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Galanin mediates features of neural and behavioral stress resilience afforded by exercise

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ABSTRACT

Exercise promotes resilience to stress and increases galanin in the locus coeruleus (LC), but the question of whether changes in galanin signaling mediate the stress-buffering effects of exercise has never been addressed. To test the contributions of galanin to stress resilience, male Sprague Dawley rats received intracerebroventricular (ICV) cannulation for drug delivery and frontocortical cannulation for microdialysis, and were housed with or without a running wheel for 21d. Rats were acutely injected with vehicle or the galanin receptor antagonist M40 and exposed to a single session of either footshock or no stress. Other groups received galanin, the galanin receptor antagonist M40, or vehicle chronically for 21d prior to the stress session. Microdialysis sampling occurred during stress exposure and anxiety-related behavior was measured on the following day in the elevated plus maze. Dendritic spines were visualized by Golgi impregnation in medial prefrontal cortex (mPFC) pyramidal neurons and quantified. Exercise increased galanin levels in the LC. Under non-stressed conditions, anxiety-related behavior and dopamine levels were comparable between exercised and sedentary rats. In contrast, exposure to stress reduced open arm exploration in sedentary rats but not in exercise rats or those treated chronically with ICV galanin, indicating improved resilience. Both exercise and chronic, ICV galanin prevented the increased dopamine overflow and loss of dendritic spines observed after stress in sedentary rats. Chronic, but not acute M40 administration blocked the resilience-promoting effects of exercise. The results indicate that increased galanin levels promote features of resilience at both behavioral and neural levels.

1. Introduction

Stress and adversity are risk factors for mental illness, but the neural mechanisms underlying stress susceptibility and resilience are not well understood (Franklin et al., 2012). Resilience involves adaptive recovery following adverse experiences, and resilient individuals experience fewer deleterious outcomes following stress. The medial prefrontal cortex (mPFC) is particularly sensitive to stress, both in terms of its function and structure (Arnsten, 2009; McEwen and Morrison, 2013), and also contributes to stress resilience (Maier and Watkins, 2010). mPFC activity is sufficient and necessary for many aspects of behavioral resilience. For example, activation of the mPFC induces resilience in behavioral tests of anxiety (Amat et al., 2005; Covington et al., 2010). Inactivation of the mPFC also reverses the anxiolytic effects of environmental enrichment (Lehmann and Herkenham, 2011) and protective effect of behavioral control over stress (Amat et al., 2005), although not all forms of resilience are PFC-dependent (Greenwood et al., 2013). Dendritic spines, the morphological hallmark of excitatory synapses, are reduced in the mPFC after stress (Leuner and Shors, 2012;

Abbreviations: ANOVA, Analysis of variance; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; ISH, in situ hybridization; ICV, intracerebroventricular; LC, locus coeruleus; mPFC, medial prefrontal cortex; VTA, ventral tegmental area.

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Radley and Morrison, 2005), whereas antidepressants (Bessa et al., 2009) or intracranial self-stimulation (Ramkumar et al., 2011) prevent stress-induced alterations in dendritic structure. Consistent with these data, clinical literature shows that resilient individuals exhibit greater mPFC activation (New et al., 2009; Peres et al., 2011), while susceptible individuals exhibit impaired mPFC-to-amygdala functional connectivity (Bremner et al., 1999; Tromp et al., 2012).

Exercise promotes behavioral resilience (Sciolino and Holmes, 2012) and reliably increases the neuropeptide galanin in noradrenergic neurons of the locus coeruleus (LC) (Holmes et al., 2006; Sciolino et al., 2012; Van Hoomissen et al., 2004). Galanin receptors (GalR1-3) are expressed throughout stress-responsive circuits, including the LC, dorsal raphe, ventral tegmental area (VTA), hypothalamus, hippocampus, amygdala, and mPFC (Hawes and Picciotto, 2004). Galanin contributes to stress resilience in behavioral tests of anxiety (Karlsson and Holmes, 2006), and galanin signaling is necessary for behavioral effects of antidepressants in animal models (Lu et al., 2005). Microarray profiling also shows that galanin expression distinguishes resilient and susceptible phenotypes (Krishnan et al., 2007). Although widely recognized for its neuromodulatory actions, galanin also has potent neurotrophic activity (Abbosh et al., 2011; Cordero-Llana et al., 2014; Hobson et al., 2008) and may alter the plasticity of neurons in regions that mediate resilience. Galanin may modulate catecholamine transmission in stress-responsive neural pathways (Holmes and Picciotto, 2006), either locally within the LC or in distal targets like the VTA or mPFC. Phasic stimulation of VTA dopamine neurons induces susceptible behavior and rapidly reverses resilience (Chaudhury et al., 2013). In contrast, galanin inhibits midbrain dopamine activity (Counts et al., 2002; Weiss et al., 1998) and generally reduces dopamine release (Jansson et al., 1989; Nordstrom et al., 1987; Tsuda et al., 1998). Thus, galanin signaling is poised to regulate resilience through multiple mechanisms throughout stress-responsive circuitry.

In the current studies, we predicted that increasing galanin levels by wheel running or repeated intracranial galanin administration would produce behavioral resilience in tests of anxiety, while chronic galanin receptor blockade would attenuate the stress-buffering effects of exercise. Based on the recognized role of mPFC dopamine as a synaptic marker of stress exposure, we also hypothesized that exercise or galanin administration would prevent stress-induced increases in dopamine overflow. Finally, we predicted that stress will reduce dendritic spine densities in the mPFC in sedentary rats treated with vehicle but not in exercising or galanin-treated rats. Collectively, the results indicate that both exercise and galanin prevent anxiety-like behaviors induced by stress and provide a potential mechanism through which galanin contributes to resilience.

2. Materials and methods

2.1. Subjects

Male Sprague–Dawley rats ($N = 81$; Harlan, Prattville AL, USA) obtained at 175–200 g and given ad libitum food and water were housed at 23 ± 3 °C with a 12:12 reverse light:dark cycle. Following 1-week habituation, rats underwent surgery and were housed individually in clear polycarbonate cages (50 × 30 × 30 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. All efforts were made to minimize animal suffering, to reduce the numbers of animals used, and to utilize alternatives to *in vivo* techniques, if available.

2.2. General experimental methods

All testing and drug administration occurred in the dark phase of the circadian cycle. The general protocol for experiments was: cannulation (day 8), surgical recovery (day 7–0), individual housing into exercise or sedentary conditions (day 1–21), footshock during microdialysis sampling (day 20), and elevated plus maze testing followed by brain extraction (day 21).

2.3. 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 (1 mm posterior, −1.5 mm lateral, 3 mm ventral) relative to bregma using the atlas of Paxinos and Watson (1998). 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, USA). Rats received flunixin meglumine (2.5 mg/kg s.c.) immediately and 24 h post-surgery.

2.4. Voluntary exercise

Rats were assigned to exercise or sedentary groups on day 1 and individually 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 (Holmes et al., 2006; Reiss et al., 2009; Sciolino et al., 2012; Van Hoomissen et al., 2004). No-wheel controls were selected to provide the best distinction between independent variables (e.g., exercise/environmental enrichment versus no exercise/no environmental enrichment). An electromagnetic counter (Mini Mitter, Bend OR, USA) detected wheel rotations and distance ran was calculated for each subject.

2.5. Drugs

In experiment 1, rats were injected with vehicle (aCSF or dH20) or the galanin receptor antagonist M40 (6 nmoles; Tocris, Minneapolis MN, USA) twenty minutes before exposure to footshock or no shock. In experiment 2, rats were injected daily with vehicle, galanin (3 nmoles, Tocris), or M40 (6 nmoles) for 21 d during assignment to exercise or sedentary conditions. Thus, unlike experiment 1, rats in experiment 2 were not microinjected before footshock in the open field. Repeated injections were used to study the trophic effects of galanin that presumably result after several weeks of exercise (Holmes et al., 2006; Sciolino et al., 2012). 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 (Lewis et al., 2004; McDonald and Crawley, 1996; Reiss et al., 2009).

2.6. 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, USA). 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.

2.7. Elevated plus maze

The '+' shaped maze was 50 cm from the floor and consisted of a pair of open (45 × 9 cm) and closed arms (45 × 9 × 38 cm) that met in a central platform (9 × 9 cm). Rats were placed on the center platform facing an open arm, as described previously (Sciolino et al., 2012). The time spent and entries on the arms was recorded for 5 min and analyzed from video by an experimenter blind to treatment. Open arm time is reported as a standard measure, although we verified that all changes in open arm time also resulted in similar changes in open arm entries and compensatory changes in closed arm time.

2.8. Microdialysis and high performance liquid chromatography (HPLC)

During the rodent's dark cycle (12–14 h before lights on), stylets were removed from cannulae in the frontal cortex and replaced with a dialysis probe (MAB 9.6.2, PES membrane of 2 mm, 0.6 mm OD, 6 kDa cutoff; SciPro, Sanborn NY, USA), as published previously (Ogbonmwan et al., 2014). 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, USA) was delivered through the in-line at 1.5 μL/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.

2.9. 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, 150 × 4.6 mm, Phenomenex Inc., Torrance CA, USA) and Max-RP cartridges (Phenomenex), as we performed previously (Masini et al., 2004). Mobile phase was

delivered at 1 mL/min and contained 100 mM sodium phosphate monobasic (Fisher, Pittsburgh PA, USA), 0.1 mM EDTA (Sigma), 0.25 mM octanesulfonic acid (Sigma), and 5% acetonitrile (JT Baker). Samples and standards were injected at 20 μL using ESA 542 at 4 °C. 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.

2.10. Brain harvesting and tissue sectioning for enzyme-linked immunosorbent assay (ELISA), 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 (Paxinos and Watson, 1998). Example cannulae tracks are shown in Fig. S1. 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 −80 °C. The hemisphere for ISH was sectioned sagittally (12 μm) and mounted on gelatin-coated slides. In Experiment 2, the frontal cortex was dissected (~5 × 13 × 9 mm of tissue), processed for Golgi, sectioned coronally (150 μm), and slide-mounted.

2.10.1. 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 0.5 M acetic acid), homogenized (15s; PowerGen 125, Fisher), heated (100 °C for 10 min), and centrifuged (30 min at 4 °C, 3000 rpm at 1500× g; Beckman Model TJ-6). Supernatant was poured off into separate tubes, evaporated in a vacuum-sealed concentrator (18 h at 60 °C and 20,000 mm Hg; Labconco Centrivap, Kansas City MO, USA), and reconstituted in 250 μL buffer. Tissue was processed according to the manufacturer's instructions for the Galanin Rat ELISA kit (S1208, Pennisula Laboratories, San Carlos CA, USA). Wells were read at 450 nm (MiniReader MR590, Dynatech Instruments Inc, Santa Monica CA, USA), 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.

2.10.2. In situ hybridization (ISH) and Densitometry

Tissue was fixed (4% formaldehyde in. 12 M PBS), dehydrated (0.25% acetic anhydride in 0.1 M 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, USA) was labeled at the 3′ end using ^{35}S-dATP (1 mCi; Perkin Elmer, Boston MA, USA), tailing buffer, $CoCl_2$, and terminal deoxynucleotransferase (Roche, Indianapolis IN, USA). Unbound radionucleotide was removed using column separation (Micro Bio-Spin P30 in Tris, Bio-Rad, Hercules CA, USA) by centrifugation (4000 rpm at 1000× g for 4 min; Micro-12, Separation Tech Inc, Sanford FL, USA) and bound radionucleotide was stabilized using 1 M dithiothreitol. Sections were covered with radiolabeled probe in hybridization buffer (25% formamide, 72 mM NaCl, 3.2 mM Tris HCl, 0.0032 mM EDTA, 0.001% sodium pyrophosphate, 0.004% sodium dodecylsulfate, 0.002 mg/mL heparan sulfate, and 2% dextran sulfate) and incubated for 24 h at 37 °C. Sections were washed in 1%SSC and 2%SSC-formamide (50:50) series at 40 °C and room temperature, distilled H_2O, and EtOH. Sections were dried and opposed to ^{35}S-sensitive film (Kodak BioMax MR, Rochester NY, USA) 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, USA) and digital camera (Nikon D5000, Micro-NIKKOR 55 mmf/2.8 lens, Melville NY, USA) under optimized conditions. The LC was traced in NIH ImageJ (http://rsb.info.nih.gov/ij/). Mean grayscale was measured in 2–4 sections/rat.

2.11. Golgi impregnation

Tissue from a subset of rats was impregnated according to Rapid GolgiStain Kit instructions (FD NeuroTechnologies, Columbia MD, USA), 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 and 2.2 mm from bregma (Paxinos and Watson, 1998), completely impregnated, not truncated, and with projections distinct and separate from other neurons as described previously (Stranahan et al., 2007). Images were obtained on a Zeiss AxioImager MTB microscope in z-stacks for later measurement in Reconstruct (http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm). Spines were imaged at 100× in secondary and tertiary dendrites in 5–7 neurons per rat, with sampling of five 10 μm segments from the apical and basal arbors. Five neurons per rat were also imaged at 10× to measure dendritic length and complexity. Data were averaged across cells from each animal for subsequent statistical analysis.

2.12. Statistics

Repeated measures analysis of variance (ANOVA) was used to examine weight, wheel running, and open field activity across time. Univariate ANOVA followed by Fisher's 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) × 100) to reduce within-subject variability and enhance statistical power. Significance was $p < 0.05$ for all analyses.

3. Results

3.1. Experiment 1. Exercise increases galanin and promotes resilience to stress in behavioral tests of anxiety

3.1.1. 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 ($p > 0.05$; Fig. 1A). Exercise also increased galanin mRNA in the LC compared to sedentary counterparts exposed to footshock stress ($t_{18} = -2.37$, $p < 0.05$; Fig. 1B–C).

3.1.2. Voluntary running increased across time, and reduced body weight

Running and body weight was recorded as follows from rats assessed for galanin (Fig. 1A) and behavior (Fig. 2A). 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. S2A). 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. S3A). The interaction of time by exercise was significant ($F_{1,8} = 15.39$, $p < 0.01$), and there was a trend for an exercise effect ($F_{1,8} = 4.59$, $p = 0.08$; Fig. S3A). Post hoc tests showed that exercise and sedentary groups were not different in body weight on day 1 ($p = 0.80$) or 14 ($p = 0.06$), but exercise rats exhibited reduced body weight on experimental day 7 ($p < 0.05$) and 21 ($p < 0.05$; Fig. S3A).

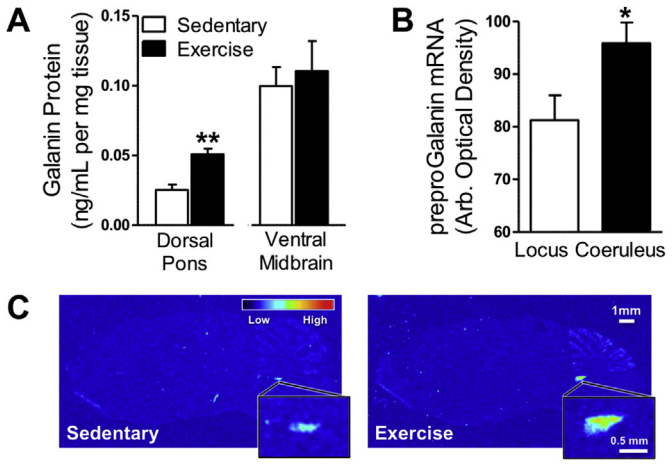

Fig. 1. Exercise increases galanin in the locus coeruleus. Galanin levels were assessed in the brain and measured in rats with access to a running wheel or no wheel for 3 weeks (see Fig. 2). Shown are means ± SEM for (A) galanin protein obtained by ELISA in tissue containing the dorsal pons or ventral 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.

Running and body weight was recorded as follows from rats assessed for galanin mRNA (see Fig. 1B) and behavior (see Fig. 2). Wheel running also increased linearly over time in rats that were microinjected before footshock exposure ($F_{1,12} = 20.56$, $p < 0.01$; Fig. S2B). The interaction of time by group ($F_{1,12} = 1.28$, $p = 0.28$) and drug effect ($F_{1,12} = 0.00$, $p = 0.96$; Fig. S2B) were 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. S3B). There was an interaction of group by time ($F_{2,19} = 5.45$, $p < 0.01$) and group ($F_{2,19} = 5.00$, $p < 0.05$; Fig. S3B). 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. S3B). Exercise reduced body weight on days 7 ($p < 0.01$), 14 ($p < 0.01$), and 21 ($p < 0.05$; Fig. S3B) compared to sedentary control. Vehicle- and M40-treated exercise rats were no different in weight at any time ($p > 0.05$; Fig. S3B).

3.1.3. Sedentary rats are susceptible to anxiety-like behavior induced by stress

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. 2A). Percent open arm time ($F_{4,27} = 3.14$, $p < 0.05$; Fig. 2B) and open arm entries ($F_{4,27} = 3.79$, $p < 0.01$; Fig. 2C) were significantly different across these groups. Exercise did not impact performance in the elevated plus maze under non-stress conditions, as there were no differences between non-stressed exercised and sedentary rats ($p > 0.05$; Fig. 2B–C). However, after exposure to stress sedentary rats exhibited anxiety-like behavior in the elevated plus maze compared to all other groups, including exercise rats treated with acute vehicle- ($p < 0.01$) or M40 ($p < 0.05$), as well as non-stressed sedentary ($p < 0.01$) and exercise controls ($p < 0.05$; Fig. 2B–C). The dose of M40 used in the present study was selected because previous research shows that M40 (1–8 nmol) effectively blocks a wide range of behaviors induced by galanin or exercise (Crawley et al., 1993; Lewis et al., 2004; McDonald and Crawley, 1996; Reiss et al., 2009). However, we found that acute M40 and vehicle-treated exercise rats were no different in open arm exploration ($p = 0.62$; Fig. 2B–C), indicating that acute blockade of galanin signaling during stress did not impact elevated plus maze behavior in exercise rats on the following day. Pilot studies revealed that acute administration of M40 (6 nmoles ICV) tended to reverse the stress-induced impairment in sedentary rats (mean ± SEM for % open time is 28.7 ± 11.9 and open entries is 5 ± 1; unpublished observation), which may indicate that galanin signaling is differently altered in sedentary versus exercise rats. No differences in general locomotion (e.g., closed arm entries, falls off the maze) was observed across groups ($p > 0.05$).

Ambulatory distance in the open field was not generally different in sedentary and exercised rats given an acute microinjection before exposure to footshock or no shock ($F_{4,29} = 0.94$, $p = 0.45$; Fig. 2D), and generally increased across time in a quadratic manner ($F_{1,29} = 49.57$, $p < 0.01$, Quadratic). The time by exercise interaction ($F_{4,29} = 3.82$, $p < 0.01$, Quadratic; Fig. 2D) was significant and follow up tests revealed that ambulation was no different at baseline (Pre: $F_{4,29} = 1.13$, $p = 0.36$) or after injection (Inject: $F_{4,29} = 1.34$, $p = 0.28$; Fig. 2D), indicating there were no differences in general locomotor activity or response to the injection between exercised and sedentary rats. However, ambulatory distance was reduced after shock exposure (Post: $F_{4,29} = 5.84$, $p < 0.01$; Fig. 2D), as shock exposed sedentary-vehicle ($p < 0.05$), exercise-vehicle ($p < 0.05$), and exercise-M40 treated rats ($p < 0.05$) exhibited less ambulation compared to non-shock counterparts.

3.2. Experiment 2. Galanin is necessary and sufficient for features of behavioral resilience and prevents neural markers of stress in the frontal cortex

3.2.1. Running increased across time, but body weight was not influenced by exercise

Running and body weight was recorded from rats that received repeated intracranial injections and were assessed for behavior (see Fig. 3) and tissue analysis (see Fig. 4). Wheel running distance increased linearly over time in rats receiving repeated microinjections and shock exposure ($F_{1,21} = 11.30$, $p < 0.01$; Fig. S2C). The time

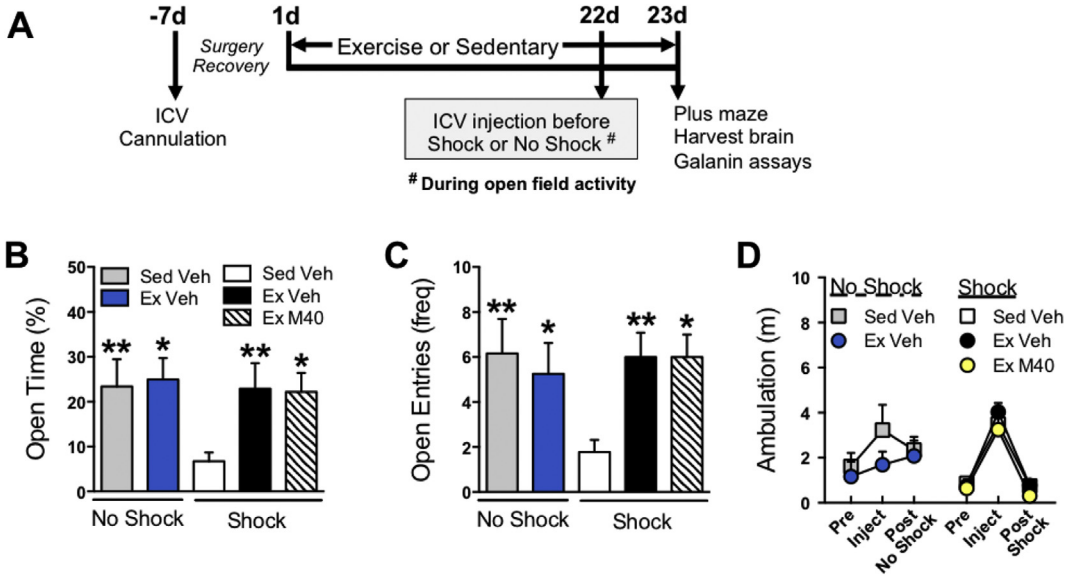

Fig. 2. Exercise blocks stress-induced behavior in an anxiety test, but this resilience behavior is not reversed by acute intra–cranial administration of M40. Rats with access to a running wheel or no wheel were administered ICV vehicle (dH20) or the galanin receptor antagonist M40 (6 nmol) immediately 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. $N = 6$ (sed-veh), 4 (ex-veh), 10 (sed-veh), 9 (ex-veh), and 5 (ex-M40). *$p < 0.05$ compared sed-veh rats exposed to footshock. Ex, exercise. Sed, sedentary. Veh, vehicle.

by drug interaction ($F_{1,21} = 0.74$, $p = 0.41$) and drug effect ($F_{1,17} = 0.73$, $p = 0.41$; Fig. S2C) were not significant. Average running on experimental day 20 was 0.84 ± 0.12 km, which was less compared to Experiment 1 likely due to the stress of repeated microinjections that briefly interrupted (e.g., for 5 min) wheel access during the active-running cycle. 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. S3C). Although the time by group interaction was significant ($F_{4,41} = 6.69$, $p < 0.01$), follow-up ANOVAs revealed no difference in body weight at each time point ($p > 0.05$; Fig. S3C).

3.2.2. Exercise-induced resilience behavior is dependent on galanin receptor activation and recapitulated by chronic ICV galanin administration

Stress-induced changes in behavior were assessed in sedentary rats treated chronically with vehicle or galanin, and in exercised rats given vehicle or M40, as shown in the timeline (Fig. 3A). The stress-buffering effects of exercise on open arm time in the elevated plus maze were mimicked in sedentary rats treated with galanin ($F_{3,37} = 11.21$, $p < 0.01$; Fig. 3B), while blockade of galanin receptors induced stress susceptibility in exercised rats ($p < 0.05$; Fig. 3B). Neither drug nor exercise altered measures that reflect general locomotion (e.g., closed arm entries, falls off the maze) ($p > 0.05$).

During the administration of footshocks in the open field on the previous day, pre-shock ambulatory distances were comparable across groups ($F_{3,36} = 0.43$, $p = 0.73$; Data Not Shown), thereby indicating no effect of exercise or drug treatment on open field habituation or general locomotor activity. After footshock, ambulatory distance increased in a manner dependent on exercise or galanin treatments ($F_{3,36} = 8.00$, $p < 0.01$). Exercise reduced stress-induced ambulation compared to sedentary controls ($p < 0.05$; one-tailed), and M40 reversed this effect in exercised rats ($p < 0.05$; Data Not Shown). Galanin increased stress-induced ambulation compared to all other groups ($p < 0.05$; Data Not Shown), which suggests that galanin promotes the use of an active defensive, coping strategy during stress.

3.2.3. Chronic ICV galanin administration and exercise each block the stress-induced increase in dopamine overflow in the frontal cortex

Stress-induced dopamine overflow in the frontal cortex was assessed in sedentary rats treated chronically with vehicle or galanin, and in exercised rats given vehicle or M40 (Fig. 3A). Baseline

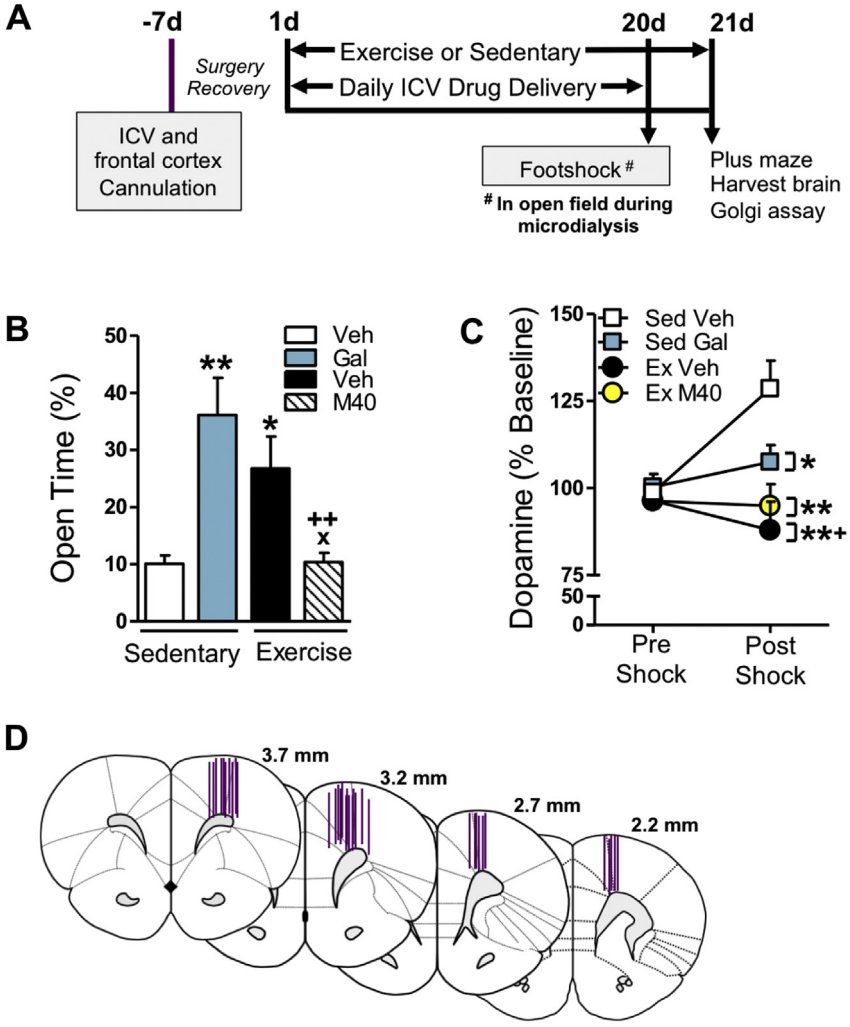

Fig. 3. Exercise blocks stress-induced behavior and -evoked dopamine, effects that are mimicked by repeated intracranial galanin and reversed partially by M40. Rats received daily administration of ICV vehicle (aCSF), galanin (3 nmol), or galanin receptor antagonist M40 (6 nmol) during exercise or sedentary conditions, and were exposed to footshock during microdialysis in an open field. Shown are (A) experimental timeline, means ± SEM for (B) open arm time in the elevated plus maze and (C) cortical dopamine levels, and (D) probe placements. $N = 9–16$ (Sed Veh), 9–10 (Sed Gal), 8–10 (veh ex), and 5–6 (Ex M40). $^{**}p < 0.01$, $^{*}p < 0.05$ compared to vehicle-treated sedentary. $++p < 0.01$, $+p < 0.05$ compared to galanin-treated sedentary. x$p < 0.05$ compared to vehicle-treated exercise. Ex, exercise. Gal, galanin. Sed, sedentary. Veh, vehicle.

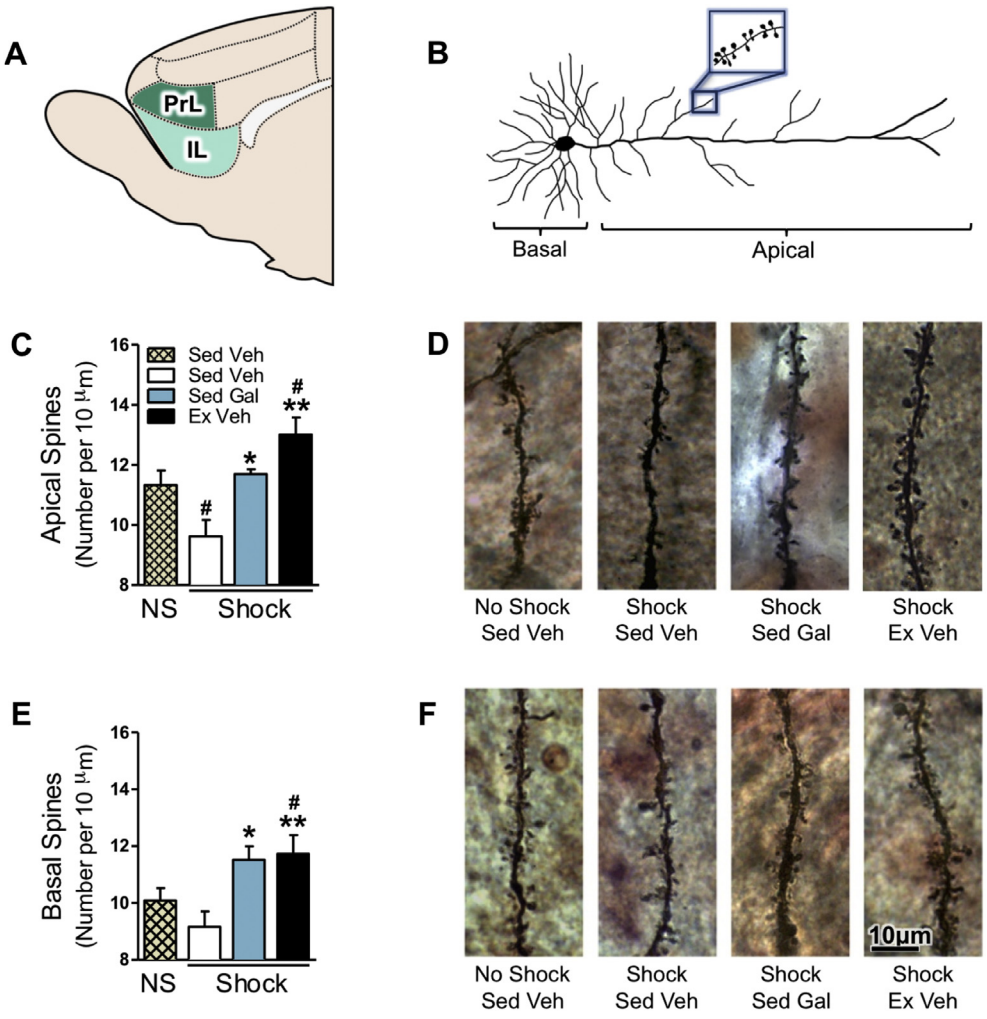

Fig. 4. Repeated intracranial galanin administration mimics exercise and prevents dendritic spine loss in mPFC pyramidal neurons after stress. Dendritic spine density was measured in mPFC pyramidal neurons in Golgi-processed tissue (imaged at 100×) obtained from a subset of rats exposed to footshock or no-shock (see Fig. 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.

dopamine levels were no different between exercise- and drug-treated 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; $F_{1,29} = 2.76$, $p = 0.05$, one-tailed; Fig. 3C). There was a significant group by time interaction ($F_{3,29} = 4.39$, $p < 0.01$) and main effect of group ($F_{3,29} = 6.51$, $p < 0.01$; Fig. 3C). Groups were no different at the pre-shock time point ($F_{3,29} = 0.33$, $p = 0.81$), but differed post-shock ($F_{3,29} = 6.65$, $p < 0.01$; Fig. 3C). Galanin ($p < 0.05$) or exercise ($p < 0.01$; Fig. 3C) prevented the stress-induced increase in dopamine overflow compared to vehicle-treated sedentary controls. There was no effect of M40 in exercise rats compared to vehicle ($p = 0.52$; Fig. 3C), indicating that the influence of exercise in dampening stress-evoked dopamine release is independent of galanin. Probe placement is shown in Fig. 3D.

3.2.4. *Chronic ICV galanin mimics the protective effects of exercise against stress-induced dendritic spine loss in mPFC neurons*

Dendritic structural plasticity was assessed in Golgi-impregnated pyramidal neurons in layer V of the prefrontal cortex, which were obtained from sedentary and exercise rats treated chronically with vehicle or galanin. Neurons were sampled from the prelimbic and infralimbic regions of the mPFC (Fig. 4A), and dendritic length and complexity, as well as dendritic spine density, were quantified in the apical and basal arbors (Fig. 4B). Apical spine density differed across groups ($F_{3,21} = 7.68$, $p < 0.01$; Fig. 4C–D). Footshock stress reduced the number of apical spines in sedentary rats ($p < 0.05$; Fig. 4C–D). Galanin treatment ($p < 0.05$) or exercise ($p < 0.01$) increased the density of spines along the apical dendrites of stressed rats relative to the sedentary control, with exercise also increasing spine density compared to the no-shock sedentary group ($p < 0.05$; Fig. 4C–D).

Basal spine density also differed across groups ($F_{3,21} = 4.77$, $p < 0.01$; Fig. 4E–F). While stress did not reduce dendritic spine density in the basal dendritic arbor of sedentary rats ($p = 0.23$), galanin treatment ($p < 0.05$) or exercise ($p < 0.05$; Fig. 4E–F) increased the density of spines along the basal dendrites of stressed rats relative to sedentary controls. No differences were observed in total dendritic length or complexity after exercise, drug, or shock manipulations ($p > 0.05$; Fig. S4).

4. Discussion

The present study employed galanin pharmacology in conjunction with an exercise model known to increase galanin expression in the LC (Holmes et al., 2006; Sciolino et al., 2012; Van Hoomissen et al., 2004). We found that exercise prevented anxiety-like behavior induced by stress. The resilience afforded by exercise was recapitulated in our behavioral tests by repeated intracranial administration of galanin and reversed by similar administration of the galanin receptor antagonist M40. However, a single dose of M40 (given prior to footshock stress) failed to reverse the stress-protective effects of exercise in anxiety tests, which demonstrates that some features of resilience do not depend on acute modulatory actions of galanin. In the chronic drug administration study, the final microinjection occurred 24 h prior to behavioral testing, diminishing the possibility that galanin reduced anxiety-related behavior through acute actions. Exercise and galanin treatment also each protected the cortex against stress-induced perturbation of dendritic structure. Combined, our data indicate that galanin is necessary and sufficient for key features of resilience/resistance displayed in a well-established rodent test of anxiety.

Exercise and galanin treatment each prevented anxiety-like behavior produced after exposure to stress. Both exercise and galanin-treated rats reacted normally 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 in the elevated plus maze. These results are likely attributable to resilience/resistance because exercised and galanin-treated rats were behaviorally protected in tests of anxiety after, but not before the initial exposure to stress. Moreover, neither exercise nor galanin groups differed in general locomotor activity (e.g., closed arm entries in plus maze, baseline ambulation in open field) or shock reactivity (Falls et al., 2010; Sciolino et al., 2012), suggesting that differences in locomotor activity or nociception are not a factor. Our data are in agreement with clinical literature that shows exercise attenuates anxiety and depression (Herring et al., 2010; U.S. Department of Health and Human Services, 2008), and literature from rodent models that show exercise promotes stress resilience in tests of anxiety (De Chiara et al., 2010; Dishman et al., 1997; Fox et al., 2008; Greenwood and Fleshner, 2013; Greenwood et al., 2008; Greenwood et al., 2007a; Maniam and Morris, 2010; Sasse et al., 2008; Sciolino et al., 2012; Zheng et al., 2006). Our data expand upon this evidence and suggest that galanin is necessary for the stress protection afforded by exercise in a standard behavioral test of anxiety.

Several lines of evidence indicate that galanin promotes behavioral resilience. First, intracranial administration of galanin does not reliably influence anxiety or depression-related behavior in the absence of stress (Holmes et al., 2005; Holmes et al., 1994; Karlsson et al., 2005; Weiss et al., 1998), but provides protection specifically under stressful conditions (Bing et al., 1993). Second, transgenic overexpression of galanin in norepinephrine neurons does not alter spontaneous behavior in tests of anxiety, but provides protection in these tests after stress exposure (Holmes et al., 2002). Third, M40 administration (8 nmoles, ICV) blocks fluoxetine-induced activity in the forced swim test (Lu et al., 2005), which is a potent stressor (Connor et al., 1997; Cryan et al., 2005). The present report is in agreement with the galanin literature and suggests that increased galanin in the LC promotes the stress resilience afforded by exercise.

We found that stress reduced dendritic spines in the mPFC in sedentary animals, whereas galanin treatment protected against this loss. This effect was observed in the apical segment of dendritic arbors but not in the basal, which is consistent with literature reporting apical segments are particularly responsive to stress (Leuner and Shors, 2012). Similarly, exercised rats exhibited increased spines in the mPFC after stress, suggesting that exercise also protects against stress-induced dendritic spine loss. 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 (Chen et al., 2013). Recent evidence links the neurotrophic and neuroprotective actions of galanin to inhibition of RhoA (Hobson et al., 2013), but additional studies will be required to fully characterize the dynamic alterations in dendritic spines following galanin treatment, with the goal to identify how galanin contributes to structural plasticity in the presence and absence of stress.

We also found that manipulations that promote resilience, including repeated exercise and galanin treatment, attenuated shock-evoked dopamine in the frontal cortex. The galanin agonist galnon also attenuates cocaine-evoked dopamine in the frontal cortex (Ogbonmwan et al., 2014), which provides further support that galanin plays a key role in regulating cortical activity. Our data are consistent with the body of literature that shows increased cortical dopamine is a reliable neurochemical signature of acute stress (Abercrombie et al., 1989; Finlay et al., 1995; Pascucci et al., 2007). Galanin administration may blunt stress-induced dopamine release through direct influences on VTA electrophysiological activity (Counts et al., 2002; Weiss et al., 1998). However, we failed to observe a difference in evoked dopamine release between M40 and vehicle-treated exercise rats. Our data therefore suggest that the resilience afforded by exercise in tests measured herein is not dependent on dopaminergic signaling in the mPFC. Although many manipulations that protect against stress do so in a manner that depends on the mPFC (Amat et al., 2005; Amat et al., 2006; Christianson et al., 2009; Lehmann and Herkenham, 2011), the resilience afforded by these manipulations may require distinct adaptations in other stress-responsive circuitry. For example, prior evidence shows that exercise protects against uncontrollable stress and enhances fear learning regardless of mPFC lesion (Greenwood et al., 2013). Indeed exercise influences the pattern of stress-induced neuronal activation in regions like the mPFC, PVN, amygdala, hippocampus, raphe, sensory/motor integration sites (Campeau et al., 2010; Collins et al., 2009; Greenwood et al., 2005; Greenwood et al., 2003), suggesting that exercise produces resilience by tuning neuronal activity in several corticolimbic circuits.

A variety of factors increase galanin gene expression in the LC, including acute and chronic stressors (see also Austin et al., 1990; Holmes et al., 1995; Kuteeva et al., 2008), voluntary or forced exercise (Holmes et al., 2006; Murray et al., 2010; O'Neal et al., 2001; Reiss et al., 2009; Sciolino et al., 2012; Van Hoomissen et al., 2004), and antidepressant treatment with fluoxetine or clomipramine (Holmes et al., 2006; Lu et al., 2005). Future research is needed to systematically identify how exercise and stress impact galanin signaling. Exercise has notably different effects in the presence and absence of stress on measures like HPA reactivity and neurogenesis (Stranahan et al., 2006; Stranahan et al., 2008). It is therefore important to consider the effect of non-manipulated stressors in the context of our data. For example, behavioral testing was likely stressful as running tended to decrease the day following behavioral testing. All of our animals were also singly housed as typically performed in exercise studies to obtain individualized information about running. Notwithstanding, we found that exercise consistently increased LC galanin levels despite that mRNA was measured after footshock and protein measured after no shock. Exercise likely dose-dependently increases galanin as prior research shows that galanin mRNA in the LC is positively associated with running

distance (Eisenstein and Holmes, 2007; Holmes et al., 2006; Sciolino et al., 2012). Although running distance was generally moderate-to-low in the present studies, probably due to surgical implantation of indwelling cannuale, we reliably observed that exercise protected against anxiety-like behavior resulting after stress. These data collectively suggest that the increases in galanin and stress resilience observed in the present study are due to exercise.

The present study is the first to show that galanin contributes to behavioral resilience that results from exercise. Exercise regulates multiple molecular and structural measures of neuroplasticity, including hippocampal neurogenesis and neurotrophic factor expression (Duman and Monteggia, 2006). These phenomena may contribute to the affective consequences of exercise, though the evidence for their specific roles is mixed (Duman et al., 2008; Duman et al., 2009; Fuss et al., 2010; Greenwood et al., 2007b; Li et al., 2008; Llorens-Martin et al., 2010; Trejo et al., 2008). The lack of research regarding the interplay between these structural and molecular signatures represents a major gap in understanding how gene–environment interactions cooperatively induce resilience or susceptibility, and this area deserves further study. Broadly, our data highlight a neurotrophic role for galanin in stress resilience, as chronic, but not acute, galanin receptor antagonism emerged as a determinant of anxious behavior. Our finding that galanin promoted stress resilience at both the behavioral and neural levels suggests a novel interpretation of galanin's role in anxiety that hinges upon its trophic function rather than its well-known neuromodulatory actions. The efficacy of chronic galanin administration alone in sedentary rats is informative in this regard. Disruption in the galanin system may therefore tip the balance towards susceptibility, as evidenced from the current report and human studies demonstrating that genetic variants in galanin and its receptors (GalR1-3) correlate with risk for anxiety and depression after stressful experiences. Polymorphisms in galanin and its receptor subtypes were found to be more predictive of neuropsychiatric disease than other well-studied polymorphisms, such as the 5-HTTLPR polymorphism (Juhasz et al., 2014). Thus, restoring the dysregulation in galanin, endogenously by exercise or exogenously by galanin-based pharmacotherapies, may offer novel approaches to the prevention or treatment of stress-related mental disorders.

5. Conclusions

The neurotrophic actions of galanin represent a potential mechanism for its impact on stress-related behaviors. Galanin may thus produce stress resilience by influencing neural plasticity in the mPFC. The present experiments revealed that manipulations that chronically enhance galanin levels buffer the impact of stress on anxiety-related behaviors and dendritic spines in the mPFC.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.09.029.

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