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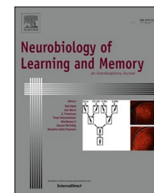

Modulation of synapse-related gene expression in the cerebellum and prefrontal cortex of rats subjected to the contextual fear conditioning paradigm

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ABSTRACT

The contextual fear conditioning (CFC) paradigm is the most productive approach for understanding the neurobiology of learning and memory as it allows to follow the evolution of memory traces of a conditioned stimulus and a specific context. The formation of long-term memory involves alterations in synaptic efficacy and neural transmission. It is known that the prefrontal cortex (PFC) exerts top-down control over subcortical structures to regulate behavioural responses. Moreover, cerebellar structures are involved in storing conditioned responses.

The purpose of this research was to determine if the response to conditioning and stressful challenge is associated with alterations in synapse-related genes mRNA levels in the PFC, cerebellar vermis (V), and hemispheres (H) of young adult male rats. Four groups of Wistar rats were examined: naïve, CFC, shock only (SO), and exploration (EXPL). The behavioural response was evaluated by measuring the total freezing duration. Real-Time PCR was employed to quantify mRNA levels of some genes involved in synaptic plasticity. The results obtained from this study showed alterations in gene expression in different synapse-related genes after exposure to stressful stimuli and positioning to new environment. In conclusion, conditioning behavioural stimuli change the expression profile of molecules involved in neural transmission.

1. Introduction

Fear is an adaptive mechanism that plays a crucial role in animal survival as it helps the subject to defend against potential dangers. One of the crucial challenges that animals have to face is how to detect and react to environmental threats. Classical conditioning involves learning a relationship between two stimuli, and it is an evolutionarily conserved learning form that animals use to counteract dangers. Pavlovian fear conditioning is a well-established learning model for exploring the neurobiological mechanisms of fear and anxiety disorders, including post-traumatic stress disorder (Indovina, Robbins, Núñez-Elizalde, Dunn, & Bishop, 2011; Morrison & Ressler, 2014).

In the fear conditioning model, the association of a stimulus with a threat evokes a typical defensive response. In particular, the contextual fear conditioning (CFC) paradigm is routinely used to study fear-based learning in rodents. It is an interesting tool that can provide new insights into the neurobiology of fear-related conditions through the identification of functional neural circuitry and molecular signalling

Abbreviations: Bdnf, Brain-Derived Neurotrophic Factor gene; CFC, Contextual Fear Conditioning; CS, Conditioned Stimulus; EXPL, Exploration; *Gapdh*, Glyceraldehyde-3- phosphate dehydrogenase gene; H, Cerebellar Hemispheres; *Ntrk2*, neurotrophic receptor tyrosine kinase 2 gene; PP1, protein phosphatase 1; *Ppp1cc*, serine/threonine protein phosphatase 1 catalytic subunit gamma gene; PFC, Prefrontal Cortex; *Reln*, Reelin gene; SO, Shock Only; *Trim32*, tripartite motif-containing 32 gene; US, Unconditioned stimulus; V, Cerebellar Vermis; *Ywhaz*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide gene.

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pathways. In addition, CFC is critical for understanding normative and pathological fear (Fanselow, 2000; Phillips & LeDoux, 1992; Sacchetti, Lorenzini, Baldi, Tassoni, & Bucherelli, 1999; Takahashi, 2011).

The CFC paradigm enables studying the behavioural response following the association of a conditioned stimulus (CS) and an unconditioned stimulus (US). In the established CFC, an emotionally neutral CS is coupled with an US (*e.g.,* foot-shock), during the acquisition phase. CS causes a conditioned fear response without presentation of the US because it acquires aversion reinforcing properties. In conditioned rats, a single training session induces, during the retrieval test, a behavioural response of immobility (*freezing*) as indicative of a fear-like state. This behaviour indicates that the animal learns and maintains the association between the new environment (CS) and the US, and this conservation lasts for a long time. A CS (*e.g.*, context or auditory stimulus or light) is chosen because it elicits either no overt or a weak response unrelated to the response that will eventually be learned. In contrast, the US (*e.g.*, a mild electrical foot shock) evokes a clear, consistent, innate, and unconditioned reaction (*e.g.,* withdrawal of the leg).

It is recognised that the formation of long-term memory, such as conditioning, entails alterations in synaptic efficacy produced by modifications in neural transmission (Lamprecht & LeDoux, 2004; Mahan & Ressler, 2012). These modifications are given by variations in gene expression and the synthesis of new proteins (Kandel, 2001). Many aspects of learning and storing memory in the mammalian brain involve cellular and molecular mechanisms, which are driven by the specific gene expression of neural circuits. In particular, fear memory is regulated by a complex neurocircuitry, consisting of the hippocampus, amygdala, and medial prefrontal cortex (Myers & Davis, 2007; Tronson et al., 2008; Maren, 2011; Orsini & Maren, 2012; Bocchio, McHugh, Bannerman, Sharp, & Capogna, 2016).

Changes in hippocampal gene expression are essential for the generation and consolidation of CFC memory traces. Several studies have shown that the dorsal hippocampus and, more generally, the mid-temporal regions are closely associated with memory development and consolidation (Federighi et al., 2013; Simbriger et al., 2021). Genetic and epigenetic modifications, especially DNA methylation and demethylation, and histone acetylation, underlie the formation and stabilization of CFC in the hippocampus and amygdala (Zovkic & Sweatt, 2013). In particular, epigenetic regulation of Brain-derived neurotrophic factor (*Bdnf)* in response to fear conditioning is described in the amygdala during the consolidation phase (Ou & Gean, 2007). BDNF is a neurotrophin that is highly expressed in the hippocampus (Blöchl & Thoenen, 1995) and is recognised as a key growth factor for the survival of cerebellar neurons and for regulating synaptic plasticity in the central nervous system (CNS) (Chaaya et al., 2021; Choi et al., 2010; Cunha, 2010; Rosas-Vidal, Do-Monte, Sotres-Bayon, & Quirk, 2014). Indeed, mice with a lack of dysfunction of the *Bdnf* gene showed defects in synaptic transmission (Liu, Lyons, Mamounas, & Thompson, 2004). A reduction in *Bdnf* expression has been observed in various neuropsychiatric as well as in neuroinflammatory (Taticchi et al., 2019) and in neurodegenerative conditions, including depression, bipolar disorder, schizophrenia, Alzheimer's and Parkinson's diseases (Conte, Sichetti, & Traina, 2020; He, Zhang, Yung, Zhu, & Wang, 2013; Hyun Kim, 2015; Zuccato & Cattaneo, 2007).

There are evidence that cerebellar and cortical structures are involved in fear learning. Recent human studies using nuclear magnetic resonance have demonstrated the involvement of the cerebellar hemispheres (H) and vermis (V) in associative learning, including CFC (Ernst et al., 2019; Lange et al., 2015). Studies in rodents have suggested that cerebellar structures are implicated in the storage of conditioned responses. Particularly, the role of the *nucleus interpositus* and the cerebellar V in fear conditioning consolidation was investigated (Kim & Thompson, 1997; Supple & Leaton, 1990). A previous study reported that the functional integrity of some regions of the cerebellum is necessary for the consolidation of fear conditioning (Sacchetti, Baldi, Lorenzini, & Bucherelli, 2002).

The prefrontal cortex (PFC) is crucial for the temporal organization of behaviour and it is thought to exert top-down control over subcortical structures to regulate appropriate behavioural responses (Giustino & Maren, 2015). Previous observations have reported the modulation of gene expression at the level of the prefrontal region in rats subjected to CFC, suggesting that this region plays a significant role in emotional behaviour, stress response, apoptosis, and learned fear (Albi et al., 2019; Morrison & Ressler, 2014; Quirk & Mueller, 2008; Santini, Quirk, & Porter, 2008; Sotres-Bayon & Quirk, 2010). However, the specific contributions and functions of the cerebellum and PFC in fear conditioning are not yet fully understood.

The objective of this study was to evaluate, in the CFC paradigm, the expression of some genes involved in synaptic plasticity, *i.e.*, *Bdnf*, serine/threonine-protein phosphatase 1 catalytic subunit gamma (*Ppp1cc),* neurotrophic receptor tyrosine kinase 2 *(Ntrk2),* reelin (*Reln),* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*Ywhaz),* and tripartite motif-containing 32 (*Trim32)* genes. These genes were chosen since it was previously demonstrated that conditioning modulates their expression in other brain regions. In particular, *Bdnf*, *Reln*, and *Ppp1cc* genes were found modulated in the hippocampus (Miller & Sweatt, 2007). The gene encoding for BDNF-receptor *Ntrk2* was chosen to evaluate relative changes in gene expression in the agonist-receptor pair. In a previous study, transcriptional analysis performed 2 days after CFC by applying the suppressive subtractive hybridization method allowed us to identify a pool of genes differentially expressed in the mid-temporal regions of CFC compared to naïve rats. Results revealed that the expression of different genes, including *Ywhaz* and *Trim32* involved in several neuronal processes, such as cell growth, apoptosis, cell migration, neurogenesis and neuron differentiation, was positively modulated by CFC in the mid-temporal regions (Federighi et al., 2013; Federighi et al., 2018).

In the present study, these genes were examined in order to investigate whether CFC, exploration (EXPL), and shock-only (SO) stimuli affect their expression in the cerebellar V, H, as well as in the PFC in order to evaluate, at the 48 h time point, the engrams during the consolidation and retention of the freezing response.

2. Materials and methods

2.1. Animals

A total of 40 male Wistar rats (70-day-old, Harlan, Udine, Italy) were employed. They were housed at 25 °C on a 12 h light/12 h dark cycle with water and food ad libitum for the duration of the treatment. The experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Florence (Italy) (Prot. N. 77/2008). In order to minimize the hormonal fluctuation, females were not employed in the present work.

2.2. Measurement of learning and memory in the Fear-Conditioning paradigm

The rat conditioning procedure was reported in previous papers (Albi et al., 2019; Federighi et al., 2013; Federighi et al., 2018). The CFC is a form of learning in which the aversive stimulus (*i.e.*, an electrical shock) is associated with a particular neutral context (*i.e.,* a square-conditioning cage).

A basic Skinner box module was used to induce freezing. Electric shocks whose number, duration and inter-trials interval were predetermined with a stimulus programming device (Scatola di comando Arco 2340, Ugo Basile, Comerio, Varese, Italy) connected to the apparatus, were delivered by stainless steel rods on the floor of the box

connected to a shock scrambler delivery apparatus (Grid Floor Shocker, model E13-08, Coulbourn). The top and two opposite sides of the box consisted of aluminium panels, while the other two were composed of transparent plastic panels. The conditioning apparatus was placed in an acoustically insulated room and kept at a constant temperature of 20 ± 1 °C. Illumination inside the room was 60 lx.

Forty rats were divided into four groups. One group (conditioned group, n = 10) was subjected to CFC, while the other group (control group, n = 10) never left the home cage (naïve) and served as control. Ten rats were subjected to a SO procedure and 10 rats were subjected to an EXPL procedure. Briefly, in CFC, the rats were taken manually from the domestic cage, and transported from the housing room to the soundproof room and, therefore, inside the conditioning apparatus. The rat remained undisturbed for 3 min to become familiar with the new environment and then subjected to a series of seven electric foot-shocks (1-sec duration, intensity 1 mA) administered at 30-sec intervals. Two minutes after the end of the stimulation, the animal was taken back to the home cage, thus spending a total of about 8 min inside the conditioning apparatus. For the SO procedure, the rat was manually removed from the domestic cage, carried to the soundproof room, and placed within the conditioning apparatus. Seven foot-shocks (1-sec duration, 1 mA intensity) were delivered to the rat at 3-sec intervals. At the end of the stimulation, the animal was returned to its home cage. The SO protocol does not allow the association of the CS with the US (Sacchetti et al., 2001; Sacchetti et al., 2002). For the EXPL procedure, which results in the memorization of the new context, the rat was manually removed from the domestic cage and brought into the soundproof chamber. Then, it was placed inside the conditioning apparatus, where it was left undisturbed for 8 min before being returned to the home cage.

Two days after conditioning, the rats of each group were subdivided into two subgroups, one subgroup (n = 5) was behaviourally tested for the measurement of retention levels and the other subgroup (n = 5) was sacrificed to obtain brain samples. Forty-eight hours after experimental procedures (CFC, EXPL and SO), one conditioned subgroup was subjected to 3 min conditioning box-free exploration, and we proceeded to measure the freezing response. The behaviour of rats was recorded using a closed-circuit television system. The total amount of freezing (in sec) during the 3 min period (total freezing) was measured manually with a stopwatch. All experimental procedures were performed between 10.00 and 13.00 h to minimize circadian influences.

2.3. Real-Time PCR

Total RNA was isolated from PFC, H and V using RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. The concentration of RNA was evaluated by measuring the absorbance at 260 nm in a spectrophotometer. One microgram of RNA was used to synthesize cDNA by Revertaid Premium First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-Time PCR (RT-PCR) was performed in a Light Cycler instrument (Applied Biosystems, Waltham, MA, USA) using a FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). The primer sequences for gene amplification were listed in Table 1. Relative gene expression was normalized to the Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) housekeeping gene. All primers were obtained from Invitrogen (Invitrogen Ltd, Waltham, MA, USA). SYBR® Green PCR amplifications were carried out in a 96-well plate in a 25 µL reaction volume containing 12.5 µL of Master Mix, 1 µL of each forward and reverse primer (400 nM), 8.5 µL water PCR grade and 2 µL of cDNA. Each sample had three replicates. The thermal profile was 95 °C for 10 min, followed by 45 cycles of denaturation for 10 sec at 95 °C, a 20-sec annealing step at 60 °C, and 1 min extension at 72 °C. mRNA relative expression levels were calculated as $2^{-\Delta\Delta Ct}$.

Table 1
Primer sequences used in the Real-Time PCR.

Accession Number	Gene name	Gene symbol	Primer sequences (F: forward; R: reverse)
NM_017008.4	Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	F: GCGAGATCCCGCTAACATCA R: CTCGTGGTTCACACCCATCA
NM _080394.3	Reelin	*Reln*	F: CGTCCTAGTAAGCACTCGCA R: GGTATCGCCTAAGCGACCTT
NM_012513.4	Brain-derived neurotrophic factor	*Bdnf*	F: TACCTGGATGCCGCAAACAT R: TGGCCTTTTGATACCGGGAC
NM_022498.2	Serine/threonine-protein phosphatase 1 catalytic subunit gamma	*Ppp1cc*	F. CCCAACTACTGTGGCGAGTT R. TTGTGATCATACCCCGTGGC
NM_013011.4	Tyrosine 3-mono-oxygenase/tryp-tophan 5-monooxygenase activation protein, zeta polypeptide	*Ywhaz*	F: GGCAGAGCGATACGATGACA R: AAGATGACCTACGGGCTCCT
NM_001012103.1	Tripartite motif containing 32	*Trim32*	F. CCGACTTGCCCAATCTCACT R. CACAACAAACTGGCCAGACG
NM_012731.3	Neurotrophic receptor tyrosine kinase 2	*Ntrk2*	F. ACGTCACCAATCACACGGAG R. TCCGTGGAGGGGATCTCATT

2.4. Statistical analysis

In order to detect any differences between the averages of the behavioural experiments, the one-way ANOVA was used and then, in the *post hoc* analysis phase, the test for multiple comparisons with Tukey's confidence interval correction was performed.

As concerns RT-PCR, fold change ($2^{-\Delta\Delta Ct}$) values, after normalization with *Gapdh*, were grouped and described for their mean ± standard error of the mean (SEM), after which the presence of outliers in the distribution was assessed using the boxplot method. Before carrying out the ANOVA followed by Tukey's *post hoc* analysis, normality requirements were assessed using the Shapiro-Wilk test and homogeneity of variances using the Levene test to assess the variance when faced with 2+ groups. The correlations matrices show the Spearman's *rho* statistic to estimate the rank-based measure of association between the different genes in different conditions.

Statistics analyses were carried out using R software (version 4.1.2, R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) and results were considered statistically significant if p-value < 0.05. Graphs reported in the present work were obtained using Microsoft Excel, 2016 and R (gplots and ggplot2 packages).

In particular, boxplots showing gene expression among different treatments and Tukey's *post hoc* analysis details are reported in the supplementary material, appendix 1 to 6, and 7, respectively.

3. Results

3.1. Fear conditioning measurement in freezing duration

At the retention test, rats of the subgroup assigned to behavioural measures exhibited marked fear conditioning according to previous

experiments (Federighi et al., 2013; Federighi et al., 2018); the *freezing* response of the CFC rats lasted about 75–80 % of the total time of exposure to the conditioning context cage. One-way ANOVA was performed to evaluate if freezing was different between the four groups: naïve (n = 5), CFC (n = 5), SO (n = 5), and EXPL (n = 5).

Freezing was statistically different between different groups, $F(3,16) = 105.6$, $p < 0.0001$, generalized eta squared 0.95. Freezing (in sec) was higher in the CFC group (139 ± 18.8) if compared to the other groups. We found that the other three groups, *i.e.,* naïve (21.6 ± 7.83), EXPL (21.6 ± 8.79) and SO (22.2 ± 12.5) have similar values. Tukey's *post hoc* analysis revealed that the increased values of the CFC group were statistically significantly higher than the values shown in the other three groups (*p*-value: < 0.0001), no other differences were observed between naïve, SO and EXPL groups (Fig. 1).

3.2. Effects of CFC, SO and EXPL on gene expression

The expression of *Bdnf, Ppp1cc*, *Ntrk2, Reln*, *Ywhaz* and *Trim32* genes was investigated by RT - PCR (Fig. 2).

The results show significant differences for almost all the analysed genes in all three areas considered (PFC, H, V). In particular, with respect to naïve, in PFC region the expression of *Bdnf* was significantly downregulated by CFC and SO (*p*-value = 0.0002 and 0.0019, respectively), while *Ppp1cc* (*p*-values = 0.0032 and 0.0046), *Ntrk2* (*p*-values = 0.0028 and 0.0003), *Reln* (*p*-values = 0.0021 and 0.0006), and *Ywhaz* gene (*p*-value = 0.0074 and 0.0001) expression resulted markedly increased. *Trim32* mRNA levels were not modified neither by EXPL and CFC nor by SO. A different trend was observed in H cerebellar area where only SO treatment induces a mild increase in *Bdnf* gene expression with respect to naïve animals (*p*-value = 0.0383). On the contrary, in the same region, significant decrease in *Ppp1cc*, *Ntrk2, Reln* and *Ywhaz* gene expression was observed in rat subjected to SO and EXPL when compared with naïve. CFC induced a decrease for *Ppp1cc* and *Ywhaz* mRNA. Again, *Trim32* gene expression was not changed by the three experimental protocols. In V cerebellar region, compared with naïve group, CFC causes a downregulation of Ntrk2 gene (p-value = < 0.05). Moreover, Ppp1cc, Ntrk2, and Ywhaz are significantly downregulated by EXPL ($p < 0.0076$, 0.0005, and 0.0048, respectively), while Bdnf is increased by the same group ($p < 0.05$).

Fig. 3 shows logarithms of Fold Change values for the considered genes in the PFC, H and V areas for CFC, SO and EXPL treatments compared to naïve. The results are clustered and visually ordered from those clusters that shows higher gene expression (at the top of the graph) to those showing lower expression than naïve subjects. The cluster has been generated using the complete aggregation method, it is possible to note that four genes clustered together when evaluated in both the PFC and H zones, although in different directions. In fact, the *Ntrk2, Reln, Ywhaz* and *Ppp1cc* genes show higher expression than naïve in the PFC and lower expression in the H for almost all treatments. However, only the CFC and SO-treated rats in the PFC and the SO and EXPL-treated rats in the H for *Ntrk2* and *Reln* showed statistically significant alterations, whereas all treatments downregulated *Ppp1cc* and *Ywhaz* in the H. The *Trim32* and *Bdnf* genes, on the other hand, clustered with each other for their behaviour in the H and V zones.

The correlation matrices in Appendix 8 show Pearson's r coefficients to show functional connectivity between the various genes and treatments within the 3 areas considered. The SO-treated *Bdnf*, *Ntrk2*, *Reln* and *Ywhaz* genes show considerable inverse correlations with respect to the remaining genes and treatments in the PFC area. In H area, all genes correlate positively with each other with the exception of CFC-treated *Trim32*, SO-treated *Ppp1cc* and EXPL-treated *Ntrk2*. In area V, an inverse correlation can be seen between the SO-treated *Bdnf*, *Ntrk2*, *Reln*, *Trim32* and *Ywhaz* genes compared to the entire EXPL-treated gene pool and the CFC-treated *Bdnf*, *Ntrk2* and *Ppp1cc*.

4. Discussion

The present study was conducted in animals 48 h after conditioning, in line with the time that was adopted in the previous study (Federighi et al., 2013; Traina et al., 2008; Traina, Federighi, & Brunelli, 2008), in which genes differentially expressed in the mid-temporal regions were identified in the animals subjected to CFC through suppressive subtractive hybridization assay (Traina et al., 2008; Traina et al., 2008). The suppressive subtractive hybridization procedure that was employed in that study is a powerful technique that has allowed to compare two mRNA populations and obtain clones of genes expressed in one population but not in the other (Traina et al., 2011). Through this method, it was possible to identify a wide variety of genes differentially expressed in the mid-temporal regions of conditioned versus naïve rats (Federighi et al., 2013). Here, the expression of some genes involved in synaptic plasticity was evaluated. Among these genes, we selected *Ywhaz* and *Trim32* as they were previously found to play a role at the level of the mid-temporal regions in CFC-treated rats (Federighi et al., 2013). The former encodes 14–3-3ζ protein and is involved in neurogenesis and neuronal migration, histamine secretion by mast cell, protein targeting to the mitochondrion, and regulation of cell death. The latter plays

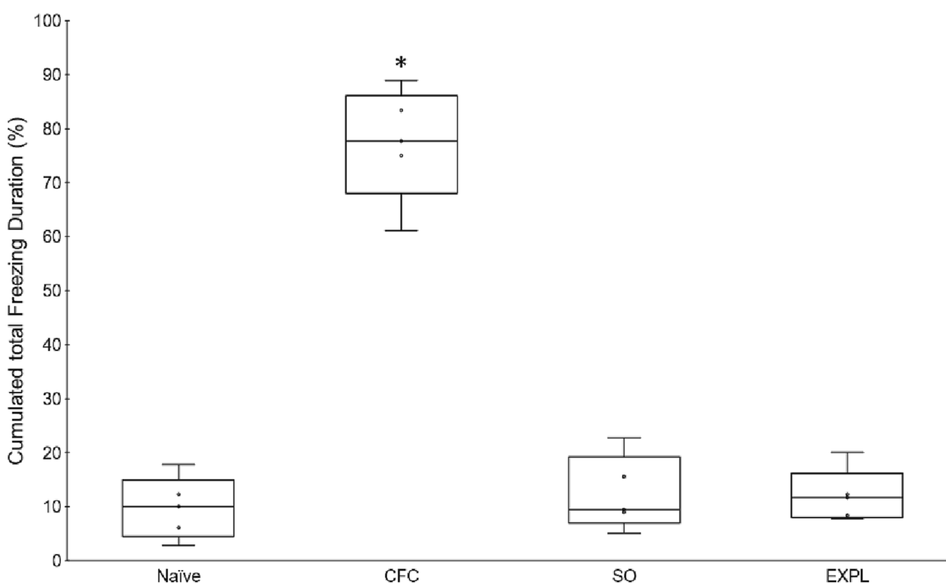

Fig. 1. The freezing duration during retrieval testing was performed 2 days after conditioning. For retrieval testing, all rats were placed for 3 min in the apparatus and the cumulated total freezing duration was measured. Freezing was defined as the complete absence of somatic motility, and respiratory movements excepted. Conditioned rats (n = 5, CFC), which had received the electric foot-shocks during the 8 min period spent inside the apparatus, exhibited freezing for about 77 % of the time, whereas the rats never before placed in the apparatus (n = 5, naïve), the rats that had received 7 electric foot-shocks condensed in 30 sec (n = 5, SO) and the rats that had freely explored the apparatus for 8 min (n = 5, EXPL) exhibited a freezing duration of 12 %. Statistical analysis: one-way ANOVA followed by Tukey's *post hoc* analysis. * $p < 0.0001$ with respect to Naïve, SO, and EXPL experimental groups.

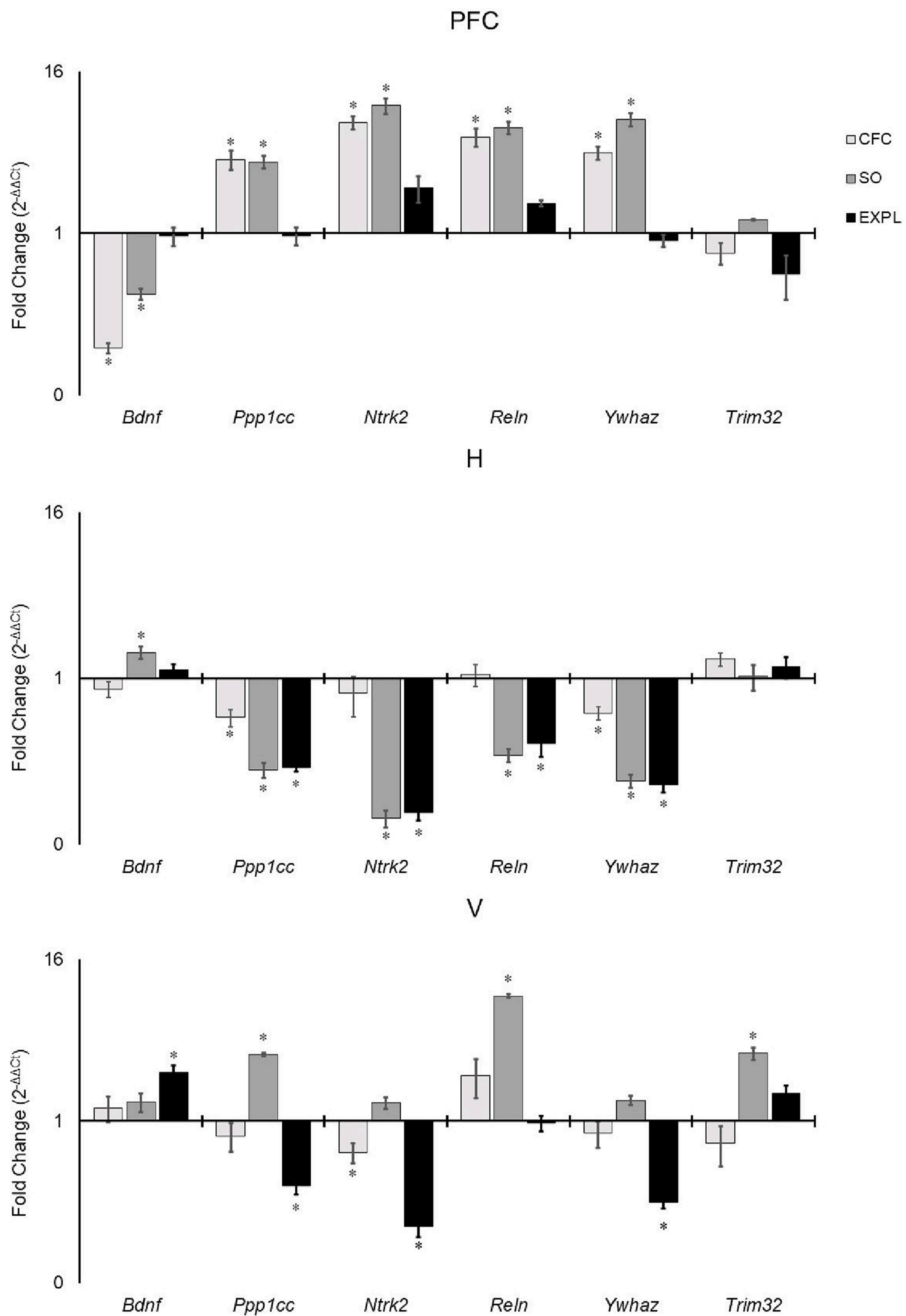

Fig. 2. Mrna levels for *Bdnf, Ppp1cc, Ntrk2, Reln, Ywhaz* and *Trim32* genes in the prefrontal cortex (PFC), cerebellar hemispheres (H) and vermis (V) of rats subjected to CFC, SO and EXPL procedure. cDNA was analysed by Real-Time PCR. Data are represented as mean ± SEM. Statistical analysis: one-way ANOVA followed by Tukey's *post hoc* analysis; * $p < 0.05$ with respect to naïve, taken as unit.

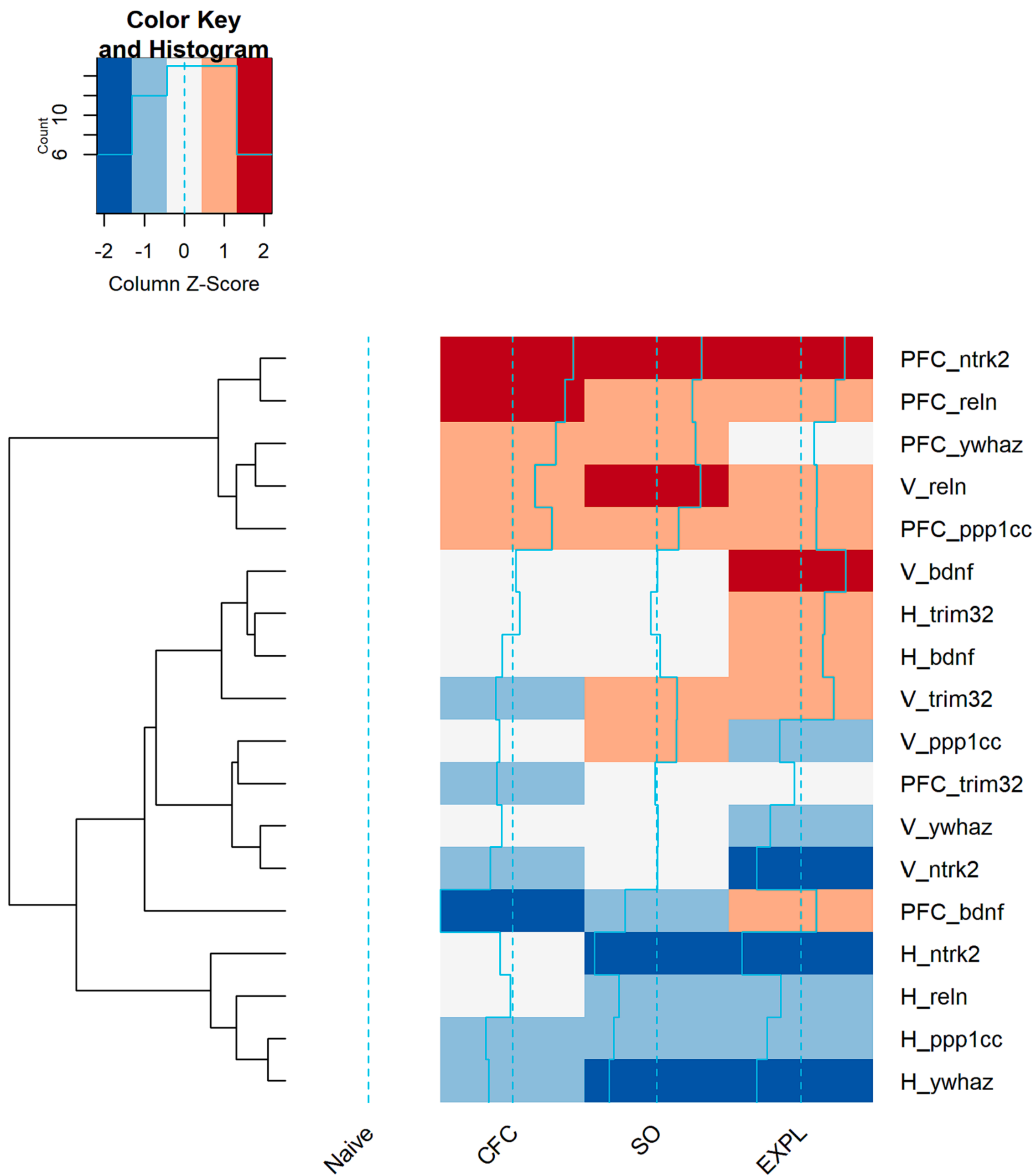

Fig. 3. Heatmap showing gene expression as logarithm of Fold Change mRNA levels for *Bdnf, Ppp1cc, Ntrk2, Reln, Ywhaz* and *Trim32* genes in the prefrontal cortex (PFC), cerebellar hemispheres (H) and vermis (V) (on the right) of rats subjected to CFC, SO and EXPL procedure (columns with Naïve as reference). On the left, the rows have been clustered using complete agglomeration method, while the continues lines represent the coefficient value.

important roles in many processes, such as negative regulation of apoptotic process and fibroblast proliferation, positive regulation of NF-kB transcription factor activity, cell cycle, cell growth, cell migration, neurogenesis, neuron differentiation, protein catabolic process, proteolysis, protein polyubiquitination, and response to tumour necrosis factor.

The expression of these and other genes, including *Bdnf*, *Reln* (protease that plays a role in the layering of neurons in the cerebral cortex and cerebellum, regulates microtubule function in neurons and neuronal migration), *Ntrk2*, and *Ppp1cc* -critical for regulation of ionic conductance and long-term synaptic plasticity- was evaluated. In particular, REELIN represents a multifunctional signal that coordinates cortical and subcortical morphogenesis during development and regulates structural plasticity by playing a role in the formation and maintenance of neurological circuits. Overexpression of *Reln* accelerates dendritic growth and maturation of the adult hippocampus. Indeed, REELIN organizes the development of the hippocampus (Stranahan, Erion, & Wosiski-Kuhn, 2013). It has also been suggested that REELIN may participate in the aetiology of various psychiatric disorders (Ovadia & Shifman, 2011). Previous studies have reported changes in brain behaviour and anatomy due to reduced *Reln* expression (Mullen, Khialeeva, Hoffman, Ghiani, & Carpenter, 2012).

In the present study, we observed a significant increase in *Reln* expression either in the PFC following CFC and SO procedures and in the V after SO stimulus, suggesting and confirming that conditioning and stressful conditions participate to the processes of modulation of structural plasticity. It has been reported that mice that overexpress *Reln* show less sensitivity to corticosterone-induced behaviours (Teixeira et al., 2011). Likely, in the present study, *Reln* overexpression could act as a protection system against environmental stressors.

At the level of the PFC, the variations in gene expression in the CFC and SO groups proceed in parallel, suggesting that it is the stress condition linked to foot shock that prevails over conditioning. This is observed for *Reln*, but also for *Bdnf*, *Ppp1cc*, *Ntrk2*, and *Ywhaz* genes. In particular, about the expression of *Ywhaz* (vesicle-mediated transport and stress-related protein), an increase in PFC was observed in the CFC and SO groups, while at the level of the H, the expression of *Ywhaz* decreases in CFC, SO and EXPL. It has been demonstrated that *Ywhaz* is expressed in brain regions that play a crucial role in neural development. Furthermore, stressful conditions are described to alter proteins that are part of a network that also includes YWHAZ (Antón-Galindo et al., 2022). In addition, YWHAZ has a role as an adapter protein of extracellular vesicles which involves the stabilization of vesicles and synapses (Drago et al., 2017). The overexpression of *Ywhaz* in the PFC could increase the formation and release of vesicles carrying protein to the synapse, and this could be a compensatory mechanism for synaptic activity in the PFC. On the other hand, protein phosphatase 1 (PP1) has been shown to limit learning and memory training. However, it is still unclear how PP1 can be modulated in memory (Hou et al., 2013).

In this study, *Bdnf* expression is significantly reduced in the prefrontal region in animals subjected to CFC, while it is increased in the V of the EXPL group of animals. The role of BDNF in learning and memory phenomena has been documented, although the exact mechanism by which BDNF modulates signalling pathways during learning has not yet been elucidated. It has been reported that BDNF can activate multiple signalling pathways that can act in concert to regulate downstream cellular effects necessary for synaptic plasticity and memory formation (Freudenthal et al., 2005; Merlo, Freudenthal, Maldonado, & Romano, 2005; Yeh, Lin, Lee, & Gean, 2002). The neurotrophin receptor NTRK2 encoded by the *Ntrk2* gene plays role in neuronal survival, proliferation, differentiation, and apoptosis. BDNF signalling through its high-affinity NTRK2 receptor is one of the main regulators of the function of fear circuits, as well as an expression of fear behaviour (Andero, Choi, & Ressler, 2014; Bathina & Das, 2015).

In the present study, we found that in both the PFC and the cerebellum, the expression of *Bdnf* and its receptor are inversely correlated. Notably, this regulatory mechanism is based on the imbalance between the turnover and production of the receptor proteins. NTRK2 receptor is highly expressed on Purkinje neurons where BDNF signalling performs various neuroprotective and neuromodulatory functions. The regulation of BDNF expression in pathological or stressful conditions is less known. CFC is critically dependent on BDNF levels in the hippocampus and hippocampal-dependent CFC is likewise selectively vulnerable to the disruption of the *Bdnf* gene (Liu et al., 2004). It is likely that the stress condition to which CFC animals are subjected determines a reduction of *Bdnf* expression also in other brain regions, as our results suggest, in particular in PFC of CFC and SO groups. Within the CFC circuit, the PFC could exert hierarchical control over subcortical structures in order to regulate appropriate behavioural responses.

A division of tasks in the PFC has already been suggested in which the prelimbic area would regulate the expression of fear while the infralimbic area of the medial PFC would regulate the suppression of fear in the rodent (Giustino & Maren, 2015; Milad & Quirk, 2012).

In this study, the analysis was conducted 2 days after conditioning. We cannot exclude that at this time the PFC, as well as the cerebellum, are not yet fully recruited in the consolidation and retention of engrams. In a recent study, Kitamura et al. (Kitamura et al., 2017) defined this time point as a “recent call” in which PFC engram cells are still immature, and perhaps simply “tagged” for later maturation of the engrams in specific areas of the brain. Future studies will evaluate the modulation of gene expression at different subsequent time points, in accordance with the timing of maturation of the engrams in specific areas of the brain.

5. Statements and declarations

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Authors’ contributions.

All authors have read and agreed to the published version of the manuscript.

Data Availability.

The experimental data used to support the findings of this study are included within the article.

Ethics approval.

The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Florence (Italy) (Prot. N. 77/2008).

Credit authorship contribution statement

Carmela Conte: Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Elisabetta Baldi:** Methodology, Validation, Investigation. **Corrado Bucherelli:** Methodology, Validation, Investigation. **Raffaella di Vito:** Formal analysis, Data curation, Writing – review & editing, Visualization. **Davide Petri:** Formal analysis, Data curation, Visualization. **Giovanna Traina:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2023.107776.

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