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Physical exercise and acute restraint stress differentially modulate hippocampal BDNF transcripts and epigenetic mechanisms in mice.

Running title: Epigenetic modulation of BDNF exons by exercise and stress

Keywords: Running, Restraint Stress, HDAC, BDNF Exons, Hippocampus

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ABSTRACT

Physical exercise and stressful experiences have been shown to exert opposite effects on behavioral functions and brain plasticity, partly by involving the action of brain-derived neurotrophic factor (BDNF). Although epigenetic modifications are known to play a pivotal role in the regulation of the different BDNF transcripts, it is poorly understood whether epigenetic mechanisms are also implied in the BDNF modulation induced by physical exercise and stress.

Here we show that total BDNF mRNA levels and BDNF transcripts 1, 2, 3, 4, 6, 7 were reduced immediately after acute restraint stress in the hippocampus of mice, and returned to control levels 24 hours after the stress session. On the contrary, exercise increased BDNF mRNA expression and counteracted the stress-induced decrease of BDNF transcripts. Physical exercise-induced upregulation of BDNF transcripts was accounted for by increase in histone H3 acetylated levels at specific BDNF promoters, while the histone H3 tri-methylated lysine 27 (H3K27) and di-methylated lysine 9 (H3K9) levels were unaffected. Acute restraint stress did not change the levels of acetylated and methylated histone H3 at the BDNF promoters. Furthermore, we found that physical exercise and restraint stress were able to differentially modulate the histone deacetylases (HDACs) mRNA levels. Finally, we report that a single treatment with HDAC inhibitors, prior to acute stress exposure, prevented the downregulation of total BDNF and BDNF transcripts 1, 2, 3, 6, partially reproducing the effect of physical exercise.

Overall, these results suggest that physical exercise and stress are able to differentially modulate the expression of BDNF transcripts by possible different epigenetic mechanisms.

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INTRODUCTION

Several studies conducted in rodents and humans have demonstrated beneficial effects of physical exercise, including enhanced mood, enhanced stress coping capabilities, improved learning and memory (Deslandes et al., 2009; Duman et al., 2008; Zschucke et al., 2013). Exercise-induced improvement in brain functions has been correlated with enhanced adult hippocampal neurogenesis, synaptogenesis, and increased activity-dependent synaptic plasticity (Farmer et al., 2004; Stranahan et al., 2007; van Praag et al., 1999; Vivar et al., 2013). The effect of exercise on brain plasticity has been associated with the augmentation of brain-derived neurotrophic factor (BDNF), a neurotrophin with a key role in synaptic plasticity, depression, learning and memory disabilities (Hopkins et al., 2011; Vaynman et al., 2004).

Conversely, stressful experiences decrease BDNF expression (Bath et al., 2013; Duman and Monteggia, 2006; Popoli et al., 2002), induce dendritic atrophy and impair hippocampal neurogenesis (Malberg and Duman, 2003; Popoli et al., 2012; Rosenbrock et al., 2005) suggesting a possible mechanism whereby exercise may counteract the deleterious effects of stress on mood and cognitive performances. Although there is some evidence that exercise could prevent stress-induced downregulation of BDNF (Adlard and Cotman, 2004; Kwon et al., 2013), the underlying molecular mechanisms are poorly understood.

The BDNF gene consists of at least eight 5’ non-coding exons alternatively spliced to one common 3’ exon that encodes for the pro-BDNF protein. All 5’ exons are controlled by distinct promoters differentially regulated by a number of cis-acting elements (Aid et al., 2007; Lyons and West, 2011). The complex structure of the BDNF gene allows the distinct BDNF exons to be differentially expressed in subcellular localizations, in specific brain regions and in response to distinct stimuli (Baj et al., 2012; Lyons and West, 2011; Musazzi et al., 2014; Russo-Neustadt et al., 2000; Zajac et al., 2010). Moreover, BDNF expression and

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release might also be altered by genetic and epigenetic variations, including histone post-translational modifications (Chen et al., 2005; He et al., 2010; Koppel and Timmusk, 2013; Qi et al., 2014).

Histone acetylation, modulated by the opposite activity of histone acetyltransferases and histone deacetylases (HDACs), is generally associated with active gene transcription. Histone methylation, regulated by histone methyltransferases and histone demethylases, can induce both activation or repression of gene transcription depending on the particular residues methylated and the extent of methylation (Robison and Nestler, 2011; Tardito et al., 2013). It has been demonstrated that several environmental manipulations can induce long-lasting epigenetic changes at the BDNF promoters (Kuzumaki et al., 2011; Lopez et al., 2013; Oztan et al., 2011). Interestingly, it has been shown that chronic social defeat stress induced enduring downregulation of BDNF transcripts by increasing the dimethylation levels of histone H3 lysine 27 (H3K27me2) at the corresponding BDNF promoters, and that antidepressant treatment reversed this downregulation by enhancing histone acetylation levels (Tsankova et al., 2006). In addition, recent evidence also suggested that acute stress modulates the overall levels of different histone post-translational modifications (Bilang-Bleuel et al., 2005; Chandramohan et al., 2007; Hunter et al., 2009). However, it is still unknown whether these rapid epigenetic changes are involved in the modulation of BDNF transcripts expression induced by acute stress. Moreover, while there is some evidence that exercise induces epigenetic modifications (Abel and Rissman, 2013; Gomez-Pinilla et al., 2011; Intlekofer et al., 2013) the issue whether exercise might prevent stress-induced downregulation of BDNF transcripts by epigenetic mechanisms has not been addressed yet.

In the present study, we assessed the levels of BDNF exon-specific transcripts, as modulated by four weeks of exercise (EXE), acute restraint stress (RS) and the combination of the two stimuli. Moreover, we asked whether EXE and RS differentially modify the levels

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of histone acetylation and methylation at specific BDNF promoters. Finally, we investigated whether pre-treatment with HDAC inhibitors has similar efficacy as exercise in preventing stress-induced downregulation of BDNF transcripts.

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MATERIALS AND METHODS

Animals

Male C57BL/6J mice 8 weeks old were purchased from Charles River (Calco, Italy) and allowed acclimatizing 1 week before being randomly divided in sedentary (SED) and physical exercise (EXE) groups. Mice were maintained in a standard 12 h light/dark cycle, temperature controlled room (21±1°C), with access to food and water *ad libitum*.

All animal procedures were conducted according to current regulations for animal experimentation in Italy (Decreto Legislativo 116/1992) and the European Union (European Communities Council Directive 2010/63/EU) and were approved by the Italian Ministry of Health (Decreto Legislativo 295/2012-A).

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Physical Exercise Protocol

In the SED groups, four mice were housed in a standard polypropylene mice cage (14x32 cm). In the EXE group, four mice were housed in standard polypropylene rat cages (25x41 cm) with free access to two running wheels (12 cm diameter, 5.5 cm width). The greater dimensions of cages for EXE mice were necessary for an adequate setup of running wheels. SED mice were purposely housed in cages without locked wheels because mice climb in locked wheels and we wanted to keep physical activity to a minimum in the SED group (Clark et al., 2012; Koteja et al., 1999; Rhodes et al., 2000). Running wheels were connected to an electronic counter and total distance run was recorded daily. Average distance run by a single mouse was calculated dividing by two the total distance recorded per wheel (2 running wheels X cage X 4 mice). The average distance run by a single mouse, in our model, is comparable to the average distance reported by others (Duman et al., 2008; Sartori et al., 2011).

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Restraint Stress protocol

After 4 weeks of specific housing cage exposure, EXE and SED mice were randomly divided in control (CTR) and restraint stressed (RS) groups. For RS, mice were individually restrained for 2 hours (h) in well-ventilated 50-ml polypropylene centrifuged tube. RS was performed during the morning period. CTR mice groups were handled for 2 min and then returned to their home cage.

Drug Treatments

Sodium butyrate (NaBu; 1.2 g/kg; 10 ml/kg), valproic acid (Val; 150 mg/kg; 10 ml/kg) (Sigma-Aldrich, Milan, Italy) and saline solution were intraperitoneally injected to mice immediately before RS procedure. Doses and timing of NaBu and Val administration were based on previous studies (Fukuchi et al., 2009; Intlekofer et al., 2013; Itzhak et al., 2013; Yildirim et al., 2003; Zhang et al., 2012), showing either an overall increase of global histone acetylation, and within BDNF promoters. CTR non-stressed mice were injected with saline solution and sacrificed 2 h later.

Corticosterone Assay

For corticosterone levels measure, trunk blood was collected on ice cooled EDTA 0.5 M pH 8.00, and plasma was separated by centrifugation and stored at – 80°C. Corticosterone levels were measured using the Corticosterone ELISA Kit (Enzo Life Sciences, Florence, Italy) according to the manufacturer’s instructions.

RNA isolation and Reverse Transcriptase

Total RNA from hippocampus was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Milan, Italy) according to manufacturer's instructions. 1 µg of total RNA was

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reverse-transcribed to cDNA using the iScript kit (Biorad, Milan, Italy) according to manufacturer's instructions.

Quantitative real-time PCR.

qPCR analysis of mRNA expression levels was performed on a 7900HT Fast PCR System (Applied Biosystems, Monza, Italy) as previously described (Conforti et al., 2013). List of primers used are reported in supporting table 1. Relative expression of mRNA for the target genes was performed by the comparative C_T ($\Delta\Delta C_T$) method using β-actin and Gapdh as control reference genes. The relative mRNA levels were expressed as fold change.

BDNF ELISA

Hippocampi were dissected and lysed in radio immune precipitation assay (RIPA) buffer. BDNF levels were measured by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BDNF Emax Immunoassay System, Promega, Milan Italy). BDNF levels measured were normalized to the total amount of proteins loaded.

Western blot

Hippocampi were dissected and lysed in RIPA buffer. Western blot analysis was performed as previously described (Tardito et al., 2009). The following antibodies were used: Rb anti-Phospo-CREB (Ser133) (1:2000), Ms anti-CREB (1:2000), Rb anti-HDAC-4 (1:1000), (Cell Signaling; distributed by Euroclone, Pero Italy), Ms anti-HDAC-5 (1:500) (Santa Cruz; distributed by DBA, Milan Italy), Ms anti-actin (1:1000) (Sigma-Aldrich).

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Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described (Conforti et al., 2013) with some modifications. Briefly, hippocampal tissue was cut into 1 mm^3 cubes and fixed in 1% formaldehyde at room temperature for 15 min. Fixation was stopped by adding glycine (125 mM final concentration). Tissue was washed in PBS, and the pellet suspended in SDS lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS). The lysate was sonicated 8 times (30 sec on, 30 sec off; power 30%) in a Bandeline Electronic sonicator (Berlin, Germany) to produce chromatin fragments of 200-800 base pair in length. Chromatin was diluted in ChIP dilution buffer (16.7 mM Tris, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS) and incubated at 4°C overnight with 4 µg of anti-pan-acetyl histone H3 (H3Ac), anti-trimethyl histone H3 Lys27 (H3K27me3), anti-dimethyl histone H3 Lys9 (H3K9me2) (Millipore, Milan, Italy), or normal rabbit or mouse IgG as control antibody (Life Technologies, Monza, Italy). Ten percent of the pre-immunoprecipitated diluted chromatin was saved as 'input' DNA for ChIP normalization. Immune complexes were incubated for 4-6 h with magnetic beads (Life Technologies) and sequentially washed with 150 mM NaCl low salt buffer and 500 mM NaCl high salt buffer, re-suspended in 120 µl of 1% SDS in 0.1 M $NaHCO_3$ and incubated at 65°C overnight. De-crosslinked samples were treated with RNase and proteinase K (Sigma-Aldrich), and DNA purified by QIAquick PCR Purification Kit (Qiagen). To quantitate histone-associated gene promoters, immunoprecipitated DNA samples were subjected to qPCR as described above.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were made using unpaired *t-test*; ONE- or TWO-way analysis of variance

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(ANOVA), and Newman-Keuls' procedure was used for multiple comparison analysis when appropriated.

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RESULTS

Body weigh gain is reduced in exercised mice

Mice were housed in small standard mouse or in large rat cages equipped with the running wheels. After 28 days, mice were subjected to restraint stress (RS) for 2h and sacrificed immediately (2h) or 22h later (24h after start of the stress protocol) (Figure 1A). Runner mice were randomly divided between control (CTR) and RS animal groups to avoid any difference in term of average distance ran daily (Figure 1B). Twenty-eight days of physical exercise (EXE) reduced the normal body weight gain compared to sedentary (SED) mice (EXE: $F_{(2,236)}=5.53$, $p<0.01$; Time: $F_{(2,236)}=90.03$, $p<0.0001$; interaction: $F_{(6,236)}=1.528$, $p=0.17$) (Figure 1C). Interestingly, stress-induced reduction of body weight was similar in both SED and EXE mice 24h after stress exposure (RS: $F_{(1,44)}=14.45$, $p=0.0008$) (Figure 1D).

Physical Exercise counteracts stress-induced decrease of BDNF

Two-way ANOVA analysis of total BDNF mRNA levels revealed a significant effect of EXE ($F_{(1,36)}=54.41$; $p<0.0001$); RS ($F_{(2,36)}=40.06$; $p<0.0001$) and a trend for the interaction between EXE and RS ($F_{(2,36)}=2.658$, $p=0.0838$) (Figure 1E). Newman-Keuls *post-hoc* analysis for the main stress effect revealed that overall BDNF mRNA levels were decreased at 2h, and returned to CTR levels at 24h (CTR vs 2h p<0.0001; 2h vs 24h $p<0.0001$; basal *vs* 24h $p>0.999$). Additional analysis between all groups showed that 2h RS-EXE mice were significantly different from both CTR-SED (p<0.05) and 2h RS-SED (p<0.05) mice, suggesting that exercise partially counteracted the RS-induced BDNF decrease. Interestingly, consistent with the changes in mRNA expression levels, we also found a significant effect of both EXE and RS in hippocampal BDNF protein levels but not an interaction (EXE: $F_{(1,12)}=55.03$, $p<0.0001$; RS: $F_{(1,12)}=19.65$, $p=0.0008$; interaction: $F_{(1,12)}=0.162$, $p=0.694$) (Figure 1F). Remarkably, an additional statistical analysis revealed that 2h RS-EXE were not

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3 significantly different from CTR-SED mice (p>0.05), suggesting that EXE counteracted RS-
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5 induced downregulation of BDNF and rescued the BDNF protein to CTR levels. This effect
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7 of exercise was not reproduced in a different brain area, such as the prefrontal and frontal
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9 cortex (PFC/FC). Indeed, while RS decreased BDNF levels in the PFC/FC, EXE did not exert
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11 any significant effect (RS: $F_{(1,28)}$=42.13,; p<0.0001, EXE: $F_{(1,28)}$=0.527, p=0.473; interaction:
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13 $F_{(1,28)}$=0.13, p=0.72) (Figure 1G), thus suggesting that EXE-induced BDNF modulation is
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15 region-specific.
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18 We verified that the changes in BDNF mRNA levels induced by physical exercise
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20 were not dependent on the different animal housing (small vs. large cages), because we found
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22 no significant difference in total hippocampal BDNF mRNA levels and in body weight gain
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24 between mice housed in small or large cages without wheels for 28 days (Supporting Figure
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26 S1).
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29 Also, this different modulation of BDNF transcription was not accounted for by
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31 different levels of corticosterone, because RS induced, in both SED and EXE mice, a similar
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33 corticosterone increase at 2h, that returned to CTR levels at 24h (EXE: $F_{(1,61)}$=0.9411,
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35 p=0.3358; RS: $F_{(2,61)}$=278.1, p<0.0001; interaction: $F_{(2,61)}$=2.593, p=0.083) (Figure 1H).
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37 Interestingly, the analysis of mineralcorticoid (MR) and glucocorticoid (GR) receptor mRNA
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39 levels revealed a significant main EXE effect only for MR ($F_{(1,34)}$=5.8903, p=0.0216), while a
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41 significant RS effect was detected for both MR and GR receptors (MR: $F_{(1,34)}$=8.034,
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43 p=0.0077; GR $F_{(1,34)}$=11.37, p=0.002) (Figure 1 I-L). Moreover, additional statistical analysis
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45 between all groups did not reveal a significant difference between CTR-SED and RS-EXE
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47 mice (p>0.05), suggesting that EXE is able to counteract also the RS-induced downregulation
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49 in the mRNA levels of MR (Figure 1 I).
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Physical exercise and stress differentially modulate the expression of distinct BDNF transcripts

As transcription of BDNF is regulated by several exons, we next sought to assess whether the BDNF mRNA changes observed in the hippocampus after RS and EXE were due to one or more different transcripts. Similar to total BDNF mRNA results, a two-way ANOVA analysis of BDNF transcripts 1, 2, 3, 4, 6 and 7 revealed a significant effect of EXE and RS for all the exons (EXE: BDNF-1 $F_{(1,36)}=31.29$, $p<0.0001$; BDNF-2 $F_{(1,36)}=41.13$, $p<0.0001$; BDNF-3 $F_{(1,36)}=41.07$, $p<0.0001$; BDNF-4 $F_{(1,36)}=30.35$, $p<0.0001$; BDNF-6 $F_{(1,36)}=38.12$, $p<0.0001$; BDNF-7 $F_{(1,36)}=25.26$, $p<0.0001$. RS: BDNF-1 $F_{(2,36)}=12.92$, $p<0.0001$; BDNF-2 $F_{(2,36)}=6.685$, $p=0.0034$; BDNF-3 $F_{(2,36)}=13.27$, $p<0.0001$; BDNF-4 $F_{(2,36)}=24.37$, $p<0.0001$; BDNF-6 $F_{(2,36)}=38.58$, $p<0.0001$; BDNF-7 $F_{(2,36)}=26.36$, $p<0.0001$); but not an interaction of the two factors. Newman-Keuls' multiple comparison tests showed that RS induced an overall downregulation in the levels of transcripts 1, 2, 3, 4, 6, 7 at 2h that returned to CTR levels at 24h (Figure 2). Interestingly, the levels of BDNF-4 were increased 24h after RS, compared to CTR and this effect was more pronounced in the EXE group (Figure 2D; $p<0.05$). We did not find any difference for BDNF-8, while BDNF-5 expression levels were very low in the hippocampus, which did not allow a reliable measure of this mRNA.

Physical exercise increases histone acetylation at BDNF promoters

To determine whether changes in epigenetic mechanisms may correlate with the different regulation of expression of BDNF transcripts after EXE and RS, we used ChIP assay to evaluate the levels of several histone modifications within BDNF promoters, following EXE and RS. In particular, we analyzed the levels of H3Ac, a marker of transcriptional activation, and the levels of H3K9me2 and H3K27me3, markers of transcriptional repression.

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Statistical analysis revealed a main significant effect of EXE for H3Ac at all BDNF promoters with the exception of BDNF-8 (EXE: BDNF-1 $F_{(1,27)}=9.362$, $p=0.005$; BDNF-2 $F_{(1,27)}=7.722$, $p=0.0099$; BDNF-3 $F_{(1,27)}=8.048$, $p=0.0085$; BDNF-4 $F_{(1,27)}=6.417$, $p=0.0017$; BDNF-6 $F_{(1,27)}=9.551$, $p=0.0046$; BDNF-7 $F_{(1,27)}=7.765$, $p=0.0096$; BDNF-8 $F_{(1,27)}=1.514$, $p=0.2291$) (Figure 3). On the contrary, we did not find any significant modification of H3K9me2 and H3K27me3 levels at the BDNF promoters (Figure 3).

Physical activity and restraint stress modulate HDACs expression

To investigate the mechanism whereby histone acetylation is increased at the BDNF promoters after EXE, we measured the levels of HDACs (HDACs 1, 2, 3, 4, 5, 7, 8, 9) in mice exposed to EXE and/or RS (Figure 4 and Supporting Figure S2). Two way ANOVA showed a significant effect of both EXE and RS for HDAC-4 (EXE: $F_{(1,36)}=4.202$, $p=0.0477$; RS: $F_{(2,36)}=13.07$, $p<0.0001$; interaction $F_{(2,36)}=0.405$, $p=0.67$), indicating that EXE overall decreased the level of HDAC-4 while RS increased it (Figure 4A). Subsequent Newman-Keuls *post-hoc* analysis for the main RS effect revealed that HDAC-4 was increased at 2h, and returned to CTR levels at 24h (Figure 4A; CTR *vs* 2h $p<0.0001$; 2h *vs* 24h $p<0.001$). Statistical analysis for HDAC-5 expression revealed a significant interaction between EXE and RS, and only a trend for EXE effect (EXE: $F_{(1,36)}=4.299$, $p=0.055$; RS: $F_{(2,36)}=1.998$, $p=0.15$; interaction: $F_{(2,36)}=4.299$; $p=0.0212$). *Post-hoc* analysis between all groups showed that levels of HDAC-5 were decreased only in EXE mice after 2h of RS (Figure 4B; $p<0.01$). Furthermore, we found a significant RS effect for HDAC-1, 2, 7 and 9 (HDAC-1: $F_{(2,36)}=4.688$, $p=0.0155$; HDAC-2: $F_{(2,36)}=3.805$, $p=0.0317$; HDAC-7: $F_{(2,36)}=4.085$, $p=0.02521$; HDAC-9: $F_{(2,36)}=3.513$, $p=0.0404$). Twenty-four hours after RS there was a slight but significant decrease of HDAC-1 and 2 (Supporting Figure S2), while HDAC-9 levels

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3 were reduced at the end of stress (Figure 4D). Moreover, for HDAC-7 levels, *post-hoc*
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5 analysis did not show any significant difference (Figure 4C).
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7 These results may suggest that RS induced a differential and time-dependent
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9 modulation of HDACs expression. To verify this hypothesis and its possible role in BDNF
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11 modulation, we performed a time-course analysis of both HDACs and total BDNF expression
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13 levels after RS. We found that BDNF mRNA levels were downregulated after 2h of RS (2h)
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15 ($p<0.0001$), returned to the CTR levels 2h later (4h); were then up-regulated 6h after the end
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17 of the stress (8h) ($p<0.05$), and finally returned towards CTR levels at 24h (Figure 4F).
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19 Interestingly, while HDAC-5, 7 and 9 were downregulated at 4h, HDAC-4 was upregulated at
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21 2h and 4h. (Figure 4G-L). We also measured a trend towards reduction of HDAC-5 and
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23 HDAC-7 levels at 8h, although these differences were not statistically significant. On the
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25 other hand, we did not find any changes in HDAC-1, 2, 3 and 8 expression levels (Supporting
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27 Figure S2). We did not find any significant difference in the hippocampal HDAC-4 and 5
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29 protein expression levels after EXE and RS (Supporting Figure S3). Together, these results
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31 suggest that the downregulation of HDAC-5, 7 and 9 measured after RS may be a
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33 compensative modification attempted to counteract the stress-induced decrease of BDNF
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35 expression.
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37 Consistent with the results of ChIP assay of H3K27me3 and H3K9me2, we did not
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39 find any changes in mRNA expression levels of enhancer of zeste homolog 2 (EZH2) and
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41 G9a/Glp, the methyltransferases that methylate H3K27 and H3K9, respectively (Supporting
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43 Figure S4).
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52 **HDAC inhibitors prevent stress-induced decrease of BDNF**
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54 To further assess whether the histone acetylation levels may modulate the stress-
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56 induced decrease of BDNF transcripts, as observed with exercise, we treated mice with the
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HDAC inhibitors sodium butyrate (NaBu) and valproic acid (Val), two compounds reported to increase histone acetylation levels at BDNF promoters (Fukuchi et al., 2009; Intlekofer et al., 2013; Zhang et al., 2012). Mice were administered with saline, NaBu or Val immediately before the RS and sacrificed 2h later. Figure 5 shows that RS decreased the total BDNF mRNA levels ($F_{(3,24)}$=8.061, p=0.0007) and of transcripts 1 ($F_{(3,24)}$=3.96, p=0.02), 2 ($F_{(3,24)}$=5.315, p=0.0059), 3 ($F_{(3,24)}$=4.164, p=0.0165), 4 ($F_{(3,24)}$=6.28, p=0.0027), 6 ($F_{(3,24)}$=6.008, p=0.0033) and 7 ($F_{(3,24)}$=8.205, p=0.0006), but not 8 ($F_{(3,24)}$=0.1056; p=0.956) (Figure 5). Interestingly, both NaBu and Val were able to prevent the overall decrease of total BDNF mRNA (NaBu p<0.05; Val p<0.01), and specifically of transcripts 1 (NaBu p<0.05; Val p<0.05), 2 (NaBu p<0.01; Val p<0.05) and 6 (NaBu p<0.05; Val p<0.01) (Figure 5 A-C and F), while only NaBu showed a significant effect in preventing the stress-induced decrease of BDNF-3 (p<0.05) (Figure 5D). Both compounds did not prevent the stress-induced decrease of BDNF-4 and 7 (Figure 5E and G).

It has been recently reported that CREB has a critical role in the HDAC inhibitor-induced expression of BDNF-4 (Koppel and Timmusk, 2013). Moreover, phosphorylation of CREB is modulated in response to stress (Alboni et al., 2011; Kwon et al., 2013). Therefore, to verify whether the different effect of EXE and HDAC inhibitors on stress-induced reduction of BDNF-4 and 7 may be mediated by differential CREB activation, we assessed the levels of phospho-CREB and total CREB after RS and EXE exposure. A two-way ANOVA revealed a main RS effect for p-CREB (p-CREB/CREB $F_{(1,25)}$=10.51; p=0.0034; p-CREB/Act $F_{(1,25)}$=8.301; p=0.0078) but not an EXE effect or interaction (EXE: p-CREB/CREB $F_{(1,25)}$=1.027, p=0.32; p-CREB/Act $F_{(1,25)}$=0.109, p=0.744; interaction: p-CREB/CREB $F_{(1,25)}$=1.344, p=0.257; p-CREB/Act $F_{(1,25)}$=2.23; p=0.14) (Figure 5L-M). Moreover, total CREB levels were similar in all the experimental groups (Figure 5N).

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DISCUSSION

In the present study we showed that 4 weeks of EXE increased the total BDNF mRNA levels in the hippocampus of mice. This modification seemed to result from augmented expression of the exon transcripts 1, 2, 3, 4, 6, 7, in turn accounted for by significant increase in the levels of acetylated histone H3 (H3Ac) at related promoters. On the contrary, 2h of RS decreased the levels of exons 1, 2, 3, 4, 6, 7 as well as of total BDNF mRNA. Interestingly, EXE prior to the onset of the stress counteracted the stress-induced downregulation of total BDNF mRNA, specific BDNF exons and BDNF protein levels. Remarkably, we found that this mechanism is specific for the hippocampus and does not occur in the PFC/FC.

Our finding that EXE counteracts the RS-induced downregulation of BDNF protein levels is consistent with an early study showing that 3 weeks of EXE override the RS-induced reduction of BDNF protein in the hippocampus of mice (Adlard and Cotman, 2004). However, the previous study did not investigate what BDNF transcripts were involved in this complementary action of EXE and RS modulations. BDNF is a complex gene that contains at least 8 alternative promoters, differentially regulated by a numbers of cis-regulatory elements that precisely control their expression during development and adulthood, in different brain regions and cells types (Lyons and West, 2011). Therefore, it is meaningful to define whether the changes observed in BDNF expression are dependent on the modulation of selected BDNF transcripts, and whether the two environmental manipulations are able to differentially modulate the same or alternative BDNF transcripts. Our results, showing that EXE and RS are both able to regulate the expression of the same BDNF exon transcripts suggest the absence of exon-specificity in the action of EXE and acute RS. However, because we have analyzed BDNF transcripts expression in the whole hippocampus, we cannot exclude that BDNF transcripts were differentially regulated within different hippocampal subfields following

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EXE and RS, as reported before for different manipulations (Baj et al., 2012; Nair et al., 2007). A future study exploring this possibility is warranted.

It is remarkable that EXE and RS differently modulated epigenetic mechanisms underlying the regulation of BDNF exons. Using ChIP assay we found that H3Ac levels were enhanced at BDNF promoters 1, 2, 3, 4, 6, 7 by EXE, but were unchanged after RS. In addition, we found that levels of HDAC-4 mRNA were slightly but significantly reduced by EXE and increased by RS. Moreover, HDAC-5 expression was specifically decreased only in EXE, but not in SED mice after RS. Interestingly, a reduction of HDAC-5 mRNA levels was previously reported also in social defeat stressed mice, but not in controls, after treatment with the antidepressant imipramine (Tsankova et al., 2006). Overall, these results suggest that changes in HDAC-5 expression may be involved in the beneficial effects of both EXE and antidepressants treatment in counteracting the stress-induced decrease of BDNF expression.

Our results, showing that RS did not modify H3Ac levels at BDNF promoters, are apparently in contrast with previous data reporting that 2h of RS decreased the levels of H3Ac within BDNF promoters 1, 4 and 6 in the rat hippocampus (Fuchikami et al., 2009). However, our data showing that only HDAC-4 mRNA, but not the protein levels, were increased immediately after the cessation of stress, may suggest that some time after the stimulus is required before the increase of HDAC-4 expression and the subsequent reduction of histone H3ac occur. In line with this hypothesis, in another model of acute stimulation, the electroconvulsive shock, a marked change in H3ac was reported only 24h after the stimulus, suggesting a delay between treatment and histone H3ac changes (Tsankova et al., 2004). On the other hand, the downregulation of HDAC-5, HDAC-7 and HDAC-9 that we measured after the RS may in turn counteract the upregulation of HDAC-4 induced by stress. Further studies will be necessary to fully elucidate these mechanisms and to understand whether the

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controversial findings may be dependent on differences in species, environmental manipulations or methodologies.

We have found that increase of BDNF mRNA levels correlated with decrease of HDAC-5, 7 and 9 at 4h. Although it is well know that HDACs are able to control BDNF expression (Fukuchi et al., 2009; Intlekofer et al., 2013; Koppel and Timmusk, 2013; Zhang et al., 2012), the specific role of distinct HDACs it still largely unknown. Moreover, it is not well knows whether and how HDACs may regulate their own expression. Therefore it is tempting to speculate that the reduction of HDACs could be an adaptive neuronal response against stressful challenges, attempting to increase the acetylation levels at specific promoters, in order to restore a normal level of gene expression for BDNF or other genes in the hippocampus (Han et al., 2014). This hypothesis is further supported by our results showing that the RS-induced reduction of BDNF expression was prevented by treatment with HDAC inhibitors.

Indeed, to the best of our knowledge, we have shown here for the first time that a single administration of HDAC inhibitors, known to increase the histone acetylation levels at the BDNF promoters (Fukuchi et al., 2009; Intlekofer et al., 2013; Zhang et al., 2012), prior to the RS session, prevented the RS-induced downregulation of BDNF transcripts. These results are consistent with our observation that EXE may prevent RS-induced downregulation of BDNF transcripts by increasing the level of H3Ac at the corresponding BDNF promoters. However, while EXE was able to counteract the decrease of all BDNF transcripts, HDAC inhibitors prevented the downregulation of BDNF-1, 2, 3, 6 but not of BDNF-4 and 7, induced by 2h of RS. A possible explanation for this discrepancy might be ascribed to a possible decrease of CREB activity, that has been reported to have a critical role in the HDAC inhibitor-induced expression of BDNF-4 (Koppel and Timmusk, 2013). However, according to previous reports (Alboni et al., 2011; Kwon et al., 2007), we found that acute RS increased

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p-CREB levels in the hippocampus, which suggests that CREB phosphorylation is not involved in the lack of efficacy of HDAC inhibitors to prevent the RS-induced downregulation of BDNF-4 and 7.

Consistently with previous reports, we found that EXE prior to RS did not modify the peak of corticosterone release (Campeau et al., 2010; Droste et al., 2003). Interestingly, it has been recently shown that, although the peak of corticosterone after RS was not different in EXE *vs* SED mice, exercised mice showed a more rapid decay of corticosterone levels, suggesting that EXE increases the negative feedback of the hypothalamic pituitary adrenal (HPA) axis (Hare et al., 2014). Intriguingly, it has been proposed that this effect of exercise may be mediated by a change in the ratio of corticosteroid receptors (Droste et al., 2003). Considering that the overexpression of MR in the forebrain was shown to modify the stress response in mice (Rozeboom et al., 2007), our data showing that EXE counteracts RS-induced decrease of MR, may suggest the possibility that MR could be involved in the faster corticosterone decline modulate by EXE (Hare et al., 2014).

Remarkably, while we found that HDAC-5 expression was already decreased at the end of the RS period in EXE mice, this downregulation was apparent only 2h later in the SED mice. Although more work will be necessary to fully clarify the time course of HDAC-5 after RS both in SED and EXE mice, our results suggest that EXE may also modulate a faster response of HDAC-5 expression to RS.

In summary, our study suggests not only that EXE and RS differentially modulate the same BDNF transcripts, but also that the epigenetic mechanisms underlying their effects are different. In particular, EXE-induced BDNF transcripts expression correlated with increased levels of H3Ac, while no posttranslational histone modifications induced by RS, at least after 2h of RS, were found. However, further studies will be necessary to fully address whether other epigenetic modifications could be associated with acute RS-induced downregulation of

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BDNF. In conclusion, our results suggest that modulation of epigenetic modifications could be a suitable treatment to enhance brain capability to cope with stressful challenges.

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FIGURE LEGENDS

Figure 1 Physical exercise prevented stress-induced downregulation of BDNF in the hippocampus. (A) Experimental time-table. (B) Total distance run daily by mice. Restraint Stress (RS) mice refer to mice subjected to RS just at the end of the sedentary (SED)/exercise (EXE) period. To calculate the average distance run by a single mouse, daily distance measured by a single electronic counter was divided by 2 (2 electronic counter per cage; four mice per cage) (n=8 number of electronic counters analyzed). (C) Total body weigh gain over the experimental period (n=5-12 mice per group). (D) RS induced body weight loss. Mice were weighted before the RS or the handle manipulation (Controls: CTR) on day 28, and re-weighted 24h later (n=12 mice per group). (E) Total hippocampal (HPC) BDNF mRNA levels were increased after 28 days of EXE, decreased after 2h of RS, and almost returned to CTR levels 24h after RS (n=5-12 mice per group). (F) BDNF protein levels in the HPC after 28 day of EXE and 2h of STR (n=4 mice per group). (G) BDNF mRNA levels in the prefrontal and frontal cortex (PFC/FC) after 28 day of EXE and 2h of RS (n=4 mice per group). (H) Plasma corticosterone levels were increased after 2h and returned to CTR levels 24h after RS, in both SED and EXE mice (n=8-17 mice per group). (I-L) MR and GR mRNA levels in the HPC after 28 day of EXE and 2h of RS (n=7-8 mice per group). Data are expressed as means ± SEM. Two-way ANOVA followed by Newman-Keuls' *post-hoc* analysis. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Figure 2 Effects of physical exercise and restraint stress in the levels of hippocampal BDNF transcripts. (A-G) BDNF mRNA levels of transcript 1 (A), 2 (B), 3 (C), 4 (D), 6 (E), 7 (F) and 8 (G) after 28 days of physical exercise (EXE), 2h of restraint stress (RS) or the combination of the two stimuli (n=5-12 mice per group). mRNA levels were measured in control non-stressed mice (CTR) and 2h or 24h after the beginning of RS. Data are expressed as means ±

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SEM. Two-way ANOVA followed by Newman-Keuls' *post-hoc* analysis. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. SED: sedentary.

Figure 3. Physical exercise increased acetylation of histone H3 at the BDNF promoters in the hippocampus. (A-G) ChIP analysis revealed that exercise (EXE) increased the H3 acetylation levels at the BDNF promoters 1 (A), 2 (B), 3 (C), 4 (D), 6 (E), and 7 (F) but not 8 (G), while restraint stress (RS) did not change the acetylation levels at any of the BDNF promoters. No differences were revealed in the H3K27 tri-methylation and H3K9 di-methylation levels at the BDNF promoters. Data are expressed as means ± SEM (n= 7-12 mice per group). Two-way ANOVA revealed a main effect only for the exercise factor * $p < 0.05$; ** $p < 0.01$. SED: sedentary; CTR: controls.

Figure 4 Effects of physical exercise and restraint stress in the hippocampal HDAC mRNA levels. (A) HDAC-4 levels were increased after 2h of restraint stress (RS) and downregulated by physical exercise (EXE). (B) HDAC-5 mRNA levels were significantly reduced in stressed mice expose to 28 days of EXE. (C) HDAC-9 mRNA levels were slightly decreased after 2h of RS. (D) HDAC-7 mRNA levels were unchanged after the two stimuli. Data are expressed as means ± SEM (n= 5-12 mice per group). Two-way ANOVA followed by Newman-Keuls' *post-hoc* analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (E) Experimental time-table. (F-L) Time course expression of total BDNF (F); HDAC-4 (H); HDAC-5 (I); HDAC-7 (L) and HDAC-9 (M) mRNA levels after 2h of RS. Data are expressed as means ± SEM (n= 8 mice per group). One-way ANOVA followed by Newman-Keuls' *post-hoc* analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Figure 5 Effects of HDAC inhibitors in stress-induced downregulation of BDNF transcripts. (A-G) HDAC inhibitors prevented RS-induced reduction of total BDNF mRNA (A) and BDNF transcripts 1 (B), 2 (C), 3 (D), 6 (F) but not BDNF transcripts 4 (E) and 7 (G). (H) RS did not modify the levels of BDNF-8 mRNA. Data are expressed as means ± SEM (n= 5-12

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mice per group). One-way ANOVA followed by Newman-Keuls' *post-hoc* analysis. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$ *vs* control (CTR). # $p < 0.05$; # # $p < 0.01$; *vs* vehicle (veh). (H-M). RS increased phosphorylation of CREB. (H) Representative immunoreactive bands from western blot of phospho-CREB (p-CREB); total CREB and β□actin. (I-L) Densitometric quantification of p-CREB and total CREB were obtained as the ratio of p-CREB/CREB; p-CREB/β□actin and CREB/β□actin. Data are expressed as means ± SEM (n= 6-8 mice per group). One-way ANOVA followed by Newman-Keuls' *post-hoc* analysis. * $p < 0.05$; ** $p < 0.01$.

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SUPPORTING FIGURE LEGENDS

Supporting Figure S1 Effects of housing conditions. Four mice were housed for 28 days in small mice or large rat cages. (A) Total BDNF mRNA levels in the hippocampus. Unpaired t-test analysis (B). Total body weight gain over the experimental period. Data are expressed as means ± SEM (n= 8 mice per group). Two-way ANOVA analysis.

Supporting Figure S2 Effects of physical exercise and restraint stress in the hippocampal HDAC levels. (A-B) Restraint stress induced reduction of HDAC-1 and HDAC-2 mRNA levels 24h after the stress session. (C-D) HDAC-3 and HDAC-8 mRNA levels were unchanged after the two stimuli. Data are expressed as means ± SEM (n= 5-12). Two-way ANOVA followed by Newman-Keuls' *post-hoc* analysis. * $p < 0.05$. (E-H) Time course expression of total HDAC-1 (E); HDAC-2 (F); HDAC-3 (G) and HDAC-8 (H) levels after 2h of restraint stress. Data are expressed as means ± SEM (n= 8 mice per group). One-way ANOVA analysis.

Supporting Figure S3 Effects of physical exercise and acute restraint stress in the HDAC-4 and HDAC-5 protein levels (A) Representative immunoreactive bands from western blot analysis of HDAC-4; HDFAC-5 and β□actin. (B-C) Densitometric quantifications of HDAC-4 and HDAC-5 were obtained as the ratio of HDAC-4/β□actin and HDAC-5/β□actin. Data are expressed as means ± SEM (n= 6-8 mice per group). Two-way ANOVA analysis.

Supporting Figure S4 Effects of physical exercise and acute restraint stress in the methyltransferase mRNA levels. (A-C) Hippocampal mRNA levels of EZH2 (A), g9a (B) and glp1 (C) were unaffected after 28 days of physical exercise and 2h of restraint stress. Data are expressed as means ± SEM (n= 5-12 mice per group). Two-way ANOVA analysis.

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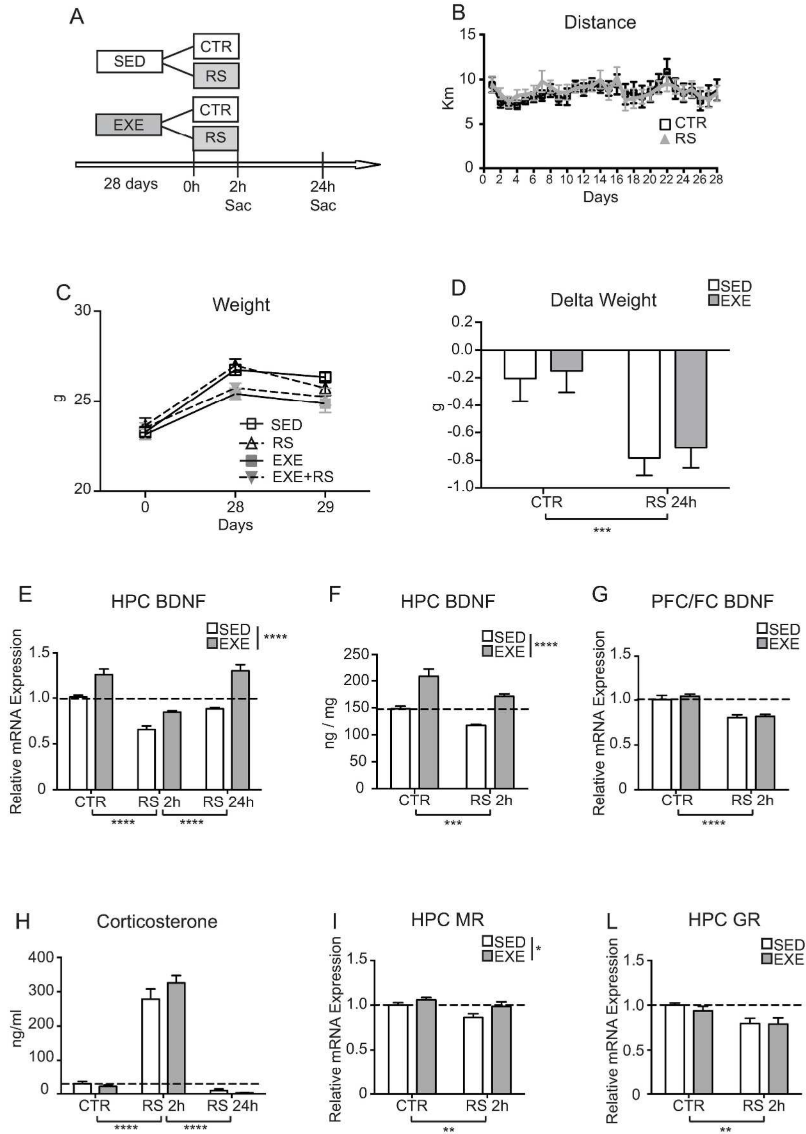

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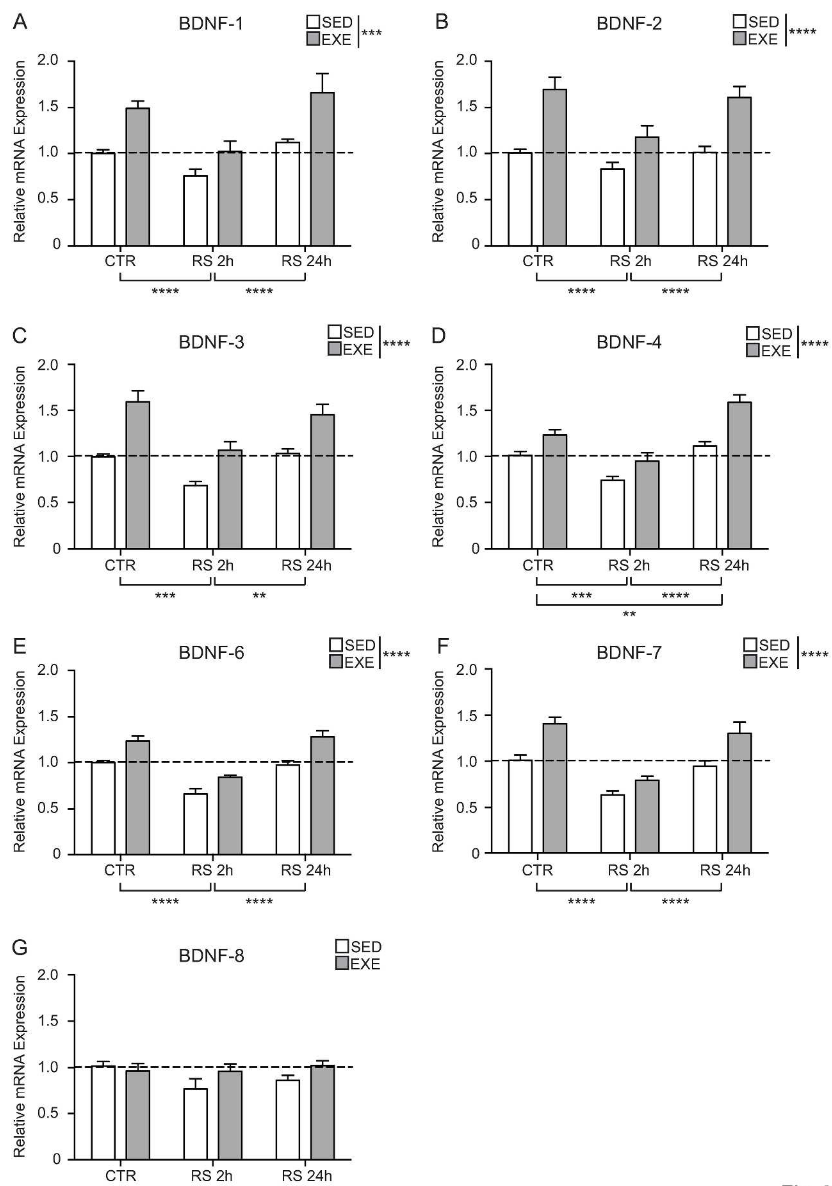

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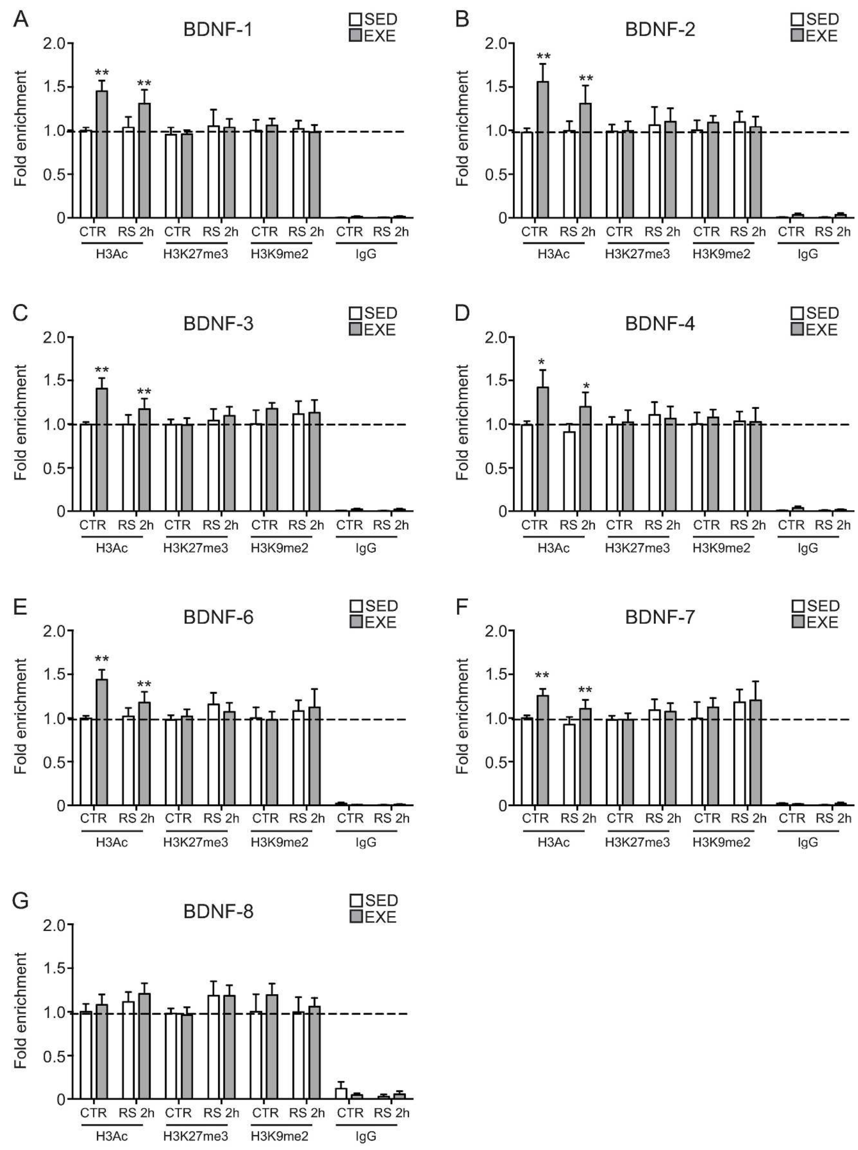

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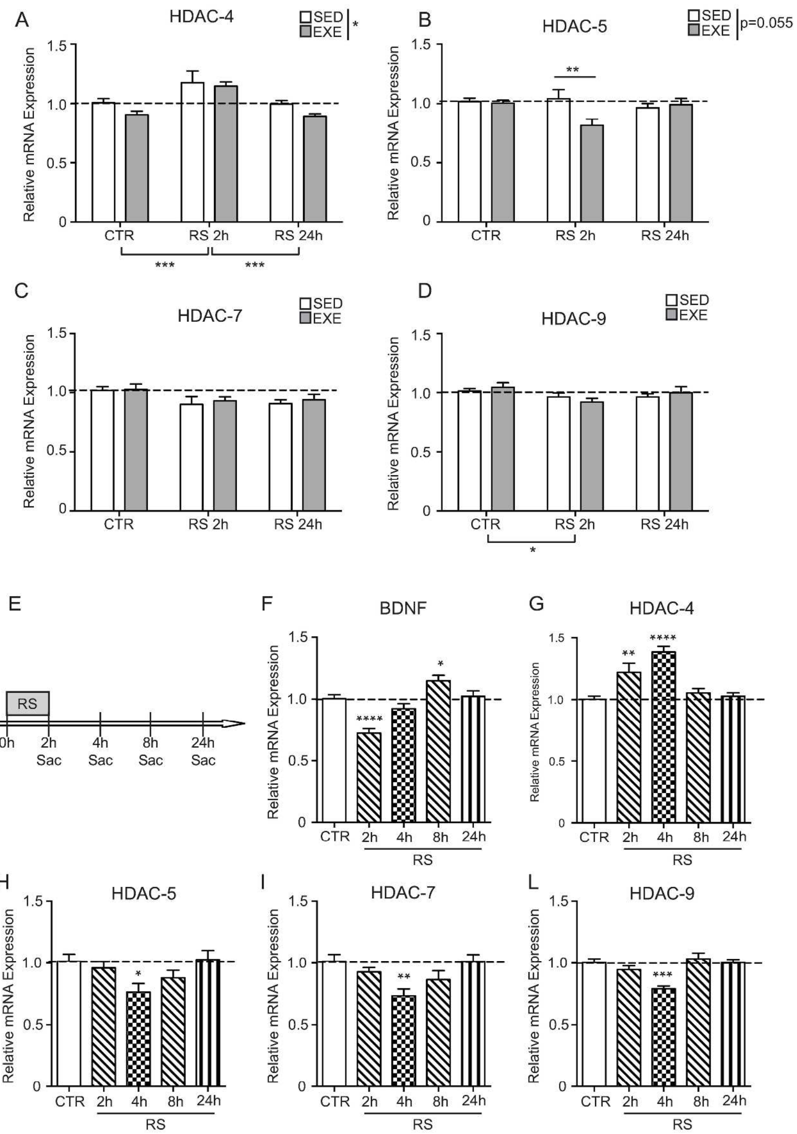

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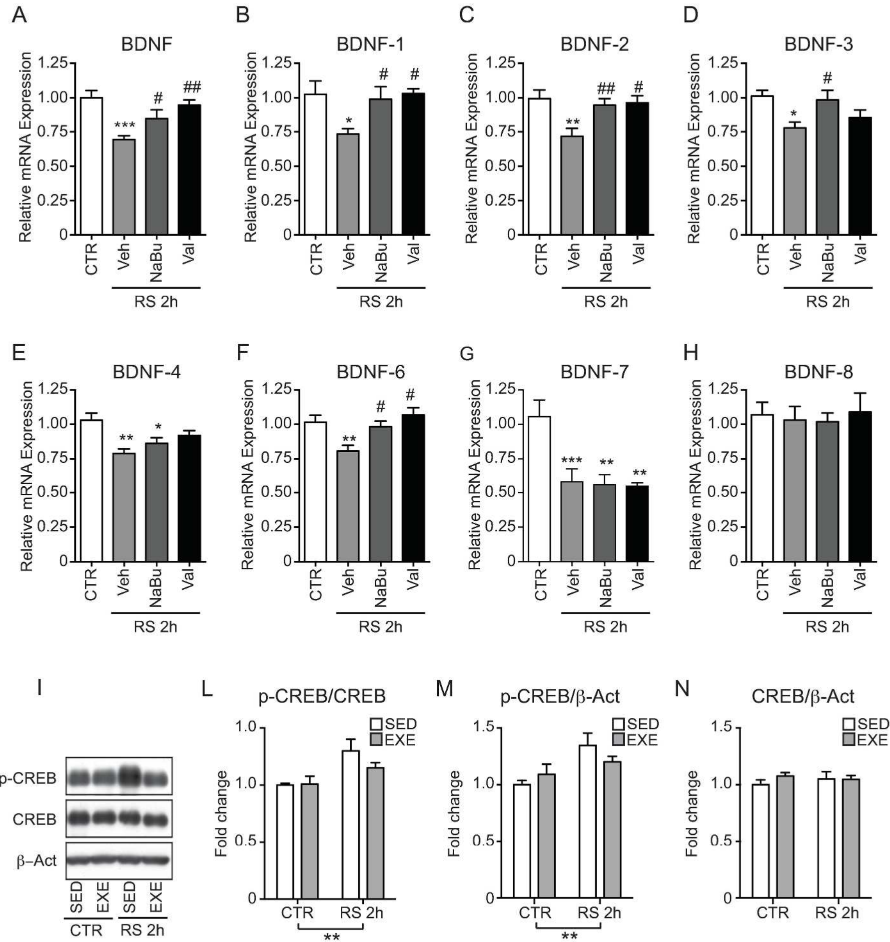

Fig. 5

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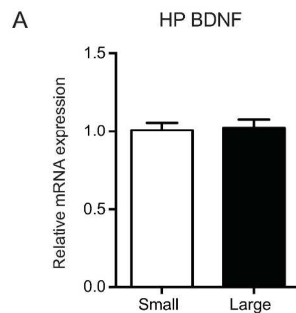

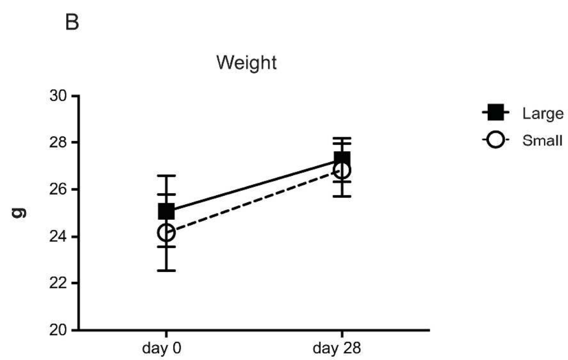

Sup. Fig. S1

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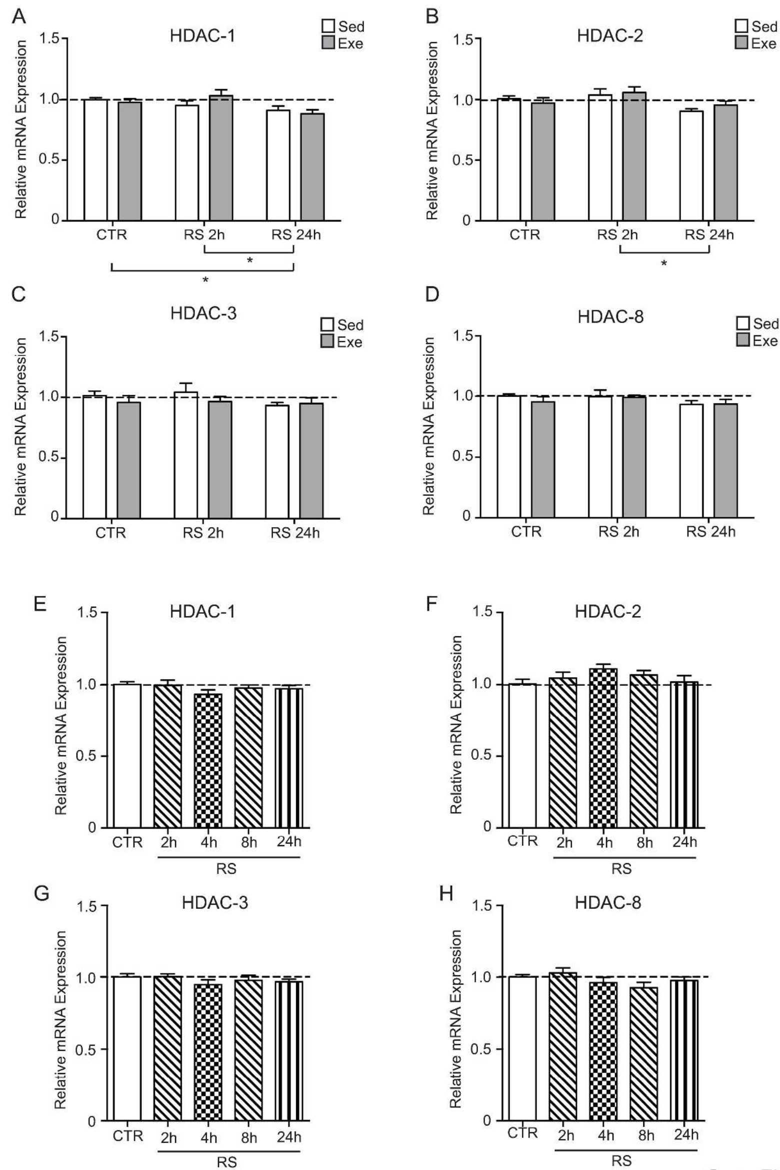

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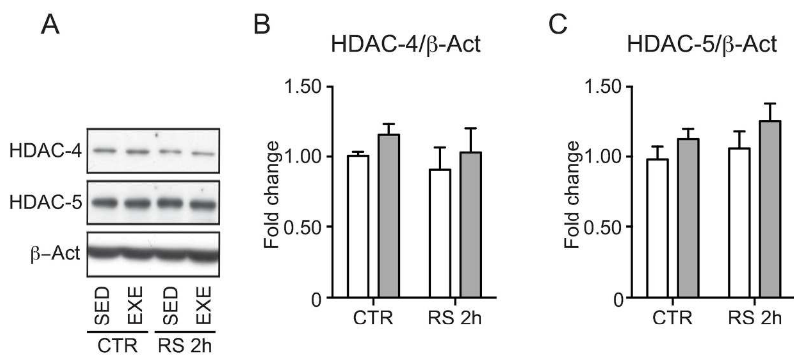

Sup. Fig. S3

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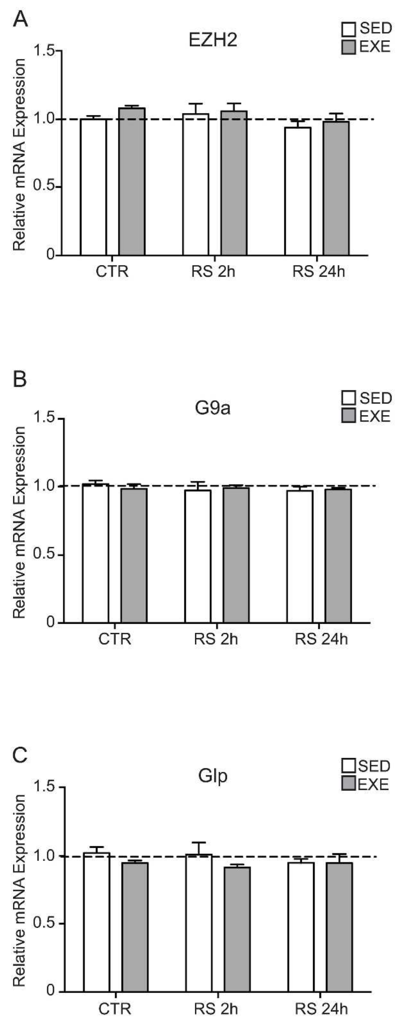

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Supporting Table 1. List and sequence of primers used in this study.

GENE	FORWARD	REVERSE
Real Time PCR		
BDNF-1	CCTGCATCTGTTGGGGAGAC	CGCCTTCATGCAACCGAAGTAT
BDNF-2	ACCTTTTCCTCCTCCTGCG	TGGATGAAGTACTACCACCTCGG
BDNF-3	TGAGACTGCGCTCCACTCCC	CGCCTTCATGCAACCGAAGTAT
BDNF-4	CAGAGCAGCTGCCTTGATGTTT	CGCCTTCATGCAACCGAAGTAT
BDNF-6	ACAATGTGACTCCACTGCCGG	CGCCTTCATGCAACCGAAGTAT
BDNF-7	ACTTACAGGTCCAAGGTCAACG	GGACAGAGGGTCGGATACAG
BDNF-8	ATGACTGTGCATCCCAGGAGAAA	CGCCTTCATGCAACCGAAGTAT
BDNF	TCGTTCCTTTCGAGTTAGCC	TTGGTAAACGGCACAAAAC
HDAC-1	GAGTTCTGTCAGTTGTCCACGG	TTCAGACTTCTTTGCATGGTGC
HDAC-2 [a]	GGGACAGGCTTGGTTGTTTC	GAGCATCAGCAATGGCAAGT
HDAC-3 [b]	GCCAAGACCGTGGCGTATT	GTCCAGCTCCATAGTGGAAGT
HDAC-4 [a]	CAATCCCACAGTCTCCGTGT	CAGCACCCCACTAAGGTTCA
HDAC-5 [a]	TGTCACCGCCAGATGTTTTG	TGAGCAGAGCCGAGACACAG
HDAC-7 [a]	GGTGGACCCCCTTTCAGAAG	TGGGTAGCCAGGAGTCTGGA
HDAC-8	AGATGCCAGAGGAACCCGC	TGCAGGGCATAGGCTTCGAT
HDAC-9 [a]	GCGAGACACAGATGCTCAGAC	TGGGTTTTCCTTCCATTGCT
EZH2	CAACCCTGTGACCATCCACG	CGACATCCAGGAAAGCGGTT
G9a [c]	GAGAGCCTGGAGGGAGATGG	TCATTGACATTTTGGCCCGACT
Glp [c]	ATTGACGCTCGGTTCTATGG	ACACTTGGAAGACCCACACC
ß-Actin	GCCAGAGCAGTAATCTCCTTCT	AGTGTGACGTTGACATCCGTA
GAPDH	CGTGCCGCCTGGAGAAACC	TGGAAGAGTGGGAGTTGCTGTTG
CHIP		
PBDNF-1 [a]	TGATCATCACTCACGACCACG	CAGCCTCTCTGAGCCAGTTACG
PBDNF-2 [a]	CCGTCTTGTATTCCATCCTTTG	CCCAACTCCACCACTATCCTC
PBDNF-3 [a]	GTGAGAACCTGGGGCAAATC	ACGGAAAAGAGGGAGGGAAA
PBDNF-4 [a]	CTTCTGTGTGCGTGAATTTGCT	AGTCCACGAGAGGGCTCCA
PBDNF-6 [a]	ACTCACACTCGCTTCCTCCT	GCACTGGCTTCTCTCCATTT
PBDNF-7	GGAAGGGTGGTGAGAGAGATATAGAG	GTGCCTAAGCCGGTGGTAAG
PBDNF-8	TGAAAGGAAAAAGGAGAGGCTTT	AGAAAATGCCACTCCCACATAAG

[a] Tsankova et al., 2006 Nat Neurosci; [b] Covington HE 3rd et al. 2009 J Neurosci.; [c] Maze et al 2014 Nat Neurosci

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