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The role of psychostimulants in addiction-related learning and memory in mice

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The role of psychostimulants in addiction-related learning and memory in mice

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Psychology by Tristan Shuman

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Chair

University of California, San Diego
2012
DEDICATION

This dissertation is dedicated to my current, past, and future family.
"I would disagree with the statement: zombiism is a devastating form of abnormal death, so death provides a good model for understanding zombiism."

Kiriana Cowansage
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ABSTRACT OF THE DISSERTATION

The role of psychostimulants in addiction-related learning and memory in mice

by

Tristan Shuman

Doctor of Philosophy in Psychology

University of California, San Diego, 2012

Professor Stephan G. Anagnostaras, Chair

Learning requires some form of long-lasting modification of neural activity that underlies a change in behavior. The mechanisms of long-lasting changes in the brain are likely to be conserved indicating that there is likely to be overlap in the mechanisms of memory and drug addiction. This dissertation will demonstrate how psychostimulants can modulate memory processes and create addiction-like memories in mice.

First, we showed that a low dose of modafinil, a psychomotor stimulant, selectively enhances contextual fear memories, while a high dose disrupts this memory. This high dose can, however, enhance water maze learning. Second, we demonstrated that modafinil can induce a conditioned place preference and can elicit locomotor sensitization in cocaine-trained animals. These results indicate that modafinil can induce and modulate both contextual and addiction-related memory processes.
To further explore the action of modafinil and other psychostimulants, we conducted a literature review to identify regions of the brain involved in the induction and expression of addiction-related memories. We found a diverse range of brain areas implicated in drug addiction but also a lack of consistency perhaps due to limitations of classical techniques.

In order to localize the neural correlate of addiction-related memories, we used a histone-GFP mouse that expresses a long-term tag of neuronal activity within a specified tagging window. We tagged neurons active during initial drug exposure and compared them to a marker of neurons active during place preference testing, using the immediate early gene zif. When cocaine was paired with the conditioning context rather than the home cage neurons in the dorsal striatum were more likely to be activated during both time points. This indicates that the dorsal striatum is one site of neural plasticity that underlies conditioned place preference to cocaine.

Finally, to localize the neural correlate of locomotor sensitization we tagged initial exposure to cocaine and compared these neurons to immediate early gene expression after a subsequent exposure to cocaine. The medial prefrontal cortex and nucleus accumbens shell appear to store a memory of the drug experience, but are unlikely to be involved in the contextual control of sensitization.
The role of psychostimulants in addiction-related learning and memory in mice

Encoding and recalling memories are a fundamental part of every aspect of life. Recalling previous experiences are imperative for avoiding negative outcomes and informing future decisions. As such, there is a clear evolutionary advantage for individuals with the ability to learn and recall past experiences. There are, however, instances where recalling previous experiences can produce harmful patterns of behavior. For example, addictive drugs can produce long-lasting changes in behavior that cause significant harm to an individual. This change in behavior is presumably caused by some “memory” of the drug-experience.

Many different types of memories have been identified. Human memories are typically divided into at least two classes: explicit memories, those that can be overtly stated such as facts or events, and implicit memories, which are observed only as an unconscious change in behavior (Squire, 1992). Explicit memories rely on structures in the medial temporal lobe, while implicit memories rely on a more diverse range of brain areas. For instance, habit learning appears to rely on the striatum, emotional learning relies on the amygdala, and priming relies on the neocortex (Squire, 1992). Furthermore, these multiple memory systems are dissociable since targeted lesions of the brain can selectively disrupt different memories (McDonald & White, 1993). Thus, memories appear to be stored in different brain areas specific to the type of learning involved.

Despite the diverse range of brain areas involved in learning and memory, the mechanisms appear to overlap. In all forms of memory, an experience must lead to a long-lasting change in neural connectivity (i.e., synaptic plasticity) that underlies behavior. Indeed, mechanisms such as long-term potentiation, long-term depression,
and chromatin remodeling have been identified that produce long-lasting changes in neural activity that are likely to govern learning and memory (Bliss & Lomo, 1973; Levenson & Sweatt, 2005; Stanton & Sejnowski, 1989). These mechanisms of synaptic plasticity are conserved across various types of learning, from spatial navigation to conditioned taste aversion to social recognition (Lynch, 2004; Martin, Grimwood, & Morris, 2000). These mechanisms are also conserved across learning in various species, from aplysia to mice to humans (Bailey, Bartsch, & Kandel, 1996; Beck, Goussakov, Lie, Helmstaedter, & Elger, 2000; Chen et al., 1996). Indeed, there is major overlap in the mechanisms involved in diverse memory systems. While the exact nature of how synaptic plasticity can encode, store, and retrieve previous experiences remains unknown, it is clear that this synaptic plasticity is required for all types of memories. This indicates that despite involving different brain areas, both explicit and implicit memories are likely to be guided by similar cellular and molecular mechanisms in the brain.

The memory of a drug experience is likely to share similar physiological substrates with that of traditional forms of learning and memory. Similar to other types of memory, exposure to an addictive drug causes long-lasting changes in the brain that continue to persist even after the individual is off the drug. These changes in neural activity and synaptic connections likely drive an individual to pursue an addicted pattern of behavior. In this manner, the drug creates a memory of the drug-experience, be it explicit or implicit, which drives addictive behavior. The synaptic plasticity underlying drug addiction may be similar to other types of memory.

To better understand how addictive drugs produce long-lasting changes in the brain, a number of animal models have been identified. Simply exposing an animal to a drug can cause dramatic changes in both the brain and behavior. By identifying the neural plasticity that is responsible for these changes in behavior, we can begin to
understand how drugs come to control the actions of drug addicts. These changes in behavior seen after drug pairing are considered addiction-related memories because they are likely to underlie the compulsive behavior observed in human addicts.

A number of addiction-related memories have been identified in order to model drug addiction in rodents, each with distinct advantages and disadvantages. Here we present four models of drug addiction-related memories that are easily induced with minimal training and passive administration of a drug: locomotor sensitization, contextual control of sensitization, conditioned locomotor response, and conditioned place preference. Locomotor sensitization is an enhanced sensitivity to the locomotor activating effects of a drug after an initial experience with the drug. For example, animals that have previous experience with cocaine will show a higher locomotor response to the drug than naïve controls. This increased response is theorized to be responsible for the increased incentive of addicts to continue taking a drug, despite dramatic negative consequences (Robinson & Berridge, 2003, 2008). A second addiction-related memory is the contextual control of locomotor sensitization. Sensitization only occurs in the context previously paired with the drug. This context-specificity is dissociable from the sensitization itself and is thought to inhibit the drug memory in inappropriate environments (Anagnostaras, Schallert, & Robinson, 2002). The third addiction-related memory is the conditioned locomotor response, which can elicit a drug-like state by simply exposing an individual to the context that was previously paired with the drug. This memory is thought to be responsible for relapse by reminding an addict about the drug-state, leading to increased craving and eventually relapse. Finally, conditioned place preference is defined by an animal choosing to explore an area that was previously paired with a drug over an area that was paired with saline. This off-drug test models drug-seeking behavior of addicts. Together, these and other
addiction-related memories are likely to produce the compulsive behavior seen in drug addicts.

This dissertation demonstrates the interplay between psychostimulants, memory, and addiction. First, we show that psychostimulants can modulate contextual and spatial memory processes, and induce addiction-related memories. To localize the action of psychostimulants in these addiction-related memories we first review evidence using classical techniques. Despite a vast literature, there is very little agreement on the site of plasticity underlying these behavioral changes induced by psychostimulants. Furthermore, these classical techniques are limited to identifying area involved in any aspect of the behavior, rather than the particular neural plasticity underlying the behavior. Thus, we utilized a novel transgenic approach to localize the specific neural representation of these memory forms. By labeling cells active during initial exposure to cocaine and during drug recall, we localized the dorsal striatum as one site of plasticity responsible for conditioned place preference. Similarly, we investigated the neural representation of locomotor sensitization and found a stable neural representation of the drug experience in the medial prefrontal cortex and nucleus accumbens shell, but these areas did not control the expression of sensitization. It is likely that the contextual control of sensitization is mediated by a brain region we did not examine, or it has a divergent neural representation that is not manifested as an increase in colocalization of cells active during training and testing.

**Modafinil enhances contextual fear memory and Morris water maze in mice**

Modafinil is a psychostimulant approved for the treatment of narcolepsy, but also widely prescribed off-label to enhance alertness and attention in patients with Alzheimer’s disease, Parkinson’s disease, and attention deficit hyperactivity disorder
(Nieves & Lang, 2002; O'Connor, 2004; Taylor & Russo, 2000). The mechanism of modafinil remains unknown, but it is clear that the drug exhibits its effects through monoamine neurotransmission, likely by acting on monoamine transporters to increase the release of dopamine and norepinephrine (Korotkova et al., 2007; Madras et al., 2006; Qu, Huang, Xu, Matsumoto, & Urade, 2008; Zolkowska et al., 2009).

In Chapter 1, we explore how modafinil can modulate memory processes by administering the drug prior to learning two popular rodent models of memory. First, we administered a high dose of modafinil prior to learning the Morris water maze task. In this task, animals are placed in a tub with opaque-colored water and an escape platform submerged just below the surface of the water. Animals must use the spatial cues in the room to locate and swim to the hidden platform. Prior to each day of training, animals received an injection of modafinil (75 mg/kg) or saline. After 7 days of training, a probe test was conducted by removing the platform and measuring the animal’s swim path. Animals that were trained on modafinil spent more time in the target quadrant than animals trained with saline. Thus, modafinil was able to enhance the learning of this spatial memory.

We then hoped to expand these findings using Pavlovian fear conditioning, a popular task that models both explicit and implicit memory. In this task, a tone is paired with a mild footshock to induce fear. A week later, the animal is exposed to the training context or the trained tone and memory is assessed by the amount of time spent freezing, an innate fear response. Contextual fear conditioning is the standard model of explicit memory because, like in humans, it initially requires the hippocampus but become independent of the hippocampus after consolidation. Similarly, tone fear conditioning models human implicit memory because it does not require the hippocampus (Anagnostaras, Gale, & Fanselow, 2001; Gale et al., 2004). Thus, we
were able to test the ability of modafinil to modulate learning on a model of both explicit and implicit memory. We administered a range of doses (0.075 – 75 mg/kg) prior to training the animals and found that modafinil was able to selectively modulate contextual fear conditioning in a dose-dependent manner. A low dose (0.75 mg/kg) of modafinil enhanced contextual fear memory, while a high dose (75 mg/kg) severely disrupted the memory. Modafinil did not affect tone fear.

Together, these findings indicate that modafinil is able to modulate memory performance and implicates the hippocampus in its locus of action because both Morris water maze and contextual fear conditioning are hippocampus-dependent tasks. There are also complex dose-dependent effects where a high dose enhances water maze learning, but disrupts context fear memory. While these findings appear contradictory, there are likely differences in the demands of each task that affect how modafinil modulates the memory. Furthermore, this study is the first evidence that very low doses of modafinil can modulate memory performance. The vast majority of studies on modafinil have exclusively examined very high doses that appear to inhibit memory performance on some tasks.

**Modafinil induces place preference and cross-sensitizes with cocaine**

While the mechanism of modafinil is unknown, it has been considered non-addictive because initial reports indicated it did not produce addiction-related memories in rodents (Deroche-Gamonet et al., 2002; Gold & Balster, 1996). These studies did, however, indicate that modafinil can elicit drug-like memories in animals previously exposed to cocaine. Thus, as with the Morris water maze and Pavlovian fear conditioning, modafinil may be able to modulate learning or retrieval processes in these addiction-related memories. Furthermore, while there have been no reports of addiction
to modafinil, it remains possible that the drug has addictive potential if given through a faster route of administration or a higher dose. This evidence led us to investigate whether modafinil could induce or modulate addiction-related memories in mice.

In Chapter 2, we examined the ability of modafinil to induce or modulate conditioned place preference and locomotor sensitization. To assess the role of modafinil in these memory processes, we used a conditioned place preference paradigm to repeatedly administer modafinil, cocaine, or a cocktail of both modafinil and cocaine in one side of a conditioning chamber. When tested off-drug, all three drug groups showed a preference for the drug-paired side indicating that each drug was rewarding to the animals. This finding that modafinil can produce reward in animals, was somewhat surprising, but in line with human experiments that show it can be modestly rewarding (Stoops, Lile, Fillmore, Glaser, & Rush, 2005). Modafinil did not, however, produce a large locomotor sensitization like cocaine and the cocktail of modafinil and cocaine did. This lack of sensitization may be responsible for the lack of addiction to modafinil as this process may be required to drive compulsive drug use (Robinson & Berridge, 2003, 2008). Finally, we found that modafinil was able to elicit a sensitized response in the cocaine-trained animals. No differences were found between the cocaine-trained animals and the animals that received the cocktail of modafinil and cocaine, indicating that modafinil did not modulate the learning of these addiction-related memories. Thus, modafinil was able to induce conditioned place preference and elicit the expression of sensitization in cocaine-trained animals. Overall, this indicates that modafinil can access the neural substrates underlying addiction-related memories in mice by inducing and eliciting these behaviors. Thus, while modafinil is considered to not be addictive, it remains possible that it could produce addiction in humans.
These findings clearly demonstrate that modafinil can access the circuitry involved in drug addiction. It is unclear, however, which brain areas are involved in these addiction-related memories. In order to understand where in the brain modafinil is working to induce and elicit these memories, we needed a better understanding of the brain areas that underlie these behaviors. Thus, we set out to review the literature on the neuroanatomy of addiction-related memories induced by psychostimulants.

**Neuroanatomy of addiction-related memories induced by psychostimulants**

A large literature exists using classical techniques such as lesions, immediate early gene expression, and targeted microinfusions to localize where addiction-related memories are stored in the brain. In Chapter 3, we review this literature focusing on four psychostimulant-induced behaviors: locomotor sensitization, contextual control of sensitization, conditioned locomotor response, and conditioned place preference. While a number of other addiction-related memories have been identified, we focused on these four because they are easily induced, use high doses of the drug, and only require passive administration of the drug. These particular behaviors are not necessarily better than others at modeling drug addiction, but are easy to study given the limited training involved. Each of these behaviors can be induced with just a single training trial, allowing for researchers to isolate the specific process underlying the induction and expression of these memories. Thus, we examined the induction and expression of these memories separately, and hypothesized that regions implicated in both phases would be critical to the storage of the memory. A number of candidate regions were implicated in each behavior, but the literature contained many contradictions that limited the strength of the findings.
For locomotor sensitization, the nucleus accumbens core, medial prefrontal cortex, and dorsal hippocampus were implicated in both the induction and expression of the memory and thus are likely areas to be involved in this memory process. The contextual control of sensitization has not been studied extensively and thus few candidate regions have been located, however tangential evidence implicates the nucleus accumbens, medial prefrontal cortex, amygdala, hippocampus, and dorsal striatum. For the conditioned locomotor response, the nucleus accumbens core is the only region strongly implicated in the storage of the memory, however the medial prefrontal cortex might also be involved. Finally, for conditioned place preference, the nucleus accumbens shell and amygdala are the most likely areas to be involved in this behavior.

While these classical techniques are useful to identify regions that are likely to be involved in these memories, they are severely limited in understanding how addiction-related memories are encoded, stored, and retrieved within a neural population. Indeed, because addiction appears to engage a diverse range of brain structures (Koob & Volkow, 2010) it is likely that these techniques would be unable to determine the specific sites of storage for these memories. It is crucial then, that we apply modern techniques to clarify and confirm the hypothesized involvement of these regions.

**Localizing the neural correlate of conditioned place preference to cocaine**

One modern tool developed to localize the storage site of memories is the TetTag mouse (Reijmers, Perkins, Matsuo, & Mayford, 2007). This transgenic mouse was developed to create a long-term tag of neurons active during initial learning of a memory, with the ability to compare that tag to immediate early gene expression of neurons activated during retrieval of a memory. In this manner, the authors examined
which individual neurons were active during both time points (i.e., overlap) and found that for contextual fear memory, neurons in the basolateral amygdala showed higher overlap than untrained animals. Similarly, neurons in the lateral amygdala showed higher overlap than untrained animals for tone fear conditioning. These findings not only confirmed these areas as the storage site of fear memories, but also established that the sparse population of neurons recruited during initial learning is stable over time and likely to be reactivated during retrieval. Thus, this reactivation of the trained neural ensemble is a hallmark of a memory’s storage site. This pattern of expression is likely to be similar in other types of memory and can be used to localize a memory trace to a specific brain region.

In Chapter 4, we use a similar transgenic mouse to localize the neural correlate of conditioned place preference to cocaine. We used a histone-GFP transgenic mouse with a cfos promoter to “tag” neurons active only during initial drug exposure. We then compared that tag to immediate early gene expression after a conditioned place preference test. During training, animals received cocaine either paired with one side of a chamber (i.e., Paired group) or in their home cage (i.e., Unpaired group). During test, they were off drug and allowed to freely explore the drug-paired side and the saline-paired side. We hypothesized that we would see higher overlap between the cells activated at training and test only in areas of the brain that are involved in the drug-paired memory trace. Neurons in the dorsal striatum were more likely to show higher overlap in the Paired group than in the Unpaired group. That is, the pairing of the drug with the training context triggered a subpopulation of cells to be activated during both initial drug exposure and CPP testing. This indicates that the dorsal striatum is one site of neural plasticity coding for the association between the drug and context.
Furthermore, this pattern of overlapping activation is likely to underlie conditioned place preference to cocaine and may be the site where this behavior is initiated.

No differences in overlap between paired and unpaired animals were found in the amygdala, hippocampus, medial prefrontal cortex, or nucleus accumbens. When compared to chance overlap (i.e., random distribution of tagged neurons) the overlap of neurons in the basolateral amygdala and hippocampus were above chance, indicating that in these brain regions, neurons active during training were more likely to be active during testing. This finding was consistent, however, between the paired and unpaired groups, indicating that this pattern is likely due to exposure to the context rather than an association between the drug and context.

**Localizing the neural correlate of locomotor sensitization to cocaine**

In Chapter 5, we investigated the neural correlate of locomotor sensitization using the same histone-GFP transgenic mouse. We tagged cells active during initial drug exposure and compared them to immediate early gene expression after a subsequent drug exposure. Again, we compared animals that were trained by receiving cocaine in the training context versus animals that received an unpaired injection. Locomotor sensitization has both a non-associative underlying component and an associative component that restricts the expression of sensitization to the drug-paired context. Thus, the underlying component of sensitization was induced in both groups, but the animals that received cocaine paired with the context showed higher locomotor activity during the subsequent test than Unpaired controls. We examined the overlap of the two tags of cellular activity in the amygdala, hippocampus, medial prefrontal cortex, nucleus accumbens, and dorsal striatum. We found higher than chance overlap in the basolateral amygdala, hippocampus, medial prefrontal cortex, and nucleus accumbens
shell, but these were consistent across both the Paired and Unpaired groups. This indicates that these areas were involved in some concurrent memory process, but are not the areas responsible for the sensitized response to the cocaine. Because the basolateral amygdala and hippocampus were implicated in the context representation of conditioned place preference, it is likely these regions are also representing the context during locomotor sensitization. Furthermore, since this paradigm involved training and testing during an on-drug state, the medial prefrontal cortex and nucleus accumbens shell are likely involved in storing a stable neural representation of the drug. We found no differences in overlap between the Paired and Unpaired animals in the brain regions examined. It is likely that the brain areas examined are not responsible for the contextual control of sensitization, and that some other brain area (i.e., a region mediating the output of locomotor behavior) would show higher overlap in Paired versus Unpaired animals. A second interpretation of this data is that increased overlap between these two time points is not the neural code for this type of memory and the underlying plasticity would be difficult to determine using this transgenic model.

Conclusions

In conclusion, we have demonstrated that psychostimulants can modulate and induce a number of memory processes that control behavior. Understanding how these drugs can modulate synaptic circuits is an important step in understanding how to manipulate memories. Hopefully, by understanding the mechanisms underlying addiction-related memories, we will be able to selectively disrupt these memory processes and control compulsive drug use.
CHAPTER 1

Modafinil and Memory: Effects of modafinil on Morris water maze learning and Pavlovian fear conditioning

By

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Abstract

Modafinil has been shown to promote wakefulness and some studies suggest the drug can improve cognitive function. Because of many similarities, the mechanism of action may be comparable to classical psychostimulants, although the exact mechanisms of modafinil’s actions in wakefulness and cognitive enhancement are unknown. The current study aims to further examine the effects of modafinil as a cognitive enhancer on hippocampus-dependent memory in mice. A high dose of modafinil (75 mg/kg, i.p.) given before training improved acquisition on a Morris water maze. When given only before testing, modafinil did not affect water maze performance. We also examined modafinil (0.075 - 75 mg/kg) on Pavlovian fear conditioning. A low dose of pre-training modafinil (0.75 mg/kg) enhanced memory of contextual fear conditioning (tested off-drug one week later) while a high dose (75 mg/kg) disrupted memory. Pre-training modafinil did not affect cued conditioning at any dose tested, and immediate post-training modafinil had no effect on either cued or contextual fear. These results suggest that modafinil’s effects of memory are more selective than amphetamine or cocaine, and specific to hippocampus-dependent memory.

Keywords: modafinil, memory, hippocampus, fear conditioning, Morris water maze
Modafinil (marketed as Provigil® in the U.S.) is a novel wake-promoting stimulant with low abuse potential used to treat excessive sleepiness and narcolepsy (Bastuji & Jouvet, 1988). Clinical testing in humans has found positive results while testing its effects on depression (Kaufman, Menza, & Fitzsimmons, 2002), schizophrenia (Turner et al., 2004), shift work sleep disorder (Roth & Roehrs, 1996), obstructive sleep apnea syndrome (Pack, Black, Schwartz, & Matheson, 2001), Parkinson’s disease (Nieves & Lang, 2002), and attention deficit hyperactivity disorder (Taylor & Russo, 2000). It is currently approved by the U.S. Food and Drug Administration (FDA) for treatment of narcolepsy, shift work sleep disorder, and obstructive sleep apnea/hypopnea syndrome. The drug is also widely prescribed off-label to enhance alertness, attention, or memory for dementia, attention deficit hyperactivity disorder, excessive daytime sleepiness, and depression (O’Connor, 2004); an illicit market exists for academic doping as well (Garreau, 2006). However, research has yet to determine if these latter effects are due solely to its wake-promoting ability or to a specific ability as a cognitive enhancer, or nootropic (Giurgea, 1982).

Recent studies indicate that modafinil has some cognitive enhancing abilities in rodents performing a variety of learning and memory tasks. Beracochea et al. (2001) found that chronic high doses of modafinil (64 mg/kg, but not 8 mg/kg or 32 mg/kg) produced a delay-dependent increase in spontaneous alternation rates on a T-maze, indicating enhanced working memory. This enhancement was only present when intertrial intervals were extended from 5s to 60s or 180s, making the task much more difficult for controls. Also on a T-maze, modafinil given chronically or acutely (64 mg/kg, but not 32 mg/kg) produced a faster adaptation to a win-stay strategy in a serial spatial discrimination reversal task while not affecting a win-shift strategy (Beracochea et al., 2002; Beracochea, Celerier, Peres, & Pierard, 2003). Since the win-shift strategy was
not impaired, this suggests that overall cognitive ability is increased, rather than just a selective inability to shift responses. In addition, control subjects performed very well on the win-shift task, but very poorly on win-stay; thus, a lack of enhancement on the win-shift task may have been due to ceiling effects or interactions between the drug and the complexity of the task.

In rats, modafinil (55 mg/kg and 100 mg/kg, but not 30 mg/kg) increased choice accuracy on a delayed nonmatching to position swim task by improving the rate of learning, indicating an enhancement of cognitive processing (Ward, Harsh, York, Stewart, & McCoy, 2004). Control animals had difficulty learning this task, which further suggests an interaction between the drug effects and task complexity. Recently, Morgan et al. (2007) reported that modafinil (64 mg/kg) enhanced choice accuracy on a 3-choice sustained attention task, but only when attention load was increased by adding a delay in stimulus onset. No effect on visual discrimination was found. Finally, modafinil (32 mg/kg, 64 mg/kg, and 128 mg/kg) did not increase choice accuracy on a five-choice serial reaction time test in rats and the two high doses increased premature responding, indicating stronger impulsivity (Waters, Burnham, O'Connor, Dawson, & Dias, 2005). This failure to find an enhancement of choice accuracy may be due to the high accuracy of controls, but even when task difficulty was increased by weakened stimulus duration and intensity, the drug did not facilitate performance. These findings indicate that modafinil does not improve visual sensitivity, but they do not rule out the drug as a cognitive enhancer. The attention load in this study may not have been large enough to see any subtle enhancement of working memory (Morgan et al., 2007).

Modafinil’s ability to rescue cognitive impairments in sleep-deprived humans is well established (Pigeau et al., 1995) but many recent studies have focused on possible cognitive enhancing effects in healthy, non-sleep deprived individuals. Turner et al.
(Turner et al., 2003) found that in healthy volunteers, modafinil (100mg or 200mg, p.o.; equivalent to about 1 – 3 mg/kg) selectively enhanced performance on cognitive tasks including digit span, visual pattern recognition memory, spatial planning, and stop-signal reaction time. Ironically, a number of cognitive tasks also showed a slowing in response latency. Modafinil (200mg) also facilitated performance on both a delayed matching task and a numeric manipulation task, indicating that the drug facilitates maintenance and manipulation of information in working memory (Muller, Steffenhagen, Regenthal, & Bublak, 2004). As seen with rodents, increasing the difficulty of these tasks produced stronger enhancement of cognitive processes. Randall et al. (Randall, Shneerson, & File, 2005; Randall, Shneerson, Plaha, & File, 2003) offered conflicting evidence when modafinil (100mg or 200mg) caused few differences in the performance of healthy volunteers on an extensive battery of cognitive tests. Only digit span and visual pattern recognition memory showed enhancement with modafinil, while numerous working memory and attention tasks were unaffected. Recent evidence suggests an interaction between the drug and IQ, which may limit the cognitive enhancing effects of the drug in subjects with high IQ scores (Randall et al., 2005).

While modafinil may generally be classified as a psychostimulant, evidence suggests it may act via a neural pathway distinct from classical psychostimulants, such as amphetamine or cocaine. C-fos and 2-deoxyglucose autoradiography studies in cats and rats, respectively, demonstrate that modafinil selectively increases activity in the anterior hypothalamic nucleus while classical psychostimulants cause broad activation and generally work through the caudate nucleus and prefrontal cortex (Engber et al., 1998; Lin, Hou, & Jouvet, 1996). Modafinil, like amphetamine and cocaine, relies on dopamine transporters (Wisor et al., 2001), but unlike classical psychostimulants, this drug does not significantly increase dopamine levels in the nucleus accumbens (Ferraro
et al., 1997), which likely accounts for its low abuse potential (Deroche-Gamonet et al., 2002). Modafinil appears to amplify the release of glutamate and serotonin while inhibiting the release of GABA (Ferraro et al., 1998; Ferraro et al., 1999; Ferraro et al., 2000). This may be achieved by blocking dopamine and/or norepinephrine transporters (Madras et al., 2006; Wisor & Eriksson, 2005). In this manner, modafinil may promote wakefulness through inhibition of the ventrolateral preoptic nucleus of the hypothalamus, a region known to promote sleep (Gallopin, Luppi, Rambert, Frydman, & Fort, 2004; Sherin, Shiromani, McCarley, & Saper, 1996). This theory, however, remains controversial (Saper & Scammell, 2004).

The current study explored the cognitive enhancing effects of modafinil on two popular memory tasks in mice, the Morris water maze and Pavlovian fear conditioning (freezing). As modafinil appears to be selective in its cognitive enhancing effects, this study tested three different types of memory (context fear memory, cued fear memory, and spatial memory) to further extract subtle effects of the drug.

The Morris water maze task requires an animal to repeatedly find a hidden platform in order to escape a pool of opaque water (Morris, 1984). Animals are placed in random starting locations and must use distal spatial cues in order to find the platform. This test of spatial memory relies heavily on the hippocampus and surrounding structures, even after long delays between the training and the lesion (Clark, Broadbent, & Squire, 2005). Thus, experiment 1 tested the effects of pre-training modafinil on spatial memory using a Morris water maze. Experiment 2 examined the effects of modafinil on Pavlovian fear conditioning. Fear conditioning relies on the association between an initially neutral conditioned stimulus (CS) and an aversive unconditioned stimulus (US). After a single pairing, the CS alone will elicit a response similar that elicited by the US. When fearful, mice display an innate tendency to exhibit freezing
behavior, defined as a lack of movement other than which to breathe. Thus, after a tone (CS) is paired with a shock (US) in a distinct context, mice will freeze in response to presentation of either the tone or contextual stimuli (Anagnostaras, Gale, & Fanselow, 2001; Fanselow, 1980). Context fear, unlike cued (tone) fear, is dependent on the hippocampus, while both are dependent on the amygdala (Anagnostaras et al., 2001; Gale et al., 2004).

In experiment 1, we began by examining the effects of a high dose of modafinil on water maze learning, which produced substantially enhanced learning. In experiment 2, we examined Pavlovian fear conditioning in order to examine hippocampus-dependent and independent learning. We began by giving injections of a high dose of modafinil either before or after training to assess its effects on memory formation and consolidation. If modafinil delivered before training affected memory of the task, this would suggest the drug facilitates the formation of memory. However, if modafinil delivered after training had enhanced memory of the task, the drug could be involved in memory consolidation. As reported below, post-training injections had no effect on memory formation and were thus discontinued in subsequent studies. A high dose of modafinil, pre-training, however produced a substantial deficit in context conditioning. Recent evidence from our laboratory (Wood & Anagnostaras, 2009; Wood, Fay, Sage, & Anagnostaras, 2007) indicates that the classical psychostimulants cocaine and amphetamine both enhance Pavlovian conditioned fear at very low doses and disrupt it at high doses; those effects were not specific to hippocampus-dependent learning. Thus, we predicted modafinil would have similar effects, which could be specific to hippocampus-dependent memory. Therefore, in experiment 3, we set out to establish a dose-response curve for modafinil using Pavlovian fear conditioning. The results are discussed in terms of modafinil’s ability to enhance or disrupt memory.
Methods and Materials

Subjects

Experiments were conducted using hybrid C57B6x129T2SvEms/J (129B6, stock from The Jackson Laboratory, West Sacramento, CA) mice that were at least 8 weeks old before testing. Mice were weaned 3 weeks after birth and group-housed (2-5 mice per cage) with unrestricted access to food and water under a 14:10 light/dark cycle. All animal care and experimental procedures were approved by the UCSD IACUC and in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Experiment 1 – The Effect of Pre-training Modafinil on Morris Water Maze Learning

Mice were placed into a water maze (made opaque with white tempera paint) and used distal cues (i.e., posters, etc.) to locate a stationary square platform hidden 1 cm below water. The water maze was made of a circular tub (height = 74 cm, diameter = 122 cm) and the water was heated to 23.5°C using a built in heater. Distal cues were arranged throughout the room and included a door, a computer, and several posters. The platform, 1 cm below the pool surface, was 12 cm square and covered with a plastic mesh to provide a textured surface for the mice to grip. The water was colored with white tempera paint so the platform would not be visible. During training, a mouse was lowered into the pool from one of four randomly assigned starting locations; the trial lasted until the mouse found the platform where it remained for 5 seconds. If the mouse did not find the platform in 60 seconds, it was placed on the platform and remained there for 20 seconds in order to ensure exposure to the reinforcement and distal cues. Probe tests consisted of 60 second trials with the platform removed. Mice were dried in a towel after completing the final trial each day.
Each training day consisted of three consecutive trials administered on days 1-4 and 6-8. In addition, a probe test (given 15 minutes after training when on the same day) was administered on days 5, 7, and 8. Location was tracked using an automated video tracking system connected to an overhead video camera (Actimetrics Inc., Evanston, IL). Probe trials were scored by computer using Water maze software, measuring percent of time in target quadrant (TQ).

The modafinil group \((n = 10)\) received modafinil (Cephalon Inc., Frazer, PA), 75 mg/kg i.p, 15 minutes before each of the first seven days of training and saline on the final day while the saline group \((n = 10)\) received saline (0.9% sodium chloride, 10 ml/kg) before each of the first seven days of training and modafinil on the final day. Modafinil was suspended 7.5 mg/ml in sterile water with 10% Tween 80.

**Experiment 2 – The Effect of Pre- and Post-Training Modafinil on Fear Conditioning**

**Conditioning context.** Four mice were tested concurrently, in individual conditioning chambers housed in a windowless room. Each chamber (32 cm wide, 25 cm high, 25 cm deep) was located within a sound attenuating chamber (63.5 cm wide, 35.5 cm high, 76 cm deep; Med-Associates Inc., Georgia, VT) and equipped with a speaker in the side wall and a stainless steel grid floor (36 rods, each rod 2-mm diameter, 8-mm center to center; Med-Associates Inc., Georgia, VT) and stainless steel drop-pan. During each trial chambers were scented with 7% isopropyl alcohol to provide a background odor and background noise (65-dB) was provided by internal fans. Each sound attenuating chamber was equipped with an overhead LED light source providing white and near infrared light, and an IEEE 1394 progressive scan video camera with a visible light filter (VID-CAM-MONO-2A; Med-Associates Inc., Georgia, VT) connected to a computer and video equipment in an adjacent room. Each chamber was connected to a solid-state scrambler, providing AC constant current shock, and an audio stimulus
generator, controlled via an interface connected to a Windows computer running Video Freeze (Med-Associates Inc., Georgia, VT), a novel program designed for the automated assessment of freezing and activity. In results that will be published more fully elsewhere, computer and human scored data had a correlation of 0.971 and a fit of computer = – 0.007 + 0.974 x human (for more detail on this calculation see, for e.g., Anagnostaras, Josselyn, Frankland, & Silva, 2000).

Alternate context. For testing tone fear the conditioning context was modified along several dimensions. White acrylic sheets were placed over the grid floor to provide a different sensory experience and a black plastic, triangular tent translucent only to near infrared light was placed inside each box, with each side of the triangle measuring 23cm. Only near infrared light was used creating a completely dark environment visible only to the video camera. Between tests, the chambers were cleaned and scented with a 5% white vinegar solution.

During each phase, freezing was measured by computer. During fear conditioning training, mice were given an i.p. injection of modafinil or saline and 15 min later placed into one of four identical chambers. After 2 minutes of baseline activity, a 30 second tone (2.8-kHz, 85dB / A-scale) was presented and coterminated with a scrambled footshock (2 seconds, 0.75mA, AC constant current) delivered through the floor of the cages. The training trial continued for 2.5 additional minutes before an additional five minute test of immediate memory (post-shock freezing) in the same chamber. Mice then received a second injection of modafinil or saline (see groups below) and were returned to their home cages. One week later, mice were returned to the training chambers for an assessment of context fear, during which no injection or shock was given. Freezing was monitored for 5 minutes and mice were then returned to their home cages. Twenty-four hours later, mice were given a tone test; they were
placed in the alternative context described above. Baseline activity was assessed for 2 minutes, after which the training tone was presented for 3 minutes and then the mice were returned to their home cages.

Mice were randomly assigned to one of three groups indicating the type of injection given before and after training, respectively: Modafinil/Saline \((n = 10)\), Saline/Modafinil \((n = 10)\), or Saline/Saline \((n = 10)\). Each injection was 75 mg/kg modafinil, i.p., or saline (0.9%, 10 ml/kg).

**Experiment 3 – Dose Response Curve with Pre-training Modafinil**

Mice were given the same training as in experiment 2, although a 0.5 mA shock was used after pilot data that suggested it would be easier to observe learning enhancements at lower levels of fear. Given that post-training modafinil at a high dose had no effect on fear conditioning, we only examined the full dose-effect curve for modafinil given pre-training.

Mice were randomly assigned to one of five groups indicating the amount of modafinil given prior to training: 0 mg/kg (saline control, \(n = 16\)), 0.075 \((n = 13)\), 0.75 \((n = 13)\), 7.5 \((n = 12)\), or 75 mg/kg \((n = 12)\), to form a full dose-effect curve. In pilot experiments, we found a high rate of lethality above 100 mg/kg so higher doses were not explored. Each dose of modafinil was suspended in sterile water with 10% Tween 80 and administered at 10 ml/kg i.p.

**Results**

**Experiment 1 – Water maze**

Figure 1 depicts the latency (fig 1A) to reach the platform for each of the training trials and the average velocity (fig 1B) for each group during training trials on day 1 and each probe test. Data were entered into a multivariate analysis of variance (MANOVA)
and the level of significance was set at a < 0.05. Post hoc comparisons were done with Fisher’s protected least significant difference (PLSD). No group differences were found during acquisition for latency [F(1,18) < 0.1, n.s.] or velocity [F(1,18) = 3.23, n.s.]. Figure 2 depicts the time spent in each quadrant during the first (2A), second (2B), and third probe tests (2C). Since mice often become disinterested and wander once they discover the platform is not there, only the first 30 seconds were analyzed. To assess learning of the task, paired comparisons between the target quadrant (TQ) and opposite quadrant (OP) were used. During probe 1, time spent in each quadrant did not differ between Modafinil mice and Saline mice [F(1,18) = 0.425, n.s.] and neither group spent significantly more time in TQ than OP [Modafinil: F(1,9) = 0.34, n.s., Saline: F(1,9) = 4.78, n.s.] indicating that neither group had learned the task. During probe 2, Modafinil mice spent more time in TQ than Saline mice [F(1,18) = 5.33, p<0.05]. Modafinil mice spent more time in TQ than OP [F(1,9) = 50.84, p < 0.001] while Saline mice did not [F(1,9) = 5.02, n.s.] indicating that Modafinil mice learned the location of the platform while Saline mice did not. When treatments were reversed in probe 3, Modafinil mice (that received saline) continued to spend more time in TQ than Saline mice (that received modafinil) although this result was out of the range of significance [F(1,18) = 4.09, p=0.058]. Modafinil mice spent more time in TQ than OP [F(1,9) = 68.72, p<0.001] while Saline mice did not [F(1,9) = 3.28, n.s.]. These results indicate that Modafinil mice learned the task by the seventh day of training while Saline mice did not, given the relatively small amount of training. Also, Modafinil mice retained this knowledge of the task when tested off drug and Saline mice remained unaware of the location of the platform when tested on drug.

Experiment 2 - Fear Conditioning with Pre- and Post-training Modafinil
Percent freezing during training and the immediate memory test is illustrated in Figure 3. Group differences were observed during training \( [F(2,39) = 22.16, p<0.05] \) and the immediate memory test \( [F(2,39) = 70.77, p<0.05] \). When compared to Sal/Sal controls, mice that received modafinil prior to training (Mod/Sal) showed a strong deficit in freezing during training \( [F(1,30) = 30.65, p<0.001] \) and the immediate memory test \( [F(1,30) = 82.52, p<0.001] \) perhaps caused by the locomotor stimulatory effects of the drug or an immediate memory defect. Group differences were also observed during the off-drug context test one week later [Figure 4a, \( F(2,39) = 5.33, p<0.05 \)]. Mod/Sal mice showed a strong deficit in context memory compared to Sal/Sal controls \( [F(1,30) = 5.35, p<0.05] \). This difference cannot be attributed to the stimulatory effects of the drug as this test was performed off drug, and modafinil did not produce an increase in activity at any dose (see, for example, Fig 1b and 6a). During the tone test (Figure 4b) no group differences were observed in baseline freezing \( [F(2,39) = 0.76, \text{n.s.}] \) or tone memory \( [F(2,39) = 0.29, \text{n.s.}] \).

**Experiment 3 – Dose Response Curve with Pre-training Modafinil**

Percent freezing differed among groups during training [Figure 5a, \( F(4,61) = 7.88, p<0.001 \)]. Mice that received 0.075 mg/kg modafinil froze significantly more than saline controls (i.e., 0 mg/kg; Fisher’s PLSD, \( p<0.05 \)) and mice that received 75 mg/kg again showed a strong deficit in freezing \( (p<0.05) \). No other doses differed significantly from saline controls. Differences were also found during the immediate memory test \( [F(4,61) = 8.10, p<0.001] \). Mice that received 0.75 mg/kg showed enhanced immediate memory (Figure 5b, \( p<0.05 \)) while mice that received 75 mg/kg mice froze significantly less than controls \( (p<0.05) \). As stated previously, this decrease in freezing may be caused by the stimulatory effects of the drug or an immediate memory defect, although we did not detect any increase in activity from any dose of modafinil (Figs 1a, 6b). Baseline activity
(measured in arbitrary units) during the first two minutes of training (Figure 6a) showed
group differences [F(4,61) = 2.63, p<0.05]. Post-hoc analysis showed that activity was
reduced in mice that received 0.075 mg/kg compared to saline controls (p<0.05). No
other doses produced significant changes in activity. Figure 6b illustrates that during the
2-second shock no differences in activity were found [F(4,61) = 1.26, n.s.] indicating that
all groups had a similar reaction to the shock. Group differences were again found
during the context test, done off-drug one week later [F(4,61) = 7.76, p<0.001].
Compared to controls, contextual memory (Figure 7a) was enhanced in mice that
received 0.75 mg/kg (p<0.05) and disrupted in those that received 75 mg/kg (p<0.05).
No other groups differed significantly from controls. During the tone test no differences
were found during the two minute baseline [F(4,61) = 1.52, n.s.] or the tone-on portion of
the test [F(4,61) = 1.40, n.s.] (data not depicted). In this experiment, a moderately high
baseline was encountered, and less tone-elicited freezing, so we further examined tone
freezing by subtracting the baseline freezing from tone-elicited freezing. This was likely
due to the fact that we reduced shock intensity from Exp 2 (0.75 to 0.5 mA) because
pilot data showed cleaner effects on context freezing. However, no significant
differences were found after subtracting the baseline from the tone-on portion of the test
[Figure 7b, F(4,61) = 1.45, n.s.]. Taken together with the results of experiment 2, where
much more robust freezing was encountered, there is little evidence that modafinil
affected tone conditioning at any dose. However, mice given saline exhibited 6.1±1.5%
freezing while those given 0.75 and 7.5 mg/kg exhibited 10.1±2.7% and 10.6±2.3%
freezing, respectively. This suggests that we may not have had enough power to detect
a potential enhancement of tone fear conditioning at those doses (Figure 7b).

Discussion
The current study examined the effects of modafinil on three types of memory (spatial memory, context fear memory, and cued fear memory) and found specific enhancements of hippocampus-dependent spatial memory and contextual fear memory, but found no effect on hippocampus-independent cued fear memory. In contrast, the classical stimulants amphetamine and cocaine produced similar enhancements and deficits that were not specific to contextual versus cued memory (Wood & Anagnostaras, 2009; Wood et al., 2007). These data suggest that modafinil has similar effects on memory as the classical psychostimulants but acts more specifically on hippocampus-dependent memory.

A high dose of pre-training modafinil (75 mg/kg) enhanced performance on Morris water maze learning. After six days of training, the modafinil group successfully learned the task while controls did not. Interestingly, the effect was not state-dependent as the group trained on modafinil continued to outperform controls during the reversal trial. This indicates that modafinil facilitated the acquisition of spatial memory, rather than retrieval or performance (Fig 2).

Using a standard fear conditioning protocol, we found that a large dose of pre-training modafinil (75 mg/kg) disrupted contextual fear memory but spared cued fear memory. We also found that the same dose of modafinil delivered post-training did not affect context or cued fear memory. This suggests that modafinil does not affect consolidation, and thus, any effects are likely due to changes in memory formation. Finally, we found that a very low dose of pre-training modafinil (0.75 mg/kg) enhanced contextual fear memory and we replicated the finding that a large dose disrupted it. We found no effect of modafinil at any dose on cued fear memory.

These results suggest that modafinil works selectively on hippocampus-dependent memory. The animal literature thus far has focused on relatively high doses
of the drug, because most of the effects have only been detected with large doses. We are the first to report a selective enhancement of memory at very low, clinically relevant, doses of modafinil. The dose that enhanced contextual fear memory is roughly equivalent to the dose used in human clinical patients (typically 100-200mg; about 1 – 3 mg/kg). This enhancement may be unique to contextual fear conditioning, but it warrants testing on other tasks, and thus, future studies investigating the cognitive effects of modafinil should include similar, more clinically relevant doses. Although many differences in human and mouse drug disposition may exist, given the little knowledge we have, we feel that a straight mg/kg conversion is the most conservative approach (Wood & Anagnostaras, 2009). Certainly higher doses used in other animal studies (e.g., 75 mg/kg, or about 60, 100 mg modafinil tablets) are not likely to be clinically relevant.

The Morris water maze task is a well-established spatial memory task in rodents (Morris, 1984). However, to ensure the drug was active during training, the typical protocol was modified to ensure all training and probe trials occurred shortly after drug administration. Therefore, trials were administered in a massed fashion, rather than distributed over a few hours. This may have made it more difficult for controls, and possibly made it more susceptible to the enhancing effects of modafinil. The fixed-location hidden platform version of the maze, as we used here, is not generally thought to involve working memory and by definition is a reference memory task (c.f., Steele & Morris, 1999). Additionally, probes 2 and 3 were administered 15 minutes after the end of a training day, while no training occurred on the day before probe 1. This may account for some of the differences between the results on the probe trials.

Throughout the animal literature modafinil’s effects appear to be related to task demands (Morgan et al., 2007). High doses of the drug have enhanced performance on a T-maze (Beracochea et al., 2001; Beracochea et al., 2002; Beracochea et al., 2003),
delayed non-match to position swim task (Ward et al., 2004), 3-choice sustained attention task (Morgan et al., 2007), and now the Morris water maze. These tasks are difficult for mice and require extensive training, and in some studies the effects were not seen until the attention or working memory demands of the tasks were increased. Thus, high doses of modafinil may only enhance performance only on tasks that involve considerable difficulty, working memory, or attention.

Contextual fear memory is characterized by a two-step associative process whereby the animal must first form a memory of the context (thought to occur in the hippocampus) and then an association between a shock and the context must be formed (thought to occur in the amygdala; see Anagnostaras et al., 2001; Wiltgen, Sanders, Anagnostaras, Sage, & Fanselow, 2006). A low dose of pre-training modafinil enhanced contextual fear memory without affecting tone memory, suggesting that the drug was able to strengthen contextual memories. This task is relatively simple, and generally not thought to involve working memory (Anagnostaras et al., 2003).

Given that the shock is very arousing and the attentional demands necessary for the task are limited, it is surprising that a drug known for increasing attention and arousal would increase contextual fear memory. It may be that at low doses, modafinil selectively enhances associative ability while at high doses, it may benefit tasks that require increased attention and arousal or working memory. Most studies done with cognitive tasks have shown dose-dependent increases with the highest doses being the most efficacious. Alternatively, the drug may enhance attention processes which manifest themselves in a similar way. This alternative is supported by the disruption of contextual fear memory at a high dose suggesting an inverted U shaped curve, a common characteristic of attention. Consistent with this, chronically stressed mice showed decreased performance on a T-maze spontaneous alternation task when given
a large dose (32 mg/kg) of modafinil (Pierard et al., 2006). Increases in attention from both stress and the drug may interact to overwhelm the animal and decrease performance. Interestingly, under stressful conditions, the lowest drug dose tested (8 mg/kg) showed the highest level of performance. In light of the current study, even lower doses of modafinil may have further facilitated T-maze performance in chronically stressed mice.

Another interesting finding presented here is the unexpected dissociation between Morris water maze learning and contextual fear conditioning, two hippocampus-dependent tasks. While a high dose of modafinil enhanced Morris water maze learning, it disrupted contextual fear conditioning. This seems to indicate that modafinil may act in different ways to produce enhancements (or deficits) on these tasks. Further study is required to understand these differences, but would begin with site-directed administration of modafinil in suspect learning and memory structures, such as the hippocampus and amygdala.

A number of alternative explanations must be considered to account for the selective enhancement and disruption of contextual fear conditioning. For instance, it is possible that modafinil may dose-dependently disrupt nociception. However, we found no evidence that modafinil altered shock sensitivity (Fig 6b). Moreover, psychostimulants (Markham, Yang, Blanchard, & Blanchard, 2006) can produce anxiogenic states that might increase fear. This is also unlikely as modafinil did not affect tone fear memory suggesting it did not promiscuously increase fear. Moreover, in clinical trials, the incidence of anxiety in patients treated with modafinil was around 5% and similar to placebo-treated controls suggesting the drug does not produce much anxiety overall (Cephalon, 2004).
Another possible explanation is that when learning occurs on-drug, the subject would perform better when tested on-drug. State-dependent learning could theoretically account for the deficit in contextual fear conditioning at high doses, but cannot explain the enhancement at the low dose. This theory is further undermined by the enhancement and deficit in the immediate memory test performed on the drug, which is similar to the effects seen off drug. Also, state dependent learning would be unlikely to affect contextual fear memory without having an effect on tone fear memory, which is what we observed. Finally, when directly tested on the Morris water maze we found no evidence of state-dependent effects of modafinil (Figure 2c).

Psychostimulants can produce locomotor activity which could in theory disrupt freezing when the animals are on drug (Wood & Anagnostaras, 2009; Wood et al., 2007). This is unlikely in the present case as we found no increase in activity at any dose of the drug (Figs 1b, 6a). Moreover any undetected hyperactivity on training is unlikely to be responsible for the deficit in contextual fear memory since this test occurred off-drug one week later. In fact, only the group that exhibited any change in activity received 0.075 mg/kg of modafinil, and that dose produced no effects in any of the memory tests. Thus, the dose-effect curves for activity and the effects on learning were so markedly different they suggest the drug’s locomotor affects and mnemonic effects are unrelated.

Wood et al. (2007) showed that a low dose of acute pre-training cocaine enhanced contextual fear memory and cued fear memory. A high dose of cocaine disrupted both forms of memory. The present study suggests that modafinil works through a similar, but more selective, mechanism as classical psychostimulants. Thus modafinil is very valuable as a cognitive performance enhancer because classical psychostimulants have often appeared to be cognitive enhancers, but their side effects and abuse potential have limit clinical use as nootropics. There are no reports of
modafinil addiction, rather modafinil appears useful in treating cocaine, amphetamine, and nicotine addiction (Brower, 2006; Ling, Rawson, & Shoptaw, 2006; Sofuoglu, Waters, & Mooney, 2008). Overall, the data suggests that modafinil is a better choice as a cognitive enhancer than classical psychostimulants for humans. In addition, the combination of its wake-promoting and cognitive enhancing effects makes it an ideal candidate for improving cognition in professions that often suffer from a lack of sleep, such as shift-workers or military personnel.
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Figure 1-1. (A) Trial latency for each day of training. Trials ended when the subject reached the platform. No differences were found in acquisition trial latency. (B) Velocity of the first day of training and each of the three probe trials. No differences were found in velocity on the acquisition trials or the probe trials. Each point represents the mean ± S.E.M.
Figure 1-2. (A) Percent time spent in each quadrant during probe 1 performed on day 5. Neither modafinil nor saline mice spent more time in the target quadrant (TQ) than other quadrants (TL: target left; TR: target right; OP: opposite). (B) Percent time spent in each quadrant during probe 2 performed on day 7. Modafinil mice learned the task, spending more time in TQ than OP, while Saline mice did not. (C) Percent time spent in each quadrant during probe 3 performed on day 8. Modafinil mice received saline prior to training and testing while Saline mice received modafinil. Despite the drug reversal, Modafinil mice continued to perform much better than Saline mice. Each point represents the mean + S.E.M.
Figure 1-3. Percent time spent freezing during (A) training and (B) the immediate memory test. The shock was presented 2 minutes and 28 seconds into training and lasted for 2 seconds. Modafinil administered before training (Mod/Sal) caused mice to freeze less than controls during both training and the immediate memory test. Each point represents the mean ± S.E.M.
Figure 1-4. Percent time spent freezing during the (A) context test and (B) tone test. Context test consisted of five minutes in training chambers with no tone or shock. Tone test consisted of five minutes in a novel chamber with a two minute baseline followed by a three minute tone presentation. Modafinil administered before training (Mod/Sal) disrupted contextual fear memory but did not affect cued fear memory. Modafinil administered after training (Sal/Mod) did not affect contextual fear memory or cued fear memory. Each point represents the mean + S.E.M.
Figure 1-5. (A) Percent time spent freezing in each minute of training. The shock was administered 2 minutes and 28 seconds into training. (B) Percent time spent freezing during the immediate memory test performed immediately after training. Mice that received 0.75 mg/kg modafinil froze more than controls while mice that received 75 mg/kg froze less than controls. Each point represents the mean ± S.E.M.
Figure 1-6. (A) Average movement, measured in arbitrary units (au), over the first two minutes of training before any tone or shock. Mice that received 0.075 mg/kg modafinil were significantly less active than controls. (B) Average movement (au) during the two second shock. No group differences were found indicating that each group had the same reaction to the shock. Each point represents the mean ± S.E.M.
Figure 1-7. (A) Percent time spent freezing during the context test. Mice that received 0.75 mg/kg modafinil showed an enhancement while those that received 75 mg/kg showed a disruption of contextual fear. (B) Percent time spent freezing during the two minute baseline was subtracted from the three minute tone to get a measure of cued fear memory. No group differences were found. Each point represents the mean ± S.E.M.
CHAPTER 2

Interactions between modafinil and cocaine during the induction of conditioned place preference and locomotor sensitization in mice: implications for addiction

By

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Abstract

Modafinil is a wake-promoting drug effective at enhancing alertness and attention with a variety of approved and off-label applications. The mechanism of modafinil is not well understood but initial studies indicated a limited abuse potential. A number of recent publications, however, have shown that modafinil can be rewarding under certain conditions. The present study assessed the reinforcing properties of modafinil using conditioned place preference and locomotor sensitization in mice. Experiment 1 examined a high dose of modafinil (75 mg/kg) as well as its interactions with cocaine (15 mg/kg). Cocaine alone and modafinil co-administered with cocaine induced sensitization of locomotor activity; modafinil alone showed little or no locomotor sensitization. Animals given modafinil alone, cocaine alone, and modafinil plus cocaine exhibited a strong and roughly equivalent place preference. When tested for sensitization using a low challenge dose of modafinil, cross-sensitization was observed in all cocaine-pretreated mice. Experiment 2 examined a low dose of modafinil that is similar to the dose administered to humans and has been shown to produce cognitive enhancements in mice. Low dose modafinil (0.75 mg/kg) did not produce conditioned place preference or locomotor sensitization. Together, these results suggest that modafinil has the potential to produce reward, particularly in cocaine addicts, and should be used with caution. However, the typical low dose administered likely moderates these effects and may account for lack of addiction seen in humans.

Keywords: Provigil, stimulant, behavior, activity, dopamine
Modafinil is a wake-promoting psychostimulant approved by the US FDA for the treatment of narcolepsy, sleep apnea/hypopnea, and shift work sleep disorder (Bastuji & Jouvet, 1988; Cephalon, 2004). The drug is also widely prescribed off-label to help patients with attention deficit disorder, excessive daytime sleepiness, dementia, and depression (O'Connor, 2004). In addition, some academic doping has emerged because modafinil may enhance memory and attention (Beracochea et al., 2001; Beracochea et al., 2002; Beracochea, Celerier, Peres, & Pierard, 2003; Garreau, 2006; Shuman, Wood, & Anagnostaras, 2009; Turner et al., 2003). Recently, modafinil has been implicated as a therapeutic for cocaine addiction (Ballon & Feifel, 2006; Dackis, Kampman, Lynch, Pettinati, & O'Brien, 2005; Hart, Haney, Vosburg, Rubin, & Foltin, 2008; Karila et al., 2008; Shearer, 2008). Modafinil is attractive because it has low addictive and cardiovascular risk compared to amphetamine or methylphenidate, and could serve as a weak or slow-onset agonist in the manner that buprenorphine has been used in the treatment of opioid addiction (Shearer, 2008).

Initial studies on modafinil indicated a minimal abuse potential. Gold and Balster (Gold & Balster, 1996) found that modafinil could not substitute for cocaine in rats but was able to act as a reinforcer in cocaine-experienced rhesus monkeys. In addition, Deroche-Gamonet and colleagues (Deroche-Gamonet et al., 2002) found little evidence of reinforcing effects of modafinil in naïve and cocaine-experienced rats. They found that modafinil (32-256 mg/kg, i.p.) did not induce a place preference for a drug-paired compartment, was not self-administered (0.28-1.7 mg/kg/infusion), and did not alter cocaine self-administration. Modafinil (64 mg/kg) did, however, enhance the reinstatement of cocaine self-administration. Together, these studies indicated that modafinil alone was not reinforcing, but could elicit reward in cocaine-experienced animals. Despite these early findings there was some evidence that modafinil alone
could be rewarding. Modafinil increases dopamine activity in the accumbens (Ferraro et al., 1997; Ferraro et al., 1996; Murillo-Rodriguez, Haro, Palomero-Rivero, Millan-Aldaco, & Drucker-Colin, 2007; Volkow et al., 2009) and human patients “self-administer” the drug in a “modified progressive ratio procedure” (Stoops, Lile, Fillmore, Glaser, & Rush, 2005). Indeed, two independent studies have contradicted earlier reports and indicated that modafinil can indeed produce place preference at high doses (64-300 mg/kg) in mice (Nguyen, Tian, You, Lee, & Jang, 2011; Wuo-Silva et al., 2011). Furthermore, locomotor sensitization to modafinil has also been observed in mice (Paterson et al., 2010; Wuo-Silva et al., 2011). Together, these recent findings indicate that modafinil, even when taken alone, may have addictive potential.

Despite its profile as a modest reinforcer there are no published case reports of addiction to modafinil, and several studies have reported that modafinil lacks a drug-induced “high” (Malcolm, Book, Moak, DeVane, & Czepowicz, 2002; Myrick, Malcolm, Taylor, & LaRowe, 2004; Rush, Kelly, Hays, Baker, & Wooten, 2002; Warot, Corruble, Payan, Weil, & Puech, 1993); for review see (Myrick et al., 2004). This may be because modafinil is used orally, and has a relatively slow peak time (2-4 hours) and long half-life (10-12 hours), compared to the stimulants of abuse (i.e., smoked or snorted cocaine and methamphetamine). The possibility remains that high doses of modafinil, especially if it were given via a rapid route of administration, such as inhalation, might be addictive. In human subjects, however, only oral ingestion of modafinil has been studied.

Modafinil’s mechanism of action is not well understood. Modafinil has been shown to induce changes in dopamine, norepinephrine, serotonin, glutamate, and GABA transmission (for review, see Minzenberg & Carter, 2008). Emerging evidence suggests the primary action of modafinil is through dopaminergic neurotransmission (Korotkova et al., 2007; Madras et al., 2006; Qu, Huang, Xu, Matsumoto, & Urade, 2008; Zolkowska et
Positron emission tomography (PET) imaging revealed that modafinil binds to over 50% of dopamine transporters and 44% of norepinephrine transporters in rhesus monkey striatum (Madras et al., 2006). Furthermore, a recent PET study in humans indicated that modafinil (200–400 mg, p.o.) binds to dopamine transporters and increases extracellular dopamine in the nucleus accumbens (Volkow et al., 2009). Overall, this profile fits with the characterization of modafinil as a modest reinforcer.

The current study uses two popular rodent models of addiction: behavioral sensitization and conditioned place preference (CPP). Behavioral sensitization is an increase in response to a drug after repeated pairings (Anagnostaras & Robinson, 1996; Anagnostaras, Schallert, & Robinson, 2002). It is hypothesized to contribute to addiction by enhancing the incentive (rewarding) salience of the drug, and cues associated with drug use, moderating the transition from casual use to compulsive drug seeking (Robinson & Berridge, 2003, 2008). CPP is a paradigm used to model drug seeking behavior. With addictive drugs, rodents show a strong preference for the drug-paired context indicating that the drug confers a conditioned reward.

Although recent reports have indicated that modafinil can be rewarding, several points remain unclear. First, it is unclear how high dose modafinil interacts with cocaine in the formation of conditioned place preference and locomotor sensitization. Experiment 1 examines this interaction by training animals on a combined behavioral sensitization and conditioned place preference (CPP) paradigm using modafinil alone, cocaine alone, or co-administered modafinil and cocaine. Second, it remains unclear if modafinil is rewarding at doses actually used by people. Thus, Experiment 2 examined whether an ultra-low dose of modafinil (that produces cognitive enhancement (Shuman et al., 2009)) also produces CPP and locomotor sensitization.
Methods and Materials

Subjects

Experiments were conducted using male and female (balanced across groups) F1 hybrid C57B6x129T2SvEms/J (129B6, stock from The Jackson Laboratory, West Sacramento, CA) mice at least 10 weeks old before testing. Mice were group-housed with unrestricted access to food and water under a 14:10 light/dark cycle. Animal care and experimental procedures were approved by the UCSD IACUC, in accordance with the NRC Guide for the Care and Use of Laboratory Animals.

Drugs

All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg. Modafinil (Sigma-Aldrich, St. Louis, MO) was suspended in 0.9% saline with 10% Tween 80 (Shuman et al., 2009). Cocaine HCl (Sigma-Aldrich) was dissolved in saline. Modafinil+Cocaine was a combined mixture suspended in 0.9% saline with 10% Tween 80.

Experiment 1: Examining the rewarding effects of high dose modafinil and its interaction with cocaine

Conditioning Context Apparatus

Four mice were tested concurrently, in individual chambers housed in a windowless room. Chambers (59-cm wide, 29-cm high, 29-cm deep) consisted of two distinct sides separated by a removable wall. The two sides were unbiased and differed in smell, floor texture, and visual stimuli, but did not differ in dimension or overall lighting. One side was lined with unscented cat litter and contained only white walls. The other side was scented with cleaner (3% Quatricide) on a smooth plastic floor with visual stimuli (stickers) on the walls. Each side was randomly designated the Saline or Drug side (counterbalanced by group). During training and all tests of sensitization, the
dividing wall allowed access to only one side. During CPP testing, the solid wall was replaced with an identical wall with a small hole allowing passage between the sides. Chambers were placed on the floor and recorded from an overhead camera. Limelight software (Actimetrics, Evanston, IL) tracked each animal at 8 frames per second and used a reference measurement to convert pixels into distance traveled. For CPP testing, it recorded locomotion and time spent in each side. Light was provided from two 150-watt bulbs distant from the chambers and background noise (65-dB) was provided by HEPA air cleaners.

Behavioral Training

Mice were randomly assigned to one of four groups indicating which drug they would receive on each training day (Fig 1A). Modafinil mice (n=12) received 75 mg/kg modafinil, Cocaine mice (n=12) received 15 mg/kg cocaine HCl (salt weight), Modafinil+Cocaine mice (n=12) received the same doses of modafinil and cocaine, and Saline control mice (n=12) received saline during drug pairing. 75 mg/kg was chosen as the modafinil dose because it has been widely used in rodents and was the highest dose we could give this mouse strain without significant mortality (unpublished data). 15 mg/kg of cocaine was chosen as a moderately high dose relevant to addiction that produces place preference and sensitization. Training lasted for seven consecutive days, during which the dividing wall was solid and allowed access to only one side of the chamber at a time. On each day, mice received an injection of saline and were immediately placed into the Saline side of the chamber. After 15 minutes, they were removed from the chamber, given a group-appropriate drug injection, and placed into the Drug side. After an additional 15 minutes, they were returned to their home cage.

Conditioned Place Preference (CPP) Testing
CPP testing occurred 3 days after the final day of training. The solid wall separating the two sides of the chamber was replaced with an identical insert with a small hole allowing passage between the two sides. All groups received the same drug treatments during testing. The testing paradigm is shown in Fig 1B. On day 10, mice were given a standard place preference test: following an injection of saline, they were placed into the small hole between the two sides of the chamber and allowed to explore both sides for 15 minutes. Time spent in each side was measured by computer.

*Sensitization Testing*

Locomotor sensitization is specific to the context in which the drug is received (Anagnostaras et al., 2002). Thus, sensitization can be measured as the difference between a challenge in the drug side and a challenge in the saline side (Martin-Iverson & Reimer, 1996). Following CPP tests, the specificity of sensitization was tested by administering a challenge dose of modafinil (0.75 mg/kg) and saline in both the drug- and saline-paired contexts (Fig 1C). This low dose of modafinil was chosen as a clinically relevant dose and because we have found it acts as a cognitive enhancer (selectively enhancing hippocampus-dependent contextual fear conditioning) without producing locomotor activity (Shuman et al., 2009). During these tests the dividing wall was solid allowing access to only one side of the chamber at a time. On Day 13, all mice from all groups received saline in the side previously paired with saline, followed three hours later by modafinil in the side previously paired with drug. On Day 14, mice received saline in the drug-paired side, followed three hours later by modafinil in the saline-paired side. Each test lasted 15 min, and locomotor activity was scored.

**Experiment 2: Examining the rewarding effects of low dose modafinil**

**Conditioning Context Apparatus**
Eight mice were tested concurrently, in individual chambers housed in a windowless room. Chambers (44-cm wide, 44-cm high, 31-cm deep, Med Associates, St. Albans, VT) consisted of two distinct sides separated by a wall with a removable hole. The two sides differed in smell, texture, and visual stimuli. Each side was randomly designated the Saline or Drug side (counterbalanced by group). During training, the hole in the dividing wall was closed which allowed access to only one side at a time. Animals were tracked using infrared beams and Activity Monitor software (Med Associates) and locomotor distance traveled was scored. Light was provided from two 150-watt bulbs distant from the chambers and background noise (65-dBA) was provided by HEPA air cleaners and an iPod speaker playing white noise.

**Behavioral Training**

Training was as in Experiment 1, but a low dose of modafinil (LoMod, 0.75 mg/kg) was substituted for the high dose. This low dose is closer to the dose given to humans (without respect to species differences) and enhances Pavlovian fear conditioning in mice (Shuman et al., 2009). Two groups were examined: mice were randomly assigned to receive either modafinil (0.75 mg/kg, n=12) or vehicle (n=12) on each day of training. Training lasted for seven consecutive days, during which the hole in the dividing wall was closed and allowed access to only one side of the chamber at a time. On each day, mice received an injection of saline and were immediately placed into the Saline side of the chamber. After 15 minutes, they were removed from the chamber, given a group-appropriate drug injection, and placed into the Drug side. After an additional 15 minutes, they were returned to their home cage.

**Conditioned Place Preference (CPP) Testing**

As with Experiment 1, CPP testing occurred 3 days after the final day of training. Mice were given a standard place preference test: following an injection of saline, they
were placed into the small hole between the two sides of the chamber and allowed to explore both sides for 15 minutes.

**Sensitization Testing**

As with Experiment 1, the specificity of sensitization was tested by administering a challenge dose of modafinil (0.75 mg/kg) and saline in both the drug- and saline-paired contexts (Fig 1C). During these tests the dividing wall was solid allowing access to only one side of the chamber at a time.

**Data Analysis**

Data were entered into a multivariate analysis of variance (MANOVA). Between-group comparisons were done with Fisher’s protected least significant difference (PLSD). The level of significance was set at \( p \leq 0.05 \) and the statement “did not differ” below reflects \( p > 0.05 \).

**Results**

**Experiment 1. Examining the rewarding effects of high dose modafinil and its interaction with cocaine**

**Locomotor Sensitization During Training**

Locomotor sensitization was assessed over the seven days of training. Locomotor activity for all groups is depicted across training as time-course data and averages (Fig 2). On the first day of training there were significant group differences \([F(3,44)=6.51, p<0.05; \text{ Fig 2A, 2E}]\). Cocaine and Modafinil+Cocaine mice, which did not differ from each other, exhibited increased locomotor activity on the first drug pairing compared to Saline controls \( (p<0.05) \), while high dose Modafinil mice did not. Group differences continued on Day 7 \([F(3,44)=27.31, p<0.05; \text{ Fig 2B, 2E}]\). In order to establish the presence of sensitization, two criteria were required: (1) the Day 7 (chronic)
response had to be higher than the Day 1 (acute) response, and (2) the Day 7 response had to be higher than the Day 7 response in Saline controls. These requirements avoid the confound of habituation occurring only in the control group leading to a lack of habituation being mistaken for sensitization (Paterson et al., 2010). Cocaine, and Modafinil+Cocaine mice, which did not differ, showed higher levels of locomotor activity than Saline animals (p-values<0.0001). High dose modafinil mice exhibited less locomotor activity than Modafinil+Cocaine or Cocaine mice, and more than Saline controls (p-values<0.05). In order to further examine sensitization, we subtracted locomotor activity on Day 1 from Day 7, shown as a time course in Fig 2C, and as an average in Fig 2F. There were significant group differences in this measure \[F(3,44)=12.3, \ p<0.05\]. Cocaine and Modafinil+Cocaine mice, while not differing from each other (Fig 2C, 2F) exhibited a large increase in activity from Day 1 to Day 7 when compared to Saline controls (p-values<0.0001). Thus, only the Cocaine and Modafinil+Cocaine groups showed significant and substantial sensitization. Visual inspection of Fig 2C and 2F suggests that the Modafinil group separated from Saline group, but it is unclear if this was due to increased responding to the drug to a lack of habituation in the Modafinil group, compared to the Saline controls. In order to explore this further, we examined the difference from zero for the Day 7 minus Day 1 subtraction on a minute-by-minute basis. Saline animals were the only ones to show a significant decrease for any minute (one sample two-tailed t-test for each minute, hypothesized mean of 0; min 1,2,5,6,8,12,13, t(11)-values<–2.22, \ p<0.06; other mins, n.s.). Cocaine mice exhibited sensitization across most minutes (min 1-2, n.s.; min 3-15, \ t(11)-values>3.14, \ p<0.01). Modafinil mice exhibited no significant differences from Day 1 to Day 7 even on a minute-by-minute basis [t(11)-values<2.18, \ p>0.05]. Finally, Modafinil+Cocaine mice exhibited significant sensitization across most minutes [min 1,
n.s.; mins 2-15, $f(11)>4.35$, $p$-values$<0.01$. Even when considered on a minute-by-minute basis there was no evidence for sensitization in the Modafinil group, only evidence for habituation in the Saline control group. Figure 2D depicts locomotor activity on the unpaired (saline) side during training. No group differences were found in the drug unpaired side ($F[3,44]=0.81$, n.s.).

*Conditioned Place Preference*

All animals received a place preference test after receiving a saline injection (Fig 1B). Data is depicted as the difference in time spent in the drug- and saline-paired sides of the chamber. Group differences were found ($F[3,44]=7.88$, $p<0.001$). Cocaine, Modafinil+Cocaine, and Modafinil groups, which did not differ from each other, showed a significant preference for the drug-paired side compared to the Saline control group ($p$-values$<0.001$; Fig 3A). Overall, these data show that cocaine and high dose modafinil induce a strong and significant place preference; no additional place preference is seen if cocaine and high dose modafinil are combined (i.e., Modafinil+Cocaine) during training.

*Specificity of Sensitization*

To examine context-specific sensitization we conducted modafinil and saline tests with the animals restricted to one side of the chamber (Fig 1C). We administered modafinil in both the drug-paired and unpaired sides of the chamber. We then subtracted the unpaired side from the drug side to obtain a measure of context specific sensitization to modafinil (Martin-Iverson & Reimer, 1996). Group differences were found (Fig 3B, $F[3,44]=6.92$, $p<0.001$), as both the Cocaine ($p<0.01$) and Modafinil+Cocaine ($p<0.001$) groups exhibited increased locomotor activity for the drug-side compared to Saline controls. Thus, administration of modafinil induced cross-sensitization in mice that previously received Cocaine or Modafinil+Cocaine. Animals that had received only modafinil during training, on the other hand, did not exhibit
sensitization in the drug-paired context. These effects were not due to conditioned hyperactivity as no group differences were found when saline was administered on both sides (Fig 3C, F[3,40]=1.88, p>0.05). This is consistent with prior work showing that a conditioned locomotor response is not readily observed within the CPP paradigm (Martin-Iverson & Reimer, 1996).

**Experiment 2: Examining the rewarding effects of low dose modafinil**

**Locomotor Sensitization During Training**

Locomotor sensitization of low dose modafinil (LoMod) was assessed over the seven days of training (Fig 4). This data is depicted minute by minute (Fig 4A) and in summary (Fig 4B). Low dose modafinil did not induce increased locomotor activity over vehicle control on either day 1 or day 7 [F(1,22) values<2.1, p-values>0.1; Fig 4B]. There were also no group differences on the drug unpaired side [F(1,22) values<1.1, p-values>0.3; Fig 4B]. Indeed, both groups exhibited a significant decline from day 7 to day 1, reflecting normal habituation [drug paired side, Day 1 vs Day 7, paired two-tailed t-tests, t(11) values>6.3, p-values<0.0001]. When difference scores were computed to estimate this reduction (Fig 4C), it is evident that mice receiving low-dose modafinil and those receiving saline showed a similar decline [F(1,22)=2.75, p>0.1].

**Conditioned Place Preference**

All animals received a place preference test after receiving a saline injection (Fig 1B). Neither group exhibited a significant place preference [Fig 4D, left; one sample t-tests vs hypothesized mean of 0, t(11) values<0.6, p-values>0.5], nor did they differ from each other [F(1,22) = 0.3, p>0.5].

**Specificity of Sensitization**

As with Experiment 1, we administered modafinil in both the drug-paired and unpaired sides of the chamber (Fig 1C). Low dose modafinil did not produce context
specific sensitization in the drug side when compared to vehicle controls \(F(1,22)=0.03, p>0.8; \) Fig 4D, middle]. In addition, when given a saline challenge, the low dose modafinil group did not produce conditioned hyperactivity in the drug side when compared to vehicle controls \(F(1,22)=0.01, p>0.09; \) Fig 4D, right].

**Discussion**

We examined the interactions between modafinil and cocaine in the induction of locomotor sensitization and conditioned place preference. We found a number of interesting results: 1) High dose modafinil (75 mg/kg) induced a robust place preference equal to cocaine, but did not alter the place preference induced by cocaine when the two were co-administered during training; 2) High dose modafinil produced very little to no locomotor sensitization and did not alter sensitization when co-administered with cocaine; 3) Mice previously sensitized to cocaine showed a hypersensitive response to low dose modafinil. 4) Low dose modafinil did not produce a place preference or locomotor sensitization. These findings are discussed in turn below.

*Modafinil induces conditioned place preference*

First, in line with recent reports (Nguyen et al., 2011; Wuo-Silva et al., 2011), the current study found that modafinil (75 mg/kg) induced a strong place preference. It was surprising, however, that modafinil and cocaine induced a similar level of place preference, since cocaine is considered to be much more reinforcing. Nonetheless, our results should not be used to conclude that modafinil is equally as rewarding as cocaine because there are likely ceiling effects involved in the CPP paradigm. Moreover, CPP can indicate whether a drug is rewarding, but is not a sensitive measure of the degree of reward given that even natural reinforcers, such as sucrose, can induce CPP (Schechter & Calcagnotto, 1993). In addition, we used a high dose of modafinil (75 mg/kg) that is
unlikely to be comparable to doses approved for use in humans, which are around 1-5 mg/kg (Shuman et al., 2009).

**Modafinil and behavioral sensitization**

Second, high dose modafinil induced little or no behavioral sensitization. Two previous studies have found modafinil induces some locomotor sensitization (Paterson et al., 2010; Wuo-Silva et al., 2011). It is unclear why sensitization occurred in the other studies, but not in the current study. One difference between the studies is the time window examined. The current study examined only the first 15 minutes after i.p. modafinil administration. Paterson et al. (Paterson et al., 2010) examined behavior for the first 30 minutes after oral administration, while Wuo-Silva et al. (Wuo-Silva et al., 2011) examined a delayed 10 min period beginning 30 min after i.p. administration. It is possible that the time window examined was not sufficient to see locomotor sensitization. This appears unlikely, however, because modafinil was sufficient to observe sensitization in cocaine-trained animals within the 15-minute test. Furthermore, previous rodent studies have shown that peak locomotor activity occurs between 20 and 40 minutes after administration (Simon, Hemet, & Costentin, 1996b; Zolkowska et al., 2009) however these studies only examined 20 minute bins and thus it is unclear where the true peak activity occurs. Examining the minute-by-minute data in Figure 2 indicates that the locomotor activity of the Modafinil mice is not increasing at the end of the 15-minute trial and thus it is unlikely to increase further.

Regardless, the current study cannot rule out that some locomotor sensitization may occur. However, even if modafinil does produce sensitization, it is far less than other addictive stimulants (Robinson & Berridge, 1993, 2003). Behavioral sensitization may be a critical factor in the transition from casual drug use to addiction (Robinson & Berridge, 2003, 2008). Thus, even if modafinil is rewarding, it may never reach the level
at which it can drive compulsive behavior. This conception could explain the seemingly
paradoxical findings that modafinil is rewarding yet there have been no reported cases of
addiction. We hypothesize that although modafinil engages many of the same
mechanisms as cocaine (Volkow et al., 2009), it fails to produce addicts because it does
not sufficiently engage the circuitry required for sensitization. In addition, the lack of
significant sensitization observed in this study may be accounted for by the lack of acute
locomotor activity produced by modafinil. We have previously reported no locomotor
activating effects of acute modafinil (0.075–75 mg/kg, (Shuman et al., 2009) and this
motor stimulant property may be required for behavioral sensitization. Indeed, we could
find few references where i.p. modafinil produced hyperactivity in a novel environment,
as opposed to dishabitation of activity in a familiar environment (Simon, Hemet, &
Costentin, 1996a; Simon, Panissaud, & Costentin, 1994; van Vliet, Jongisma, Vanwersch,
Olivier, & Philippens, 2006; Zolkowska et al., 2009). This is in sharp contrast to cocaine,
which readily elicits locomotor activity even at very low doses in a novel environment
(Wood, Fay, Sage, & Anagnostaras, 2007).

High dose modafinil did not alter the acute response to cocaine or the formation
of locomotor sensitization and conditioned place preference when the two drugs were
coadministered. The acute (Day 1) and chronic (Day 7) locomotor response were
slightly enhanced, although not significantly. While both drugs have actions on
dopaminergic, adrenergic, and serotonergic neurotransmission, the relative potency or
pharmacodynamic differences between their actions may account for the differences in
the locomotor and rewarding properties of the drugs. This is consistent with the current
finding that modafinil does not blunt the acute locomotor effect of cocaine, and in fact,
appears to slightly augment this response (Fig 2, 3A). This finding, however, could be a
result of the high dose used and may not generalize to lower doses. Indeed, future
studies should focus on interactions at a range of cocaine and modafinil doses to explore possible dose-related effects.

*Modafinil cross-sensitization with cocaine*

Thirdly, we found that a low dose of modafinil cross-sensitized in cocaine-trained mice. This observation is consistent with work indicating that modafinil can cause reinstatement of cocaine self-administration (Deroche-Gamonet et al., 2002) and conditioned place preference (Bernardi, Lewis, Lattal, & Berger, 2009). Those experiments, however, were done at high doses (64-128 mg/kg), and the current results expand these findings to our low challenge dose of 0.75 mg/kg. Modafinil, therefore, activates the pathway that is sensitized in animals with previous experience with cocaine. This is also consistent with what is known about the mechanism of modafinil. Cocaine sensitization can reduce autoreceptor activity and increase D1 receptor sensitivity (Ackerman & White, 1990; De Vries, Schoffelmeer, Binnekade, Raaso, & Vanderschuren, 2002; Pierce, Duffy, & Kalivas, 1995). Modafinil may activate this circuitry by increasing dopamine in the accumbens (Ferraro et al., 1997; Ferraro et al., 1996; Murillo-Rodriguez et al., 2007; Volkow et al., 2009).

*Low dose modafinil*

Finally, we found that a low dose of modafinil (0.75 mg/kg) does not produce a place preference or locomotor sensitization. This low dose is far lower than the doses that are generally tested in mice, but is sufficient to express locomotor sensitization (Fig 4B) and enhance learning (Shuman et al., 2009). It is unclear how a rodent dose of modafinil translates to human use, but we have argued that the equivalent dose is unlikely to be 100 times larger than what is given in humans (100-200 mg or 1-3 mg/kg; (Shuman et al., 2009)). This low dose may be a better model of human modafinil administration that the very high doses seen in previous studies. Furthermore, this
difference in dose could account for the lack of addiction in human subjects. It is possible, then, that modafinil could be very rewarding if given at high doses or through an alternate route of administration. Regardless of which dose is a better model of human use, future rodent studies should focus on a wide range of doses. Significantly, the cost of modafinil is presently very high (up to $20 per 100 mg tablet) indicating that high dose administration in humans is very unlikely.

*Modafinil and reward*

Together, the current data reinforce the notion that modafinil can be rewarding in mice, but only when given at a high dose or in cocaine-experienced subjects. Acute reward, however, is not necessarily the sole component of addiction. We found that modafinil produced very little, if any, behavioral sensitization like other addictive stimulants (Robinson & Berridge, 1993, 2003). In addition, there appear to be no withdrawal symptoms or a negative motivational state associated with drug termination that could drive negative reinforcement (Cephalon, 2004). Perhaps modafinil has relatively weak potency and slow absorption, especially orally as it is taken, compared to the stimulants of abuse. Thus, despite having a modest reward profile, modafinil is unlikely to drive addictive behavior.

Modafinil is suggested as a pharmacotherapy for cocaine addiction. Agonist therapy has emerged as a promising candidate for treating addiction (Shearer, 2008) and relies on weak activation to reduce craving and withdrawal. Modafinil meets this profile, as it appears to be a weak dopaminergic agonist. Weak or slow agonist therapy has generally been effective for Heroin (with buprenorphine) and nicotine (with varenicline) addiction, but has not been established as effective for psychostimulant addiction (Grabowski, Rhoades, et al., 2004; Grabowski, Shearer, Merrill, & Negus, 2004). Animal studies indicate that modafinil may not be a useful long-term therapeutic,
as it can enhance reinstatement of cocaine self-administration (Deroche-Gamonet et al., 2002), induce place preference (Fig 4), and cross-sensitize with cocaine (Fig 4), however, these effects have not been seen in human cocaine addicts (Vosburg, Hart, Haney, Rubin, & Foltin, 2010). Thus, while modafinil holds some promise as a therapeutic to reduce cocaine craving, more studies are needed to identify ways to minimize the potential for triggering relapse.
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The dissertation author was the primary investigator and author of this paper.
Figure 2-1. Schematic of procedure for Experiment 1. (A) On days 1-7, mice first received an injection of saline and were placed into the Saline Side of the chamber for 15 minutes. Immediately after, mice receive a group-specific drug injection (modafinil, cocaine, modafinil+cocaine, or saline) and were placed in the Drug Side of the chamber for 15 minutes. This data is depicted in Figure 2. (B) During conditioned place preference testing a small hole between the two sides of the chamber allowed free access to either side of the chamber. On day 10, all mice received an injection of saline and were placed into the chamber for 15 minutes. This data is depicted in Figure 3A. (C) In order to test the context and drug specificity of sensitization, all mice received modafinil and saline on each of the Saline and Drug Sides. On day 13, mice first received an injection of saline and were placed into the Saline Side for 15 minutes. Immediately after, mice received an injection of modafinil (0.75 mg/kg) and were placed into the Drug Side. Conversely, on day 14, mice first received an injection of saline and were placed into the Drug Side for 15 minutes. Immediately after, mice received an injection of modafinil (0.75 mg/kg) and were placed into the Saline Side. This data is depicted in Figure 3B and 3C. For Experiment 2, animals were then trained identically to part A above with either low dose modafinil (LoMod) or saline.
Figure 2-2. Induction of Sensitization. (A) Time course of drug action on day 1 of training. Cocaine and Mod+Coc groups showed increased locomotor activity over controls. Modafinil alone failed to induce substantial locomotion. (B) Time course of drug action on day 7 of training. All three drug groups showed an increased response over the saline control group. (C) Time course of locomotor sensitization. The difference in response from Day 1 to 7 is shown. Cocaine and Mod+Coc mice showed a significant increase in locomotor activity from Day 1 to Day 7. Modafinil mice did not differ from control mice. (D) Locomotor activity after saline treatment, by day. No group differences were observed. (E) Average locomotor activity after drug treatment, by day. Cocaine and Mod+Coc groups were enhanced over control mice. (F) Difference in locomotor activity of drug training trials between Day 1 and Day 7. Cocaine and Mod+Coc mice were enhanced over control mice. Modafinil mice did not differ from control mice. Each point represents the mean + S.E.M. (* p<0.05).
Figure 2-3. Place preference and cross-sensitization testing. (A) After a saline injection, all three drug groups spent significantly more time in the drug-paired side of the chamber. The difference in time spent in drug-paired side and saline-paired side is shown. (B) Locomotor activity in the drug-paired side minus the saline paired side after an injection of a challenge dose of modafinil (0.75 mg/kg). Both cocaine- and modafinil+cocaine-trained mice showed higher locomotor activity in the Drug Side of the chamber, when compared to Saline controls. Thus, the sensitization observed in these groups cross-sensitized to modafinil administration. (C) Locomotor activity in the drug-paired side minus the saline paired side after an injection of saline. No significant group effects were found. Each point represents the mean ± S.E.M. (* versus saline side, p<0.01).
Figure 2-4. Low dose modafinil does not induce sensitization or place preference. (A) Time course of drug action on Day 1 (left) and Day 7 (right) of training. Low-dose Modafinil (LoMod) and Saline groups did not differ. (B) Average locomotor activity after saline treatment (left) and drug treatment (right), by day. No group differences were found. (C) Difference in locomotor activity of drug training trials between Day 1 and Day 7 shown as minute-by-minute (left) or averages (right). Both groups showed equivalent decreases from Day 1 to Day 7. (D) Left, conditioned place preference test displayed as the difference in time spent in the Drug and Saline sides. Both groups of mice spent a roughly equal amount of time in each side of the chamber. Middle, locomotor activity in the drug-paired side minus the saline paired side after an injection of a challenge dose of modafinil (0.75 mg/kg). Neither the LoMod or Saline group showed higher locomotor activity in the drug paired side. Right, locomotor activity in the drug-paired side minus the saline paired side after an injection of saline. Neither the LoMod or Saline group showed higher locomotor activity in the drug paired side. Each point represents the mean ± S.E.M.
CHAPTER 3

Neuroanatomy of behaviors induced by exposure to psychostimulants

By

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Introduction

Drug addiction is a major social and economic problem that affects millions of people. It destroys people’s lives and can often lead to death. The National Institute on Drug Abuse estimates that drug abuse costs the United States up to $100 billion each year in treatment, healthcare, crime, and lost productivity (NIDA InfoFacts). While many people experiment with addictive drugs, only a small percentage ever become addicted. Research on addiction has therefore been geared to understanding the shift in behavior from casual drug use to compulsive drug taking. Drug addiction has generally been defined by compulsive drug use, compulsive drug seeking, and a high tendency to relapse (Jaffe, 1980). Drug seeking and relapse are two behaviors that occur when the addict is not on the drug, implying that the drug produces long lasting physical changes in the brain. In this manner, a memory of the drug must be formed and remembered, whether consciously or not, in order to drive addictive behavior. Identifying this memory form has been the centerpiece of recent studies in the field with the eventual goal of developing amnesic treatments to treat addiction. This review will identify some of these memory processes associated with addictive drugs and attempt to localize the brain areas critical for the induction and expression of these memories with a special focus on psychostimulants.

Theories of Drug Addiction

Three main theories have emerged as the best attempts to understand the motivation that drives addicts to compulsive drug use. The opponent-process theory is based on the notion that numerous drug exposures will produce withdrawal symptoms that will drive an addict to take the drug. It was originally presented (Solomon & Corbit, 1974; Solomon, 1980) as a two-process model of affective response to a stimulus (i.e.,
drug) with an excitatory a-process that was followed by an unpleasant b-process. Solomon theorized that the a-process was a physiological response to the drug and showed tolerance with repeated exposures, while the opponent b-process was actually strengthened. Thus, after multiple exposures to the drug the a-process produces a minimal increase in hedonic state (manifest as drug tolerance) that is followed by a large decrease, which would theoretically lead to further drug seeking. This theory is consistent with addicts’ reports of decreased positive affect during drug exposure and increased withdrawal with successive drug experiences. Recently, Koob and Le Moal (1997, 2001, 2008a) have suggested potential neural correlates of these processes, theorizing that the a-process represents activation of the mesolimbic reward circuitry that can become downregulated with repeated drug use. Furthermore, they argue that homeostatic processes continue to drive an anti-reward process that never returns to normal homeostatic levels. They also argue that after dependence and withdrawal, strengthened noradrenergic signaling drives an increased stress response through corticotropin releasing factor (CRF) in the amygdala (Koob and Le Moal, 2008b).

An alternative theory of addiction is based on repeated exposures of a drug increasing the drive to take that drug. The incentive-sensitization theory asserts that the physical changes that occur in the brain cause a sensitization of the incentive qualities of the drug (Robinson and Berridge, 1993, 2003). This increase in the “wanting” or incentive salience of the drug is what leads to escalating and eventually compulsive use, in addition to changes in executive control. It is important to note that the incentive properties of the drug are separate from the hedonic value of the drug, which is also consistent with addicts’ reports of decreased pleasure during drug exposure yet increased drive to obtain the drug. The best evidence for this theory has come from studies using repeated drug exposures to produce increased attributions of incentive. A
number of studies have shown that prior drug treatment can increase the three key features of incentive stimuli (Berridge, 2001): approach behavior (Harmer & Phillips, 1998), Pavlovian instrumental transfer (Wyvell & Berridge, 2001), and conditioned reinforcement (Taylor & Horger).

Other theories of addiction have focused on aberrant learning mechanisms where associations between the drug, the behavioral and physiological response, and the reward become overlearned. The reward-prediction error hypothesis posits that phasic dopamine acts to bind the rewarding effects of a drug with the response, and the huge influx of dopamine allows for a gross overlearning of these associations, leading to further use (Hyman, 2005; Hyman et al., 2006). This theory is certainly not mutually exclusive from the incentive sensitization theory and relies on many of the same mechanisms and assumptions (e.g., dopamine release in the nucleus accumbens). In fact, all three theories are likely to contribute in some way to the complex state of addiction.

**Addiction and Memory**

Regardless of the theory of addiction, it is clear that addictive drugs create long lasting physical changes, or memories, which continue to influence behavior well after the drug has left the system. In order to investigate what memories are formed during drug exposure that drive the shift to compulsive drug taking many researchers have turned to rodent models. Indeed, a number of behavioral memories can be seen with simple repeated exposures of psychostimulants in rodents. Psychostimulants enhance locomotor behavior and generally work by increasing monoamine transmission. While these drugs are very similar, a number of differences have emerged that distinguish
them from one another. Thus, some actions of these drugs will not overlap and these cases will be highlighted.

The first and perhaps best-studied memory induced by chronic psychostimulants is behavioral sensitization, an increase in the psychomotor stimulant properties of a drug after multiple exposures. Behavioral sensitization is typically studied by administering a constant dose of a psychostimulant (e.g., cocaine, amphetamine) over multiple days and measuring locomotor activity or stereotyped behaviors. Often a drug challenge test is given after a short incubation period in order to get rid of any tolerance to the drug. During this test, sensitized animals will show a response two to three times greater than an acute dose. Behavioral sensitization, however, is generally specific to the context paired with the drug meaning that a drug challenge test administered in a novel environment will not express sensitization (Anagnostaras & Robinson, 1996). This context specificity has been shown to be a dissociable memory form as electroconvulsive shock can selectively disrupt it while leaving sensitization intact (Anagnostaras et al., 2002b). A third memory form is a Pavlovian conditioned response, where mere exposure to a drug-paired context can elicit behavior similar to the drug itself. Thus, locomotor hyperactivity is generally observed in the drug-paired environment even when the animal is in a drug-free state. Finally, in conditioned place preference (CPP) a drug is repeatedly paired with one of two distinct environmental contexts while saline is paired with the other. On test day, the barrier between the two contexts is removed and the animal (off-drug) is allowed to explore either context. Animals generally show a strong preference for the drug-paired context with all drugs of abuse.

Each of these memory forms (locomotor sensitization, context specificity, conditioned response, conditioned place preference) can be seen with simple pairings of
psychostimulants. Other memory processes can also be seen with more intricate experimental design (e.g., self-administration) however this review will focus on the four previously mentioned. The induction and expression of these memory processes are often mediated by different circuits and will thus be considered separately. While there is some disagreement, each of these memory forms appear to be dissociable from each other and will generally be treated as independent of one another. Indeed, this review will demonstrate that these behaviors each rely on a unique set of interconnected circuitry indicating that the actions of psychostimulants produce diverse physical and behavioral changes that contribute to addiction.

Techniques

This review will focus on the neuroanatomical basis of these memory processes with the hope of better understanding how addiction-related memory processes work together to shape addictive behavior. Currently, the two most prominent strategies used to examine the neuroanatomy of behaviors are localized brain lesions and immediate early gene expression. Lesions are useful because they allow researchers to determine whether or not a particular behavior depends on a specific area of the brain. When a behavior is selectively knocked out, it indicates that the lesion area is at least a part of the pathway regulating that behavior. Classically, lesions have been either electrolytic (injecting current into an area of the brain in order to overexcite neurons and cause cell death) or excitotoxic (injecting an excitatory chemical, generally NMDA, into an area of the brain in order to cause overexcitation and cell death). Electrolytic lesions can have better spatial resolution because of less chemical leaking, but also destroy fibers of passage often leading to less clear results. Recently, many researchers have turned to reversible lesions by infusing chemicals (e.g., lidocaine) to inactivate certain areas of the
brain for brief periods of time during either training or testing. Also, lesions are sometimes impractical due to various unrelated effects, and in these cases, many researchers turn to localized injections of specific agonists or antagonists. These confer more information about the neurochemical basis of behavior but complex interactions often make interpretation difficult.

Immediate early genes (IEGs), such as fos or zif268, are cellular markers that respond transiently to neuronal activation and can thus be used to localize active areas of the brain following certain behaviors (Sheng & Greenberg, 1990). Recently, these genes have been shown to play a key role in learning and memory. Therefore it is not surprising that these genes have been linked to reward learning and the persistent changes found with chronic drugs of abuse (Hiroi et al., 1997; Zhang et al., 2006; Solecki et al., 2008). Acute psychostimulants induce major IEG expression throughout the brain and this makes it difficult to isolate specific brain areas involved in the induction of drug-induced behavioral changes. Thus, IEG expression studies are most helpful in localizing the circuits involved in the expression of these behaviors. This is easily accomplished by simply measuring IEG levels immediately following the expression of the behavior.

**Behavioral Sensitization**

The locomotor activating and stereotypy-producing response of many drugs of abuse can be increased or sensitized after multiple drug exposures. This behavioral sensitization has generally been studied in classic psychostimulants such as amphetamine and cocaine, as well as the opiates, morphine and heroin. Proponents of the incentive-sensitization theory of addiction argue that sensitization is the critical factor in the transition to compulsive drug seeking in addicts (Robinson and Berridge, 2003,
Sensitization has been shown to last up to one year in the rat (Paulson, Camp, & Robinson, 1991) and thus proponents contend that this process could account for the very long lasting changes seen in addicts. They do, however, draw an important distinction between behavioral sensitization and more specifically locomotor sensitization. While most studies on sensitization explore locomotor activity or stereotypy, the critical component is incentive sensitization. Although studies on sensitization are not directly measuring incentive sensitization, the similarities between the locomotion circuitry and the motivation circuitry allow for insight into both systems.

**Associative versus Non-Associative Views of Sensitization**

Sensitization can be seen as both an associative process and as a non-associative process. The associative view holds that conditioned stimuli (CS; e.g., context) become paired with the drug unconditioned response (UR; e.g., locomotor hyperactivity) to elicit a conditioned response (CR). Sensitization, in this view, is conceived as an increasingly larger CR that manifests as increased expression of the drug response despite the UR remaining unchanged. The main evidence in favor of this view comes from the notion that sensitization can be attenuated by administering the drug in a novel environmental context. Also, a conditioned response can be observed by placing an animal in an environment previously paired with the drug. Even without administering the drug, locomotor activity is increased.

Despite clear associative control, there are several lines of evidence contradicting a purely associative view of sensitization. First, while a conditioned response is generally observed, it is relatively small and lasts only a few minutes, which likely cannot account for the difference between naïve and sensitized animals. The associative view counters that this conditioned response is driven by both extrinsic and intrinsic cues elicited by the drug, and thus, a CR observed on drug will be much larger
and may be able to account for the difference. Another piece of evidence problematic for the associative view is that the CR can be extinguished, while leaving sensitization largely intact (Stewart and Vezina, 1991; Anagnostaras and Robinson, 1996). Also, certain experimental procedures do not produce a CR to the environmental context, but do produce sensitization (Vezina & Stewart, 1990). In both of these cases the associative view relies on intrinsic cues alone eliciting a strong enough CR to account for all of the sensitization effect. This appears unlikely and there is very little direct evidence for this claim.

Sensitization can also be seen as a non-associative change in the neural substrates mediating the response to a drug. In this view, the unconditioned response becomes increasingly larger and this can occur without conditioned effects. Evidence for this view comes from numerous studies showing structural and neurochemical changes in related circuitry. After repeated exposure to amphetamine, cocaine, or morphine, long lasting physical changes occur in the morphology of the nucleus accumbens and prefrontal cortex (Robinson and Kolb, 1997, 1999a, b). Neurons in these areas have longer dendrites and have a higher density of dendritic spines. In addition, these drugs lead to an augmented response of dopamine in the nucleus accumbens (Kolta et al., 1985; Robinson et al., 1988; Pierce & Kalivas, 1997). Importantly, many of these changes can occur in the absence of conditioned effects. They can even occur when there is no unconditioned response to the drug such as in vitro studies (Robinson and Becker, 1982), anesthetized animals (Wang and Hsiao, 2003), or targeted intracranial injections (Vezina & Stewart, 1990). These changes in related circuitry are referred to as neural sensitization. While a number of neural changes have been identified, they are diffusely distributed around the brain and do not generally map on to the profile of behavioral sensitization (Pierce and Kalivas, 1997;
Vezina, 2004). Also, it is clear that some associative processes can control the expression of behavioral sensitization (Anagnostaras & Robinson, 1996; Anagnostaras et al., 2002b). Thus, these neural changes must be taken together with the associative influences in order to fully understand the development and expression of sensitization.

**Major Projections Involved in Regulating Psychostimulants**

Figure 1 outlines the major projections thought to be involved in the actions of drugs of abuse. This motive circuit has been highly implicated in the production and expression of locomotor sensitization. It is considered a cortico-striatal-thalamic loop because it connects the prefrontal cortex (PFC) with the striatum, which projects to the thalamus and finally back to the PFC and motor cortex for behavioral output. In the striatum, after receiving input from the PFC, the ventral tegmental area (VTA) sends a large dopaminergic projection to the nucleus accumbens (NAc), an area of the brain strongly implicated in reward. The NAc then projects to the ventral pallidum (VP) and out of the striatum to the thalamus. In addition to these main projections, a number of feedback connections are located within the loop. For example, the VTA also projects dopamine to the PFC and VP. Also, other structures have been implicated in the regulation of this loop, such as the amygdala and hippocampus (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000; Degoulet et al., 2008). Three main neurotransmitter systems work together to regulate this loop. Projections from the VTA are dopaminergic, while the NAc and VP are generally GABAergic, and the PFC, thalamus, and amygdala all rely on glutamatergic signaling. Each neurotransmitter system has complex interactions and such diverse signaling all converging at the same location (i.e., NAc) makes it difficult to determine how specific inputs affect the system.

**Induction of Behavioral Sensitization**
Systemic injections of amphetamine and cocaine produce robust sensitization, though targeted intracranial injections are useful for identifying the location of neural activation involved in the induction of sensitization. A number of studies have shown that infusions of amphetamine directly into the VTA sensitize an animal to a later systemic injection (Kalivas & Weber, 1988; Vezina & Stewart, 1990; Cador et al., 1995, 1999; Bijjou et al., 1996), and this is not true of any other related circuitry (Dougherty Jr & Ellinwood Jr, 1981; Vezina & Stewart, 1990; Hooks et al., 1992; Cador et al., 1995). In addition, intra-VTA injections of amphetamine can sensitize an animal to a later injection into the NAc (Perugini & Vezina, 1994), the main output of the VTA. Thus, the VTA projection to the NAc appears to be the critical site of action of amphetamine in the induction of behavioral sensitization.

The evidence for the involvement of the VTA in behavioral sensitization to cocaine is less clear. An initial experiment reported that multiple infusions of cocaine into the VTA were insufficient to produce behavioral sensitization to systemic cocaine (Steketee, 1998a). However, that same study reported that intra-VTA infusion of the specific dopamine reuptake inhibitor GBR-12909 did produce sensitization to systemic cocaine. These unexpected results led to a hypothesis that other areas involved in regulating this circuit (e.g., PFC) were necessary for the induction of sensitization to cocaine. Further evidence for this view was provided by the notion that intra-VTA SCH-23390, a dopamine D1 receptor antagonist, prevented the neural changes (such as increased levels of dopamine) induced by systemic cocaine in the NAc, but did not prevent behavioral sensitization (Steketee, 1998b). This highlights the dissociation between the neural changes in the mesoaccumbens system induced by psychostimulants and behavioral sensitization, further indicating that other parts of the circuit likely modulate the projection of the VTA to the NAc. In contrast, intra-VTA SCH-
23390 prevents both the neural changes and behavioral sensitization to systemic amphetamine (Vezina, 1996). Thus, there appears to be substantive differences between mechanisms by which amphetamine and cocaine induce sensitization. These studies, however, conflict with a similar study that indicated that intra-VTA infusions of cocaine did sensitize an animal to a later systemic cocaine injection (Cornish & Kalivas, 2001). The reasons for these disparate results are unclear as both experiments employ the same experimental design and animals were acquired from the same source. One explanation offered by Cornish and Kalivas (2001) is that in Steketee (1998b) the baseline responding was elevated and this may have occluded any sensitization. Also, the exact distribution of the intra-VTA infusions was slightly different and could have led to the different results. Nonetheless, the authors agree that the induction of sensitization to cocaine is likely dependent on other areas of related circuitry.

Targeted lesions are another commonly used method in exploring the areas of the motive circuit involved in the induction of sensitization. Pre-training electrolytic lesions of the NAc shell, in particular the dorsomedial region, reduce the induction of behavioral sensitization (Todtenkopf et al., 2002a). Also, multiple studies have shown that ibotenic acid lesions of the prefrontal cortex prevent the induction of behavioral sensitization to both amphetamine and cocaine (Wolf et al., 1995; Tzschentke & Schmidt, 1998; Cador et al., 1999). 6-hydroxydopamine (6-OHDA) lesions to the medial PFC also prevent the induction of sensitization to amphetamine (Bijjou et al., 2002). This indicates that while the mesoaccumbal projection is the main site of action of psychostimulants, presumably glutamatergic input from the PFC is also required. In addition, two studies have found that ibotenic acid and 6-OHDA lesions of the amygdala prevent the induction of behavioral sensitization (Wolf et al., 1995; Bijjou et al., 2002). This is in contrast, however, to the findings of Cador et al. (1999), which found that
ibotenic acid lesions of the amygdala did not affect the induction of behavioral sensitization. There are no clear indications as to why these studies produced conflicting results although the lesions in Wolf et al. (1995) are more extensive which could account for the difference. The fimbria-fornix, which passes information from the hippocampus to the striatum, has also been implicated in the induction of behavioral sensitization to amphetamine (Yoshikawa et al., 1991), however multiple studies have found this not to be the case (Wolf et al., 1995; Browman et al., 1996). These differences could be due to differences in the strength of the drug as Yoshikawa et al. (1991) used the stronger methamphetamine form of the drug. Another study indicated that the hippocampus may play a role in the induction of sensitization through connections other than the fimbria-fornix as complete hippocampal lesions prevented the induction of sensitization, while fimbria-fornix lesions had at most a minor effect (Coutureau et al., 2000). This study was difficult to interpret, however, because both the fimbria-fornix and the hippocampal lesions produced hyperactivity compared to sham controls. Together, these studies indicate that while the VTA is the main locus of action of psychostimulants in the induction of sensitization, signaling from a number of other regions is still necessary. In particular, the PFC, amygdala, hippocampus, and NAc shell appear to be required for the induction of behavioral sensitization.

*Expression of Behavioral Sensitization*

The motive circuit appears to play a role in both the induction and expression of behavioral sensitization. Despite this, the components of this circuit involved in induction and expression are quite different as a number of studies have indicated certain components are critical for induction but not expression, and vice versa. For example, infusions of amphetamine into the NAc can elicit the expression of sensitization from both intra-VTA and systemic induction (Perugini & Vezina, 1994; Cador et al., 1995).
This indicates that the NAc is important for the expression, but not the induction of sensitization. Not surprisingly, psychostimulants delivered directly to the NAc produce locomotor hyperactivity. This indicates that the NAc regulates locomotor activity, and is likely involved in the expression of behavioral sensitization. In addition, the expression of sensitization appears to have multiple phases. Early research pointed to increased expression of behavioral sensitization after a period of withdrawal (Robinson and Becker, 1986) and a number of studies have shown large differences in both the behavioral and neural sensitization evoked by short and long periods of withdrawal.

Short periods of withdrawal from both cocaine and amphetamine are characterized by a reduced sensitivity in D2 autoreceptors in the VTA, while long periods of withdrawal are not (Ackerman & White, 1990; Yamada et al., 1991, Pierce et al., 1995; De Vries et al., 2002). Also, short withdrawal periods have produced D1 receptor supersensitivity in the NAc. Therefore, reduced autoreceptor activity and increased D1 receptor sensitivity may be at least partially responsible for the short-term expression of behavioral sensitization, perhaps by altering the gain of signaling in the mesoaccumbal system (Pierce & Kalivas, 1997). In addition, short periods of withdrawal have been associated with increased levels of dopamine (Kalivas & Duffy, 1993), tyrosine hydroxylase (Beitner-Johnson & Nestler, 1991), and dopamine transporters (Lu & Wolf, 1997; Shilling et al., 1997) in the VTA. These changes following a short period of withdrawal, however, are transient and generally do not persist longer than one week (De Vries et al., 2002).

The expression of behavioral sensitization after long periods of withdrawal appears to be dependent on increases in dopaminergic signaling in the NAc. Unlike after short periods of withdrawal, there is consistent evidence that long-term sensitization evokes increased levels of extracellular dopamine in the NAc in response
to psychostimulants (Robinson et al., 1988; Kalivas & Duffy, 1993; Hooks et al., 1993; Pierce & Kalivas, 1995; Heidreder et al., 1996) and this increase appears to best correlate with the expression of sensitization. A number of cellular adaptations have been proposed as the mechanism by which extracellular dopamine is increased including an augmented vesicular pool of dopamine, increased calcium conductance, and changes in glutamatergic signaling (Pierce & Kalivas, 1997; Vanderschuren & Kalivas, 2000; Vezina, 2004). In addition to increased dopamine in the NAc, changes in dendritic morphology (Robinson & Kolb, 1997, 1999a,b), increased extracellular glutamate (Pierce et al., 1996), and glutamatergic receptor subunit composition (Lu & Wolf, 1999; Lu et al., 1999) have all been observed after long periods of withdrawal, however these mechanisms have not been well examined and their contribution to the expression of sensitization is unclear. Thus, the expression of sensitization is divided into an early and a late component with different neural profiles. Early expression of sensitization appears to be mediated by short-term changes in dopamine receptor sensitivities in the mesoaccumbal projection, while long-term expression of sensitization is controlled by increased extracellular dopamine in the NAc in response to psychostimulants.

In order to further elucidate the role of the motive system in the expression of behavioral sensitization, a number of studies have used immediate early gene expression to identify candidate regions. The earliest studies focused on changes in Fos immunoreactivity in the striatum after repeated exposures to amphetamine. After the expression of sensitization, increased levels of Fos were observed in the striatum (Norman et al., 1993; Jaber et al., 1995). Further studies expanded these finding to include a finer resolution and pinpoint substructures within the striatum. A number of studies compared chronic psychostimulant treatment to acute injections and found that
chronic administrations increased expression of Fos specifically in the core region of the NAc (Hedou et al., 2002; Hope et al., 2006; Conversi et al., 2008; Nordquist et al., 2008). This profile of expression of behavioral sensitization included both short and long periods of withdrawal from the drug, but is also contradicted by other reports (Todtenkopf et al., 2002b; Vanderschuren et al., 2002). This contradiction may involve differences in the protocol used in the induction of sensitization as each of these studies used pretreatments that may have altered the associative components of sensitization (Nordquist et al., 2008). They also used smaller doses that may have led to a lowered threshold of activation. Thus, while the NAc dorsomedial shell is important for induction of sensitization, the core region appears to be involved in its expression. Additional areas of the motive circuit have been implicated in these studies such as the PFC (Hedou et al., 2002), caudate putamen (Conversi et al., 2008), and the intermediate zone of the shell of the NAc (Todtenkopf et al., 2002a), however these findings have not been replicated.

Another interesting finding that is consistent across these studies is the notion that increases in Fos immunoreactivity produced by psychostimulant sensitization coincided specifically with enkephalin-positive neurons in the striatum (Jaber et al., 1995; Hope et al., 2006; Mattson et al., 2007). Enkephalin is an endogenous opioid that is generally found in striatal medium spiny neurons that also express D2 dopamine receptors. These neurons have thus been hypothesized to mediate the expression of sensitization, perhaps through the actions of ERK/MAPK-dependent activation of CREB through enhanced glutamatergic innervation from the cortex (Sgambato et al., 1998; Mattson et al., 2007). Also, this appears to be a mechanism by which both psychostimulants and opiates may elicit similar effects.
Lesions studies further indicate divergent patterns of activation between the induction and expression of behavioral sensitization. Two studies have indicated that the PFC is not necessary for the expression of behavioral sensitization to cocaine and amphetamine using post-training ibotenic acid lesions (Li & Wolf, 1997; Li et al., 1999). These results, however, are in direct conflict with another report that implicates the dorsal PFC in the expression of behavioral sensitization (Pierce et al., 1998). This study performed post-training lesions of the PFC and found that the expression of sensitization was blocked, as well as the increase of glutamate in the NAc core seen in sensitized animals. These disparate results may be explained by differences in protocols as Pierce et al. (1998) allowed a two week withdrawal period before administering the lesions, while the two other studies did not. It is possible that the PFC does not play an important role in short-term expression of behavioral sensitization, yet is required for long-term expression. Some sort of consolidation process could occur where expression is initially mediated solely by the mesoaccumbal projection but is then consolidated to other areas of the motive circuit. Recently, the role of the hippocampus in expression of sensitization to amphetamine was explored using lidocaine to induce reversible lesions of the dorsal and ventral hippocampus. Lesions of the dorsal hippocampus, but not the ventral hippocampus, disrupted expression (Degoulet et al., 2008). This finding is not entirely surprising as the dorsal hippocampus plays an important role in spatial and contextual learning (Moser et al., 1993; Anagnostaras et al., 1999, 2001, 2002a) and may mediate the expression of sensitization through associative influences. A number of other regions involved in the induction of behavioral sensitization are not involved in its expression. Lesions of the fimbria-fornix, amygdala, and periventricular thalamus do not prevent the expression of sensitization (Todtenkopf et al., 2002c). Thus, the
expression of behavioral sensitization appears to rely mostly on the NAc core region, as well as input from the dorsal hippocampus and perhaps the dorsal PFC.

**Contextual Control of Behavioral Sensitization**

The induction and expression of behavioral sensitization can be powerfully modulated by the context in which training and testing occurs. Repeated amphetamine delivered in the home cage produces minimal behavioral sensitization, yet the same procedure delivered in a novel environment produces robust sensitization (Badiani et al., 1995a,b). This lack of sensitization in the home cage could be accounted for by the absence of conditioned response, however pre-exposure to the novel context for 6-8 hours before amphetamine administration eliminates the conditioned response without altering sensitization (Crombag et al., 2001). In addition, other procedures that do not produce a conditioned response still induce sensitization (Anagnostaras and Robinson, 1996). Thus, the conditioned response is dissociable from the ability of a novel environmental context to facilitate sensitization. Furthermore, the ability of a novel environmental context to facilitate sensitization is unique to contextual cues and cannot be induced by discrete cues, such as a light, tone, or odor, that reliably predict drug exposure (Crombag et al., 2000).

While most experiments have focused on external environmental cues, it appears that interoceptive drug cues may also provide control over the expression of sensitization. Carey et al. (2005) demonstrated that the stimulus properties of a drug act as a contextual gating mechanism. The 5-HT$_{1A}$ agonist 8-OHDPAT (8OH) and D$_1$/D$_2$ agonist apomorphine were each paired with cocaine during the induction of sensitization. In both cases, sensitization was only observed when tested in the presence of the cocaine-paired drug. This study, however, is difficult to interpret because the inhibitory
nature of the 8OH and apomorphine disrupt the acute effects of the drug. The authors argue that because the drugs produce similar inhibition they can be used to effectively evaluate sensitization, but the interactions between these drugs could lead to unknown effects.

**Excitatory Conditioning Model**

The expression of behavioral sensitization can be entirely controlled by the context in which testing occurs. In this manner, sensitized animals tested in a novel environment do not express behavioral sensitization (Post et al., 1981; Anagnostaras and Robinson, 1996). A number of different models have been used to explain this phenomenon. One of the earliest explanations for this context specific expression of sensitization was an excitatory conditioning model. As discussed above, this model relies on an increasing conditioned response elicited by conditioned cues. Thus, when tested in a novel context there is no conditioned response and therefore no sensitization. This is unlikely to be the case as numerous studies have dissociated the conditioned response from the expression of sensitization (Stewart and Vezina, 1991; Anagnostaras and Robinson, 1996).

**Inhibitory Conditioning Model**

A second model to account for context specificity is an inhibitory conditioning model (Stewart and Vezina, 1988, 1991; Vezina and Leyton, 2008). This model posits that the lack of sensitization seen in unpaired groups is due to inhibitory processes produced by exposures to the context explicitly unpaired with the drug. Indeed, cues that are explicitly unpaired with an unconditioned stimulus can act as conditioned inhibitors (Rescorla, 1969). Furthermore, Stewart and Vezina (1991) showed that extinction to an explicitly unpaired context revealed intact sensitization. Thus, they concluded that conditioned inhibition is indeed responsible for the lack of sensitization.
seen in unpaired groups. A number of findings are problematic for this model, including multiple studies that have found no sensitization after extinction in unpaired animals (Ahmed et al., 1993; Anagnostaras and Robinson, 1996). Also, explicit unpairing is not necessary to suppress the expression of sensitization. Indeed, amphetamine induced in a novel context and then tested in a second novel context is highly context specific (Anagnostaras and Robinson, 1996; Anagnostaras et al., 2002b).

**Occasion-Setting Model**

A third model to explain the context specificity of behavioral sensitization theorizes that the environmental context acts as a modulator or occasion-setter of sensitization (Anagnostaras and Robinson, 1996; Anagnostaras et al., 2002b). Occasion-setters are stimuli that do not themselves elicit a conditioned response, but do allow for the expression of other conditioned or unconditioned responses (Rescorla, 1985; Holland, 1992). Anagnostaras et al. (2002) explored this possibility by sensitizing rats to amphetamine and administering electroconvulsive shock (ECS), a procedure known to produce a profound retrograde amnesia (Duncan, 1949). Recent studies have indicated that reactivating a memory can make that memory susceptible to disruption. Thus, 3-4 days post-training ECS was administered after a brief reactivation of the memory of the training context. After ECS, sensitization was no longer context specific as the unpaired group, which had received amphetamine only in an alternate context, showed equal sensitization to the paired group, which had received amphetamine only in the testing context. The unpaired control group that received sham ECS retained context specificity and no effect on the conditioned response was found in any group. Therefore, the ECS selectively disrupted the context specificity of behavioral sensitization, indicating that the mechanism of context specificity is inhibitory. This evidence is consistent with the inhibitory model proposed by Vezina and Stewart (1988,
1991) however, as discussed above, it is unlikely that inhibitory conditioning alone can account for context specificity. Thus, inhibition appears to gate the expression of sensitization in different contexts, perhaps through an occasion-setting mechanism linked to the expectations of the animal.

*Enhancement of Sensitization by Novelty*

The ability of a novel context to enhance the induction of sensitization has been examined further using IEG expression. Indeed, amphetamine delivered in a novel environment induces larger IEG expression than when it is administered in the home cage (Badiani et al., 1998). This enhanced pattern of expression does not, however, increase dopamine overflow in the striatum, indicating that novelty induces its effects through a mechanism other than the direct action of amphetamine on the mesoaccumbal projection. A number of structures have been implicated, including the dorsomedial caudate-putamen and medial PFC. These structures produce a super-additive increase in c-fos mRNA expression when a novel environment is paired with amphetamine (Ostrander et al., 2003). Also, corticostriatal projections alone can induce c-fos expression specifically in enkephalin-positive striatal neurons (Parthasarathy & Graybiel, 1997). This population of neurons has been implicated in the enhanced response to amphetamine in a novel environment and sensitization (Ferguson & Robinson, 2004). Thus, cortical input to the striatum may underlie at least part of the contextual control over the induction of sensitization.

*Expression of Context Specificity*

Two recent studies have attempted to localize the expression of context specific sensitization using IEG expression. Rademacher et al. (2007) administered saline and amphetamine in two distinct environments on alternating days and then tested the animals by injecting either cocaine or amphetamine in the saline- or drug-paired context.
After testing, Fos and synaptophysin, a vesicular marker of synapses, were measured. Amphetamine delivered in the drug-paired context induced Fos in the hippocampus, amygdala, NAc core and shell, and dorsolateral striatum (putamen), whereas amphetamine administered in the saline-paired context elicited significantly lower expression in each area studied. Thus, it appears that the context specific control of sensitization lies in the inability of the unpaired group to engage multiple sites of action as seen in the paired animals. This design, however, lacked an acute control and thus makes it difficult to interpret any changes in expression of the unpaired group. Interestingly, the unpaired group did find decreased synaptophysin immunoreactivity in the CA1 region of the hippocampus and dorsolateral striatum when compared to all other groups. This indicates that the hippocampus and dorsolateral striatum may act to bind contextual information with the unconditioned stimulus and gate the expression of sensitization. Two pathways may be involved including one through the medial PFC (Jay and Witter, 1991) or a direct connection from the subiculum to the striatum (Lisman and Grace, 2005).

Mattson et al. (2008) used a similar procedure to investigate IEG expression in the gating of sensitization to cocaine, but instead used two different Fos-related antigens to study how specific neuronal ensembles were activated in response to cocaine in a saline- or drug-paired context. FosB is a long lasting isoform that can be used to identify neurons that have been chronically activated, while c-fos is a short acting isoform used to identify neurons that have been recently activated. Combining these techniques allowed Mattson and colleagues to investigate whether specific ensembles of neurons were activated during training and testing in both the saline- and drug-paired environments. They found that specific ensembles of neurons in both the NAc and caudate-putamen were activated during training and testing and that the percentage of
cells activated during both was higher in the drug-paired context than in the saline-paired context. This indicates that the drug-paired context was able to stimulate more of the specific neurons activated during training, a trademark of a stable neural correlate of memory (Reijmers et al., 2007). The authors argue that this activation of specific neural ensembles underlies the context specificity of the expression of sensitization. In this manner, contextual information may be transmitted through glutamatergic afferents into different sets of neurons in the striatum (Pennartz et al., 2004; O’Donnell, 2003).

Taken together, these two IEG studies indicate that the contextual gating of behavioral sensitization may be accomplished through afferent projections to the striatum providing contextual and interoceptive cues that allow expression. Thus, an inhibitory occasion-setting mechanism appears to prevent the expression of sensitization in a novel or unpaired context through a gating system in the striatum controlled by afferent glutamatergic projections.

### Conditioned Response

Many behavioral studies in animals have established the ability of contextual stimuli to elicit a conditioned response (CR) similar to the unconditioned response (UR) associated with drug exposure (Pavlov, 1927; Post et al., 1981; Beninger & Hahn, 1983; Carey, 1986; Tirelli & Terry, 1998). Like the UR, a conditioned response can take the form of increased locomotor activity, stereotypy, and rotational behavior. This CR appears to enhance sensitization during paired exposures, but can be eliminated through extinction and is dissociable from sensitization and context specificity (Anagnostaras and Robinson, 1996). In addition, certain sensitization protocols do not produce a locomotor CR, such as intra-VTA amphetamine administration (Vezina & Stewart, 1990) or a conditioned place preference protocol (Martin-Iverson & Reimer,
1996), further dissociating sensitization and the conditioned response. Thus, in most cases systemic administration of the drug in the distinct test environment is necessary, but not sufficient to produce the conditioned response. Contrary to the context-specific control of sensitization that requires a unique environment be paired with a drug, discrete cues alone can elicit a CR. A conditioned response to discrete cues, however, is not accompanied by activation of Fos in the limbic system. Conversely, a conditioned response to a generalized environment does produce activation in this system, indicating that these similar Pavlovian conditioned responses may work through different mechanisms (Hotsenpiller et al., 2002). Alternatively, the capacity of a discrete cue to elicit a response may simply be lower than a generalized environmental context, and this may lead to sub-threshold activation. There is little evidence, however, that similar Pavlovian processes necessarily activate the same circuits. Accordingly, while the circuitry of Pavlovian fear conditioning has been well mapped, there may be very little overlap between the circuitry activated by a conditioned response elicited by a shock-paired cue and a drug-paired cue.

**Induction of Conditioned Response**

The involvement of the nucleus accumbens in the induction of conditioned locomotor activity was first established by the notion that intra-accumbal infusions of cocaine did produce a conditioned response (Hemby et al., 1992). Lesion studies have also implicated the NAc in the induction of the conditioned response as 6-OHDA lesions of the nucleus accumbens administered pre-training abolish the conditioned locomotor response to amphetamine (Gold et al., 1988). Sellings and Clarke (2006) extended these findings to selectively implicate the NAc core region as pre-training bilateral 6-OHDA lesions of the core, but not the medial shell, abolished the conditioned response to amphetamine. Importantly, these lesions did not reduce the acute response to the
drug. Taken together, these studies indicate that the NAc is critically involved in the induction of the conditioned response to amphetamine, likely through the actions of the core region.

Expression of Conditioned Response

A number of studies have investigated the neuroanatomy of the conditioned response using Fos expression. The critical difference in Fos expression is between a paired group, which receives drug in the test chamber and then saline in another context, and an unpaired group that receives saline in the test environment and drug in an alternate context. On test day, saline is administered in the testing environment. A number of areas have consistently shown increased Fos expression in the paired group, including the amygdala, lateral septum, and medial PFC (Brown et al., 1992; Hotsenpiller et al., 2002; Franklin & Druhan, 2000; Mead et al., 1999; Rademacher et al., 2007). In addition, the NAc, claustrum, and paraventricular nucleus (PVN) of the thalamus have been strongly implicated (Brown et al., 1992; Franklin & Druhan, 2000; Hotsenpiller et al., 2002; Rademacher et al., 2007), but these results have not been entirely consistent (Mead et al., 1999; Hotsenpiller et al., 2002).

The amygdala is a likely candidate to be involved in the conditioned response because of its critical role in Pavlovian fear conditioning and other emotional learning (Maren, 2001; Cardinal et al., 2002). In addition, Fos studies indicate that it is activated during the expression of the conditioned locomotor response. Despite this, the amygdala does not appear to play a role in the induction or expression of the conditioned response as pre-training lesions do not block conditioning (Brown & Fibiger, 1993). Furthermore, Mead et al. (1999) administered the AMPA receptor antagonist NBQX before each drug-context pairing with amphetamine and found that the conditioned response was never established. Interestingly, the conditioned Fos
activation in the amygdala was still present. This dissociation between amygdala activation and the conditioned response further indicates that the amygdala is not involved in the conditioned locomotor response to amphetamine. The expression of Fos in the medial PFC, on the hand, was abolished along with the behavioral conditioned response. This indicates that glutamatergic signaling in the medial PFC plays an important role in the development of the conditioned response. Consistent with this notion, discrete cues previously paired with cocaine induce a large increase in glutamate in the NAc (Hotsenpiller et al., 2001). This indicates that NAc afferents from the medial PFC may drive the conditioned locomotor response.

Evidence for the involvement of the NAc in the expression of the conditioned response is fairly inconsistent in Fos expression studies with two reports indicating increased expression only in the core region (Franklin & Druhan, 2000; Hotsenpiller et al., 2002), one reporting increased expression in the caudal region of both the core and the shell (Rademacher et al., 2007), and two negative findings (Brown et al., 1992; Mead et al., 1999). The finding that the caudal region of the NAc may be selectively involved may help to explain this disparity. In this manner, the negative findings may have been caused by a dilution of the effect across the larger structures. Lesion studies have reinforced these findings as post-training 6-OHDA lesions of the nucleus accumbens disrupt the conditioned locomotor response to amphetamine (Gold et al., 1988). Thus, the NAc is critical for the expression of the conditioned response, however further investigation is necessary to pinpoint the specific subregions involved.

Unfortunately, the conditioned response has not been as extensively studied as other related behaviors and thus, making conclusions about the neuroanatomy involved is difficult. Currently, it appears that the NAc and medial PFC are critically involved,
however a number of other structures have been implicated and may prove to play an important role as well.

**Conditioned Place Preference**

Conditioned place preference (CPP) is a paradigm used to model drug seeking behavior in animals. In CPP, a drug is repeatedly paired with one of two separate environmental contexts while saline is paired with the other. On test day, the barrier between the two contexts is removed and the animal (off-drug) is allowed to explore either context. With psychostimulants, rodents show a strong preference for the drug-paired context indicating that the drug offers a conditioned reward. This associative process may appear similar to the conditioned response, however a number of studies have indicated that these processes are dissociable. For instance, some drug-pairing protocols induce CPP but do not produce a conditioned response (Martin-Iverson & Reimer, 1996), while intra-accumbens cocaine produces a conditioned response without CPP (Hemby et al., 1992). In addition, specific lesions can selectively disrupt CPP, while leaving the CR intact (Brown & Fibiger, 1993). Also, similar to the conditioned response, CPP appears to be dissociable from locomotor sensitization (Rowlett et al., 1994; Seymour & Wagner, 2008, unpublished data). Thus, this associative process is likely to have a distinct neural basis from each of the other behavioral processes discussed.

*Induction of Conditioned Place Preference*

Targeted microinfusions of psychostimulants have been used to assess the locus of action in the induction of CPP. There is clear involvement of the NAc in the induction of CPP to amphetamine as intra-accumbal infusions produce CPP (Carr & White, 1983, 1986; Josselyn & Beninger, 1993; Schildein et al., 1998; Liao et al., 2000). It is less
clear, however, which subregions of the NAc are involved, as there are disparate data selectively implicating both the core and the shell regions (Schildein et al., 1998; Liao et al., 2000). While both Carr & White (1983) and Josselyn & Beninger (1993) do not distinguish between the core and shell regions of the NAc, visual inspection reveals that in both experiments infusions were administered almost exclusively into the NAc core region. Nonetheless, both the core and shell regions appear to be important for amphetamine-induced CPP. Fewer studies have examined infusions of cocaine into the NAc with two reporting CPP (Aulisi & Hoebel, 1983; Liao et al., 2000) and one reporting a lack of CPP (Hemby et al., 1992). These contrasting findings may be explained by apparent differences in the location of infusions within the NAc as Liao et al. (2000) implicates a selective role for the shell region. Further examination of Hemby et al. (1992) reveals infusion sites almost exclusively in the NAc core region. This indicates that, in contrast to amphetamine, the NAc shell region may play a selective role in the induction of CPP to cocaine, though further investigation is clearly necessary. Other brain areas implicated by targeted infusions of amphetamine are the ventral pallidum (Gong et al., 1996), olfactory tubercle (Ikemoto, 2003), and the central nucleus of the amygdala (O'Dell et al., 1999). While O'Dell et al. (1999) find a selective involvement of the central amygdala only in the induction of CPP to amphetamine and not to cocaine, this is likely a result of insufficient power. The range of amphetamine doses used was much larger than cocaine leading to more power and a significant main effect only for amphetamine. Despite this, a significant post-hoc analysis indicates that cocaine likely has a similar effect and infusion into the central nucleus of the amygdala appears to be sufficient to induce CPP. Carr and White (1986), however, report that intra-amygdala amphetamine does not induce CPP. This lack of CPP may be a consequence of the diffuse pattern of infusions rather than selectively targeted subregions of the amygdala.
Several studies have confirmed the role of the amygdala in the induction of CPP, however there is disagreement on the specific subregions involved. Excitotoxic lesions of the lateral amygdala (Hiroi & White, 1991), basolateral amygdala (Fuchs et al., 2002; cf. Hiroi & White, 1991), and the entire amygdala (Brown & Fibiger, 1993) disrupt the development of CPP. In addition, reversible inactivation of the basolateral amygdala during training disrupts CPP (Hsu et al., 2002). Thus, there is a clear role of the amygdala in the induction of CPP, however the exact neural location is still unclear.

The role of the medial PFC in CPP is controversial. An early report indicated that ablation of the medial frontal cortex disrupted induction of CPP to cocaine (Isaac et al., 1989). In a series of experiments, Tzschentke and Schmidt (1998, 1999) followed up on this notion and showed that quinolinic acid lesions of the prelimbic area of the medial PFC blocked the induction of CPP by cocaine. Interestingly, lesions of the medial PFC did not affect CPP induced by amphetamine. These results, however, are at odds with Zavala et al. (2003) who report normal CPP after prelimbic lesions. The authors indicate that more salient cues, such as texture and odor, were used in addition to visual stimuli, and this may have engaged other neural systems allowing for the induction of CPP. They also claim that there were no differences in the extent of the lesions, however visual examination indicates that this may be the case. Thus, there is disagreement on the extent of the involvement of the medial PFC in the induction of CPP to cocaine.

Two studies have examined the role of the NAc through 6-OHDA lesions. These studies used lesions of either the core or shell region, and correlated the residual dopamine in each region to CPP induction and expression. They found that dopamine lesions of the shell region, but not the core, abolished the induction of CPP induced by both i.v. cocaine and i.p. amphetamine (Sellings & Clarke, 2003; Sellings et al., 2006). In both of these cases they also found a significant positive correlation of DAT binding in
the shell with CPP. This effect was not found for i.p. cocaine, however it does trend in the same direction. Taken together, these data indicate that dopamine in the NAc shell plays a major role in the induction of CPP to psychostimulants.

The hippocampus is another area implicated in CPP. Excitotoxic lesions of the dorsal, but not the ventral, hippocampus disrupt the development of CPP to cocaine (Meyers et al., 2003). Thus, the dorsal hippocampus is a critical structure in the induction of CPP.

Together these studies indicate that the NAc shell, amygdala, and dorsal hippocampus are critical for the induction of CPP. The NAc core, medial PFC, ventral pallidum, and olfactory tubercle are also implicated but further exploration is needed.

Expression of Conditioned Place Preference

Similar to induction, the expression of CPP is highly correlated with DAT binding in the shell region of the NAc (Sellings & Clarke, 2003). Also, post-training 6-OHDA lesions of the NAc shell disrupt the expression of CPP (Sellings & Clarke, 2003). Thus, dopamine in the NAc shell plays a critical role in the expression of CPP to psychostimulants.

A number of IEG experiments have also explored the neural basis of CPP. During expression of CPP there is increased activity in the hippocampus, amygdala, NAc, ventral pallidum, medial PFC, and lateral hypothalamic orexin neurons (Miller & Marshall, 2004; Rademacher et al., 2006; Aston-Jones et al., 2009; Chiang et al., 2009). In addition, there is a particularly high correlation between CPP and Fos immunoreactivity in the basolateral amygdala (Rademacher et al., 2006; cf. Chiang et al., 2008). The enhanced neural response in the medial PFC has been further studied using double labeling of Fos and GAD67, a marker of inhibitory interneurons. Miller and Marshall (2004) report increased overlap of Fos and GAD67 in the prelimbic region of the
medial PFC after the expression of CPP compared to an unpaired group, indicating that the enhanced response in this area is due to increased inhibition. Furthermore, using a retrograde tracer colocalized with Fos activity, Miller and Marshall (2005) report that the increased activation of the basolateral amygdala is due to efferent connections with the prelimbic cortex and NAc core. In contrast, the increased activation of the prelimbic cortex does not increase its efferent connections. Thus, these experiments indicate that excitatory drive during CPP is driven by increased projections from the basolateral amygdala to the NAc rather than from the prelimbic cortex.

Post-training lesions of the amygdala, however, have yielded incongruent results. Hiroi & White (1991) found the lateral amygdala to be critical for the expression of CPP, while Hsu et al. (2002) found that inactivation of the basolateral amygdala abolished CPP. Further complicating the matter, Fuchs et al. (2002) reported no deficit in CPP after post-training excitotoxic lesions of the basolateral amygdala. These data are not easily reconciled, but they may be due to procedural differences producing differential engagement of various learning processes. Alternatively, these lesions could have produced extra-structural or intra-structural damage that could account for the differences (Fuchs et al., 2002). Another possibility is that differences between cocaine and amphetamine could account for the lack of agreement since the only study showing no role of the amygdala (Fuchs et al., 2002) uses cocaine, while the others use amphetamine. Nonetheless, the amygdala appears to play an important role in the expression of CPP, however more research is necessary to elucidate the mechanisms involved.

There appears to be no lesion studies examining the role of the PFC or hippocampus in the expression of CPP to either cocaine or amphetamine. This is surprising given the amount of attention it has gathered in other studies using IEG
expression and in other drug-related behaviors. Nonetheless, the NAc shell and amygdala are involved in the expression of CPP while the contributions of the medial PFC, hippocampus, ventral pallidum, and lateral hypothalamus need to be further studied.

Conclusions

Table 1 summarizes the neuroanatomical structures involved in both the induction and expression of each behavior discussed. The induction of sensitization appears to rely on the NAc, VTA, medial PFC, amygdala, and hippocampus. On the other hand, the expression of sensitization relies on the NAc core, dorsal hippocampus, and perhaps the medial PFC and dorsal striatum. This divergent profile further indicates that neural changes occur during the induction of sensitization rather than simple associative processes that would likely lead to a similar neuroanatomical profile between induction and expression.

Context specific environmental control over sensitization has not been thoroughly explored, but the NAc, medial PFC, amygdala, hippocampus, and dorsal striatum have all been implicated in both its induction and expression. Clearly, more research is needed to further explore this associative memory.

The Pavlovian conditioned response appears to be mediated by the NAc core and the medial PFC in both its induction and expression. In addition, the lateral septum, claustrum, and PVN of the thalamus may be involved, but the evidence for this is not particularly compelling. This neuroanatomical profile is quite similar to the expression of sensitization, which is not surprising as both processes have a similar behavioral output. Also, this is somewhat expected as the conditioned response has been hypothesized to minimally contribute to the expression of sensitization. Thus, while it is tempting to use
this notion to argue that the conditioned response may underlie the expression of sensitization, the dramatically different profile of induction disputes this argument. Indeed, as one would expect with an associative process, there is high overlap between the induction and expression of the conditioned response, yet this overlap is not seen with sensitization.

A number of controversies plague a thorough understanding of the neuroanatomy involved in conditioned place preference. The induction of CPP is clearly dependent on the NAc shell, dorsal hippocampus, and amygdala, however the NAc core, medial PFC, ventral pallidum, and olfactory tubercle have all been implicated as well. The expression of CPP is quite similar as the NAc shell and amygdala are clearly required, yet the medial PFC, hippocampus, ventral pallidum, and lateral hypothalamus may also be involved. As with the conditioned response, this high overlap between induction and expression is indicative of an associative process.

Figures 2 and 3 visually represent the induction and expression, respectively, of these four behavioral memories. Interestingly, while there is some overlap between the structures involved in each of these behavioral processes, none of them appear to be completely mediated by the similar structures. This indicates that these processes are distinct and dissociable, a concept that fits well with the behavioral data (Hemby et al., 1992; Rowlett et al., 1994; Anagnostaras & Robinson, 1996; Anagnostaras et al., 2002b; Seymour & Wagner, 2008). Furthermore, psychostimulants appear to produce lasting neural and behavioral changes throughout the motive circuit. This implies that any treatment aimed at drug addiction cannot simply target one brain area, but a more diverse treatment pattern will be needed. Certainly, a greater understanding of how addiction is formed and maintained is crucial for eventually treating this condition.
Future Directions

While these behaviors elicited by psychostimulants have been well studied, considerable more work needs to be done to further elucidate these circuits. While IEG studies are useful in identifying a large number of candidate regions involved in a certain behavior, they generally cannot determine causal relationships. Lesion studies, on the other hand, can determine these causal relationships, but they must be targeted to the correct region and are generally slow in producing results. Thus, novel techniques should be employed in order to nail down the circuitry involved in these behaviors.

Recent advances in molecular genetics have produced exciting new ways to examine neural circuitry in rodents. Reijmers et al. (2007) used a transgenic mouse engineered to provide multiple markers of neuronal activity at different time points and found that coactivation of neurons during both training and testing correlated with behavioral memory. This exciting finding demonstrated a stable neural correlate of memory intrinsic to the activation of individual neurons. In this manner, coactivation between training and testing can be used as a neural marker of memory. This technique could prove extremely useful in the study of behaviors elicited by psychostimulants and will likely lead to a more precise understanding of the neuroanatomical basis of these memories.
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This chapter, in part, is currently being prepared for submission for publication of the material. Shuman, T. & Anagnostaras, S.G. The dissertation author was the primary investigator and author of this material.
Table 3-1. Areas involved in the induction and expression, respectively, of behavioral sensitization, context specificity of sensitization, conditioned response, and conditioned place preference. “Yes” indicates strong evidence that this area is required for induction or expression. “Yes?” indicates that there is some evidence for this area’s involvement, but it has not been directly manipulated or there is conflicting evidence. “No” indicates that this area is not necessary for its respective behavior. “-” indicates that no data is available.

<table>
<thead>
<tr>
<th>Region</th>
<th>Sensitization</th>
<th>Context Specificity</th>
<th>Conditioned Response</th>
<th>CPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAc core</td>
<td>Yes</td>
<td>Yes?</td>
<td>Yes</td>
<td>Yes?</td>
</tr>
<tr>
<td>NAc shell</td>
<td>Yes</td>
<td>Yes?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>VTA</td>
<td>Yes (AMPH only?)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>mPFC</td>
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Figure 3-1. Simplified diagram of the major projections involved in behaviors elicited by psychostimulants. AMYG, amygdala; DHip, dorsal hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcS, nucleus accumbens shell; PVN, periventricular nucleus; VP, ventral pallidum; VTA, ventral tegmental area. The major neurotransmitter system for each projection is also shown. DA, dopamine; GABA, gamma-aminobutyric acid; Glu, glutamate.
Figure 3-2. Induction. Areas of the motive circuit implicated in the *induction* of each of the four behaviors. Red circles indicate areas involved in behavioral sensitization. Green circles indicate areas involved in context specificity. Brown circles indicate areas involved in the conditioned response. Blue circles indicate areas involved in conditioned place preference. Solid circles represent strong evidence that this area is required for induction. Dashed circles indicate that there is some evidence for this areas involvement, but it has not been directly manipulated or there is conflicting evidence. Sens, sensitization; Cont Spec, context specificity; Cond Resp, conditioned response; CPP, conditioned place preference.
Figure 3-3: Expression. Areas of the motive circuit implicated in the expression of each of the four behaviors. Red circles indicate areas involved in behavioral sensitization. Green circles indicate areas involved in context specificity. Brown circles indicate areas involved in the conditioned response. Blue circles indicate areas involved in conditioned place preference. Solid circles represent strong evidence that this area is required for expression. Dashed circles indicate that there is some evidence for this areas involvement, but it has not been directly manipulated or there is conflicting evidence. Sens, sensitization; Cont Spec, context specificity; Cond Resp, conditioned response; CPP, conditioned place preference.
CHAPTER 4

Localizing the stable neural correlate of conditioned place preference to cocaine

By

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Abstract

Cocaine administration induces many changes in the brain that are likely to underlie addiction. The predominant model of drug-seeking behavior in mice is conditioned place preference (CPP), in which an animal will choose to spend its time in a chamber that has previously been paired with cocaine. We used a transgenic mouse that allows for labeling cells that are active at both the initial drug exposure as well as during testing at a later time. This TetTag Histone-GFP mouse line contains a doxycycline-dependent, cfos driven GFP bound to histone proteins to act as a long-term tag of neural activity. We used this transgenic mouse to mark neurons that were active during drug exposure and compared this tag to neurons that were active during a CPP test using the immediate early gene zif268. In animals that were trained with cocaine, we found that cells in the dorsal striatum were more likely to be active during both training and testing when compared to control mice that received cocaine only in their home cage. This reactivation of a subpopulation of cells during the off-drug test is theorized to be driving memory recall and is likely to underlie the behavioral choice to spend more time in the drug-paired chamber. These results implicate the dorsal striatum as an important region where drug-induced neural plasticity leads to drug seeking behavior.

Keywords: conditioned place preference, memory, cocaine, dorsal striatum
Drug addiction is defined by a pattern of behavior characterized by compulsive drug use, compulsive drug seeking, and a high tendency to relapse (see Chapter 3). Addictive drugs, like the psychostimulant cocaine, engage a large number of brain areas and induce long-lasting synaptic changes that presumably underlie the compulsive pattern of behavior seen in addicts. It is unclear, however, where in the brain this plasticity occurs in order to drive addicted behavior.

A number of rodent behaviors have been proposed to model individual aspects of drug addiction. Drug use is often modeled with self-administration and locomotor sensitization; drug seeking is often modeled with conditioned place preference and cue-elicited self-administration; relapse is generally modeled with conditioned locomotor response and the reinstatement of self-administration (Chapter 3). One commonality between these models is that they create a memory for the drug experience that is recalled during testing. In this manner, each of these behaviors model an addiction-related memory that is likely to contribute to the pattern of behavior seen in drug addicts.

The behavioral changes induced by these addiction-related memories are generally dissociable (Chapter 3), indicating that addictive drugs cause a distributed pattern of synaptic changes, rather than one primary change, that leads to addicted behavior (Koob & Volkow, 2010). It is useful, then, to isolate each change in behavior to investigate the neural plasticity necessary to drive each specific addicted-like behavior. This chapter will focus on identifying the neural circuitry involved in cocaine-seeking behavior using conditioned place preference to cocaine. In conditioned place preference, one side of a chamber is paired with cocaine, while the other side of the chamber is paired with saline. During testing, animals are off-drug and when allowed to explore both sides of the chamber they spend more time in the cocaine-paired side.
Thus, an association between the contextual cues and the drug experience appears to drive the behavioral response to prefer the cocaine-paired side of the chamber.

A number of candidate regions are likely to be involved in conditioned place preference to cocaine. The amygdala is a strong candidate region because it has been shown to store associations responsible for emotional memories in both the lateral and basolateral regions (Reijmers, Perkins, Matsuo, & Mayford, 2007). The hippocampus is also highly likely to be involved because the task involves recalling contextual cues, which are believed to be stored in the hippocampus (Anagnostaras, Gale, & Fanselow, 2001). The medial prefrontal cortex is implicated because it is involved in executive function and is dysregulated in addicts (Goldstein & Volkow, 2002). The nucleus accumbens is involved in both the acute response to cocaine as well as reward learning (Ikemoto & Panksepp, 1999) which makes it likely to be involved in the CPP memory. Finally, the dorsal striatum can integrate emotional information with goal-oriented behavior (Balleine, Delgado, & Hikosaka, 2007; O’Doherty et al., 2004), which is likely to be involved in conditioned place preference. There is also considerable evidence for the involvement of the dorsal striatum in cue-elicited drug seeking behavior in self-administering rats, which is likely to have overlapping circuitry to conditioned place preference (Vanderschuren, Di Ciano, & Everitt, 2005; Vanderschuren et al., 2002).

The neural circuitry of conditioned place preference has been heavily investigated using classical neuroanatomical techniques. Lesion studies, immediate early gene expression, and targeted microinfusions have implicated the lateral amygdala and nucleus accumbens shell in psychostimulant-induced place preference (see Chapter 3). These classical neuroanatomical techniques, however, have produced many conflicting results likely because lesion studies and targeted microinfusions are often confounded by variability in the location and diffusion of the manipulation. Furthermore,
these techniques are unable to determine how the brain might adapt to these manipulations and utilize an alternative neural representation. One example of this from the memory literature is that animals can learn contextual information after hippocampal lesions by engaging an alternate circuitry that is not used in normal context learning (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Liu et al., 2012; Maren, Aharonov, & Fanselow, 1997). These adaptations can occlude effects produced by lesions and microinfusions. Similarly, immediate early gene expression studies are difficult to interpret because only one time point is presented. An increase in expression is generally considered to indicate involvement of the brain area, but it is entirely unclear what role these neurons are playing in the memory process. In fact, an emerging literature indicates that memories increase synaptic connections only at specific synapses and may reduce unrelated activity in order to increase signal-to-noise ratio (Fu, Yu, Lu, & Zuo, 2012; Lai, Franke, & Gan, 2012; Okuno et al., 2012). This neural refinement is difficult to observe using classical immediate early gene labeling.

By conceptualizing conditioned place preference as an associative memory process, we can utilize novel techniques developed to study other forms of associative memory and begin to understand how the memory of conditioned place preference is coded. The coding of associative memories is presumed to occur in the strengthening of connections between a subpopulation of neurons. This network of neurons can then be reactivated in response to sensory cues to recall that memory. By this logic, a memory is stored in the subset of neurons that are activated during both initial encoding of memory and during recall.

Evidence for this theory was first provided using fluorescent in-situ hybridization to label neurons active during two distinct context exposures (Guzowski, McNaughton, Barnes, & Worley, 1999). When animals were exposed to two different contexts, two
distinct subsets of neurons in the hippocampus were activated. When animals were exposed to the same context twice, the same subset of neurons in the hippocampus was activated. Thus, contextual cues elicited a stable representation of the environment that could be activated again at a later time point. More recently, Reijmers et al. (2007) created a transgenic TetTag mouse with a long-term tag of neurons active during learning, and compared this tag to neurons active during retrieval using the immediate early gene zif268. They trained animals using Pavlovian fear conditioning and compared the activity of individual neurons during training and testing of contextual and tone fear conditioning. For contextual fear memories, they found a significant increase in overlapping neurons in trained versus untrained animals, but only in the basolateral amygdala, the region hypothesized to underlie context-shock associations (LeDoux, 2000). Similarly, for tone fear memories, they found a significant increase in overlapping neurons only in the lateral amygdala, the region hypothesized to underlie tone-shock associations (LeDoux, 2000). Thus, increased overlap between neurons that were activated at training and test was observed only in the brain region where CS-US connections were stored. This established neuronal overlap between training and testing as a hallmark of the storage site of Pavlovian fear memories. Moreover, this hallmark of memory storage is likely to be consistent across other types of associative memories.

Here we used a modified TetTag mouse to localize the neural correlate of conditioned place preference to cocaine. This novel histone-GFP transgenic mouse tags neurons active during a doxycycline-dependent tagging window to mark only neurons that are active during initial drug exposure. We then compared these neurons to those active during testing using the immediate early gene zif268 (ZIF). During training, animals received cocaine either paired with one side of a chamber (i.e., Paired
group) or in their home cage (i.e., Unpaired group). During test, they were off drug and allowed to freely explore the drug-paired side and the saline-paired side. We identified nine regions of interest that are theorized to be involved in conditioned place preference to cocaine including the amygdala, hippocampus, medial prefrontal cortex, nucleus accumbens, and dorsal striatum (see Chapter 3). We hypothesized that a region underlying conditioned place preference will show a higher overlap between cells active at training and testing when compared to control animals. Indeed, we found that the dorsal striatum induced a higher overlap in cells active during both training and testing in Paired versus Unpaired animals. That is, the pairing of the drug with the training context triggered a subpopulation of cells to be activated during both initial drug exposure and CPP testing. Furthermore, since the only difference in training was in the association between drug and context, this subpopulation of cells is likely to store this association and underlie the neural representation responsible for conditioned place preference behavior.

**Methods and Materials**

**Subjects**

Histone-GFP (hiGFP) mice have been previously described (Tayler et al., 2011) and expressed two hemizygous transgenes (Fig 1A). The first transgene contained a c-fos promoter driving the expression of a tetracycline transactivator protein (cfos-tTA). A second, independent transgene contained a tetO promoter driving the expression of a GFP-tagged histone protein (tetO-HIST1H2BJ/GFP). HiGFP animals were maintained on a C57BL/6J background. Approximately equivalent numbers of male and female hiGFP mice were raised and maintained on a diet enriched with doxycycline (40 mg/kg). In the presence of doxycycline, production of tTA does not drive the tetO promoter to
produce hiGFP proteins (Fig 1A, right). When doxycycline is removed from the diet, neural activity drives the cfos promoter to produce tTA, which activates the tetO promoter to produce hiGFP proteins (Fig 1A, left). These hiGFP proteins are a permanent marker of active cells. Mice were housed 2-5 per cage, given free access to food and water, and maintained on a 14:10 LD cycle. All experimental activities were conducted during the light phase and animals were at least 90 days old prior to the start of the experiment. Animal care and experimental procedures were approved by the UCSD IACUC, in accordance with the NRC Guide for the Care and Use of Laboratory Animals.

Drugs

All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg. Cocaine HCl (Sigma-Aldrich) was dissolved in 0.9% saline.

Conditioning Apparatus

Chambers were as described in Chapter 2, Experiment 2. Briefly, 2-3 mice were tested concurrently, in individual chambers housed in a windowless room. Chambers (44-cm wide, 44-cm high, 31-cm deep, Med Associates, St. Albans, VT) consisted of two distinct sides separated by a wall with a removable hole. The two sides differed in smell, texture, and visual stimuli. Each side was randomly designated the Saline or Drug side (counterbalanced by group). During training, the hole in the dividing wall was closed which allowed access to only one side at a time. During testing, the hole in the dividing wall was open which allowed free access to either side of the chamber. Animals were tracked using infrared beams and Activity Monitor software (Med Associates). Light was provided from two 150-watt bulbs distant from the chambers and background noise (65-dBA) was provided by HEPA air cleaners and an iPod speaker playing white noise.

Procedure
Figure 1B outlines the behavioral protocol. All animals were raised and maintained on a doxycycline-enriched diet to prevent activation of the hiGFP transgene. Subjects were gently handled for three minutes per day over a period of 9 days to acclimate the animals to experimenter interaction. On the first three days, animals were handled in the vivarium. On the next three days, animals were carted to the conditioning room to be handled. On the final three days, animals were carted to the conditioning room, handled, and then received an injection of saline to habituate the animals to the injection procedure. One day after completing the handling phase, animals were given a habituation place preference test. During the habituation phase, the door between the two chambers was open. Animals were placed in the middle of the chamber and allowed to freely explore both sides for 30 minutes. Animals were immediately returned to their home cage.

Three days after habituation, animals were switched to a regular diet without doxycycline. This 3-day gap between habituation and removing doxycycline is essential to clear the animal’s system of unwanted tTA protein that can take up to 3 days to diminish. In the absence of doxycycline, active neurons are tagged with the hiGFP protein. Thus, removing doxycycline from the diet opens the hiGFP tagging window.

Three days after removing doxycycline from the diet, subjects were trained on a conditioned place preference task. During the training phase, the door between the two chambers was closed and subjects were confined to one side or the other. Two groups were trained: a Paired group received cocaine in the Drug side of the chamber while an Unpaired group received cocaine in the home cage. It is important to note that both groups received the same context and drug exposure. The only difference between the groups was whether the drug was paired with the conditioning context (Paired group), or with the home cage (Unpaired group).
All subjects first received an injection of saline and were placed into the Saline side of the chamber for 30 minutes. Subjects immediately received an injection of cocaine (Paired group) or saline (Unpaired group) and were placed into the Drug side of the chamber. After 30 minutes, animals were given a final injection of either cocaine (Unpaired group) or saline (Paired group) and returned to their home cage. Immediately after training animals were placed on a high doxycycline diet (1 g/kg) for the duration of the experiment. This high doxycycline diet closes the hiGFP tagging window within 5 hours after training. Thus, only neurons active during training were tagged with the hiGFP label.

One week after training, animals received a place preference test. During the test phase, the door between the two chambers was open allowing free access to both sides. Animals were placed in the middle of the chamber and allowed to explore both sides for 30 minutes. One hour after testing, animals were sacrificed and perfused to allow for immunohistochemistry of ZIF activation.

**Immunohistochemistry**

Brains were perfused with 4% paraformaldehyde (PFA), removed from the animal and post-fixed in 4% PFA overnight. Twenty-four hours later, brains were switched to phosphate buffered saline (PBS). Within one day, brains were sliced into 50 µm sections using a Leica vibratome and incubated overnight in blocking buffer (10% normal goat serum, 0.5% TritonX-100 in PBS) at 4°C. Sections were then incubated overnight in blocking buffer with primary antibody (ZIF rabbit antibody – Cell Signaling) diluted 1:750 at 4°C. Sections were then washed in PBS followed by a 2-hour incubation in blocking buffer with secondary antibody (Cy3 conjugated goat-anti-rabbit) diluted 1:500 at room temperature. Slices were washed in PBS and then immersed in 10x Tris Buffered Saline with DAPI, a nuclear DNA stain, diluted 1:500 at room
temperature. Slices were washed in PBS and mounted onto slides using ProLong Gold antifade reagent and sealed with a coverslip.

**Analysis of immunohistochemistry data**

Images were taken using a Nikon C2 confocal microscope with a 20x air objective. Between 4 and 12 images of individual brain regions for each subject were averaged. The following coordinates in respect to bregma were imaged: lateral amygdala (-1.58, -1.82, -2.06), basolateral amygdala (-1.58, -1.82, -2.06), dentate gyrus (-1.58, -1.82, -2.06), CA3 (-1.58, -1.82, -2.06), CA1 (-1.58, -1.82, -2.06), medial prefrontal (infralimbic) cortex (+1.94, +1.70), nucleus accumbens core and shell (+1.42, +1.18), dorsal striatum (+0.98, +0.74). Lateral amygdala and basolateral amygdala images contained a flattened stack of 6-8 images 1.15µm apart. A representative image from the Paired group in the dorsal striatum is presented in Figure 1C.

All cell counts were done using ImageJ software by a blind scorer. The nuclear staining of these markers allowed for automated cell counting of DAPI and ZIF-DAPI overlap. Counting of GFP-DAPI overlap and GFP-ZIF overlap was done by hand.

**Statistics**

All analysis was conducted using only signals that overlapped with DAPI. Chance level of overlap was calculated as: (number of GFP+ / total DAPI) * (number of ZIF+ / total DAPI) * 100%. Normalized overlap was calculated as: (number of overlapping neurons / total DAPI) / (Chance Overlap).

All statistics presented are t-tests for independent samples. Because each comparison is testing a separate hypothesis (i.e., each brain regions involvement in the memory), alpha correction is not necessary (O'Keefe, 2003; Weber, 2007). Thus, while repeated t-tests will lead to an escalated overall chance of finding a significant result, this is not true for each individual brain area. Furthermore, for each brain area we
looked for a clear pattern of significant findings that were consistent with a memory storage hypothesis.

**Results**

*Habituation CPP Pre-test*

Prior to training, animals were given a CPP pre-test to habituate the animals to the testing environment and to confirm that there was no underlying bias for either side of the chamber. Data are shown as drug-side preference (Fig 2A; proportion of time spent in the drug-paired side). A result of 0.5 represents no bias toward either side, with higher numbers indicating a bias toward the drug side. Both groups showed no bias for either side (one-sample t-test versus 0.5, Paired: \( p=0.66 \), Unpaired: \( p=0.97 \)) and did not differ from each other (t[21]=0.381, \( p=0.707 \)).

*CPP Test*

After training, animals were given a CPP test to determine if they preferred the drug-paired side of the chamber. Data are shown as drug-side preference (Fig 2B). The Paired group spent more time in the drug-paired side than the Unpaired group (t[21]=2.292, \( p=0.032 \)). The Paired group showed a preference for the drug-paired side (t[11]=2.791, \( p=0.018 \)) while the Unpaired group showed no preference (t[10]=0.557, \( p=0.59 \)).

*GFP Expression – Cells activated by training*

GFP expression was used as a marker of cells active during training. Figure 3 shows the percent of total cells (DAPI+) that were activated during training (GFP+). There was a large variance in expression between brain regions, but there were no differences between the Paired and Unpaired groups in any of the nine brain regions examined (t-values\( \leq 1.730 \), \( p \geq 0.10 \)).
ZIF Expression – Cells activated by testing

ZIF expression was used as a marker of cells active during testing. Figure 4 shows the percent of total cells (DAPI+) that were activated during testing (ZIF+) across the nine brain regions of interest. In the medial prefrontal cortex (mPFC), the Paired group had significantly less ZIF expression than the Unpaired group ($t[20]=-2.245$, $p=0.036$). In the dorsal striatum, there was a trend for less ZIF expression in the Paired group than in the Unpaired group ($t[21]=-1.930$, $p=0.067$). In all other brain regions examined, there were no differences between the Paired and Unpaired groups ($t$-values $\leq 1.482$, $p \geq 0.15$).

GFP-ZIF Overlap

We compared the overlap between GFP and ZIF to assess the number of cells active during both training and testing. Figure 5 shows the number of overlapping cells as a percent of total cells (DAPI) within each brain region. In the dorsal striatum, the Paired group had more overlapping cells than the Unpaired group ($t[21]=2.752$, $p=0.012$). In all other brain regions examined, there were no differences between the Paired and Unpaired groups ($t$-values $\leq 1.622$, $p \geq 0.12$).

Given the levels of expression of GFP and ZIF, we calculated the percent of cells that would overlap by chance (i.e., random arrangement of active cells within all cells – see Methods). We then normalized overlap in each brain region by dividing the overlap by chance. Thus, for normalized overlap, a score of 1 indicates that the overlap was at chance, consistent with a random assignment of active neurons during testing. A number above 1 indicates that cells active during training were more likely than other cells to be active during testing. A number below 1 indicates that cells active during training were less likely than other cells to be active during testing.
Figure 6 shows the normalized overlap for each brain region examined. In the dorsal striatum, the Paired group showed significantly higher normalized overlap than the Unpaired group ($t[21]=2.910, p=0.008$). No other brain regions showed differences between the two groups ($t$-values≤1.318, $p$≥0.20). A number of regions did, however, have higher than chance overlap evidenced by normalized overlap above chance. The basolateral amygdala, CA3, and CA1 all had normalized overlap significantly above chance in both the Paired and Unpaired groups (one-sample t-tests against hypothesized value of 1; $t$-values≥2.665, $p$<0.05). Thus, the cells active during training were more likely than other cells to be active during testing in these regions. Conversely, the nucleus accumbens core and shell had normalized overlap significantly below chance in both the Paired and Unpaired groups ($t$-values≤-2.265, $p$<0.05). Hence, cells active during training were less likely than other cells to be active during testing in the nucleus accumbens. Likewise, in the dorsal striatum, the Unpaired group had normalized overlap significantly below chance ($t[10]=-6.673, p<0.001$). The Paired group, on the other hand, was not different from chance ($t[11]=-1.452, p=0.174$) and was significantly higher than the Unpaired group ($t[21]=2.903, p=0.008$). Together, these data demonstrate that the cells active during training in the BLA, CA3, and CA1 were more likely to be reactivated during testing. Cells active during training in the nucleus accumbens were less likely to be active during testing. Finally, cells active during training in the dorsal striatum were less likely to be active during testing, but only in the Unpaired group. That is, the pairing of the drug with the context during training was able to overcome the bias away from activating the cells that were active during training. 

Finally, we further examined the dorsal striatum in the context of how the place preference memory trace may be coded. In the dorsal striatum, the Paired group had slightly more neurons active during training (Fig 7A), which could have driven the
increase in overlap. Thus, we examined overlap as a percent of GFP+ cells. Despite having marginally less ZIF expression (Fig 7B), the Paired group had significantly more overlap as a percent of GFP+ cells than the Unpaired group (Fig 7D; t[21]=2.303, p=0.032). Thus, even though there was less overall activation in the Paired group during testing, there was a still higher reactivation of the cells that were active during training.

Discussion

We investigated the neural coding of conditioned place preference by comparing the activation of individual cells during training and testing. Cells in the dorsal striatum were more likely to be reactivated during testing when the drug was paired with the training context than when paired with the home cage. That is, a subpopulation of cells, that were active during training, was again active during testing, but only in the Paired animals. Critically, both groups received the same context and drug exposure, with the only difference being the association between the drug and context. Therefore, it is likely that this association between the drug and the context is the only experience that could have caused these changes in the neural response of the dorsal striatum. Furthermore, an increase in overlap has been shown to be a hallmark of the storage site of associative memories (Guzowski et al., 1999; Reijmers et al., 2007). Presumably, recalling a memory involves reactivating a subpopulation of cells that were active during training, eliciting a representation of the training event. In the current study, during conditioned place preference, a subpopulation of cells in the dorsal striatum that were active during training was again activated during test. This increase in overlap between training and testing indicates that the dorsal striatum is likely to be one area underlying conditioned place preference to cocaine.
While the dorsal striatum has not previously been implicated in conditioned place preference (Tzschentke, 1998, 2007; Chapter 3), there is significant evidence that the dorsal striatum is involved in cue-elicited drug seeking behavior. These two tasks are very different but are both used to model drug seeking behavior in mice. Cue-elicited drug seeking involves gradually training an animal to self-administer cocaine, and pairing a discrete cue with the presence of drug availability. Using a second-order conditioning paradigm, this task can probe drug seeking behavior in an off-drug state (Everitt & Robbins, 2000), similar to conditioned place preference. A significant series of experiments by Everitt and colleagues have implicated the dorsal striatum in this cue-elicited drug seeking. Indeed, infusions of a dopamine or AMPA/kainate antagonist into the dorsal striatum significantly decreased drug seeking behavior (Belin & Everitt, 2008; Vanderschuren et al., 2005). Furthermore, the connection between the nucleus accumbens and dorsal striatum (likely through the substantia nigra) is critical for this drug-seeking behavior (Belin & Everitt, 2008). In line with these findings, the drug-associated cue induces release of extracellular dopamine in the dorsal striatum, but only when the cue is contingent on responding (Ito, Dalley, Robbins, & Everitt, 2002). Taken together, these studies indicate that the dorsal striatum is responsible for integrating sensory cues with drug-seeking actions. It is not surprising, then, that the dorsal striatum would be involved in conditioned place preference given that in this task the animal must integrate contextual cues to decide which side of the chamber to explore. Thus, our findings are consistent with a similar mechanism driving drug seeking behavior in both conditioned place preference and cue-elicited drug seeking, despite obvious differences in protocols. This is surprising given the training involved in each of these tasks. Cue-elicited drug seeking requires many hours of action-contingent drug exposure while conditioned place preference requires just one passive exposure to the drug. The notion
that these two behaviors rely on the same underlying mechanism is particularly compelling because it indicates that blocking this mechanism could disrupt all drug-seeking behavior in rodents. Furthermore, it implies that future treatments in humans could be targeted at a singular mechanism despite the large differences in drug experiences seen in addicts.

We found no differences in GFP expression between our Paired and Unpaired groups. This was not surprising, as both groups received equivalent exposure to the context and the drug. We did, however, observe differences in ZIF expression after testing. When cocaine was paired with the training context, animals had reduced ZIF expression in the mPFC and marginally reduced ZIF in the dorsal striatum (Fig 4). The mPFC is implicated in the loss of executive function seen in addicts (Goldstein & Volkow, 2002) and thus it was not surprising to see reduced activity in this region. The decrease in cells active during testing did not, however, coincide with any changes in overlap or normalized overlap with GFP (Figs 5, 6). This indicates that while there was reduced activity in the Paired animals during testing, this activity was reduced randomly and not in any coordinated fashion. This distributed reduction in activity could play a role in reduced inhibition of other brain regions but the lack of coordinated activity indicates that is unlikely to be able to activate a specific memory trace. Thus, while the mPFC may be involved in the conditioned place preference to cocaine, it is unlikely to be able to directly activate the memory trace and drive behavior. We also observed a marginal reduction in ZIF expression in the dorsal striatum in the Paired group. This reduction, however, coincided with an increase in the overlap between GFP and ZIF (Fig 7). Thus, cells in the Paired group that were activated during training were more likely to be activated again during testing, compared to the Unpaired group. At the same time, other cells that were not activated during training were less likely to be activated during testing. This
pattern of activity is consistent with increased activation of cells involved in the
association between drug and context, and a down-regulation of cells unrelated to this
association. Both of these processes increase the signal-to-noise ratio between a small
population of neurons that are presumably encoding this association between the drug
and context. We hypothesize, then, that when presented with the appropriate context
and an action-oriented decision, the dorsal striatum is able to integrate these processes
by reactivating a subpopulation of neurons active during training and reducing the
activity of other unrelated cells.

To account for differences in expression levels between brain areas, we
compared the observed overlap to the amount of chance overlap, given random
activation of all cells. Consistent with previous studies implicating the amygdala and
hippocampus in conditioned place preference, we found higher than chance overlap in
the BLA, CA3, and CA1 regions. In these brain regions a small subpopulation of cells
active during training were again activated during testing. We did not, however, observe
differences between animals that received cocaine paired with the training context or
with the home cage. This indicates that in these regions a neural representation was
encoded during training and then retrieved during testing, but was unrelated to the drug-
context association. Each of these brain areas is implicated in associative context
conditioning (Anagnostaras et al., 2001; Reijmers et al., 2007), and are likely to be
encoding the context or other associations (e.g., associating an injection with the
context). Thus, since both groups of mice were given equivalent exposure to the context
during both training and testing, this data indicates that the stable representation of the
context (or other associations) is likely stored in these brain areas. Conversely, we
found lower than chance overlap in the nucleus accumbens. That is, cells in the NAc
that were activated during training were less likely than other cells to be activated again
during testing. This bias away from the trained cells indicates that the nucleus accumbens responds differentially to distinct brain states. This is not surprising given that the nucleus accumbens is involved in the acute drug response. Thus, during training, animals received cocaine and activated a distinct “drug” population of cells. When tested off-drug, these “drug” cells were not activated, leading to a bias away from the trained cells, and a below-chance level of overlap (Fig 6). These results predict that if animals received drug during both training and testing, then overlap in this area would increase to at or above chance. Indeed, when animals are on-drug during both training and testing using a sensitization paradigm, overlap between training and testing is at or above chance in this region (Chapter 5).

In the dorsal striatum, similar to the nucleus accumbens, the Unpaired group had overlap below chance presumably caused by the distinct brain states during training and testing. The Paired group, however, was not below chance. This indicates that the association between drug and context was sufficient to activate a subpopulation of cells in the dorsal striatum responding to the association between drug and context. By comparing animals with equivalent exposure to the context and drug, we isolated the association between these stimuli that is reactivated during training. In this manner, we identified the dorsal striatum as the only brain region that we examined that codes for the association between drug and context that can drive conditioned place preference behavior.

One possible problem interpreting these findings is that it seems unlikely that a memory would be coded by an overlap between training and testing that is at or even slightly below chance activation. This interpretation, however, ignores the fact that there is a bias away from trained cells in the Unpaired group. That is, the dorsal striatum is responding differentially to distinct brain states between training (i.e., on-drug) and
testing (i.e., off-drug). Despite this, in the Paired group, a subpopulation of cells that were active during training were again active during testing. For a memory to be retrieved, a subpopulation of cells needs to be reactivated. For most brain regions, baseline reactivation is at chance, and therefore, by reactivating a subpopulation of neurons active during training, this will drive the overlap to be above chance. In the dorsal striatum, however, the baseline reactivation is far below chance. Thus, by activating a subpopulation of neurons that were active during training, this drives the overlap to only be at chance. The question, then, is whether a small subpopulation of neurons, can drive a behavior. A number of studies looking at reactivation of neurons have found the coding of memories to be very sparse, with the proportion of overlapping cells very low (Han et al., 2009; Koya et al., 2009; Liu et al., 2012; Reijmers et al., 2007; Zhou et al., 2009). Thus, despite below chance overlap, the reactivation of a small neural population in the dorsal striatum can likely drive the stable representation of conditioned place preference to cocaine.

These results predict a number of testable hypotheses implicating the dorsal striatum as the site of storage for conditioned place preference behavior. First, there must be some underlying change in neural connections that underlies the increase in overlap between training and testing. Blocking this plasticity, then, should lead to reduced drug seeking behavior. To test this in mice, one could block cellular plasticity by locally infusing protein synthesis inhibitors or dopamine antagonists into the dorsal striatum during training. Our data would predict that these treatments would block cocaine seeking in these animals. Second, our results indicate that a subpopulation of cells in the dorsal striatum encode the context-drug associations underlying cocaine seeking. Therefore, inactivation of this subpopulation of cells in the dorsal striatum should lead to reduced cocaine seeking. This can be tested in mice using optogenetic
or transgenic approaches to specifically inactivate cells that were activated during training. Furthermore, by understanding the changes in neural connectivity caused by drugs of abuse we can begin to understand the underlying cause of addiction. Localizing a stable neural correlate of drug seeking behavior could be the first step in designing treatments to help addicts avoid relapse. It is critical, then, that we continue to explore the underlying changes associated with drug seeking and all behavioral changes associated with addiction.
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Figure 4-1. (A) Schematic of double transgenic hiGFP mouse. Without doxycycline, cellular activity drives the cfos promoter to express tTA (tetracycline transactivator protein), which binds to TetO in order to drive expression of the histone-GFP fusion protein. When doxycycline is present in the diet, tTA cannot activate the TetO-histoneGFP transgene and no tagging occurs. (B) Outline of behavioral procedure. Animals were raised and handled on-DOX and received a habituation CPP pre-test in order to establish that there was no pre-existing preference for either side of the chamber. Three days after habituation, animals were switched to regular diet to open the GFP tagging window. Three days later, animals were trained by pairing cocaine with either one side of the conditioning chamber (Paired group) or the home cage (Unpaired group). Animals were immediately returned to a DOX diet. Six days later, animals were given a CPP test to assess their preference for the drug-paired side. One hour after testing, animals were sacrificed for immunohistochemistry. (C) Representative image from the dorsal striatum in the Paired group. GFP positive cells (green) were active during training, while ZIF positive cells (red) were active during testing. DAPI (blue) was used as a marker of all cells. Far left image represents an overlay of all three markers. White arrows point to two cells positive for all three markers. White bar represents scale of 100 micrometers.
Figure 4-2. Behavioral tests. (A) During the habituation pretest, animals in both groups spent equivalent time in both the drug- and saline-paired sides. Neither group had a preference for either side. (B) After training, Paired animals showed a preference for the drug-paired side while Unpaired animals had no preference. Data are represented as mean ± SEM. * indicates p<0.05.
Figure 4-3. Cells active during training represented as the total number of GFP-positive cells (GFP+) as a percent of all cells (%DAPI). No differences were found between the Paired and Unpaired group in any of the brain regions examined. Data are represented as mean ± SEM. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
Figure 4-4. Cells active during testing represented as the total number of ZIF-positive cells (ZIF+) as a percent of all cells (%DAPI). The Paired group had significantly less expression than the Unpaired group in the mPFC and marginally less expression in the dorsal striatum. No other differences approached significance. Data are represented as mean ± SEM. * indicates $p<0.05$. # indicates $p<0.07$. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
Figure 4-5. Cells active during both training and testing represented as the number of overlapping cells as a percent of all cells (%DAPI). In the dorsal striatum, the Paired group had significantly more overlap than the Unpaired group. No other differences between Paired and Unpaired were found. Data are represented as mean ± SEM. * indicates p<0.05. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
To compare the observed overlap to what would be expected by chance, we divided the percent of overlapping cells (Overlap) by the Chance Overlap. Chance is represented by a normalized overlap of 1. In the dorsal striatum the Paired group had significantly more normalized overlap than the Unpaired group. No other differences between the Paired and Unpaired groups were found. Both the Paired and Unpaired groups had above chance overlap in the BLA, CA3, and CA1. Both groups had below chance overlap in the NAc core and shell. Only the Unpaired group had below chance overlap in the dorsal striatum. Data are represented as mean ± SEM. ** indicates p<0.01. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
Figure 4-7. Expression in the Dorsal Striatum. (A) GFP+ cells represents the cells active during training. No differences between Paired and Unpaired were found. Same data as in Figure 4-3. (B) ZIF+ cells represents the cells active during testing. Paired animals had marginally less ZIF expression than the Unpaired animals. (C) Despite expressing marginally less ZIF+ cells, the number of overlapping cells was higher in the Paired group than the Unpaired group. Same data as in Figure 4-5. (D) Isolating only cells that were active during training, the Paired group still had significantly more overlap than the Unpaired group. Thus, even though there was less overall activation in the Paired group during testing, there was a still higher reactivation of the cells that were active during training. Data are represented as mean ± SEM. * indicates p<0.05.
CHAPTER 5

Localizing the stable neural correlate of locomotor sensitization to cocaine

By

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Abstract

Drug addiction involves a gradual shift from casual drug taking to the compulsive pattern of behavior seen in addicts. One mechanism that could lead to this change in behavior is sensitization, an increase in the effect of a drug after repeated administrations. In mice, animals that have been previously exposed to cocaine show an elevated locomotor response over naïve controls. This elevated response, however, is specific to the context in which initial exposure occurred and is presumably caused by a change in the neural connectivity of neurons. To isolate this synaptic plasticity we used the TetTag Histone-GFP transgenic mouse to mark neurons that were active during initial drug exposure and compared this tag to neurons that were active during a second drug exposure using the immediate early gene zif268. We identified nine candidate brain regions hypothesized to underlie this drug memory and found that cells in the basolateral amygdala (BLA), CA3, CA1, medial prefrontal cortex (mPFC), and nucleus accumbens (NAc) shell that were activated during initial exposure were more likely than other cells to be reactivated during the second exposure. Similar to Chapter 4, we found evidence that the BLA, CA3, and CA1 are involved in contextual memory. Furthermore, the mPFC and NAc shell appear to store a stable neural representation of the drug experience. These results, however, also occurred in control animals that expressed significantly less sensitization, indicating that they are not directly responsible for differences in the expression of sensitization. Thus, the brain regions examined may not be directly involved in storing the contextual control of sensitization, or there might be a divergent form of neural coding underlying this memory.

Keywords: locomotor sensitization, memory, cocaine, TetTag
Drug addiction involves a shift from casual drug use to a compulsive pattern of behavior that overpowers an addict's self-control. One prominent theory of addiction posits that this shift in behavior is caused by an increase in the incentive salience (i.e., the wanting or craving) of a drug (Robinson & Berridge, 1993, 2003, 2008). In this manner, positive value attributed to the drug experience continually increases and leads an addict to take the drug despite severe negative consequences. This increase could be mediated by sensitization, an increased response to a drug after repeated exposures. Thus, the incentive-sensitization theory of addiction holds that sensitization of the incentive properties of the drug intensifies an addict's response to the drug, leading to a robust increase in craving and compulsive behavior (Robinson & Berridge, 1993, 2003, 2008). As a result, sensitization may be the key process underlying addiction.

The study of psychostimulant sensitization has become a popular model of addiction and can be studied in rodents using psychomotor activation (i.e., locomotor activity, stereotypy) as a proxy for the drug value because they share common, or at least overlapping, circuitry (Wise & Bozarth, 1987). Locomotor sensitization to cocaine is established by repeatedly administering the drug paired with a training context. Each subsequent administration results in increased locomotor activity compared to both the animal’s acute response and to naïve control animals. Traditionally, sensitization has been studied using a large number of administrations (i.e., 5-10), but can also be induced with just one training injection (Valjent et al., 2010). In order to express sensitization, however, the drug must be administered in the same context that it was originally paired with. When sensitized animals receive an injection in a novel environment, they have a similar response to naïve controls (Anagnostaras & Robinson, 1996; Anagnostaras, Schallert, & Robinson, 2002). This contextual control of sensitization is an inhibitory memory process that blocks the increased response to the
drug and can be disrupted with electroconvulsive shock treatment, causing sensitization to be expressed in a novel environment (Anagnostaras et al., 2002). Thus, when studying sensitization, it is important to understand that there are at least two processes occurring. There is an underlying sensitization of the unconditioned response to the drug, as well as a contextual control that can inhibit the sensitization in novel environments (Anagnostaras et al., 2002). The sensitization of the unconditioned response is considered to be non-associative because the neural substrate mediating the response (i.e., neural sensitization) to the drug can occur in vitro (Robinson & Becker, 1982), in anesthetized animals (Wang & Hsiao, 2003), or with targeted intracranial injections (Vezina & Stewart, 1990). Contextual control of sensitization, on the other hand, is an associative process that requires context information to allow the expression of sensitization. Together, these two processes are responsible for the expression of locomotor sensitization. In addition, excitatory conditioning (i.e., a conditioned locomotor response to the paired context) may also contribute to the increased locomotor activity observed during locomotor sensitization. This response is generally transient (i.e., it lasts only 5-15 minutes versus sensitization which can last much longer) and relatively weak compared to sensitization (Anagnostaras et al., 2002), which indicates that it is not a major factor in the expression of behavioral sensitization.

In Chapter 3, we reviewed studies exploring the neuroanatomy of locomotor sensitization using classical techniques such as lesions, immediate early gene expression, and targeted microinfusions. We found that the induction of sensitization was dependent on the ventral tegmental area (VTA), prefrontal cortex (PFC), amygdala, hippocampus, and nucleus accumbens (NAc) shell. The expression of this sensitization is dependent on the NAc core and input from the dorsal hippocampus. Furthermore, the contextual control of sensitization is hypothesized to come through a corticostriatal
afferent that can gate expression of the behavior (Chapter 3). From these findings it is difficult to pinpoint a specific region of the brain that holds a stable neural correlate of locomotor sensitization. Using more modern techniques, a new view of sensitization has emerged implicating the nucleus accumbens shell and medial prefrontal cortex. Both of these regions show increased spine density in animals that are sensitized (Robinson & Kolb, 1999) which has been hypothesized to underlie the sensitized response. Furthermore, selectively inactivating neurons in the NAc shell that were activated by induction of sensitization eliminated the expression of sensitization (Koya et al., 2009). Finally, resetting the connection between mPFC into the NAc shell using optogenetic techniques can block the expression of locomotor sensitization (Pascoli, Turiault, & Luscher, 2012). Thus, the NAc shell is heavily implicated in the expression of locomotor sensitization, perhaps mediated by input from the mPFC. All of these studies, however, are limited to identifying regions that are necessary for the expression of the behavior, and cannot pinpoint any region that stores or initiates the behavior. The current study circumvents this problem by studying cellular activation in an endogenous system that can identify the neural correlate of specific memories.

Locomotor sensitization can be conceptualized as a memory (i.e., a long-lasting change in behavior) that must be stored and retrieved in order to drive addiction-related behavior. This memory, however, has both associative and non-associative components that are necessary for expression, and these two different processes are likely to have divergent neural representations in the brain. The neural representation of associative memories is likely to involve the reactivation of a subpopulation of neurons that was active during initial encoding. This has been demonstrated by tagging neurons active during both training and testing of a memory, and only areas implicated in memory storage show a higher overlap than controls animals (Guzowski, McNaughton,
In Chapter 4, we found cells in the dorsal striatum to be more likely to be reactivated during testing when the drug was paired with the conditioning context than when the drug was paired with the home cage. Furthermore, we can compare the amount of overlapping expression to a random pattern of expression dispersed between all cells to see if cells active during training are more or less likely to be activated during testing. In chapter 4, we found that during conditioned place preference, the BLA, CA3, and CA1 were all above chance overlap, while the NAc core and shell were below chance. This indicated that the BLA, CA3, and CA1 were coding for contextual cues and the NAc core and shell were coding for the drug-state.

The neural representation of non-associative memories is less clear. In the literature, we cannot find any examples of tagging neurons during the encoding and retrieval of non-associative memories. It is possible that the neural representation is similar to associative memories, in that it is stored in a particular subpopulation of neurons that are activated during training and then reactivated during testing. On the other hand, it is also possible that non-associative memories are stored using a divergent mechanism and will not show the higher overlap characteristic of a brain region storing an associative memory. In this case, memory storage may be coded with other cellular or molecular modifications that lead to the behavioral output. For instance, it is possible that locomotor sensitization could be stored using a cellular mechanism that increases signaling throughout a particular brain region, rather than in specific cells. This would lead to an increased response without necessitating a reactivation of specific cells.

Here we used the TetTag histone-GFP system to investigate the neural representation responsible for locomotor sensitization to cocaine. This novel transgenic
mouse tags neurons active during a doxycycline-dependent tagging window to mark only neurons that are active during initial drug exposure. We then compared these neurons to those active during a subsequent exposure using the immediate early gene zif268 (ZIF). During training, animals received cocaine either paired with a conditioning context (i.e., Paired group) or in their home cage (i.e., Unpaired group). During test, they received an injection of cocaine in the conditioning context. We identified nine regions that are theorized to be involved in locomotor sensitization to cocaine including the amygdala, hippocampus, medial prefrontal cortex, nucleus accumbens, and dorsal striatum (see Chapter 3). We hypothesized that both groups would have underlying neural sensitization, but the behavior will only be expressed in the Paired group during test. Thus, a region underlying the sensitization of the unconditioned response will show equivalent overlap between cells active at training and testing in the Paired and Unpaired groups. On the other hand, in a region that stores the contextual gating of sensitization, we expect to see a higher overlap in the Paired animals than the Unpaired controls. This would indicate that a subpopulation of neurons activated during training were reactivated during testing, and are responsible for the difference in behavior. We found above chance overlap in the BLA, CA3, CA1, mPFC, and NAc shell but there were no differences between the Paired and Unpaired groups, despite clear differences in behavior. This indicated that the BLA, CA3, and CA1 are again underlying the neural representation of the context (see chapter 4), and that the mPFC and NAc shell contain a stable neural representation of the drug experience. Thus, the mPFC and NAc shell are likely responsible for the underlying sensitization, but not the expression of sensitization. It is unlikely that any of the brain regions examined are responsible for the contextual inhibition of locomotor sensitization.
Methods and Materials

Subjects

Histone-GFP (hiGFP) mice have been previously described (Tayler et al., 2011; Chapter 4). Briefly, hiGFP mice expressed two transgenes, a c-fos promoter driving a tetracycline transactivator (cfos-tTA) and a tetO promoter driving a GFP-tagged histone protein (tetO-HIST1H2BJ/GFP). Approximately equivalent numbers of male and female hiGFP mice were raised and maintained on a diet enriched with doxycycline (40 mg/kg). In the presence of doxycycline, production of tTA does not drive the tetO promoter to produce hiGFP proteins. When doxycycline is removed from the diet, neural activity drives the cfos promoter to produce tTA, which activates the tetO promoter to produce hiGFP proteins. These hiGFP proteins are a permanent marker of active cells. Mice were housed 2-5 per cage, given free access to food and water, and maintained on a 14:10 LD cycle. All experimental activities were conducted during the light phase and animals were at least 90 days old prior to the start of the experiment. Animal care and experimental procedures were approved by the UCSD IACUC, in accordance with the NRC Guide for the Care and Use of Laboratory Animals.

Drugs

Cocaine HCl (Sigma-Aldrich) was dissolved in 0.9% saline. All drugs were administered intraperitoneally (i.p.) in a volume of 10 ml/kg.

Conditioning Apparatus

Chambers were as described in Chapters 2 and 4, with the exception that only one side of the chamber was used and the barrier between the two sides was always in place. Briefly, 2-3 mice were tested concurrently, in individual chambers (22-cm wide, 44-cm high, 31-cm deep, Med Associates, St. Albans, VT) housed in a windowless room. Animals were tracked using infrared beams and Activity Monitor software (Med
Associates) and locomotor distance traveled was scored. Light was provided from two 150-watt bulbs distant from the chambers and background noise (65-dBA) was provided by HEPA air cleaners and an iPod speaker playing white noise.

**Procedure**

All animals were raised and maintained on a doxycycline-enriched diet to prevent activation of the hiGFP transgene. Subjects were gently handled for three minutes per day over a period of 9 days to acclimate the animals to experimenter interaction. On the first three days, animals were handled in the vivarium. On the next three days, animals were carted to the conditioning room to be handled. On the final three days, animals were carted to the conditioning room, handled, and then received an injection of saline to habituate the animals to the injection procedure. Prior to training, animals were habituated to the conditioning chambers for two days. During each habituation day, animals were given an injection of saline and placed in the chamber for 30 minutes and then returned to their home cage.

Three days after habituation, animals were switched to a regular diet without doxycycline. In the absence of doxycycline, active neurons are tagged with the hiGFP protein. Thus, removing doxycycline from the diet opened the hiGFP tagging window.

Three days after removing doxycycline from the diet, subjects were trained using a one-trial sensitization protocol. Animals were first pre-exposed to the conditioning chamber to reduce baseline locomotor activity and then received the drug treatment. Two groups were trained: a Paired group (n=8) received cocaine (15 mg/kg) in the chamber while an Unpaired group (n=8) received the same dose of cocaine in the home cage. It is important to note that both groups received the same context and drug exposure. The only difference between the groups was whether the drug was paired with the conditioning context (Paired group), or with the home cage (Unpaired group).
During training, animals first received an injection of saline and were placed into the chamber for 30 minutes. Subjects then received an injection of cocaine (Paired group) or saline (Unpaired group) and were placed back into the same chamber for 60 minutes. After the training, animals were given a final injection of either cocaine (Unpaired group) or saline (Paired group) and returned to their home cage. Immediately after training animals were placed on a high doxycycline diet (1 g/kg) for the duration of the experiment. This high doxycycline diet closed the hiGFP tagging window within 5 hours after training and thus, only neurons active during training were tagged with the hiGFP label.

One week after training, animals received a second drug exposure. The procedure for this test phase was the same as during training, except a lower challenge dose (5 mg/kg) of cocaine was used. This challenge dose isolated the retrieval of sensitization, as it does not induce sensitization in this paradigm (unpublished data). Using a lower dose also prevented locomotor activity from achieving ceiling levels which can occlude sensitization (unpublished data). Animals were pre-exposed by receiving an injection of saline and being placed in the chamber for 30 minutes. All animals then received an injection of cocaine and were returned to the conditioning chamber for another 60 minutes, and returned to their home cage. Thirty minutes after the completion of testing, animals were sacrificed and perfused to allow for immunohistochemistry of ZIF activation.

**Immunohistochemistry**

Immunohistochemistry was performed identical to Chapter 4. Brains were perfused with 4% paraformaldehyde (PFA), removed from the animal and post-fixed in 4% PFA overnight. Twenty-four hours later, brains were switched to phosphate buffered saline (PBS). Within one day, brains were sliced into 50 μm sections using a Leica
vibratome and incubated overnight in blocking buffer (10% normal goat serum, 0.5% TritonX-100 in PBS) at 4°C. Sections were then incubated overnight in blocking buffer with primary antibody (ZIF rabbit antibody – Cell Signaling) diluted 1:750 at 4°C. Sections were then washed in PBS followed by a 2 hour incubation in blocking buffer with secondary antibody (Cy3 conjugated goat-anti-rabbit) diluted 1:500 at room temperature. Slices were washed in PBS, immersed in 10x Tris Buffered Saline with DAPI, a nuclear DNA stain, diluted 1:500 at room temperature. Slices were washed in PBS and mounted onto slides using ProLong Gold antifade reagent and sealed with a coverslip.

Analysis of immunohistochemistry data

Analysis of immunohistochemistry was identical to Chapter 4. Images were taken using a Nikon C2 confocal microscope with a 20x air objective. Between 4 and 6 images of individual brain regions for each subject were averaged. The following coordinates in respect to bregma were imaged: lateral amygdala (-1.58, -1.82, -2.06), basolateral amygdala (-1.58, -1.82, -2.06), dentate gyrus (-1.58, -1.82, -2.06), CA3 (-1.58, -1.82, -2.06), CA1 (-1.58, -1.82, -2.06), medial prefrontal (infralimbic) cortex (+1.94, +1.70), nucleus accumbens core and shell (+1.42, +1.18), dorsal striatum (+0.98, +0.74). Lateral amygdala and basolateral amygdala images contained a flattened stack of 6-8 images 1.150µm apart.

All cell counts were done using ImageJ software by a blind scorer. The nuclear staining of these markers allowed for automated cell counting of DAPI and ZIF-DAPI overlap. Counting of GFP-DAPI overlap and GFP-ZIF overlap was done by hand.

Statistics

Statistics were performed identical to Chapter 4. All analysis was conducted using only signals that overlapped with DAPI. Chance level of overlap was calculated as:
(number of GFP+ / total DAPI) * (number of ZIF+ / total DAPI) * 100%. Normalized overlap was calculated as (number of overlapping neurons / total DAPI) / (Chance Overlap).

All statistics presented are t-tests for independent samples. Because each comparison is testing a separate hypothesis (i.e., each brain regions involvement in the memory), alpha correction is not necessary (O'Keefe, 2003; Weber, 2007). Thus, while repeated t-tests will lead to an escalated overall chance of finding a significant result, this is not true for each individual brain area. Furthermore, for each brain area we looked for a clear pattern of significant findings that were consistent with a memory storage hypothesis.

Results

Sensitization Training

Figure 2A shows the locomotor activity during training. The Paired and Unpaired group did not differ during the 30 minute pre-exposure period (t[14]= 1.745, p>0.10). The Paired group showed a significant acute response to the cocaine compared to the Unpaired group (t[14]= 6.237, p<0.001).

Sensitization Testing

Figure 2B shows the locomotor activity during testing presented as distance traveled per minute. The Paired group had significantly higher locomotor activity during the 30 minute pre-exposure period while off-drug (t[14]= 2.732, p=0.02). The Paired group also showed a significantly higher response to the cocaine during the first 10 minutes after drug administration (t[14]= 2.973, p=0.01). To explore the group difference during the 30-minute pre-exposure periods while off-drug, we subtracted the distance traveled of animals during training from the distance traveled during testing (Fig 2C).
Neither group is significantly different from 0 (t-values<1.91, p>0.05), which indicates that this difference was likely caused by Paired animals not fully habituating to the conditioning context and also expressing conditioned locomotor activity. To isolate the specific contribution of sensitization between the groups (as opposed to differences in habituation and conditioned activity) we measured the drug response as an increase over the pre-exposure baseline (Fig 2D). We subtracted the average distance traveled during pre-exposure from the average of the first 10 minutes after drug administration at test. The Paired animals showed a higher drug response than the Unpaired animals (t[14]= 2.238, p=0.04), even when accounting for differences in baseline activity.

**GFP Expression – Cells activated by training**

GFP expression was used as a marker of cells active during training. Figure 3 shows the percent of total cells (DAPI+) that were activated during training (GFP+). There was a large variance in expression between brain regions, however there were no differences between the Paired and Unpaired groups in any of the nine brain regions examined (t-values≤1.518, p≥0.15).

**ZIF Expression – Cells activated by testing**

ZIF expression was used as a marker of cells active during testing. Figure 4 shows the percent of total cells (DAPI+) that were activated during testing (ZIF+). There were no differences between the Paired and Unpaired groups in any of the nine brain regions examined (t-values≤1.436, p≥0.17).

**GFP-ZIF Overlap**

We compared the overlap between GFP and ZIF to assess the number of cells active during both training and testing. Figure 5 shows the number of overlapping cells as a percent of total cells (DAPI+) within each brain region. There were no differences
between the Paired and Unpaired groups in any of the nine brain regions examined (t-values≤1.239, \( p ≥ 0.24 \)).

Given the levels of expression of GFP and ZIF, we calculated the percent of cells that would overlap by chance (i.e., random arrangement of active cells within all cells – see Methods). We then normalized overlap in each brain region by dividing the overlap by chance. Thus, for normalized overlap, a score of 1 indicates that the overlap was at chance, consistent with a random assignment of active neurons during testing. A number above 1 indicates that cells active during training were more likely than other cells to be active during testing. A number below 1 indicates that cells active during training were less likely than other cells to be active during testing.

Figure 6 shows the normalized overlap for each brain region examined. No brain regions showed differences between the two groups (t-values≤1.532, \( p ≥ 0.14 \)). Both the DG and CA3 appear to be trending toward significance, but the trend is almost entirely driven by a single outlier in each group that has extremely low chance overlap due to very low GFP and ZIF expression. Regardless, neither group reached a marginally significant result (\( p ≥ 0.14 \)). A number of regions did, however, have higher than chance overlap evidenced by a normalized overlap above 1. The BLA, CA3, and CA1, and mPFC all had normalized overlap significantly above chance in both the Paired and Unpaired groups (one-sample t-tests against hypothesized value of 1; t-values≥2.938, \( p ≤ 0.02 \)). In the NAc Shell, both the Paired and Unpaired groups appeared above chance, but this did not quite reach significance in the Paired group (Paired: \( t[7]=2.301, \ p=0.055 \), Unpaired: \( t[7]=3.651, \ p=0.008 \)). The two groups did not differ, and when combined they are well above chance (\( t[15]=4.160, \ p=0.001 \)). This was also true of the BLA, CA3, CA1, and mPFC (t-values≥3.831, \( p ≤ 0.002 \)). Thus, in the BLA, CA3, CA1, mPFC, and NAc shell, the cells active during training were more likely than other cells to
be reactivated during testing. No other brain regions examined differed from chance in the Paired, Unpaired, or combined groups (t-values<2.077, p>0.05).

Discussion

We examined the neural representation of locomotor sensitization to cocaine by labeling cells active during initial drug exposure and comparing them to cells active during a subsequent exposure. During testing, animals that had received cocaine in the conditioning context showed an increased drug response compared to animals that had received cocaine in the home cage. Despite this difference in behavior, the groups did not differ in expression of GFP, ZIF, or overlap between the two time points.

Examining the neural representation of the underlying sensitization to cocaine

When we normalized the overlap in each region to chance overlap, we found five regions of the brain that had higher than chance overlap between these two time points in both the Paired and Unpaired groups: BLA, CA3, CA1, mPFC, and NAc shell. In these regions, cells that were activated during initial drug exposure were more likely than other cells to be reactivated during the subsequent drug administration. This colocalization of active cells indicates that some stable neural representation of training and testing is represented in these structures. The exact information coded by each of these structures cannot be determined from this study alone, but a comparison with Chapter 4 provides some insight. For instance, when a similar protocol was followed using a conditioned place preference paradigm (Chapter 4), the BLA, CA3, and CA1 also showed a higher than chance overlap. In that experiment, animals received cocaine in the conditioning context during training and were tested by being exposed to the context off-drug. The only stimuli similar between training and testing in both groups were the exposures to the conditioning context. Thus, the BLA, CA3, and CA1 likely
contain a neural representation of the conditioning context or related associations (i.e.,
injections, handling, etc.). Similar to the experimental design in Chapter 4, mice in the
current study were exposed to the same context during training and testing. As
expected, the same brain regions showed higher than chance overlap, indicating again
that these regions are likely coding for the context and related associations of the
conditioning paradigm. Unlike Chapter 4, however, in the current study cocaine was
administered during the test. Thus, both the training and testing sessions involved
receiving the drug so any increase in overlap above chance is likely caused by a
representation of the context (as in the BLA, CA3, and CA1) or of the drug experience.
The mPFC and NAc are unlikely to be involved in representing the conditioning context
because they did not show higher than chance overlap in the conditioned place
preference paradigm (Chapter 4). It is likely, then, that the mPFC and NAc shell are
coding for a stable representation of the drug experience.

Both the Paired and Unpaired groups received cocaine during training and likely
both developed the underlying mechanism of locomotor sensitization. The Paired group,
however, was able to behaviorally express significantly more sensitization during the test
than the Unpaired group (Fig 2D). This context specificity of sensitization is controlled
by associative contextual input (Anagnostaras et al., 2002). Thus, there are two
mechanisms that must be represented in the brain during sensitization, an underlying
neural representation of the previously experienced drug, and a sensitization-gating
mechanism controlled by contextual input. We found that neurons in the mPFC and NAc
shell are likely to code for the stable neural representation of the drug experience. It is
likely then, that the underlying neural representation of sensitization occurs in one or
both of these regions. At a minimum, these regions encode an experience of the drug
that can be recalled during a subsequent exposure to the drug. While the current data
cannot differentiate between a neural representation of the drug experience and a sensitization mechanism (i.e., what drives the higher locomotor response), it is highly likely that these two neural representations are overlapping. There is significant evidence from recent studies indicating that manipulating this region can block locomotor sensitization to cocaine. For instance, inactivating the subpopulation of neurons active during training in the NAc shell can block the expression of sensitization (Koya et al., 2009). Also, resetting the input from the mPFC into the NAc shell abolishes previously learned sensitization (Pascoli et al., 2012). The current data, taken together with these studies, provides strong evidence that the mPFC and NAc shell are the main sites within the striatal circuitry contributing to the induction and storage of locomotor sensitization.

We found that the underlying neural representation of a drug experience and of sensitization is likely stored in the mPFC and NAc shell. These regions appear to store the non-associative component of locomotor sensitization. These data are the first evidence that a non-associative memory is likely encoded with a neural representation similar to associative memories, triggered by reactivating a subpopulation of neurons that was active during initial learning. It remains possible, however, that sensitization is independent of this neural representation of the drug experience and is merely an output of this drug representation. This experiment was not designed to isolate a neural representation of sensitization between the two groups (i.e., a difference in overlap between the groups that would underlie the sensitization process), rather to isolate the representation of contextual control over the expression of sensitization. In fact, it would be extremely difficult to isolate a sensitization mechanism between groups, because it would require exposing two different groups to equivalent contextual and drug experiences while inducing sensitization in one group, but not the other. By definition, the drug experiences (i.e., that produces sensitization versus not producing
sensitization) would be different between the two groups and would confound any findings linking a neural representation to the process of sensitization. Likewise, it is unlikely that we would be able to tag neurons in an equivalent drug state without inducing some form of underlying sensitization. Other modern techniques, such as optogenetically manipulating neurons, suffer the same limitation. Simply manipulating the neural population encoding a drug experience could trigger the expression or suppression of sensitization, whether or not that region is actually representing the sensitization process, by directly or indirectly activating the entire drug circuit. Thus, while it is clear that the NAc shell and mPFC are representing a memory of the drug experience, and likely sensitization, these two cannot be separated with these techniques. This limitation is particular to non-associative memories because a specific association between stimuli cannot be isolated between groups. Thus, while non-associative memories are likely to be encoded using a similar neural representation as associative memories, they cannot be isolated in the same manner and thus cannot be identified by an increase in overlap between two different groups (with equivalent experience differing only in the association between them).

It is somewhat surprising that cells in the NAc core region did not show higher than chance overlap. This area is involved in reward learning and was hypothesized to be involved in the expression of sensitization (Chapter 3). For instance, a number of studies have found that chronic psychostimulant administration increases expression of Fos specifically in the core region of the NAc (Conversi, Bonito-Oliva, Orsini, Colelli, & Cabib, 2008; Hedou, Jongen-Reło, Murphy, Heidbreder, & Feldon, 2002; Hope, Simmons, Mitchell, Kreuter, & Mattson, 2006; Nordquist et al., 2008). It is likely then, that while the core region may be involved in the expression of the sensitized response, the stable representation of the drug experience is contained in the NAc shell region and
mPFC. Thus, in sensitized animals cocaine likely activates a larger, but random array of cells in the NAc core, and a specific pattern of cells in the NAc shell. This indicates that a possible mechanism of locomotor sensitization lies in the specific pattern of activation in the NAc shell driving the increased global activity in the core region, which could act as an output of the behavioral sensitization. Indeed, drug information is hypothesized to be relayed from the NAc shell through the NAc core to the behavioral output regions (Haber, Fudge, & McFarland, 2000). The contextual control of sensitization, then, would have to be a downstream inhibitor of the output since we did not observe differences in the activity of Paired and Unpaired animals in the NAc core after testing. On the other hand, since the current experiment only had one prior exposure to the drug, it is possible that the increase in NAc core activity requires many exposures, and is a result of a sensitized response in another region.

*Examining the neural representation of the contextual control of sensitization*

We did not find any differences in immediate early gene expression between animals that received cocaine in the testing environment (i.e., Paired group) and those that received cocaine in their home cage (i.e., Unpaired group). These two groups received equivalent drug and context exposure, but the Paired animals expressed higher locomotor activity to cocaine during testing. Presumably, the only difference between these two groups lies in the contextual control of sensitization, controlled by the association between the drug and context experiences. Thus any differences in cellular activity or overlap could only be attributed to this underlying association between the context and drug. We did not find any differences in GFP, ZIF, or overlapping expression, despite clear differences in behavior, in any of the areas examined. At least two explanations can account for the lack of significant differences in activity markers between the two groups. The simplest explanation is that the association between
context and drug is represented in a brain structure that we did not examine. Due to the nature of this experiment it was not possible to examine every brain region implicated in drug action. Furthermore, a number of structures (such as the VTA and substantia nigra) are very difficult to image in mice without additional staining methods. Thus, one of these other brain structures could be responsible for inhibiting the expression of sensitization in an unpaired context and, if examined, would show a higher overlap between training and testing in the Paired versus Unpaired group. Based on the finding that cells in the NAc shell and mPFC showed higher than chance overlap in both the Paired and Unpaired groups, it is likely that the inhibitory control of sensitization is downstream of those structures. A number of candidate regions are downstream of the NAc shell and mPFC including the ventral pallidum, thalamus, and motor cortex (see Chapter 3). One of these regions could be the site of storage for the associative control of sensitization.

A second explanation for these data is that the contextual control of locomotor sensitization is not coded by increased overlap between training and testing. While associative memories for fear (Reijmers et al., 2007) and context (Guzowski et al., 1999) are coded by an increase in overlap between training and testing (see also Chapter 4) it is unclear if this is true for all memories, or even for all associative memories. The contextual control of sensitization is considered associative because the drug response is paired with the context, but the underlying neural representation is not known. Thus, it remains possible, that the contextual control of sensitization has an alternative representation within the brain, and is not manifested by an increase in overlapping activity between the two groups. This explanation seems unlikely given the associative nature of the contextual control of sensitization and the current data showing no differences in each of the markers of cellular activity between the two groups. Even if
the neural representation for this memory is not seen with increased overlap between training and testing, there should be some difference in expression between the training or testing. The fact that there are no differences in any of the markers of cellular activity indicate that the regions examined are unlikely to be involved in the contextual control of sensitization.

Finally, it is also possible that this experiment did not have sufficient power to detect differences in cellular activity. Our samples were fairly small in both groups (n=8), and the variance could have occluded small differences in activity or overlap. Furthermore, this memory form is likely to be stored in a very sparse population of cells that need to be activated at both training and testing to allow sensitization to be expressed. Thus, it is possible we missed an effect because the sensitivity of the measured overlap between the two signals was not sufficient. This explanation is fairly unlikely, however, given that there were no marginally significant trends in the data. Furthermore, there were no patterns of activity changes that could correspond with a learning process. In the CPP experiment, there was a clear pattern to the changes in the dorsal striatum that were evident in the ZIF expression, raw overlap, normalized overlap, and overlap as a percent of GFP. The current experiment did not find any similar patterns that could account for the changes in behavior, even when considering the lack of power.
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Figure 5-1. Outline of behavioral procedure. Animals were raised and handled on-DOX and were habituated to the conditioning chamber by receiving an injection of saline and being placed in the conditioning chamber. Three days after habituation, animals were switched to regular diet to open the GFP tagging window. Three days later, animals were trained by receiving cocaine (15 mg/kg) paired with the conditioning chamber or paired with the home cage. Animals were immediately returned to a DOX diet to close the GFP tagging window. Six days later, animals were given a Sensitization Test by exposing the animal to a lower dose of cocaine (5 mg/kg) in the conditioning context. One hour after testing, animals were sacrificed for immunohistochemistry.
Figure 5-2. Induction and expression of locomotor sensitization. (A) During training, all animals received an injection of saline and were placed in the context for a 30 minute pre-exposure. Animals were immediately given cocaine (Paired group) or saline (Unpaired group) and returned to the conditioning context for 60 min. Animals received another injection of either saline (Paired group) or cocaine (Unpaired group) and were returned to their home cage. No differences were found between the Paired and Unpaired groups during the first 30 mins of training. The Paired animals showed a strong response to the cocaine. (B) During testing, all animals received an injection of saline and were placed in the context for a 30 min pre-exposure. All animals then received cocaine (5 mg/kg) and were placed in the conditioning context for 60 minutes. Paired animals had higher locomotor activity during the pre-exposure and during the first 10 minutes after drug administration. (C) To further explore the difference between groups during the pre-exposure, we subtracted the locomotor activity during training from the activity during testing. The Paired group had a higher difference in locomotor activity than the Unpaired group, but neither group was significantly different from training. (D) To isolate the relative contribution of sensitization, we subtracted the baseline activity from the activity in the first 10 minutes after drug administration. Paired animals had a higher response to the drug, even when accounting for the higher baseline. Data are represented as mean ± SEM. * indicates p<0.05 compared to Unpaired group.
Figure 5-3. Cells active during training represented as the total number of GFP-positive cells (GFP+) as a percent of all cells (%DAPI). No differences were found between the Paired and Unpaired group in any of the brain regions examined. Data are represented as mean ± SEM. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
Cells Active During Testing

Figure 5-4. Cells active during testing represented as the total number of ZIF-positive cells (ZIF+) as a percent of all cells (%DAPI). No differences were found between the Paired and Unpaired group in any of the brain regions examined. Data are represented as mean ± SEM. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
Cells Active During Training and Testing

![Bar chart showing cells active during both training and testing as a percent of all cells (%DAPI). No differences between the Paired and Unpaired groups were found. Data are represented as mean ± SEM. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.]

Figure 5-5. Cells active during both training and testing represented as the number of overlapping cells as a percent of all cells (%DAPI). No differences between the Paired and Unpaired groups were found. Data are represented as mean ± SEM. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
Figure 5-6. To compare the observed overlap to what would be expected by chance, we divided the percent of overlapping cells (Overlap) by the Chance Overlap (GFP/DAPI * ZIF/DAPI). Chance is represented by a normalized overlap of 1. No differences between the Paired and Unpaired groups were found. Both the Paired and Unpaired groups had above chance overlap in the BLA, CA3, CA1, mPFC and NAc shell. No other regions differed from chance in either group. Data are represented as mean ± SEM. LA, lateral amygdala; BLA, basolateral amygdala; DG, dentate gyrus of the hippocampus; CA3, hippocampus; CA1, hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; DS, dorsal striatum.
GENERAL DISCUSSION

This body of work provides evidence that psychostimulants can modulate and induce memory processes in order to drive behavior in mice. We studied two psychostimulants, modafinil and cocaine, and examined how these drugs are able to shape contextual, spatial, and drug-related memories. Determining how these drugs target specific circuits in the brain will help us minimize their addictive potential as well as find new treatments that can help break the compulsive pattern of drug use seen in addicts.

We first examined how modafinil can modulate contextual and spatial memory using the Morris water maze and Pavlovian fear conditioning. By administering the drug prior to a learning experience we were able to modulate the learning process. A high dose of modafinil enhanced spatial navigation memory, but also disrupted contextual fear memory. A low dose, on the other hand, enhanced contextual fear memory. It is unclear why the high dose had divergent effects on these two hippocampus-dependent tests, but it is possible that the higher locomotor and motivational demands of the water maze could require more stimulant effects. Furthermore, while both tasks require the hippocampus, modafinil could be modulating learning in other brain regions such as the basolateral amygdala in fear conditioning or the dorsal striatum in the water maze. Regardless of the mechanism it is clear that modafinil was able to modulate the learning process involved in each of these memories. These results were similar to those seen in other psychostimulants (Wood & Anagnostaras, 2009; Wood, Fay, Sage, & Anagnostaras, 2007), which indicated that these drugs could be acting through a similar mechanism.

Because modafinil was able to modulate memory processes, we wanted to expand these findings to drug-related memories and explore modafinil’s therapeutic
implications. Modafinil has been suggested as a pharmacological treatment for cocaine addiction so we examined how modafinil and cocaine interact in the induction of two popular rodent models of addiction: conditioned place preference (CPP) and locomotor sensitization. We administered modafinil, cocaine, and a combination of modafinil and cocaine during place preference training and the induction of sensitization. Since a high dose of modafinil was able to disrupt contextual memory, a key component of CPP, it was possible that modafinil could block the preference for the cocaine-paired side. Instead, we found that modafinil alone was able to induce a place preference and did not modify the cocaine preference. Modafinil did not induce a significant locomotor sensitization but was sufficient to express sensitization in cocaine-trained animals. Thus, this study provided more evidence that modafinil and cocaine work through a similar mechanism as modafinil was able to tap into the neural changes underlying sensitization to cocaine. Unfortunately the neural changes underlying conditioned place preference and sensitization are not known. Because of this, we do not know which specific circuits modafinil is acting on in order to induce conditioned place preference and express sensitization. This lack of crucial information drove us to review the neural circuitry involved in addiction-related memories. By understanding how psychostimulants induce conditioned place preference, locomotor sensitization, and conditioned locomotor activity, we can better understand the mechanism of psychostimulants and gain insight into how these drugs can cause addiction.

In Chapter 3, we reviewed the neuroanatomy of these drug-related memories and identified a number of brain regions that were implicated in each memory. Unfortunately, most of the studies were done using classical techniques (i.e., lesions, immediate early gene expression, microinfusions) that have a number of limitations. These limitations led to a lack of coherence in the literature and a number of conflicting
results. Fortunately, a number of new techniques have recently been developed to help probe the neuroanatomical projections underlying these behaviors. Advances in transgenic, optogenetic, and imaging techniques have made it possible to probe neural circuits with more temporal and spatial precision. We decided to use a novel transgenic approach to probe the neural representation of addiction-related memories, beginning with conditioned place preference.

The neural representation of conditioned place preference has not been extensively studied using modern techniques. It is likely, however, that because CPP is an associative memory, it will have a similar neural code to other associative memories. Previous evidence indicated that associative memories are stored in a subset of neurons that are activated during initial learning, undergo some neural plasticity, and then are reactivated during retrieval (Guzowski, McNaughton, Barnes, & Worley, 1999; Reijmers, Perkins, Matsuo, & Mayford, 2007). Thus, we set out to find a region in the brain where neurons are activated during initial CPP training and are then reactivated during testing. We used the TetTag hiGFP transgenic mouse to tag cells active during training and testing. We found that a subpopulation of cells in the dorsal striatum were more likely than controls to be activated during training and then reactivated during testing. Critically, the control group had equivalent exposure to the drug and the context, which means that the only difference between the two groups was in the association between the drug and context. These data suggest that the dorsal striatum is critical to the storage of this association.

While we have localized a subpopulation of neurons active during learning and retrieval of conditioned place preference to cocaine, it is unclear if these results generalize to other psychostimulants or drugs of abuse. Because the behavioral patterns of both addicts and rodent models of addiction are similar across drugs of
abuse, it is likely that a similar mechanism is driving the behavior, despite each drug working on a variety of different receptors. Furthermore, drugs of abuse have a similar characteristic of increasing extracellular dopamine in the nucleus accumbens and dorsal striatum (Di Chiara & Imperato, 1988). It is likely that this consistent mechanism is involved in the induction of conditioned place preference. While we have localized the dorsal striatum as one of the key storage site of this associative memory, the nucleus accumbens is still critically involved (see Chapter 3) perhaps because this region projects information into the dorsal striatum (Haber, Fudge, & McFarland, 2000). Thus, the role of the dorsal striatum is likely to integrate the drug and context information in order to drive behavior. It is likely then, that the dorsal striatum is the location where the conditioned stimulus (CS; context information) and the unconditioned stimulus (US; drug information) converge and form the association underlying the behavior (similar to the role of the BLA in contextual fear conditioning). All drugs of abuse could activate the critical parts of this circuit through drug-related input from the nucleus accumbens and contextual input from the hippocampus and perhaps BLA. Thus, this CS-US association converging in the dorsal striatum is likely to underlie conditioned place preference to all drugs of abuse. Furthermore, this mechanism could also be responsible for the conditioned place preference that can be induced by natural reinforcers such as sugar, mating, and social interaction (Schechter & Calcagnetti, 1993) because these stimuli also produce increased dopamine in the nucleus accumbens (Ikemoto & Panksepp, 1999). Thus, we argue that the dorsal striatum is likely to be the storage site of conditioned place preference memories for a variety of reinforcers.

While the hiGFP mouse is a powerful tool to examine the neural representation of associative memories, it is unclear if this technique can be applied to non-associative memories that may have a different pattern of neural representation between induction
and expression of the memory. We attempted to localize the neural representation of locomotor sensitization using the hiGFP mouse by tagging neurons active during initial exposure to cocaine and again during a subsequent drug exposure. This memory includes both a non-associative sensitization process, as well as an associative process that limits the expression of sensitization to the trained environment. Thus, we compared a group that was given cocaine paired with the context to a group that was given cocaine paired with the home cage. While the animals showed clear behavioral differences in behavior (i.e., higher locomotor response in the Paired group), they did not have any differences in GFP expression, ZIF expression, or overlap between the two signals in any of the 9 brain regions hypothesized to be involved in this behavior. Thus, the difference in the expression of sensitization is unlikely to be coded by an association between the drug and context memories in any of these regions. It is possible, however, that we did not examine a broad enough set of brain regions and if we examined all regions of the brain, we would find the storage site. Alternatively, the neural representation of the inhibitory control of sensitization may not be stored in a subset of cells that are reactivated during memory retrieval. There are a number of alternative mechanisms that could account for a brain region being the site of storage without reactivating a distinct subset of neurons. We did, however, find a number of brain regions in which a small subpopulation of cells that were activated during training were again reactivated during testing, including the BLA, CA3, CA1, mPFC, and NAc shell. These regions are hypothesized to be involved in some form of memory involved in sensitization, but did not differ between Paired and Unpaired animals indicating that they are not involved in the expression of sensitization.

A number of interesting comparisons can be made between the two hiGFP experiments presented in Chapters 4 and 5. By comparing the training procedures we
can identify the expression patterns that were likely caused by similar or divergent circumstances. For instance, in both experiments animals received a context exposure during training, and then another exposure to the same context during testing. In both cases, a memory for the context was formed during training and then reactivated during testing. Thus, there is likely to be similar areas of the brain that show higher than chance overlap responsible for this memory representation. Indeed, we found a subpopulation of cells in the BLA, CA3, and CA1 that were activated at above chance levels during both training and testing in both experiments. While there were other similarities between the two experiments (e.g., the injections, the time of day, the handling procedure) that could account for these parallel findings, it is likely that these regions are storing context memory and other associations (Guzowski et al., 1999; Reijmers et al., 2007). Similarly, we can compare the expression of overlap in the nucleus accumbens between these two experiments. In CPP, animals were trained on-drug and tested off-drug. In contrast, the sensitization-trained animals were trained and tested on-drug. We see a striking difference in the amount of overlapping cells in the NAc between the two experiments. In CPP, these regions show lower than chance overlap, presumably due to brain-state dependent activation patterns. During training cocaine activates a distinct set of drug-responsive cells that are specifically not activated during testing, presumably because it occurs in an off-drug state. Conversely, when we administered the drug during both training and testing, overlap in these regions increased significantly in both the overall overlap (i.e., as a percent of total cells – Chapter 4, Fig 5; Chapter 5, Fig 5) and compared to chance (Chapters 4 and 5, Fig 6). This indicates that the activation of cells in the nucleus accumbens is dependent on the drug state. Furthermore, we have isolated the NAc shell and mPFC as regions where a specific subpopulation of cells were activated during initial drug exposure, and then reactivated during a subsequent
drug exposure. This indicates that a stable neural representation of the drug experience is contained in these regions. Thus, by comparing the overlapping expression of activity markers between experiments, we can gain insight into where and how these memories are stored in the brain.

This work is the first example that we are aware of colocalizing expression of active cells to locate a memory trace. This technique has the potential to add a significant amount to our understanding of how memories are represented in the brain. A number of techniques have been developed to manipulate specific parts of a neural representation. Using a combination of viral and transgenic approaches Liu et al. (2012) was able to activate a specific subpopulation of neurons in the DG that had been active during training. The authors used optogenetics to reactivate this subpopulation of neurons and by doing so reactivated a fear memory. Similarly, Garner et al. (2012) was able to activate a synthetic memory trace using activity-driven insertion of a DREADD (designer receptor exclusively activated by designer drug) receptor to drive the neural representation of a previously learned context. These are pioneering techniques that can be used to probe the circuits involved in encoding, storing, and retrieving memories. We argue that examining the colocalization of cells active during training and testing can be just as powerful of a technique. While this approach cannot yet manipulate the circuits involved in these memories, it can localize a subpopulation of neurons that are activated during training and then reactivated during testing. It also avoids the limitations of artificially reactivating a subpopulation of neurons by ensuring that the activation of the memory is naturalistic. When using synthetic techniques the brain may be able to compensate for artificial activation or inhibition, leading to confounded results. Furthermore, these techniques make it difficult to distinguish between specific memories because activation of one aspect of the memory could indirectly reactivate the entire
memory trace. Using the hiGFP mice, we were able to compare a Paired and Unpaired group to probe the exact association that underlies conditioned place preference. While we did not explore it further, by making small changes to the procedure we could have investigated the specific involvement of the BLA, CA3, and CA1 that led to higher than chance overlap in both groups. Thus, the hiGFP is a powerful tool to examine the neural representations of associative memories and should be explored using other systems and memories.

In Chapters 4 and 5, we found evidence for the role of the dorsal striatum in storing the association between drug and context information underlying conditioned place preference to cocaine. We also found evidence that the BLA, CA3, and CA1 store contextual information while the NAc shell and mPFC store information about the drug experience. Using this information, we can now update our hypotheses laid out in Chapter 3 about the contributions of each brain region to these behaviors. In Chapter 3, we hypothesized that the dorsal hippocampus, amygdala, and NAc shell were very likely to be involved in the conditioned place preference to cocaine. Indeed, we did find evidence for the involvement of the dorsal hippocampus (CA3 and CA1) and amygdala (BLA) as these regions are likely coding for the contextual information that elicits the induction and expression of conditioned place preference. We did not find evidence for the involvement of the NAc shell in the storage of this memory, however this region may still be critically involved in the induction of the memory by inputting drug information into the dorsal striatum (Haber et al., 2000). The dorsal striatum had not been previously implicated in conditioned place preference and was not included in the original hypothesized circuit. We found striking evidence for the role of the dorsal striatum in storing the connection between the contextual and drug information that underlies conditioned place preference to cocaine. We hypothesize that the dorsal striatum is the
region in which contextual input from the dorsal hippocampus and perhaps amygdala converges with drug input from the nucleus accumbens (Fig 6-1). This region is likely to contain the neural plasticity that drives conditioned place preference to cocaine via outputs to the ventral pallidum.

Similarly, we can now update the hypothesized regions involved in locomotor sensitization (Fig 6-2). We found evidence that a stable neural representation of the drug experience is contained within the mPFC and NAc shell. These regions are likely involved in the underlying sensitization, but are not involved in the contextual control of sensitization. We hypothesize that sensitization is contained within the NAc shell and mPFC but is controlled by contextual input downstream of these regions in the ventral pallidum, thalamus, or motor cortex. This contextual control of sensitization likely arises from contextual input from the dorsal hippocampus and BLA. In remains unclear, however, how this contextual information can inhibit the expression of sensitization.
Figure 6-1. Illustration of hypothesized CS-US convergence in conditioned place preference. A stable representation of the context is contained in the dorsal hippocampus and basolateral amygdala. During training context information converges with drug information from the nucleus accumbens and substantia nigra to form an association in the dorsal striatum. This underlying association can then be reactivated by context alone during the conditioned place preference test, driving behavioral output.
Figure 6-2. Illustration of hypothesized circuit involved in locomotor sensitization to cocaine. A stable representation of the drug is contained in the mPFC and NAc shell, while a stable representation of the context is contained in the dorsal hippocampus and basolateral amygdala. Locomotor sensitization likely activates the drug representation to drive the sensitized response, but can be inhibited by the context through connections with the dorsal hippocampus and BLA. BLA, basolateral amygdala; DHip, dorsal hippocampus; mPFC, medial prefrontal cortex; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; VTA, ventral tegmental area; VP, ventral pallidum; Thal, thalamus.
REFERENCES


Crombag, H. S., Badiani, A., Chan, J., Dell’Orco, J., Dineen, S. P., & Robinson, T. E. (2001). The ability of environmental context to facilitate psychomotor sensitization to amphetamine can be dissociated from its effect on acute drug responsiveness and on conditioned responding. *Neuropsychopharmacology, 24*(6), 680-690.


Steketee, J. D. (1998a). Injection of SCH 23390 into the ventral tegmental area blocks the development of neurochemical but not behavioral sensitization to cocaine. *Behav Pharmacol*, **9**(1), 69-76.


