GALANIN MEDIATES THE RESILENCE AFFORDED BY EXERCISE: BEHAVIORAL, NEUROCHEMICAL, AND NEUROANATOMICAL EVIDENCE

by

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(Under the Direction of Philip Holmes)

ABSTRACT

Exercise is effective treatment for symptoms of mental disorders such as anxiety and substance abuse. However, the neural mechanisms that support these benefits are only beginning to be identified and dissected. Our laboratory has repeatedly shown that exercise increases levels of the peptide galanin in the locus coeruleus (LC). The effects of galanin share many qualities with those of exercise. Both manipulations are protective against the behavioral impact of stress and cocaine in rodent models of mental disease. We hypothesized that the behavioral protection provided by exercise is mediated by galanin, and similarly that galanin receptor agonists would protect against stress- and drug-induced perturbation of behavior. We also predicted that exercise or administration of a galanin agonist would block neural markers of stress or cocaine in the frontal cortex, a region particularly vulnerable to these challenges. Male Sprague Dawley rats were used in all experiments, and a subset underwent stereotaxic brain cannulation for drug delivery (ICV) and/or *in vivo* microdialysis (intra-frontal cortex). Rats were assigned to sedentary or exercise conditions and then exposed to stress or cocaine as detailed in the experiments. We measured behavior in tests of anxiety, including the elevated plus maze, open field, and shock probe defensive burying test. We measured galanin levels after exercise using *in situ* hybridization and ELISA. We also performed *in vivo* microdialysis and Golgi-impregnation in the frontal cortex. The results showed that exercise increased galanin levels in

the locus coeruleus. Exercise and administration of a galanin receptor agonist (galnon or galanin) protected against the behavioral impacts of stress and cocaine. These manipulations also blunted the stress- and cocaine- induced increase in dopamine in the frontal cortex. Further, exercise protected against the loss of dendritic spines after stress. The behavioral protection induced by exercise was mimicked by repeated intracranial galanin administration, and blocked by repeated administration of the galanin receptor antagonist M40. Collectively, our data implicate an important role for galanin in behavioral resilience. Exercise, like galanin therapy, may protect against the aberrant neural plasticity seen in comorbid disorders like anxiety and drug addiction.

INDEX WORDS: Anxiety; brain; cocaine; cortex; dendritic spines; dopamine; environmental enrichment; exercise; galanin; galnon; locus coeruleus; mPFC; neuroplasticity; resilience; stress;

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DEDICATION

This work is dedicated to Jean DiPirro and Brendan Walsh for your endless support, kindness, and belief in me. At the root of things, this work is also dedicated to Nunzia and Benedetto Sciolino.

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CHAPTER 1

GENERAL INTRODUCTION

Exercise promotes brain health and effectively treats symptoms of mental disorders, such as depression, anxiety, and substance abuse. Although historically it is an old observation that exercise improves mental health, not until the last few decades have the supporting mechanisms been identified and dissected using tools available to modern neuroscience. Exercise is multi-mechanistic, causing change to many transmitter systems and trophic factors, in the brain. These changes are found among neural circuits that control arousal, emotion, memory, motivated behavior, and the response to stress. One such change that our laboratory has shown repeatedly is that exercise increases the peptide galanin in the locus coeruleus (LC).

Galanin is highly expressed in the LC, and is colocalized in most norepinephrine neurons in this region. Galanin receptors (GalR1, GalR2, GalR3) are densely located throughout neural circuits that are affected by stress and drugs of abuse (i.e., frontal cortex, amygdala, ventral hippocampus, ventral tegmental area, nucleus accumbens, periaqueductal gray, dorsal raphe, locus coeruleus, sympathetic nerves, and vagus nerve). The actions of galanin share many qualities with those of exercise. Both manipulations are neurotrophic, neuroprotective, and neurorestorative in disease models. Both manipulations also promote resilience to stress and attenuate anxiety-like behavior (see Chapter 2 for review in tests of anxiety) and responses to some drugs of abuse.

In the studies described herein, we used galanin pharmacology in conjunction with an exercise model known to endogenously increase galanin expression in the LC. We hypothesized that the behavioral protection provided by exercise is mediated by galanin, and similarly that galanin agonists would also protect against stress- and drug-induced perturbation of behavior. Stress and drugs of abuse change the structure and function of the mPFC

substantially. The mPFC also plays a central role in regulating stress responses and addictive behaviors. Thus, we hypothesized that exercise or a galanin agonist would protect the cortex against these changes.

The purpose of the first study (Chapter 3) was to characterize the stress protection afforded by exercise in behavioral tests of anxiety, and to verify that exercise increases galanin in the LC. The purpose of the second study (Chapter 4) was to assess whether the synthetic galanin receptor agonist galnon would protect against cocaine-induced behavior and neurochemistry in the frontal cortex. Galnon was selected because it exhibits similar benefits to galanin, but can be administered systemically as a non-peptide agonist that crosses the blood-brain barrier. The purpose of the third study (Chapter 5) was to determine whether galanin receptor activation is necessary for the behavioral protection afforded by exercise. Another purpose was to compare the protection afforded by exercise to repeated intracranial galanin administration by examining stress-induced behavior, as well as neurochemistry and neuroplasticity in the frontal cortex.

CHAPTER 2

LITERATURE REVIEW

EXERCISE OFFERS ANIOLYTIC POTENTIAL: A ROLE FOR STRESS AND BRAIN NORADRENERGIC-GALANINERGIC MECHANISMS

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Abstract

Although physical activity reduces anxiety in humans, the neural basis for this response is unclear. Rodent models are essential to understand the mechanisms that underlie the benefits of exercise. However, it is controversial whether exercise exerts anxiolytic-like potential in rodents. Evidence is reviewed to evaluate the effects of wheel running, an experimental mode of exercise in rodents, on behavior in tests of anxiety and on norepinephrine and galanin systems in neural circuits that regulate stress. Stress is proposed to account for mixed behavioral findings in this literature. Indeed, running promotes an adaptive response to stress and alters anxiety-like behaviors in a manner dependent on stress. Running amplifies galanin expression in noradrenergic locus coeruleus (LC) and suppresses stress-induced activity of the LC and norepinephrine output in LC-target regions. Thus, enhanced galanin-mediated suppression of brain norepinephrine in runners is supported by current literature as a mechanism that may contribute to the stress-protective effects of exercise. These data support the use of rodents to study the emotional and neurobiological consequences of exercise.

Anxiety and its treatment

Fear and anxiety-related behavior are adaptive responses that span across the phylum and serve to protect the organism from threat (1). However, mental pathology occurs when these responses are excessive, persistent, and clinically impairing in humans, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IVR) (2). Anxiety is the most prevalent type of mental disorder in the general population (3). Anxiety defines a class of disorders that contain an assortment of diagnoses (i.e., panic, agoraphobia, phobias, obsessive-compulsive disorders, posttraumatic or acute stress disorder, and generalized anxiety disorder), each of which possess a unique prevalence, pattern of symptoms, course, and treatment (2). Anxiety disorders exact a pervasive toll on the individual and impair numerous aspects of quality-of-life (i.e., induce physical, social, emotional, and occupational dysfunction) (4). The lifetime prevalence of DSM-IVR anxiety is about 31% and the 1 yr prevalence is about 19% in the US alone, according to World Mental Health surveys (3). The annual cost of treating anxiety in the US is $42.3 billion as assessed by the most recent national survey in 1990 (5, see also 6). Thus, anxiety disorders incur substantial cost to both the individual and society.

Pharmacotherapy is often a first-line of treatment for anxiety (7, 8). Yet, current drug therapies for anxiety have many limitations, including the high financial expense, delay in onset, limited efficacy, unwanted side effects, dependence, and stigma associated with consuming and depending on pharmaceuticals (9, 10). A need to develop novel treatments to fulfill these shortcomings exists. Physical inactivity is a risk factor for mental pathology (11-13) and physical activity improves psychological risk factors (14), which suggests that involvement in physical activity contributes to normal mental health. Exercise may offer additional benefits that leading anxiety therapies cannot (e.g., social acceptance of exercise as a healthy behavior, low financial costs, limited side effects, physical health benefits). Evidence accumulated extensively over the past 30 years suggests that physical activity is a promising candidate for the treatment of anxiety.

Physical activity protects against the onset of anxiety and treats anxiety symptoms in healthy people and medical patients, despite age, sex, or other medical conditions (15, 16). The effectiveness of exercise is comparable to or better than many standard forms of anxiety treatment (17-19). Quantitative reviews suggest that exercise reduces anxiety with a small-to-moderate magnitude of effectiveness that can be seen after short- and long-term treatment (16, 17, 19-21). Population data show that as the weekly frequency of exercise increases the risks for anxiety decreases (12). Moderate-to-high intensities of exercise yield larger treatment effects on anxiety than low intensities, as further supported by intervention studies (20). However, a dose-response relation or specific duration or mode of exercise that is especially well suited for treating anxiety is yet to be confirmed using randomized controlled trials, likely because such studies are presently scarce (13, 17, 22). Nonetheless, there is evidence that acute aerobic exercise and training produces immediate and lasting improvements in anxiety symptoms (19, 23), whereas the beneficial effects of a resistance exercise may depend on characteristics of the exercise regimen (e.g., intensity, duration) (24). Together, these data suggest that physical activity can serve as an alternative or compliment to current treatments for anxiety.

The neurobiological mechanisms that support the anxiolytic potential of exercise are unclear. Rodent models of anxiety are essential to permit mechanism-driven investigation into the neural basis of exercise. The contribution of rodent models was key to establish that exercise has neurogenic, neurotrophic, and neuroplastic effects that underlie improvements in learning and memory (for review see 25). However, it is presently controversial whether voluntary exercise reduces anxiety-like behavior in rodents. Evaluation of such evidence is critical at this juncture, as it will help determine whether rodent models should be used to understand the role exercise has on anxiety and its underlying neurobiology. Thus, the primary aim of this review is to evaluate current evidence of voluntary wheel running in behavioral tests of anxiety in rodents. We conclude that rodent models can indeed be used to understand the

effects of exercise on anxiety and propose that the behavioral efficacy of exercise depends on stress. We also identify variables that may impact the relationship between voluntary exercise and anxiety-like behavior, while drawing attention to limitations in the literature and recommending research to further understand this relationship. A secondary aim is to examine how wheel running alters neurotransmission involving norepinephrine and galanin in circuits that regulate stress and anxiety. We propose that wheel running promotes an adaptive response to stress via norepinephrine-galanin mediated brain mechanisms.

This review will focus on evidence from studies that used voluntary exercise. Evidence from other experimental paradigms (e.g., treadmill running, swimming) will not be included due to the confounded influence of stress and well-documented difference between free-choice wheel running and forced exercise in motor and affective behavior (26-31), brain signaling systems (27, 29, 30, 32, 33), and other physiological systems sensitive to stress (34, 35). Stress increases wheel running in a manner that is blocked by an anxiolytic drug (36), suggesting that voluntary exercise is not itself a stressor. Exercise also protects against stress at the neurobiological, neuroendocrine, and neuroimmune level (for review see 37, 38, 39). Although voluntary exercise has qualities of a stressor (e.g., activates sympathetic nervous system, HPA axis, and other stress-responsive brain circuitry), it deserves unique classification and examination from other stressors because it is engaged voluntarily and is neuroprotective, predictable, controllable, and rewarding (40-44).

Tests and models of anxiety in rodents

The distinction between tests and models of anxiety is an important consideration in this review because their use, alone or in combination, may influence the interpretation of behavioral outcomes produced by voluntary exercise. Tests and models are tools that are user-defined by their application in research and do not possess “hereditary titles” (45). Tests of anxiety are commonly used once in a study as a bioassay or screen to characterize anxiolytic drugs or to

phenotype rodents (e.g., genetic knockouts). Tests of anxiety are optimized under specific environmental parameters, validated mainly by benzodiazepines, and include the Geller and Vogel conflict, defensive burying, elevated plus maze, fear potentiated startle, hole board, open field, social interaction, and ultrasonic vocalization (for review see 46, 47). Although it is relevant to note that many of these tests can become models and induce persistent anxiety-relevant features in studies that measure the lasting consequence of exposure to the test itself, they are more commonly used as a test on one occasion with no further testing. Thus, as generally used in the biomedical literature and in every study examined in the present review, tests of anxiety allow a means to collect dependent variables to characterize behavior (hereafter referred to as *baseline responding*). *Models* of anxiety exhibit validity (e.g., construct, etiological, face validity) that tests do not necessarily possess and can produce relatively stable and persistent anxiety-related traits after induction by an experimental manipulation (hereafter referred to as *evoked responding*). Common stress-evoked models include uncontrollable stress, chronic unpredictable stress, and maternal deprivation. However, tests of anxiety are unfittingly referred to as 'models' of psychopathology throughout biomedical when used as a screen (for reviews see 48, 49). This terminology misuse muddles the theoretical purpose (independent *vs.* dependent variable) and implicitly assumes that tests possess forms of validity that were not necessarily evoked. Thus, this misnomer may affect how one assigns value to data and places results into logical frameworks.

The bulk of basic research employing voluntary wheel running used tests of anxiety as screens to measure baseline responding (i.e., without the use of a model or assessing evoked responding after exposure to an experimental stressor) (26, 27, 30, 50-62). However, several reports measured the influence of wheel running on evoked responding in tests of anxiety by exposing rodents to a stressor (63-72) or stress-based models of anxiety (73-75). Based on evidence available to date, we propose that the behavioral efficacy of exercise in tests of anxiety is influenced by stress, including stress-based models of anxiety. The stress response

is any event that moves an organism away from homeostasis and can be adaptive when elicited short-term in a threatening environment. However, stress can become maladaptive and contribute to the development/exacerbation of anxiety when excessive, uncontrollable, and persistent (76-78). In the present review, we try to avoid making assumptions about emotional states and complex cognitive processes in rats that may not exist and/or cannot be directly measured (48, 79, 80), but focus more on the variables that are manipulated and measured in the experiments. We therefore refer to stress in the context of an independent variable rather than assuming that it has produced anxiety in rodents.

Effects of wheel running on anxiety-like behavior and fear learning

Wheel running offers anxiolytic-like potential in a manner dependent on stress

The effect of exercise is at odds when assessing baseline responding in tests of anxiety (see Table 2.1). Chronic wheel running produces anxiolytic-like (30, 31, 50-52, 59, 66, 81), anxiogenic-like (26, 54, 57, 58), and null (55) effects in rodents. Discrepancies between these reports on exercise and affect can be attributed to differences in experimental parameters, including social rearing conditions (40, 82), time of day of behavioral testing (53), type of sedentary comparison group (no wheel *vs*. locked wheel) (60), duration or distance of wheel running (26, 67, 68, 83), sex of subjects (84), time of testing relative to the last wheel access (51), as well as aversiveness of the testing environment, handling history, and genetic background (46, 85). Although these experimental parameters likely moderate the relationship of wheel running and emotion, no single variable is expected to reliably account for inconsistent effects of exercise across tests of anxiety when stress was not experimentally manipulated. Instead, wheel running produces inconsistent effects on baseline responding in tests of anxiety likely due to a variety of internal and/or external variables that ultimately influence the impact of stressors on the organism.

Wheel runners are resistant to the toll of stressors or stress-evoked models of anxiety (see Table 2.2). It is important to note that benefit of exercise on evoked responding in an array of tests of anxiety is mainly due to stress-induced impairment in sedentary, but not exercise rodents (63-65, 67-71, 73-75). Reliable detection of the beneficial consequences of wheel running may result only when stress is experimentally manipulated because the effects of exercise interact with stress to alter responding in a manner that behavioral screens can detect. Experimental evidence that bolsters this conclusion suggests that differences between exercise and sedentary rats on evoked responding in tests of anxiety emerge after exposure to repeated injection/drug stress, but not in the absence of such stress (63, 65). For example, rats that were allowed access to a running wheel for 3 wk exhibited anxiolytic-like behaviors across several tests if the rat had a history of repeated stress, but failed to produce these effects in exercised rats tested under baseline conditions of stress or intense stress evoked by a high dose of an anxiogenic drug (see Figure 2.1). The central thesis of this review is that the anxiolytic-like benefit of chronic voluntary exercise emerges after exposure to mild-to-moderate intensity stress, wherein the level of stress an animal experiences is deliberately induced by an experimenter or inherent in the experimental design (e.g., aversiveness of the housing or testing environment, rearing and handling conditions) and/or modified by other factors that influence stressor responsiveness (e.g., genetics, maternal history) (see Figure 2.2). Of note, to our knowledge there is no evidence to suggest that physically active animals would be more anxious than sedentary animals following extremely high levels of stress, although this is a possibility in select populations (e.g., regularly running high amounts) based on prior evidence (57, 58). The thesis of this review is further evaluated below by examining the impact of exercise on baseline responding in tests of anxiety, followed by examining the impact of exercise on stress-evoked responding.

Effects of wheel running on baseline responding in tests of anxiety

Affect-modulated startle. The startle reflex is a muscular contraction to an abrupt stimulus (e.g., tone, light, air puff) that likely serves to avert injury from attack (for review see 86). Select isoforms of anxiety, including posttraumatic stress disorder and obsessive compulsive disorder, are distinguished by exaggerated baseline startle and/or diminished ability to inhibit startle (87, 88). Emotive-laden stimuli can be used to enhance or diminish startle (89-91). Whether acute exercise reduces such measures of startle in humans is presently unclear. An acute exercise session was not sufficient to alter baseline startle or affect-modulated startle in healthy individuals, nor did it alter prepulse inhibition in those with high-trait anxiety (92-95). Yet, the impact of a chronic exercise regimen on the startle reflex remains to be elucidated.

While some reports suggest that a history of wheel running reduces baseline startle (59, 66), others show that running does not alter this measure (55, 84, 96). Prepulse inhibition of the acoustic startle response is consistently unaltered by wheel running (55, 66, 97). In line with the idea that a history of wheel running produces beneficial effects on startle, mice that ran on a wheel for 2 wks exhibited reduced acoustic startle amplitude relative to sedentary counterparts (66). Reductions in baseline startle were not seen after short durations of wheel running (3 d after), but were detected 1 wk after and persisted as long as the mice were allowed to run (up to 12 wks). Thus, reduced startle after wheel running likely occurs from adaptations that result from repeated running. Further, wheel running and sedentary rodents both exhibit comparable reductions in startle as acoustic stimuli are repeatedly presented, which suggests that wheel running exerts an influence on startle independent of habituation (55, 66). Reports that show reduced baseline startle after wheel running originate from a laboratory that uniquely tested for startle during the light phase of the light:dark cycle, whereas those showing null effects stem from laboratories that tested during the dark phase; c.f. Cacciaglia et al. (96) and Pietropaolo et al. (55, 84). Diurnal variations in startle may explain why these reports are at odds, as startle exhibits circadian rhythmicity (98-100). Specifically, startle is about half the amplitude in the

light versus the dark phase, and the effects of pharmacological agents may be more evident when startle is measured in the light phase (101-104). Thus, testing in the light phase may produce better experimental conditions to reveal treatment-induced reductions in startle by wheel running. However, it is also possible that divergent effects of wheel running are due to inherent or external levels of stress that affect startle (105).

Exploration. Most research on exercise characterized anxiety-like behavior using tests that rely heavily on locomotion. Exploration-based tests are time-restricted to elicit a typical response and include examples like the elevated plus and zero mazes, hole board, dark:light box, and open field tests (for review of tests see 46, 47). Responding in exploration-based tests relies on unconditioned, spontaneous behavior in a novel testing apparatus that is designed to elicit approach-avoidance conflict. These tests likely evoke a degree of neophobia, exploration, fear, and motivation, although the weight of each is probably different in each test. Attesting to the differences across tests of exploration, the degree of overlap is suggested to be very low (estimated at approximately < 20% overlap) (106). However, an important unifying theme of all exploration-based conflict tests is the reliance on locomotor activity (107, 108). File offers particularly useful advice in a review on the use of tests of exploratory behavior to study anxiolytic agents, writing, "…the use of tests of exploratory behavior to screen for new potential "benzodiazepine-like" compounds is somewhat hazardous, unless accompanied by other tests and carefully interpreted" (108). Applying and extending this advice to understand exercise, we are reminded that such tests are GABA-ergic sensitive (and possibly preferential) and warned against overreliance or oversimplification of anxiety-like behavior from exploration-based tests (as they are often confounded by locomotor activity and result from numerous impinging drives). Nonetheless, exploration-based tests are important to profile anxiolytics, including exercise regimens.

Dark:light tests. Rodent behavior in the dark:light box is driven by the conflict to avoid brightly illuminated spaces (reside in dark compartment) against the need to explore a novel environment (reside in light compartment) (for review see 46, 47, 109). The defensive withdrawal test is a validated variant of the dark:light box, which has a proportionally smaller dark enclosure (110-119). Typically, anxiolytic-like responding is defined by decreased latency to enter, increased time spent, and/or increased entries in the lit compartment.

In dark:light tests, wheel running reduces (50, 60), enhances (57, 58, 96), or does not alter (65, 120) baseline anxiety-like behavior. Systematic factors likely account for differing results and may include the control group comparison, amount of wheel running, and level of stress the animal experiences. The sedentary comparison group likely contributes to reliable detection of anxiolytic-like effects of exercise in the dark:light test, as every report demonstrating this effect compared behavior against a sedentary group without a blocked wheel. For example, Chaouloff and colleagues (60) showed that the beneficial effects of wheel running in the dark:light box were present only when compared to sedentary controls that did not have a blocked wheel. Comparisons to no-wheel sedentary controls probably maximizes the difference between experimental groups, as a blocked wheel offers some degree of exercise (e.g., hanging, climbing; 121) and environmental enrichment (122, 123). The amount of wheel running may also contribute to the detection of anxiogenic behavior in the dark:light box, which emerges in mice that ran approximately more 8 km per day (see Table 2.1). In support of this explanation, qualities of the training regimen (27) and the amount of neurogenesis in the hippocampus is a necessary factor that determines the affective consequence of exercise in the dark:light box (57). Also, strong associations exist between the number of cells exhibiting a marker of hippocampal neurogenesis (DCX) and anxiogenic-like behavior in dark:light test (inverse correlation between time in lit side and exits from dark side; positive correlation between initial latency to exit lit side and endexploration) (58). Together, these data suggest that wheel running has the potential to exert benefits on baseline anxiety-like behavior in the

dark:light box, although additional factors (e.g., high amounts of running, sedentary control group, stress) likely moderate baseline anxiety.

Elevated mazes. Rodent behavior in the elevated maze is theorized to be the product of the endogenous drive to avoid unprotected open spaces versus the motivation to explore a novel environment (for review see 124, 125, 126). The elevated plus and zero maze are comparable in concept, sensitivity to detect anxiolytic/anxiogenic agents, and design, except the elevated zero maze has the O-shape modification that eliminates the potential confound of a central hub (127-129). Typical anxiolytic-like behavior in the elevated mazes consists of increased open arm time and entries, as well as a concomitant decrease in time spent on the closed arms.

The effects of wheel running in the elevated maze are mixed when baseline responding is measured in tests of anxiety. For instance, baseline anxiety-like behavior in the elevated mazes was reduced (31, 50-52, 84), increased (26, 54, 84, 96), or not changed (53, 55, 56, 65, 130) in runners that were not exposed to an experimental stressor. It is possible that inconsistent evidence in the elevated maze results from effects of exercise on locomotion or differences across studies in running distance. A subset of studies showed that wheel running reduces locomotor activity (e.g., distance traveled, number of total, closed, or full arm entries) in the elevated maze (31, 50, 51, 58, 96), and traditionally doses of drugs that impair locomotion confound interpretation of anxiety-like properties (131). However, the effect of exercise on baseline anxiety-like behavior in the elevated plus maze is still mixed even after excluding studies with locomotor confounds. High amounts of running likely contribute to detection of anxiogenic behavior in the elevated maze (see also section 4.1.1.1 57, 58, 96), although a minority of reports also show anxiolytic-like effects after high amounts of running (51). Collectively, we conclude that wheel running exerts anxiolytic potential in the elevated mazes,

but the effect of exercise is likely influenced by additional factors (e.g., distance of wheel running, stress).

Comparing the effects of wheel running across studies using the elevated maze is difficult because the behaviors measured are diverse, such that reports that conclude the same effect of wheel running produce different alterations in dependent variables. Therefore, it is recommended that future studies demonstrate alterations in complementary behaviors (e.g., increased open arm time corresponds with decrease closed arm time) in the elevated mazes, which will add confidence in conclusions about exercise that are based on data generated from these tests. Future studies interested in teasing apart the affective consequences of exercise in the elevated maze, should establish a dose-response relationship by testing log-base distance and durations of wheel running. Such research would add valuable insight to evaluate whether there is a threshold or an optimal level of characteristics that define exercise (e.g., distance, duration, frequency) that are needed to acquire beneficial emotional consequences. Indeed, a minimal duration of wheel running is necessary to see changes in the elevated maze (26) and restricted wheel access is also effective in reducing inherent levels of anxiety-like behavior in the elevated plus maze (31, 52). The efficiency the elevated maze offers allows the "dose-response" question of exercise to be tested with relative ease, which is of high translational relevance in recommending exercise regimens.

Hole board. The hole board test permits quantification of both directed (towards holes in floor board of arena) and general (in entire arena) exploratory activity in a novel testing arena (46, 109, 132-135). An anxiolytic-like response in this test is generally defined by an enhancement of hole-directed behavior. To date, only a couple of reports tested the effects of wheel running in the hole board test, both of which reported no effect of 4 weeks of wheel running in this assay. Wheel runners and sedentary controls were not different in the expression of head dipping in the Bossier's four hole board test (120). In the modified hole

board test that contains 23 centrally-located holes, exercised mice did not reliably differ on anxiety-related measures (equal time spent on or entries onto the hole board, but enhanced board latency) relative to sedentary mice (50). Exercised mice also exhibited reduced line crosses in the hole board test, which suggests that locomotor effects can be dissociated from head-dipping exploration (50). Not enough data are available from the hole board test to credibly interpret the effects of wheel running. It remains to be determined whether stress and characteristics of running (e.g., duration, frequency) will systematically influence behavior in this test.

Open field. The open field is a spacious arena used to characterize spontaneous locomotor activity and exploratory behaviors relevant to the study of anxiety (for review see 46, 109, 136). Similar to other approach-avoidance tests, rodent behavior in the open field is speculated to result from the need to avoid the center, unprotected portion of arena versus the impetus to explore a new environment. Most investigations show that rodents given access to a running wheel later exhibit reduced locomotor activity in the novel open field (26, 51, 53, 54, 56-58, 66), although some reports show no effect on locomotion upon initial exposure to the open field (27, 55, 81, 84, 120, 130). Comparing across studies, running-induced decreases in locomotion in the open field persist across characteristics of the subject (species, strain, sex, housing), exercise regimen (duration, distance ran, restricted, shared, resistance), and experimental test (duration, lighting, measure of locomotion). Evidence does not suggest that the open field is more aversive to wheel runners (66, 73, 75). Among the reports that show wheel running does not alter locomotion, wheel running failed to alter anxiety-relevant behaviors (e.g., center time or entries) in the open field relative to sedentary controls (27, 55, 81). Reduced locomotion in the open field is not likely due to running-induced fatigue because runners resume exercise after behavioral testing (unpublished observation), exhibit enhanced performance in the rotorod test (66), and are no different from sedentary controls on locomotion

in an activity or home cage (57, 81). Also, a strong positive correlation between open field locomotor activity and running distance exists during the active portion of the day, such that increases in locomotion are associated with increases in running distance (84). The fact that wheel running decreases locomotor activity limits meaningful interpretation of the effects of exercise on emotion-relevant behavior in the open field. Indeed, it is well accepted that inferring emotion from exploratory behavior is inaccurate when confounds in locomotion exist. Thus, we suggest that the open field is not well suited to infer the emotional consequences of wheel running, but is appropriate to observe alterations in locomotion or demonstrate locomotor confounds that could influence other tests of emotion.

Novelty. Though all of the paradigms reviewed above typically involve an element of novelty as an aversive stimulus, some paradigms place particular emphasis on novelty as the independent variable, and therefore fit appropriately into a separate category of tests. Novelty is speculated to provoke fear in rodents as measured by reduced exploration and enhanced avoidance in tests that evoke an approach-avoidance conflict (137-139). Several reports suggest that wheel running minimizes the effects of novelty on spontaneous behavior (61, 62, 64, 120). During exposure to a small novel cage or container, exercised rats exhibit more resting (i.e., more lying and/or stationary behaviors) and less non-resting behaviors (i.e., rearing, walking, grooming), all of which are displayed in an undisturbed rodent during the daytime (61, 62, 64, 120). Exercise also reduced the effects of novelty on HPA and autonomic functioning, as rats allowed to run exhibited reduced plasma ACTH and corticosterone, heart rate, and body temperature after exposure to a novel cage/container compared to sedentary rats (61, 64, 140). However, runners and sedentary controls did not statistically differ in the latency to consume novel chocolate pellets (55). Interpretation of this result is limited because the stress of novelty *per se* was not induced (i.e., similar mean latency to consume standard chow and novel chocolate in exercise and sedentary conditions). Further, the mean latency to

consume the novel chocolate tended to be lower in the exercise condition relative to a sedentary control, which may be meaningful because the research was based on a small sample (*n* = 5-6) and statistical analyses that included other groups. More research is necessary to determine whether wheel running reduces food neophobia, preferably as assessed by measures with less nutritional/energetic confounds (e.g., latency to *approach* the novel food). Together these data suggest that wheel running promotes adaptive coping to the stress of a novel environment.

Social interaction. Under normal conditions, rodents spontaneously engage in social interaction, whereas isolation produces an array of behavioral and neurochemical abnormalities (for review see 141, 142, 143). In the social interaction test, a pair of rodents is allowed to interact in an arena and the time spent engaged in active social behaviors with an unfamiliar mate is measured (144, 145). Anxiogenic drugs reduce social interaction, whereas this behavior is increased by anxiolytics (146, 147). Wheel running also has the potential to increase social interaction. For example, Salam et al. (66) showed that exercised mice that were group housed exhibited increased time/frequency sniffing, following, grooming, and climbing a novel conspecific, relative to non-exercising mice. However, Burghardt et al. (26) showed that exercise and sedentary rats kept singly housed were no different in the time spent in contact or active pursuit of a novel conspecific . Differences in the social history of the subject (single vs. group housed) or social mate (potentially non-matched vs. matched for social history) may account for differences in social interaction after wheel running. Indeed, isolation in the juvenile period or adulthood increases aggression and alters social interaction and exploratory behavior in rodents (142, 148-150). Adolescent rodents that were socially reared prefer a compartment previously paired with similarly housed partners, whereas isolates do not exhibit this preference (150). In any case, although data on wheel running and social

interaction is limited, they are consistent with the conclusion that exercise offers anxiolytic potential.

Structured threat. Threat initiates defensive behaviors that are analogous across human and non-human animals (151-153). Defensive behaviors are speculated to be perturbed in those with anxiety disorder, and accordingly are modified by anxiolytics (154-159). Structured tests of threat (e.g., shock probe defensive burying, anxiety/defense test battery; for reveiws see 160, 161) initiate an array of defensive behaviors that are not necessarily measured in standard tests of anxiety (157). Consistent with the idea that the effects of exercise and stress interact, evidence shows that exercise failed to reliably alter measured behavior in the shock probe defensive burying test in rats that were exposed to no experimental stressor (65). More research is needed to comprehensively understand the conditions under which exercise alters defensive behavior and structured tests of threat should prove useful.

Effects of wheel running on stress-evoked responding in tests of anxiety

Affect-modulated startle. Wheel running consistently produces a stress-protective effect in the acoustic startle test of anxiety. For example, wheel running mitigated light-induced and mCPP-induced potentiation of acoustic startle in mice (63, 66). Startle data are consistent with the hypothesis that the anxiolytic-like benefit of wheel running emerges after exposure to mild-to-moderate intensity stress. For example, exercise-induced reductions in startle were dependent on the dose of the anxiogenic agent mCPP, such that only the highest dose (1mg/kg i.p.) increased startle in exercised mice relative to vehicle (63). Since wheel running alters factors that modulate startle, such as arousal (162-164), attention (165, 166), and motivation (42, 44, 167-169), it is particularly relevant to determine whether these factors influence the effects of wheel running on startle. Collectively, these data suggest that chronic wheel running

has the ability to modulate startle in a manner that is beneficial and potentially stress-dependent.

Exploration. The ability of wheel running to ameliorate the effects of stress in exploration-based tests of anxiety is clear. In the dark:light box, wheel running prevented anxiety-like behavior (time in lit area and number of lit compartment entries) that sedentary rats exhibited after exposure to maternal deprivation (74). Wheel running also facilitated locomotor habituation in the defensive withdrawal test in rats exposed to repeated injection stress or pharmacological stress using the anxiogenic β-carboline FG7142 (7.5 mg/kg x 10 d) (65). However, a high dose of FG7142 (30 mg/kg i.p. x 1 d) dramatically suppressed locomotor activity and produced intense immobility and avoidance in this test regardless of whether rats ran on a wheel, which could imply that the beneficial effects of exercise are not sufficient to overcome intense stressors. In the elevated plus maze, rats allowed to run did not show enhanced anxiety-like behavior (reduced open arm time, open entries, and head dips) that their sedentary counterparts exhibited after exposure to either repeated injection stress or maternal deprivation (65, 74). Similarly, in the open field wheel running mitigated the deficits in locomotion induced by chronic mild stress or social stress (73, 75). Stress increased the time spent in the center portion of the open field in wheel runners (relative to no-stress), whereas stress produced the opposite or anxiety-like effect in sedentary rats (170). These data clearly demonstrate that wheel running offers stress resilience in an array of exploration-based tests of anxiety.

Shuttle box escape and freezing after shock-elicited fear. Uncontrollable or inescapable stress is a model of anxiety that evokes deficits of shuttle box escape and exaggerated freezing in tests conducted 24-72 hr later, whereas controllable or escapable stress does not (for reviews see 77, 171). Uncontrollable stress induced by shock (e.g., 100 shocks / session) produces

behavioral sequelae that generalize to environments separate from the fear context (unlike fear conditioning) and sensitize neural systems that mediate fear. Wheel running does not alter shock-elicited fear *per se*, but blocks the behavioral impairment later displayed after uncontrollable stress (for review see 39). Wheel running is repeatedly shown to ameliorate the effects of uncontrollable stress induced by shock on shuttle box escape and freezing (37, 68-71). Of note, the benefit of exercise after uncontrollable stress is displayed after 6 wks of wheel running, but not before then (67-69, 172), which suggests that some benefits of exercise become apparent after long durations. Wheel running also protected against the shuttle box escape deficit and exaggerated freezing produced by an acute dose of the selective serotonin reuptake inhibitor fluoxetine (71). The effects of wheel running after shock-elicited fear are robust and suggest that exercise offers stress resilience.

Structured threat. Burying in the shock probe test is an active defensive behavior that is increased by stress or anxiogenic manipulations (145). The advantage of this measure, in contrast with the majority of those discussed above, is that it assesses active as well as passive behavioral responses to aversive stimuli. We observed that wheel runners do not exhibit the increase in burying that sedentary rats display after repeated injection stress or pharmacological stress using the anxiogenic β-carboline FG7142 (65). Furthermore, the effect of exercise in this paradigm may depend on the level of evoked stress. A high dose of FG7142 (30 mg/kg i.p. x 1 d) produced intense immobility and hindered other defensive behaviors in the shock probe test regardless of whether rats ran on a wheel (see Figure 2.1). These findings once again support the model proposed herein that the anxiolytic potential of exercise depends on stress.

Wheel running improves fear learning

Exercise improves learning and memory and prevents cognitive decline in humans and non-human animals (for review see 173, 174). Fear conditioning is associative learning that

permits an organism to use relevant cues in the environment to predict threat (for reviews see 175, 176-178). In the context of this review, it is relevant to evaluate whether the effects of exercise on fear drive or produce the enhancement of aversively-motivated learning. Collectively, the effects of exercise on aversively motivated learning are separable from fear/anxiety-relevant behaviors. Therefore, we conclude that wheel running enhances fear conditioning across paradigms through learning and memory (see Table 2.3), and not fear processes *per se*.

Converging evidence suggest that rodents with a history of wheel running exhibit improved aversively-motivated learning in a contextual fear conditioning paradigm, as assessed by increased freezing to a context that was previously paired with shock (53, 60, 81, 83, 179-183). Enhanced contextual fear conditioning occurs across wheel running durations that range from ~2 to 8 weeks, which suggest that the learning effects of exercise are long-lasting. However, exercise-induced adaptations may need to be established prior to contextual fear conditioning, as wheel running (1, 4, or 6 wk) did not alter freezing to the shock-paired context if it occurred after fear-conditioning (96, 181). Further, wheel running (1 or 6 wk) did not alter extinction of fear-conditioned freezing, regardless of whether running was pre- or post-fear conditioning (181). Van Hoomissen and colleagues proposed that wheel running alters the speed of memory retrieval comparable to exercise-training in humans (184), as running selectively increased freezing to context in the beginning of the fear conditioning test (179, 180).

A minority of reports did not generate an enhancement of contextual fear conditioning after running (26, 55, 96, 185). Of these Wojtowicz et al. (185) trended towards demonstrating exercise-induced facilitation of fear conditioning. However, the lack of an effect observed in the other two reports are likely explained by factors previously shown to alter fear conditioning and wheel running, such as the time of testing (53) or distance ran (high vs. low running) (83). Higher amounts of freezing are selectively exhibited in sedentary controls when tests of contextual fear conditioning occur at the beginning relative to the end of the light cycle (53). As

such, increased freezing in sedentary mice in Pietropaolo et al. (55) may not be specific to learning because testing was uniquely conducted in the dark of the light:dark cycle, which could preclude detection of enhanced freezing in wheel runners that is indicative of learned fear. The null finding in Burghardt et al. (26) may be attributed to large individual variation in running, which is supported by subsequent data from the authors showing variation in running concealed gains in contextual fear learning (83).

Several lines of evidence support the conclusion that wheel running increases fear conditioned freezing due to associative learning of the shock-context pair. First, wheel running reduced or did not alter freezing to a novel context never paired with shock, but selectively increased freezing to the context paired with shock (75, 180, 181). Second, enhanced contextual freezing after exercise cannot be attributed to confounds in freezing or nociceptive detection/sensitivity because wheel running and sedentary animals exhibit similar pre-conditioning freezing, shock reactivity, and activity burst durations (59, 83, 96, 179, 181, 183). Third, wheel running facilitated learning under non-optimal conditions (e.g., minimal duration of context pre-exposure) in a manner independent of freezing in a context not paired with an aversive stimulus (181). Although intrinsic differences in fear may influence fear learning in exercised rodents (83), the reviewed evidence suggests that it is unlikely that differences in fear *per se* produce the enhancement of fear learning after exercise. These data suggest that wheel running enhances contextual fear conditioning via learning and memory processes.

Wheel running also enhances fear learning in tests of passive avoidance (see also 29, 186) and fear-potentiated startle (59). Mice given access to a running wheel exhibit enhanced startle amplitude to a tone previously paired with shock relative to sedentary mice (59). Wheel running may particularly influence learning and consolidation, as wheel runners exhibit improved fear-potentiated startle when running is restricted to periods most likely to affect learning (2 wk before conditioning) or consolidation (2 wk after conditioning), but not retrieval or performance (2 wk before testing) compared to sedentary counterparts (59). However, wheel running does

not alter *freezing* to a tone previously paired with shock (53, 55, 81, 183, 185). Differences between cued conditioned freezing and other forms of fear conditioning may result from several factors, including the behavioral measure of fear learning (freezing vs. startle), strength of conditioning, or strength of input from different neural regions mediating these responses (e.g., hippocampus, regions of the amygdala, locus coeruleus, dorsal raphe), as previously hypothesized (59, 83, 179, 181). Because anxiety is characterized by an inability to inhibit fear responding and a bias to attend to threat-related cues (187, 188, for review see 189), it is important for future research to focus on whether wheel running assists in extinguishing learned fear and distinguishing safety signals from threat.

Conclusions and future directions. Rodents are sensitive to the benefits of voluntary wheel running across tests of anxiety, which supports the utility of rodent models to investigate the mechanisms underlying the benefits of exercise on emotion. The evidence reviewed herein shows a clear benefit of exercise on evoked responding (e.g., after exposure to a stressor or stress-based model of anxiety) and mixed effects for the benefit of exercise on baseline responding in tests of anxiety. Although it remains possible that conflicting evidence of exercise on baseline responding results from variation in behavior across tests of anxiety or laboratories, we identify specific variables that could contribute inconsistent effects in the literature. Evaluation of experimental variables (e.g., manipulated stressors, non-manipulated variables that act as stressors) that influence the effects of exercise through meta-analysis will be warranted as additional research accumulates. Further, wheel running improves fear conditioning through learning and memory processes, which minimizes the possibility that exercise-induced alterations in fear *per se* drives such learning. In sum, evidence to date suggests that wheel running exerts anxiolytic potential in a manner that depends on stress (Figure 2.2).

The important influence of stress in the reviewed data is in line with previous evidence showing that stress is a risk factor for anxiety and comorbid disorders (190, 191). The influence of specific types of stress (physical vs. psychological) or intensities of stress (no, mild, moderate, and severe) on exercise outcomes remains to be validated by meta-analytic techniques. Induction of persistent anxiety is an essential design element that is needed to further characterize the ability of exercise to buffer the toll of stressful life events. Thus far, only a few reports investigated the effects of wheel running in an established stress-evoked of anxiety (i.e., chronic mild stress, repeated social stress, maternal deprivation, uncontrollable stress) (73-75). An extensive review of the advantages and disadvantages of preclinical models of anxiety can be found elsewhere (49). Models that are genetically (e.g., High Anxiety Behavior strain, Syracuse strain, serotonin transporter knockouts) and pharmacologically based are particularly well-suited to offer mechanistic insight into the protective effects offered by exercise (192-197). For translational purposes, it will be relevant to explore the biological underpinning of short- and long-access running, as they may have distinct affective consequences (198).

The absence of a clear dose-response of exercise (intensity, duration, frequency) on anxiety deserves consideration. In humans, a dose-response relationship between exercise and anxiety has yet to be established (13). Similarly, as assessed by correlation in rodents, no reliable association exists between running distance and responding in tests of anxiety, including the elevated plus maze (26, 84), open field (26, see also 84), prepulse inhibition of acoustic startle (84), or shuttle box escape and freezing after uncontrollable stress (69). This does not preclude the idea that a dose-response exists for wheel running and anxiety, but forces one to examine whether this response is linear and/or affected by other factors such as stress, reward, attention, or learning.

The reviewed evidence supports the use of wheel running as a tool in the study of exercise and anxiety. Understanding the specific neurobiological mechanisms for exercise-

mediated improvements in anxiety should focus on specific behaviors that are well defined operationally. The evidence reviewed above indicates that measures of acoustic startle in fear-potentiated paradigms, defensive burying in the shock probe test, and freezing and shuttle box escape in uncontrollable stress paradigms show particular promise. A symptom-driven approach will show clear links between the neural alterations of wheel running and specific anxiety-relevant behavior. Recognizing that rodent models are limited in their ability to reproduce the collection of symptoms seen in humans with anxiety will minimize anthropomorphic leaps and encourage a coherent understanding of the functional neurobiology underlying exercise.

Effects of wheel running on neurotransmission in regions controlling stress and anxiety

Several plausible neural mechanisms have been proposed to mediate the affective consequence of wheel running, including alterations in monoamine (31, 32, 68, 69, 74, 199-202), endocannabinoid (73), glutamate (203, 204), GABA (30, 205), and galanin (179, 202) signaling systems. A summary of alterations induced by wheel running in neural circuitry controlling stress and anxiety is provided (see Table 2.4). Neuroanatomical structures that transmit the stress-protective benefit of wheel running may be obtained from measures of immediate early gene expression (see Table 2.5). In particular, wheel running attenuates stress-induced elevations of cFos in stress-responsive circuitry, including the prelimbic and infralimbic cortex, lateral septum, subiculum, bed nucleus of the stria terminalis, periventricular nucleus, preoptic area, dorsal medial hypothalamus, dorsal raphe, cuneiform nucleus, and locus coeruleus (68, 69, 201, 206).

Although many mechanisms have been proposed, only a single report to our knowledge provides causal evidence for the effects of exercise on anxiety. For example, exercise-induced alterations in anxiety-like behavior in the open field, dark:light test, and elevated O-maze were reversed by blockade of hippocampal neurogenesis (57). In contrast, irradiation of the

hippocampus did not block the enhancement of contextual fear conditioning that was exhibited in runners, which suggests that intact hippocampal neurogenesis is not required to mediate fear learning (182). The enhancement of contextual fear conditioning after wheel running was prevented by either chronic administration of the non-selective β-adrenergic receptor blocker propranolol (179) or removal of cortical afferents to basal limbic structures (180). These studies bolster the idea that enhanced fear learning after exercise is likely attributed to improved learning capacity and not fear *per se*. Although beyond the scope of the present review, it is important to point out that voluntary exercise also exerts stress resilience through neuroendocrine (40), neuroimmune (207), and neuroplastic and neuroprotective (41, 208) mechanisms, as reviewed in detail elsewhere. For a comprehensive discussion, the remainder of this review will focus on evidence showing that wheel running alters norepinephrine and galanin systems in circuitry controlling stress and anxiety, as other well-supported mechanisms of exercise (i.e., serotonergic) are reviewed elsewhere (Greenwood and Fleshner, 2008, 2011). We propose that a noradrenergic-galaninergic mechanism plays an important role in conferring the stress buffering capacity of exercise. To support this hypothesis, it is important to briefly review how alterations in norepinephrine and galanin systems contribute to anxiety in a manner dependent on stress.

Norepinephrine and galanin systems control anxiety-like responding in a manner dependent on stress

Norepinephrine is implicated in the pathogenesis of anxiety, and similarly drugs that target the norepinephrine system (i.e., norepinephrine serotonin reuptake inhibitors) are currently a popular first-line of therapy for anxiety (209, 210). Norepinephrine putatively influences anxiety in a manner that depends on conditions of stress (211). Norepinephrine can induce both anxiogenic and anxiolytic effects, and therefore a balanced noradrenergic tone produces appropriate vigilance and stressor responsiveness. Norepinephrine is a

neuromodulatory transmitter that likely influences anxiety by optimizing excitatory and inhibitory tone in the brain via action at adrenergic α_1, α_2, and β receptor subtypes (211, 212). The ascending noradrenergic system contains cell bodies in the medulla and pons that project throughout the brain (213). The locus coeruleus is an important noradrenergic nucleus that projects to numerous other regions that regulate stress and anxiety, including the frontal and cingulate cortices, amygdala, olfactory bulb, septum, hippocampus, hypothalamus, periaquiductal gray, and raphe nuclei (214-216).

The majority of norepinephrine neurons in the locus coeruleus also contain the peptide/trophic factor galanin (217-220). Under conditions of high activation, amperometric experiments show that the soma of locus coeruleus neurons release norepinephrine from large dense core vesicles (221), which presumably co-contain peptides (e.g., galanin). *In situ* hybridization evaluated at the light and electron microscope level coupled with tannic-acid experiments show that galanin is synthesized and released in large dense-core vesicles from dendrites in the locus coeruleus (222). Galanin acts on at least three G-protein coupled galanin receptors in the brain (GalR1-3), all of which are present in the locus coeruleus, and are differentially expressed in midbrain and limbic structures that respond to stress (223-231). Activation of galanin receptors is predominantly inhibitory and induces hyperpolarization/outward current by increasing K^+ or decreasing Ca^{++} conductance (for review see 232). Electrophysiological data show that galanin inhibits activity of the locus coeruleus in brainstem slice preparations of the rat (233-236). Collectively, these data support an auto-inhibitory role of galanin on locus coeruleus neurons.

Stress and rodent models of pathology alter prepro-galanin expression and galanin receptor density in the locus coeruleus as well as the amygdala and hypothalamus (237-242). Additionally, polymorphisms in the prepro-galanin promoter are associated with the severity of symptoms in women with anxiety disorders (243, 244). Both galanin and the non-selective galanin receptor agonist galnon dose-dependently reduce anxiety-like behavior in several

rodent assays when administered systemically or intracerebroventricularly (i.c.v.) (245, 246). Whereas a non-specific galanin receptor antagonist like M35 or M40 blocks these effects or produces the opposite effect by increasing anxiety-like behavior (246, 247). Interestingly, systemic administration of the selective GalR3 receptor antagonist SNAP37889 or, SNAP398299 produced anxiolytic-like behavior in several rodent assays (248), which suggests that galanin receptor subtypes each uniquely influence anxiety. Although galanin modulates anxiety, the effects of galanin may depend on the brain region of interest, test used to measure anxiety, and the degree of stress the animal experiences (for review see 249, 250, 251).

Accumulated evidence suggests that the anxiety-related effects of galanin are specifically evoked under conditions of high stress or norepinephrine activity. For example, galanin administered i.c.v. failed to alter anxiety-like behavior under non-stressed conditions in C57BL/6J mice in tests of exploration (252). Administration of the galanin receptor antagonist M40 in the central nucleus of the amygdala was unable to alter behavior in rodents tested for baseline responding in tests of anxiety or evoked responding after experimental exposure to acute restraint stress or the α_2 adrenergic receptor antagonist yohimbine (253). However, restraint stress combined with yohimbine was necessary to increase galanin levels in the central nucleus of the amygdala, and only under these conditions did M40 block the effect of stress on anxiety-like behavior (253). M40 injected into the lateral septum or bed nucleus of the stria terminalis blocked anxiety-like behaviors in the shock probe defensive burying, elevated plus maze, and social interaction tests in rats that were exposed to an electrified shock prod or restraint stress (254, 255). Further, transgenic mice that overexpress galanin in norepinephrine and epinephrine-synthesizing neurons exhibit normal behaviors in an array of tests of anxiety under baseline conditions, but after exposure to yohimbine stress exhibit an anxiolytic-like behavior in the dark:light task compared to wild type controls (256). These data support the conclusion that enhanced galanin drive in noradrenergic circuitry is induced by stress to dampen enhanced noradrenergic drive. It is notable that the anxiolytic potential of galanin and

wheel running bear a striking similarity: both manipulations do not reliably alter baseline levels of anxiety, yet consistently reduce stress-evoked responding in tests of anxiety-like behavior. This parallels data presented in the first part of the review and highlights the interesting possibility that wheel running exerts anxiolytic-like potential via neural mechanisms that involve interaction between the norepinephrine and galanin systems.

A proposed noradrenergic-galaninergic brain mechanism underlying the stress protective effects of wheel running

Research from our laboratory and a few others show that wheel running regulates norepinephrine activity. Suggesting that chronic exercise inhibits noradrenergic locus coeruleus neurons, rats allowed regular access to a running wheel exhibit reduced expression of cFos in locus coeruleus neurons that express tyrosine hydroxylase immunoreactivity after uncontrollable stress (201). *In vivo* microdialysis data show that wheel running dampens the enhanced norepinephrine release after footshock stress in the frontal cortex (202). As the cortex receives norepinephrine terminals exclusively from the locus coeruleus (257-259), it is reasonable to assume that such changes after exercise result from alterations in locus coeruleus projection terminals. However, runners were not different from sedentary counterparts in the expression of the rate-limiting norepinephrine synthetic enzyme tyrosine hydroxylase in the locus coeruleus or other regions (e.g., subcoeruleus, A5, ventral lateral medulla) (201, 202, 240, 260), which supports an important role of galanin in mediating the effects of exercise on the locus coeruleus. Wheel running also failed to alter mRNA expression for the α_2 adrenergic autoreceptor in the locus coeruleus after six weeks of running (37). The lack of such an effect does not preclude the possibility that wheel running constrains locus coeruleus noradrenergic activity via this autoreceptor, as alterations in locus coeruleus adrenergic α_2 receptors may occur after transcription (e.g., increased α_2 receptor affinity and/or expression of this protein after wheel running). However, it does suggest that exercise-induced inhibition of locus coeruleus activity

may involve other, non-noradrenergic mechanisms. Further, wheel running increased β adrenergic receptor binding in the frontal cortex after footshock stress, yet the opposite effect was seen in the absence of an experimental stressor (261). These data show that wheel running alters noradrenergic signaling in neural circuits that are sensitive to stress and support the possibility that wheel running exerts anxiolytic-like potential through use of the neuromodulator galanin in these circuits.

We have repeatedly observed that galanin expression is augmented in the locus coeruleus by wheel running. Rats that ran on a wheel for 3-4 weeks exhibited increased density of prepro-galanin mRNA in the locus coeruleus relative to sedentary rats (65, 179, 260, 262, 263). It is interesting to note that both voluntary and forced exercise increase prepro-galanin mRNA in the locus coeruleus (240). Supporting the functional relevance of these data, plasma galanin secretion is also increased after an acute bout of exercise in humans (264). Increases in galanin expression in the locus coeruleus is correlated with increases in the distance ran on a wheel, suggesting a dose-dependent effect of exercise on brain galanin (65, 167, 262). Galanin expression in the locus coeruleus is also increased by acute and chronic stress (237, 265), an animal model of depression (238), psychotherapeutic treatment (262, 266, 267), and opiate administration and withdrawal (268), which suggests that galanin is recruited as a counter-regulatory mechanism to dampen noradrenergic tone. Elevated galanin levels after exercise may remain elevated after stressor exposure, as exercised rats that were exposed to shock exhibited increased prepro-galanin mRNA in the locus coeruleus relative to sedentary rats (202). These data collectively show that wheel running increases galanin in noradrenergic brain circuits that are sensitive to stress.

We propose that enhanced galanin resulting from wheel running constrains activation of locus coeruleus neurons during stress to beneficially alter anxiety-like behavior (see Figure 2.3). Specifically, we suspect that enhanced galanin signal after exercise acts at somatic or somatodendritic galanin receptors in the locus coeruleus to dampen norepinephrine release to

target areas that influence anxiety, including the frontal cortex and amygdala. Indeed, *in vitro* data shows that galanin inhibits the firing rate of locus coeruleus mainly through somatodendritic GalR1 and GalR3 receptors (233, 269), and to a lesser extent GalR2 (224, 228, 269, 270). Wheel running may also exert anxiolytic potential via galanin-mediated inhibition of norepinephrine neurons in regions downstream to the locus coeruleus. The fact that wheel running induced galanin gene expression in the hippocampus of rats (271) supports the idea that multiple levels of the neural axis mediate the anxiolytic-like potential of wheel running. Indeed, presynaptic GalR1 and GalR2 receptors are expected to modulate release of norepinephrine in forebrain regions, including the hippocampus and cortex (269, 272, 273). Wheel running alters norepinephrine levels (70) and increases mRNA expression for the α_{1b} adrenergic receptor in the dorsal raphe nucleus in a time-dependent manner compared to sedentary conditions (200), which lends further evidence to conclude that wheel running-induced adaptations are mediated via noradrenergic signaling in target regions of the locus coeruleus. These results demonstrate that wheel running alters norepinephrine and galanin systems in neural circuits that are sensitive to stress, but the functional significance is yet to be elucidated.

It is also possible that the anxiolytic potential of wheel running is influenced by galanin-mediated alterations in 5-HT. The dorsal raphe may be an especially important site for serotonergic-galaninergic interactions after wheel running, because galanin is synthesized in these neurons (220). The functional consequences of GalR1 receptor homodimers (274) and heterodimers with monoamine receptors (275, 276) in regions controlling stress remain to be explored, as well as the ability of wheel running to modify these novel receptor combinations. Future studies should establish whether the anxiolytic potential of wheel running can be attenuated in a manner that is necessary and/or sufficient for brain galanin and whether such effects are time-course specific. Experiments elucidating the anxiolytic potential of wheel

running could utilize selective galanin receptor (ant) agonists, galanin receptor knockout mice, and galanin overexpressing mice (e.g., driven by a dopamine β-hydroxylase promoter).

Final Remarks

The evidence reveals that voluntary wheel running offers anxiolytic potential and stress resiliency. Specific neural mechanisms that underlie such benefits are offered. Wheel running increases galanin in norepinephrine systems that are sensitive to stress. Precisely how wheel running affects interactions between the brain galanin and norepinephrine systems to alter anxiety is under speculation, but alterations likely occur at multiple levels of stress responsive circuitry. We hypothesize that the impact of exercise on galanin in the locus coeruleus is particularly relevant for noradrenergic output to stress responsive targets and subsequent anxiety-like behavior. Candidate norepinephrine and galanin receptor subtypes that transmit the beneficial effect of wheel running are elucidated based on functional neuroanatomical evidence and descriptive reports of wheel running on norepinephrine and galanin neurotransmitter systems. Evidence to date support the use of rodent models to investigate these and other neural mechanisms underlying the emotional consequences of exercise.

Table 2.1

Summary of the effects of wheel running on baseline responding in tests of anxiety

Behavioral test	Exercise is anxiolytic?		Wheel access (d)	Distance ran (km/d)	Sex	Strain/species	Housed	Sed Ctrl	Reference
Acoustic startle	Y		14 [a]	4.5	M	C57BL/6J	G	L	(Falls et al., 2010)
	Y		14 [a]	5	M	C57BL/6N	G	L	(Salam et al., 2009)
	–		28	10	M	C57BL/6NCrl	S	L	(Cacciaglia et al., 2011)
	–		60 [a]	nd	F	C57BL6/J	G	L	(Pietropaolo et al., 2006)
	–		90	4	F, M	WT mice	S	L	(Pietropaolo et al., 2008)
	N		21	8	M	C57BL/6J	S	L	(Fuss et al., 2010a)
	Y		24	6-7	M	C57Bl6/N	S	A/L	(Dubreucq et al., 2010b)
Dark:light box	N		26	9-12	M	C57BL/6J	S	L	(Fuss et al., 2010b)
	–		28 [a]	3-4	M,F	129xC57Bl6	G	A	(Garcia-Mesa et al., 2011)
	Y		28	4	M	C57BL/6N	S	A	(Binder et al., 2004)
	N		28	10	M	C57BL/6NCrl	S	L	(Cacciaglia et al., 2011)
Defensive withdrawal	–		21	4	M	Sprague	S	A	(Sciolino et al., in prep)
	–		12 [a]	5	M,F	Sprague	G	A	(Brocardo et al., 2011)
	–		19 [a]	2	M	Long Evans	G	A	(Hopkins and Bucci, 2010b)
	N		20	1-2	M	Sprague	S	A	(Grace et al., 2009)
	–		21	4	M	Sprague	S	A	(Sciolino et al., in prep)
	Y		28	4	M	C57BL6/N	S	A	(Binder et al., 2004)
Elevated plus maze	Y		28 [b]	5	M	Sprague	G-S	A	(Hopkins and Bucci, 2010a)
	Y		21-28	12-14	M	C57Bl/6	S	A	(Duman et al., 2008)
	–		28 [a c]	<1-5	M	Wistar	G	A	(Garcia-Capdevila et al., 2009)
	Y		30 [b]	1.5	M	C57Bl/6	G	L	(Gorton et al., 2010)
	N		28 or 56	6	M	Sprague	S	L	(Burghardt et al., 2004)
	–		60 [a]	nd	F	C57BL6/J	G	L	(Pietropaolo et al., 2006)
	Y, N		90	4	F, M	WT mice	S	L	(Pietropaolo et al., 2008)
	N [d]		21	8	M	C57BL/6J	S	L	(Fuss et al., 2010a)
Elevated zero maze	N		25	9-12	M	C57BL/6J	S	L	(Fuss et al., 2010b)
	N		28	10	M	C57BL/6NCrl	S	L	(Cacciaglia et al., 2011)
Hole board	–		28	4	M	C57BL/6N	S	A	(Binder et al., 2004)
	–		28 [a]	3-4	M,F	129xC57Bl6	G	A	(Garcia-Mesa et al., 2011)
	Y		28 [a]	3-4	M,F	129xC57Bl6	G	A	(Garcia-Mesa et al., 2011)
Novel cage/container	Y		28	4-7	M	Sprague	S	A	(Collins et al., 2009)
	Y		28	6	M	Sprague	S	A	(Droste et al., 2007)
	Y		42	2	M	Sprague	S	A	(Masini et al., 2011)
Novel food	–		60 [a]	nd	F	C57BL6/J	G	L	(Pietropaolo et al., 2006)
	–	↔	12 [a]	5	M,F	Sprague	G	A	(Brocardo et al., 2011)
	Y	↓	14 [a]	5	M	C57BL/6N	G	L	(Salam et al., 2009)
	nd	↓	19 [a]	2	M	Long Evans	G	A	(Hopkins and Bucci, 2010b)
	–	↓	20	1-2	M	Sprague	S	A	(Grace et al., 2009)
	N	↓	21	8	M	C57BL/6J	S	L	(Fuss et al., 2010a)
	N	↓	21	9-12	M	C57BL/6J	S	L	(Fuss et al., 2010b)
	N [e]	↓ [e]	21-28	12-14	M	C57BL6/J	S	A	(Duman et al., 2008)
	–	↓	28 [a c]	<1-5	M	Wister	G	A	(Garcia-Capdevila et al., 2009)
Open field	nd	↔	28 [a]	3-4	M,F	129xC57Bl6	G	A	(Garcia-Mesa et al., 2011)
	– [f]	nd	28	4	M	C57BL/6N	S	A	(Binder et al., 2004)
	N	↓	28 or 56	6	M	Sprague	S	L	(Burghardt et al., 2004)
	–	↔	37	3-5	M	CB_1 KO & WT mice	S	L	(Dubreucq et al., 2010a)
	–	↔	56 [b]	<1	F	Long Evans	G	L	(Leasure and Jones, 2008)
	Y	↑	56	3	M	Sprague	S	A	(Dishman et al., 1996)
	–	↔	60 [a]	nd	F	C57BL6/J	G	L	(Pietropaolo et al., 2006)
	nd	↔	90	4	F, M	WT mice	S	L	(Pietropaolo et al., 2008)
Shock probe defensive burying	–		21	4	M	Sprague	S	A	(Sciolino et al., in prep)
Social interaction	Y		14 [a]	5	M	C57BL/6N	G	L	(Salam et al., 2009)
	–		56	6	M	Sprague	S	L	(Burghardt et al., 2004)
Stress-induced hyperthermia	Y		14 [a]	5	M	C57BL/6N	G	L	(Salam et al., 2009)

Table 2.1. Abbreviations: –, no effect on anxiety; ↑, increased locomotion; ↓, reduced locomotion; ↔, did not alter locomotion ; A, absent wheel controls; CB_1, cannabinoid type I receptor; F, female; G, group housing; KO, knockout; L, locked wheel controls; M, male; nd, no data; N, anxiogenic; S, single housing; Sed Ctrl, sedentary controls; WT, wild type; Y, anxiolytic. Footnotes: [a], wheel was shared; [b], exercise was restricted; [c], resistance created in wheel; [d], effect depends on hippocampal neurogenesis; [e], effect depends on time of testing relative to last wheel access; [f], measured object recognition during test. Notes: Open field reported as changes in center time/entries/distance from the walls followed by changes in locomotion. Wheel access is reported at behavioral testing and underlining indicates behaviorally ineffective durations. Mean distance ran is reported around the time of behavioral testing and was divided by the number of subjects per cage when the wheel was shared.

Table 2.2

Summary of the effects of wheel running on stress-evoked responding in tests of anxiety.

Behavioral test	*Stressor*	*Stressor regimen*	*Exercise relieves stressor?*	*Wheel access (d)*	*Distance ran (km/d)*	*Sex*	*Strain / species*	*Housing*	*Sed Ctrl*	*Reference*
Acoustic startle	mCPP (0.1-1 mg/kg)	1x	Y [a]	14 [b]	4.5	M	C57BL/6N	G	L	(Fox et al., 2008)
Dark:light box	Maternal deprivation	PND 2-14 (180 min/d)	Y	84 [b]	<1	F	Sprague	G	nd	(Maniam and Morris, 2010)
Defensive withdrawal	FG7142 (30 mg/kg)	1x	–	21	4	M	Sprague	S	A	(Sciolino et al., in prep)
	Saline inject or FG7142 (7.5 mg/kg)	10 d (1x/d)	Y	21	4	M	Sprague	S	A	(Sciolino et al., in prep)
Elevated plus maze	FG7142 (30 mg/kg)	1x	Y	21	4	M	Sprague	S	A	(Sciolino et al., in prep)
	Saline inject or FG7142 (7.5 mg/kg)	10 d (1x/d)	Y	21	4	M	Sprague	S	A	(Sciolino et al., in prep)
	Maternal deprivation	PND 2-14 (180 min/d)	Y	84 [b]	<1	F	Sprague	G	nd	(Maniam and Morris, 2010)
	Chronic mild stress	28 d (1x/d)	– (Y)	14,28,or 42	nd	M	Sprague	S	A	(Zheng et al., 2006)
Open field	Repeated social stress	3 d (3 hr/d)	Y (Y) [d]	15	nd	M	C57/Bl6	G	A	(De Chiara et al., 2010)
	Uncontrollable tail shocks	1 session	Y (–)	42	3	M	Fisher344	S	nd	(Greenwood and Fleshner, In Press)
Shock probe defensive burying	FG7142 (30 mg/kg)	1 x	–	21	4	M	Sprague	S	A	(Sciolino et al., in prep)
	Saline inject or FG7142 (7.5 mg/kg)	10 d (1x/d)	Y	21	4	M	Sprague	S	A	(Sciolino et al., in prep)
Shuttle box escape, freezing	Uncontrollable foot shocks	1 hr session	Y	14 or 42	2 or 3	M	Fisher344	S	A	(Greenwood et al., 2007)
	Uncontrollable tail shocks	1 session	Y	21 or 42	2 or 3	M	Fisher344	S	L	(Greenwood et al., 2005a)
	Uncontrollable tail shocks	1 session	Y	42	4	M	Sprague	S	L	(Greenwood et al., 2003a)
	Fluoxetine (10 mg/kg)	1x	Y	42	3	M	Fisher344	S	A	(Greenwood et al., 2008b)
	Uncontrollable foot shocks	20-45 min session	Y	63-84 [e]	1.5	F	Sprague	S	A	(Dishman et al., 1997)

Table 2.2. Abbreviations: –, did not reduce effects of stressor; A, absent wheel controls; F, female; G, group housing; L, locked wheel controls; M, male; mCPP, metachlorophenylpiperazine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; S, single housing; Sed Ctrl, sedentary controls; Y, reduced the toll of stressor. Footnotes: [a], exercise produces anxiolytic-like response when stressor is not present; [b], exercise wheel was shared; [c], exercise was restricted; [d], exercise effects were dependent on cannabinoid CB_1 receptors; [e], resistance created in wheel. Notes: Wheel access is reported at the time of behavioral testing, wherein underlining indicates behaviorally ineffective durations. Mean distance ran is reported at behavioral testing and divided by the number of subjects per cage when the wheel was shared. Open field data are reported first as changes in center time/entries followed by changes in locomotion in parentheses. All drugs listed above were given via an intraperitoneal route.

Table 2.3

Summary of the effects of wheel running on fear learning.

Behavioral test	*Exercise improves fear conditioning?*	*Wheel access (d)*	*Distance ran (km/d)*	*Sex*	*Strain / species*	*Housed*	*Sed Ctrl*	*Reference*
Fear potentiated startle	Y	14 [a]	4.5	M	C57BL/6J	G	L	(Falls et al., 2010)
Passive avoidance	Y	28 [a]	1 [b]	M	C57BL/6J	G	L	(Samorajski et al., 1985)
	–	28 [a]	2-3 [c]	M	BALB/c	G	L	(Liu et al., 2009)
	Y [df] and – [e]	19 [a]	2	M	Long Evans	G	A	(Hopkins and Bucci, 2010b)
Fear conditioned freezing	Y [dg]	21	7	M	Long Evans	S	A	(Van Hoomissen et al., 2004)
	Y [dh]	22	7	M	Long Evans	S	A	(Van Hoomissen et al., 2011)
	Y [di]	26	6-7	M	C57Bl6/N	S	A/L	(Dubreucq et al., 2010b)
	– [d]	28	10	M	C57BL/6NCrl	S	L	(Cacciaglia et al., 2011)
	Y [d] and – [e]	30	2-5	M	Long Evans	S	A	(Baruch et al., 2004)
	Y [d]	42	4	M	Fisher 344	S	A	(Greenwood et al., 2009)
	– [ej]	38-41	5	M	CB_1 WT mice	S	L	(Dubreucq et al., 2010a)
	– [dek]	46	6-7	M	Long Evans	S	nd	(Wojtowicz et al., 2008)
	Y [dl]	54	6	M, F	C57BL6/J	S	A	(Clark et al., 2008)
	– [d]	56	6	M	Sprague	S	L	(Burghardt et al., 2004)
	Y [dm]	56	8	M	Sprague	S	L	(Burghardt et al., 2006)
	– [de]	60 [a]	nd	F	C57BL6/J	G	L	(Pietropaolo et al., 2006)

Table 2.3. Abbreviations: –, did not alter fear conditioning; A, absent wheel controls; CB_1, cannabinoid type I receptor; F, female; G, group housing; L, locked wheel controls; M, male; N, impaired fear conditioning; nd, no data; S, single housing; Sed Ctrl, sedentary controls; WT, wild type; Y, improved fear conditioning. Footnotes: [a], wheel was shared; [b], km/hr; [c], km/12 hr; [d], conditioning to context; [e], conditioning to cue; [f], effect reversed when testing occurred in PM or end of light portion of the light:dark cycle; [g], effect abolished by non-selective β-adrenergic receptor blocker propranolol; [h], effect was reversed by olfactory bulbectomy; [i], effect present only when compared to no wheel controls, not locked wheel controls; [j], improved deficits of CB_1 receptor knockout mice; [k], trended towards improving fear conditioning, wherein time spent freezing was positively correlated with the number of cells expressing the young neuron marker PSA-NCAM in the dentate gyrus; [l], effect was not dependent on hippocampal irradiation; [m], effect present only after high, but not low running. Notes: Wheel access is reported at behavioral testing. Distance ran is reported as the mean at behavioral testing and was divided by the number of subjects per cage when the wheel was shared.

Table 2.4

Summary of the effects of wheel running on neurotransmission in regions controlling stress and anxiety.

Cannabinoid (CB)
Potentiated reductions in striatal sIPSC, but not sEPSC, frequency that were induced by a cannabinoid agonist (HU210), through presynaptic action and in a manner and dependent on exercise duration (De Chiara et al., 2010); these effects were slowly reversible after discontinuation of running (De Chiara et al., 2010). Potentiated reductions in striatal sIPSC frequency that were induced by the group I metabotropic glutamate receptor agonist S-DHPG in a manner dependent on the CB_1 cannabinoid receptors (De Chiara et al., 2010).
Dopamine (DA)
Dopamine levels were reduced in the Arc, but unchanged in the LC, DR, CeA, BLA, CA1, PVN, PAG, NAc, CPu, and PFC (Dishman et al., 1997; Gorton et al., 2010).
DOPAC levels or the ratio of DOPAC to DA levels were not different in the LC, DR, CeA, CA1, Arc, PAG, or PFC whereas only DOPAC/DA levels were reduced in the PVN (Dishman et al., 1997; Soares et al., 1999).
Galanin (Gal)
Prepro-Gal mRNA was increased in LC (Holmes et al., 2006; Van Hoomissen et al., 2004) and hippocampus (Tong et al., 2001). Prepro-galanin mRNA expression in LC was altered after footshock or chronic pharmacological stress (Sciolino et al., Under Review; Soares et al., 1999).
Gamma-aminobutyric acid (GABA)
GAD67 levels were regionally increased (CA1-3, DG, BNST, motor cortex, NAc core) or decreased (Pir), but unaltered in the PL, IL, sensory cortex, NAc shell, CPu, LS, and amygdala (Hill et al., 2010).
GABA levels were unaltered in striatum (Dishman et al., 1996).
$GABA_A$ receptor density was reduced in striatum (Dishman et al., 1996). Downregulated gene expression of $GABA_A$ and glutamate decarboxylase GAD65 in the hippocampus (Molteni et al., 2002). mRNA for $GABA_A$ receptor subunits were increased in hippocampal CA1 (α5, β1), CA2 (α5, β1, δ), CA3 (α5), and DG (α5, β1) (Hill et al., 2010). mRNA for select $GABA_A$ receptor subunits were reduced in PL (β3), Pir (β3, γ2), IL (α2), NAc core and shell (α2), CPu (α2), LS (α2), BNST (γ2), PVN (α2), and CA3 (α2) (Hill et al., 2010). mRNA for select $GABA_A$ receptor subunits were not different in the BLA and CeA (α2, β3, γ2) or sensory cortex (α2, β3) (Hill et al., 2010).
Glutamate (Glu)
AMPA GluR1 mRNA was decreased and increased in VTA after 1 and 23 d of exercise, respectively (Makatsori et al., 2003). AMPA receptor (GluR1, GluR2/3) and Glu receptor anchoring protein (SAP-97, GRIP-1, PSD-95) immunocontent was increased in cortical postsynaptic density, whereas immunocontent for kainite (GluR6/7) and NMDA receptors (Dietrich et al., 2005) was not altered. NMDA receptor subunit NR1 was unaltered in VTA (Makatsori et al., 2003). Phosphorlyated NMDA subunits (phosphor-NMDAR1, NMDAR2B) and binding of the NMDA receptor antagonist MK801 were increased in cortical postsynaptic densities (Dietrich et al., 2005). Unregulated gene expression of NMDAR2A and NMDAR2B in the hippocampus (Molteni et al., 2002).
Norepinephrine (NE)
Reduced the number of cFos immunoreactive cells after uncontrollable stress that were colocalized with tyrosine hydroxylase in the LC, A5 cell group, and rostral ventrolateral medulla (Greenwood et al., 2003b). TH mRNA in LC was unaltered (Soares et al., 1999).
NE levels were increased in spinal cord and pons medulla (Dunn et al., 1996) and LC and DR (Dishman et al., 1997), which was correlated with increased freezing in contextual fear conditioning. NE levels were no different in the CeA, hippocampus, Arc, PVN, and PAG after footshock (Dishman et al., 1997). Reduced footshock-induced increases in NE levels in the PFC (Soares et al., 1999).
MHPG and DHPG levels were unaltered in spinal cord, pons medulla, hippocampus, & frontal cortex (Dunn et al., 1996).
α_{1B} mRNA was increased in DRN regions, not in the MR, depending on exercise length; this effect was not correlated with distance ran (Greenwood et al., 2005b). α_2 receptor mRNA was unaltered in the locus coeruleus (Greenwood and Fleshner, 2008). β adrenergic B_{MAX} (lower receptor number) and K_d (enhanced affinity/sensitivity) were decreased in the frontal cortex at baseline, but these effects were reversed after footshock (Yoo et al., 1999).
Serotonin (5-HT)
5-HT levels were reduced in the CeA, but unchanged in the LC, DR, CA1, Arc, PVN, PAG, NAc, CPu, PFC, and BLA (Dishman et al., 1997; Gorton et al., 2010). Attenuated tail shock-induced activity of 5-HT neurons in the rostral-mid DRN in a manner dependent on the duration of exercise (Greenwood et al., 2005a; Greenwood et al., 2003a).
5-HIAA levels were reduced in the CeA, but not different in the LC, DR, Arc, PVN, and PAG after both uncontrollable and controllable stress (Dishman et al., 1997). 5-HIAA levels were reduced in CA1 only after uncontrollable stress (Dishman et al., 1997). Ratio of 5-HIAA to 5-HT levels were reduced in PVN, but not different in the LC, DR, CeA, CA1, Arc, and PAG (Dishman et al., 1997).
5-HT transporter mRNA in MR and DRN subregions was reduced; this effect was not correlated with distance ran (Greenwood et al., 2005b).
$5\text{-}HT_{1A}$ receptor mRNA was increased in the MR and in subregions of the dorsal and lateral DRN in a manner that was dependent on exercise length, whereas $5\text{-}HT_{1A}$ receptor mRNA was not altered in ventral DRN subregions; these effects were not correlated with distance ran (Greenwood et al., 2005b; Greenwood et al., 2003a). Reversed maternal deprivation induced reductions in $5\text{-}HT_{1A}$ mRNA in hippocampus (Maniam and Morris, 2010). $5\text{-}HT_{1B}$ receptor mRNA was reduced in select ventral DRN subregions in a manner dependent on exercise length, whereas $5\text{-}HT_{1B}$ receptor mRNA was not changed in the MR or dorsal/lateral DRN; this effect was not correlated with distance ran (Greenwood et al., 2005b).

Table 2.4. Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate; Arc, arcuate; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; CPu, caudate putamen; DG, dentate gyrus; DHPG, 3, 4-dihydroxyphenylglycol; S-DHPG,*S*-3,5-dihydroxyphenylglycine; DOPAC, 3,4-dihydroxyphenylacetic acid; DRN, dorsal raphe nucleus; GAD67, glutamic acid decarboxylase; IL, infralimbic cortex; LC, locus coeruleus; LS, lateral septum; MHPG, 3-methoxy-4-hydroxyphenylglycol; MR, median raphe; NAc, nucleus accumbens; NMDA, N-methyl-D-aspartate; PAG, periaqueductal gray; PFC, prefrontal cortex; Pir, piriform cortex; PL, prelimbic cortex; PVN, paraventricular nucleus; sEPSC, spontaneous excitatory postsynaptic current; sIPSC, spontaneous inhibitory post synaptic current; SubC, subcoeruleus; TH, tyrosine hydroxylase; VLM, ventral lateral medulla; VTA, ventral tegmental area.

Table 2.5

Summary of the effects of wheel running on immediate early gene expression in brain regions controlling stress and anxiety.

Altered Fos immunoreactivity in a region dependent manner by increasing Fos in the CeA and DG and decreasing Fos in the BLA, without changing this measure in CA1 or CA3; these effects were not colocalized with enkephalin or parvalbumin (Burghardt et al., 2006).
Did not alter the amount of cFos mRNA in LC (Soares et al., 1999), the number of cFos immunoreactive neurons in the NAc or PVN (Collins et al., 2009), nor the amount of cFos immunoreactivity in the A7 region, subcoeruleus, CeA, BLA, or lateral habenula (Greenwood et al., 2005a; Greenwood et al., 2003b).
Increased cFos immunoreactivity in the DG, but not CA1 or CA3 (Fuss et al., 2010a).
Reduced cFos mRNA in PVN after a saline injection (Campeau et al., 2010).
Regionally attenuated footshock stress induced increases in cFos expressing cells / immunoreactivity in DRN, PVN, Bar, LC, A5, ventral medial and lateral medulla, and caudal raphe nucleus (Greenwood et al., 2005a; Greenwood et al., 2003a; Greenwood et al., 2003b). Regionally influenced the expression of cFos immunoreactivity in the BNST in a manner that was dependent on the duration of wheel running (Greenwood et al., 2005a).
Attenuated auditory stress induced increases in cFos mRNA in PVN, rostral and ventral LS, antereoventral area of anterior BNST, medial POA, DM, Me, ventral subiculum, DR, CnF, PL, and IL (Campeau et al., 2010).
Augmented both novelty and forced swim induced increases in the number of cFos immunoreactive neurons in the DG (Collins et al., 2009)

Table 2.5. Abbreviations: Bar, Barrington's nucleus; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; CnF, cuneiform nucleus; DG, dentate gyrus; DM, dorsal medial hypothalamic nucleus; DRN, dorsal raphe nucleus; IL, infralimbic cortex; LC, locus coeruleus; LS, lateral septal nucleus; Me, medial amygdaloid nucleus; mRNA, messenger ribonucleic acid; NAc, nucleus accumbens; PL, prelimbic cortex; POA, preoptic area; PVN, paraventricular nucleus; SubC, subcoeruleus. Note that most effects from Campeau et al. (2010) were seen after intermittent and continuous exercise

Figure 2.1

Stress alters the benefit of exercise in tests of anxiety.

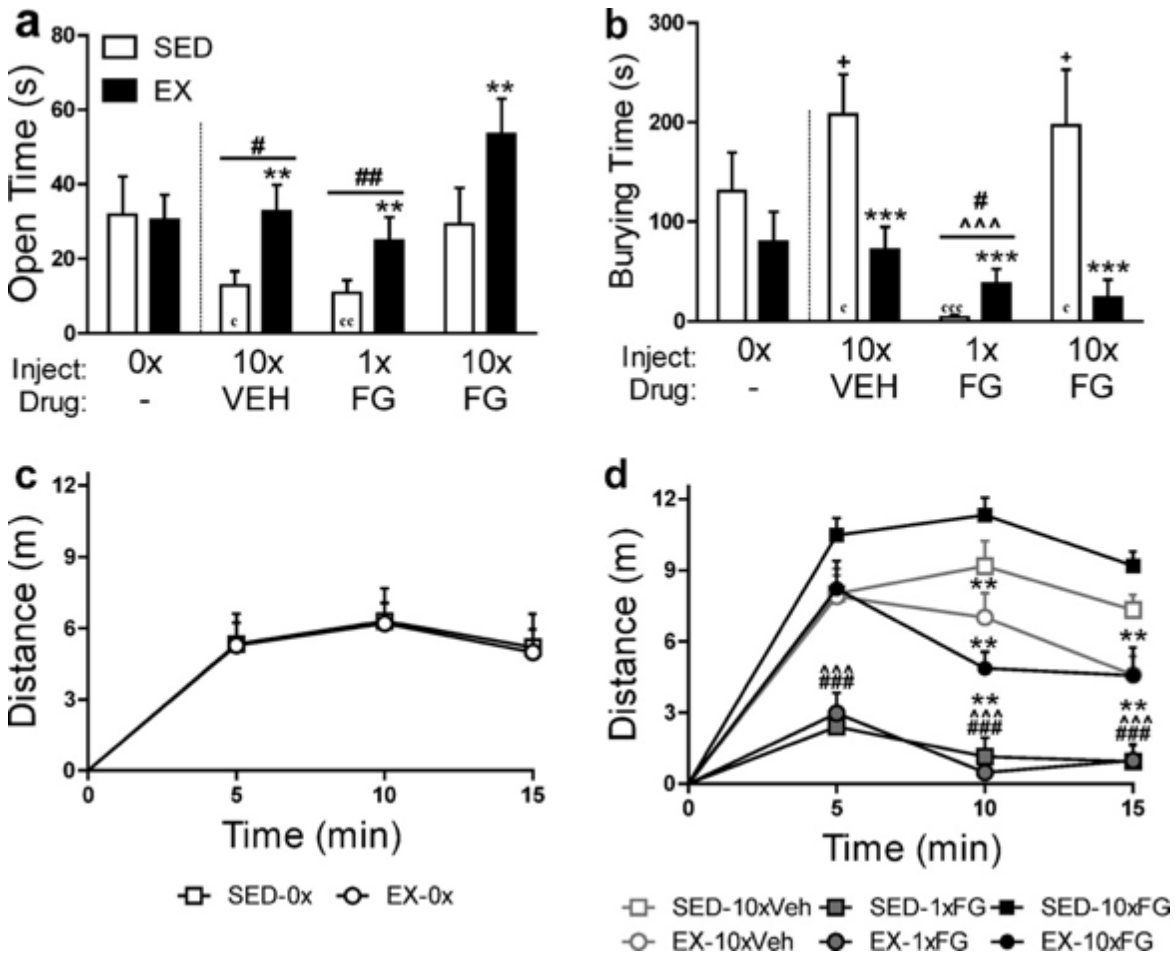

Figure 2.1. Exercise produces anxiolytic-like behavior in the (a) elevated plus maze and (b) shock probe defensive burying test and facilitates locomotor habituation in the (c–d) open field only after exposure to repeated injection or pharmacological stress using the anxiogenic β-carboline FG7142 (7.5 mg/kg i.p. × 10 days), but not in the absence of these stressors or in the presence of stress induced by a high, acute dose of FG7142 (30 mg/kg i.p. × 1 day). Results obtained from Sciolino et al. (2012). Data are reported as mean ± SEM (n = 8–10). ***p < 0.001, **p < 0.01 vs. sedentary; ### p < 0.001, ## p < 0.01, # p < 0.05 vs. chronic FG7142; + p < 0.05 vs. exercise rats treated with chronic vehicle, both sedentary and exercise rats treated with acute FG7142, and exercise rats treated with chronic FG7142; ^^^p < 0.001 vs. chronic vehicle; ccc p < 0.001, cc p < 0.01, c p < 0.05 vs. pooled no inject groups. Abbreviations: EX, exercise; FG, FG7142; SED, sedentary; VEH, vehicle.

Figure 2.2

A stress-based model to explain the influence of wheel running in tests of anxiety.

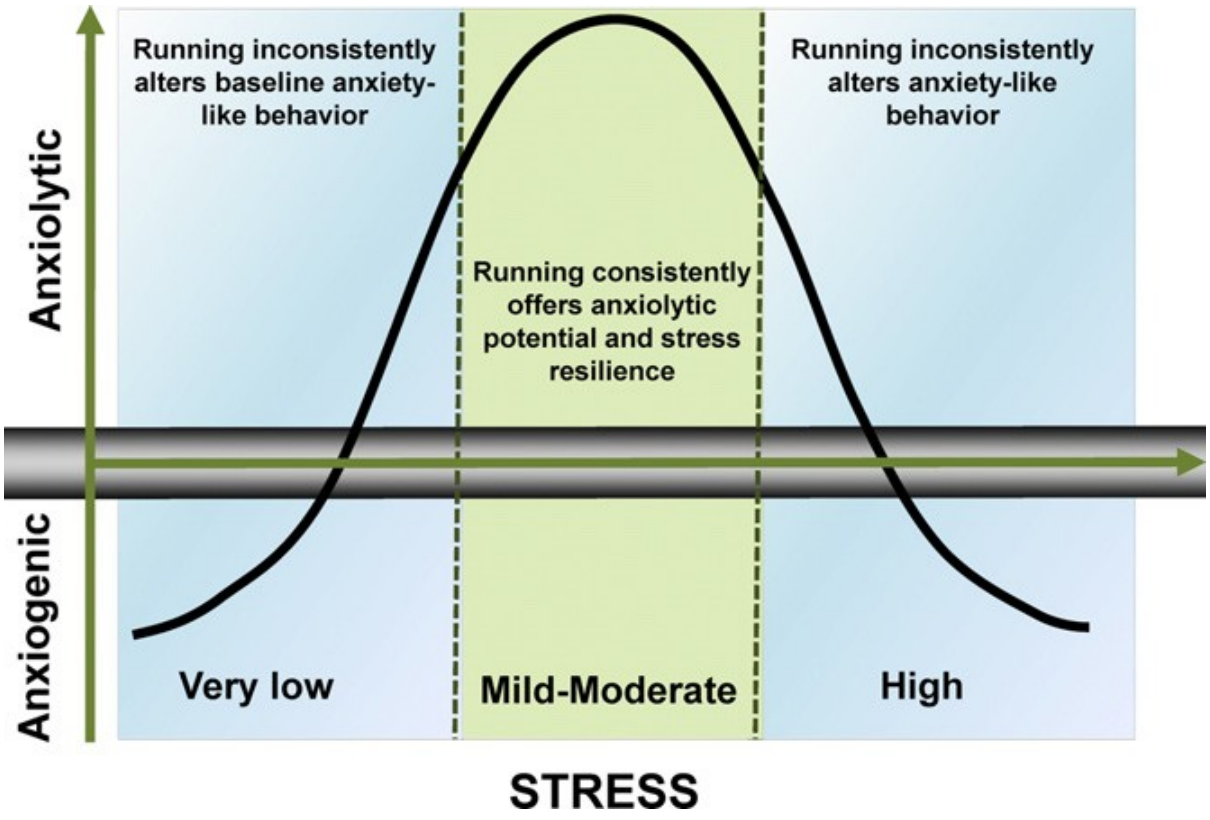

Figure 2.2. The relationship between voluntary wheel running and performance in tests of anxiety is non-monotic and influenced by the level of stress the animal is experiencing. Anxiolytic-like effects of exercise are expected to occur because wheel running interacts with stress to alter behavior. The impact of mild-to-moderate levels of stress reveals anxiolytic-like benefits of exercise, whereas null or anxiogenic findings occur outside of this range (in blue area). The level of stress an animal experiences can be deliberately induced by an experimenter or inherent in the experimental design (e.g., aversiveness of the housing or testing environment, rearing and handling conditions) and/or modified by other factors that influence stressor responsiveness (e.g., genetics, maternal history). This model is adapted from the Yerkes-Dodson law (Broadhurst, 1957; Yerkes and Dodson, 1908).

Figure 2.3

A stress-based neural model of functional noradrenergic circuitry that is implicated in mediating the anxiolytic-like potential of wheel running.

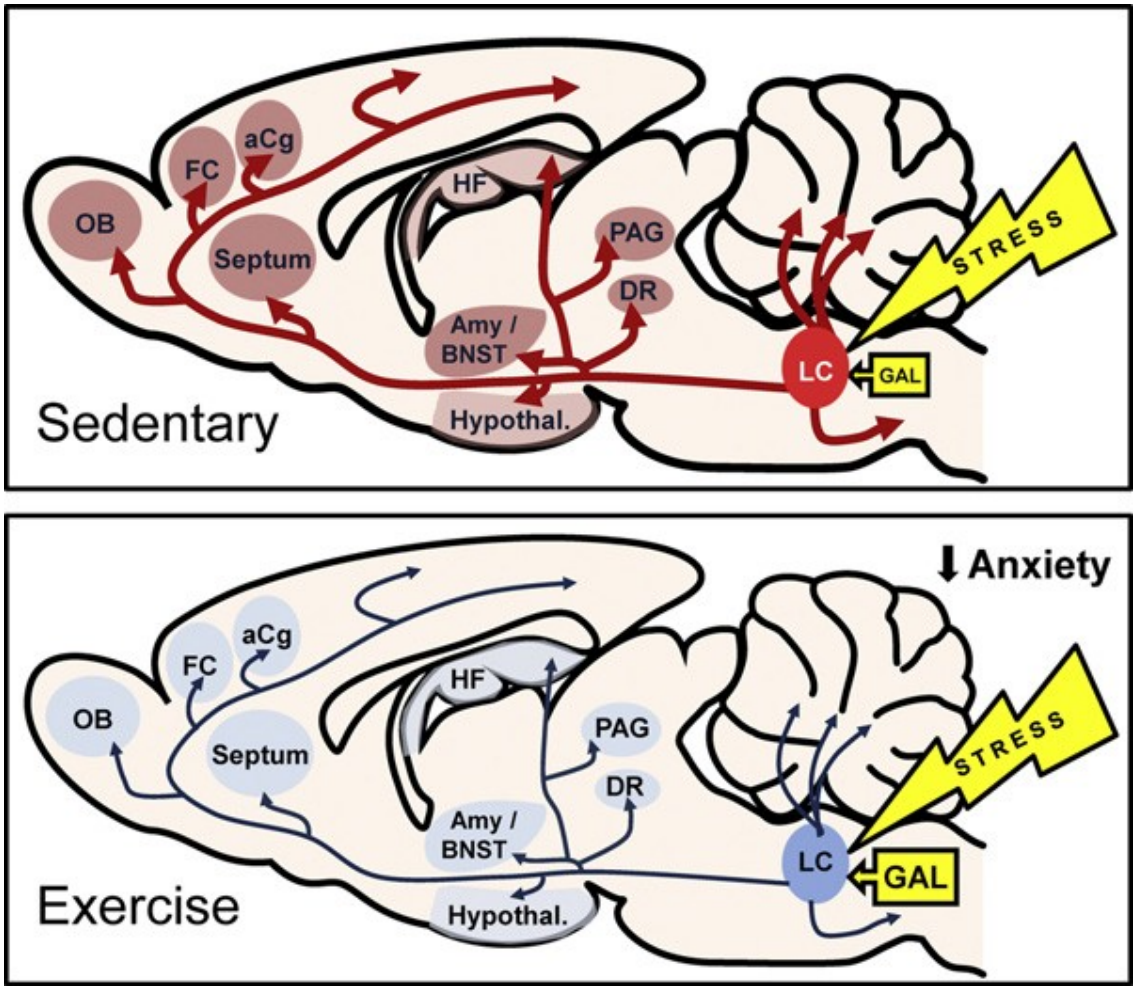

Figure 2.3. Stress increases norepineprhine output from the locus coeruleus to brain circuitry control- ling anxiety-like behavior in sedentary rodents. However, rodents given repeated access to a running wheel exhibit increased expression of galanin, a peptide colocalized with norepineprhine in the locus coeruleus relative to sedentary controls. Therefore, we propose that enhanced galanin-mediated suppression of noradrenergic output from the locus coeruleus in wheel runners is a mechanism that can account for the attenuation of anxiety-like behavior after stressor exposure. Abbreviations: aCg, anterior cingulate; Amy, amygdala; BNST, bed nucleus of the stria terminalis; DR, dorsal raphe; FC, frontal cortex; GAL, galanin; HF, hippocampal formation; Hypothal., hypothalamus; LC, locus coeruleus; OB, olfactory bulb; PAG, periaqueductal gray area.

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CHAPTER 3

VOLUNTARY EXERCISE OFFERS ANXIOLYITC POTENTIAL AND AMPLIFIES GALANIN GENE EXPRESSION IN THE LOCUS COERULEUS OF THE RAT

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Sciolino, N.R., R.K. Dishman and P.V. Holmes. 2012. *Behavioural Brain Research.* 233:191-200. Reprinted here with permission of the publisher.

Abstract

Although exercise improves anxiety in humans, it is controversial whether exercise is anxiolytic in rodents. We tested the hypothesis that stress influences the effect of exercise on anxiety-like and defensive behaviors. To explore the neurobiological mechanisms of exercise, we also examined whether exercise alters gene expression for the stress-related peptide galanin. Rats were housed in the presence or absence of a running wheel for 21 d. A subset of these rats were (1) not injected or received a single high, dose of the β-carboline FG7142 (inverse agonist at the benzodiazepine receptor site) immediately prior to testing or (2) were injected repeatedly with vehicle or FG7142 during the last 10 d of exercise. On day 22, anxiety-like and defensive behaviors were measured in the elevated plus maze, shock probe defensive burying, and defensive withdrawal tests. Locus coeruleus prepro-galanin mRNA was measured by in situ hybridization. Exercise and sedentary rats that were not injected exhibited similar behavior in all tests, whereas FG7142 injected immediately prior to the test battery produced intense avoidance and immobility consistent with an anxiety-like response. However, exercise produced anxiolytic-like and active defensive behaviors in the test battery relative to the sedentary condition in rats injected repeatedly with vehicle or FG7142. Exercise also increased prepro-galanin mRNA in the locus coeruleus relative to sedentary controls. These data suggest that the emergence of enhanced adaptive behavior after chronic voluntary exercise is influenced by stress. Our data support a role for galanin in the beneficial consequences of wheel running.

Introduction

Physical activity improves anxiety symptoms in healthy people and medical patients [1-3]. However, it is controversial whether wheel running – a common experimental means to voluntarily permit exercise – indicates treatment potential in rodent models of anxiety. For example, although chronic wheel running produces anxiolytic-like effects in some experiments [4-11], both null [12] and anxiogenic effects have also been reported [13-16]. Research designed to resolve this discrepancy is critical because it will help to determine the value of rodent models to study the anxiolytic potential of exercise and its underlying neurobiology.

A potential source for divergence in this literature may result because of the moderating influence of stress. Discrepant findings under baseline conditions may result because non-manipulated variables (e.g., environmental aversiveness of the testing environment, handling and housing conditions) operate as stressors and interact with the effects of exercise. Lending support to this hypothesis, wheel running exerts a clear anxiolytic-like profile after exposure to stress. For instance, wheel running reduces the behavioral toll of several acute stressors, including intense illumination [4], social defeat [17], tail shock [18, 19], the serotonergic receptor agonist metachlorophenylpiperazine [20], the selective serotonin reuptake inhibitor fluoxetine [21], and uncontrollable footshock [22, 23]. Wheel running also buffers the behavioral deficits induced by stress-based models of psychopathology, including chronic mild stress [24], maternal deprivation [25], and social defeat [26]. Further, a role for exercise in stress-resilience has been documented for several neurobiological, neuroendocrine, and neuroimmune responses and in other behavioral paradigms [for review see 27, 28, 29]. Thus, it is expected that the anxiolytic actions of exercise are consistently observed when stress is experimentally manipulated because exercise interacts with stress to produce an anxiolytic-like response during testing.

Another potential source for divergence in this literature may result because enhanced defensive behavior (e.g., heightened sensory processing, immobility, flight, defensive

threat/attack behaviors) in wheel runners competes with the display of anxiolytic-like behavior. Indeed, ‘anxiogenic’ responding in wheel runners was suggested to result because they exhibit enhanced defensive behaviors in tests of anxiety [16, 30]. However, this hypothesis is yet to be examined using tests that are optimized to detect active defensive behaviors, while also assessing behaviors specific to anxiety. Structured tests of threat, such as the shock probe defensive burying test, permit detection of defensive behaviors that otherwise are undetected or uninitiated in other, standard tests of anxiety [for reveiws see 31, 32]. Active defensive behaviors are theorized to be preserved across species and perturbed in anxiety disorders [33, 34] and these behaviors are also altered by anxiolytic drugs [35, 36]. Thus, understanding the effects of exercise on active defensive behaviors is of relevance for understanding and treating anxiety.

The molecular mechanisms underlying the affective consequences of voluntary exercise are not well understood [37]. Research in our laboratory over the past decade supports the hypothesis that wheel running mitigates the effects of stress by norepinephrine-galanin mediated mechanisms that involve the locus coeruleus. The locus coeruleus is an important noradrenergic nucleus that mediates stress and anxiety [38]. The majority of locus coeruleus neurons also contain the peptide galanin [39-42]. Wheel running increases prepro-galanin mRNA in the locus coeruleus [43-45] and dampens stress-induced norepinephrine release in a region the locus coeruleus targets (i.e.., frontal cortex) [46] relative to sedentary conditions. Plasma galanin is increased after an acute bout of exercise in humans [47], supporting the potential clinical relevance of exercise-induced regulation of galanin. Chronic stress and a model of mental pathology also increase the expression of prepro-galanin mRNA in the locus coeruleus relative to controls [41, 48], which suggests that alterations in locus coeruleus galanin occur as a common molecular adaptation to compensate for the toll of stress or psychopathology.

The primary aim of the present experiments was to evaluate the influence of voluntary wheel running on anxiety-like and defensive behavior as a function of stress. We tested the hypothesis that chronic wheel running would produce anxiolytic-like behaviors in rats that were exposed to stress. We also hypothesized that repeated wheel running would not reliably influence anxiety-like behavior in rats that were not exposed to stress. Anxiety-like and defensive behaviors were measured in an array of behavioral tests to characterize the effects of exercise, including the elevated plus maze, shock probe defensive burying, and defensive withdrawal tests. The secondary aim was to verify that exercise upregulates galanin gene expression, as expected from previous experiments using different behavioral manipulations [43-45].

Material and methods

Subjects

Seventy-seven male Sprague-Dawley rats (200-250 g; Harlan, Prattville, AL) were used at approximately 2 months of age at the beginning of testing. Rats had *ad libitum* access to food and water and were individually housed in clear polycarbonate cages (50 x 30 x 30 cm) with wood chip bedding. Rats were housed under constant temperature (23 ± 1ºC) and lighting (12:12 reverse light:dark) with lights off at 7 or 9 am. Rats were allowed to habituate to the animal facility for at least 5 d before the initiation of any experimental procedures. All rats were weighed on experimental day 1, 11, and 21. Rats receiving chronic injections were also weighed on experimental day 16 to ensure accurate drug dosing. All procedures were carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals and formal approval to conduct the experiments was obtained from the University of Georgia Animal Care and Use Committee.

General experimental methods

Experiment 1: Validation of a lack of an effect of chronic exercise on anxiety-like behavior at baseline (i.e., no stressor exposure)

Rats were randomly selected and assigned to either exercise or sedentary conditions on experimental day 1 and remained under these conditions for 21 d until wheels were locked just after lights off on experimental day 22. Half of the rats were then randomly assigned to receive no stress (i.e., were not injected to test the hypothesis of a null effect of exercise in the absence of stress), and the remaining half was assigned to receive an single, high dose (30 mg/kg i.p.) of the β-carboline compound FG7142 immediately prior to behavioral testing (to validate the sensitivity of our tests of anxiety). The design of experiment 1 was as follows: sedentary/0x FG (*n* = 10), exercise/0x FG (*n* = 10), sedentary/1x FG (*n* = 8), exercise/1x FG (*n* = 10). Rats were tested in the test battery (elevated plus maze, shock probe defensive burying test, defensive withdrawal test) on experimental day 22.

Experiment 2: Effects of chronic exercise on anxiety-like behavior after exposure to repeated injection or pharmacological stress

A separate group of rats were randomly selected and assigned to either exercise or sedentary conditions on experimental day 1 and remained under these conditions for 21 d until wheels were locked just after lights off on experimental day 22. On experimental day 12, half of the rats were randomly assigned to receive daily, repeated intraperitoneal injections of vehicle (10 d on experimental days 12-21) or chronic FG7142 (7.5 mg/kg x 10 d on experimental days 12-21). The design of experiment 2 was as follows: sedentary/10x Vehicle (*n* = 10), exercise/10x Vehicle (*n* = 10), sedentary/10x FG (*n* = 9), exercise/10x FG (*n* = 10). Rats were tested in the test battery (elevated plus maze, shock probe defensive burying test, defensive withdrawal test) on experimental day 22.

Experiment 3: Effects of exercise on galanin gene expression in the locus coeruleus of rats that were exposed to either no injection stress or repeated injections of FG7142

As a positive control to verify previous findings from this laboratory that exercise upregulates galanin gene expression in the locus coeruleus [43-45], *in situ* hybridization was performed in the brains of all unstressed rats. Directly after testing in experiment 1, rats that were not exposed to stress were rapidly decapitated and brains were harvested and frozen. In order to determine the generalizability of exercise-induced upregulation of galanin when potentially stressful behavioral manipulations are involved, brains were similarly harvested from rats repeatedly injected with FG7142 from experiment 2.

Exercise

The homecage of rats assigned to sedentary conditions was without a wheel, whereas the exercise condition had a stainless steel running wheel (Mini Mitter, Bend, OR) that permitted 24-hr free access. Wheel rotations were measured for each subject by an electromagnetic counter and were recorded by an experimenter at the same time each day (2-3 hr post the onset of the dark phase of the light: dark cycle). Daily distance ran was determined by multiplying the number of wheel rotations by the wheel circumference (105 cm).

Drugs

The β-carboline FG7142 (partial inverse agonist at the benzodiazepine site of the GABAA receptor; Tocris Bioscience, Ellisville, MI) was selected because it mimics the effects of stress and produces robust anxiety across several species [for review see 49]. FG7142 was prepared fresh daily by suspending the drug in vehicle (distilled H2O containing 1 drop Tween80 / 5 mL) and vortexting. Injections were administered in a volume of 1 mL/kg in a room separate from the animal holding room 2-3 hr after the onset of the dark phase of the light: dark cycle. Doses were selected based on previous research that reported anxiety-like effects [50-

52]. For experiments requiring acute administration of FG7142, rats were injected and immediately underwent behavioral testing. Substantiating that the effects of acute FG7142 are behaviorally active for the entire test battery, previous evidence in the rodent shows that FG7142 quickly (as early as 10 min after) and lastingly (up to 1 hr after injection) alters behavior [50, 53-58]. For experiments requiring chronic administration of FG7142, our injection paradigm did not influence the behavioral acquisition or maintenance of wheel running (see Figure. 3.1). Rats were also visually inspected for seizures 1 hr post each repeated FG7142 injection to verify that the doses employed were subconvulsant [59, 60].

Behavioral testing

Testing occurred during the dark phase of the light: dark cycle (started ~2 hrs after lights off and ended ~2 hrs before lights on). Rats were tested in the defensive withdrawal, shock probe defensive burying, and elevated plus maze tests, in that order, such that testing lasted about 50 min. Pilot experiments confirmed that serial testing produced acceptable baseline responding (i.e., non-injected controls exhibited about 10-15% of the time spent on the open arms of the elevated plus maze, 15-30% of the time spent immobile and burying in the shock probe defensive burying test, and 15-20 m traveled in the defensive withdrawal test). In particular, the single shock received in the defensive burying test likely did not alter subsequent elevated plus maze behavior, as baseline responding in the maze in rats that *were* shocked (present report) is similar to data previously reported by the investigators from rats that *were not* shocked and of the same strain [61]. Although the possibility of carry-over-effects from serial testing cannot be ruled out [62-65], the evidence indicated above suggests that the influence of serial testing in the present report is minimal. Background noise of 79 dB was emitted during testing by a white-noise generator. Behavioral equipment was wiped clean with Vimoba disinfectant (Quip Laboratories, Wilmington, DE) and allowed to dry between subjects.

Elevated plus maze

The apparatus was a wooden "+"-shaped maze elevated 50 cm from the floor and consisted of two opposite open arms (45 x 9 cm), two opposite closed arms (45 x 9 x 38 cm), and a central platform (9 x 9 cm). Illumination in the apparatus (~15 lux) was generated by placing a 15 W bulb ~1 m above the central platform. Testing was performed as previously described [61]. Each rat was placed in the center of the maze facing opposite the experimenter and towards an open arm of the maze. Behavior was video recorded for 5 min. An experimenter blind to group assignment, quietly remained in the testing room behind a divider and scored behavior. Measured behaviors were open and closed arm time and entries.

Shock probe defensive burying test

The test cage (50 x 30 x 60 cm; polycarbonate) was filled with 5 cm of fresh bedding (Sani Chips, Harlan, Prattville, AL) and contained a shock probe that extended 6 cm into the cage and 2 cm above the bedding. The probe was a glass rod (1 cm diameter) wrapped with 2 alternating copper wires (18 G wires spaced 5 loops/cm) that were connected to a shock generator (Coulbourne Instruments, Whitehall, PA). Illumination in the apparatus (~15 lux) was generated by a 15 W bulb that was centered ~1 m above the floor of the cage. Upon testing, rats were placed in the cage at a position that was farthest from the probe and in a direction opposite the probe. After receipt of a single shock (3 mA), the shock generator was turned off and behavior was recorded for 15 min, as described previously [66]. An experimenter blind to group assignment quietly remained in the testing room and scored behavior. A video record was also obtained for each rat for behavioral analysis at a later date due to the complicated nature of scoring the wealth of relevant behavior in this test. Measured behaviors included the onset of initial probe contact and burying, time spent engaging in burying, immobility, and rearing behaviors, shock reactivity based on a four-point scale used previously [67] and the frequency of probe bites and non-shock probe returns [see 32, 68].

Defensive withdrawal test

The test chamber (ENV 515-16, Med Associates, St. Albans, VT) was a clear polycarbonate cage (44.5 x 44 x 30 cm) that contained a dark enclosure that was impenetrable to visible light (22 x 12.5 x 15 cm with a 6 x 7 cm door; Cyro Acrylite GP, Rideout Plastics, San Diego, CA**)**. Ultraviolet beam interruptions were used to track the coordinate position and movement of the rat. Illumination in the light portion of the apparatus (250 lux) was generated by a 40 W bulb located 70 cm above the floor of the chamber. Each rat was placed in the center of the apparatus, facing opposite the experimenter, and allowed to explore the chamber for 15 min. Behavior was automatically collected (ENV-520, SOF-810, Med Associates) for latency to enter and time spent in the dark enclosure, frequency of dark enclosure transitions, and distance traveled in the entire test chamber, as described previously [69].

Tissue sectioning

Sedentary and exercised rats (*N*=39) from the no-injection and chronic FG7142 groups were decapitated immediately after the last behavioral test. Brains were rapidly harvested, blocked (at a coronal plane caudal to thalamus), frozen on dry ice, and stored at -80ºC until cryostat sectioning (-22ºC Microm; Waldorf, Germany). Tissue was cut into 12 µm coronal sections, collected between -9.8 to 10.04 mm from bregma, and subsequently thaw-mounted onto gelatin-coated glass microscope slides (2 sections/slide), which were stored at -80ºC until further processing. Anatomical location was also verified in adjacent sections using .1% thionin stain and a rat brain atlas [70].

In situ hybridization and densitometry

Tissue was processed as previously described [71]. For pretreatment, tissue was fixed in 4% formaldehyde in .12M phosphate buffered saline (PBS), rinsed in PBS, soaked in .25% acetic anhydride in .1M triethanolamine HCl and .9% NaCl, dehydrated in a series of EtOH

washes, delipidated in chloroform, and washed in EtOH. An oligonucleotide probe (Human galanin: 5'-G AAG GTA GCC AGC GCT GTT CAG GGT CCA GCC TCT CTT CTC CTT T - 3'; Oligos etc, Wilsonville, OR) was labeled at the 3' end with ^{35}S-dATP (1 mCi; Perkin Elmer, Boston, MA), tailing buffer, CoCl2, and terminal deoxynucleotransferase (Roche, Indianapolis, IN). Unbound radionucelotide was removed using column separation (Micro Bio-Spin P30 in Tris, Bio-Rad, Hercules, CA) and bound radionucelotide was stabilized using 1M dithiothreitol. Tissue from rats in all experimental groups was processed concurrently in the same assay. Sections were covered with radiolabeled probe in hybridization buffer (25% formamide, 72mM NaCl, 3.2mM Tris HCl, .0032mM EDTA, .001% sodium pyrophosphate, .004% sodium dodecyl sulfate, .002 mg/mL heparin sulfate, and 2% dextran sulfate) and incubated for 24 hrs at 37ºC. Sections underwent a series of washes in 1% SSC and 2% SSC-formamide (50:50) at 40ºC and room temperature as well as in distilled H2O and EtOH. Sections were allowed to dry and subsequently opposed to ^{35}Ssensitive film (Kodak BioMax MR, Rochester, NY) for 14 d. Films were developed in Kodak GBX fixer and developer and air dried.

Film images were captured under optimized conditions using a light table (Northern Light D95, Imaging Research Inc., Piscataway, NJ) and digital camera equipped with a macro lens (Nikon D5000, Micro-NIKKOR 55mmf/2.8 lens, Melville, NY). Images were processed on a Macintosh computer (Apple, Inc., Cupertino, CA) using NIH Image (Bethesda, MD, http://rsb.info.nih.gov/nih-image/). Images of the locus coeruleus were selected and measured using a uniform area of the dorsal portion of the locus coeruleus. Mean grayscale brightness values were obtained from 2-4 sections per subject. Densitometry was performed on original images that were in no way digitally manipulated. Example photomicrographs were uniformly transformed across groups to a color scale using NIH Image.

Statistics

Intraclass correlation coefficients using Cronbach α were calculated to verify strong inter-rater reliability in behavioral coding from a random subset of videos, which ranged from α = .84 - 1. A two-way (drug x time) or three-way (exercise x drug x time) analysis of variance (ANOVA) was performed with time point as the repeated measure to assess running distance or body weight and distance traveled during the defensive withdrawal test, respectively. Greenhouse-Geisser corrections were applied to repeated factors violating sphericity [72]. Cronbach α (model II intraclass correlation) was calculated to determine internal consistency of body weight and running distance across the experiments. Separate 2 (exercise) x 2 (drug) ANOVAs were performed for all other behavioral measures to evaluate the effects of exercise. Bonferroni post-hoc tests were performed for significant interaction effects. Partial eta squared (η^2) effect size calculations were performed for each ANOVA to gauge the amount of variance our manipulations accounted for in the dependent measures evaluated. Using Cohen's standards, η^2 values above .01, .06 , and .14 are commonly considered small, medium, and large effects, respectively [73-75]. To assess the effects of exercise on locus coeruleus prepro-galanin mRNA expression, separate *t*-tests were performed in rats that received no injections and repeated injection of FG7142. To generate statistical power needed to avoid a type II error, linear regression analysis was performed with data obtained from exercise rats that were exposed to no injection stress and chronic FG7142 groups (matched for prior housing and testing experience) to gauge the relationship between running distance and galanin message. All analyses were performed using SPSS statistical software (SPSS Incorporated, Chicago, IL).

Results

Experiment 1

Wheel running and body weight increased across time in rats that were not injected or injected with FG7142 immediately prior to testing

Distance ran in wheels increased linearly across experimental days and was internally consistent across time ($F_{1.14, 20.47} = 26.32$, $p < .01$; $\eta^2 = .61$; Cronbach $\alpha = .96$; Data not shown). Wheel running was not affected by drug treatment ($p > .05$; Data not shown). Average distance ran during week one, two, and three of running was 1.62 ± .17, 3.32 ± .41, and 4.53 ± .64 km/d (Data not shown), respectively. All rats gained weight across experimental days ($F_{1.31, 44.62} = 831.80$, $p < .01$; $\eta^2 = .96$; Cronbach $\alpha = .78$; Data not shown). Exercised rats maintained a lower body weight relative to sedentary rats ($F_{1.31, 44.62} = 44.40$, $p < .01$; $\eta^2 = .57$; Data not shown) on experimental day 11 ($p < .01$) and day 22 ($p < .01$), but were initially similar in body weight on experimental day 1 ($p > .05$).

Acute FG7142 increased anxiogenic behavior in a battery of tests when given immediately prior to testing

In the elevated plus maze, injection of an acute, high dose of FG7142 immediately prior to testing did not reliably alter the amount of time spent on the open arms of the maze ($p = .09$; Table 3.1), but reduced the frequency of open arm entries ($F_{1, 34} = 6.43$, $p < .05$; $\eta^2 = .16$; Table 3.1) relative to the no injection condition. In addition, injection of acute FG7142 immediately prior to testing also increased the amount of time spent on the closed arms of the maze ($F_{1, 34} = 6.32$, $p < .05$; $\eta^2 = .16$; Table 3.1) and reduced the frequency of closed arm entries relative to the no injection condition ($F_{1, 34} = 29.10$, $p < .01$; $\eta^2 = .46$; Table 3.1).

In the shock probe defensive burying test, injection of an acute, high dose of FG7142 immediately prior to testing reduced the amount of time spent burying ($F_{1, 33} = 9.15$, $p < .01$; $\eta^2 = .22$; Table 3.1) and rearing ($F_{1, 33} = 37.49$, $p < .01$; $\eta^2 = .53$; Table 3.1) and concomitantly

increased the amount of time spent immobile ($F_{1, 33} = 11.38$, $p < .01$; $\eta^2 = .26$; Table 3.1) relative to the no injection condition. Acute injection of FG7142 did not alter any other measure in the shock probe defensive burying test relative to the no injection condition ($p > .05$; Table 3.1).

In the defensive withdrawal test, injection of an acute, high dose of FG7142 immediately prior to testing reduced the distance traveled across the entire duration of the test relative to the no injection condition ($F_{1.62, 56.62} = 5.58$, $p < .01$; $\eta^2 = .14$; Table 3.1). In addition, injection of FG7142 immediately prior to testing increased the time spent in the dark box ($F_{1, 34} = 8.82$, $p < .01$; $\eta^2 = .01$; Table 3.1) and latency to enter the dark box ($F_{1, 34} = 8.93$, $p < .01$; $\eta^2 = .05$; Table 3.1) and reduced the frequency of dark box transitions ($F_{1, 34} = 43.57$, $p < .01$; $\eta^2 = .15$; Table 3.1) relative to the no injection condition.

Exercise failed to alter anxiety-like behavior across a battery of tests in rats not exposed to stress or exposed to intense stress via injection of FG7142 immediately prior to testing

Exercise did not alter any measure of anxiety-like or defensive behavior in elevated plus maze, shock probe defensive burying, and defensive withdrawal tests relative to sedentary conditions in rats that were not injected or injected with a high dose of FG7142 immediately prior to testing ($p > .05$; Table 3.1).

Experiment 2.

Wheel running and body weight increased across time in rats repeatedly injected with vehicle or FG7142

Distance ran in wheels increased linearly across experimental days and was internally consistent across time ($F_{2, 36} = 23.45$, $p < .01$; $\eta^2 = .61$; Cronbach $\alpha = .95$; Figure. 3.1). Wheel running was not affected by drug treatment ($p > .05$; Figure. 3.1). Average distance ran during

week one, two, and three of running was 1.92 ± .14, 3.78 ± .40, and 4.12 ± .44 km/d, respectively. All rats gained weight across experimental days ($F_{1.40, 40.63} = 261.12$, $p < .01$; $\eta^2 = .96$; Cronbach $\alpha = .77$; Data not shown). Exercised rats maintained a lower body weight relative to sedentary rats ($F_{1.40, 40.63} = 13.46$, $p < .01$; $\eta^2 = .32$; Data not shown) on experimental day 11 ($p < .01$) and day 22 ($p < .01$), but were initially similar in body weight on experimental day 1 ($p > .05$). Rats that received repeated FG7142 were no different in weight from rats that received repeated vehicle ($p > .05$; Data not shown).

Exercise produced anxiolytic-like behavior in the elevated plus maze in rats repeatedly injected with vehicle or FG7142

In rats that received repeated injections of vehicle or FG7142, exercise increased the time spent on the open arms of the maze ($F_{1, 35} = 7.78$, $p < .01$; $\eta^2 = .18$; Figure. 3.2a) and frequency of open arm entries ($F_{1, 35} = 7.81$, $p < .01$; $\eta^2 = .18$; Figure. 3.2b) relative to sedentary controls. Exercise reduced the time spent on the closed arms of the maze ($F_{1, 35} = 6.89$, $p < .01$; $\eta^2 = .16$; Data not shown), but did not alter the frequency of closed arm entries relative to sedentary conditions in rats that received repeated injections of vehicle or FG7142 ($p > .05$; Figure. 3.2c).

Main effects of repeated FG7142 were also detected for time spent on the open ($F_{1, 35} = 5.53$, $p < .05$; $\eta^2 = .14$; Figure. 3.2a) and closed arms of the maze ($F_{1, 35} = 5.27$, $p < .05$; $\eta^2 = .13$; Data not shown) as well as the frequency of open arm entries ($F_{1, 35} = 8.66$, $p < .01$; $\eta^2 = .20$; Figure. 3.2b).

Exercise increased anxiolytic-like behaviors in the shock probe defensive burying test in rats repeatedly injected with vehicle or FG7142

In rats that received repeated injections of vehicle or FG7142, exercise reduced the amount of time spent burying ($F_{1, 35} = 18.20$, $p < .01$; $\eta^2 = .34$; Figure. 3.3a) and rearing ($F_{1, 35} =$

6.64, $p < .01$; $\eta^2 = .16$; Data not shown) and concomitantly increased the amount of time spent immobile ($F_{1, 35} = 9.82$, $p < .01$; $\eta^2 = .22$; Figure. 3.3b) compared to sedentary conditions. Further, the effects of exercise on immobility time were abolished by repeated FG7142 administration ($F_{1, 35} = 4.78$, $p < .05$; $\eta^2 = .12$; Figure. 3.3b). Exercise increased the frequency of probe bites ($F_{1, 35} = 4.76$, $p < .05$; $\eta^2 = .18$; Figure. 3.3c) and non-shock probe returns ($F_{1, 35} = 4.79$, $p < .05$; $\eta^2 = .12$; Data not shown) relative to sedentary conditions in rats that received repeated injections of vehicle or FG7142. Shock reactivity, onset of initial probe contact, or time to initiate burying was comparable between exercise and sedentary rats ($p > .05$; Data not shown).

Exercise facilitated locomotor habituation and defensive withdrawal in rats repeatedly injected with vehicle or FG7142

In rats that received repeated injections of vehicle or FG7142, exercise reduced the amount of distance traveled ($F_{1.62, 56.62} = 5.58$, $p < .01$; $\eta^2 = .14$; Figure. 3.4a) in the defensive withdrawal test in a manner that was time-dependent compared to sedentary conditions. Exercised rats that were repeatedly injected with either vehicle or FG7142 were no different from sedentary counterparts in distance traveled at 5 min ($p > .05$; Figure. 3.4a), but exhibited reduced distance traveled at both 10 ($p < .01$; Figure. 3.4a) and 15 min ($p < .01$; Figure. 3.4a) of the test. In addition, exercise increased the amount of time spent in the dark box ($F_{1, 35} = 8.70$, $p < .01$; $\eta^2 = .20$; Figure. 3.4b) and reduced the frequency of dark box transitions ($F_{1, 35} = 6.89$, $p < .01$; $\eta^2 = .17$; Figure. 3.4c) relative to sedentary conditions in rats that received repeated injections of vehicle or FG7142. Exercise did not alter the latency to enter the dark box relative to the sedentary group in rats that received repeated injections of vehicle or FG7142 ($p > .05$; Data not shown).

Experiment 3

Exercise increases prepro-galanin mRNA expression in locus coeruleus, which is positively correlated with the amount of running on an exercise wheel

Exercise increased prepro-galanin mRNA expression in the locus coeruleus relative to sedentary conditions in rats that were not injected ($t18 = -2.73$, $p < .01$; Figure. 3.5a). However, exercise did not reliably alter prepro-galanin mRNA expression in the locus coeruleus relative to sedentary conditions in rats that were repeatedly injected with FG7142 ($p > .05$; Figure. 3.5a). Regression analysis revealed a positive correlation between prepro-galanin mRNA in the locus coeruleus and distance ran during week three ($\beta = .44$, $t17 = 2.03$, $p = .05$; $R^2 = .20$; Figure. 3.5b), but not during week one or two ($p > .05$; Data not shown), which suggests that the increased variance (coefficient of variation, c_v = 33%, 48%, and 60% during week one, two, and three, respectively) in running during week three contributes to the positive correlation between running distance and locus coeruleus prepro-galanin mRNA.

Discussion

The present study demonstrates that voluntary wheel running elicited anxiolytic-like behavior in rats with a history of stress relative to their sedentary counterparts in several behavioral tests. However, under baseline stress conditions (i.e., no experimental stressor administered), exercise failed to differentially alter anxiety-like behavior. Additionally, regardless of whether rats exercised intense avoidance and immobility, at the loss of other defensive behaviors, was generated by severe stress induced by an acute, high dose of the β-carboline FG7142 administered immediately prior to testing. Further, exercise increased the expression of prepro-galanin mRNA in the locus coeruleus in rats that were not exposed to an experimental stressor.

The present data indicate alterations in emotion-related behavior rather than locomotion or pain sensitivity, as exercise and sedentary rats were no different in several measures of

these variables (i.e., number of closed arm entries in the elevated plus maze, onset of initial probe contact or shock reactivity in the shock probe defensive burying test, or latency to enter the dark box in the defensive withdrawal test). Our data generated in rats that were exposed to stress (i.e., repeated injection with vehicle or FG7142) are consistent with other reports that show exercise exerts anxiolytic potential after stressor exposure [17-26]. Of note, chronic injections alone appear to be the critical factor leading to the appearance of the anxiolytic-like effect of voluntary exercise. Perhaps the ability to run after the stress of injection was the critical factor allowing the anxiolytic-like effect of exercise to be revealed. Although this possibility cannot be conclusively determined from our data set, this interpretation is supported by previous research showing that stress increases wheel running in a manner that is ameliorated by the anxiolytic drug diazepam [76]. Further, our data generated under *baseline* conditions of stress are in line with previous findings that show exercise does not alter anxiety-like behavior in the unstressed rodent [9, 12, 30, 77-82]. However, anxiolytic-like effects are also reported in exercised rodents that were not exposed to an experimental stressor [5, 6, 10, 11, 13]. We expect that the inconsistent effects of wheel running in the literature on baseline levels of anxiety-like behavior result from differing levels of stress (e.g., due to handling or other environmental variables) and/or other factors (e.g., genetics) that influence stress reactivity. Alternatively, no effect of exercise in the non-stressed (and highly stressed) rat could mean that a longer wheel access is necessary to demonstrate the stress reducing effects of voluntary exercise. Supporting this, wheel running that lasts 6 wks, but not less, is necessary to see the stress-reducing effects of exercise on “learned helplessness” behaviors (i.e., exaggerated freezing and shuttle box escape deficit after uncontrollable stress) [18, 19, 22, 83]. However, many previous reports reveal that 3 weeks of wheel running (as used in the present experiment) or less produces anxiolytic-like [4, 5, 8, 20, 24, 26, 84], anxiogenic [13-15], and null [77, 81] effects in several other tests of anxiety, suggesting that this duration of exercise is sufficient to detect its influence on anxiety-related behaviors using a variety of paradigms.

The present report showed that a high dose of the β-carboline FG7142 (30 mg/kg i.p. x 1 d) produced intense immobility and avoidance in the shock probe defensive burying and defensive withdrawal tests regardless of whether rats ran on a wheel, which could imply that the beneficial effects of exercise may not be sufficient to overcome intense stressors. The lack of an acute vehicle group in experiment 1 limits our ability to determine whether injection/handling or drug *per se* produced the anxiogenic effect. In other words, because rats from the no-stress control group were not injected the difference between acute FG7142 and non-injected groups is likely inflated. However, we expect that the anxiety-like behaviors detected in experiment 1 are most likely attributable to the high dose of FG7142 because of the extensive previous evidence that acute FG7142 mimics the effects of stress on behavioral, physiological, neuroendocrine, neuroimmune, and neurobiological responses and produces robust anxiety-like behavior [for review see 49]. Regardless of the source of the anxiogenic effects in the acute FG7142 injected rats, the present design suited the primary goal of the present report, which was to evaluate whether the emotional consequences of exercise are influenced by stress. The group assignment used in the present report permitted detection of a null effect of exercise in the absence of stress in non-injected rats as well as validation of the sensitivity of our tests of anxiety using the anxiogenic FG7142 manipulation. Additionally, it is important to note that we did not observe increases in anxiety-like behavior in rats given repeated injections of FG7142, whereas rats that received a single injection in Experiment 1 showed increased anxiety-like behavior. It is possible that the behavioral profile of rats given a single verse repeated injections of FG7142 differed because they experienced different levels of stress during testing (i.e., drug was present in sufficient plasma concentrations to be behaviorally active during testing in rats receiving a single inject of FG7142, but not for those receiving repeated injections prior to testing). Further, this group difference could be due to compensatory changes in brain signaling systems after repeated administration of FG7142 (e.g., upregulation of the GABAA receptor complex and/or the β-adrenergic receptor), as supported by prior neurochemical and behavioral

evidence [57,58,82,83]. Future research is needed to systematically evaluate how FG7142 alters the brain galanin system, as this is the first report to our knowledge that examined brain galanin expression after FG7142 administration.

The increased frequency of probe bites by exercised rodents in the shock probe test may represent either exploratory behavior, which is consistent with an anxiolytic-like effect of exercise, or a form of aggressive behavior. Considering anxiety and aggression are inversely related and that the neural systems that regulate these two behaviors can be distinct [84,85], it is reasonable that under different circumstances anxiolytic-like effects of exercise may increase aggressive behavior. In line with this interpretation, exercised rats were previously reported to be more aggressive and displayed a greater degree of biting and struggling during handling by an experimenter than sedentary rats [16]. However, wheel running reduced another form of aggression (i.e., conspecific aggression) in a manner that was dependent on whether rats had access to a wheel or whether they were locked [86]. Further, a line of mice that were bred for high running capacity exhibited elevated predatory aggression towards crickets, but reduced maternal and intermale aggression compared to control lines [87]. These findings suggest that the effects of wheel running on aggression are complex and possibly dependent on whether aggression is offensive or defensive.

The shock probe defensive burying test was sensitive to detect an enhanced learning capacity of runners, as indicated by the greater frequency of probe returns and bites in exercised rats after the probe was turned off. This suggests that exercised rats had an enhanced drive to re-approach the probe after the initial shock and, thus, had the opportunity to learn that (1) the probe was no longer electrified and (2) there would be no negative consequence of further probe contacts or bites. Indeed, improved learning after exercise has been extensively documented in humans and non- human animals in both fear and non-fear dependent paradigms [8,9,11,37,45,81,88–94].

Exercise increased galanin gene expression in the locus coeruleus in rats that were not exposed to an experimental stressor (see Figure. 3.5c). Prior research from our laboratory shows that 3–4 weeks of wheel running increases the expression of prepro- galanin mRNA in the locus coeruleus in rats that were exposed to other experimental manipulations that may involve varying degrees of stress [43–45]. The present result thus confirms the generalizability of exercise-induced galanin gene expression across a variety of paradigms. Further, in the present report we reproduced correlation data that suggests chronic exercise is driving the enhancement in locus coeruleus galanin [43,95]. In particular, we show that increases in prepro-galanin mRNA in the locus coeruleus are statistically correlated with increases in the distance ran on a wheel during the third week of running, but not before then. However, in the present report no exercise-induced increase in locus coeruleus galanin gene expression was observed in rats that were repeatedly injected with FG7142. This result does not preclude the possibility that the galanin peptide itself was increased by exercise in this group or that mRNA levels were maximal (i.e., at a ceiling) or subject to negative feedback caused by galanin levels exceeding some threshold. Direct measures of galanin peptide will be required to test these hypotheses in future experiments. Furthermore, future research using galanin receptor antagonists and agonists will be needed to determine whether galanin is necessary and/or sufficient for the anxiolytic effect of exercise. Supporting the idea that stress and exercise interact to alter the genetic expression of galanin, we have previously shown that wheel runners exhibit increased expression of prepro-galanin mRNA expression in the locus coeruleus after acute footshock [46] and that forced exercise (a stress-maintained behavior) produced this same effect [96]. Elevated galaninergic tone in the locus coeruleus may function as a counter-regulatory mechanism that serves to dampen noradrenergic activity in wheel runners. Indeed, exercise attenuates stress-induced norepinephrine release in the frontal cortex [46]. Galanin has been shown to inhibit the activity of locus coeruleus norepinephrine neurons [97–100] and to reduce norepinephrine release in target sites in a manner that is dependent on the galanin receptor

[101,102]. Collectively, these data support the possibility that wheel running mitigates the effect of stress via norepinephrine-galanin mediated mechanisms.

Additional brain regions that are targeted by the locus coeruleus may be involved in generating the anxiolytic-like and stress- buffering capacity offered by wheel running. Brain mapping studies for immediate early gene expression have already begun to identify such regions and show that wheel running attenuates stress- induced elevations of cFos in numerous structures relevant to the study of stress and anxiety, including the prelimbic and infralimbic cortex, lateral septum, subiculum, bed nucleus of the stria terminalis, paraventricular nucleus of the hypothalamus, striatum, preoptic area, dorsal medial hypothalamus, dorsal raphe, cuneiform nucleus, and locus coeruleus [18,19,103,104]. In addition, brain-mapping studies that utilize markers that accumulate over time (e.g., the truncated, splice variant of ΔFosB; 35–37 kDa size) may help to identify anatomical locations that are responsible for the long-term neural adaptations required to express the affective benefits of wheel running.

Conclusions

A prolonged, voluntary exercise regimen produced anxiolytic- like effects in rats that also had a history of repeated stress, but failed to produce these effects in exercised rats tested under base- line conditions of stress or intense stress elicited by a high dose of a B-carboline. These data support the idea that chronic exercise exerts anxiolytic-potential in a manner that depends on the presence or absence of stress. Wheel running increased galanin gene expression in the locus coeruleus, suggesting galanin plays a role in exercise-mediated regulation of stress responsivity. Our data caution against interpreting exercise-induced increases in defensive behavior as anxiogenic, and are consistent with the conclusion that a chronic exercise regimen produces beneficial effects on anxiety.

Table 3.1

Anxiety-like behavior of rats with a history of voluntary exercise or sedentary conditions that were not exposed to an experimental stressor or received an acute injection of the β-carboline FG7142

	Sedentary 0x FG	Sedentary 1x FG	Exercise 0x FG	Exercise 1x FG
Elevated plus maze				
Open arm time (s)	31.75 ± 10.33	10.85 ± 3.39^{ns}	30.37 ± 6.83	24.81 ± 6.29^{ns}
Closed arm time (s)	251.31 ± 12.34	276.03 ± 4.51$^{\#}$	248.82 ± 6.79	266.77 ± 6.72$^{\#}$
Open arm entries (freq)	3.70 ± 1.23	.75 ± .25$^{\#}$	3.60 ± .82	2.20 ± .63$^{\#}$
Closed arm entries (freq)	13.60 ± 2.21	5.25 ± 1.39$^{\#\#}$	17.20 ± 1.03	7.60 ± 1.65$^{\#\#}$
Shock probe defensive burying				
Burying time (s)	130.59 ± 38.98	4.15 ± 2.39$^{\#\#}$	79.85 ± 30.28	37.73 ± 14.58$^{\#\#}$
Immobility time (s)	136.69 ± 63.39	334.85 ± 38.82$^{\#\#}$	181.15 ± 42.84	378.87 ± 77.34$^{\#\#}$
Probe bites (freq)	.20 ± .20	.00 ± .00	1.00 ± .62	.10 ± .10
Rearing time (s)	155.09 ± 26.10	32.08 ± 6.40$^{\#\#}$	117.83 ± 16.61	26.73 ± 7.56$^{\#\#}$
Non-shock probe returns (freq)	2.50 ± 1.36	.13 ± .13	2.90 ± 1.75	.40 ± .22
Shock reactivity (scale)	1.90 ± .10	1.88 ± .35	2.30 ± .21	2.00 ± .00
Latency to initiate probe contact (s)	64.30 ± 41.06	16.88 ± 3.86	27.30 ± 7.36	65.89 ± 33.88
Latency to initiate burying (s)	256.60 ± 108.53	684.75 ± 134.73	285.60 ± 105.21	265.56 ± 120.80
Defensive withdrawal				
Distance traveled 0-5 min (m)	5.33 ± 1.28	2.41 ± .57$^{\#\#}$	5.27 ± .96	2.97 ± .86$^{\#\#}$
Distance traveled 6-10 min (m)	6.30 ± 1.38	1.14 ± .81$^{\#\#}$	6.20 ± .86	.47 ± .13$^{\#\#}$
Distance traveled 11-15 min (m)	5.19 ± 1.40	.94 ± .73$^{\#\#}$	4.98 ± .96	.97 ± .64$^{\#\#}$
Dark box time (s)	565.45 ± 78.70	807.53 ± 42.41$^{\#\#}$	585.01 ± 31.21	740.55 ± 87.46$^{\#\#}$
Dark box transitions (freq)	18.50 ± 3.81	2.63 ± 1.35$^{\#\#}$	20.40 ± 2.66	2.40 ± .82$^{\#\#}$
Latency to enter dark box (s)	20.94 ± 3.61	48.60 ± 11.87$^{\#\#}$	20.98 ± 3.50	31.76 ± 5.85$^{\#\#}$

Data are mean ± S.E.M. (n = 8-10); $^{\#\#}p < .01$, $^{\#}p < .05$ vs. No injection (0x FG7142); $^{ns}p = .09$ vs. No injection.

Figure 3.1

Daily distance ran in a running wheel increased across experimental days in a similar manner for all treatment groups.

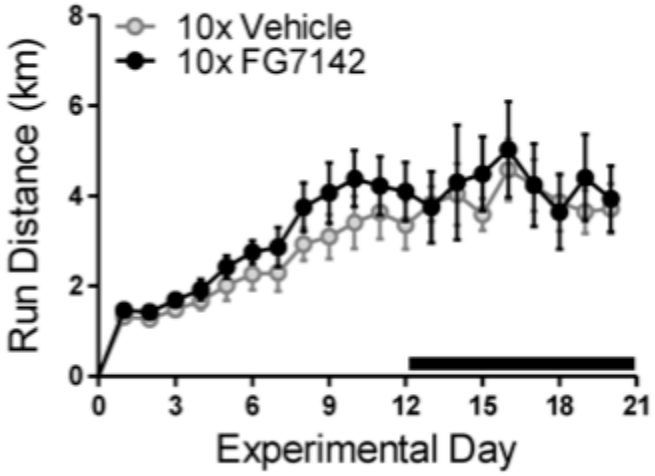

Figure 3.1. Note that the bold line indicates the experimental days on which repeated (10x) injections of vehicle or FG7142 occurred. Data are mean ± SEM (n = 9-10).

Figure 3.2

Exercise produces anxiolytic-like behavior in the elevated plus maze in rats exposed to repeated injection or FG7142.

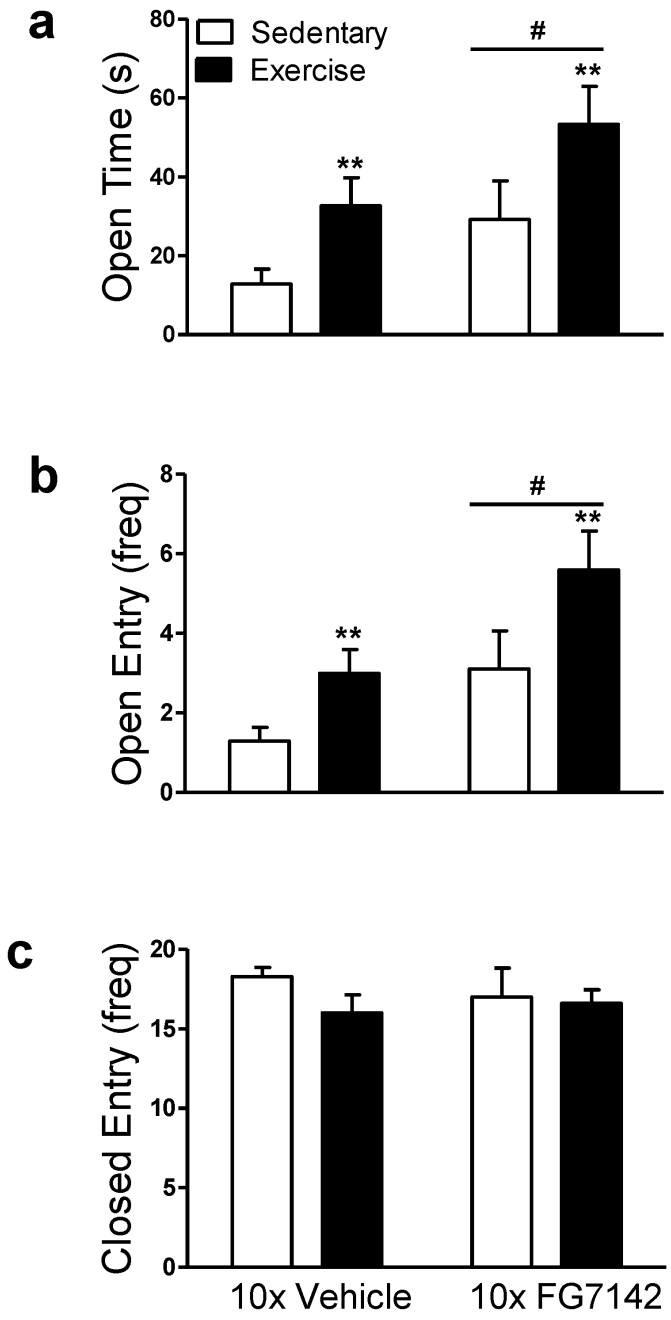

Figure 3.2. Exercised rats that were repeatedly injected (10x) with vehicle or FG7142 exhibit increased (a) open arm time and (b) more open arm entries compared to sedentary rats. Rats repeatedly injected with FG7142 exhibit increased (a) open arm time and (b) open arm entries relative to vehicle-treated rats. (c) Neither exercise manipulation nor repeated injection stress altered (c) closed arm entries. Data are mean ± SEM (n = 9-10). **p < .01 vs. Sedentary; $^{\#\#}p$ < .01, $^{\#}p$ < .05 vs. Vehicle.

Figure 3.3

Exercise increases both anxiolytic-like and active defensive behaviors in the shock probe defensive burying test in rats exposed to repeated injection or FG7142.

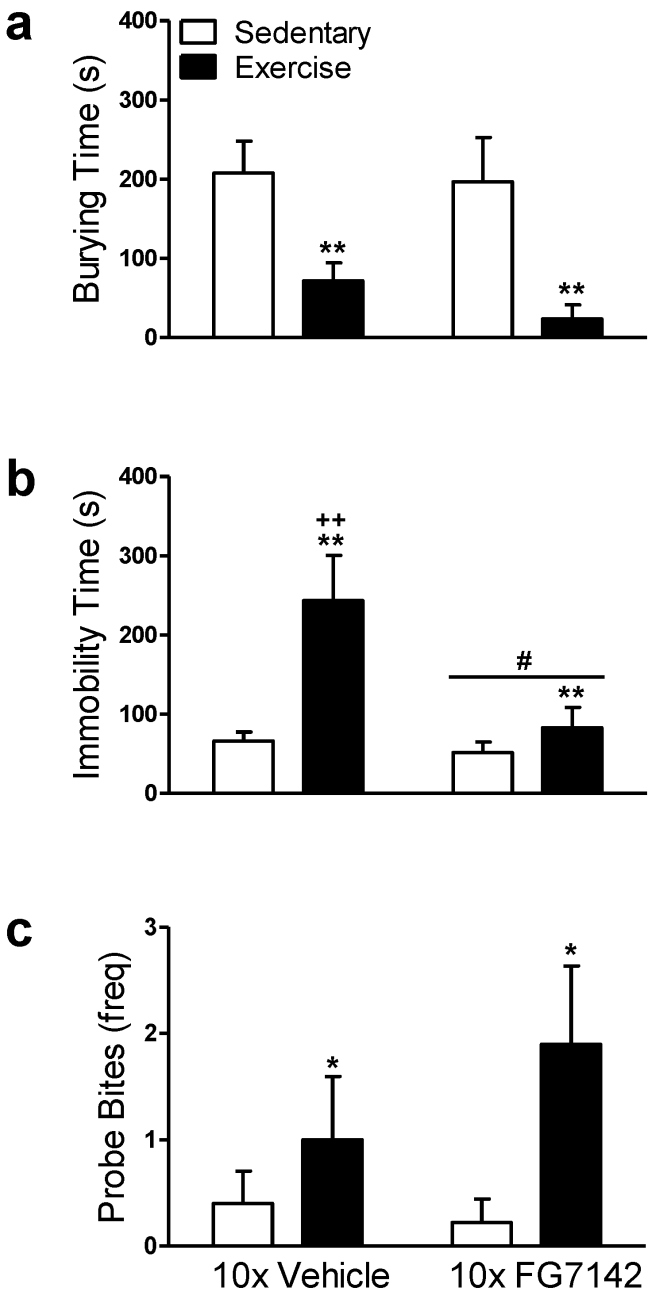

Figure 3.3. Exercised rats that were repeatedly injected (10x) with vehicle or FG7142 spent less (a) time burying and more (b) time immobile while simultaneously exhibiting a greater frequency of (c) probe bites compared to sedentary rats. Repeated FG7142 abolished the increase in (b) time spent immobile in exercise rats. Rats repeatedly injected with FG7142 exhibit reduced (b) time spent immobile relative to vehicle-treated rats. Data are mean ± SEM (n = 9-10). $^{**}p < .01$, $^{*}p < .05$ vs. Sedentary; $^{\#}p < .05$ vs. Vehicle; $^{++}p < .01$ vs. All other groups.

Figure 3.4

Exercise facilitates locomotor habituation and defensive withdrawal in rats exposed to repeated injection or FG7142.

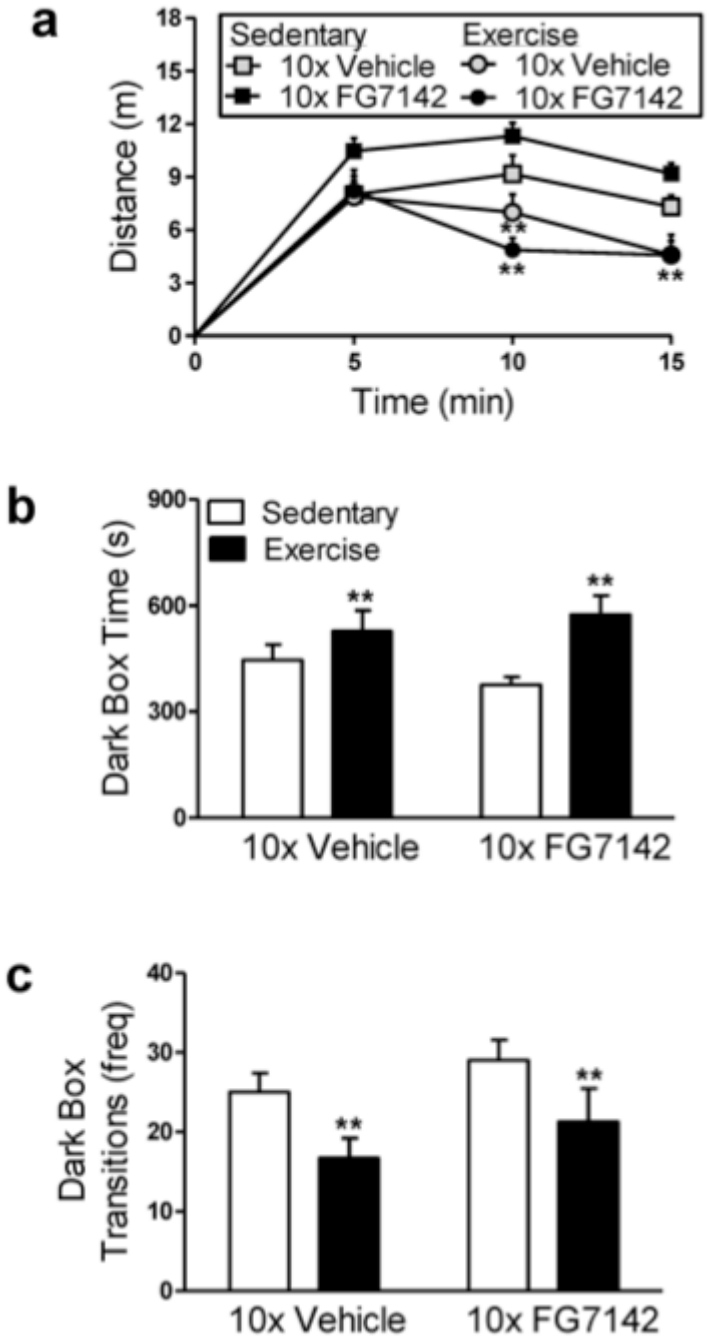

Figure 3.4. Exercised rats that were repeatedly injected (10x) with vehicle or FG7142 exhibited similar initial (0-5 min) (b) distance traveled, but thereafter (6-15 min) exhibited reduced distance traveled compared to sedentary rats. Exercised rats that were repeatedly injected with vehicle or FG7142 exhibit increased (a) time spent in the dark box and fewer (b) dark box transitions compared to sedentary rats. Data are mean ± SEM (n = 9-10). **p < .01 vs. Sedentary.

Figure 3.5.

Exercise increased prepro-galanin mRNA expression in the locus coeruleus.

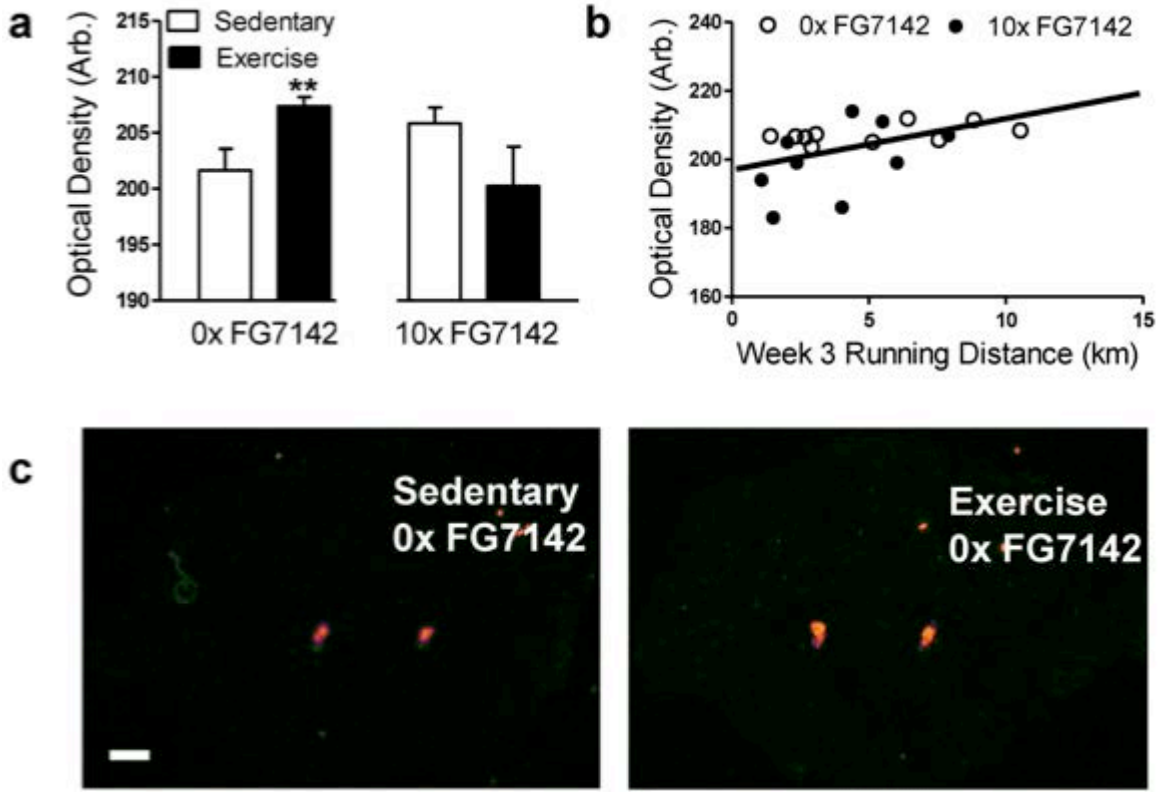

Figure 3.5. Exercise rats that were not injected (0x FG7142) exhibit increased (a) optical density for prepro-galanin mRNA in the locus coeruleus compared to sedentary counterparts. Suggesting that long durations of running are needed to increase galanin gene expression, the (b) optical density for prepro-galanin mRNA in the locus coeruleus was positively correlated with distance ran at 3 weeks. (c) The representative photomicrographs show 35S-oligonucleotide binding directed towards prepro-galanin mRNA in the brain of rats that were not injected and either forced to remain in sedentary conditions (left) or allowed access to a running wheel (right) for three weeks. Sections were collected at -10.04 mm from bregma. Scale bar indicates 1 mm. Data are mean ± SEM (n = 9-10). **p < .01 vs. Sedentary 0x FG7142.

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CHAPTER 4

THE GALANIN RECEPTOR AGONIST GALNON ATTENUATES COCAINE-INDUCED REINSTATEMENT AND DOPAMINE OVERFLOW IN THE FRONTAL CORTEX

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Abstract

Relapse represents one of the most significant problems in the long-term treatment of drug addiction. Cocaine blocks plasma membrane monoamine transporters and increases dopamine (DA) overflow in the brain, and DA is critical for the motivational and primary reinforcing effects of the drug as well as cocaine-primed reinstatement of cocaine seeking in rats, a model of relapse. Thus, modulators of the DA system may be effective for the treatment of cocaine dependence. The endogenous neuropeptide galanin inhibits DA transmission, and both galanin and the synthetic galanin receptor agonist galnon interfere with some rewarding properties of cocaine. The purpose of this study was to further assess the effects of galnon on cocaine-induced behaviors and neurochemistry in rats. We found that galnon attenuated cocaine-induced motor activity, reinstatement, and DA overflow in the frontal cortex at a dose that had no effect on baseline motor activity, stable self-administration of cocaine, baseline extracellular DA levels, or cocaine-induced DA overflow in the nucleus accumbens (NAc). Similar to cocaine, galnon had no effect on stable food self-administration but reduced food-primed reinstatement. These results indicate that galnon can diminish cocaine-induced hyperactivity and relapse-like behavior, possibly in part by modulating DA transmission in the frontal cortex.

Introduction

Cocaine addiction, defined as intense craving and compulsive use of cocaine despite negative consequences, is a major problem in our society. Current treatment options (e.g. behavioral therapy) are not very effective, and there are currently no FDA-approved or generally accepted pharmacotherapies for cocaine dependence. Because relapse is a major obstacle in the treatment of drug addiction, one promising strategy is to develop therapies that block the ability of triggers such as the drug itself, drug-associated cues, or stress to precipitate relapse (1, 2). Cocaine blocks plasma membrane monoamine transporters, which in turn increases extracellular levels of DA, norepinephrine, and serotonin in the brain. It is well established that DA is critical for mediating the motivational and reinforcing effects of cocaine, and blocking its transmission attenuates drug-seeking behavior during reinstatement, a model of relapse (1, 3). NE and 5-HT play modulatory roles and are also implicated in reinstatement.

One intriguing molecule that modulates DA transmission and behavioral responses to addictive drugs is the neuropeptide galanin. Galanin and its G protein-coupled receptors (GalR1-3) are expressed within the mesocorticolimbic circuit implicated in drug addiction (4, 5). Galanin receptors can also be activated by galnon, a synthetic non-peptide agonist that crosses the blood-brain barrier and binds to GalR1 and GalR2 (6). In general, galanin reduces DA release (7-10), and both galanin and galnon attenuate responses to drugs of abuse (11). For example, intracerebroventricular administration of galanin attenuates morphine conditioned place preference (12), and opiate withdrawal is decreased by galanin overexpression or galnon and exacerbated by genetic knockout of galanin or GalR1 (13, 14). Moreover, galanin knockout mice are hypersensitive to morphine and cocaine conditioned place preference, and these phenotypes are abolished by galnon administration (15). By contrast, complete knockout of galanin has minimal effect on cocaine self-administration in mice using several doses and schedules of reinforcement (15, 16). However, several important aspects of drug responses have not been examined after galanin receptor activation, including relapse-like behavior and

DA transmission. In this study, we examined the consequences of galnon administration on drug seeking during the maintenance and reinstatement phases of operant cocaine self-administration, as well as on cocaine-induced changes in DA overflow in the frontal cortex and the nucleus accumbens (NAc).

Materials and methods

Subjects

Male Sprague-Dawley rats (151-175 g) were used for all experiments. Self-administration experiments were conducted at Emory University (*N*=43 rats purchased from Charles River, Wilmington, MA) and motor activity and microdialysis experiments were conducted at the University of Georgia (*N*=107 rats purchased from Harlan, Prattville, AL). Rats were individually housed in clear polycarbonate cages (50 x 30 x 30 cm) and given *ad libitum* access to food and water unless otherwise specified in a temperature and humidity controlled animal facility and maintained on a 12-hour reverse light/dark cycle. Testing occurred during the dark phase with background noise emitted by a white noise generator. Animals were allowed to acclimate to the vivarium for one week prior to surgery. Rats were treated in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Experiments were approved by Emory and University of Georgia IACUCs.

Drugs

Cocaine was obtained from NIDA and dissolved in 0.9% physiological saline. Galnon (Bachem, Torrance, CA, USA) was sonicated in 1% DMSO. Galnon is a non-selective galanin receptor agonist (K_d =1 μM) with a half-life of approximately 60 min (17). Galnon was injected 20-30 min before cocaine based on effective pretreatment schedules in rodents (15, 18-20). All injections were performed in a volume of 1 ml/kg.

Stereotaxic cannulation surgery

Rats were anesthetized with isoflurane administered by vaporizer with oxygen delivered through a nose cone, and the surgical site was shaved and cleaned with Betadine. Rats were positioned in a stereotaxic apparatus, a longitudinal incision was made along the scalp, and 3 screws were anchored to the skull. Unilateral guide cannulae (MAB 6.6.IC; SciPro, Sanborn, NY) were implanted targeting the frontal cortex (3.2 mm anterior, 2.2 mm lateral, -1.5 mm ventral, relative to bregma) or NAc shell (1.7 mm anterior, 0.6 mm lateral, -6.5 mm ventral) according to the atlas of Paxinos and Watson (21). Cannulae and a plastic guard were fixed to the skull using fast-drying epoxy. Rats received banamine (2.5 mg/kg, s.c.) immediately and 24 h after surgery. At the end of experiments, 4 µl of dye (2 mg/ml India ink) was injected to verify cannulae placement, determined by inspection of dye and termination of the cannulae track in 12 µm Nissl-stained coronal cryosections.

Motor activity

Rats were placed in the center of an open arena (44.5 x 44 x 30 cm; ENV 515-16, Med Associates, St. Albans, VT) that contained fresh bedding as described (22). Infrared beam breaks were used to track the coordinate position and movement of the rat (ENV-520, Med Associates) every 50 ms using default settings (SOF-810). Behavior was recorded continuously during the 140 min test, pausing in 20 min intervals for all rats to collect a dialysis sample or to inject drug. Behavior was sampled for 40 min before treatment with galnon (2, 5, or 10 mg/kg i.p.) or vehicle, collected for another 20 min before cocaine (10 mg/kg i.p.), and recorded for 60 min post-cocaine. Ambulation was initiated after 3 beam breaks and measured as ambulatory distance traveled in continuous (< 500 ms without rest) movement outside a 2x2 area of x-y beams. Non-ambulatory movement was defined as movements that did not achieve criteria for ambulation, and thus was the continuous movement within a 2x2 area of x-y beams. Rats were not habituated to the open field before testing because pilot experiments showed that novelty-

induced increases in ambulation diminished steadily across baseline testing, after which time locomotor exploration was nearly zero (i.e., see 40 min in Fig. 4.1A).

In vivo microdialysis

Microdialysis was performed in all rats during the motor activity test (see above), as we described previously (23). A microdialysis probe was inserted into cannulae targeting the frontal cortex (MAB 9.6.2, 0.6 mm OD, 6 kDa cutoff PES membrane; SciPro, Sanborn, NY) or NAc (S-8020, 0.36 mm OD, 20 kDA cutoff PAN membrane; Synaptech, Marquette, MI) the night before testing and extended 2 mm beyond the guide cannulae. Probes were connected to tubing (PE50, VWR, Westchester, PA) before experimentation. Rats were allowed to freely explore an open field while artificial CSF (pH 7.4; 0.13 M NaCl, 3.1 mM $KHCO_3$, 1 mM $MgCl_2$, 2 mM $NaHCO_3$, 2.5 mM dextrose, 1.2 mM $CaCl_2$; Sigma Chemicals, St. Louis, MO) was delivered through the probe at 2 µl/min. Dialysate was collected every 20 min during the 140 min open field test, including before galnon (2, 5, or 10 mg/kg i.p.) or vehicle injection at time -40, before cocaine at time 0, and for 60 min post-cocaine. These procedures were employed to allow DA levels to equilibrate and become Ca^{++}-dependent (i.e., probes inserted 24 hours before testing, baseline sampling lasted 40 min before drug manipulation), as shown previously (24). Dialysate was transferred to sterile microcentrifuge vials (Fischer Scientific, Suwanee, GA) pre-filled with 0.1% phosphoric acid and DHBA (10% sample volume, 0.08 ng/ul) or 0.1 M perchloric acid in EDTA (1 mM of final solution volume). Different preservatives were used in a counterbalanced manner to determine if analyte detection could be improved, but preservatives did not alter DA content in our study (data not shown). Samples were transported in an opaque container on ice and stored at -80° C. Tubing was flushed with 70% EtOH, distilled H_20, air, and aCSF between rats.

High performance liquid chromatography

Samples were thawed and injected into the HPLC system that consisted of two ESA 584 pumps (ESA, Chelmsfor, MA) with a pre-column filter (Synergi Max-RP 4u Security Guard, 150 x 4.6 mm, Phenomenex Inc., Torrance, CA) and Max-RP cartridges (Phenomenex). Mobile phase, containing 100 mM sodium phosphate monobasic (Fisher), 0.1 mM EDTA (Sigma), 0.25 mM octanesulfonic acid (Sigma), and 5% acetonitrile (JT Baker) was delivered at 1 ml/min. Samples and standards were housed and sampled using an ESA 542 autosampler maintained at 4º C. Dialysate injection was 20 µl, and a duplicate set of standards was run every 12 samples. Peaks were detected over 30 min using an ESA CoulArray electrochemical detector (-150 mV on initial electrode, 200 mV on a subsequent electrode). The position and height of peaks for DA were compared with reference standard solutions (Sigma; diluted in aCSF). Peak areas from chromatograms were integrated and analyzed by CoulArray Data Station Software 3.05.

Food training

To facilitate acquisition of drug self-administration, rats were first trained to lever press for food (45 mg pellets) in an operant chamber (Med Associates, St Albans, VT) prior to surgery. Each chamber was equipped with a house light and two retractable levers with a stimulus light above each lever. Animals were trained on a fixed ratio 1 (FR1) schedule with a 20-s time out. One lever press on the active lever resulted in the delivery of one food pellet. Presses on the inactive lever had no programmed consequences. Food training sessions lasted 8 h, or until the animal met criteria, defined as at least 70% active lever selection and at least 100 food pellets obtained. Most rats met criteria on the first day of food training, but a few required 2–3 days.

Jugular catheter surgery

All instruments and implants were sterilized prior to surgery. Rats were surgically implanted with a catheter into the right jugular vein after food training, as described (25, 26).

Rats were anesthetized with isoflurane administered by vaporizer with oxygen delivered through a nose cone, and the surgical site was shaved and cleaned with Betadine. Catheter tubing was threaded subcutaneously from the back and guided over the shoulder into the right jugular vein, and tubing was sutured down. Rats received meloxicam (1 mg/kg, s.c.) immediately following surgery, and allowed to recover for 1 week prior to cocaine self-administration. Catheters were flushed daily with 0.05 ml gentamicin (3 mg/ml) and 0.1 ml heparinized saline (30 ml in sterile saline) to help maintain patency. Catheter patency was verified prior to cocaine self-administration by administering 0.08-0.12 ml of the short-acting barbiturate methohexital sodium (10 mg/ml, IV; Eli Lilly, Indianapolis, IN, USA), which rapidly produces moderate sedation.

Cocaine self-administration

Daily cocaine self-administration sessions were run for 2 h on a FR1 schedule. At the start of each session, both active and inactive levers were extended, and rats received a non-contingent infusion of cocaine (0.5 mg/kg). During training, each press of the active lever resulted in a cocaine infusion (0.5 mg/kg, 167 μl/kg) accompanied by a discrete flashing light above the lever. Following a 20-s timeout period (during which time active lever presses were recorded but did not result in drug infusion), the stimulus light was extinguished and responses were again reinforced. Responses on the inactive lever had no programmed consequences. To prevent overdose, the session was terminated early if the number of cocaine infusions exceeded 40. The effects of galnon were assessed once rats reached a stable level of responding (number of drug infusions varied by <20% of the mean and preference for the active lever was at least 75% for 3 consecutive days, with a minimum of 5 total days of cocaine self-administration). Rats received an injection of vehicle or galnon (2 mg/kg, i.p.) 30 min prior to the self-administration session. Each rat received both pretreatments in a counterbalanced fashion.

Extinction

Following completion of the maintenance phase of cocaine self-administration, lever pressing was extinguished in daily 2-h sessions during which presses on the previously active lever no longer resulted in delivery of cocaine or presentation of cocaine-paired cues. Behavior was considered extinguished when active lever presses over 3 consecutive days was <30% of the average number of active lever presses during the last 3 days of maintenance.

Cocaine-primed reinstatement

Rats were pretreated with vehicle or galnon (2 mg/kg, i.p.) the day after extinction criteria were met. Thirty min later, they were given a non-contingent priming injection of cocaine (10 mg/kg, i.p.) and placed in operant chambers under extinction conditions (i.e., presses on the "active" lever had no programmed consequences) for 2 h. Each rat received both pretreatments in a counterbalanced fashion, separated by extinction sessions until they met criteria (described above).

Food self-administration

Separate groups of rats were used for the food self-administration and reinstatement experiments. Rats were maintained on a restricted diet of 16 g of normal rat chow per day, given in the evening at least 1 h after self-administration sessions ended. Parameters of food self-administration were identical to the cocaine self-administration experiments, except that rats received a food pellet instead of a cocaine infusion for each active lever press, and sessions lasted 1 h and were terminated if the number of reinforcers obtained exceeded 60. Once rats reached maintenance criteria, rats received an injection of vehicle or galnon (2 mg/kg, i.p.) 30 min prior to the self-administration session. Each rat received both pretreatments in a counterbalanced fashion.

Food-primed reinstatement

After extinction training was completed (extinction criteria were identical to those used for cocaine-primed reinstatement), rats were pretreated with vehicle or galnon (2 mg/kg, i.p.). Thirty min later, they were placed in the operant chambers for the reinstatement session. Three food pellets were delivered non-contingently in the first 10 sec of the session and levers were presented. Responses on either of the levers had no programmed consequence. Throughout the 60 min food reinstatement session, a food pellet was delivered every 3 min non-contingently, and responses upon the formerly active and inactive levers were recorded. Each rat received both pretreatments in a counterbalanced fashion, separated by extinction sessions as described above.

Statistics

Self-administration data were analyzed by ANOVA followed by Newman-Keuls post hoc tests. Motor activity was combined from rats implanted with cannulae in the cortex and NAc because there were no differences between these groups. Motor activity were analyzed using repeated measures ANOVA, and area under the curve (AUC) was performed as follow-up tests for significant interaction effects to detect the source of group differences during relevant experimental phases, specifically during habituation (Phase I: -60 to -20 min), pretreatment (Phase II: -20 to 0 min), and cocaine phases (Phase III: 0 to 60 min). Analyte levels are reported in nmol/ml for descriptive purposes. Repeated measures ANOVA was used to analyze % DA overflow (post-cocaine analyte at 0, 20, 40, or 60 min / lowest baseline analyte at -40 or -20 min) X 100) to reduce within-subject variability. To test *a priori* hypotheses and minimize a type II error, AUC for % DA was performed to assess the effects of galnon during the cocaine phase and *t*-tests were performed to assess the effects of galnon at baseline (time 0). Based on standard criteria (2 standard deviations ± mean), occasional outliers (e.g., <1% of values) were removed and missing values (e.g., ~6% of values due to loss of the microdialysis sample during

transfer or collection) were replaced by the group mean to prevent loss of statistical power. In addition, one rat in the galnon 10 mg/kg group was removed entirely from locomotor activity analyses because this subject achieved outlier criteria. The number of subjects per group differed in the behavioral and dialysis studies because HPLC data were not available for all subjects and we focused our analysis on the vehicle and 2 mg/kg galnon dose. Data were analyzed using SPSS (IBM PAWS Statistical Software, Chicago, IL) and graphed using Prism 5.0 (GraphPad, La Jolla, CA).

Results

Galnon reduces cocaine-induced motor activity.

The effect of galnon (2, 5, or 10 mg/kg) on baseline and cocaine-induced motor activity were assessed in an open field. Ambulatory distance changed across time in a cubic fashion ($F_{1,100}$=130.97, p=0.00; Cubic) (Fig. 4.1A). Distance traveled reduced across time compared to initial exploration (-40 vs. 0 min; p=0.00), was stimulated immediately after cocaine administration (10 min; p=0.00), and then steadily declined at 40 min (p=0.00), 50 min (p=0.00), and 60 min post-cocaine (p=0.00) (Fig. 4.1A) compared to the initial effects of cocaine at 10 min. Although the main effect of galnon was not significant for ambulatory distance ($F_{3,102}$=1.08, p=0.36), the drug by time effect was significant ($F_{3,102}$=3.94, p=0.01) (Fig. 4.1A). Follow-up AUC analyses reveled that ambulatory distance was no different across groups during habituation in phase I (-60 to -20 min; $F_{3,102}$=0.39, p=0.76), after pretreatment with vehicle or galnon in phase II (-20 to 0 min; $F_{3,102}$=0.92, p=0.44), overall post-cocaine in phase III (1 to 60 min; $F_{3,102}$=1.56, p=0.21), or initially post-cocaine administration in phase IIIa (1 to 30 min; $F_{3,102}$=0.43, p=0.73). However galnon reduced cocaine-induced ambulatory distance later in phase IIIb compared to vehicle (31 to 60 min; $F_{3,102}$=3.08, p<0.05), at doses of 2 mg/kg (p<0.05) and 5 mg/kg (p<0.05) with a trend at the 10 mg/kg dose (p=0.13) (Fig. 4.1B).

Non-ambulatory movement was also different across time ($F_{1,102}$=150.30, p=0.00; Cubic) (Fig. 4.1C). Non-ambulation was less frequent across time compared to initial ambulation (-40 vs. 0 min; p=0.00), more frequent immediately after cocaine administration (10 vs. 0 min; p=0.00), and then became less frequent at 40 min (p=0.00), 50 min (p=0.00), and 60 min post-cocaine (p=0.00) (Fig. 4.1C) compared to initial effects of cocaine at 10 min. Although the main effect of galnon was not significant for non-ambulatory movement ($F_{3,102}$=0.74, p=0.53), the drug by time effect was significant ($F_{3,102}$=4.02, p=0.01) (Fig. 4. 1C). Follow-up AUC analyses reveled that non-ambulatory movement was no different across groups during habituation in phase I (-60 to -20 min; $F_{3,102}$=0.94, p=0.43), after pretreatment with vehicle or galnon in phase II (-20 to 0 min; $F_{3,102}$=0.07, p=0.97), overall post-cocaine in phase III (1 to 60 min; $F_{3,102}$=1.36, p=0.26), or initially post-cocaine administration in phase IIIa (1 to 30 min; $F_{3,102}$=0.34, p=0.80). However galnon reduced cocaine-induced ambulatory distance later in phase IIIb compared to vehicle (31 to 60 min; $F_{3,102}$=2.90, p<0.05), at the 5 mg/kg dose (p<0.01) but not significantly at the 2 mg/kg (p=0.26) or 10 mg/kg doses (p=0.15) (Fig. 4.1D). Based on these results and other data showing galnon suppresses general motor activity and consummatory behavior at higher doses (Abramov et al., 2004; our unpublished data), we chose the 2 mg/kg dose for the remainder of the experiments.

Galnon attenuates cocaine-induced dopamine overflow in the frontal cortex but not the nucleus accumbens.

DA in the frontal cortex was measured in rats treated with vehicle and galnon 2 mg/kg during motor activity testing, as shown in the timeline (Fig. 4.2A). Groups were no different in DA levels at baseline/before pretreatment (vehicle 77.81±18.87 nmol/ml, galnon 73.39±22.33 nmol/ml, p>0.05). DA levels were subsequently examined as percent change from baseline because between-subject variability in both groups was large (e.g., ~20 nmol/mL), which likely resulted from possible between-subject differences in probe recovery (although not measured in

our study) and/or variability across multiple HPLC assays. Galnon pretreatment did not reliably alter DA levels relative to vehicle (0 min; t_{22}=1.59, p=0.11) (Fig. 4.2B). DA overflow increased following cocaine administration, and was attenuated by galnon (Fig. 4.2B). Two-way repeated measures ANOVA revealed a significant effect of time ($F_{1,22}$=5.09, p<0.05; Quadratic) and galnon ($F_{1,22}$=7.27, p=0.01), but not a galnon x time interaction ($F_{1,22}$=0.76, p=0.39) (Fig. 4.2B). AUC analyses reveled that galnon reduced post-cocaine DA levels relative to vehicle (t_{22}=3.03, p<0.01) (Fig. 4.2C).

DA in the nucleus accumbens was measured in a separate group of rats treated with vehicle and galnon 2 mg/kg during motor activity testing (Fig. 4.3A). Groups were no different in DA levels at baseline/before pretreatment (vehicle 30.60±3.31 nm/ml, galnon 28.12±3.54 nmol/ml, p>0.05). Galnon pretreatment did not reliably alter DA levels relative to vehicle (0 min; t_{13}=-1.13, p=0.28) (Fig. 4.3B). Cocaine increased dopamine overflow ($F_{1,13}$=15.26, p<0.01; Quadratic), but there was no main effect of galnon ($F_{1,13}$=1.85, p=0.20). There was a significant interaction of galnon by time ($F_{1,13}$=10.36, p<0.01; Cubic) (Fig. 4.3B), and follow-up tests revealed that galnon and vehicle-treated rats were no different in post-cocaine DA levels at 20 min (p=0.29), but were different at 40 (p<0.05) and 60 min post-cocaine (p<0.05) (Fig. 4.3B). Galnon and vehicle-treated rats were not significantly different in post-cocaine DA levels as assessed by AUC (t_{13}=-1.65, p=0.12) (Fig. 4.3C), suggesting that galnon delayed cocaine-induced dopamine overflow.

Galnon has no effect on cocaine self-administration but blocks cocaine-primed reinstatement of cocaine seeking.

After reaching maintenance criteria for cocaine or food self-administration, rats were pretreated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a self-administration session, and we found that galnon had no effect on operant responding for drug (one way repeated measures ANOVA: active lever, $F_{2,14}$=0.24, p=0.79; inactive lever, $F_{2,14}$=0.03, p=0.97) (Fig.

4.4A, 4.2B). Rats were next subjected to non-reinforced sessions until meeting extinction criteria, and were then pretreated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a reinstatement test following a non-contingent priming injection of cocaine (10 mg/kg, i.p.). In contrast to its inability to alter cocaine self-administration, we found that galnon attenuated cocaine-primed reinstatement of cocaine seeking (Fig. 4.4C). One way repeated measures ANOVA showed a main effect of treatment on active lever presses ($F_{2,12}$=16.07, p<0.001). Post hoc tests revealed that vehicle-pretreated rats robustly reinstated following cocaine prime compared to extinction (p<0.001), while galnon-pretreated rats did not significantly reinstate (p>0.05). Galnon-pretreated rats also displayed significantly fewer active lever presses than vehicle-pretreated animals (p<0.01). Inactive lever presses were low and did not differ between groups ($F_{2,12}$=1.35, p=0.30) (Fig. 4.4D).

Galnon attenuates food-primed reinstatement of food seeking.

To determine whether the effects of galnon on cocaine-primed reinstatement were specific to drug-induced relapse-like behavior, we also evaluated the consequences of galnon on food self-administration and reinstatement. Similar to what we found with cocaine, galnon (2 mg/kg, i.p.) had no effect on reinforced food self-administration (one way repeated measures ANOVA: active lever, $F_{2,18}$=0.12, p=0.88; inactive lever, $F_{2,18}$=3.45, p=0.054) (Fig. 4.5A, 4.3B) but partially attenuated food-primed reinstatement of food seeking ($F_{2,18}$=12, p<0.001) (Fig. 4.5C). Posthoc tests revealed that, while both vehicle- (p<0.001) and galnon- (p<0.05) pretreated rats significantly reinstated following a food prime compared to extinction, galnon-pretreated animals displayed significantly less active lever pressing than vehicle-pretreated animals (p<0.05). Inactive lever presses were low and did not differ between groups ($F_{2,18}$=0.93, p=0.41) (Fig. 4.5D).

Discussion

Our study is the first to report that galanin receptor activation decreases relapse-like behavior at a dose that had no impact on locomotion following habituation to a novel environment or reinforced cocaine/food self-administration. The behavioral effects of galnon were accompanied by a complete suppression of cocaine-evoked DA overflow in the frontal cortex, but not the NAc. These results are consistent with, and extend, previous reports showing that galanin receptor signaling diminishes the rewarding properties of cocaine and other drugs of abuse, and support targeting the galanin system as a potential treatment for addiction.

The effects of galnon on motor activity

Galanin signaling has little impact on basal motor activity and attenuates drug-induced ambulation under some conditions. For instance, galnon failed to alter general locomotion at doses below 5 mg/kg (16, 27, 28), but at high doses (e.g. 10 mg/kg and above) it impairs motor activity and food consumption under some conditions (27) (our unpublished data). Consistent with these data, we found that doses of galnon ranging from 2-10 mg/kg had no effect on exploratory activity before cocaine administration. We also found that galnon decreased cocaine-induced motor activity during the later phase of the time course examined. In contrast to our present findings in rats, locomotor activity following cocaine was not altered in mice pretreated with galnon (1-4 mg/kg) or in galanin knockout mice (16). The explanation for these discrepant findings is unknown, but is most likely attributable to species differences. Consistent with our results, galnon (2 mg/kg) also suppresses morphine-induced locomotor activity in galanin knockout mice (28). These data collectively suggest that galnon modestly attenuates hyperlocomotion following acute administration of cocaine or morphine, and low does of galnon do not reliably alter baseline locomotion. To avoid a general locomotor effect of galnon, we chose the lowest dose (2 mg/kg) for the remaining experiments in this study, which aimed to examine the effects of galnon on reward-related behaviors and neurochemistry.

The effects of galnon on cocaine and food self-administration and reinstatement

In general, galanin receptor signaling opposes the rewarding properties of cocaine and other drugs of abuse. For example, conditioned place preference to cocaine and morphine is facilitated in galanin knockout mice, while galanin or galnon suppresses the rewarding effects of these drugs (11, 12, 15, 16, 28). However, nicotine conditioned place preference is *attenuated* in galanin knockout mice (29), suggesting the nature of the drug influences the valence of galanin on reward. The effect of galnon on conditioned place preference likely does not extend to the reinforcing properties of cocaine as measured by operant self-administration; neither galanin knockout nor galnon altered cocaine self-administration in mice (15, 16). Our data showing that galnon did not alter stable operant responding for cocaine during the maintenance phase are consistent with these results.

The effect of galanin receptor activation on relapse-like behavior has not been studied, and we employed the reinstatement paradigm to address this gap in the literature. It is important to note that reinstatement responding differs from the maintenance phase of self-administration sessions because it is run under extinction conditions (i.e., active lever presses have no programmed consequences), and represents non-reinforced drug-seeking behavior thought to model aspects of relapse (1). We found that galnon abolished cocaine-primed reinstatement. There was no significant difference between extinction and reinstatement responding following galnon pretreatment, while robust reinstatement was observed following vehicle pretreatment. This reduction in drug seeking appears to be specific for reinstatement as opposed to a general suppression of motor activity or operant behavior because the dose of galnon used had no effect on baseline exploratory activity, cocaine self-administration, food self-administration, or inactive lever presses during any phase. Moreover, galnon slightly attenuated cocaine-induced motor activity, which unlikely accounts for the robust loss of reinstatement behavior because reinstatement was reduced for a non-drug reinforcer (food) that does not produce hyperactivity.

The effects of galnon on cocaine-induced changes in dopamine overflow

Cocaine-primed reinstatement of cocaine seeking requires DA transmission in the prefrontal cortex, but not the NAc (3, 30-33). Galanin can reduce DA release in some brain regions (7-10), but the consequences of galanin receptor activation on cocaine-induced DA overflow have not been investigated. We employed *in vivo* microdialysis in the frontal cortex and NAc to determine whether changes in extracellular DA accompanied galnon's ability to attenuate the behavioral effects of cocaine. The region of frontal cortex selected for microdialysis sampling primarily represents motor cortex. This area receives dense dopaminergic innervation from ventral tegmental area neurons (34) and was selected because it is a reliable target for catecholamine recovery based on our previous microdialysis experiments (23). The region is also highly sensitive to acute cocaine challenge as indicated by magnetic resonance imaging of regional cerebral blood volume in rats (35). In these studies, cocaine elicited equivalent increases in peak blood volume in motor cortex, dorsal striatum, and nucleus accumbens. DA overflow in the motor cortex thus serves as a proxy for cocaine-induced activation of the mesocortical system. We found that galnon had no immediate impact (e.g., 20 min later) on DA levels following habituation to a novel environment. We also found that galnon prevented cocaine-induced increases in locomotor activity and DA overflow in the frontal cortex. These data suggest that attenuated cocaine-induced DA overflow in the frontal cortex may contribute to the ability of galnon to reduce cocaine-induced locomotor activity and possibly aspects of relapse-like behavior, although the latter hypothesis needs to be directly evaluated by collecting dialysate in the prefrontal cortex during reinstatement testing.

The exact mechanisms underlying the changes in DA transmission in the present study are not clear. One possibility is that galnon modulates cocaine-induced DA release by suppressing the activity of ventral tegmental area DA neurons that project to the frontal cortex or by acting directly on mesocortical DA terminals. Indeed, galanin typically inhibits neuronal activity (36), and galanin receptors are present in both brain regions (5). Alternatively, galnon

may act indirectly by altering the activity of other brain nuclei (i.e., locus coeruleus) that, in turn, project to and control the activity of mesocortical DA neurons (11). In support of this hypothesis, galnon suppresses morphine-induced neuronal activity in the locus coeruleus, and transgenic overexpression of galanin in noradrenergic neurons reduces behavior associated with morphine withdrawal (13). Although future experiments are needed to identify the loci and mechanisms of action that mediate the benefit of galnon on cocaine-induced behavior, these data collectively suggest that galnon is well positioned to alter catecholamine transmission across circuits targeted by drugs of abuse.

Galnon is a synthetic non-peptide galanin receptor agonist that crosses the blood brain barrier and has equal binding affinity for GalR1 and GalR2 (6, 37). It is important to note that high concentrations of galnon (e.g., 10 uM) *in vitro* also produce agonist activity at other GPCRs and activate intracellular G-proteins independent of receptor activation (38). However, at doses comparable to those used in the present study, the behavioral effects of galnon are similar to galanin (39) and blocked by co-administration of a galanin receptor antagonist (6, 27, 40). Thus, the effects of galnon in the present study are in all likelihood mediated, at least in part, by galanin receptor signaling. Future experiments using selective antagonists for GalR1 and GalR2 will be necessary to assess the contribution of each receptor subtype.

In the present study, we show that galnon decreases cocaine-induced DA overflow in the frontal cortex, but does not alter DA in the NAc. A simple explanation is that mesocortical DA neurons are more sensitive to inhibition by galanin receptor activation than mesolimbic DA neurons. Relative to other areas (e.g., NAc, ventral tegmental area), all three galanin receptors are highly expressed in the prefrontal cortex (5), and this region is changed substantially in both structure and function by cocaine administration (41-43). Our results are consistent with data from slice preparations showing that galanin reduces DA release (7, 8, 10), although one study found increased DA utilization in the striatum following galanin administration (9). The failure of galnon to alter DA overflow in the NAc may result from complex interactions within mesolimbic

circuitry. For example, because cortical DA transmission opposes subcortical DA transmission (44-47), suppression of cortical DA overflow by galnon could help preserve normal accumbal DA levels. In support of this idea, our finding that the increase in cocaine-induced DA overflow in the NAc following galnon pretreatment is delayed (to ~40 min post-cocaine) compared with the peak in vehicle pretreated animals (~20 min post-cocaine), implicates a secondary circuit rather than a direct excitatory influence of galnon on mesolimbic DA neurons. Collectively, these data support a model in which galnon primarily affects mesocoritcal DA neurons that drive cocaine-primed reinstatement.

Conclusions

In summary, we report that galnon reduces reward-seeking behavior during reinstatement and cocaine-induced DA overflow in the frontal cortex. However, the results should be interpreted with caution, and further studies are required to define the underlying mechanisms. The modulatory action of galanin on other drug-induced behaviors appears to involve GalR1 and GalR2 receptor subtypes (14, 48). In addition, although all existing evidence points to a central effect of galanin and galnon (12, 13), peripheral galanin receptors cannot be ruled out due to our systemic route of galnon administration. Although further experiments are required to identify the galanin receptor subtype and neuroanatomical substrates involved, the data presented here suggest that the galaninergic system is a candidate target for anti-relapse therapies.

Figure 4.1

Galnon reduces cocaine-induced hyperactivity.

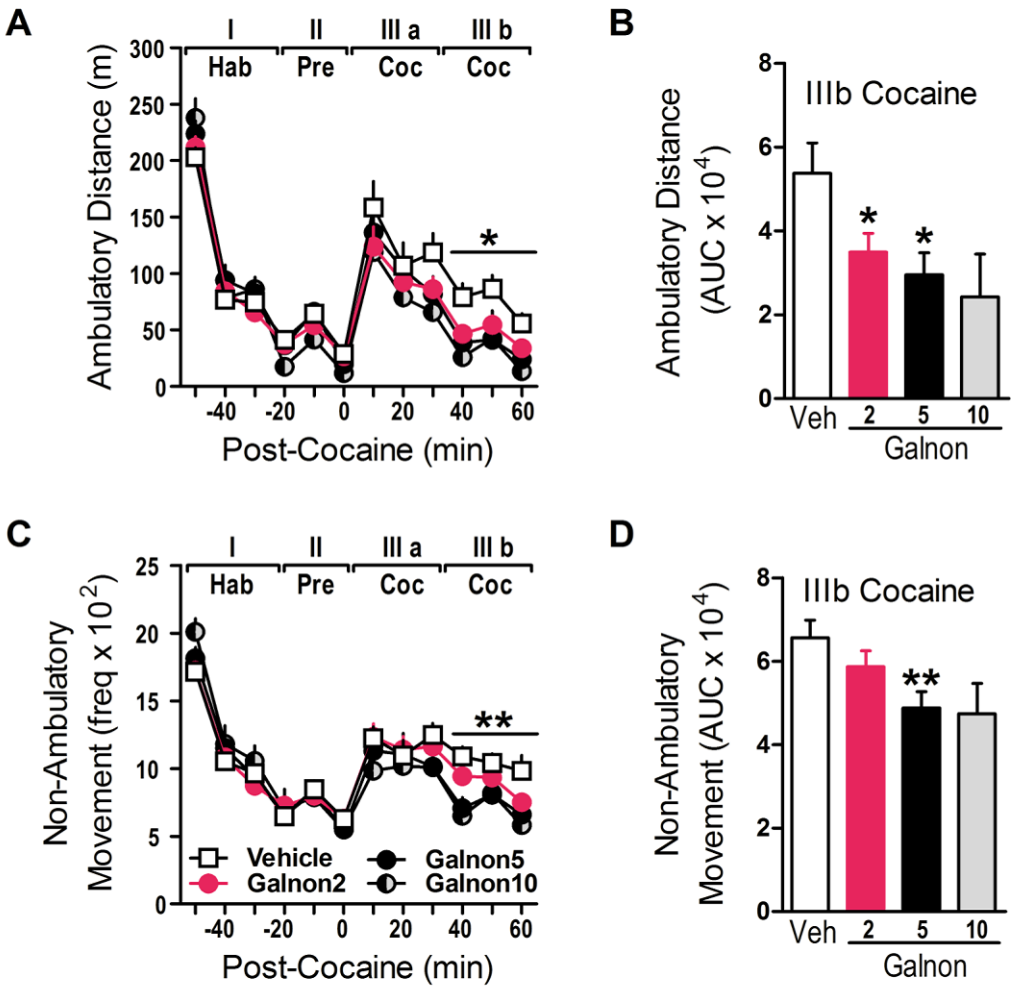

Figure 4.1. Rats were placed in an open field, administered vehicle or galnon (2, 5, or 10 mg/kg, i.p.), and injected with cocaine (10 mg/kg, i.p.) 20 min later. Shown are the mean ± SEM for (A) ambulation time and (B) frequency of non-ambulatory movement following cocaine administration. N = 46 (vehicle), 32 (galnon 2 mg/kg), 23 (galnon 5 mg/kg), and 5 (galnon 10 mg/kg). **p<0.01, *p<0.05 compared to vehicle. Hab, habituation (phase I). Gal, galnon (phase II). Coc, cocaine (phase III). Veh, vehicle.

Figure 4.2

Galnon attenuates cocaine-induced increases in dopamine overflow in the frontal cortex.

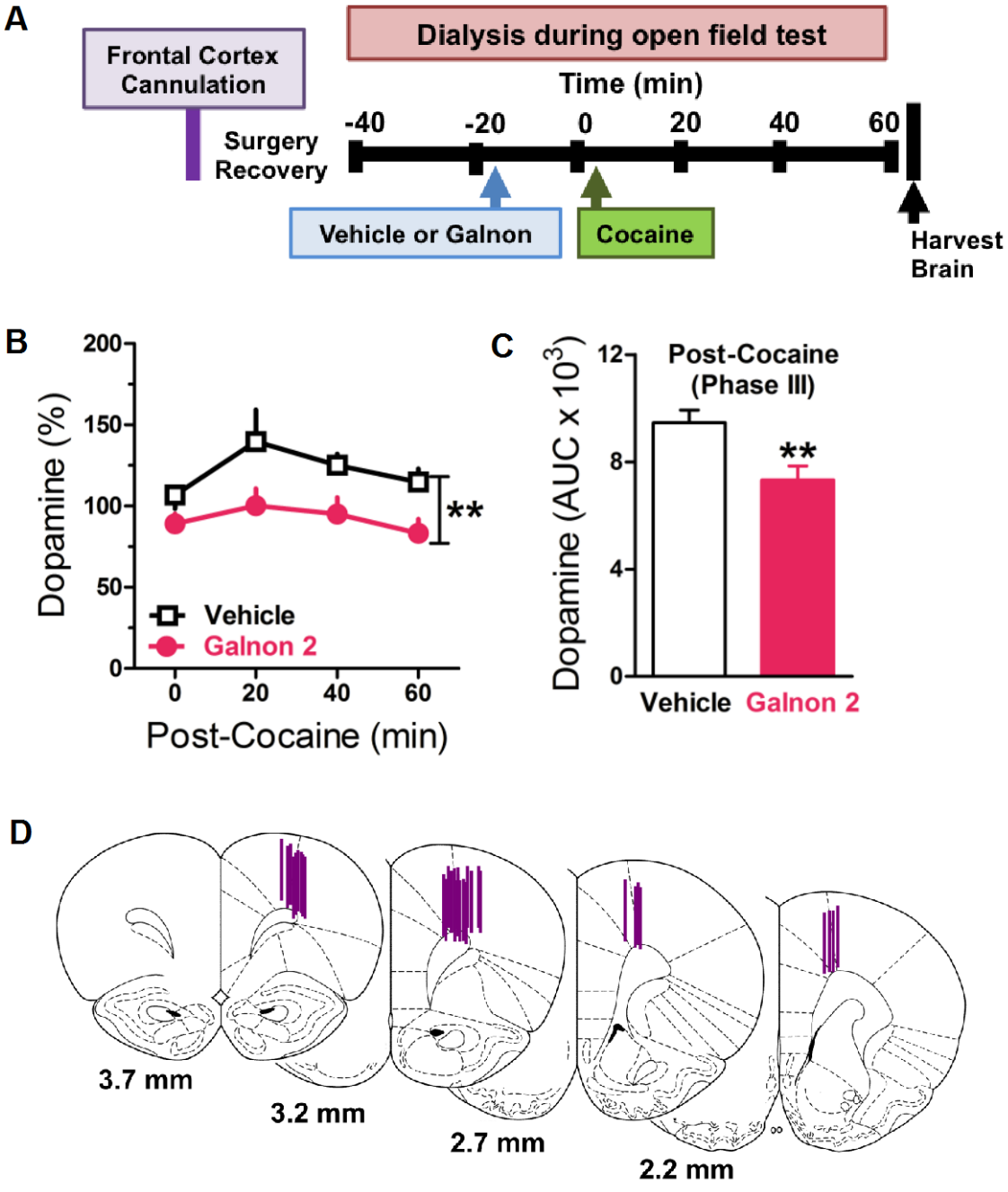

Figure 4.2. Microdialysis samples were collected through probes targeting the frontal cortex from rats administered vehicle or galnon (2, 5, or 10 mg/kg, i.p.), and injected with cocaine (10 mg/kg, i.p.) during behavioral testing (see Fig. 4. 1). Shown are (A) experimental timeline, (B) mean ± SEM extracellular DA levels (% baseline) for vehicle (n=14) and galnon 2 mg/kg (n=9), and (C) probe placements. **p<0.01, *p<0.05 compared to vehicle..

Figure 4.3

Galnon does not alter cocaine-induced increases in dopamine overflow in the nucleus accumbens.

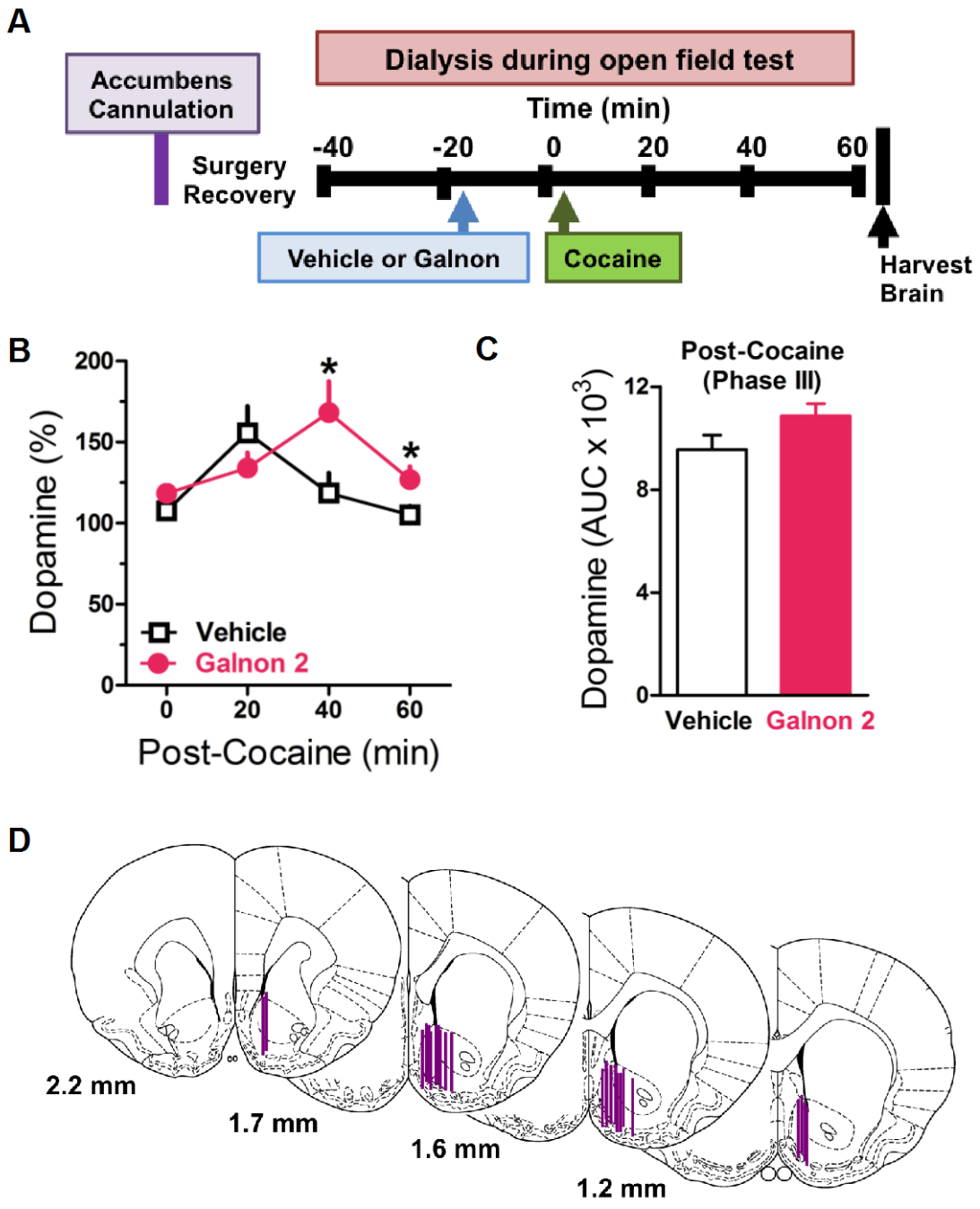

Figure 4.3. Microdialysis samples were collected through probes targeting the NAc shell from rats administered vehicle or galnon (2, 5, or 10 mg/kg, i.p.), and injected with cocaine (10 mg/kg, i.p.) during behavioral testing (see Fig. 4. 1). Shown are (A) experimental timeline, (B) mean ± SEM extracellular DA levels (% baseline) for vehicle (n=8) and galnon 2 mg/kg (n=7), and (C) probe placements.

Figure 4.4

Galnon has no effect on cocaine self-administration but attenuates cocaine-primed reinstatement.

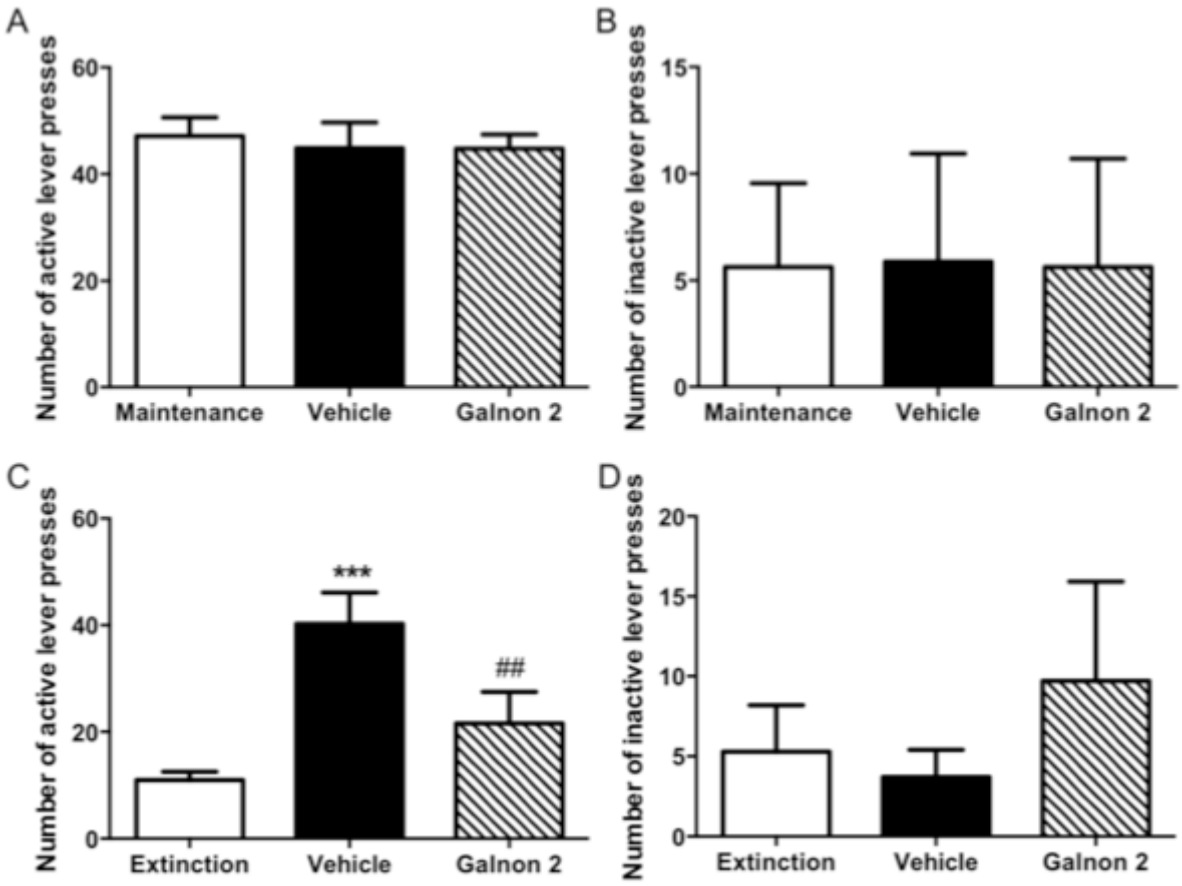

Figure 4.4. Following the establishment of stable maintenance responding for cocaine on a FR1 schedule (maintenance values reflect an average of the last 3 days of maintenance sessions), subjects (n=8) were treated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a self-administration session. Shown are the mean ± SEM of (A) active and (B) inactive lever presses during the 2 h test session. Lever pressing was then extinguished (extinction values reflect an average of the last 3 days of extinction), and rats (n=7) were pretreated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a cocaine-primed (10 mg/kg, i.p.) reinstatement test. Shown are the mean ± SEM (C) active and (D) inactive lever responses during the 2 h session. ***$p<0.001$ compared to extinction, $^{\#\#}p<0.01$ compared to vehicle.

Figure 4.5

Galnon has no effect on food self-administration but attenuates food-primed reinstatement.

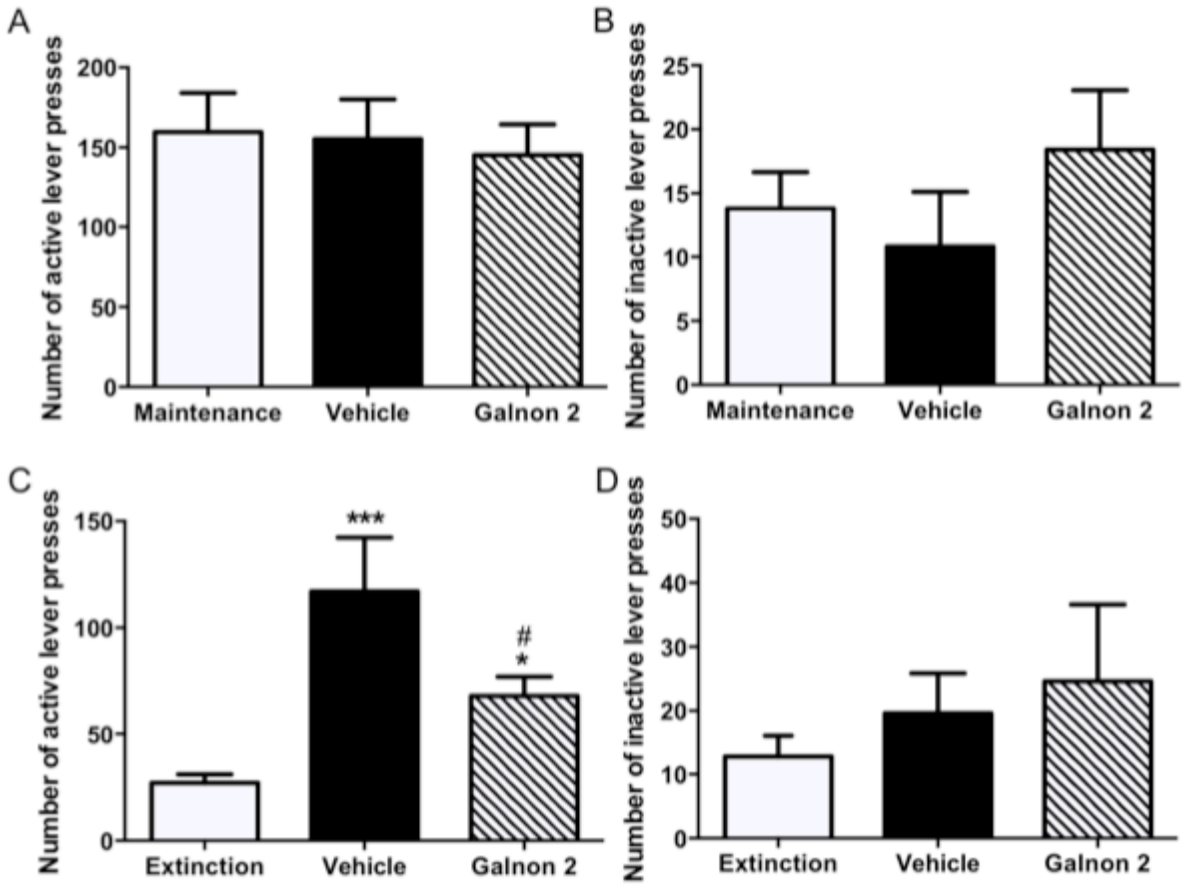

Figure 4.5. Following the establishment of stable maintenance responding for food pellets on a FR1 schedule (maintenance values reflect an average of the last 3 days of maintenance sessions), subjects (n=10) were treated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a self-administration session. Shown are the mean ± SEM of (A) active and (B) inactive lever presses during the 2 h test session. Lever pressing was then extinguished (extinction values reflect an average of the last 3 days of extinction), and rats (n=10) were pretreated with vehicle or galnon (2 mg/kg, i.p.) 30 min prior to a food-primed reinstatement test. Shown are the mean ± SEM (C) active and (D) inactive lever responses during the 2 h session. *$p<0.05$ compared to extinction, ***$p<0.001$ compared to extinction, $^{\#}p<0.05$ compared to vehicle.

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CHAPTER 5

GALANIN MEDIATES THE NEURAL AND BEHAVIORAL STRESS RESILIENCE AFFORDED BY EXERCISE

Sciolino, N.R., J.M. Smith, A.M. Stranahan, K.G. Freeman, G.L. Edwards, D. Weinshenker, and P.V. Holmes. To be submitted to *Neuropsychopharmacology*.

Abstract

Exercise promotes resilience to stress and increases galanin in the locus coeruleus (LC). We hypothesized that the behavioral benefit of exercise would be mimicked by galanin administration and reversed by galanin receptor blockade. We also predicted that exercise or galanin administration would block neural signatures of stress, including stress-induced catecholamine release and dendritic spine loss in the cortex. Male Sprague Dawley rats were cannulated for drug delivery (intra-cerebroventricle; ICV) and/or frontocortical microdialysis, and housed with or without a running wheel for 21d. Rats were acutely injected with vehicle or M40 and exposed to footshock or no stress during open field-testing. Other groups were stressed and received galanin, the galanin receptor antagonist M40, or vehicle for 21d. Exploratory behavior was measured in the elevated plus maze and open field during microdialysis. Dendritic spines in mPFC pyramidal neurons were visualized by Golgi impregnation and quantified. Exercise increased galanin levels in the LC compared to sedentary conditions. Under non-stressed conditions, behavior and dopamine levels were no different between exercised and sedentary rats. Vehicle-treated sedentary rats exposed to stress exhibited reduced open arm exploration compared to exercising animals or ICV galanin-treated sedentary rats. Repeated, but not acute, M40 administration blocked the anxiolytic effects of exercise. Both exercise and ICV galanin prevented stress-induced dopamine overflow and loss of dendritic spines observed in sedentary rats. Galanin mediates behavioral resilience in tests of anxiety and prevents stress-evoked cortical change. Like exercise, galanin therapy shows promise as a treatment for deleterious consequences of stress.

Introduction

Stress is well known to predict mental illness, but the neural mechanisms that determine whether an individual becomes susceptible or resilient are not well understood (1). Resilience is the adaptive ability to recover from challenge, which minimizes the deleterious outcomes following stress. The medial prefrontal cortex (mPFC) is particularly sensitive to stress, both in terms of its function and structure (2, 3), and also contributes to stress resilience (4). mPFC activation is necessary for the anxiolytic effects of environmental enrichment (5) and behavioral control over stress (6, see also 7). Activation of the mPFC is also sufficient for resilience, as mPFC stimulation induces resilience in behavioral tests of affect (6, 8). Dendritic spines, the morphological hallmark of excitatory synapses, are reduced in the mPFC and hippocampus after stress (9, 10), whereas antidepressants (11) or intracranial self-stimulation (12) restores dendritic structure after stress. Consistent with these data, clinical literature shows that evoked mPFC activity is enhanced in resilient people (13, 14), but reduced in anxious individuals that exhibit impaired mPFC-to-amygdala functional connectivity (15, 16).

Exercise effectively promotes behavioral resilience (17), in part by increasing neurotrophic factor expression (18). Exercise reliably increases the neuropeptide galanin in noradrenergic neurons of the locus coeruleus (LC) (19-21). Galanin receptors (GalR1-3) are present throughout stress-responsive regions, including the LC, dorsal raphe, ventral tegmental area (VTA), hypothalamus, hippocampus, amygdala, and mPFC (22). Galanin mediates stress resilience in behavioral tests of affect (23), and galanin signaling is necessary for the antidepressant-like effects observed in a stress paradigm (24). Microarray profiling also shows that galanin expression distinguishes resilient and susceptible phenotypes (25). Although widely recognized for its orexigenic and anticonvulsant effects, galanin has potent neurotrophic activity (26, 27) and may alter the plasticity of neurons in regions that subserve resilience. Galanin may also exert therapeutic benefit through inhibition of catecholamine transmission in stress-responsive neural pathways (28), either locally within the LC or in distal targets like the VTA.

Optogenetic phasic stimulation of VTA dopamine neurons induces susceptible behavior and rapidly reverses resilience (29). In contrast, galanin inhibits midbrain DA activity (30, 31) and generally reduces dopamine release (32-34). Thus, galanin signaling is poised to regulate resilience through multiple mechanisms throughout stress-responsive circuitry.

Collectively, these data indicate that both exercise and galanin promote resilience to stress and suggest a causal relationship between the two. The purpose of this study was to determine whether galanin is necessary and/or sufficient for stress resilience. We predicted that increasing galanin by long-term access to wheel running or repeated intracranial galanin administration would produce behavioral resilience, while chronic galanin receptor blockade would attenuate stress resilience induced by exercise. We also predicted that exercise or galanin administration would protect the cortex against general markers of stress, including stress-induced structural change and dopamine overflow.

Materials and Methods

Subjects

Male Sprague-Dawley rats (N=81; Harlan, Prattville, AL) obtained at 175-200 g and given ad libitum food and water were housed at 23±3ºC with a 12:12 reverse light:dark cycle. Following 1-week habituation, rats housed individually in clear polycarbonate cages (50x30x30 cm). Procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 2013) and approved by University of Georgia IACUC.

General Experimental Methods

All testing occurred in the dark phase of the circadian cycle. The general protocol for experiments was: cannulation (day -8), surgical recovery (day -7 to 0), exercise or sedentary

housing (day 1 to 21), footshock during microdialysis sampling (day 20), and elevated plus maze testing followed by brain extraction (day 21).

Stereotaxic Cannulation Surgery

Rats were anesthetized with isoflurane (1-5%) and guide cannulae were positioned and implanted stereotactically. In Experiment 1, unilateral 22G cannulae targeted the lateral ventricle for acute drug delivery (1mm posterior, -1.5mm lateral, 3mm ventral) relative to bregma using the atlas of Paxinos and Watson (35). In Experiment 2, unilateral cannulae targeted the lateral ventricle for chronic drug delivery and another targeted the frontal cortex for microdialysis (3.2 mm anterior, 2.2 mm lateral, 1.5 mm ventral; MAB6.6.IC; SciPro, Sanborn, NY). Rats received flunixin meglumine (2.5 mg/kg s.c.) immediately and 24 hours post-surgery.

Voluntary exercise

Rats were assigned to exercise or sedentary groups on day 1 and received continuous, free access to a wheel or no wheel in the homecage. Rats were housed in these conditions until the experiment ended (day 21 or 23), as this exercise duration effectively increases galanin (19-21, 36). No-wheel controls were selected to provide the best distinction between independent variables (e.g., exercise/environmental enrichment vs. no exercise/no environmental enrichment). An electromagnetic counter (Mini Mitter, Bend, OR) detected wheel rotations and distance ran was calculated for each subject.

Drugs

In experiment 1, rats were injected with vehicle (aCSF or dH20) or the galanin receptor antagonist M40 (6nmoles; Tocris, Minneapolis, MN) twenty minutes before exposure to footshock or no shock. In experiment 2, rats were injected daily for 21 d in with vehicle, galanin (3 nmoles, Tocris), or M40 (6 nmoles) starting at 12-3 pm. Repeated injections were used to

study the trophic effects of galanin that presumably result after several weeks of exercise (19, 20). Microinjections were administered in a volume of 5 µL using 27G needles extending 1 mm beyond the guide cannulae. Doses of galanin and M40 were chosen based on behavioral effectiveness in rodents (36-38).

Footshock stress in the open field

Rats were exposed to footshock or no shock during *in vivo* microdialysis in an open field (ENV-520, -4145, SOF-810; Med Associates, St. Albans, VT). Footshock consisted of 20 shocks across 20 min (1 mA, 0.5 s long, 60 s ITI) and was delivered through the grid floor in the open field. Rats were placed in the open field and ambulatory distance was recorded before (60 min), during (20 min) and after footshock (40 min). A dialysis sample was collected every 20 min. In Experiment 1, an ICV microinjection was also administered 20 min before exposure to footshock or no shock.

Elevated plus maze

Rats were placed in the center of a '+' shaped maze with a pair of open arms (45 x 9 cm) and closed arms (45 x 9 x 38 cm) that met in a central platform (9 x 9 cm) which stood 50 cm from the floor, as described previously (20). Time spent and entries on the arms was recorded for 5 min and analyzed from video by an experimenter blind to treatment.

Microdialysis

The night before open field activity, stylets were removed from the cannulae in the frontal cortex and replaced with a dialysis probe (MAB 9.6.2, PES membrane of 2 mm, .6 mm OD, 6 kDa cutoff; SciPro, Sanborn, NY). Rats were returned to their homecages and tested in microdialysis experiments the next day. Rats were connected to PE50 tubing and freely explored an open field while aCSF (Sigma Chemicals, St. Louis, MO) was delivered through the

in-line at 1.5µL/min. Dialysate was transferred from the out-line every 20 min into sterile microcentrifuge vials filled with DHBA (10% sample volume, 0.08 ng/uL) and 0.1% phosphoric acid. Vials were placed in a dark container on ice, and stored at -80°C until use. Samples were analyzed by HPLC with electrochemical detection using a system with ESA 584 pumps (ESA, Chelmsfor, MA) with a pre-column filter (Synergi Max-RP4u Security Guard, 150 x 4.6 mm, Phenomenex Inc., Torrance, CA) and Max-RP cartridges (Phenomenex).

Brain harvesting and tissue sectioning for enzyme-linked immunosorbent assay (ELISA) and in situ hybridization (ISH)

Rats were microinjected with Fast Green dye (2 µL/mL; 1-4 µL) and decapitated directly after behavioral testing (Day 21 or 23). Brains were sectioned coronally (12 µm; Microm, Waldorf, Germany) to verify cannulae placement, which was considered on target by dye in the ventricles and correct cannulae track placement (35). Example cannulae tracks are shown in Fig. 5.8. In Experiment 1, brains were sectioned into hemispheres. One hemisphere was used for ELISA and the other for in ISH in a counterbalanced manner. Tissue was frozen using dry ice and stored at -80ºC. The hemisphere for ISH was sectioned sagittally (12 µm) and mounted on gelatin-coated slides. In Experiment 2, the frontal cortex was dissected (~5x13x9 mm of tissue), processed for Golgi, sectioned coronally (150µm), and slide-mounted.

Enzyme-Linked Immunosorbent Assay (ELISA)

Tissue from the dorsal pons and ventral midbrain was weighed after freezing in isopentane chilled with dry ice, and protein was extracted as we previously described (Primeaux and Holmes, 2000). Tissue was put in test tubes containing buffer (250 µL of 2.5% aprotinin in .5M acetic acid), homogenized (15s; PowerGen 125, Fisher Scientific), heated (100ºC for 10 min), and centrifuged (30 min at 4°C, 3000 rpm at 1500xg; Beckman Model TJ-6). Supernatant was poured off into separate tubes, evaporated in a vacuum-sealed concentrator (18 hr at 60°C

and 20,000mm Hg; Labconco Centrivap), and reconstituted in 250µL buffer. Tissue was processed according to the manufacturer's instructions for the Galanin Rat ELISA kit (S1208, Pennisula Laboratories, San Carlos, CA). Wells were read at 450 nm (MiniReader MR590, Dynatech Instruments Inc., Santa Monica, CA), averaged across duplicates, and a curve of best fit was used to calibrate to standards. Data are reported as galanin protein in ng/mL per mg tissue.

In Situ Hybridization (ISH) and Densitometry

Tissue was fixed (4% formaldehyde in.12M PBS), dehydrated (0.25% acetic anhydride in 0.1M triethanolamine HCl and 0.9% NaCl, followed by 70-100% EtOH washes), and delipidated in chloroform (Murray *et al*, 2010; Sciolino *et al*, 2012). An oligonucleotide probe (Human prepro-galanin: 5'-G AAG GTA GCC AGC GCT GTT CAG GGT CCA GCC TCT CTT CTC CTT T - 3'; Oligos etc, Wilsonville, OR) was labeled at the 3' end using ^{35}S-dATP (1 mCi; Perkin Elmer, Boston, MA), tailing buffer, $CoCl_2$, and terminal deoxynucleotransferase (Roche, Indianapolis, IN). Unbound radionucleotide was removed using column separation (Micro Bio-Spin P30 in Tris, Bio-Rad, Hercules, CA) by centrifugation (4000 rpm at 1000xg for 4min; Micro-12, Separation Tech Inc, Sanford, FL) and bound radionucleotide was stabilized using 1M dithiothreitol. Sections were covered with radiolabeled probe in hybridization buffer (25% formamide, 72 mMNaCl, 3.2 mM Tris HCl, 0.0032 mM EDTA, 0.001% sodium pyrophosphate, .004% sodium dodecylsulfate, .002mg/mL heparan sulfate, and 2% dextran sulfate) and incubated for 24hr at 37ºC. Sections were washed in 1%SSC and 2%SSC-formamide (50:50) series at 40ºC and room temperature, distilled H_2O, and EtOH. Sections were dried and opposed to ^{35}S-sensitive film (Kodak BioMax MR, Rochester, NY) for 14 d. Films were developed in Kodak GBX fixer and developer. Images were captured on a light table (Northern Light D95, Imaging Research Inc., Piscataway, NJ) and digital camera (Nikon D5000, Micro-NIKKOR 55mmf/2.8 lens, Melville, NY) under optimized conditions. The locus coeruleus was

traced in NIH ImageJ (http://rsb.info.nih.gov/ij/). Mean grayscale was measured in 2-4 sections/rat.

High Performance Liquid Chromatography

Samples were thawed and injected into a system of ESA 584 pumps (ESA, Chelmsfor, MA) with a pre-column filter (Synergi Max-RP4u Security Guard, 150x4.6mm, Phenomenex Inc., Torrance, CA) and Max-RP cartridges (Phenomenex), as we performed previously (Masini *et al*, 2004). Mobile phase was delivered at 1mL/min and contained 100mM sodium phosphate monobasic (Fisher), 0.1mM EDTA (Sigma), 0.25mM octanesulfonic acid (Sigma), and 5% acetonitrile (JT Baker). Samples and standards were injected at 20µL using ESA 542 at 4ºC. Peaks were detected over 30 min using ESA CoulArray (-150 and 200 mV on the initial and final electrodes). Position and height of dopamine peaks were compared to standards (Sigma; diluted in aCSF). Standards were run in duplicate/12 samples. Dopamine detection limit was 13.7 nmol/mL. Peak chromatogram area was integrated and analyzed by CoulArray 3.05.

Golgi impregnation

Tissue from a subset of rats was impregnated according to Rapid GolgiStain Kit instructions (FD NeuroTechnologies, Columbia MD), sectioned and mounted as described above, counterstained in 1% Neutral Red, and coverslipped. Neurons were selected and imaged if they met the following criteria: layer V pyramidal neuron of the prelimbic/infralimbic region between 4.7 to 2.2 mm from bregma (35), completely impregnated, not truncated, and with projections distinct and separate from other neurons as described previously (39). Images were obtained on a Zeiss AxioImager M2 microscope in z-stacks for later measurement in Reconstruct (http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm). Spines were imaged at 100x in secondary and tertiary dendrites in 5-7 neurons per rat, wherein 25 enriched 10 µm segments were measured in apical and basal portions, with care to sample equally

across neurons. Five neurons per rat were also imaged at 40x to measure dendritic length and bifurcation number. Data were averaged across cells and group.

Statistics

Repeated measures analysis of variance (ANOVA) was used to examine weight, wheel running, and open field activity across time. t-tests were used to assess whether exercise and sedentary rats differed in behavior under non-shock conditions. Univariate ANOVA followed by LSD post hoc tests was used to examine behavior, dopamine, and dendritic structure. Dopamine is reported in nmol/mL for descriptive purposes, and analyzed as percent change after stress (average analyte at post-stress time points) / (lowest analyte at baseline) X 100) to reduce within-subject variability and enhance statistical power. Significance was $p<0.05$ for all analyses.

Results

Experiment 1. Exercise increases galanin and confers behavioral resilience to stress

Exercise increases galanin in the locus coeruleus. Exercise increased galanin protein in the dorsal pons ($t_6= -4.51$, $p<0.01$), with no effect in the ventral midbrain compared to sedentary counterparts under non-stress conditions ($p>.05$; Fig. 5.1A). Exercise also increased galanin mRNA in the LC compared to sedentary counterparts ($t_{18}= -2.37$, $p<0.05$; Fig. 5.1B-C).

Voluntary running increased across time, and reduced body weight. Wheel running distance increased over time in rats microinjected with vehicle before exposure to no shock ($F_{1,3}=48.97$, $p<0.01$; Fig. 5.5A). Average running distance peaked on day 20 at 4.24±1.58 km. Body weight increased linearly across time in rats exposed to no shock ($F_{1,8}=316.90$, $p<0.01$; Fig. 5.6A). The interaction of time by group was significant ($F_{1,8}=15.39$, $p<0.01$), and there was a trend for a group effect ($F_{1,8}=4.59$, $p=0.08$; Fig. 5.6A). Follow-up tests showed that exercise

and sedentary groups were not different in body weight on day 1 ($p=0.80$), but exercise rats exhibited reduced body weight on experimental day 7 ($p<0.05$), 14 ($p=0.06$), and 21 ($p<0.05$; Fig. 5.6A).

Wheel running also increased linearly over time in rats that were microinjected before footshock exposure ($F_{1,12}=20.56$, $p<0.01$; Fig. 5.5B). The interaction of time by group ($F_{1,12}=1.28$, $p=0.28$) and group effect ($F_{1,12}=0.00$, $p=0.96$; Fig. 5.5B) was not significant. Running peaked on day 20 at 2.07±0.25 km. Body weight increased linearly across time in rats exposed to footshock ($F_{1,19}=214.89$, $p=0.00$; Fig. 5.6B). There was an interaction of group by time ($F_{2,19}=5.45$, $p<0.01$) and group ($F_{2,19}=5.00$, $p<.05$; Fig. 5.6B). Groups were not different in weight on day 1 ($F_{2,19}=1.17$, $p=0.33$), but were different on day 7 ($F_{2,19}=4.07$, $p<0.05$), 14 ($F_{2,19}=7.26$, $p<0.01$), and 21 ($F_{2,19}=5.06$, $p<0.05$; Fig. 5.6B). Exercise reduced body weight on days 7 ($p<0.01$), 14 ($p<0.01$), and 21 ($p<0.05$; Fig. 5.6B) compared to sedentary control. Vehicle- and M40- treated exercise rats were no different in weight at any time ($p>0.05$; Fig. 5.6B).

Sedentary rats are susceptible to stress-induced changes in behavior. Behavior in anxiety-related tests was measured in sedentary and exercised rats given an acute microinjection before exposure to footshock or no shock, as shown in the timeline (Fig. 5.2A). Under non-stress conditions exercised and sedentary rodents are not reliably different in spontaneous tests of anxiety (17). Based on this literature and to avoid a type II error, behavior was analyzed separately for rats exposed to shock and no shock. Under non-shock conditions, exercise rats were not different on any measure in the elevated plus maze from sedentary counterparts administered vehicle ($p>0.05$; Fig. 5.2B-C). Ambulatory distance in the open field measured on the previous day increased linearly across time in rats exposed to no shock ($F_{1,8}=8.51$, $p<0.05$; Fig. 5.2D). The time by group interaction ($F_{1,8}=0.11$, $p=0.75$) and group effect ($F_{1,8}=0.65$, $p=0.44$; Fig. 5.2D) was not significant.

Percent open arm time in the elevated plus maze differed across groups after footshock ($F_{2,18}$=6.72, p<0.01; Fig. 5.2E). Exercise rats treated with acute vehicle- (p<0.01) or M40 (p<0.05; Fig. 5.2E) exhibited increased percent open arm time relative to vehicle-treated sedentary rats. Vehicle- and M40-treated exercise rats were no different in percent open arm time (p=0.62; Fig. 5.2E). Open arm entries also differed across groups after footshock ($F_{2,18}$=10.86, p<0.01; Fig. 5.2F). Acute vehicle- (p<0.01) and M40-treated exercise rats (p<0.01; Fig. 5.2F) exhibited a greater number of open arm entries relative to vehicle-treated sedentary rats. Vehicle- and M40-treated exercise rats were no different in the number of open arm entries (p=0.67; Fig. 5.2F). The number of closed arm entries and falls off the maze were no different in these groups after exercise, shock, or drug manipulation (p>0.05). Ambulatory distance in the open field increased in a quadratic manner across time in rats exposed to shock ($F_{1,21}$=94.92, p<0.01; Fig. 5.2G). Ambulation increased after the ICV injection compared to baseline (p<0.01; Fig. 5.2G). Post-shock ambulation decreased relative to after the ICV injection (p<0.01), and was comparable to baseline ambulation (p=0.47; Fig. 5.2G). The time by group interaction ($F_{2,21}$=0.26, p=0.77) and group effect ($F_{2,21}$=0.89, p=0.43; Fig. 5.2G) was not significant for ambulatory distance.

Experiment 2. Galanin is necessary and sufficient for behavioral resilience and prevents stress-evoked dopamine overflow and dendritic spine loss in the cortex.

Running increased across time, but body weight was not influenced by exercise. Wheel running distance increased linearly over time in rats receiving repeated microinjections and shock exposure ($F_{1,21}$=11.30, p<0.01; Fig. 5.5C). The time by group interaction ($F_{1,21}$=0.74, p=0.41) and group effect ($F_{1,17}$=0.73, p=0.41; Fig. 5.5C) was not significant. Average running on experimental day 20 was 0.84±0.12 km. Body weight increased linearly over time ($F_{1,41}$=67.93, p<0.01), but there was no significant effect of group assignment ($F_{4,41}$=0.62, p=0.65; Fig. 5.6C). Although the interaction of time by group was significant ($F_{4,41}$=6.69, p<0.01), follow-up

univariate ANOVAs revealed no significant group difference in body weight at each time point ($p>0.05$; Fig. 5.6C).

Exercise-induced resilience is mimicked by chronic ICV galanin administration and dependent on galanin receptors. Behavior in anxiety-related tests was measured after footshock exposure in sedentary rats repeatedly microinjected with vehicle or galanin, and exercise rats given vehicle or M40, as shown in the timeline (Fig. 5.3A). Percent open time in the elevated plus maze differed across these footshock-exposed groups ($F_{3,37}=11.21$, $p<0.01$; Fig. 5.3B). Galanin-treated sedentary and vehicle-treated exercise rats exhibited increased percent open arm time relative to vehicle-treated sedentary ($p<0.05$) and M40-treated exercise rats ($p<0.05$; Fig. 5.3B). Open arm entries also differed across groups ($F_{3,38}=4.77$, $p<0.01$; Fig. 5.3C). Galanin-treated sedentary and vehicle-treated exercise rats exhibited more open arm entries relative to vehicle-treated sedentary ($p<0.05$) and M40-treated exercise rats ($p<0.05$; Fig. 5.3C). Neither drug nor exercise altered closed arm entries or falls off the maze ($p>0.05$). Footshock increased ambulatory distance in the open field ($F_{1,36}=20.05$, $p<0.01$; Fig. 5.3D). There was a significant time by group interaction ($F_{3,36}=5.72$, $p<0.01$) and group effect ($F_{3,36}=6.75$, $p<0.01$; Fig. 5.3D). Pre-shock ambulatory distance was no different across groups ($F_{3,36}=0.43$, $p=0.73$), but post-shock distance was different ($F_{3,36}=8.00$, $p<0.01$; Fig. 5.3D). Vehicle-treated exercise rats exhibited reduced ambulatory distance after footshock compared to M40-treated exercise rats ($p<0.05$) and vehicle-treated sedentary rats ($p<0.05$; one-tailed; Fig. 5.3D). Galanin-treated sedentary rats exhibited increased ambulatory distance after footshock compared to all groups ($p<0.05$; Fig. 5.3D).

Chronic ICV galanin administration, like exercise, blocks the increase in cortical dopamine after stress. Dopamine in the frontal cortex was measured before and after footshock, as shown in the timeline (Fig. 5.3A). Baseline dopamine levels were no different

between groups (sedentary-vehicle 14.18±23.68 nmol/mL, sedentary-galanin 23.31±25.34 nmol/mL, exercise-vehicle 24.28±23.09 nmol/ml, exercise-M40 19.96±13.10 nmol/ml; *p*>0.05). Footshock increased dopamine levels compared to baseline (Pre-shock; $F_{1,29}$=2.76, p=.05, one-tailed; Fig. 5.3E). Shock increased dopamine overflow in vehicle-treated sedentary rats (t_8=-3.91, p<0.01), but not galanin-treated sedentary (t_9=-1.31, p=0.22), vehicle-treated exercise (t_7=0.85, p=0.43), or M40-treated exercise rats (t_5=0.15, p=0.89; Fig. 5.3E). There was also group by time interaction effect ($F_{3,29}$=4.39, p<.01) and overall effect of group assignment ($F_{3,29}$=6.51, p <.01; Fig. 5.3E). Groups were no different at the pre-shock timepoint ($F_{3,29}$=0.33, p =.81), but differed post-shock ($F_{3,29}$=6.65, p <.01; Fig. 5.3E). Galanin-treated sedentary (p<0.05), vehicle-treated exercise (p<0.01), and M40-treated exercise rats (p<0.01; Fig. 5.3E) exhibited reduced dopamine overflow compared to vehicle-treated sedentary rats at the post-shock timepoint. Vehicle-treated exercise rats also exhibited reduced dopamine levels post-shock compared to galanin-treated sedentary rats (p<0.05; Fig. 5.3E). However, vehicle- and M40-treated exercise rats were no different in dopamine levels post-shock (p=0.52; Fig. 5.3E). Probe placement is shown in Fig. 5.3F.

Exercise and chronic ICV galanin administration both prevent stress-induced dendritic spine loss in mPFC neurons. Dendritic markers of plasticity were measured in Golgi-processed tissue obtained from a subset of rats exposed to footshock or no-shock (see Fig. 5.3A). Neurons were sampled from select regions of the mPFC (Fig. 5.4A), and spine density was measured in apical and basal portions of pyramidal neurons (Fig. 5.4B). No differences were observed in bifurcation number and total dendritic length after exercise, drug, or shock manipulations (p>0.05; Fig. 5.7).

Apical spine density was different across groups ($F_{3,21}$=7.68, p<0.01; Fig. 5.4C). Footshock- exposed, vehicle-treated sedentary rats exhibited fewer number of apical spines

compared to non-shock counterparts ($p<0.05$; Fig. 5.4C). Footshock-exposed, galanin-treated sedentary ($p<0.05$) and vehicle-treated exercise rats ($p<0.01$; Fig. 5.4C) exhibited a greater number of apical spines compared to vehicle-treated sedentary counterparts. Footshock-exposed, vehicle-treated exercise rats also exhibited a greater number of apical spines compared to non-shock sedentary counterparts ($p<0.05$; Fig. 5.4C). Representative apical segments are shown in Fig. 5.4D.

Basal spine density differed across groups ($F_{3,21}= 4.77$, $p<0.01$; Fig. 5.4D). The number of basal spines was not statistically different in footshock exposed vehicle-treated sedentary rats compared to non-shock counterparts ($p=0.23$; Fig. 5.4D). Footshock-exposed, galanin-treated sedentary ($p<0.05$) and vehicle-treated exercise rats ($p<0.01$; Fig. 5.4D) exhibited a greater number of basal spines compared to vehicle-treated sedentary counterparts. Footshock-exposed, vehicle-treated exercise rats exhibited a greater number of basal spines compared to non-shock sedentary counterparts ($p<0.05$; Fig. 5.4D). Representative basal segments are shown in Fig. 5.4F.

Discussion

In the present study, we used galanin pharmacology in conjunction with an exercise model known to endogenously increase galanin expression in the LC (19-21). We showed that these manipulations protect against the behavioral, neurochemical, and neuroanatomical impact of stress. We found that galanin is necessary and sufficient for stress resilience in behavioral tests of affect. Our study is the first to report that long-term galanin treatment for 3 weeks reduces anxiety-related behaviors and mimics the stress protection afforded by exercise. Galanin is essential for the behavioral benefits of exercise observed in these experiments. The galanin receptor antagonist reversed the stress protection produced by exercise after repeated ICV administration, but not when administered acutely and immediately before stress. We also found that exercise and galanin treatment both protect the cortex against stress-induced

perturbation of dopamine and dendritic structure. Combined, our data indicate that stress-protective features of exercise are mediated by galanin.

Exercise and galanin treatment diminished the behavioral impact of stress in a manner better characterized as resilience rather than resistance to stress. Exercised and galanin-treated rats reacted to footshock (e.g., vocalized, defecated, and urinated during shock), and responded to stress initially by altered locomotor behavior in the open field. However, these groups of rats also exhibited reduced anxiety-like behavior the day following stress. These results are likely attributable to resilience, rather than anti-anxiety effects per se, because exercise and galanin groups differed from controls in anxiety-related behaviors after, but not before stress. Moreover, neither exercise nor galanin groups differed in general locomotor activity in the present report (e.g., closed arm entries in plus maze, baseline ambulation in open field) or shock reactivity (20, 40), suggesting that differences in locomotor activity or nociception are not a factor. Our data are in agreement with literature showing exercise attenuates anxiety and depression in humans (41), and evidence indicating that galanin buffers the behavioral impact of stress (23). Our data extend these results and indicate that galanin is necessary for the behavioral resilience afforded by exercise.

Increased dopamine in the frontal cortex is a reliable neurochemical signature of acute stress (42-44), which we observed to be attenuated by manipulations promoting resilience (e.g., exercise and galanin treatment). Galanin administration may blunt stress-induced dopamine release through direct influences on VTA electrophysiological activity (30, 31). In the present study, we failed to observe a difference in evoked dopamine release between M40 and vehicle-treated exercise rats. Our data therefore suggest that the behavioral resilience afforded by exercise is independent of dopaminergic signaling, yet dependent on galanin signaling in the brain.

We also show that both galanin and exercise protect against stress-induced spine loss in the mPFC. We observed an effect of stress in the apical segment of dendritic arbors but not in the basal segment in vehicle-treated rats, which is consistent with literature reporting apical segments are particularly responsive to stress (9). The molecular mechanisms that underlie the change in dendritic morphology caused by stress, and the protection provided by manipulations affording resilience, are beginning to be elucidated. Stress-related depletion of dendritic spines in the hippocampus involves activation of RhoA (45). Conversely, galanin may maintain dendritic spines by differentially regulating RhoA. Recent evidence supporting this hypothesis shows that galanin stimulates neurite outgrowth in primary sensory neurons through GalR2-mediated inhibition of RhoA and subsequent cofilin activation (46). GalR2-signaling may also stabilize neurites via maintenance of microtubule integrity by promoting aggregation of MAP2 and β-tubulin (47), a process that may also involve inhibition of RhoA (48).

The present study is the first to show that galanin is both necessary and sufficient for the behavioral resilience afforded by exercise. Exercise also increases neurogenesis and the expression of other neurotrophic factors (i.e., BDNF, VGF) (49), which also contribute to the behavioral effects of exercise. The lack of research regarding the interplay of these signals represents a major gap in understanding how gene-environment interactions promote resilience, and this area deserves further study. Broadly, our data implicate a neurotrophic basis for resilience and suggest further that galanin plays a key role in resilience. Disruption in the galanin system may tip the balance towards stress-susceptibility. Indeed, variants in genes for galanin and its receptors (GalR1-3) confer risk for anxiety and depression in people exposed to stress, and are better than other well-studied polymorphisms in predicting stress-related neuropsychiatry (50). Thus, galanin, like exercise, may offer therapeutic potential, especially in susceptible individuals that suffer from stress-related mental disorders.

Figure 5.1

Exercise increases galanin in the locus coeruleus.

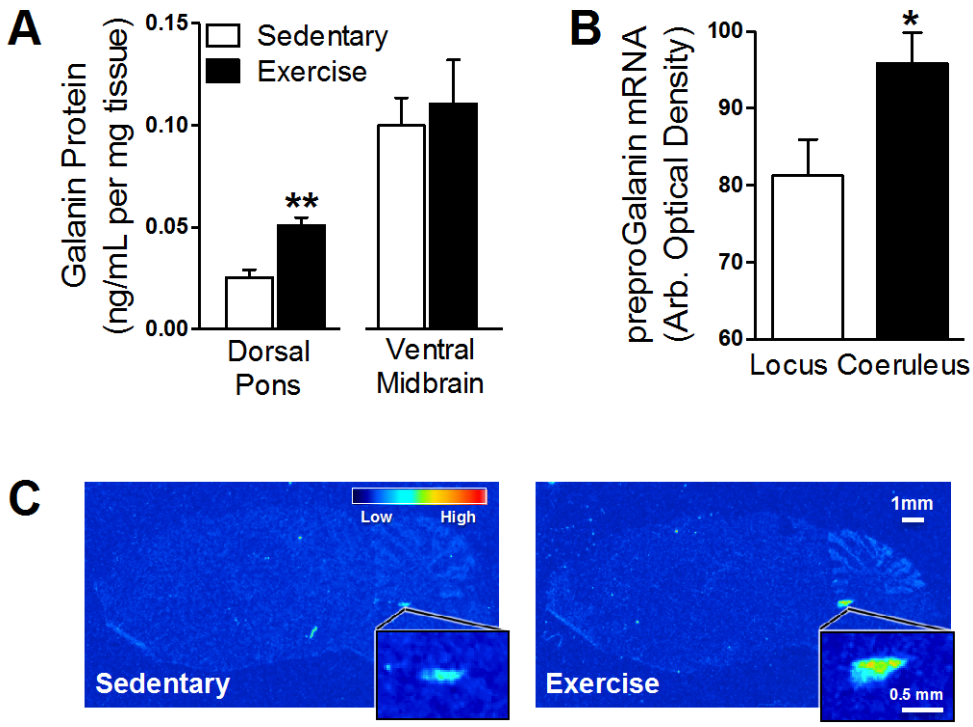

Figure 5.1. Galanin levels were assessed in the brain and measured in rats with access to a running wheel or no wheel for 3 weeks (see Fig. 5. 2). Shown are means ± SEM for (A) galanin protein obtained by ELISA in tissue containing the dorsal pons or ventral midbrain from exercise and sedentary rats (n=4-5) exposed to no shock. Also shown are (B) means ± SEM for galanin mRNA measured by ISH in tissue containing the locus coeruleus (LC) from exercise and sedentary rats (n=10) exposed to footshock, and (C) images of galanin mRNA in representative sagittal brain sections from exercise (top) and sedentary rats (bottom). Spectrum scale shows the intensity of (C) galanin mRNA expression. Insets show (C) galanin mRNA in the LC. *$p<0.05$ compared to sedentary.

Figure 5.2

Exercise produces resilience to stress that is not reversed by acute intracranial administration of M40.

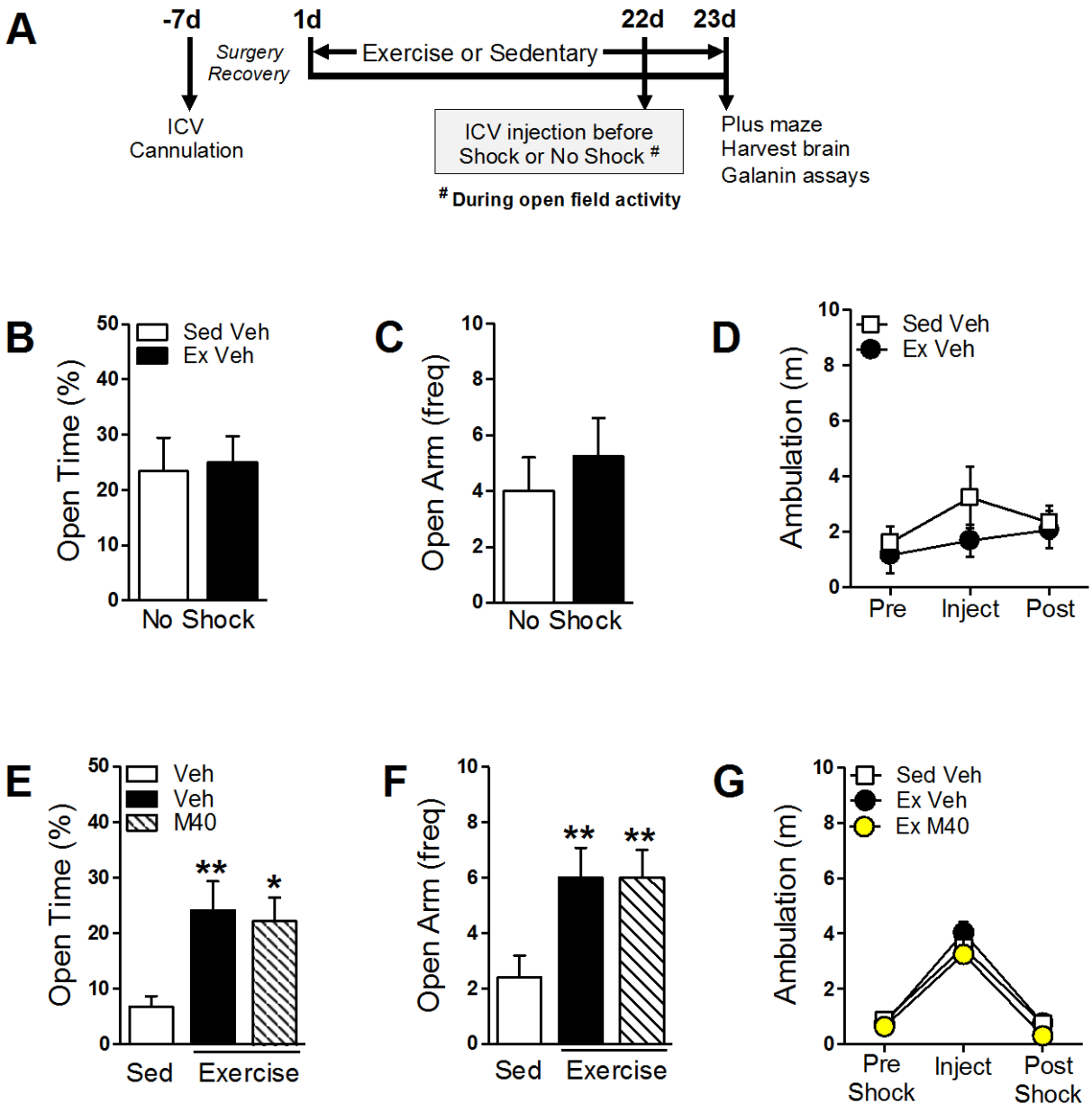

Figure 5.2. Rats with access to a running wheel or no wheel were administered ICV vehicle (dH20) or the galanin receptor antagonist M40 (6 nmol) immediately before exposure to footshock or no-shock during open field activity. Shown are (A) experimental timeline, means ± SEM for (B) open arm time and (C) open arm entries in the elevated plus maze, and (D) ambulatory distance during open field activity from no-shock exposed rats. N = 6 (sed-veh), 4 (ex-veh). Also shown are the means ± SEM for (E) open arm time and (F) open arm entries in the elevated plus maze, and (G) ambulatory distance during open field activity from shock-exposed rats. N = 10 (sed-veh), 9 (ex-veh), and 5 (ex-M40). $^{**}p<0.01$, $^{*}p<0.05$ compared to sedentary. Ex, exercise. Sed, sedentary. Veh, vehicle.

Figure 5.3

Exercise produces behavioral resilience that is mimicked by repeated intracranial galanin administration and reversed by M40.

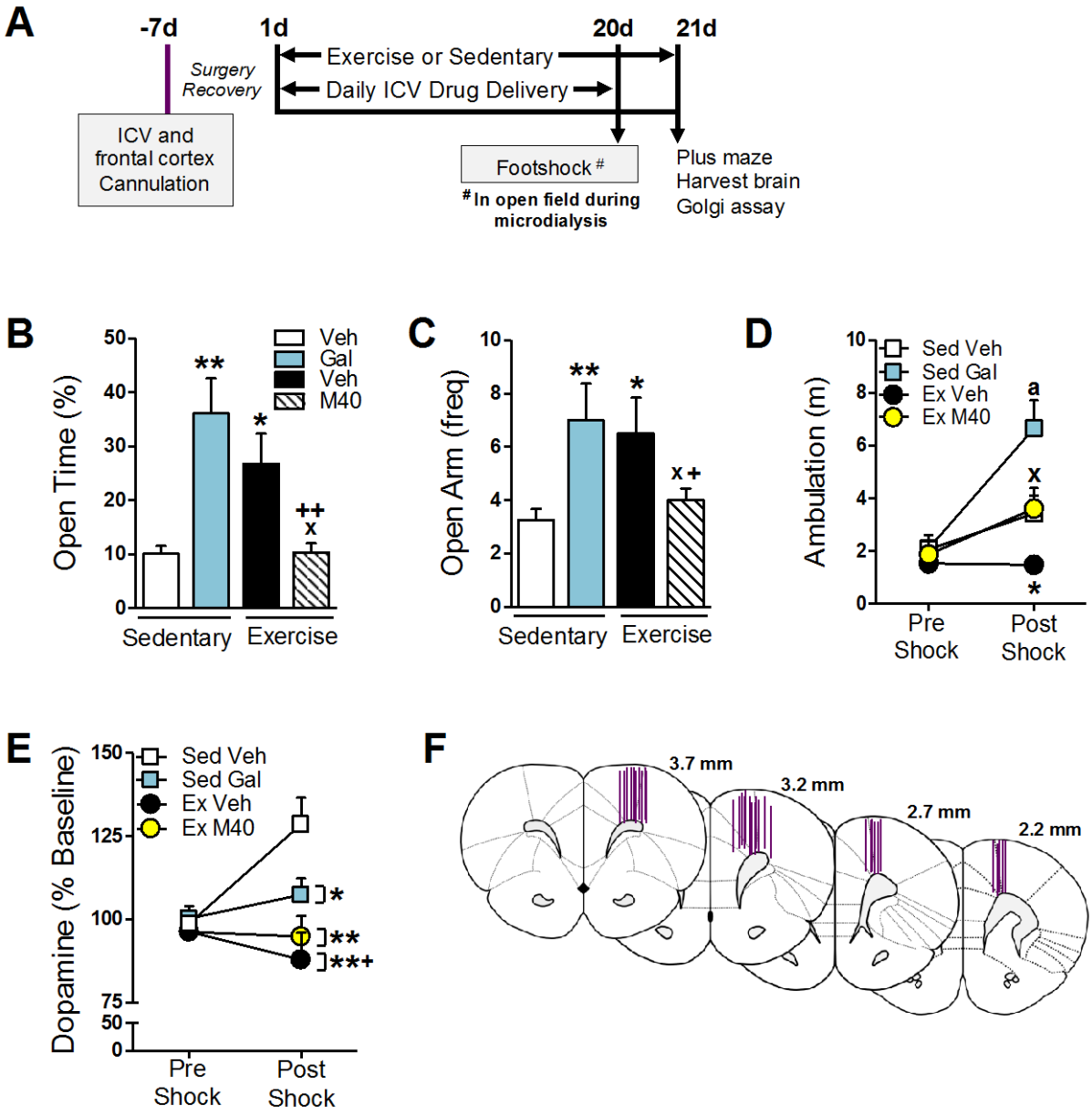

Figure 5.3. Rats received daily administration of ICV vehicle (aCSF), galanin (3 nmol), or galanin receptor antagonist M40 (6 nmol) during exercise or sedentary conditions, and were exposed to footshock during microdialysis in an open field. Shown are (A) experimental timeline, means ± SEM for (B) open arm time and (C) open entries in the elevated plus maze, (D) ambulatory distance during open field activity, (E) cortical dopamine levels, and (F) probe placements. N = 9-16 (vehicle sedentary), 9-10 (galanin sedentary), 8-10 (vehicle exercise), and 5-6 (M40 exercise). $^{**}p<0.01$, $^{*}p<0.05$ compared to vehicle-treated sedentary. $^{++}p<0.01$, $^{+}p<0.05$ compared to galanin-treated sedentary. $^{X}p<.05$ compared to vehicle-treated exercise. $^{a}p<0.05$ vs. all groups. Ex, exercise. Gal, galanin. Sed, sedentary. Veh, vehicle.

Figure 5.4

Repeated intracranial galanin administration mimics exercise and prevents dendritic spine loss in mPFC pyramidal neurons after stress.

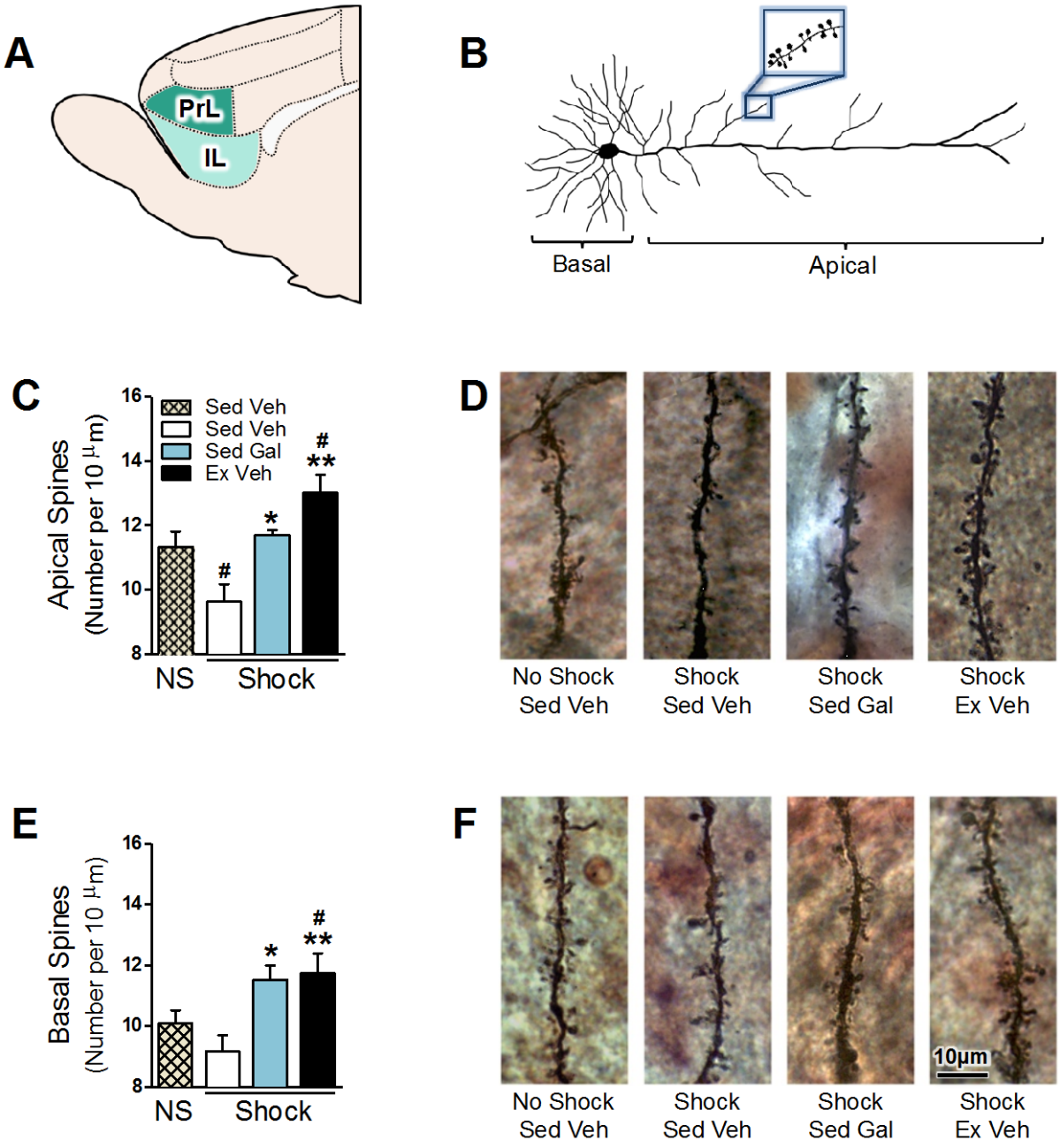

Figure 5.4. Dendritic spine density was measured in mPFC pyramidal neurons in Golgi-processed tissue (imaged at 100x) obtained from a subset of rats exposed to footshock or no-shock (see Fig. 5. 3). Shown are (A) regions of the mPFC where neurons were selected, and (B) pyramidal neuron cartoon showing the apical and basal segments assessed for spine density. Shown also are (C) mean ± SEM for apical spine number, (D) representative apical segments, (E) means ± SEM for basal spine number, and (F) representative basal segments. N = 6 (sed-veh no shock), 7 (sed-veh shock), 3 (sed-gal shock), and 6 (ex-veh shock). #$p<0.05$ compared to no-shock exposed vehicle-treated sedentary. **$p<0.01$, *$p<0.05$ compared to shock exposed vehicle-treated sedentary. Ex, exercise. Gal, galanin. IL, infralimbic. NS, no-shock. PrL, prelimbic. Sed, sedentary. Veh, vehicle

Figure 5.5

Wheel running increased across the experiments.

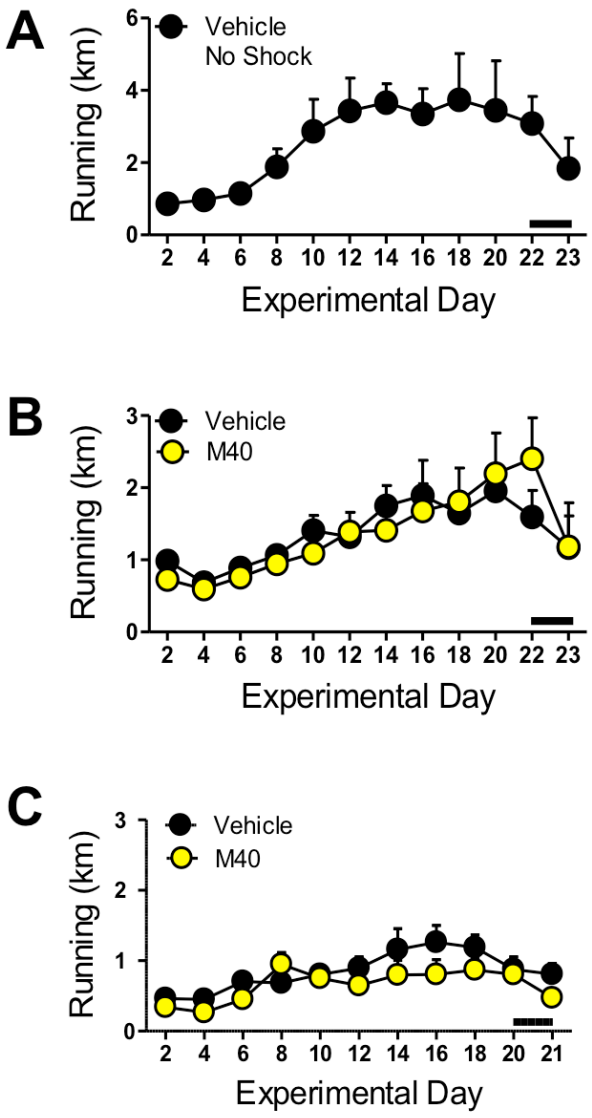

Figure 5.5. Daily wheel running was collected during exercise conditions in (A) experiment 1 from rats (n=4) tested under non-stress conditions and administered acute ICV vehicle, (B) experiment 1 from rats (n=5-9) administered an ICV injection of vehicle or M40 before footshock, and (C) experiment 2 from rats (n=6-16) receiving repeated ICV administration of vehicle or M40 and exposed to footshock. The black bar indicates the days of experimental testing. Veh, vehicle.

Figure 5.6

Weight gain progressed across the experiment.

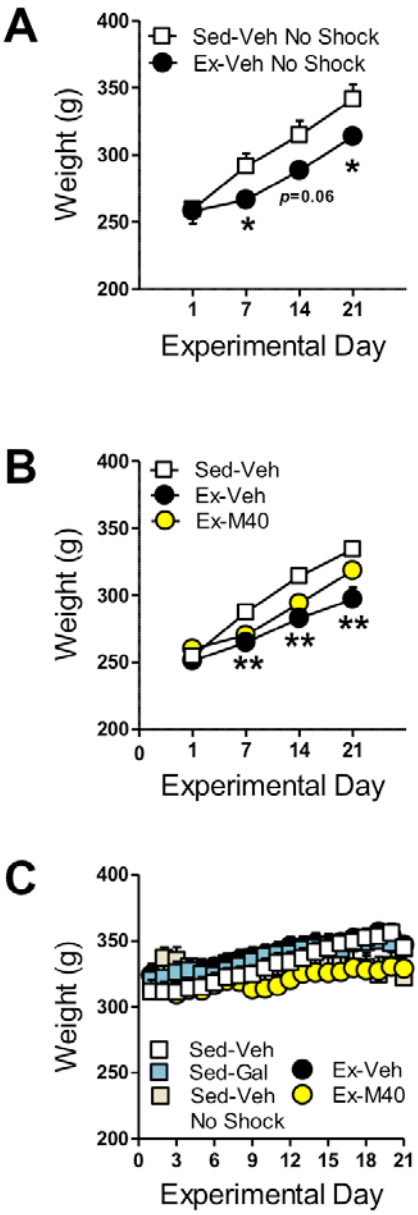

Figure 5.6. Body weight was collected during exercise and sedentary conditions in (A) experiment 1 from rats (n=4) tested under non-stress conditions and administered acute ICV vehicle, (B) experiment 1 from rats (n=5-10) administered an ICV injection of vehicle or M40 before footshock, and (C) experiment 2 from rats (n=6-16) receiving repeated ICV administration of vehicle or M40 and exposed to footshock. $^{**}p<0.01$, $^{*}p<0.05$ compared to sedentary counterpart. Ex, exercise. Gal, galanin. Sed, sedentary. Veh, vehicle.

Figure 5.7

Exercise, galanin, and stress failed to alter both the total dendritic length and number of bifurcations in mPFC pyramidal neurons.

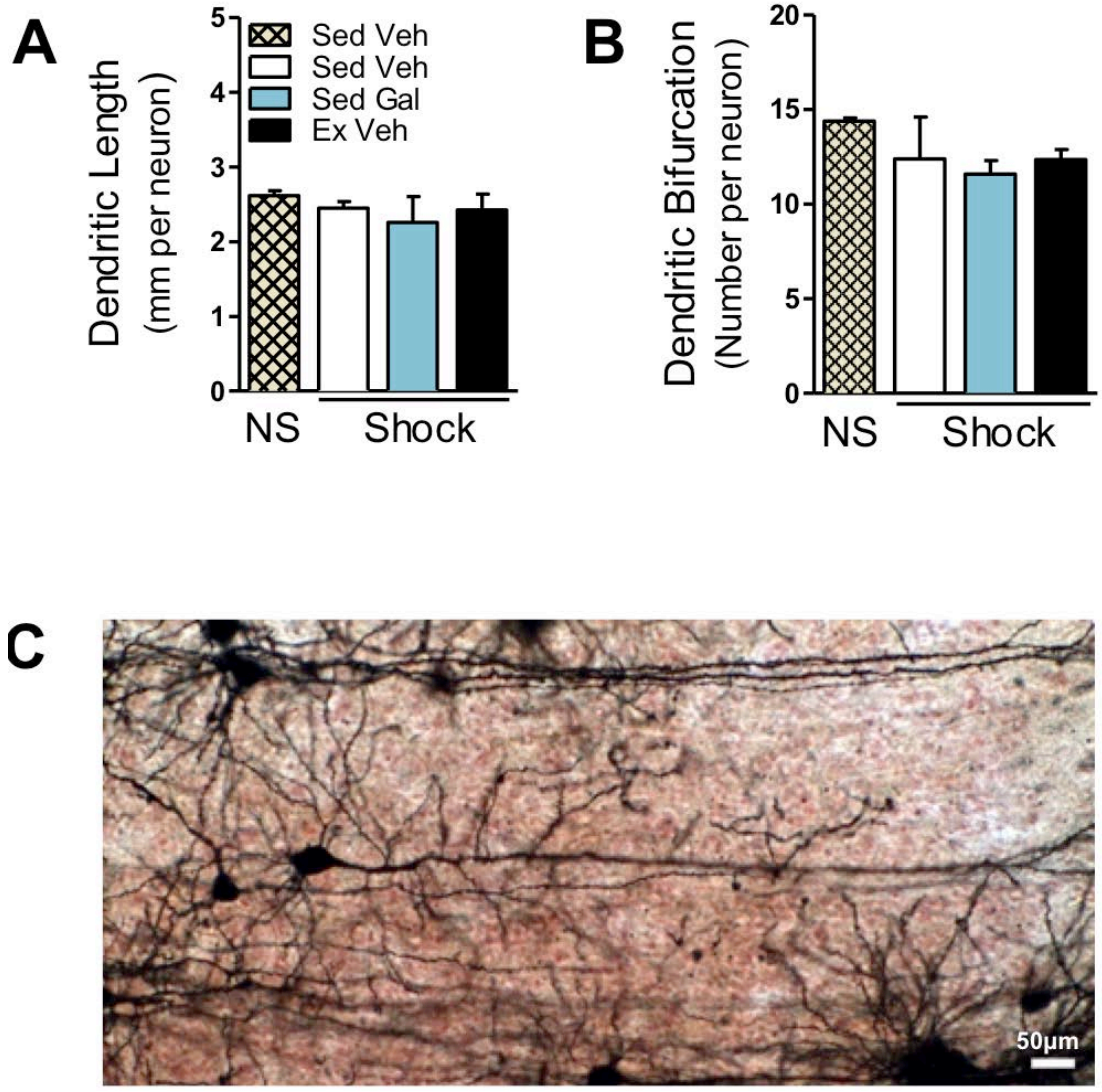

Figure 5.7. Dendritic size and complexity was measured in mPFC pyramidal neurons of layer V in Golgi-processed tissue imaged at 40x and obtained from a subset of exercise and sedentary rats exposed to footshock or no stress during open field activity (see Fig. 5. 3). Shown are (A) means ± SEM for total dendritic length and (B) total dendritic bifurcation, and (C) representative image of a sampled neuron. N = 4 (sed-veh no shock), 4 (ed-veh shock), 3 (sed-gal shock), and 4 (ex-veh shock). Ex, exercise. Gal, galanin. NS, no-shock. Sed, sedentary. Veh, vehicle.

Figure 5.8.

Cannulae placement in thionin-stained sections.

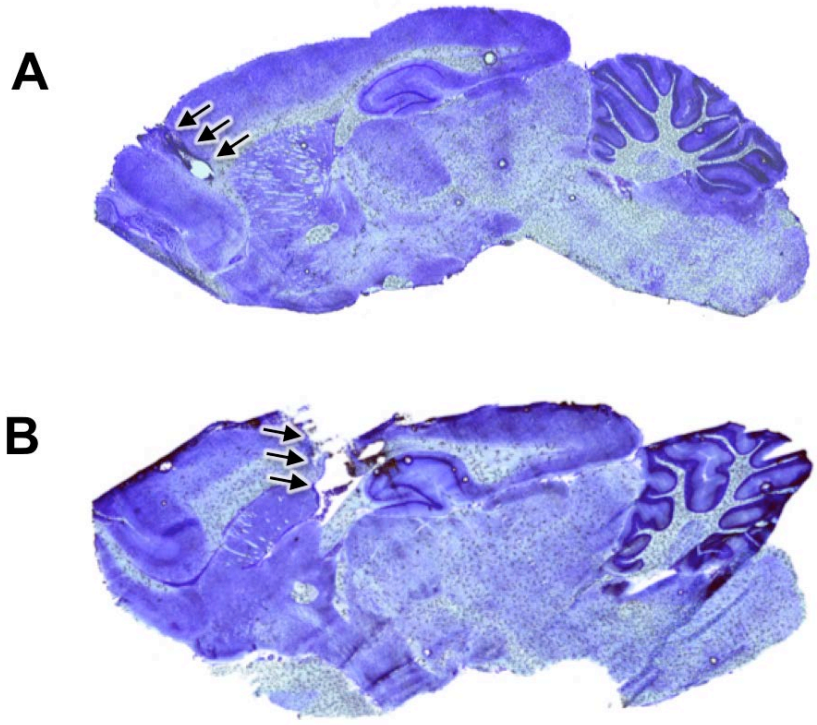

Figure 5.8. Shown are thionin-stained brain sections that show placement of (A) dialysis probes in the frontal cortex, and (B) cannula that targeted the lateral ventricle. Thin arrows indicate the track line.

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CHAPTER 6

GENERAL CONCLUSIONS

The experiments presented herein show that regular voluntary exercise increases galanin levels in the locus coeruleus. Manipulations that promote galanin (e.g., exercise and administration of galanin agonists) protect against stress- and cocaine- induced changes in behavior. These manipulations also protect against stress- and cocaine-induced catecholamine release in the frontal cortex. Similarly, exercise protects against the stress-induced loss of dendritic spines in the mPFC. The behavioral stress resilience afforded by exercise was blocked by repeated, but not acute, intracranial galanin receptor antagonism. These data collectively suggest that galanin is necessary and sufficient for features of resilience incurred by exercise, which may result in part by protecting cortical integrity.

In chapter 3, we showed that a prolonged, voluntary exercise regimen produced anxiolytic- like effects in rats with a history of repeated stress. However, exercise failed to produce these effects in rats tested under non-stress conditions or intense stress elicited by a high dose of a β-carboline. These data support the idea that chronic exercise exerts anxiolytic-potential in a manner that depends on stress. Wheel running increased galanin gene expression in the locus coeruleus, suggesting galanin plays a role in exercise-mediated regulation of stress responsivity. Our data caution against interpreting exercise-induced increases in defensive behavior as anxiogenic, and are consistent with the conclusion that a chronic exercise regimen produces beneficial effects on anxiety.

In chapter 4, we report that galnon reduces reward-seeking behavior during reinstatement and cocaine-induced DA overflow in the frontal cortex. However, further studies are required to define the underlying mechanisms. Galnon is a synthetic non-peptide galanin receptor agonist that crosses the blood brain barrier and has equal binding affinity for GalR1

and GalR2. The modulatory action of galanin on other drug-induced behaviors appears to involve both receptor subtypes. All existing evidence points to a central effect of galanin and galnon, but peripheral galanin receptors cannot be ruled out due to our systemic route of galnon administration. Although further experiments are required to identify the galanin receptor subtype and neuroanatomical substrates involved, the data presented here suggest that the galanin system is a candidate target for anti-relapse therapies.

In Chapter 5, we used galanin pharmacology in conjunction with an exercise model known to endogenously increase galanin expression in the LC. We found that intracranial (ICV) galanin treatment for 3 weeks reduces anxiety-related behaviors and mimics the stress protection afforded by exercise. Intracranial administration of the galanin receptor antagonist M40 for 21 d, but not 1 d, reversed the stress protection produced by exercise. We also found that exercise and galanin treatment both protect the cortex against stress-induced perturbation of dopamine and dendritic structure. Our data suggest that manipulations that promote galanin assist the cortex to respond to stress. Although correlative data demonstrate that exercise increases galanin in the LC in a “dose-dependent” fashion, this study is the first to show that galanin is both necessary and sufficient for stress resilience following exercise.

Collectively, these experiments suggests that exercise or galanin treatment may be therapeutic for susceptible brains predisposed to mental illness, possibly by normalizing mPFC dysfunction. Although our data implicate an important role for galanin in resilience and cortical integrity, such protection likely occurs at multiple levels across the neural axis. Future research is needed to define the signaling pathway and circuits that underlie the protective capacity of galanin. The exercise-sedentary comparison offers promise and power to elucidate the role of neurotrophins in health and disease.