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Environmental Enrichment Increased *Bdnf* Transcripts in the Prefrontal Cortex: Implications for an Epigenetically Controlled Mechanism

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Abstract—Environmental enrichment (EE) is a condition characterized by its complexity regarding social contact, exposure to novelty, tactile stimuli and voluntary exercise, also is considered as a eustress model. The impact of EE on brain physiology and behavioral outcomes may be at least partly underpinned by mechanisms involving the modulation of the brain-derived neurotrophic factor (BDNF), but the connection between specific *Bdnf* exon expression and their epigenetic regulation remain poorly understood. This study aimed to dissect the transcriptional and epigenetic regulatory effect of 54-day exposure to EE on BDNF by analysing individual BDNF exons mRNA expression and the DNA methylation profile of a key transcriptional regulator of the *Bdnf* gene, exon IV, in the prefrontal cortex (PFC) of C57BL/6 male mice (sample size = 33). *Bdnf* exons II, IV, VI and IX mRNA expression were upregulated and methylation levels at two CpG sites of exon IV were reduced in the PFC of EE mice. As deficit in exon IV expression has also been causally implicated in stress-related psychopathologies, we also assessed anxiety-like behavior and plasma corticosterone levels in these mice to determine any potential correlation. However, no changes were observed in EE mice. The findings may suggest an EE-induced epigenetic control of BDNF exon expression via a mechanism involving exon IV methylation. The findings of this study contribute to the current literature by dissecting the *Bdnf* gene topology in the PFC where transcriptional and epigenetic regulatory effect of EE takes place.

Key words: environmental enrichment, epigenetics, BDNF, corticosterone, anxiety, methylation.

INTRODUCTION

Environmental enrichment (EE) is a housing condition equipped with a variety of objects, running wheels and social interaction. The constant offer of novelty, social contact and different activities are key factors of enrichment that have rewarding effects and as such may also induce "eustress" (Nithianantharajah and Hannan, 2006). These stimuli bring benefits to animals such as improvement in mood and sensory, cognitive and motor functions. (Nithianantharajah and Hannan, 2006; Simpson and Kelly, 2011). The dynamic and complex characteristics of EE are able to stimulate learning, exploratory behavior, social interaction and can elicit anxiolytic effects (van Praag et al., 2000; Sale et al., 2014; Rae et al., 2018). Indeed, EE is known to reverse stress-related behaviors (Francis et al., 2002) and to increase social interaction (Rae et al., 2018), although enhanced aggressiveness and anxiety behaviors after EE have also been reported (McQuaid et al., 2012; McQuaid et al., 2013).

One of the key molecular markers involved in the aforementioned effects of EE is the brain-derived neurotrophic factor (BDNF) which is a small, secreted protein, member of the neurotrophin family of growth factors (Leibrock et al., 1989). It is cleaved from its proform into its mature form via highly regulated molecular mechanisms. The mature form is the biologically active form implicated in neural plasticity and neurogenesis (Rogers et al., 2019). Some studies have demonstrated an inherent ability of EE to increase BDNF levels in several brain regions (Young et al., 1999; Angelucci et al., 2009). Enhanced BDNF levels in the cortex, hippocampus, basal forebrain and hindbrain of enriched rats have pivotal roles in numerous protective effects of EE (Ickes et al., 2000). For instance, increased glial- and brain-derived neurotrophic factor in the hippocampus is thought

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to provide resilience to hippocampal injuries (Young et al., 1999) and improvement in memory and learning processes (Hirase and Shinohara, 2014). Also, EE has been shown to increase ramification, length, and number of dendritic spines (Greenough, Volkmar and Juraska, 1973; Connor, Wang and Diamond, 1982; Leggio et al., 2005), leading to hippocampal neurogenesis (Kempermann, Kuhn and Gage, 1997; van Praag, Kempermann and Gage, 2000) that contributes to the beneficial effects of EE on cognition. The impact of EE on anxiety behavior in rats and cognitive enhancement in humans requires the activation of hippocampal BDNF signalling (Janke et al., 2015; Hakansson et al., 2017). Despite the increasing evidence for the involvement of hippocampal BDNF on the EE-related effects (Kuzumaki et al., 2011), there is scarce evidence that addresses the role of the prefrontal cortex (PFC) in this context.

The role of the PFC on emotional and cognitive behavior has been widely studied (Friedman and Robbins, 2022). It is primarily involved in decision making and emotional regulation by exerting top-down inhibitory control over regions involved in reward and emotion (Miller and Cohen, 2001; Dixon et al., 2017). In this context, the exon IV of the *Bdnf* gene is known to have an important role in the modulation of inhibitory PFC functions in mice as demonstrated in Sakata et al (2009) using a PFC promoter IV-specific knockout mice.

Bdnf gene contains a single coding exon (exon IX) and eight non-coding exons in rodents (Nair and Wong-Riley, 2016), each of which can be connected to the coding exon and form at least nine different transcripts (Liu et al., 2006). Interest has been directed to the epigenetic regulation at the *Bdnf* promoters of the exons and the resulting enduring changes of their expression, which are influenced by environmental factors since early life (Roth et al., 2009). In fact, rats exposed to voluntary exercise – a key EE factor - showed hypomethylation of *Bdnf* promoter IV in the hippocampus resulting in enhanced mRNA and BDNF protein expression (Gomez-Pinilla et al., 2011), reinforcing the impact of EE on the regulation of *Bdnf* promoters which can influence BDNF expression and as a result, potentially behaviors.

Of all these exons, *Bdnf* exon IV promotor methylation has gained interest as it controls BDNF expression (Zheleznyakova et al., 2016). *Bdnf* exon IV is involved in mechanisms of stress and pathophysiology of depression (Sakata et al., 2010) and EE seems to be particularly beneficial in reversing depression-like behavior in mice with reduced exon IV expression (Jha et al., 2016; Dong et al., 2018). Interestingly, patients with major depression disorder with hypomethylation of a specific CpG site in the *Bdnf* exon IV showed lower response to antidepressants (Tadic et al., 2014), clearly highlighting the importance of *Bdnf* exon IV epigenetic modification on antidepressant efficacy. In agreement, a study in patients with major depressive disorder (Lopez et al., 2013) showed increased expression in peripheral *Bdnf* mRNA in patients classified as responders to chronic citalopram treatment, accompanied by a decrease in H3K27me3 (trimethylation at lysine 27 of histone H3) at promotor IV of *Bdnf* gene after the treatment with the antidepressant. As such, exon IV methylation of the *Bdnf* gene has gained ground as a biological marker to predict responses to antidepressants (Lieb et al., 2018).

Epigenetic changes are mechanisms which organisms can adapt in accordance with environmental stimuli, prompting to subsequent phenotypical alterations and known to contribute to the vulnerability or resistance in several brain disorders (Sweatt, 2009). DNA methylation is an epigenetic mechanism used by cells to control gene expression. Several mechanisms exist to control gene expression in eukaryotes, such as DNA methylation in the promoter region, which usually promotes transcription silencing (Brenet et al., 2011) whereas histone modifications, like DNA acetylation, generally provide a permissive environment enhancing the gene transcription (Moore et al., 2013). The influence of DNA methylation on gene expression is generally linked to two main mechanisms. DNA methylation may suppress gene expression preventing transcription factors to bind to their respective sites in promoters if a methyl-cytosine is present (Moore et al., 2013). Alternatively, transcription can be repressed with the help of proteins that binds to methylated DNA, the Methyl CpG-binding proteins. (Tate and Bird, 1993).

Moreover, environmental stimuli have been shown to alter *Bdnf* methylation profile with clear consequences on its expression. For instance, traumatic experiences, such as continuous psychosocial stress, was shown to induce *Bdnf* hypermethylation in exon IV and concomitant reduction in its expression in the dorsal hippocampal CA1 region of adult rats (Roth et al., 2011). Similarly, adult rats exposed to stress (maternal maltreatment) during early life (postnatal days 1–7) exhibited significant methylation at both exons IV and IX (Roth et al., 2009) demonstrating a sensitivity of exon IV on methylation induced by stressful experiences.

With respect to EE, there is evidence that this strategy can promote global or specific loci changes in DNA methylation which involve modifications in the expression of DNA methyltransferases in human and rodents (Barrès et al., 2012; Griñán-Ferré et al., 2016). Most of the studies describing the involvement of *Bdnf* gene methylation in EE-related mechanisms have been observed in the hippocampus. For instance, Morse et al (2015) concluded that EE exposure for 5 weeks 1 h-per day reversed histone methylation changes in the hippocampus of aged rats in an object learning test, and this was concomitant with an increase in total *Bdnf* mRNA levels. Similarly, Zajac et al, (2010) found increased total *Bdnf* gene expression in male mice exposed to EE (4 weeks exposure, 1 h-per day, 3x/week) and Kuzumaki et al, (2011) also showed BDNF mRNA upregulation in the hippocampus after 3–4 EE weeks. Even though is stablished that adult hippocampal neurogenesis can be enhanced by EE in the dentate gyrus (DG) (van Praag, Kempermann and Gage, 2000) this mechanism can be blocked in heterozygous knockout animais (*Bdnf* $^{+/-}$) like in Rossi et al, (2006) or even show no differential expression of *Bdnf* in the DG but with the possibility to be upregulated in other hippocampal regions (Zhang et al., 2018). Other than hippocampus, knowledge regarding

the effects of EE on epigenetic modulation of *Bdnf* gene in the PFC is still scarce. Thus, the current study aimed to dissect the *Bdnf* gene expression topology profile induced by EE in the PFC and the epigenetic mechanism potentially underlining these expression changes.

As such, we dissected the transcriptional and epigenetic regulatory effect of long exposure to EE on the PFC *Bdnf gene* by analysing individual exon gene expression in the exons I, II, IV, VI and IX and DNA methylation profile of exon IV of the *Bdnf* gene of C57BL/6 mice. Given the role of exon IV on control of emotional behaviour (Sakata et al., 2010; Chen et al., 2011), we also assessed the effect of EE on anxiety like behaviour and HPA activity. There is indeed a close link between HPA activity and BDNF levels as demonstrated by alterations of BDNF levels induced by corticosterone administration in several brain regions (Schaaf et al., 2000; Lin et al., 2022). Findings from these studies would shed light on the molecular mechanism underlining the well-established beneficial effect of EE on mental health wellbeing.

EXPERIMENTAL PROCEDURES

Animals

Adult male C57BL/6 mice (thirty-three animals; PND 65–75 at the beginning of the experiments; Biomedical Sciences Institute, University of Sao Paulo) were housed in groups of five per cage with free water and food access in a room with controlled temperature (24 ± 2 °C) and humidity conditions. Animals were maintained under a 12/12 h light/dark cycle in an inverted cycle (lights off between 10:00 am and 10:00 pm). Red lights were used to handle the animals during the dark phase, when objects were exchanged in the EE cage. Procedures were approved by the Ethical Committee for Animal Use (CEUA) of the University of Sao Paulo, registered under protocol no. 5664120118. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

Housing conditions

The non-enriched groups (NE) were housed in standard housing conditions (polypropylene cages, 27.5 cm length × 16.5 cm width × 13 cm height) while the enriched groups (EE) were housed in transparent polycarbonate cages larger than the standard ones (42 cm length × 28 cm width × 21.5 cm height) with a range of stimuli such as pipes, ramps, ladders, houses and running wheels (objects were changed/moved three times a week) as previously described in Rueda et al. (2012), Marianno et al. (2017), and Rae et al. (2018). The bedding conditions and access to food and water were the same for both groups.

Experimental design

Thirty-three mice (15 NE and 18 EE) were maintained in non-enriched or enriched housing for 54 consecutive days. Mice were tested on day 32 in the elevated plus-maze (EPM) to investigate the animal anxiety-like effect in EE and NE. Blood samples were collected (see a detailed description in blood sampling and corticosterone concentration) immediately after the test. The rationale for measuring corticosterone around 32 days of EE was to compare with previous study showing that 30-days EE decreased corticosterone levels and prevented anxiety-like behavior induced by stressful conditions in rats (Islas-Preciado et al., 2016). On day 54, after euthanasia, the brains were removed. All the procedures were carried out during the animal light phase (between 7:00 and 10:00 am). See Fig. 1 for an illustration of the design of the experimental protocol.

Animals were not tested in the EPM at the end of the experiment to avoid any interference of novelty exposure stress on gene expression.

Elevated plus maze (EPM)

The EPM was used to assess anxiety-like responses in rodents on day 32. The apparatus consists of two open arms (33.5 cm × 7 cm) bordered by a 0.5 cm high wall to prevent the animals from falling, and two closed arms (33.5 cm × 7 cm) with walls 20 cm high, which is at a height of 50 cm from the floor. The test was carried out during the light period of the cycle (between 7:00 – 10:00 am), in a room with approximately 100 lux (Komada, Takao and Miyakawa, 2008). After 1 hour of habituation in the experimentation room, each mouse was placed in the central area and freely explored the apparatus for 5 minutes. The following parameters were evaluated (Plus MZ software): open arms entries and time spent in the open arms, closed arms entries and anxiety index [1 − (frequency of entries in the open arms + permanence rate in the open arms)/2] (Cohen et al., 2008). "Entry" was considered only when the animal put the four legs in the respective arm of the apparatus.

Blood sampling and corticosterone concentration

Corticosterone levels were measured on Day 32. Approximately 150 μl of blood from the caudal vein was collected in heparinized microtubes (500 U/ml, in the proportion of 10% of the total volume of blood collected). The tubes were centrifuged for 15 minutes at 2000*g* at 4 °C and the plasma was collected and stored at −80 °C until corticosterone levels were measured using the biochemical kit (Enzo Life Sciences® – corticosterone ELISA kit), following the manufacturer's protocol.

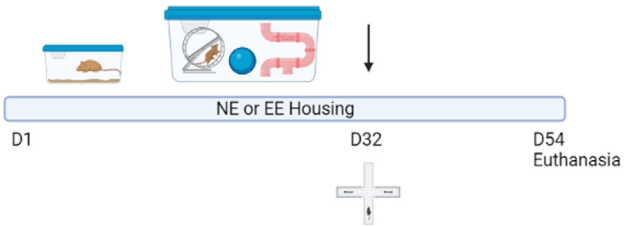

Fig. 1. Experimental design: the animals were kept in non-enriched (NE) or enriched (EE) housing 24 h/day throughout the experiment for 54 days. Arrow: blood sample collection, cross: elevated plus-maze test.

Brain tissue collection

Animals were euthanized on day 54 during the light cycle (7:00–10:00 am) at approximately the same time to minimize any circadian effects. The brains were removed, and the PFC was freshly dissected. The dissection was carried out under a microscope using a mouse brain matrix ASI-Instruments® (Houston, TX), based on the atlas of brain structures (Franklin and Paxinos, 1997), with divisions 1 mm apart, to slice the brain in coronal sections and brain punches (1.2 mm from Harris Micro-Punch, Ted Pella). The sections were immersed in *RNA later* stabilization solution (Sigma®) and stored at −80 °C.

Protein extraction and western blot assay – analysis of BDNF protein levels

BDNF protein levels were analysed in the PFC of EE (54 days) and standard housed mice to assess if any transcriptional changes in *Bdnf* exons translate into changes in BDNF protein levels in the PFC. The PFC samples were homogenized by friction with a conical plastic pestle (Thermo Fisher Scientific, MA, USA) in a 1.5 ml microtube, in lysis buffer (Tris-HCl 1 M pH 7.5, 10% SDS, glycerol, 3 M NaCl, deionized water, 500 mM NaF, 500 mM beta-glycerophosphate, protease inhibitor cocktail (Protease and Phosphatase Inhibitor) Cocktail - Thermo Fisher Scientific, Inc) and kept on ice. The samples were then sonicated at 50% of amplitude for approximately 5 seconds (Ultrasonic processor Vibra cell VC-505 – Sonics and Materials, Inc), incubated on ice for 20 minutes and centrifuged at 4 °C for 20 minutes at 13,000 *g*. The supernatant was collected, and the protein concentration was determined by the Bradford method (Biorad protein assay, Bio-Rad Laboratories, Inc) (Bradford, 1976). The extracted protein was combined with Laemmli buffer (Bio-Rad Laboratories, Inc, supplemented with 5% mercaptoethanol) and incubated at 95 °C for 5 min. The protein samples (10 μl of 2μg/μl protein) were separated by size on a 12.5% polyacrylamide SDS-PAGE gel (sodium dodecyl sulphate–polyacrylamide gel) at 90 V using Mini-Protean® Tetra Cell device (Bio-Rad Laboratories, Inc) and then transferred to the Nitrocellulose membrane (EMD Millipore Corporation). Ponceau's immunoblot method was used to ensure the load of equal proteins (Salinovich and Montelaro, 1986). The membrane was blocked with 5% bovine serum albumin (BSA) diluted in 1x TBS-T buffer (50 mM Tris–HCL, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and incubated overnight at 4 °C. In the following day the membranes were incubated with BDNF antibody (Santa Cruz Biotechnology, rabbit polyclonal IgG; N-20, sc-546, Lot#B0811) 1: 1000 and left overnight again at 4 °C. In the following day the membrane was probed with a secondary antibody (1: 2000 dilution, anti-rabbit Ac, Santa Cruz Biotechnology) for 2 h at room temperature and then developed on a ChemiDoc MP photo-documenter (Bio-Rad Laboratories, Inc). The samples were analysed with ImageLab® software (Bio-Rad Laboratories, Inc). The relative density of each band was normalized to the value of β-actin (dilution 1: 40000, Santa Cruz Biotechnology). Four samples were used for the NE and six samples for the EE group for the BDNF assay.

Bdnf gene expression assays and epigenetic analysis

The non-coding exons I, II, IV, VI and the coding exon IX were analysed, in accordance with previous studies (Fuchikami et al., 2011; Karpova, 2014; Xu et al., 2018). Our main target was exon IV considering its role in inhibitory functions in the PFC (Sakata et al., 2009).

DNA methylation profile of exon IV was analysed by pyrosequencing (PSQ) targeting 12 CpG sites.

DNA/RNA extraction

DNA/RNA isolation from the tissue samples were carried out as described in Rae et al., (2018). AllPrep DNA/RNA Mini kit (Qiagen UK) was used to simultaneously isolate DNA and RNA from the tissue samples. The thawed samples were processed in nuclease-free 2 ml safe lock tubes with a 5 mm stainless still bead (Qiagen UK) and 600 μl of RLT + lysis buffer (RLT buffer + 1% β-mercaptoethanol) per < 30 mg tissue. The samples were macerated using a tissue disruptor (TissueLyser II QIAGEN®) by subjecting them to 2–3 times 20 pulses per sec, 2 minutes each. The lysis product was centrifuged at 8000x*g* for 2 min at room temperature, the supernatant was passed through a DNA spin column to bind the DNA to the column. Equal volume of 70% EtOH was added to the pass-through liquid, mixed by repeat pipetting and then passed through RNeasy spin column. The column with the RNA bound to the matrix was washed once using Wash buffer RW1. On-column DNase 1 treatment was carried out for 15 minutes at room temperature using Qiagen RNase-free DNase 1 as per the manufacturer's protocol, followed by one more wash with RW1 and 2x RPE buffer. RNA was eluted from the column with RNase-free water. The DNA spin column was washed once each with washing buffers AW1 and 2 and DNA was eluted using EB buffer provided in the kit. The DNA and RNA were quality-checked and quantitated using a nanoscale spectrophotometer (NanoDrop® 2000 Thermo Scientific). RNA was stored at −80 °C and DNA at + 4 °C.

Reverse transcription and real-time polymerase chain reaction (PCR) (RT-qPCR)

QuantiNova Reverse Transcription kit (QIAGEN®) was used to carry out cDNA synthesis from the of RNA samples. In brief, 1 μg RNA was used per sample for cDNA synthesis. A mastermix of Reverse Transcription reactions (RT-Mix) was prepared for all samples as per the protocol. A final volume of 15ul of RNA and gDNA Removal Mix was prepared, incubated for 2 min at 45 °C and immediately transferred to ice. 5 μl of RT-Mix was added to individual RNA samples followed by incubation for 3 min at 25 °C, 10 min at 45 °C and finally 5 min at 85 °C.The resultant cDNA was stored at −20 °C.

The target DNA sequence was identified and obtained from UCSC Genome Browser GRCm38-mm10. For

qPCR, primer sets were designed using NCBI Primer Blast tool available in public domain. The qPCR was carried out with reagents from QuantiNova SYBR Green PC kit (QIAGEN®) on a 384 well plate, in triplicates, for a final volume of 5 μl per well containing 1 μl of cDNA each on a QuantiStudio 7 Applied Biosystems machine. Housekeeping gene Gapdh was used to normalise the expression of the target exons. The Real-time cycle conditions were as follows: activation step of 2 min at 95 °C, followed by step 2 of 95 °C for 5 sec and 60 °C for 10 sec for 40 cycles and a final an automated melt-curve step. *δ-ctct* method was used to get the fold-change in respective exon mRNA expression.

The following primers were used:**

Exon I:	Forward: 5′TGTCTCTCAGAATGAGGGCG3′
	Reverse: 5′CATCCACCTTGGCGACTACA3′
Exon II:	Forward: 5′CATTGAGCTCGCTGAAGTTGG3′
	Reverse: 5′CCCAGTATACCAACCCGGAG3′
Exon IV:	Forward: 5′ATGGAGCTTCTCGCTGAAGG3′
	Reverse: 5′CGAGTCTTTGGTGGCCGATA3′
Exon VI:	Forward: 5′GCGTGACAACAATGTGACTCC3′
	Reverse: 5′TCTGGCTCTCGCACTTAGC3′
Exon IX:	Forward: 5′CGACATCACTGGCTGACACT3′
	Reverse: 5′CAAGTCCGCGTCCTTATGGT3′

Bisulfite conversion and pyrosequencing for DNA methylation

Epitect Bisulfite Conversion kit (QIAGEN®) was used for the bisulfite conversion of the DNA as per the manufacturer's protocol prior to pyrosequencing to determine methylation status of targeted regions. The Bisulfite solution was prepared by briefly incubating at 60 °C, followed by vertexing to dissolve the reagents completely. A reaction volume of 140 μl was prepared containing 40 μl of 500 ng DNA, 85ul of Bisulfite Solution and 15 μl DNA protect buffer. The reaction was carried out in a thermocycler as follows: denaturation for 5 min at 95 °C, incubation for 10 min at 60 °C, followed by denaturation for 5 min at 95 °C and incubation for 10 min at 60 °C. All reagents were equilibrated to room temperature prior to use. The mixture was transferred to a 1.5 ml nuclease-free tube to which 310 μl Buffer BL and 250 μl of absolute ethanol were added, mixed well and the entire volume was transferred to a MinElute DNA spin column, centrifuged for 1 min at full-speed to bind the DNA to the column. The column was washed with BW wash buffer. The desulfonation of the bound DNA was carried out using 500 μl Buffer BD for 15 min at room temperature followed by two washed with BW buffer, one wash with 100% alcohol. The DNA was eluted from the column using 40 μl of EB buffer. The single-stranded bisulfite-converted DNA (BS-DNA) was stored at −20 °C.

Pyrosequencing and methylation profile analysis

The pyrosequencing procedure was carried out as previously described by Coley et al., 2012. The primers were designed using the PyroMark Primer Assay 2.0 software (Qiagen). A region containing 12 CpG sites (from UCSC Genome Browser, GRCm38-mm10) primarily based on previous studies with rodents (Lubin et al., 2008; Roth et al., 2009) was targeted for this study (Fig. 3).

Two μl of BS-DNA was used for a total reaction volume of 50 μl per sample, the amplification was carried out using HotStart*plus* *Taq*polymerase (Qiagen). The PCR cycles were as follows: 15 min at 95 °C, followed by 50 cycles of denaturation for 30 sec at 95 °C, annealing at 53 °C for 30 sec and extension at 72 °C for 30 sec and a final extension at 72 °C for 10 min.

Forty μl each of the amplicons were used for sequencing. The amplified, biotin-labelled DNA suspended in binding buffer and charged Sepharose beads (Amersham Plc) were captured using a hedgehog and vacuum, released on to the annealing buffer containing sequencing primer, annealed for 2 min at 80 °C prior to sequencing using a PSQ MD machine and Pyromark Gold Q96 reagents (QIAGEN®).

Details of the primers used was as follows:

Forward	5′GGTAGAGGAGGTATTATATGATAGT3′
Reverse	Bio-5′ATTTCCCCTTCTCTTCAATTA3′
Sequence 1	5′AGGAGGTATTATATGATAGTT3′
Sequence to Analyse	TA**Y**GTTAAGG TAG**Y**GTGGAG TTTTTT**Y**GTG GATTTTTATT TATTTTTTTA TTTAT**Y**GAGG AGAGGATTGT TTT**Y**GTTGT**Y** GTTTTTTTTA TTTATTTT**Y**G G**Y**GAGTTAGT ATGAAATTTT TTAGTTT
Sequence 2	5′TTTAGTTTTTGTTTAGATTAAATGG3′
Sequence to Analyse	AGTTTTT**Y**GT TGAAGG**Y**GTG **Y**GAGTATTAT TTT**Y**GTTATG TAATTTTTAT TATTAATAA

Statistical analysis

Shapiro Wilk test was used to test for normality and the unpaired t-student tests were used to compare the mean of data of the groups (NE and EE). Data are expressed as box-and-whisker-plots (median, 25th and 75th quartile; whiskers 5th-95th percentiles). Differences with $p < 0.05$ was considered statistically significant. All analyses were performed on the Statistica 12 software, and the graphs were plotted in Graphpad Prism 9.0.

RESULTS

Gene expression

Analysis of individual exon gene expression revealed a significant increase in EE compared to the respective controls, as following: exon I ($t = 2.43$, df = 10, $p = 0.03$, Fig. 2A); exon II ($t = 1.54$, df = 10, $p = 0.01$, Fig. 2B); exon IV ($t = 8.54$, df = 10, $p < 0.0001$, Fig. 2C); exon VI ($t = 4.43$, df = 10,

p = 0.0013, Fig. 2D); exon IX (t = 6.03, df = 10, p = 0.0001, Fig. 2E). The individual exon gene expression was calculated as fold change of EE against control NE. The NE was presented as individual NE samples against the average fold-change of all NE (n = 6) to show any variation in expression among the NE samples.

BDNF protein levels

BDNF protein levels were measured after 54 days of EE. No statistical difference was found between EE and NE mice (t = 0.50, df = 8, p = 0.63; Fig. 2F).

Methylation status of *Bdnf* exon IV

CpG islands are stretches that have a higher CG density compared with other genome regions which are generally not methylated (Bird et al., 1985). Most CpG islands are located in gene promoters (Saxonov et al., 2006) which in turn are highly conserved in mice and human genomes (Illingworth et al., 2010). CpG islands in promoter regions regulate gene expression through transcriptional silencing, thus, methylation in these islands can be crucial for the gene expression (Lim et al., 2019).

In our study, the increase in the *Bdnf* exon IV gene expression led us to speculate a possible hypomethylation status of CpG sites of a CpG island.

Twelve CpG sites of the exon IV of *Bdnf* gene were analysed. One animal from the NE group failed the pyrosequencing software quality control and was excluded from the analysed data. Multiple unpaired t-tests detected a statistically significant decrease in DNA methylation at CpG sites 5 and 10 in the EE group vs NE (t = 2.29, df = 9, p = 0.047; t = 2.44, df = 9, p = 0.04). Methylation levels of the other CpG sites were unchanged (Fig. 3).

Elevated Plus-Maze

Mice in enriched cage for 32 days showed higher number of entries in the closed arms compared to the non-enriched mice (t = 3.22, df = 30, p = 0.003, Fig. 4C). No differences were found between the two groups in the EPM test regarding anxiety-related parameters, i.e., % of time spent in open arms (t = 1.60, df = 30, p = 0.12), % of entries in open arm (t = 1.54, df = 30, p = 0.14), and anxiety index (t = 1.83, df = 30, p = 0.08) (Fig. 4A, B, D). One animal from the EE group jumped from the EPM and was excluded from the statistical analyses.

Corticosterone levels

Corticosterone levels were measured after 32 days of EE. No differences were found between EE and NE mice (t = 0.61, df = 10, p = 0.56, Fig. 4E).

DISCUSSION

This study aimed to dissect the transcriptional and epigenetic regulatory effect of 54-day exposure to EE on prefrontal cortex (PFC) by analysing individual exon gene expression (exon I, II, IV, VI, XI) and DNA methylation profile of exon IV of the *Bdnf* gene in C57BL/6 mice. The data showed a significant exon specific upregulation of *Bdnf* mRNA levels in the PFC concomitant with reduction in DNA methylation in certain CPG sites of exon IV of the *Bdnf* gene. Given that DNA methylation commonly causes reduced gene expression or silencing, we suggest that the hypomethylation of CpG sites 5 and 10 in exon IV in the PFC of EE mice is responsible for the increase in exon IV *Bdnf* expression. These findings are suggestive of an epigenetic control of *Bdnf* gene expression (at least at the level of exon IV), by EE exposure in the PFC and add to the current literature by further dissecting the impact of EE on *Bdnf* expression and DNA methylation in individual exons.

Increases in BDNF protein or *Bdnf* mRNA levels in the hippocampus of animals exposed to EE housing have already been reported (Falkenberg et al., 1992; Young et al., 1999; Zhang et al., 2016; Rojas-Carvajal et al., 2020). Nonetheless, studies assessing EE effects on BDNF expression in the frontal cortex are scarcer and inconsistent, with findings pointing to increase (Gelfo et al., 2011), decrease (Rueda et al., 2012) or no effect (Chen et al., 2005). The lack of consistency among the studies are related to differences in time of exposure to EE, species (rats in Gelfo et al., 2011; Chen et al., 2005 and mice in Rueda et al., 2012) and variability of enrichment factors (Simpson and Kelly, 2011). Interestingly and in contrast with EE, maltreatment stress in infancy (stress-abusive mother) was shown to increase *Bdnf* DNA methylation in exon IV and IX, an effect which was concomitant with a reduced total *Bdnf* mRNA (exon IX) in the adult PFC (Roth et al., 2009), demonstrating a contrasting epigenetic effect of stress on PFC *Bdnf* depending on stress type (e.g., environmental enrichment ‘‘eustress” vs maternal maltreatment stress).

The current study showed significant EE-induced changes in CpG sites within BDNF exon IV in the PFC which was concomitant with upregulation of BDNF gene expression of certain exons. Likewise, Zajac et al. (2010) described an EE-induced upregulation in BDNF gene expression in the hippocampus. Interestingly, this upregulation was independent of the extent of DNA methylation along the BDNF gene sequence based on analysis of overall levels of methylation of 4 CpG sites of a CpG island (Zajac et al.,2010). Also, Tomiga et al (2021) detected an exercise-induced hypomethylation in the *Bdnf* promoter IV in the hippocampus at different CpG sites, and Tadic et al (2014) found hypomethylation in the *Bdnf* promoter IV in blood cells of depressed patients in yet different CpG sites, highlighting that the position of CpG methylation status in the Bdnf promoter of exon IV may change depending on the brain regions or environmental conditions (e.g., psychosocial stress, enriched ambient, exercise). Despite the slight differences in the methylation status of CpG positions in the Bdnf promoter IV and exon IV, the vast majority of studies in the literature have consistently reported a strong negative correlation between the transcript levels and methylation status (i.e., increased transcription and

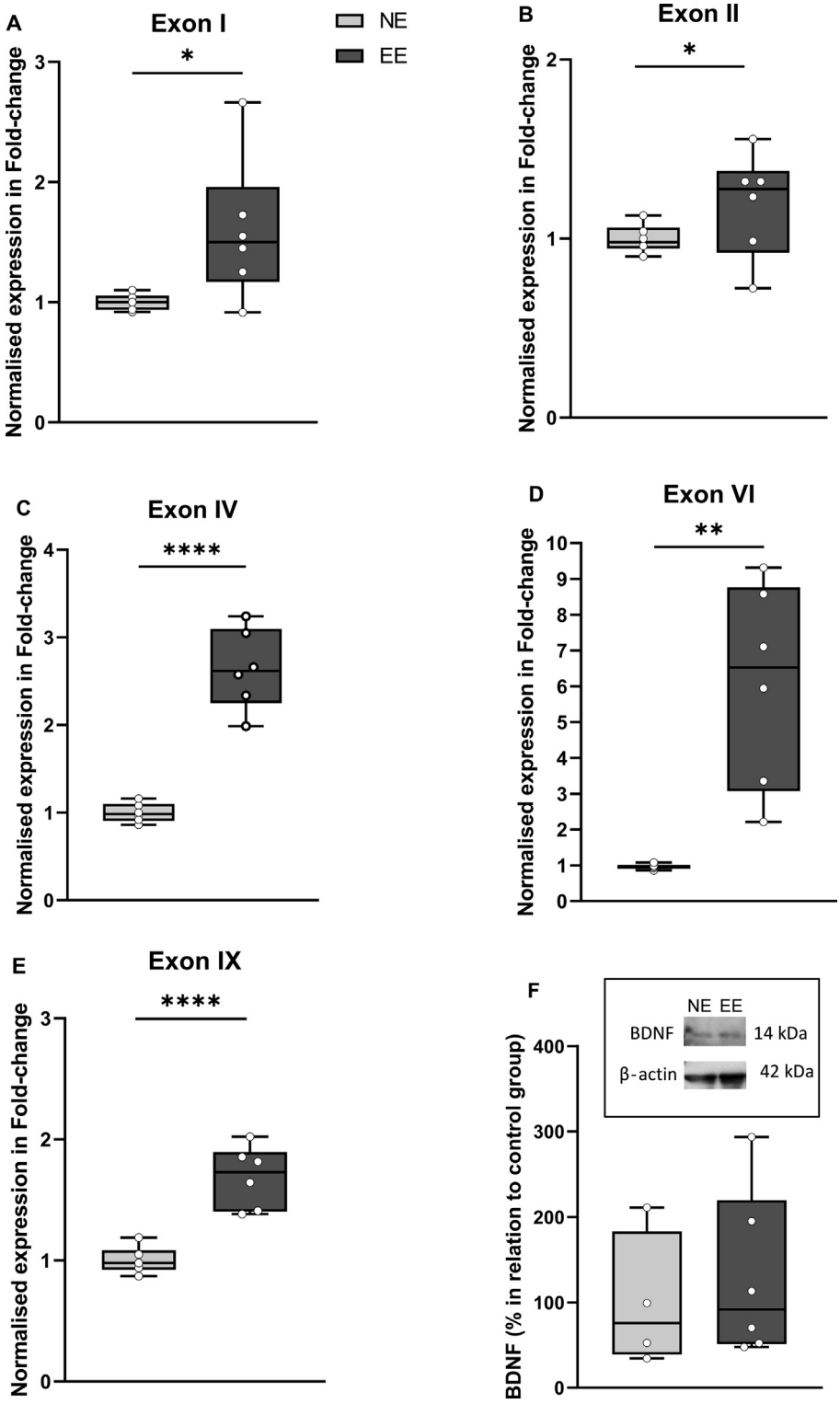

Fig. 2. *Bdnf* exons expression (I, II, IV, VI, IX; n = 6/NE and n = 6/EE) **(A–E)** (the expression is presented as fold change against NE where NE is presented as individual samples against the average fold-change of all the samples); BDNF levels (optical density normalized in relation to control) and a representative image of western blot (n = 4–6/group) **(F)** in the PFC of mice housed in non-enriched (NE) or enriched (EE) conditions for 54 days. Data are expressed as box-and-whisker-plots (median, 25th and 75th quartile; whiskers 5th–95th percentiles). Unpaired t-student test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

hypomethylation or decreased transcription and hypermethylation).

All in all, our findings corroborate with a significant body of literature demonstrating that exon IV *Bdnf* expression can be altered by environmental stimuli via epigenetic mechanisms (Martinowich et al., 2003; Aid et al., 2007; Roth et al. 2009; Sakharkar et al., 2016). Indeed, Roth et al. (2011) reported that traumatic experiences such as continuous psychosocial stress can elicit hypermethylation in exon IV and reduce *Bdnf* gene expression in the hippocampus of rats. Interestingly, a previous study from the same group demonstrated that negative maternal behavior and increases in methylation of exon IV in the PFC perpetuated across generations, suggesting a transgenerational epigenetic effect on the exon IV induced by negative psychosocial environmental factors (Roth et al., 2009). In addition, *Bdnf* exon IV promoter methylation has been considered as a marker for treatment response to antidepressant in patients with major depressive disorder (Lieb et al., 2018), suggesting a key role for exon IV epigenetic regulation in shaping mood features and treatment efficacy. The behavioral impact of this hypomethylation of exon IV detected in our study is not clear and warrants further investigation but given the association of exon IV methylation with depression, one can only speculate that it is likely to be involved in mood enhancement and wellbeing. Patients with depression showed increased methylation levels of promotor IV compared to healthy controls (Kang et al., 2013; Januar et al., 2015; Kang et al., 2015). Hence, it is not perhaps surprising that interventions that upregulate BDNF pathways exert beneficial antidepressant effect.

Whether EE-induced *Bdnf* exon IV regulation in the PFC affects emotional behaviour and mental state is not clear but there is evidence to suggest that it may involve alterations to PFC activity. Exon IV is particularly sensitive to neuronal activity (Martinowich et al., 2003) and is rapidly transcribed in

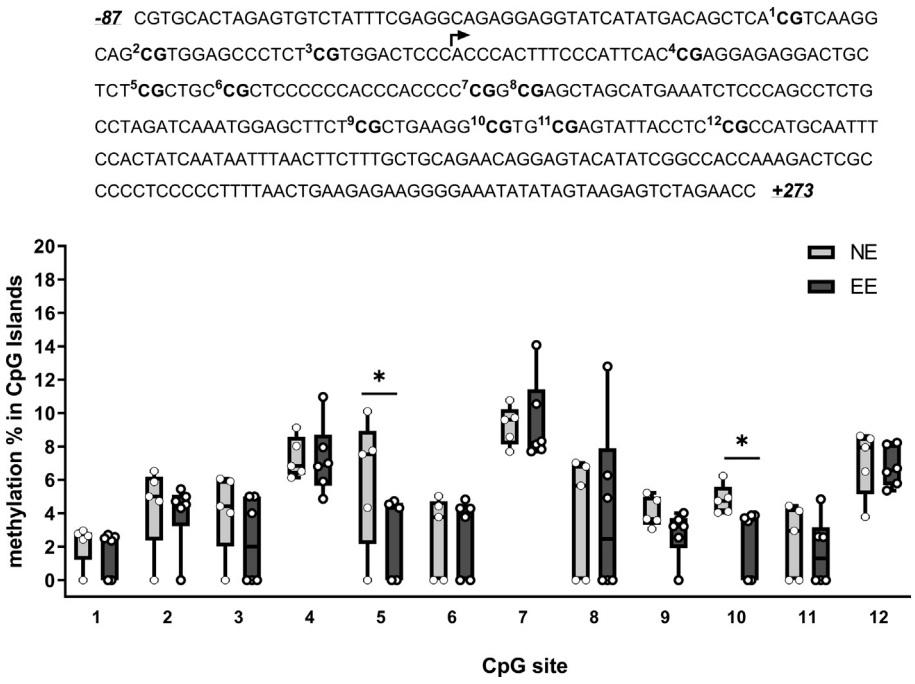

Fig. 3. Methylation status in a CpG island of *Bdnf* exon IV in the PFC of mice housed in non-enriched (NE) or enriched (EE) conditions for 54 days. The targeted sequence map and position of CpG sites relative to the transcription start site (bent arrow) of exon IV are shown on the top. Data are expressed as box-and-whisker-plots (median, 25th and 75th quartile; whiskers 5th–95th percentiles). Multiple unpaired t-student tests, $*p < 0.05$, $n = 5–6$/group.

response to stress (Marmigère et al, 2003). It contains an anchorage site for CREB which is involved in the regulation of BDNF transcription by a mechanism involving calcium influx, mediated by a calcium-responsive transcription factor (CaRF) (Tao et al., 1998; Tao et al., 2002; Dias et al., 2003). Interestingly, promoter IV *Bdnf* mutant mice which would inevitably affect epigenetic regulation of *Bdnf* expression, was shown to exhibit altered PFC function, by mechanisms related to GABAergic interneurons dysfunction (Sakata et al., 2009), clearly suggesting a link between epigenetic regulation of this gene and PFC activity. Deficits in GABAergic inhibitory neurotransmission in the PFC has been associated with psychiatric disorders, especially schizophrenia and post-traumatic syndrome disorder (PTSD) (Bremner et al., 2000; Egerton et al., 2017), thus pointing towards a potential mechanism associating exon IV and its epigenetic regulation with the pathophysiology as well as the treatment of stress-related mood disorders. Whether the hypomethylation of exon IV induced by EE observed in our study offers a protection against mental health disorders remains to be determined.

In concordance with our findings, Kuzumaki et al (2011) also showed that EE can increase *Bdnf* mRNA expression in the hippocampus, demonstrating a similar EE-induced epigenetic mechanism controlling exon IV in both the PFC and the hippocampus. Indeed, methylation process affects the interaction between DNA and chromatin proteins or transcription factors, blocking the gene transcription and expression.

Exons I, II and VI were also upregulated in the PFC of EE mice. Unlike exon IV, exons I and II does not seem to be involved in stress responses since its expression is not affected by an acute stress (Molteni et al., 2009). An upregulation of exons I, II, IV and VI suggest a generalized increase in mRNA transcripts induced by EE in the PFC, despite the variation in the distribution of Bdnf splice variants in the soma and dendrites (Chiaruttini et al., 2008). While exon I and IV were localized in the somatic cellular, exons II and VI were found in dendrites in response to pilocarpine (Chiaruttini et al., 2008). Similar to our study, voluntary physical exercise – also considered an EE – induced a specific enhancement in exon VI expression in the somata and dendrites of hippocampal regions (Baj et al., 2012). Nevertheless, the behavioural impact of this enhancement of exon VI transcription in the PFC warrants further investigation.

Although EE induces altered gene expression of different exons related to *Bdnf* transcription, it did not significantly alter the protein levels of BDNF. Changes in mRNA levels does not always go hand in hand with changes in protein levels and the mechanism to which mRNA levels correlates with differences in protein expression commonly vary among different studies. Some cases report a poor correlation – around 40% of protein levels explained by mRNA levels (Tian et al., 2004; Vogel et al., 2010; Schwanhäusser et al., 2011) or even less than 20% (Ingolia et al., 2009) – while others can show high correlation - around 80% (Li, Bickel, and Biggin, 2014). As such, whether regulation at the translational level influences on global protein abundance or whether it is restricted to a subset of genes remains unclear (de Klerk and 't Hoen, 2015).

Despite the profound changes in *Bdnf* gene expression in the PFC of mice housed in EE conditions, exposure to EE did not alter anxiety like behaviour in the EPM test. The fact that EE did not modify the anxiety-like behavior may not be surprising considering that the beneficial effects of EE on anxiety-like behavior is more evident when the animals are challenged with a stressor or in animal models of anxiety susceptibility (Renoir et al, 2011; Ravenelle et al., 2013; Koe et al, 2016), which is clearly not the case in our model. Interestingly, Sakata et al., (2010) failed to detect any changes in anxiety behavior as measured by EPM in mice deficient in exon IV suggesting that changes in *Bdnf* gene expression may not contribute to alteration in anxiety levels, at least at the level of exon IV. Moreover, the ability of EE to alter basal anxiety levels depends on the age in which animals are exposed to the EE, length of time of exposure and animal strain; for instance, Chapillon et al (1999) found lower

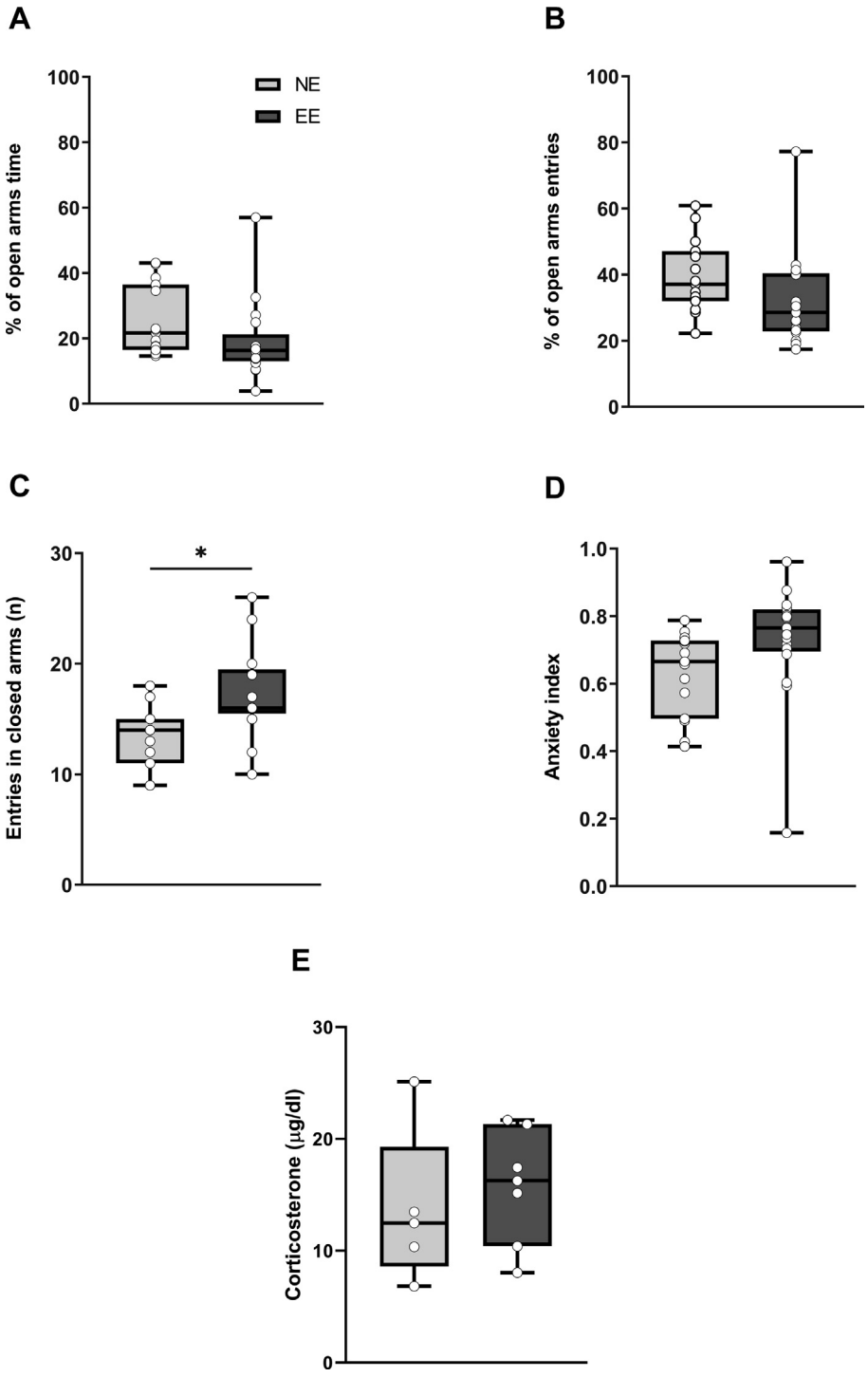

Fig. 4. Anxiety like-behavior evaluated in the elevated plus-maze (**A–D**; $n = 15$/NE and $n = 17$/EE) and plasma corticosterone levels (**E**; $n = 5$/NE and $n = 7$/EE) in mice housed in non-enriched (NE) or enriched (EE) conditions for 32 days. Data are expressed as box-and-whisker-plots (median, 25th and 75th quartile; whiskers 5th–95th percentiles). Unpaired t-student test; $^*p < 0.05$.

trait anxiety profile in BALB/c adult mice reared in EE since infancy while in C57BL/6 mice reared in the same condition, did not modify the level of trait anxiety, corroborating our findings. Moreover, we found that 32 days EE also did not alter the plasma corticosterone levels, suggesting that EE did not trigger a hypothalamic–pituitary–adrenal axis (HPA) response. This agrees with previous studies which were unable to detect a HPA axis stimulation following one (Lopes et al., 2018) or four weeks (McQuaid, Audet and Anisman, 2012) of EE. Together, these findings highlight that our EE model was unlikely to affect stress levels in mice. One ought to be, however, a bit cautious as anxiolytic effects of EE have been reported using other experimental tests, such as open-field and dark-light box (Chourbaji et al., 2005; Jha et al., 2016). Thus, even though we did not find evidence of anxiety-like changes in our EE protocol using the EPM test, this possibility cannot be rule out.

Epigenetic modifications represent key mechanisms by which negative environmental factors (e.g., stressors) induce enduring changes in gene expression which participate in the onset of various psychiatric disorders. *Bdnf* is one of the key genes which are known to undergo long-lasting epigenetic changes in response to negative environmental challenges, especially when these occur during early development (Boulle et al., 2012). As such deficiency in epigenetically controlled BDNF signalling seems to play a central role in the course and development of various neurological and psychiatric disorders (Boulle et al., 2012). Thus, strategies that modify and reverse the impact of negative environmental challenges on epigenetic regulation at specific *Bdnf* exons may represent a promising strategy for the treatment of psychiatric disorders. The current findings from this study suggest EE exposure as one such strategy through a *Bdnf* exon specific epigenetic mechanism. However, by limiting our analysis to exon IV it was not possible to provide a global picture of the methylation status of other exons. More studies are warranted to determine its functional and behavioral significance which would determine its translational value.

AUTHOR CONTRIBUTIONS

GAC: experimental design, behavioral experiments, molecular assays, data analysis, paper writing; **NKGTS, PM:** biochemical experiments and data analysis; **PC:** primers design, molecular and epigenetic assays, paper writing; **AB:** experimental design, paper writing and epigenetic assays; **RC:** experimental design, paper writing, experiment supervision.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 1 was created with BioRender.com.

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