constants (D; weighted τ) were calculated using the equation: $\tau_w=[I_f/(I_f+I_s)]\tau_f+[I_s/(I_f+I_s)]\tau_s$ (Stocca and Vicini, 1998). *Significant main effect of region, $p<0.05$.

Table 2.1. Striatal DA tissue content three weeks after a neurotoxic regimen of METH. Values are average (±SEM) DA content (ng DA/mg protein) in striatal tissue determined via HPLC-ECD analysis of 1-mm^3 tissue punches from dorsolateral or ventromedial striatum. Values are ng DA/mg protein. *Significantly different from saline (p<0.05).

Treatment	Striatal DA Tissue Content
	Dorsolateral
Saline (n=8)	314±26
METH (n=8)	98±15*
	Ventromedial
Saline (n=8)	282±26
METH (n=8)	94±26*

CHAPTER 3

CHANGES IN NEURAL CIRCUITRY REGULATING RESPONSE-REVERSAL LEARNING AND *ARC*-MEDIATED CONSOLIDATION OF LEARNING IN RATS WITH METHAMPHETAMINE-INDUCED PARTIAL DOPAMINE LOSS

Abstract

Methamphetamine-induced neurotoxicity results in long-lasting depletions of monoamines and changes in basal ganglia function. We previously reported that rats with methamphetamine-induced neurotoxicity no longer engage dorsomedial striatum during a response reversal-learning task, as their performance is insensitive to acute disruption of dorsomedial striatal function by local infusion of an *N*-methyl-D-aspartate receptor antagonist or an antisense oligonucleotide against the activity-regulated cytoskeleton-associated (*Arc*) gene. However, methamphetamine-pretreated rats perform the task as well as controls. Therefore, we hypothesized that the neural circuitry involved in the learning had changed in methamphetamine-pretreated rats. To test this hypothesis, rats were pretreated with a neurotoxic regimen of methamphetamine or with saline. Three to five weeks later, rats were trained on the reversal-learning task and *in situ* hybridization for *Arc* was performed. A significant correlation between *Arc*

expression and performance on the task was found in nucleus accumbens shell of methamphetamine-, but not saline-, pretreated rats. Consistent with the idea that the correlation between *Arc* expression in a brain region and behavioral performance implicates that brain region in the learning, infusion of an antisense oligonucleotide against *Arc* into the shell impaired consolidation of reversal learning in methamphetamine-, but not saline-, pretreated rats. These findings provide novel evidence suggesting that methamphetamine-induced neurotoxicity leads to a shift from dorsal to ventral striatal involvement in the reversal-learning task. Such reorganization of neural circuitry underlying learning and memory processes may contribute to impaired cognitive function in individuals with methamphetamine-induced neurotoxicity or others with striatal dopamine loss, such as patients with Parkinson's disease.

Introduction

Methamphetamine (METH) abuse continues to have considerable societal impact, with 12 million Americans reporting use in their lifetime (2011 National Survey on Drug Use and Health, SAMHSA). METH abuse in humans causes decreases in the dopamine transporter (DAT) (Wilson et al., 1996) and serotonin transporter (SERT) (Sekine et al., 2006). Further, recent data indicate that people with a history of hospitalization for METH abuse are at higher risk of developing Parkinson's disease (Callaghan et al., 2012).

The monoamine loss resulting from METH abuse in humans can be recapitulated in rodents. METH-induced neurotoxicity causes partial depletions of

dopamine (DA) and serotonin (5-HT) (Wagner et al., 1980). As in human METH abusers (Dean et al., 2013), this partial monoamine loss is associated with cognitive deficits, including impairments in odor and object recognition, attentional set-shifting (Marshall and O'Dell, 2012), sequential motor learning (Chapman et al., 2001; Daberkow et al., 2005), formation of stimulus-response associations (Son et al., 2011), and inhibitory control over behavior (Son et al., 2013). The deficits in basal ganglia-mediated behaviors may arise secondary to impaired phasic DA neurotransmission in the partially denervated striatum (Howard et al., 2011; Howard et al., 2013a).

Arc (activity-regulated cytoskeleton-associated gene) is an effector immediate-early gene involved in synaptic plasticity and memory consolidation (Shepherd and Bear, 2011). Hippocampal *Arc* expression correlates with performance and is necessary for memory consolidation on the spatial version of the Morris water maze (Guzowski et al., 2000; Guzowski et al., 2001). Similarly, our lab has reported correlations between *Arc* mRNA in dorsomedial striatum (DMS) and performance on a striatally-mediated response reversal-learning task in normal (Daberkow et al., 2007, 2008), but not METH-pretreated (Daberkow et al., 2008), rats, suggesting that although METH-pretreated rats perform as well as normal rats on the reversal-learning task, they may rely on different brain circuitry to perform the task (Daberkow et al., 2008; Pastuzyn et al., 2012). To test this hypothesis, we looked for correlations between *Arc* mRNA expression in different brain regions and reversal learning in METH-pretreated rats relative to controls. We found a significant correlation in the nucleus accumbens (NAc) shell

in METH-pretreated rats that did not exist in saline-pretreated rats. Further, disruption of *Arc* signaling in the NAc shell of METH-, but not saline-, pretreated rats impaired consolidation of the reversal learning. Taken together with our previously published observations (Pastuzyn et al., 2012), these data suggest that METH-induced neurotoxicity is associated with reorganization of neural circuitry engaged in a learning and memory task typically dependent on DMS, and that correlations between *Arc* mRNA expression in brain regions and behavioral performance may be a viable *ex vivo* approach for mapping neural circuitry engaged in learning and memory tasks.

Materials and Methods

Animals. Male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC, USA; 275-300 g) were singly housed in tub cages on a 12:12 hr light cycle. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Utah and followed the *Guide for the Care and Use of Laboratory Animals*.

Methamphetamine pretreatment. Rats were treated with a neurotoxic regimen of (±)-METH-HCl (4x10 mg/kg, free base, at two-hour intervals, s.c.; NIDA, Research Triangle Park, NC, USA) over the course of one day as previously described (Daberkow et al., 2008; Son et al., 2013). The day after treatment, rats were returned to their home cages and given free access to food and water until training began.

Dopamine and serotonin transporter autoradiography. DAT and SERT autoradiography was performed as detailed previously (Boja et al., 1992; Barker-Haliski et al., 2012b; Pastuzyn et al., 2012; Son et al., 2013). For striatal sections, the buffer contained fluoxetine (Sigma-Aldrich, St. Louis, MO, USA) to block binding to the SERT, whereas for prefrontal cortical (PFC) sections, fluoxetine was omitted from the buffer, as PFC sections incubated in buffer containing fluoxetine showed no staining (data not shown). Slides were apposed to film (Kodak Biomax MR film; Eastman Kodak, Rochester, NY, USA) for 24 hr.

Arc *correlations with response-reversal learning: Reversal-learning task.* Response-reversal learning on a T-maze was conducted as previously described (Daberkow et al., 2007; Barker-Haliski et al., 2012a; Pastuzyn et al., 2012). Beginning three weeks after METH pretreatment, rats (METH-pretreated, *n*=9; saline-pretreated, *n*=10) were food restricted and habituated to the food reward and maze. The turn bias of each rat was determined, followed by acquisition training for three days and then reversal learning. During reversal learning, rats had to turn in the opposite direction from acquisition to receive the reward. The criterion for learning on both acquisition and reversal tasks was 9/10 correct turns in a row. Five minutes after reaching criterion on reversal, rats were exposed to CO_2 for one minute and then sacrificed by decapitation. Brains were quickly removed, flash frozen in 2-methylbutane (Mallinckrodt Baker, Phillipsburg, NJ, USA) on dry ice, and stored at -80°C until sectioning.

Radioactive in situ *hybridization histochemistry.* Frozen brains were sectioned (12-μm; Cryocut 1800; Leica, Wetzlar, Germany). Sections from PFC

(mm from Bregma: +3.7 to +2.2), striatum (+1.6 to -0.92 mm), and dorsal hippocampus (-2.3 to -3.6 mm) were thaw-mounted onto Superfrost Plus slides (VWR, Aurora, CO, USA) and stored at -20°C. Infusion cannula placements were determined by eye at this time and recorded on schematic diagrams from a rat brain atlas (Paxinos and Watson, 1998).

To assess *Arc* mRNA expression, slides containing striatal, PFC, or hippocampal sections were postfixed and delipidated as previously described (Ganguly and Keefe, 2001). Detection of *Arc* mRNA was accomplished using a full-length ribonucleotide probe (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b; Howard et al., 2013b). The plasmid containing the cDNA for *Arc* (Lyford et al., 1995) was linearized with *Eco*RI. The antisense ribonucleotide probe was transcribed using ^{35}S-UTP (striatum) or ^{33}P-UTP (PFC and hippocampus) (PerkinElmer, Waltham, MA, USA) and T7 RNA polymerase (Roche, Indianapolis, IN, USA). Radioactive *in situ* hybridization was performed as previously described, with slightly modified final washing procedures (Ganguly and Keefe, 2001). Slides were apposed to film (Biomax MR) for four-six days.

Image analysis. Densitometric analysis of digitized film images was conducted using NIH ImageJ software, yielding background-subtracted average gray values in several brain regions from one hemisphere of each of the four sections on the slide. The regions analyzed were: cingulate (Cg1), prelimbic (PLC), infralimbic (ILC), ventral orbitofrontal (vOFC), and lateral orbitofrontal (lOFC) cortices; DMS and dorsolateral striatum (DLS) and nucleus accumbens (NAc) core and shell; CA1, CA3, upper and lower blades of dentate gyrus (DG),

and hilus of the dorsal hippocampus. For cortical regions, all cortical layers were analyzed.

Effect of Arc *antisense oligonucleotide infusion in nucleus accumbens shell.* Two weeks after saline (*n*=11) or METH (*n*=13) pretreatment, rats were anesthetized with ketamine/xylazine (90/10 mg/kg, i.p.) and placed in a stereotaxic instrument (Stoelting Co., Wood Dale, IL, USA). A dual 26-gauge guide cannula (Plastics One, Roanoke, VA, USA) was lowered to end bilaterally just dorsal to NAc shell (mm from Bregma: +2.2 AP; ±1.0 ML; -6.4 DV) and secured. Bilateral infusions into NAc shell during behavioral experiments were made through 33-gauge infusion cannulae extending 1.1 mm beyond the end of the guides into NAc shell.

The reversal task was the same as described above, except that two hours before undergoing reversal learning, either an *Arc* antisense oligonucleotide, *Arc* nonsense oligonucleotide, or 0.1M PBS (vehicle) was infused into NAc shell. The *Arc* antisense and nonsense oligonucleotides were prepared and infused (1 µL of oligonucleotide; 1 nmol/µL in 0.1M PBS, pH 7.4 or PBS) at 0.33 µL/min bilaterally into NAc shell, as previously described for DMS (Pastuzyn et al., 2012). The design of the oligonucleotides was based on the prior work of Guzowski and colleagues (2000). Further, the concentration of oligonucleotide and volumes and rates of infusion were also based on that work, as well as that of other labs showing restricted delivery of the antisense oligonucleotide to specific brain regions, including the nucleus accumbens core, lateral amygdala, and anterior cingulate cortex (Ploski et al., 2008; Holloway and

McIntyre, 2011; Lv et al., 2011). Postinfusion, rats rested in their home cages for two hours, were trained to criterion (9/10 correct trials) on reversal, and were returned to their home cages overnight. “Reversal retention” occurred 24 hr later, during which rats were rewarded for turning in the reversal direction learned the previous day, until criterion (9/10 correct trials) was reached. No further infusions were made on the reversal-retention day.

Statistical analysis. Unpaired *t*-tests were used to compare RTI-55 autoradiographic signals and trials to criterion on the reversal-learning task for the saline and METH pretreatment groups on which *ex vivo* analysis of *Arc* mRNA expression was completed. Trials to criterion on the reversal-learning task were also correlated with *Arc* mRNA expression. A two-factor MANOVA (pretreatment x day) followed by *post hoc* analysis with paired *t*-tests across acquisition days was used to assess any effect of METH pretreatment on trials to criterion on the three days of response acquisition. A two-way ANOVA was used to evaluate the effects of pretreatment (saline or METH) and treatment (infusion of *Arc* antisense, *Arc* nonsense, or PBS) on trials to criterion on the reversal-learning and reversal-retention tasks. All statistical tests were run using JMP v.9.0 (SAS Institute Inc., Cary, NC, USA).

Results

DAT and SERT autoradiography. The administration of METH resulted in significant reductions in striatal and NAc DAT binding (Fig. 3.1a). DAT depletions did not differ between rats used for *ex vivo* analysis of *Arc* mRNA expression and

those used to examine the effects of *Arc* antisense oligonucleotide infusion into NAc shell, as two-way ANOVA revealed no main effect of "group" (*ex vivo Arc* or *Arc* antisense group: DMS, p=0.99; DLS, p=0.7; NAc core, p=0.97; NAc shell, p=0.5) and no group x pretreatment (METH or saline) interaction in DMS (p=0.8), DLS (p=0.9), NAc core (p=0.97), or NAc shell (p=0.5). There were main effects of pretreatment in all four striatal regions. Unpaired, one-tailed *t*-tests revealed a significant decrease in DAT binding in rats pretreated with METH in DMS (t=12.1, p=0.0001), DLS (t=9.2, p=0.0001), NAc core (t=5.0, p=0.0001), and NAc shell (t=3.3, p=0.002).

Pretreatment with the binge regimen of METH also resulted in reductions in SERT binding in PFC (Fig. 3.1b). As with DAT, SERT depletions were not significantly different between the two groups of rats, so the data were collapsed for the purpose of this analysis (no effect of group or group x pretreatment interaction, respectively, in Cg1 (p=0.5; p=0.5), PLC (p=0.3; p=0.3), ILC (p=0.4; p=0.4), vOFC (p=0.6; p=0.6), or lOFC (p=0.4; p=0.4)). Unpaired, one-tailed *t*-tests revealed significant decreases in SERT binding in all regions of PFC examined in METH-pretreated rats: Cg1, t=9.1, p=0.0001; PLC, t=6.1, p=0.0001; ILC, t=3.96, p=0.0003; vOFC, t=10.4, p=0.0001; lOFC, t=8.8, p=0.0001.

Effect of METH-induced neurotoxicity on trials to criterion for acquisition and reversal of response learning. As previously reported (Daberkow et al., 2008; Pastuzyn et al., 2012), there was no effect of METH pretreatment on acquisition of the response-learning task ($F_{(1,17)}$=0.2, p=0.6) and no pretreatment x acquisition day interaction ($F_{(2,16)}$=0.3, p=0.7). There was a main effect of

acquisition day ($F_{(2,16)}$=8.4, p=0.03), with the rats overall taking significantly fewer trials to reach criterion on the third day of acquisition relative to both the second (t=-3.2, p=0.003) and first (t=-3.7, p=0.0008) days (data not shown). Rats with METH-induced monoamine depletions also did not differ from saline-pretreated controls in the numbers of trials to criterion on the reversal day (t=-0.9, p=0.4; data not shown).

Arc *mRNA expression*. Analysis of the film autoradiograms revealed no significant differences between the levels of *Arc* mRNA expression in METH- vs. saline-pretreated rats in DMS (t=0.2, p=0.8), DLS (t=0.2, p=0.8), NAc core (t=0.5, p=0.6), or NAc shell (t=1.6, p=0.1).

As in striatum, there was no significant effect of METH pretreatment on the levels of *Arc* mRNA expression as reflected in the radioactive *in situ* hybridization signal in Cg1 (t=-1.1, p=0.3), PLC (t=-1.2, p=0.2), ILC (t=0.2, p=0.8), vOFC (t=-0.9, p=0.4), or lOFC (t=-0.8, p=0.4). In hippocampal subregions, there were also no significant differences between the intensity of the *Arc* mRNA signals in the METH- vs. the saline-pretreated rats in CA1 (t=-1.6, p=0.1), upper blade of the DG (t=-1.5, p=0.1), and lower blade of the DG (t=1.1, p=0.3). However, the intensity of the *Arc* mRNA *in situ* hybridization signal was significantly greater in METH- vs. saline-pretreated rats in CA3 (t=-2.5, p=0.02) and hilus (t=-4.3, p=0.0007).

Previous work suggests that although *Arc* mRNA expression is induced in multiple brain regions in animals learning a particular behavior, the degree of that induction in a given brain area only correlates with measures of learning if that

brain area is involved in the learning (Guzowski et al., 2001; Daberkow et al., 2007). Consequently, we did not include a caged control group in the present studies, because our prior work showed that reversal learning induces *Arc* throughout the brain (Daberkow et al., 2007), and we were testing whether there was a correlation between *Arc* in various brain regions and behavior, not whether there simply was an induction of *Arc*. Furthermore, our prior work suggests that rats with METH-induced monoamine depletions no longer rely on “normal” striatal circuitry for response-reversal learning (Daberkow et al., 2008; Pastuzyn et al., 2012). Therefore, we used *ex vivo* analysis of *Arc* mRNA expression across multiple brain regions that might be involved in reversal learning in an attempt to reveal neural substrates being used by the METH-pretreated rats as they learned the reversal response.

Although PFC has been implicated in reversal learning (for review, see Ragozzino, 2007), we found no significant correlations in either saline- or METH-pretreated rats between *Arc* mRNA expression in PFC regions and trials to criterion on the reversal-learning task (Table 3.1). We further speculated that the METH-pretreated rats might be relying on a spatial strategy to solve the reversal task, and thus looked for correlations between *Arc* mRNA expression in hippocampal subregions and trials to criterion. Again, no significant correlations were found in either saline- or METH-pretreated rats (Table 3.1).

In contrast to the lack of significant correlations in the PFC and hippocampus, significant correlations were apparent in striatum, and the region in which the correlations were observed varied as a function of METH pretreatment.

As previously reported (Daberkow et al., 2007, 2008), *Arc* mRNA expression in DMS, but not DLS, was significantly negatively correlated with trials to criterion on the reversal-learning task in saline-pretreated rats (Fig. 3.2). No such significant correlation (p=0.9) was apparent for DMS of METH-pretreated rats, again consistent with our prior observations (Daberkow et al., 2008). However, in NAc shell, there was a significant negative correlation between *Arc* mRNA expression and performance in METH-pretreated rats (R^2=0.44, p=0.0497) that was not apparent in saline-pretreated rats (p=0.2). Thus, prior exposure to a neurotoxic regimen of METH is associated with a change in the brain regions in which *Arc* mRNA expression correlates with behavioral performance, suggesting that the METH-pretreated rats might be relying on NAc shell, rather than DM striatum, in this task.

Effect of Arc *antisense on response-reversal learning and its retention.* Prior work from our lab (Pastuzyn et al., 2012) and others (Guzowski et al., 2000; Ploski et al., 2008; Czerniawski et al., 2011; Hearing et al., 2011; Holloway and McIntyre, 2011; Maddox and Schafe, 2011) has shown that disruption of *Arc* in a brain area known to be involved in completion of a particular learning/memory task disrupts consolidation of the memory. Thus, to further examine whether the circuitry mediating response-reversal learning and consolidation of that learning in METH-pretreated rats had shifted to rely on NAc shell, we determined whether local infusion of an *Arc* antisense oligonucleotide into NAc shell had differential effects on retention of the reversal learning in METH- vs. saline-pretreated rats.

Figure 3.2 illustrates the locations of the tips of the infusion cannulae in NAc shell for each rat.

Consistent with our prior observations (Daberkow et al., 2008; Pastuzyn et al., 2012), METH- and saline-pretreated rats did not differ in trials to criterion during the acquisition days (data not shown). Infusion of an *Arc* antisense oligonucleotide did not alter performance on the day of reversal learning (Fig. 3.4a), as a two-way ANOVA on pretreatment (saline, METH) x treatment (*Arc* antisense, *Arc* nonsense, PBS) for trials to reach criterion on the reversal task revealed no significant main effect of pretreatment ($F_{(1,1)}$=0.05, p=0.8) or infusion ($F_{(2,2)}$=1.0, p=0.4) and no significant interaction ($F_{(2,2)}$=0.1, p=0.9).

Rats were tested for retention of the reversal learning 24 hr later. Two-way ANOVA on pretreatment x treatment for trials needed to reach criterion on the reversal-retention test revealed a main effect of pretreatment (Fig. 3.4b; $F_{(1,18)}$=5.29, p=0.03), a trend towards an effect of treatment ($F_{(2,18)}$=2.81, p=0.09), and a significant pretreatment x treatment interaction ($F_{(2,2)}$=6.2, p=0.009). Tukey HSD *post hoc* analysis of the significant interaction revealed that infusion of *Arc* antisense into NAc shell during the reversal learning did not impair retention of the reversal learning in the saline-pretreated rats, as the trials to criterion on the retention day were not different from those in the saline-pretreated rats infused with a nonsense oligonucleotide (p=0.97) or PBS (p=0.995). Conversely, infusion of the *Arc* antisense oligonucleotide into NAc shell did impair retention of reversal learning in the METH-pretreated rats. METH-pretreated rats infused with the *Arc* antisense oligonucleotide during reversal learning took significantly more trials to

reach criterion on the retention test the following day relative to METH-pretreated rats infused with the nonsense oligonucleotide (p=0.01) or PBS (p=0.03), as well as relative to the saline-pretreated rats infused with the *Arc* antisense oligonucleotide (p=0.003). Taken together with our prior results showing that infusion of *Arc* antisense into DMS impairs retention of reversal learning in saline-, but not METH-, pretreated rats (Pastuzyn et al., 2012), the accumulating evidence suggests that the neural circuitry in which consolidation of reversal learning occurs is altered as a consequence of METH-induced neurotoxicity.

Discussion

Previous results suggest that the correlation between *Arc* mRNA expression in a brain region and behavioral performance on a task, rather than the simple presence of gene expression, reflects the necessity of synaptic modifications in that brain region for learning and its consolidation (Guzowski et al., 2001; Daberkow et al., 2007; Hearing et al., 2011; Pastuzyn et al., 2012). The present findings provide additional support for this view by showing again that disruption of *Arc* in a brain region impairs consolidation of learning only if a significant correlation between *Arc* expression in that brain region and behavioral performance was observed. The present work also confirms earlier results showing a loss of the normal correlation between *Arc* mRNA expression in DMS and response-reversal learning in rats with METH-induced neurotoxicity (Daberkow et al., 2008). The present study extends those findings by demonstrating the appearance of a novel correlation between *Arc* expression in

NAc shell and behavioral performance as a consequence of prior METH exposure and subsequent sensitivity of reversal-learning consolidation to infusion of an *Arc* antisense oligonucleotide into NAc shell. These findings suggest that prior neural injury—in this case, METH-induced neurotoxicity—leads to alterations in the neural circuitry engaged when an animal performs a learning and memory task, and that this change in circuitry can be monitored by evaluating the correlation between *Arc* mRNA expression in various brain regions and behavioral performance. This approach may therefore serve as an *ex vivo* imaging approach to interrogate neural circuits engaged in learning and memory tasks and how those circuits are affected by CNS insult.

The reversal learning examined in the present study is typically dependent on the functional integrity of DMS, as infusion of an NMDA receptor antagonist or an antisense oligonucleotide against *Arc* into DMS in normal animals disrupts learning and consolidation of that learning, respectively (Palencia and Ragozzino, 2004; Pastuzyn et al., 2012). Despite this apparent specific role of DMS in response-reversal learning, *in situ* hybridization histochemical staining revealed expression of *Arc* mRNA throughout the brain. Thus, as previously suggested (Guzowski et al., 2001; Daberkow et al., 2007, 2008), evidence is accumulating that it is not simply the presence of *Arc* mRNA induction in a brain region that implicates plasticity processes in that region in the learning/memory formation; rather, it appears to be the *correlation* between *Arc* and the measure of learning that is the hallmark implicating synaptic plasticity processes in a brain

region as being critical for the particular learning being examined and its consolidation.

Evidence that the correlation between *Arc* mRNA expression in a brain region and behavioral performance is the critical dependent measure for using *Arc* mRNA expression to identify brain regions involved in the learning/memory being examined comes from studies using site-specific infusions of antisense oligonucleotides to disrupt *Arc* function. For example, Guzowski and colleagues (2001) reported a significant inverse correlation between hippocampal *Arc* expression and latency to escape in a spatial, but not cued, version of the Morris water maze. Antisense-mediated knockdown of *Arc* in hippocampus during learning on the spatial task impaired memory consolidation, as evidenced by impaired retention of the previously learned spatial location (Guzowski et al., 2000). Likewise, prior work by Hearing and colleagues (2008) revealed a significant correlation between *Arc* mRNA expression in DLS and context-induced lever pressing (cocaine-seeking) during a one-hour extinction test. Infusion of an *Arc* antisense oligonucleotide during that one-hour extinction session impaired consolidation, as evidenced by greater lever pressing in the antisense-infused rats when assessed 24 and 48 hr later (Hearing et al., 2011). We also previously reported that normal rats show a significant inverse correlation between *Arc* mRNA in DMS and trials to criterion on the reversal-learning task (as reported herein and Daberkow et al., 2007, 2008) and that infusion of an *Arc* antisense oligonucleotide into DMS impairs consolidation of reversal learning in those normal animals (Pastuzyn et al., 2012). Importantly, in

METH-pretreated rats the correlation between *Arc* mRNA expression in DMS and reversal learning is lost (as reported herein and Daberkow et al., 2007, 2008), and infusion of an *Arc* antisense oligonucleotide into the DMS does not impair consolidation of the reversal learning in these METH-pretreated rats (Pastuzyn et al., 2012). Similarly, in the present study, infusion of the *Arc* antisense oligonucleotide into NAc shell of normal animals—a brain region in which *Arc* mRNA expression does not correlate with reversal learning—does not impair retention of the learned reversal, even though there is *Arc* expression in this region. Taken together, these findings suggest the interpretation that the correlation between *Arc* in a brain region and the index of learning can be used to map, *ex vivo*, the neural circuitry engaged in the particular learning and memory task.

We have previously reported that the correlation between *Arc* in DMS and reversal learning normally observed in intact rats is lacking in rats with METH-induced neurotoxicity, despite the fact that they have apparently normal response-reversal learning (Daberkow et al., 2008). Therefore, in the present work, we performed a broader evaluation of *Arc* mRNA in different brain regions and reversal learning in METH-pretreated rats. We discovered a novel correlation in METH-pretreated rats between *Arc* in NAc shell and reversal learning. In this case, infusion of *Arc* antisense into NAc shell during the reversal learning impaired consolidation of that learning. Although we did not directly verify that the antisense oligonucleotide-mediated knockdown of *Arc* remained confined to the NAc shell, two lines of evidence suggest that the effect of the antisense

oligonucleotide observed in the METH-pretreated rats is likely due to loss of Arc function in the NAc shell. First, several prior studies have infused biotinylated *Arc* antisense oligonucleotides into specific brain regions similar in size to the NAc shell at concentrations, volumes, and rates of infusion similar to those used here and have reported that the infused oligonucleotide remains restricted to the region in which it was infused (Ploski et al., 2008; Holloway and McIntyre, 2011; Lv et al., 2011). Second, although restriction of infused *Arc* antisense to the NAc shell has not been directly examined, the prior work by Lv and colleagues (2011) reported dissociable effects of *Arc* antisense infusion into the NAc shell vs. core on morphine-induced conditioned place preference. Taken together, these data suggest that infusion of *Arc* antisense oligonucleotide in the present study likely specifically disrupted Arc function in the NAc shell, thereby disrupting reversal learning in the METH-pretreated rats.

The fact that METH-pretreated rats appear to rely on different striatal circuitry to perform the reversal task relative to intact controls is consistent with literature showing differences in neural circuitry activated during learning paradigms between normal individuals and individuals with CNS injury/disease, such as Parkinson's disease (*e.g.*, Moody et al., 2004; Beauchamp et al., 2008; Rieckmann et al., 2010). For example, previous fMRI analysis of Parkinson's disease patients who performed as well as controls on a probabilistic weather prediction task revealed that they activated medial temporal lobe during the task, whereas controls showed normal activation of basal ganglia circuitry (Moody et al., 2004). It is therefore critical to assess not just behavioral performance, but

also the neural circuitry underlying behavioral performance, in order to fully appreciate the impact of CNS insult, as the neural circuitry mediating the behavior may be altered even if gross behavioral performance appears intact.

In the case of the present studies, the basis for this reorganization of task-related processing is unknown, but may be secondary to the METH-induced DA depletions. As confirmed in the present work, exposure to high doses of METH results in partial DA loss (Wagner et al., 1980). This loss is associated with impairment of phasic DA signaling (Howard et al., 2011; Howard et al., 2013a), along with loss of transcriptional activation and normal subcellular distribution of *Arc* mRNA in dorsal striatum (Barker-Haliski et al., 2012b), both of which are critical for synaptic plasticity underlying basal ganglia-mediated learning and memory processes (Calabresi et al., 2007; Schultz, 2007). As previously reported (*e.g.*, Ricaurte et al., 1980; Haughey et al., 1999; Wallace et al., 1999; Johnson-Davis et al., 2002), in the present study, METH-induced DA loss in the NAc, particularly in the shell, was less extensive. Based on previous evidence from our lab, there appears to be a threshold (~40% depletion) necessary for behavioral impairments to be evident (Daberkow et al., 2005). Perhaps the ~25% depletion in NAc shell observed in the present study was insufficient to prevent this brain region from being used by METH-pretreated rats in the behavioral task. While we have observed disruption of DA transients in the NAc core of METH-pretreated rats (Howard et al., 2013a), whether there is less significant disruption of phasic DA signaling in NAc shell at these levels of METH-induced DA loss remains to be determined.

Both DMS and NAc shell are motor outputs for the basal ganglia, and NAc shell is also often touted as being involved in motivated and goal-directed behavior (Ikemoto, 2007; Humphries and Prescott, 2010). Therefore, DA-mediated plasticity may be relatively preserved in NAc shell compared to DMS after METH-induced neurotoxicity, allowing DA-mediated synaptic modifications there to subserve consolidation of response-reversal learning. Furthermore, studies have shown that there are differences in how rostral and caudal NAc shell modulate behavior (Reynolds and Berridge, 2001). Our infusions targeted the rostral NAc shell, and given the differences in behavioral output of rostral and caudal NAc shell, as well as the anatomical inputs/outputs to the two regions (*e.g.*, Usuda et al., 1998; Groenewegen et al., 1999), it will be interesting in future studies to examine the relative contributions of the rostral vs. caudal NAc shell to reversal learning in normal and METH-pretreated rats.

As noted above, the lack of effect of METH pretreatment on the levels of *Arc* mRNA expression in dorsal striatum in the present study appears to be at odds with our prior work showing decreased *Arc* mRNA in such animals (Daberkow et al., 2008; Barker-Haliski et al., 2012b). This apparent difference likely arises from the approaches and, more so, the dependent measures, used in the different studies. In our former studies, we used fluorescent *in situ* hybridization (FISH) and determined the numbers of striatonigral vs. striatopallidal neurons with *Arc* mRNA signal in different subcellular compartments. In the present study, the radioactive *in situ* hybridization signal gives us a broad determination of *Arc* mRNA expression across both populations

of striatal efferent neurons and in all subcellular compartments. Our work with FISH has shown that basal *Arc* mRNA transcription is increased in both striatal efferent neuron populations in rats with METH-induced neurotoxicity, but that the animals do not further induce *Arc* mRNA in response to behavioral activation (Daberkow et al., 2008; Barker-Haliski et al., 2012b). Further, METH-induced DA loss is associated with loss of *Arc* mRNA specifically in the cytoplasm of striatonigral efferent neurons (Daberkow et al., 2008; Barker-Haliski et al., 2012b). It is this latter effect that is apparent in our prior work (Daberkow et al., 2008; Barker-Haliski et al., 2012b), as the dependent measure reported is the number of neurons with *Arc* mRNA signal in the cytoplasm. In the present study, because the radioactive *in situ* hybridization approach incorporates signal in both populations of neurons and in all subcellular compartments, the METH-induced loss of cytoplasmic *Arc* mRNA signal in one subpopulation of neurons is not apparent. That said, what the FISH and radioactive *in situ* approaches have in common is that both reveal the correlation between *Arc* mRNA expression and behavior (findings herein and in Daberkow et al., 2007, 2008).

The results of this study suggest that there is a change, following METH-induced neurotoxicity, in the brain circuitry used in a behavioral task. Further, they provide additional support for the proposition that a correlation between *Arc* mRNA expression in a brain region and the measure of learning on a task implicates synaptic plasticity processes in that brain region as being critical for learning and its consolidation. Since humans with a history of METH abuse also can have partial monoamine loss, they may be forced to rely on potentially “less

ideal" neural circuits to perform particular cognitive tasks, and this lack of normal cognitive processes may contribute to cognitive deficits seen (Dean et al., 2013), especially in more complicated tasks that may require engagement of several brain regions at once. Better understanding the impact of neurotoxicity on synaptic plasticity mechanisms should allow for the development of targeted therapies to address impaired cognitive function in individuals with METH-induced neurotoxicity or others with striatal dopamine loss, such as patients with Parkinson's disease.

The authors declare no conflict of interest.

This work was supported by DA024036 (KAK) and DA032502 (EDP).

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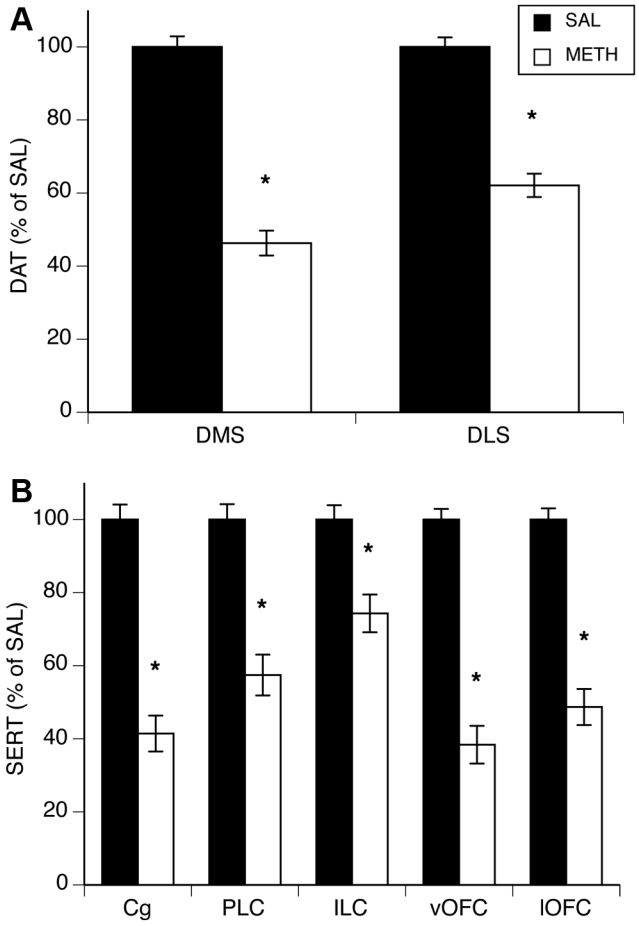

Figure 3.1. METH neurotoxicity results in decreases in DAT and SERT binding. Graphs showing METH-induced decreases in (A) striatal DAT and (B) prefrontal cortical SERT as revealed by [^{125}I]RTI-55 binding. Since DAT and SERT binding between rats in the *Arc* correlation experiment and *Arc* antisense infusion experiment were not significantly different, binding values from both sets of rats are combined into one graph. Saline (SAL)-pretreated, n=21; METH-pretreated, n=22. *Significantly different from SAL, p<0.001.

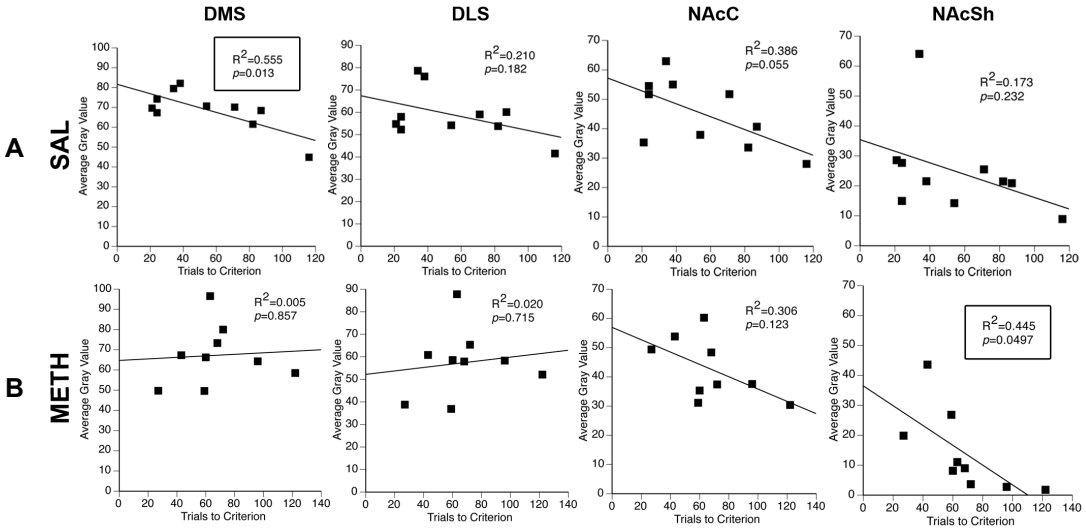

Figure 3.2. Correlations between *Arc* mRNA in striatal subregions and trials to criterion on the response reversal-learning task. *Arc* mRNA expression was determined by densitometric analysis of film autoradiograms using ImageJ and is expressed as background-subtracted average gray values (arbitrary units). Significant correlations (as indicated by box around R^2 and *p* values) were in DM striatum of (A) saline (SAL)-pretreated rats (R^2=0.56, *p*=0.013) and NAc shell of (B) methamphetamine (METH)-pretreated rats (R^2=0.44, *p*=0.0497). METH-pretreated rats were given a neurotoxic regimen of (±)-METH•HCl (4 x 10 mg/kg free base, s.c., at two-hour intervals) approximately seven weeks prior to reversal learning. DMS, dorsomedial striatum; DLS, dorsolateral striatum; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell.

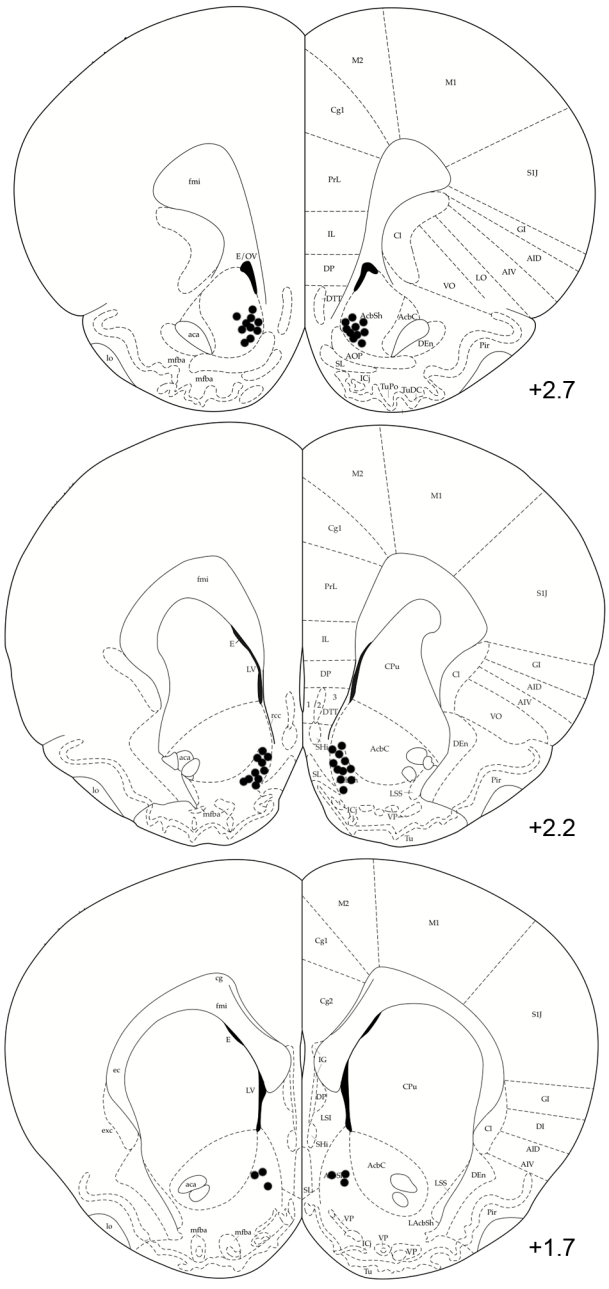

Figure 3.3. Placement of infusion sites in nucleus accumbens shell. Diagrams (modified from Paxinos and Watson, 1998) showing placement of infusion sites (black dots) in the nucleus accumbens shell of rats infused with an *Arc* antisense or *Arc* nonsense oligonucleotide or with PBS. Numbers indicate mm from Bregma.

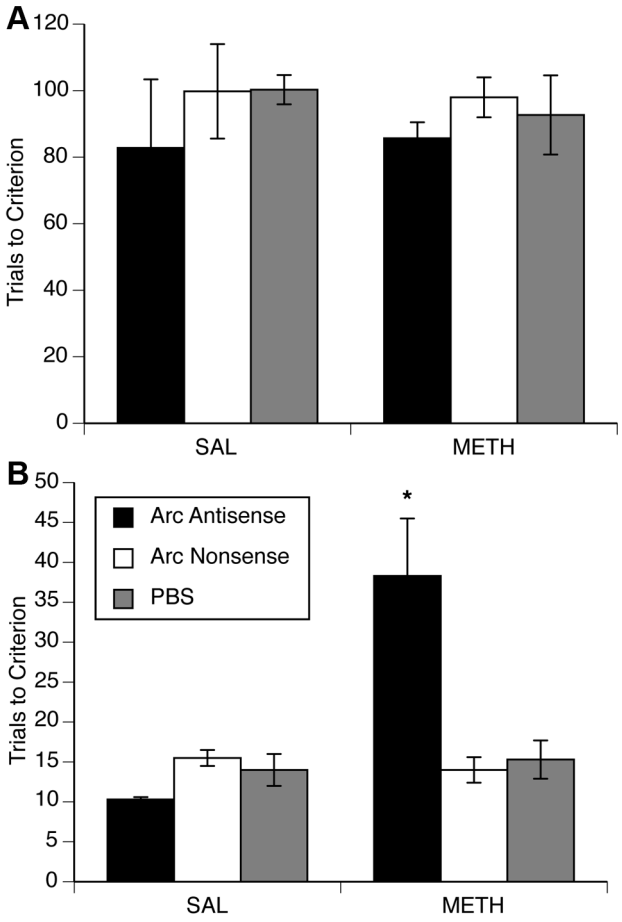

Figure 3.4. Knockdown of *Arc* impairs consolidation of reversal learning in METH-, but not saline-, pretreated rats. Rats were infused with an *Arc* antisense oligonucleotide, *Arc* nonsense oligonucleotide, or PBS into NAc shell two hours prior to response-reversal learning on a T-maze. (A) None of the compounds had any effect on reversal learning in saline- or METH-pretreated rats. (B) Rats were tested on reversal retention 24 hr after reversal learning. Knockdown of *Arc* mRNA in NAc shell via an *Arc* antisense oligonucleotide impaired reversal retention in METH-, but not saline-, pretreated rats. Values are average trials to criterion (9/10 correct consecutive trials; ±SEM, *n*=3-6/group) on the reversal-learning task (A) or on the reversal-retention test 24 hr later (B). *Significantly different from all other groups, all *p* values<0.05.

Table 3.1. Correlations between *Arc* mRNA expression in subregions of prefrontal cortex and hippocampus and trials to criterion on the response reversal-learning task. Values are R^2 and respective *p* values obtained from multivariate analysis of the relation between *in situ* hybridization histochemical staining for *Arc* mRNA in each brain region and trials to criterion on a response reversal-learning task for rats pretreated with saline (4 x 1 mL/kg at two-hour intervals, *n*=10) or a neurotoxic regimen of (±)-METH (4 x 10 mg/kg, at 2-hr intervals, *n*=9) at least seven weeks prior to testing and sacrifice. Cg, cingulate cortex; PLC, prelimbic cortex; ILC, infralimbic cortex; vOFC, ventral orbitofrontal cortex; lOFC, lateral orbitofrontal cortex; CA1, *Cornu Ammonis* 1; CA3, *Cornu Ammonis* 3; upper DG, upper blade of dentate gyrus; lower DG, lower blade of dentate gyrus.

Brain Region	Saline-pretreated		METH-pretreated	
	R^2	*p*-value	R^2	*p*-value
Prefrontal Cortex				
Cg	0.03	0.7	0.03	0.6
PLC	0.08	0.4	0.0002	0.97
ILC	0.2	0.1	0.003	0.9
vOFC	0.01	0.8	0.001	0.9
lOFC	0.001	0.9	0.05	0.6
Hippocampus				
CA1	0.08	0.4	0.01	0.8
CA3	0.03	0.6	0.4	0.07
Upper DG	0.002	0.9	0.07	0.5
Lower DG	0.001	0.9	0.1	0.3
Hilus	0.06	0.5	0.2	0.3

CHAPTER 4

PHASIC-LIKE STIMULATION OF THE MEDIAL FOREBRAIN BUNDLE AUGMENTS STRIATAL GENE EXPRESSION DESPITE METHAMPHETAMINE-INDUCED PARTIAL DOPAMINE DENERVATION

Abstract

Methamphetamine-induced partial dopamine depletions are associated with impaired basal ganglia function, including decreased *preprotachykinin* mRNA expression and impaired transcriptional activation of activity-regulated, cytoskeleton-associated (*Arc*) gene in striatum. Recent work implicates deficits in phasic dopamine signaling as a potential mechanism linking methamphetamine-induced dopamine loss to impaired basal ganglia function. The present study thus sought to establish a causal link between phasic dopamine transmission and altered basal ganglia function by determining whether the deficits in striatal neuron gene expression could be restored by increasing phasic dopamine release. Three weeks after pretreatment with saline or a neurotoxic regimen of methamphetamine, rats underwent phasic- or tonic-like stimulation of ascending dopamine neurons. Striatal gene expression was examined using *in situ*

hybridization histochemistry. Phasic-like, but not tonic-like, stimulation induced immediate-early genes *Arc* and *zif268* in both groups, despite the partial striatal dopamine denervation in methamphetamine-pretreated rats, with the *Arc* expression occurring in presumed striatonigral efferent neurons. Phasic-like stimulation also restored *preprotachykinin* mRNA expression. These results suggest that disruption of phasic dopamine signaling likely underlies methamphetamine-induced impairments in basal ganglia function, and that restoring phasic dopamine signaling may be a viable approach to manage long-term consequences of methamphetamine-induced dopamine loss on basal ganglia functions.

Introduction

Methamphetamine (METH) is an addictive psychostimulant that is neurotoxic to dopamine (DA) neurons. Markers of DA innervation in both the dorsal and ventral striatum are reduced in brains of chronic METH abusers (Wilson et al., 1996), and individuals with a history of hospitalization associated with METH use are more likely to develop Parkinsonism (Callaghan et al., 2012). Thus, it is likely that some proportion of individuals who abuse METH will experience a period of partial DA loss as a consequence of their METH abuse prior to the development of Parkinsonism. Prior studies have suggested a relation between METH-induced DA loss and cognitive impairment in individuals with a history of METH abuse, as reduction of dopamine transporter (DAT) binding in the caudate-putamen correlates with motor and cognitive impairments

in METH users (Volkow et al., 2001). Furthermore, METH use is associated with cognitive decline, as executive functions, learning, and memory, which are dependent on intact striatal circuitry (Brown et al., 1997; Packard and Knowlton, 2002), are impaired in METH users (Scott et al., 2007; Dean et al., 2013, but see Hart et al., 2012). Similarly, in rodents, exposure to METH causes significant striatal DA denervation (Hotchkiss and Gibb, 1980; Ricaurte et al., 1980; Ricaurte et al., 1982). Associated with this depletion are learning impairments, including impaired recognition of novel stimuli (Schröder et al., 2003; Marshall et al., 2007; Herring et al., 2008; O'Dell et al., 2011), impaired motor-sequence learning (Chapman et al., 2001; Daberkow et al., 2005), and altered reversal learning (Izquierdo et al., 2010; Pastuzyn et al., 2012). Despite the association between METH-induced DA denervation and learning impairments, the molecular mechanisms linking these phenomena are not fully understood.

Approximately 95% of striatal neurons are GABAergic medium spiny neurons (MSNs), which are found in two roughly equal subtypes. Striatonigral (“direct pathway”) MSNs express D1 DA receptors, as well as the neuropeptides substance P (and its *preprotachykinin* (*ppt*) precursor) and dynorphin (Gerfen et al., 1990; Surmeier et al., 1996). Striatopallidal neurons (“indirect pathway”) express D2 DA receptors, as well as the neuropeptide enkephalin (and its *preproenkephalin* (*ppe*) precursor) (Gerfen et al., 1990; Le Moine et al., 1990; Surmeier et al., 1996). Partial striatal DA loss induced by either METH or 6-hydroxydopamine (6-OHDA) results in a reduction in basal *ppt* expression in striatum, but no change in *ppe* expression (Nisenbaum et al., 1996; Chapman et

al., 2001; Johnson-Davis et al., 2002). Furthermore, METH-induced neurotoxicity is associated with loss of *Arc* (activity-regulated, cytoskeleton-associated gene) transcription in response to behavioral activation (Daberkow et al., 2008; Barker-Haliski et al., 2012a), and only the impairment in the numbers of striatonigral neurons with *Arc* mRNA in the cytoplasm correlates significantly with the degree of METH-induced striatal DA loss (Barker-Haliski et al., 2012a). Taken together, these data suggest that partial DA loss, such as that induced by METH, affects striatal efferent neuron function and that this dysfunction may predominantly affect striatonigral neurons.

METH-induced neurotoxicity selectively impairs phasic-like DA signaling (Howard et al., 2011), which is thought to preferentially affect striatonigral neurons (Chergui et al., 1997; Gonon, 1997; Onn et al., 2000). METH-induced partial DA depletion is associated with diminished amplitude of DA signals evoked using phasic-like stimulation of the DA neurons ascending through the medial forebrain bundle (MFB) (Howard et al., 2011), as well as the amplitude of endogenous, spontaneously occurring phasic DA transients (Howard et al., 2013). On the other hand, tonic DA levels, which are thought to be sufficient to activate D2 receptors expressed by striatopallidal neurons (Richfield et al., 1989; Dreyer et al., 2010), are not disrupted following METH pretreatment, as assessed by microdialysis (Cass and Manning, 1999). Interestingly, enhancing phasic-like, but not tonic-like, DA signaling via electrical stimulation of DA neurons results in increased *zif268* expression in D1 DA receptor-containing striatonigral neurons

(Chergui et al., 1997). Therefore, METH-induced dysfunction in direct pathway MSNs may be due to impairments in phasic, but not tonic, DA signaling.

If reduced phasic DA signaling is related to the gene expression deficits observed following METH-induced partial DA denervation, then augmenting phasic DA signaling should restore normal gene expression in the partially DA denervated striatum. We tested this hypothesis by pretreating rats with a neurotoxic regimen of METH and then, three weeks later, stimulating DA neurons ascending through the MFB in either a phasic- or tonic-like pattern. We then assessed striatal expression of *ppt* and *ppe* and the immediate-early genes (IEGs) *Arc* and *zif268*. We show that phasic-like stimulation increases IEG expression in both METH- and saline-pretreated rats and that the increase in *Arc* mRNA expression is preferentially in direct pathway neurons. Furthermore, the phasic-like stimulation restores *ppt* expression in METH-pretreated rats without altering *ppe* expression. These findings suggest that restoring phasic DA signaling may ameliorate basal ganglia dysfunction arising consequent to partial DA depletion, such as that induced by METH.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (*n*=30, 240-350 g at time of pretreatment) were purchased from Harlan (Indianapolis, IN, USA) and housed in a light- and temperature-controlled vivarium. Access to food and water was provided *ad libitum*. All procedures conformed to the NIH *Guide for the Care and*

Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Illinois State University.

Drugs. (±)-Methamphetamine hydrochloride was provided by the National Institute on Drug Abuse (Rockville, MD, USA). METH doses were calculated as free base. All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

METH pretreatment. The "binge" neurotoxic METH regimen was conducted as previously described (Howard et al., 2011). Briefly, animals were housed in plastic tub cages (50 cm length X 40 cm width X 20 cm height: four rats/cage). METH was dissolved in 0.9% saline and all injections were made subcutaneously. Four injections of either METH (7.5 mg/kg) or saline were administered at two-hour intervals. Temperature was monitored rectally using a Thermalert TH-5 (Physitemp, Clifton, NJ, USA) prior to, immediately after the first injection, and every hour thereafter, continuing two hours after the final injection of METH. Health was assessed at least every hour, and if rats showed signs of overheating, they were placed in a separate tub on ice for ~10 min.

Electrical stimulation and in vivo *voltammetry.* Three weeks after METH or saline pretreatment, animals to be stimulated were anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tajunga, CA, USA). Four holes were drilled into the skull to allow for lowering of the stimulating electrode, two carbon fiber microelectrodes (CFM), and a Ag/AgCl reference electrode. The twisted, bipolar stimulating electrode (Plastics One, Roanoke, VA, USA) was placed dorsal to the MFB (-4.6 AP; +1.4 ML; -7.0 DV)

(Paxinos and Watson 1986) and was incrementally lowered until a robust DA signal was recorded in the striatum at the CFMs placed in the dorsomedial (DM) and dorsolateral (DL) striatum (+1.2 AP; +1.0 and +4.0 ML, respectively; -4.5 DV; Paxinos and Watson 1986) at 6^{o} angles to allow side by side ipsilateral placement. Changes in DA concentration were recorded using fast-scan cyclic voltammetry (FSCV), where a triangular waveform (-0.4 V to 1.3 V and back at 400 V/s) was applied to the tip of the CFM every 100 ms (Cahill et al., 1996) using an EI400 bipotentiostat (Ensman Instruments, Bloomington, IN, USA) that was computer-controlled using TH-1 software (ESA, Chelmsford, MA, USA). Current recorded at each CFM was converted to concentration using *in vitro* calibration immediately following experiments (Logman et al., 2000). The purpose of recording with FSCV at a CFM in this experiment was to identify DA neurons ascending through the MFB for stimulation with phasic- and tonic-like pulse trains, and to ensure optimal stimulating electrode placement. Nonstimulated control rats were anesthetized with urethane, but did not undergo surgical manipulations or any stimulation.

Following optimization of the stimulating electrode placement, no stimulation was given for one hour to reduce optimization- and handling-induced striatal gene expression (Daberkow et al., 2007). The experimental stimulation protocol was then begun. Electrical stimulation was optically isolated (NL 800, Neurolog, Medical Systems, Great Neck, NY, USA) and synchronized with FSCV recordings. The stimulation protocol was chosen based on previous work noting enhanced expression of mRNA for the IEG *zif268* following phasic-like (“burst”),

but not tonic-like (“regular”), stimulation (Chergui et al., 1997). Stimulation was comprised of constant-current, biphasic pulses (2 ms and 300 μA each phase) and was delivered in either a phasic-like (5 pulses at 30 Hz repeated every 1 s for 60 s; *n*=5 METH-pretreated; *n*=5 saline-pretreated) or tonic-like (300 pulses at 5 Hz; *n*=5 METH-pretreated; *n*=5 saline-pretreated) pattern. Stimulation trains were 60 s in duration and were repeated a total of 15 times, with 2 min separating each stimulation train, for a total stimulation session time of 45 min. Immediately following stimulation sessions, animals were euthanized and brains were rapidly extracted. Nonstimulated control rats were sacrificed 180 min after being anesthetized with urethane (*n*=5 METH-pretreated; *n*=5 saline-pretreated). Brains were flash frozen in 2-methylbutane (EMD Millipore, Billerica, MA, USA) on dry ice and stored at -80^{o}C.

Dopamine transporter (DAT) autoradiography. To determine the extent of METH-induced DA depletions, frozen brains were sectioned (12-μm coronal sections) using a cryostat (Cryocut 1800; Leica, Wetzlar, Germany). Sections were thaw-mounted onto Superfrost Plus (VWR, Aurora, CO, USA) slides. Slides were stored at -20°C until needed. DA transporter (DAT) autoradiography was performed as detailed previously by others and us (Boja et al., 1992; O'Dell et al., 2011; Pastuzyn et al., 2012). Briefly, slides were incubated in buffer containing fluoxetine to block radioligand binding to the serotonin transporter in striatum. Slides then were incubated in the continued presence of fluoxetine with [^{125}I]RTI-55 (PerkinElmer, Waltham, MA, USA). Slides were then rinsed, dried under a

stream of warm air, and exposed to film (Kodak Biomax MR; Eastman Kodak, Rochester, NY, USA) for 24 h before being developed.

Radioactive in situ *hybridization.* To assess striatal IEG expression, frozen slides containing striatal sections were postfixed and delipidated as previously described (Ganguly and Keefe, 2001). As detailed previously, detection of *ppt* (Chapman et al., 2001; Johnson-Davis et al., 2002; Horner et al., 2005), *ppe* (Ganguly and Keefe, 2000; Chapman et al., 2001; Ganguly and Keefe, 2001; Daberkow et al., 2007), *zif268* (Keefe and Adams, 1998), and *Arc* (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012a) mRNAs was accomplished using ribonucleotide probes. Antisense ribonucleotide probes were transcribed from linearized plasmids using ^{35}S-UTP (PerkinElmer) and SP6 (*ppe* and *ppt*) or T7 (*zif268* and *Arc*) RNA polymerases (Roche, Indianapolis, IN, USA). The radioactive *in situ* hybridization was performed as previously described (Ganguly and Keefe, 2001) with slight modifications to final washing procedures. The last four washes on the second day were either at 55°C (*Arc* and *ppt*) or room temperature (*ppe* and *zif268*). Slides were dipped in ddH_2O, air-dried, and exposed to film. Exposure times were: *ppt*, *zif268*, and *Arc*, two weeks; *ppe*, three days.

Fluorescent in situ *hybridization.* To assess pathway-specific expression of *Arc* mRNA, slides were postfixed and delipidated as for radioactive *in situ* hybridization above. As previously described (Daberkow et al., 2007; Barker-Haliski et al., 2012a), expression of *Arc* and *ppe* mRNAs in striatum was determined by performing double-label fluorescent *in situ* hybridization (FISH)

using probes directed against *Arc* and *ppe* mRNAs. *Arc* and *ppe* antisense ribonucleotide probes were synthesized using digoxigenin-UTP (DIG-UTP) and fluorescein-UTP (FITC-UTP) with T7 and SP6 RNA polymerases using DIG or FITC labeling kits (Roche), respectively. Slides were hybridized and detected as previously described (Daberkow et al., 2007, 2008; Barker-Haliski et al., 2012b), except that a 1:50,000 solution of SYTOX Green (Molecular Probes; Life Technologies, Grand Island, NY, USA) was used as a nuclear stain instead of DAPI. As controls, a set of slides was run in parallel either without ribonucleotide probes or without antibodies. Lack of signal on these slides was taken as evidence of the specificity of the *in situ* hybridization histochemical labeling.

Image analysis. Films from DAT autoradiography and radioactive *in situ* hybridization histochemistry were developed, and images were digitized using a video camera (CCD72S; Dage-MTI, Michigan City, IN, USA) and fiber optic light box. The intensity of the light was adjusted so that it fell within the linear range of the camera, as determined by a photographic step tablet (Eastman Kodak Co.). Densitometric analysis of the digitized images was then accomplished using NIH ImageJ software, yielding average gray values in DM and DL striatum for both hemispheres. Two rostral (+1.6 mm from Bregma) and two middle striatal sections (+0.7 mm from Bregma) were analyzed per rat. For each hemisphere, the average gray value of the corpus callosum was subtracted from the average gray value of DM and DL striatum to correct for background staining. Decreases in DAT in METH-pretreated rats were calculated as a percent of DAT levels in saline-pretreated rats.

FISH images were collected using a Leica DM4000B automated upright microscope (63X oil immersion objective) connected to a Leica EL6000 external light source with a mercury metal halide bulb and a Leica DFC300 FX digital color camera. Surveyor computer software (Objective Imaging Ltd.; Cambridge, UK) was used to control the automated stage, perform multichannel scanning, and capture the fluorescent images. A 2 x 2 montage (0.38 mm^2) was captured in the area of striatum with the most *Arc* expression, as identified from the film autoradiograms of *Arc* mRNA expression. In the case of rats with no apparent stimulation-induced *Arc* mRNA expression on the film autoradiograms, montages of FISH staining were captured in DL striatum, as DL striatum was the location where the majority of rats stimulated in a phasic-like manner showed *Arc* expression. The total numbers of *Arc*-positive/*ppe*-negative (*i.e.*, presumed striatonigral neurons) and *Arc*-positive/*ppe*-positive (*i.e.*, striatopallidal neurons) neurons in each image were counted by an experimenter blinded to the treatment groups. Furthermore, the average signal intensity of *Arc* expression in each individual neuronal population (striatonigral and striatopallidal) was measured in ImageJ by first individually outlining all *Arc*-positive/*ppe*-positive neurons in an image and measuring signal intensity in those cells. Then, the *Arc*-positive/*ppe*-negative neurons in the image were outlined and the average *Arc* signal intensity of those neurons was measured.

Statistical analysis. Rectal temperatures were compared using repeated measures MANOVA with pretreatment as a factor and time as a repeated measure. *Post hoc* one-way ANOVAs with Tukey-Kramer HSD tests were used

to further interrogate the significant pretreatment x time interaction for the body-temperature data. For radioactive *in situ* hybridization and DAT autoradiography, average gray values were compared between pretreatment (METH or saline) and stimulation (no stimulation, tonic, or phasic) groups by two-way ANOVA. For FISH, the numbers of *Arc*-positive/*ppe*-negative and *Arc*-positive/*ppe*-positive cells were compared between pretreatment and stimulation groups by two-way ANOVA. The relative amount of *Arc* signal in the striatal neuron populations was also examined by calculating a "difference score," which was the average gray value of the *Arc*-positive/*ppe*-negative population minus the average gray value of the *Arc*-positive/*ppe*-positive population for each animal. This "difference score" was compared between pretreatment and stimulation groups by two-way ANOVA. *Post hoc* Tukey-Kramer HSD tests were performed when ANOVA revealed significant interactions or main effects. Statistical tests were performed using JMP (v. 9.0) or SAS (v. 9.3) software.

Results

METH pretreatment and DAT autoradiography. A treatment x time repeated measures MANOVA revealed that the METH "binge" pretreatment paradigm differentially altered body temperature across METH- and saline-treated rats (Fig. 4.1a; significant effect of time, $F_{(7,30)}=4.45$, $p=0.0032$; significant effect of treatment $F_{(1,30)}=53.43$, $p<0.0001$; significant time x treatment interaction, $F_{(7,30)}=35.20$, $p<0.0001$). *Post hoc* analysis revealed that METH-treated animals were significantly hyperthermic relative to saline-treated animals

two hours after METH injection and every hour thereafter (p<0.001 at 120-420 min after METH). METH pretreatment resulted in significant decreases in striatal DAT three to five weeks later, as assessed by [^{125}I]RTI-55 autoradiography (Fig. 4.1b-c). A two-way ANOVA revealed a main effect of pretreatment (DM striatum, $F_{(1,24)}$=17.1, p<0.001; DL striatum, $F_{(1,24)}$=20.3, p=0.0001), but no significant main effect of stimulation and no significant pretreatment x stimulation interaction (p>0.05).

Effect of phasic- and tonic-like stimulation of the MFB on Arc *expression.* Stimulation of the MFB resulted in changes in striatal *Arc* expression in both saline- and METH-pretreated rats (Fig. 4.2a). A two-way ANOVA (pretreatment x stimulation) for *Arc* mRNA expression in DM striatum revealed a main effect of stimulation (Fig. 4.2b; $F_{(2,24)}$=5.29, p<0.05), but no significant effect of pretreatment (p>0.05) and no significant stimulation x pretreatment interaction (p>0.05). *Post hoc* analysis of the main effect of stimulation revealed that phasic-like stimulation increased *Arc* mRNA expression in the DM striatum both relative to nonstimulated controls (p=0.03) and rats receiving tonic-like stimulation (p<0.05). However, *Arc* mRNA expression in rats receiving tonic-like stimulation was not different from the nonstimulated control group (p>0.05).

Similarly, in DL striatum, there was a main effect of stimulation (Fig. 4.2b; $F_{(2,24)}$=7.24, p<0.01), but no main effect of pretreatment (p>0.05) and no significant stimulation x pretreatment interaction (p>0.05). *Post hoc* analysis again revealed that phasic-like stimulation increased *Arc* mRNA expression relative to that seen in nonstimulated controls (p<0.01) and rats receiving tonic-

like stimulation (p=0.03). As in DM striatum, there was no significant difference in *Arc* mRNA expression in rats receiving tonic-like stimulation relative to nonstimulated controls (p>0.05). Thus, regardless of whether rats had partial DA loss induced by METH pretreatment, phasic-like activation increased the expression of *Arc* mRNA in DM and DL striatum.

Effect of phasic- and tonic-like stimulation of the MFB on zif268 *expression.* Stimulation of the MFB resulted in changes in striatal *zif268* expression in both saline- and METH-pretreated rats that were similar to those observed for *Arc* (Fig. 4.2c). A two-way ANOVA (pretreatment x stimulation) in DM striatum revealed a main effect of stimulation (Fig. 4.2d; $F_{(2,24)}$=9.46, p<0.001), but no effect of pretreatment (p>0.05) and no significant interaction (p>0.05). *Post hoc* analysis of the main effect of stimulation revealed that *zif268* expression was significantly greater in rats receiving phasic-like stimulation relative to both rats receiving tonic-like stimulation (p<0.001) or no stimulation (p<0.001). The expression of *zif268*, however, was not different between rats receiving tonic-like stimulation and rats receiving no stimulation (p>0.05).

Likewise, in DL striatum, there was a significant main effect of stimulation (Fig. 4.2d; $F_{(2,24)}$=8.12, p<0.01), but no main effect of pretreatment (p>0.05) and no significant interaction (p>0.05). Again, *post hoc* analysis revealed that *zif268* expression in rats receiving phasic-like stimulation was significantly greater than that in rats receiving tonic-like stimulation (p<0.01) or no stimulation (p<0.01). The expression of *zif268* was not significantly different between rats receiving tonic-like stimulation and nonstimulated controls (p>0.05). Thus, as was the case

for *Arc*, phasic- but not tonic-like stimulation of the MFB increased *zif268* expression in both METH- and saline-pretreated rats.

Effects of phasic- and tonic-like stimulation of the MFB on ppt *expression.* We and others (Nisenbaum et al., 1996; Chapman et al., 2001; Johnson-Davis et al., 2002) have previously reported that partial DA loss, such as that induced by METH pretreatment, results in a long-term decrease in *ppt* expression. Consistent with this prior work, in the present study, METH-pretreatment was associated with a decrease in *ppt* mRNA expression (Fig. 4.3a, c, d). Two-way ANOVA of *ppt* mRNA expression in DM striatum revealed a main effect of pretreatment (Fig. 4.3c; $F_{(1,24)}$=4.87, p<0.05), with *ppt* expression being lower overall in METH-pretreated rats. The ANOVA also revealed a main effect of stimulation (Fig. 4.3b; $F_{(2,24)}$=4.6, p<0.05), but no significant interaction (p>0.05). *Post hoc* analysis of the main effect of stimulation revealed that phasic-like stimulation significantly elevated *ppt* mRNA expression in DM striatum relative to rats receiving tonic-like stimulation of MFB (p<0.02).

In DL striatum, there was a trend towards an effect of pretreatment (Fig. 4.3d; $F_{(1,24)}$=3.34; p=0.08) and a main effect of stimulation (Fig. 4.3b; $F_{(2,24)}$=3.67, p<0.05), but no significant interaction (p>0.05). *Post hoc* analysis revealed strong trends indicating that phasic-like stimulation increased *ppt* expression in DL striatum relative to both that seen in rats receiving tonic-like stimulation (p=0.057) and control rats that did not receive stimulation (p=0.084). As with the other genes, *ppt* mRNA expression was not different between rats receiving tonic-like stimulation vs. no stimulation controls (p=0.98).

Effects of phasic- and tonic-like stimulation of the MFB on ppe *expression.* Neither METH pretreatment nor stimulation of the MFB in either a phasic-like or tonic-like pattern resulted in any significant changes in striatal *ppe* mRNA expression in saline- or METH-pretreated rats (Fig. 4.3e-f). Thus, in both DM and DL striatum, there were no significant main effects of pretreatment or stimulation and no significant interactions (all $p>0.05$).

Effects of phasic- and tonic-like stimulation of the MFB on Arc *expression in subpopulations of striatal neurons.* Stimulation of the MFB did not cause any changes in the numbers of *ppe*-negative (presumed striatonigral) and *ppe*-positive (striatopallidal) neurons positive for *Arc* mRNA expression ($p>0.05$; Fig. 4.4a-b). However, it was clear during blinded image analysis that the relative amount of signal for *Arc* mRNA was different between striatonigral and striatopallidal neurons (Fig. 4.4c-f). Thus, we measured the relative average gray value of *Arc* expression in the two efferent neuron populations separately and calculated a “difference score” (see Materials and Methods) reflecting the difference in the average gray value of the *Arc* signal in *ppe*-negative vs. *ppe*-positive neurons. Two-way ANOVA of this "difference score" revealed a significant main effect of stimulation ($F_{(2,24)}=9.3$, $p=0.001$), but no main effect of pretreatment ($p>0.05$) and no significant interaction ($p>0.05$). *Post hoc* analysis of the main effect of stimulation revealed that greater *Arc* mRNA expression in *ppe*-negative neurons was induced by phasic-like stimulation relative to either tonic-like stimulation ($p<0.001$) or no stimulation ($p<0.02$).

Discussion

Consistent with prior reports in the literature, the present results show that partial striatal DA loss, such as that induced by a neurotoxic regimen of METH, is associated with decreased *ppt* mRNA expression in the striatum (Nisenbaum et al., 1996; Chapman et al., 2001; Johnson-Davis et al., 2002). Furthermore, the present results confirm prior work (Chergui et al., 1997) that phasic-like stimulation of the MFB increases *zif268* expression in striatum. Additionally, the present work extends these previous studies by showing that phasic-like stimulation also increases *Arc* and *ppt* mRNA expression in striatonigral neurons and, importantly, that this effect of phasic-like stimulation is effective in driving gene expression in presumed striatonigral efferent neurons even in the setting of partial DA loss induced by METH. Thus, the present data reinforce the idea that phasic-like DA neuron activity appears to selectively affect the function of striatonigral efferent neurons and suggest that sufficient circuitry remains in rats with partial striatal DA loss to restore striatal function by enhancing phasic DA neurotransmission

The present data suggest that augmentation of phasic-like DA neurotransmission in animals with partial striatal DA loss may generally restore the ability of residual DA neurons to regulate striatonigral efferent neuron gene expression. As noted above, partial DA loss, such as that induced by 6-OHDA or a neurotoxic regimen of METH, is associated with an impairment of phasic-like, but not tonic-like, DA signals (Bergstrom and Garris, 2003; Howard et al., 2011). It has been proposed, based on electrophysiological (Onn et al., 2000) and

computer modeling (Dreyer et al., 2010) studies, that D1 DA receptors, and thus striatonigral efferent neurons (Gerfen and Surmeier, 2011), are most sensitive to the higher levels of extracellular DA resulting from phasic DA activity. Alternatively, it has been suggested that D2 DA receptors, which are selectively expressed by striatopallidal efferent neurons, are largely saturated by DA under tonic extracellular DA levels and thus are largely insensitive to phasic increases in extracellular DA levels (Dreyer et al., 2010). Taken together, these data suggest that the decrease in phasic DA neurotransmission associated with partial DA loss selectively impairs D1 receptor activation and, therefore, normal gene expression in striatonigral neurons. Our present results further suggest that augmenting phasic-like DA neurotransmission can restore the degree of D1 dopamine receptor activation and, thus, striatonigral neuron function.

Consistent with this model, expression of *ppe* mRNA in D2 DA receptor-expressing striatopallidal neurons was not altered either by METH-induced neurotoxicity or by phasic- or tonic-like stimulation of the MFB in this study. Previous studies from our lab and others that found a decrease in *ppt* expression in the setting of partial striatal DA loss also reported no change in *ppe* mRNA expression (Nisenbaum et al., 1996; Chapman et al., 2001; Johnson-Davis et al., 2002). Interestingly, expression of *ppe* mRNA in striatopallidal neurons only changes (increases) in the setting of extensive (~80-90%) denervation of the striatum (Gerfen et al., 1991; Nisenbaum et al., 1996), and this same degree of striatal DA denervation is necessary before a decrease in tonic, extracellular levels of DA occurs (Abercrombie et al., 1990; Castañeda et al., 1990). Given

that METH neurotoxicity resulted in an ~25% striatal DA denervation (Fig. 4.1c), METH-induced neurotoxicity is not associated with changes in tonic extracellular levels of DA (Robinson et al., 1990; Cass and Manning, 1999). It is therefore not surprising that the METH-pretreated rats in this study, as in previous reports, did not have changes in *ppe* expression.

One confound of the current study is nonselective stimulation of axons in the MFB. The MFB is highly heterogeneous and contains nondopaminergic neurons that project to both striatum and the cortex (Nieuwenhuys et al., 1982). Additionally, the striatum receives glutamatergic afferents from various cortical areas (McGeorge and Faull, 1989; Ramanathan et al., 2002), and electrical stimulation of the cortex augments striatal IEG expression in monkeys (Parasathay and Graybiel, 1997) and rats (Fu and Beckstead, 1992; Liste et al., 1995). Therefore, it is possible that the gene expression measured here was partially induced through a nondopaminergic pathway, potentially relayed through the cortex. However, electrical stimulation of MFB released DA in the striatum as measured by FSCV (data not shown), the stimulating electrode was optimized within the MFB to elicit this DA release, and previous work has demonstrated that the D1 antagonist SCH23390 impairs expression of *zif268* (*NGFI-A)* caused by MFB stimulation (Chergui et al., 1997). Furthermore, as is apparent in Figures 4.2 and 4.3, cortical activation of *Arc* and *zif268* expression is notable in animals that received phasic- or tonic-like stimulation, whereas only animals receiving phasic-like stimulation showed increased striatal IEG expression. We noted no correlation between gene expression in striatum and that in the cortex directly

overlying striatum in either stimulation group (data not shown), suggesting that the cortical and striatal gene expression are not linked. Thus, these data suggest that the striatal gene expression is likely induced by electrical stimulation of DA neurons ascending through the MFB, although we cannot definitively rule out a contribution of other circuits to the effects at present.

Somewhat analogous to METH-induced neurotoxicity, Parkinson's disease (PD) is characterized in part by loss of striatal DA nerve terminals (Hornykiewicz and Kish, 1987) and dysfunction in striatal gene products (Nisbet et al., 1995). Importantly, cognitive impairments have been recognized in PD patients during the preclinical stage (Abbruzzese et al., 2009). While the pathology underlying these cognitive impairments is not understood, theoretical models indicate that deficits in D1 receptor signaling may play a role (Frank et al., 2004; Guthrie et al., 2009; Wiecki and Frank, 2010). It is thus informative that decreases in indices of D1 DA receptor-expressing striatonigral neuron function, including transcriptional activation of IEGs essential for consolidation of long-term memories (Barker-Haliski et al., 2012a), and deficits in basal ganglia-mediated learning and memory functions are apparent in rats with partial DA depletions (less than 80%), such as those induced by METH (Chapman et al., 2001; Daberkow et al., 2005; Daberkow et al., 2008; Son et al., 2011; Pastuzyn et al., 2012). Taken together, these data suggest that deficits in phasic DA signaling and downstream striatonigral gene expression alterations may be involved in the cognitive disabilities apparent in both the setting of METH-induced neurotoxicity and the preclinical stages of PD. The present data further suggest that

approaches to augment residual phasic DA signaling in the context of partial DA denervation or to replicate such signaling in the setting of more extensive DA denervation may prove fruitful in managing cognitive deficits associated with deficits in DA signaling, such as those observed in individuals with a history of METH abuse or PD.

Our findings demonstrate that augmenting phasic DA signaling in the partially DA denervated striatum enhances striatal gene expression to the same extent as in the intact striatum. Deficits in striatonigral neuron gene expression induced by large DA-depleting brain lesions are reversed with administration of levodopa (L-DOPA) (Zeng et al., 1995; Westin et al., 2001). Given that L-DOPA increases vesicular content of DA (Pothos et al., 1996) and electrically evoked DA release in intact rats (Garris et al., 1994; Rodríguez et al., 2007), it seems likely that it will augment phasic DA signaling in the context of partial striatal DA loss. Whether the postsynaptic consequences of partial DA denervation can be reversed with L-DOPA treatment or administration of other agents that enhance phasic DA signaling, such as amphetamine (Ramsson et al., 2011; Daberkow et al., 2013), which is used as a cognitive enhancer in treating attention-deficit hyperactivity disorder and drug addiction (Brady et al., 2011; Steiner and Van Waes, 2013), is currently being examined.

The authors declare no conflicts of interest. This work was supported by DA024036 (KAK, CDH, MBH, and PAG) and DA032502 (EDP).

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Figure 4.1. Effect of methamphetamine pretreatment on body temperature and dopamine innervation in the dorsal striatum. (A) METH treatment resulted in significantly increased body temperatures relative saline-treated controls. Arrows indicate time of METH injections. Data are average rectal temperatures (°C; mean±SEM, *n*=15 for saline-treated and *n*=15 for METH-treated). *Significantly different from saline-treated rats at the same time point. BL, baseline; ***p*<0.001, ****p*<0.0001. (B) Representative images of [^{125}I]RTI-55 striatal DAT binding in a saline- and METH-pretreated rat three to five weeks after METH pretreatment. (C) METH pretreatment resulted in partial dopamine denervation in both the dorsomedial (DMS) and dorsolateral (DLS) striatum three to five weeks after METH pretreatment as assessed by [^{125}I]RTI-55 binding to dopamine transporters. Data are presented as percent of saline-pretreated values (±SEM, *n*=15 for saline-pretreated and *n*=15 for METH-pretreated). *Significant effect of METH pretreatment. ***p*<0.001, ****p*<0.0001.

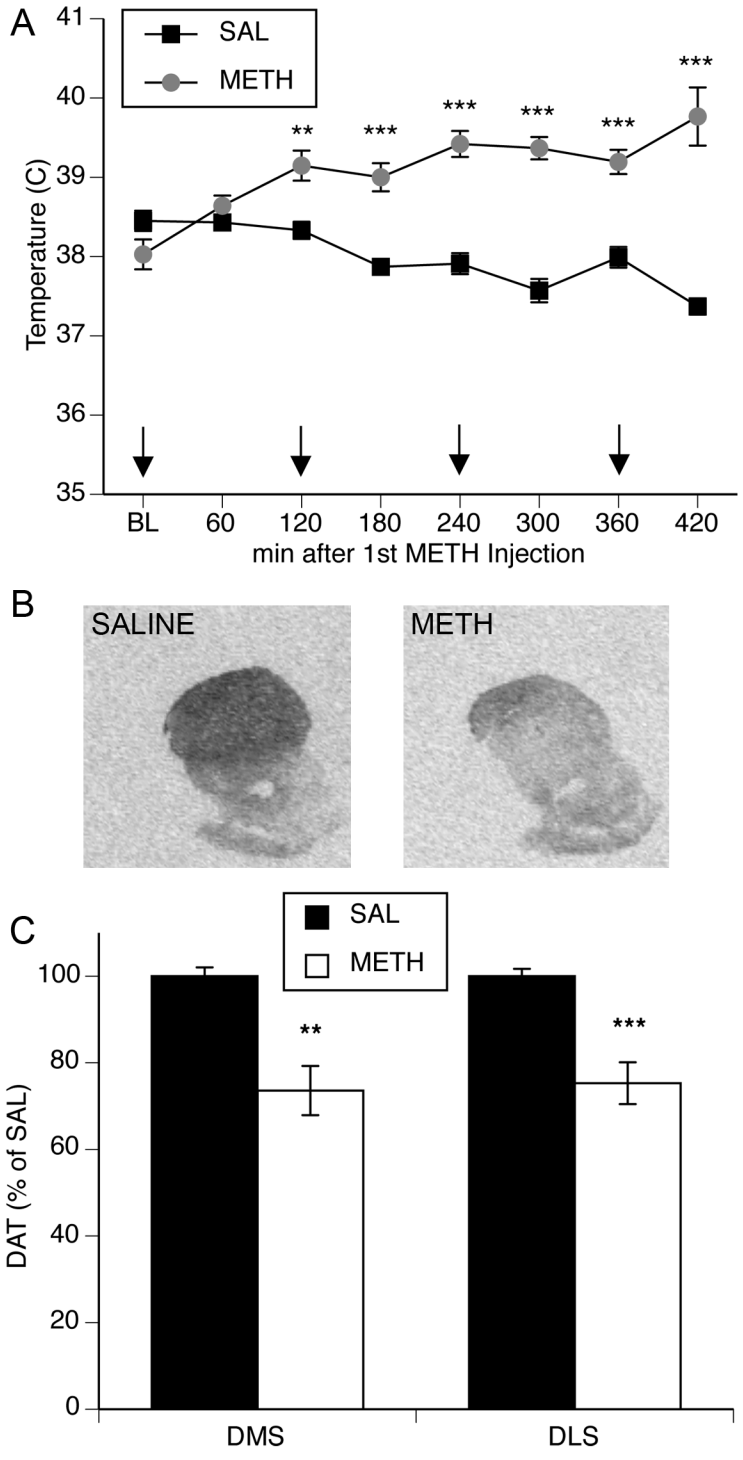

A
SAL
METH
Temperature (C)
41
40
39
38
37
36
35
**
***
***
***
***
***
BL
60
120
180
240
300
360
420
min after 1st METH Injection
B
SALINE
METH
C
SAL
METH
DAT (% of SAL)
100
80
60
40
20
0
**
***
DMS
DLS

Figure 4.2. Effect of MFB stimulation on expression of *Arc* and *zif268* in striatum of METH- and saline-pretreated rats. (A, C) Representative images of striatal *Arc* (A) or *zif268* (C) mRNA expression in saline- (top row) and METH- (bottom row) pretreated rats. Circles on the *Arc*/saline-pretreated/no stimulation image represent the regions of interest (ROIs) measured in both dorsomedial (DMS) and dorsolateral (DLS) striatum for all genes in this study. (B, D) Quantification of *Arc* (B) and *zif268* (D) expression in stimulation groups collapsed across pretreatment group. Data are average gray values (arbitrary units; mean±SEM; n=10 for each stimulation group per subregion, per gene product). *Significantly different from both no stimulation (No Stim) and Tonic groups, p<0.05. **Significantly different from both No Stim and Tonic groups, **p<0.01. ***Significantly different from both No Stim and Tonic groups, p<0.001.

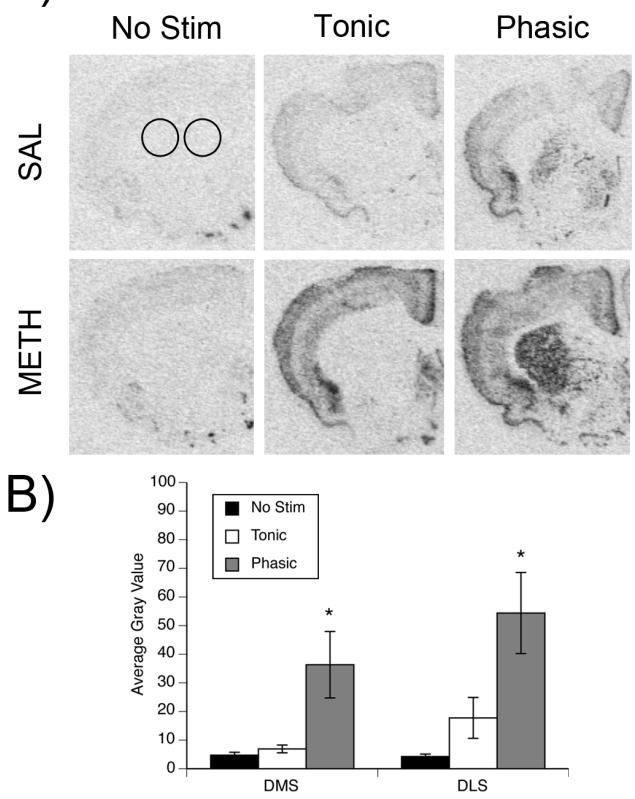

A) Arc
No Stim
Tonic
Phasic
SAL
METH
B)
No Stim
Tonic
Phasic
Average Gray Value
DMS
DLS
C) zif268
No Stim
Tonic
Phasic
SAL
METH
D)
No Stim
Tonic
Phasic
Average Gray Value
DMS
DLS

Figure 4.3. Effect of MFB stimulation on expression of *preprotachykinin* and *preproenkephalin* in striatum of METH- and saline-pretreated rats. (A) Representative images of striatal *ppt* mRNA expression in saline- (top row) and METH- (bottom row) pretreated rats. (B) Quantification of *ppt* expression in stimulation groups collapsed across pretreatment groups. Data are average gray values (arbitrary units; mean±SEM, n=10 for each stimulation group per subregion). *Significantly different from Tonic group, p<0.05. (C, D) Quantification of *ppt* expression separated by pretreatment group to demonstrate the effect of METH pretreatment on *ppt* expression. Data are average gray values (arbitrary units; mean±SEM, n=5 for saline- and METH-pretreated animals per stimulation group for both DMS and DLS). $^{\#}$Main effect of pretreatment, p<0.05. (E) Representative images of striatal *ppe* mRNA expression in saline- (top row) and METH- (bottom row) pretreated rats. (F) Quantification of *ppe* expression in dorsomedial striatum. Data are average gray values (arbitrary units; mean±SEM, n=5 for saline- and METH-pretreated animals per stimulation group). Data from dorsolateral striatum are not shown, as there was no subregion difference.

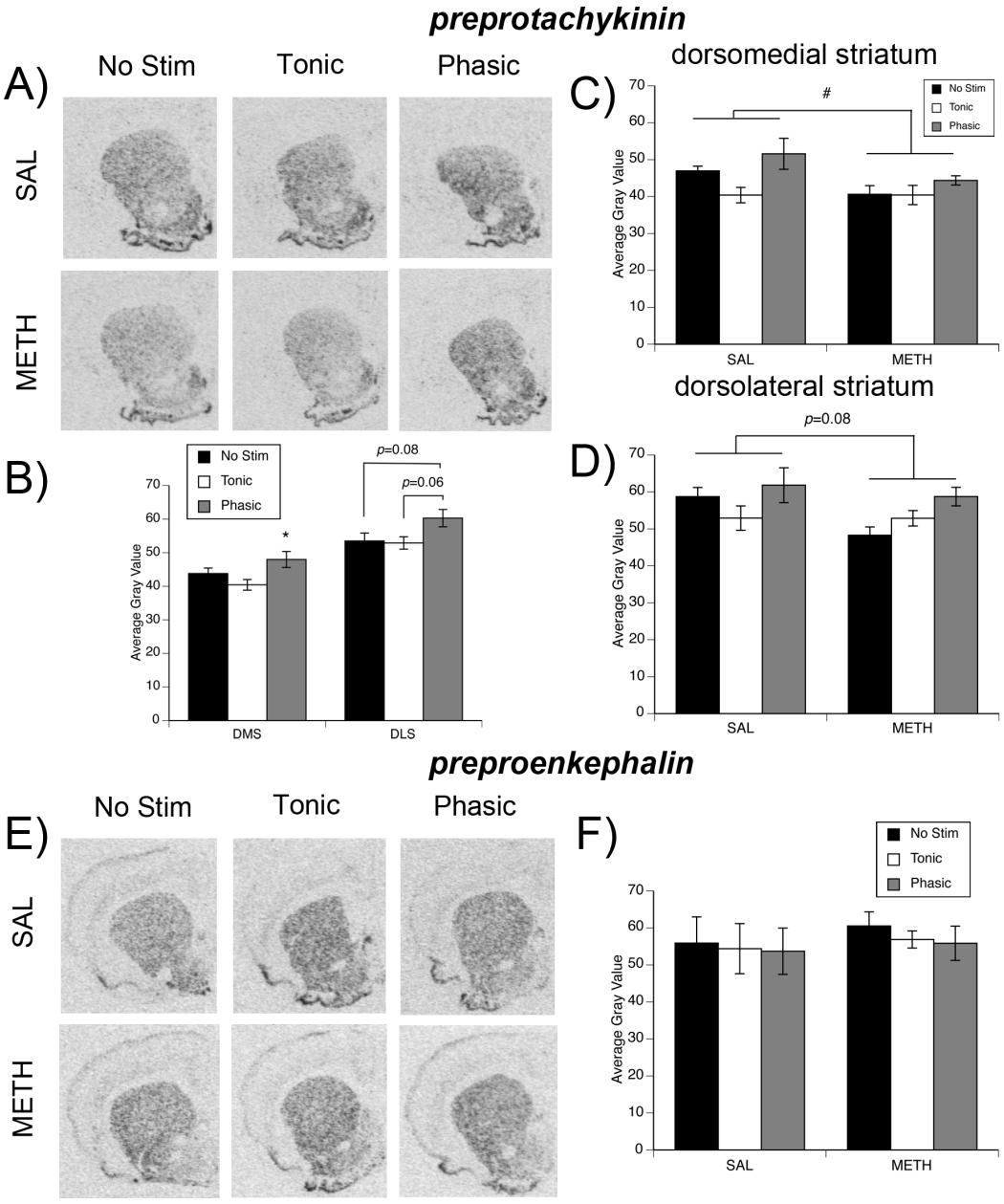
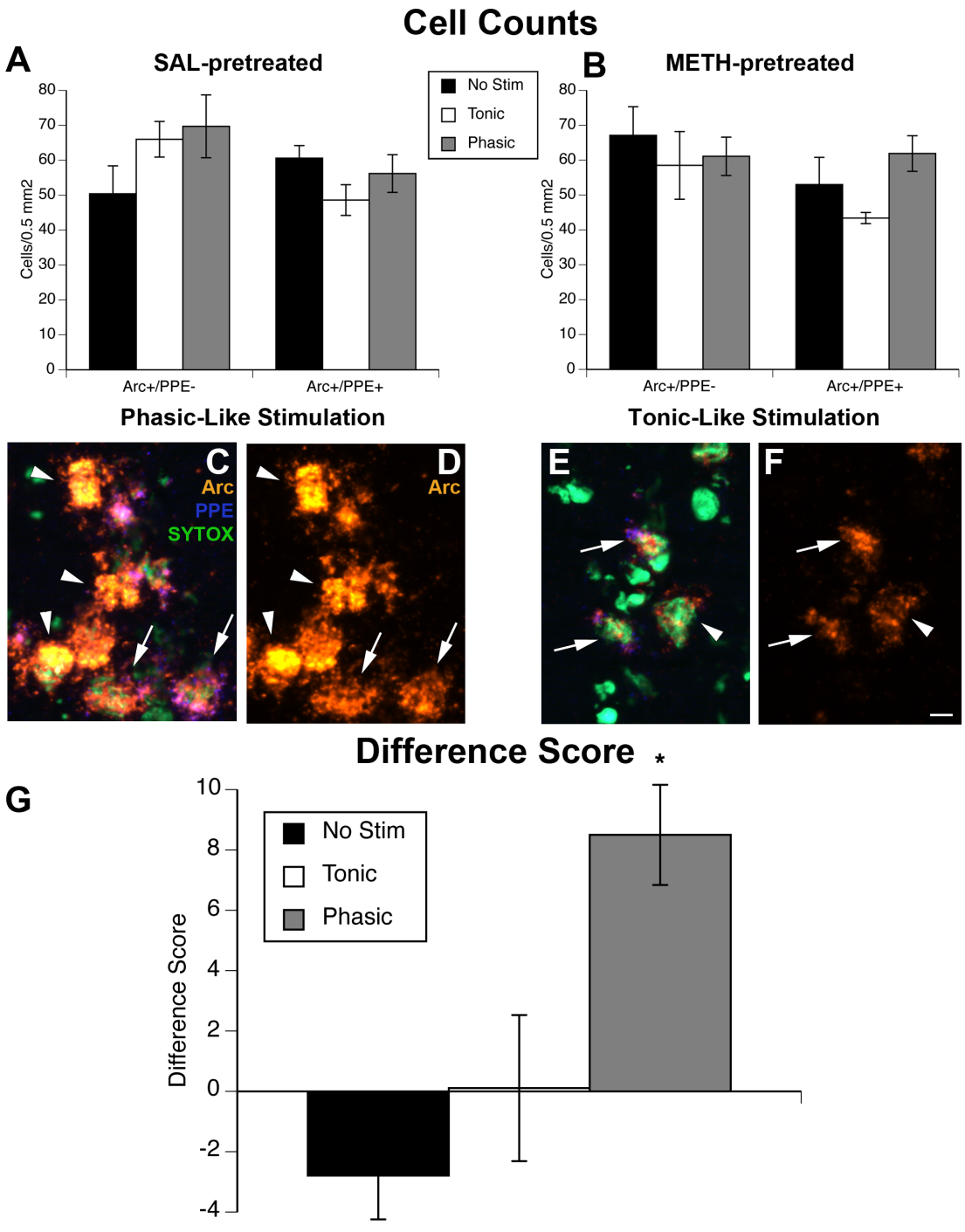

preprotachykinin
A)
No Stim
Tonic
Phasic
SAL
METH
B)
No Stim
Tonic
Phasic
p=0.08
p=0.06
*
Average Gray Value
DMS
DLS
C)
dorsomedial striatum
#
No Stim
Tonic
Phasic
Average Gray Value
SAL
METH
D)
dorsolateral striatum
p=0.08
Average Gray Value
SAL
METH
preproenkephalin
E)
No Stim
Tonic
Phasic
SAL
METH
F)
No Stim
Tonic
Phasic
Average Gray Value
SAL
METH

Figure 4.4. Phasic-like stimulation of dopamine neurons increases *Arc* expression in striatonigral neurons. (A-B) Numbers of *Arc*-positive/*ppe*-negative and *Arc*-positive/*ppe*-positive cells/0.5 mm^2 in the striatum of A) saline (SAL)- and B) METH-pretreated rats receiving no stimulation (No Stim) or tonic-like or phasic-like stimulation of the MFB. (C-F) Representative fluorescent *in situ* hybridization images of *Arc* mRNA expression in presumed striatonigral neurons (*Arc*-positive/*ppe*-negative; arrowheads) and striatopallidal neurons (*Arc*-positive/*ppe*-positive; arrows) in rats that received phasic-like (C-D) or tonic-like (E-F) stimulation. *Arc* is orange, *ppe* is blue, SYTOX nuclear stain is green. Images in C and E show all three channels, while images in D and F show only the *Arc* channel. Scale bar=10 μm. (G) Graph showing the "difference score" collapsed across pretreatment ($p>0.05$), defined as the difference between the average gray value of *Arc*-positive/*ppe*-negative and *Arc*-positive/*ppe*-positive neurons. *Significantly different from Tonic and No Stim groups, $p<0.05$, $p<0.001$, respectively.


Cell Counts
A
SAL-pretreated
B
METH-pretreated
No Stim
Tonic
Phasic
Cells/0.5 mm2
Arc+/PPE-
Arc+/PPE+
Phasic-Like Stimulation
Tonic-Like Stimulation
C
Arc
PPE
SYTOX
D
Arc
E
F
Difference Score
*
G
No Stim
Tonic
Phasic
Difference Score

CHAPTER 5

L-DOPA RESCUES DOPAMINE RESPONSES EVOKED BY PHASIC-LIKE STIMULATION OF THE MEDIAL FOREBRAIN BUNDLE IN RATS WITH METHAMPHETAMINE-INDUCED PARTIAL MONOAMINE LOSS

Abstract

Methamphetamine (METH) abuse causes long-lasting damage to monoamine systems in the brain, which can be modeled in rats using a "binge" regimen of METH. METH-pretreated rats display partial depletion of dopamine (DA) in the striatum and show abnormal striatal gene expression, altered circuitry used in a striatally-mediated task, and decreased striatal phasic DA transmission. Recent research has indicated that L-DOPA may restore normal function to the striatum after METH-induced neurotoxicity. Here, we investigated whether L-DOPA could restore phasic DA neurotransmission following METH neurotoxicity. Rats were pretreated with a neurotoxic regimen of METH (4x10 mg/kg, s.c., at two-hour intervals) or with saline. Three weeks later, rats were anesthetized and fast-scan cyclic voltammetry was used to measure DA release in the dorsomedial and dorsolateral striatum caused by stimulation of the medial forebrain bundle. Rats were injected with benserazide (40 mg/kg, i.p.) and L-DOPA (50 mg/kg, i.p.)

and stimulation-induced DA release was recorded for two hours. L-DOPA restored phasic-like DA release in METH-pretreated rats to saline-pretreated control levels. Surprisingly, response to L-DOPA was not uniform throughout the striatum, as some recording sites displayed large activation to L-DOPA and some did not, even if recorded simultaneously within the same striatal subregion. Also, chemometric analysis indicated no detectable basal DA changes after L-DOPA, indicating specific phasic DA activation. These findings suggest that the decreased phasic DA release seen in METH-pretreated rats can be partially rescued with L-DOPA, which is promising for future translational studies in recovering METH addicts.

Introduction

Methamphetamine (METH) abuse is an ongoing societal issue. In addition to the highly addictive nature of METH, users abstaining from the drug also face cognitive impairments (Marshall and O'Dell, 2012; Dean et al., 2013), which are linked to long-term changes in the brain induced by METH including loss of markers of dopamine (DA) innervation in striatum (Wilson et al., 1996; Volkow et al., 2001; McCann et al., 2008). “Binge” regimens of METH in rodents cause similar DA depletion to those seen in human METH abusers (Seiden et al., 1976; Morgan and Gibb, 1980; Ricaurte et al., 1980; Wagner et al., 1980; Marshall and O'Dell, 2012). Additionally, METH-induced DA loss is related to diminished learning performance in rats on several behavioral tasks (Friedman et al., 1998; Chapman et al., 2001; Schröder et al., 2003; Achat-Mendes et al., 2005; Belcher

et al., 2005; Daberkow et al., 2005; Izquierdo et al., 2010; O'Dell et al., 2011; Reichel et al., 2012), though the mechanisms underlying these impairments are not clear.

Secondary to monoamine loss is basal ganglia dysfunction, which is evidenced by altered basal (Chapman et al., 2001; Johnson-Davis et al., 2002) and behavior-induced striatal gene expression (Daberkow et al., 2008; Barker-Haliski et al., 2012) and changes in circuitry mediating behavior (Son et al., 2011; Pastuzyn et al., 2012). While the exact mechanism behind striatal dysfunction is unclear, METH-induced reduction in phasic DA signaling (Howard et al., 2011; Howard et al., 2013b), a mode of DA neurotransmission important in striatal gene expression (Chergui et al., 1997), has been proposed to subserve these changes. In fact, enhancing phasic DA signals in METH-pretreated animals normalizes gene expression deficits, indicating a link between reduced DA signaling and basal ganglia dysfunction (Howard et al., 2013a).

Therefore, restoring normal phasic signaling may be a means of ameliorating basal ganglia dysfunction following METH neurotoxicity. An obvious candidate to restore phasic DA signaling is L-3,4-dihydroxyphenylalanine (L-DOPA), the biochemical precursor to DA and gold-standard treatment of Parkinson's disease. Chronic L-DOPA reestablishes normal gene expression following METH neurotoxicity, indicating a restoration of phasic DA (unpublished observations). Additionally, electrochemical measurements in intact (Wightman et al., 1988; Garris et al., 1994b; Rodríguez et al., 2007) and severely DA-depleted rats (Keller Jr. et al., 1988) indicate L-DOPA can augment electrically-

evoked DA. Therefore, to test whether L-DOPA restores normal phasic DA following METH-induced partial DA depletions, L-DOPA (50 mg/kg, i.p.) was administered to METH- and saline-pretreated rats, and phasic DA signals were electrically evoked and measured using fast-scan cyclic voltammetry. L-DOPA restored phasic signals to baseline, control values in METH-pretreated animals. Unexpectedly, L-DOPA-mediated increases in phasic signals varied based on striatal recording site, even within the same animal and brain region. Additionally, chemometric analysis revealed no overt changes in basal DA levels within the first hour of L-DOPA treatment in METH- or saline-pretreated rats, despite increases in evoked signals. These data indicate that L-DOPA normalizes METH-induced basal ganglia dysfunction through specific remediation of phasic DA signals.

Materials and Methods

Animals. Adult male Sprague-Dawley rats (250-300 g, METH-pretreated, *n*=12, 18 recordings; saline-pretreated *n*=12, 17 recordings) were purchased from Harlan (Indianapolis, IN, USA) and were housed in a light- and temperature-controlled vivarium. Access to food and water was provided *ad libitum*. All procedures conformed to the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Illinois State University.

Drugs. (±)-Methamphetamine hydrochloride was provided by the National Institute on Drug Abuse (Rockville, MD, USA). L-3,4-dihydroxyphenylalanine

methyl ester hydrochloride and benserazide hydrochloride were both purchased from Sigma-Aldrich (St. Louis, MO, USA) and were dissolved in 0.9% sterile saline for injections.

Methamphetamine pretreatment. Rats were treated with a neurotoxic "binge" regimen of METH (4 x 10 mg/kg, s.c., at two-hour intervals) as previously described (Howard et al., 2011). Briefly, rats were housed in plastic tub cages (50 cm length x 40 cm width x 20 cm height; 4-8 rats/tub). Rectal temperature was taken every hour for eight hours during the injection day using a Thermalert TH-5 (Physitemp, Clifton, NJ, USA). Rats were rehoused in the vivarium the following morning.

In vivo *voltammetry.* Rats were anesthetized with urethane (1.5 g/kg, i.p.) and placed in a stereotactic frame (David Kopf Instruments, Tajunga, CA, USA). Four holes were drilled to allow insertion of two recording electrodes, a stimulating electrode, and a reference electrode. Evoked DA concentration was recorded using fast-scan cyclic voltammetry (Cahill et al., 1996) by applying a triangular waveform to carbon fiber recording microelecrodes, which were fabricated as previously described (Ramsson et al., 2011b). Recording electrodes were lowered at a 6^{o} angle ipsilaterally into the dorsomedial (DMS) and dorsolateral (DLS) striatum (mm from Bregma: +1.2 AP, +1.0 and +4.0 ML, respectively, and -4.5 DV; Paxinos and Watson, 1986). The Ag/AgCl reference electrode (Stoelting, Wood Dale, IL, USA) was placed in the contralateral cortex relative to the working electrodes. Voltammetry was controlled using TH-1 software (ESA, Chelmsford, MA, USA) with either an EI400 (Ensman

Instruments, Bloomington, IN, USA) or UEI (University of North Carolina at Chapel Hill, Department of Chemistry Electronics Facility, Chapel Hill, NC, USA) bipotentiostat. Immediately following *in vivo* experiments, recorded current was converted to concentration by *in vitro* calibration (Logman et al., 2000).

Electrical stimulation. A twisted bipolar stimulating electrode (Plastics One, Roanoke, VA, USA) was placed dorsal to the medial forebrain bundle (mm from Bregma: -4.6 AP, +1.4 ML, -7.0 DV; Paxinos and Watson, 1986) and was optimized to maximize evoked DA response in the striatum. During experiments, DA was evoked using phasic-like stimulus parameters (300 μA, 4 ms biphasic pulses, 24 pulses at 60 Hz). Stimulation was optically isolated and controlled using constant-current generators (Neurolog NL800, Digitimer Limited, Letchworth Garden City, UK).

Experimental design. Following optimization of recording and stimulating electrodes, 24 pulse, 60 Hz stimulation was applied every 5 min for a total of 155 min. Fifteen minutes following beginning of recordings, the peripheral L-amino acid decarboxylase inhibitor benserazide hydrochloride (40 mg/kg, i.p.) was administered, followed 20 min later by L-DOPA (50 mg/kg, i.p.) (Abercrombie et al., 1990; Rodríguez et al., 2007). Recordings were collected for two hours following L-DOPA injection. Maximal DA response at the oxidation potential for dopamine was determined, and evoked responses were analyzed for release and uptake rates (see following section). Additionally, basal DA was assessed using a chemometric approach (see following section). Immediately following

experiments, rats were euthanized and brains were rapidly removed and flash frozen using 2-methyl butane (EMD Millipore, Billerica, MA, USA) on dry ice.

DAT autoradiography. Fresh-frozen brains were sectioned (12 µm), thaw-mounted onto Superfrost Plus slides (VWR, Aurora, CO, USA), and stored at -20°C. DAT binding in striatum was determined with [^{125}I]RTI-55 (PerkinElmer, Waltham, MA, USA; Boja et al., 1992) binding, as previously reported (Barker-Haliski et al., 2012; Pastuzyn et al., 2012; Son et al., 2013). Slides were apposed to film (Biomax MR; Eastman Kodak Co., Rochester, NY, USA) for 24 hr and developed. Images were digitized and densitometric analysis accomplished using NIH ImageJ, yielding average, background-subtracted gray values in DMS and DLS. Two rostral (+1.6 mm from Bregma; Paxinos and Watson, 1986) and two middle (+0.7 mm) striatal sections and four prefrontal cortical sections were analyzed per rat. DAT binding in METH-pretreated rats was converted to percent of average levels in saline-pretreated rats.

Kinetic analysis. Evoked DA responses are a combination of two counteracting mechanisms; release and uptake. These rates were assessed using a mathematical model established by Wightman et al. (1988):

$$d[DA]/dt = [DA]_p * f - k[DA] \quad (1)$$

where $[DA]_p$ is the concentration of DA release per stimulus pulse, k is the first-order term for DA uptake rate, and f is the frequency of the electrical stimulation. Experimental data were fit to simulated curves to determine k and $[DA]_p$ by using

a non-linear regression based on a simplex-minimization algorithm (Wu et al., 2001).

Principal component regression. In addition to evoked DA concentrations, changes in basal DA were assessed using principal component regression (Keithley et al., 2009). Here, training sets were collected prior to the beginning of experiments, wherein DA was evoked using various electrical stimulation parameters (60 Hz, 60 pulses; 60 Hz, 24 pulses; 60 Hz, 12 pulses; 30 Hz, 24 pulses; 30 Hz, 12 pulses; and 30 Hz, 6 pulses; all at 300 μA; Heien et al., 2005). In addition to the DA component, training sets included background drift, which is crucial for assessing DA changes (Hermans et al., 2008), and when detectable, pH changes. Because electrode background changes were dynamic over time, voltammograms for drift just prior to drug administration best predicted changes after L-DOPA administration. Therefore, pH and background components were collected from predrug files, and predrug files were thus not analyzed for DA changes.

Unaccounted variance in experimental data sets that cannot be encompassed by the training set (Q) must fall below a calculated threshold (Q_α) to appropriately predict concentration changes (Keithley and Wightman, 2011). To avoid “resetting” Q at the beginning of each five minute bin, which has previously been described as inappropriate (Keithley and Wightman, 2011), Q was cumulatively assessed across all post-L-DOPA recordings. This conservative approach resulted in exaggerated Q values, and changes in DA concentration were only included when these Q values fell below Q_α. Predicted DA changes

with Q values under Q_α were averaged by pretreatment and subregion. To obtain a measure of basal/tonic DA changes, 20 sequential data points in each PCR output were averaged at 2 s, 1202 s, and 2398 s after the beginning of the file when L-DOPA was injected. These time points were chosen based on the latest time at which Q values crossed Q_α (see Results).

Assessment of predrug responses as "fast" and "slow" release sites. Previous work using FSCV has reported two distinct profiles of release sites that are differentially regulated by D2 autoinhibition (Moquin and Michael, 2009). Termed "fast" and "slow" release sites, these types of DA release have different release and uptake dynamics, different response to uptake inhibitors, different short-term plasticity to subsequent stimulation, and different responses to D2 antagonism (Moquin and Michael, 2009; Taylor et al., 2012) Therefore, we investigated our predrug responses based on criteria defining these two types of release sites (Taylor et al., 2012). Predrug responses were analyzed for (1) duration to increase following stimulus onset, (2) short-term facilitation (amplitude of signal at 200-400 ms normalized to amplitude at 0-200 ms during stimulation), (3) duration of "overshoot" or time following stimulation cessation until signal maximum ($[DA]_{max}$), and (4) rate of uptake as assessed using results of kinetic analysis. These characteristics were averaged for each pretreatment, subregion, and site type (*i.e.*, our "high" and "low" release sites) group and compared.

*Statistical analysis***.** Levels of DAT binding between pretreatment groups (saline and METH) in each brain region were compared by one-way ANOVA. Maximal DA response ($[DA]_{max}$), DA release rate ($[DA]_p$), and DA uptake rate (k)

were compared in each subregion with a three-way repeated measures pretreatment (saline or METH) x release site type ("high" or "low") x time (0, 20, or 40 min post-L-DOPA) MANOVA. Once high and low release sites were determined to respond differently to L-DOPA, further comparisons were made by a two-way ANOVA on pretreatment x time, with *post hoc* tests run when significant effects or interactions were revealed. Changes in basal DA were assessed with a two-way repeated measures pretreatment x time MANOVA with follow-up *post hoc t*-tests when significant effects of time or an interaction were discovered. Characteristics of "fast" and "slow" release types (Moquin and Michael, 2009; Taylor et al., 2012) were compared using three-way ANOVA, with pretreatment, subregion, and site-type (*i.e.*, our "high" and "low" release sites) as factors. Statistics were performed using JMP (v.9.0) and SAS (v.9.3) software (SAS Institute Inc., Cary, NC, USA).

Results

DAT autoradiography. Pretreatment with a neurotoxic regimen of METH resulted in decreases in striatal DAT binding, as revealed by [^{125}I]RTI-55 autoradiography (Fig. 5.1). Unpaired *t*-tests showed that METH-pretreated rats had significantly less striatal DAT binding than SAL-pretreated rats in both DMS (t=9.17, p=0.0001) and DLS (t=8.45, p=0.0001).

Effect of L-DOPA on evoked DA amplitude in saline- and METH-pretreated rats. An injection of 50 mg/kg L-DOPA resulted in increased DA signals evoked by phasic-like stimulation of the medial forebrain bundle in DMS and DLS of both

saline- and METH-pretreated rats as measured by fast-scan cyclic voltammetry. In Fig. 5.2, typical predrug responses and evoked traces 40 min after L-DOPA are shown respectively in left and right panels for DMS and DLS of saline-pretreated (Fig. 5.2A and 5.2C, respectively) and DMS and DLS of METH-pretreated (Fig. 5.2B and 5.2D, respectively) rats.

However, not all recording sites reacted in the same way to L-DOPA. Some sites demonstrated complete lack of response to L-DOPA, even when a simultaneous recording within the same rat and same brain region showed robust activation. Fig. 5.3 demonstrates traces collected within the same subregion (DMS) in a saline- (Fig. 5.3A and 5.3B) and METH-pretreated rat (Fig. 5.3C and 5.3D) before, 20, and 40 min after L-DOPA injection. As seen in the figure, within the same subregion (~0.5 mm apart) of the same animal, DA response to L-DOPA was highly variable. More interestingly, recordings with larger predrug evoked responses appeared to respond more robustly to L-DOPA. Therefore, all data were separated into “high release” and “low release” sites based on evoked predrug concentration for subsequent analysis. Inclusion criteria for high release sites in saline-pretreated animals was predrug evoked responses >0.4 μM. As METH-pretreated groups, on average, had lower predrug evoked responses, inclusion criteria for high release sites in METH-pretreated animals was >0.3 μM. The release site type criterion for the METH-pretreated group was set based on expected differences in evoked response between pretreatment groups based on previous work (Howard et al., 2011; Howard et al., 2013b), and accordingly, the criterion difference between saline- and METH-

pretreated groups (0.4 vs. 0.3 μM or 25%) was similar to differences in average evoked predrug differences between saline- and METH-pretreated animals in the current study (29.5% reduction in METH pretreated animals).

As shown in Fig. 5.4, L-DOPA caused robust and long-lasting increases in evoked DA amplitude in high release, but not low release sites in DMS of both METH- and saline-pretreated animals (Fig. 5.4A). In DLS of METH- and saline-pretreated animals, increases were noted in high release sites, but were diminished relative to DMS and low release sites showed minimal response to L-DOPA (Fig. 5.4B). Importantly, in high release sites, METH pretreatment reduced evoked responses, and this decrement was temporarily remediated by L-DOPA. Three timepoints were selected for subsequent analysis: the stimulation event just prior to drug injection (time 0), 20 min, and 40 min following L-DOPA administration. A repeated measures MANOVA on evoked DA concentration in DMS at 0 (baseline), 20, and 40 min post-L-DOPA revealed a significant effect of pretreatment ($F_{(1,14)}$=17.7, p=0.0009), release site type ($F_{(1,14)}$=60.1, p=0.0001), time ($F_{(2,13)}$=34.7, p=0.0001), a time x pretreatment interaction ($F_{(2,13)}$=6.68, p=0.0101), time x release site type interaction ($F_{(2,13)}$=16.2, p=0.0003), and time x pretreatment x release site type interaction ($F_{(2,13)}$=3.86, p=0.048; Fig. 5.4C), suggesting that the high and low release sites had significantly different responses to L-DOPA. Similarly, in DLS, a repeated measures MANOVA on evoked $[DA]_{max}$ revealed a significant effect of pretreatment ($F_{(1,20)}$=11, p=0.0035), release site type ($F_{(1,20)}$=72.5, p=0.0001), time ($F_{(2,19)}$=19.8, p=0.0001), a time x pretreatment interaction ($F_{(2,19)}$=29.3, p=0.0001), time x

release site type interaction ($F_{(2,19)}$=12.7, p=0.0003), and time x pretreatment x release site type interaction ($F_{(2,19)}$=14.1, p=0.0002; Fig. 5.4D). Therefore, high and low release sites responded differently to L-DOPA in both DMS and DLS, which justifies separating high and low release sites for subsequent analysis.

Effect of L-DOPA on average evoked responses, DA uptake, and DA release rates. Fig. 5.5 shows average characteristics of electrically evoked DA signals before and following L-DOPA administration, where maximal DA responses ($[DA]_{max}$; top four panels), DA release ($[DA]_p$; middle four panels), and DA uptake rate (k; bottom two panels) were averaged by pretreatment groups and release site type (high vs. low) and are shown for DMS (left) and DLS (right). At high release sites in DMS (Fig. 5.5A), two-way repeated measures MANOVA revealed a significant effect of pretreatment ($F_{(1,7)}$=23, p=0.002), a significant effect of time ($F_{(2,6)}$=34.6, p=0.0005), and a time x pretreatment interaction ($F_{(2,6)}$=7.24, p=0.025). *Post hoc* t-tests showed that there was a significant effect of pretreatment (SAL>METH) at 0 (t=3.6, p=0.0087), 20 (t=4.48, p=0.0029), and 40 (t=4.76, p=0.0021) min post-L-DOPA. A student's t-test on evoked DA values at high release sites in SAL-pretreated rats at 0, 20, and 40 min post-L-DOPA revealed that evoked DA levels at 20 and 40 min were both significantly greater than at 0 min (20: t=3.9, p=0.0034; 40: t=9.3, p=0.0001) and 40 min was greater than 20 min (t=5.4, p=0.0004). At high release sites in METH-pretreated rats, a student's t-test revealed a strong trend for evoked DA levels at 40 min to be greater than at 0 min (t=2.0, p=0.07). At low release sites in DMS (Fig. 5.5C),

two-way repeated measures MANOVA revealed that there was no effect of pretreatment (p=0.11), time (p=0.11), or time x pretreatment interaction (p=0.67).

At high release sites in DLS (Fig. 5.5B), two-way repeated measures MANOVA revealed a strong trend towards an effect of pretreatment ($F_{(1,5)}$=6.21, p=0.055), a significant effect of time ($F_{(2,4)}$=29, p=0.0042), and a time x pretreatment interaction ($F_{(2,4)}$=37.1, p=0.0026). *Post hoc* t-tests showed that there was no effect of pretreatment at baseline (p=0.21), but there was at 20 min post-L-DOPA (t=3.13, p=0.026), and there was a trend towards an effect at 40 min post-L-DOPA (t=2.29, p=0.071). A student's t-test on evoked DA values at 0, 20, and 40 min post-L-DOPA showed that there were no significant differences between DA levels at the different times at high release sites in either METH- or saline-pretreated rats (all p>0.05). At low release sites in DLS (Fig. 5.5D), a two-way repeated measures MANOVA revealed a significant effect of pretreatment ($F_{(1,15)}$=4.63, p=0.048), no effect of time (p=0.35), and a strong trend towards a time x pretreatment interaction ($F_{(2,14)}$=3.6, p=0.055).

Kinetic analysis of evoked DA traces was performed. A three-way repeated measures MANOVA (pretreatment x release site type x time) on the amount of DA released per pulse ($[DA]_p$) in DMS revealed a significant effect of pretreatment ($F_{(1,14)}$=10.9, p=0.0052), release site type ($F_{(1,14)}$=30.4, p=0.0001), time ($F_{(2,13)}$=33.5, p=0.0001), time x pretreatment interaction ($F_{(2,13)}$=8.96, p=0.0036), time x release site type interaction ($F_{(2,13)}$=22.4, p=0.0001), and time x pretreatment x release site type interaction ($F_{(2,13)}$=4.02, p=0.044). Based on

these significant interactions, we split the $[DA]_p$ from high and low release sites for subsequent statistical analysis.

At high release sites in DMS (Fig. 5.5E), a two-way repeated measures MANOVA revealed a significant effect of pretreatment ($F_{(1,7)}$=6.61, p=0.037), time ($F_{(2,6)}$=38.3, p=0.0004), and a time x pretreatment interaction ($F_{(2,6)}$=8.4, p=0.018). *Post hoc* t-tests revealed that SAL>METH at 20 (t=2.69, p=0.031) and 40 (t=2.97, p=0.021) min post-L-DOPA, but not at baseline (p=0.16). A student's t-test on $[DA]_p$ values at high release sites in SAL-pretreated rats at 0, 20, and 40 min post-L-DOPA revealed that $[DA]_p$ at 40 min was significantly greater than at 0 min (t=4.1, p=0.0026) and 20 min (t=2.4, p=0.043). Student's t-test revealed no significant differences between $[DA]_p$ at the three different timepoints at high release sites in METH-pretreated rats. At low release sites in DMS (Fig. 5.5G), a two-way repeated measures MANOVA revealed a trend towards an effect of pretreatment ($F_{(1,7)}$=4.47, p=0.072), but no effect of time (p=0.33) or time x pretreatment interaction (p=0.23).

At high release sites in DLS (Fig. 5.5F), a two-way repeated measures MANOVA revealed a trend towards an effect of pretreatment ($F_{(1,5)}$=4.18, p=0.096), a significant effect of time ($F_{(2,4)}$=26.9, p=0.0048), and a time x pretreatment interaction ($F_{(2,4)}$=11.2, p=0.023). *Post hoc* t-tests showed that there was a trending effect for SAL>METH at 20 (t=2.46, p=0.057) and 40 (t=2.01, p=0.1) min post-L-DOPA, but no significant effect of pretreatment at baseline (p=0.22). A student's t-test on $[DA]_p$ values at high release sites in SAL-pretreated rats at 0, 20, and 40 min post-L-DOPA revealed that $[DA]_p$ at 40 min

was significantly greater than at 0 min (t=2.3, p=0.0455). At high release sites in METH-pretreated rats, a student's t-test revealed a trend for $[DA]_p$ levels at 40 min to be greater than at 0 min (t=2.1, p=0.08). At low release sites in DLS (Fig. 5.5H), a two-way repeated measures MANOVA revealed a significant time x pretreatment interaction ($F_{(2,12)}$=4.26, p=0.04), but no effect of pretreatment (p=0.26) or time (p=0.68). *Post hoc* t-tests revealed no significant effect of pretreatment at any time point (all p>0.05).

A three-way repeated measures MANOVA (pretreatment x release site type x time) was performed on values for k, or DA uptake. In DMS (Fig. 5.5I), there was a strong trend for a significant effect of pretreatment ($F_{(1,14)}$=4.55, p=0.051) and time ($F_{(2,13)}$=14.6, p=0.0005), but no other significant effects or interactions (p>0.05). In DLS (Fig. 5.5J), there was only a significant effect of time ($F_{(2,17)}$=6.23, p=0.0093). Because of this, we could not statistically separate high and low release sites for analysis of k, suggesting that the high and low release sites did not differ in response to L-DOPA because of DA uptake rate. *Post hoc* student's t-tests revealed that values for k at 0, 20, and 40 min post-L-DOPA were not significantly different in either DMS or DLS (all p>0.05).

Analysis of evoked responses based on "fast" and "slow" release site characteristics. We analyzed predrug responses to investigate if our "low" and "high" release sites corresponded to previously described "fast" and "slow" release sites (Taylor et al., 2012; Fig. 5.6). Much to our surprise, we report very different evoked responses than those described by Taylor et al. (2012); for example, we see no true "fast" release sites, as all evoked responses in the

current work had some overshoot following the end of stimulation (Fig. 5.2 and 5.3). Additionally, release sites in the current work demonstrated at least some short-term facilitation as opposed to short-term depression exhibited by “fast” release sites (Taylor et al., 2012). Three-way ANOVA revealed that our “high” and “low” release sites showed no difference in duration to increase ($F_{(7,33)}$=2.51, p=0.1331), short-term facilitation ($F_{(7,33)}$=0.00, p=0.9769), duration of overshoot ($F_{(7,33)}$=0.83, p=0.3690), or uptake rate (k; $F_{(7,33)}$=0.44, p=0.5096). However, METH pretreatment did increase duration to increase ($F_{(7,33)}$=4.51, p=0.0412) and duration of overshoot ($F_{(7,33)}$=6.75, p=0.0139), as well as reduce uptake rate ($F_{(7,33)}$=4.56, p=0.0383). There were no significant interactions for any measure (all p values>0.05). Therefore, effects of L-DOPA were not attributable to “fast” or “slow” release sites in the current study.

*Effect of L-DOPA on evoked and basal DA concentration in saline- and METH-pretreated rats***.** L-DOPA-induced change in basal DA concentration was assessed using principal component regression (PCR; Keithley et al., 2009). As shown in Fig. 5.7, increases in basal current recorded following L-DOPA administration were not attributable to changes in DA. When PCR was used to separate the influences of DA, pH, and electrode drift, increases in current were dramatically reduced before (Fig. 5.7A) and after L-DOPA administration (50 min postdrug shown in Fig. 5.7B). Importantly, PCR accurately assessed DA changes as evoked response current, which are thought to be primarily attributable to DA changes (Chergui et al., 1994), shows good agreement with PCR output (Fig. 5.7, INSET).

Predicted DA changes collected from PCR output were averaged by subregion and pretreatment (Fig. 5.8). As stated in Materials and Methods, changes in basal DA were only included if Q values remained below Q_α. In general, all Q values tended to pass Q_α ~60 min after L-DOPA administration; therefore, later time points were excluded. Additionally, in the DMS of METH-pretreated animals, Q values crossed threshold in all but two experiments prior to 60 min postdrug; therefore, data were analyzed through 40 min postdrug. We chose three time points to analyze: 2 s after the beginning of the file in which L-DOPA was administered, at 20 min and 2 s (1202 s), and 2 s before the end of 40 min (2398 s). Additionally, recordings with Q values that quickly crossed threshold were excluded. L-DOPA did marginally affect basal DA across all pretreatment groups (trending effect of time, $F_{(2,20)}$=3.28, p=0.0586) though this appeared to be marginally related to pretreatment (trending effect of pretreatment, $F_{(1,21)}$=3.16, p=0.0897). Changes in basal DA were also not different between subregions (no significant effect of subregion, $F_{(1,21)}$=0.02, p=0.8854; no significant time x pretreatment interaction, $F_{(2,20)}$=1.02, p=0.3780; no significant time x subregion interaction, $F_{(2,20)}$=0.44, p=0.6526; no significant time x pretreatment x subregion interaction, $F_{(2,20)}$=0.87, p=0.4359; two-way repeated measures MANOVA; Fig. 5.8). Though trends for increases in basal DA were noted, lack of significant increases in basal DA indicates L-DOPA may be preferentially acting by augmenting phasic DA release.

Discussion

This study demonstrated the novel finding that L-DOPA remediates evoked phasic DA signaling impaired by neurotoxic METH pretreatment. Unexpectedly, L-DOPA-induced increase of phasic signaling was not uniform throughout the striatum, and augmented phasic DA signaling occurred irrespective of overt increases in basal DA tone, indicating a specific enhancement of phasic activity in both nonlesioned controls and at the partial lesion degree seen following METH neurotoxicity. These data indicate that L-DOPA may partially restore normal striatal functioning by restoring deficits in phasic DA signaling in METH-pretreated animals.

L-DOPA restores normal electrically-evoked DA signaling despite partial DA depletion induced by METH neurotoxicity. In agreement with previous work from us and others, pretreatment of rats with a neurotoxic dose of METH impairs electrically-evoked phasic DA signals (Howard et al., 2011; Loewinger et al., 2012; Howard et al., 2013b; Fig. 5.4A-D, Fig. 5.5A-D). These deficits were restored to control levels in “high release” sites of METH-pretreated animals in the current study, though no obvious changes in “low release” sites were noted in either DMS or DLS. Our previous work has indicated that electrically-evoked phasic signals are indicative of the status of “spontaneous” DA signals, also called DA transients (Howard et al., 2013b). However, direct measures of naturally-occurring DA transients following L-DOPA administration are yet to be reported.

METH-induced partial DA depletions blunted L-DOPA-induced augmentation of DA release in the current study (Fig. 5.4 and 5.5). Following severe 6-OHDA-induced DA depletion, peak DA concentration in dialysate fails to reach those of intact striata after lower (Gerlach et al., 2004), higher (Keller Jr. et al., 1988), and identical L-DOPA doses to those used here (Miller and Abercrombie, 1999). This is likely due to fewer terminals packaging and releasing newly synthesized DA in the 6-OHDA-treated rat. However, relative to pre-L-DOPA baseline, increases in DA release in severely lesioned striata are vastly larger, which is indicative of compensation in DA neurons following large DA tissue loss (Abercrombie et al., 1990). Since METH-pretreated rats have a relatively mild lesion (~60%) compared to 6-OHDA-treated rats, the lack of a relatively larger increase in DA release in METH vs. saline rats following L-DOPA likely indicates lack of active compensation of DA neurons.

L-DOPA restores DA release rates following METH-induced partial DA depletion. Remediation of phasic DA signal amplitude occurred with simultaneous increases in exocytotic DA release rate (Fig. 5.5). Previous reports have also demonstrated L-DOPA-induced increases in DA release as monitored by voltammetry (Stamford et al., 1984; Keller Jr. et al., 1988; Wightman et al., 1988; May and Wightman, 1989b; Garris et al., 1994b). Additionally, L-DOPA has been shown to dramatically (>300%) increase quantal size in midbrain DA neurons (Pothos et al., 1996; Pothos et al., 1998b). While the modest (~100-140%) increases in DA release reported here may be linked to dose differences,

a potential role of D2 autoreceptors on quantal size in intact preparation (Pothos et al., 1998a) may also lead to these differences.

Prior to L-DOPA, DA release rate is diminished by partial DA depletion, which is consistent with previous work in METH pretreated rats (Howard et al., 2011; Howard et al., 2013b) and work in a rat model of Parkinson's disease (Garris et al., 1997; Bezard et al., 2000; Bergstrom and Garris, 2003), but is in contrast to studies demonstrating compensation of DA release following DA denervation (Stachowiak et al., 1987; Snyder et al., 1990; Zigmond et al., 1990; Zoli et al., 1998; McCallum et al., 2006; Perez et al., 2008; Bergstrom et al., 2011). The reason for these discrepant results is beyond the scope of the current work (see Bergstrom et al., 2011).

"High" and "low" release sites exist side-by-side in striatum. DA release within the striatum is known to be heterogeneous, with not all sites responding in the same way to stimulation or a cue (May and Wightman, 1989b, a; Garris et al., 1994a; Brown et al., 2011). Besides regional variation, differences in release within a region have also been described (Rodriguez et al., 2006; Moquin and Michael, 2009; Wang et al., 2010; Moquin and Michael, 2011; Taylor et al., 2012). However, to our knowledge, the finding that some release sites showed a pronounced amplification in amplitude post-L-DOPA, while some do not, is a novel one. These high and low release sites reported herein do not appear to follow the guidelines for "fast" and "slow" release sites laid out by Michael and colleagues (Moquin and Michael, 2009; Taylor et al., 2012). By positioning two CFMs within the same brain region in a rat, we were able to rule out injection site

contributing to lack of L-DOPA effect. Further, DLS seemed to possess more low release sites than DMS; as differences have been described between the function and connectivity of DMS and DLS (*e.g.*, Yin et al., 2004, 2006; Humphries and Prescott, 2010), it is not entirely surprising that DMS and DLS have different contingents of types of release sites. Further, while saline- and METH-pretreated rats seemed to have numerous high release sites in DMS, in DLS, high release sites were less frequent, even in saline-pretreated rats (experimenter observations). The mechanism for different actions of L-DOPA at these different sites is unknown, but may be related to D2 autoreceptor density (Moquin and Michael, 2009; Taylor et al., 2012).

L-DOPA preferentially enhances phasic DA release. The accepted mechanism of L-DOPA-mediated increases in striatal DA is that L-DOPA enhances both phasic and tonic DA signaling (Breitenstein et al., 2006) and that L-DOPA augments basal DA via increased tonic DA release (Simuni and Hurtig, 2002). Here, results of principal component regression indicate increases in evoked DA responses *without* overt increase in basal DA levels (Fig. 5.8), though average (nonsignificant) increases of ~30 nM were noted nearly 40 min after L-DOPA administration in METH-pretreated animals. Years of microdialysis studies have demonstrated increases in basal DA after L-DOPA, typically at higher doses than those used here (Koshimura et al., 1992). However, microdialysis studies have also demonstrated no change or decreases in basal DA at similar doses used in the current study (Abercrombie et al., 1990; Kannari et al., 2000; Rodríguez et al., 2007), or at lower doses (Touchet and Bennett Jr, 1989).

Importantly, microdialysis lacks temporal resolution to differentiate the respective influences of tonic and phasic DA signaling on DA tone.

Increases in basal DA seen in previous studies utilizing real-time electrochemical approaches have demonstrated increases in currents thought to reflect DA or DA metabolites following L-DOPA (Rodríguez et al., 2007). However, these studies demonstrated changes in basal DA using amperometry and chronoamperometry, which are not selective to DA. In fact, as discussed by Rodríguez et al. (2007), nonselective electrochemical approaches may oxidize L-DOPA, as increases in background current mirror changes in L-DOPA availability in the brain and occur irrespective of increased DA as monitored by microdialysis.

To the contrary, other studies have shown that L-DOPA preferentially increases DA release to high (≥30 Hz) stimulus frequency (May and Wightman, 1989b; Oksman et al., 2009), which coincides with physiological firing rates during burst firing of DA neurons (Grace and Bunney, 1984; Hyland et al., 2002). Furthermore, L-DOPA does not alter [^{11}C]raclopride binding in monkeys (Antonini et al., 1994) and humans (Flöel et al., 2008) when at rest (an index of basal DA), whereas augmented release was noted during formation of motor memory, which is thought to reflect phasic DA activity (Flöel et al., 2008). Furthermore, a recent study found that L-DOPA was able to restore the reward prediction error in elderly subjects (Chowdhury et al., 2013), which is thought to be mediated by phasic DA release (Schultz, 2013; Steinberg et al., 2013).

The lack of increased basal DA observed in this study is puzzling, as increasing DA release while lowering DA uptake should result in increased basal DA levels. However, feedback loops in striatal circuitry may subserve the lack of basal increases. Additionally, “low release” sites monitored in the current study may display greater D2 autoreceptor regulation of DA release (Taylor et al., 2012), which may explain lack of increases in DA release and basal DA levels in these sites. The use of anesthesia in this work is also a concern, as DA cell firing is blunted under anesthesia (Kelland et al., 1990). Finally, increases in DA tone may occur below detection limits of voltammetry, although this is unlikely, as “efflux” of DA following amphetamine is clearly detectable in both anesthetized (Ramsson et al., 2011a) and freely-behaving rats (Daberkow et al., 2013).

Implications for L-DOPA as a cognitive enhancer. Phasic DA signaling is known to be important in cue-based learning (Zweifel et al., 2009), encoding errors in reward prediction (Schultz, 1998, 2007, 2013), and “wanting” of rewards (Berridge, 2007). We have previously proposed reduced phasic DA signaling as a possible link between METH neurotoxicity and METH-induced cognitive impairments (Howard et al., 2011; Howard et al., 2013b). Based on the current work, it is possible that L-DOPA may remediate some impaired cognitive processes following METH neurotoxicity by normalizing phasic DA signaling. Work in both healthy (Knecht et al., 2004; Pessiglione et al., 2006; Chowdhury et al., 2013) and parkinsonian (Frank et al., 2004; Graef et al., 2010) patients has revealed cognitive-enhancing properties of L-DOPA, though L-DOPA treatment is also associated with impaired reversal learning and learning from negative

outcomes in Parkinson's patients (Cools et al., 2001; Cools et al., 2004; Frank et al., 2004). This bidirectional effect of L-DOPA on cognitive processes has been suggested to be caused by enhanced "Go" or direct pathway activity, which is specifically facilitated by phasic DA (Dreyer et al., 2010), and simultaneous impairments in "No Go" or indirect pathway activation (Frank et al., 2004; Wiecki and Frank, 2010), which is influenced by DA tone (Dreyer et al., 2010). Therefore, future work should investigate the effects of L-DOPA on these important forms of learning in the setting of METH neurotoxicity.

Conclusions. The results of this study demonstrate for the first time that L-DOPA can restore phasic DA signaling known to be deficient in METH-pretreated rats (Howard et al., 2011; Howard et al., 2013b) without impacting basal DA tone. As human METH abusers are known to have cognitive impairments as a result of partial monoamine loss (Dean et al., 2013), restoration of normal dopaminergic signaling in the brain could improve cognitive function and allow abstinent METH abusers to have a better chance of staying abstinent and functioning better in society.

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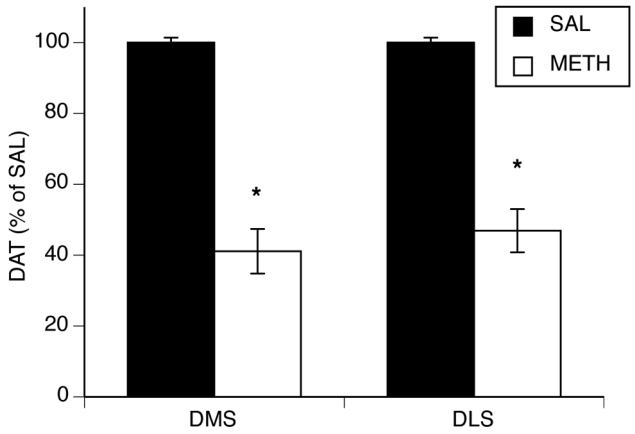


Figure 5.1. A neurotoxic regimen of METH results in decreases in DAT and SERT binding. [^{125}I]RTI-55 autoradiography revealed that METH neurotoxicity causes decreases in striatal DAT binding. Values in METH-pretreated rats are expressed as a percent of values in saline-pretreated rats. *Effect of pretreatment, p=0.0001.

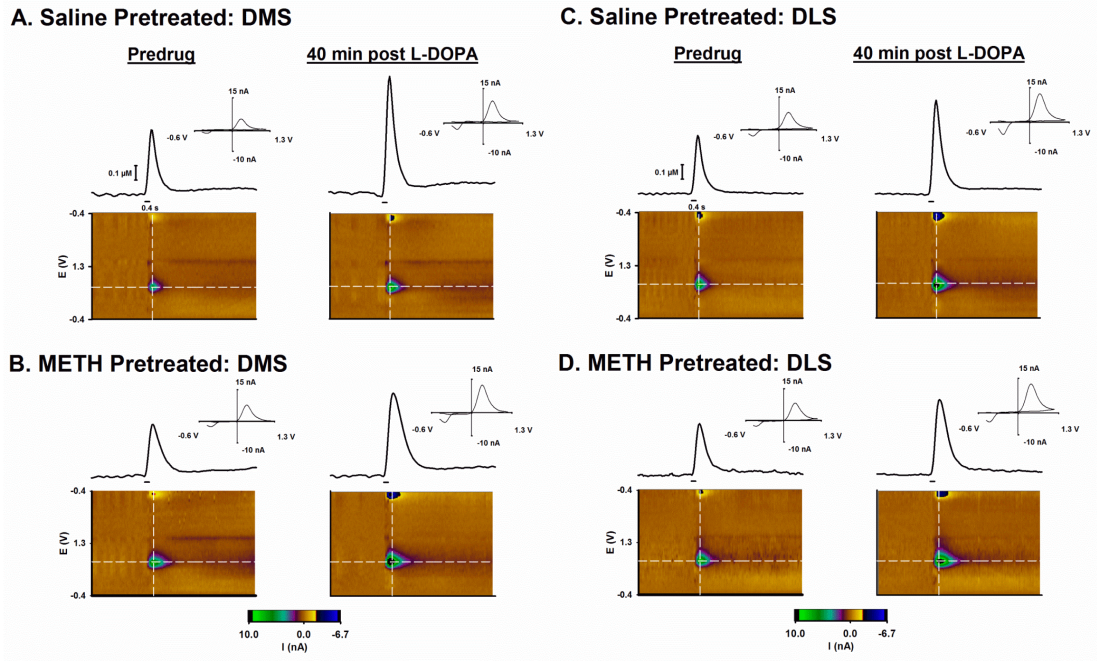


Figure 5.2. Representative evoked DA traces in response to L-DOPA in saline- and METH-pretreated animals. Evoked DA responses are shown from baseline (predrug, left, thick black trace) and 40 min (right) after L-DOPA in saline- (A and C) and METH- (B and D) pretreated rats in both DMS (A and B) and DLS (C and D). Under evoked responses, stimulation duration is shown as a straight black line. Cyclic voltammograms indicate the measured analyte is DA (INSET). Colorplots (below evoked trace) are shown with time as the abscissa and voltage as the ordinate. The z axis (color) represents measured current during each voltage scan. DA traces are taken from peak DA oxidation potentials (horizontal dotted white lines) and cyclic voltammograms are taken from maximal evoked responses (vertical white dotted lines).

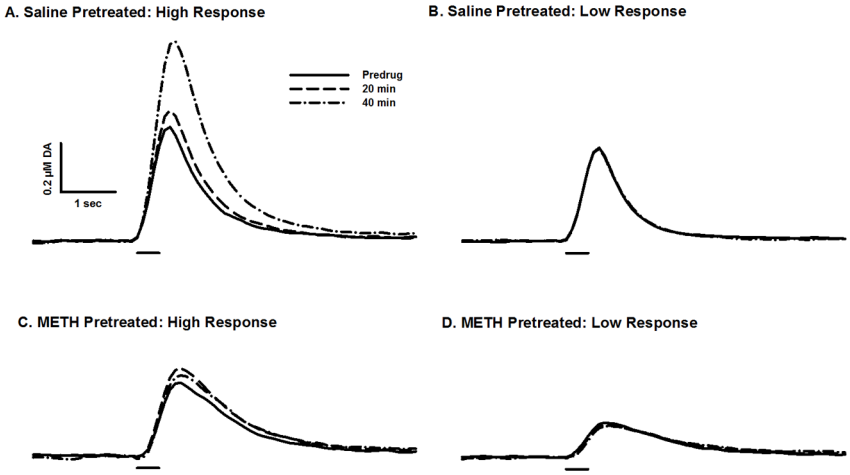


Figure 5.3. L-DOPA preferentially increases evoked responses in sites of higher predrug DA release in DMS and DLS of saline- and METH-pretreated rats. Traces show amplitude of evoked DA release at baseline and 20 and 40 min after L-DOPA in both a saline- (A and B) and METH- (C and D) pretreated rat. CFMs were positioned within 0.5 mm of each other in the same saline- or METH-pretreated rat, demonstrating that high and low release sites can be found side-by-side within the same brain region of the same rat. The high release site in each rat shows a robust increase in evoked $[DA]_{max}$ after L-DOPA, while the low release sites show no activation after L-DOPA. Example traces are from DMS.

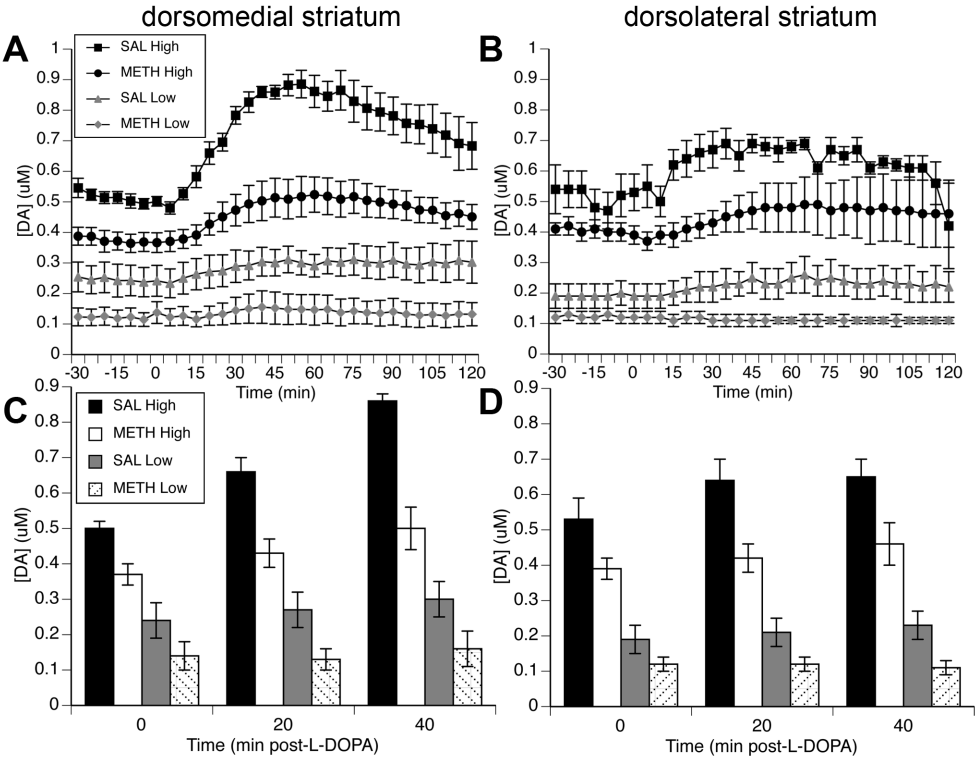


Figure 5.4. Timecourse of L-DOPA effects on evoked DA signals amplitude. (A) L-DOPA increased evoked responses in high release sites in DMS of saline- (black squares) and METH-pretreated animals (black circles), but had no effect on low release sites of saline- (gray squares) or METH-pretreated animals (gray circles; L-DOPA injected at time 0). (B) L-DOPA increased evoked responses in high release sites in DLS of saline- and METH-pretreated animals, but these responses were blunted relative to those seen in DMS (A). (C) Average evoked response by pretreatment and site type revealed that L-DOPA had differential actions on high release sites in DMS (black, saline-pretreated high release sites; gray, METH-pretreated high release sites; white, saline-pretreated low release sites; patterned, METH-pretreated low release sites). (D) Similar to DMS, L-DOPA had differing actions on low and high release sites of DLS.

Figure 5.5. L-DOPA increases DA release but has no impact on DA uptake rates in the DMS and DLS of saline- and METH-pretreated animals. Evoked $[DA]_{max}$, $[DA]_p$, and *k* at 0, 20, and 40 min post-L-DOPA in both high and low release sites in DMS and DLS. Graphs compare saline- and METH-pretreated rats. (A-D) Repeated measures MANOVA revealed a significant effect of time in DMS high (p=0.0005) and DLS high (p=0.0042) sites. There was a trending effect of time in DMS low sites (p=0.11). **Significant effect of pretreatment, p<0.01. *Significant effect of pretreatment, p<0.05. #Trend towards effect of pretreatment, p<0.11. (E-H) Repeated measures MANOVA revealed a significant effect of time in DMS high (p=0.0004) and DLS high (p=0.0048) sites. *Significant effect of pretreatment, p<0.05. #Trend towards effect of pretreatment, p<0.1. (I-J) Repeated measures MANOVA revealed a significant effect of time in DMS (p=0.0005) and DLS (p=0.0093). *k* for high and low release sites was not significantly different, so sites are collapsed into one graph for DMS and one for DLS.

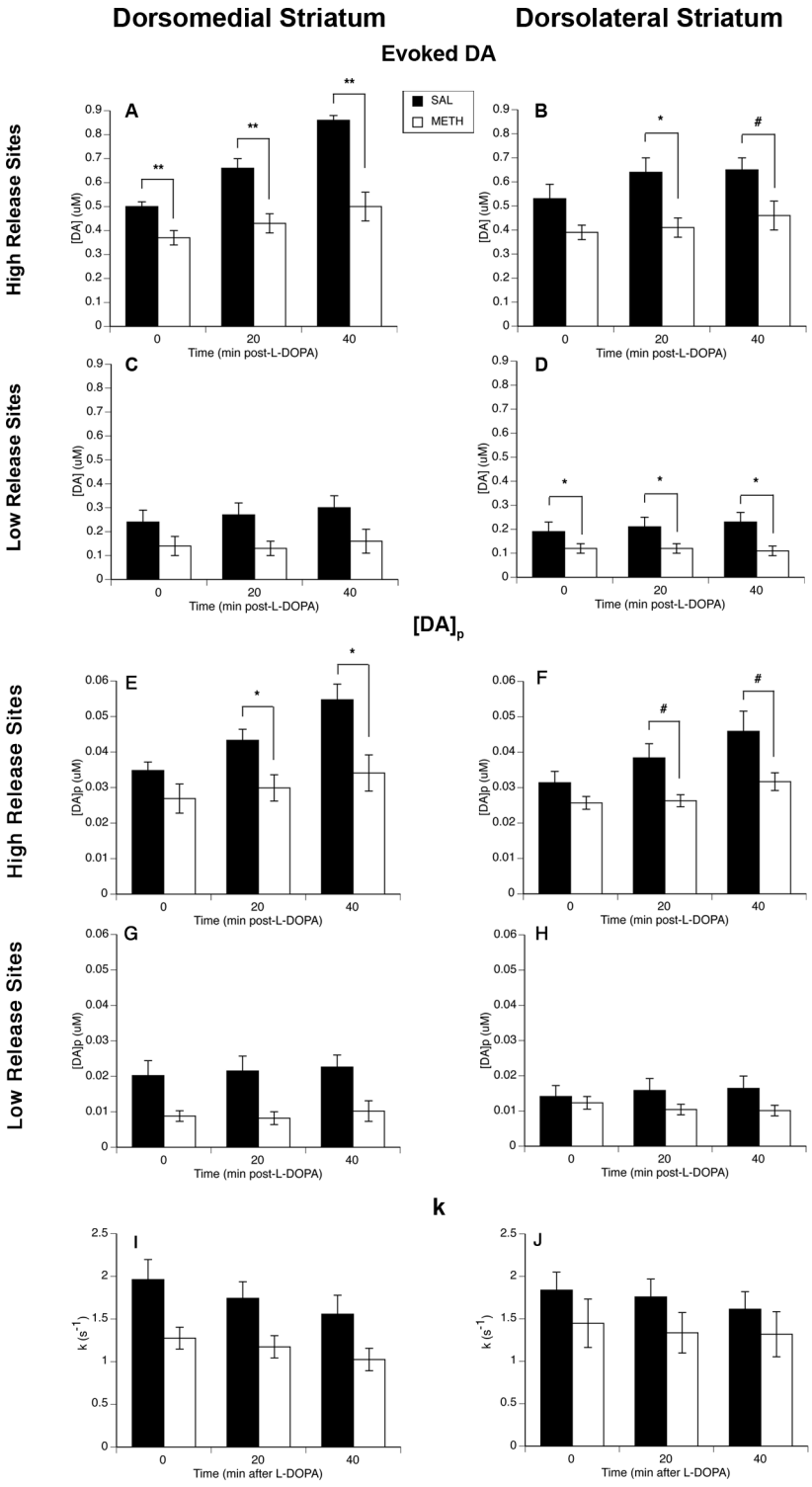
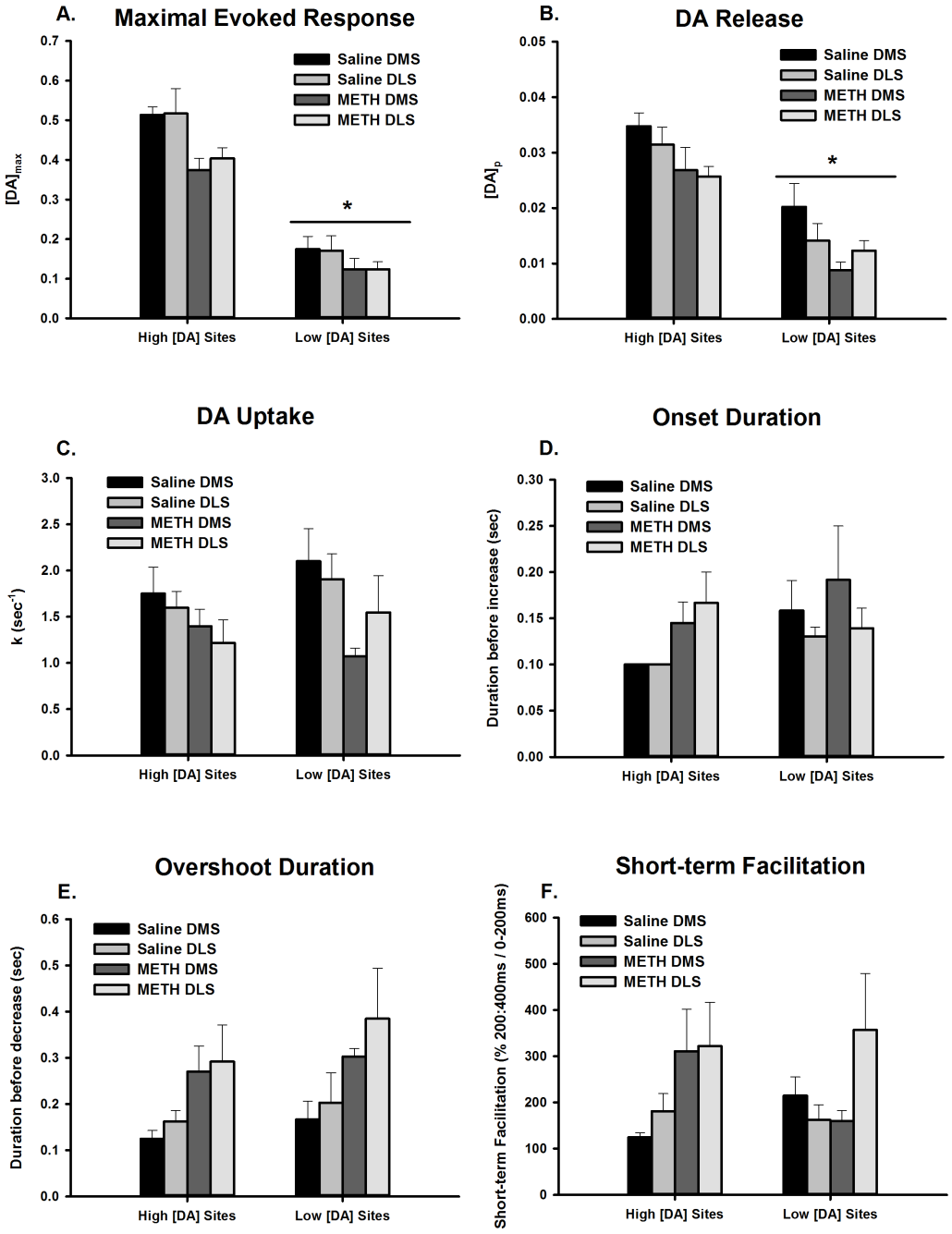

Dorsomedial Striatum
Dorsolateral Striatum
Evoked DA
SAL
METH
High Release Sites
Low Release Sites
A
B
C
D
[DA] (uM)
Time (min post-L-DOPA)
[DA]p
E
F
G
H
[DA]p (uM)
k
I
J
k (s-1)
Time (min after L-DOPA)

Figure 5.6. Amplitude of evoked phasic-like DA signals and DA release are greater in high release sites, but other parameters of evoked responses are not different between site types. (A-B) When separated by high and low release sites, both maximal concentration of evoked phasic-like DA signals (A: $[DA]_{max}$) and DA release (B: $[DA]_p$) are reduced. *Significantly different from high release sites, $p<0.05$. (C-F) DA uptake (C), duration to increase following stimulation (D), duration of overshoot following the end of stimulation (E), and short-term facilitation (F) are not statistically different between high and low release sites.


A.
Maximal Evoked Response
[DA]max
Saline DMS
Saline DLS
METH DMS
METH DLS
*
High [DA] Sites
Low [DA] Sites
B.
DA Release
[DA]p
Saline DMS
Saline DLS
METH DMS
METH DLS
*
High [DA] Sites
Low [DA] Sites
DA Uptake
C.
k (sec-1)
Saline DMS
Saline DLS
METH DMS
METH DLS
High [DA] Sites
Low [DA] Sites
Onset Duration
D.
Duration before increase (sec)
Saline DMS
Saline DLS
METH DMS
METH DLS
High [DA] Sites
Low [DA] Sites
Overshoot Duration
E.
Duration before decrease (sec)
Saline DMS
Saline DLS
METH DMS
METH DLS
High [DA] Sites
Low [DA] Sites
Short-term Facilitation
F.
Short-term Facilitation (% 200:400ms / 0-200ms)
Saline DMS
Saline DLS
METH DMS
METH DLS
High [DA] Sites
Low [DA] Sites

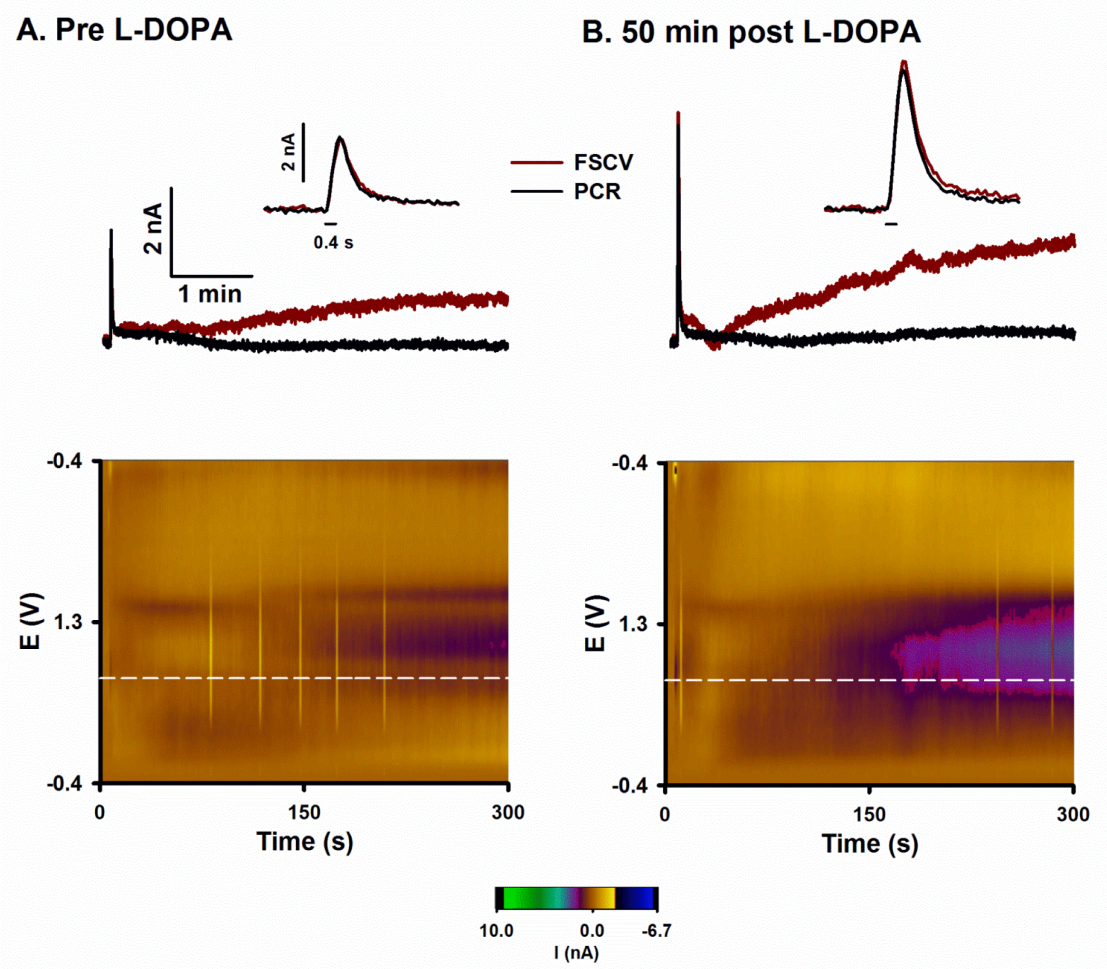


Figure 5.7. Principal component regression removes interferents from voltammetric recordings. Principal component regression indicated that increases in current across the peak oxidation potential for DA (red line, FSCV) were not attributed to changes in DA (black line, PCR). After subtracting pH and drift, dramatic increases in current were negated. Additionally, principal component analysis did not detract current attributable to DA from the DA component, as evoked responses show good agreement between raw voltammetric recordings and the DA component of principal component regression output (INSET).

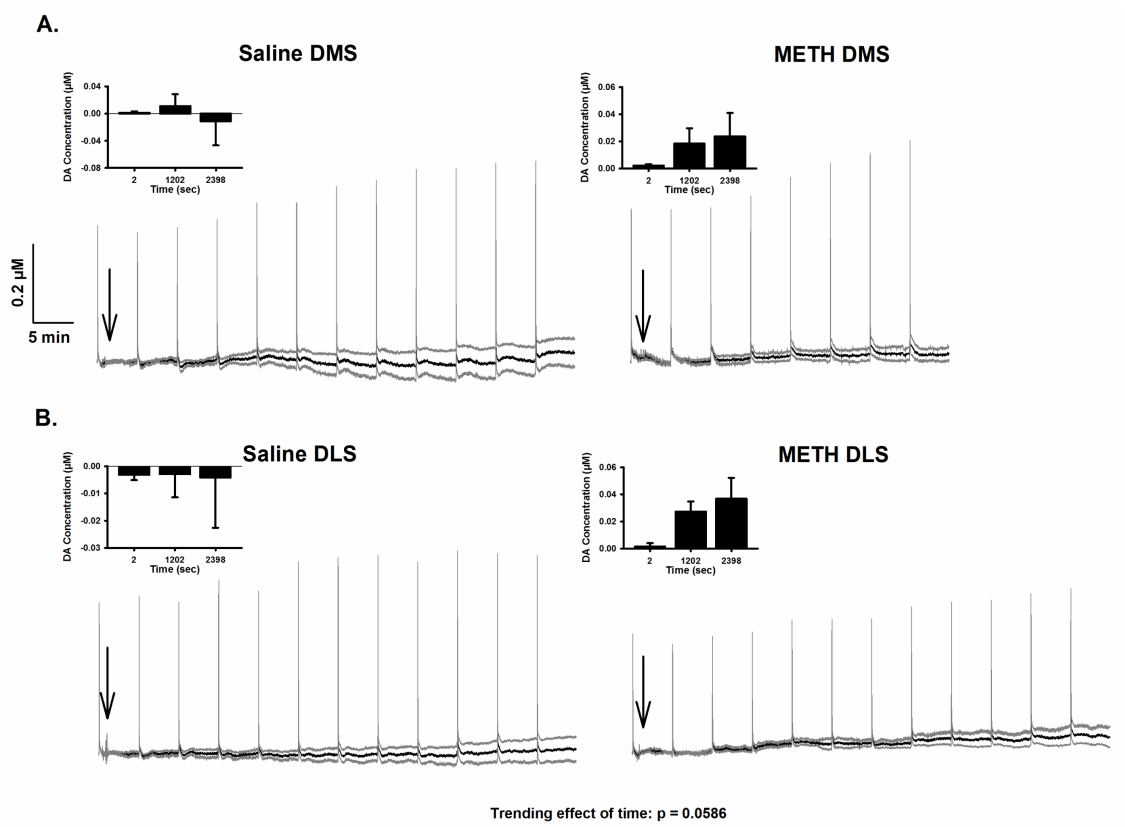


Figure 5.8. L-DOPA selectively increases phasic DA signaling. Traces show average principal component regression output (black line) ± SEM (grey lines). Traces show first hour following L-DOPA administration (arrow). METH DMS is shown only for the first 40 min (see Results). Current was averaged between 20 points at 2 sec (predrug), 1202 sec, and 2398 sec and then averaged between recordings (INSET, data are mean±SEM).

CHAPTER 6

CONCLUSION

This dissertation has presented data for three main conclusions: first, methamphetamine (METH)-induced neurotoxicity results in an alteration of circuitry underlying response-reversal learning. Second, the effector immediate-early gene *Arc* (activity-regulated cytoskeleton-associated gene) is critical for consolidation of learning in both dorsomedial (DM) striatum and nucleus accumbens (NAc) shell when that brain region is necessary for the learning being examined on the behavioral task. Third, if phasic dopamine (DA) neurotransmission is restored in METH-pretreated rats, either artificially or pharmacologically, rescued METH-induced deficits in striatal gene expression and evoked DA release are observed.

Rats that are pretreated with a neurotoxic regimen of METH have long-lasting partial DA loss (Seiden et al., 1976; Morgan and Gibb, 1980; Ricaurte et al., 1980; Wagner et al., 1980). This partial DA loss causes deficits on some learning and memory tasks, but no noticeable impairment on others. For example, METH-pretreated rats perform as well as saline-pretreated rats on a response-reversal T-maze task, in that they take the same number of trials to reach criterion (Daberkow et al., 2008; Pastuzyn et al., 2012; Chapters 2 and 3).

However, METH-pretreated rats have no correlation between *Arc* mRNA expression and performance on reversal learning in DM striatum as saline-pretreated rats do (Daberkow et al., 2007, 2008; Chapter 3). Thus, knocking down *Arc* expression in DM striatum of saline-pretreated rats using an *Arc* antisense oligonucleotide impairs memory consolidation on the response reversal-learning task, but has no effect on memory consolidation in METH-pretreated rats (Pastuzyn et al., 2012; Chapter 2). Instead, METH-pretreated rats have a correlation between *Arc* and performance in NAc shell, which does not exist in saline-pretreated rats (Chapter 3). Correspondingly, when *Arc* is knocked down via the *Arc* antisense oligonucleotide in NAc shell, it impairs memory consolidation in METH-, but not saline-, pretreated rats (Chapter 3). These data suggest a number of things. First, METH-pretreated rats no longer rely on DM striatum to perform the response reversal-learning task, as evidenced by the lack of effect of NMDA receptor blockade or *Arc* antisense on learning and memory consolidation in these rats. Second, METH pretreatment appears to induce a change in circuitry underlying response-reversal learning, such that METH-pretreated rats rely on NAc shell rather than DM striatum to consolidate reversal learning memories. Finally, the correlation of *Arc* expression with a measure of learning, rather than simply the induction of *Arc*, appears to indicate the necessity of encoding processes in that brain region for the learning taking place on the task, as has been previously suggested (Guzowski et al., 2001).

METH-pretreated rats have a deficit in phasic DA neurotransmission, but not in tonic DA neurotransmission (Cass and Manning, 1999; Howard et al.,

2011; Loewinger et al., 2012; Howard et al., 2013a; Chapter 5). We previously hypothesized that restoring phasic DA neurotransmission in METH-pretreated rats may restore proper functioning to the striatum (Keefe and Horner, 2010; Barker-Haliski et al., 2012). We have now shown that stimulating the medial forebrain bundle (MFB) in a phasic-like manner can rescue impaired *preprotachykinin* (*ppt*) expression in striatum of METH-pretreated rats, whereas stimulating the MFB in a tonic-like manner has no effect on gene expression (Howard et al., 2013b; Chapter 4). Phasic-like stimulation of the MFB also induces immediate-early gene expression (*Arc* and *zif268*) to the same extent in both METH- and saline-pretreated rats despite the partial loss of DA terminals in METH-pretreated rats, suggesting that the striatum of METH-pretreated rats has the capability for proper gene expression if phasic DA neurotransmission is provided.

L-3,4-dihydroxyphenylalanine (L-DOPA) is the most common treatment for the symptoms of Parkinson's disease and works by increasing the amount of DA in the brain. We hypothesized that L-DOPA could rescue phasic DA neurotransmission in METH-pretreated rats simply by providing more DA for neurons to release. When L-DOPA was given to anesthetized rats and the MFB was stimulated in a phasic-like manner, fast-scan cyclic voltammetry for DA at sites in DM and dorsolateral (DL) striatum revealed that L-DOPA increased the amplitude of DA release evoked by phasic-like stimulation of the MFB in METH-pretreated rats back to evoked levels seen in saline-pretreated rats (Chapter 5). Interestingly, this did not occur at all recording sites in DM and DL striatum

irrespective of pretreatment with METH or saline—some sites responded robustly to L-DOPA (“high release sites”), whereas other sites did not increase evoked DA release after L-DOPA (“low release sites”). At this time, the basis for the differences between these sites in the same brain region of the same animal is unknown. Finally, using principal component regression, we were able to separate out the tonic/basal component of the DA recording from the phasic component. We found that 50 mg/kg L-DOPA specifically increased phasic-like DA release in both METH- and saline-pretreated rats without altering basal release, suggesting that one dose of L-DOPA can impact just phasic DA neurotransmission, the mode of firing impaired in METH-pretreated rats.

In conclusion, METH-pretreated rats have altered circuitry underlying reversal learning and deficits in striatal gene expression and phasic DA neurotransmission. The impairment in gene expression and DA neurotransmission can be rescued by restoring phasic-like DA neurotransmission, either via artificial electrical stimulation or pharmacologically using L-DOPA. These findings suggest that it may be possible to restore function to the striatum as a whole in METH-pretreated rats and, consequently, the normal circuitry used in behavioral tasks. Restoration of striatal function has obvious implications for translational therapeutic use of L-DOPA in recovering METH addicts, and could help them function better while they are trying to abstain from this highly addictive drug. In the future, it would be interesting to test whether L-DOPA could restore striatal function in METH-pretreated rats on the response-reversal T-maze task. If L-DOPA restores phasic DA release in DM

striatum of METH-pretreated rats back to normal and they could once again use DM striatum on the reversal task, then NMDA receptor blockade or *Arc* antisense in DM striatum would be expected to impair reversal learning or retention of learning, respectively. These findings would prove that METH-pretreated rats have regained use of DM striatum through rescue of phasic DA signaling.

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