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Date:
2019-01-01

Citation:
Rogers, J., Chen, F., Stanic, D., Farzana, F., Li, S., Zeleznikow-Johnston, A. M., Nithianantharajah, J., Churilov, L., Adlard, P. A., Lanfumey, L., Hannan, A. J. & Renoir, T. (2019). Paradoxical effects of exercise on hippocampal plasticity and cognition in mice with a heterozygous null mutation in the serotonin transporter gene. British Journal of Pharmacology, 176 (17), pp.3279-3296. https://doi.org/10.1111/bph.14760.

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Paradoxical effects of exercise on hippocampal plasticity and cognition in mice with a heterozygous null mutation in the serotonin transporter gene

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.14760

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Background and Purpose: Exercise is known to improve cognitive function, but the exact synaptic and cellular mechanisms remain unclear. We investigated the potential role of serotonin transporter 5-HTT in mediating these effects.

Experimental approach: Hippocampal long-term potentiation (LTP) and neurogenesis were measured in standard-housed and exercising (wheel running) wild-type (WT) and 5-HTT heterozygous (HET) mice. We also assessed hippocampus-dependent cognition using the Morris water maze (MWM) and a two-choice spatial pattern separation touchscreen task.

Key Results: 5-HTT HET mice had impaired hippocampal LTP regardless of the housing conditions. Exercise increased hippocampal neurogenesis in WT mice. However, this was no longer observed in 5-HTT HET animals, even though both genotypes used the running wheels to a similar extent. We also found that standard-housed 5-HTT HET mice displayed better cognitive flexibility than WT littermate controls in the MWM reversal learning task. However, 5-HTT HET mice no longer exhibited this phenotype after exercise. Similar cognitive deficits, specific to 5-HTT HET mice in the exercise condition, were also revealed on the touchscreen spatial pattern separation task, especially when the cognitive flexibility load was at its highest.

Conclusions & Implications: Our study is the first evidence of reduced hippocampal LTP in 5-HTT HET mice. We also show that functional 5-HTT is required for exercise-induced increases of adult neurogenesis. Paradoxically, exercise had a negative impact on hippocampus-dependent cognitive tasks, especially in the 5-HTT HET mice touchscreen location discrimination probe. Taken together, our results suggest unique complex interactions between exercise and altered 5-HT homeostasis.

Keywords: serotonin transporter, exercise, synaptic plasticity, neurogenesis, cognition

Introduction

The gene x environment (G x E) interaction approach has helped to identify environmental and genetic factors that may contribute to the manifestation of psychiatric disorders (Caspi, Hariri, Holmes, Uher & Moffitt, 2010; Halldorsdottir & Binder, 2017). Serotonin (5-HT) signaling regulates hippocampus-dependent cognitive and emotional processing that have been associated with the psychobiology of mental illness (Barnes & Sharp, 1999). The serotonin transporter (5-HTT) is essential to this regulation, by terminating 5-HT action in the synaptic cleft through 5-HT reuptake into the presynaptic terminal (Jacobs & Azmitia, 1992). For these reasons, the interaction between a functional tandem repeat polymorphism in the 5-HTT gene, the 5-HTT-linked polymorphic region (5-HTTLPR), and environmental factors have been exhaustively investigated (Caspi, Hariri, Holmes, Uher & Moffitt, 2010; Halldorsdottir & Binder, 2017; Karg, Burmeister, Shedden & Sen, 2011). 5-HTT expression and 5-HT reuptake capacity are lowered in carriers of the short (S) allele (Lesch et al., 1996); and the interaction between experiencing early life stress and carrying the S allele may enhance the risk of depression (Caspi et al., 2003), although this finding remains controversial (Culverhouse et al., 2018). Recent evidence has suggested that early positive environments also interact with S allele carriers, in this case enhancing aspects of cognition (Homberg & Lesch, 2011; van Ijzendoorn & Bakermans-Kranenburg, 2015; van Ijzendoorn, Belsky & Bakermans-Kranenburg, 2012). This evidence may account for the controversy in clinical studies, and suggests that population variability in 5-HT reuptake capacity may increase sensitivity to environments in general (Moore & Depue, 2016).

Measuring early versus adult environmental exposures in clinical studies has been an obstacle to progress in the G x E field (Fox & Beevers, 2016; Keller, 2014; Song & Gleeson, 2018; Tost, Champagne & Meyer-Lindenberg, 2015). In rodents, the G x E interaction approach has been more successful due to experimental control over the environment (Nithianantharajah & Hannan, 2006). Voluntary physical activity (exercise) has been found to improve cognition in human and animal models (Stimpson, Davison & Javadi, 2018; Suo et al., 2016). Additionally, exercise is

known to enhance hippocampal synaptic plasticity and adult neurogenesis, although the precise mechanisms remain unclear (Ohline & Abraham, 2018; Patten, Yau, Fontaine, Meconi, Wortman & Christie, 2015). 5-HT is a candidate molecular mediator of exercise-induced alterations to hippocampal cellular and molecular plasticity (Rogers, Renoir & Hannan, 2019). The extracellular concentration of 5-HT ([5-HT]ext) may be the critical factor, because exercise increases the [5-HT]ext in the hippocampus (Gomez-Merino, Bequet, Berthelot, Chennaoui & Guezennec, 2001). It has been established that mice lacking brain 5-HT have impaired exercise-induced cell proliferation, indicating that the pro-proliferative effect of exercise requires the release of 5-HT (Klempin, Beis, Mosienko, Kempermann, Bader & Alenina, 2013). Serotonin neurons project throughout the neuraxis of the brain from the raphe nuclei, and 5-HT signals through 14 metabotropic receptors and a sole ionotropic receptor, all present in the hippocampus (Jacobs & Azmitia, 1992; Palacios-Filardo & Mellor, 2019). Supporting the idea that the [5-HT]ext is a critical factor, 5-HT signaling through the sole ionotropic 5-HT receptor, the 5-HT_3 receptor, but not the metabotropic 5-HT_{1A} receptor, was reported to be critical for exercise-induced adult neurogenesis in the hippocampus (Kondo, Kondo, Nakamura, Ishida & Shimada, 2014; Rogers et al., 2016). The evidence for 5-HT regulating functional plasticity in the hippocampus is not as clear (Araragi & Lesch, 2013; Palacios-Filardo & Mellor, 2019); however, recent evidence *in vivo* suggests that mechanisms exist where 5-HT facilitates long-term potentiation (LTP) between neurons in the CA1 region of the hippocampus (Cai et al., 2013; Teixeira et al., 2018).

In this current study, we examined whether the partial disruption of 5-HTT during development was sufficient to alter the cellular and behavioural effects of voluntary exercise in adulthood. 5-HTT heterozygous (HET) mice have been established as a relevant model of 5-HTTLPR S allele carriers and widely used to study G x E interactions both during development and in the adult brain (Carola et al., 2008; Kästner, Richter, Lesch, Schreiber, Kaiser & Sachser, 2015; Schraut et al., 2014; Spinelli et al., 2013). For instance, early life stress exposure causes 5-HTT HET mice to display depressive-like behaviour (Carola et al., 2008), mirroring both the clinical findings

(Caspi et al., 2003) and the phenotype of constitutive 5-HTT knock-out (KO) rodents (Kalueff, Olivier, Nonkes & Homberg, 2010). Paradoxically, arguments for a conceptual change in the current deficit-oriented connotation of the 5-HTTLPR variants have recently being put forward, mainly based on recent findings that humans and nonhuman primates carrying the s variant of the 5-HTTLPR outperform subjects carrying the long allele in an array of cognitive tasks (Homberg & Lesch, 2011). In some cases, 5-HTT HET mice could exhibit enhanced cognition (especially on tasks requiring cognitive flexibility), although modest effect size and inconsistent findings have been reported so far. (Brigman et al., 2010; Homberg & Lesch, 2011; Ineichen et al., 2012).

Importantly, 5-HTT HET mice have reduced 5-HT reuptake in a gene-dose dependent fashion, manifesting as a 2-3 fold increase in [5-HT]ext compared to a 7-9 fold increase in [5-HT]ext in 5-HTT KO animals (Mathews, Fedele, Coppelli, Avila, Murphy & Andrews, 2004). This difference, in addition to other gene-dosage changes in the raphe nuclei of 5-HTT HET mice, may explain the behavioural differences between the two 5-HTT mutants (Murphy & Lesch, 2008). Finally, all aspects of adult neurogenesis in young-adult 5-HTT HET mice are normal at baseline (Schmitt et al., 2007), but, no study has yet measured CA1 LTP in the hippocampus of 5-HTT mutant mice.

In addition of studying the effects of exercise on synaptic and cellular plasticity mechanisms in adult 5-HTT HET mice, we further investigated potential effects on hippocampus-dependent cognitive tasks. Our data suggest that serotonin reuptake capacity is critical for the stimulating effect of exercise on hippocampal neurogenesis. We report for the first time an impaired hippocampus LTP in 5-HTT HET mice (regardless of housing conditions). We also observed enhanced cognitive flexibility in 5-HTT HET standard-housed mice. Finally, exercise did not enhance pattern separation ability in WT mice, despite a large increase in neurogenesis.

Methods

Animals and housing

Animal studies are reported in compliance with the ARRIVE guidelines (McGrath & Lilley, 2015). Wild-type (WT) and 5-HTT HET littermate mice were generated on a C57BL/6 background using 5-HTT HET breeding pairs. 5-HTT heterozygous (HET) mice have been established as a relevant model of 5-HTTLPR S allele carriers. Post-weaning, mice were group-housed (4-5 mice per cage) by genotype in individually ventilated containers until 5 weeks of age. At this stage, mice were put in standard-housing (SH) open-top cages (34 x 16 x 16 cm) on a 12-hour light/12-hour dark cycle (lights on at 7 am) with *ad libitum* access to food and water. Mice of both sexes were used in all experiments except for the touchscreen cohort (for which only males were used). At 8 weeks of age, mice were randomly assigned (4-5 mice per cage) to continue in SH cages or to change to exercise cages (EX) (40 cm x 28 cm x 18 cm) containing two running wheels (12 cm diameter), with animals again being separated by genotype. Several cohorts of animals were required to complete this study. Animals used for the Morris water maze (MWM) test and synaptic/cellular plasticity analyses were assessed after 5 weeks of exercise, because this corresponds to the generation of new adult-born neurons in the dentate gyrus. A different cohort of mice was used for touchscreen testing. For that specific experiment, exercise access began at the end of pre-training, which meant that those mice had 4 weeks of exercise access when the performance probe began. All experiments were conducted following the guidelines of the Florey Institute's Animal Ethics Committee and the National Health and Medical Research Council (NHMRC), and with the experimenters blind to genotype.

Distance run

Using two different cohorts of animals, we measured total distance run in both single-housing and grouped-housing conditions. In the first cohort, automated running wheel chambers (30 cm x 19 cm x 11 cm; 12 cm diameter wheel) were used to assess the total distance run by single-housed individual mice upon completion of five weeks of exercise (upon completion of the MWM, but prior to the synaptic and cellular plasticity experiments). The number of wheel rotations in a 24-hour period was recorded using activity monitor software (Lafayette Instrument, Lafayette, Indiana, USA) that was then converted into distance run (km). In the cohort used for the touchscreen experiment, the running wheels were attached to the cage lid to enable wired bicycle speedometers (Sigma Sport Asia, Neustadt, Germany) to detect magnets attached to the wheels. These detectors functioned as odometers by recording the number of daily revolutions and converting this into a distance in km. Through this method, we were able to calculate the distance run per box and then divide it by the number of mice group-housed in that cage to obtain the average per mouse.

Morris water maze test (MWM)

To assess hippocampal-dependent spatial learning and spatial memory, we conducted the reference version of the MWM as described previously (Rogers et al., 2016). Mice were trained with four trials per day for six days to learn the location of a hidden escape platform. To assess spatial learning, we performed a search strategy analysis to assess the rate of allocentric map formation as extensively described elsewhere (Rogers, Churilov, Hannan & Renoir, 2017). From this previous work, we have established that the % spatial search strategy is the outcome measure of spatial learning that reliably predicts formation of spatial memory on the MWM. Other measures of spatial learning, namely the latency and path length to the escape platform have been included for completeness. During a 1-minute retention probe on the seventh day, the platform was removed, and intact spatial memory was demonstrated by a preference for the quadrant of the pool that had previously contained the escape goal. The mice then underwent two

days of reversal learning, during which the escape platform was moved to the opposite side of the pool. We again assessed spatial learning as the rate of allocentric map formation using a second search strategy analysis (again reporting the latency and path length to the escape goal for completeness). On the final day the platform was removed, and mice again could demonstrate spatial memory for the new escape goal position by showing a preference for the quadrant of the pool that contained the hidden platform during the reversal learning training in a 1-minute reversal probe.

Two-choice spatial location discrimination touchscreen test

To assess spatial pattern separation, we employed the two-choice spatial location discrimination (LD) task as part of the rodent touchscreen platform (Campden Instruments Ltd, Leicestershire, England), which has been described previously (Oomen et al., 2013). Male mice (5 weeks of age) were housed in standard cages and maintained on a reversed 12-hour light/12-hour dark cycle. Animals were weighed daily from 6 to 8 weeks of age to establish free-feeding baseline weights, then food was restricted to approximately 85% free-feeding weight (8 to 9 weeks of age). Weights were continually monitored throughout the touchscreen experiment and the level of restriction adjusted accordingly. The touchscreen platform is essentially an appetitive operant conditioning paradigm that enables complex cognitive testing, so food restriction is an essential part of the experimental procedure as mice require motivation (i.e. obtaining a food reward) to learn how the system operates prior to training to solve the task of interest (Oomen et al., 2013). As our mice were group-housed, the amount of food required to maintain each individual mouse's weight was calculated and then made available to the entire group after testing. If individual mice were gaining/losing weight away from their 85% free-feeding weight, those mice were individually housed during the daily feeding session (for 1 to 2 hours until food was consumed) until their weight had stabilized. At that time, group-housed feeding resumed as

above. Mice then underwent pre-training in the touchscreen chambers (~9 to 11 weeks of age) as exhaustively described elsewhere (Oomen et al., 2013). Strawberry milk (Iced Strawberry Milk, Nippy's Ltd, Melbourne, Australia) was used as the reward (Zeleznikow-Johnston, Burrows, Renoir & Hannan, 2017). All testing occurred in the dark-cycle, under red light, 6 days per week. An extensive overview of the mouse touchscreen apparatus can be found elsewhere (Horner et al., 2013).

At the completion of pre-training, mice were allocated to cages with exercise wheels. The two-choice spatial LD task has previously been performed in both young-adult and aged mice who had exercise wheel access from a similar time point (Creer, Romberg, Saksida, van Praag & Bussey, 2010). Importantly, the combination of exercise and food restriction in both sets of WT mice had no adverse consequences to the animals' health. Thus, we were confident to proceed with the task training with exercising mice of both genotypes. Animals were then trained on the LD test using the intermediate separation between stimuli (response windows 2 and 5 were lit) until the task training criterion was met. In the acquisition phase, either the left (response window 2) or right square (response window 5) was designated as the correct conditioned stimulus (CS+). Once an animal achieved 7/8 consecutive correct responses, the reversal phase of the task began, where the location of the CS+ designation was reversed. Animals were given daily sessions until both the acquisition and reversal criterions (7/8 consecutive correct responses) were achieved, a maximum number of 60 trials were completed, or the maximum session time of 60 minutes had elapsed (whichever came first). Animals successfully completed the training once they completed an acquisition and reversal criterion in a session, across 3/4 consecutive sessions. To allow all animals within the cohort to complete training before probing for pattern separation, animals that had achieved the training criterion received reminder sessions every 2 days, until the entire cohort was ready to move to the next stage. For the performance probe phase, we maintained the animals at the same testing pace to ensure the stage of neurogenesis was consistent across the cohort during the assessment of pattern separation. To examine different spatial loads on pattern separation, two separations were employed: high

separation (response windows 1 and 6) or low separation (response windows 3 and 4). Performance on the low separation is known to require adult neurogenesis (Clelland et al., 2009). Animals were tested for 2 days on either low or high separation, then alternated with 2 days on the other separation, for a total of 6 sessions on each separation.

Immunohistochemistry

Peroxidase

The rate of adult-born cell survival was assessed using peroxidase 5-bromodeoxyuridine (BrdU) immunohistochemistry as previously described (Rogers et al., 2016). Mice were injected with BrdU i.p. at a dose of 50 mg kg^{-1} of body weight for 7 consecutive days at the beginning of housing, with access to exercise. After 5 weeks of exercise access and assessment of the nightly distance run, mice were euthanized using an IP injection of Lethabarb (325 mg/mL Pentobarbitone Sodium; Virbac, Peakhurst, NSW, Australia) to deeply anaesthetize the animals prior to intracardial perfusion with 0.9% saline, followed by 4% paraformaldehyde (PFA) solution in 0.1 M phosphate buffer (pH 7.3). Following perfusion, tissue was obtained and stored at −80 °C for immunohistochemistry as previously described (Ransome & Turnley, 2007). Serial coronal sections from -1.34 to -2.54 mm Bregma were cut at 40 µm thickness and were collected in a 1 in 6 series spaced 240 µm apart, thereby sampling the entire length of the dorsal hippocampus. Peroxidase immunohistochemistry was then performed exactly as described previously (Rogers et al., 2016). The absolute numbers of BrdU+ cells were counted to quantify the effect of exercise on adult-born cell survival. This measure was used as an indicator of dentate gyrus cellular plasticity because it is a known surrogate for the rate of adult neurogenesis in the hippocampus.

Fluorescence

Sections were washed 3 times in 0.1 M PBS, incubated in Omnipur deionized formamide (Merck, Darmstadt, Germany; #4610-OP) for 2 hours at 65 °C, and then washed 3 times in PBS again. Sections were then incubated in 2N HCl (Merck) at 37 °C for 30 min, followed by 0.1 M sodium tetraborate decahydrate (Sigma, Saint Louis, MO, USA) at room temperature (RT) for 20 min, and 6 washes in PBS prior to commencement of triple-fluorescence immunohistochemistry. Primary antibodies used, and dilutions [in PBS containing 0.3% Triton X-100 and 0.5% BSA (Sigma)], were: sheep anti-bromodeoxyuridine (BrdU) polyclonal antibody (1:500; Exalpha Biologicals Inc, Shirley, MA, USA; Cat# A205P); rabbit anti-doublecortin (DCX) polyclonal antibody (1:500; Abcam, Cambridge, UK; Cat# ab18723; RRID:AB_732011) and mouse anti-neuronal nuclei (NeuN) monoclonal antibody, clone A60, (1:400; Millipore, Temecula, CA, USA; Cat# MAB377; RRID:AB_2298772). After 20 hours incubation in primary antisera at RT, sections were washed in PBS followed by TNT buffer (0.1 M TRIS-HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween20, Sigma) and incubated with TNB blocking solution (TNT with 0.5% Dupont blocking powder; NEN Life Science Products, Inc., Boston, MA, USA) for 1 hour. To visualise the immunoreactivity, sections were incubated for 90 min at RT with the following secondary antibodies: Alexa Fluor 594-conjugated AffiniPure donkey anti-rabbit (1:500; RRID: AB_2340621); Alexa Fluor 647-conjugated AffiniPure donkey anti-mouse (1:500; RRID: AB_2492288); and biotin-SP-conjugated AffiniPure donkey anti-sheep (1:750; Jackson ImmunoResearch Laboratories, West Grove, PA; RRID: AB_2340716), all diluted in TNB. Sections were then washed in TNT buffer and PBS, incubated in Alexa Fluor 488-conjugated streptavidin (1:500; Jackson; RRID: AB_2337249) diluted in PBS for 90 min, washed 3 times in PBS, mounted onto superfrost slides (Fisher Scientific) and coverslipped using a fluorescent mounting medium (Dako, Glostrup, Denmark).

Fluorescence image acquisition

After immunohistochemistry, sections were examined using a Zeiss Observer.Z1 microscope (Oberkochen, Germany), equipped with a dark field condenser and epi-polarization, epifluorescence with appropriate filter combinations. Images were acquired using a ×20 objective lens (Plan-Apochromat, N.A. 0.8), an AxioCam ICc5 digital camera (Zeiss), and ZEN 2012 (blue edition) software (Zeiss). The entire z-plane of the dentate gyrus from each section was captured at intervals of 1.0 um using z-stack, tiles and stitching functions of the Zen 2012 software, allowing for 3-dimensional quantification of BrdU/DCX or BrdU/NeuN double-immunoreactive cell bodies.

Fluorescence cell quantification

Quantification of the number of BrdU/DCX or BrdU/NeuN double-immunoreactive cells in the granular cell layer and subgranular zone of the dentate gyrus was performed with the researcher blinded to the identity of the slides. Five sections from each animal were examined, ranging from 1.34 to 2.30 mm, caudal to bregma. Manual 3-dimensional quantification was performed on acquired images by focusing through the entire z-dimension of each field of view, using the z-position and measurement functions of the Zen 2012 software.

Micro-electrode array (MEA) electrophysiology

Acute hippocampal slice preparations

After the end of the distance run assessment, mice were put into a closed container containing a cotton soaked with 1 ml isoflurane and decapitated while under deep anaesthesia. The whole brains were quickly removed and place in ice-cold, oxygenated (95% O_2, 5% CO_2) cutting solution (composition in mmol/L: 206 sucrose, 3 KCl, 0.5 $CaCl_2$, 6 $MgCl_2$-H_2O, 1.25 NaH_2PO_4, 25 $NaHCO_3$ and 10.6 D-glucose). Transverse hippocampus slices (350 µm) were prepared with a VT 1200S tissue slicer (Leica) and quickly transferred to 34^oC artificial CSF (aCSF) (composition in mmol/L: 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.3 6 $MgCl_2$-H_2O, 1.25 NaH_2PO_4, 25

$NaHCO_3$ and 10 D-glucose) for 30 min. After recovery of 1 hour equilibrium in oxygenated aCSF at RT, the slices were transferred to a submersion recording chamber, as detailed below. In all cases, a Multichannel Systems MEA2100 system and associated software was utilized for all data collection and analysis (MCS GmbH, Reutlingen, Germany).

MEA recordings

One acute hippocampal slice was placed on a MEA chip comprising of 60 titanium electrodes (30 µm diameter) spaced 200 µm apart (60MEA200/30iR-Ti: MCS GnbH, Reutlingen, Germany). The slice was immobilized using a harp slice grid (ALA Scientific Instruments, New York, USA). The slice was continuously perfused with carbonated aCSF (3ml/min at 32^{o}C) during the whole recording session. The recording sites were collected from the layer of stratus radiatum (stimulation site) as major targeted dendritic synaptic sites of Schaffer collateral projections from the CA1/CA3 border are present there. Schaffer collaterals were stimulated by injecting a biphasic current waveform (100 µs) through one selected electrode at 0.033 Hz. Care was taken to place the stimulating electrode in the same region from one slice to the other. The peak-to-peak amplitude of field excitatory postsynaptic potentials (fEPSPs), at the proximal stratum radiatum of CA1, were analysed by using LTP-Analyzer (MCS GmbH, Reutlingen, Germany). Following a 20-min incubation period, slices were continuously stimulated with medium-strength stimuli. When stable evoked fEPSPs were detected (for at least 20 mins), the stimulus threshold was determined, and a stimulus strength-evoked response curve (i.e. input-output, *I-O curve*) was recorded by gradually increasing stimulus intensity until the maximal EPSP (peak amplitude of ESPS that appeared) was obtained. Only electrodes showing paired-pulse facilitation (PPF) were chosen for LTP stimulation and recording. PPF was obtained by giving two consecutive stimulations separated by an inter-stimulation interval (ISI) of 20 ms. The intensity of the test stimulus was set to be 50% of the intensity which generates a maximal response. A successful PPF had over a 150% increase in amplitude of the second EPSP compared to the first EPSP. After recording at least 20 min of stable baseline of EPSPs, LTP was

induced by applying 3 bursts of high frequency stimulus (HFS; 3x100 Hz, 500 ms width with 20 s intertrain interval) at the tested stimulation intensity, then fEPSPs were recorded for 60 mins. LTP was expressed as the percentage increase of fEPSP over the baseline fEPSP (between 3 and 60 mins post HFS). PTP was expressed as the percent increase of fEPSP over the baseline fEPSP (over 2 mins post HFS). Due to little variability in the basal size of fEPSPs among recorded slices (slices with either very small EPSPs or little linear relationship in the I-O curve were discarded), comparisons between the basal fEPSP sizes among the different groups were not performed.

Statistical Analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Two-way analysis of variance (ANOVA) with genotype and treatment (i.e. exercise) as between-group factors was performed for most of the statistical analysis. Any significant interactions or main effects were followed by Tukey's post hoc comparisons. For the LD touchscreen task summary data (i.e. trials to criterion as the dependent variable), repeated measures ANOVAs were performed with separation and phase included as within-group factors. Genotype and treatment were again included as between-group factors. In this case, we performed Bonferroni pair-wise comparisons when main effects or interactions were significant. Repeated measures ANOVAs were also performed as above for the MWM latency to platform and path length to platform data, except training day was included as the within-group factor. For both the MWM search strategy analysis and for the trial-by-trial LD task analysis, a random-effects logistic regression model was used, with animals treated as random-effects, as both outcome measures were binary in nature (Rogers, Churilov, Hannan & Renoir, 2017; Rogers et al., 2016; Zeleznikow-Johnston, Burrows, Renoir & Hannan, 2017; Zeleznikow-Johnston, Renoir, Churilov, Li, Burrows & Hannan, 2018). Effect sizes were estimated as odds ratios (OR) with corresponding 95% confidence intervals (CI) to quantify the

precision of the estimated effects. Individual search strategies (spatial or nonspatial, MWM) or individual response choices (correct or incorrect, LD task) were nested within each individual animal. For the MWM analysis, the overall effect of day (learning) on the search strategy was assessed, and the independent variables of genotype, treatment and start location were used as covariates for adjustment purposes. For the LD task trial-by-trial analysis, the overall effect of separation on the response choice was assessed and the independent variables of genotype, treatment and phase were used as covariates for adjustment purposes. For both sets of regression analyses, appropriate interaction terms were introduced and subgroup analyses were only performed if significant interactions were obtained. For the MWM retention probe, a group was determined to have quadrant preference (and thus intact spatial memory) only if the 95% CI of the time in target mean point estimate did not overlap with chance (i.e. the mice preferred the area of the pool formerly containing the escape goal). For completeness, a second measure of quadrant preference has been included, using the time in all four quadrants. In that analysis, a repeated measure ANOVA was performed using time in quadrant as the within-group factor, despite the fact that the outcome measure violates a key assumption required to perform an ANOVA (i.e. the independence of cases). Using pairwise comparisons, whether the time in the target quadrant was significantly different to the time in each of the remaining three quadrants was assessed. If that was the case, that group was said to have intact spatial memory. In all cases, the significance threshold was set at $\alpha = 0.05$. No significant sex differences were detected, so all data presented is pooled from both sexes.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

Results

5-HTT HET mice exercise a comparable amount to WT mice

Part of our exercise intervention protocol involved the group housing of animals to avoid social isolation as a potential experimental confound. This presents a challenge to evaluate the distance run by individual mice, which we obtained in distinct ways for each cohort (see methods). For our MWM cohort, we performed an unpaired two-tailed t-test and found no significant differences between the genotypes, with WT mice running 3.0 ± 0.43 km/night (n = 11) and 5-HTT HET mice running an average of 3.5 ± 0.45 km/night (n = 12). During the two-choice spatial pattern separation task we also identified no significant differences between exercising WT and 5-HTT HET mice. Individual WT mice ran an average of 3.9 ± 0.47 km/night, while individual 5-HTT HET mice ran 2.8 ± 0.64 km/night [WT cages, 3 x (n = 3), 1 x (n =2); HET cages, 3 x (n = 3)]. Using both measurements, we identified no significant differences between the genotypes and identified that the nightly distance run was similar using single-housing versus group-housing assessments.

Hippocampal CA1 long-term potentiation is impaired in 5-HTT HET mice and exercise has no significant effect in either genotype

We assessed synaptic plasticity by recording hippocampal CA1 LTP in both standard-housed and exercising 5-HTT HET and WT mice (Figure 1). Input/output curves (Figure 1A) and pair-pulsed ratios (Figure 1B) revealed no significant differences between the four groups, so we proceeded to assess LTP with confidence that we could appropriately interpret those results. We revealed an overall significant effect of genotype when analyzing the average LTP for the first hour after the tetanus (Figure 1C, 1D), but there was no effect of treatment or a genotype x treatment interaction. Post-hoc comparisons revealed that 5-HTT HET mice in standard-housing

did not have significantly different % LTP over baseline compared to WT standard-housed control animals (Figure 1D). Furthermore, exercise access in 5-HTT HET mice did not significantly alter the % LTP over baseline compared to 5-HTT HET standard-housed animals (Figure 1D). Notably, WT exercising mice were not significantly different to WT control mice (Figure 1D). Collectively, these data suggest 5-HTT HET mice have reduced LTP compared to WT mice, and that exercise access had no significant effect.

Exercise increases adult-born cell survival in WT mice but not in the 5-HTT HET mice

We assessed the effect of exercise on hippocampal adult-born cell survival in the dentate gyrus by counting BrdU-positive (BrdU+) cells in the dentate gyrus (Figure 2). The number of BrdU+ cells is a combination of the number of cells initially labelled by BrdU (i.e. adult-born cell proliferation), as well as their survival over the 5 weeks of our study. We observed a significant genotype x treatment interaction, as well as overall effects of both treatment and genotype. Our pairwise comparisons revealed that, in WT mice, exercise induced a dramatic increase in adult-born cell survival compared to controls (Figure 2A, 2B). However, exercise had no effect on numbers of BdrU-positive cells in 5-HTT HET mice (Figure 2B). Also of note, baseline levels of adult-born cell survival were not significantly different in WT standard-housed mice compared to standard-housed 5-HTT HET mice (Figure 2B).

In order to assess the effect of exercise on hippocampal neurogenesis specifically, we quantified the percentage (%) of BrdU+ cells in the dentate gyrus that co-labelled for neuronal nuclei (NeuN), a marker for mature neurons. We also co-labelled for doublecortin (DCX), a marker for immature adult-born neurons. As expected, we found limited or no co-expression between BrdU and DCX, with less than 1% of the BrdU-+ cells in the dentate gyrus co-labelled with DCX. However, when we quantified the number of BrdU+ cells that co-labelled for NeuN, we revealed a high level of co-expression between BrdU and NeuN, with around 80% of BrdU+ cells co-labelling with NeuN (Figure 2C). Importantly, we showed that neither exercise nor genotype

significantly altered the conversion or differentiation rate of adult-born cells to adult-born neurons [WT-SH (77.2 ± 4.6%); WT-EX (83.4 ± 3.2%); HET-SH (79.2 ± 4.1%); HET-EX (79 ± 3.3%); all mean ± SD]. Altogether, these co-labelling results suggest that a high proportion of the adult-born cells generated in the dentate gyrus during the week of BrdU injection, at the beginning of the exercise intervention, committed to a neuronal lineage, and that by 5 weeks of age, most had matured into adult-born neurons.

Standard-housed 5-HTT HET mice show enhanced cognitive flexibility on the Morris water maze

We explored the significance of the altered hippocampal plasticity in 5-HTT HET mice by using hippocampal-dependent behavioural paradigms involving cognitive flexibility (Figures 3, 4 & 5). The MWM assesses the formation of an allocentric map to an escape goal location (Figure 3; Supplementary Figure 1). After mice form an allocentric map, a reversal learning paradigm, with the location of the escape goal changed, can then be used to test the cognitive flexibility of the animals. Reversal learning establishes whether mice can flexibly remap the altered escape goal location from its previous location during spatial learning. We recently showed that analysis of whether the search strategy selection employed on any given trial is spatial or nonspatial (Figure 3A) is the most reliable indicator of allocentric map formation during MWM training (Rogers, Churilov, Hannan & Renoir, 2017). Adjusting for genotype, treatment and start location, we found a significant increase in the odds to adopt a spatial search strategy over training, confirming that all mice had formed an allocentric map to the escape goal (Figure 3B). During this analysis, we identified no significant differences between genotypes or treatments. In addition, there was no effect of genotype or treatment on the average velocity during the acquisition phase (data not shown). Analysis of traditional measures of spatial learning, latency to platform (Supplementary Figure 1A) and path length to platform (Supplementary Figure 1B) corroborated the search strategy analysis results. In both cases, mice were learning across

training and there were no significant differences between the experimental groups. On the seventh day of training, the escape goal was removed, allowing us to confirm the existence of the allocentric map (Figure 3C). In contradiction to the search strategy assessment, exercising WT animals did not display the expected quadrant preference (Figure 3C). All other groups demonstrated the existence of spatial memory for the escape goal, as the 95% CI of the time in target quadrant mean did not overlap with chance (Figure 3C). These results were confirmed using a more traditional assessment of quadrant preference, by determining whether the time spent in the target quadrant was significantly different to time spent in each of the remaining three quadrants (Supplementary Figure 1C). All mice then received two days of reversal learning and we performed a second search strategy analysis (Figure 3D). Indicative of the challenging protocol, adjusting for genotype, treatment and start location, we did not observe a significant increase in the odds to adopt a spatial search strategy during reversal learning (Figure 3D). In addition, we again identified no significant differences between genotypes or treatment group. However, in contrast to the search strategy results, analysis of both latency to platform (Supplementary Figure 1D) and path length to platform (Supplementary Figure 1E) indicated that mice were learning during the MWM reversal training. Importantly, we again identified no significant differences between genotypes or treatments. On the final day of this second phase of the MWM, we evaluated spatial memory for the new escape goal location using quadrant preference (Figure 3E; Supplementary Figure 1F). Using either measure of spatial memory formation, 5-HTT HET standard-housed mice exhibited quadrant preference despite the fact WT control animals were unable to form a spatial memory for the new escape goal location. This suggested that 5-HTT HET mice have enhanced cognitive flexibility (Figure 3E; Supplementary Figure 1F). Notably, exercising mice of both genotypes were unable to update their allocentric map with the new hidden platform location (Figure 3E; Supplementary Figure 1F).

Exercise alters spatial pattern separation ability on a touchscreen task

We used a two-choice LD touchscreen task to assess the spatial pattern separation ability in both 5-HTT HET and WT mice (Figures 4 and 5). After training, pattern separation was probed under two conditions: stimuli presented with an increased degree of separation (high separation) or a decreased degree of separation (low separation), which is more taxing on pattern separation (Figure 4A). In the training phase of the LD task, animals were first exposed to an acquisition phase, and once they reached a criterion, were then exposed to the reversal phase. In contrast to the seminal studies using the LD task (Clelland et al., 2009; Coba et al., 2012; Creer, Romberg, Saksida, van Praag & Bussey, 2010), our mice were able to achieve the criterion for both the acquisition and reversal phases during the probe, regardless of the degree of separation (Figures 4 and 5). Interestingly, with all else being equal, we identified a significant decrease in the summary data for percentage correct throughout all probe sessions between controls and exercising 5-HTT HET mice (Figure 4B). Using trials to criterion as the outcome measure for task performance, we revealed a significant effect of separation, which indicated, as expected, that all else being equal during the probe, mice found the low separation more challenging than the high separation. Additionally, we observed no significant effect of phase, which indicated that overall, mice did not find the reversal phase more challenging than the acquisition phase. Furthermore, using a trial-by-trial analysis of the choice selection mice made on the LD task, we corroborated these overall effects of separation and phase on the likelihood that mice chose the correct answer on the LD task. Mice were 152% more likely to make a correct choice when the separation was high. Surprisingly, that analysis indicated that mice found the reversal phase slightly less difficult. There was a significant effect of phase, as the adjusted odds of selecting the correct answer increased by approximately 6% per trial during the reversal phase. However, we also obtained a significant interaction between separation, phase, genotype and treatment. This allowed us to perform subgroup analyses equivalent to the pairwise comparisons made following the repeated measures ANOVA, performed using the trials to criterion summary data.

For the acquisition phase data, we observed significant genotype x separation and treatment x separation interactions, but we observed no significant differences between genotype or

treatment (Figure 4C). Our pairwise comparisons revealed significant differences in performance in the low and the high separation conditions in all groups except the standard-housed WT animals (Figure 4C). This indicated that, all else being equal, standard-housed WT mice did not find the low separation more challenging than the high. Our trial x trial subgroup analysis allowed us to restrict analysis to the acquisition phase and to obtain the effect of separation on the likelihood of making a correct choice within each experimental group. During the acquisition phase, WT exercising mice, 5-HTT HET standard-housed mice and 5-HTT HET exercising mice had significantly increased likelihoods of selecting the correct answer of 40%, 66%, and 270%, respectively, when the separation was high (Figure 4D). Furthermore, exercising 5-HTT HET mice had significantly increased likelihoods compared to the other two groups. The likelihood of selecting the correct answer on any given trial was not significantly different for standard-housed WT mice in either separation (Figure 4D).

In contrast to the acquisition phase, during the reversal phase (Figure 4E) our post-hoc analysis revealed that there was a significant difference in all groups between the two separations. We identified significant interactions between separation, genotype and treatment, as well as separation and treatment during the reversal phase. We also observed a significant overall effect of genotype, but not treatment. However, during the reversal phase, our pairwise comparisons revealed a significant difference between standard-housed and exercising 5-HTT HET mice, specifically when the separation was low, but not when the separation was high (Figure 4E). Our trial x trial subgroup analysis also allowed us to restrict analysis to the reversal phase and to again obtain the effect of separation on the likelihood of making a correct choice within each of our experimental groups. During the reversal phase, WT standard-housed mice, WT exercising mice, 5-HTT HET standard-housed mice, and 5-HTT HET exercising mice had significantly increased likelihoods of selecting the correct answer of 65%, 37%, 28% and 72%, respectively, when the separation was low (Figure 4F).

In a second set of analyses, we also analysed the data using the phase as the repeated measures factor for both the high separation dataset and the low separation dataset (Figure 5). Using the high separation phase dataset, we identified a significant phase x genotype interaction (Figure 5A). We observed no other significant interactions or main effects. Our pairwise comparisons revealed that only exercising 5-HTT HET mice found the reversal phase significantly harder than the acquisition phase (Figure 5A). Our trial x trial subgroup analysis also allowed us to restrict analysis to the high separation and to obtain the effect that phase had on the likelihood of making a correct choice within each of our experimental groups. The likelihood of selecting the correct answer was only significantly increased in standard-housed WT mice, but not in the remaining experimental groups (Figure 5B). WT standard-housed mice were 32% more likely to select the correct answer during the reversal phase compared to the acquisition phase when the separation was high (Figure 5B).

Using the low separation phase dataset, we identified a significant phase x genotype x treatment interaction in addition to observing significant main effects of both genotype and treatment (Figure 5C). Our pairwise comparisons revealed that only exercising 5-HTT HET mice found the reversal phase to be significantly harder than the acquisition phase (Figure 5C). It was also identified that, in the reversal phase, 5-HTT HET mice with exercise access were significantly worse than 5-HTT HET animals that were standard-housed (Figure 5C). Our trial x trial subgroup analysis also allowed us to restrict analysis to the low separation and to again measure the effect that phase had on the likelihood of making a correct choice within each of our experimental groups. In this case, standard-housed WT mice were 15% less likely to select the correct answer during the reversal phase, while exercising HET mice were approximately 15% more likely to select the correct answer (Figure 5D). For the remaining groups, there was no significant difference in the likelihood to select the correct answer in either phase when the separation was low.

Finally, as control data, we obtained both the total number of trials performed (Figure 5E) and the average response latency (Figure 5F) for the duration of the probe testing. For both datasets, we identified no significant effects of either genotype or treatment or genotype x treatment interactions.

Discussion

In this study, 5-HTT HET mice were used to explore the potential role for 5-HTT in mediating exercise-induced hippocampal plasticity. We found evidence that serotonergic signaling is involved in mediating some of the effects of chronic voluntary exercise. Firstly, at the level of synaptic plasticity, LTP was impaired in 5-HTT HET mice regardless of access to exercise; and, at the level of cellular plasticity, we showed that the neurogenic effect of exercise found in WT mice did not occur in 5-HTT HET mice. Additionally, standard-housed 5-HTT HET animals displayed enhanced cognitive flexibility on the MWM. That phenotype was abolished with access to exercise as exercised 5-HTT HET mice also exhibited impaired spatial pattern separation. Importantly, there was no difference between 5-HTT HET and WT mice when assessing the distance run in the running-wheels. Taken together, our results suggest unique and complex interactions between exercise and altered 5-HT homeostasis, which will be discussed in further detail below.

Neurogenesis

The function of the 5-HTT is central to the regulation of serotonin activity in the brain through 5-HT reuptake from the synaptic cleft (Barnes & Sharp, 1999). The partial removal of 5-HT reuptake capacity in 5-HTT HET mice causes a 2-3 fold increase in [5-HT]ext (Kim et al., 2005; Mathews, Fedele, Coppelli, Avila, Murphy & Andrews, 2004). The resulted increase in [5-HT]ext might affects the ability of 5-HT to mediate signaling pathways during development that are critical for the creation of correct forebrain architecture in adult mice (Gaspar, Cases & Maroteaux, 2003). The consequences of the increase in [5-HT]ext between 5-HTT HET mice and WT mice is subtle yet significant, and has been extensively studied (Araragi & Lesch, 2013; Murphy & Lesch, 2008). Previous work has established that exercise raises the [5-HT]ext in the hippocampus of WT C57BL/6 mice (Gomez-Merino, Bequet, Berthelot, Chennaoui &

Guezennec, 2001). In the present study, exercise-induced changes to neurogenesis were absent in 5-HTT HET mice housed with running wheels. This finding is consistent with a previous report confirming that 5-HT is critical for the pro-proliferative effect of exercise (Klempin, Beis, Mosienko, Kempermann, Bader & Alenina, 2013). Another study revealed that 5-HT plays a critical role in exercise-induced neurogenesis through the 5-HT_3 receptor (Kondo et al. 2014). On the other hand we recently showed the 5-HT_{1A} receptor may not be required for exercise-induced increases to adult-born cell survival (Rogers et al., 2016). We hypothesise that the exact mechanism by which exercise increased hippocampal neurogenesis may involve paracrine signaling using the [5-HT]ext increases induced by exercise in the dentate gyrus (Gomez-Merino et al. 2001). However, due to the 2-3 fold increase in hippocampal [5-HT]ext, 5-HTT HET mice may be unable to benefit from utilising this signaling pathway, potentially accounting for the absence of exercise-induced changes to neurogenesis identified. These changes most likely result from complicated interactions with alterations to serotonin neuron signaling that are unique in exercising 5-HTT HET mice compared to exercising WT animals.

Long term potentiation (LTP)

We show for the first time, that CA1 hippocampal LTP is reduced in 5-HTT HET mice regardless of access to voluntary exercise. Notably, the *in vivo* spontaneous firing rate of 5-HTT HET mice has been reported to be reduced by 36% in the raphe nuclei, and 5-HT_{1A} receptor activity has also been found desensitised, while no alterations appear to occur in the forebrain (Gobbi, Murphy, Lesch & Blier, 2001; Lira et al., 2003). This is consistent with the reduction of somatodendritic 5-HT_{1A} receptor numbers in the raphe nuclei but no change in postsynaptic 5-HT_{1A} receptors in the hippocampus. Previous *in vitro* electrophysiological studies in 5-HTT HET mice have revealed no genotype effect, as CA1 pyramidal neurons could still be hyperpolarised by a 5-HT mimic (La Cour, Boni, Hanoun, Lesch, Hamon & Lanfumey, 2001).. This study also

revealed that, compared to WT mice, there was no differences in CA1 pyramidal neuron properties of 5-HTT HET mice, including resting membrane potential and input resistance.

Considering the present results in the context of existing literature, it may be that the reduction in LTP observed in 5-HTT HET mice was due not to any deficits in the properties of CA1 pyramidal cells, but instead may have resulted from overall alterations in serotonin signaling, which may have even occurred from developmental stages. Interestingly, this sheds light on the controversy surrounding 5-HT's role in modulating LTP, as current evidence suggests that both reduction and overexpression of [5-HT]ext positively modulate synaptic plasticity *in vitro* and *in vivo* (Araragi & Lesch, 2013). Recent evidence using optogenetic manipulation of hippocampal serotonin input demonstrated that 5-HT controls synaptic transmission in CA1, especially the input from CA3 to CA1, and is thus critical to LTP (Teixeira et al., 2018). The loss of 5-HT homeostasis in 5-HTT HET mice may fundamentally alter how 5-HT controls this synaptic transmission in the hippocampus. This finding provides further evidence that 5-HT signaling is critical to CA1 LTP. On that note, using Wistar Kyoto depression model rats, a previous study found that 5-HTT and 5-HT_{1A} receptors may be associated with synaptic plasticity in the hippocampal CA1 region (Han et al., 2018). And another study showed that induction of the perceptual correlate of human LTP was associated with genetic variability in the gene encoding the 5-HTT (Matre, Olsen, Jacobsen, Klein & Gjerstad, 2013). However, the frequency stimulation conditioning applied to one arm to induce LTP in that latter study, might not be relevant to hippocampal synaptic plasticity we focused on in our study.

The LTP reduction appeared more pronounced in exercised 5-HTT HET mice, which could be due to the differential elevation of extracellular 5-HT resulting from exercise in in the context of reduced 5-HTT levels. It is plausible that specific effects of exercise on LTP in 5-HTT HET mice failed to reach significance due to a limited sample size. However our initial power analysis resulted in a total sample size of 5 mice per group. Also, we still revealed a main effect of genotype. On the other hand, in our study, exercise had no effect on LTP in wild-type mice.

Notably, previous work has demonstrated that the exercise-induced increases in LTP are only observed with longer periods of running (i.e. 56 days compared to 35 days in our study) (Patten et al., 2013).

Behaviour

We report that standard-housed 5-HTT HET mice exhibit enhanced cognitive flexibility on the reversal learning phase of the MWM (without enhanced hippocampus-dependent spatial learning *per se*). To our knowledge, this is the first study assessing 5-HTT HET mice reversal learning using the MWM. Indeed, previous investigation of 5-HTT mutant mice using the MWM, focused on the spatial learning phase only and found no differences between WT and 5-HTT HET mice (Karabeg et al., 2013). This is consistent with our acquisition of allocentric search strategies data, which also revealed no genotype effect. Interestingly, Karabeg et al. (2013) revealed that learning performance in the MWM was only altered after complete ablation of 5-HTT (i.e. in the 5-HTT homozygous knockout mice). However, it is worth noting that the 5-HTT homozygous KO mice were described in this study to have 'stopped swimming', which would have confounded clear interpretation on any learning performances. For this reason, we chose to focus on 5-HTT HET and not assess 5-HTT homozygous KO mice. In addition to not performing/swimming in the MWM, there are reasons to suspect that 5-HTT homozygous KO mice might not engage with running wheels in a similar fashion to WT animals. Indeed, 5-HTT KO animals are known to have a dysregulated stress response and exhibit anxiety- and depressive-like behaviours (Rogers, Li, Lanfumey, Hannan & Renoir, 2017). While the 5-HTT HET enhanced cognitive flexibility phenotype is in line with previous studies (although not using the MWM) (Brigman et al., 2010; Ineichen et al., 2012), the fact that exercised 5-HTT HET mice no longer displayed intact spatial memory on the MWM reversal learning probe was unexpected. Since chronic voluntary exercise is known to increase the circadian peak of plasma corticosterone and modulate stress response in mice (Otawa, Arai & Atomi, 2007), it is quite

plausible that 5-HTT HET mice may respond differently to exercise, especially when combined with the acute stress component of swimming in the MWM.

Reversal learning in 5-HTT HET mice was not enhanced on the spatial pattern separation touchscreen task, which does not corroborate the MWM findings discussed above. This is despite previous work demonstrating enhanced cognition on reversal learning in a visual discrimination touchscreen task in 5-HTT HET mice (Brigman et al., 2010). Exercise specifically caused decreased performance in 5-HTT HET mice during the reversal phase on the low separation part of the task (with the highest cognitive load), data that was not collected in previous spatial pattern separation touchscreen studies (Clelland et al., 2009; Coba et al., 2012; Creer, Romberg, Saksida, van Praag & Bussey, 2010). Exercise in WT mice has previously been shown to enhance cognition on the spatial pattern separation task used in this study, an effect associated with exercise-induced increases to adult neurogenesis (Clelland et al., 2009; Creer, Romberg, Saksida, van Praag & Bussey, 2010). However, in our study, we found no cognitive enhancement in exercised WT mice, despite these animals having 2-3 fold exercise-induced increases to neurogenesis, a similar effect size to the equivalent previous study (Creer, Romberg, Saksida, van Praag & Bussey, 2010). Further assessment of the effects of our exercised paradigm on HPA axis in WT mice would also be interesting. Additional tests relevant to the behavioral and physiological component of 'emotionality' in mice should be considered in follow up studies. We have previously published anxiogenic-like effects of our exercise paradigm in some testing environments. This includes an overall reduction of total distance travelled in mice which underwent chronic exercise (Rogers et al., 2016). In line with that, voluntary exercise induced anxiety-like behavior in adult C57BL/6J mice has been previously shown to correlate with hippocampal neurogenesis (Fuss et al., 2010). However, we did not reveal any group difference when measuring response latencies in the touch-screen experiments.

It is worth noting that those previous touchscreen studies were done using 'Med Associates' boxes, with sugar pellet rewards, whereas our study took place in the newly commercialised

chambers (Cambden Instruments) with liquid rewards, which may have influenced task difficulty (Horner et al., 2013; Oomen et al., 2013). Indeed, in the newly commercialized chambers our group now routinely performs the Trial-Unique Non-Matching to Location (TUNL) task (Zeleznikow-Johnston, Burrows, Renoir & Hannan, 2017; Zeleznikow-Johnston, Renoir, Churilov, Li, Burrows & Hannan, 2018), a task that was deemed too difficult to be performed by mice in the previous generation of chambers (Oomen et al., 2013). Despite this, overall, our results show that exercise clearly affects cognitive flexibility in 5-HTT HET mice, on both the MWM and the spatial pattern separation touchscreen task. Further investigation of the effects of chronic voluntary exercise on the hypothalamic-pituitary-adrenal (HPA) axis ('stress axis') of the 5-HTT mutants would be interesting, especially since 5-HTT HET mice exposed to repeated stress have shown impairments, relative to controls, in a probabilistic reversal learning task (Spinelli et al 2013).

Limitations and Conclusions

At first glance, our behavioural results are somewhat surprising, as a substantial body of clinical and preclinical literature has established positive behavioural effects of exercise in both WT mice and transgenic mouse models (Rogers, Renoir & Hannan, 2019; Stimpson, Davison & Javadi, 2018; Suo et al., 2016). Furthermore, extensive research has established CA1 LTP and neurogenesis as candidate mechanisms underpinning enhanced cognitive functioning in WT and transgenic mice following exercise (Ohline & Abraham, 2018; Patten, Yau, Fontaine, Meconi, Wortman & Christie, 2015). This body of work makes our observation that standard-housed 5-HTT HET performed better on MWM reversal learning, despite disruptions to CA1 LTP, somewhat paradoxical. Surprisingly, exercising WT mice did not display intact spatial memory on the first retention probe, despite our previous work which demonstrated that the selection of allocentric search strategies predicts spatial memory formation (Rogers, Churilov, Hannan & Renoir, 2017; Rogers et al., 2016). Thus, we interpret the reversal learning data for exercising

WT mice with caution, despite evidence that exercise-induced neurogenesis is critical for MWM reversal learning (Garthe & Kempermann, 2013). The touch-screen performance of standard-housed WT mice in our study was enhanced compared to other studies using the two-choice spatial LD task (Coba et al., 2012; Creer, Romberg, Saksida, van Praag & Bussey, 2010). Using a trial-by-trial analysis (instead of summary data for trials to criterion), we observed that standard-housed WT mice did not find, during the acquisition phase, the low separation more difficult than the high separation, which provides an explanation for the difference in performance between the studies. In those previous experiments, WT animals were single-housed, which may also account for the differences to our study, in addition to the potential differences in task difficulty discussed above.

Exercise might act in WT mice through specific mechanisms that are altered in 5-HTT HET animals (e.g. stress response etc.). 5-HTT HET mice have been used as a mouse model of 5-HTTLPR S allele carriers in studies that have manipulated both the early life and adult life environments (Carola et al., 2008; Kästner, Richter, Lesch, Schreiber, Kaiser & Sachser, 2015; Schraut et al., 2014; Spinelli et al., 2013). In those studies, 5-HTT HET mice were vulnerable to negative environments but found positive environments beneficial. Early environmental influences act on the genome and shape adaptability to environmental changes later in life (Tost, Champagne & Meyer-Lindenberg, 2015). Thus, an 'environmental prediction error' may be programmed in early life that creates an environmentally sensitive or insensitive disposition in the adult brain, in a high-risk high resilience program (Uher & McGuffin, 2008). Our hypothesis is that an 'environmental prediction error' is created by tonic 5-HT neuron signaling through the [5-HT]ext in the early developmental period (Gaspar, Cases & Maroteaux, 2003). We hypothesise that adult 5-HTT HET mice are then more sensitive to their environmental context in general (Moore & Depue, 2016) and that the heightened sensitivity to the stress component of daily exercise access may perhaps result in the adverse cognitive effects observed. Also, raphe nuclei medial frontal cortex connectivity determine whether stressor type and duration are deemed controllable or uncontrollable (Amat, Baratta, Paul, Bland, Watkins & Maier, 2005), so

alterations to this signaling pathway could account for these hypothesised changes in exercising 5-HTT HET mice. It is hoped this present study will help inform future clinical research investigating the interaction between 5-HTT activity and the effects of exercise. The precise mechanism of how exercise impacts the cognitive function of 5-HTT HET mice remains unclear but is an exciting avenue for future research. This may help inform future clinical studies investigating the interaction between the 5-HTTLPR polymorphism and environmental factors.

Author Contributions

J.R., T.R. and A.J.H. conceived the study; J.R. and T.R. designed the study; J.R., F.C., F.F., S.L, D.S. performed the experiments; J.R., T.R, F.C. and D.S acquired and analysed the data; T.R. J.R. and A.J.H wrote the manuscript; J.N. and A.M.Z. helped with the touchscreen experiment; L.C. helped with statistical analyses; P.A.A. helped with the Micro-electrode array (MEA) electrophysiology set up; L.L. provided the 5-HTT HET mice

Acknowledgements

Carey Wilson helped in editing the manuscript. This work was supported by an ARC Discovery Early Career Research Award (TR). AJH is an NHMRC Principal Research Fellow. The Florey Institute of Neuroscience and Mental Health acknowledges the support from the Victorian Government's Operational Infrastructure Support Grant.

Conflicts of Interest

The authors have no conflict of interest to report.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Immunoblotting and Immunohistochemistry, Animal Experimentation, and Design & Analysis and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Legends

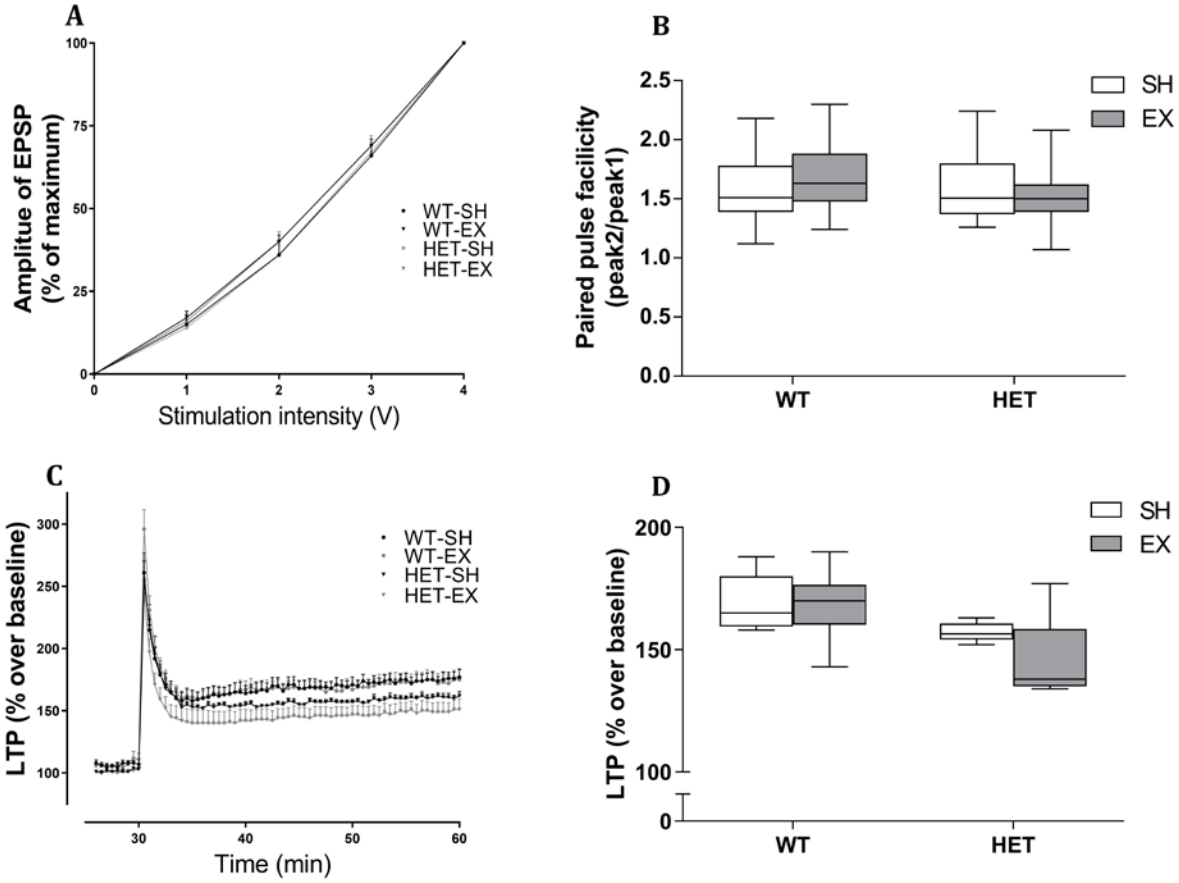


Figure 1: Long-term potentiation (LTP) is impaired in 5-HTT mutant mice and exercise has no significant effect in either genotype. A) Stimulus strength-evoked response curves, input-output curves, demonstrate that there were no significant differences between our experimental groups with the amplitudes of excitatory post-synaptic potentials (EPSP) obtained at any stimulation intensity. (B) Only electrodes showing paired-pulse facilitation were chosen for LTP stimulation, and again there were no significant differences between our experimental groups. (C) Minute-by-minute recordings of LTP expressed as the percentage increase over baseline of field excitatory postsynaptic potentials (fEPSPs). (D) The average LTP response, during the hour after the

tetanus, was reduced in 5-HTT HET mice when compared to WT animals. Data were analysed by two-way ANOVA followed by Tukey's pairwise comparisons; $p < 0.05$; data is expressed as mean ± SEM;WT, wild-type mice; HET, 5-HTT heterozygous mice; SH, standard-housing; EX, exercise; WT-SH, n = 5 (11 slices); WT-EX, n = 6 (13 slices); HET-SH, n = 6 (14 slices); HET-Ex, n = 5 (11 slices); our aim was to have n = 6 mice in each group, but technical difficulties meant we were unable to get viable recordings from two mice.

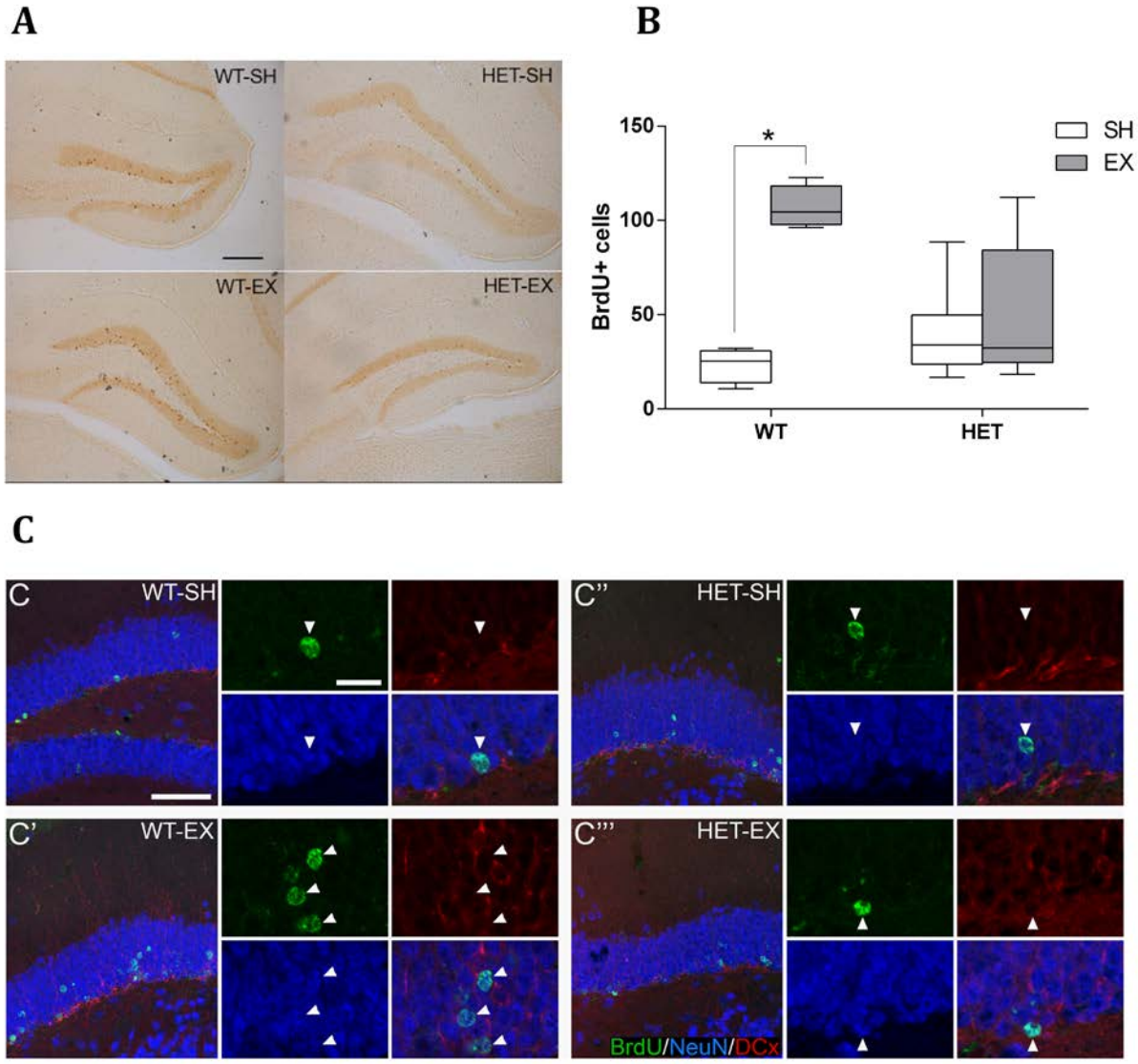


Figure 2: Exercise increases adult neurogenesis in WT but not 5-HTT HET mice. (A) Representative micrographs of the level of adult-born cell survival from each experimental group. The scale bar represents 100 µm. (B) As expected, WT mice with exercise access had a significantly higher rate of adult-born cell survival than standard-housed controls. However, 5-HTT HET mice with exercise access did not have a significantly increased rate of adult-born cell survival compared to 5-HTT HET standard-housed mice. Finally, 5-HTT HET animals in standard-housing did not have significantly different rates of adult-born cell survival than WT standard-housed animals. (C-C’’’) Triple-immunofluorescence photomicrographs showing the distribution of bromodeoxyuridin- (BrdU, green), Neuronal Nuclei- (NeuN, blue) and doublecortin (DCX, red)-like immunoreactivity in the dentate gyrus of the hippocampal formation of (C) standard-housed WT, (C’) exercising WT, (C’’) standard-housed 5-HTT HET and (C’’’) exercising 5-HTT HET mice. The right four panels in each of C-C’’’ are single-channel micrographs of BrdU (green), NeuN (blue) and DCX (red) immunoreactivity, and triple fluorescence, shown at higher magnification to demonstrate BrdU/NeuN co-labelled cells (arrowheads), and the lack of BrdU/DCX co-labelled cells. Scale bars: C = 100 µm, applies C-C’’’; C inset (BrdU) = 20 µm, applies to all inset panels in C-C’’’. All data were analysed by two-way ANOVA followed by Tukey’s pairwise comparisons: *$p < 0.05$; data is expressed as mean ± SEM; WT, wild-type mice; HET, 5-HTT heterozygous mice; SH, standard-housing; EX, exercise; BrdU, Bromodeoxyuridine; Peroxidase immunohistochemistry: WT-SH, n = 4; WT-EX, n = 5; HET-SH, n = 6; HET-Ex, n = 5; our aim was to have n = 6 mice in each group, but technical difficulties meant we were unable to obtain viable slices from four mice.

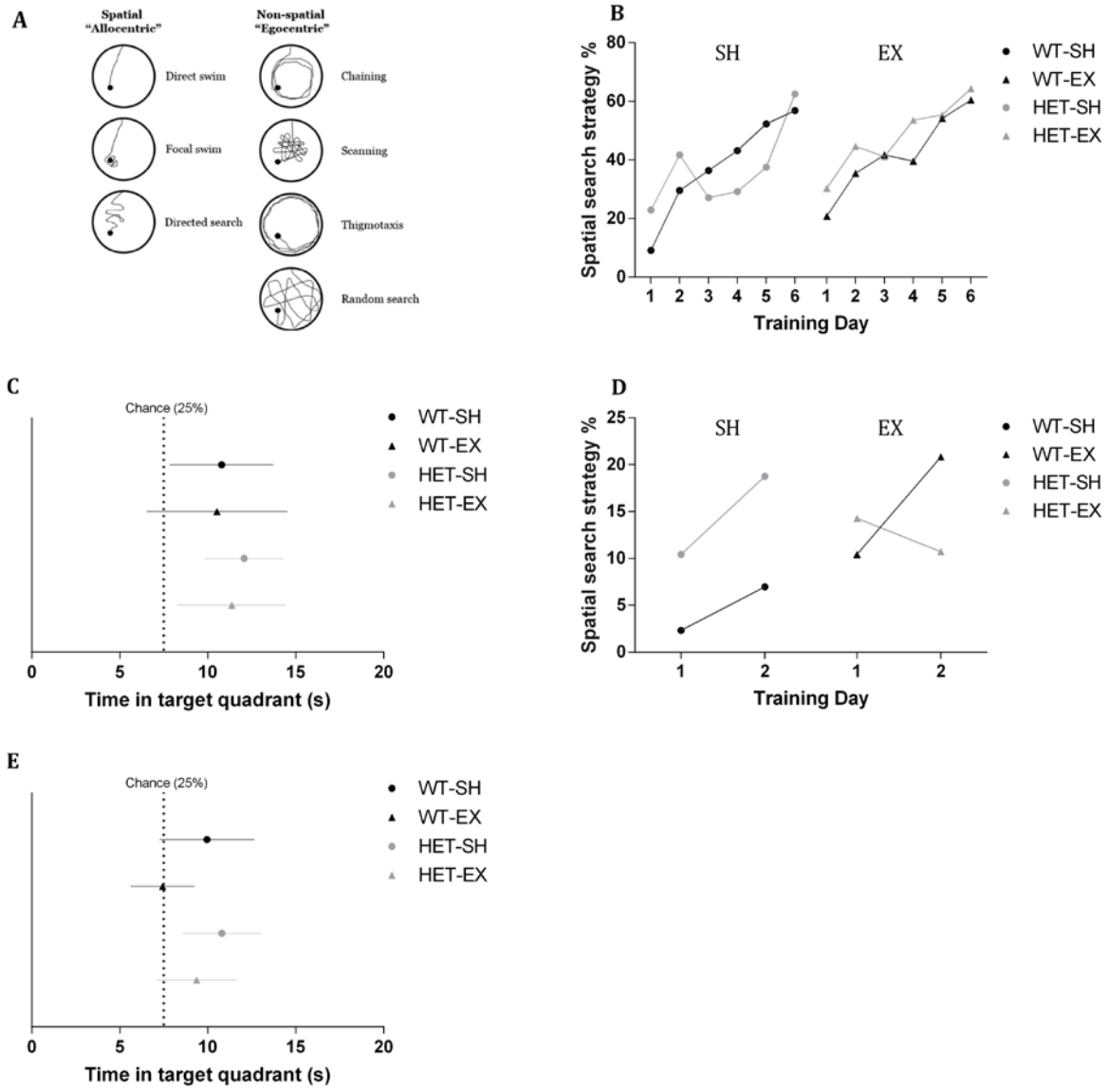


Figure 3: Standard-housed 5-HTT HET mice exhibit cognitive flexibility during Morris water maze (MWM) reversal learning, but housing with exercise access abrogates this ability. (A) The strategy mice employed while navigating to the escape goal locations was assessed objectively by entering time-tagged coordinates into a Matlab algorithm (adapted from (Rogers, Churilov,

Hannan & Renoir, 2017)). The type of strategy is dichotomized into spatial or nonspatial categories and this classification used to assess spatial learning on the MWM (B) Analysis of spatial search strategy indicated that spatial learning occurred during training [day: OR 1.43, 95% CI (1.32; 1.55), $p < 0.05$] and that there were no significant differences in the ability of any group of mice [genotype: OR 1.17, 95% CI (0.68; 1.99), $p > 0.05$] [treatment: OR 1.51; 95% CI (0.88; 2.58), $p > 0.05$] to form an allocentric map to the escape location. No subgroup analyses were performed as we found no significant interactions between any of the independent variables with spatial search strategy selection. (C) Spatial memory on the retention probe is demonstrated by preference for the target quadrant (i.e. the 95% CI of the point estimate not overlapping with the chance performance of 7.5 s), which WT exercising mice surprisingly fail to demonstrate. Spatial memory is present in WT controls and all 5-HTT HET mice. (D) Analysis of spatial search strategy indicated that, overall, mice were not able to form an updated cognitive map to the new escape goal location, as no significant learning effect was present [day: OR 1.61, 95% CI (0.84; 3.08), $p > 0.05$]. There was no significant effect of genotype [OR 1.36, 95% CI (0.61; 3.01), $p > 0.0.5$] or treatment [OR 1.56, 95% CI (0.70; 3.48), $p > 0.05$] and no subgroup analyses were performed, as there were again no significant interactions between any of the independent variables. (E) Spatial memory on the reversal learning probe indicated that spatial memory of the new escape goal location was demonstrated only in 5-HTT HET mice that were standard-housed. Control animals failed to demonstrate spatial memory, suggesting that 5-HTT HET-SH mice have enhanced cognitive flexibility during the MWM. Search strategy data was analysed using mixed-effect regression models. Spatial memory was analysed by assessing whether the 95% CI of the time in target quadrant point estimate overlapped with chance performance (i.e. 7.5s). Search strategy data are expressed as a percentage of spatial strategies selected within all trials on a given day; spatial memory data are expressed as mean ± 95% CI; WT, wild-type mice; HET, 5-HTT heterozygous mice; SH, standard-housing; EX, exercise; WT-SH, n = 11; WT-EX, n = 12; HET-SH, n = 12; HET-Ex, n = 14; our aim was to have n = 12 mice in each group, but our breeding strategy resulted in the number of mice listed.

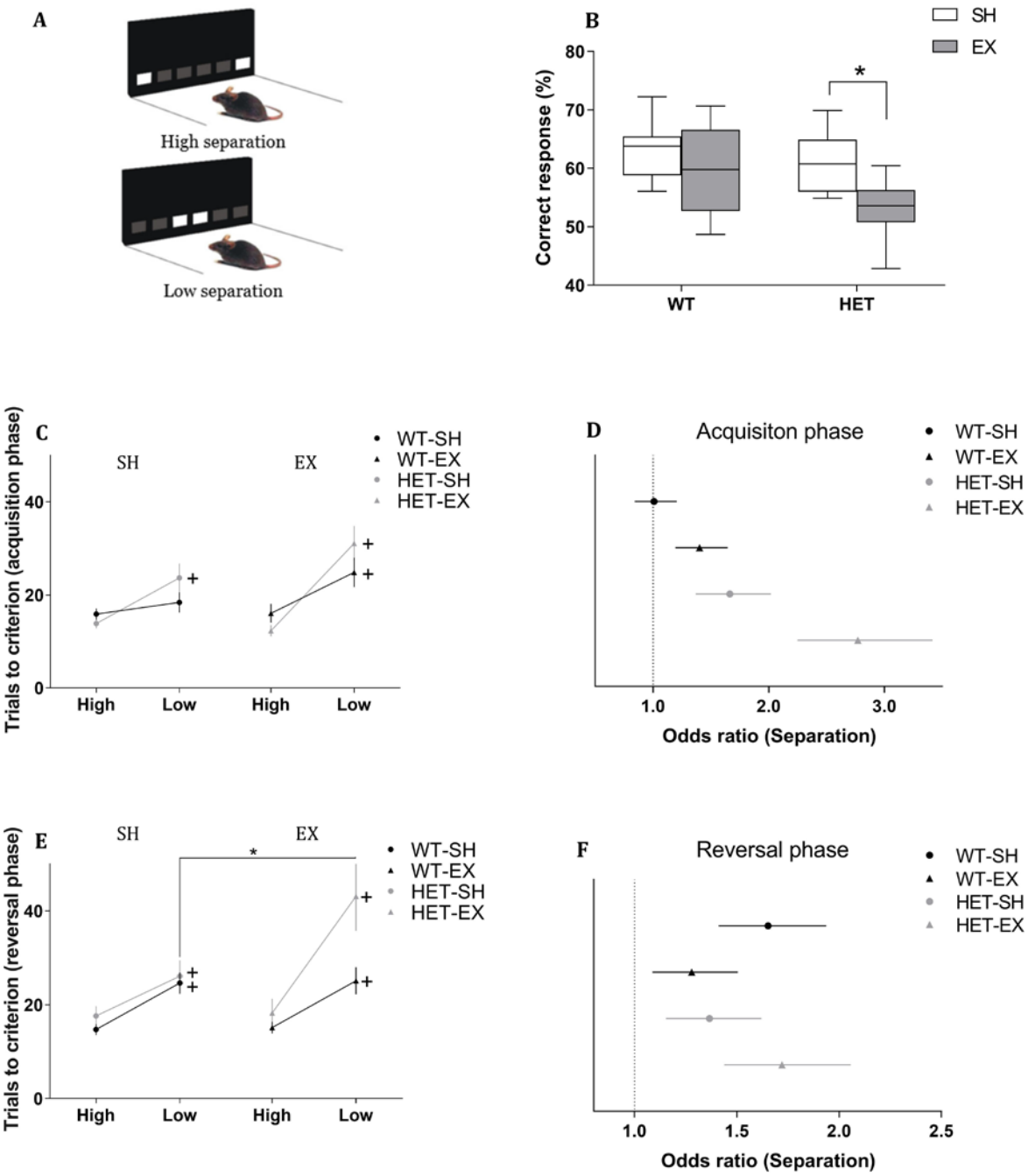


Figure 4: Exercise access impairs the performance of 5-HTT HET mice on elements of touchscreen location discrimination probe requiring increased cognitive flexibility. (A) Two-choice spatial discrimination touchscreen task, where mice were trained to discriminate the correct spatial location between two illuminated squares, located in two of six possible locations, either close together or far apart (to vary the pattern separation load) (adapted from (Coba et al., 2012)). (B) All else being equal, exercising 5-HTT HET mice had a significantly lowered % correct response throughout the entire probe compared to standard-housed 5-HTT HET mice (C) During the acquisition phase of the probe, standard-housed WT controls did not find the low separation significantly harder than the high separation. All other groups found the low separation significantly harder than the high. (D) The likelihood of selecting the correct response during the acquisition phase on a trial-by-trial basis was increased when the separation was high in WT-EX [separation: OR 1.40, 95% CI (1.19; 1.65), $p < 0.05$], HET-SH [separation: OR 1.66, 95% CI (1.37; 2.02), $p < 0.05$] and HET-EX [separation: OR 2.77, 95% CI (2.25; 3.42), $p < 0.05$], but not WT-SH [separation: OR 1.01, 95% CI (0.84; 1.21), $p > 0.05$]. Exercising 5-HTT HET mice had significantly increased odds ratios compared to standard-housed 5-HTT HET mice (E) In contrast, during the reversal phase of the probe, standard-housed WT controls did find the low separation significantly harder than the high, which indicated that the reversal phase had an increased difficulty level when employing low separation. Exercising 5-HTT HET mice were significantly worse at the low separation than 5-HTT HET standard-housed mice. (F) The likelihood of selecting the correct answer on a trial-by-trial basis was significantly increased when the separation was high in all experimental groups WT-SH [separation: OR 1.65, 95% CI (1.41; 1.94), $p < 0.05$], WT-EX [separation: OR 1.28, 95% CI (1.09; 1.51), $p < 0.05$], HET-SH [separation: OR 1.37, 95% CI (1.15; 1.62), $p < 0.05$], and HET-EX [separation: OR 1.72, 95% CI (1.44; 2.06), $p < 0.05$]. Trials to criterion data were analysed by a repeated measure two-way ANOVA: + significant difference between separation $p < 0.05$; * significant difference between housing within genotype $p < 0.05$; data is expressed as mean ± SEM. Choice selection data were analysed by mixed-effect regression models; data is expressed as odds ratios with 95% CIs. WT,

wild-type mice; HET, 5-HTT heterozygous mice; SH, standard-housing; EX, exercise; WT-SH, n = 11; WT-EX, n = 11; HET-SH, n = 9; HET-Ex, n = 9; Our aim was to have n = 12 mice in each group, but our breeding strategy resulted in the number of mice listed; Acq, acquisition phase; Rev, reversal phase.

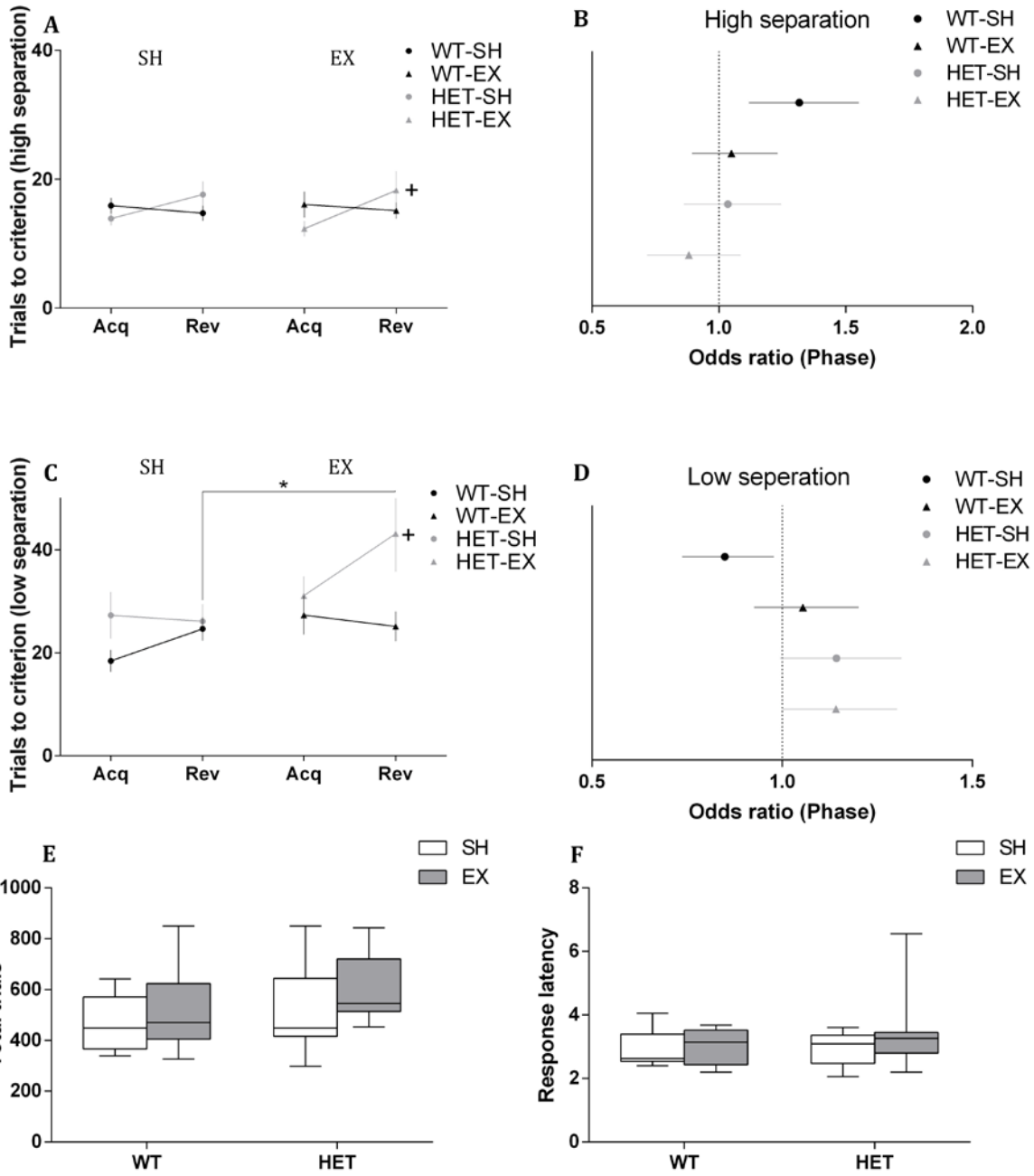


Figure 5: Exercise access impairs the performance of 5-HTT HET mice on further elements of touchscreen location discrimination probe requiring increased cognitive flexibility. (A) Using phase as the repeated measure, when mice were exposed to the high separation, only exercising 5-HTT HET mice found the reversal phase significantly harder than the acquisition phase. B) When the separation was high, only standard-housed WT mice had significant increases in their likelihood to obtain a correct answer, with over a 30% increase during the reversal phase [phase: OR 1.32, 95% CI (1.12; 1.55), $p < 0.05$]. C) When mice were exposed to the low separation, only exercising 5-HTT HET mice found the reversal phase significantly harder than the acquisition phase. (D) The likelihood of selecting the correct answer on a trial-by-trial basis was significantly decreased when the separation was low in the acquisition phase in WT standard-housed mice [phase: OR 0.85, 95% CI (0.74; 0.98), $p < 0.05$], but significantly increased in the reversal phase in exercising 5-HTT HET mice [phase: OR 1.14, 95% CI (1.00; 1.30), $p < 0.05$]. (E) No significant differences were identified in the total number of trials performed throughout the probe. (F) The average response latency was used as a control measure for touchscreen tasks, and we identified no significant differences between the groups. Trials to criterion data were analysed by a repeated measure two-way ANOVA: + significant difference between phase $p < 0.05$; * significant difference between housing within genotype $p < 0.05$; data is expressed as mean ± SEM. Choice selection data were analysed by mixed-effect regression models; data is expressed as odds ratios with 95% CIs. WT, wild-type mice; HET, 5-HTT heterozygous mice; SH, standard-housing; EX, exercise; WT-SH, n = 11; WT-EX, n = 11; HET-SH, n = 9; HET-Ex, n = 9; our aim was to have n = 12 mice in each group, but our breeding strategy resulted in the number of mice listed; Acq, acquisition phase; Rev, reversal phase.

Supplementary Figure 1: Standard-housed 5-HTT HET mice exhibit spatial memory during Morris water maze (MWM) reversal learning, but housing with exercise access abrogates this ability. (A) Analysis of latency to platform indicated that there was a significant effect of

training, but that there were no significant differences in the ability of any group to find the escape location during the standard MWM training phase. (B) Analysis of the path length mice took to the escape platform also indicated that spatial learning had taken place, but again there were no significant differences in the ability of any group to find the escape location. (C) Spatial memory on the retention probe is demonstrated by quadrant preference (i.e. the time in the target quadrant being significantly increased compared to the three remaining quadrants), which WT exercising mice failed to demonstrate. Spatial memory is present in WT controls and all 5-HTT HET mice. (D) Analysis of latency to platform indicated that there was a significant effect of training during MWM reversal learning, but that there were no significant differences in the ability of any group to find the escape location. (E) Analysis of path length to platform also indicated that there was a significant effect of training during MWM reversal learning, but that there were no significant differences in the ability of any group to find the escape goal. (F) The reversal learning probe indicated that spatial memory for the new escape goal location was demonstrated only in standard-housed 5-HTT HET mice. WT control animals failed to demonstrate spatial memory, suggesting that 5-HTT HET-SH mice have enhanced cognitive flexibility. Latency to platform, path length to platform and time in quadrant data were analysed by a repeated-measure two-way ANOVA; $^{\gamma}p < 0.05$ (T vs A, T vs A, & T vs O); Data is expressed as mean ± SEM; WT, wild-type mice; HET, 5-HTT heterozygous mice; SH, standard-housing; EX, exercise; T, target quadrant; A, adjacent quadrant; O, opposite quadrant; WT-SH, n = 11; WT-EX, n = 12; HET-SH, n = 12; HET-Ex, n = 14; our aim was to have n = 12 mice in each group, but our breeding strategy resulted in the number of mice listed.

Bullet point summary

What is already known

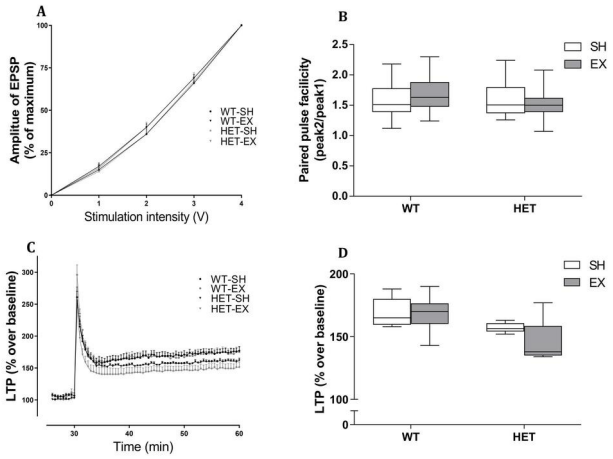
- Voluntary physical activity (exercise) increases hippocampal neural plasticity in healthy wild-type mice
- Exercise enhances hippocampus-dependent cognition
- Serotonin transporter (5-HTT) heterozygous (HET) mice have increased vulnerability to stress

What this study adds

- 5-HTT HET mice displayed impaired hippocampal LTP which was not corrected by exercise.
- Exercise-induced increase in neurogenesis was blunted in 5-HTT HET mice
- Exercise impaired 5-HTT HET mice touchscreen location discrimination probe on elements requiring increased cognitive flexibility

Clinical significance

- Functional serotonin transporter seem to be required for exercise-induced increased in hippocampal neurogenesis
- Altered 5-HTT function might heighten sensitivity to the stress component of daily exercise
- The clinical influence of the 5-HTTLPR polymorphism could result from dysregulation in synaptic plasticity



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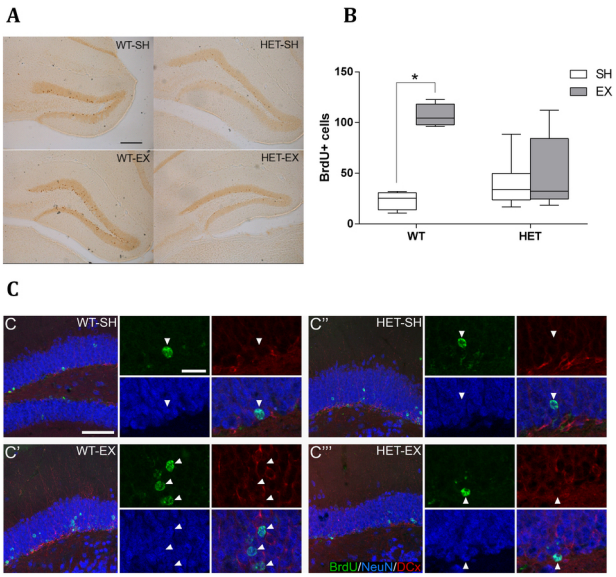

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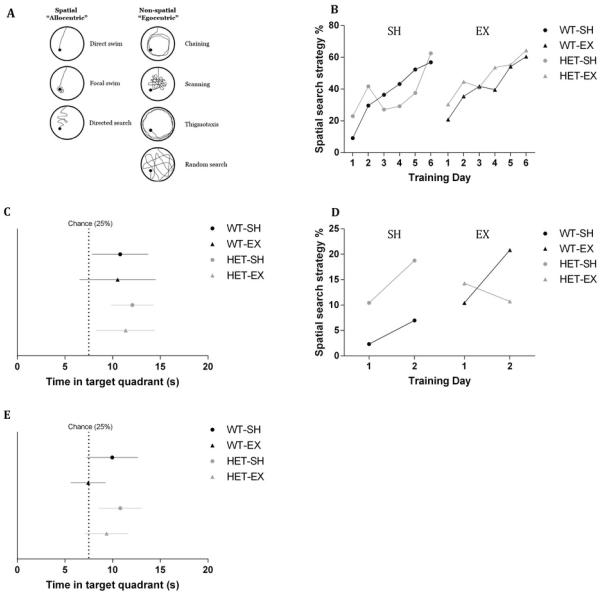

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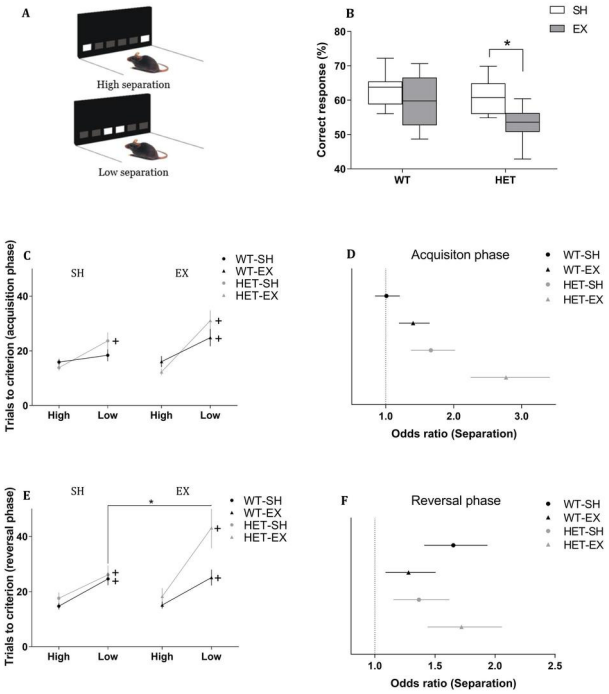

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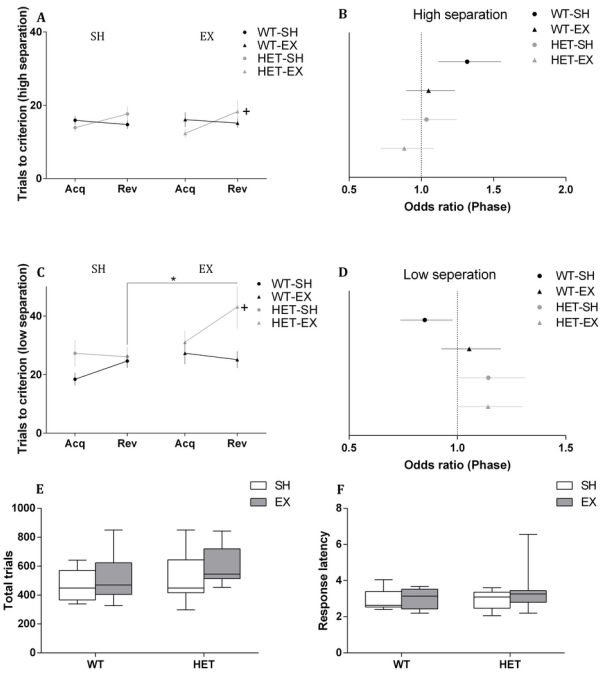

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