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Research article

A novel knockout mouse model of the noncoding antisense *Brain-Derived Neurotrophic Factor* (*Bdnf*) gene displays increased endogenous Bdnf protein and improved memory function following exercise

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ABSTRACT

Brain-derived neurotrophic factor (Bdnf) expression is tightly controlled at the transcriptional and post-transcriptional levels. Previously, we showed that inhibition of noncoding *Bdnf* antisense (*Bdnf*-AS) RNA upregulates Bdnf protein. Here, we generated a *Bdnf*-antisense knockout (*Bdnf*-AS KO) mouse model by deleting 6 kilobases upstream of *Bdnf*-AS. After verifying suppression of *Bdnf*-AS, baseline behavioral tests indicated no significant difference in knockout and wild type mice, except for enhanced cognitive function in the knockout mice in the Y-maze. Following acute involuntary exercise, *Bdnf*-AS KO mice were re-assessed and a significant increase in *Bdnf* mRNA and protein were observed. Following long-term involuntary exercise, we observed a significant increase in nonspatial and spatial memory in novel object recognition and Barnes maze tests in young and aged *Bdnf*-AS KO mice. Our data provides evidence for the beneficial effects of endogenous *Bdnf* upregulation and the synergistic effect of *Bdnf*-AS knockout on exercise and memory retention.

1. Introduction

BDNF is a neurotrophin that performs diverse and vital functions in the brain and central nervous system (CNS). *BDNF* is involved in the development and patterning of the nervous system as well as plastic changes in learning and memory and its loss is implicated in neurological disorders (Leal et al., 2014; Miranda et al., 2019; Notaras and van den Buuse, 2019). In humans, mature brain-derived neurotrophic factor (BDNF) binds tropomyosin kinase B (trkB), activating a complex signaling cascade that results in increased survival, growth and neuroplasticity in hippocampal neurons (Huang and Reichardt, 2003; Yoshii and Constantine-Paton, 2010). BDNF is found in hippocampal synapses where it is a potent regulator of neurons that play a key role in learning and memory, in part by altering synaptic spines (Wurzelmann et al., 2017; Zagrebelsky et al., 2020). The loss of neuroprotective factors is a key feature of neurodegeneration, particularly in Alzheimer's disease (AD) (Connor et al., 1997; Phillips et al., 2015). We and others have previously reported that BDNF is significantly downregulated in the post-mortem brains of patients with AD (Du et al., 2018; Modarresi et al., 2012). Reductions in BDNF result in increased oxidative stress, reduced neuroplasticity, diminished neuronal health and impaired recovery after damage, factors which contribute to neurodegeneration (Miranda et al., 2019). The pleiotropic effects associated with BDNF modulation have become a focal point of neurodegenerative and cognitive longevity research. Several studies have examined the therapeutic potential of intravenous or intrathecal administration of BDNF, however, the poor pharmacokinetic properties of BDNF protein administration have necessitated a more effective alternative approach to enhance *BDNF* signaling (A controlled trial of recombinant methionyl human BDNF in ALS: The BDNF Study Group (Phase III), 1999; Beck et al., 1994; Ochs et al., 2000). In addition to targeting *BDNF* receptor agonists such as *TrkB*, *p75*NTR and sortilin (Zagrebelsky et al., 2020), the delivery of the *BDNF* gene via Adeno-associated virus (AAV) vectors to the entorhinal cortex has also been attempted with limited success (Kells et al., 2008; Nagahara et al., 2018). Intranasal delivery of AAV-constructs containing BDNF fused to peptides that can cross the cell membrane have been

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attempted to directly deliver BDNF to the brain. However, although modest benefits have been seen, the difficulty in regulating dose, distribution throughout the body and potential immunogenicity to the AAV vector itself, have proven to be problematic (Arregui et al., 2011; Ma et al., 2016).

Previously, we showed that epigenetic modulation of the *Bdnf* locus can be achieved with synthetic antisense oligonucleotides (AntagoNATs), resulting in the upregulation of endogenous Bdnf (Modarresi et al., 2012). Modifying expression at the *Bdnf* locus to upregulate endogenous Bdnf is a physiologically relevant therapeutic approach as gene expression is tightly controlled such that downstream molecules are elevated to homeostatic brain levels. AntagoNATs upregulate *Bdnf* by blocking the activity of *Bdnf*-AS, a regulatory noncoding natural antisense RNA transcript (NAT) transcribed from the opposite strand of the *Bdnf* locus (Modarresi et al., 2012). NATs are a conserved class of noncoding RNA (ncRNA) molecules that can function as powerful suppressors that reduce the expression of a coding gene transcribed from the same locus. Downregulation can be achieved by recruiting repressive epigenetic enzymes to the locus from which the NAT is expressed (Faghihi and Wahlestedt, 2009; St Laurent and Wahlestedt, 2007). NATs have been identified for more than 40% of genes and they are expressed in a temporal and cell-type specific pattern (St Laurent and Wahlestedt, 2007). Importantly, ncRNAs are abundant in the brain, suggesting that they have an important role in the regulation of the nervous system (Faghihi and Wahlestedt, 2009; St Laurent and Wahlestedt, 2007). NATs are also differentially expressed in several neurodegenerative disorders (Faghihi et al., 2008; Magistri et al., 2015) including Parkinson's disease (Yamanaka et al., 2015) and Fragile X mental retardation syndrome (Khalil et al., 2008). Previously, we demonstrated that NATs play a pivotal role in AD pathophysiology (Faghihi et al., 2008, 2010), and can function as regulators of important AD-related genomic loci (Faghihi et al., 2008; Kang et al., 2014; Modarresi et al., 2011; Yamanaka et al., 2015), including Bdnf (Modarresi et al., 2012).

The *Bdnf* locus is regulated in part by *Bdnf*-AS, an antisense transcript that can suppress the expression of Bdnf mRNA and protein (Modarresi et al., 2012). We studied the physiological role of *Bdnf*-AS suppression by generating an *in vivo Bdnf*-AS knockout mouse model. However, the *Bdnf*-AS and *Bdnf* coding regions overlap and a direct knockout of *Bdnf*-AS alone is not possible. Therefore, we produced a mouse model for antisense RNA knockout by deleting the *Bdnf*-AS promoter. This novel mouse model lacking the *Bdnf*-AS promoter, termed *Bdnf*-AS KO mice, enabled us to investigate the effects of antisense RNA knockout for the first time *in vivo*. We utilized this model to determine the effects of the absence of *Bdnf*-AS transcription on Bdnf expression, learning and memory at baseline and in response to acute aerobic exercise. We also demonstrate that *Bdnf*-AS-mediated regulation of the *Bdnf* locus might be responsible for the reported beneficial effects of exercise on memory (Liu and Nusslock, 2018). Our data suggests that *Bdnf*-AS may be an important regulator of the exercise-induced cognitive benefits observed with concomitant Bdnf upregulation.

2. Results

2.1. Bdnf-AS promoter knockout mouse

We identified two splice variants of the *Bdnf*-AS transcript in mice, each with two exons. Both splice variants had an approximately 800 nucleotide-long overlapping region with the *Bdnf* transcript (Modarresi et al., 2012). However, the *Bdnf*-AS promoter is located downstream of the *Bdnf* gene, so we chose this as our target to knockdown *Bdnf*-AS. To design the optimal mouse embryo targeting strategy for the removal of the *Bdnf*-AS promoter region without disrupting *Bdnf* expression, we PCR-amplified 2-, 4-, 6-, and 8-kilobase segments downstream of the mouse *Bdnf* gene. The PCR products were then cloned into a promoter-less basic luciferase vector and firefly luciferase activity was measured using a dual luciferase assay as an indicator of promoter activity. (Supplementary Figure- 1). Insertion of the 6 kilobase region resulted in luciferase activity above the background, similar to the weak endogenous *Bdnf*-AS promoter activity previously measured in the brain. The 6-kilobase region amplified downstream of the mouse *Bdnf* gene was eliminated from the mouse genome using the FRT-LoxP flanked Neo cassette that was later removed by FRT-LoxP recombination and the deletion was confirmed by sequencing (Figure- 1A). The mouse embryo was targeted with this vector at the Ingenious targeting laboratory and two sets of primers were used to screen the mice for neomycin cassette deletion. The expected PCR product in wild type mice was 479 bp, while mice with the neomycin cassette deletion produced a 359 bp size product (Figure- 1B). A single band of 359 bp indicated mice that are homozygous (HO) for the *Bdnf*-AS promoter knock out, two bands (479 bp and 359 bp) indicated heterozygous (HZ) mice and a single band of 479 bp indicated wild type (WT) mice.

2.2. Baseline expression of Bdnf and Bdnf-AS in Bdnf-AS knockout mice

We measured the expression of *Bdnf* and *Bdnf*-AS in the hippocampus, cerebellum and prefrontal cortex of 4-week old wild type (WT) and homozygous *Bdnf*-AS promoter knock out (*Bdnf*-AS KO HO) mice. No significant differences in *Bdnf* mRNA expression were observed in any of the brain regions in both groups of mice (Figure- 1C). Considering the significance of this neurotrophin in brain development and throughout life, the homozygous knockout of the antisense transcript promoter in mice might exert compensatory mechanisms for maintaining steady physiological *Bdnf* levels in order to sustain life *in utero*. Our previous studies focused on short-term blocking technologies to knockdown *Bdnf*-AS in wild type mice, where we observed a robust upregulation in *Bdnf*. Presumably, after long-term *Bdnf*-AS depletion, an *in vivo* compensatory mechanism might prevail to prevent continuous Bdnf upregulation which could lead to adverse effects including epileptogenesis (Hu and Russek, 2008). *Bdnf*-AS expression in mice homozygous for *Bdnf*-AS KO had an 85–90% reduction in *Bdnf*-AS that was weakly detected with gene specific reverse primers. *Bdnf* antisense was significantly reduced ($p \leq 0.0001$) in the prefrontal cortex, hippocampus and cerebellum of *Bdnf*-AS KO HO mice as compared to wild type (Figure- 1D). The negligible *Bdnf*-AS expression we observed was likely a result of low levels of promoter-less expression, indicative of promoter leakage (Glover-Cutter et al., 2009). We measured *Bdnf* and *Bdnf*-AS in 15-week old mice and confirmed this data was consistent with the observed expression levels in 4-week old mice (Supplementary Figure- 2A, B). We were therefore able to specifically suppress the promoter activity of *Bdnf* antisense, indicating that *Bdnf*-AS knockout mice (*Bdnf*-AS KO) indeed lack *Bdnf*-AS expression, while leaving *Bdnf* mRNA unaffected.

2.3. Baseline behavior in Bdnf-AS KO mice and control wild-type littermates

At baseline, both groups of *Bdnf*-AS KO homozygous and wild-type mice had normal grooming behavior, similar mating activity and did not exhibit any alterations in life span (data not shown). Furthermore, we did not detect altered *Bdnf* mRNA expression despite observing reduced *Bdnf*-AS levels during normal habitual activities in mice (data not shown). Therefore, we studied changes in *Bdnf* expression following exercise to investigate a potential altered phenotype in the *Bdnf*-AS KO HO mice.

2.4. Baseline weight assessment and muscle strength in Bdnf-AS KO HO mice are similar to WT mice

Bdnf signaling was previously linked to weight loss and enhanced mitochondrial biogenesis in skeletal muscle (Wood et al., 2018). We measured weight gain in *Bdnf*-AS KO HO and WT mice over time and observed similar slopes in graphs of weight increase over age in *Bdnf*-AS KO HO and WT mice (Supplementary Figure- 3 A, B). We also measured

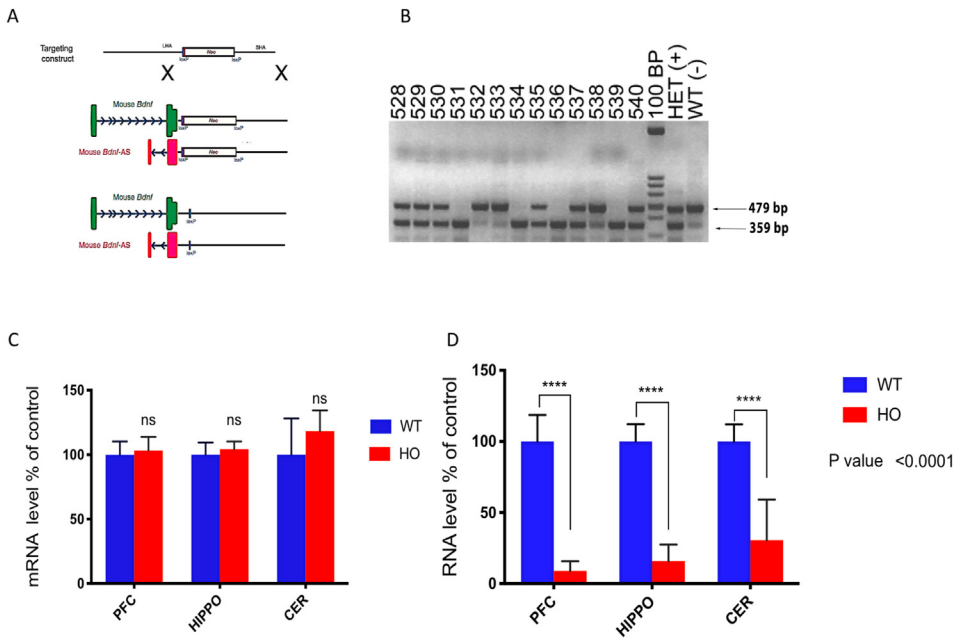

Figure 1. *Development of Bdnf-AS promoter knockout mice.* (A) *Bdnf*-AS promoter targeting construct and deletion strategy. Green boxes – mouse *Bdnf* exons, red boxes – mouse *Bdnf*-AS exons, empty boxes – Neomycin (Neo) cassette. Arrows indicate the direction of transcription. (B) PCR amplification of mouse tail DNA confirming Neo cassette deletion. Lanes are labeled with mouse identification numbers, 100 bp is the molecular weight marker lane, HET (+) is a mouse confirmed as heterozygous for the Neo cassette deletion, WT (-) indicates the WT mouse. *Bdnf* mRNA (C) and *Bdnf*-AS RNA (D) levels were measured in the prefrontal cortex (PFC), hippocampus (HIPPO) and cerebellum (CER) of 4-week old homozygous *Bdnf*-AS promoter knockout (HO) and wild type (WT) mice. Real time PCR data, WT n = 5, HO n = 5, ns – not statistically significant, ****$p \leq 0.0001$. Error bars represent standard error of the mean (SEM).

grip strength and as expected, we observed that females had slightly lower grip strength than males in both genotypes. There was no significant alteration in muscle grip strength in *Bdnf*-AS KO homozygous mice compared to wild type mice in any of the tested groups (Supplementary Figure- 4 A, B).

2.5. Baseline nonspatial and spatial memory are unchanged in Bdnf-AS KO HO compared to WT mice

Novel Object Recognition (NOR) and object location tests are performed to evaluate physiological and pathological processes of various brain regions in rodents and can be used jointly or individually to assess memory function (Denninger et al., 2018). No significant differences in discrimination and recognition indices (calculated using Eqs. 1 and 2) were observed between the *Bdnf*-AS KO HO and WT groups (Supplementary Figure- 5 A, B), indicating that knockout *Bdnf*-AS has no significant effect on the hippocampus and other brain regions involved in NOR memory (Lueptow, 2017). The Barnes maze test is commonly used to evaluate short- and long-term spatial memory (Gawel et al., 2019). We tested similar groups of *Bdnf*-AS KO HO mice and WT littermates and observed that these mice moved similar distances during the 3 days of acquisition (Supplementary Figure- 6A). Additionally, there was no difference between the two groups in latency to first approach and finding the goal box (Supplementary Figure- 6B). Probe phase results, including error hole pokes and duration of time spent in the target zone were also similar at baseline (Supplementary Figure- 6 C, D), indicating no significant alterations in learning in *Bdnf*-AS KO homozygous mice compared to wild type in baseline conditions without exercise. Collectively, these findings indicate that any alterations in memory performance in mice are independent of hippocampal involvement at baseline.

2.6. Baseline anxiety is unchanged in Bdnf-AS KO HO mice as compared to WT mice

To evaluate anxiety levels in the *Bdnf*-AS KO HO mice, we performed the elevated plus maze. The percentage of time spent inside of and frequency of entries to the open arms were recorded and Eq. 3 was used to calculate the anxiety index (Walf and Frye, 2007). Our data indicate that there are no significant differences between anxiety index and nose poke frequency to open arms in the two groups of mice (Supplementary Figure- 7, A, B, C). Zero (O maze) is also commonly used to evaluate anxiety in mice by measuring an anxiety index similar to the elevated plus maze (Walf and Frye, 2007). We did not observe a significant difference in anxiety levels between *Bdnf*-AS KO HO and wild type mice (data not shown). Collectively, data from these studies indicate that anxiety levels were not altered in *Bdnf*-AS KO HO mice compared to WT littermates.

2.7. Baseline prefrontal cortex function is improved in Bdnf-AS KO HO mice

Y-maze is widely used in assessing short-term memory and the effects of prefrontal cortex functioning in transgenic mice (Maggi et al., 2018). Our data indicate that *Bdnf*-AS KO homozygous mice had significantly ($p \leq 0.0001$) better memory function (about 30% improvement) as indicated by their spontaneous alternation performance (SAP) (calculated using Eq. 4) as depicted in Figure- 2A. When we adjusted for sex, the same trends in SAP were observed and we saw a significant difference between male ($p \leq 0.001$) and female ($p \leq 0.01$) *Bdnf*-AS KO HO mice as compared to wild type littermates (Figure- 2B), suggesting this finding is not sex-dependent. As expected, total arm entries for arms A, B, and C did not differ significantly in the *Bdnf*-AS KO HO mice (Figure- 2C). At baseline, we observed that *Bdnf*-AS KO HO mice performed better in the Y-maze, indicating stronger prefrontal cortex baseline function as compared to wild type mice. An improvement in short-term memory without a corresponding increase in *Bdnf* mRNA could be the result of differences in the expression of the Bdnf receptor TrkB, which is more abundant in the mouse prefrontal cortex (Allen Institute for Brain Science., © 2021). These results indicate that our *Bdnf*-AS KO HO mice had improved short-term memory at baseline.

2.8. Bdnf is upregulated after a single bout of involuntary exercise in Bdnf-AS KO HO mice

Exercise-induced upregulation of *Bdnf* and its downstream physiological effects such as increased neural plasticity and associated improvements in learning and memory have been widely reported (da Costa Daniele et al., 2020; Pedersen, 2019). To characterize the effect of exercise on our *Bdnf*-AS KO HO mice, two groups of *Bdnf*-AS KO HO and WT mice underwent acute involuntary exercise as per the timeline depicted

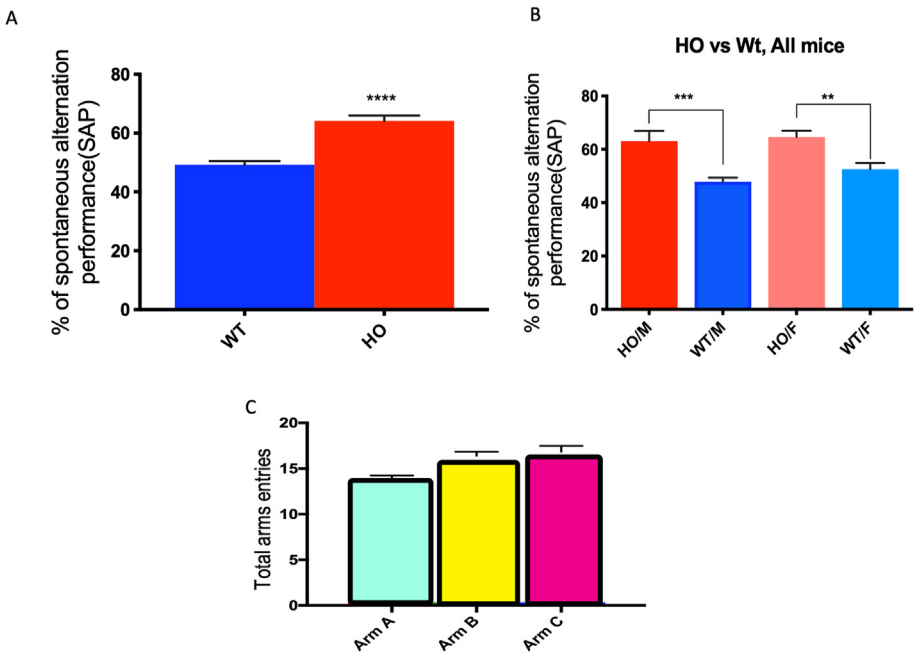

Figure 2. *Y maze test without exercise shows improved hippocampal function in Bdnf-AS KO HO mice.* (A) Spontaneous alternation performance (SAP) in *Bdnf*-AS KO HO (HO, n = 45, M = 23, F = 22) was significantly increased as compared to WT 6- to 8-week old mice (WT, n = 42, M = 20, F = 22) mice, M – male, F - female. (B) SAP data in (A) was adjusted for sex. (C) Total number of arm entries for arms A, B, and C were measured for all test subjects. ns – not statistically significant, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Error bars represent SEM.

in Figure- 3 A. We use an involuntary exercise protocol to mimic the effect of acute exercise to measure changes in *Bdnf* mRNA in exercise-naive wild type and KO HO mice. Previous publications have shown differing changes in Bdnf following chronic and even a single prior exposure to exercise. Therefore, we eliminated this potential confounding factor by selecting an acute involuntary exercise model to measure

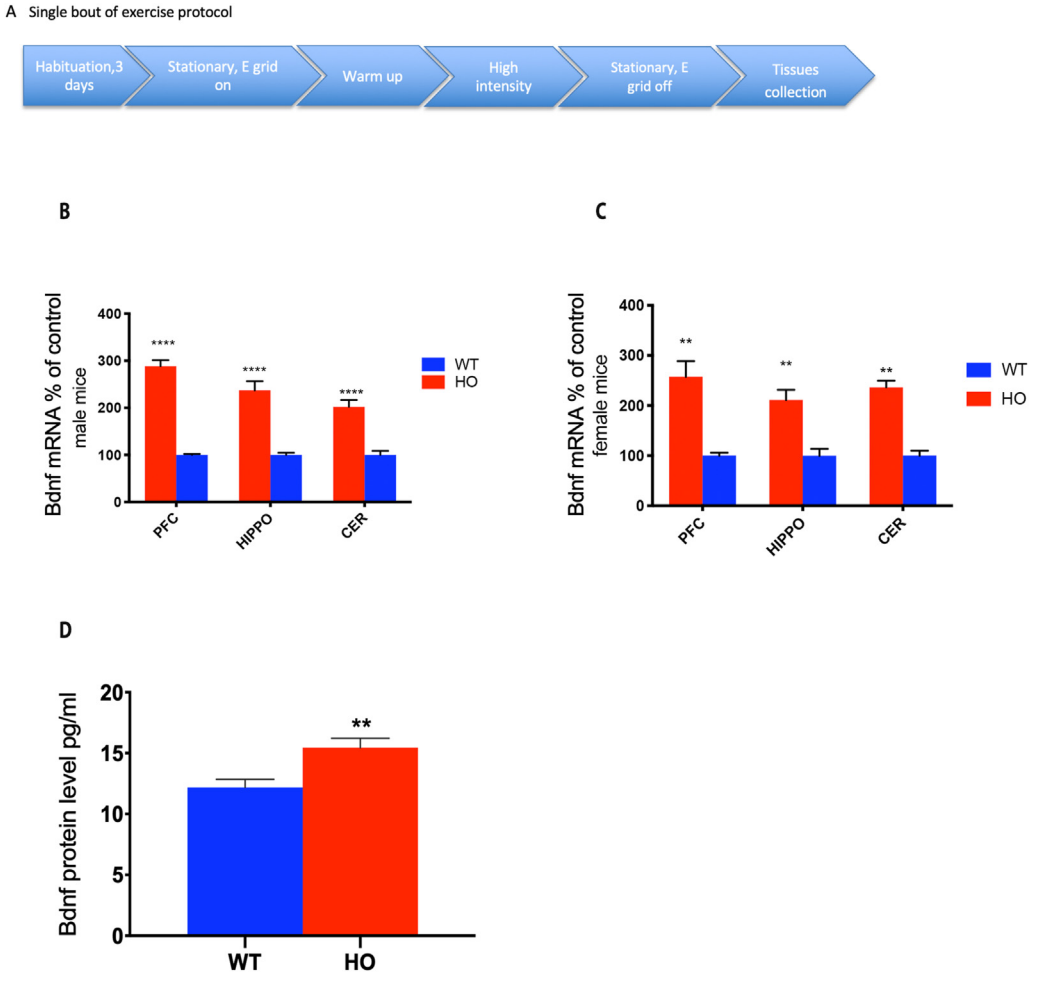

Figure 3. *Bdnf upregulation after a single bout of exercise in WT and Bdnf-AS KO HO mice.* (A) Timeline for the single bout of exercise experiments in 6–8 week old mice. Post-exercise *Bdnf* mRNA levels are measured in three brain regions [prefrontal cortex (PFC), hippocampus (HIPPO) and cerebellum (CER)] of homozygous *Bdnf*-AS promoter knockout mice and wild type (WT) (B) male and (C) female mice. Real time PCR data, n = 7 WT (4 male, 3 female) and n = 11 HO (8 male, 3 female). (D) Bdnf is measured in the hippocampus of homozygous *Bdnf*-AS KO HO and wild type (WT) mice following exercise, measured by ELISA, n = 3 WT, 3 HO. **p ≤ 0.01, ****p ≤ 0.0001. Error bars represent SEM.

changes in Bdnf mRNA and protein to determine if there is a difference between WT and KO HO mice (Venezia et al., 2017). We collected three brain regions (hippocampus, cerebellum and prefrontal cortex) and utilized real-time PCR (RT-PCR) to measure the relative quantity of *Bdnf*. Following a single bout of exercise, a roughly 3-fold increase ($p \leq 0.0001$) in the prefrontal cortex, 2.5 fold increase ($p \leq 0.0001$) in the hippocampus and 2-fold increase ($p \leq 0.0001$) in the cerebellum was observed in *Bdnf* mRNA in male *Bdnf*-AS KO HO mice as compared to wild type mice (Figure- 3B). Following a single bout of exercise in female mice, a 2.5-fold increase ($p \leq 0.01$) in the prefrontal cortex, 2 fold increase ($p \leq 0.01$) in the hippocampus and 2.3-fold increase ($p \leq 0.01$) in the cerebellum was observed in *Bdnf* mRNA in *Bdnf*-AS KO HO mice as compared to wild type (Figure- 3 C). Although *Bdnf* mRNA was significantly upregulated in all three brain regions in male and female KO HO mice, the level of upregulation is less potent in females. This difference in *Bdnf* mRNA upregulation following a single bout of involuntary exercise can potentially be explained by sex-dependent differences in *Bdnf* regulation resulting from differences in the regulation of energy homeostasis (McAllan et al., 2018). We also subjected another cohort of *Bdnf*-AS KO HO and WT (male and female) mice to similar involuntary exercise for 55 min and euthanized them 16 h later. These mice were subjected to the same acute involuntary exercise, however they were sacrificed 16 h following exercise to capture changes in protein expression which naturally require a longer period to take effect. We extracted protein from the hippocampus of euthanized mice and measured Bdnf protein on fresh samples by ELISA. We observed marked upregulation [about 25%, ($p \leq 0.01$)] of Bdnf protein in the hippocampus of *Bdnf*-AS KO HO mice following a single bout of involuntary exercise as compared to wild-type littermates (Figure- 3 D). Although *Bdnf* mRNA in the hippocampus was upregulated by 2-fold, we observed a smaller, yet still significant increase in Bdnf protein. This finding is in line with our previous findings that a more potent upregulation in *Bdnf* transcription is required for increased Bdnf protein levels (Modarresi et al., 2012). We observed a consistent and significant increase in Bdnf mRNA and protein expression in *Bdnf*-AS KO HO mice as compared to wild type littermates following a single bout of involuntary exercise.

2.9. Exercised Bdnf-AS KO HO mice had improved spatial and nonspatial memory as compared to WT littermates

We had previously measured baseline performance in Novel Object Recognition (NOR) (Supplementary Figure 5) and found no significant difference in discrimination and recognition indices between WT and KO HO mice. Our acute involuntary exercise experiments (Figure 3) indicated that Bdnf mRNA and protein are upregulated in KO HO following exercise. Therefore, we hypothesized that longer-term exercise exposure would alter memory in KO HO mice as compared to WT. To test this, we exposed young homozygous and wildtype (6–8 week old) mice to involuntary treadmill exercise using the schema depicted in Figure- 4 A. We designed this protocol to mimic a longer-term chronic exercise regimen in exercise-naïve mice and to also control for the amount and duration of the exercise as any prior exercise can result in altered Bdnf responses in mice (Venezia et al., 2017). After two weeks of involuntary daily exercise to assess changes in nonspatial memory (Cohen and Stackman, 2015), we measured the discrimination index (Eq. 1) and found that *Bdnf*-AS KO HO mice performed significantly better ($p \leq 0.01$) than their WT littermates (Figure- 4 B). Interestingly, the WT mice had a negative discrimination index, indicating a stronger preference to explore familiar rather than novel objects (Cohen and Stackman, 2015). The recognition index (Eq. 2) was 2-fold higher ($p \leq 0.01$) in homozygous *Bdnf*-AS KO HO mice compared to their wild type littermates (Figure- 4 C). The improved NOR in *Bdnf*-AS KO HO (as assessed by discrimination and recognition indices) suggests a coordinated increase in memory performance that results in significant alterations in memory function and consequently performance on the NOR. These mice were returned to their conditions and continued a daily exercise schedule for an additional two weeks, prior to performing the Barnes Maze test to assess changes in long-term spatial memory (Gawel et al., 2019). We monitored acquisition phase data (training) to

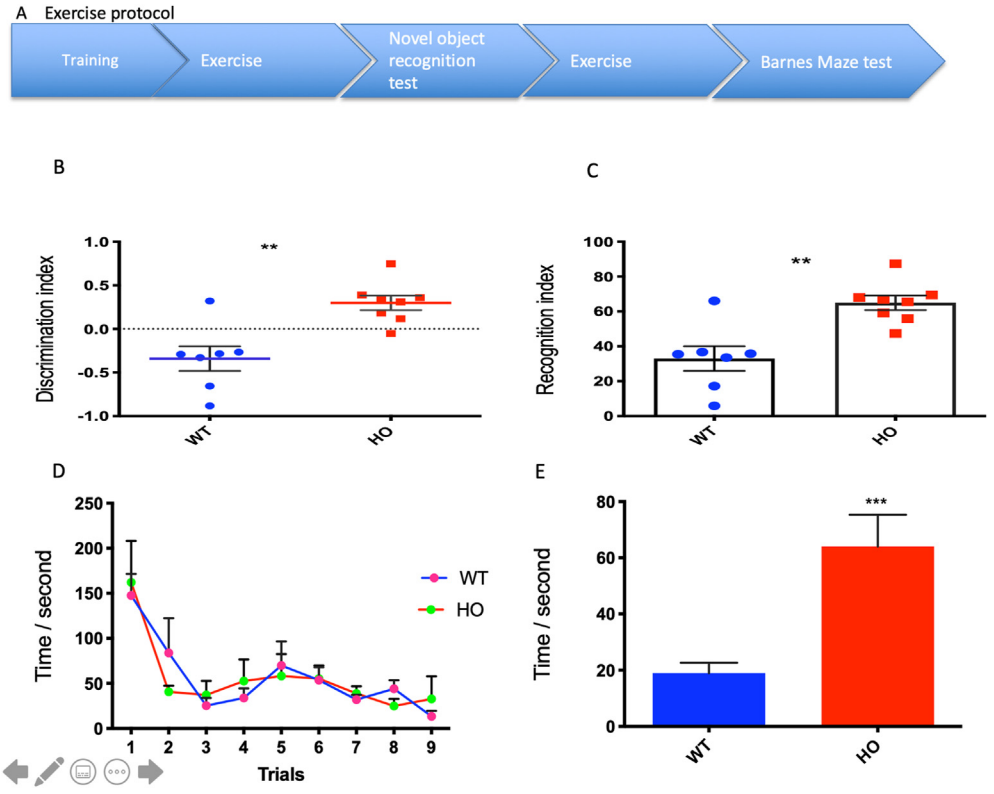

Figure 4. *Memory improvements are observed in exercised Bdnf-AS KO HO mice as compared to WT littermates.* (A) Exercise and testing protocol in 6–8 week old mice. The training phase consisted of three days of low speed exercise at 5–7 m/min for 10 min. Exercise phase included daily 30-minute involuntary treadmill exercise at a maximum speed of 13 m/min for 2 weeks, excluding weekends. Novel object recognition test (NOR) was performed after two weeks of exercise. Daily 30-minute exercise continued after NOR for an additional 2 weeks followed by the Barnes Maze test. Discrimination (B) and recognition (C) indices were measured in the NOR test in *Bdnf*-AS KO HO mice (HO) and WT littermates (WT) following the protocol in (A). (D) Changes in time to finding the goal box in the Barnes Maze test in *Bdnf*-AS KO HO mice (HO) and WT littermates (WT) during the acquisition phase. (E) Time in the target zone in the Barnes Maze test in *Bdnf*-AS KO HO mice ($n = 8$) and WT littermates ($n = 9$). $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Error bars represent SEM.

show that both groups had similar training and found the goal box in a timely manner (Figure- 4 D), assuring us that our data were free of training bias. We found that homozygous *Bdnf*-AS KO HO mice spent three times longer in the target zone as compared to their wild type littermates, indicating significant ($p \leq 0.001$) improvements in long-term hippocampal-dependent changes in spatial memory following exercise (Figure- 3 E). Additionally, we exercise-trained two cohorts of aged (15–17 month old) mice, using an abbreviated training protocol to account for the natural inability of the older mice to perform aerobic exercise for the same duration as their younger counterparts. The results were roughly equal in magnitude and significance to our long-term exercise studies in young mice with significantly better performance as assessed by both recognition and discrimination indices in the object location memory (OLM) subthreshold test in the aged mice (Supplementary, Figure- 8 A, B). Collectively, our data suggests that exercise leads to improved nonspatial and spatial memory in *Bdnf*-AS KO homozygous mice compared to WT over a broad age range.

3. Discussion

Forty percent of long noncoding RNAs are expressed specifically in the brain, a number of which have been shown to play fundamental roles in neurological development, plasticity and degenerative processes (Briggs et al., 2015). Here, we presented the first knockout animal model that provides direct proof that Bdnf can be specifically targeted and altered *in vivo* by ablating antisense transcript expression. The *Bdnf* locus harbors a powerful 'brake' that regulates its expression through epigenetic mechanisms (Tao et al., 1998). This complex gene structure is also controlled by a noncoding antisense RNA, *Bdnf*-AS, that suppresses the expression of Bdnf mRNA and protein (Modarresi et al., 2012). Highlighting the suppressive role of *Bdnf*-AS *in vivo* is our finding that mice that lack *Bdnf*-AS respond strongly, with marked *Bdnf* elevation, in response to exercise stimulus.

The neurological benefits of aerobic exercise have received much attention in the last two decades however the biological mechanisms behind such benefits have yet to be fully elucidated (Cabral et al., 2019). To date, upregulation of a series of trophic factors, including BDNF, have been found to be essential for neurocognitive improvements observed post-acute exercise in animal models (Chen and Russo-Neustadt, 2009; Choi et al., 2018; Vaynman et al., 2004). In our study, Bdnf increases several-fold in multiple brain regions only after exercise and this correlates with improved spatial learning and memory in a series of behavior tests in our animal model. We showed that physical exercise, widely studied as a potential modulator of neurodegeneration elevates Bdnf expression more efficaciously in situations when the *Bdnf*-AS mediated transcription is disabled. A recent report showed increased neurogenesis and cognition upon administration of circulating factors from the blood of exercised mice into sedentary elderly mice, suggesting a novel liver-brain axis (Horowitz et al., 2020). Indeed, a number of mechanisms and signaling factors, including altered metabolism could be responsible for altering Bdnf expression following exercise. Therefore, it is possible that the effects of exercise could be compounding and more potent than originally thought. Furthermore, both exercise and BDNF upregulation have been touted as ways to combat neurodegeneration, therefore a combination of the two might produce a synergistic therapeutic effect that limits cognitive decline.

BDNF plays a central, universal role in cognitive function and an understanding of the regulation of BDNF mRNA and antisense transcription following exercise will be extremely valuable in the context of neurodegenerative research. Additionally, crossing the *Bdnf*-AS KO HO mouse with an Alzheimer's disease mouse model could provide key information on the molecular mechanisms that underlie the reported exercise-induced therapeutic effects of Bdnf in neurodegenerative processes as both preventative and therapeutic measures, providing key insights into the significance of this approach for pharmacologic intervention.

BDNF is essential for neuronal plasticity, survival and recovery and also plays an important role in cognitive decline during normal aging and in the course of AD (Choi et al., 2018; Connor et al., 1997). Using a sophisticated targeting strategy, we were able to generate a mouse that did not express a non-coding antisense RNA previously shown to be important in the regulation of the *Bdnf* locus. The *Bdnf*-AS-KO HO mouse had normal weight, grip strength, life span and behaviors, except for improved hippocampal cognition, as compared to wild type mice at baseline. However, following involuntary exercise, we measured a significant increase in endogenous *Bdnf* in our *Bdnf*-AS KO homozygous mice that was not observed in wild type littermates. The degree of *Bdnf* mRNA upregulation differed in the prefrontal cortex, hippocampus and the cerebellum. Although we observed the greatest increase in mRNA in the prefrontal cortex it is unclear if the quantifiable difference alters protein translation or is regulated by other mechanisms. The increased Bdnf correlates with behavior studies showing improved nonspatial and spatial learning and memory in a battery of tests following involuntary exercise in *Bdnf*-AS KO HO mice. This study highlights the importance of mouse models of noncoding RNAs and also presents a valuable tool that can be used to further study the mechanism of Bdnf regulation in the nervous system, particularly in the context of aerobic exercise.

3.1. Limitations of the study

The animal model used in this study is entirely novel and is a powerful tool for further study of the *in vivo* mechanisms that regulate the function of noncoding RNAs. However, due to time and funding limitations, we were unable to include several interesting studies which would have provided direct evidence for the clinical relevance of our work. Crossing our mice with different models of neurodegenerative disease would have allowed us to determine if the knockout phenotype could help slow or reverse neurodegeneration in various disease contexts. Furthermore, although our error bars were small and our data were significant, repeating tests in larger sample sizes and performing immunohistochemistry to observe potential changes in neurogenesis in the hippocampus would have enhanced our findings. However, further understanding of the kinetics and temporal changes in *Bdnf* throughout the life of the *Bdnf*-AS KO HO mice will necessitate larger, more well-funded studies which we sincerely hope our colleagues will be able to spearhead.

4. Star methods

4.1. Resource availability

4.1.1. Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Mohammad Ali Faghihi (mfaghihi@expressgene.us).

4.1.2. Materials availability

This study generated new and unique luciferase constructs used to target and knockout the *Bdnf*-AS promoter. The knockout mouse model targeting the promoter of the *Bdnf*-AS gene is also novel and unique to this study. Upon acceptance for publication, targeting constructs will be deposited to Addgene and the knockout mouse will be deposited to the Knockout Mouse Project (KOMP).

4.1.3. Data and code availability

All primer sequences are available in the Methods section.

4.2. Experimental model and subject details

Novel *Bdnf*-AS promoter knockout mice were generated for this study using targeting constructs that ablate the *Bdnf*-AS promoter. Transgenic mice were generated with this construct in a C57Bl/6 background which

is outlined in the methods section of this manuscript. All animal procedures and behavioral tests were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami Miller School of Medicine. Mice were housed in groups of 3–5 per cage to reduce social isolation stress and all cages had free access to food and water at all times in addition to extra enrichment with a cotton pad. Animal cages were maintained under 12-hour day and night light cycles. All behavioral tests were done from 9 am to 6 pm and mice were returned to their holding room before 6pm. No night studies were performed. For all behavioral tests, animals were moved into the behavior room for 30–60 min prior to testing to allow the mice to acclimate to the room conditions.

4.3. Method details

4.3.1. Knockout of the Bdnf-AS promoter

To produce mouse *Bdnf*-AS knock out mice, we first cloned 2, 4 and 6 kilobases upstream of *Bdnf*-AS into a promoter-less basic luciferase vector (Promega, CA). The expression of luciferase was calculated as a reflection of promoter activity. We designed the *Bdnf*-AS promotor region (6 kb) together with short and long arm homology regions into vectors.

4.3.2. Generation of the knockout Bdnf-AS mouse model

We outsourced transgenic mouse development to the Ingenious Mouse Targeting Company (Ingenious, USA). Briefly, vectors with long and short homology arms with loxP at both sides of neomycin cassettes were used to transfect embryonic stem cells. Targeted embryonic stem cells were then injected into blastocysts to generate chimeric mice. Mice with a high percentage black coat color were crossed to C57Bl/6 mice to remove the Neomycin cassette. Primers NDEL1, NDEL2 and WT1 were used to screen mice for deletion of Neomycin cassette. The PCR product for the wild type mouse is 479 bp. After Neomycin deletion, one set of Loxp-FRT sites remained (160 bp). Two sets of primers FLP1 and FLP2 were used to screen mice for the presence of FLP transgenic mice. Amplified products for primer sets of FLP1 and FLP2 were 725 bp. Tail DNA samples from positive mice were amplified with new sets of primers (B1 and NEOGT) to confirm integration of the short homology arm. NEGT is located inside the Neo cassette and B1 is upstream of the short homology arm, outside the region used for the targeting construct. B1/NEGT amplifies a fragment of 6.09 kb in length. Confirmed somatic Neo deleted mice were set up for mating with C57BL/6 wild type mice to generate germline Neo deleted mice. FLP1 and FLP2 primers were used to confirm FLP deletion in mice. Confirmed FLP absence germline Neo deleted mice were mated to each other to generate homozygous germline Neo deleted mice. Forward primer NDEL1: 5′-TGG CAT TAA AAC TTA ACC ACA TCA GAA GCC -3′ Reverse primers: NDEL2: 5′-CTT TGA CCA CCT TCA GAG ATG TGA CAG -3’; WT1: 5′-AAT CAA ATA GAG CCT GTC AGC CCT GG -3’.

4.3.3. Colony maintenance and genotyping of knock out mice

We received a limited number of mice from Ingenious laboratory. The mice were quarantined upon receipt as per the University of Miami's policies. Following quarantine, breeding cages were set up to expand the mouse colony. All pups from breeding cages were genotyped by using 1 μg tail DNA, using Ingenious laboratory's primers sequences and PCR protocol. New breeding cages were set up in order such that parents with the same genotype were in breeding cages (WT + WT and HO + HO) to avoid possible genotyping mistakes. All mouse pairs for breeding cages were confirmed by genotyping prior to being placed in breeding cages.

4.3.4. RNA extraction, cDNA synthesis and RT-PCR

After expanding the mouse colony, we collected tissues from different brain regions from both wild type and *Bdnf*-AS KO homozygous mice. The tissue was mechanically homogenized and RNA was extracted with TRIzol (Thermo Fisher, CA), before being applied onto an RNeasy RNA column (Qiagen, MD). RNA was eluted into 30 μl ddH_2O and RNA concentration was determined by Nanodrop (Thermo Fisher, CA). cDNA was synthesized using 800 ng total RNA and random hexamers (Thermo Fisher, CA). We performed real-time PCR using gene specific Taqman primers and probes for *Bdnf*, *Bdnf*-AS, *beta-actin* and *Gapdh* as housekeeping genes (Thermo Fisher, CA). Sequences for primers used in this study are included in Supplementary Table 1. The *Bdnf* expression data was normalized to the average cycle threshold (Ct) of the two housekeeping genes.

4.3.5. Protein extraction and ELISA

Total protein was extracted immediately from the dissected brain region of one brain hemisphere into 200 μl lysis buffer of M-PER mammalian protein extraction reagent (Thermo Fisher, CA) supplemented with 100X Halt™ protease inhibitor cocktail (Thermo Fisher, CA). Bdnf protein was quantified by ELISA using the Promega BDNF Emax Immunoassay System (Promega, CA) as per the manufacturer's protocol. The complete protein extraction and ELISA protocols are available in the Supplementary methods.

4.3.6. Behavioral studies

All animal procedures and behavioral tests were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami Miller School of Medicine. Mice used in each experiment were experimentally naïve and were housed in groups of 3–5 per cage to reduce social isolation stress and all cages had free access to food and water at all times in addition to extra enrichment with a cotton pad. Animal cages were maintained under 12-hour day and night light cycles. All behavioral tests were done from 9 am to 6 pm and mice were returned to their holding room before 6pm. No night studies were performed. For all behavioral tests, animals were moved into the behavior room for 30–60 min prior to testing to allow the mice to acclimate to the room conditions.

4.3.7. Weight and grip strength assessment

Body weight was measured once a week for 24 consecutive weeks in 17 males (8 WT, 9 KO HO) and 15 females (7 WT, 8 KO HO) starting at 4 weeks of age. Muscle strength was measured in *Bdnf*-AS KO HO mice and WT littermates using a muscle strength test device, Bioseb model Bio-GS3. Grip strength was measured in a total of 27 WT and 32 *Bdnf*-AS KO HO male and female mice from various age groups. The strength of the front paws and 4 legs were recorded 5 times each. We then normalized all recorded values to weight for each mouse.

4.3.8. Novel object recognition (baseline)

The test includes three phases; habituation to open field, acquisition and recognition phases. The testing apparatus consisted of two plexiglass boxes that measure 30 × 30 × 20 cm^3. The testing apparatus was cleaned after each trial with 50% ethanol and was dried completely to eliminate olfactory cues. During acquisition, mice are introduced to two identical objects located 6 cm away from each side wall. During recognition, one object is replaced by a new object in the same location whereas in object location memory and one object is moved to a new location. We tested *Bdnf*-AS KO homozygous and wild type mice at the age of 8 weeks with a standard 10-minute novel object recognition test (NOR) in baseline conditions without involuntary exercise. Mice had undergone habituation to the field for 10 min twice a day for 3 days. During the acquisition phase, they were introduced to two identical objects for 10 min each. One hour later, during the recognition phase, mice were exposed to one familiar and one new object for 10 min. All trials were recorded and data were calculated using Noldus Ethovision 8.5 software. Exported data were used to calculate the discrimination index (DI) and recognition index (RI) using the following equations:

$$\text{Discrimination Index (DI)} = \frac{\text{Time exploring novel object} - \text{time exploring familiar object}}{\text{Time exploring novel object} + \text{time exploring familiar object}} \quad (1)$$

$$\text{Recognition Index (RI)} = \frac{\text{Time exploring novel object} \times 100}{\text{Time exploring novel object} + \text{time exploring familiar object}} \quad (2)$$

4.3.9. Elevated plus maze and O maze without exercise (baseline)

To evaluate the level of anxiety in this novel knockout mouse, we performed two behavior tests commonly used to assess anxiety levels. We tested 30 homozygous *Bdnf*-AS KO and 40 wild type mice between the ages of 10–12 weeks for this study. Elevated plus maze is a cross shape apparatus with 4 arms that join at the center. Two opposing arms with sidewalls and the other two arms and center parts are open. The apparatus is elevated 45 cm from the ground. The subject is introduced to center facing a closed arm without previous habituation to the maze. Time spent in open arms was compared to the total time in closed arms. Frequency of entries to open arms compare to closed arm were also measured. Mice show a preference for parts of the maze that have a side wall. Trials were recorded and data produced using Noldus EthoVision 8.5 software. The anxiety index is calculated as follows (Eq. 3):

$$\text{Anxiety index} = 1\text{- ([open arm time/total time]+[open arm entries/total number of entries]/2)} \quad (3)$$

4.3.10. Zero-maze

The zero-maze consists of a circular apparatus with two open and two sides with lateral walls. We tested 26 homozygous *Bdnf*-AS KO and 36 wild type mice between 10-12 weeks for this test.

$$\text{Spontaneous Alternation Percentage} = \frac{\#\text{ of Spontaneous Alternation} \times 100}{\text{Total number of movements} - 2} \quad (4)$$

4.3.11. Barnes Maze test (baseline)

We tested 5 *Bdnf*-AS KO homozygous and 5 wild type mice at 8 weeks to evaluate base line differences. All mice went through three phases of tests including habituation, acquisition and probe (24 h after last trial of acquisition). The Barnes maze apparatus is a table 100 cm in diameter and 100 cm high that has 20 holes; each hole is located 3 cm from the peripheral border. A small hidden box is fixed under one hole and the whole area of the table is divided into 4 zones by two diagonal lines. The zone including the hidden box is the target zone. Each mouse was placed in the center of the table under a dark paper box for 15 s, before being exposed to the open table surface. For three days, each mouse had 3 min per trial, 3 trials per day and a 20-minute intertrial rest period. The acquisition phase consisted of 3 trials per day for each mouse. Each trial ended when either the mouse identified the goal box or at 3 min, whichever came first. The probe was performed 24-hours after the last trial, by removing the goal box and turning the table 360°. The time spent in the target zone and number of error pokes to each hole is calculated using Noldus Ethovision software version 8.5.

4.3.12. Y-maze

Y maze has been widely used in many cohort studies to evaluate spatial working memory in rodents. It can be assessed by introducing mice to the maze without previous exposure. Mice will explore the maze because of the inherent curiosity of exploring a new environment. The maze has a three armed designed with 120-degree angles between arms. Homozygous *Bdnf*-AS KO (n = 45) and wild type (n = 42) male and female mice of various ages between 9-15 weeks old were tested. Animals were brought to the testing room 60 min before actual testing to acclimate to room conditions. Mice were exposed to the maze for 5 min without previous exposure and no habituation experience. Mice were in the center of the maze and were allowed to freely explore the maze. Mice could freely explore the maze for 5 min and the sequences of arms entries were recorded in order to assess spontaneous alternation performance (SAP). The sequences of arms entries were recorded in order to assess spontaneous alternation performance for each mouse, which is 3 consecutive non-similar movements. All trials were recorded by Noldus Ethovision version 8.5. The sequences of arm entries were recorded in order to assess spontaneous alternation performance for each mouse (Eq. 4), which is 3 consecutive non-similar movements (e.g. ABCCBABCABB, the SAP would be ABC, CBA, ABC, BCA, CAB, out of total move of 11, so spontaneous alternation performance would be 55%.

4.3.13. Single bout of acute involuntary treadmill exercise for animals of all ages

We exposed homozygous (*Bdnf*-As KO HO) and wildtype (WT) mice to involuntary treadmill exercise as per a previously published protocol (Venezia et al., 2017) in 7 wildtype and 11 KO mice from both gender (male and female), at 7–8 weeks of age. All mice were brought to the experiment room 30 min before to acclimate to room conditions, then placed on a stationary treadmill for 10 min with electric grid on for 3 consecutive days to habituate to the device. On day 4, a single bout of exercise with electrical grid on was performed as follows: No running for 1 min; Warm up: start treadmill 5 m/min increasing 1 m/min for 5 min; High intensity-increasing speed 1 m/min slowly to a maximum of 13 m/min for 50 min with 0% grading. Tactile stimulations were applied to the mice prior to touching the E grid, encouraging them to run and reduce the episode of the electrical stimulus from the grid. After running, all mice were kept on the stationary treadmill for 70 more minutes and

euthanized immediately afterwards. The brain and peripheral tissues were dissected on a cold surface and snap frozen on dry ice for further processing of RNA and protein. We repeated this study to collect fresh tissues for protein purification, but this time animals were back to their home cage after exercise and were euthanized 16 h later to allow for changes in protein expression. Dissected brain regions were not frozen and protein was extracted on fresh samples.

4.3.14. Habituation in involuntary exercise

All mice were brought to the experiment room 60 min before the start of behavioral trials to acclimate to room conditions. The mice were then put on a stationary treadmill for 10 min with the electric grid on for 3 consecutive days to habituate to the device. On the 4th day, a single bout of involuntary exercise was performed as follows. Mice were placed on a grid without running for 1 min, then began the warm-up phase on the treadmill at a speed of 5 m per minute with incremental increases of 1 m/min for 5 min. Afterwards, the high intensity phase was initiated by increasing speed 1 m/min up to a final speed of 13 m/min for 50 min with 0% grading. Tactile stimulations were applied to the mouse's back prior to touching the E grid to encourage them to run and reduce the episode of touching the electrical stimulus grid. After the running steps, all mice were kept on a stationary treadmill for 70 min, euthanized and various brain regions were collected and snap frozen on dry ice. We repeated this study to collect fresh tissue for protein purification and returned the animals back to their home cages after exercise prior to euthanizing 16 h later. Dissected brain regions for ELISA were not frozen and protein was extracted immediately following dissection. Detailed methods are included in the Supplementary methods.

4.3.15. Involuntary exercise and daily treadmill running in young mice

In order to evaluate effect of exercise on memory function of this novel strain, we repeated the treadmill running protocol on a new group of mice without previous exposure to the treadmill. This group included 9 WT and 8 KO HO male and female mice. The protocol was designed to introduce them to the device by sitting on a stationary treadmill for 3 consecutive days, then running on the treadmill daily at a starting speed of 5 m/min for 10 min per day for 3 days. All mice ran on the treadmill 25-30 min daily for two more weeks, increasing speed incrementally to reach a final speed of 13 m/min, before behavior tests were performed using the following protocol:

Day 1–2: 5min for 5m/min- 5 min increase from 5 to 7 m/min, total 10 min, total 10 min daily.

Day 3–4: 5min for 5m/min- 5 min increase from 5 to 7 m/min- 5 min for 7 m/min, total 15 min daily.

Day 5–7: 5min for 5m/min- 3 min increase from 5 to 7 m/min - 3 min increase from 7 to 10 m/min - 9 min for 10 m/min, total 20 min daily.

Day 8–9: 5min for 5 m/min - 5 min increase from 5 to 10 m/min - 15 min for 10 m/min, total 25 min daily.

Day 10: 5min for 5m/min- 5 min increase from 5 to 10 m/min - 20 min for 10 m/min, total 30 min daily.

Day 11: the same running protocol plus habituation to open field (one trial).

Day 12: one trial of 10 min habituation plus running on treadmill with the same protocol for 30 min.

We had two more days of habituation to open field with 10-minute trials twice a day without exercise on the treadmill.

Day 15: one episode of exercise with the same protocol followed by subthreshold novel object acquisition for 3 min exposure to two identical objects in the afternoon (before 6 pm). All mice were placed back in their home cages overnight in the holding room.

Day 16: Recognition test was first performed with 5 min testing on one familiar object and one new object. Test subjects had one episode of exercise in the afternoon several hours after behavior tests were completed.

Day 17–21: one trial of daily exercise for 25 min.

Day 22: treadmill exercise and Barnes maze habituation, 4min, one trial for each mouse.

Day 23: Barnes maze training day 1, two trials for each mouse with at least 20 min intertrial, no running.

Day 24: Barnes maze training day 2, 3 trials for each mouse with 20 min intertrial.

Day 25: Barnes maze training day 3, 2 trials with the same protocol.

Day 26: Barnes maze training day 4; exercise for 25 min followed by 3 h intertrial and one trial of 5 min Barnes maze training.

Day 27: one trial of Barnes maze training (5min).

Day 28: exercise followed by Barnes maze probe experiment; 6 h intertrial.

On probe day, the hidden box was removed, and the maze was turned 360°. The probe trial consists of 5 min exploration time for each mouse. The duration of time spent in the target zone and number of errors and pokes to holes in target zone was calculated. Mice were not exercised during weekends and did not go longer than 3 days without exercise. There was at least 3 h intertrial between treadmill and any behavior tests to allow the mice to get conditioned to the new environment before testing.

4.3.16. Involuntary exercise and daily treadmill running in aged mice

We repeated the study for aged mice groups, including 13 homozygous knockout and 12 wild type litter mates. Homozygous mice were between 15-17 months old and wild type mice were between 10-17 months old including both genders in each group. Exercise and open field protocols were as follows: We first habituated the mice to the treadmill and then to the open field chamber every day up to the end of the study with a minimum 3–4 h intertrial. Test subjects were brought to the exercise room 30 min before trials to acclimate to room conditions. Exercise and open field protocols were performed as follows:

Day 1: 10 min on stationary treadmill, no open field.

Day 2: 10 min stationary treadmill, E grid on +1st open field habituation/10 min.

Day 3: 10 min stationary treadmill with E grid on +2nd open field habitation/10 min.

Day 4:10 min stationary treadmill with E grid on +3rd open field habitation/10 min.

Day 5: 3 min treadmill habituation and 7 min moving 4 m/min +4th open field habituation/10min.

Day 6: treadmill 1 min habituation, 5 min moving in speed 4.6 m/min followed by 5 min with speed of 5.4 m/min+ 5th open field habituation/10 min. Plots of moved distance values showed that the subjects were well habituated to the open field.

Day 7: single bout of exercise on treadmill as follows: Habituation 3 min, warm up speed 5 m/min/5 min, increase speed 5–7 m/min for 5 min, increase 7–10 m/min for 10 min, full speed of 12 m/min for 24 min. Total running time was 47 min with gradually increasing speed to avoid exhaustion. Theses mice were older and therefore some were not able to fully perform the running protocol, so they were given a few minutes break and were temporarily removed from the moving treadmill. The total running time for each mouse was recorded.

Day 8: no treadmill running, subthreshold novel object recognition test was for 3 min exposure to 2 identical object, 45 min ISI followed by 5 min recognition testing with one familiar object and one new object in the same arena.

Day 9: treadmill running consisted of 1 min habituation, 11 min speed increase 5–10 m/min.

Day 10: treadmill running: 1 min habituation, 11 min speed increase 5–12 m/min.

Day 11–14: treadmill running: 1 min habituation, 11min speed increasing 5–12 m/min + open field habituation 10 min (4 extra daily trials).

Day 15: 1min habituation, 11 min speed increasing 5–12 m/min + subthreshold Object location memory test (NOL test was done 3 h post exercise, before 6 pm). Animals were exposed to two identical objects for 3 min, following by 45 min ISI, then 5 min recognition test with the same objects while one object was moved to a new location. As expected, results varied, although homozygous knockout mice did spend more time around novel objects as compared to wildtype but no significant meaningful changes in discrimination and recognition indices between two groups was measured. We did not count weekends as part of study days. All mice were weighed before and after the study with the same scales used for all cohorts used in the exercise studies.

4.3.17. Novel object recognition following involuntary exercise

An intermittent short-term daily exercise regimen was designed, consisting of a 30-minute daily exercise plan, 5 days a week, for two weeks in 11 WT and 13 *Bdnf*-AS-KO HO male and female mice.

4.3.18. Barnes maze test following involuntary exercise

This test was performed similar to baseline tests, preceded by involuntary exercise conditioning.

4.3.19. RNA extraction from mouse tissues

All mouse tissue dissections were performed on a cold surface and were collected in 2ml Eppendorf tubes that were snap frozen on dry ice. Collected tissues were kept at -80 °C for further processing. For RNA extraction, 1 ml Trizol and one metal bullet were added to each tube containing tissues, that were mechanically homogenized using a Retsch-MM400 homogenizer for 2–3min, with a pull frequency of 30/second. Chloroform was then added to each sample (1/5 of initial Trizol volume) and vortexed prior to centrifugation at high speed for 20 min at 4 °C. The aqueous layer was collected in a new Eppendorf tube and equal volumes of 70% ethanol were added to each tube prior to being passed through columns using the RNeasy mini kit (Qiagen, catalog # 74106).

4.3.20. Real-time PCR (RT-PCR) and gene expression detection

We collected brain regions from mice at specific ages as mentioned in the main text. RNA extraction was performed using 1 ml TRIzol prior to application onto columns as per the manufacturer's instructions (RNeasy mini kit column purification, Qiagen catalog # 74106). We designed gene specific primers for mouse *Bdnf*-AS. The primers and probes are designed to span the overlapping region between mouse *Bdnf* and mouse *Bdnf* antisense, which produces an 800bp amplicon. We used 20–50 ng of total RNA with 3 repeats for each gene. For reverse transcriptase reactions, we used 0.6 μl of 10μm primers plus 0.25 units of 40X reverse transcriptase and 5 μl of RNA-to Ct -1-step kit master mix from Applied Biosystems (catalog # 4392938) to amplify the sense (Bdnf) transcript and reverse primers to amplify *Bdnf*-AS transcript in 384-well plates. PCR program was as follows: 48 °C for 30 min, 95 °C for 15 min and hold at 4 °C.

We also used reverse primer for housekeeping genes *Gapdh* and *beta-actin* in reverse transcription (RT) reactions. After a brief spin to collect evaporated PCR drops on the tube cap, 0.5 μl of primer and probe were added to each well including the no RT and no template controls. For control genes, G*apdh* (Life technologies Assay ID# Mm99999915_g1) and *beta-actin* (Life technologies Assay ID# Mm02619580_g1) were used as per the manufacturer's protocol. RT-PCR reactions cycling conditions were: 50 °C for 2min, 95 °C for 10min, 40 cycles of 95 °C for 15 s and 60 °C for 1min on the Applied Biosystem Quant Studio 6 Flex real-time PCR machine. Mouse *Bdnf*, mouse *Bdnf*-AS were measured alongside *beta-actin* and G*apdh* as housekeeping genes. Target gene expressions was normalized to the average of the Ct values of the housekeeping genes. To ensure validity of RT-PCR data, two additional control reactions were included: a no RT reaction lacking reverse transcriptase enzyme and a no template control which has water in place of RNA. A two-step RT-PCR was performed with total RNA extracted from mouse brain regions excised after a single bout of treadmill exercise. cDNA was synthesized with 800ng of total RNA with random hexamers and using (Taqman reverse transcriptase reagent, Life technologies catalog# N808-0234). The RT-PCR reaction was performed using 1600 ng cDNA per well and 3 replications for each target gene including housekeeping genes. RT_PCR reactions were performed using commercial mouse *Bdnf* Taqman primer and probe assays (Applied Biosystems, Assay ID# 04230607) and gene expression master mix (Applied Biosystems, catalog #4369016) with 384-well Taqman dd Ct on QuantStudio6 Flex machine with the same cycling conditions mentioned above. Mouse Bdnf gene expression was normalized to housekeeping gene expression RT-PCR data.

4.3.21. Data analysis

Gene expression of target and housekeeping genes were calculated from Cycle threshold (Ct) values from RT-PCR. Each sample was performed in triplicate and Ct values were used to calculate RQ ($2^{-\Delta\Delta Ct}$). Graph Pad Prism software was used to prepare graphs of relative quantity values, which were used to compare between groups by Student's t-test or two-way ANOVA. Data are reported as the mean with SEM and p-value ≤ 0.05 to indicate significant data.

4.3.22. ELISA to measure protein from mouse hippocampus

Mouse brains were dissected, and the prefrontal cortex, hippocampus and cerebellum were excised and placed in Eppendorf tubes before being snap frozen on dry ice. For protein processing, hippocampal tissue was collected and kept cold on ice and immediately immersed in 200 μl lysis buffer (M-PER mammalian protein extraction reagent, Thermo Fisher catalog #78501 containing 100X proteinase inhibitors cocktail, HaltsTM protease inhibitor cocktail (catalog # 78438). Sonicated tissues were kept cold to preserve protein integrity during homogenization (35 min). Samples were centrifuged at 1400 x g for 10 min to sediment cell debris and the supernatant was collected into a new Eppendorf tube. Protein titration was performed using the Pierce BCA protein assay kit (Thermo Fisher catalog# 23225 and 23227). We utilized the hippocampus after treadmill exercise for two separate studies. First, we measured protein levels 70 min after a single bout of exercise which did not show meaningful changes in level of BDNF protein. We repeated this study a second time and euthanized animals immediately following exercise on the treadmill after resting overnight in the home cage. Protein extraction and titration were done based on manufacturer's instructions. ELISA was performed using fresh protein extracted from hippocampal tissues of 3 wild type mice versus 3 homozygous mice using 500ng of protein per well and 3 technical replicates per sample for a total of 3 technical and 3 biological replicates. The ELISA BDNF Emax® ImmunoAssay System, (Promega, catalog# G7610 and G761). We optimized the ELISA assay with 1:10 serial dilutions of mouse protein (0.1 μg–1000ng) to detect the optimal concentration for maximum detection Bdnf. A concentration of 0.5 μg protein had the best Bdnf signal in all brain regions and was used for all protein studies. The standard curve was for ELISA included blank wells for background detection. Plates were read on the Perkin Elmer Envision 2104 Multilabel plate reader at 450nm. All signals from the sample wells were normalized to the background and calculated based on the closest standard range.

4.4. Quantification and statistical analysis

We performed all statistical analyses using GraphPad Prism software. We include all statistical data in the individual figure legends. Protein

and mRNA data were analyzed by one or two-way analysis of variance (ANOVA) when appropriate ($p < 0.05$ considered statistically significant).

Declarations

Author contribution statement

Farzaneh Modarresi, Mohammad Ali Faghihi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Roya Pedram Fatemi, Seyedeh Fatemeh Razavipour: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Natalie Ricciardi, Madina Makhmutova, Nathalie Khoury, Marco Magistri, Claude-Henry Volmar, Claes Wahlestedt: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflicts of interest.

Additional information

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Supplementary Table 1

Primer sequences for Taqman assays used in this study.

	Sequence
mBDNF-AS probe	5′-ACG GTC ACA GTC CTA GAG AAA GTC CC-3′
mBDNF-AS Reverse Primer	5′-TCA CAG CGG CAG ATA AAA AG-3′
mBDNF-AS forward primer	5′-TCA GTT GGC CTT TGG ATA CC-3′
Mouse ACT forward primer	5′ GCTCTTTTCCAGCCTTCCT 3′
Mouse ACT Reverse Primer	5′ CGGATGTCAACGTCACACTT 3′
Mouse GAPDH forward primer	5′ GTGGCAAAGTGGAGATTGTTG 3′
Mouse GAPDH reverse primer	5′ ACCAGTAGACTCCACGACATA 3′
Mouse GAPDH reverse primer 2	5′ GAATTTGCCGTGAGTGGAGT 3′
MBdnf, primer and probe, qPCR	Life Technologies Assay ID# Mm01334042
GAPDH, primer and probe, qPCR	Life Technologies Assay ID# Mm99999915_g1
Bactine, primer and probe, qPCR	Life Technologies Assay ID# Mm02619580_g1

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