

Brain-Derived Neurotrophic Factor Val66Met Human Polymorphism Impairs the Beneficial Exercise-Induced Neurobiological Changes in Mice

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Several studies have shown that exercise improves cognitive functions and emotional behaviors. Positive effects of exercise have been associated with enhanced brain plasticity, adult hippocampal neurogenesis, and increased levels of brain-derived neurotrophic factor (BDNF). However, a substantial variability of individual response to exercise has been described, which may be accounted for by individual genetic variants. Here, we have assessed whether and how the common human BDNF Val66Met polymorphism influences the neurobiological effects modulated by exercise in BDNF Val66Met knock-in male mice. Wild-type (BDNF^{Val/Val}) and homozygous BDNF Val66Met (BDNF^{Met/Met}) male mice were housed in cages equipped with or without running wheels for 4 weeks. Changes in behavioral phenotype, hippocampal adult neurogenesis, and gene expression were evaluated in exercised and sedentary control mice. We found that exercise reduced the latency to feed in the novelty suppressed feeding and the immobility time in the forced swimming test in BDNF^{Val/Val} but not in BDNF^{Met/Met} mice. Hippocampal neurogenesis was reduced in BDNF^{Met/Met} mice compared with BDNF^{Val/Val} mice. BDNF^{Met/Met} mice had lower basal BDNF protein levels in the hippocampus, which was not recovered following exercise. Moreover, exercise-induced expression of total BDNF, BDNF splice variants 1, 2, 4, 6 and fibronectin type III domain-containing protein 5 (FNDC5) mRNA levels were absent or reduced in the dentate gyrus of BDNF^{Met/Met} mice. Exercise failed to enhance PGC-1 α and FNDC5 mRNA levels in the BDNF^{Met/Met} muscle. Overall these results indicate that, in adult male mice, the BDNF Val66Met polymorphism impairs the beneficial behavioral and neuroplasticity effects induced by physical exercise.

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INTRODUCTION

Several studies have shown that regular physical exercise is beneficial for physical and mental health at all ages, including the elderly. Exercise improves mental health by reducing anxiety, depression, negative mood and by improving self-esteem and cognitive functions (Deslandes *et al*, 2009; Duman *et al*, 2008; Schuch *et al*, 2016; Voss *et al*, 2013). Moreover, some lines of evidence strongly suggest that individuals who are physically active during adulthood tend to have better cognitive performances later in life (Mattson, 2015). Exercise-induced improvement in brain function has been associated with increased adult hippocampal neurogenesis, synaptogenesis, and enhanced activity-dependent synaptic plasticity (Christie *et al*, 2008; Farmer *et al*, 2004;

Stranahan *et al*, 2007; van Praag *et al*, 1999; Vivar *et al*, 2013). One of the major mechanisms proposed whereby exercise affects brain plasticity and function is the increase of brain-derived neurotrophic factor (BDNF), a neurotrophin implicated in anxiety, depression, learning, and memory disabilities (Hopkins *et al*, 2011; Ieraci *et al*, 2015; Intlekofer *et al*, 2013; Phillips *et al*, 2014; Vaynman *et al*, 2004; Voss *et al*, 2013). However, the mechanism underlying the exercise-induced BDNF expression in the hippocampus is still poorly understood.

Recently, it has been reported that exercise enhances the expression of fibronectin type III domain-containing protein 5 (FNDC5) in the muscle, in a peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α)-dependent manner (Wrann *et al*, 2013). FNDC5 is a transmembrane protein released in the blood stream after proteolytic cleavage (Bostrom *et al*, 2012). Importantly, overexpression of FNDC5 into liver via adenovirus infection significantly enhances BDNF expression in the hippocampus (Wrann *et al*, 2013). Interestingly, BDNF in turn is able to reduce the expression of FNDC5 suggesting a homeostatic feedback

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loop (Wrann *et al*, 2013). However, whether BDNF genetic variants may alter this pathway is still unknown.

Despite the well-documented positive effects of exercise in humans, substantial variability in the individual response has been reported. This variability may be accounted for by genetic factors that interfere with the benefit of exercise on cognitive functions and mood.

A number of common single-nucleotide polymorphisms (SNPs) have been identified in the human BDNF gene. The rs6265 SNP leads to a Valine to Methionine substitution at position 66 (Val66Met) in the pro-domain of BDNF, which impairs the activity-dependent release of BDNF. Val66Met SNP is associated with increased susceptibility to cognitive deficits and neuropsychiatric disorders (Egan *et al*, 2003; Hariri *et al*, 2003; Lim *et al*, 2014; Notaras *et al*, 2015; Verhagen *et al*, 2010). Despite the strong evidence that exercise increases BDNF expression, only a few human studies have been conducted to address the potential role of Val66Met SNP in moderating exercise response, showing inconclusive results, probably due to confounding factors (Brown *et al*, 2014; Gujral *et al*, 2014; Hopkins *et al*, 2012; Lemos *et al*, 2016; Mata *et al*, 2010; Nascimento *et al*, 2015). Animal models may offer a useful tool to better address such question in a well-controlled environment and genetic setting.

A knock-in mouse carrying the human BDNF Val66Met SNP has been generated (Chen *et al*, 2006). BDNF^{Met/Met} homozygous mice recapitulate the phenotypic hallmarks of humans carrying the BDNF Met allele. Indeed, BDNF^{Met/Met} mice display reduced hippocampal volume, reduced dendritic arborization in the dentate gyrus (DG), reduced BDNF levels, and NMDA receptor-dependent synaptic plasticity in the hippocampus (Chen *et al*, 2006; Ninan *et al*, 2010). Furthermore, we have recently found decreased expression and dendritic trafficking for selected BDNF transcripts in BDNF^{Met/Met} hippocampus (Mallei *et al*, 2015). Interestingly, BDNF^{Met/Met} mice are unresponsive to treatment with some antidepressant drugs, in particular it has been reported that chronic fluoxetine treatment and acute ketamine administration do not promote anxiolytic and antidepressant-like effects, respectively, in BDNF^{Met/Met} mice (Chen *et al*, 2006; Liu *et al*, 2012). Thus, this mouse may provide an ideal model to explore whether and by what mechanism(s) the Val66Met SNP moderates the effects of environmental and pharmacological treatments.

In this study, we assessed the effect of exercise on anxious- and depressive-like behaviors in BDNF^{Val/Val} wild-type and BDNF^{Met/Met} mutant adult male mice. We explored the possible mechanisms underlying the exercise-induced behavioral changes by measuring the levels of neurogenesis and BDNF expression in the hippocampus of BDNF^{Val/Val} and BDNF^{Met/Met} mice exposed to exercise. Finally, we investigated whether exercise-induced PGC-1 α and FNDC5 expressions were altered in BDNF^{Met/Met} mice.

MATERIALS AND METHODS

Animals

Adult male 3- to 4-month-old BDNF^{Met/Met} and BDNF^{Val/Val} mice were used for all studies (Chen *et al*, 2006). Mice were maintained in a standard 12 h light/dark cycle, temperature

controlled room (21 \pm 1 $^{\circ}$ C), with free access to food and water.

All experimental procedures involving animals were performed in accordance with the European Community Council Directive 86/609/EEC, and were approved by Italian legislation on animal experimentation (Decreto Legislativo 116/1992).

Behavioral Tests

Behavioral tests were conducted between 0900 and 1600 h. See Supplementary Materials and Methods for more details.

BrdU Treatment and Immunohistochemistry

Detailed information can be found in Supplementary Materials and Methods.

BDNF ELISA

BDNF levels were measured by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BDNF Emax Immunoassay System, Promega).

Western Blot

Detailed western blot procedures can be found in Supplementary Materials and Methods.

RNA Isolation and Quantitative Real-Time PCR

Detailed information is reported in Supplementary Materials and Methods.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Data are presented as mean \pm standard error of the mean (SEM). Statistical analyses were made using unpaired *t*-test or two-way analysis of variance (ANOVA), and Fischer's LSD *post hoc* test was used for multiple comparison analysis.

RESULTS

Physical Exercise-Induced Reduction of Body Weight Gain

Adult male BDNF^{Val/Val} and BDNF^{Met/Met} mice underwent 4 weeks of free voluntary exercise. BDNF^{Val/Val} and BDNF^{Met/Met} displayed similar daily running distance ($P=0.3001$; Figure 1a). However, we found a main body weight gain effect for exercise and genotype (exercise: $F_{(1,186)}=9.204$; $P=0.0028$; genotype: $F_{(1,186)}=3.962$; $P=0.048$; interaction: $F_{(1,186)}=0.647$; $P=0.422$). Exercise significantly reduced the body weight gain in both genotypes although this effect was apparently less evident in BDNF^{Met/Met} mutant mice (Figure 1b). The exercise-induced reduction of body weight was not due to differences in starting weight (Supplementary Figure S1). Interestingly, the daily food consumption was affected by both the exercise and genotype factors (exercise: $F_{(1,56)}=11.13$; $P=0.0015$; genotype:

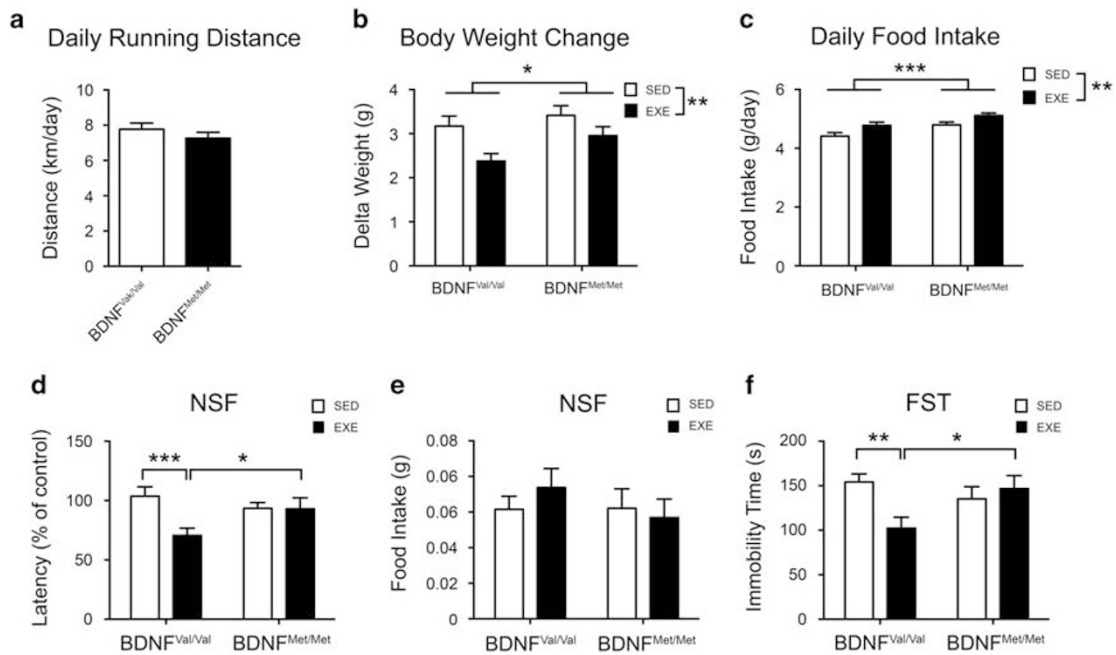


Figure 1 BDNF Val66Met polymorphism impairs physical exercise-induced beneficial effect of physical exercise on anxious- and depressive-like phenotype in mice. (a) Total distance run daily by mice. Daily running distance was calculated by dividing for 2 the distance measured by a single electronic counter (two electronic counter per cage; four mice per cage). Data are expressed as means \pm SEM ($n=35-39$ number of electronic counters analyzed per group). Unpaired *t*-test. (b) Total body weight gain over 4 weeks of physical exercise treatment ($n=45-52$ mice per group). (c) Daily food consumption. Amount of daily food intake per mice was calculated by dividing for 4 the total amount of food consumed per cage (four mice per cage) ($n=14-16$ number of cage analyzed per group). (d, e) Novelty suppressed feeding (NSF) test. (d) Exercise reduces the latency to feed in NSF test only in BDNF^{Val/Val} mice. (e) There is no difference in the amount of food consumed in the home cages among all the groups. (f) Forced swimming test (FST). Exercise decreases the immobility time only in BDNF^{Val/Val} mice. Data are expressed as means \pm SEM ($n=18-24$ mice per group). Two-way ANOVA followed by Fischer's LSD *post hoc* analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. EXE, exercise; SED, sedentary.

$F_{(1,56)} = 12.52$; $P = 0.0008$; interaction: $F_{(1,56)} = 0.068$; $P = 0.7949$). Running mice consumed more food than the corresponding sedentary group and BDNF^{Met/Met} ate more food than BDNF^{Val/Val} mice (Figure 1c).

Physical Exercise-Induced Beneficial Behavioral Changes Are Impaired in the BDNF^{Met/Met} Mutant Mice

First, we assessed whether the BDNF Val66Met SNP moderates the exercise-induced decrease of anxiety- and depression-like behaviors in mice, using the novelty suppressed feeding (NSF) and the forced swimming test (FST), respectively. The results from the NSF test showed a significant main effect for exercise and for the interaction between exercise and genotype (exercise: $F_{(1,80)} = 5.207$; $P = 0.025$; genotype: $F_{(1,80)} = 0.704$; $P = 0.404$; interaction: $F_{(1,80)} = 4.923$; $P = 0.0293$) (Figure 1d). Fisher *post hoc* analysis revealed that exercise reduces the latency to feed only in the BDNF^{Val/Val} mice (Figure 1d). Moreover BDNF^{Val/Val} runner mice exhibited a significant reduction of latency time compared with running BDNF^{Met/Met} mice (Figure 1d). As a control, there were no significant changes in food consumption measured in the home cage for a period of 5 min immediately after the NSF test between genotypes (sedentary and running) (exercise: $F_{(1,80)} = 0.124$; $P = 0.725$; genotype: $F_{(1,80)} = 0.678$; $P = 0.413$; interaction: $F_{(1,80)} = 0.788$; $P = 0.377$) (Figure 1e).

In the FST, there was a main effect for the interaction exercise \times genotype (exercise: $F_{(1,80)} = 2.701$; $P = 0.104$;

genotype: $F_{(1,80)} = 1.074$; $P = 0.303$; interaction: $F_{(1,80)} = 6.591$; $P = 0.0121$). The immobility time was reduced in the BDNF^{Val/Val} exercise mice group compared with the sedentary BDNF^{Val/Val} ($P = 0.0019$) and the BDNF^{Met/Met} exercise ($P = 0.0127$) mice groups (Figure 1f). Altogether, the data from NSF and FST tests suggest that the BDNF Val66Met polymorphism impairs the beneficial behavioral effects induced by exercise.

Neurogenesis in the DG Is Diminished in the BDNF^{Met/Met} Mutant Mice

Increase of neurogenesis in hippocampal DG is recognized as a major effect of exercise in mice. Therefore, we assessed whether the exercise-induced behavioral changes observed in the two genotypes were related to variations of neurogenesis in the DG. To measure the survival of newborn neurons, mice were injected with BrdU once-daily for 7 days and killed 3 weeks after the last injection (Figure 2a). We found that exercise increased the number of surviving newborn neurons in the DG of both BDNF^{Val/Val} and BDNF^{Met/Met} mice (exercise: $F_{(1,32)} = 84.54$; $P < 0.0001$). We also found a main effect for the genotype (genotype: $F_{(1,32)} = 5.23$; $P = 0.029$; interaction: $F_{(1,32)} = 1.655$; $P = 0.208$), indicating that the overall neurogenesis is lower in the BDNF^{Met/Met} mice and this difference was bigger in exercised mice ($P = 0.0167$) (Figure 2b-d). In order to measure an endogenous marker of neurogenesis, we quantified the total number of neuroblasts expressing doublecortin (DCX) in the

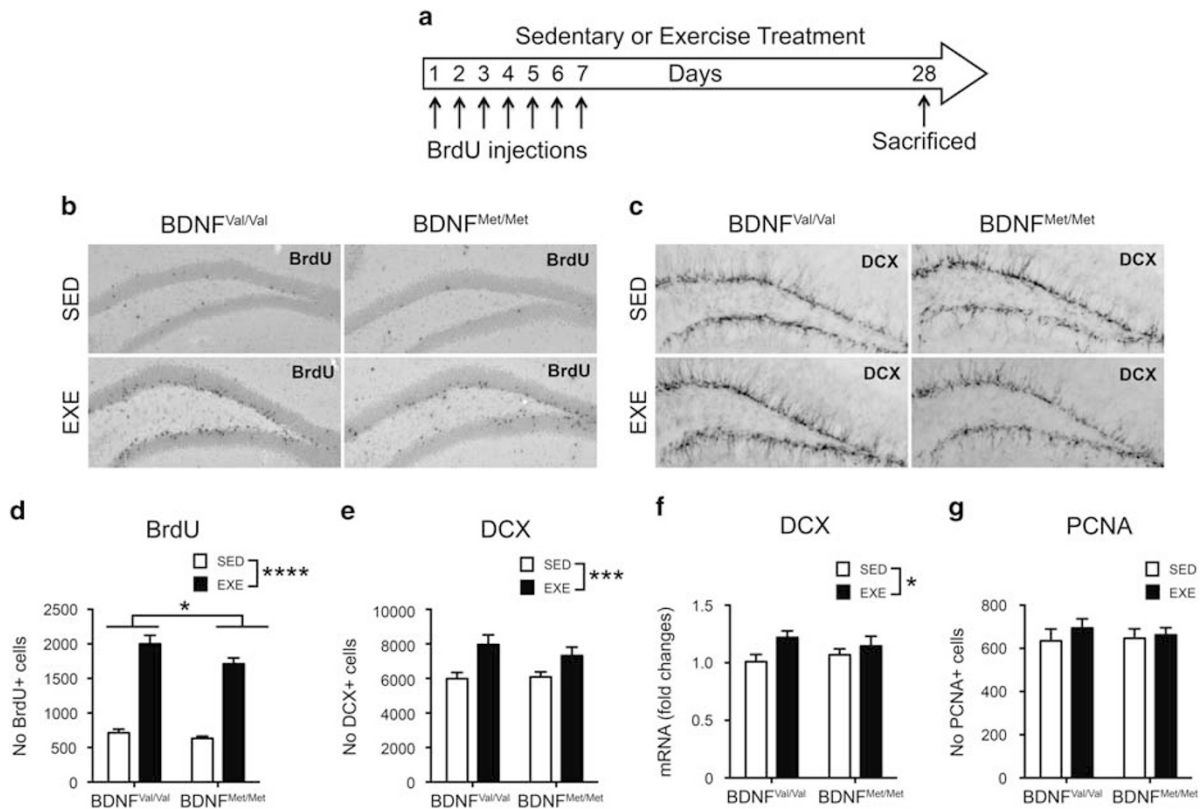


Figure 2 BDNF Val66Met polymorphism reduces hippocampal neurogenesis in mice. (a) Schematic representation of the experimental procedure. Mice were injected with BrdU for 7 days and killed 21 days after the last injection. (b, c) Images of BrdU and DCX immunostaining in the DG. (d) Total amount of BrdU-positive cells in the DG ($n = 9$ mice per group). (e, f) Exercise significantly increases the total amount of DCX-positive cells ($n = 9$ mice per group) and DCX mRNA levels ($n = 6-7$ mice per group) in the DG. (g) There is no significant difference in the number of PCNA-positive cells among all the groups ($n = 6$ mice per group). Data are expressed as means \pm SEM. Two-way ANOVA. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$. EXE, exercise; SED, sedentary.

DG. A two-way ANOVA revealed only a significant overall effect for exercise (exercise: $F_{(1,32)} = 13.48$; $P = 0.0009$; genotype: $F_{(1,32)} = 0.377$; $P = 0.543$; interaction: $F_{(1,32)} = 0.709$; $P = 0.406$) (Figure 3c-e). A similar result was obtained by measuring the DCX mRNA levels in the DG (exercise: $F_{(1,22)} = 4.452$; $P = 0.0465$; genotype: $F_{(1,22)} = 0.009$; $P = 0.924$; interaction: $F_{(1,22)} = 1.002$; $P = 0.328$) (Figure 2f). To assess whether the number of proliferating cells was also differentially affected we performed an immunohistochemistry staining for the proliferating cell nuclear antigen (PCNA), an endogenous marker of ongoing cell proliferation. We did not find significant changes in the number of PCNA-positive cells measured throughout the DG (exercise: $F_{(1,20)} = 0.693$; $P = 0.415$; genotype: $F_{(1,20)} = 0.052$; $P = 0.822$; interaction: $F_{(1,20)} = 0.24$; $P = 0.629$) (Figure 2g). Overall, the combined data suggest that exercise mainly favors survival of new neuron after 4 weeks, but not their proliferation, and this effect is bigger in BDNF^{Val/Val} mice.

Physical Exercise-Induced BDNF Expression in the Hippocampus Is Impaired in the BDNF^{Met/Met} Mutant Mice

It has been proposed that exercise-induced behavioral changes are mediated by elevation of BDNF and its receptor TrkB in the hippocampus (Liu et al, 2008; Vaynman et al, 2004). Therefore, we examined whether the BDNF Val66Met

polymorphism affects the exercise-induced augmentation of hippocampal BDNF and TrkB levels in mice. Analysis of total BDNF mRNA levels revealed that the overall BDNF levels in whole hippocampus were increased by exercise (exercise: $F_{(1,37)} = 6.633$; $P = 0.0141$). However, BDNF mRNA levels were lower in BDNF^{Met/Met}, both in sedentary and in exercised mice (genotype: $F_{(1,37)} = 7.988$; $P = 0.0076$; interaction: $F_{(1,37)} = 1.496$; $P = 0.2291$) (Figure 3a). Next, we evaluated the effect of exercise on the hippocampal BDNF protein levels in the BDNF^{Val/Val} and BDNF^{Met/Met} mice, using the ELISA method. As previously reported (Bath et al, 2012), we found that BDNF protein levels in the BDNF^{Met/Met} mice were significantly lower compared with BDNF^{Val/Val} mice (genotype: $F_{(1,44)} = 52.26$; $P < 0.0001$). Interestingly, although exercise significantly increased the overall BDNF protein levels in the whole hippocampus of both genotypes (exercise: $F_{(1,44)} = 8.217$; $P = 0.0063$; interaction: $F_{(1,44)} = 1.775$; $P = 0.189$), this increase was limited in the BDNF^{Met/Met} mice, in which BDNF level remained significantly lower than in BDNF^{Val/Val} sedentary mice (BDNF^{Val/Val} sedentary 114.6 ± 6.398 vs BDNF^{Met/Met} exercise 73.67 ± 10.04 ; $P < 0.01$) (Figure 3b). Finally, we measured the hippocampal TrkB protein levels by western blot. We found that TrkB protein levels were overall higher in BDNF^{Met/Met} mice and modulated by exercise (exercise: $F_{(1,44)} = 4.623$; $P = 0.0371$; genotype: $F_{(1,44)} = 5.463$; $P = 0.0024$; interaction: $F_{(1,44)} = 0.615$; $P = 0.437$) (Figure 3c and d).

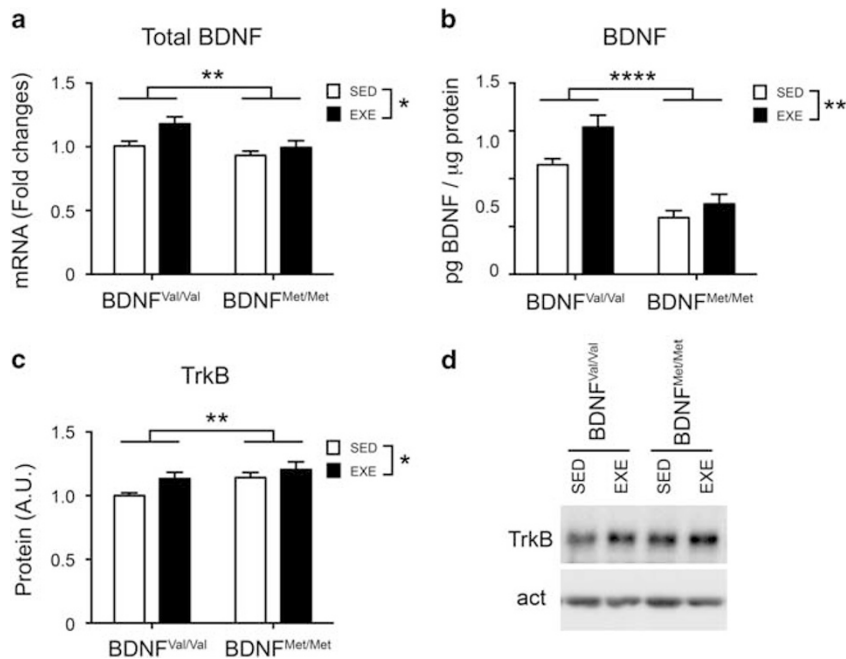


Figure 3 BDNF Val66Met polymorphism impairs BDNF and TrkB expression in mice hippocampus. (a, b) Total hippocampal BDNF mRNA ($n = 10$ – 11 mice per group) and protein levels ($n = 12$ mice per group) are increased after 28 days of physical exercise and reduced in BDNF^{Met/Met} mice. (c) TrkB protein levels in the hippocampus (HPC) are upregulated by physical exercise. (d) Representative western blot pictures from TrkB and β -actin ($n = 12$ mice per group). Data are expressed as means \pm SEM. Two-way ANOVA. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. EXE, exercise; SED, sedentary.

The hippocampus is a non-homogenous region expressing different cell types and markers in the DG, CA1, CA2, and CA3 regions. Moreover, the BDNF gene has a complex structure and its transcription is regulated by several non-coding 5' exons alternatively spliced to one 3' common exon that encodes for the pro-BDNF protein. The complex BDNF gene structure allows the distinct BDNF transcripts to be differentially modulated during development and adulthood, in different brain regions, cell types, and subcellular compartments. (Aid *et al*, 2007; Lyons and West, 2011). In particular, it has been demonstrated that BDNF transcripts are specifically segregated within the soma (BDNFs 1 and 4) or the dendrites (BDNFs 2 and 6), therefore working as a spatial code (Baj *et al*, 2012, 2013; Mallei *et al*, 2015).

Therefore, we sought to explore whether (1) the exercise-induced expression of BDNF was specific for a particular hippocampal sub-region and (2) what are the BDNF splice variants responsible for the variation of total BDNF mRNA expression after exercise. To this aim, we dissected the DG from the remaining part of the hippocampus (collectively indicated as CA). The accuracy of our dissection was evaluated by measuring the mRNA levels of tryptophan 2,3-dioxygenase (TDO2) and TYRO3 protein tyrosine kinase 3 (Tyro3), two markers specifically expressed in the DG and in the CA regions, respectively (Hagihara *et al*, 2009), which revealed a clear separation of the two areas (Supplementary Figure S2). First, we assessed the total BDNF mRNA levels, finding that BDNF mRNA expression was higher in the DG compared with the remaining CA regions and that BDNF expression was modulated by exercise only in the DG area (area: $F_{(1,44)} = 106.8$; $P < 0.0001$; groups: $F_{(3,44)} = 14.38$; $P < 0.0001$; interaction: $F_{(1,44)} = 5.119$; $P = 0.004$). Specifically, exercise increased BDNF mRNA levels only in the DG

of BDNF^{Val/Val} mice ($P < 0.0001$) (Figure 4a). Next, we evaluated the levels of BDNF-1; BDNF-2, BDNF-4, and BDNF-6, which are the major transcript splice variants expressed in the hippocampus. We found that BDNF-1 and BDNF-2 were roughly equally expressed in the DG and CA regions and that their expression was increased by exercise in the two regions in both BDNF^{Val/Val} and BDNF^{Met/Met} mice, although at lower extent in BDNF^{Met/Met} mice (BDNF-1: area: $F_{(1,44)} = 0.113$; $P = 0.738$; groups: $F_{(3,44)} = 14.59$; $P < 0.0001$; interaction: $F_{(1,44)} = 4.604$; $P = 0.007$; BDNF-2: area: $F_{(1,44)} < 0.0001$; $P = 0.9948$; groups: $F_{(3,44)} = 24.10$; $P < 0.0001$; interaction: $F_{(1,44)} = 4.164$; $P = 0.0111$) (Figure 5b and c). For BDNF-4, we found only a main effect for the groups (area: $F_{(1,44)} = 2.982$; $P = 0.0912$; groups: $F_{(3,44)} = 4.569$; $P = 0.0072$; interaction: $F_{(1,44)} = 0.8618$; $P = 0.468$) and a further comparison among the groups revealed that BDNF-4 mRNA levels were significantly different in the BDNF^{Val/Val} exercised mice compared with BDNF^{Val/Val} sedentary and BDNF^{Met/Met} exercised mice in the DG (Figure 4d). BDNF-6 mRNA levels were differentially expressed in the different areas and groups (area: $F_{(1,44)} = 240$; $P < 0.0001$; groups: $F_{(3,44)} = 12.58$; $P < 0.0001$; interaction: $F_{(1,44)} = 4.466$; $P = 0.008$). In particular, we found that BDNF-6 was markedly higher in the DG compared with the CA regions ($P < 0.0001$); the BDNF-6 levels were lower in the DG of BDNF^{Met/Met} than in BDNF^{Val/Val} ($P = 0.0146$); and exercise significantly enhanced BDNF-6 expression exclusively in the DG of BDNF^{Val/Val} mice ($P = 0.0004$) (Figure 4e). Finally, we measured the mRNA levels of TrkB, and found a higher TrkB expression in the CA regions with respect to DG (area: $F_{(1,44)} = 43.36$; $P < 0.0001$; groups: $F_{(3,44)} = 2.699$; $P = 0.0572$; interaction: $F_{(1,44)} = 0.411$; $P = 0.746$) (Figure 4f).

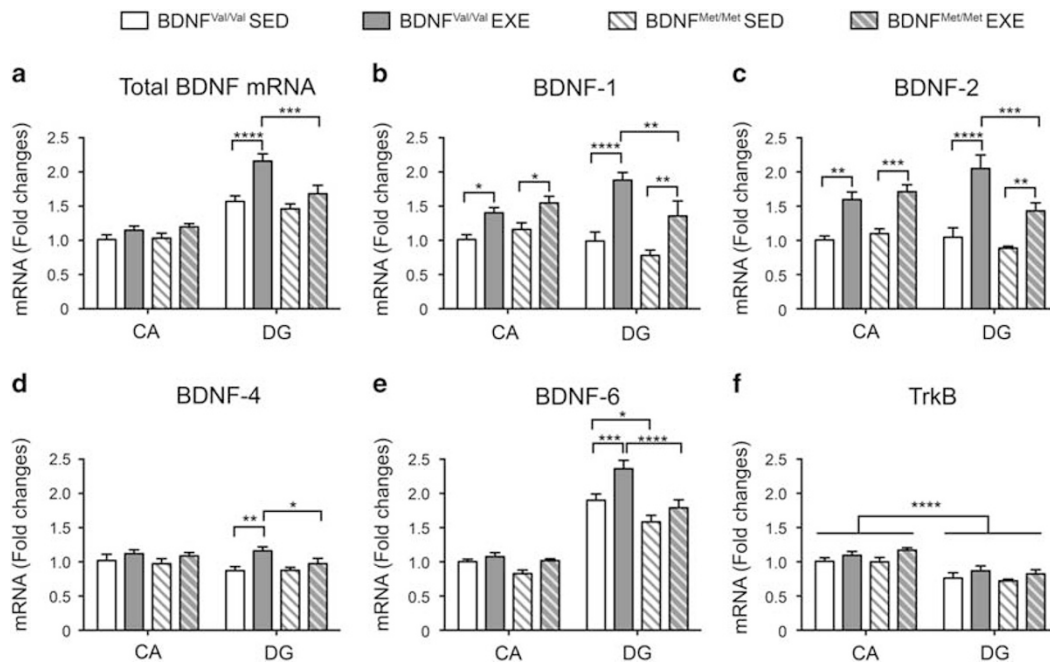


Figure 4 Effect of exercise on BDNF transcripts and TrkB gene expression in the DG of BDNF^{Val/Val} and BDNF^{Met/Met} mice. (a, d, e) Physical exercise significantly increases the mRNA levels of total BDNF, BDNF-4 and -6 only in BDNF^{Val/Val} mice in the DG. (b, c) BDNF Val66Met polymorphism reduces the physical exercise-induced upregulation of BDNF transcripts 1 and 2 in the DG. (f) There are no significant differences in the TrkB mRNA levels among all different groups. Data are expressed as means \pm SEM ($n=6-7$ mice per group). Two-way ANOVA followed by Fischer's LSD *post hoc* analysis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. CA, cornu ammonis; DG, dentate gyrus; EXE, exercise; SED, sedentary.

Physical Exercise-Induced PGC-1 α and FNDC5 Expressions Are Impaired in the BDNF^{Met/Met} Mutant Mice

Recently, it has been reported that exercise stimulates the production and release of factors in skeletal muscle that are able to affect the hippocampal BDNF expression (Wrann *et al*, 2013). In particular, exercise increases the expression of the co-activator transcriptional factor PGC-1 α , which in turn controls the expression of FNDC5 in the skeletal muscle. FNDC5 is then cleaved, and the product released in the blood stream is able to stimulate the hippocampal BDNF expression with an as yet unidentified mechanism (Bostrom *et al*, 2012; Wrann *et al*, 2013). Therefore, we decided to explore whether the BDNF Val66Met polymorphism affects also the basal and exercise-induced mRNA expression of PGC-1 α and FNDC5 in the gastrocnemius muscle. In agreement with previous reports, the mRNA levels of PGC-1 α and FNDC5 were enhanced in BDNF^{Val/Val} wild-type runner mice compared with BDNF^{Val/Val} sedentary mice (PGC-1 α : exercise: $F_{(1,41)} = 0.755$; $P = 0.39$; genotype: $F_{(1,41)} = 0.139$; $P = 0.71$; interaction: $F_{(1,41)} = 8.285$; $P = 0.0063$; *post hoc* $P = 0.0129$; FNDC5: exercise: $F_{(1,41)} = 1.55$; $P = 0.22$; genotype: $F_{(1,41)} = 0.1178$; $P = 0.676$; interaction: $F_{(1,41)} = 6.622$; $P = 0.014$; *post hoc* $P = 0.0114$) (Figure 5a and b). Surprisingly, the basal level of PGC-1 α and FNDC5 was higher in the muscle of BDNF^{Met/Met} mice than in BDNF^{Val/Val} mice (PGC-1 α : $P = 0.0216$; FNDC5: $P = 0.033$), and was not further modulated by exercise (PGC-1 α : $P = 0.16$; FNDC5: $P = 0.335$) (Figure 5a and b).

Next, we measured the mRNA levels of these genes also in the different regions of the hippocampus. We found that

mRNA levels of FNDC5 were differentially expressed in the two regions and in the groups (FNDC5: area: $F_{(1,44)} = 34.71$; $P < 0.0001$; groups: $F_{(3,44)} = 2.909$; $P = 0.045$; interaction: $F_{(3,44)} = 0.481$; $P = 0.6972$). A further comparison among the groups revealed a significant difference only between the sedentary and exercise BDNF^{Val/Val} mice in the DG ($P = 0.0262$) (Figure 5d). On the contrary, the levels of PGC-1 α were not significantly modified in any of the different conditions (PGC-1 α : area: $F_{(1,44)} = 39.17$; $P < 0.0001$; groups: $F_{(3,44)} = 0.788$; $P = 0.507$; interaction: $F_{(3,44)} = 0.127$; $P = 0.944$) (Figure 5c).

Altogether, these results indicate that FNDC5 mRNA expression levels were differentially altered in both the gastrocnemius muscle and hippocampus in basal condition and following exercise exposure in the BDNF^{Met/Met} respect to the BDNF^{Val/Val} mice, while PGC-1 α levels were modulated only in the muscle.

DISCUSSION

Physical-Exercise Induced Anxiolytic- and Antidepressant-Like Response Is Impaired in BDNF^{Met/Met} Mutant Mice

In the present study, we reported for the first time that exercise-induced anxiolytic- and antidepressant-like response is impaired in adult male BDNF^{Met/Met} mutant mice. These deficiencies were associated with reduced neurogenesis and BDNF levels in the BDNF^{Met/Met} hippocampus. Remarkably, we have found that exercise-induced FNDC5 expression in the muscle and DG is also impaired in BDNF^{Met/Met} mutant mice. These differences in behavior and

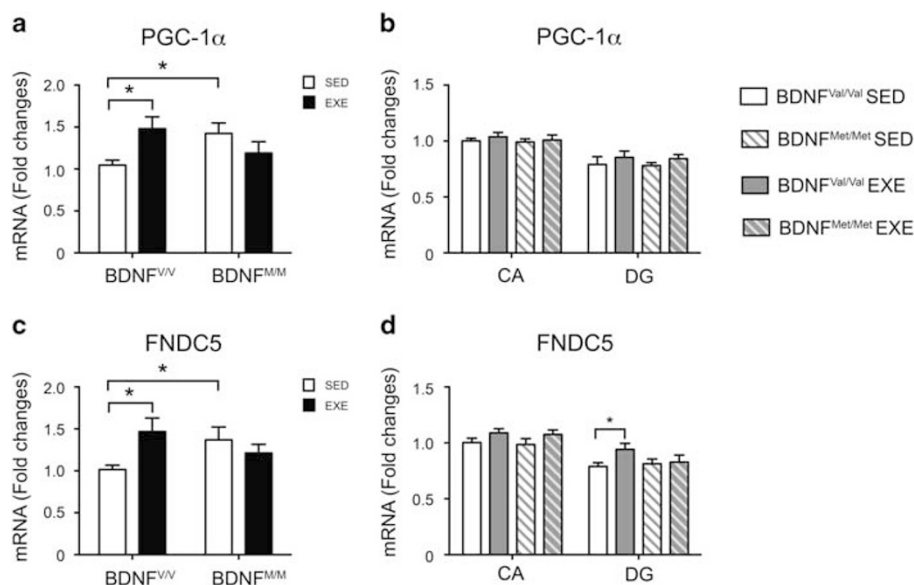


Figure 5 Effect of physical exercise on PGC-1 α and FNDC5 gene expression in BDNF^{Val/Val} and BDNF^{Met/Met} mice. (a, c) Physical exercise significantly increases the mRNA levels of PGC-1 α and FNDC5 only in the gastrocnemius muscle of BDNF^{Val/Val} mice ($n=9-13$ mice per group). (b) There are no significant differences in the PGC-1 α mRNA levels among all different groups. (d) Physical exercise significantly increases mRNA levels of FNDC5 in the DG only in BDNF^{Val/Val} mice ($n=6-7$ mice per group). Data are expressed as means \pm SEM. Two-way ANOVA followed by Fischer's LSD *post hoc* analysis. * $P < 0.05$. CA, cornu ammonis; DG, dentate gyrus; EXE, exercise; SED, sedentary.

gene expression were not due to different extent of exercise, as the daily average distance run by BDNF^{Val/Val} wild-type and BDNF^{Met/Met} mutant mice was similar.

While substantial evidence indicate that exercise affects the hippocampal function by increasing BDNF levels, few reports have thoroughly investigated the involvement of the BDNF pathway in the outcome of exercise (Duman *et al*, 2008; Li *et al*, 2008). Here, we reported that exercise fails to induce anxiolytic- and antidepressant-like effects in the Val66Met human polymorphism knock-in mutant mice. These results are consistent with previously published data showing that exercise was not effective in decreasing FST immobility in the heterozygous knock-out BDNF mice (Duman *et al*, 2008). Moreover, results from the present study complement and extend previous data showing that anxiolytic- and antidepressant-like effects of chronic fluoxetine and acute ketamine treatments, respectively, were absent in BDNF^{Met/Met} mutant mice (Chen *et al*, 2006; Liu *et al*, 2012), suggesting a more general ineffectiveness of pro-adaptive anxiolytic and antidepressant treatments in BDNF^{Met/Met} mice. The therapeutic implication of the failure of BDNF^{Met/Met} mutant mice to show the behavioral changes induced by exercise is significant. Exercise is an affordable treatment that produces extensive favorable effects, both in the central nervous system and in the whole body. However, the behavioral benefits vary in different subjects, suggesting that different factors may interact to produce distinct consequences. Indeed, some studies have addressed whether the BDNF Val66Met polymorphism moderates the response to exercise in human, producing mixed and inconclusive results, probably depending on the ethnic group, gender, age, and type and frequency of the exercise considered (Brown *et al*, 2014; Gujral *et al*, 2014; Hopkins *et al*, 2012; Lemos *et al*, 2016; Mata *et al*, 2010; Nascimento *et al*, 2015).

Although further studies will be necessary to fully elucidate the role of BDNF Val66Met polymorphism in exercise-induced changes in humans, our results indicate that the presence of BDNF Val66Met polymorphism in homozygosis impairs the anxiolytic and antidepressant beneficial effects of exercise in adult male mice.

The present results showing that exercise failed to promote behavioral changes and to increase hippocampal BDNF levels in the BDNF^{Met/Met} mice are in line with the neurotrophic hypothesis of depression that postulates a central role of the BDNF pathway in the therapeutic efficacy of antidepressant treatments. Indeed, almost all the pharmacological and environmental antidepressant treatments used to date increase BDNF expression, and the genetic ablation of BDNF in animal models prevents the response to antidepressant treatments (Lindholm and Castren, 2014; Monteggia *et al*, 2004; Monteggia *et al*, 2007; Musazzi *et al*, 2009). Our observations are also in line with recent studies showing that exercise increases peripheral levels of BDNF only in wild type, but not in humans carrying the Val66Met SNP (Lemos *et al*, 2016; Nascimento *et al*, 2015), suggesting an additional possible common feature between mice and humans carrying the Val66Met SNP.

Interestingly, we found that TrkB protein levels were overall augmented in the BDNF^{Met/Met} hippocampus of sedentary mice. Increased levels of TrkB have been previously reported also in the hippocampus of heterozygous BDNF^{+/-} and may be due to a compensatory effect aimed at preventing hippocampal dysfunction (Carreton *et al*, 2012). Moreover, it has been shown that prolonged BDNF exposure downregulates the expression of TrkB levels (Chen *et al*, 2005; Sommerfeld *et al*, 2000); therefore, the upregulation of TrkB in BDNF^{Met/Met} mice may be a direct consequence of diminished BDNF protein levels. However, further studies

will be necessary to elucidate whether the TrkB protein differences observed in the whole hippocampus are equally distributed among the different sub-regions or whether they are more pronounced in the DG respect to the CA regions.

A Role for FNDC5 in the Lack of Physical Exercise-Induced Stimulation of BDNF Expression in BDNF^{Met/Met} Mutant Mice

Recently, it has been reported that exercise increases FNDC5 levels in both the quadriceps muscle and hippocampus. Interestingly, overexpression of FNDC5 in the periphery, by adenoviral vector infection, has been shown to be sufficient to induce upregulation of BDNF in the hippocampus, suggesting that the signal carried by a secreted circulating form of FNDC5 is able to cross the blood–brain barrier and induces hippocampal gene expression changes (Wrann *et al*, 2013). In line with these results, we found upregulation of FNDC5 only in the BDNF^{Val/Val} gastrocnemius and DG after 4 weeks of exercise. Remarkably, in BDNF^{Met/Met} mice exercise failed to enhance the FNDC5 mRNA levels in the gastrocnemius and DG, and this was paralleled by a reduced increase of BDNF levels in the DG after exercise. Further studies will be necessary to reveal the specific contribution of local vs systemic FNDC5 to exercise-induced hippocampal BDNF expression, by using selective deletion of FNDC5 in different tissues. However, the present results suggest that the compromised upregulation of hippocampal BDNF levels is linked to the failed increase of FNDC5 in BDNF^{Met/Met} mice.

A Role for Neurogenesis and BDNF Expression in the Beneficial Effect of Physical Exercise

Hippocampal neurogenesis has been proposed as a major mechanism in the therapeutic efficacy of chronic antidepressants (Santarelli *et al*, 2003). However, some recent studies have suggested both neurogenesis-dependent and -independent mechanisms underlying the anxiolytic and antidepressant action, as some studies showed that increased neurogenesis induced by specific treatments is not critical for behavioral changes (Hill *et al*, 2015; Jedynak *et al*, 2014; Meshi *et al*, 2006; Sahay *et al*, 2011). Here, we reported that exercise significantly increased the total number of newborn neurons in both BDNF^{Val/Val} and BDNF^{Met/Met} mice, and that BDNF^{Met/Met} mice had overall fewer BrdU-positive cells. Our data could suggest that exercise-induced increases of neurogenesis may not be critical for the behavioral changes of exercise in BDNF^{Met/Met} mice, which is consistent with the neurogenesis-independent mechanism hypothesis reported in some behavioral paradigms.

Interestingly, similar data were also reported after chronic fluoxetine treatments in BDNF^{Met/Met} mice, in which there was an increase in newborn neuronal survival after antidepressant administration without behavioral benefit (Bath *et al*, 2012; Chen *et al*, 2006).

We observed that the exercise-increased total BDNF mRNA expression in the BDNF^{Val/Val} hippocampus was mainly due to the upregulation of BDNF in the DG. This increase was attributable to the augmented levels of BDNF transcripts 1-2-4-6. Interestingly, we found that in sedentary mice BDNF-6 was reduced in the DG of BDNF^{Met/Met} mice,

confirming our previous data showing an overall reduction of BDNF-6 in the BDNF^{Met/Met} compared with BDNF^{Val/Val} hippocampus (Mallei *et al*, 2015). Remarkably, the upregulation of total and specific BDNF transcripts induced by exercise was absent or reduced in BDNF^{Met/Met} mice. These results suggest that modulation of BDNF in the DG has an important role in the behavioral effect produced by exercise. Previous evidence showing that specific ablation of BDNF in the DG, but not in the hippocampal CA1 region, attenuates antidepressant efficacy is consistent with this hypothesis (Adachi *et al*, 2008).

In summary, our results demonstrate that BDNF^{Met/Met} mice do not benefit from the anxious- and antidepressant-like effects of chronic exercise and show a likely mechanism underlying this impaired response. These results highlight the importance of genetic factors in moderating the response to exercise, and how much genetic variations should be taken into consideration in clinical trials. Moreover, our study highlights the importance of using animal models unresponsive to pharmacological and environmental treatments, like the knock-in mouse for the human BDNF Val66Met polymorphism, to provide further evidence on the mechanisms underlying the beneficial effects of exercise. A better understanding of the relationship between the genetic profile and changes in response to exercise will allow developing more personalized environmental and pharmacological treatments.

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The authors declare no conflict of interest.

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