EFFECTS OF PHYSICAL EXERCISE AND EXERCISE-INDUCED ADULT HIPPOCAMPAL NEUROGENESIS ON CONDITIONED PLACE PREFERENCE FOR COCAINE IN MICE

BY

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DISSERTATION

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ABSTRACT

Treatments for drug abuse and addiction remain largely ineffective. Recent studies in both the human and rodent literature suggest that exercise-based interventions for drug addiction have positive outcomes. Exercise has many effects in the brain. The mechanism by which exercise achieves positive outcomes relevant to drug abuse and addiction is not known. One idea is that exercise extends plasticity to the brain that helps to weaken drug-context associations underlying drug abuse and addiction. It is known that exercise increases hippocampal neurogenesis. However, recently, conflicting reports have been published showing hippocampal neurogenesis to either be involved in or not contributory to learning and memory, and so the role of hippocampal neurogenesis in weakening learned drug-context associations remains unclear. The goal of my dissertation is to identify and evaluate select potential mechanisms causally related to accelerated extinction of conditioned place preference (CPP) for cocaine from exercise in male C57BL/6J mice. Since the conditioned place preference paradigm represents the major behavioral assay I employ in the proposed experiments, Chapter 1 presents a review of the relevant conditioned place preference literature. Chapter 2 examines the hypothesis that timing of exercise relative to conditioning has opposing effects on cocaine CPP in male C57BL/6J mice, and that exercise induces hippocampal neurogenesis. The main findings were that wheel running accelerated extinction of CPP when running occurred entirely after drug conditioning, whereas running delayed extinction when administered before conditioning, and that running approximately doubled adult hippocampal neurogenesis. Chapter 3 assesses the relative contribution of running versus enrichment to the neurogenic and pro-cognitive effects of an enriched environment in male C57BL/6J mice. The main finding was that an enriched environment devoid of running wheels did not significantly up-regulate hippocampal neurogenesis or improve behavioral performance on a spatial learning task, although running approximately doubled adult hippocampal neurogenesis. The finding that only running bestowed significant neurogenic and behavioral effects leaves open the possibility environmental enrichment promotes other types of neural plasticity that may make it a suitable substitute for running as an intervention in other domains. Therefore,
Chapter 4 investigates the possibility that environmental enrichment, like running, can accelerate extinction of CPP. Results suggest that environmental enrichment does not effectively accelerate extinction of CPP, nor does it significantly increase hippocampal neurogenesis. Chapter 5 directly tests the hypothesis that new neurons from running are necessary for accelerated extinction of cocaine CPP from running. Chapter 6 attempts to answer the question of whether there are neuropeptides that are differentially induced in runners versus sedentary C57BL/6J mice in the amygdala and hippocampus after exposure to a drug-associated context that could underlie the accelerated extinction of CPP from exercise. Neuropeptidomic profiles in the hippocampus suggest differences between sedentary and runner animals, but not in response to the contextual cues. Chapter 7 discusses future projects and directions, including an experiment to test the hypothesis that increased locomotor activity in the testing apparatus disrupts CPP, an experiment that will identify key temporal parameters that influence the effect of running on cocaine CPP by testing both whether one week of running wheel exposure followed by three weeks of sedentary housing or three weeks of sedentary housing followed by one week of running wheel exposure before testing can accelerate extinction of CPP for cocaine, an optogenetics experiment testing the role of new neurons in exercise-induced extinction of CPP for cocaine by hyperpolarizing new hippocampal neurons immediately prior to CPP testing, and the need to house mice in an empty “holding cage” for several hours prior to testing them for CPP to avoid the possible confound of extraneous stress impacting CPP test performance in future experiments involving complex housing conditions. Results of my dissertation further our knowledge of how wheel running may accelerate extinction of CPP by demonstrating that the effect is likely related to plasticity induced in the brain, independent of neurogenesis, and likely caused by the exercise as opposed to environmental enrichment component of running.
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CHAPTER 1

Measuring and analyzing conditioned place preference
Abstract
Conditioned place preference (CPP) is a standard preclinical behavioral model used to study the rewarding and aversive effects drugs of abuse. The objective of this Chapter is to present the various ways of measuring and analyzing CPP, with the intent of illustrating both the advantages and disadvantages of key methodological variations. There are a variety of apparatuses used for CPP: biased and unbiased, and with either three or two-compartment conditioning chambers. There are a number of different ways of quantifying CPP, each with advantages and disadvantages. One can use percentage of time spent on the drug-paired compartment, a comparison of pre-versus post-test scores, or post-test raw scores to arrive at values for CPP. One alternate way of depicting CPP is by raw post-test scores, between-subjects. An important issue to consider when measuring and analyzing CPP is the use of appropriate control groups, with one common control group being a saline-only-receiving group of animals. A less popular control group is a randomized drug-receiving control group, for which injections of drug and vehicle have no association with a particular CPP compartment. An appropriate control for associative learning is a comparison between animals conditioned with drug to one texture and animals conditioned with drug to the alternate texture. Such a group is a between-subjects control group for associative learning. When done correctly (i.e. when apparatus bias is reported, when control groups are used, etc.), CPP can serve as a valuable tool for elucidating the role of drug-to-context associations in drug use and abuse.
Conditioned place preference (CPP), a standard preclinical behavioral model that aims to measure the rewarding and aversive effects of drugs (Calcagnetti et al., 1995; Bardo and Bevins, 2000; Tzschentke, 2007; Prus et al., 2009), is a type of associative learning paradigm that is traditionally considered a form of classical Pavlovian conditioning. It has been used to study the rewarding and aversive effects drugs of abuse as well as of natural reinforcers such as food, interaction with pups, brain stimulation, and wheel running (Cunningham et al., 2003; Mattson and Morrell, 2005; Tzschentke, 2007). When CPP is used to investigate the rewarding effects of drugs, the primary motivational aspects of a drug represent the unconditioned stimulus (US). During conditioning sessions, the drug is repeatedly paired with an environmental stimulus, so that the environment itself becomes the conditioned stimulus (CS) and eventually becomes capable of eliciting the unconditioned response (UR). The UR is a rewarding effect on the animal and associated approach behavior (or, if the drug is aversive, a negative effect on the animal’s subjective state and withdrawal or escape behavior) (Prus et al., 2009). Approach behavior is one component of addiction. Drug-reward learning is assumed to be an underlying component of addiction (Bardo et al., 1998; Aguilar et al., 2009).

There are a number of reasons to employ CPP in lieu of other procedures that assess drug reward, such as self-administration. Over the last decade, CPP has been increasingly used for extinction and reinstatement procedures as a model of relapse, whereas previously, self-administration procedures predominated (Tzschentke, 2007). CPP is sensitive to both reward and aversion, so the procedure is versatile with regard to the types of drugs one can investigate with it (Bardo and Bevins, 2000). The CPP procedure is methodologically simple, requiring little in the way of expensive equipment and surgical preparations, in stark contrast to self-administration procedures (Cunningham et al., 2006a). Remarkably, a single conditioning session has been shown to elicit CPP to certain drugs, such as morphine, cocaine, and amphetamine (Mucha et al., 1982; Bardo et al., 1986; Bardo and Neisewander, 1986; Bardo et al., 1999). Such single-trial CPP avoids issues of tolerance and sensitization (Bardo and Bevins, 2000). At the same time, CPP remains versatile and suitable for studying tolerance and sensitization to the rewarding effects of drugs if one wishes to examine these, as pre-treatment regimens
can be employed (Tzschentke, 2007). In addition, with CPP, one can concurrently track animals’ locomotor changes that are induced by the drug, and drug-induced locomotor sensitization can also be readily measured (Bardo and Bevins, 2000). Furthermore, the CPP procedure is adaptable to many species, including transgenic mice (Bardo and Bevins, 2000; Tzschentke, 2007). CPP has also recently acquired more face validity as an experimental protocol of drug reward in humans when Childs and de Wit (2009) were able to demonstrate for the first time amphetamine-induced CPP in humans. Prior to this, CPP could be criticized for not having been validated for measuring drug reward in primates or humans, with the one exception of a study that showed that rhesus monkeys can be conditioned to show preference for a cocaine-associated context (Foltin and Evnas, 1997). Since the 1980s, when CPP studies began to be published at an exponential rate, CPP has become a firmly established, widely used tool in behavioral pharmacology and in particular in addiction research (Tzschentke, 1998).

There are a variety of apparatuses used for CPP. Most CPP studies use either three or two-compartment conditioning chambers (Tzschentke, 1998). The three-compartment apparatus, features a neutral middle compartment that an animal does not experience until testing and that is usually used as the ‘start’ box for CPP. When given the choice, rodents generally spend more time in a novel over a familiar environment (Hughes, 1968). In the course of CPP conditioning, animals receive the same total number of exposures to the drug-paired as to the vehicle-paired compartment. However, it is conceivable that the administered drug could block normal familiarization to the drug-paired environment, so that the drug-paired compartment gains familiarity at testing, when the animal is evaluated for CPP (which is generally done in a drug-free state) (Bardo and Bevins, 2000). Proponents of the three-compartment CPP apparatus purport that it eliminates the possibility of a novelty-seeking confound because the neutral middle compartment is the most novel compartment of all. Accordingly, if a test animal were to primarily choose the context it spends time on during preference testing based on novelty rather than based on a drug-to-context association, the animal would be predicted to spend the most time in the novel compartment. Conversely, significant place preference for the drug-paired compartment in a three-chamber CPP apparatus would suggest that an animal’s behavior was not reflecting primarily novelty-seeking but instead, genuine
preference for the drug-paired compartment. In a three-compartment CPP apparatus, the relative novelty of the drug-paired compartment is going to be less than that of the neutral compartment simply because it has not been experienced as often (it is absent during conditioning trials), but the drug-paired compartment does still retain some novelty during testing, since the animal is for the first time experiencing the drug-paired compartment in absence of the drug. Thus, one could argue that even with a three-compartment CPP apparatus, the possibility of a novelty-seeking confound, while greatly reduced, is not completely eliminated. Furthermore, while using a three-compartment apparatus to measure CPP may avoid a novelty-seeking confound, it can introduce additional confounds. For example, it is not clear what to do if treatment groups differ for time spent in the center compartment, because it is unclear whether it is fair to then compare times spent on the other sides between these groups. Additionally, in many three-compartment apparatuses, the size of the training and testing arenas differ. This is problematic because an animal habituates to the size of a compartment either during an explicit habituation session that occurs in many CPP experiments before conditioning itself—or, in the absence of such sessions—over the conditioning sessions themselves. The addition of a center compartment during testing alters the size of the CPP apparatus that an animal has, up until that point, become familiarized with. Therefore, CPP testing in a three-compartment apparatus may be subject to a novelty effect. It is unclear in what direction such an effect would bias results. Lastly, a three-compartment apparatus may add noise to the time that an animal spends exploring the two sides if an animal enters into one side and is then afraid to go back through the door that separates the center compartment from the two conditioning compartments.

A second major variation of CPP apparatuses is a two-compartment apparatus. A two-compartment CPP apparatus does not feature a neutral center compartment during testing. Proponents of the two-compartment apparatus prefer it over the three-compartment apparatus in part because it forces each animal to make a choice. This forced choice paradigm allows for relatively straightforward data collection. While an animal does have the free choice to spend time on the drug-paired compartment or on the vehicle-paired compartment, there is no “neutral zone” for the animal. A two-compartment CPP apparatus does not avoid the possibility of a novelty-seeking confound.
When tested in drug-free state, as is customary in CPP testing, the animal is given access to the CS+ compartment, which may take on new novelty because the animal encounters it in a drug-free state for the first time on test day. However, as outlined previously, this novelty confound also applies to the three-chamber apparatus. The neutral center compartment in a three-compartment CPP apparatus reduces the likelihood of such a confound because it is relatively more novel than the previously drug-paired compartment. One way to show that a novelty-seeking confound is not biasing data is to include reinstatement testing in addition to regular CPP testing. In reinstatement testing, an animal receives a priming injection of the drug before being placed into the testing apparatus. If it were the case that CPP for a previously drug-paired side was due to a novelty-seeking effect, because testing was the first time that an animal experienced the previously drug-paired side in the absence of drug, then a reinstatement dose of the drug would be expected to reduce or abolish the previously displayed preference for the drug-paired compartment. During reinstatement testing, the animal would again be experiencing the compartment as it did during conditioning, with the drug ‘on board,’ and the compartment would lose the novelty effect that it had during preference testing. The sudden abolishment of CPP at reinstatement testing is not typically observed, so a two-compartment CPP procedure is not generally subject to a novelty confound (Morales et al., 2007).

With both three-compartment and two-compartment CPP apparatuses, one can use a biased or an unbiased apparatus, where the term “biased” refers to an apparatus in which drug-naïve animals show a large and consistent preference for one of the stimulus alternatives (Cunningham et al., 2003). Bias towards one side of a CPP apparatus is usually determined at an animal’s first exposure to the CPP chambers during a pre-test that occurs prior to any conditioning, when the animal is still drug-naïve and has not experienced any pairings of an apparatus side with a drug or with vehicle. Among the advantages of a biased apparatus is that it is easy to elicit CPP if a drug is paired with the initially non-preferred compartment. In fact, there are certain drugs, including nicotine, for which CPP has reliably been more effectively shown using a biased apparatus (Calcagnetti and Schechter, 1994; Brielmaier et al., 2008). Among the disadvantages of a two-compartment apparatus is the fact that place conditioning may only be apparent when
the drug is paired with the initially non-preferred compartment. This brings into question whether a biased apparatus is truly measuring preference. This is in fact the case with nicotine; in several studies, nicotine has been shown to elicit CPP when paired with the initially non-preferred side, whereas it was unable to elicit CPP when it was paired with the initially preferred side (Calcagnetti and Schechter, 1994; Le Foll et al., 2005). Cocaethylene is another drug that has been shown to produce CPP when paired with the initially nonpreferred side, whereas it was unable to elicit CPP when it was paired with the initially preferred side (Schechter, 1995). Thus, assigning a CPP compartment as the drug-paired compartment will only successfully elicit CPP for certain drugs like nicotine and cocaethylene if baseline preference is accounted for and subject assignment is manipulated accordingly (i.e. non-random assignment). This can lead to false positive results. In cases where a drug is anxiolytic and is paired with the initially non-preferred compartment, repeated pairings of the drug with the non-preferred compartment can work against this initial aversion and increase relative preference for the non-preferred compartment without eliciting absolute preference over the initially preferred compartment (Papp and Moryl, 1994). This indicates that drugs like nicotine are able to counteract the aversive effects of a stimulus but may not have rewarding properties in and of themselves, so an unbiased apparatus may be more suitable to measure rewarding properties of drugs, since anxiolytic effects are not synonymous with true reward effects.

An unbiased apparatus poses fewer methodological challenges than a biased apparatus, regardless of whether one uses a three-compartment or a two-compartment apparatus. The definition of an unbiased apparatus is one in which a group of untrained animals on average does not show preference for one cue over another (Cunningham et al., 2003). This does not preclude the inevitable reality that individual animals will show a slight preference for one compartment even in an apparatus that is unbiased, as a group of animals is generally normally distributed with respect to their preference for an unbiased apparatus (Cunningham et al., 2006a). What makes an unbiased apparatus unbiased is that a group of animals as a whole does not display a significant preference for one of the compartments. An unbiased CPP apparatus is not generally subject to ceiling or floor effects. One of its drawbacks is that it may make detection of CPP more difficult than when a biased apparatus is used and the drug is paired with the initially
non-preferred compartment (Calcagnotti and Schechter, 1994; Brielmaier et al., 2008). Importantly, this property of an unbiased apparatus can be viewed as an advantage, as it makes it a more stringent test for conditioned place preference than a biased apparatus.

A CPP apparatus can have one or multiple cues that distinguish the respective conditioning compartments. These single or compound cues can be of several modalities (olfactory, visual, spatial, auditory, and tactile) and are either distal (for instance, visual cues) or proximal (for instance, tactile cues) to the individual animal (Bardo and Bevins, 2000). Because CPP is adaptable to a variety of species besides laboratory rodents, one can tailor the specific cue modality to the species. Chickens’ relatively high visual acuity lends itself to a CPP apparatus that relies primarily on visual cues to distinguish conditioning compartments, whereas rodents’ poor visual acuity makes tactile stimuli more appropriate, especially for albino rodents (Hughes et al., 1995). A study that directly compared the effectiveness of single-cue conditioning chambers versus dual-cue conditioning chambers in the same modality (in this case, floor color and ceiling color) to elicit CPP for a variety of doses of cocaine (5-20 mg/kg i.p.) as well as methamphetamine (1-2 mg/kg i.p.) found that both types of CPP chambers elicited significant CPP at all doses tested (Shimosato and Ohkuma, 2000). However, the major difference observed between the apparatus types was that the dual-cue conditioning apparatus elicited significantly greater CPP to the low (5 mg/kg) dose of cocaine than the single-cue chamber proved capable of eliciting (Shimosato and Ohkuma, 2000). In addition to explicit contextual cues like floor texture, spatial location cues (i.e. the conditioning compartment’s relative locations during conditioning) appear to be critical for CPP. It is thought that the spatial location can become an additional predictor of drug (Cunningham et al., 2006b). In one study, animals were trained to distinguish compartments using spatial locations and visual cues consisting of black objects printed on white paper (of 92 brightness): either an irregular pattern of black (6.4 mm diameter) circles or wide black lines printed at (6.4 mm) intervals that ran as stripes parallel to the end panels of the CPP box. Spatial location cues affected CPP for ethanol in mice compared to CPP when spatial location was not an additional cue (Cunningham et al., 2006b). When the conditioning compartments differed in terms of tactile stimuli, though, spatial location cues did not appear to matter, in contrast to when spatial location cues were paired with
visual stimuli (Cunningham et al., 2006b). Tactile (floor) cues were powerful enough cues so as to be able to elicit robust CPP for ethanol in mice regardless of whether their spatial location during training was kept consistent across conditioning sessions (Cunningham et al., 2006b). In fact, when tactile cues were used in combination with visual or spatial cues, CPP for ethanol was not enhanced relative to CPP elicited when tactile cues alone were used, a finding that suggests that tactile stimuli are particularly effective conditioned stimuli, at least for CPP for ethanol in mice (Cunningham et al., 2006b). This may differ for other animal models and other drugs of abuse.

Aside from the apparatus, there are a number of different ways of quantifying CPP, each with its own distinct advantages and disadvantages. One way of quantifying CPP is as percentage of time spent on the drug-paired side during a testing session. In this analysis, animals that are conditioned with drug to one context (for example, HOLE floor) are not considered to be a distinct group from animals that were conditioned with drug on the alternate context (for example, GRID floor). The utility of this approach is that one can collapse across conditioning subgroups when one expresses CPP as percentage of time spent on the drug-paired floor, so then the group means are based on twice the number of mice than the actual number of mice in each individual conditioning subgroup (drug+GRID floor vs. drug+HOLE floor). The disadvantage of this way of measuring CPP is that it necessarily requires a group that undergoes a control procedure (i.e. a group that only receives saline on both textures, or, alternatively, a group that receives unpaired drug-context exposures) to which the experimental conditioning group can be compared (Cunningham et al., 2003). These control groups have to be included in order to show that a significant CPP at testing is not merely the result of repeated exposure to the CPP apparatus but is a true shift in preference that is the result of a learned drug-context association. This method of expressing CPP loses the ability to detect whether an overall significant conditioning effect is driven by one particular conditioning subgroup (Cunningham et al., 2003). For instance, it is easy to imagine this effect at work in a scenario in which one CPP compartment is dark, and one is light. Rodents naturally prefer dark compartments under bright lighting conditions, while they tend to avoid light compartments (Roma and Riley, 2006). Mice would be expected to prefer the dark compartment when a drug is paired with the dark compartment, but they
would perhaps show less of a preference for the drug-paired side if the drug-paired side is the initially non-preferred (light) compartment. If CPP is expressed as percentage of time spent on the drug-paired side, one might still see an overall conditioning effect of the drug (if the CPP is so potent that it overrides the aversion to the light compartment in mice that receive drug paired with the light compartment), but in this depiction of CPP, one would lose the ability to discern which CPP compartment was “driving” the overall CPP effect. In practice, this is rarely an issue, because the drug reward is usually exclusively paired with the non-preferred side (i.e. would not be paired with the light side) to avoid the problem, but it is nonetheless an issue to consider.

A second way of expressing CPP is a comparison of pre-test versus post-test score. With this method, one conducts a pre-test of innate preference in drug-naïve animals when they have not yet experienced any compartment. The utility of this approach is that it accounts for animals’ pre-existing bias for or against a compartment. Conversely, though, this approach suffers from the limitation that an individual mouse’s pretest bias is a relatively weak predictor of its post-test bias when a neutral stimulus is given \((r=\sim0.4)\) (Cunningham et al., 2003). For instance, when a mouse is injected with the neutral stimulus saline and given access to a grid floor and to a hole floor compartment, the amount of time it spends in one compartment on the pre-test is only a weak predictor of the amount of time that it will spend in the same compartment during a follow-up test (Cunningham et al., 2003). The problem is that if the pre-test measure is subtracted from the post-test score when it has no reliability, then it is simply adding a large amount of noise to the signal, and therefore makes CPP more difficult to detect.

Another shortcoming of the pre-test versus post-test score approach is that an animal’s bias may change over the course of repeated exposures to the compartments independently of any learned drug-to-context associations. Such an evolving bias would not be controlled for with the simple pre- vs. post-test measure of CPP. One example of an evolving bias that would be independent of a learned drug-to-context association is a scenario in which the floor of the home cage that animals are returned to between conditioning sessions resembles one conditioning chamber texture more closely than the other conditioning chamber texture. This set-up could influence animals’ bias towards the more unfamiliar chamber texture from pre-test to post-test independently of a drug-to-
context association that was learned over the course of conditioning sessions only. In this case, a pre- vs. post-test score could reflect a bias in preference for a texture, rather than CPP.

An alternative way of depicting CPP is by raw post-test scores, between-subjects. This method compares the (raw) post-test times between animals that are randomly assigned to a drug treatment (i.e. GRID+ versus HOLE+ animals) in a counterbalanced fashion. In a CPP experiment that uses this method, animals that receive drug on the grid floor (considered the GRID+ conditioning subgroup) will be expected to spend more time on the grid floor than the mice that receive the same drug on the hole floor (considered the HOLE+ conditioning subgroup), because a learned preference for the drug-paired floor is expected to develop (Cunningham et al., 2006a). Animals are matched for their exposure to the drug (i.e. animals receive the drug either on GRID or on HOLE texture), their exposure to the floor textures (i.e. all animals are exposed to GRID and HOLE floors the same total number of times), and there is a saline-only control group (Cunningham et al., 2006a). This method holds the distinct advantage over other methods of depicting CPP of including a valid learning control. Both the animal group that receives drug on GRID floor and the animal group that receives drug on HOLE floor is presented with a specific drug-to-context pairing. Learning of specific drug-to-context associations takes place in both groups of animals. Because this drug-to-context association is reversed between the two groups (i.e. one group receives drug on GRID floor; the other group receives the drug on HOLE floor), any difference between the two conditioning subgroups in time spent on the textures at testing has to be due to the drug-to-context learning that took place, as this is the only variable that differs between the two groups. A two-way ANOVA can detect significant effects of conditioning subgroup and significant drug by conditioning subgroup interactions, should they exist (Cunningham et al., 2003). Importantly, the development of a bias for one texture in a treatment group over time (from pre-to post-test) would not compromise the analysis of CPP if CPP is determined as a between-subjects comparison between experimental groups. If all the animals in one conditioning group (i.e. GRID+) were to develop a bias towards one texture, due to, for instance, the texture of running wheels that the animals have access to resembling the grid floor (and driving bias towards the more novel hole
floor), then both animals assigned to the drug-on-Grid group and the drug-on-Hole group would develop this bias over time. If these two groups are directly compared in the analysis of CPP, and they are both developing a bias towards one texture, then when the difference score is computed, this bias is subtracted out. The method of arriving at CPP (as outlined by Cunningham et al., 2003) is elegant precisely because it avoids problems of confounding biases in preference that might develop. A downside to this approach of depicting CPP is that one cannot collapse across conditioning subgroups (GRID+ and HOLE+), so that the group means are based on half the number of mice than in a design that uses percentage of time spent on the drug-paired side or pre- minus post-test scores as the primary dependent variable.

Besides the type of apparatus and way of measuring CPP, another important issue to consider when measuring and analyzing CPP is the use of appropriate control groups. One common control group is a vehicle-only-receiving group of animals (Le Foll et al., 2005; Brielmaier et al., 2008; Li et al., 2008; Yang et al., 2008; Nechifor et al., 2010; Chauvet et al., 2011). This control group of animals experiences the same number of conditioning sessions in the CPP apparatus as the experimental groups, but always receives vehicle (usually saline) injections on both CPP textures. This type of control group represents a valid pharmacological control, because it directly controls for the possibility that the administered drug itself (rather than the paired CS-US) may be changing experimental group animals’ preference. This type of control group also controls for a bias that may evolve over time, after repeated exposure to the CPP apparatus, because vehicle-control animals can be tested for bias in a pre-test, and again at the time that experimental group animals are tested for CPP. If there is a discrepancy between the vehicle-control animals’ relative preferences for the CPP compartments between the pre-and post-tests, then a bias that developed over time becomes evident. While it boasts the advantage of being able to detect a bias that evolves over time with exposure to the CPP apparatus, an all-vehicle control group by itself is not a valid learning control, because animals that receive vehicle on both CPP textures are not learning a specific drug-to-context association. Hence, with a vehicle control group alone, it remains possible that CPP could reflect a bias that develops in preference due to
learning in the experimental group animals, rather than due to the learning of particular stimulus-drug associations (the intended construct).

A less frequently used control group in CPP experiments than an all-vehicle control group is a group of animals that is treated the same as the experimental animals, except that the injections of drug and vehicle have no consistent or exclusive association with a particular CPP compartment. This type of control group is explicitly unpaired (Guo et al., 2008). Unlike an all-vehicle control group, such a randomized drug-receiving control group does not boast the advantage of being a proper pharmacological control for the effects of the conditioning drug, because such control group animals are still receiving the drug the same total number of times as animals in the experimental group. Any effect of drug itself on biases in preference would not be detected. This shortcoming may explain its relatively rare use as a control in the CPP literature. Like an all-vehicle control group, a randomized drug-receiving control group also offers no appropriate control for associative learning, because animals are not learning any unique drug-to-context associations.

An appropriate control for associative learning would be a comparison between animals that are conditioned with drug to one texture (for example, hole, denoted by HOLE+) and animals that are conditioned with drug to the alternate texture (for example, grid, denoted by GRID+). Both the animal group that receives drug on GRID and the animal group that receives drug on HOLE floor is presented with a unique drug-to-context scenario. Learning takes place in both groups of animals. In contrast to a design in which a control group of animals receives vehicle on both floor textures, the between-subjects approach serves as its own control, because it presents the opportunity to learn a unique drug-to-context association to both conditioning subgroups. As already presented in the discussion of how to quantify CPP as a between-subjects comparison of post-test scores, because this drug-to-context association is reversed between the two groups (i.e. one group receives drug on GRID floor; the other group receives the drug on HOLE floor), any difference between the two conditioning subgroups in time spent on the textures has to be due to the drug-to-context learning that took place, as this is the only variable that differs between the two groups. Such a design does not offer a valid
pharmacological control, though. One can remedy this by including an all-vehicle control group to guard against a possible pharmacological confound.

A discussion of CPP would not be complete without briefly addressing controversies surrounding aspects of CPP. There is some controversy over whether CPP is truly a form of classical conditioning, because it does not allow for the dissociation of approach behavior between context-and cue-dependent memories of drug reward (Tzschentke, 1998). In other words, it is not entirely clear whether an animal during testing in a drugged state (i.e. reinstatement testing) approaches the drug-paired compartment due to state-dependent learning (i.e. the subjective effects of the drug) or whether an animal approaches the drug-paired compartment because it remembers the context paired with the drug. However, a study that assessed CPP for cocaine in rats in both the un-drugged and drugged state found no discernable difference between CPP for cocaine in the two states, so it appears that animals’ CPP is motivated by context-dependent memories, rather than by cue-dependent memories of the drug (Nomikos and Spyraki, 1988). It has also been questioned whether the drug-paired compartment ever actually becomes the CS per se; that is, if it is capable of eliciting rewarding effects resembling those of the drug of abuse (Prus et al., 2009). This would affect the validity of the CPP paradigm as a model of drug-context associations thought to be rooted in Pavlovian learning.

Several other issues complicate the interpretation of CPP. CPP is a complex task that, like many learning tasks, can be affected by a variety of factors. A recent study showed that social environment has the potential to interact with strength of CPP (Watanabe et al., 2011). Rats that had a cage mate that underwent identical methamphetamine CPP conditioning showed a social enhancement effect compared to rats that had a cage mate that underwent alternate CPP conditioning (i.e. a cage mate that was injected with saline on the day that the rat of interest was injected with methamphetamine) (Watanabe et al., 2011). Stressful handling has been shown to attenuate expression of conditioned place aversion (CPA) but not CPP for ethanol in mice, which suggests that separate neural circuits may underlie CPA and CPP (Bechtholt et al., 2004). The idea that separate neural circuits may underlie CPA and CPP pertains to use of a biased CPP procedure; as discussed, when a drug is paired with the initially less-
preferred cue, the CPP of the drug may be superimposed onto the pre-existing CPA, and if the two are driven by distinct neural circuits, it is not clear whether the biased apparatus, biased assignment procedure is really measuring CPP or a reduction of CPA. Even with several key methodological aspects under contention, CPP has made valuable contributions to the research literature and continues to gain in use. A comprehensive analysis of the literature showed that between the years of 1998 and 2007 alone, over 1000 new studies employing the CPP procedure were published in peer-reviewed journals (Tzschentke, 2007).

In summary, there are a variety of ways to measure and quantify CPP. One can use a three-compartment apparatus or a two-compartment apparatus, an unbiased or a biased apparatus. A host of discriminative stimuli can be used for the conditioning chambers. An all-vehicle control group, a randomized drug-receiving control group, or a between-subjects assignment procedure that serves as a control can be used. Finally, percentage of time spent on drug-paired compartment, or a comparison of pre-versus post-test scores, or post-test raw scores can be used to arrive at a value for CPP (Parker, 1992). Parameters to choose when planning a CPP experiment include drug type, drug dose, number of conditioning sessions, and route of administration (generally, passive administration by the experimenter) (Nomikos and Spyraki, 1988; Calcagnetti and Schechter, 1994; Brielmaier et al., 2008). For instance, a study that directly tested whether cocaine could elicit CPP when administered subcutaneously or by i.p. injections found that only i.p.-delivered cocaine elicited CPP (Mayer and Park, 1993). All of the usual factors to consider that affect learning tasks, such as circadian rhythms, also apply to CPP. For instance, Kurtunco et al. (2004) showed that 10/mg i.p. cocaine produced robust CPP in mice when conditioning and testing both took place in the daytime, whereas CPP for cocaine was significantly reduced when conditioning and testing both took place during the night. When dealing with mice in particular, the specific strain of animal that one wishes to use should also influence CPP parameters, such as number of conditioning sessions required to elicit CPP, because interference by high locomotor activity has been shown to be linked to weaker CPP in particular mouse strains bred for high activity (Cunningham et al., 2004).
Careful consideration of the inherent advantages and limitations of using different procedures should dictate what exact CPP design to employ. There is no one correct way of measuring CPP, but an unbiased apparatus with randomized, counterbalanced assignment to conditioning subgroups and inclusion of an all-vehicle control group eliminates most of the confounding factors that can complicate interpretation of CPP. When it is employed correctly (i.e. when apparatus bias is reported, when control groups are used, etc.), CPP can serve as a valuable tool for elucidating the role of drug-to-context associations in drug use and abuse. CPP has become one widely used behavioral model of drug use. As such, it can contribute unique information about the affective properties of drugs that drive the drug-context associations thought to underlie drug addiction, and it can complement what we learn from other models of drug use, such as self-administration experiments.
References


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Wheel running can accelerate or delay extinction of conditioned place preference for cocaine in male C57BL/6J mice, depending on timing of wheel access.

Abstract

Aerobic exercise may represent a useful intervention for drug abuse in predisposed individuals. Exercise increases plasticity in the brain that could be used to reverse learned drug associations. Previous studies report that exposing mice to a complex environment including running wheels after drug conditioning abolishes conditioned place preference (CPP) for cocaine, whereas running can enhance CPP when administered before conditioning. The purpose of the present study was to test the hypothesis that timing of exercise relative to conditioning has opposing effects on cocaine CPP. Male C57BL/6J mice experienced 30 days of running or sedentary treatments either before or after cocaine conditioning. Control animals always received saline and never cocaine but otherwise underwent the same conditioning and exercise treatments. Animals were administered BrdU injections at the onset of conditioning or exercise and euthanized at the end of the study to quantify survival of new neurons in the hippocampus as a marker of plasticity. Wheel running accelerated extinction of CPP when running occurred entirely after drug conditioning, whereas running delayed extinction when administered before conditioning. A single conditioning day after running was sufficient to abolish the accelerated extinction observed when all conditioning preceded running. Running approximately doubled adult hippocampal neurogenesis, whereas cocaine had no effect. Results suggest exercise-induced plasticity can facilitate learning that context is no longer associated with drug. However, if drug exposure occurs after exercise, running-induced plasticity may strengthen drug associations. Results provide insight into the interaction between exercise and drug conditioning that could have implications for drug abuse treatments.
Introduction

Additional treatments for cocaine abuse are critically needed. Compliance with current treatment programs is weak, and abstinence is typically ephemeral. For example, in a recent study, fifty-two percent of cocaine users dropped out of a National Institute for Drug Abuse treatment trial within three months (Ghitza et al., 2010). Studies have suggested that aerobic exercise could be useful as an intervention for maintaining abstinence in individuals willing to substitute exercise for drug reward (Sinyor et al., 1982). Exercise promotes brain plasticity and activates some of the same brain structures as those involved in reward and addiction. In rodents, the neural plasticity marker ΔFosB is upregulated in striatum and nucleus accumbens after wheel running to a comparable degree as after chronic cocaine exposure (Brene et al., 2007). In addition, mice bred for high levels of voluntary wheel running display increased brain activity marker c-Fos in brain structures implicated in reward. Among these are the lateral hypothalamus, medial frontal cortex, and striatum (Rhodes et al., 2003). Taken together, this evidence suggests that exercise may serve as a substitute reward, but whether substituting exercise for drugs helps to ameliorate addiction is still unknown.

Plasticity from exercise could be used to modify associations between drug reward and contextual cues. For example, exercise increases adult hippocampal neurogenesis, and new neurons have been hypothesized to display greater ability to mold to new experiences than pre-established neurons (van Praag et al., 1999). In addition to being a site where exercise exerts effects on the brain, the hippocampus may influence reward circuitry because of its anatomical connections. The hippocampus receives input from the nucleus accumbens and ventral tegmental area and sends output to the nucleus accumbens (Eisch and Harburg, 2006). Manipulations of hippocampal dentate gyrus granule cells have been shown to influence dopaminergic signaling in the nucleus accumbens and ventral tegmental area, areas important to drug reward (Eisch and Harburg, 2006).

Consistent with the hypothesis that exercise can enhance plasticity and facilitate extinction of conditioned place preference (CPP), a recent study using C57BL/6 mice found that environmental enrichment including running wheels completely abolished CPP for cocaine when the enrichment was administered after conditioning (Solinas et al.,
2008). The same group found that rearing C57BL/6 from weaning in an enriched environment abolished cocaine CPP when the animals were conditioned and tested as adults (Solinas et al., 2009). Similarly, in Lewis rats, forced treadmill running during adolescence weakened cocaine CPP when conditioning occurred after running (Thanos et al., 2010). On the other hand, wheel running strengthened cocaine CPP when adult Long-Evans rats were given wheel access before conditioning (Smith et al., 2008). To the best of our knowledge, no other studies have investigated effects of running on cocaine CPP. The purpose of this study was to determine (1) whether the influence of wheel running on cocaine CPP depends critically on the timing of wheel access relative to drug conditioning and (2) whether changes in CPP correlate with changes in adult hippocampal neurogenesis in response to the exercise and cocaine treatments.

**Methods**

**Animals**

Ninety male C57BL/6J mice were obtained at 5 weeks of age (The Jackson Laboratory, Bar Harbor, ME) and housed 4 per cage in a climate-controlled environment on a 12 h light/dark cycle (lights off at 9:00 a.m.) for 12 days. Dimensions of cages without running wheels were 29 x 19 x 13 cm (L x W x H). Mice were individually housed for 1 week before starting the experimental procedures. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All measures were taken to minimize the number of mice used as well as the pain and suffering of the animals.

**Drugs**

Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline and was administered at a dose of 10 mg/kg via intraperitoneal (i.p.) injections in a volume of 10 ml/kg. Dose was chosen based on the literature and was prepared according to the salt not the base form (Zombeck et al., 2008).

**Place conditioning chambers**

The place conditioning chambers were modeled after Cunningham et al. (2006) and were the same as in previous studies from our laboratory (Zombeck et al., 2008; Johnson et al., 2010). They consisted of 20 identical black acrylic boxes (30 cm x 15 cm x 15 cm), with removable clear plastic tops. The floors were interchangeable and
consisted of three types of distinct textures: HOLE, GRID, and HOLE/GRID. The HOLE/GRID floor consisted of half GRID and half HOLE textures. Distance traveled and location of mice within CPP boxes were recorded by TopScan video tracking software (Clever Sys Inc., Vienna, VA) (Zombeck et al., 2008; Johnson et al., 2010).

**Conditioned place preference (CPP) procedure**

We followed Cunningham et al. (2003; 2006). Runners and Sedentary animals were counterbalanced with respect to the conditioned stimulus, and experienced cocaine on GRID (CS+GRID) or cocaine on HOLE (CS+HOLE) texture and saline on the alternate texture. During testing, animals explored the same size chamber as during conditioning except with the HOLE/GRID floor type. Hence the animals were forced to spend time on either HOLE or GRID side, and duration on HOLE is equivalent to the total duration of the test (30 min) minus duration on GRID (see Place Conditioning Chambers above). Conditioned place preference was determined by comparing the duration spent on HOLE (or GRID, statistics would be the same) between groups, CS+HOLE versus CS+GRID. The design ensures that any difference in duration spent on textures between groups (CS+GRID versus CS+HOLE) is due to drug-to-context learning, as this is the only variable that differs between the two groups. Biases in baseline preference for textures cannot produce false positives with this method because duration spent on one texture (HOLE or GRID) is compared between subgroups CS+GRID and CS+HOLE, both of which would be expected to display the bias if one developed. Hence, when the difference score is computed, any bias is subtracted out. The two groups are also matched for drug exposure which is important because drug exposure itself could affect the development of biases in preference. Moreover, each group serves as the other group’s learning control, because both groups learned to associate one texture with cocaine and the alternate texture with saline. This is important because as compared to using a control in which all animals receive saline on both textures, the experience of learning itself could bias preferences for the textures (Cunningham et al., 2003; Cunningham et al., 2006).

On the days of CPP habituation, pretesting, conditioning, testing, and cocaine priming (see below), mice were moved to a testing room, where lights were turned off at 9:00 A.M. Mice were kept in the room for one hour before testing began. Between
sessions, the chambers were cleaned with disinfectant. Animals were returned to home cages with or without wheels immediately after testing. Hence, Runners had continuous access to running wheels throughout behavioral testing except when in the conditioning chambers.

**Habituation**

To familiarize the mice with the place conditioning chambers, animals were placed on a flat surface without a texture in the conditioning chambers in the morning (900 h; for 30 min) and in the afternoon (1500 h; for 30 min) for one day without any injection treatment (Cunningham et al., 2006).

**Pretesting**

To determine individual biases in preference for the textures prior to drug pairing, animals were weighed, received a 10 ml/kg i.p. saline injection, and were immediately placed in the apparatus with HOLE/GRID floor in the morning (900 h; for 30 min) and afternoon (1500 h; for 30 min) (Cunningham et al., 2006).

**Conditioning**

Four conditioned stimulus (CS+) trials (i.e., cocaine paired with one floor texture: HOLE or GRID) and four CS- trials (i.e. vehicle paired with the alternate floor texture) were administered over four days. Each day, one CS+ trial and one CS- trial was administered in the morning and afternoon. The order of exposure to CS+ and CS- was counterbalanced within each group. Experimental animals were weighed, received an injection of 10 mg/kg i.p. cocaine (CS+ trial) or vehicle (CS- trial), and were immediately placed on the appropriate floor texture. Control animals underwent the identical procedure, except that they always received vehicle (saline) on both floor textures.

**Testing**

Testing took place twice daily: in the morning (900 h; 30 min) and in the afternoon (1500 h; 30 min) for four consecutive days. Prior to each testing session, each mouse was weighed, injected i.p. with 10 ml/kg saline, and placed into the center of the HOLE/GRID conditioning chamber.

**Cocaine priming**

Cocaine priming began two days after the final CPP test session and consisted of two daily (morning and afternoon) 30 min exposures to the HOLE/GRID texture for four
consecutive days. Experimental animals were weighed and injected with 10 mg/kg i.p. cocaine immediately before being placed into the HOLE/GRID conditioning chamber, whereas control animals received a saline injection before all priming sessions.

**Running wheels and sedentary treatment**

Dimensions of running wheel cages were 36 x 20 x 14 cm (L x W x H), with a 23 cm diameter wheel mounted in the cage top. Running wheel rotations were monitored continuously in 1 min increments throughout the experiments via magnetic switches interfaced to a computer. Mice assigned to the Sedentary group were deliberately not housed in cages with locked wheels because mice climb in locked wheels and we intended to keep physical activity to a minimum in the Sedentary group (Koteja et al., 1999; Rhodes et al., 2000; Rhodes et al., 2003).

**Experimental design**

**Experiment 1**

At 54 days of age, animals (n = 30 total) underwent habituation, pretesting, and cocaine CPP (n = 20, Cocaine group) or CPP without cocaine, (n = 10, Control group) as detailed above (Figure 2.1). During the four conditioning days, mice received daily injections of 50 mg/kg Bromodeoxyuridine (BrdU) to label dividing cells. The day after the four conditioning days, mice were placed individually in cages either without (Sedentary, n = 10 Cocaine and 5 Control) or with running wheels (Runner, n = 10 Cocaine and 5 Control) for 30 days. On the second day of Sedentary or Runner treatment, mice received one additional BrdU injection. After 30 days, mice underwent four consecutive days of CPP testing, followed one day later by four consecutive days of CPP testing after cocaine priming. After testing each day, mice were returned to their cages with or without running wheels so that Runners had continuous access to wheels throughout the

**Experiment 2**

At 54 days of age, animals underwent habituation and pretesting (n = 30 total). The next day, mice were individually placed into cages either without (Sedentary, n = 15) or with running wheels (Runner, n = 15) for 30 days. During the first 10 days of Sedentary or Runner treatment, mice received daily injections of 50 mg/kg BrdU. After 30 days, the mice underwent cocaine CPP (n = 20, Cocaine group, 10 Runners and 10 Control group)
Sedentary) or CPP without cocaine \((n = 10, \text{Control group, 5 Runners and 5 Sedentary})\) for four days. Mice then underwent four consecutive days of CPP testing, followed one day later by four consecutive days of CPP testing after cocaine priming. After conditioning or testing each day, mice were returned to their cages with or without running wheels so that Runners had continuous access to wheels throughout the testing period.

**Experiment 3**

At 54 days of age, animals \((n = 30 \text{ total})\) underwent habituation, pretesting, and cocaine CPP \((n = 20, \text{Cocaine group})\) or CPP without cocaine \((n = 10, \text{Control group})\) for three consecutive days. The next day, mice were placed individually in cages either without (Sedentary, \(n = 10 \text{ Cocaine and 5 Control}\)) or with running wheels (Runner, \(n = 10 \text{ Cocaine and 5 Control}\)) for 30 days. The first 10 days of Sedentary or Runner treatment, mice received daily injections of 50 mg/kg BrdU. After 30 days, mice underwent one additional day of CPP conditioning to simulate a relapse episode, followed by four consecutive days of CPP testing, and then, one day later, by four consecutive days of CPP testing with cocaine priming. After the last conditioning or testing session each day, mice were returned to their cages with or without running wheels so that Runners had continuous access to wheels throughout the testing period.

**Immunohistochemistry**

**Tissue preparation**

Following behavioral testing, all the mice \((n = 90)\) were anesthetized with 100 mg/kg sodium pentobarbital (i.p.) and then perfused transcardially with 4% paraformaldehyde in phosphate buffer solution (PBS; 0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were postfixed overnight and then transferred to 30% sucrose in PBS. Brains were sectioned using a cryostat into 40 µm thick coronal sections. Sections were placed into tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) in 24 well plates and stored at -20 °C. Two separate one-in-six series of these sections (i.e. series of sections throughout the rostro-caudal extent of the brain with 240 µm increments separating each section, approximately nine sections) were stained in the following way.
**BrdU-DAB**

**Purpose:** To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free floating sections were washed in tissue buffering solution (TBS; 1.3% Trizma hydrochloride, 0.19% Trizma base, 0.9% sodium chloride) and then treated with 0.6% hydrogen peroxide in TBS for 30 min. To denature DNA, sections were treated for 120 min with a solution of 50% de-ionized formamide and 2X SSC buffer, rinsed for 15 min in 2X SSC buffer, then treated with 2 N hydrochloric acid for 30 min at 37 °C, then 0.1 M boric acid in TBS (pH 8.5) for 10 min at room temperature. Sections were then treated (blocked) with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus) for 30 min, and then incubated in primary antibody against BrdU made in rat (AbD Serotec, Raleigh, NC, USA, catalog number OBT0030) at a dilution of 1:100 in TBS-X plus for 72 h at 4 °C. Sections were then washed in TBS, blocked with TBS-X plus for 30 min and then incubated in biotinylated secondary antibody against rat made in goat (Vector, Burlingame, CA, USA, catalog number BA-9400) at 1:250 in TBS-X plus for 100 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA, USA, catalog number PK-6100) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO, USA, catalog number D4418-505ET).

**Double-fluorescent label**

**Purpose:** To determine the proportion of BrdU-positive cells in the dentate gyrus that differentiated into neurons. Sections were treated as for BrdU-DAB except a cocktail was used for the primary antibody step. Rat anti-BrdU (1:100; AbD Serotec, Raleigh, NC, USA, catalog number OBT0030) was combined with mouse anti-neuronal nuclear protein (NeuN) (1:50; Millipore, Billerica, MA, USA, catalog number MAB377) for 72 h at 4 °C. Secondary antibodies were conjugated with fluorescent markers (Cy2-green anti-mouse, and Cy3-red anti-rat; Jackson ImmunoResearch, West Grove, PA, USA, catalog numbers 115-225-166 and 112-165-167, respectively) at a dilution of 1:250 and also delivered as a cocktail.

**Image Analysis**

**BrdU-DAB**
The entire granule layer (bilateral), represented in the 1-in-6 series, was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope and taking multiple photographs, via camera interfaced to computer, under 10x (total 100x) magnification. Positively labeled cells in these photographs were counted to generate estimates of total number of labeled cells. The total volume of the dentate gyrus represented in the series was measured so that the counts could be expressed per µm³ dentate gyrus sampled.

**Double label**

A Leica SP2 laser scanning confocal microscope (using a 40x oil objective, pinhole size 81.35 µm diameter) was used to determine the proportion of dentate gyrus BrdU-positive cells that differentiated into neurons (NeuN+). Dentate gyrus BrdU-positive cells were identified as either co-expressing NeuN or not. Each BrdU-positive cells in the granular layer (represented in the 1-in-6 series) was analyzed by focusing through the tissue in the z-axis to establish co-labeling with NeuN. The number of new neurons per µm³ per mouse was calculated as the number of BrdU cells per µm³ multiplied by average proportion BrdU cell co-expressing NeuN for the designated group.

**Statistical analysis**

Data were analyzed using SAS (version 9.1) or R (version 2.7.2) statistical software. In all analyses, \( P < 0.05 \) was considered statistically significant. Each experiment (1, 2, and 3) was analyzed separately. Conditioned place preference data were analyzed the following way. Control animals that never received cocaine were analyzed separately from cocaine-treated animals. First, the duration spent on the HOLE texture was analyzed by three-way repeated measures ANOVA with conditioned stimulus (CS+HOLE versus CS+GRID; between-subjects), exercise history (Runner versus Sedentary; between subjects), day of testing (1-4; within-subjects) and all interactions entered as factors. Testing session, whether at 9:00 h or 15:00 h, was also included as a factor in initial models but was never significant and therefore was removed from the final linear models. In addition, the CPP data were analyzed separately within each group using standard methods (Cunningham et al., 2006). Within each group, the duration spent on the HOLE texture was compared between the CS+HOLE and CS+GRID groups (Cunningham et al., 2006). Number of new neurons in the granule layer of the dentate
gyrus were analyzed by two-way ANOVA with exercise history (Runner versus Sedentary), cocaine treatment (cocaine versus vehicle) and all interactions entered as factors. The correlation between distance traveled and number of new neurons was estimated by simple linear regression. The proportion of BrdU-labeled cells in the granule cell layer that co-expressed NeuN was analyzed by logistic regression, where proportion (binomial response) was modeled as a linear function of drug treatment (cocaine versus saline), exercise group (Runner versus Sedentary), and all interactions entered as factors.

Results

Baseline preference

During the pretest, before the animals ever experienced cocaine, and before any of the animals ran on wheels, animals spent approximately 50% of their time on each side, an average of 15.1 minutes (± 0.32 SE) on the HOLE texture. Running for 30 days slightly changed the baseline preference of Control animals, as was evidenced at CPP testing, when Control animals that had never received cocaine but had run showed a bias toward the HOLE texture that they had not displayed during the initial pretest. Sedentary Control animals that never received cocaine spent approximately 50% of their time, an average of 14.8 minutes (± 0.74 SE), on the HOLE texture, whereas Runners spent approximately 60% of their time, 18.0 minutes (± 0.74 SE) on HOLE ($F_{1,246} = 10.8, P = 0.001$). This bias in preference observed in Runners does not compromise analysis of cocaine CPP because the bias was present in both groups of Runners (CS+ GRID and CS+ HOLE) being compared to establish CPP (see Methods, CPP procedure).

Locomotor activity in CPP chambers

During conditioning, animals administered cocaine traveled an average of 56.7 meters (± 1.26 SE) per conditioning session, whereas animals administered saline traveled 35.0 meters (± 1.26 SE) per conditioning session ($F_{1,413} = 617.5, P < 0.0001$). Runners moved a similar distance in the apparatus as compared to Sedentary animals after saline or cocaine administration.

Wheel running

Running increased from days 1-20 and then maintained a plateau for remaining days (e.g., for experiment 1, day was significant, $F_{29,1311} = 23.7, P < 0.0001$) (Figure 2.2).
Cocaine had no influence on wheel running (Figure 2.2). Average level of running across all animals in all experiments was 6.2 kilometers (± 0.41 SE).

**Experiment 1: Running after CPP**

Sedentary animals displayed significantly stronger CPP than Runners, as indicated by a significant interaction between texture group (whether conditioned with cocaine on HOLE or GRID) and exercise group (Runner or Sedentary) \((F_{1,128} = 7.7, P = 0.007)\). In addition, CPP extinguished faster in Runners as compared to Sedentary animals as indicated by a significant interaction between exercise group and day \((F_{3,128} = 2.7, P = 0.046)\) (Figure 2.3A).

Posthoc analyses revealed that Sedentary animals displayed significant CPP on days 1-3 (all \(P < 0.05\)), whereas Runners displayed significant CPP only on day 1 \((P < 0.05)\). The strength of preference on day 1 was slightly lower in Runners as compared to Sedentary animals \((P = 0.08)\).

During cocaine priming, Sedentary animals displayed significantly stronger CPP than Runners, as indicated by a significant interaction between exercise group and day \((F_{3,114} = 4.4, P = 0.006)\) and a significant three-way interaction between exercise group, texture group and day \((F_{3,114} = 3.0, P = 0.035)\) (Figure 2.3B). Posthoc analyses indicated that Sedentary animals displayed significant CPP only on day 1 of cocaine priming \((P < 0.05)\), whereas Runners never displayed significant CPP during cocaine priming, and the strength of preference on day 1 was significantly lower in Runners as compared to Sedentary animals \((P = 0.03)\).

**Experiment 2: Running before CPP**

Runners displayed significantly stronger CPP than Sedentary animals, as indicated by a significant interaction between texture group and exercise group \((F_{1,128} = 10.3, P = 0.002)\). In addition, CPP tended to extinguish faster in Sedentary animals as compared to Runners, as suggested by an interaction between exercise group and day that approaches significance \((F_{3,128} = 2.5, P = 0.066)\) (Figure 2.3C). Posthoc analyses revealed that Runners displayed significant CPP on all days 1-4 (all \(P < 0.05\)), whereas Sedentary animals displayed significant CPP only on days 1-2 \((P < 0.05)\) and the strength of preference on day 2 was significantly lower in Sedentary animals as compared to Runners \((P = 0.027)\).
During cocaine priming, Runners displayed significantly stronger CPP than Sedentary animals, as indicated by a significant interaction between exercise group and day ($F_{3,128} = 5.2, P = 0.002$) and a significant interaction between texture group and day ($F_{3,128} = 5.8, P = 0.0009$) (Figure 2.3D). Posthoc analyses indicated that Runners displayed significant CPP on days 1-4 of cocaine priming ($P < 0.05$), whereas Sedentary animals displayed significant CPP only on the first day of cocaine priming, and the strength of preference on day 1 tended to be lower in Sedentary animals as compared to Runners ($P = 0.07$).

Experiment 3: Running before CPP with simulated relapse episode

Runners and Sedentary animals displayed significant CPP for all testing days, as indicated by a significant effect of texture group ($F_{1,128} = 46.1, P < 0.0001$) but no effects of exercise group or day or any interactions were significant (Figure 2.3E). During cocaine priming, Runners displayed significantly stronger CPP than Sedentary animals, as indicated by a significant interaction between exercise group and day ($F_{3,128} = 5.0, P = 0.003$) (Figure 2.3F). Posthoc analyses indicated that Runners displayed significant CPP on days 1-4 of cocaine priming ($P < 0.05$), whereas Sedentary animals displayed significant CPP only on the first day of cocaine priming, and the strength of preference on day 1 tended to be lower in Sedentary animals as compared to Runners ($P = 0.07$).

Hippocampal neurogenesis

Runners displayed approximately a 2-fold increase in the number of new neurons (BrdU cells co-labeled with NeuN) as compared to Sedentary animals in each of the 3 experiments (Experiment 1, $F_{1,24} = 29.7, P < 0.0001$; Experiment 2, $F_{1,26} = 31.3, P < 0.0001$; Experiment 3, $F_{1,32} = 23.0, P < 0.0001$) (Figure 2.4). Average distance traveled in the wheel over the 30 days in Runners was significantly correlated with number of new neurons ($F_{1,43} = 7.9, P = 0.007$). Cocaine had no effect on neurogenesis ($P > 0.05$). Analysis of logistic regression revealed that running significantly increased the proportion of BrdU cells that differentiated into neurons in all three experiments (Deviance = 4.6, $P = 0.03$). The percentage of BrdU cells that differentiated into neurons as indicated by co-labeling with NeuN was 92% ± 1.2 SE in Sedentary animals and 94% ± 0.8 SE in Runners. Cocaine had no effect on proportion of BrdU cells that differentiated into neurons, and no interactions with cocaine or experiment were
significant \((P > 0.05)\). Given the high percentage of BrdU cells that differentiated into neurons, results were similar when total number of BrdU cells was analyzed instead of total number of new neurons \(i.e., \) BrdU cells co-labeled with NeuN). Running approximately doubled the number of BrdU cells in the granule layer in each of the three experiments \(\text{Experiment 1}, F_{1,24} = 16.7, P = 0.0004; \text{Experiment 2}, F_{1,26} = 22.6, P < 0.0001; \text{Experiment 3}, F_{1,32} = 32.1, P < 0.0001\).

**Discussion**

The main finding from the study is that wheel running has opposing effects on cocaine CPP depending on when running is administered relative to drug conditioning (Figure 2.3). Results have implications for drug rehabilitation programs considering exercise as an intervention for maintaining abstinence \(\text{Sinyor et al., 1982}\). We speculate that when exercise is administered before drug conditioning, the plasticity engendered from running strengthens the learning of the drug-to-context association, such that when tested, the runners are resistant to extinction. This interpretation is consistent with many previous reports that running enhances learning and memory \(\text{e.g., van Praag et al., 1999; Colcombe and Kramer, 2003; Eisenstein and Holmes, 2007; Clark et al., 2008; Griffin et al., 2009; Creer et al., 2010}\). On the other hand, when exercise is administered after drug conditioning, the plasticity engendered from running cannot strengthen drug learning because the plasticity was increased after the drug learning took place. However, the plasticity from running could be recruited later to facilitate the new learning that the context is no longer associated with the drug, and hence accelerated extinction of CPP was observed in runners. Taken together, results suggest that exercise could be a useful intervention to facilitate extinction of conditioned drug associations during abstinence. However, the benefit of exercise could be reversed if a relapse episode occurs after running primes the brain for plasticity.

One of the important findings from the study was that running after drug conditioning no longer accelerated extinction of CPP for cocaine when a single conditioning session was administered after running (Figure 2.3E). The single additional conditioning trial abolished the benefits of exercise in facilitating extinction despite the fact that 75\% of conditioning occurred prior to exercise exposure, when no new plasticity generated from running could help in acquisition of cocaine-to-context learning. In
accordance with Smith et al.’s (2008) study, our results showed that even one drug conditioning day after exercise results in delayed CPP extinction, as compared to the results seen in studies where running combined with a complex environment precedes drug conditioning (Solinas et al., 2008; Chauvet et al., 2011). Moreover, the priming dose of cocaine elicited significant CPP across all cocaine priming days only in the animals that had run prior to cocaine conditioning (Figure 2.3D, F). The fact that these animals’ CPP never extinguished even during cocaine priming suggests that their drug-to-context learning was especially strong, possibly due to enhanced plasticity that was present at the time of drug conditioning, e.g., BDNF, IGF-1, angiogenesis, synaptogenesis (Neeper et al., 1995; Carro et al., 2001; Dietrich et al., 2008; Gomez-Pinilla et al., 2008; Clark et al., 2009).

When administered before conditioning, exercise strengthened cocaine CPP and delayed extinction (Figure 2.3C). As discussed above, this result is consistent with a vast literature demonstrating pro-cognitive effects of exercise on many different forms of learning and memory (e.g., van Praag et al., 1999; Colcombe and Kramer, 2003; Eisenstein and Holmes, 2007; Clark et al., 2008; Griffin et al., 2009; Creer et al., 2010). Although there are relatively few studies examining effects of exercise on CPP for drugs of abuse, there is no a priori reason to believe effects of exercise or enrichment would be any different for drug learning as opposed to other forms of associative learning. Consistent with this proposition and our results (Figure 2.3C), early studies found that housing Sprague-Dawley rats in an enriched environment (without running wheels) from weaning until adulthood strengthened CPP for amphetamine (Bowling and Bardo, 1994; Bardo et al., 1995), and a more recent study found that wheel running administered before conditioning strengthened CPP for cocaine in Long-Evans rats (Smith et al., 2008). However, two other studies found the exact opposite result (Solinas et al., 2009; Thanos et al., 2010). Solinas et al. (2009) recently reported that housing C57BL/6J mice from weaning until 2-3 months of age with toys and running wheels completely abolished cocaine CPP when conditioning and testing was administered after the enriched housing, as compared to mice housed in standard conditions, which displayed significant CPP. The Solinas et al. (2009) study is very similar to ours, with the same strain of mice and running wheels. The only difference between the Solinas et al. (2009) study and ours was
that their mice were housed in cages with toys and running wheels from weaning to adulthood, whereas our animals were housed only with running wheels starting in adulthood. However, we would have expected the longer duration of enrichment to produce a greater enhancement in CPP learning and retention, and certainly not to abolish cocaine CPP. Solinas et al. (2009) argued that enrichment abolished CPP not by altering learning but by reducing the rewarding effects of cocaine, which may be a possibility.

The other study that found that exercise before conditioning attenuated CPP for cocaine (Thanos et al., 2010) was conducted in adolescent rats and used forced treadmill running as the form of exercise. Hence, this study has numerous differences from our study, the most important of which is probably the forced running which can induce stress, and is not considered a rewarding form of exercise in animals (Greenwood et al., 2011).

Our speculation that plasticity from running facilitated learning in our experiments is consistent with our observation of increased adult hippocampal neurogenesis in Runners as compared to Sedentary animals in all three experiments (Figure 2.4). A role for the hippocampus in associative learning has been established (Ferbinteanu and McDonald, 2001; Fuchs et al., 2005; Rudy and Matus-Amat, 2005). Moreover, several papers have provided direct evidence that new neurons can enhance associative learning (Winocur et al., 2006; Wojtowicz et al., 2008; Hernandez-Rabaza et al., 2009; Drew et al., 2010). New neurons have been hypothesized to display greater ability to mold to new experiences than older established neurons because their processes and connections are not yet solidified (van Praag et al., 1999). Hence, it is conceivable that in the case where exercise is administered before conditioning, new neurons generated from running could be recruited during drug learning and lead to strengthening of associative learning, making the behavior more difficult to extinguish. Moreover, it is also conceivable that when exercise is administered after conditioning, new neurons could be recruited during extinction to more rapidly acquire the new association that the context is no longer paired with drug. However, at present, the connections between neurogenesis and the CPP outcomes are correlations only. Many other changes occur in the brain from exercise, including synaptogenesis, increases in trophic factors, growth factors, neurotransmitter concentrations, angiogenesis, and changes in dendritic morphology, among others that could account for our findings (Meeusen and De Meirleir,
Moreover, exercise is known promote resistance to stress, which could contribute to the behavioral outcomes depending on how stressful the CPP testing was perceived to be by the animals (Greenwood and Fleshner, 2008). One way to directly test the role of new neurons in the behavioral outcomes is to repeat the study with animals that are unable to generate new neurons from exercise, through use of irradiation or transgenic mouse models (Saxe et al., 2006; Clark et al., 2008; Dupret et al., 2008; Deng et al., 2009).

Previous literature suggests that cocaine exposure reduces adult hippocampal neurogenesis (Noonan et al., 2010; Sudai et al., 2011). However, we did not observe such a result in any of the three experiments (Figure 2.4). One explanation is the difference in duration of cocaine exposure between studies. In our study, animals were exposed to cocaine for a total of 8 days, 4 days during conditioning and 4 during cocaine priming, whereas cocaine exposure of up to 15 days is characteristic of studies that have found a significant cocaine-induced reduction in hippocampal neurogenesis. Moreover, most of the studies reporting a decrease in neurogenesis from cocaine employed operant conditioning methods, where the rats have to perform a lever press to receive an intravenous infusion of drug, as opposed to classical conditioning methods used here where animals are administered intraperitoneal injections (Noonan et al., 2010; Sudai et al., 2011).

The present study is the first to show an opposing effect of wheel running on cocaine CPP that depends critically on the timing of exercise relative to drug conditioning. The mechanisms by which running facilitates or extinguishes CPP are not known. One possible explanation is that plasticity generated from running, including new hippocampal units, could facilitate learning of drug-to context associations or learning that drug is no longer associated with context. A better understanding of the mechanisms that mediate effects of exercise on drug-to-context learning could lead to improved treatments for drug addiction.
Figure 2.1. Schematic diagram of the experimental design. The black arrows indicate when CPP conditioning sessions were administered. The white arrows indicate when CPP testing took place. The gray arrows indicate when CPP testing took place after a cocaine priming dose had been administered. The boxes indicate when the runner/sedentary conditions were administered relative to conditioning and CPP testing. Each experiment included 30 animals (20 conditioned with cocaine and 10 saline controls, divided equally into runner and sedentary groups). In all three experiments, animals experienced 1 day of habituation to reduce novelty effects, and 1 day of CPP pretesting to establish baseline texture preferences over the 2 days immediately preceding the conditioning (experiments 1 and 3) or runner/sedentary treatments (experiment 2). Animals in all three experiments experienced 30 days of uninterrupted running or sedentary treatment, and a total of 4 days of CPP conditioning. The three experiments differed in the timing of the four CPP conditioning sessions relative to the runner/sedentary treatment phase. Animals were returned to cages with or without running wheels immediately after conditioning and testing, to avoid the potential confound of animals experiencing withdrawal from running during the testing procedures.
Figure 2.2. Wheel running over the course of the study. Average distance run (km/day) (± SE) for a representative experiment (experiment 1), shown separately for cocaine-treated ($n = 10$, solid circles) and Control mice ($n = 5$, open circles) that never experienced cocaine. Wheel running data for experiments 2 and 3 were similar (data not shown). Increased wheel running over the first 20 days is typical for C57BL/6J mice.
Figure 2.3. Conditioned place preference for cocaine during testing and cocaine priming. Mean difference in duration (min) ± SE spent on the HOLE texture between animals receiving cocaine on HOLE texture (CS+HOLE) and animals receiving cocaine on GRID texture (CS+GRID) plotted separately for Runners and Sedentary animals. Each bar represents data for 10 animals (n = 5 CS+HOLE animals and n = 5 CS+GRID animals). A), C), and E) show testing data for experiments 1, 2 and 3, respectively whereas B), D), and F) show cocaine priming data for experiment 1, 2, and 3, respectively. The stars indicate significant place preference at \( P < 0.05 \).
Figure 2.4. Adult hippocampal neurogenesis. A) Photographs of the dentate gyrus stained for BrdU-DAB, showing representative sections from each of the four groups. Black dots are nuclei stained positive for BrdU indicating newly divided cells. B) Photographs of a representative section through the dentate gyrus of a Runner mouse double-stained green for NeuN (mature neuronal marker) and red for BrdU. Panels to the right show the tissue illuminated for each color separately and combined zoomed in around the BrdU cells, indicating two episodes of neurogenesis. C) Average number of new neurons per volume dentate gyrus shown as a percentage of the average Sedentary Control animal. Runners are shown as grey bars and Sedentary as white bars. Data are shown separately for Control animals (never treated with cocaine,  \( n = 5 \) Sedentary and 5 Runners) versus Cocaine-treated (\( n = 10 \) Sedentary and 10 Runners) for each experiment. Standard error bars are shown.
References


Aerobic exercise is the critical variable in an enriched environment that increases hippocampal neurogenesis and water maze learning in male C57BL/6J mice

Abstract

Previous studies have shown that housing mice with toys and running wheels increases adult hippocampal neurogenesis and enhances performance on the water maze. However, the relative contribution of running versus enrichment to the neurogenic and pro-cognitive effects is not clear. Recently, it was demonstrated that enrichment devoid of running wheels does not significantly enhance adult hippocampal neurogenesis in female C57BL/6J mice. However, novel toys were not rotated into the cages, and dietary enrichment was not included, so it could be argued that the environment was not enriched enough. In addition, only females were studied, and animals were group-housed, making it impossible to record individual running behavior or to determine the time spent running versus exploring the toys. Therefore, we repeated the study in singly housed male C57BL/6J mice and enhanced enrichment by rotating novel tactile, visual, dietary, auditory, and vestibular stimuli into the cages. Mice were housed for 32 days in one of 4 groups: running-only, enrichment-only, running plus enrichment, and standard cage. The first 10 days BrdU (bromodeoxyuridine) was administered to label dividing cells. The last 5 days mice were tested on the water maze, and then euthanized to measure number of BrdU cells co-labeled with NeuN (neuronal nuclear marker) in the dentate gyrus. Mice in the running-only group ran, on average, greater distances than animals in the running plus enrichment group. The combination of enrichment and running did not significantly increase hippocampal neurogenesis any more than running alone did. Animals in the running-only condition were the only group to show enhanced acquisition on water maze relative to standard cage controls. We confirm and extend the conclusion that environmental enrichment alone does not significantly increase hippocampal neurogenesis or bestow spatial learning benefits in male C57BL/6J mice, even when the modalities of enrichment are very broad.
Introduction

The idea that experiences during development and as an adult can have long-lasting effects on the chemistry, morphology and physiology of the brain with consequences for behavior and psychology is well established and has led to some of the great discoveries of modern neuroscience. For example, it is now widely believed that cognitive performance in humans is enhanced and protected against decline (associated with a variety of conditions including neurodegenerative disease, inflammation, brain trauma, and normal aging) by behaving in a way that challenges the brain with new learning and exploration experiences and/or by staying aerobically fit (Nilsson et al., 1999; Arendash et al., 2004; Colcombe et al., 2004; Griesbach et al., 2004; van Praag et al., 2005; Cruise et al., 2011; Kohman et al., 2011; Voss et al., 2011). However, the relative contribution of physical exercise versus stimulation from learning and new experiences to the cognitive benefits is not well understood (Kobilo et al., 2011).

Knowing how the different components of enrichment (e.g., physical exercise versus sensory exploration) influence the various domains of cognition (e.g., episodic memory, associative learning, spatial learning, executive control, long term memory, short term memory, working memory etc.) could help optimize or tailor treatments for the specific cognitive deficits presented in human subjects.

One common way to model experience-dependent plasticity in animals is to compare rodents housed in an enriched environment as compared to an environment that is deprived of sensory and motor stimuli. A plethora of studies using rats and mice has documented changes in the morphology and physiology of the brain using the environmental enrichment model (e.g., Volkmar and Greenough, 1972; Greenough et al., 1973; Greenough et al., 1978; Greenough et al., 1985; Black et al., 1987; Sirevaag et al., 1988; Black et al., 1991; Kempermann et al., 1997; Johansson and Belichenko, 2002; Kumar et al., 2012). An inevitable limitation in most of these studies is that multiple sensory, social, and physical activity factors are typically combined in the environmental enrichment treatment, making it difficult to determine which of the various enrichment factors or the interaction contributes to the observed effects on biochemistry, neurophysiology and morphology.
Previous studies have shown that housing mice either in an enriched environment that contains toys and running wheels or in an environment with only running wheels increases adult hippocampal neurogenesis and enhances performance on spatial learning tasks (Kempermann et al., 1997; van Praag et al., 1999a; van Praag et al., 1999b; Rhodes et al., 2003; Clark et al., 2008). However, the relative contribution of running versus the sensory stimulation from the toys to the increases in neurogenesis and cognition remains unclear. A study was recently conducted that for the first time attempted to separate the different components of enrichment to evaluate their effects on adult hippocampal neurogenesis (Kobilo et al., 2011). In that study, female C57BL/6J mice were housed in large cages in groups of 10. There were 4 treatment groups: 1) toys only (consisting of a constant set of toys and tunnels), 2) running wheels only (consisting of 10 running wheels), 3) toys and running wheels, and 4) empty cage without toys or running wheels. The mice housed with access to running wheels showed increased neurogenesis as compared to those without running wheel access. Neurogenesis levels of mice housed with both toys and running wheels were comparable to neurogenesis levels of mice housed with only wheels. Finally, mice housed with only toys displayed similar levels of neurogenesis as compared to those housed in an empty cage (Kobilo et al., 2011). Taken together, these data suggest that animals derived no neurogenic benefit from the addition of toys to their environment. However, one possibility is that mice habituated to the presence of the toys. Although the authors rearranged the toys spatially in the cage on a weekly basis, novel toys were never introduced and dietary enrichment was not included. In addition, the mice were group-housed, making it impossible to record individual running behavior or to determine the time spent running versus exploring the toys.

Therefore, we aimed to test the hypothesis that an enriched environment (without running wheels) can increase hippocampal neurogenesis if novel objects are continually introduced and the stimuli activate several different sensory modalities, including tactile, visual, dietary, auditory, and vestibular stimuli. We compared mice housed in a standard environment, an enriched environment (consisting of all the modalities listed above), running wheels only, or both enriched plus running wheels. Mice were singly housed so we could record time spent running and individual distance traveled to use as a covariate in the analysis of neurogenesis. Moreover, in the enriched plus running group, the cage
was divided with the enrichment stimuli on one side and the running wheel on the other, with a small partition through which a mouse could cross between the two compartments. This enabled us to record where the animals spent their time by video-tracking. With this approach, we attempted to ascertain the extent to which increases in neurogenesis can be attributed to time spent running on wheels versus engaging with other sensory stimuli (e.g., toys, dietary, vestibular, etc.). We predicted that we would find the highest levels of hippocampal neurogenesis and enhanced cognitive performance in the enriched environment plus running wheel group, followed by the running wheel-only group, the enriched environment-only group, and, finally, the group housed in the standard cage environment.

Methods

Animals

A total of 32 male C57BL/6J mice were used in this study. C57BL/6J was chosen because this strain has been widely used in studies of the effects of environmental enrichment and wheel running on adult hippocampal neurogenesis and water maze performance (van Praag et al., 1999a; van Praag et al., 2005; Clark et al., 2008). The mice were obtained at 5 weeks of age from The Jackson Laboratory (Bar Harbor, ME). Initially, they were housed 4 per cage in standard cages 29 x 19 x 13 cm (L x W x H) for 14 days. On day 15, mice (7 weeks of age) were housed individually in custom-made acrylic home cages with dimensions 67.0 cm x 18.5 cm x 16 cm (L x W x H) and clear plastic lids conducive for video tracking from above (Figure 3.1). A clear plastic divider with a small entrance large enough for a mouse to crawl through divided each cage in half so that each side measured 33.5 cm x 18.5 cm x 16 cm (L x W x H). Each mouse could freely move between both sides of its cage, and duration spent on each side was recorded by continuous video tracking (Figure 3.1). Animals in adjacent cages could see each other through the clear sides of the cages but could not otherwise interact. Mice were kept in a climate-controlled environment on a 12 h light/dark cycle (lights off at 10:00 a.m.). Food and water were delivered from both sides so that time spent on one cage side was not artificially inflated by time spent seeking food or water and so the mouse was visible in all areas of the cage when viewed from above (except under EE conditions as described below). Corncob bedding (Harlan 7097) was provided at
approximately 2 cm depth. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All measures were taken to minimize the number of mice used as well as the pain and suffering of the animals.

Treatment groups (see Figure 3.1)

1 Standard Environment (CONT)

Mice were singly housed in the custom-made cages described above. Both sides of the cage were empty except for bedding, regular mouse chow, and plain tap water.

2 Environmental Enrichment (EE)

Animals were housed in the same cages as above except one side of the cage contained bedding, toys, sweet drinking solutions, and food treats. The other side was empty except for bedding, regular mouse chow, and plain tap water. Certain toys were always present on the enriched side and never rotated. They were 1 plastic igloo, 1 wooden gnaw stick, cotton nesting material, a plastic ball that contained a bell, and a handful of straw. In addition, two of the following toys were rotated into the cage every 4 days in an attempt to engage multiple sensory modalities: auditory (ticking plastic clock, squeeze toy, rattle), visual (mirror, small dome), vestibular (see-saw, smooth winding tunnel), tactile (foam ball, small plastic hedgehog animal toy, towel piece, smooth tunnel, tunnel lined with bubble wrap, tunnel lined with Velcro material, and a tunnel lined with foam) (Fig 1). In addition, every 4 days, the animals received a new drinking solution provided in standard water bottles. They included: apple juice (11.7% sugar), cranberry juice (12.5% sugar), grape juice (16.7%), Kool-Aid (1 of 4 flavors: grape, lemonade, orange, or strawberry; 10.5% sugar), and a syrup consisting of 40% sucrose solution (wt/volume) in plain tap water (Suzuki et al., 2006). Finally, a novel solid food treat was provided every 4 days. Each rotation included one of the following: 5 peanuts, 5 cashews, 1 cracker, 5 Cheerios, 2 mini-carrots, 5 chocolate chips, 1 teaspoon of peanut butter, or 10 unroasted sunflower seeds. The treat was always provided in a plastic weigh-boat placed on top of the igloo.

3 Running wheels (RUN)

One side of the cage was empty except for bedding, regular mouse chow, and plain tap, whereas the other side, in addition to bedding, food and water, contained a
saucer-shaped running disc (Bio-Serv, Frenchtown, NJ, USA) tilted at an angle and attached to a Schwinn pedometer to monitor distance, speed, and cumulative duration of running. Pedometers were recorded and reset daily.

4 Environmental Enrichment with Running Wheels (EE+RUN)

One side of the cage contained toys, drinking solutions and food treats as described for the EE group, and the other cage side contained the saucer-shaped running disc as described for the RUN group.

Video Tracking

Eight video cameras mounted on the ceiling of the animal room provided continuous video feed into two separate computers running TopScan video tracking software (Clever Sys Inc, Reston, VA, USA). The video coverage allowed continuous monitoring of all 32 animals at the same time in 32 individual cages (as described above). White lights were placed under the tables holding the cages to produce diffuse light during lights on and were controlled with a timer for 12:12 L:D cycle. Red lights were placed in various positions in the room overhead to illuminate the cages during the dark phase for continuous video tracking. (Mice cannot see red light). The duration spent on each side of the cages was continuously recorded as defined by the location of the center of mass of the animals (1 mm resolution). In the EE condition (as described above), mice were video-tracked only on the empty side because of interference with tunnels and toys that could hide the mouse from view.

Experimental Design

Mice were housed under the 4 conditions (CONT, EE, RUN, or EE+RUN) for 32 days (n=8 per group). The first 10 days, mice received 50 mg/kg bromodeoxyuridine (BrdU) injections to label dividing cells. The last 5 days, mice were tested for performance on the water maze task. Testing took place during the light phase of the light/dark cycle. Animals were returned to cages immediately after testing. Hence, mice in the EE, RUN, and EE+RUN groups had continuous access to enrichment and/or running throughout the behavioral testing period. The following day after testing, animals were euthanized to measure adult hippocampal neurogenesis.

Behavioral Performance

Morris water maze
Mice were trained on water maze with 2 two trials per day for 5 days. A trial lasted either 60 s or until the mouse reached the platform and remained on the platform for 10 s. If a mouse did not reach the platform in 60 s, it was gently guided there by hand. Mice were placed back in their cage and allowed to rest for 30 s between trials. One hour after training on day 5, the platform was removed and mice were tested with a probe trial (60 s). Dimensions and parameters followed Clark et al. (2008). The maze consisted of a circular tub, 100 cm in diameter and 20 cm deep. A square platform made of white plastic mesh 8.5 x 8.5 cm was placed in the middle of one quadrant and submerged 0.5 cm below the surface of the water. Sixty ml of Crayola white tempera paint was added to the water to make the water sufficiently opaque to hide the platform from sight. White was chosen to provide contrast for video tracking from above (black mouse on white background). Water temperature was maintained at 25-26 ºC. Topscan (Clever Sys Inc, Reston, VA, USA) video tracking software was used to measure path length, swim speed, and duration spent in the different quadrants of the maze.

**Immunohistochemistry**

**Tissue preparation**

All the mice (n = 32) were anesthetized with 100 mg/kg sodium pentobarbital (i.p.) and then perfused transcardially with 4% paraformaldehyde in phosphate buffer solution (PBS; 0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were postfixed overnight and then transferred to 30% sucrose in PBS. Brains were sectioned using a cryostat into 40 µm thick coronal sections. Sections were placed into tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) in 24 well plates and stored at -20 ºC. Two separate one-in-six series of these sections (i.e. series of sections throughout the rostro-caudal extent of the brain with 240 µm increments separating each section, approximately nine sections) were stained in the following way.

**BrdU-DAB**

Purpose: To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free floating sections were washed in tissue buffering solution (TBS; 1.3% Trizma hydrochloride, 0.19% Trizma base, 0.9% sodium chloride) and then treated with 0.6% hydrogen peroxide in TBS for 30 min. To denature DNA, sections were treated for 120
min with a solution of 50% de-ionized formamide and 2X SSC buffer, rinsed for 15 min in 2X SCC buffer, then treated with 2 M hydrochloric acid for 30 min at 37 °C, then 0.1 M boric acid in TBS (pH 8.5) for 10 min at room temperature. Sections were then treated (blocked) with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus) for 30 min, and then incubated in primary antibody against BrdU made in rat (AbD Serotec, Raleigh, NC, USA, catalog number OBT0030) at a dilution of 1:100 in TBS-X plus for 72 h at 4 °C. Sections were then washed in TBS, blocked with TBS-X plus for 30 min, and then incubated in biotinylated secondary antibody against rat made in goat (Vector, Burlingame, CA, USA, catalog number BA-9400) at 1:250 in TBS-X plus for 100 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA, USA, catalog number PK-6100) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO, USA, catalog number D4418).

**Double-fluorescent label**

Purpose: To determine the proportion of BrdU-positive cells in the dentate gyrus that differentiated into neurons. Sections were treated as for BrdU-DAB except a cocktail was used for the primary antibody step. Rat anti-BrdU (1:100; AbD Serotec, Raleigh, NC, USA, catalog number OBT0030) was combined with mouse anti-neuronal nuclear protein (NeuN) (1:50; Millipore, Billerica, MA, USA, catalog number MAB377) for 72 h at 4 °C. Secondary antibodies were conjugated with fluorescent markers (Cy2-green anti-mouse, and Cy3-red anti-rat; Jackson ImmunoResearch, West Grove, PA, USA, catalog numbers 115-225-166 and 112-165-167, respectively) at a dilution of 1:250 and also delivered as a cocktail.

**Image Analysis**

**BrdU-DAB**

The entire granule layer (bilateral), represented in the 1-in-6 series, was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope and taking multiple photographs, via camera interfaced to computer, under 10x (total 100x) magnification. Following Clark et al. (2011), to obtain unbiased estimates of BrdU cell numbers, total counts were multiplied by 0.85, under the assumption that 15% of the nuclei counted would intersect with the plane of the section. This was estimated based on the observation that the average size of BrdU nuclei was 6
microns, which is 15% of 40 microns, the thickness of the section. In addition, the volume of the granule layer was measured by tracing the granule layer using ImageJ software (NIH, Bethesda, MD). A comparison of the volumes between groups assumes that there were no group differences in shrinkage of tissue.

*Double label*

A Leica SP2 laser scanning confocal microscope (using a 40x oil objective, pinhole size 81.35 µm diameter) was used to determine the proportion of dentate gyrus BrdU-positive cells that differentiated into neurons (NeuN+). Dentate gyrus BrdU-positive cells were identified as either co-expressing NeuN or not. Each BrdU-positive cells in the granular layer (represented in the 1-in-6 series) was analyzed by focusing through the tissue in the z-axis to establish colabeling with NeuN. The total number of new neurons per mouse was calculated as the number of BrdU cells multiplied by average proportion BrdU cell co-expressing NeuN for the designated group. Total number of new neurons per cubic micron was estimated as total numbers divided by volume sampled.

*Statistical analysis*

Data were analyzed using SAS (version 9.1) or R (version 2.7.2) statistical software. In all analyses, $P < 0.05$ was considered statistically significant. Average duration (hr) spent running and average distance run (km/day) were analyzed using 1-way ANOVA with treatment (RUN versus EE+RUN) entered as the factor. Average percent time spent on the EE side was analyzed by 1-way ANOVA with treatment (RUN versus EE+RUN) entered as the factor. Number of BrdU-positive cells, number of new neurons (BrdU+/NeuN+), volume of the dentate gyrus, and number of new neurons per volume, were analyzed using 2-way ANOVA with factors exercise (runner versus sedentary) and enrichment (enriched versus not). Tukey posthoc tests were used to evaluate pair-wise differences between group means. Numbers of new neurons and number of new neurons per volume in the EE+RUN and RUN groups were also analyzed by analysis of covariance, with average distance traveled as the covariate and treatment (EE+RUN versus RUN) as the factor, with the interaction also entered in the model. The proportion of BrdU-labeled cells in the granule cell layer that co-expressed NeuN was analyzed by logistic regression, where proportion (binomial response) was modeled as a
linear function of treatment condition (CONT, EE, RUN, EE+RUN). For water maze acquisition, average path length (m) and latency (sec) to reach the platform per day was analyzed by repeated measures analysis with days as the within-subjects factor and treatment (CONT, EE, RUN, EE+RUN) as the between-subjects factor. For the probe trial, duration (sec) in the target quadrant was analyzed using 1-way ANOVA with treatment (CONT, EE, RUN, EE+RUN) as the factor.

**Results**

**Body mass**

Average body mass of the animals at the end of the experiment was 24.5 g (± 0.28 S.E.). No differences between groups or differences from the beginning to the end of the study within individuals were detected.

**Wheel Running**

On average, over the 30 day period, mice in the RUN group spent 2.3 hrs/day (± 0.14) running and rotated the disc an equivalent of 6.5 km/day (± 0.49). Mice in the EE+RUN group ran 1.8 hrs/day (± 0.48), and rotated the disc an average of 5.6 km/day (± 1.50). These differences were not statistically significant (Figure 3.2).

**Environmental Enrichment**

Mice in the EE group spent an average of 81% (± 0.02) of their time during the 30-day period on the enriched side, whereas mice in the EE+RUN group spent an average of 68% (± 0.02) on the enriched side ($F_{1,14}=24.7, p=0.0002$) (see Figure 3.2E-2F). Fluid intake was similar in the EE and EE+RUN groups. On average, mice in the EE and the EE+RUN groups drank 10.9 ml (± 2.39) of apple juice, 6.08 ml (± 0.92) of cranberry juice, 8.24 ml (± 1.39) of grape juice, 8.92 ml (± 0.57) of 40% sucrose, and 10.29 ml (± 0.67) of Kool-Aid (collapsed across each flavor) per day. Mice always ate the treats provided within the 4 day rotations. This was accomplished usually within the first day. Many mice were observed moving the weigh boat to the igloo soon after it was placed in the cage.

**Hippocampal Neurogenesis**

Running produced a 2-fold increase in the total number of BrdU-positive cells and total number of new neurons (BrdU+/NeuN+) in both the RUN and EE+RUN groups relative to their control groups, CONT and EE. Environmental enrichment had no effect.
This was indicated by a significant main effect of running on total number of BrdU-positive cells ($F_{1,28}=30.3, p<0.0001$) and total number of new neurons ($F_{1,28}=33.6, p<0.0001$) but no significant effect of enrichment or the interaction between enrichment and running (Figure 3.3A-F). The RUN group displayed the highest numbers of new neurons, followed by EE+RUN, EE, and then CONT. All posthoc pair-wise differences between groups were significant ($p<0.05$) except EE versus CONT, and EE+RUN versus RUN.

The percentage of BrdU-positive cells differentiated as neurons as indicated by co-expression of NeuN (mature neuronal nuclear marker) and BrdU was 80% ($\pm 3.6$), 81% ($\pm 3.5$), 85% ($\pm 2.4$), and 81% ($\pm 2.5$) for the CONT, EE, RUN and EE+RUN groups, respectively. These differences were not statistically significant.

Running and environmental enrichment both increased the volume of the granule layer and the effects were additive. This was indicated by a significant main effect of running ($F_{1,28}=14.4, p=0.0007$) and enrichment ($F_{1,28}=6.0, p=0.02$) but no significant interaction. Running increased volume by approximately 20% whereas enrichment increased volume by approximately 12%. The EE+RUN group displayed the largest volume ($0.65 \, \mu m^3 \pm 0.041$), followed by RUN ($0.57 \, \mu m^3 \pm 0.035$), EE ($0.52 \, \mu m^3 \pm 0.015$), and then CONT ($0.46 \, \mu m^3 \pm 0.023$). Post-hoc comparisons indicated that EE+RUN volume was significantly larger than CONT and EE. No other pair-wise differences were significant.

Running increased the total number of new neurons per volume granule layer ($F_{1,28}=15.2, p=0.0006$). Environmental enrichment had no main effect. However, a significant interaction between running and enrichment was observed ($F_{1,28}=5.8, p=0.02$). The interaction was due to enrichment tending to decrease density of new neurons in runners whereas enrichment tended to increase density in sedentary animals. The RUN group displayed the greatest density of new neurons ($7.6 \pm 0.77$ new neurons per cubic mm x $10^3$), followed by EE+RUN ($6.0 \pm 0.45$), EE ($5.2 \pm 0.34$), and then CONT ($4.3 \pm 0.39$). Posthoc comparisons indicated that density of new neurons was significantly greater in RUN than CONT and EE groups. No other pair-wise differences were significant.
Average distance traveled on the disc was significantly correlated with numbers of new neurons in the RUN and EE+RUN groups \((F_{1,12}=26.3, \ p<0.0001)\) (Figure 3.3G). The slope of the relationship between distance run and total number of new neurons was similar between the two groups. After correcting for the influence of distance run by analysis of covariance, total number of new neurons remained similar between the RUN and EE+RUN groups. Average distance traveled on the disc was significantly correlated with numbers of new neurons expressed per cubic mm volume sampled in the RUN and EE+RUN groups \((F_{1,12}=19.4, \ p=0.0009)\). The slope of the relationship between distance run and numbers of new neurons per volume was steeper for the RUN than the EE+RUN group. This was reflected in a significant main effect of group \((F_{1,12}=5.1, \ p=0.04)\) and the interaction between group and distance \((F_{1,12}=9.4, \ p=0.01)\).

**Behavioral Performance: Morris Water Maze**

All animals learned the Morris water maze as indicated by decreasing path length \((F_{4,139}=28.6, \ p<0.0001)\) and latency \((F_{4,139}=25.0, \ p<0.0001)\) across days (Figure 3.4). A significant interaction between day and treatment group was detected for both path length \((F_{12,139}=3.1, \ p=0.0007)\) and latency \((F_{12,139}=2.7, \ p=0.003)\) to reach the hidden platform. The main effect of treatment was not significant. Animals in the RUN group showed the steepest learning curve as compared with the other groups, as indicated by significant posthoc differences between RUN and all the other groups on day 2 \((p<0.05)\). On the probe test, all groups spent significantly more time in the target quadrant as compared with any other quadrant \((p<0.0001\) for each group), but no differences between groups were detected.

**Discussion**

Results support and extend results from Kobilo et al. (2011) suggesting that aerobic exercise is the critical variable in environmental enrichment that increases adult hippocampal neurogenesis and performance on the water maze in male C57BL/6J mice. The implication for humans is that physical exercise and not simply mental stimulation may be necessary to enhance total number of new neurons in the hippocampus and to confer associated cognitive gains in the domain of spatial learning.

One of the novel features of our study was the ability to clearly record how much time the animals spent on the enrichment side versus the other side of the cage. We
discovered that when enrichment was provided, mice spent nearly all their time on that side (Figure 3.2E-F). When both a running disc and enrichment was provided, the mice spent slightly less time on the enrichment side, mostly because they were running on the other side. Many of the animals were observed to make nests in the igloo with the straw and cotton bedding, and therefore, the enrichment side was typically the designated sleeping quarters. Hence, it is not surprising that the majority of time was spent on that side. It was not possible to track individual interactions with the toys due to mice hiding in the toys and tunnels provided.

Mice in the EE+RUN group had access to a running wheel but failed to display the facilitated acquisition of the water maze that mice in the RUN group did (Figure 3.4A). This result may seem surprising given that both groups of animals had access to running wheels and ran, on average, at similar levels. Moreover, both groups displayed increased total numbers of new neurons relative to the CONT and EE groups. The finding of a significant post-hoc difference between the RUN and the other groups on day 2 of water maze testing is typical of the modest but significant pro-cognitive effects typically seen as a result of exercise on learning in the water maze and other tasks (Kempermann et al., 1997; van Praag et al., 1999a; van Praag et al., 1999b; Rhodes et al., 2003; Clark et al., 2008). However, given the small effect size, the results should be interpreted with caution. One possible interpretation is that the enhanced performance of the RUN group relative to the EE+RUN group in the water maze could be attributed to the increased density of new neurons. Mice in the EE+RUN group displayed slightly larger volume of the granule layer and fewer new neurons per volume than the RUN group, although these differences were not statistically significant by post-hoc tests. It is not clear what might have contributed to the slightly increased volume in the EE+RUN group relative to the RUN group, because total numbers of new neurons were the same between EE+RUN and RUN groups. It could be larger cells or more space between cells or more processes extending from the cell bodies (Redila and Christie, 2006). The EE+RUN group also displayed a significantly flatter slope than the RUN group for the relationship between distance run and number of new neurons per volume. An alternative possibility is that the new neurons in the RUN group could have displayed greater plasticity because they had not yet integrated into the circuitry as much, compared to the EE+RUN group, in which
new neurons could have been already heavily recruited into the processing of the enrichment stimuli (Clark et al., 2012).

Our study also included novel foods as an enrichment manipulation. While all mice received standard rodent chow as their primary diet, mice in the EE and EE+RUN groups also had access to special treats, such as sunflower seeds, peanuts, cashews, peanut butter, carrots, and juice (see Experimental Procedures). While there is evidence that a diet high in sucrose, fat, or calories decreases neurogenesis and hippocampal BDNF levels (Molteni et al., 2002; Lindqvist et al., 2006; Stangl and Thuret, 2009), it is not known how much dietary sucrose, fat, or calories are required to produce these effects. In addition, to the best of our knowledge, no one has documented a reduction of hippocampal neurogenesis due to a more varied diet. In fact, our rotation of treats included various items high in polyunsaturated fatty acids (cashews, peanuts, sunflower seeds), and there is some evidence that a mixed diet that includes polyunsaturated fatty acids may actually enhance hippocampal neurogenesis compared to an isocaloric control diet, at least in serotonin-transporter knock-out rats (Schipper et al., 2011). Therefore, we doubt that the diet component of our environmental enrichment obliterated a significant increase in enrichment-induced neurogenesis that would have been apparent without the dietary manipulation.

It was previously believed that running and enrichment increase neurogenesis via dissociable pathways (Kempermann et al., 1998; van Praag et al., 1999b; Olson et al., 2006). Running, it was thought, mainly increases proliferation of cells, whereas enriched environment increases the survival of cells that had already proliferated (Kempermann et al., 1998; van Praag et al., 1999b; Olson et al., 2006). However, recent studies have demonstrated that the proliferative effect of running is ephemeral and that running mainly increases neurogenesis by increasing the survival of cells, not by increasing proliferation (Kronenberg et al., 2006; Snyder et al., 2009; Clark et al., 2010; Fuss et al., 2010). Therefore, the interpretation of the present results, based on the updated literature, is that aerobic exercise is needed to increase the survival of new neurons in the dentate gyrus. Stimulation from toys and treats has no effect on neurogenesis or cognition, at least in C57BL/6J mice.
In our study, environmental enrichment devoid of running wheels failed to up-regulate hippocampal neurogenesis and improve cognitive performance, even when the modalities of the enrichment were broad. We concluded that aerobic exercise is required to increase neurogenesis and water maze performance in male C57BL/6J mice. However, environmental enrichment can be implemented in many different ways, and it remains a possibility that alternative stimuli or a different environment than that which was included here could produce a different outcome. For example, mice in our study were singly housed, and it is possible that effects of an enriched environment are contingent on interaction with cage mates (i.e. social enrichment) (Schapiro, 2002). However, the RUN group displayed increased hippocampal neurogenesis relative to the CONT despite both groups being singly housed. Furthermore, Kobilo et al. (2011) included large group housing in the enrichment condition and still failed to find an effect of enrichment on neurogenesis in female C57BL/6J mice.

The data in this study demonstrate the relative importance of running over sensory stimulation for increasing adult hippocampal neurogenesis and water maze learning, but that does not imply that enrichment without aerobic exercise has no effect on the brain. For instance, the original studies by Greenough and colleagues used large cages filled with toys without running wheels and found differences in blood vessel density, dendritic spines, and synapses in the visual cortex and cerebellum (Volkmar and Greenough, 1972; Greenough et al., 1973; Greenough et al., 1978; Greenough et al., 1985; Turner and Greenough, 1985; Greenough et al., 1986; Black et al., 1987; Sirevaag and Greenough, 1987; Sirevaag et al., 1988; Black et al., 1991). Therefore, despite the fact that we did not observe a neurogenic effect of environmental enrichment in our study, the possibility remains that environmental enrichment modulates other brain plasticity mechanisms capable of bestowing cognitive benefits. Future studies are needed to discover which brain mechanisms respond to environmental enrichment and whether they are required for gains in cognitive performance akin to those associated with running-induced hippocampal neurogenesis in this study.

In summary, we confirmed and extended the results of Kobilo et al. (2011) by finding that even rotating novel objects that stimulate various sensory modalities into enriched cages in C57BL/6J mice does not produce increases in neurogenesis or
performance enhancement on the water maze if the environment does not include the opportunity to run. Hence, the implication is that mere interaction with a stimulating environment does not confer the same neurogenic or cognitive benefits as voluntary aerobic exercise does.
Figures

Figure 3.1. Treatment groups: (A) standard control (CONT); (B) environmental enrichment (EE); (C) running (RUN); (D) environmental enrichment and running (EE + RUN).
Figure 3.2. Wheel running and duration spent on the EE side. (A) Average distance run on wheels (km) per day in the RUN and EE + RUN groups. (B) Average distance run on wheels (km/day) collapsed across the entire 32 day period in the RUN and EE + RUN
groups. (C) Average time spent running on wheels (h) per day in the RUN and EE + RUN groups. (D) Average time spent running on wheels (h/day) collapsed across the entire 32 day period in the RUN and EE + RUN groups. (E) Average percent time spent on the EE side per day in the EE and EE + RUN groups. (F) Average percent time spent on the EE side collapsed across the entire 32 day period in the EE and EE + RUN groups. Standard error bars shown.
Figure 3.3. Neurogenesis. Representative sections through the dentate gyrus stained for BrdU-DAB from a mouse in the (A) CONT, (B) EE, (C) RUN, and (D) EE + RUN groups. (E) Photographs of a representative section through the dentate gyrus of a mouse double-stained red for BrdU (bromodeoxyuridine) and green for NeuN (mature neuronal marker). The panels below show the tissue illuminated for each color separately and combined, zoomed in around the BrdU cell, indicating an episode of neurogenesis. (F) Average number of new neurons in the dentate gyrus in the four groups. Standard error bars shown. (G) Number of new neurons in the RUN and EE + RUN groups plotted against average distance run (km/day) across the 32 day study.
Figure 3.4. Water maze. (A) Average path length (m) to the hidden platform plotted against days with the four treatment groups plotted separately. (B) Average duration (s) spent in the target quadrant of the probe test in the four groups. Standard error bars shown.
References
adult C57BL/6J mice correlating with hippocampal neurogenesis. Hippocampus 20:364-376.


A comparison of wheel running versus environmental enrichment on extinction and reinstatement of conditioned place preference for cocaine in male C57BL/6J mice

(currently unpublished)
Introduction

Relapse is a prominent feature of drug addiction and constitutes a major obstacle to recovery (Volkow and Baler, 2013). Contextual cues that have come to be associated with drug use (e.g., drug paraphernalia, places where drugs are taken or people they are taken with) can serve as powerful triggers for relapse even after long periods of abstinence (Back et al., 2014; Perry et al., 2014). Therefore, finding interventions that help extinguish behavior elicited by drug-paired cues is critical for effective addiction treatment.

Recent evidence from some rehabilitation programs suggests that incorporating aerobic exercise into the life routine during abstinence can greatly improve long-term substance use outcomes (Brown et al., 2010; Roessler, 2010; Smith and Lynch, 2011). In one such study of human subjects, drug-dependent individuals who participated in a 12-week group exercise intervention had better substance use outcomes, including decreased propensity for relapse, than individuals who did not attend a majority of exercise sessions (Brown et al., 2010).

The conditioned place preference (CPP) paradigm has been used to model drug-context associations and approach behavior in rodents (Bardo and Bevins, 2000; Tzschentke, 1998; Tzschentke, 2007). Consistent with the human data, we recently reported that running after conditioning accelerates extinction of CPP in male C57BL/6J mice (Mustroph et al., 2011). We conditioned mice to a dose of 10 mg/kg cocaine and then housed mice for 30 days before testing either in standard cages that allowed no exercise beyond normal cage ambulation or with a freely accessible running wheel in the cage (Mustroph et al., 2011). CPP of runner mice extinguished faster than that of sedentary controls, and we found that this occurred in conjunction with increased hippocampal neurogenesis among runner mice (Mustroph et al., 2011).

While exercise appears to be a useful intervention to extinguish the salience of drug-paired cues, it requires high compliance and will not be a viable option for everyone. Individuals in poor physical shape or with low motivation to exercise would not be good candidates for an intervention centering around exercise. Environmental enrichment might be a useful alternative. In humans, enhancing environmental enrichment could include seeking out new experiences or learning opportunities, or developing a richer,
more complex sensory/learning environment. In rodent models, an enriched environment usually includes the addition of toys, tunnels, larger cages, opportunities for more complex social interactions, and often includes access to running wheels (Chauvet et al., 2009; Chauvet et al., 2011; de Carvalho et al., 2010; El Rawas et al., 2009; Solinas et al., 2008; Xu et al., 2007).

Recent evidence in rodents suggests environmental enrichment can reduce the strength of CPP to various drugs (Chauvet et al., 2009; Chauvet et al., 2011; de Carvalho et al., 2010; El Rawas et al., 2009; Solinas et al., 2008; Xu et al., 2007). However, in all of these studies, the environmental enrichment included running wheels. To the best of our knowledge, no study has examined the effectiveness of environmental enrichment alone, i.e. devoid of running wheels, at accelerating extinction of CPP for cocaine. Hence, it is not possible to determine from the existing CPP literature whether the reduction in CPP seen from enriched environments is due to running or other components of enrichment (e.g., toys, tunnels) that were included in these studies.

In our previous study, we only included running wheels, and we found accelerated extinction of CPP (Mustroph et al., 2012; Mustroph et al., 2011). However, that in and of itself does not imply that the exercise was the critical variable for achieving reduction in CPP, because the addition of the running wheel also added substantial sensory enrichment from the presence of the wheel. In other words, it is not clear whether we would have observed the same effect of accelerated extinction of CPP if we had included a treatment that was matched for the sensory experience of having a wheel but without the physical activity component. Some studies use a wheel that is prevented from rotating as a control for the non-aerobic, sensory component of a running wheel (Devaud et al., 2012; Lynch et al., 2010; Peterson et al., 2014; Zlebnik et al., 2014a; Zlebnik et al., 2014b). However, the degree of sensory stimulation from running may not be well-matched to (i.e. may exceed) a stationary running wheel. Clearly, presence of the locked wheel makes for a slightly enriched sensory experience as compared to an empty cage. However, a better control might be a complex environment that would provide more of a sensory experience than a stationary wheel would but that does not allow for the opportunity of physical exercise.
Hence, the goal of this study was to directly compare the effectiveness of environmental enrichment alone as compared to wheel running alone for accelerating extinction of CPP for cocaine. Because exercise is associated with a host of whole animal physiological outcomes (e.g., reduced body weight, reduced fat, higher aerobic capacity, increased physical strength, and coordination) that we believe impact the brain in ways that environmental enrichment without exercise does not (Ballor and Poehlman, 1994; Goldshmit et al., 2008; Green and Houston, 1975; Rubenstein et al., 2000; Wexler, 1983), we hypothesized that exercise would be the critical variable in accelerating extinction of CPP for cocaine. Moreover, while some of the physiological changes in the brain from exercise also occur from enrichment, other changes are specific to exercise. For example, both exercise (Dietrich et al., 2008; Eadie et al., 2005; Neeper et al., 1995; Neeper et al., 1996; Pysh and Weiss, 1979) and environmental enrichment (Birch et al., 2013; Kondo et al., 2012; Lauterborn et al., 2013; Leggio et al., 2005; Ramirez-Rodriguez et al., 2013) increase extracellular brain derived neurotrophic factor, spine morphology, dendritic branching, and synaptogenesis. One of the most dramatic changes in the brain from exercise is increased adult hippocampal neurogenesis. We and others have found that environmental enrichment alone does not increase neurogenesis, at least not to levels comparable to exercise (Kobilo et al., 2011; Mustroph et al., 2012). Exercise-induced neurogenesis provides a major source of plasticity, and convincing evidence in the literature shows that neurogenesis promotes decay of established memories (Akers et al., 2014). We believe that new neurons may also promote extinction of learned place association (Akers et al., 2014). Hence, we predicted that running in association with increased hippocampal neurogenesis would be required for accelerated extinction of cocaine CPP and that environmental enrichment alone would not be sufficient to reduce cocaine CPP.

Materials and methods

Animals

Eighty male C57BL/6J mice were obtained at 5 weeks of age (The Jackson Laboratory, Bar Harbor, ME) and housed 4 per cage in a climate-controlled environment on a 12 h light/dark cycle (lights off at 10:00 a.m.) for 14 days. C57BL/6J was chosen because this was the strain used in our previous study, where we reported wheel running
accelerated extinction of cocaine CPP when running was administered after conditioning (Mustroph et al., 2011). Dimensions of cages without running wheels were 29 x 19 x 13 cm (L x W x H). Mice were individually housed for 1 week before starting the experimental procedures. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All measures were taken to minimize the number of mice used as well as the pain and suffering of the animals.

*Drugs*

Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline and was administered at a dose of 10 mg/kg via intraperitoneal (i.p.) injections in a volume of 10 ml/kg. Dose was chosen based on the literature and was prepared according to the salt not the base form (Johnson et al., 2010; Mustroph et al., 2011; Zombeck et al., 2008).

*Place conditioning chambers*

The place conditioning chambers were modeled after Cunningham et al. (2006) and were the same as in previous studies from our laboratory (Johnson et al., 2010; Mustroph et al., 2012; Zombeck et al., 2008). They consisted of 20 identical black acrylic boxes (30 cm x 15 cm x 15 cm), with removable clear plastic tops. The floors were interchangeable and consisted of three types of distinct textures: HOLE, GRID, and HOLE/GRID. The HOLE/GRID floor consisted of half GRID and half HOLE textures. Distance traveled and location of mice within CPP boxes were recorded by TopScan video tracking software (Clever Sys Inc., Vienna, VA) (Johnson et al., 2010; Mustroph et al., 2012; Zombeck et al., 2008).

*Conditioned place preference (CPP) procedure*

We followed Cunningham et al.’s (2003; 2006) procedure. Runners, Environmentally Enriched, and Sedentary animals were counterbalanced with respect to the conditioned stimulus, and experienced cocaine on GRID (CS+GRID) or cocaine on HOLE (CS+HOLE) texture and saline on the alternate texture. During testing, animals explored the same size chamber as during conditioning, except with the HOLE/GRID floor type. Hence the animals were forced to spend time on either HOLE or GRID side, and duration on HOLE is equivalent to the total duration of the test (30 min) minus
duration on GRID (see Place Conditioning Chambers above). Conditioned place preference was determined by comparing the duration spent on HOLE (or GRID, statistics would be the same) between groups, CS+HOLE versus CS+GRID. The design ensures that any difference in duration spent on textures between groups (CS+GRID versus CS+HOLE) is due to drug-to-context learning, as this is the only variable that differs between the two groups. Biases in baseline preference for textures cannot produce false positives with this method because duration spent on one texture (HOLE or GRID) is compared between subgroups CS+GRID and CS+HOLE, both of which would be expected to display the bias if one developed. The two groups are also matched for drug exposure, which is important, because drug exposure itself could affect the development of biases in preference. Moreover, each group serves as the other group’s learning control, because both groups learn to associate one texture with cocaine and the alternate texture with saline. This is important because as compared to using a control, in which all animals receive saline on both textures, the experience of learning itself could bias preferences for the textures (Cunningham et al., 2003; Cunningham et al., 2006).

On the days of CPP habituation, pretesting, conditioning, testing, and cocaine priming (see below), mice were moved to a testing room, where lights were turned off at 10:00 A.M. Mice were kept in the room for one hour before testing began. Between sessions, the chambers were cleaned with disinfectant. Animals were returned to home cages with or without wheels immediately after testing. Hence, runners had continuous access to running wheels throughout behavioral testing except when in the conditioning chambers.

Habituation

To familiarize the mice with the place conditioning chambers, animals were placed on a flat surface without a texture in the conditioning chambers in the morning (1000 h; for 30 min) and in the afternoon (1600 h; for 30 min) for one day without any injection treatment (Cunningham et al., 2006).

Pretesting

To determine individual biases in preference for the textures prior to drug pairing, animals were weighed, received a 10 ml/kg i.p. saline (i.e. vehicle) injection, and were
immediately placed in the apparatus with HOLE/GRID floor in the morning (1000 h; for 30 min) and afternoon (1600 h; for 30 min) (Cunningham et al., 2006).

Conditioning

Four conditioned stimulus (CS+) trials (i.e., cocaine paired with one floor texture: HOLE or GRID) and four CS- trials (i.e. vehicle paired with the alternate floor texture) were administered over four days. Each day, one CS+ trial and one CS- trial was administered in the morning and afternoon. The order of exposure to CS+ and CS- was counterbalanced within each group. Animals were weighed, received an injection of 10 mg/kg i.p. cocaine (CS+ trial) or vehicle (CS- trial), and were immediately placed on the appropriate floor texture.

Testing

Testing took place twice daily: in the morning (1000 h; 30 min) and in the afternoon (1600 h; 30 min) for four consecutive days. Prior to each testing session, each mouse was weighed, injected i.p. with 10 ml/kg saline, and placed into the center of the HOLE/GRID conditioning chamber.

Cocaine priming

Cocaine priming began the day after the final CPP test session and consisted of two daily (morning and afternoon) 30 min exposures to the HOLE/GRID texture for four consecutive days. Animals were weighed and injected i.p. with 10 mg/kg cocaine immediately before being place into the HOLE/GRID conditioning chamber.

Experimental design

At 56 days of age, animals (n = 80 total) underwent habituation, pretesting, and cocaine CPP as detailed above (Figure 4.1). The day after the four conditioning days, mice were placed individually in cages either with running wheels (Runner, n = 27), toys (Environmentally Enriched, n = 27), or without running wheels or toys (Sedentary, n = 26) for 30 days. During the first 10 days of Runner, Environmentally Enriched, or Sedentary treatment, mice received daily i.p. injections of 50 mg/kg Bromodeoxyuridine (BrdU) to label dividing cells. After 30 days, mice underwent four consecutive days of CPP testing, followed by four consecutive days of CPP testing after cocaine priming. After testing each day, mice were returned to their cages with or without running wheels and with or without toys so that Runners had continuous access to wheels throughout the
testing period and Environmentally Enriched animals had continuous access to toys throughout the testing period.

*Treatment groups*

**Sedentary (SED)**

Mice in the sedentary group were singly housed in 36 x 20 x 14 cm (L x W x H) cages. The cages were empty except for bedding, regular mouse chow, and plain tap water. Mice assigned to the Sedentary group were deliberately not housed in cages with locked wheels because mice climb in locked wheels, and we intended to keep physical activity in the Sedentary group to a minimum (Koteja et al., 1999; Rhodes et al., 2003; Rhodes et al., 2000).

**Environmental Enrichment (EE)**

Mice in the environmentally enriched group were housed in the same cages as above except the cages also contained the same toys as in a previous enrichment paper from our lab (Mustroph et al., 2012). Certain toys were always present in the cage and never rotated. They were: 1 plastic igloo, 1 wooden gnaw stick, cotton nesting material, a plastic ball that contained a bell, and a handful of straw. In addition, two of the following toys were rotated into the cage every 4 days in an attempt to engage multiple sensory modalities: auditory (ticking plastic clock, squeeze toy, rattle), visual (mirror, small dome), vestibular (see-saw, smooth winding tunnel), tactile (foam ball, small plastic hedgehog animal toy, towel piece, smooth tunnel, tunnel lined with bubble wrap, tunnel lined with Velcro material, and a tunnel lined with foam).

**Running (RUN)**

Mice in the RUN group were housed in the same-sized cages as the Sedentary and Environmentally Enriched animals, with bedding, regular mouse chow, and plain tap water provided, except the cages also contained a 23 cm diameter wheel mounted in the cage top. Running wheel rotations were monitored continuously in 1 min increments via magnetic switches interfaced to a computer.

*Immunohistochemistry*

*Tissue preparation*

The day following behavioral testing, all the mice (*n* = 80) were anesthetized with 100 mg/kg sodium pentobarbital (*i.p.*) and then perfused transcardially with 4%
paraformaldehyde in phosphate buffer solution (PBS; 0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were postfixed overnight and then transferred to 30% sucrose in PBS. Brains were sectioned using a cryostat into 40 µm thick coronal sections. Sections were placed into tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) in 24 well plates and stored at -20 °C. Two separate one-in-six series of these sections (i.e. series of sections throughout the rostro-caudal extent of the brain with 240 µm increments separating each section, approximately nine sections) were stained in the following way.

*BrdU-DAB*

Purpose: To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free-floating sections were washed in tissue buffering solution (TBS; 1.3% Trizma hydrochloride, 0.19% Trizma base, 0.9% sodium chloride) and then treated with 0.6% hydrogen peroxide in TBS for 30 min. To denature DNA, sections were treated for 120 min with a solution of 50% de-ionized formamide and 2X SSC buffer, rinsed for 15 min in 2X SCC buffer, then treated with 2 M hydrochloric acid for 30 min at 37 °C, then 0.1 M boric acid in TBS (pH 8.5) for 10 min at room temperature. Sections were then treated (blocked) with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus) for 30 min, and then incubated in primary antibody against BrdU made in rat (AbD Serotec, Raleigh, NC, USA, catalog number OB0030) at a dilution of 1:100 in TBS-X plus for 72 h at 4 °C. Sections were then washed in TBS, blocked with TBS-X plus for 30 min, and then incubated in biotinylated secondary antibody against rat made in goat (Vector, Burlingame, CA, USA, catalog number BA-9400) at 1:250 in TBS-X plus for 100 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA, USA, catalog number PK-6100) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO, USA, catalog number D4418-505ET).

*Double-fluorescent label*

Purpose: To determine the proportion of BrdU-positive cells in the dentate gyrus that differentiated into neurons. Sections were treated as for BrdU-DAB, except a cocktail was used for the primary antibody step. Rat anti-BrdU (1:100; AbD Serotec, Raleigh, NC, USA, catalog number OBT0030) was combined with mouse anti-neuronal nuclear
protein (NeuN) (1:250; Millipore, Billerica, MA, USA, catalog number MAB377) for 48 h at 4° C. Secondary goat antibodies were conjugated with fluorescent markers (Cy2-green anti-mouse, Cy3-red anti-rat; Jackson ImmunoResearch, West Grove, PA, USA, catalog numbers 115-225-166 and 112-165-167, respectively) at a dilution of 1:250 and also delivered as a cocktail.

Image Analysis

BrdU-DAB

The entire granule layer (bilateral), represented in the 1-in-6 series, was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope and taking multiple photographs, via camera interfaced to computer, under 10x (total 100x) magnification. Following Clark et al. (2009), to obtain unbiased estimates of BrdU cell numbers, total counts were multiplied by 0.85, under the assumption that 15% of the nuclei counted would intersect with the plane of the section. This was estimated based on the observation that the average size of BrdU nuclei is 6 microns, which is 15% of 40 microns, the thickness of the section. In addition, the volume of the granule layer was measured by tracing the granule layer using ImageJ software (NIH, Bethesda, MD). A comparison of the volumes between groups assumes that there were no group differences in shrinkage of tissue.

Double-label

A Leica SP2 laser scanning confocal microscope (using a 40x oil objective, pinhole size 81.35 µm diameter) was used to determine the proportion of dentate gyrus BrdU-positive cells that differentiated into neurons (NeuN+). Dentate gyrus BrdU-positive cells were identified as either co-expressing NeuN or not. Each BrdU-positive cell in the granular layer (represented in the 1-in-6 series) was analyzed by focusing through the tissue in the z-axis to establish co-labeling with NeuN. The total number of new neurons per mouse was calculated as the number of BrdU cells multiplied by average proportion BrdU cell co-expressing NeuN for the designated group. Total number of new neurons per cubic micron was estimated as total numbers divided by volume sampled.

Statistical analysis
Data were analyzed using SAS (version 9.3) statistical software. In all analyses, $P < 0.05$ was considered statistically significant. Conditioned place preference data were analyzed the following way. First, the duration spent on the HOLE texture was analyzed by three-way repeated measures ANOVA with conditioned stimulus (CS+HOLE versus CS+GRID; between-subjects), treatment history (Runner versus Environmentally Enriched versus Sedentary; between-subjects), day of testing (1-4; within-subjects) and all interactions entered as factors. In previous papers from our lab (Mustroph et al., 2011), testing session (in this study, whether at 10:00 h or 16:00 h) was included as a factor in initial models but was never significant, and it was therefore removed from the final linear models. In addition, the CPP data were analyzed separately within each group using standard methods (Cunningham et al., 2006). Within each group, the duration spent on the HOLE texture was compared between the CS+HOLE and CS+GRID groups (Cunningham et al., 2006).

Number of BrdU-positive cells in the granule layer of the dentate gyrus, number of new neurons (BrdU+/NeuN+) in the granule layer of the dentate gyrus, volume of the dentate gyrus, and number of new neurons per volume, were analyzed using one-way ANOVA with treatment history (runner versus environmentally enriched versus sedentary) as a factor. Tukey posthoc tests were used to evaluate pair-wise differences between group means. Numbers of new neurons and number of new neurons per volume in the RUN group were also analyzed by analysis of covariance, with average distance traveled as the covariate. The proportion of BrdU-labeled cells in the granule cell layer that co-expressed NeuN was analyzed by logistic regression, where proportion (binominal response) was modeled as a linear function of treatment condition (SED, EE, RUN).

Results

Baseline preference

During the pretest, before the animals ever experienced cocaine, and before any of the animals ran on wheels or experienced toys, animals spent approximately 50% of their time on each side, an average of 15.1 minutes (± 0.37 SE) on the HOLE texture. Sedentary animals spent approximately 53% of their time, an average of 16.0 minutes (± 0.53 SE) on the HOLE texture, Environmentally Enriched animals spent approximately 47% of their time, an average of 14.1 minutes (± 0.47 SE) on the HOLE texture, and
Runners spent approximately 50% of their time, 15.2 minutes (± 0.51 SE) on the HOLE texture. There was no significant bias in preference observed in any of the groups, but even a bias would not compromise analysis of cocaine CPP because the bias would be present in both groups of, for example, Runners (CS+ GRID and CS+ HOLE) being compared to establish CPP (see Methods, CPP procedure).

**Locomotor activity in CPP chambers**

During testing, Runners, Environmentally Enriched, and Sedentary animals, when administered saline, traveled an average of 48.6 meters (± 0.81 SE) per testing session, whereas during cocaine priming, when administered cocaine, Runners, Environmentally Enriched, and Sedentary animals traveled an average of 86.80 meters (± 1.64 SE) per testing session. During testing and cocaine priming, Runners moved a similar distance in the apparatus as compared to Sedentary and Environmentally Enriched animals after saline or cocaine administration.

**Wheel running**

Running increased from days 1-24 and then maintained a plateau for the remaining days (e.g., day was significant, $F_{36,873} = 26.77, P < 0.0001$) (Figure 4.2). Average level of running across all animals was 3.5 kilometers/day (± 0.16 SE).

**CPP**

A significant main effect of texture ($F_{1,219} = 14.92, P = 0.0001$) was observed, indicating successful conditioning. In addition, a significant interaction between texture and day ($F_{3,219} = 7.71, P < .0001$) was observed (Figure 4.3A). Posthoc tests indicated that preference for Hole texture extinguished over the testing days in animals that had been trained with cocaine to Hole texture, whereas preference for Hole texture increased over the testing days in animals that had been trained with cocaine to Grid texture (i.e. extinction of preference for the drug-paired side was observed). There was no main effect of treatment, nor were there any significant interactions involving treatment. All groups showed significant CPP on the first day ($P < 0.05$ by t-tests for all groups). However, as indicated by t-tests only, the Sedentary and Environmentally Enriched groups exhibited significant CPP on days one and two of testing, suggesting a trend towards more rapid extinction of CPP in Runner animals.
During cocaine priming, CPP of Sedentary animals and Environmentally Enriched animals exhibited a trend towards stronger CPP than Runners, but this trend was not statistically significant. There was a trend towards a significant main effect of day (i.e. decreased CPP over the days of CPP priming) that approached but failed to reach statistical significance ($F_{3,219} = 2.22, P = 0.087$).

**Hippocampal neurogenesis**

Running increased neurogenesis, as indicated by a significant main effect of treatment on total number of BrdU-positive cells ($F_{2,33}=10.47, P=0.0003$) and total number of new neurons ($F_{2,33}=12.65, P<0.0001$). Posthoc comparisons indicated that total numbers of new neurons were significantly greater in the Runner than in the Sedentary and Environmentally Enriched groups. Runners displayed approximately a 1.5-fold increase in the total number of new (BrdU+) cells and new neurons (BrdU+/NeuN+) as compared to Sedentary and Environmentally Enriched animals (Figure 4.4). Animals in the Runner group displayed the greatest numbers of new neurons ($8.9 \pm 0.53$ new neurons per cubic mm x $10^3$), followed by animals in the Environmentally Enriched group ($6.2 \pm 0.53$ new neurons per cubic mm x $10^3$), and then animals in the Sedentary group ($5.3 \pm 0.53$ new neurons per cubic mm x $10^3$). Posthoc comparisons indicated that density of new neurons was significantly greater in the Runner than in the Sedentary and Environmentally Enriched groups. No other pair-wise differences were significant.

The percentage of BrdU-positive cells differentiated into neurons as indicated by co-expression of NeuN (mature neuronal nuclear marker) and BrdU was 91.4% ($\pm 3.5$), 94.1% ($\pm 4.1$), and 96% ($\pm 3.3$) for the Sedentary, Environmentally Enriched, and Runner groups, respectively. These differences were not statistically significant.

Average distance traveled in the wheel over the 30 days in Runners showed a trend towards being significantly correlated with numbers of new neurons ($F_{1,12}= 2.34, P = 0.1570$). When the outlier was removed, the correlation was significant ($F_{1,11}= 15.74, P = 0.0033$) (Figure 4.4).

Neither running nor environmental enrichment increased the volume of the granule layer, as indicated by lack of a significant main effect of treatment ($F_{2,33}=1.93, P = 0.16$). Animals in the Environmentally Enriched group displayed the largest volume of the granule layer ($0.60 \mu m^3 \pm 0.012$), followed by the Runner group ($0.59 \mu m^3 \pm 0.012$),
and then the Sedentary group (0.56 μm³ ± 0.012). These differences in volume of the granule layer of the dentate gyrus were not statistically significant.

**Discussion**

The main finding of this study is that environmental enrichment alone, devoid of running wheels, was not sufficient to accelerate extinction of CPP for cocaine in mice relative to animals housed in a standard cage environment. In previous chapters, we established that wheel running accelerates extinction of cocaine CPP, and indeed, the effect has been reproduced multiple times in our laboratory (Chapters 2, 5, and 6). However, in the present study, although we observed a trend of running enhancing CPP extinction, the effect was not statistically significant. We have some possible explanations for why the running effect was not as strong as it was in previous studies, but it is not because of low sample size, as we repeated the experiment twice and had at least 26 animals total in each group, hence plenty of statistical power relative to variability and effect sizes from previous studies.

In light of the non-significant findings in this study between treatments, we are hesitant to overinterpret our work. However, if one considers environmental enrichment as not effective and running from previous studies as effective at accelerating CPP extinction, then our findings support the view that environmental enrichment alone is not a suitable substitute for exercise for accelerating extinction of drug-context associations. Hence, the application for humans would be that engaging individuals in toys, games, and other forms of intellectual distraction may not be as effective as exercise in terms of reducing the strength of drug-context associations that underlie relapse to drug use. We believe that running creates a unique microenvironment in the brain that is not recapitulated by environmental enrichment alone. Our running effect seen repeatedly in our other studies (Chapters 2, 5, and 6) is consistent with previous studies showing that access to running wheels alone or environmental enrichment that includes running wheels facilitates extinction of CPP for cocaine (Chauvet et al., 2011; Mustroph et al., 2011; Rozeske et al., 2011; Solinas et al., 2008; Thanos et al., 2010) and self-administration of cocaine (Chauvet et al., 2009). The present study adds to these findings by suggesting that it is probably the running rather than the environmental enrichment that was the
critical variable necessary to facilitate extinction of CPP behavior in studies that employed environmental enrichment in conjunction with running wheels.

The reason for why we were not able to replicate the effect of running on extinction of CPP for cocaine (Mustroph et al., 2011) here is unclear. One thought we had was that because the animals were housed in cages with environmental enrichment, it took longer to capture these animals. Often, we needed to move multiple objects out of the way and chase the animals down. This was unavoidable, but it may have disturbed both the enriched mice as well as the running mice housed next to them, introducing more variability in the data.

Our present findings are consistent with the broader literature of environmental enrichment effects on CPP. One study that did include running wheels in the enrichment manipulation found that environmental enrichment did not reduce the rewarding effects of amphetamine (a drug with pharmacological mechanisms similar to cocaine) in the CPP paradigm (Thiriet et al., 2011). In this study, animals were exposed to environmental enrichment prior to conditioning. Accurate comparison between this study and ours requires careful consideration of the timing of the enrichment manipulation relative to conditioning. Our behavioral data is still consistent with Thiriet et al.’s (2011) because of our own previous observation (Mustroph et al., 2011) that access to running wheels before conditioning has the opposite effect on CPP as access to running wheels after conditioning. Therefore, we would expect environmental enrichment with running wheels to not reduce (and even to increase) CPP when it is implemented before rather than after conditioning, as in Thiriet et al.’s (2011) study. Our present results are also not inconsistent with studies that found environmental enrichment reduces CPP for cocaine (Zakharova et al., 2009) and heroin (El Rawas et al., 2009), because these studies also put mice in an enriched environment during early stages of life, before conditioning, rendering the findings not directly comparable to our study. The findings of these studies employing environmental enrichment in the context of the CPP paradigm are thus not contradictory to our data.

The trend for more rapid extinction of CPP from running than from environmental enrichment suggests that the physiology in the brain that results from exercise and accelerates extinction must be specific to running, i.e. it does not occur from
environmental enrichment. Running and environmental enrichment produce many similar changes in the brain, such as dendritic complexity, trophic support, vascularization, and LTP (Artola et al., 2006; Beauquis et al., 2010; Birch et al., 2013; Clark et al., 2009; Dietrich et al., 2008; Duffy et al., 2001; Eadie et al., 2005; Kondo et al., 2012; Lauterborn et al., 2013; Leggio et al., 2005; Neeper et al., 1995; Neeper et al., 1996; Pysh and Weiss, 1979; Ramirez-Rodriguez et al., 2013; van Praag et al., 1999a). One of the major plasticity changes that happens in the brain from running but not from environmental enrichment is adult hippocampal neurogenesis (van Praag et al., 1999a; van Praag et al., 1999b). Exercise-induced neurogenesis may be a mechanism contributing to the accelerated extinction of CPP, particularly since neurogenesis happens massively with exercise and not at all with environmental enrichment alone, as suggested in this study (Figure 4.4) and others (Kobilo et al., 2011; Mustroph et al., 2012). Exercise may contribute to accelerated CPP extinction because it sets up a cognitive reserve of new neurons that integrate into existing circuitry (Kempermann, 2008; Mirochnic et al., 2009). The reserve of new neurons built up during running may allow animals to better learn during testing that the previously drug-associated context is no longer associated with drug. The idea that exercise-induced hippocampal neurogenesis may underlie the accelerated extinction of CPP seen from exercise is in line with very recent research showing that postnatal hippocampal neurogenesis mediates degradation of established memories, which exhibits as forgetting (Akers et al., 2014). In this study, postnatal hippocampal neurogenesis was manipulated in adult mice by the naturalistic intervention we employ, voluntary wheel running, as well as (in separate experiments) by the pro-neurogenic drugs memantine and fluoxetine (Akers et al., 2014). Increased hippocampal neurogenesis enhanced forgetting of tasks that the authors had trained mice in, relative to sedentary, untreated or non-runner control mice (Akers et al., 2014). Conversely, when the authors blocked hippocampal neurogenesis, either via ganciclovir administration in the nestin-thymidine kinase mouse model or (in a separate experiment) the DNA alkylating agent temozolomide, they observed increased memory persistence of tasks they had trained mice on, relative to untreated control mice, suggesting that hippocampal neurogenesis promotes forgetting (Akers et al., 2014).
Consistent with our results that exercise promotes CPP extinction, during cocaine priming, animals with running wheels tended to display the least CPP reinstatement, even showing a slight aversion to the drug-paired texture, whereas sedentary and environmentally enriched animals trended towards a preference for the drug-paired texture. This result is consistent with the idea that running is uniquely protective against drug-context associations during drug priming. Our findings are consistent with another study that found that environmental enrichment does not reduce cocaine-induced reinstatement of CPP (Chauvet et al., 2009). Our data are also not inconsistent with a study that found that environmental enrichment prevents cocaine-induced reinstatement of CPP for cocaine (Solinas et al., 2008), because this study included running wheels in the environmental enrichment condition. Running wheels render the study’s environmental enrichment condition not directly comparable to our environmental enrichment condition. In this study, running wheels may have driven the reduction in reinstatement of CPP.

The possibility remains that we did not maximize enrichment in the present study and that this, rather than the fact that environmental enrichment does not up-regulate hippocampal neurogenesis, is why we did not observe a significant effect of environmental enrichment on CPP. For instance, our environmental enrichment condition lacked social contact between mice. However, we have evidence that the environmental enrichment we provided at least matches the sensory stimulation from a running wheel, and so if environmental enrichment alone is capable of reducing CPP, we should have observed an effect of environmental enrichment in this study. We have evidence that our enrichment condition is actually more interesting for mice than a blocked running wheel. Mice do not interact with a blocked wheel as much as they do with our toys; when mice are given access to two cage sides, one with the environmental enrichment and one with a running wheel, they spend 68% on the environmentally enriched cage side (Mustroph et al., 2012). This evidence contradicts the idea that our environmental enrichment condition was somehow lacking in enrichment and allows us to continue to favor the hypothesis that we did not observe a reduction of CPP from environmental enrichment in this study because environmental enrichment is incapable of inducing the same changes
as exercise in the brain. The necessary exercise-induced brain change for weakening drug-context associations and CPP may be increased hippocampal neurogenesis.

There is a need to conduct a direct test of the role of neurogenesis in CPP extinction. Future work should concentrate efforts on investigating the mechanisms by which running achieves its effect on CPP. This may allow us to identify alternative interventions that share this mechanism with running but that may themselves have higher compliance rates than exercise. The goal is to find an intervention for drug-context associations that has as broad an appeal as possible to individuals susceptible to relapse behavior.

Lastly, I would like to address the failure to replicate the running effect on CPP in the environmental enrichment experiment. One possible explanation for our failure to replicate the running effect on CPP in the environmental enrichment experiment is based on the anecdotal observation that taking the mice out of the enriched cages for CPP testing required disrupting the mice’s home cage environments and often involved removing the mouse from inside the plastic igloo and subsequently chasing it through toys. Furthermore, several mice in the enriched condition had to be dislodged from the winding tunnel by taking the tunnel apart into its component pieces before the mouse could be retrieved for CPP testing. This may have caused a series of disturbances loud and distressing enough for all mice, including runner mice, to perceive. In fact, locomotor activity during testing was, at an average of 48.7 m (± 0.81) per session, higher in this study than in a similar study we recently conducted, in which locomotor activity during testing averaged only 24.5 m (±0.43 SE) per session. Gremel and Cunningham (2007) and Vezina and Stewart (1987) found that increased locomotor activity during test sessions disrupts expression of CPP. In our study, mice were more active than usual during test sessions. On the basis of the work by Gremel and Cunningham (2007) and Vezina and Stewart (1987), we expected CPP in our animals to be decreased, because of the high locomotor activity exhibited during testing. Instead, we observed robust CPP, even in runner animals. The reason that the CPP of even runners stayed robust may be due to the cause of the increase in locomotor activity in our study. In Vezina and Stewart’s (1987) study, increased locomotor activity during the test session was due to increases in apparatus size at the time of testing, and in Gremel and Cunningham’s
(2007) study, increased locomotor activity during the test session was due to ethanol pretreatment in a mouse strain in which ethanol has substantial locomotor stimulating effects. In contrast, in our study, we think that increased locomotor activity during the test session was due to stress. Stress has a uniquely aggravating effect on drug-related behaviors in both humans and animals. In cocaine-dependent individuals, exposure to even a brief stressor increases feelings of cocaine craving and anxiety and is associated with greater likelihood of relapse (Sinha et al., 2003). In animals, stress has been shown to induce reinstatement of extinguished CPP, including CPP for cocaine (Bahi and Dreyer, 2014; Cruz et al., 2010; Karimi et al., 2014; Lu et al., 2002; Wang et al., 2000). In our study, the CPP-reinstating effect of stress may have overpowered the mitigating effects of exercise and of increased locomotor activity on CPP, resulting in a failure to observe reduced CPP from increased locomotor activity during testing, as the work of Gremel and Cunningham (2007) and Vezina and Stewart (1987) would have us expect. More importantly, stress may have resulted in our failure to replicate the effect of running on CPP behavior that we observed in three independent experiments (Chapters 2, 5, and 6). To remedy this shortcoming, in future experiments involving complex housing conditions, we will house mice in an empty “holding cage” for several hours prior to testing them for CPP to avoid the possible confound of extraneous stress impacting CPP test performance.
Figure 4.1. Schematic diagram of the experimental design. The black arrows indicate when CPP conditioning sessions were administered. The white arrows indicate when CPP testing took place. The grey bars indicate when CPP testing took place after a cocaine priming dose was administered. The boxes indicate when the sedentary/environmental enrichment/running conditions were administered relative to conditioning and CPP testing. The experiment contained 80 animals. Animals experienced 1 day of habituation to reduce novelty effects and 1 day of CPP pretesting to establish baseline texture preferences over the 2 days immediately preceding the conditioning. Animals in all 3 groups experienced 30 days of uninterrupted sedentary (n=26), environmental enrichment (n=27), or running treatment (n=27) and a total of 4 days of CPP conditioning. Animals were returned to cages with or without running wheels and with or without toys immediately after conditioning and testing to avoid the potential confound of animals experiencing withdrawal from running or environmental enrichment during the testing procedures.
Figure 4.2. Wheel running over the course of the study. Distance run (km/day) (± SE) for mice in the runner group. Increased wheel running over the first 20 days is typical for C57BL/6J mice.
Figure 4.3. Conditioned place preference for cocaine during testing and cocaine priming. Mean difference in duration (min) ± SE spent on the HOLE texture between animals receiving cocaine on HOLE texture (CS+HOLE) and animals receiving cocaine on GRID texture (CS+GRID) plotted separately for sedentary, environmental enrichment, and runner animals. Each bar represents data for the following animals: sedentary mice $n = 13$ CS+HOLE animals and $n = 13$ CS+GRID animals, environmental enrichment mice $n = 16$ CS+HOLE animals and $n = 11$ CS+GRID animals, and runner mice $n = 11$ CS+HOLE animals and $n = 15$ CS+GRID animals. A) shows testing data, whereas B) shows cocaine priming data. The stars indicate significant place preference at $P<0.05$. 
Figure 4.4. Adult hippocampal neurogenesis. Photographs of the dentate gyrus stained for BrdU-DAB, showing representative sections from each of the three groups from a mouse.
in the A) Sedentary B) Environmental Enrichment and C) Runner group. Black dots are nuclei stained positive for BrdU indicating newly divided cells. D) Photographs of a representative section through the dentate gyrus of a runner mouse double-stained green for NeuN (mature neuronal marker) and red for BrdU (bromodeoxyuridine). The panels below show the tissue illuminated for each color separately and combined, zoomed in around the BrdU cell, indicating an episode of neurogenesis. E) Total numbers of new neurons shown separately by treatment group (sedentary versus environmental enrichment versus runner). Standard error bars are shown. A significant effect of treatment was detected by one-way ANOVA, and total numbers of new neurons show a significant trend of exercise-induced neurogenesis. F) Number of new neurons in the Runner group plotted against average distance run (km/day) across the 30 days.
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CHAPTER 5

New neurons are not necessary for exercise to abolish conditioned place preference for cocaine

(Submitted to European Journal of Neuroscience)
**Introduction**

Relapse is a major obstacle to recovery from drug abuse and makes drug dependence a chronic problem even after long periods of abstinence (Somoza et al., 2013). Relapse is triggered, in part, by exposure to drug-associated cues, such as places where subjects used the drug, which can induce strong emotional reactions of drug craving (O'Brien et al., 1998; Volkow et al., 2006). In order to attenuate relapse, it would be useful to find interventions that extinguish drug-to-context associations.

Recent evidence suggests exercise can reduce the incidence of relapse in humans (Brown et al., 2010), but underlying mechanisms are not well understood. Exercise may act as a substitute reward (Sinyor et al., 1982) and/or facilitate extinction of learned associations between drug rewards and contextual cues. Rodent work supports the idea that exercise reduces the strength of drug-context associations. For example, Thanos et al. (2010) found that 6 weeks of exercise exposure prior to place conditioning with cocaine significantly attenuated conditioned place preference (CPP) in both male and female Lewis rats. Solinas and colleagues (2008) found that 30 days of exposure to an enriched environment that included running wheels after conditioning completely abolished CPP for cocaine in male C57BL/6J mice. In a previous study, we showed that exercise facilitates extinction of CPP for cocaine in male C57BL/6J mice when running wheels are made available after conditioning (Mustroph et al., 2011). Taken together, the human and rodent evidence suggest exercise may reduce relapse by facilitating extinction of drug-context associations, but the neurobiological mechanisms are not known.

The hippocampus is major site in the brain where effects of exercise, associative learning, and extinction converge. First, the hippocampus, specifically the dentate gyrus subregion, plays a critical role in binding information from different sensory modalities into unique memories, which are required for expression of CPP (Chauvet et al., 2011; Hernandez-Rabaza et al., 2008; Johnson et al., 2010; Meyers et al., 2003). fMRI studies in human subjects show that heavy drug users display bilateral activation of the hippocampus when they are shown images of drug paraphernalia (Ames et al., 2013; Michaelides et al., 2012). The hippocampus also plays an important role in extinction learning (Quirk and Mueller, 2008). For example, re-exposure to an environment to which mice had been previously conditioned results in significant activation of CREB-
mediated gene expression in the hippocampus (Mamiya et al., 2009). Moreover, extinction of CPP for cocaine is associated with acetylation of specific histone residues in the hippocampus (Malvaez et al., 2013).

In addition to its role in associative learning and extinction, the hippocampus, specifically the dentate gyrus, is also a major site of activation during an acute bout of exercise (Clark et al., 2011a) and a center for plasticity and remodeling in response to chronic exercise in humans and rodents. In the hippocampus, exercise has been shown to increase markers of neuroplasticity, including brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), FGF-2, EGF, VEGF, angiogenesis, gliogenesis, synaptogenesis, and neurogenesis (Clark et al., 2009; Dietrich et al., 2008; Gomez-Pinilla et al., 2008; Neeper et al., 1995). In randomized trials in humans, exercise increases the volume of the hippocampus, specifically the dentate gyrus (Biedermann et al., 2012; Chaddock et al., 2010; Erickson and Kramer, 2009; Erickson et al., 2011; Leavitt et al., 2013). Furthermore, such exercise-induced morphological changes in the hippocampus have consistently shown strong correlations with improvements in cognitive functions (Colcombe and Kramer, 2003; Creer et al., 2010; Griffin et al., 2009; O'Callaghan et al., 2009).

One of the most remarkable features of hippocampal remodeling in response to exercise is the generation of entire new granule neurons in the dentate gyrus (van Praag et al., 1999). Exercise-induced adult hippocampal neurogenesis is likely supported by many of the other physiological changes documented in the region, including increased vasculature (Clark et al., 2009), and concentration of growth and trophic factors (Gomez-Pinilla et al., 2008; Neeper et al., 1995). In mice, housing animals with access to a running wheel increases adult neurogenesis from 2 to 5-fold, depending on the strain, resulting in up to a 10% larger granule cell layer (Clark et al., 2011b). The magnitude of the increase in neurogenesis in response to exercise is greater than any other environmental stimulus known to enhance neurogenesis, including SSRIs (Kodama et al., 2004; Malberg and Duman, 2003; Malberg et al., 2000; Marlatt et al., 2013; Tanti et al., 2013), dietary restriction (Lee et al., 2000; Lee et al., 2002), learning a task (Ambrogini et al., 2000; Anderson et al., 2011; Curlik et al., 2013; Dalla et al., 2007; Gould et al.,
Several papers have provided direct evidence that new neurons enhance associative learning (Drew et al., 2010; Hernandez-Rabaza et al., 2009; Winocur et al., 2006). New neurons are hypothesized to represent highly plastic units because they have not yet fully integrated into existing circuitry (van Praag et al., 1999). Therefore, they have been posited to display greater ability to mold to new experiences than established, mature neurons (van Praag et al., 1999). As we have conjectured in a previous paper, when exercise is administered as an intervention in the CPP paradigm and implemented after conditioning, new neurons could be recruited during extinction and allow animals to more rapidly acquire the new association, presented during testing that context is no longer associated with drug (Mustroph et al., 2011). Having relatively more highly plastic units available at a time when the animal is learning that context is no longer associated with drug could facilitate extinction of place preference.

Computational modeling supports the idea that acquisition of a task in which novel aspects arise in a familiar context (such as re-learning that a previously drug-paired texture is no longer associated with drug) is enhanced by hippocampal neurogenesis (Garth et al., 2009; Wiskott et al., 2006). Moreover, in a study using mice, a lack of newborn hippocampal neurons resulted in a strong persevering preference for an old, unrewarded response in a hippocampus-dependent learning task, indicating failure to develop an appropriate new preference to a changed situation (Garth et al., 2009). In CPP testing, mice learn to abandon a previously acquired association in favor of a new association. Hence, it makes sense that new neurons might also contribute to CPP extinction.

Although prior studies have established a correlation between adult hippocampal neurogenesis and extinction of cocaine CPP (Mustroph et al., 2011), to the best of our knowledge, no previous study has directly tested the causal role of new neurons in extinction of CPP behavior. Clearly, there are many alternative mechanisms for how exercise could facilitate extinction of cocaine CPP besides new neurons, including altering the reward circuit in the brain, or increasing plasticity through new or modified synapses, glial cells, vasculature, metabolism, and other biochemical and physiological
alterations. Therefore, the goal of this study is to directly test the hypothesis that intact neurogenesis is required for exercise to abolish cocaine CPP using the nestin-thymidine kinase transgenic mouse model, in which neurogenesis is selectively reduced.

**Materials and Methods**

**Animals**

Eighty-two male Nestin-thymidine kinase (nestin-TK) transgenic mice were used in this experiment. Nestin-TK transgenic mice were originally obtained from Steven G. Kernie (Columbia University, Department of Pathology and Cell Biology). The nestin-TK mice express a modified version of the herpes simplex virus thymidine kinase (HSV-TK) driven by the nestin promoter and its second intron regulatory element (Yu et al., 2008). This transgenic mouse line was generated by pronuclear injection into fertilized murine eggs on a C57BL/6J genetic background (Yu et al., 2008). The mouse line allows temporally regulated, inducible ablation of early dividing neural progenitors by systemic administration of the pro-drug ganciclovir (Yu et al., 2008). Mice were bred in the Beckman Institute’s animal facility, where a colony has been established. Genotype of each mouse was verified by tail snip, followed by DNA extraction, PCR, and gel electrophoresis (with GADPDH used as control gene). Mice were weaned at 3 weeks and housed 4 per cage in a climate-controlled environment on a 12 h light/dark cycle (lights off at 9:00 a.m.) for 4 weeks. Dimensions of cages without running wheels were 29 x 19 x 13 cm (L x W x H) (Harlan Tekland, Madison, Wisconsin, USA). Mice were individually housed for 2 weeks before starting the experimental procedures and remained singly-housed throughout the experiment. Transgenic nestin-TK mice were used because previous work has established that pro-drug valganciclovir has no effect on hippocampal neurogenesis of non-transgenic (C57BL/6J) mice (Schloesser et al., 2009). All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All measures were taken to minimize the number of mice used as well as the pain and suffering of the animals.

**Experimental design**

At approximately 65 days of age (range: 59 days-71 days), all animals underwent habituation, pretesting, and cocaine CPP conditioning (see Conditioned place preference section below and Figure 5.1). During the first 10 days after cocaine CPP conditioning,
all mice received daily injections (i.p.) of 50 mg/kg Bromodeoxyuridine (BrdU) to label dividing cells. The day after the four conditioning days, mice were placed in cages either without (Sedentary) or with running wheels (Runner) and received either a diet of valganciclovir-infused (Val) or normal rodent chow (Control) for 28 days. Sample sizes were as follows: Control Sedentary \((n=26)\), Control Runner \((n=18)\), Val Sedentary \((n=20)\), Val Runner \((n=18)\). After 28 days, mice underwent four consecutive days of CPP testing. Runners had continuous access to wheels, and animals continued to receive their designated diets during these days of testing.

*Running wheels and sedentary treatment*

Dimensions of running wheel cages were 36 x 20 x 14 cm (L x W x H), with a 23 cm diameter wheel mounted in the cage top. Running wheel rotations were monitored continuously in 1 min increments via magnetic switches interfaced to a computer. Mice assigned to the Sedentary group were deliberately not housed in cages with locked wheels because mice climb in locked wheels and we intended to keep physical activity in the Sedentary group to a minimum (Koteja et al., 1999; Rhodes et al., 2003; Rhodes et al., 2000).

*Valganciclovir administration*

After the conditioning phase of the experiment was completed (see CPP section below), mice were switched from a diet of standard rodent chow (Harlan Teklad, Madison, Wisconsin, USA) provided *ad libitum* to a diet of rodent chow infused with valganciclovir (900 mg/kg dose) (Custom Animal Diets, Bangor, PA, USA) or control chow (Custom Animal Diets, Bangor, PA, USA), also made available *ad libitum*. Valganciclovir is the valine ester pro-drug of ganciclovir (Pescovitz et al., 2000). Upon ingestion, the vast majority of valganciclovir is rapidly converted to ganciclovir by hydrolysis (Jung and Dorr, 1999). Valganciclovir was used in this study because its bioavailability is 10-fold higher than that of ganciclovir (Pescovitz et al., 2000). Chow (valganciclovir-infused and control) was weighed every 7 days and replenished to maintain an *ad libitum* supply that would allow a suggested target valganciclovir consumption of 200 mg/kg (Blaiss et al., 2011).

*Drugs*
Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline and was administered at a dose of 10 mg/kg via intraperitoneal (i.p.) injections in a volume of 10 ml/kg. Dose was chosen based on the literature and was prepared according to the salt not the base form (Johnson et al., 2010; Mustroph et al., 2011; Zombeck et al., 2008).

Conditioned place preference
We used the same procedure as previously published by our lab (Johnson et al., 2010; Mustroph et al., 2011) based on Cunningham’s apparatus and experimental design (Cunningham et al., 2006).

Habituation
To familiarize the mice with the place conditioning chambers, animals were placed on a flat surface without a texture in the conditioning chambers in the morning (1000 h; for 30 min) and in the afternoon (1600 h; for 30 min) for one day without any injection treatment.

Pretesting
To determine individual biases in preference for the textures prior to drug pairing, animals were weighed, received a 10 ml/kg i.p. saline injection, and were immediately placed in the apparatus with HOLE/GRID floor in the morning (1000 h; for 30 min) and afternoon (1600 h; for 30 min).

Conditioning
Four conditioned stimulus (CS+) trials (i.e., cocaine paired with one floor texture: HOLE or GRID) and four CS- trials (i.e. vehicle paired with the alternate floor texture) were administered over four days. The assignment to HOLE or GRID was counterbalanced in each group. Each day, one CS+ trial and one CS- trial was administered in the morning and afternoon. The order of exposure to CS+ and CS- was counterbalanced within each group. Animals were weighed, received an injection of 10 mg/kg i.p. cocaine (CS+ trial) or vehicle (CS- trial), and were immediately placed on the appropriate floor texture.

Testing
Testing took place daily (morning and afternoon; 30 min) on days 29-32 after the last conditioning session. Prior to each testing session, each mouse was weighed, injected i.p. with 10 ml/kg saline, and placed into the center of the HOLE/GRID conditioning
chamber. All testing was conducted by experimenters blinded to the group assignment of the animals.

Immunohistochemistry

Tissue preparation—Following behavioral testing, all the mice \((n = 82)\) were anesthetized with 100 mg/kg sodium pentobarbital (i.p.) and then perfused transcardially with 4% paraformaldehyde in phosphate buffer solution (PBS; 0.287% sodium phosphate monobasic anhydrous, 1.102% sodium phosphate dibasic anhydrous, 0.9% sodium chloride in water). Brains were postfixed overnight and then transferred to 30% sucrose in PBS. Brains were sectioned using a cryostat in 40 µm thick coronal sections. Sections were placed into tissue cryoprotectant (30% ethylene glycol, 25% glycerin, 45% PBS) in 24 well plates and stored in -20°C. Two separate one-in-six series of these sections (i.e. series of sections throughout the rostro-caudal extent of the brain, with 240µm increments separating each section, approximately nine sections) were stained in the following ways.

**BrdU-DAB**

**Purpose:** To detect BrdU-positive (newly divided) cells in the dentate gyrus. Free-floating sections were washed in tissue buffering solution (TBS; 1.3% Trizma hydrochloride, 0.19% Trizma base, 0.9% sodium chloride) and then treated with 0.6% hydrogen peroxide in TBS for 30 min. To denature DNA, sections were treated for 120 min with a solution of 50% de-ionized formamide and 2X SSC buffer, rinsed for 15 min in 2X SSC buffer, then treated with 2 M hydrochloric acid for 30 min at 37 °C, then 0.1 M boric acid in TBS (ph 8.5) for 10 min at room temperature. Sections were then treated (blocked) with a solution of 0.3% Triton-X and 3% goat serum in TBS (TBS-X plus) for 30 min, and then incubated in primary antibody against BrdU made in rat (AbD Serotec, Raleigh, NC, USA, catalog number OB0030) at a dilution of 1:100 in TBS-X plus for 72 h at 4°C. Sections were then washed in TBS, blocked with TBS-X plus for 30 min, and then incubated in biotinylated secondary antibody against rat made in goat (Vector, Burlingame, CA, USA, catalog number BA-9400) at 1:250 in TBS-X plus for 100 min at room temperature. Sections were then treated using the ABC system (Vector, Burlingame, CA, USA, catalog number PK-6100) and stained using a diaminobenzidine kit (Sigma, St. Louis, MO, USA, catalog number D4418-505ET).
Double-fluorescent label

**Purpose:** To determine the proportion of BrdU-positive cells in the dentate gyrus that differentiated into neurons. Sections were treated as for BrdU-DAB except a cocktail was used for the primary antibody step. Rat anti-BrdU (1:100; AbD Serotec, Raleigh, NC, USA, catalog number OBT0030) was combined with mouse anti-neuronal nuclear protein (NeuN) (1:250; Millipore, Billerica, MA, USA, catalog number MAB377) for 48 at 4° C. Secondary goat antibodies were conjugated with fluorescent markers (Cy2-green anti-mouse and Cy3-red anti-rat; Jackson ImmunoResearch, West Grove, PA, USA, catalog numbers 115-225-166 and 112-165-167, respectively) at a dilution of 1:250 and also delivered as a cocktail.

**Image analysis**

*BrdU-DAB*—All image analyses were conducted with experimenters blinded to the assignment group of the animals’ tissue. The entire granule layer (bilateral), represented in the 1-in-6 series, was photographed by systematically advancing the field of view of the Zeiss brightfield light microscope and taking multiple photographs, via camera interfaced to a computer, under 10x (total 100x) magnification. Positively labeled cells in these photographs were counted to generate estimates of total numbers of labeled cells. The total volume of the dentate gyrus represented in the series was measured so that the counts could be expressed per µm³ dentate gyrus sampled.

*Double-label*—All image analyses were conducted with experimenters blinded to the assignment group of the animals’ tissue. A Leica SP2 laser scanning confocal microscope (using a 40x oil objective, pinhole size 81.35 µm diameter) was used to determine the proportion of dentate gyrus BrdU-positive cells that differentiated into neurons (NeuN+). Dentate gyrus BrdU-positive cells were identified as either co-expressing NeuN or not. Each BrdU-positive cell in the granule layer (represented in the 1-in-6 series) was analyzed by focusing through the tissue in the z-axis to establish co-labeling with NeuN. The number of new neurons per µm³ per mouse was calculated as the number of BrdU cells per µm³ multiplied by average proportion of BrdU cells co-expressing NeuN for the designated group.

**Statistical analysis**
Data were analyzed using SAS (version 9.2) statistical software. In all analyses, $P < 0.05$ was considered statistically significant.

**CPP**-- To evaluate whether the animals used in this study had an initial average bias for HOLE or GRID textures, the average duration (min) spent on Hole texture was compared to 15 min (half the duration of the test) using a one-sample t-test. Conditioned place preference data were analyzed the following way: First, the duration spent on the HOLE texture was analyzed by 4-way repeated-measures ANOVA with conditioned stimulus (CS+HOLE versus CS+GRID; between-subjects), exercise history (Sedentary versus Runner; between-subjects), diet received (Control versus Val; between-subjects), and day of testing (1-4; within-subjects) and all interactions entered as factors. Testing session, whether at 10:00 h or 16:00 h, was also included as a factor in initial models but was never significant and therefore was removed from the final linear models. Microanalysis of CPP on the initial test (day 1, a.m.) was analyzed by a similar repeated measures procedure except the within-subjects factor was bin number (1-6, consisting of duration on HOLE in 5 minute bins over the 30 min test). Posthoc comparisons of CPP were conducted using unpaired t-tests comparing CS+HOLE versus CS+GRID within Runner/Sedentary-Val/Control treatment groups.

**Body mass, food consumption, distance traveled in the CPP boxes, and wheel running**-- These variables (except wheel running) were analyzed by two-way ANOVA with exercise history (Sedentary versus Runner), diet received (Control versus Val), and all interactions entered as factors. Total wheel running distance traveled over the course of the study was analyzed by an unpaired t-test comparing Control versus Val.

**Neurogenesis**-- Total number of BrdU cells in the granule layer and total number of new neurons (BrdU cells co-labeled with NeuN) were analyzed by two-way ANOVA with exercise history (Sedentary versus Runner), diet received (Control versus Val), and all interactions entered as factors. The proportion of BrdU-labeled cells in the granule cell layer that co-expressed NeuN was analyzed by logistic regression, where proportion (binomial response) was modeled as a linear function of exercise history (Sedentary versus Runner), diet received (Control versus Val), and all interactions entered as factors.

**Results**

*Body mass*
Average body mass of the animals at the end of the experiment was 24.74 g (± 0.29 S.E.) in the Sedentary-Control group, 24.92 g (± 0.57 S.E.) in the Sedentary-Val group, 24.58 g (± 0.26 S.E.) in the Runner-Control group, and 24.00 (± 0.35 S.E.) in the Runner-Val group. No significant differences in body mass between groups or differences from the beginning to the end of the study within individual animals were detected.

Chow consumption and dose of Valganciclovir received

Sedentary animals ate 91% as much chow as runners ($F_{1,63}=3.43, P = 0.0011$). There were no differences in consumption of the valganciclovir chow versus normal chow (Figure 5.2A). Because they ate more chow, runners received an average daily dose of 215 mg/kg/day (± 4.2 SE), whereas sedentary animals received 187 mg/kg/day (± 5.4 SE) ad libitum valganciclovir consumption (Figure 5.2B).

Wheel running

Running increased over the first 2-3 weeks and thereafter maintained a plateau (Figure 5.2C). No differences in running were observed for animals fed valganciclovir chow versus normal chow. Runner mice receiving regular rodent chow ran 4.3 km/day (± 0.54 SE), and Runner mice receiving valganciclovir-infused rodent chow ran 4.2 km/day (± 0.49 SE).

Hippocampal neurogenesis

Running increased neurogenesis, as indicated by a significant main effect of exercise on total number of BrdU-positive cells ($F_{1,32}=22.5, P<0.0001$) and total number of new neurons ($F_{1,32}=19.1, P=0.0001$). Valganciclovir reduced neurogenesis, as indicated by a significant main effect of diet on total number of BrdU-positive cells ($F_{1,32}=40.99, P<0.0001$) and total number of new neurons ($F_{1,32}=42.7, P<0.0001$). A significant interaction between diet and running on total number of BrdU-positive cells ($F_{1,32}=6.12, P=0.0189$) and total number of new neurons ($F_{1,32}=5.9, P=0.0209$) was observed (Figure 5.4A-D). All posthoc pair-wise differences between groups were significant ($P<0.05$), except for Sedentary-Control vs. Runner-Val, and Sedentary-Val vs. Runner-Val. Levels of neurogenesis were reduced by a proportionally greater amount in Runners versus Sedentary animals. Among Sedentary animals, the valganciclovir-fed
mice displayed 42% as many cells as normal chow-fed mice. Among Runners, valganciclovir-fed mice displayed 35% as many cells as normal chow-fed mice.

The percentage of BrdU-positive cells differentiated as neurons as indicated by co-expression of NeuN (mature neuronal nuclear marker) and BrdU was: 83.5% (± 2.6), 81.3% (± 3.1), 87.8% (± 2.3), 88.0% (± 2.3) for the Sedentary-Control, Sedentary-Val, Runner-Control, and Runner-Val groups, respectively. Analysis of logistic regression revealed that running significantly increased the proportion of BrdU cells that differentiated into neurons (Deviance = 4.4, $P = 0.04$). Diet and the interaction between diet and running were not significant.

Neither running nor diet had a significant effect on volume of the dentate granule layer, nor were any significant interactions observed. Runner-Control animals displayed the largest volume (0.55 µm$^3$ ± 0.020), followed by the other groups: Runner-Val (0.50 µm$^3$ ± 0.020), Sedentary-Control (0.50 µm$^3$ ± 0.020), and then Sedentary-Val (0.49 µm$^3$ ± 0.020).

**CPP Baseline preference**

During the pretest, before the animals ever experienced cocaine, and before any of the animals ran on wheels, animals spent approximately 50% of their time on each side, i.e., there was no significant bias in preference observed in any of the groups.

**Locomotor activity in CPP chambers**

During testing, animals traveled an average of 24.4 meters (± 0.51 SE) per testing session. Runners moved a similar distance in the apparatus as compared to Sedentary animals, and animals receiving valganciclovir-infused chow moved a similar distance in the apparatus as compared to animals receiving control chow.

**CPP Testing**

Running completely abolished CPP in both animals fed normal control chow and valganciclovir-infused chow, whereas Sedentary animals fed either chow type displayed significant CPP that only extinguished by day 2 or 3 (Figure 5.3A). This differential extinction of CPP, completely absent in Runners versus present in Sedentary animals, was indicated by a significant interaction between texture group (whether conditioned with cocaine on HOLE or GRID), exercise history (Runner or Sedentary), and day of testing ($F_{2,144} = 9.51$, $P = 0.0001$). Posthoc analyses revealed that Sedentary animals fed
normal chow displayed significant CPP on days 1 and 2, which was extinguished by day 3. Sedentary animals fed valganciclovir chow displayed significant CPP on day 1, which was extinguished by day 2 and 3. Runners never showed significant CPP on any day. There was no significant main effect or any significant interactions involving diet (Val or Control rodent chow) on CPP behavior.

**Within-session extinction on test day 1**-- Microanalysis of the first CPP a.m. test session in 5-minute bins shows that Sedentary animals start out with significant CPP, whereas Runners do not, and that the level of preference is maintained across the 30 minute session, as was indicated by a significant main effect of exercise history (Runner or Sedentary) \( (F_{1,20} = 106.04, P < 0.0001) \) and no effect of bin number, diet, or any interactions (Figure 5.3B). This indicates that CPP was completely abolished in Runners, rather than extinguished during the first test.

**Discussion**

In our study, the transgenic runners fed valganciclovir displayed levels of neurogenesis below sedentary control levels (Figure 5.3C), even though they displayed normal levels of running (Figure 5.2C). This provided the ideal conditions for directly testing the hypothesis that increased neurogenesis from running is necessary for running to abolish cocaine CPP. The transgenic runners with compromised hippocampal neurogenesis displayed the same abolishment of CPP as runners with intact hippocampal neurogenesis (Figure 5.3A).

The main finding of the study is that elevated neurogenesis is not required for running to abolish cocaine CPP. This result is important because the functional significance of new neurons in behavior is currently a highly contested topic, and the mechanisms underlying benefits of exercise for reducing relapse in drug abuse are not well understood. With respect to implications for drug abuse treatment regimens, results suggest that increases in neurogenesis are not necessary for a treatment to have desirable outcomes.

There are several alternative explanations for why reducing neurogenesis had no significant influence on CPP behavior. None are mutually exclusive, and we do not believe the cumulative evidence necessarily favors one over the other. The first explanation is that exercise-induced neurogenesis plays no direct role in extinction of
cocaine CPP behavior from exposure to running wheels. Demonstration of CPP in our model requires that the animal forms a positive (rewarding) association between the conditioned stimulus (CS+) (the texture) and the unconditioned stimulus (US) (cocaine). The CPP task encompasses both reward and learning components. The test requires that the animal remembers that the CS+ was associated with the US for the 28 days between conditioning and testing. Hence, exercise could have—independently of neurogenesis—altered the perception of the reward from cocaine-associated cues without affecting learning per se, or it could have affected learning without affecting reward, or some combination of both. After 28 days, the animal might remember that the cues were associated previously with cocaine, but these cues might now be perceived as less valuable, possibly due to the substitution of the exercise reward for the cocaine reward (Fontes-Ribeiro et al., 2011; Lynch et al., 2013; Ozburn et al., 2008; Werme et al., 2000). In that case, changes in the brain underlying the exercise-induced abolishment of cocaine CPP might occur in classic reward circuit regions, such as the nucleus accumbens, lateral hypothalamus, extended amygdala, or ventral midbrain, not necessarily the hippocampus (De Chiara et al., 2010; Olsen, 2011; Shapiro et al., 2011; Werme et al., 2000; Zlebnik et al., 2014). In the current study, the idea that running acts as a substitute reward is supported because runners lack CPP even on Day 1 of testing (Figure 5.4A), suggesting that running blunted the rewarding effect of the cocaine cue. Moreover, the microanalysis of the first session indicated CPP was absent in runners even from the very beginning of the test (Figure 5.4B). There are many reasons to suppose why exercise might have altered reward. In line with this theory are the findings that in humans, exercise diminishes sensitivity to monetary rewards (Bothe et al., 2013) and reduces neuronal responses in brain regions consistent with reduced pleasure of food (Evero et al., 2012), findings that support the idea that exercise blunts the salience of alternative rewards in general. There is also biological evidence that exercise produces effects that substitute for drug reward. Exercise increases circulating endocannabinoid levels (Ferreira-Vieira et al., 2014; Heyman et al., 2012; Raichlen et al., 2013; Rola et al., 2004; Sparling et al., 2003). Furthermore, running induces similar changes in the brain as cocaine. In Lewis rats, 30 days of running and 7 days of i.p. cocaine administration (10 mg/kg, identical to the dose used in our study) both upregulate mRNA of the endogenous opioid dynorphin in medial
caudate putamen, part of the brain reward pathway, to comparable levels (Werme et al., 2000). This suggests a common mechanism of induction between running and cocaine and the possibility of common neuronal adaptations in brain regions to running and cocaine (Werme et al., 2000) that might allow running to substitute for a drug reward.

The hypothesis that running serves as a substitute reward explains the abolished CPP we observed from running in the present study. Evidence from our previous paper, however, favors the learning hypothesis over the reward substitution hypothesis. In our previous study, runner mice exhibited robust CPP on Day 1 of testing, but CPP then extinguished more rapidly than the CPP of sedentary mice (Mustroph et al., 2011). The facilitated extinction of CPP from running in that study—rather than its complete absence from the start—suggests that an exercise-induced process promoted learning over the course of testing in runners. Furthermore, in an experiment in which exercise was made available before conditioning, CPP was particularly robust, and did not extinguish even after 4 days, whereas CPP of sedentary animals extinguished by day 2 (Figure 5.4A) (Mustroph et al., 2011). If exercise was a reward substitute, then running should have weakened CPP regardless of whether it had been implemented before or after conditioning. On the other hand, if exercise affected CPP by enhancing plasticity and learning, then it should strengthen CPP when it occurs before learning (conditioning), but when it occurs after conditioning but before extinction, exercise should facilitate extinction learning during CPP testing, which is in fact what was observed (Mustroph et al., 2011). The current data suggesting that running reduced the salience of the drug reward cue prompted us to closely examine the behavioral results of our previous study. There is compelling evidence that, in addition to promoting acquisition of the new drug-cue association, which manifested as accelerated CPP extinction, running in our original study did blunt the salience of the drug reward cue. Runners in the earlier study exhibit a reduced CPP on Day 1 of testing compared to sedentary animals (Mustroph et al., 2011), suggesting that running substitutes as a drug reward. Likely, running in the present study blunted the reward of the drug-associated cue on test Day 1 to a large enough degree that the effect of running on learning during the subsequent testing days, in the form of accelerated CPP extinction, was not evident to us because there simply was no significant CPP to begin with.
The result that wheel running abolished CPP for cocaine, observed in the current study, differs from what has been suggested by our earlier study reporting wheel running merely accelerates—rather than completely abolishes—CPP extinction (Mustroph et al., 2011). The reason for the difference is not entirely clear. However, it is possible that the effect of wheel running may differ depending on the mouse strain, running output, or duration of wheel running before testing. Our earlier study (Mustroph et al., 2011) used non-transgenic C57BL/6J mice, not nestin-TK transgenic mice (on a C57BL/6J background). The site of integration of the transgene in the transgenic mice may explain in part why wheel running had a different effect on CPP in this study than it did in our previous study. Mice in our previous study also averaged more wheel running, namely 6.2 km/day (±0.41 SE) rather than 4.3 km/day (± 0.32 SE) and ran for 30 rather than 28 days.

Given the hippocampus’ central role in the conditioned associations thought to underlie drug addiction (Everitt and Robbins, 2005) and the growing understanding that it is a major locus in the brain for change induced from exercise (Carro et al., 2001; Clark et al., 2009; Dietrich et al., 2008; Gomez-Pinilla et al., 2008; Neeper et al., 1995), we strongly believe that plasticity in the hippocampus contributes to the observed effects of exercise on CPP behavior. This plasticity could be any brain changes that occur in response to exercise, including but not limited to increased extracellular brain-derived neurotrophic factor, spine morphology, dendritic branching, and synaptogenesis (Dietrich et al., 2008; Eadie et al., 2005; Neeper et al., 1995; Neeper et al., 1996). Adult hippocampal neurogenesis is only one mechanism by exercise in the brain.

Our results demonstrate that exercise can abolish cocaine CPP without new neurons, but it is still possible that new hippocampal neurons are preferentially involved to bring about this behavior in the same way that we know from lesion studies that the hippocampus is not necessary for contextual fear conditioning but is still preferentially involved when intact (Gewirtz et al., 2000; Matus-Amat et al., 2004; Sanders et al., 2003). As with all approaches that intend to reduce neurogenesis by inducing the apoptosis of newly dividing cells, side effects of our method as well as compensatory mechanisms are critical to consider when interpreting results. In our study, there were two possible outcomes of the experiment. Outcome #1: runner mice with reduced neurogenesis display
the same CPP as runner mice with intact neurogenesis. Outcome #2: runner mice with reduced neurogenesis display different CPP compared to runner mice with intact neurogenesis. We observed outcome scenario #1, which is the strongest possible result we could have observed, because it establishes that new neurons are not necessary for exercise to abolish cocaine CPP. Note that if we had observed outcome scenario #2, different CPP in runner mice in whom neurogenesis had been reduced, than in runner mice with intact neurogenesis, then we could have only tentatively concluded that new neurons are necessary for the behavioral outcome. This is because side effects or compensatory responses from the treatment used to reduce neurogenesis rather than the lost neurogenesis itself could have contributed to the behavioral outcomes. We speculate that many of the previous papers that used systemic administration of antimitotic agents (Doetsch et al., 1999; Shors et al., 2002) or focal irradiation (Mizumatsu et al., 2003) to reduce neurogenesis, including some of our own papers (Clark et al., 2008), may have found reduced task performance in treated animals because of enduring side effects related to inflammation or toxicity of the treatment used to reduce neurogenesis rather than because of the loss of neurogenesis per se (Singer et al., 2009). Previous papers suggest that ganciclovir can induce toxicity related to the way it is administered. Administering ganciclovir via chow appears to be the desired method, as opposed to i.p., s.c. via minipump, or i.c.v., administration techniques which can induce systemic toxicity or require implantation surgery, with risk of surgical complications (Singer et al., 2009). In our study, valganciclovir had no detectable influence on wheel running behavior (Figure 5.2C) or body mass (Figure 5.2D), and the mice displayed normal locomotor activity and CPP behavior (Figure 5.4A).

Our study and the more recent studies that are using highly specific methods to reduce neurogenesis are finding new neurons are not required for behavioral performance on a variety of hippocampus-involved tasks (Dupret et al., 2008; Groves et al., 2013; Saxe et al., 2006; Zhang et al., 2008). We believe the data obtained in our study along with the meta-analysis of other work (Groves et al., 2013) should be used to revise our thinking about the role of new neurons in behavioral performance. The revised view should consider the possibility that when animals are learning hippocampus-dependent tasks, that new neurons may be involved or even preferentially recruited if present, but if
new neurons are not available, older, established neurons can compensate and display sufficient plasticity required for learning.

Performance in the CPP test could require pattern separation. In a CPP test, many cues (the pattern of the walls, the size of the textured areas) are identical between the test compartments. The one subtle feature that differs is the floor texture. During testing, the animal must differentiate between the many similar and one differing feature. Dentate gyrus function is thought to play a critical role in pattern separation (Groves et al., 2013). A recent study and meta-analysis of the literature suggests that adult hippocampal neurogenesis is not essential for hippocampus-dependent spatial pattern separation (Groves et al., 2013). If hippocampal neurogenesis is not essential for pattern separation in the CPP task, it makes sense that runners with compromised hippocampal neurogenesis would perform equivalently on the CPP task as runners with intact hippocampal neurogenesis (Figure 5.4A).

Given the high incidence of relapse in drug abuse, effective treatment is likely going to require a large collection of life changes to be made. Nevertheless, any reduction in drug-cue strength may be helpful when recovering from drug abuse in an environment in which drug use occurred. Here, we provide evidence that exercise reduces drug-cue strength, and that it accomplishes this independently of new neurons. This finding is an important step in optimizing treatment regimens relevant for drug dependence, since it shows that maximizing neurogenesis from running will likely impart no additional benefit to treatment outcomes.
Figure 5.1. Schematic diagram of the experimental design. The black arrows indicate when twice-daily CPP conditioning sessions were administered. The white arrows indicate when CPP testing took place. The boxes indicate when the runner/sedentary conditions and valganciclovir/control chow were administered relative to conditioning and CPP testing. The experiment contained 82 animals (20 Sedentary mice receiving Valganciclovir, 18 Runner mice receiving Valganciclovir, 26 Sedentary mice receiving Control Chow, and 18 Runner mice receiving Control Chow). Animals experienced 1 day of habituation to reduce novelty effects and 1 day of CPP pretesting to establish baseline texture preferences over the 2 days immediately preceding the conditioning. Animals experienced 28 days of uninterrupted running or sedentary treatment and a total of 4 days of CPP testing. Animals were returned to cages with or without running wheels immediately after testing to avoid the potential confound of animals experiencing withdrawal from running during the testing procedures.
Figure 5.2. A. Chow consumption, valganciclovir dose received, wheel running, and body weights over the course of the study. Chow consumption (g/day) (± SE) shown separately for sedentary mice receiving control chow, runner mice receiving control chow, sedentary mice receiving valganciclovir chow, and runner mice receiving valganciclovir chow. Chow consumption was higher among runners. B. Valganciclovir dose received
(mg/kg/day) (± SE) shown separately for sedentary and runner mice receiving valganciclovir chow. Runner mice exceeded the desired dose of 200 mg/kg/day of valganciclovir. C. Distance run (km/day) (± SE) shown separately for mice receiving control chow and mice receiving valganciclovir-infused chow. Wheel running data for mice receiving control chow and mice receiving valganciclovir chow were similar. Increased wheel running over the first 18 days is typical for C57BL/6J mice. D. Body weights (g) (± SE) shown separately for sedentary mice receiving control chow, runner mice receiving control chow, sedentary mice receiving valganciclovir chow, and runner mice receiving valganciclovir chow during conditioning (i.e. before runner/sedentary and valganciclovir/control chow assignments were made) and during testing (i.e. at end of runner/sedentary and valganciclovir/control chow phase of the experiment). Body weights slightly increased similarly in all groups from conditioning to testing.
Figure 5.3. Adult hippocampal neurogenesis. A) Photographs of the dentate gyrus stained for BrdU-DAB, showing representative sections from each of the four groups. Black dots
are nuclei stained positive for BrdU, indicating newly divided cells. B) Photographs of a representative section through the dentate gyrus of a runner mouse receiving control chow double-stained green for NeuN (mature neuronal marker) and red for BrdU. Panels to the right show the tissue illuminated for each color separately and combined, zoomed in around the BrdU cells, indicating an episode of neurogenesis. C) Total number of new neurons shown separately by exercise group (sedentary versus runner) and diet group (control versus valganciclovir). Standard error bars are shown. Numbers of new neurons per dentate gyrus volume show a significant trend of exercise-induced neurogenesis (effect of running) and a significant reduction of neurogenesis due to diet (effect of valganciclovir). A significant interaction between exercise and diet was observed. D) Number of new neurons in runners plotted against average distance run (km/day) across the 28 days of uninterrupted running, graphed separately for runners receiving control chow and valganciclovir chow.
Figure 5.4. Conditioned place preference for cocaine during testing. A) Mean difference in duration (min) ± SE spent on the HOLE texture between animals receiving cocaine on HOLE texture (CS+HOLE) and animals receiving cocaine on GRID texture (CS+GRID) plotted separately for sedentary animals and runners receiving control chow or valganciclovir chow. Each bar represents data for the following animals: sedentary mice
receiving control chow \( n = 13 \) CS+HOLE animals and \( n = 13 \) CS+GRID animals, sedentary mice receiving valganciclovir chow \( n = 10 \) CS+HOLE animals and \( n = 10 \) CS+GRID animals, runner mice receiving control chow \( n = 9 \) CS+HOLE animals and \( n = 9 \) CS+GRID animals, and runner mice receiving valganciclovir chow \( n = 10 \) CS+HOLE animals and \( n = 8 \) CS+GRID animals. The stars indicate significant place preference at \( P < 0.05 \). B) Mean difference in duration (min) ± SE spent on the HO\(_{\text{LE}}\) texture between animals receiving cocaine on HO\(_{\text{LE}}\) texture (CS+HO\(_{\text{LE}}\)) and animals receiving cocaine on GRID texture (CS+GRID) during the first CPP testing session plotted in 5-minute intervals and separately for sedentary animals and runners receiving control chow or valganciclovir chow. Each point represents data for the following animals: (grey circles) sedentary mice receiving control chow \( n = 13 \) CS+HOLE animals and \( n = 13 \) CS+GRID animals, black circles) sedentary mice receiving valganciclovir chow \( n = 10 \) CS+HO\(_{\text{LE}}\) animals and \( n = 10 \) CS+GRID animals, (white triangles) runner mice receiving control chow \( n = 9 \) CS+HOLE animals and \( n = 9 \) CS+GRID animals, and (grey triangles) runner mice receiving valganciclovir chow \( n = 10 \) CS+HO\(_{\text{LE}}\) animals and \( n = 8 \) CS+GRID animals.
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Identifying neuropeptides differentially induced in sedentary versus runner male C57BL/6J mice in the amygdala and hippocampus after exposure to a drug-associated context

(currently unpublished)
Introduction

Neuropeptides, hormones, and cytokines modulate many behaviors by influencing the activity of neural circuits (Fekete and Lechan, 2006), and they are expected to play a role in running-induced responses to drugs (Fricker and Sweedler, 2010; Romanova et al., 2009). One mechanism by which running accelerates the extinction of drug-to-context associations may be by modifying expression of neuropeptides. Knowledge of specific neuropeptides induced in the amygdala and hippocampus in response to a context previously associated with drug is therefore essential. It will allow for targeting of these peptides in the search for more effective interventions for drug dependence and relapse to drug use. The objective of this Chapter was to determine whether we can detect any neuropeptides, defined as species with a distinct mass peak and a mass between 600 and 5000 Daltons, or neuropeptide profiles that are differentially induced by cocaine conditioning and exercise in the hippocampus and amygdala using an unbiased peptidomics technology. Our approach employed matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on brain samples collected from the hippocampus and amygdala immediately after mice were exposed to a cocaine- or vehicle-associated context.

The time point of sacrifice was chosen to capture peptide release following exposure to the conditioned stimulus (CS+). It is important to note that relative abundances of peptides are measured, not peptide release. However, changes in response to an acute stimulus (i.e., within 5-10 minutes) that do not involve transcription and translation are probably due to movement of the molecules from inside the cell, where they are relatively stable, to outside the cell, where they are easily degraded.

Our MALDI-TOF MS approach is discovery-driven because it allows us to identify novel neuropeptides. The rationale was that successful completion of the proposed research will contribute new information regarding peptide regulation under different running exposures in key brain regions involved in CPP for cocaine (Ferbinteanu and McDonald, 2001; Fuchs et al., 2005; Rudy and Matus-Amat, 2005). Our expectation was that a distinct set of neuropeptides in the amygdala and hippocampus that are modulated by cocaine conditioning and running and that occur in parallel with CPP would be uncovered. Such a finding will be important because it will
identify novel and much-needed molecular targets for interrupting the drug-to-context associations that are thought to contribute to drug dependence and relapse.

Running induces robust peptide changes in the brain. For instance, running increases expression of dynorphin in brain reward pathways (Brene et al., 2007). Interestingly, cocaine increases mRNA expression of dynorphin in brain reward pathways to a comparable degree as exercise, so the reward value of running and cocaine may actually be comparable (Werme et al., 2000). A study from the Rhodes laboratory showed that amygdala expression of c-Fos (and presumably downstream peptide expression as well, given that c-Fos is a transcription factor) in response to exposure to a previously cocaine-paired context was negatively correlated with animals’ CPP for cocaine (Zombeck et al., 2008).

Our experimental design included the amygdala and hippocampus as brain regions from which samples were collected because the amygdala and hippocampus share neural connections. The amygdala directly projects to the hippocampus and vice versa, and both brain regions are implicated in context conditioning (Amaral, 1986; Ito et al., 2008; Phillips and LeDoux, 1992). PET and fMRI studies in people have shown that cue-elicited craving activates both amygdala and hippocampus (Volkow et al., 2004). Furthermore, cocaine users exposed to drug paraphernalia exhibit increased amygdala blood flow (Childress et al., 1999; Grant et al., 1996; Kiltz, 2001). In addition, the primate amygdala has been shown to represent the positive value of stimuli during learning (Paton et al., 2006). Moreover, the amygdala (specifically, the basolateral amygdala) plays a key role in appetitive place conditioning (Everitt et al., 1991; Ito et al., 2006), of which CPP is one form. Specifically, the amygdala is required for acquisition and reconsolidation of cocaine-associated cues in CPP (Lee et al., 2005; Lee et al., 2006), and as such, is a clear candidate brain region for examination of peptide changes induced by cocaine conditioning. The basolateral amygdala was chosen as the region for sampling based upon its established role in CPP (Everitt et al., 1991; Ito et al., 2006). The dentate gyrus of the hippocampus was selected for sampling, given it is where hippocampal neurogenesis occurs (van Praag et al., 1999a; van Praag et al., 1999b), and hippocampal neurogenesis is a key plasticity change induced by exercise.
I sought to determine whether differences in conditioned place preference for cocaine between animals that remained sedentary and animals that had had access to running wheels for 30 days after conditioning are associated with changes in levels of detectable peptides in the hippocampus and amygdala. It was our expectation to find changes in levels of detectable peptides or—if specific peptide peaks could not be identified—a different peptide expression profile (of multiple peptide peaks) between sedentary and runner animals in the hippocampus. With respect to effects of re-exposure to a cocaine-associated context, I expected to find changes in levels of detectable peptides in the amygdala between animals re-exposed to a cocaine-associated context and animals re-exposed to a saline-associated context before sacrifice. In the absence of changes in levels of detectable peptides in the amygdala between animals re-exposed to cocaine-associated context and animals re-exposed to a saline-associated context before sacrifice, it was my expectation that we would observe a different peptide expression profile (of multiple peptide peaks) between animals re-exposed to a cocaine-associated context and animals re-exposed to a saline-associated context before sacrifice.

Since re-exposure to a cocaine-associated context has been shown to re-invoke the same brain changes and behavior as the actual experience of acute cocaine, including expression of Fos in the amygdala and locomotor activity (Brown et al., 1992), I would expect specific peptides to be up- or down-regulated in the amygdala in response to a cocaine-paired context. I would expect peptides to be up- or down-regulated in runner animals re-exposed to a cocaine-associated context relative to animals that remained sedentary, and I would expect these peptides up- or down-regulated in the hippocampus to be protective against the effects of the changed expression levels of peptides in the amygdala induced by re-exposure to a cocaine-associated context, because the hippocampus is known to project to the amygdala and to modify eventual amygdala output (Huebner et al., 2014). I would not expect re-exposure to a saline-associated context to lead to peptide expression levels in the amygdala between sedentary and runner animals, but I would expect to see differences in peptide expression levels or peptide expression profiles in the hippocampus between sedentary and runner animals.

Materials and Methods
**Animals**

Male C57BL/6J mice (n=48) were obtained at 5 weeks of age (The Jackson Laboratory, Bar Harbor, ME) and housed 4 per cage in a climate-controlled environment on a 12 h light/dark cycle (lights off at 10:00 a.m.) with food and water available *ad libitum* for 10 days. Dimensions of cages were 29 x 19 x 13 cm (L x W x H). Mice were individually housed for 9 days before starting the experimental procedures. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All measures were taken to minimize the number of mice used as well as the pain and suffering of the animals.

**Drugs**

Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was dissolved in 0.9% saline and administered at a dose of 10 mg/kg via intraperitoneal (*i.p.*) injections in a volume of 10 ml/kg. Dose was chosen based on the literature and was prepared according to the salt not the base form (Che et al., 2006; Zombeck et al., 2008).

**Running Wheels and Sedentary Treatment**

Dimensions of running wheel cages were 36 x 20 x 14 cm (L x W x H), with a 23 cm diameter wheel mounted in the cage top. Running wheel rotations were monitored continuously in 1 min increments via magnetic switches interfaced to a computer. Mice assigned to the Sedentary group were deliberately not housed in cages with locked wheels because mice climb in locked wheels (Koteja et al., 1999; Rhodes et al., 2003; Rhodes et al., 2000). Our objective was to keep random physical activity in the Sedentary group to a minimum.

**Experimental Design**

Mice were conditioned to cocaine using the procedure below (Figure 6.1). The day after CPP testing, animals underwent one re-exposure to context session in the morning (1000 h; for 30 min). Mice were either re-exposed to the CPP texture they had been conditioned to associate with cocaine (*n*=24) or the CPP texture they had been conditioned to associate with vehicle (*n*=24). All animals were weighed, injected *i.p.* with 10 ml/kg saline, and placed into the center of either a HOLE or GRID conditioning chamber. All mice had, up to this point, received identical treatment, including number of total cocaine context exposures. An important difference existed between the groups.
During the conditioning phase, the experimental group animals were conditioned to experience cocaine on the texture they were re-exposed to before sacrifice. Control group animals had not experienced cocaine on the texture they were re-exposed to before sacrifice. Mice were deliberately not placed in the dual-texture chamber at context re-exposure, because the goal was to isolate the conditioning effect. If mice were placed in a dual-texture chamber at re-exposure, then they would be exposed to both a cocaine context cue and a neutral cue. Furthermore, time spent on the cocaine vs. the neutral context cue would vary between mice. In contrast, in our design, if re-exposure to just one context was accompanied by differences in peptide expression, these differences must be attributable to the effect of that specific cue (cocaine or neutral context).

In order to avoid circadian fluctuations, all animals were euthanized within a 2 hour period. In order to sacrifice all mice within the 2 hour window, animals were split into groups of 10 and their conditioning and subsequent testing and context re-exposure staggered by 1 day so that their sacrifice was also staggered by 1 day.

**Conditioned Place Preference**

**Habituation**

To familiarize the mice with the place conditioning chambers, animals were placed on a flat surface without a texture in the conditioning chambers in the morning (1000 h; for 30 min) and in the afternoon (1600 h; for 30 min) for one day without any injection treatment (Cunningham et al., 2006).

**Pretesting**

To determine individual biases in preference for the textures prior to drug pairing, animals were weighed, received a 10 ml/kg i.p. saline injection, and were immediately placed in the apparatus with HOLE/GRID floor in the morning (1000 h; for 30 min) and afternoon (1600 h; for 30 min) (Cunningham et al., 2006).

**Conditioning**

Four conditioned stimulus (CS+) trials (i.e., cocaine paired with one floor texture: HOLE or GRID) and four CS- trials (i.e., vehicle paired with the alternate floor texture) were administered over four days. Each day, one CS+ trial and one CS- trial was administered in the morning and afternoon. The order of exposure to CS+ and CS- was counterbalanced within each group. Experimental animals were weighed, received an
injection of 10 mg/kg *i.p.* cocaine (CS+ trial) or vehicle (CS- trial), and were immediately placed on the appropriate floor texture. Control animals underwent the identical procedure, except that they only received vehicle (saline) on both floor textures.

**Testing**

Testing took place once in the morning (1000 h; 30 min) 31 days after the last conditioning session. Prior to the testing session, each mouse was weighed, injected *i.p.* with 10 ml/kg saline, and placed into the center of the HOLE/GRID conditioning chamber.

**Tissue Sampling and Extraction of Peptides**

Immediately following the 30 minute context re-exposure session, mice were anesthetized with 150 mg/kg sodium pentobarbital (*i.p.*) and then perfused transcardially with ice-cold saline. Their brains were carefully removed and immediately flash-frozen in liquid nitrogen for preservation and stored at -80°C. The next day, frozen brains were mounted using distilled water on a cryostat. Samples of the bilateral basolateral amygdala and bilateral dentate gyrus of the hippocampus were isolated via biopsy punches of 1.25 mm diameter (World Precision Instruments, Sarasota, FL). Coordinates of the punches taken were determined according to the mouse brain atlas (Franklin & Paxinos, 2008). Coordinates of punches taken from basolateral amygdala were: from -0.94 to -1.94 bregma. Coordinates of punches taken from dentate gyrus of the hippocampus were: from -1.94 to -2.94 bregma. For peptide extraction, tissue samples from both hemispheres of the amygdala for each animal were placed into 19 µL 0.25% acetic acid and incubated for 24 h before spotting for MALDI-TOF MS. Tissue samples from both hemispheres of the dentate gyrus of the hippocampus were placed into 14 µL 0.25% acetic acid extraction solution and incubated for 24 h before spotting for MALDI-TOF MS.

**Analysis of Peptide Profiles by MALDI-TOF MS**

For MALDI-TOF MS analysis, the following tissue samples from mice from each of the 4 groups were used: *n* = 11 sedentary control group (saline-context re-exposed) mice, *n* = 12 sedentary experimental group (cocaine-context re-exposed) mice, *n* = 13 runner control group (saline-context re-exposed) mice, and *n* =12 runner experimental group (cocaine-context re-exposed) mice. 5 µL of each hippocampus sample from 48
animals was added to 2 µL 10mM acidic peptide from *Aplysia* (serving as internal standard; MW 2960). 5 µL of each amygdala sample from 47 animals was added to 1 µL 10mM acidic peptide from *Aplysia* (serving as internal standard). One animal’s amygdala could not be sampled due to brain shape distortion from immersion in liquid nitrogen.

Next, 0.7 µL of each sample’s solution was co-crystallized with 0.7 µL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix (13 mg/ml in 60% acetonitrile) per spot and spotted as five technical replicates for each animal on a gold-coated MALDI target (Bruker Daltonics). After this, pooled samples were created by combining the unused portions of the individual extracts as follows: the remaining tissue sample liquid in each vial plus an additional 10 µL dH20 added per vial (used to wash out the vial contents) from 3 animals of each group of the 4 groups was transferred to a new vial. The remaining 11 tissue samples were combined into two separate vials, one containing 6 animals’ tissue samples, one containing 5 animals’ tissue samples for later sequencing efforts. In contrast to individual samples, no acidic peptide from *Aplysia* was added to the grouped samples as an internal standard prior to spotting on a target. 0.7 µL of each grouped sample plus 0.7 µL CHCA matrix was spotted as three technical replicates on a gold target. Since pilot experiments had used DHB as matrix, 0.7 µL of each grouped sample was co-crystallized with 0.7 µL of freshly prepared concentrated DHB matrix (50 mg/ml in a 50% acetonile/water mix) and spotted as three technical replicates on a gold-coated MALDI target (Bruker Daltonics). Positive ion mass spectra for each tissue sample were acquired in the 600-5,000 m/z region using a Bruker Ultraflextreme mass spectrometer (Bruker Daltronics) in reflectron mode with high-precision calibration, by automatic run for the individual and grouped tissue samples co-crystallized with CHCA, and manually for the grouped samples co-crystallized with DHB.

Remaining peptide mixtures were used for later LC-ESI analysis, as pooling increases tissue amount required for LC-ESI analysis. No acidic peptide from *Aplysia* was added as internal standard to the LC-ESI samples.

*Statistical data analysis*

Data were analyzed using SAS (version 9.2) statistical software. In all analyses, $P < 0.05$ was considered statistically significant.
CPP-- To evaluate whether the animals used in this study had an initial average bias for HOLE or GRID textures, the average duration (min) spent on Hole texture was compared to 15 min (half the duration of the test) using a one-sample t-test. Conditioned place preference data were analyzed the following way: First, the duration spent on the HOLE texture was analyzed by 2-way ANOVA with conditioned stimulus (CS+HOLE versus CS+GRID; between-subjects), exercise history (Sedentary versus Runner; between-subjects) and all interactions entered as factors.

*Peptide Profiles by MALDI-TOF MS*-- The raw MALDI MS data were imported into ClinProTools 2.2 software (Bruker Daltonics), with a m/z 600-5000 mass filter, corrected with the TopHat method for a baseline flatness of 0.1. Smoothing was performed with Savitzky-Golay algorithm at a width of 1.0 m/z over 4 cycles to smooth isotopic envelopes, normalized to total ion count. Putative candidate peptide peaks likely to differ significantly between treatment groups were identified by PCA of raw (i.e. not normalized to internal standard peptide intensity) MALDI-TOF MS data, where the peptide peaks considered for PCA were restricted to a signal-to-noise ratio of 6:1. Independent PCA was performed: all groups in the hippocampal samples, the sedentary versus runner experimental (cocaine-context-re-exposed) groups in the hippocampal samples, all groups in the amygdala samples, and the sedentary control (vehicle-context-re-exposed) versus sedentary experimental (cocaine-context-re-exposed) groups in the amygdala samples. (Data collected in previous pilot experiments indicated that peptide peaks would be differentially detected among the sedentary versus runner experimental (cocaine-context-re-exposed) groups in the hippocampus, and among the sedentary control (vehicle-context-re-exposed) versus sedentary experimental (cocaine-context-re-exposed) groups in the amygdala.) PCA reduces the dimensionality of the data, with the resultant principal components (PCs) each representing a set of linearly uncorrelated m/z values. The intensity value of each of the candidate peptide peaks in each animal’s tissue samples was then restricted to a signal-to-noise ratio of 3:1 and expressed as a ratio of the intensity of internal standard peptide present in the sample (sum of the peaks 2959, 2844, and 2731 due to fragmentation) and 2x2 between-within ANOVA tests run on the peptide peak expression levels, where treatment (exercise or sedentary) and context at re-exposure (vehicle or cocaine) were between-groups factors and iteration (i.e. sample
spot) was a within-subjects variable (5 per animal). All peak intensity values were then transformed to log(intensity) + 0.5 in order to normalize them.

**Results**

*Baseline preference*

During the pretest, before the animals ever experienced cocaine, and before any of the animals ran on wheels or experienced toys, animals spent approximately 53% of their time on each side, an average of 16.0 minutes (± 0.42 SE) on the HOLE texture. There was no significant bias in preference observed, but even a bias would not compromise analysis of cocaine CPP because the bias would be present in both groups of, for example, Runners (CS+ GRID and CS+ HOLE) being compared to establish CPP (see Methods, CPP procedure).

*Wheel running*

Running increased from days 1-27 and then maintained a plateau for the remaining days (e.g., day was significant, $F_{31,723} = 13.44$, $P < 0.0001$) (Figure 6.2). Average level of running across all animals was 5.6 kilometers/day (± 0.20 SE).

*CPP*

A significant main effect of texture ($F_{1,44} = 21.22$, $P < 0.0001$) was observed, indicating successful conditioning. In addition, a significant main effect of exercise ($F_{1,44} = 8.58$, $P = 0.0054$) was observed, indicating that CPP of animals that had had running wheels was significantly lower than CPP of sedentary animals (Figure 6.3). In addition, the interaction between texture and exercise ($F_{1,44} = 2.89$, $P = 0.096$) trended towards significance (Figure 6.3A).

*Peptide Profiles in Brain Regions by MALDI-TOF MS*

Based on PCA plots, the following peaks were selected for further analysis: 1099.87, 1186.9, 1315.18, 1662.5, 1648.6 for a comparison among all groups in the hippocampus, 1099.88, 1211.88, 1285.77, 1315.18, 1648.55, 1662.51 for a comparison of the sedentary experimental (cocaine-context-re-exposure) versus runner experimental (cocaine-context-re-exposure) in the hippocampus, 1135.18, 1187.13, 1315.38, 1478.48, and 2305.47 for a comparison of all groups in the amygdala, and 1135.17, 1147.24, 1429.26, 1662.41, and 3012.12 for a comparison of the sedentary control (vehicle-
context-re-exposure) versus sedentary experimental (cocaine-context-re-exposure) groups in the amygdala.

Statistical analysis of the peak intensities in the hippocampal samples by 2 x 2 ANOVA (factors: exercise treatment and context at re-exposure) run on all four treatment groups revealed four peptides that exhibited significantly differential expression by exercise group: 1099.87 ($F_{1,44} = 9.93, P=0.0029$), 1186.9 ($F_{1,44} = 9.42, P=0.0037$), 1315.18 ($F_{1,44} = 8.13, P=0.0066$), and 1648.6 ($F_{1,44} = 8.29, P=0.0061$) (Figure 6.1). All of these peptides were more highly expressed in sedentary than in runner mice and were highly correlated with each other (Tables 6.1-6.2). Peak 1662.5 showed a trend towards a significant effect of exercise ($F_{1,44}=3.96, P=0.0527$). There was no significant effect of context or any significant interaction between exercise and context in these peptide peaks in the hippocampus.

Statistical analysis of the peak intensities in the hippocampal samples by 2 x 2 ANOVA (factors: exercise treatment and context at re-exposure) run on the Cocaine-context-Run versus Cocaine-context-Sedentary groups also revealed four peptides that exhibited significantly differential expression by exercise: 1099.88 ($F_{1,22} = 8.10, P=0.0094$), 1315.18 ($F_{1,22} = 5.92, P=0.0236$), 1648.55 ($F_{1,22} = 7.67, P=0.0112$), 1662.51 ($F_{1,22} = 5.06, P=0.0348$) (Figure 6.2). Peak 1099.88 was more highly expressed in runner than in sedentary animals, whereas peaks 1315.18, 1648.55, and 1662.51 were more highly expressed in sedentary than in runner animals, and peaks were highly correlated with each other (Tables 6.3-6.4). Peak 1285.77 showed a trend towards a significant effect of exercise ($F_{1,22}=2.06, P=0.1655$). There was no significant effect of context or any significant interaction between exercise and context in these peptide peaks in the hippocampus.

Statistical analysis of peak intensities in the amygdala samples by 2 x 2 ANOVA following the PCA run on all four treatment groups and PCA run on just the Vehicle-context-Sedentary versus Cocaine-context-Sedentary groups yielded no peptides that were differentially expressed between groups. Peaks were correlated with each other (Tables 6.5-6.8).
The mass peaks differentially detected in the hippocampus by MALDI-TOF MS and verified by ANOVA tests to differ significantly between groups will be confirmed by LC-ESI and identified via sequencing.

**Discussion**

The main findings of this study are that we successfully improved upon our previous pilot experiments to design a MALDI-TOF MS acquisition method with which we identified peptide peaks differentially expressed in the hippocampus of mice that had had access to running wheels and mice that had remained sedentary after conditioning. Using this technology, we are as yet unable to establish learning effects on peptide expression levels. We do not yet understand how context exposure prior to sacrifice differentially induces peptide release in the amygdala or hippocampus.

Other studies besides ours have set out to identify neuropeptides differentially modulated in response to a drug and a drug-related behavior in distinct brain regions by mass spectrometry techniques. They include studies that have set out to identify putative biomarker neuropeptides associated with drug-related behavior such as locomotor sensitivity to cocaine (Romanova et al., 2010) and amphetamine-induced sensitization (Romanova et al., 2012). These studies have yielded subsets of peptides with significant expression differences in distinct brain regions, but these studies pre-treated animals with an acute dose of cocaine (Romanova et al., 2010) or amphetamine (Romanova et al., 2012). In contrast, our context manipulation was subtle and did not involve a pre-treatment with acute cocaine prior to sacrifice. Rather, it was our expectation that mere re-exposure to an environment associated with cocaine might in fact activate the same pathways as an acute dose of the drug itself. This difference in subtlety of our manipulation from previous studies may explain our lack of finding an effect of context exposure prior to sacrifice on peptides in either of the brain regions examined.

We show significant CPP in both sedentary and runner animals, with the CPP of sedentary animals being lower than that of runners. This is the ideal behavioral outcome we hoped to achieve. We sought to re-expose animals to contexts either associated with cocaine or with vehicle at a time point when the two groups were diverging in CPP, recalling that previous work (Chapter 2) suggests that this occurs on Day 2 of CPP testing (i.e. the day chosen for context re-exposure and sacrifice in our present study).
We anticipated that cocaine-conditioned mice exposed to the cocaine-paired context would display a different set and/or levels of peptides in hippocampus and amygdala compared to cocaine-conditioned mice exposed to the saline-associated context. In addition, we expected to observe a modulation of the peptide profiles in hippocampus and amygdala in mice that ran for 30 days between conditioning and testing.

Future directions include using tandem mass spectrometry (MS/MS) and the expertise of the Sweedler group to identify the mass peaks we detected here and sequencing of the peptides. Our approach of assaying samples by MALDI-TOF MS to characterize neuropeptides only used a fraction of the samples. Remaining samples have been pooled for LC-ESI MS to identify peptides present, so that peaks in MALDI-TOF MS spectra can be unambiguously identified. Differential isotope labeling, which allows for relative neuropeptide quantification (Rubakhin et al., 2005), will be employed on remaining hippocampal samples, with animals split up by exercise group. Pending further analyses of amygdala data by MALDI-TOF MS, differential isotope labeling will be used on remaining amygdala samples by either exercise or context re-exposure group. LC-ESI with isotope labeling can identify some peaks that will not have been detected by MALDI-TOF MS (Rubakhin et al., 2005). One limitation of differential isotope labeling is that some peaks have a blocked N-terminal that will not label, so a label-free comparison of peak heights can also be considered. For sequencing, first-stage separation was done by the Sweedler group, and samples have been sent to the Kelleher group at Northwestern University, which specializes in whole proteome analysis of mammalian cells, for sequencing analysis. This analysis will allow us to identify currently unknown peptides shown to differ in expression in the hippocampus between animals that had run and animals that had remained sedentary.

Concurrently with this, we will continue to pursue further analyses on the MALDI-TOF MS data we collected. We plan to use a modeling function in the ClinProTools program on peak heights that are relativized to internal standard expression in sample to identify the number and masses of peaks that differ significantly between treatment groups in the hippocampus and amygdala samples. In addition, some settings for analyses will be refined. Signal-to-noise ratio for peak detection will be increased from 3:1 to 5:1 to make peak detection more stringent. The peak identification algorithm
will be changed from SNAP to centroid. We will not smooth spectra. We also plan to undertake formal statistical tests for outliers to exclude 0 values that appear in technical replicates if the tests indicate that these values are true outliers rather than indications of the absence of a particular peak. We would keep “true 0” values, defined to be a signal below the 5:1 signal-to-noise threshold, if this is the value obtained for all technical replicates of a sample. We are aware that a missing value may not necessarily represent a detection limit problem as much as that a peptide may be more susceptible to salt and get sodiated as opposed to protonated or absorbed to plastic. We are confident that such modification of our settings will enable us to detect differential peptide expression by context exposure in the amygdala, as intimated by results of prior pilot work. It may also allow us to detect currently unobserved context-induced alterations in the hippocampus. We may also attempt to correlate wheel running output or the magnitude of CPP of individual animals with peptide expression levels.

In the unlikely event that we fail to detect peptides differentially induced by exposure to a cocaine-paired context, we could consider: (1) repeating the experiment with more conditioning trials (10 instead of 4), (2) changing strength of the unconditioned stimulus (US) by increasing cocaine dose (from 10 mg/kg to 20 mg/kg), (3) altering the strength of the conditioned stimulus (CS+) by adding discriminative modalities (i.e., pair a light, odor, or noise with texture), and (4) pooling brain areas to eliminate variation between regions. The above-described methodological modifications, Steps 1-4, could be accomplished in a single repeat experiment.

Another useful future experiment might broaden discovery of differentially expressed neuropeptides through the use of lipid extractions. Lipids/low-molecular weight peptides may be induced by exercise or context re-exposure in the hippocampus and/or amygdala. It is possible that certain small molecules like lipid metabolites are undetectable by our method. Such an approach would require extraction of lipids first in acidic and then organic solvents from brain samples that are no longer available. Therefore, this approach would require another group of animals to be conditioned, tested, and sacrificed for sampling of brain regions. Finally, in the future, we would opt to employ a non-degrading internal standard synthetic peptide/polymer of amino acids rather than acidic peptide present in Aplysia. This would avoid internal standard
degradation and the associated difficulty of determining which mass peaks represent internal peptide degradation fragments.

We remain uncertain regarding the identity of the differentially expressed hippocampal mass peaks identified in treatment groups. However, the extant literature suggests some candidate peptides. In one study, peptides found to increase in the hippocampus from exposure to twice-daily cocaine (10 mg/kg; identical to the dose we used for conditioning) for 5 days, relative to saline-treated mice, in Cpefat/fat, which lack functional carboxypeptidase E activity due to a point mutation, and wild-type mice included: an unknown peptide of mass 3020.50 Dalton that was increased by a ratio of 1.60 (±0.20) in drug vs. saline-treated mice in both mouse strains, and a chromogranin B peptide 516-537 fragment of mass 2684 Dalton (Che et al., 2006). We may observe these mass peaks or fragments of these peaks in our samples, particularly after we employ the modeling function in the ClinProTools program discussed earlier.

We are familiar with data regarding cocaine-induced alterations in the human amygdala. Postmortem analyses of human drug abusers show increased expression of Homer 1b/c protein, which comprises the postsynaptic density domain of metabotropic glutamate receptors mGluR1 and mGluR5, and increased expression of dynamin-3 protein in the amygdala (Okvist et al., 2011). Dynamin-3 is the protein to which Homer 1b/c binds, and is localized to dendritic spine tips (Gray et al., 2003). Dynamin-3’s localization to dendritic spines and the postsynaptic density may indicate its possible role in remodeling of dendritic spines. These data allude to dysregulated glutamatergic signaling in the amygdala of cocaine abusers. This further suggests that cocaine treatment in our mice may show aberrant (i.e. differentially expressed) peptide peaks in the amygdala of mice that received cocaine. All of the mice in our study received cocaine, since they all were conditioned. This would suggest that we would not observe differences in peptide peaks by groups in the amygdala in our study. However, the possibility also remains that mere exposure to a cocaine-paired context immediately prior to sacrifice was significantly consequential. Indeed, perhaps it was sufficient to induce a signaling cascade that led to a reemergence or heightened expression of the dysregulation in glutamatergic proteins in the amygdale that was originally started by cocaine. If proteins like dynemin-3 are involved in dendritic remodeling due to cocaine, they may, by
extension, also be involved in the dendritic remodeling that would occur from learning about a context. It is thus conceivable that Homer 1 b/c or dynamin-3 or a related protein is differentially expressed in the amygdala in mice that were re-exposed to a cocaine-paired context prior to exercise, if these peptides are involved in the dendritic remodeling occurring during evaluation of the salience of a stimulus.

Besides the protracted time from death to analysis of peptides in the subjects, another potential limitation of the results of this postmortem study of human drug abusers to informing our study is that the study’s subjects were not solely cocaine abusers, but instead abused multiple drugs (7 used heroine, 8 cocaine, 7 both heroine and cocaine, and 7 were drug-naïve controls) (Okvist et al., 2011). However, it should also be noted that similar alterations in the glutamatergic system were seen in the amygdalas of 29 exclusively heroine-using subjects analyzed postmortem, suggesting that the dysregulating effect on the glutamatergic system may not actually be limited to cocaine but instead an effect pertinent to all drugs of abuse (Okvist et al., 2011). An exciting correlate of this possibility is that any intervention that reduces alterations in proteins of the glutamatergic system may hold promise as an effective, centrally acting intervention for the effects of all drugs of abuse.

Exercise effects in the hippocampus—if not the amygdala—are robust. This is evident by our ability to detect clear differences in the expression levels of mass peaks in the hippocampus by exercise groups in spite of a signal to noise ratio of 3 and without exclusion of outlying results. We believe that our efforts will yield both candidate known and novel neuropeptides identified in a drug-and context-dependent manner in specific brain regions in exercised versus sedentary animals. Our findings are important, as they identify novel and much-needed molecular targets for curbing the drug associations that contribute to drug dependence and relapse.

These outcomes are expected to have an important positive impact. Once mechanisms of action and neuropeptides induced by exercise are identified, these can be targeted for development of more effective treatments to help maintain abstinence from drug abuse and dependence. We acknowledge that our findings of neuropeptides will represent correlations, not causation. If we find interesting peptides, in future work, we could potentially block the peptides via antagonist drugs. Alternatively, if no drugs are
available, we could perhaps develop interference methods at the RNA level. Evidence of neuropeptide profiles induced by exposure to exercise and a drug-paired context is an important step forward for the field.
Figure 6.1. Schematic diagram of the experimental design. The H designates a habituation day to reduce novelty effects. The P designates a pretesting day to establish baseline texture preferences preceding the conditioning. T denotes CPP testing day. C+ and C- refer to context re-exposure (C+ = cocaine-associated texture; C- = vehicle-associated texture). Black arrow indicates sacrifice immediately after context re-exposure session. Second black arrow indicates brain punches taken from brain the day after sacrifice. The runner/sedentary conditions lasted for 30 days. The experiment included 48 animals (50 originally; 2 died of natural causes). Animals were returned to cages with or without running wheels immediately after testing, to avoid the potential confound of animals experiencing withdrawal from running prior to context re-exposure the next day.
Figure 6.2. Wheel running over the course of the study. Average distance run (km/day) (± SE). Running increased from days 1-27 and then maintained a plateau for the remaining days. Increased wheel running over the first 25 days is typical for C57BL/6J mice. Arrows indicate the testing day and the re-exposure day, both of which show reduced wheel running activity, since animals were removed from wheels for portions of these days.
Figure 6.3. Conditioned place preference for cocaine during testing. Mean difference in duration (min) ± SE spent on the HOLE texture between animals receiving cocaine on HOLE texture (CS+HOLE) and animals receiving cocaine on GRID texture (CS+GRID) plotted separately for Runners and Sedentary animals. The white bar represents data for 23 animals; the grey bar represents data for 25 animals (n = 11 sedentary CS+HOLE animals, 12 runner CS+HOLE animals, and n = 12 sedentary CS+GRID animals, 13 CS+GRID animals). The stars indicate significant place preference at $P < 0.05$. 
Figure 6.4. Hippocampus: All groups’ peptides selected from PCA plots for further analysis. Mean intensity of mass peaks relative to acidic peptide of *Aplysia* (internal standard) plotted separately for the top five candidate peptides most likely to differ between the four groups. Intensity values are the average of five technical replicates of a hippocampal sample from each of the 48 animals in the study (25 runner, 23 sedentary animals). The stars indicate significant expression differences of 4 of the 5 mass peaks between sedentary and runner animals at $P < 0.05$. 
Figure 6.5. Hippocampus: Sedentary-cocaine-context versus exercise-cocaine-context candidate peptides selected from PCA plots for further analysis. Mean intensity of mass peaks relative to acidic peptide of *Aplysia* (internal standard) plotted separately for the top six candidate peptides most likely to differ between animals in the sedentary-cocaine-context versus animals in the exercise-cocaine-context groups. Intensity values are the average of five technical replicates of a hippocampal sample from each of the 24 sedentary animals in the study that were re-exposed to a cocaine-associated context before sacrifice and 24 runner animals that were re-exposed to a cocaine-associated context before sacrifice. The stars indicate significant expression differences of 4 of the 6 mass peaks between sedentary and runner animals re-exposed to a cocaine-associated context prior to sacrifice at $P < 0.05$. 

![Graph showing intensity relative to acidic peptide internal standard for m/z values of 1099.88, 1112.88, 1285.77, 1315.18, 1648.55, and 1662.51. The graph includes bars for both sedentary and runner groups, with stars indicating significant differences at $P < 0.05$.](image-url)
Table 6.1. Hippocampus: correlations between peptides in runners. Correlations between each of the top five mass peaks likely to differ in runners, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of hippocampal samples relative to acidic peptide from *Aplysia* (internal standard) of all 25 runner animals in the study were run. Numbers in bold font are mass peaks. Numbers in plain font represent $R^2$ values between mass peaks.

<table>
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Table 6.2. Hippocampus: correlations between peptides in sedentary mice. Correlations between each of the top five mass peaks likely to differ in sedentary mice, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of hippocampal samples relative to acidic peptide from *Aplysia* (internal standard) of all 23 sedentary animals in the study were run. Numbers in bold font are mass peaks. Numbers in plain font represent $R^2$ values between mass peaks.

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Table 6.3. Hippocampus: correlations between peptides in runners that were re-exposed to a cocaine-associated context. Correlations between each of the top six mass peaks likely to differ in runners re-exposed to a cocaine-associated context, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of hippocampal samples relative to acidic peptide from *Aplysia* (internal standard) of all 13 runner animals in the study that were re-exposed to cocaine-associated context were run. Numbers in bold font are mass peaks. Numbers in plain font represent $R^2$ values between mass peaks.

<table>
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Table 6.4. Hippocampus: correlations between peptides in sedentary mice that were re-exposed to a cocaine-associated context. Correlations between each of the top six mass peaks likely to differ in sedentary animals re-exposed to a cocaine-associated context, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of hippocampal samples relative to acidic peptide from *Aplysia* (internal standard) of all 12 sedentary animals in the study that were re-exposed to cocaine-associated context were run. Numbers in bold font are mass peaks. Numbers in plain font represent $R^2$ values between mass peaks.

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Table 6.5. Amygdala: correlations between peptides in mice that were re-exposed to a cocaine-associated context. Correlations between each of the top give mass peaks likely to differ in sedentary and runner mice re-exposed to a cocaine-associated context, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of amygdala samples relative to acidic peptide from *Aplysia* (internal standard) of all 25 animals in the study that were re-exposed to cocaine-associated context were run. Numbers in bold font are mass peaks. Numbers in plain font represent $R^2$ values between mass peaks.
Table 6.6. Amygdala: correlations between peptides in mice that were re-exposed to a vehicle-associated context. Correlations between each of the top give mass peaks likely to differ in sedentary and runner mice re-exposed to a vehicle-associated context, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of amygdala samples relative to acidic peptide from *Aplysia* (internal standard) of all 23 animals in the study that were re-exposed to vehicle-associated context were run. Numbers in bold font are mass peaks. Numbers in plain font represent R² values between mass peaks.

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Table 6.7. Amygdala: correlations between peptides in sedentary mice that were re-exposed to a cocaine-associated context. Correlations between each of the top five mass peaks likely to differ in sedentary mice re-exposed to a cocaine-associated context, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of amygdala samples relative to acidic peptide from *Aplysia* (internal standard) of all 12 sedentary animals in the study that were re-exposed to cocaine-associated context were run. Numbers in bold font are mass peaks. Numbers in plain font represent $R^2$ values between mass peaks.
Table 6.8. Amygdala: correlations between peptides in sedentary mice that were re-exposed to a vehicle-associated context. Correlations between each of the top five mass peaks likely to differ in sedentary mice re-exposed to a vehicle-associated context, with peptides selected from PCA plots for further analysis. Correlations of average intensity values across five technical replicates of amygdala samples relative to acidic peptide from *Aplysia* (internal standard) of all 11 runner animals in the study that were re-exposed to vehicle-associated context were run. Numbers in bold font are mass peaks. Numbers in plain font represent $R^2$ values between mass peaks.

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References


CHAPTER 7

Future Directions
In this dissertation, I have clarified mechanisms by which voluntary wheel running influences conditioned place preference (CPP) for cocaine. I showed that wheel running accelerated extinction of CPP when running occurred entirely after drug conditioning, whereas running delayed extinction when administered before conditioning, while approximately doubling adult hippocampal neurogenesis in both scenarios. I then showed that an enriched environment devoid of running wheels did not significantly up-regulate hippocampal neurogenesis or improve learning. I next investigated the possibility that environmental enrichment, like running, can accelerate extinction of CPP, and obtained results that suggest that environmental enrichment does not effectively accelerate extinction of CPP. I also showed that new neurons from running are not necessary for abolished cocaine CPP from running. Lastly, I showed evidence that neuropeptidomic profiles in the hippocampus differ between runner and sedentary mice. Taken together, these chapters present evidence that voluntary wheel running creates unique hippocampal plasticity not approximated by environmental enrichment and suggests that this plasticity can be harnessed to weaken CPP for cocaine in mice. Furthermore, since exercise can strengthen or weaken CPP depending on when it is implemented, exercise appears to work by promoting learning. This dissertation has laid the groundwork for the pursuit of several logical avenues of future research.

In July-August 2014, I will follow up on intriguing pilot data I collected (not included in this dissertation) which suggest that exposure to running wheels for one week after conditioning is sufficient to accelerate extinction of CPP for cocaine. In the original experiment, I compared the effectiveness of one week of wheel access to four weeks of wheel access at reducing CPP for cocaine in mice and found that just one week of wheel access accelerated extinction of CPP for cocaine, relative to sedentary mice. At the present time, it is unclear whether one week of wheel access would still effectively reduce CPP if the total duration between conditioning and testing was kept at four weeks. I will be identifying key temporal parameters that influence the effect of running on cocaine CPP, testing both whether one week of running wheel exposure followed by three weeks of sedentary housing or three weeks of sedentary housing followed by one week of running wheel exposure before testing can accelerate extinction of CPP for cocaine. Originally undertaken to establish the duration of running needed for effective
intervention in CPP, my follow-up experiment will establish the proximity of one week of running to conditioning and CPP testing needed for effective intervention in CPP.

Since the start of this dissertation, the field of neuroscience has advanced new tools with which questions remaining from the research I conducted can be pursued. In Chapter 5, I present evidence that new neurons from running are not necessary for abolished CPP from running. However, as I discussed in that chapter, it is still possible that new hippocampal neurons are preferentially involved to bring about this extinction of CPP behavior in the same way that we know from lesion studies that the hippocampus is not necessary for contextual fear conditioning but is still preferentially involved when intact (Gewirtz et al., 2000; Matus-Amat et al., 2004; Sanders et al., 2003). Since we ablated neurogenesis via valganciclovir administration starting a full 28 days before CPP testing in the experiment in Chapter 5, compensatory mechanisms are critical to consider when interpreting results. The possibility remains that when animals are learning hippocampus-dependent tasks, new neurons may be involved or even preferentially recruited if present, but if new neurons are not available, older, established neurons can compensate and display sufficient plasticity required for the learning. The possibility that new neurons may be involved or even preferentially recruited when present to contribute to exercise-induced extinction of CPP for cocaine can be examined with optogenetics techniques. Optogenetics employs light-sensitive protein pumps targeted for expression in certain cells to control the activity of certain neurons (Kim et al., 2013). In particular, specific cell types, such as newly formed neurons, can be selectively targeted to express light-driven chloride pumps such as halorhodopsin (NpHR) (Kim et al., 2013; Zhao et al., 2008). Hyperpolarizing the subset of new neurons in the hippocampus immediately prior to the time of CPP testing by shining green light of wavelength 532 nm would noninvasively and temporarily silence (i.e. inactivate) these new neurons in freely moving animals and would require an animal’s brain to perform CPP tests without allowing for sufficient time for any compensatory mechanisms that developed in response to the ablation of new neurons to be activated (Zhao et al., 2008). An example of the form that such compensatory mechanisms could take is increased numbers of synapses between pre-existing cells during the 28 days before CPP testing, during which new neurons were eliminated with valganciclovir administration. An experiment
employing optogenetics techniques to inactivate new neurons immediately prior to CPP testing would add to and extend the insight gleaned from this dissertation work about the role of new neurons in CPP.
References