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Sex and BDNF Val66Met Polymorphism matter for exercise-induced increase in neurogenesis and cognition in middle-aged mice

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Highlights:

- BDNF Val/Val mice performed better than BDNF Met/Met mice in middle-age
- Aerobic training (AT) increased cognitive performance in BDNF Met/Met mice
- AT increased neurogenesis in middle-aged BDNF Val/Val mice only
- Female BDNF Val/Val mice had better cognitive flexibility than males regardless of AT
- AT increased more mature new neurons in middle-aged female mice

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Abstract:

Females show greater benefits of exercise on cognition in both humans and rodents, which may be related to brain-derived neurotropic factor (BDNF). A single nucleotide polymorphism (SNP), the Val66Met polymorphism, within the human *BDNF* gene, causes impaired activity-dependent secretion of neuronal BDNF and impairments to some forms of memory. We evaluated whether sex and BDNF genotype (Val66Met polymorphism (Met/Met) versus Wild Type (Val/Val)) influenced the ability of voluntary running to increase neurogenesis and cognition in mice. C57BL/6J (13 months) mice were randomly assigned to either a control or an aerobic training (AT) group (running disk access). Mice were trained on the visual discrimination and reversal paradigm in a touch screen-based technology to evaluate cognitive flexibility. BDNF Val/Val mice outperformed BDNF Met/Met mice on both cognitive tasks. Female BDNF Val/Val mice showed greater cognitive flexibility compared to male mice regardless of AT. Despite running less than BDNF Val/Val mice, AT improved both cognitive tasks in BDNF Met/Met mice. AT increased neurogenesis in the ventral hippocampus of BDNF Val/Val mice of both sexes and increased the proportion of mature type 3 doublecortin-expressing cells in the dorsal hippocampus of female mice only. Our results indicate AT results in improved cognitive performance in BDNF Met/Met mice and increased hippocampal neurogenesis in BDNF Val/Val mice in middle age. Furthermore, middle-aged female mice may benefit more from AT than males in terms of neuroplasticity, an effect that was influenced by the BDNF Val66Met polymorphism.

Keywords: Voluntary running, female, hippocampus, prefrontal cortex, Cognitive flexibility

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Normal aging is associated with reductions in a number of cognitive domains, including episodic memory and executive functions (Levine et al., 2021). Coupled with cognitive decline are further changes in the brain, notably the hippocampus, a region that is important for learning and memory (Sang et al., 2021). Hippocampal volume decreases with age, which is associated with higher risk for cognitive impairment and dementia (Raz et al., 2005; Jack et al., 2010). Sex differences in cognitive decline with normal aging are seen as females have greater cognitive decline in executive functions with age (Levine et al., 2021). It is crucial to find a viable, non-invasive and feasible strategy to prevent decline and improve cognition in older age. Recently the World Health Organisation suggested engaging in physical activity to slow down cognitive decline (*Risk Reduction of Cognitive Decline and Dementia*, 2019). However, given the sex differences seen in cognitive decline with normal aging and in dementia risk (Irvine et al., 2012; Levine et al., 2021), it is important to understand how sex may affect the role of exercise to offset cognitive decline with aging, particularly in middle-age as this is seen as an important time for intervention (Barha and Liu-Ambrose, 2020).

Physical exercise, in the form of aerobic training (AT), promotes cognition including prefrontal cortex (PFC)-dependent executive functions, and increases hippocampal volume in older adults with or without mild cognitive impairment (MCI; a prodromal state to Alzheimer's disease(AD)) (Baker et al., 2010; Liu-Ambrose et al., 2018; Erickson et al., 2011; Makizako et al., 2015; ten Brinke et al., 2015). AT also increases hippocampal-dependent learning and memory in rodents, but fewer studies have investigated AT effects on executive functions in rodents (Barha et al., 2017b). Evidence suggests AT effects on cognition may vary by sex, where human females may benefit more than human males in terms of executive functions in older age (Barha et al., 2017a, 2017b; Middleton et al., 2008). Interestingly, in rodents, a meta-analysis suggested sex differences in cognitive benefit dependent on the type of cognition as females showed greater benefits on spatial tasks whereas males showed greater benefits on non-spatial and conditioned avoidance tasks (Barha et al., 2017b). These sex differences may be related to the ability of AT to influence neurotrophic factors such as brain-derived neurotrophic factor (BDNF) (Barha et al., 2019a).

Exercise, sex, and estrogens influence BDNF (Barha et al., 2017a, 2017b; Chan and Ye, 2017). BDNF levels are increased after exercise in humans (plasma and serum) and rodents (hippocampus) (Barha et al., 2017a, 2017c; Erickson et al., 2011; Short et al., 2022; Triviño-Paredes et al., 2016; Voss et al., 2013). BDNF is a neurotrophin strongly involved in the neurogenic process, and modulates the effects of exercise on brain outcomes (Berchtold et al., 2001; Cowansage et al., 2010; Triviño-Paredes et al., 2016; Voss et al., 2013). BDNF levels are also negatively associated with more cognitive impairment in AD (Erickson et al., 2010; Siuda et al., 2017), and positively associated with increased hippocampal volume and spatial memory performance (Erickson et al., 2010; Voss et al., 2013). Sex differences in BDNF levels after exercise have also been noted, with human and rodent females showing a greater increase in BDNF levels after exercise compared to males, which are related to ovarian hormone levels (Barha et al., 2017a, 2017b, 2017c; Berchtold et al., 2001).

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A single nucleotide polymorphism (SNP) is found within the pro-domain region of the human BDNF gene, resulting in an amino acid substitution of valine (Val) to methionine (Met) at codon 66, termed the Val66Met polymorphism. The BDNF Val66met polymorphism reduces activity dependent neuronal secretion of BDNF (Egan et al., 2003; Miranda et al., 2019; Park et al., 2017), and a robust body of literature suggests it is associated with impaired hippocampal-dependent memory (Chen et al., 2008; Dincheva et al., 2014; Egan et al., 2003; Kennedy et al., 2015; Lamb et al., 2015). However, less is known about other cognitive domains such as PFC-dependent executive functions and, intriguingly, BDNF Met/Met have compromised PFC activity (Pattwell et al., 2012). Under the umbrella term for PFC-dependent cognition, cognitive flexibility is a part of executive functioning and is a fundamental domain needed in order to adapt our behavior to cope with novelty, and thus facilitate adaptation (Diamond, 2013; Logue and Gould, 2014; Armbruster et al., 2012; Dajani and Uddin, 2015). Notably, cognitive flexibility is one of the first domains affected in pathological and non-pathological aging (Corbo and Casagrande, 2022; Guarino et al., 2020), and impairments in cognitive flexibility could be a predictor of later onset of AD, perhaps due to the early degeneration of the PFC in AD (Traykov et al., 2007; Salat et al., 2001).

The hippocampus is a highly plastic structure including the presence of adult neurogenesis in the dentate gyrus (Christie and Cameron, 2006). Hippocampal neurogenesis exists in all mammalian species, including humans (Christie and Cameron, 2006; Eckenhoff and Rakic, 1988; Eriksson et al., 1998). Although a couple of studies suggest that hippocampal neurogenesis is non-existent or rare in humans (Franjic et al., 2022; Sorrells et al., 2018), the vast majority of studies, using a variety of techniques, have shown that adult hippocampal does in fact occur (Boldrini et al., 2018; Moreno-Jiménez et al., 2021; Spalding et al., 2013; Zhou et al., 2022). Intriguingly, adult hippocampal neurogenesis decreases with age, is impaired in AD, increases with exercise, promotes cognitive flexibility, and is modulated by both sex and BDNF levels (Anacker and Hen, 2017; Berdugo-Vega et al., 2021; Choi et al., 2018; Kuhn et al., 1996; Marrocco et al., 2017; Mu and Gage, 2011; van Praag et al., 1999; Yagi and Galea, 2019). BDNF is required for the survival of new neurons, and increasing BDNF in AD mouse models increases hippocampal neurogenesis and cognition to levels that mimic the positive cognitive effects of exercise on AD (Sairanen et al., 2005; Choi et al., 2018). Sex differences are seen in both hippocampal structure and function, including neurogenesis, (Yagi and Galea, 2019) and exercise may increase neurogenesis to a greater degree in females than in males (Ma et al., 2012; Ransome and Hannan, 2013). Thus it is important to consider sex in studies examining the influence of BDNF polymorphisms, exercise and neurogenesis.

In the present study, we examined sex differences in cognitive flexibility and neurogenesis in response to AT in middle-aged BDNF Val/Val and BDNF Met/Met mice. To our knowledge, no previous study has addressed the effect of AT on cognitive flexibility assessed through a translational tool in middle-aged male and female rodents carrying the BDNF val66met polymorphism. Thus, we evaluated the effect of voluntary running in middle-aged male and female BDNF Val/Val and Met/Met mice on cognitive flexibility. We expected that the BDNF Val/Val mice would show superior cognition and neurogenesis, particularly in females,

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compared to the Met/Met mice and that AT would have differential effects on cognition and neurogenesis based on genotype and sex.

Methods:

Subjects

C57BL/6J knock-in mice in which the human BDNF Val66Met SNP was inserted into the mouse BDNF gene were obtained via the University of Calgary from a stock that was originally from Francis Lee. Mice were bred and aged to 13 months old in our breeding facilities during which time they were group housed (2 per cage) in a temperature and humidity controlled colony room (21 ± 1 °C; 50 ± 10% humidity), with a 12-h light/dark cycle (lights were on at 07:00 h). A total of n=34 BDNF Val/Val mice (18 males, 16 females) and n=28 BDNF Met/Met mice (13 males, 15 females) were used. Mice were given food (Purina chow) and water ad libitum before assignment to the experimental groups. All experimental procedures were approved by the Animal Care Committee at the University of British Columbia and were performed according to the Canadian Council on Animal Care guidelines.

Exercise Intervention

Middle-age male and female mice (13 months old) were single-housed in Digital Ventilated Cages® (Techniplast, Italy) with 12-h inverted light/dark cycle (lights were off at 07:00 h) and randomly assigned to the control sedentary or the Aerobic Training (AT) group. Finally, eight groups were formed for sex X genotype X AT intervention as follows: Male BDNF Val/Val control (n=8), male BDNF Val/Val AT (n=10), female BDNF Val/Val control (n=8), female BDNF Val/Val AT (n=8); male BDNF Met/Met control (n=6), male BDNF Met/Met AT (n=7), female BDNF Met/Met control (n=8), female BDNF Met/Met AT (n=7). The AT group was housed with a running wheel (Bio-serv Igloo fast-trac, circumference 479 mm) and the distance ran was recorded daily. Access to the running wheel was maintained throughout the experiment. Control sedentary mice were housed without a running wheel.

Behavioral assessment

Mice were food-restricted to 85-90% of their free-fed body weight to increase motivation to complete the task (based on Buscher et al., 2017). Mice reached target weight in ~7 days and during habituation to the food-restriction regimen, mice were exposed to 8 µl of the strawberry milkshake reward (Neilson Dairy, Saputo Inc.) placed in a small container inside the home cages. To assess cognitive flexibility, mice were trained on the Pairwise (Visual) Discrimination and reversal paradigm of the Bussey-Saksida touchscreen system (Lafayette Instrument, U.K.).

Apparatus

The Bussey-Saksida touchscreen system consisted of four, individual trapezoidal chambers (238mm wide at screen x 170mm tall x 532 mm deep) equipped with a milk receptacle, an infra-red nose-poke detector, a light and tone generator, and an external reward dispenser connected to the reward tray inside the chamber. On the opposite wall to the tray was a touch-sensitive screen covered with a metal template dividing the monitor into two squares to

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restrict the animal's choice field. The divided touch-sensitive screen displayed the two stimuli to be discriminated against. The base was composed of non-shock perforated floors with a waste-collector tray underneath. All chambers were placed inside sound-proofing boxes equipped with a small fan and a video camera to record each session.

Experimental timeline

The experimental timeline is shown in Figure 1. Mice were randomly assigned to either control sedentary or voluntary AT groups. Mice were allowed two weeks with the running wheels (or no running wheels) before the cognitive training protocol commenced. The running wheel was introduced in the home cage after the two acclimation weeks elapsed. After 3 weeks of voluntary AT, each animal was weighed for 3 consecutive days to have a basal average weight and then were kept at 85-90% of their free-feeding weight. Milk reward was introduced in their home cages before behavioural training commenced. Mice were trained for approximately 15 days and then promoted to the visual discrimination acquisition phase. Mice learned to visually discriminate the stimulus associated with the milk reward for a total of 13 sessions (1 session/day). Finally, mice were subjected to the reversal paradigm for 14 days. Twenty-four hours after the last behavioral session, mice were perfused and brain and blood were collected for future determinations. Adrenals were also extracted and weighed.

Training protocol

Pre-training

The pre-training protocol served to train mice how to operate the touchscreens, and was performed with minor changes to Lafayette Instrument's manual in the following 5 stages: (i) habituation (A and B), (ii) initial touch, (iii) must touch, (iv) must initiate, and (v) punish incorrect. After reaching criterion for a particular stage, each mouse progressed to the next pre-training stage in the following session. To avoid over-training, upon reaching criterion for 'punish incorrect', mice were given 2 days rest followed by a reminder session until all subjects reached criterion at 70% [based on Horner et al., 2013]. In this way, all animals began Visual Discrimination training on the same day. Pretraining took 15-22 days.

Visual Discrimination Acquisition

After pre-training completion, visual discriminatrion training began as mice learned to select the correct stimulus displayed on the screen to elicit the reward delivery response. The trial began with the display of two novel stimuli, one on each side of the screen. The task requires mice to discriminate between these stimuli and to learn which stimulus is "correct", or associated with the reward delivery, (S^+) and which one was "incorrect" (S^-). Placement of S^+ on the screen was determined pseudorandomly, as the S^+ image would not appear on the same side more than three consecutive times. S^+ and S^- images were counterbalanced across groups. The images used were "marble" and "fan" (see Figure 1), as this pair of pictures is recommended for the Visual Discrimination and Reversal task in mice (Horner et al., 2013). The mouse must 'nose poke' the S^+ image to elicit the strawberry milk delivery, which was accompanied by the illumination of the reward-tray light and a tone. Once the tray was entered and reward collected, the tray light turned off and a 10 sec ITI began, followed by reillumination of the reward tray. The animal had

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to nose poke and exit the reward tray to start a new trial, with the same two images displayed on the screen. Nose poking the incorrect image (S^-) resulted in a 5 sec time-out period. The criterion for visual discrimination training was 21/30 correct trials (70% of accuracy) in 60 minutes on two consecutive sessions. Mice were trained for 13 sessions in the Visual Discrimination paradigm. To avoid over-training, mice that reached criterion were rested for two days and then given a reminder trial on the third day. If the mouse performed at least 70% of accuracy on the reminder session, another two days of resting were given. If performance was below 70% of accuracy on the re-train session, the mouse kept training until the criterion of 70% accuracy in two sessions in a row was reached again.

Reversal test

Cognitive flexibility can be modeled in rodents through reversal learning paradigms and touchscreen-based technology has been proven as a highly translatable tool to explore cognition among rodents, humans and other species (Izquierdo et al., 2017; Turner et al., 2017). The correct and incorrect stimuli are switched such that the previously unrewarded stimulus (S^-) must now be selected (S^+) to elicit the reward delivery response, and the previously rewarded stimulus (S^+) is now incorrect (S^-). Mice were tested in the Reversal task for 16 sessions. The procedure, trials, ITI, time-out periods and criterion of accuracy remained the same as in Visual Discrimination.

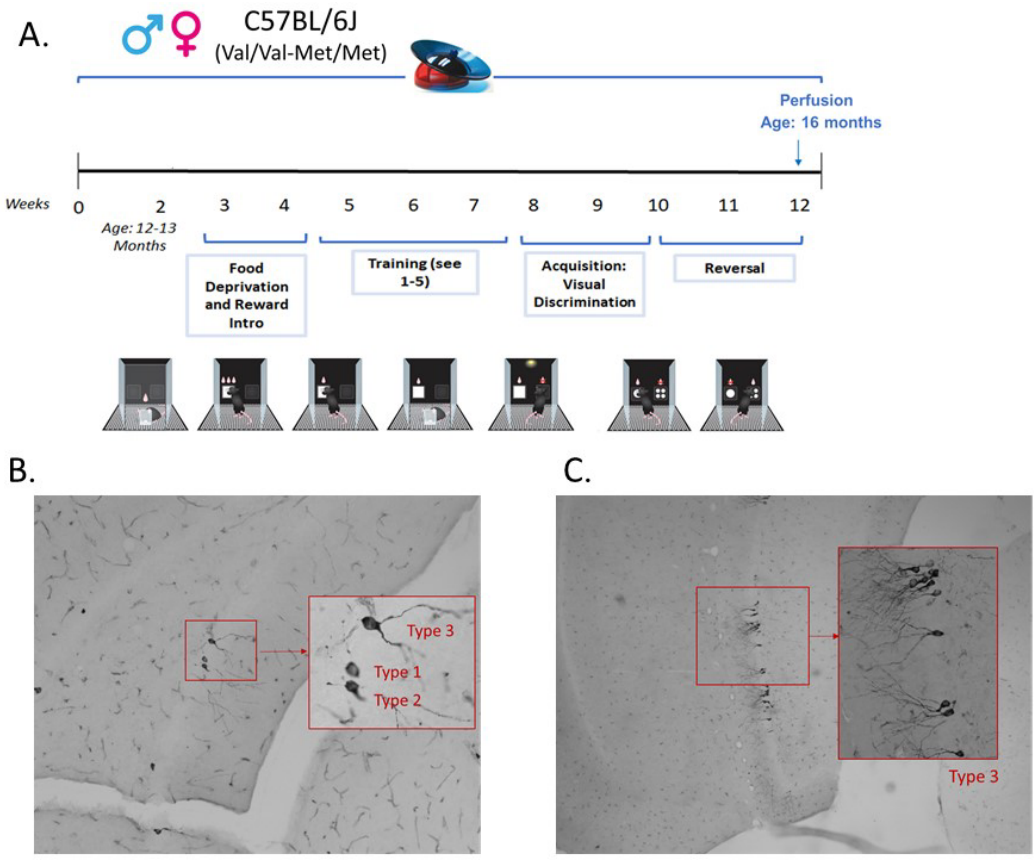

Figure 1. A) Experimental timeline of C57BL/6J mice (with either the brain derived neurotrophic factor (BDNF) Met/Met genotype or the BDNF Val/Val genotype), behaviour and testing. B)

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Large photomicrograph (taken with a 20x objective) of an example of a type 1, type 2 and type 3 doublecortin (DCX) expressing cells from a female mouse. Inset taken with a 40x objective. C) Photomicrograph (taken with a 20x objective) of type 3 DCX expressing cells from a female mouse. Inset taken with a 20x objective.

Tissue collection

Twenty-four hours after the last behavioural session, mice were perfused by administering an overdose of sodium pentobarbital (500 mg/kg, i.p.). Blood samples were collected from the thoracic cavity, and mice were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (Sigma-Aldrich). Brains were extracted and postfixed in 4% paraformaldehyde overnight, then transferred to 30% sucrose (Fisher Scientific) solution for cryoprotection and remained in the solution until sectioning. Adrenals were extracted and weighed. Brains were sliced into 30-µm coronal sections using a Leica SM2000R microtome (Richmond Hill, ON, Canada). Sections were collected in series of ten throughout the entire rostral-caudal extent of the hippocampus and stored in antifreeze solution consisting of ethylene glycol, glycerol, and 0.1 M PBS at -20°C until immunostaining.

Doublecortin Immunohistochemistry

Doublecortin (DCX) is a microtubule binding protein expressed in progenitor cells and immature neurons, and is used as a marker for adult neurogenesis (Brown et al., 2003). Briefly, sections were rinsed 3□×□10□min in 0.1□M phosphate buffered saline (PBS), treated with 3% hydrogen peroxide in dH2O for 30□min, rinsed again 3□×□10□min in 0.1□M PBS, and incubated at 4□°C in primary antibody solution: 1:1000, goat anti-doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). 24□h later, sections were rinsed 5□×□10□min and incubated at 4□°C in an secondary antibody solution with 1:500, rabbit anti-goat (Vector Laboratories, Burlingam, CA,USA). Another 24□h later, sections were rinsed 5 x□10□min and incubated in Avidin-Biotin complex (AB; 1:1000; Vector) for 4□h at room temperature. Sections were then rinsed 3 x 10 min in 0.1M PBS and then washed in 0.175□M sodium acetate buffer 2 times for 2□min, developed using diaminobenzidine in the presence of nickel (DAB Peroxidase Substrate Kit). Sections were then washed 2 x 30 seconds in 0.175□M sodium acetate buffer, rinsed 3 x 10 mins in 0.1M PBS and mounted on slides, and dried. Sections were then dehydrated, cleared with xylene, and coverslipped with Permount (Fisher).

Microscopy

Microscopy analyses were conducted by an experimenter blind to the group conditions. DCX-expressing cells were quantified in 2 dorsal and 2 ventral sections, using the 40x objective on an Olympus microscope. Cells were classified as DCX-expressing if the stain was dark and cells were present in the inner granule cell layer of the dentate gyrus (Figure 1B-C). Cells were counted separately in the dorsal and ventral regions of the dentate gyrus, as these regions are thought to be functionally distinct. The dorsal region is involved in spatial reference memory whereas the ventral region is involved in working memory, stress and anxiety and has input to the PFC (Fanselow and Dong, 2010; Moser and Moser, 1998; Twining et al., 2020).

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Forty cells positively labelled for DCX (20 from the dorsal and 20 from the ventral) were further examined and categorized by morphology. Cells in a proliferative state were classified as type 1 (no or short processes), cells in an intermediate state were classified as type 2 (medium processes with no branching), and cells in a post mitotic state were classified as type 3 (strong dendritic branching into the molecular layer) (Plümpe et al., 2006; see Figure 1B-C). Two Female Val/Val mice were excluded from these analyses due to tissue loss.

Statistical Methods

All data were analysed using Statistica software (v. 9, StatSoft, Inc., Tulsa, OK, USA). Dependent variables of interest were anlaysed using an analysis of variance (ANOVA) with sex (male, female), genotype (BDNF Val/Val, BDNF Met/Met) and exercise (sedentary, AT) as between-group variables, and within-subjects variables of either testing block (1-8), region (dorsal hippocampus, ventral hippocampus) or DCX expressing cell type (type 1,2,3). Post-hoc comparisons used Newman-Keuls. Pearson product-moment correlations were conducted between dependent variables of interest. A priori we were interested in whether or not sex and genotype influenced the effects of AT on hippocampal neurogenesis and cognitive flexibility. All effects were considered statistically significant if $p \leq 0.05$, trends are discussed if $p \leq 0.10$. Outliers were eliminated if higher or lower than 2 standard deviations from the mean. Animals that did not complete 30 trials at least in one session were excluded from the cognitive performance analyses. Data are presented as mean $\pm$ standard error and effect sizes for significant results were reported as partial eta squared (η_p^2) or Cohen's d, as appropriate.

Results:

BDNF Val/Val ran more than BDNF Met/Met mice. Relative adrenal mass was higher in females, irrespective of AT

BDNF Val/Val ran significantly more than BDNF Met/Met mice regardless of sex (main effect of genotype: [$F(1, 29)=13.68$ $p<0.05$, $\eta_p^2=0.32$; see Table 1). No other significant main or interaction effects were found (all p's >0.65).

Table 1 shows the adrenal mass expressed in percentage of body weight. Females had larger adrenals in comparison to males regardless of AT ($p<0.001$). Moreover, there were differences per genotype with a higher relative adrenal weight in BDNF Val/Val females than in the Met/Met counterparts ($p<0.001$); interaction Genotype x Sex ($F_{1, 42}= 12.327$, $p=0.001$, $\eta_p^2= 0.23$). There were also main effects of genotype ($F_{1, 42}= 22.980$, $p<0.001$, $\eta_p^2= 0.35$) and sex ($F_{1, 42}= 48.093$, $p<0.001$, $\eta_p^2=0.53$)). There were no other significant main effects or interactions (all $p>0.533$).

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Female BDNF Val/Val mice had significantly lower body mass than female BDNF Met/Met mice as well as both BDNF Val/Val and Met/Met males (Table 1) (genotype by sex interaction [$F_{1, 41}$=4.578, p=0.038, η_p^2= 0.100]; post hoc, all p<0.0017). There were also main effects of genotype (p<0.0006) and sex (p=0.013).

		Val/Val		**Met/Met**	
		Ctrl	***AT***	***Ctrl***	***AT***
Cumulative Running (km)	**Males**	N/A	247.903 ± 82.339	N/A	24.932 ± 7.562
	Females	N/A	288.208 ± 64.019	N/A	42.753 ± 26.210
Adrenal Mass Ratio (/body weight)	**Males**	0.000192 ± 0.000040	0.000198 ± 0.000057	0.000155 ±0.000008	0.000169 ± 0.000014
	Females	0.000513 ± 0.000049	0.000468 ± 0.000036	0.000541 ± 0.000352	0.000297 ± 0.000034
Average Body Mass (g)	**Males**	37.800 ± 3.397	36.167 ± 2.151	42.167 ± 0.946	38.857 ± 1.2617
	Females	26.750 ± 2.136	26.000 ± 1.065	41.125 ± 5.485	37.857 ± 2.087

Table 1. Cumulative running in kilometres (Km), adrenal mass ratio, and body weight across all groups. BDNF Val/Val mice ran significantly more than BDNF Met/Met mice Adrenal weight expressed as a proportion of body weight. Females had higher relative adrenal weight compared to males, regardless of AT. Female BDNF Val/Val mice had a higher relative adrenal weight than female BDNF Met/Met mice . Female BDNF Val/Val mice had the lowest body weight compared to all other groups. Data presented as mean ± standard error of the mean. Ctrl= Sedentary controls; AT= Aerobic training.

BDNF Val/Val mice took fewer days to reach critierion on Visual Discrimination trials and AT reduced days to reach criterion in BDNF Met/Met mice only.

Figure 2A shows the days to reach training criterion for visual discrimination. The analysis revealed that sedentary BDNF Val/Val mice reached the training criterion in fewer days than sedentary BDNF Met/Met (p<0.001, Cohen's d= 1.909; interaction of genotype by AT ($F_{1,56}$= 13.333, p<0.001, η_p^2= 0.19). However, BDNF Met/Met mice showed a reduction in days to reach criteria with AT (p=0.003, Cohen's d=1.269) but the same AT benefit was not observed in

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BDNF Val/Val mice (p=0.229). There were also main effects of genotype ($F_{1,56}$= 48.533, p<0.001, η_p^2= 0.46) and AT($F_{1,56}$= 4.082, p=0.048, η_p^2= 0.07) but no other significant main effects or interaction effects were observed.

Across the blocks of visual discrimination trials, BDNF Val/Val mice learned to discriminate the visual stimulus better than BDNF Met/Met mice (on blocks 2, 4-7 (all p's <0.02; genotype by block: $F_{6,258}$= 4.139, p=0.0005, η_p^2= 0.087; Figure 2B). A priori, we expected AT mice to perform better than sedentary mice and they did on the fourth and last block of sessions (p's <0.0007; AT by block: $F_{6,258}$= 1.849, p=0.09, η_p^2= 0.041). There were also main effects of blocks ($F_{6,258}$= 33.409, p<0.0001, η_p^2= 0.437) and genotype ($F_{1,43}$= 5.138, p=0.028, η_p^2= 0.107) but no other significant effects (all p's >0.23).

Female BDNF Val/Val showed increased cognitive flexibility compared to males, regardless of AT. AT improved cognitive flexibility in Met/Met mice regardless of sex

Female BDNF Val/Val mice had better cognitive flexibility (reversal performance), regardless of AT, than males of the same genotype (on blocks 5, 6 and 8 (all p's<0.05; genotype by sex by block ($F_{7,301}$= 2.259, p=0.030, η_p^2= 0.050) with no significant differences between the sexes of BDNF Met/Met mice (all p's >0.610). In addition, the BDNF Val/Val mice performed better on reversal learning than BDNF Met/Met mice, particularly in females (Block 4-8 all p's <0.012) than in males (Block 7, p=0.016). In addition, BDNF Val/Val mice outperformed BDNF Met/Met mice on the reversal task particularly under sedentary conditions (Blocks 4-8, p's <0.002) than under AT (Block 7 p=0.040). Although Met/Met mice did not reach criterion for visual discrimination or reversal, better cognitive flexibility (reversal performance) was observed in AT BDNF Met/Met mice compared to sedentary (block 8 p=0.025; genotype by AT by block ($F_{7,301}$= 2.287, p=0.028, η_p^2= 0.050; Figure 2D). AT did not significantly influence reversal training in BDNF Val/Val mice across blocks (all p's>0.14). There were also significant main effects of genotype ($F_{1,43}$= 9.193, p=0.004, η_p^2= 0.176) and block ($F_{7,301}$= 43.043, p<0.001, η_p^2= 0.500) and an interaction of blocks by genotype ($F_{7,301}$= 6.190, p<0.0001, η_p^2= 0.125) but no other significant effects (all p's>0.210).

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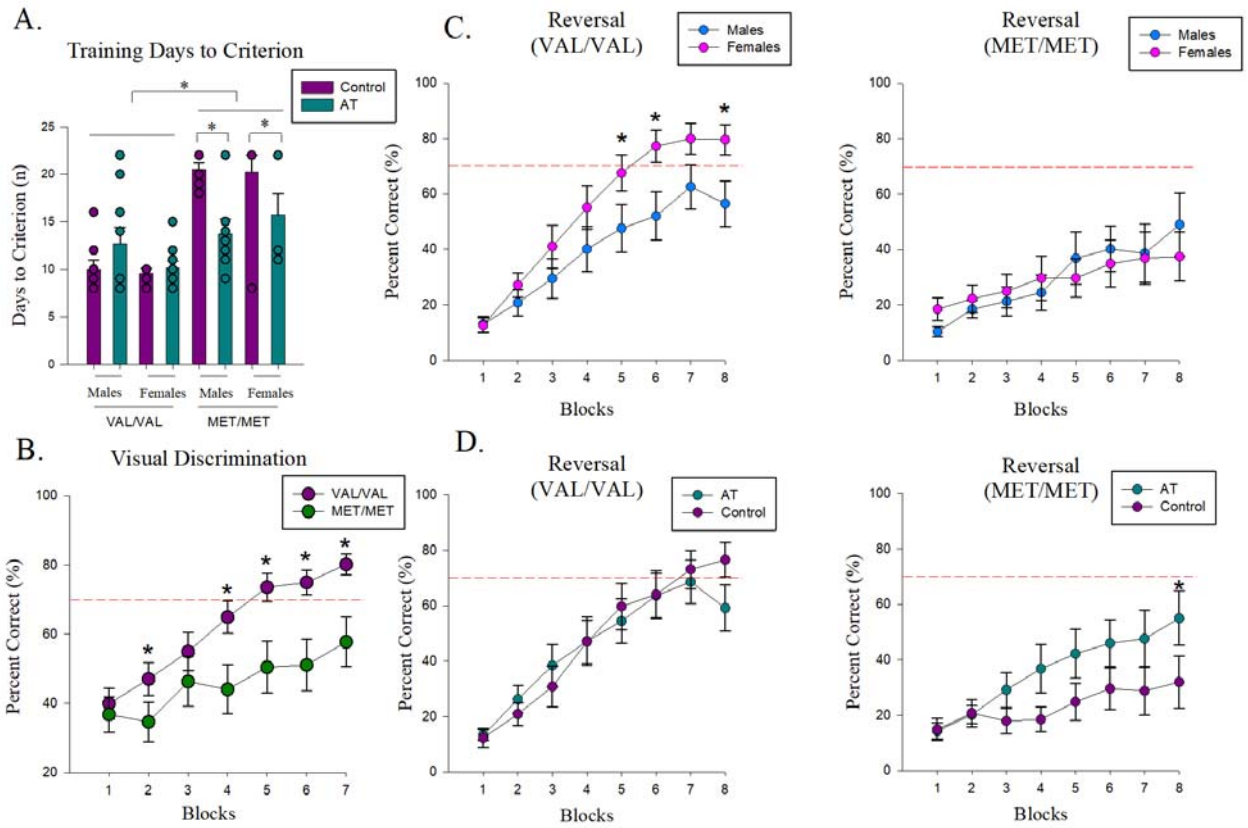

Figure 2. A) BDNF Met/Met mice took more days to reach training criterion than BDNF Val/Val mice, regardless of sex. Aerobic training (AT) reduced days to reach criterion in BDNF Met/Met mice only. (*p<0001 vs Met/Met). B)Visual discrimination training. BDNF Val/Val mice learned to discriminate the visual stimulus better than BDNF Met/Met mice. C-D) Reversal training (cognitive flexibility). C) Female BDNF Val/Val mice had better cognitive flexibility, regardless of AT, than males of the same genotype (on blocks 5, 6 and 8 (all p's<0.05; genotype by sex by block ($F_{7,301}$= 2.259, p=0.030, η_p^2= 0.050). No differences were observed in BDNF Met/Met mice (p's >0.610). D) Although Met/Met mice did not reach criterion for visual discrimination or reversal, AT increased cognitive flexibility (reversal performance) in BDNF Met/Met mice compared to sedentary (block 8 p=0.025; genotype by AT by block ($F_{7,301}$= 2.287, p=0.028, η_p^2= 0.050). No significant differences were observed in BDNF Val/Val mice (all p's>0.14). Red dotted lines indicate the criterion of 70% correct trials.

Voluntary running increased the total density of doublecortin-expressing cells in the ventral region of BDNF Val/Val mice

AT increased the total density of DCX-expressing cells in the ventral region compared with sedentary controls in the BDNF Val/Val mice (p=0.0097; Cohen's d= 0.393) but not in the BDNF Met/Met mice (p=0.935; interaction between genotype, AT, and hippocampal region [F(1,53)=4.373, p=0.041, η_p^2=0.076; see Figure 3A-B]. Further a trend was found for an interaction of genotype by sex by region [F(1,53)=3.976, p=0.051, η_p^2=0.069], but no

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meaningful post-hoc significant outcomes were found, and main effects of hippocampal region [$F(1,53)=39.958$, $p<0.0001$, $\eta_p^2=0.430$]). In addition, a trend for a main effect of AT [with the AT group having more DCX-expressing cells : $F(1,53)=3.454$, $p=0.069$, $\eta_p^2=0.061$]) was found but no other significant main or interaction effects (p's >0.34).

Voluntary running increased the proportion of Type 3 doublecortin-expressing cells in the dorsal region of females; Val/Val mice had more type 3 doublecortin-expressing cells

The morphology of the DCX-expressing cells indicates the maturity of the new neurons (Plümpe et al., 2006) and we next examined the proportion of type 1, 2 and 3 DCX-expressing cells. Type 1 DCX-expressing cells were significantly higher in the ventral compared to the dorsal region, and conversely, type 3 DCX-expressing cells were significantly higher in the dorsal compared to the ventral region (post-hocs, all p's<0.05; a region by type of DCX-expressing cell interaction ([$F(2,104)=8.2627$ $p=0.0005$, $\eta_p^2=0.137$]). There was a significantly greater proportion of type 1 DCX-expressing cells compared to both type 2 or 3 in both BDNF Val/Val and Met/Met mice (all p's<0.0001; type by genotype interaction ($F(2,104)=329.675$ $p<0.0001$, $\eta_p^2=0.8638$]) (see Figure 3C). Despite the genotype by sex by group interaction effect ([$F(1,52)=39219$ $p<0.0001$, $\eta_p^2=0.999$]), there were no significant differences between the groups of interest in the post hoc analysis.

Type 3 DCX-expressing cells were increased in the AT group compared to sedentary control in female, but not male mice (a priori p=0.001, Cohen's d=0.2491; Figure 3D). Furthermore, proportional maturity varied by genotype in the dorsal region only. Met/Met mice had a greater proportion of type 1 cells compared to the Val/Val mice (p=0.003, Cohen's d=0.626; Figure 3C), whereas the opposite was true for the dorsal type 3 DCX expressing cells where Val/Val mice had significantly more type 3 DCX expressing cells compared to the Met/Met mice (p=0.003, Cohen's d=0.752; Figure 3E).

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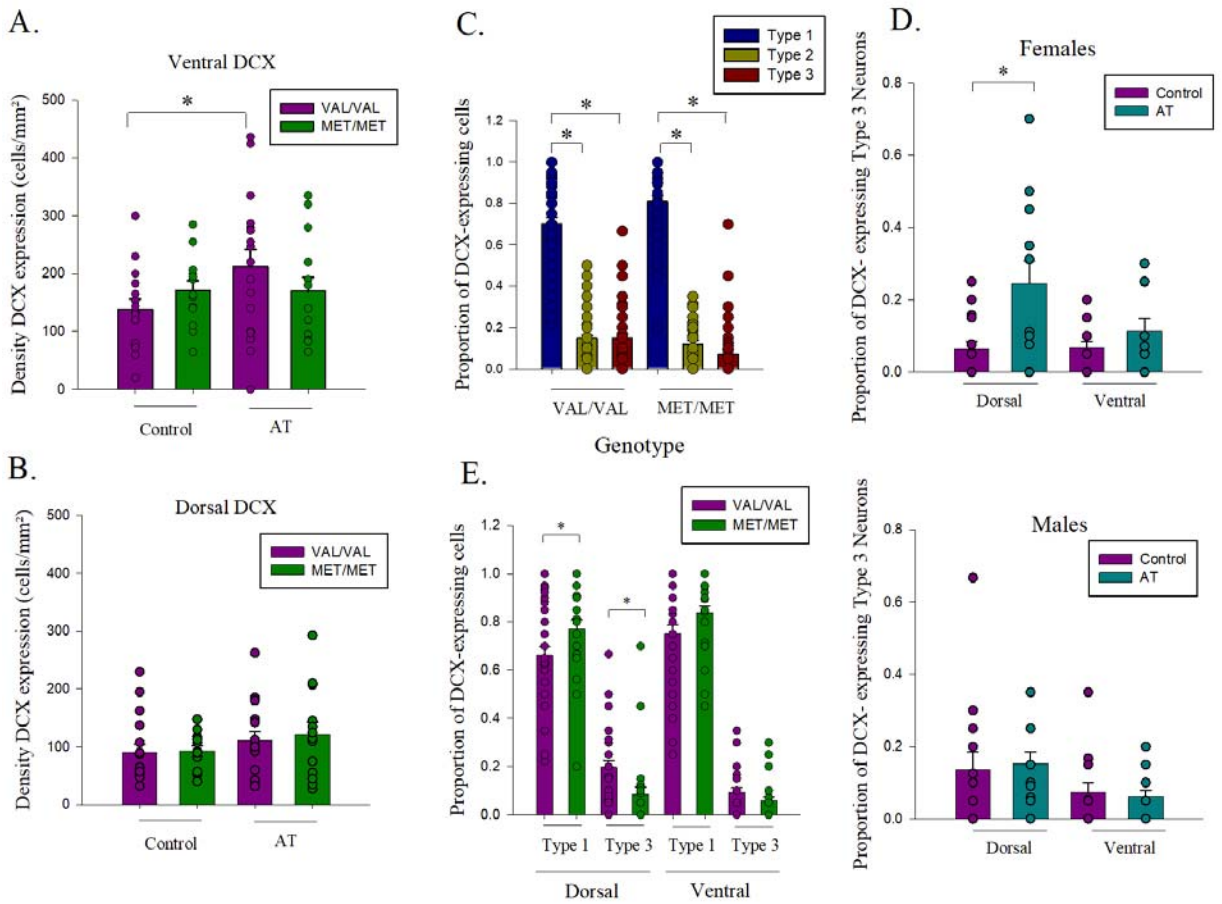

Figure 3. A-B) Density of doublecortin (DCX)-expressing cells by BDNF genotype and aerobic training (AT) in the ventral (A) and dorsal (B) region of the dentate gyrus. The density of doublecortin-expressing cells was increased with AT in the ventral (A), but not dorsal (B) , region of the hippocampus in the BDNF Val/Val mice. C-E) Maturity of doublecortin (DCX) expressing cells. C) There was a significantly greater proportion of proliferative type 1 DCX expressing cells compared to both type 2 and type 3 in both the BDNF Val/Val and Met/Met mice. D) The proportion of type 3 DCX-expressing cells increased with AT in female but not male mice. E) BDNF Met/Met mice had a greater proportion of type 1 DCX-expressing cells in the dorsal region of the hippocampus compared to the BDNF Val/Val mice. Whereas, the BDNF Val/Val mice had a greater proportion of type 3 DCX-expressing cells compared to the Met/Met mice, in the dorsal hippocampus.

Correlations

No significant correlations by sex or genotype on any of the dependent measures were observed that survived Bonferroni correction.

Discussion

BDNF Val/Val mice performed better than BDNF Met/Met mice on the visual discrimination task regardless of sex or AT treatment. Female BDNF Val/Val mice performed better than males on the cognitive flexibility task, regardless of AT, whereas AT benefited both cognitive

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flexibility and visual discrimination in BDNF Met/Met mice regardless of sex. AT significantly increased neurogenesis in the ventral region of the hippocampus in middle-aged BDNF Val/Val mice (wild-type), but not in the mice with the BDNF Val66Met polymorphism (BDNF Met/Met mice), regardless of sex. However, when we examined the specific effect of voluntary AT on the maturity of these DCX-expressing cells, AT increased the proportion of type 3 DCX-expressing cells in the dorsal region in female mice compared to males, regardless of genotype, but better cognitive performance in reversal was only noted in the BDNF Val/Val female mice. Furthermore, BDNF Val/Val mice were found to have a greater proportion of dorsal type 3 DCX-expressing cells, along to better visual discrimination, compared to the BDNF Met/Met mice. In summary, our results indicate that in middle-age, female mice may benefit more in terms of neuroplasticity and cognitive flexibility with AT, an effect that can be influenced by the BDNF Val66Met polymorphism.

Cognitive flexibility performance in middle-age is associated with sex and genotype

Here we found that BDNF Val/Val middle-aged mice performed better on both visual discrimination and cognitive flexibility than BDNF Met/Met middle-aged mice, regardless of sex or AT. Previous studies have found in middle-age humans that individuals with the BDNF Met/Met genotype show a steeper decline in executive function and episodic memory (Boots et al., 2017), but others have found less decline in cognitive flexibility assessed through set-shifting in patients with Parkinson's Disease (van der Kolk et al., 2015) or in healthy aging using the Digital Symbol Substitution Test (Barha et al., 2019b). The inconsistencies among studies may be due to tasks given (set shifting, processing speed, flexibility), health status (Parkinson's versus cognitive healthy aging), and/or relative proportions of males to females. Indeed, in our study we found that female BDNF Val/Val mice showed greater cognitive flexibility than males regardless of AT. These findings are at least partially consistent with previous literature where human females had a higher cognitive baseline compared to males in middle-age for executive functions (Levine et al., 2021) and where white female individuals with the BDNF Val/Val Genotype had better digit symbol substitution scores than white males (Barha et al., 2019b) than males. These findings suggest that female sex is associated with better cognitive flexibility in both humans and rodents, that may depend on genotype. The sex by genotype differences in cognitive flexibility may be partially modulated by differences in gene expression in the CA3 region (Marrocco et al., 2017), as the CA3 region, along with the dentate gyrus contributes to cognitive flexibility (Berdugo-Vega et al., 2021; Webler et al., 2019).

AT improved cognitive performance in BDNF Met/Met mice but increased neurogenesis in BDNF Val/Val mice

In our study middle-aged BDNF Met/Met mice did not run as much as middle-aged BDNF Val/Val mice, regardless of sex. Previous reports have shown no differences in the amount of running between BDNF Val/Val and BDNF Met/Met in young adult mice (Sandrini et al., 2019; Ieraci et al., 2016), Therefore, it is possible that age influences the running pattern in BDNF Met/Met.

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Despite the fact that BDNF Met/Met mice did not run as much as BDNF Val/Val mice, and performed worse on both cognitive tasks, we found greater AT efficacy to promote both visual discrimination and cognitive flexibility in the BDNF Met/Met mice, regardless of sex. These findings may also play into the findings that individuals with the BDNF Met/Met genotype have a slower rate of cognitive decline (Barha et al., 2019b, van der Kolk et al., 2015) in different human populations. It is possible that given exercise is an effective strategy to ward off cognitive decline, perhaps this is benefiting this population more so than others. Thus, it may be that the slower rates of decline in executive functions seen in Met carriers, may be related to the greater cognitive benefits of AT in Met carriers. Other studies have found that AT enhances cognition in older human females compared to older males (Liu-Ambrose et al., 2018), and although we did not find a sex by genotype difference in these middle-aged mice, it is possible that our mice were too young to see this aging effect. A previous meta-analysis reported greater AT benefits on non-spatial memory tasks in healthy male older rodents compared to female rodents (Barha et al., 2017b). Similarly, Short et al., (2022) found beneficial effects of AT on cognitive flexibility in male, but not in female, middle-aged mice. As noted, BDNF Met/Met mice ran significantly less than Val/Val mice, it is possible that different genotypes require more AT to improve cognition, and future studies could explore longer AT interventions in the Val/Val mice.

BDNF Val/Val mice showed an increase in ventral DCX-expressing cells compared to sedentary controls, whereas BDNF Met/Met showed minimal running and no significant increase in DCX-expressing cells compared to sedentary controls. Interestingly, the lack of an AT effect on neurogenesis in BDNF Met/Met is consistent with previous research done in younger male mice (Ieraci et al., 2016), even though these younger male mice did run as much as the younger BDNF Val/Val mice (Ieraci et al., 2016). Taken together with our results, genotype seems to play a greater role, independent of the amount of running, to thwart the AT-induced increase in neurogenesis. A possible explanation for this genotype effect is the impaired release, binding affinity and intracellular trafficking of neural BDNF that is seen with the Val66Met polymorphism (Chen et al., 2008; Chiaruttini et al., 2009). As BDNF increases the survival of new neurons and thus increases neurogenesis (Sairanen et al., 2005), less BDNF secretion (due to lack of AT or impairments intrinsic to the genotype) could reduce the amount of neurogenesis in the BDNF Met/Met mice. Indeed, crucial BDNF transcripts for dendritic trafficking and optimal neuroplasticity are differentially expressed in the hippocampus of BDNF Met/Met and Val/Val young mice (Mallei et al., 2015) and future studies should confirm if similar effects occur in middle-aged rodents. Our findings that AT improved cognitive flexibility in BDNF Met/Met mice but increased neurogenesis in BDNF Val/Val mice, also suggest that AT does not have uniform effects to promote neuroplasticity or cognition based on genotype.

Female mice showed more mature DCX-expressing cells than male mice with AT

AT increased the proportion of dorsal type 3 DCX-expressing cells, an effect that was driven by the female mice, regardless of genotype, agreeing with previous literature where AT increases neurogenesis (van Praag et al., 1999), though sex differences were not examined in that study. As the dorsal hippocampus is involved in memory (Fanselow and Dong, 2010), and AT exercise provides cognitive benefits in a variety of species (Barha et al., 2017b; Erickson et al., 2011;

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Makizako et al., 2015; ten Brinke et al., 2015), increased neurogenesis in this region may be expected. Type 3 DCX-expressing cells are classified as late progenitor cells and are mature DCX-expressing cells (Plümpe et al., 2006). Intriguingly, the running-induced increase in neurogenesis was driven by female mice, consistent with previous results indicating that voluntary or treadmill running increased neurogenesis to a greater degree in younger adult female mice compared to males (Ma et al., 2012; Ransome and Hannan, 2013). One caveat to the past findings is that typically younger female rodents run more than male rodents (Ransome and Hannan, 2013), but in our sample of middle-aged C57B6 mice, there was no sex difference in the total amount of running, suggesting that despite similar levels of AT, a greater neurogenic response was seen in middle-aged female mice compared to middle-aged male mice. Our finding that neurogenesis was increased in AT female mice is consistent with our behaviour results wherein the females outperformed the males on both cognitive tasks. Future studies are needed to further investigate these sex differences found in response to AT in middle-age.

A greater proportion of type 3 DCX-expressing cells was also found to be present in the dorsal region of the hippocampus of BDNF Val/Val compared to the Met/Met mice. As mentioned above, type 3 DCX-expressing cells are mature or late progenitor cells (Plümpe et al., 2006), therefore BDNF Val/Val mice contain a higher proportion of mature DCX-expressing cells compared to Met/Met mice. This may be due to greater neural release of BDNF in BDNF Val/Val mice compared to the BDNF Met/Met mice. Intriguingly, DCX-expressing cells are more likely to be activated with cognitive flexibility in male rats (Webler et al., 2019), suggesting that this increase may have contributed to the better flexibility in BDNF Val/Val mice in the present study, albeit in females. Another intriguing finding is that Met/Met mice had more type 1 DCX-expressing neurons compared to Val/Val mice, with the reverse for Type 3 DCX-expressing cells. This suggests that the BDNF Met/Met mice have fewer new neurons surviving to maturity, or have slower maturation, as Met/Met individuals have impaired BDNF/TrKB signaling that strongly influences the survival and maturation of hippocampal neurons (Donovan et al., 2008). Given that one study has noted that maturation of DCX-expressing cells may be differentially involved in cognitive flexibility (Webler et al., 2019), and that in our studies female BDNF Val/Val mice had superior cognitive flexibility and greater proportion of mature DCX cells these may be linked. The contribution of different ages of new neurons to cognitive flexibility across sex and genotype may be an important route for future studies.

Future directions and Limitations

The BDNF Met/Met mice in our study did not run as much as the BDNF Val/Val mice and performed worse on both cognitive tasks. In the future, one strategy might be to start cognitive training earlier or extend it longer (to give more time to reach training criteria) and take measures to ensure more running in the BDNF Met/Met. This also might suggest that interventions in BDNF Met/Met individuals need to start earlier in one's lifespan. Previous studies found that after AT, BDNF levels increased more in females compared to males (Barha et al., 2017a, 2017b, 2017c). However, as we did not measure hippocampal BDNF levels, we cannot positively link our results of improved female cognition and neurogenesis to higher BDNF levels compared to males and future studies should be encouraged to examine BDNF levels.

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Although both males and females show increased cortisol levels in older age, this effect is three times more pronounced in females compared to males (Otte et al., 2005). Furthermore, increased cortisol levels in older age are linked to poorer cognition and smaller hippocampal and prefrontal cortical volumes (Stomby et al., 2016). Additionally, individuals with the BDNF Met/Met genotype show reduced cortisol release in response to stress, particularly in females (Fiocco et al., 2020; Jiang et al., 2017) compared to individuals with the BDNF Val/Val genotype. However in the present study we did not observe any significant differences in relative adrenal mass with AT. Thus, future studies could further examine this effect and additionally measure corticosterone level differences in response to AT and the BDNF Val66Met polymorphism.

Conclusions

Female mice with the wild type BDNF Val/Val alleles showed greater cognitive flexibility regardless of AT, compared to all other groups. AT increased neurogenesis and improved cognitive measures differentially depending on genotype, as AT improved visual discrimination and cognitive flexibility in the BDNF Met/Met mice, but increased neurogenesis in the ventral region of BDNF Val/Val mice. AT also increased the proportion of mature DCX expressing cells in the dorsal region of the hippocampus in female mice, regardless of genotype. Thus, AT may exert its effects on neuroplasticity and cognition differentially by genotype. This study also indicates that middle-aged female mice may benefit more from AT in terms of improved cognitive flexibility and neuroplasticity, an effect that can be influenced by the BDNF Val66Met polymorphism.

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