Hippocampal long-term potentiation is modulated by exercise-induced alterations in dopamine synaptic transmission in mice selectively bred for high voluntary wheel running

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Abstract –

BACKGROUND: High-runner (HR) mice, selectively bred for increased voluntary wheel-running, exhibit heightened motivation to run. Exercise has been shown to influence hippocampal long-term potentiation (LTP) and memory, and is neuroprotective in several neurodegenerative diseases.

OBJECTIVE: This study aimed to determine the impact of intense running in HR mice with wheel access on hippocampal LTP, compared to HR mice without wheels and non-selected control (C) mice with/without wheels. Additionally, we investigated the involvement of D1/D5 receptors and the dopamine transporter (DAT) in LTP modulation and examined levels of these proteins in HR and C mice.

METHODS: Adult female HR and C mice were individually housed with/without running wheels for at least two weeks. Hippocampal LTP of extracellular field excitatory postsynaptic potentials (fEPSPs) was measured in area CA1, and SKF-38393 (D1/D5 receptor agonist) and GBR 12909 (DAT inhibitor) were used to probe the role of D1/D5 receptors and DAT in LTP differences. Western blot analyses assessed D1/D5 receptor and DAT expression in the hippocampus, prefrontal cortex, and cerebellum.

RESULTS: HR mice with wheel access showed significantly increased hippocampal LTP compared to those without wheels and to C mice with/without wheels. Treatment with SKF-38393 or GBR 12909 prevented the heightened LTP in HR mice with wheels, aligning it with levels in C mice. Hippocampal D1/D5 receptor levels were lower, and DAT levels were higher in HR mice compared to C mice. No significant changes were observed in other brain regions.

CONCLUSIONS: The increased hippocampal LTP seen in HR mice with wheel access may be related to alterations in dopaminergic synaptic transmission that underlie the neurophysiological basis of hyperactivity, motor disorders, and/or motivation.

Introduction

Aerobic exercise enhances cognitive function, particularly memory, in both humans (Erickson et al., 2011; Hillman et al., 2008) and rodents (Fordyce & Wehner, 1993; van Praag et al., 1999), in healthy and diseased states (da Costa Daniele et al., 2020; Gaitán et al., 2021; Huuha et al., 2022; Jahangiri et al., 2019). Exercise-enhanced improvements in memory are mediated, in part, through changes in hippocampal function that involve increased neurogenesis, release of trophic factors, and angiogenesis, along with changes in neurotransmitter function and structural alterations (for review, see (Cooper et al., 2018)). The effects of voluntary wheel running on hippocampal function has been studied most extensively in the dentate gyrus (Christie et al., 2008; Farmer et al., 2004), the site of dramatic exercise-induced adult neurogenesis, but neurochemical and structural changes occur in area CA1 as well (Czurkó et al., 1999; Lin et al., 2018; Pietrelli et al., 2018; Stranahan et al., 2007). Voluntary running has also been shown to enhance long-term potentiation (LTP), a cellular model for learning and memory, in the rodent dentate gyrus (Dahlin et al., 2019; van Praag et al., 1999) and area CA1 of the hippocampus (Dong et al., 2022).

The effects of extremely high levels of running can be studied in High Runner (HR) mice, a set of four replicate lines that have been selectively bred for more than 100 generations for high voluntary wheel-running behavior as a model for voluntary exercise. HR mice are highly motivated to run, running almost three times farther per day than those from four non-

selected control (C) lines (Rhodes et al., 2005; Swallow, Carter, & Garland, Jr., 1998; Waters et al., 2013). In addition, HR mice have elevated home-cage activity when wheels are not provided (Copes et al., 2015; J. et al., 2001; Malisch et al., 2009). Previous work by Johnson et al. (Johnson et al., 2003) and Rhodes, van Praag et al. (2003) demonstrated that HR mice with wheel access produce higher levels of brain-derived neurotrophic factor (BDNF) and maximal neurogenesis in the dentate gyrus compared to their control (non-selected) counterparts. Though access to a running wheel enhanced spatial memory in control animals, as shown in studies of other strains of mice (Alomari et al., 2013; van Praag et al., 2005), performance on the Morris Water Maze task was significantly worse in HR mice that had wheel access compared to those that did not have access, and compared to control mice with wheel access (Rhodes, van Praag, et al., 2003). Reasons for this memory deficit in HR mice are still unclear but may be related to the maximal ceiling effects in neurogenesis seen in these mice (Rhodes, van Praag, et al., 2003), problems in attention to visual cues of the task, an increase in corticosterone levels (Malisch et al., 2007), and/or differences in dopaminergic neurotransmission. Alterations in LTP in HR mice, or other rodents selectively bred for high running (e.g., Roberts et al., 2017; Waters et al., 2013; Wikgren et al., 2012) have not been examined, however.

Given that HR mice were bred for their motivation and ability to run on a wheel and are hyperactive in home cages, it is likely that dopaminergic transmission is altered in these mice. Indeed, Rhodes and Garland observed reduced running behavior in HR mice treated with methylphenidate (Ritalin; a norepinephrine-dopamine reuptake inhibitor), such that their speed was comparable to that of the C untreated mice (Rhodes et al., 2005; Rhodes & Garland, 2003). In contrast, Ritalin increased the running activity in the C mice, just as it increases motor activity in individuals who are not affected by attention deficit hyperactivity disorder (ADHD). The HR

mice also showed behavioral responses to a D_1/D_5 receptor antagonist (SCH23390) that differed from those in the C mice: the HR mice were less sensitive to the D_1/D_5 antagonist (as measured by running speed), whereas their sensitivity to a D_2 receptor antagonist was comparable to that in the C mice (Rhodes & Garland, 2003). The reduced sensitivity to the D_1/D_5 receptor antagonist may be caused by reduced expression of the receptor in key regions of the brain, reduced affinity of the receptor in the HR mice, changes in downstream signal transduction mechanisms, and/or differences in synaptic levels of dopamine. The latter possibility--changes in dopamine levels in the HR mice—was examined by looking at the DOPAC/DA ratio in two brain regions that receive abundant dopaminergic input, the nucleus accumbens and caudate/putamen (Rhodes et al., 2005). Differences in dopamine levels in HR vs C mice were not observed in these regions, nor in area CA1 of the hippocampus (Waters et al., 2013), suggesting that the differences in dopaminergic transmission are occurring downstream of dopamine release.

Alterations in dopaminergic transmission in HR mice are likely to alter synaptic plasticity, particularly in the hippocampus, and therefore contribute to the deficits in learning and memory previously observed by Rhodes and colleagues (Rhodes, van Praag, et al., 2003). Several groups have demonstrated that dopamine (Frey et al., 1990; Li et al., 2003a; Lisman & Grace, 2005) and D_1/D_5 agonists in particular (Hansen & Manahan-Vaughan, 2014; Otmakhova & Lisman, 1996; Swanson-Park et al., 1999) can enhance LTP in area CA1 of standard strains of laboratory mice or rats. More recently, several groups have demonstrated the necessity of D_1/D_5 receptors in the dorsal area CA1 (dCA1) in novelty-enhanced LTP (Li et al., 2003a; Takeuchi et al., 2016), spatial memory (O'Carroll et al., 2006), and linked contextual memory (Chowdhury et al., 2022); activation of D1/D5 Rs also appears to be sufficient for memory of linked contexts

(Chowdhury et al., 2022). In these cases, the majority of the dopaminergic input to dCA1 seems to be from the locus coeruleus (Takeuchi et al., 2016), rather than the ventral tegmental area.

Although enhanced neurogenesis and BDNF levels following voluntary exercise on wheels have been examined in the HR mice (Rhodes, van Praag, et al., 2003), changes in hippocampal LTP and dopaminergic transmission have not been characterized. In this study, we examined effects of high voluntary running on LTP in area CA1 of HR mice housed with and without a running wheel and compared the magnitudes of LTP to those observed in control mice, with and without access to running wheels. Given the high levels of BDNF seen in exercising mice (Cooper et al., 2018; Johnson et al., 2003; Kobilo et al., 2011; Rhodes, van Praag, et al., 2003), we predicted that LTP would be greater in the HR mice with wheels. Considering the differences in dopaminergic function in these mice (Rhodes et al., 2005; Rhodes & Garland, 2003; Waters et al., 2013), and the influences of DA neurotransmission on LTP (Li et al., 2003a; Lisman & Grace, 2005) we also wanted to determine whether hippocampal LTP is influenced by D_1/D_5 receptor activation, or by elevating synaptic dopamine concentrations through inhibition of the dopamine transporter (DAT). Given previous reports of reduced transcripts for D1 receptors in the striatum of HR mice (Mathes et al., 2010) and increased D1 receptor transcripts in the cerebellum (Caetano-Anollés et al., 2016), we examined potential differences in the levels of D_1/D_5 receptors and DAT in hippocampus, cerebellum, and prefrontal cortex.

Methods

Animals

Female High-Runner (HR) and non-selected control (C) mice were obtained from Dr. Theodore Garland's laboratory at the University of California, Riverside. Starting in 1993, four

replicate lines of HR mice were bred from a founder population of Hsd:ICR *Mus domesticus* and were selectively chosen for their high voluntary running activity. An additional four replicate control (C) lines were maintained by breeding without regard to how much they ran (Swallow, Carter, & Garland, 1998). Animals used for this study were mature adults between two and seven months of age, with age spread randomized across conditions, and were obtained from Generation 72 or later of the selection experiment. At this point in the experiment, all four HR lines had reached selection limits (plateaus) (Careau et al., 2013). In order to make comparisons to previous learning, memory, and dopaminergic work with these mice (Rhodes, van Praag, et al., 2003; Rhodes et al., 2005; Rhodes & Garland, 2003), we used females, which run farther distances per day than males (Careau et al., 2013). Mice were sampled from all four HR lines and from all four C lines, but line designation was not considered during statistical analyses (see below). They were individually housed with or without a running wheel (7.5-inch WARE Flying Saucer Exercise Wheel for Small Animals; see Supplementary Movie 1) for at least two weeks before being sacrificed for experiments. These wheels did not have the ability to record running behavior. All animals were housed with a 12-hour light/dark cycle with food (Purina Rodent Chow Lab Diet #5001) and water available *ad libitum*. All experiments were conducted in accordance with the Pomona College Institutional Animal Care and Use Committee (IACUC).

Hippocampal Slice Preparation for Electrophysiology

Transverse hippocampal slices (450 μm) were prepared as described previously (Madison & Edson, 1997). Mice were anesthetized with isoflurane and decapitated. The brain was then immediately removed and submerged into ice-cold, oxygenated (with 95% O_2/ 5% CO_2) sucrose cutting solution that contained (in mM): 210 sucrose, 20 glucose, 26 $NaHCO_3$, 2.5 KCl, 1.25

NaH_2PO_4, 0.5 $CaCl_2$, and 3.0 $MgCl_2 \cdot 6H_2O$. The hippocampus was rapidly dissected on a chilled platform with ice-cold, oxygenated cutting solution. A manual tissue chopper (Stoelting) was used to prepare 450-μm slices, which were initially submerged in ice-cold, oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose. Hippocampal slices were then placed into an oxygenated holding chamber at room temperature (23-24°C), atop filter paper moistened with oxygenated aCSF. Slices were allowed to recover for at least two hours prior to recording.

Hippocampal Slice Recording

Individual slices were transferred from the holding chamber to a submerged-slice recording chamber, pinned to a Sylgard substrate, and superfused with oxygenated aCSF at room temperature (23-24°C) at the rate of 2 mL/min. A concentric bipolar stimulating electrode (Fred Haer, Inc.) and a glass recording microelectrode filled with aCSF were placed on the Schaffer collateral fibers in the stratum radiatum of area CA3 and CA1, respectively. Stimulus pulses (0.1 ms duration) were delivered at 0.033 Hz with the stimulus intensity set to elicit field excitatory postsynaptic potentials (fEPSPs) with slopes that were approximately 50% of the maximum slope. Recordings were amplified at 1000X gain using a Dagan IX amplifier and digitized at 1 kHz using an AD Instruments PowerLab and analyzed using AD Instruments Scope software. Paired-pulse facilitation (PPF) was examined for five min by applying two stimuli separated by a 50-ms interpulse interval. The average PPF ratio was calculated as the fEPSP slope of the second response divided by that of the first response, for the ten trials. Baseline fEPSP recordings were then obtained for a minimum of 30 min. LTP was induced using a single theta burst stimulation (TBS) consisting of 10 trains of 5 pulses given at 100 Hz, with trains delivered

at the theta frequency of 5 Hz. Following LTP induction, responses were recorded for at least 60 min. Data were normalized with respect to the mean values of fEPSP slopes recorded during the 5 min prior to the TBS. For experiments involving drug superfusion, baseline measurements were taken for an additional 30 min following a solution change from aCSF to aCSF containing the drug. The solution was switched back to aCSF at 5 min post-TBS, and fEPSP recordings continued for at least 55 min.

Drugs

The dopamine D_1/D_5 receptor agonist, (±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8,-diol (SKF-38393; Tocris Bioscience and Sigma-Aldrich), was prepared as a 100 mM stock solution in dimethylsulfoxide (DMSO) at room temperature. The stock solution was aliquoted, stored at -20 °C, and diluted 1:1000 in oxygenated aCSF immediately before use for the final working concentration (100 μM SKF-38393), which was selected based on previous work conducted by (Mockett et al., 2004). The DAT inhibitor GBR 12909 was obtained from Tocris, prepared as a 5mM stock solution in DMSO, and diluted 1:10,000 in aCSF for a final bath concentration of 0.5 μM (Sriram et al., 2002), a concentration that does not promote nicotinic acetylcholine receptor-induced norepinephrine release (Szasz et al., 2007). DMSO, 0.1% or lower in aCSF, did not affect the fEPSP slope nor the magnitude or duration of LTP when applied to the bath.

Tissue Collection for Western Blots

HR and C mice, with and without access to a running wheel, were anesthetized with isoflurane and immediately decapitated. The brain was then immediately removed and

submerged in ice-cold, oxygenated (with 95% O_2/5% CO_2) sucrose cutting solution. The hippocampus, prefrontal cortex, and cerebellum were rapidly dissected on a chilled platform with ice-cold, oxygenated cutting solution. A manual tissue chopper (Stoelting Co.) was used to prepare 450-μm slices from each brain region. Slices from each brain region were snap-frozen upon being placed into Eppendorf LoBind microcentrifuge tubes (Sigma-Aldrich) that were pre-chilled on dry ice, and samples were stored at -80 °C. To maintain consistency of protein concentrations across samples, an equal number of brain slices (e.g., 4-5) were placed in each tube.

Total Protein Extraction

A motorized pestle mixer (Argos Technologies) was used to homogenize the frozen tissue samples in an ice-cold lysis buffer containing 1.0 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM ethylene glycol tetraacetic acid (EGTA), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and Mini cOmplete Protease Inhibitor Cocktail (Sigma Aldrich) in phosphate-buffered saline. The samples were then sonicated on ice at 20% power with 10 short pulses using the Branson 450 Digital Sonifier (Marshall Scientific), incubated on ice for 30 min, and centrifuged at 13,200 *g* for 10 min at 4 °C. The supernatant was collected and stored at -80 °C.

Total protein quantification

Total protein concentration of the supernatant was quantified spectrophotometrically using Gen5 Microplate Reader and Imager software (BioTek Instruments) via the DC Protein Assay (Bio-Rad), following the manufacturer's protocol. Bovine serum albumin (BSA; Bio-

Rad) standard curves were prepared on the same assay plates and diluted sample concentrations were then interpolated using second order polynomial nonlinear regression fitting in Prism version 5.0 (GraphPad Software).

Western Blot Analysis

Prepared tissue samples containing 100 μg of total protein were heated at 95 °C in an equal volume of 2x Laemmli's sample buffer (Bio-Rad) containing β-mercaptoethanol for 5 min, then placed on ice and centrifuged. The samples and molecular weight markers (3:1 ratio of Bio-Rad Kaleidoscope Precision Plus Protein Standards and Thermo Fisher MagicMark XP Western Protein Standard) were then loaded into a 10-well precast 4-15% TGX polyacrylamide gel (Bio-Rad) and separated by size via SDS polyacrylamide gel electrophoresis (SDS-PAGE) in 1x Tris-glycine-SDS running buffer (Bio-Rad) at 200 V for 30 min. Proteins on the gel were electroblotted onto a nitrocellulose membrane (Bio-Rad) at 25 V for 7 min using the semidry Trans-Blot Turbo Transfer System (Bio-Rad) in cold Trans-Blot Turbo Transfer Buffer (Bio-Rad). Following the transfer, the membrane was washed in Tris-buffered saline with 0.1% Tween (TBST; 2 x 5 min) and blocked in TBST containing 4% BSA (Bio-Rad) for 15 min at room temperature to inhibit nonspecific binding. The membrane was then incubated overnight with shaking at 4 °C in the primary antibody, including D1 (1:200, Thermo Fisher Scientific, PA5-77388), DAT (1:500, Thermo Fisher Scientific, PA5-78382), and GAPDH (1:1000, Thermo Fisher Scientific, PA1-987), diluted in TBST containing 4% BSA. Following incubation in the primary antibody, the membrane was washed in TBST (3 x 5 min) and then incubated at room temperature with shaking for 2 h in horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2000, Invitrogen, #31460) diluted in TBST containing 4% BSA.

Following secondary-antibody incubation, the membrane was washed with TBST (3 x 5 min) and proteins of interest were visualized using Clarity Western ECL Substrate (Bio-Rad) with a 1-min exposure using a chemiluminescence imaging system (ImageQuant LAS 500; General Electric). Immunoreactive signals were quantified by densitometry using ImageJ software (version 1.51m9). Original images were converted to 8-bit greyscale images and the total area of pixels in the band region from each lane were measured under non-saturating conditions. Bands at 80 kDa, 60 kDa, and 37 kDa—corresponding to the D1 receptor, DAT, and GAPDH, respectively—were detected and quantified. Subsequent analysis compared the areas of target proteins normalized to that of GAPDH for each brain region.

Statistical analyses

All data are presented as mean ± standard error of mean (SEM) and were analyzed using Prism version 10.0 (GraphPad Software) and R (R Core Team (2024). For LTP experiments, sample sizes refer to the number of slices used, not the number of individual mice. Differences between mean normalized fEPSP slopes during the 0- to 10-min before and 50- to 60-min period following TBS were assessed using two-way analysis of variance (ANOVA) where mouse genotype (HR vs. C) and exercise (with vs. without wheel) were entered as the two factors. Western blot data were analyzed using two-way ANOVAs, with mouse genotype and exercise as the two factors; sample sizes refer to the number of mice. Levene tests for homogeneity of variance were conducted for all of the ANOVAs. Note that, unlike in previous studies of these mice, we did not use replicate line as a nested random effect within linetype (i.e., HR versus C lines), due to the relatively small number of animals involved. For all data sets,

differences were considered significant for p values < 0.05 (shown in figures as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

Results

The selectively bred HR and Control mice used for these experiments were mature adult females obtained from Generation 72 or later of the High Runner mouse selection experiment for voluntary wheel running, which began in 1993 (Swallow, Carter, & Garland, 1998). At generation 72, their female siblings ran an average of 13,600 revolutions per day (HR mice) vs 4,300 revolutions per day (Control mice) on days 5-6 of running wheel access, and had body weights of 22 vs 25 gm (HR vs Control, respectively).

Hippocampal LTP

To assess whether access to a running wheel would affect LTP in area CA1 of HR and C mice, synaptic responses were recorded for 60 minutes following application of one full TBS. In HR mice with wheel access, the mean fEPSP slope at 50-60 minutes post-TBS (normalized to the mean slope during baseline) was approximately 40% greater than that in HR mice without wheel access (magnitude of LTP with wheel access, 84 ± 9.9% above baseline, n = 13 slices from 10 mice; without wheel access, 44.7 ± 9.5% above baseline, n = 9 slices from 7 mice. In contrast, C mice with or without wheel access did not differ in LTP magnitude (magnitude of LTP with wheel access, 60.3 ± 8.4% above baseline, n = 9 slices from 8 mice; without wheel access, 60.7 ± 13.3% above baseline, n = 8 slices from 8 mice; n.s.; **Figure 1a,b**). Overall, the magnitude of LTP was greater in mice with wheel access than in those with no wheel access

(two-way ANOVA; main effect of wheel access: $F_{1,35} = 4.335$, P = 0.0447). However, this effect was entirely due to the HR mice, as indicated by the borderline significant genotype x wheel access term ($F_{1,35} = 4.033$, p = 0.0524; **Figure 1a,b**).); LTP magnitudes were higher in HR mice with wheel access than with no wheel access, whereas control mice did not differ in their LTP magnitudes between wheel access treatments. There was no main effect of genotype on the magnitude of LTP ($F_{1,35} = 0.472$, p = 0.4967). Differences in LTP in the HR mice with wheel access cannot be attributed to differences in the input-output (I-O) relationships between the groups, as there were no significant differences in the baseline responses (0.13 ± 0.04 mV/ms, 0.16 ± 0.04 mV/ms, 0.11 ± 0.03 mV/ms, and 0.17 ± 0.04 mV/ms for HR mice with wheel, HR without wheel, C mice with wheel, and C without wheel, respectively), which were set at 50% of the maximal I-O response. Moreover, although PTP during the 0-10 min period following TBS was higher in HR and C mice with wheel access than in those without, these differences were not statistically significant (magnitude of PTP in slices from HR mice with wheel access, 114.8 ± 13% above baseline, *n* = 13 slices; HR without wheel, 82.4 ± 13% above baseline, *n* = 9 slices; C with wheel, 114 ± 18% above baseline, *n* = 9 slices; C without wheel, 108 ± 23% above baseline, *n* = 8 slices; n.s.; Fig. 1d).

To examine whether the high voluntary wheel running results in presynaptic changes in neurotransmitter release properties, paired-pulse facilitation (PPF), a pre-synaptic-mediated form of potentiation, was measured before measuring baseline responses for HR and C mice with and without wheel access. The mean PPF magnitudes were similar between mice housed with and without wheel access within each genotype (**Figure 1c**), suggesting that neither exercise nor the genotype of the mouse alters presynaptic mechanisms of glutamate release. Therefore, postsynaptic changes likely underlie the effects of exercise on LTP in HR mice.

Role of dopamine in LTP differences

Previous studies have shown that pharmacological manipulations aimed at increasing dopaminergic neurotransmission in the hippocampus could improve cognitive functions (Chowdhury et al., 2022; Yamasaki & Takeuchi, 2017). To examine the effect of D_1/D_5 receptor activation and its possible interactions with exercise treatment in the HR mouse brain, hippocampal slices from HR and C mice with and without wheel access were superfused with the selective D_1/D_5 receptor agonist, SKF-38393, before and during LTP recordings. As observed by Mockett et al. (2004), the agonist did not affect basal synaptic transmission, as mean fEPSP slopes were not significantly altered upon SKF-38393 superfusion (data not shown). With regard to LTP, the effects of SKF-38393 strongly depended on access to wheels as well as the genotype. In slices from HR mice with wheel access, SKF-38393 significantly reduced LTP by approximately 42%, to a magnitude similar to that observed in HR mice without wheel access (magnitude of LTP in the presence of SKF-38393, 40.6 ± 13% above baseline, n = 6 slices from 6 mice; in the absence of SKF-38393, 84 ± 10% above baseline, n = 13 slices from 10 mice. However, the agonist had no effect on LTP in slices from HR mice without wheel access (magnitude of LTP in the presence of SKF-38393, 45 ± 6.1% above baseline, n = 7 slices from 7 mice; in the absence of SKF-38393, 39 ± 14% above baseline, n = 7 slices from 7 mice; n.s.; **Figure 2a,b**). These differences were reflected by the results from two-way ANOVA; main effect of wheel access, $F_{1,32} = 4.983$, $p = 0.0327$; main effect of drug, $F_{1,32} = 6.057$, $p = 0.0194$; interaction of wheel access x drug, $F_{1,32} = 4.710$, $p = 0.0375$. The significance of the main effects and the interaction are due to the strong LTP response of the HR mice with wheel access; the presence of the D1/D5 agonist the LTP response appears to cancel the effect of wheel access.

In contrast to results observed in HR mice, the dopamine receptor agonist had no effect on LTP in C mice, regardless of their wheel access. The magnitude of LTP with wheel access in the presence of SKF-38393 was 67 ± 17% above baseline (n = 7 slices from 7 mice); with wheel access in the absence of SKF-38393, LTP was 61 ± 10% (n = 7 slices from 7 mice); without wheel access in the presence of SKF-38393, LTP was 43 ± 7.7% above baseline (n = 5 slices from 5 mice); and without wheel access in the absence of SKF-38393, LTP was 60.7 ± 14% above baseline (n = 8 slices from 8mice). Two-way ANOVA revealed no significant differences between these groups: main effect of wheel access, $F_{1,24} = 0.866$, $p = 0.808$; main effect of drug, $F_{1,24} = 0.061$, $p = 0.808$; interaction of wheel access x drug, $F_{1,24} = 1.908$, $p = 0.180$); **Figure 3a,b,c**.

Effects of drugs or conditions on post-tetanic potentiation (PTP) can reveal influences of such factors on presynaptic release mechanisms. No significant differences in PTP during the 0-10 min period following TBS were observed among the groups (magnitude of PTP in slices from HR mice with wheel access, 114 ± 18% above baseline, n = 13 slices; HR with wheel + SKF-38393, 71 ± 14% above baseline, n = 6 slices; HR with wheel + GBR, 78 ± 23%; HR without wheel, 84 ± 13% above baseline, n = 9 slices; HR without wheel + SKF-38393, 64 ± 15% above baseline, n = 8 slices; C with wheel, 114 ± 18% above baseline, n = 7 slices; C with wheel + SKF-38393, 82 ± 14% above baseline, n = 8 slices; C without wheel, 108 ± 23% above baseline, n = 8 slices; C without wheel + SKF-38393, 114 ± 29% above baseline, n = 5 slices); **Figures 2a,b,d; 3a,b,d**.

Rhodes and Garland (2003) have observed that the running behavior of HR mice is altered acutely by enhancing synaptic dopamine levels through blockade of the dopamine transporter (DAT). To determine whether LTP is also modulated by enhanced synaptic levels of

dopamine in HR mice with wheel access, slices were treated with the DAT inhibitor GBR 12909 before and during the TBS used to induce LTP. GBR 12909 (0.5 μM) did not affect basal synaptic transmission (data not shown), but significantly reduced the magnitude of LTP in slices from HR mice with wheel access (magnitude of LTP in the presence of GBR 12909, 33.9 ± 9% above baseline, $n = 6$ slices from 4 mice; in the absence of GBR 12909, 84 ± 10% above baseline, $n = 13$ slices from 10 mice; main effect of drug, $F_{2,22} = 7.242$, $p = 0.00383$, one-way ANOVA (**Figure 2a**). Bonferroni's multiple comparison test of HR mice without wheel vs these mice with GBR or with SKF confirmed that LTP was significantly lower with either of the two drugs ($p < 0.01$); furthermore, the LTP magnitude did not differ between the two drug treatments.

D_1/D_5 receptors and DAT expression in HR vs C mice

Considering that structural alterations precede functional changes of synapses, Western blot analysis was used to assess whether genotype and/or wheel access affected the expression of proteins involved in dopamine neurotransmission. Tissues harvested from the hippocampus, prefrontal cortex, and cerebellum of HR and C mice with and without wheel access were processed for total protein extraction and blotted with primary antibodies directed against D_1-receptors and DAT.

When using the antibody against the D_1 receptor, an 80 kDa band was consistently observed (Suppl. Figure S1) and selected to represent the D_1 receptor based on its proximity to the observed molecular weights reported by the manufacturer and by other published studies (~75-90 kDa; Levey et al., 1993; Salyer et al., 2011; Zhang et al., 2015; Stojanovic et al., 2017). In HR mice, normalized expression of D_1 receptors in the hippocampus was significantly reduced

compared to levels in C mice; levels of D_1 receptors were approximately 30% lower in HR mice without wheel and 20% lower in HR mice with wheel, compared to C mice without a wheel (two-way ANOVA; main effect of genotype: $F_{1,12} = 55.790$, $p < 0.001$). There was no apparent main effect of wheel access ($F_{1,12} = 2.510$, $p = 0.139$) and no significant interaction between genotype and wheel access ($F_{1,12} = 1.800$, $p = 0.203$; **Figure 4a; Suppl Figure 1a**). On the other hand, hippocampal levels of normalized DAT were elevated by approximately 21% in HR mice, with or without wheel access (two-way ANOVA; main effect of genotype: $F_{1,12} = 11.014$, $p < 0.001$). There was no apparent main effect of wheel access ($F_{1,12} = 0.004$, $p = 0.9494$) and no significant interaction between genotype and wheel access ($F_{1,12} = 0.002$, $p = 0.9646$; **Figure 4a; Suppl Figure 1b**). No differences in normalized mean D_1-receptor or DAT levels were observed in the prefrontal cortex (**Figure 4b**) or cerebellum (**Figure 4c**).

Discussion

Though many previous investigations have shown that voluntary running enhances LTP in the dentate gyrus of C57Bl/6 mice (van Praag et al., 1999) and various rat strains (Farmer et al., 2004; Dahlin et al., 2019; for review, see Loprinzi, 2019), and in area CA1 of C57Bl/6 mice (Dong et al., 2022), alterations in synaptic plasticity had not been examined in HR mice, which are a unique, selectively bred and genetically divergent model for high voluntary wheel-running behavior (Garland et al., 2011; Hillis et al., 2020; Wallace & Garland, 2016). Access to a running wheel significantly enhanced LTP in area CA1 of HR mice; however, it did not alter LTP in slices prepared from mice from non-selected (control) lines, as observed in CA1 of C57BL/6 mice by van Praag et al. (1999). The enhanced LTP in the HR mice with wheel access is likely due to postsynaptic changes in their CA1 synapses, as differences in PPF and PTP in

these animals were not observed. We predicted that activation of D_1/D_5 Rs in slices from the HR mice without wheel access would enhance LTP to the levels seen in their exercising counterparts, suspecting that the elevated LTP in the heavily exercising mice was due to excess dopamine release. Surprisingly though, the D_1/D_5 R agonist influenced LTP levels only in the HR mice with wheel access, suppressing it to levels seen in the non-wheel HR mice and the C mice. This anomalous effect was also seen with the DAT inhibitor GBR 12909, which similarly reduced LTP in the highly active HR mice to control levels. The HR mice, regardless of wheel access, also had reduced levels of D_1 Rs and elevated DAT levels in hippocampus, with no changes observed in brain regions associated with decision-making or motor coordination (the prefrontal cortex and cerebellum, respectively).

The increased LTP in the HR mice with wheel access is not surprising, given the high levels of BDNF in the hippocampus of these animals (Johnson et al., 2003; Rhodes, van Praag, et al., 2003) and potential alterations in dopaminergic transmission (Bronikowski et al., 2004; Mathes et al., 2010; Rhodes et al., 2001; Rhodes et al., 2005; Rhodes & Garland, 2003; Waters et al., 2013). The increase in BDNF may be a consequence of release of exerkines, such as IGF-1, irisin, apelin, cathepsin B, or beta-hydroxybutyrate, during the high-endurance cardiovascular activity (for review, see Cooper et al., 2018; Rendeiro & Rhodes, 2018; Vints et al., 2022). Previous groups have shown in rat hippocampi that BDNF reduces the threshold for inducing LTP following theta burst stimulation, perhaps by reducing the size of the after-hyperpolarization (AHP) during the theta stimuli (Kramár et al., 2004), and that BDNF activation of TrkB receptors is necessary for TBS-induced LTP (Kang et al., 1997). The lack of increase in LTP in the control mice with wheels suggests that high BDNF levels may be a contributing factor for the elevated LTP in the HR mice; examination of BDNF concentrations in area CA1

after chronic intense exercise could support this hypothesis. Additional neural mechanisms might also be involved. One such mechanism could be the enhanced neurogenesis in the dentate gyrus of HR mice with wheels (Rhodes, van Praag, et al., 2003), which could influence synaptic plasticity downstream in the tri-synaptic pathway. Other mechanisms could involve changes in neurotransmission in the HR mice, particularly dopaminergic transmission.

Dopamine receptors in the hippocampus, particularly D_1/D_5 receptors, play an important role in many cognitive functions, including spatial memory (Kempadoo et al., 2016; Li et al., 2003b; McNamara et al., 2014; Silva et al., 2012), novelty-induced memory (Duszkiewicz et al., 2019; Li et al., 2003a), and linked contextual memory (Chowdhury et al., 2022). Given the neuromodulation of LTP by dopamine in area CA1, particularly by activation of D_1/D_5 receptors (Otmakhova & Lisman, 1996), we expected the D_1/D_5 agonist SKF 38393 to enhance LTP in control mice modestly, and perhaps more robustly in HR mice without wheel access, bringing them closer to the enhanced levels seen in the HR mice with wheel access. Instead, we saw the opposite effects in the HR mice. The lack of effect of the agonist in the control mice was not too surprising, given that some groups have not seen effects of D_1/D_5 stimulation on early LTP in CA1 in other strains of mice or rats (Mockett et al., 2004; Papaleonidopoulos et al., 2018). The anomalous effects of the agonist in HR mice could be explained, in part, by activation of D_1/D_5 receptors on inhibitory interneurons in CA1. Gangarossa et al. (2012) and Puigermal et al. (2017) have shown that D_1/D_5 receptors are likely located on interneurons in stratum radiatum; thus, activation of the D_1/D_5 Rs may promote an increase in GABA release, thereby suppressing LTP. It is unclear why this effect was observed in the HR mice with wheel access but not in the control mice, but perhaps some aspect of dopaminergic transmission is altered with the intense running behavior. For example, there could be altered expression of D1/D5 receptors in the

interneurons of the HR mice. Indeed, reduced levels of hippocampal D_1 Rs were observed in HR mice, regardless of their wheel access (Fig. 4). Whether the reduction of D_1 receptors was caused by changes in expression in interneurons or principal neurons was below the level of resolution of our assays, however. In any case, it is possible that a combination of factors, such as activation of D_1/D_5 receptors and TrkB receptors on interneurons of HR mice, contribute to the restoration of LTP to normal levels.

As seen with running behavior (Rhodes et al., 2001), the effects of the D_1/D_5 receptor agonist in the intensely exercising HR mice were mirrored by the inhibition of DAT by GBR 12909: the resulting increases in endogenous synaptic levels of dopamine suppressed the elevated LTP seen in the HR mice with wheel access. As with the D_1/D_5 receptors, we do not know if the changes in DAT levels in the hippocampus of HR mice are due to increases in DAT at synapses onto interneurons or pyramidal cells. GBR 12909 activity at dopaminergic terminals onto interneurons, however, could explain the reduction in LTP seen in the HR mice with wheels.

The reduced levels of D_1/D_5 receptors and elevated levels of DAT were restricted to the hippocampus, and not observed in the prefrontal cortex or cerebellum. Thus, the difference in running behaviors in the HR mice treated with D_1/D_5 receptor drugs or DAT inhibitors (Rhodes et al., 2001; Rhodes & Garland, 2003) may not be due to alterations in the decision to run (i.e., prefrontal cortical activity) or motor coordination (cerebellar function). Instead, the differences are likely attributable to differences in striatal dopamine function, which were not examined in this study, or hippocampal function. Also of note are the enlarged hippocampi of HR mice, regardless of whether they have long-term wheel access (Schmill et al., 2023). Given the very high motivation of the HR mice to run, and the increased intermittency of their running patterns

(Girard et al., 2001), which might be interpreted as impulsivity (see also Khan et al., 2024 on behavior in a food reward task), it will be crucial to look at differences in dopaminergic transmission in the mesolimbic pathway of these animals in future studies. While the HR mice are highly motivated to run, their conditioned place preference (CPP) for dopaminergic drugs like cocaine or methylphenidate is not different from CPP in C mice (Schmill et al., 2021). This mouse model may provide a better understanding of the role of dopamine in natural rewards vs drug rewards.

In addition to the high motivation for running that they display and involvement of reward pathways (e.g., see Rhodes, Garland, et al., 2003; Rhodes, Garland and Gammie, 2003), the hyperactivity and possible impulsivity of HR mice have several features in common with symptoms seen in attention deficit hyperactivity disorder (ADHD; Thapar & Cooper, 2016). Given the need for additional models of ADHD that have strong face, construct, and predictive validity (for review, see Kantak, 2022; Regan et al., 2022), the HR mice could be considered for this purpose. In terms of face validity, the HR mice show hyperactivity in their familiar environment (Copes et al., 2015; Malisch et al., 2009; Rhodes et al., 2001) as well as in novel environments; use of HR mice as an ADHD model though would require demonstration of inattention and/or impulsivity through assays such as the 5-choice serial reaction time test and go/no go tests, respectively. In terms of construct validity, ADHD is a complex, heterogeneous disorder in which many of the neurobiological substrates have not been identified, but the altered dopaminergic transmission in HR mice is consistent with altered dopaminergic transmission in ADHD individuals. The predictive validity of HR mice as an ADHD model is supported by the observation that their high running behavior is normalized to control levels by treatment with methylphenidate (Rhodes et al., 2005; Rhodes & Garland, 2003) and that high LTP levels are

normalized to control levels after treatment with the selective DAT inhibitor, GBR 12909 in the present study. It would also be helpful to determine whether the compromised performance on the Morris water maze task in HR mice with long-term wheel access (Rhodes, van Praag, et al., 2003) can be prevented by pretreatment with methylphenidate or GBR 12909.

In addition to examining D_1/D_5 receptor and DAT function in the reward pathway of HR mice, it would be useful to examine changes in $D_2/D_3/D_4$ receptor function in hippocampus and striatum. Bronikowski et al. (2004) observed differences in D_2 and D_4 gene expression in the hippocampus of HR mice, but did not measure protein levels or receptor function; nor did they examine changes in dopamine receptor gene expression in the striatum. Subsequent studies have revealed no changes in gene expression for D_1/D_5 or D_2 receptors or DAT in the striatum (Saul et al., 2017), but this does not rule out possible changes in other proteins involved in dopaminergic transmission, such as downstream kinases and phosphatases involved in signal transduction or enzymes involves in dopamine synthesis or degradation.

Identifying differences in HR vs C mice that could underly their increased motivation to run could allow the development of strategies to enhance motivation for exercise in individuals suffering from neurodegenerative or neuropsychiatric conditions in which increased exercise appears to be therapeutic. The mesolimbic dopamine pathway, and particularly the nucleus accumbens (NAc), plays a key role in motivation (Koob & Volkow, 2010), including voluntary running behavior (Nestler et al., 2001). Using c-fos staining to label active neurons, (Rhodes, Garland, et al., 2003) observed very high neuronal activation in the NAc of HR mice, particularly when they were denied access to their running wheel; the extent of activation was directly proportional to the distances they ran on the day before the wheel was blocked. Work by (Roberts et al., 2014) in rats selectively bred for low vs high voluntary running (LVR vs HVR)

has further revealed differences in NAc neurons between the two groups: the LVR mice show less development of medium spiny neurons, the neurons in the NAc that express dopamine receptors and receive inputs from the ventral tegmental area. In addition, the HVR rats demonstrated plasticity of dopamine-related transcripts with running, whereas the LVR rats did not. These and other studies (Knab et al., 2009; Mathes et al., 2010; Rhodes & Garland, 2003) continue to point to the importance of dopamine systems in motivation for exercise. Given the tendency for such motivation to decline with age (for review, see Ingram, 2000; Marck et al., 2016), it is critical to more fully characterize the neurobiological substrates of this complex behavior. Ample evidence shows that increased activity levels can be beneficial in managing age-related diseases such as Alzheimer's and Parkinson's (Binda et al., 2021; Ingram, 2000; Dao et al., 2016; for review, see da Costa Daniele et al., 2020), perhaps in part due to changes in dopaminergic mechanisms.

Enhancing motor activity may also be effective in management of other neurological conditions, such as depression, anxiety, and responses to stress. Many studies in humans (Carek et al., 2011) and rodents (Mul et al., 2018) have revealed antidepressant properties of voluntary exercise, via mechanisms dependent on BDNF (Duman et al., 2008) and neurogenesis (Ernst et al., 2006). Voluntary wheel running can also ameliorate depressive responses to stress (Nowacka-Chmielewska et al., 2022), such as chronic social defeat stress (Mul et al., 2018)), as well as anxiety in mice (Duman et al., 2008; Salam et al., 2009). Such studies are not without controversy, however, as some groups (Fuss et al., 2010; Renoir et al., 2012)) have observed enhanced depression and/or anxiety in mice following high levels of running activity. Of concern in the current study was the need to singly-house the mice to ensure access to their running wheels; the single housing may have imposed some amount of stress, and such isolation-

induced stress may be greater in female vs male mice (Arzate-Mejía et al., 2020; Challa et al., 2023). It will be important to continue these studies using both sexes for this and other reasons.

In conclusion, our results suggest that the enhanced synaptic plasticity seen in the hippocampus of intensely active HR mice when housed with wheels (Fig. 1 and 2) is underlain by alterations in dopaminergic function at these synapses. Our studies have focused on area CA1 of the hippocampus, as dopaminergic innervation to this region seems particularly relevant to novelty-enhanced memory and memory for linked contexts. It would be helpful to look further at LTP in the dentate gyrus of HR mice to get a more direct measure of the effects of maximal neurogenesis, as seen in highly active HR mice (Rhodes, van Praag, et al., 2003) on LTP. Similarly, it would be helpful to examine dopamine receptor and DAT levels in these subregions of the HR hippocampus, and in interneurons vs principal neurons. In any case, the reduced levels of D1/D5 receptors and elevated DAT levels observed in hippocampus were consequences of the selective breeding for high running (genotype) rather than environmental experience (access to and intensive use of a running wheel). These observed alterations in dopaminergic neurotransmission in the selectively bred HR mice continue to support their use as a model for ADHD and/or to better understand animals' motivation for higher physical activity.

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Data availability:

The data supporting the findings of this study are available upon request from the corresponding author.

Figure legends

Fig. 1. **High voluntary wheel running enhances LTP in HR mice without affecting paired pulse facilitation.** (a) Scatter plots show normalized mean fEPSP slope ± SEM recorded from hippocampal slices of control (C) and HR mice with and without wheel access. A full TBS was delivered at 0 min (C with wheel: n = 9; C no wheel: n = 8; HR with wheel: n = 13; HR no wheel: n = 9). Representative fEPSPs recorded during baseline (1) and 1 h post-TBS (2) are superimposed (scale bars: 0.5 mV, 5 ms). (b) Bar graph: Mean degree of potentiation (± SEM), measured as a percent increase of normalized fEPSP slope from baseline, 50-60 min post-TBS. LTP was significantly enhanced in hippocampal slices from HR mice with wheel access (same n values as above; two-way ANOVA: wheel access, $F_{1,35} = 4.335$, $P = 0.0447$; genotype, $F_{1,35} = 0.472$, $p = 0.4967$; genotype x wheel access, $F_{1,35} = 4.033$, $p = 0.0524$). (c) Mean PPF ratios (± SEM) measured during baseline recordings in aCSF. Neither wheel access nor genotype affected PPF (same *n* values as above; two-way ANOVA). Traces (scale bars: 0.5 mV, 5 ms) are representative fEPSPs elicited using a paired pulse-stimulation protocol during baseline recordings. (d) Mean magnitude of PTP (± SEM) in the first ten minutes post-TBS. Neither wheel access nor genotype affected PTP (same *n* values as above; two-way ANOVA).

Fig. 2. **Treatment with a D_1/D_5 receptor agonist or DAT inhibitor selectively decreases LTP in HR mice with wheel access. (a,b)** Scatter plots show normalized mean fEPSP slope ± SEM recorded from hippocampal slices of HR mice without (a) and with (b) wheel access. A full TBS was delivered at 0 min. Representative fEPSPs (scale bars: 0.5 mV, 5 ms) recorded during baseline (1) and 1 h post-TBS (2) are superimposed. **(c)** Bar graph shows mean degree of potentiation (± SEM), measured as a percent increase of normalized fEPSP slope from baseline,

50-60 min post-TBS. SKF-38393 reduced LTP in HR mice with wheel access while having no effect on HR mice without wheel access (HR with wheel: n = 13; HR with wheel + SKF: n = 6; HR with wheel + GBR: n = 6; HR no wheel: n = 7; HR no wheel + SKF: n = 7; two-way ANOVA: wheel access, $F_{1,32} = 4.983$, p = 0.0327; SKF, $F_{1,32} = 6.057$, *p = 0.0194; wheel access x SKF, $F_{1,32} = 4.710$, *p = 0.0375; main effect of GBR, $F_{2,22} = 7.242$, *p = 0.00383, one-way ANOVA. Bonferroni's post hoc comparison of HR with wheel vs HR with wheel + SKF or + GBR, ** $p < 0.01$; there are no significant differences in LTP responses in the presence of SKF vs GBR in the HR mice with wheel access.

Fig. 3. **The D_1/D_5 receptor agonist has no significant effect on LTP in control mice with or without wheel access.** Scatter plots show normalized mean fEPSP slope ± SEM recorded from hippocampal slices of C mice without and with wheel access. A full TBS was delivered at 0 min. Representative fEPSPs (scale bars: 0.5 mV, 5 ms) recorded during baseline (1) and 1 h post-TBS (2) are superimposed. Bar plots show mean degree of potentiation (± SEM), measured as a percent increase of normalized fEPSP slope from baseline, 50-60 min post-TBS. SKF-38393 had no effect on LTP in slices from control mice with and without wheel (control with wheel: n = 7; C with wheel + SKF: n = 7; C no wheel: n = 8; C no wheel + SKF: n = 5; two-way ANOVA: wheel access, $F_{1,24} = 0.866$, p = 0.808; drug, $F_{1,24} = 0.061$, p = 0.808; wheel access x drug, $F_{1,24} = 1.908$, p = 0.180).

Fig. 4. **HR mice have fewer D_1 receptors and more DAT in the hippocampus than control mice. (a)** Representative Western blots and densitometric quantification of proteins from HR and C mice, with and without wheel access. HR mice have reduced hippocampal levels of D1

receptors and elevated hippocampal levels of DAT compared to C mice (n = 4 mice per group; 2-way ANOVA for D1/GAPDH: main effect of genotype: $F_{1,12} = 55.790$, ***$p < 0.001$; wheel access: $F_{1,12} = 2.510$, $p = 0.139$; interaction between genotype and wheel access: $F_{1,12} = 1.800$, $p = 0.203$; 2-way ANOVA for DAT/GAPDH: main effect of genotype: $F_{1,12} = 11.014$, ***$p < 0.001$; wheel access: $F_{1,12} = 0.004$, $p = 0.9494$; genotype x wheel access: $F_{1,12} = 0.002$, $p = 0.9646$). **(b-c)** In the prefrontal cortex (b) and cerebellum (c), no significant differences in D1 receptor protein or DAT, based on genotype or wheel access, were observed (n = 4 mice per group; 2-way ANOVAs). For all blots, GAPDH was used as a loading control. Molecular weight markers (kDa) were used to determine the apparent molecular weights. Data are presented as mean ± SEM.

Supplementary Data

Movie 1. An HR mouse on a WARE Flying Saucer Exercise Wheel for Small Animals™.

Supplemental Figure 1. Original scans of immunoblotted protein samples from the hippocampus. (a-c) Blots probed with primary antibodies directed against D1 receptor **(a)**, DAT **(b)**, and GAPDH **(c)**. Dotted boxes outline the area of images shown in Figure 4a of the main text.

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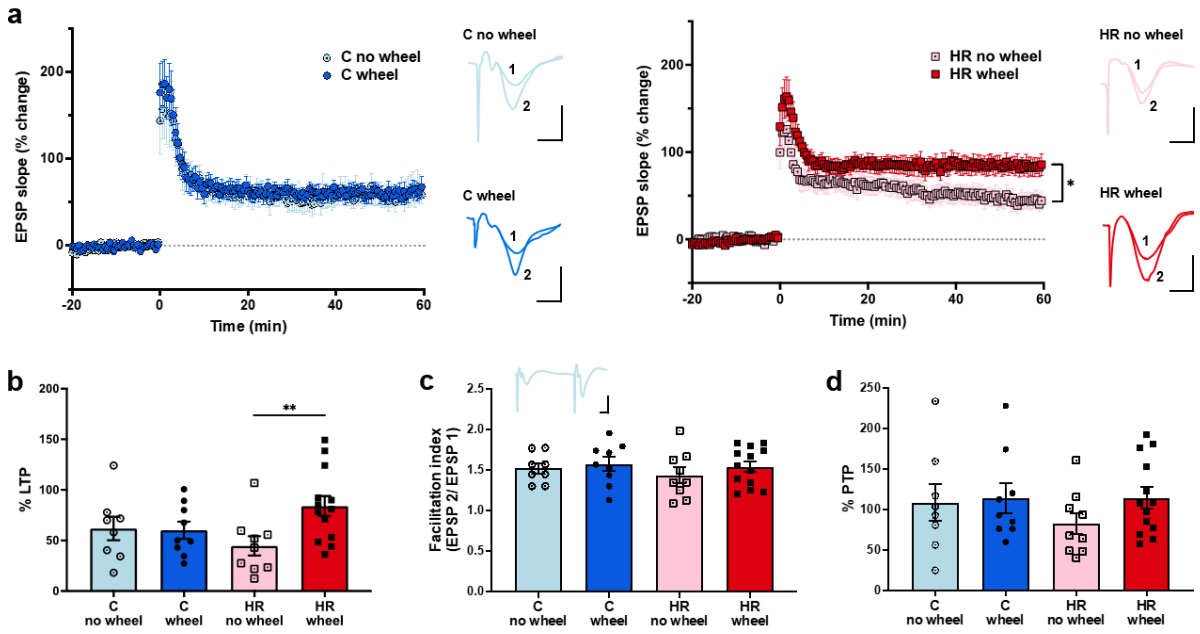
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C no wheel
C wheel
EPSP slope (% change)
Time (min)
HR no wheel
HR wheel
b
% LTP
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c
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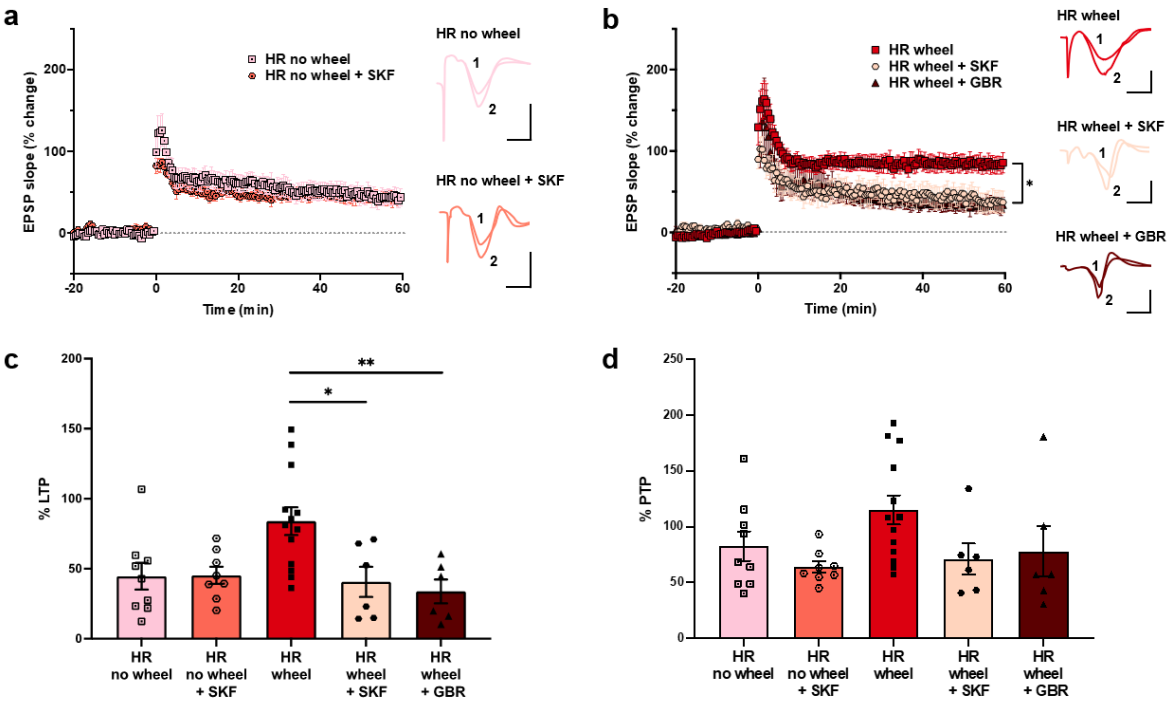
d
% PTP

Figure 1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HR no wheel + SKF
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Time (min)
HR no wheel
HR no wheel + SKF
b
HR wheel
HR wheel + SKF
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EPSP slope (% change)
Time (min)
HR wheel
HR wheel + SKF
HR wheel + GBR
c
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HR no wheel + SKF
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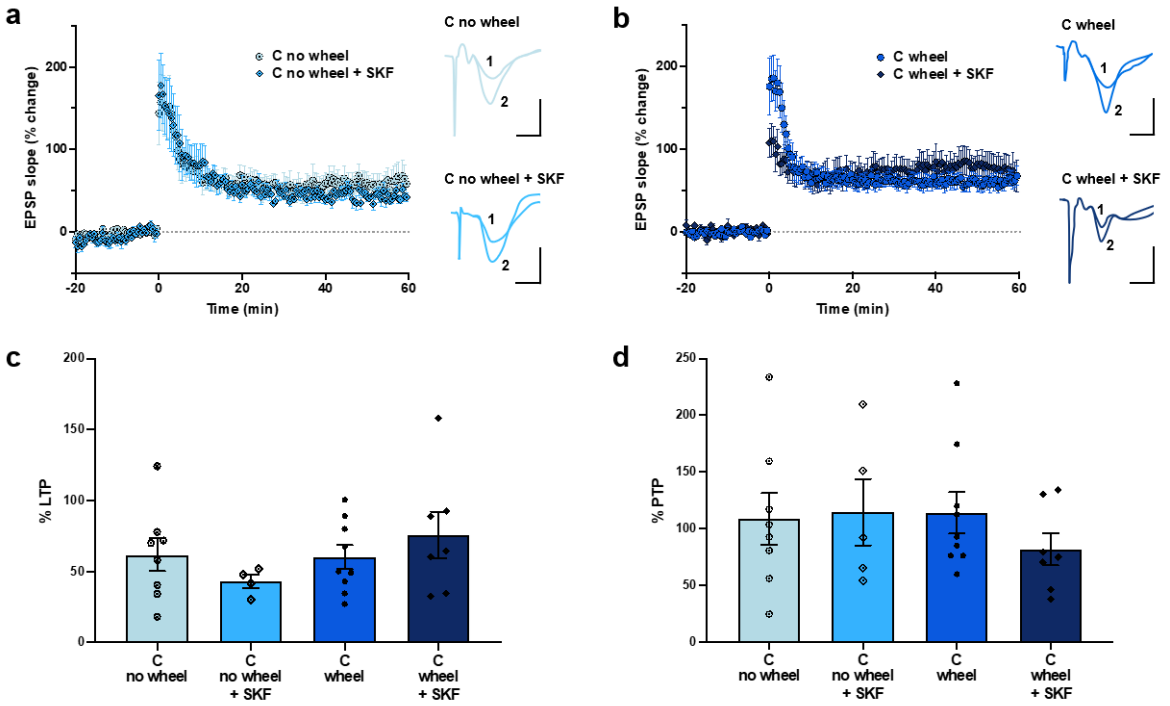
Figure 2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C no wheel + SKF
EPSP slope (% change)
Time (min)
C no wheel
C no wheel + SKF
b
C wheel
C wheel + SKF
EPSP slope (% change)
Time (min)
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C wheel + SKF
c
% LTP
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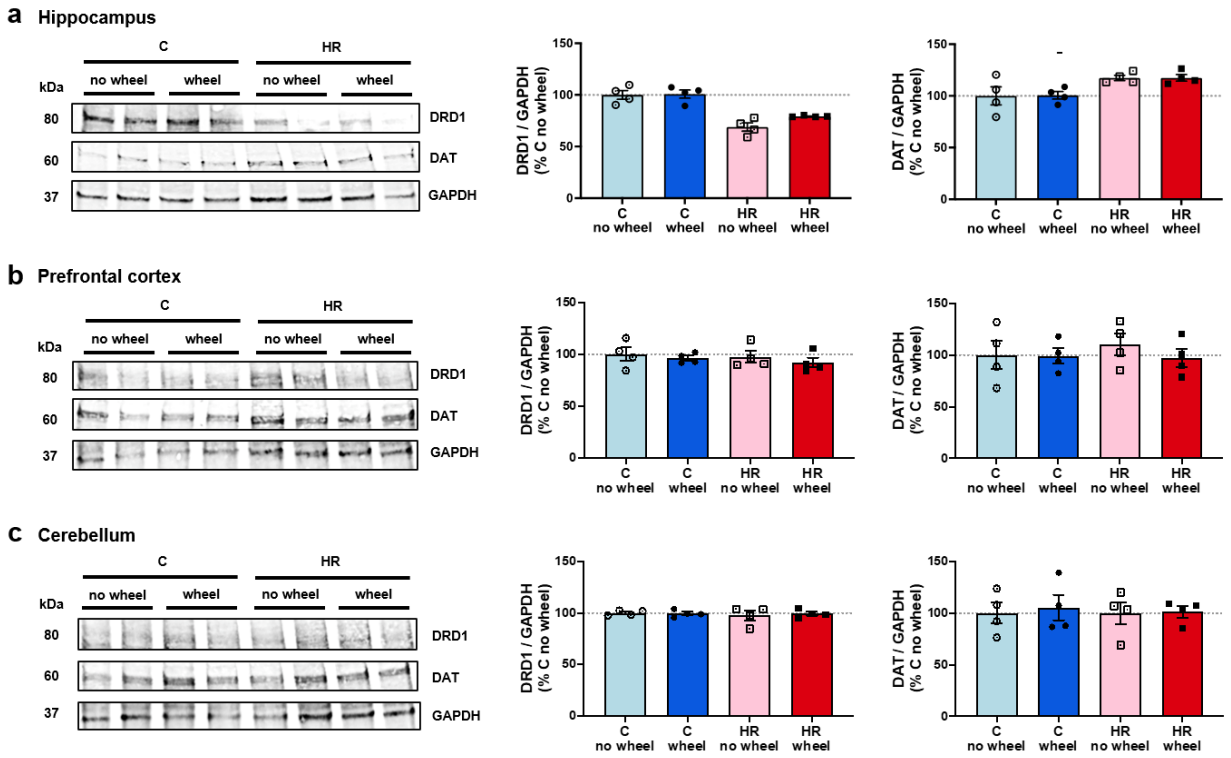
Figure 3


a Hippocampus
C
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kDa
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80
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37
DRD1
DAT
GAPDH
DRD1 / GAPDH (% C no wheel)
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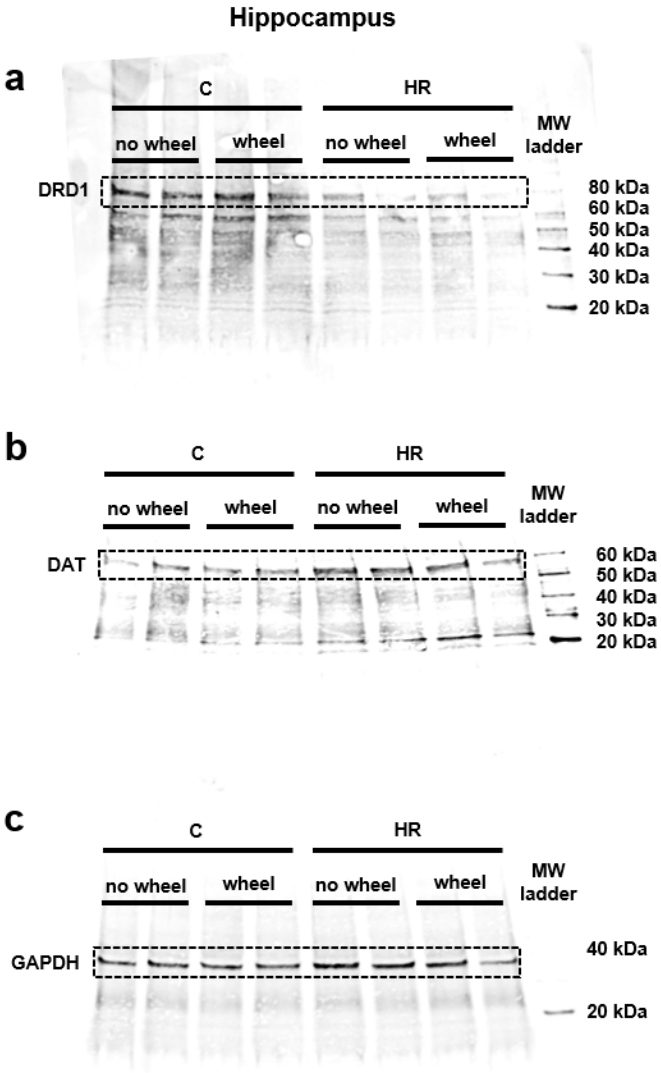
C wheel
HR no wheel
HR wheel
b Prefrontal cortex
c Cerebellum

Figure 4



Supplementary Figure S1