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Running-induced anxiety is dependent on increases in hippocampal neurogenesis

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Abstract

Exercise, specifically voluntary wheel running, is a potent stimulator of hippocampal neurogenesis in adult mice. In addition, exercise induces behavioral changes in numerous measures of anxiety in rodents. However, the physiological underpinnings of these changes are poorly understood. To investigate the role of neurogenesis in exercise-mediated anxiety, we examined the cellular and behavioral effects of voluntary wheel running in mice with a reduction in hippocampal neurogenesis, achieved through conditional deletion of ataxia telangeictasia-mutated and rad-3 related protein (ATR), a cell cycle checkpoint kinase necessary for normal levels of neurogenesis. Following hippocampal microinjection of an adeno-associated virus expressing Cre recombinase to delete ATR, mice were exposed to four weeks of voluntary wheel running and subsequently evaluated for anxiety-like behavior. Wheel running resulted in increased cell proliferation and neurogenesis, as measured by bromodeoxyuridine and doublecortin, respectively. Wheel running also resulted in heightened anxiety in the novelty-induced hypophagia, open field, and light-dark box tests. However, both the neurogenic and anxiogenic effects of wheel running were attenuated following hippocampal ATR deletion, suggesting increased neurogenesis is an important mediator of exercise-induced anxiety.

Keywords

neurogenesis; anxiety; exercise; wheel running; hyponeophagia

Introduction

Exercise is associated with a wide array of health benefits (van Praag, 2008, for review). In addition to the physical benefits, exercise can improve cognition (van Praag et al., 1999; Van der Borght et al., 2007; Winter et al., 2007; Nichol et al., 2009), alleviate symptoms of neurodegenerative diseases (Tillerson et al., 2003; Nichol et al., 2009), and aid in recovery from mood disorders (Blumenthal et al., 1999; Babyak et al., 2000; Duman et al., 2008).

Rodents exhibit a strong penchant for voluntary wheel running, which activates brain reward pathways (Brene et al., 2007) and is used as a model to investigate the mechanisms

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underlying the benefits of exercise in humans. Voluntary running results in a robust increase in hippocampal neurogenesis in adult mice. Indeed, running has been shown to increase proliferation of neural progenitor cells (Kronenberg et al., 2003), and increase both the number and percentage of newborn cells which become mature neurons (van Praag et al., 1999). Running and neurogenesis have been directly correlated, further highlighting the potent influence of exercise on hippocampal neurogenesis (Allen et al., 2001; Rhodes et al., 2003; Clark et al., 2011). A multitude of changes are implicated in the therapeutic effects of exercise, including heightened synaptic plasticity (van Praag et al., 1999; Farmer et al., 2004), angiogenesis (Van der Borght et al., 2009) and growth factor expression (Neeper et al., 1996; Kitamura et al., 2003). As evidenced by the importance of newborn neurons in cognition and mood regulation (Sahay et al., 2011; Surget et al., 2011), neurogenesis is an additional mechanism which may contribute to the therapeutic effects of exercise.

In light of clinical evidence for the mood-improving effects of exercise (Deslandes et al., 2009, for review), we set out to investigate the underlying mechanisms associated with these effects. A number of studies have investigated the effects of voluntary wheel running on mood and anxiety-related behaviors in rodents, the results of which are inconsistent. While some studies find decreased anxiety (Dishman et al., 1996; Salam et al., 2009), others demonstrate increased anxiety (Burghardt et al., 2004; Fuss et al., 2010). Yet others demonstrate either increased or decreased anxiety depending on the specific anxiety measure used and the timing of the measurement (Binder et al., 2004; Duman et al., 2008). A recent study demonstrated that running-induced anxiety was prevented by x-ray irradiation, a technique to ablate neurogenesis (Fuss et al., 2010), supporting a direct role for neurogenesis in the development of exercise-induced anxiety. Alternatively, Revest and colleagues (Revest et al., 2009) find increased anxiety following a partial knockdown of neurogenesis. These conflicting observations regarding the relationship between rate of neurogenesis and anxiety state support the need for additional research in this arena.

We previously showed partial suppression of hippocampal neurogenesis following hippocampal deletion of ATR, a cell cycle checkpoint kinase (Onksen et al., 2011). Here, we explore the possibility that deletion of ATR from the hippocampus results in resistance to the neurogenic effects of voluntary exercise and examine the behavioral implications of that resistance.

Methods

Animals

Mice homozygous for the Cre/*lox*-conditional allele of ATR ($ATR^{f/f}$) on a 129S2/SvPas/C57BL/6 mixed background were generated as previously described (Ruzankina et al., 2007). Briefly, mice were generated from a D3 ES cell line originally derived from 129Sv blastocysts. Homologously recombined ES cells were injected into C57BL/6 blastocysts and chimeric offspring were mated with mice from the C57Bl/6 strain to produce mice of all three geneotypes; $ATR^{f/f,}$ $ATR^{f/-,}$ $ATR^{-/-}$. $ATR^{f/f}$ were obtained from intercrosses and ultimately maintained as $ATR^{f/f}$ from approximately 4 generations of homozygous crosses of $ATR^{f/f} \times ATR^{f/f}$. $ATR^{f/f}$ mice receiving hippocampal microinjection of AAV.Cre and having ATR deleted throughout the hippocampus are subsequently referred to as $ATR^{\Delta Hipp}$, while control mice injected with AAV.eGFP retain the $ATR^{f/f}$ designation.

Stereotaxic surgery was performed at 7-8 weeks of age and all behavioral experiments were conducted on adult males and females at least 6 weeks following stereotaxic surgery. Mice were housed in groups of 3-4 following surgery and subsequently single-housed at least one week prior to being placed in to cages containing running wheels. Cages with running wheels (Mini Mitter, Bend OR) measured 20×36 cm with an 11.5 cm diameter wheel

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mounted to the cage top. Wheel rotations were monitored continuously via VitalView (Mini Mitter). Sedentary control animals were housed in identical cages with immobilized wheels. Animals were maintained on a 12 hr light/dark cycle (lights on 7:00 AM to 7:00 PM) with food and water available *ad libitum* in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee.

Experimental Design

Two cohorts of $ATR^{f/f}$ mice were used in this study. Mice in both cohorts received hippocampal micro-injections at 8 weeks of age. For a schematic of the experimental design see Figure 1a. Mice in cohort #1 were used for the novelty-induced hypophagia (NIH) test and were therefore single housed and trained to consume peanut butter chips in their home cages prior to being placed in cages containing running wheels. Cohort #2 was used to evaluate behavior in the open field, marble-burying and light-dark box tests (separated by 3 days). All behavioral testing was performed during the 4th week of wheel running. All mice were injected with 200 mg/kg bromodeoxyuridine (BrdU, Sigma) 24 hours following the last behavioral test and subsequently perfused 24 hours following BrdU injection. Mice had *ad libitum* access to running wheels until they were perfused (except when removed from the cages for behavioral testing). Data from both cohorts was pooled to examine effects of AAV injection and voluntary running on neurogenesis.

Adeno-associated virus

Adeno-associated viruses (AAV) expressing Cre recombinase (AAV2/9.CMV.PI.CRE, titer $2.84*10^{13}$ gc/ml) and eGFP (AAV2/9.CMV.eGFP, titer $3.74*10^{13}$ gc/ml) were generated by the University of Pennsylvania Vector Core. The expression cassette consists of the AAV2 terminal repeats flanking the CMV promoter-PI-Cre recombinase/eGFP sequences packaged into AAV9. The vectors were purified using CsCl sedimentation method. Quantification of vector genome copies (gc) was performed by Q-PCR. AAVs were diluted in sterile PBS for microinjections.

Hippocampal Injections

$ATR^{f/f}$ mice (6-8 weeks) were anesthetized with isoflurane and secured in a stereotaxic frame (Kopf, Tujuna, CA). Holes were drilled bilaterally in the skull at sites of injection. Stereotaxic coordinates to target the dorsal and the ventral hippocampus are (from Bregma) anterior-posterior -2.1, lateral +/-1.4, dorso-ventral -2.0, and anterior-posterior -2.9, lateral +/-3.0, dorso-ventral -3.8. 0.5 ul of $1*10^{10}$ gc/ul AAV was injected at each site through a 33 gauge needle on a 5 ul Hamilton syringe using a KDS310 Nano Pump (KD Scientific, Holliston, MA) mounted to the stereotaxic frame, at a rate of 0.1 ul/min. The needle remained in place for 4 additional minutes at each injection site. The skin was sutured and the animal recovered on a heating pad before returning to the home cage.

Immunohistochemistry

Mice were anesthetized with nembutol (10 mg/kg) and transcardially perfused with cold 0.1 M phosphate-buffered saline (PBS) for 5 minutes, followed by cold 4% paraformaldehyde (PFA) in PBS for 10 minutes. Brains were postfixed overnight in PFA at 4°C and subsequently stored at 4°C in 30% sucrose. Brains were frozen on dry ice, sectioned coronally at a thickness of 40 μm, and transferred to PBS + 0.5% Sodium Azide at 4°C prior to processing for immunohistochemistry.

For BrdU analysis, sections were wet-mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) for stereological analysis using a modified version of the optical fractionator method (West et al., 1991). Mounted sections were incubated in 0.1 M citric acid, pH 6.0,

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for antigen retrieval. Subsequently, slides were incubated in trypsin, 2N HCl, then primary antibody (mouse anti-BrdU, 1:200, Becton Dickinson) with 0.5% Tween 20 overnight. On the second day, slides were incubated for 1 hour in secondary antibody (biotinylated anti-mouse IgG, Vector Laboratories), 1 hour in avidin-biotin-HRP (1:100, Vector Laboratories), and labeled cells were visualized using diaminobenzidine (Sigma). PBS-washed slides were then counterstained and coverslipped with Permount. Systematic sampling using a predetermined periodicity (every ninth slice) and a random starting point was employed for quantification. All BrdU-IR cells in every 9th section of the hippocampus, along the complete extent of its rostralcaudal axis, were counted with a 100x oil immersion lens, omitting cells in the upper-most focal plane to avoid double-counting. Every 9th coronal slice between -0.9 mm and -4.0 mm from bregma were analyzed to ensure complete sampling. Within each section, all immuno-reactive cells within the granule cell layer (GCL) and the subgranular zone (SGZ) were counted. The SGZ was defined as the layer within two cells of the GCL. Cells within clusters were counted by distinguishing nuclear borders while focusing down through the tissue using an objective with a narrow depth of focus.

For doublecortin (DCX) analysis, free-floating sections of a 1/9 series through the hippocampus were blocked for 1 hour with 3% Normal Horse Serum, 0.5% Tween 20 and 0.2% Triton in PBS (blocking solution). Sections were then incubated for 72 hours at 4°C in primary antibody (Goat anti-DCX, Santa Cruz #8066) diluted 1:500 in blocking solution. Sections were washed and incubated for 1 hour in secondary antibody (Horse anti-goat, Vector Laboratories) diluted 1:200 in blocking solution. Sections were then washed and treated with 0.75% H_2O_2 for 20 minutes prior to incubation in avidin-biotin-HRP (1:200, Vector Laboratories). Labeled cells were visualized using nickel-enhanced diaminobenzidine. Free-floating sections were mounted onto slides and dried overnight before dehydrating and coverslipping with Permount. DCX-IR cells in every 9th section were counted with a 100x oil immersion lens, omitting cells in the upper-most focal plane. All cells located within the GCL and SGZ were counted.

Novelty-induced Hypophagia

For 1 week prior to the training period, and through the experiment, mice were single housed. On 11 consecutive training days, mice were exposed to highly palatable food (peanut butter chips, Nestle, Glendale CA) in a plastic dish in their home cage. On each training day mice were acclimated to the presence of a plastic cage divider for 1 hour, consistent with previous NIH studies (Gur et al., 2007; Onksen et al., 2011). Peanut butter chips were then placed in the cage for 15 minutes and latency to initiate feeding was measured. Subsequent to training, mice were placed in cages containing running wheels and left for 28 days. On days 25 and 26, a home cage test was performed in which mice were removed from wheel-containing cages and placed into a standard mouse cage, allowed to acclimate to the cage and divider for 1 hour, and latency to feed was subsequently measured. On day 27, latency to feed in a novel environment was measured. Mice were removed from wheel-containing cages, placed for 1 hour in the same standard mouse cage with divider that was used for home cage tests, and subsequently placed in a novel, anxiety provoking environment where latency to feed was measured. The novel environment consisted of an empty cage with no bedding, set inside a white box with a bright light and a novel odor (pine sol). Following each test session, mice were returned to their running wheel-containing cages.

Open Field

Open field behavior was monitored in a white box measuring 41×35 cm in dim light conditions (40 lux). Activity was recorded by an overhead camera and analyzed with TopScanLite 2.0 (Clever Sys, Inc., Reston, VA) Parameters assessed were time spent and

distance travelled in the center and outer zones. The center zone was defined as 25% of the total area.

Light-Dark Box

The light-dark box consisted of two adjacent chambers, connected by a 5×5 cm opening. Each chamber measured 17×20 cm. The dark chamber was black and covered. The light chamber was white and illuminated with bright light (750 lux). Each test session lasted 5 minutes and was initiated by placing the mouse in the corner of the light chamber. Behavior was recorded by an overhead camera and analyzed with TopScanLite 2.0. Parameters assessed were transitions between the two chambers, time spent in each chamber, and distance travelled in the light chamber.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 and JMP software. Data are reported as mean ± SEM. Studies involving two variables were analyzed by two-way ANOVA, with time as a repeated measure where appropriate. Bonferroni *post hoc* tests were performed to compare individual treatment groups. Data from the NIH test was analyzed by three-factor ANOVA to account for gene, exercise, and test environment.

Results

ATR deletion attenuates the neurogenic effect of voluntary wheel running

To determine if ATR deletion confers resistance to the neurogenic effects of wheel running, we microinjected AAV.Cre or AAV.eGFP into the hippocampus of $ATR^{f/f}$ mice and assigned them to either the running or the sedentary condition. The pattern of AAV spread and subsequent eGFP expression is depicted in photomicrographs of the dorsal (Fig. 1b) and ventral (Fig. 1c) hippocampus. We previously demonstrated a 78% reduction of ATR in the hippocampus following microinjection of AAV.Cre (Onksen et al., 2011). Six weeks following AAV injection, mice were single housed in running wheel-containing cages. Running distance plateaued in the 2nd week in both $ATR^{f/f}$ and $ATR^{\Delta Hipp}$ mice (Fig. 1d), which is consistent with other studies involving wheel running (Clark et al., 2009). No differences were observed in wheel running distance between treatment groups ($ATR^{f/f}$, 7.73±2.49 km/day; $ATR^{\Delta Hipp}$, 8.31±2.14 km/day; gene main effect, $F_{(1,72)}$=0.404, p=0.53; time main effect, $F_{(1,72)}$=16.57, p<0.001).

Following 28 days of running, immunohistochemical analysis was performed to measure cell proliferation and number of immature neurons. BrdU (200 mg/kg) was injected 24 hours prior to perfusing the animals. An average of 8.29±0.61 brain sections per mouse were analyzed for BrdU quantification, and 8.31±0.56 sections per mouse were analyzed for doublecortin (DCX) quantification. Primary statistical analysis revealed no significant effects of sex on BrdU or DCX counts. For BrdU, three-factor ANOVA (sex, gene, exercise as independent factors) revealed no main effect of sex ($F_{(1,53)}$=0.636, p=0.43), no sex × gene interaction ($F_{(1,53)}$=0.191, p=0.66) and no sex × exercise interaction ($F_{(1,53)}$=0.0005, p=0.98). For DCX, three-factor ANOVA (sex, gene, exercise as independent factors) revealed no main effect of sex ($F_{(1,54)}$=0.035, p=0.85), no sex × gene interaction ($F_{(1,54)}$=0.196, p=0.66), and no sex × exercise interaction ($F_{(1,54)}$=0.747, p=0.39). Based on these results, male and female mice were pooled for subsequent analyses. Two-way ANOVA of BrdU-positive cells in the hippocampus (Fig. 2a,b) revealed main effects of gene ($F_{(1,50)}$=28.89, p<0.0001) and exercise ($F_{(1,50)}$=23.33, p<0.0001), as well as a significant interaction ($F_{(1,50)}$=5.26, p<0.05). *Post tests* indicate that wheel running resulted in a significant increase in cell proliferation in $ATR^{f/f}$ runners compared to all other treatment groups (+89% vs. $ATR^{f/f}$ sedentary, +206% vs. $ATR^{\Delta Hipp}$ sedentary, +102% vs.

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ATR$^{\Delta Hipp}$ runners, each $p<0.001$). Unlike ATR$^{f/f}$ runners, ATR$^{\Delta Hipp}$ runners did not exhibit significantly increased cell proliferation compared to their sedentary counterparts. In addition, as previously observed, ATR deletion resulted in significantly reduced cell proliferation among sedentary mice (-38%, $p<0.05$). Two-way ANOVA of DCX-positive cells in the hippocampus (Fig. 2c,d) revealed significant main effects of gene ($F_{(1,51)}=32.65$, $p<0.0001$) and exercise ($F_{(1,51)}=39.73$, $p<0.0001$), as well as a significant interaction ($F_{(1,51)}=10.08$, $p<0.01$). Similar to our BrdU observations, *post tests* indicated DCX expression was significantly increased in ATR$^{f/f}$ runners compared to all other groups (+172% vs. ATR$^{f/f}$ sedentary, +406% vs. ATR$^{\Delta Hipp}$ sedentary, +146% vs. ATR$^{\Delta Hipp}$ runners, each $p<0.001$). ATR$^{\Delta Hipp}$ runners did not exhibit significantly increased DCX-expressing cells compared to their sedentary counterparts. *Post tests* did not show a significant effect of ATR deletion among sedentary mice. However, we previously demonstrated an effect of ATR deletion to reduce DCX-expressing cells, so we therefore performed a Student's t-test comparing sedentary ATR$^{f/f}$ and ATR$^{\Delta Hipp}$ mice to verify this effect had again been achieved, and indeed it had (-46%, $t_{(30)}=4.00$, $p<0.001$). The effects of ATR deletion and wheel running were similarly observed in both the dorsal and ventral hippocampus (Supp. Fig. S1). Taken together, BrdU and DCX data indicate that mice lacking hippocampal ATR exhibit a reduced neurogenic response to wheel running.

Running-induced anxiety in the NIH test is attenuated in ATR$^{\Delta Hipp}$ mice

We recently observed reduced feeding latency in a novel environment in mice with reduced levels of neurogenesis (Onksen et al., 2011). The NIH paradigm consists of training mice to consume a palatable food in their home cage, and subsequently measuring their latency to consume this same food in a novel, anxiety provoking environment. Thus, reduced neurogenesis may contribute to reduced anxiety in this particular paradigm. As others have demonstrated heightened anxiety as a result of voluntary exercise, we sought to determine whether our voluntary wheel running paradigm would result in increased anxiety in the NIH test, and whether this anxiogenic phenotype would be altered by reduced neurogenesis.

Six weeks following hippocampal microinjection of AAV to delete ATR, mice were single housed and subsequently trained to consume peanut butter chips in their home cage. No differences were observed in training performance between ATR$^{f/f}$ and ATR$^{\Delta Hipp}$ mice (Fig. 3a), as measured by two-way ANOVA of feeding latency on days 6-11, with day as a repeated measure (day main effect, $F_{(5,150)}=15.91$, $p<0.0001$; gene main effect, $F_{(1,150)}=0.339$, $p=0.565$). Training was followed by 28 days of housing in running-wheel-containing cages.

On the 25th and 26th days of running, mice were given access to peanut butter chips in a home cage environment. Latency was measured on the 2nd home environment exposure. Our unpublished observations indicated exercise to be anxiogenic when mice are initially removed from running wheels and tested for feeding latency in a home cage. Thus, we measured feeding latency during the second day of home cage feeding, during which all treatment groups performed similarly (Fig. 3b). Normalization of home cage behavior allows for clear interpretation of any anxiety phenotypes observed in the novel test. On the 27th day of running, feeding latency in a novel, anxiety-provoking environment was measured.

Three-factor ANOVA of feeding latency revealed significant main effects of gene ($F_{(1,51)}=7.64$, $p<0.01$), exercise ($F_{(1,51)}=7.94$, $p<0.01$) and environment ($F_{(1,51)}=23.31$, $p<0.001$), in addition to a significant gene × exercise × environment interaction ($F_{(1,51)}=24.29$, $p<0.001$). *Post test* comparisons indicated significantly greater latency in the ATR$^{f/f}$ runners compared to all other treatment groups in the novel environment (Fig. 3b, $p<0.05$). Among ATR$^{\Delta Hipp}$ mice, there was no difference in latency between runners and

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their sedentary counterparts in the novel environment (Fig. 3b), indicating ATR deletion blocked the anxiogenic effects of running on feeding latency. As with cellular effects, no main effect of sex, no sex × gene interaction and no sex × exercise interactions were revealed, therefore male and female mice were pooled for these and subsequent behavioral analyses.

Running-induced anxiety in an open field is attenuated in ATR$^{\Delta Hipp}$ mice

Voluntary wheel running results in increased anxiety in an open field test, as measured by reduced time spent in the center zone. This anxiogenic phenotype was attenuated in ATR$^{\Delta Hipp}$ mice. Two-way ANOVA of time spent in the center zone (Fig. 4a) revealed a near-significant effect of exercise ($F_{(1,24)}$=3.67, p=0.067). Bonferroni *post tests* to compare individual treatment groups indicated significantly reduced time spent in the center zone in ATR$^{f/f}$ runners compared to their sedentary counterparts (-67%, $t_{(13)}$=2.445, p<0.05). ATR$^{\Delta Hipp}$ runners did not differ from either sedentary control group. In addition to time spent in the center zone, total distance traveled in the open field was measured (Fig. 4b). We observed a significant main effect of exercise ($F_{(1,24)}$=5.75, p<0.05) and of ATR deletion ($F_{(1,24)}$=9.77, p<0.01), with no exercise × gene interaction ($F_{(1,24)}$=0.06, p=0.81). This is indicative of an overall inhibitory effect of exercise and an overall stimulatory effect of ATR deletion on exploratory behavior in the open field.

Running-induced anxiety in the light-dark box test is attenuated in ATR$^{\Delta Hipp}$ mice

As an additional measure of anxiety, we evaluated behavior in the light-dark box following voluntary wheel running. Two-way ANOVA revealed a significant main effect of exercise ($F_{(1,25)}$=12.82, p<0.01) on light-to-dark transitions (Fig. 5a). There was no effect of gene ($F_{(1,25)}$=0.004, p<0.95) and no exercise × gene interaction ($F_{(1,25)}$=0.19, p<0.67) on this outcome measure, suggesting the effect of exercise on transitions is not dependent on a robust stimulation of neurogenesis. Analysis of distance travelled in the light compartment revealed a trend towards an anxiogenic effect of running being blunted in ATR$^{\Delta Hipp}$ mice (Fig. 5b). Two-way ANOVA revealed a near-significant main effect of exercise ($F_{(1,25)}$=3.97, p=0.057) and a non-significant, but trending, exercise × gene interaction ($F_{(1,25)}$=3.105, p=0.09). *Post tests* revealed a significant decrease in distance travelled in the light compartment in ATR$^{f/f}$ runners compared to their sedentary counterparts (p<0.05). This effect was not apparent in ATR$^{\Delta Hipp}$ mice. No effects of running or ATR deletion were observed on time spent in the light compartment (exercise main effect $F_{(1,25)}$=0.01, p<0.92; gene main effect $F_{(1,25)}$=1.14, p=0.30; exercise × gene interaction $F_{(1,25)}$=0.001, p=0.97). Reduced distance traveled in the light compartment in the absence of any changes in total time spent in the compartment may be indicative of reduced exploration.

Discussion

Exercise is associated with many health benefits in humans, ranging from the cognitive to the physiological (van Praag, 2008). Preclinical research has established exercise as a potent enhancer of hippocampal neurogenesis (van Praag et al., 1999), which may underlie its health benefits. Interestingly, exercise leads to changes in anxiety-like behavior in rodents. The potential role of neurogenesis in modulating anxiety-related behaviors following exercise is only now being explored. In this study, we utilized a transgenic mouse in which Cre recombinase-inducible deletion of ATR from the hippocampus of adult mice leads to reduced levels of hippocampal neurogenesis. Our previous characterization of hippocampal ATR deletion examined effects on basal neurogenesis only. Here, we found that ATR deletion attenuated the neurogenic effect of wheel running. Additionally, wheel running resulted in heightened anxiety in the novelty-induced hypophagia and trends in anxiolytic behavior in the open field, and light-dark box tests. Some of the anxiety phenotypes induced

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by wheel running were absent following ATR deletion (NIH), while others were attenuated (open field and light-dark box). While our findings are in agreement with work by others (Fuss et al., 2010), there are conflicting reports in the literature regarding effects of exercise on anxiety (Dishman et al., 1996; Binder et al., 2004; Burghardt et al., 2004; Duman et al., 2008; Salam et al., 2009). These discrepancies may arise from a multitude of factors including variation in housing conditions and exercise parameters. However, the most relevant factor is likely differences in running distance and neurogenic response to running across mouse strains (Clark et al., 2011), which will subsequently influence behavioral outcome measures.

We utilized a 4 week, *ad libitum* access wheel running paradigm, as we previously observed increased cell proliferation and BDNF mRNA in the hippocampus in naïve $ATR^{f/f}$ mice using this paradigm (J.L.O., unpublished observations). Wheel running exerted a potent stimulatory effect on neurogenesis in $ATR^{f/f}$ mice, as indicated by increased BrdU and DCX immunostaining to measure cell proliferation and immature neurons, respectively (Fig. 2). This effect of wheel running was attenuated in $ATR^{\Delta Hipp}$ mice; levels of neurogenesis in $ATR^{\Delta Hipp}$ runners were comparable to those of sedentary $ATR^{f/f}$ controls. Our neurogenesis data suggests $ATR^{\Delta Hipp}$ mice retain some neurogenic capacity in response to a strong stimuli. However, the overall magnitude of the increase remains well below that which is observed in the control group, allowing for investigation of the causal role of excessive neurogenesis in the behavioral changes observed following exercise.

To identify behavioral implications of a blunted neurogenic response to exercise, we examined the anxiety state of the animals. We first examined behavior in the NIH paradigm following exercise. In the novel environment, we observed a large increase in latency to feed in $ATR^{f/f}$ runners compared to sedentary $ATR^{f/f}$ controls (Fig. 3b). This increase was not apparent in the $ATR^{\Delta Hipp}$ runners, suggesting heightened neurogenesis may underlie the effect observed in $ATR^{f/f}$ mice. Because ATR deletion does not influence feeding latency in the home cage test, it is possible that the role of neurogenesis in exercise-induced anxiety is context-dependent and is most relevant in novel environments. Previously, we observed a statistically significant reduction in latency to feed in a novel environment following ATR deletion (Onksen et al., 2011), whereas here we observe only a trend in the NIH test (Fig. 3b) and ATR deletion alone does not reduce all measures of anxiety examined in the present study. We hypothesize that the single-housing conditions necessary for the wheel running studies result in alterations to anxiety state (Kwak et al., 2009) such that certain differences are less likely to be observed.

In addition to the NIH paradigm, we examined anxiety-like behavior in the open field and light-dark box tests. In the open field test (Fig. 4), $ATR^{f/f}$ runners spent less time in the center zone compared to their sedentary counterparts, indicative of increased anxiety. While there were no significant interactions between exercise and ATR deletion in the hippocampus, the $ATR^{\Delta Hipp}$ runners spent similar amounts of time in the center of the open field as the sedentary group, suggesting that ATR and/or the increase in neurogenesis associated with running in wild-type mice is required for some anxiolytic properties of exercise.

In the light-dark box test (Fig. 5), running resulted in a trend for fewer transitions between the compartments. This effect was present in both $ATR^{f/f}$ and $ATR^{\Delta Hipp}$ runners. However, the overall distance traveled in the light compartment was reduced in $ATR^{f/f}$ mice exposed to running, whereas no such reductions were observed between runners and sedentary mice in which ATR had been deleted in the hippocampus ($ATR^{\Delta Hipp}$). Among previous reports using the light-dark or dark-light tests, some report changes in both transitions and time spent in the light compartment as measures of anxiety (Chaouloff et al., 1997; Frye et al.,

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2008; Varadarajulu et al., 2011), while others report one or the other (Binder et al., 2004; Correa et al., 2008; Fuss et al., 2010; Pilhatsch et al., 2010). Variability may be dependent on differences in mouse strain or parameters of the testing environment, including size and lighting conditions. Thus, we analyzed transitions, distance traveled in the light compartment, and time in the light compartment in our mice.

Taken together, this data from multiple anxiety tests suggests mice with increased neurogenesis exhibit heightened anxiety. Because increased neurogenesis is associated with heightened cognition in rodent models (Sahay et al., 2011), the phenotype exhibited by $ATR^{f/f}$ runners may be due to increased awareness of novel surroundings and subsequent caution in exploring the environment, evidenced by general trends for reduced distance traveled in the open field and transitions in the light-dark box. An additional hypothesis to be considered is that reduced neurogenesis and the behavioral changes observed herein are parallel effects of ATR deletion. This, however, is an unlikely possibility, as previous research has demonstrated an essential role for ATR in cell proliferation, and no role for ATR within mature neurons on numerous behavioral and circadian outcome measures (Brown and Baltimore, 2003; Ruzankina et al., 2007), suggesting deletion of ATR from the neural stem/progenitor cells directly affects neurogenesis and the subsequent behavioral changes are a result of that altered neurogenesis. In addition, irradiation–induced deficits in neurogenesis and associated behavioral alterations in the open field test were ameliorated by running, further suggesting the link between neurogenesis and behavioral changes in anxiety paradigms (Naylor et al., 2008).

In conclusion, we have shown here that hippocampal neurogenesis is an important determinant of some anxiety-like behaviors in mice. Findings of heightened anxiety would seem at odds with clinical evidence for the beneficial effects of moderate exercise. However, the data presented herein, in addition to recent literature highlighting the prevention of running-induced anxiety by x-ray irradiation (Fuss et al., 2010), strengthens the hypothesis that neurogenesis directly influences the anxiety-like behavior induced by running. As further explanation of exercise effects which seem counterintuitive to the beneficial effects observed in humans, running distances achieved by rodents may not be representative of the exercise behavior that is beneficial to the treatment of mood and anxiety disorders in humans and may indicate that too much neurogenesis can be detrimental to certain behaviors (Saxe et al., 2007). Indeed, moderate exercise is recommended as therapy for depressed mood in humans (Greer and Trivedi, 2009), often in conjunction with pharmacotherapy, and excessive exercise can have negative consequences (Peluso and Guerra de Andrade, 2005; Purvis et al., 2010; Czepluch et al., 2011). Thus, while rodent models of exercise are a valuable tool for studying neurogenesis, caution must be exercised in applying the results of unlimited voluntary wheel running to the human population. Future work should focus on developing rodent models of exercise which accurately mirror moderate exercise in humans, and on understanding the mechanisms through which newborn neurons in the hippocampus influence anxiety-like behaviors. This is especially pertinent in the context of efforts to develop therapeutic compounds that stimulate hippocampal neurogenesis for the treatment of mood disorders.

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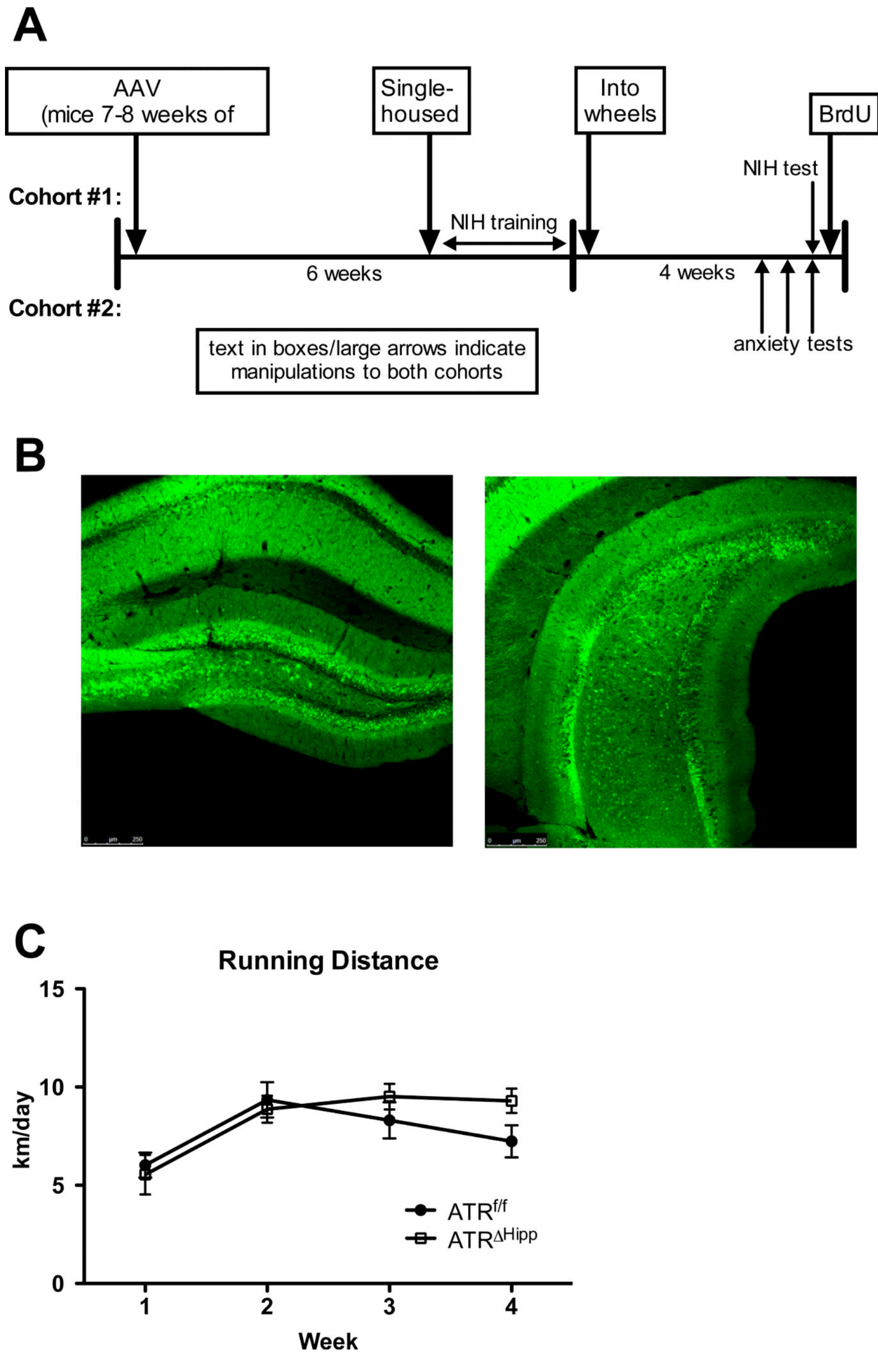

Figure 1.

Experimental design. **A** Two cohorts of mice were used in this study, the first for novelty-induced hypophagia and the second for open field and light-dark box tests. Both cohorts were injected with AAV at 7-8 weeks of age, single-housed approximately 5 weeks later, and then placed in running wheels for 4 weeks. Behavioral testing occurred in the final week of running. BrdU (200 mg/kg, IP) was injected 24 hours following the last behavioral test and mice were perfused 24 hours following BrdU injections. **B,C** Photomicrographs depicting eGFP expression in the dorsal (**B**) and ventral (**C**) hippocampus 8 weeks following AAV.eGFP injection. **D** Average weekly running distance. No significant differences were

observed in running distance between $ATR^{f/f}$ and $ATR^{\Delta Hipp}$ mice. Error bars represent SEM.

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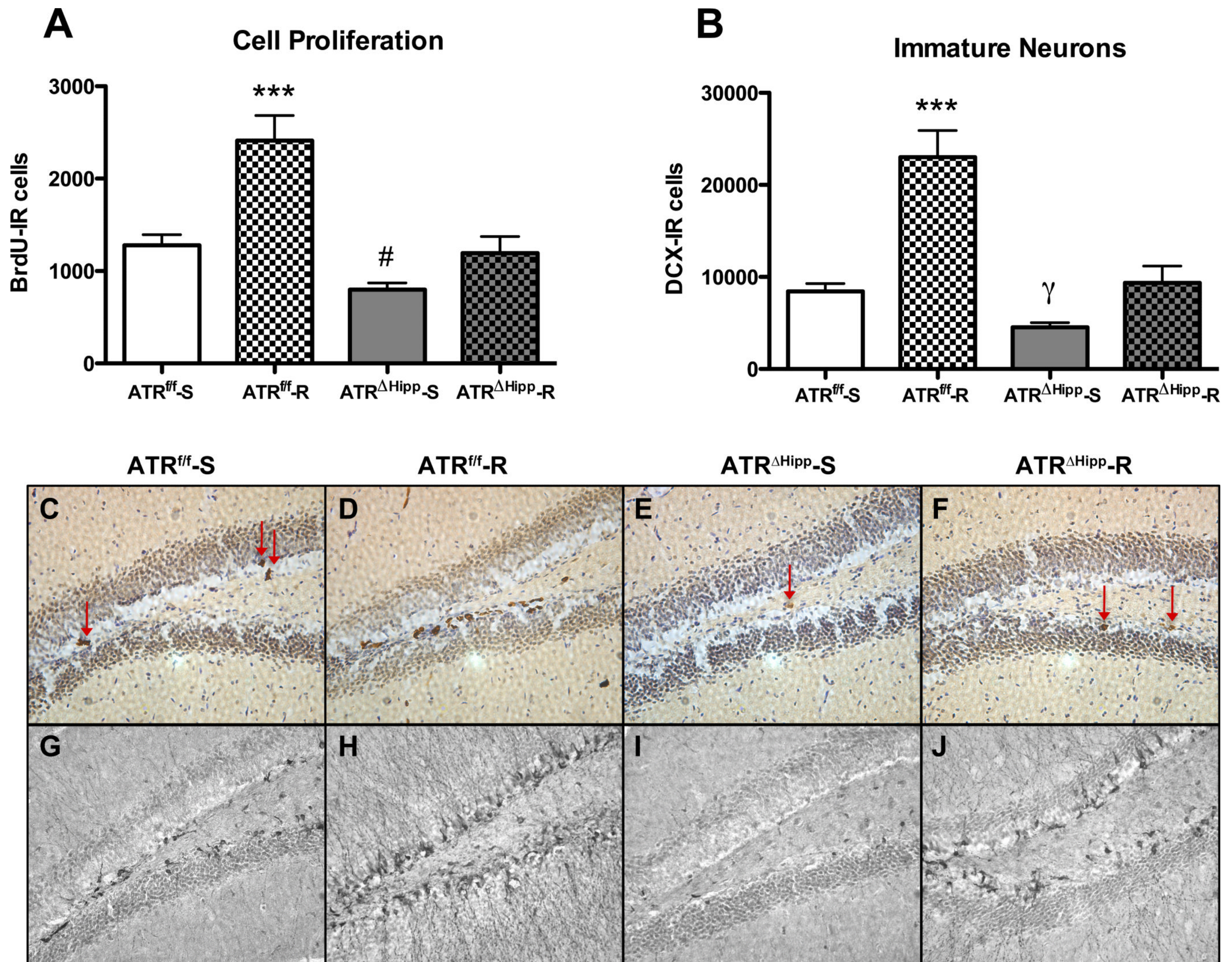

Figure 2.

Effects of ATR deletion and voluntary wheel running on neurogenesis. **A** Voluntary exercise resulted in a significant increase in BrdU-expressing cells in ATR$^{f/f}$ mice (***, p<0.001 vs. ATR$^{f/f}$-S, ATR$^{\Delta Hipp}$-S and ATR$^{\Delta Hipp}$-R groups, n=11-16 per treatment group). Among sedentary controls, ATR deletion resulted in significantly reduced BrdU-expressing cells (#, p<0.05 vs. ATR$^{f/f}$-S group). **B** Voluntary exercise resulted in a significant increase in DCX-expressing cells in ATR$^{f/f}$ mice (***, p<0.001 vs. ATR$^{f/f}$-S, ATR$^{\Delta Hipp}$-S and ATR$^{\Delta Hipp}$-R groups, n=11-16 per treatment group). Among sedentary controls, ATR deletion resulted in reduced DCX-expressing cells (γ, p<0.001 vs. ATR$^{f/f}$-S group *by Student's t-test*). **C-F.** Representative photomicrographs of BrdU immunohistochemistry in each of 4 treatment groups: ATR$^{f/f}$-S (**C**), ATR$^{f/f}$-R (**D**), ATR$^{\Delta Hipp}$-S (**E**) and ATR$^{\Delta Hipp}$-R (**F**). **G-J.** Representative photomicrographs of DCX immunohistochemistry in each of 4 treatment groups: ATR$^{f/f}$-S (**G**), ATR$^{f/f}$-R (**H**), ATR$^{\Delta Hipp}$-S (**I**) and ATR$^{\Delta Hipp}$-R (**J**). Error bars represent SEM.

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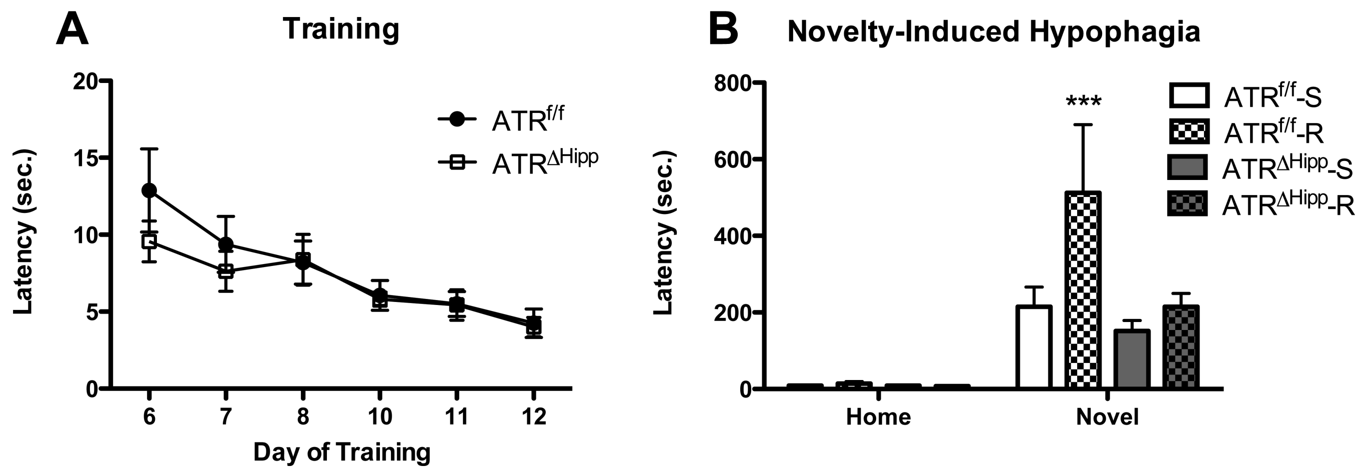

Figure 3.

Effects of ATR deletion and voluntary wheel running on behavior in the NIH test. **A** Home cage feeding latency prior to placement in running wheels. No gene effect observed. **B** Home cage and novel environment feeding latencies following wheel running. In the home test all mice exhibited comparable feeding latencies. On the novel test day ATR$^{f/f}$ runners exhibited significantly greater latency compared to each of the other treatment groups (***, $p<0.001$ vs. ATR$^{f/f}$-S, ATR$^{\Delta Hipp}$-S and ATR$^{\Delta Hipp}$-R, n=4-8 per group). Error bars represent SEM.

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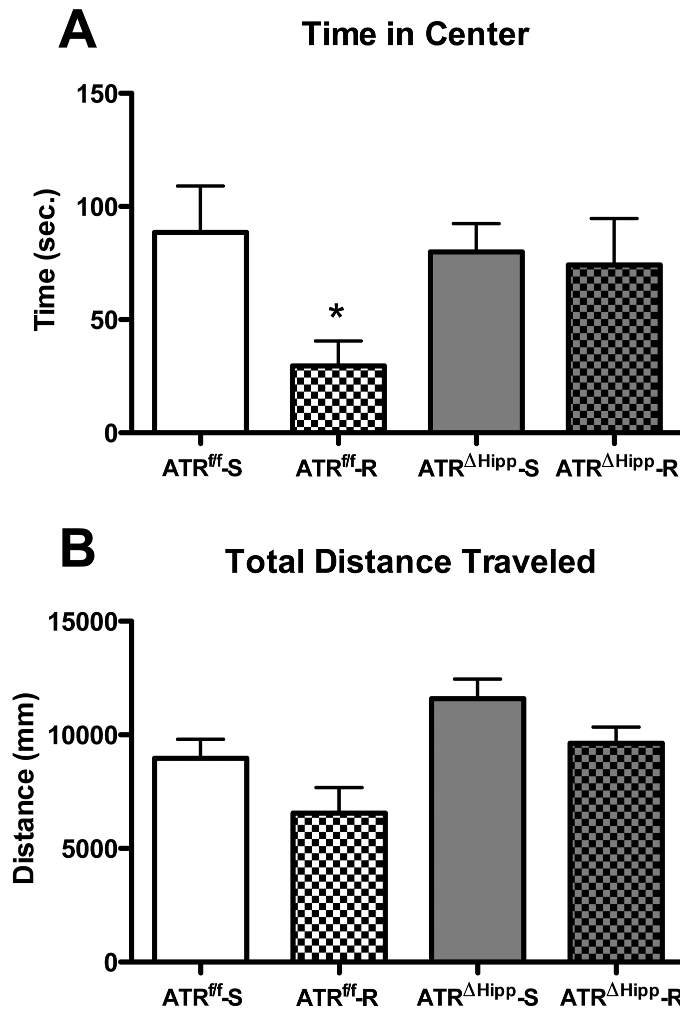

Figure 4.

Effects of ATR deletion and voluntary wheel running on open field behavior. **A** Time in center zone. $ATR^{f/f}$ runners spent less time in the center zone compared to their sedentary counterparts (*, $p<0.05$ vs. $ATR^{f/f}$-S). **B** Total distance traveled. Running exerted a main effect of reduced total distance traveled compared to sedentary mice. ATR deletion exerted a main effect of increased total distance traveled compared to control $ATR^{f/f}$ mice. There were no significant differences between individual groups. N=6-8 per group. Error bars represent SEM.

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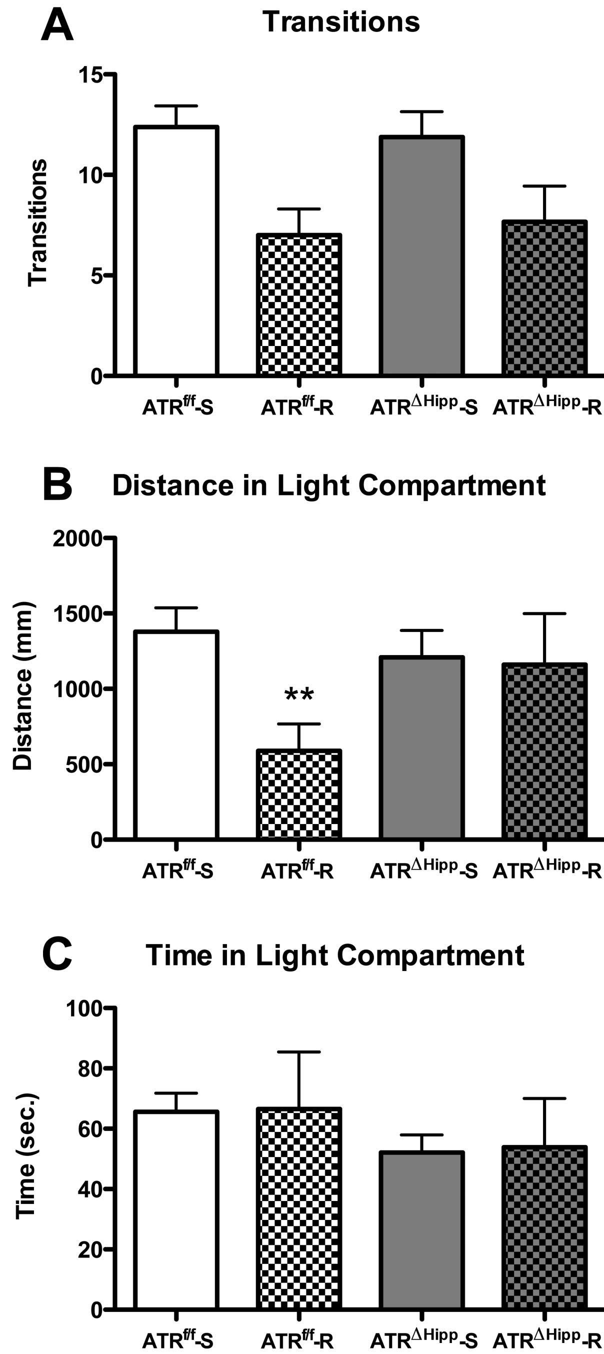

Figure 5.

Effects of ATR deletion and voluntary wheel running on light-dark box behavior. **A** Running exerted a main effect of reduced transitions compared to sedentary mice. **B** $ATR^{f/f}$ runners traveled a shorter distance in the light compartment compared to their sedentary counterparts (**, $p<0.01$ vs. $ATR^{f/f}$-S). This effect was not observed in $ATR^{\Delta Hipp}$ mice. **C** No differences were observed in total time spent in the light compartment. N=6-8 per group. Error bars represent SEM.