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RESEARCH REPORT

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Brief exposure to enriched environment rapidly shapes the glutamate synapses in the rat brain: A metaplastic fingerprint

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Abstract

Environmental enrichment (EE) has been shown to produce beneficial effects in addiction disorders; however, due to its configurational complexity, the underlying mechanisms are not yet fully elucidated. Recent evidence suggests that EE, acting as a metaplastic agent, may affect glutamatergic mechanisms underlying appetitive memory and, in turn, modulate reward-seeking behaviours: here, we have investigated such a possibility following a brief EE exposure.

Adult male Sprague–Dawley rats were exposed to EE for 22 h and the expression of critical elements of the glutamate synapse was measured 2 h after the end of EE in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and hippocampus (Hipp) brain areas, which are critical for reward and memory. We focused our investigation on the expression of NMDA and AMPA receptor subunits, their scaffolding proteins SAP102 and SAP97, vesicular and membrane glutamate transporters vGluT1 and GLT-1, and critical structural components such as proteins involved in morphology and function of glutamatergic synapses, PSD95 and Arc/Arg3.1.

Our findings demonstrate that a brief EE exposure induces metaplastic changes in glutamatergic mPFC, NAc and Hipp. Such changes are area-specific and involve postsynaptic NMDA/AMPA receptor subunit composition,

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Arc/Arg3.1, Activity-regulated cytoskeleton-associated protein; CNS, Central nervous system; CP-AMPARs, Calcium permeable-AMPA receptors; Cx, Context; EE, Environmental enrichment; GLT-1, Glutamate transporter 1; GRIP, Glutamate receptor interacting protein; Hipp, Hippocampus; LTD, Long-term depression; LTP, Long-term potentiation; mGluR5, Metabotropic glutamate receptor 5; mPFC, Medial prefrontal cortex; NAc, Nucleus accumbens; NMDA, N-methyl-D-aspartate; PSD, Postsynaptic density; PSD95, Postsynaptic density protein 95; SAP102, Synapse-associated protein 102; SAP97, Synapse-associated protein 97; TIF, Triton insoluble fraction; TSF, Triton soluble fraction; vGluT1, Vesicular glutamate transporter 1.

Cristiano Chiamulera and Fabio Fumagalli share the seniorship.
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as well as changes in the expression of their main scaffolding proteins, thus influencing the retention of such receptors at synaptic sites.

Our data indicate that brief EE exposure is sufficient to dynamically modulate the glutamatergic synapses in mPFC-NAc-Hipp circuits, which may modulate rewarding and memory processes.

KEYWORDS

environmental enrichment, glutamatergic synapses, learning, memory, metaplasticity, reward

1 | INTRODUCTION

Environmental enrichment (EE) has been shown to produce beneficial and therapeutic effects in several preclinical models of central nervous system disorders. Due to a combination of social, cognitive and sensorimotor stimulations, EE leads to a variety of positive effects, ranging from cellular and molecular (e.g., neurogenesis, changes in the expression of genes and receptors subunits, neurotransmitters and brain-derived neurotrophic factor levels) to cognitive and behavioural changes (e.g., enhanced learning and memory performance, altered emotional state), in both health and disease models (Benaroya-Milshtein et al., 2004; Chapillon et al., 1999; Falkenberg et al., 1992; Kempermann et al., 1997; Lee et al., 2003; Leggio et al., 2005; Moser et al., 1997; Rampon et al., 2000; Roy et al., 2001; Tang et al., 2001; van Praag et al., 2000; Young et al., 1999).

EE-induced neuroplasticity is also involved in the protective and therapeutic effects on addiction disorders, with experimental evidence suggesting stress-related mechanisms as possible targets of the anti-addictive effect of EE (Crofton et al., 2015; Solinas et al., 2010). However, given the configurational complexity of environmental stimulation, the underlying mechanisms still need further investigation.

EE exposure duration and components (i.e., social, sensorimotor, cognitive) seem critical parameters for its effects. Indeed, although chronic and acute exposure to a complex EE (with social, cognitive and sensory-motor components) reduces drug/sucrose-taking and -seeking behaviours (Grimm et al., 2008, 2013, 2016; Margetts-Smith et al., 2021; Solinas et al., 2008), we however recently demonstrated that a brief EE exposure in rats (22 hours, without motor and social components) potentiates conditioned context (Cx)-induced sucrose-seeking (a phenomenon called *renewal*) and Cx-memory reconsolidation after reactivation (Pintori et al., 2022).

Thus, the wide variety and complexity of EE features (e.g., length of exposure, location, type of stimulation and many others) limited the translation into clinical practice so far. Therefore, understanding the mechanisms underlying acute brief EE manipulation could be useful to develop new and innovative configurations of EE – with translational value and feasibility – to improve existing therapeutic approaches against addiction and addiction-related central nervous system (CNS) disorders.

Based on data about EE-induced enhancement of memory and learning abilities (Cortese et al.,2018; Leggio et al.,2005; Tang et al.,2001), we suggested that this brief EE exposure may act as a *proactive interference agent*, influencing subsequent memory processes (i.e., renewal, Cx-memory reactivation/reconsolidation) (Pintori et al., 2022). From a neurobiological point of view, this proactive action might potentially act as a *metaplastic effect* (defined as "the plasticity of synaptic plasticity" (Abraham & Bear, 1996)), as recently suggested by electrophysiological evidence (Chiamulera et al., 2020; Eckert & Abraham, 2013; Schmidt et al., 2013). Importantly, the ability of EE to affect long-term potentiation (LTP) and long-term depression (LTD) seems related to alterations of glutamatergic signalling particularly at the level of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Duffy et al., 2001; Eckert et al., 2010; Foster & Dumas, 2001; Li et al., 2006; Thomas et al., 2008).

In the present paper, we aim to investigate whether brief EE exposure influences the homeostasis of the glutamate synapse, which could be correlated to the behavioural effects previously reported (Pintori et al., 2022). To this end, adult male Sprague–Dawley rats were exposed to EE for 22 h and two hours after the end of EE exposure we analysed the expression of glutamate determinants such as vesicular glutamate transporter (vGluT1), which participates in the regulation of presynaptic glutamate release and glutamate transporter (GLT-1), which is responsible for glutamate reuptake, together with the expression of the main subunits of NMDA (GluN1, GluN2A, GluN2B) and AMPA (GluA1

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and GluA2) receptors and their related scaffolding protein SAP102 (synapse-associated protein 102), SAP97 (synapse-associated protein 97) and GRIP (glutamate receptor interacting protein). Further, we set out to explore the potential structural effects of brief EE exposure by measuring the expression levels of the integral protein of the glutamate synapse postsynaptic density protein 95 (PSD95) and the cytoskeletal protein activity-regulated cytoskeleton-associated protein (Arc/Arg3.1). These analyses were performed in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), and hippocampus (Hipp), both in the whole homogenate, which provides information essentially about translational changes, as well as in the postsynaptic density (PSD), which, instead, informs primarily about the synaptic localization and composition of critical determinants of the glutamatergic synapse.

2 | MATERIAL AND METHODS

2.1 | Animals

Twelve adult male Sprague–Dawley rats (Charles River, Italy) 275-300 g were single-housed in a temperature and humidity-controlled environment (19–23°C, 60 ± 20%) on a 12-h light/dark cycle, with light ON at 7:30 pm. Water and food were available ad libitum. All animal care and experimental procedures are reported in compliance with the European Union regulations and Directive 2010/63/EU and were approved by the ethical committee (OPBA) of the University of Verona and by the Ministry of Health (authorization n. 780/2019-PR).

2.2 | Brief environmental enrichment exposure

After two weeks of acclimatisation period in the animal facility, half of the rats were exposed for 22 h to Environmental Enrichment (EE group). EE consisted of a novel housing cage (35.6 × 48.5 × 21.8 cm, Optirat Gen II, Animal Care System) with new bedding, where rats were single-housed (i.e., no social component) for 22 hours (from 11:00 am to 09:00 am, light OFF at 7:30 am) with various objects (toys with different materials, shapes and colours, i.e., one plastic ball, one ladder and one wood brick) for sensory stimulation. In order to exclude novelty effects (Ball et al., 2006; Rebec et al., 1997), the second half of the rats (NoEE group) were exposed to the same housing conditions, i.e., single-housed in a novel housing cage (35.6 × 48.5 × 21.8 cm, Optirat Gen II, Animal Care System) with new bedding and without any Environmental Enrichment object.

2.3 | Western blot assays

Two hours after the end of EE exposure (11:00 am), rats were sacrificed, the brains were rapidly removed, and following the brain atlas of Paxinos and Watson (Paxinos & Watson, 2007), 1-mm thick slices containing the medial prefrontal cortex (mPFC, bregma +3.20 mm), the nucleus accumbens (NAc, bregma +1.70 mm), and the hippocampus (Hipp, bregma −3.30 mm) were dissected by using a 1-mm Coronal Brain Matrix (SouthPointe Surgical Supply, Florida, USA), frozen on dry ice and stored at −80°C. After the dissection of brain areas, proteins were isolated and analysed in the whole homogenate and PSD as previously described with minor modifications (Piva et al., 2020). Briefly, mPFC, NAc and Hipp were homogenized in a Teflon-glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 1 mM $MgCl_2$, 1 mM $NaHCO_3$ and 0.1 mM PMSF, in presence of commercial cocktails of protease (Roche, Monza, Italy) and phosphatase inhibitors (Sigma-Aldrich, Milan, Italy). An aliquot of each homogenate was then sonicated and stored at −20°C. The remaining homogenate was centrifuged at 800*g* for 5 min; the obtained supernatant was then centrifuged at 13000*g* for 15 min, obtaining a pellet. This pellet was resuspended in a buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100000*g* for 1 h. The resulting supernatant, referred as Triton X-100 soluble fraction (TSF, extra-synaptic fraction), was stored at −20°C; the pellet, referred as PSD or Triton X-100 insoluble fraction (TIF, post-synaptic density), was homogenized in a glass–glass potter in 20 mM HEPES, protease and phosphatase inhibitors and stored at −20°C in presence of glycerol 30%. Total proteins have been measured in the homogenate and TIF fractions according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as a calibration standard.

Western blots were run as previously described (Mottarlini et al., 2022). Briefly, equal amounts of proteins of the homogenate (10 μg) and TIF fraction (8 μg) were run on a sodium dodecyl sulfate-8% polyacrylamide gel under reducing conditions and then electrophoretically transferred onto nitrocellulose membranes (GE Healthcare, Milan, Italy). Blots were blocked for 1 h at room temperature with I-Block solution (Life Technologies Italia, Italy) in TBS 0.1% Tween-20 buffer and incubated with antibodies against the proteins of interest. The conditions of the primary antibodies were the following:

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anti-vGLUT-1 (1:1000, Cell Signaling Technology Inc. RRID: AB_2797887), anti-GLT-1 (1:5000, AbCam. RRID: AB_1566262), anti-GluN1 (1:1000, Cell Signaling Technology Inc. RRID: AB_659874), anti-GluN2B (1:1000, Cell Signaling Technology Inc; RRID: AB_1264223), anti-GluN2A (1:1000, Cell Signaling Technology Inc. RRID: AB_2112295), anti-SAP102 (1:1000, AbCam; RRID: AB_1860292), anti-GluA1 (1:1000, Cell Signaling Technology Inc. RRID: AB_2732897), anti-GluA2 (1:2000, Cell Signaling Technology Inc. RRID: AB_10622024), anti-SAP97 (1:1000, AbCam), anti-PSD95 (1:2000, Cell Signaling Technology Inc. RRID: AB_2292883), anti-GRIP (1:1000, Synaptic System. RRID: AB_887728), anti-Arc/Arg3.1 (1:500, BD Transduction Lab. RRID: AB_399886) and anti-β-Actin (1:10000, Sigma-Aldrich. RRID: AB_476744). Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories) and analysed with Image LabTM software (Bio-Rad) by evaluating the band density. Immunocomplexes representing SAP97 and SAP102 have been analysed by quantifying both bands observed at the predicted molecular weight. Results were standardized using β-actin as the control protein, which was detected by evaluating the band density at 43 kDa. Each set of proteins shown in Figures 1–3 was run in the same WB assay, thus only one band of β-Actin is presented. Gels were run three times each and the results, expressed as percentage of the NoEE group, represent the average from three different western blots by using a specific correction factor: correction factor gel B = average of (OD protein of interest/OD β-actin for each sample loaded in gel A)/(OD protein of interest/OD β-actin for the same sample loaded in gel B); correction factor gel C = average of (OD protein of interest/OD β-actin for each sample loaded in gel A)/(OD protein of interest/OD β-actin for the same sample loaded in gel C) (Caffino et al., 2020). Cropped immunoblots are reported in Supplementary Information (SI) (Figures S1–S6).

2.4 | Statistical analysis

All the numerical data are given as mean ± SEM. Data were tested for normal distribution using Shapiro–Wilk's test. Data were collected in individual animals (independent determinations) and molecular changes produced by Environmental Enrichment exposure were analysed separately for each brain area using unpaired Student's *t*-test. Non-parametric test (i.e., Mann–Whitney U-test) was chosen when data were found not to be normally distributed, while *t*-test with Welch's correction was chosen when there was significant variance in homogeneity. Differences were considered significant at $p < 0.05$. All analyses were performed using the GraphPad software package (Prism, version 9; GraphPad, San Diego, California, USA).

3 | RESULTS

3.1 | Effects of brief EE exposure on medial prefrontal cortex protein levels

Western blot analysis of the NMDA receptors in the PSD of the mPFC after EE exposure showed increased expression of the GluN2A subunit ($t_{[10]} = 4.86$, $p < 0.001$, +45%) with no changes for the other subunits GluN1 and GluN2B. This result led to an increased GluN2A/GluN2B ratio ($t_{[10]} = 2.44$, $p < 0.05$, +20%). The analysis of the expression of the scaffolding proteins related to NMDA receptors revealed an increase of SAP102 after EE exposure ($t_{[10]} = 2.39$, $p < 0.05$, +22%) with no changes for PSD95. The analysis of AMPA receptors showed that EE exposure decreased GluA1 subunit expression ($t_{[10]} = 2.83$, $p < 0.05$, −22%) while increasing GluA2 levels ($t_{[10]} = 3.34$, $p < 0.01$, +25%), a result that led to a significant decrease of GluA1/GluA2 ratio ($t_{[10]} = 7.47$, $p < 0.0001$, −38%). Considering the scaffolding proteins related to AMPA receptors, both SAP97 ($t_{[10]} = 2.60$, $p < 0.05$, +31%) and GRIP (U = 0, $p < 0.01$, +18%) protein levels significantly increased after EE exposure. Finally, we found Arc/Arg3.1 significantly increased in the mPFC of the EE group as compared to the NoEE group ($t_{[10]} = 3.27$, $p < 0.01$, +30%) (Figure 1a).

The analysis of the whole homogenate showed a significant increase of vGluT1 ($t_{[10]} = 2.49$, $p < 0.05$, +22%) and GLT-1 expression ($t_{[10]} = 2.57$, $p < 0.05$, +16%) as compared to NoEE group. In line with the results of the PSD, only the expression of the GluN2A subunit of NMDA receptors was significantly increased ($t_{[10]} = 2.83$, $p < 0.05$, +20%), an effect that was accompanied by SAP102 up-regulation ($t_{[10]} = 2.96$, $p < 0.05$, +22%) and, at variance from the PSD, by PSD95 up-regulation ($t_{[10]} = 4.82$, $p < 0.001$, +20%) as compared to NoEE group. Analysing AMPA receptors, the pattern of expression was quite different from the PSD with increased expression of GluA1 subunit ($t_{[10]} = 3.53$ $p < 0.01$, +22%) and no effects on GluA2 subunit whereas the related scaffolding proteins showed significantly increased expression [SAP97 ($t_{[10]} = 3.90$, $p < 0.01$, +21%) and GRIP (U = 2, $p < 0.01$, +15%)], in line with data in the PSD. Finally, no changes in Arc/Arg3.1 expression were observed when compared to the NoEE group (Figure 1b).

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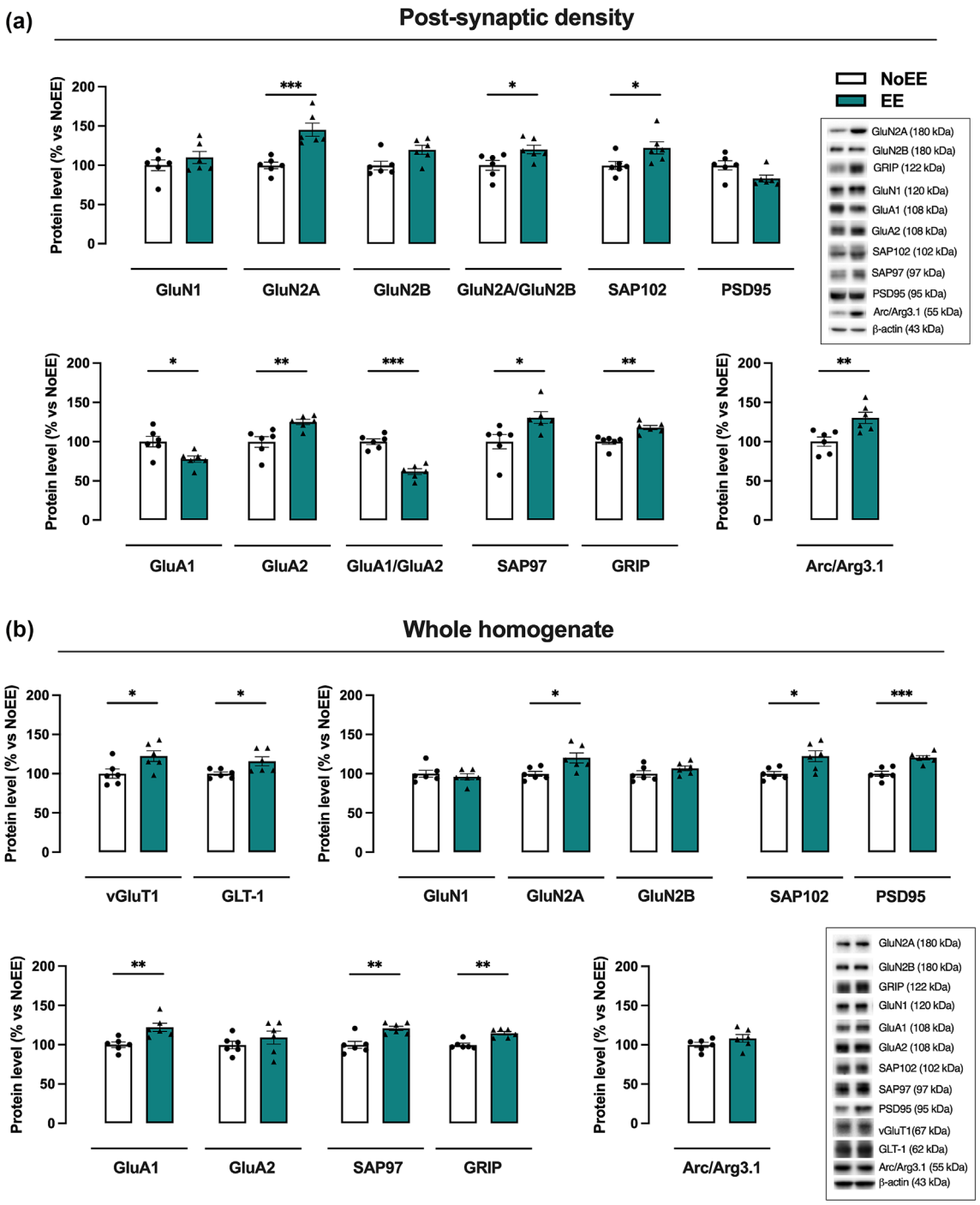

FIGURE 1 Effects of brief EE exposure on medial prefrontal cortex protein levels. Quantification of vGluT1 and GLT-1, of NMDA receptor subunits, GluN1, GluN2A, and GluN2B, of the AMPA receptor subunits, GluA1 and GluA2, of scaffolding proteins, SAP102, SAP97, GRIP, and PSD95, and the cytoskeletal marker Arc/Arg3.1 in the post-synaptic density (a) and in the whole homogenate (b) of the medial prefrontal cortex (mPFC) 2 hours after the end of EE exposure. In the right insert, representative immunoblots are shown for vGluT1, GLT-1, GluN1, GluN2A, GluN2B, SAP102, GluA1, GluA2, SAP97, GRIP, Arc/Arg3.1, and β-actin in the post-synaptic density or the homogenate of mPFC. Data are shown as the mean + SEM and are expressed as percentage of the NoEE group. NoEE = white columns; EE = teal columns. $N = 6$ rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NoEE; unpaired two-tailed Student's *t*-test or Mann–Whitney test.

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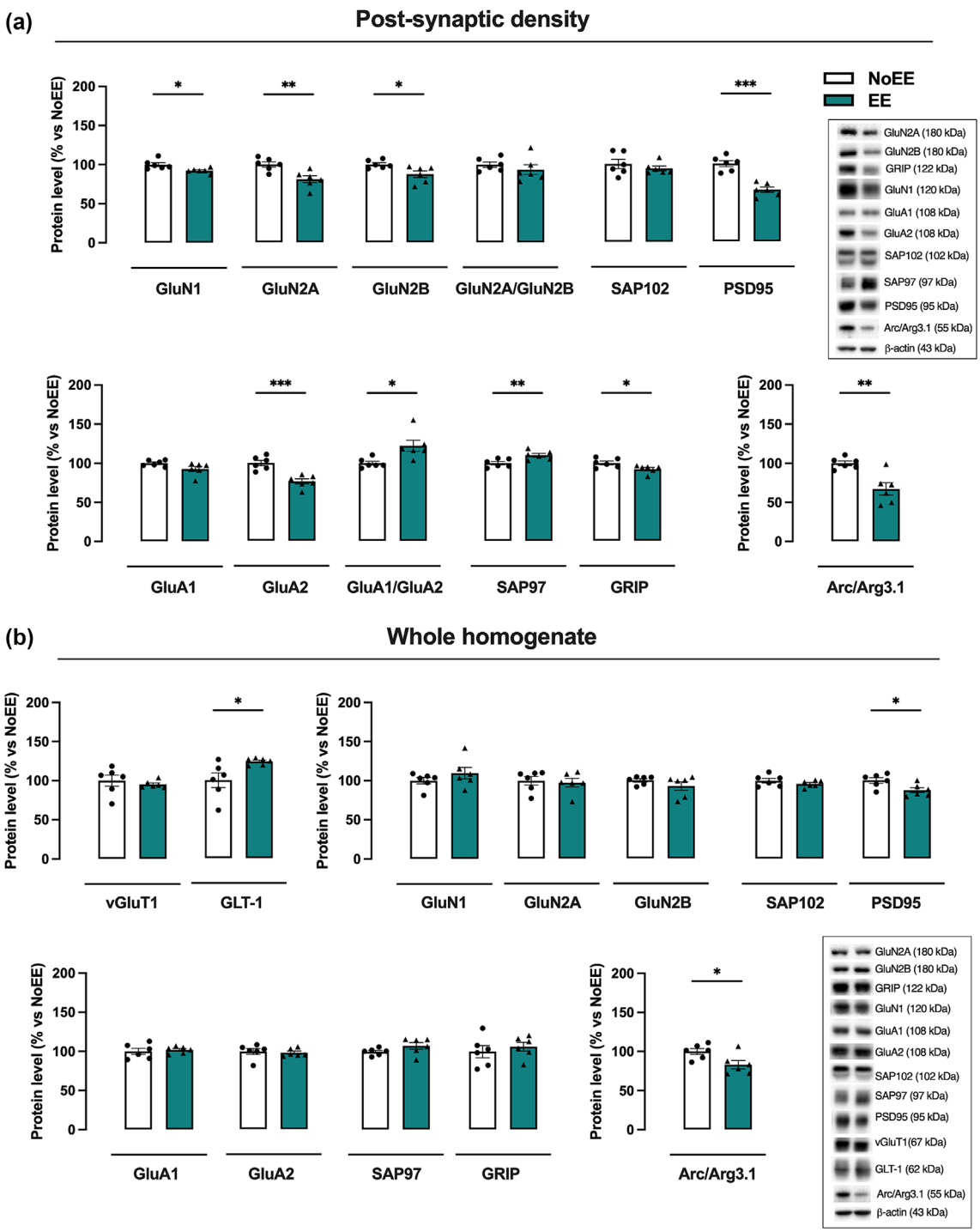

FIGURE 2 Effects of brief EE exposure on nucleus accumbens protein levels. Quantification of vGluT1 and GLT1, of NMDA receptor subunits, GluN1, GluN2A, and GluN2B, of the AMPA receptor subunits, GluA1 and GluA2, of scaffolding proteins, SAP102, SAP97, GRIP, and PSD95, and the cytoskeletal marker Arc/Arg3.1 in the post-synaptic density (a) and in the whole homogenate (b) of nucleus accumbens (NAc) 2 hours after the end of EE exposure. In the right insert, representative immunoblots are shown for vGluT1, GLT-1, GluN1, GluN2A, GluN2B, SAP102, GluA1, GluA2, SAP97, GRIP, Arc/Arg3.1, and β-actin in the post-synaptic density or the homogenate of NAc. Data are shown as the mean + SEM and are expressed as percentage of the NoEE group. NoEE = white columns; EE = teal columns. $N = 6$ rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NoEE; unpaired two-tailed Student's *t*-test or Mann–Whitney test.

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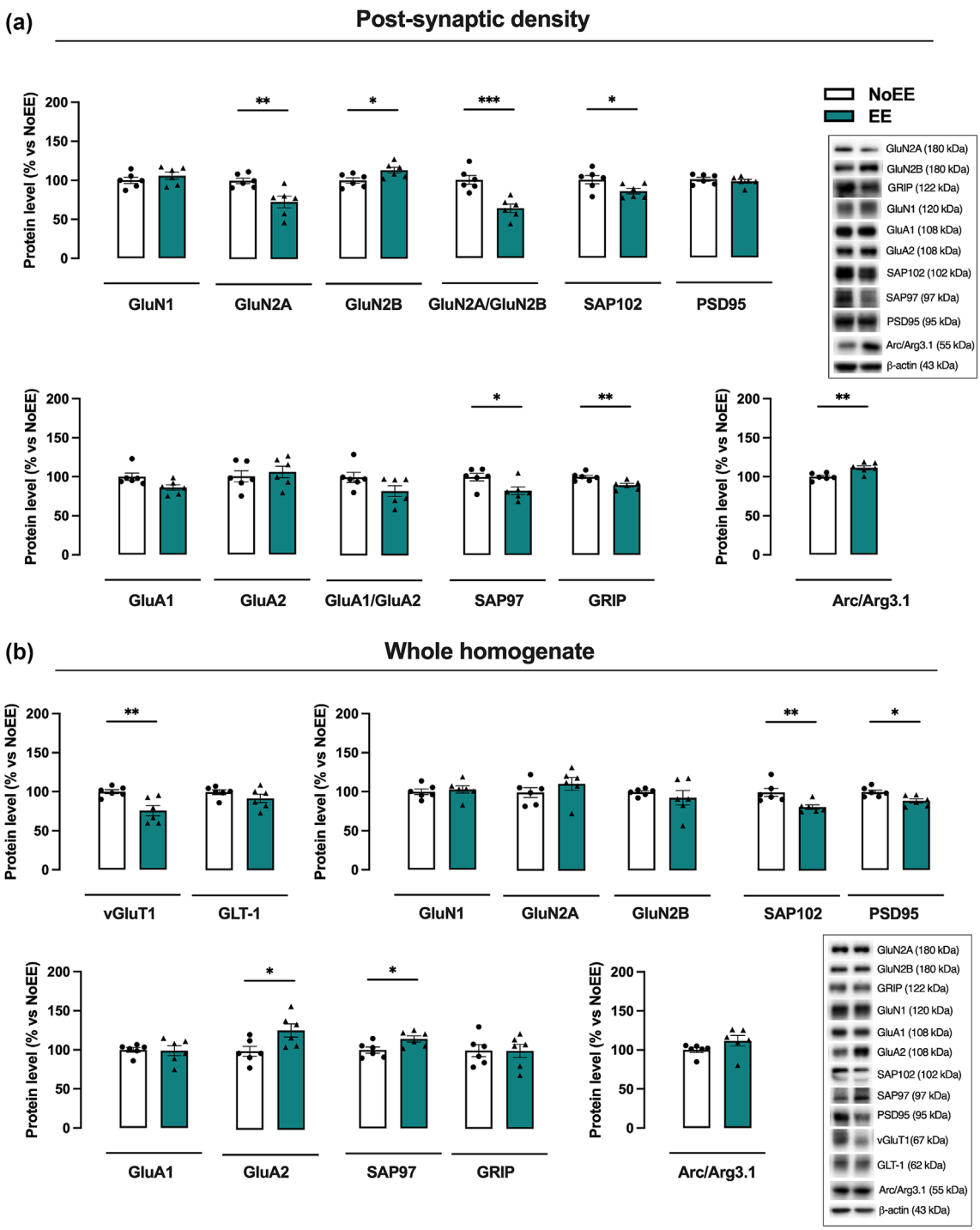

FIGURE 3 Effects of brief EE exposure on hippocampus protein levels. Quantification of vGluT1 and GLT-1, of NMDA receptor subunits, GluN1, GluN2A, and GluN2B, of the AMPA receptor subunits, GluA1 and GluA2, of scaffolding proteins, SAP102, SAP97, GRIP, and PSD95, and the cytoskeletal marker Arc/Arg3.1 in the post-synaptic density (a) and in the whole homogenate (b) of hippocampus (Hipp) 2 hours after the end of EE exposure. In the right insert, representative immunoblots are shown for vGluT1, GLT-1, GluN1, GluN2A, GluN2B, SAP102, GluA1, GluA2, SAP97, GRIP, Arc/Arg3.1, and β-actin in the post-synaptic density or the homogenate of Hipp. Data are shown as the mean + SEM and are expressed as a percentage of the NoEE group. NoEE = white columns; EE = teal columns. $N = 6$ rats/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NoEE; unpaired two-tailed Student's *t*-test or Mann–Whitney test.

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3.2 | Effects of brief EE exposure on nucleus accumbens protein levels

Western blot analysis in the PSD of the NAc after EE exposure revealed an overall downregulation of NMDA receptor subunits as compared to NoEE group (GluN1: $t_{(10)} = 2.77$, $p < 0.05$, −8%; GluN2A: $t_{(10)} = 3.44$, $p < 0.01$, −19%; GluN2B: $t_{(10)} = 2.68$, $p < 0.05$, −13%); accordingly, the GluN2A/GluN2B ratio was not changed in the EE group. The analysis of the NMDA-related scaffolding proteins revealed a significant decrease of PSD95 after EE exposure ($t_{(10)} = 6.48$, $p < 0.0001$, −33%), with no changes in SAP102 expression. Analysis of AMPA receptors shows a significant reduction of GluA2 levels after EE exposure ($t_{(10)} = 4.95$, $p < 0.001$, −24%), with no GluA1 alterations. This result led to an increased GluA1/GluA2 ratio ($t_{[6.19]} = 2.95$, $p < 0.05$, +22%). The analysis of AMPA-related scaffolding proteins showed a significant increase of SAP97 ($t_{(10)} = 3.17$, $p < 0.01$, +10%) and a reduction of GRIP expression (U = 5, $p < 0.05$, −8%) after EE exposure. Finally, Arc/Arg3.1 expression was significantly decreased in the EE group as compared to the NoEE group ($t_{[6.37]} = 3.91$, $p < 0.01$, −33%) (Figure 2a).

When analysing the whole homogenate, we found no changes in vGluT1 levels and a significant increase of GLT-1 expression as compared to the NoEE group ($t_{[5.33]} = 2.56$, $p < 0.05$, +24%). With respect to NMDA receptors and related scaffolding proteins, no changes were observed except for a significant decrease of PSD95 ($t_{(10)} = 2.62$, $p < 0.05$, −13%). Similarly, no changes were observed for AMPARs and related scaffolding proteins. Finally, Arc/Arg3.1 protein levels were significantly decreased as compared to the NoEE group ($t_{(10)} = 2.57$, $p < 0.05$, −17%) (Figure 2b).

3.3 | Effects of brief EE exposure on hippocampal protein levels

Western blot analysis in the PSD of the Hipp after EE exposure showed no changes in the main NMDA subunit GluN1 whereas the expression of GluN2A and GluN2B were, respectively, significantly decreased ($t_{(10)} = 3.33$, $p < 0.01$, −27%) and increased ($t_{(10)} = 2.56$, $p < 0.05$, +13%). This result led to a significant reduction of the GluN2A/GluN2B ratio ($t_{(10)} = 4.64$, $p < 0.001$, −36%). The analysis of the NMDA-related scaffolding proteins after EE exposure revealed a significant decrease of the scaffolding protein SAP102 ($t_{(10)} = 2.28$, $p < 0.05$, −15%), with no changes for PSD95. At variance from NMDA receptors, no changes in the AMPARs subunits GluA1 and GluA2 expression were observed after EE exposure, with the consequence of an unaltered GluA1/GluA2 ratio. However, we found SAP97 and GRIP levels significantly decreased in the EE group as compared to the NoEE group ($t_{(10)} = 2.52$, $p < 0.05$, −17%; $t_{(10)} = 3.39$, $p < 0.01$, −11%; respectively). Finally, Arc/Arg3.1 significantly increased after EE exposure ($t_{(10)} = 3.57$, $p < 0.01$, +12%) (Figure 3a).

Examining the whole homogenate, vGluT1 levels were decreased ($t_{(10)} = 3.45$, $p < 0.01$, −24%) with no changes for GLT-1 as compared to the NoEE group. Notably, the expression of NMDA receptors was not altered after EE exposure, except for a significant decrease for both SAP102 ($t_{(10)} = 3.17$, $p < 0.01$, −19%) and PSD95 ($t_{(10)} = 3.07$, $p < 0.05$, −11%). Differently from NMDA receptors, the GluA2 subunit of AMPA receptors was significantly increased ($t_{(10)} = 2.56$, $p < 0.05$, +27%) while no GluA1 changes were observed. The analysis of AMPA-related scaffolding proteins showed a slight but significant increase of SAP97 as compared to the NoEE group ($t_{(10)} = 2.58$, $p < 0.05$, +14%). Finally, Arc/Arg3.1 expression was not altered after EE exposure (Figure 3b).

4 | DISCUSSION

In the present study, we investigated the effects of brief EE exposure on various components of glutamatergic transmission to understand whether potential metaplastic effects underlie EE modulation of sucrose-seeking behaviour previously reported (Pintori et al., 2022). Our findings reveal that 22 hours of EE exposure differently affects glutamate homeostasis in the brain areas involved in reward and reward-related memory. These results demonstrate that a brief EE exposure is sufficient to cause different adaptive mechanisms that may influence glutamate receptor availability at the synapse; such mechanisms are peculiar for each brain region.

4.1 | Effects of brief EE exposure in the medial prefrontal cortex

In the mPFC, brief EE exposure selectively increased GluN2A levels, leading to a significant increase in GluN2A/GluN2B ratio. The analysis of the AMPA receptor, instead, revealed a decrease of GluA1 and an increase of GluA2 subunit expression leading to a decrease in GluA1/GluA2 ratio. This suggests that brief EE exposure alters the composition of these receptors which might contribute to changes in the homeostasis of the glutamate system.

According to the literature, GluN2A/GluN2B ratio change correlates with modifications of the threshold for

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LTP and LTD (Chiamulera et al., 2020). For instance, while lack of sensory experiences, such as light deprivation, decreases the GluN2A/GluN2B ratio, restoring visual experience rapidly increases the GluN2A/GluN2B ratio, raising the LTP/LTD threshold in the visual cortex (Philpot et al., 2003; Quinlan et al., 1999). This bidirectional change of

LTP/LTD threshold induced by rapid modification of NMDAR subunit composition has been proposed as a demonstration of metaplasticity (Kirkwood et al., 1996; Philpot et al., 2003). Similarly, we hypothesize that brief EE may cause an increase in LTP/LTD threshold, by inducing a rapid switch of NMDARs with a higher proportion of GluN2A subunits, thus reducing Ca^{2+} influx.

In line with an 'overstimulation hypothesis', these AMPA/NMDA receptor changes are coupled with an increase of the related scaffolding protein levels both in the PSD fraction (SAP102, SAP97, GRIP) and in the whole cortical homogenate (SAP102, SAP97, GRIP, PSD95). Particularly, it is interesting to note that while GluA1 expression is reduced in the PSD, the expression of the specific scaffolding protein of this receptor, SAP97, is significantly elevated, presumably as an adaptive mechanism to anchor more tightly this receptor at the membrane. Considering their role in the formation, trafficking and stabilization of NMDARs and AMPARs at excitatory synapses (Kim et al., 2005; Vickers et al., 2006), these EE-induced scaffolding protein changes could be involved in increased membrane stability of new glutamate receptor configuration induced by EE exposure. Consistently with this hypothesis, Arc/Arg3.1 increased in the PSD after EE exposure, an observation in line with its involvement in a simultaneous strengthening of stimulated synapses and maintaining weakness at non-potentiated synapses through AMPARs trafficking modulation (Zhang & Bramham, 2021). Finally, the overstimulation hypothesis is further accounted for by the upregulation of vGluT1, which promotes glutamate release (Li et al., 2020), and that may be partly countered by the increased levels of GLT-1, which is chiefly responsible for glutamate reuptake (Shigeri et al., 2004).

Taken together, these neuroplastic changes occurring at glutamatergic synapses induced by brief EE exposure, could reflect a limitation of mPFC synaptic excitability (and in turn a reduction of its inhibitory action on subcortical regions) by further stimuli (i.e., reward-associated stimuli) as a consequence to previous strong cortical activation. In line with a reduction of cortical inhibitory activity, we previously reported that brief EE exposure enhanced rather than inhibited context-induced sucrose-seeking (Pintori et al., 2022). Electrophysiological experiments would be needed to better understand the change of synaptic properties in the mPFC under EE conditions.

4.2 | Effects of brief EE exposure in the nucleus accumbens

Brief EE exposure caused post-synaptic glutamatergic signalling changes in the NAc that are opposite compared to mPFC. In particular, EE induced an overall reduction of NMDAR subunits, both accessory (GluN2A, GluN2B) and obligatory (GluN1), in the PSD. Notably, no changes were observed in the expression of these subunits in the whole homogenate, suggesting that receptor synthesis is not affected while receptor retention is reduced as a consequence of brief EE exposure. This possibility is corroborated by the fact that the expression of SAP102, the scaffolding protein specific for NMDA receptors, is not increased to compensate for reduced expression, thus leading to unstable synapses. These results suggest that NMDA-mediated neurotransmission is depotentiated in the NAc, evidence also strengthened by reduced expression of PSD95 that, besides being a scaffolding protein, is an integral protein of the glutamate synapse centrally involved in multiple aspects of synaptic function (Vallejo et al., 2017). Moreover, since structural changes of dendritic spines are sustained by PSD95 and Arc/Arg3.1 (Newpher et al., 2018), their reduced expression suggests that a brief EE exposure might have reduced the density of dendritic spines contributing to the toning down of the NMDA receptors-mediated glutamate neurotransmission.

A different pattern can be observed for AMPA receptors. We found a significant reduction only in the expression of the GluA2 subunit, thus leading to an increase in GluA1/GluA2 ratio. This enhancement of GluA2-lacking AMPA receptors reflects a higher Ca^{2+} permeability AMPA-mediated in the excitatory synapse, representing a metaplastic phenomenon, as it is established that the upregulation of calcium-permeable AMPARs (CP-AMPAR) is able to change the threshold for different forms of plasticity (Liu & Zukin, 2007). While CP-AMPARs are expressed at low levels under basal conditions, their expression in the excitatory neurons increased in response to neuronal activity and neuronal insults. Importantly, CP-AMPARs in the NAc are involved in drug-seeking behaviours (see review (Neuhofer & Kalivas, 2018)). For instance, it has been demonstrated that the increase of NAc CP-AMPARs plays a critical role in the incubation of cocaine craving (Caffino et al., 2021; Conrad et al., 2008; Lee et al., 2013; Wolf & Ferrario, 2010). These data show that even a brief EE exposure may influence, through changes in accumbal glutamate homeostasis, salience attribution and behavioural response to reward-associated stimuli (i.e., cue, context), ultimately driving maladaptive reward-related behaviours. Consistently, rats exposed to EE in adolescence (PND21–55) showed enhanced

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amphetamine-induced DA NAc release and locomotor stimulation (Bardo et al., 1999).

In line with this evidence, the enhancement of CP-AMPAR levels observed in the NAc after brief EE exposure is consistent with the potentiation of Cx-induced sucrose-seeking previously reported (Pintori et al., 2022). Of note, the excessive calcium influx via EE-induced increase of CP-AMPARs can contribute to NMDA receptor inactivation, as previously demonstrated (Legendre et al., 1993; Paoletti et al., 2013; Sibarov & Antonov, 2018), thus further reducing the postsynaptic response.

We also found no changes in glutamate release as shown by the lack of effect on vGluT1. In addition, EE exposure leads to increased GLT-1 levels presumably to limit glutamate spillover and to prevent activation of extrasynaptic glutamate receptors that can impair synaptic plasticity (Scimemi et al., 2009).

4.3 | Effects of brief EE exposure in the hippocampus

The analysis of the Hipp revealed a different situation in comparison to mPFC and NAc. Brief EE exposure only slightly altered glutamatergic postsynaptic signalling in this brain area. The most relevant effect is the reduced GluN2A/GluN2B ratio in the PSD, due to a decrease of GluN2A and an increase of GluN2B receptor subunits expression together with a reduction in SAP102 expression. This change of synaptic NMDA receptor configuration may reflect a decrease of the LTP/LTD threshold, in line with the reduced presynaptic glutamate release as evidenced by a significant down-regulation of vGluT1. Our evidence is consistent with the literature showing that EE exposure (both long- and short-term) facilitates the LTP in CA1 (see review (Ohline & Abraham, 2019)). It is plausible that this EE-induced NMDAR hippocampal change may contribute to an enhancement of learning and memory abilities, such as spatial recognition. Moreover, GluN2B-containing receptors in the dorsal Hipp are necessary for drug context-induced cocaine-seeking behaviour (Xie et al., 2013), as well as memory reconsolidation (Wells et al., 2016), and relapse even after a long period of abstinence from cocaine self-administration (Werner et al., 2020). In line with this speculation, we previously reported that brief EE exposure improves discrimination efficiency (an index of instrumental learning and memory) and increases sucrose-seeking behaviour during both contextual-memory reactivation session and renewal test (Pintori et al., 2022), suggesting that the increased hippocampal GluN2B-containing receptor expression might be the underlying mechanism of such behaviours.

Differently from mPFC and NAc, EE did not change postsynaptic AMPA receptor expression and subunit configuration in the Hipp, while reducing the expression of AMPA receptor scaffolding proteins SAP97 and GRIP in the PSD, suggesting reduced AMPA receptor retention at the synaptic site. Together with a more mobile pool of GluN2B-containing NMDA receptors (Groc et al., 2006) and supported by increased Arc/Arg3.1 levels (Chowdhury et al., 2006), this effect might contribute to altering the synaptic strength of the excitatory synapse thus leading to a less stable hippocampal synapse.

These changes, together with the decreased GluN2A/GluN2B ratio, may represent a 'canonical' metaplastic mechanism to set up glutamatergic synapses to respond more robustly to future stimulations/experiences, and subsequence learning (Chiamulera et al., 2020).

5 | LIMITATIONS

The study has some limitations such as, firstly, the focus on ionotropic glutamate signalling and not, for instance, on the potential role of metabotropic mGluR5 receptors as suggested by EE-induced mGluR5 enhancement of hippocampal LTP (Buschler & Manahan-Vaughan, 2017). Therefore, ad-hoc molecular studies are needed on the entire glutamatergic components, as well as on other neurochemical pathways (e.g., brain-derived neurotrophic factor, serotonin) and brain regions (e.g., amygdala) relevant to rewarding and learning. Further, we did not investigate the mechanism underlying the expression change, whether due to changes in synthesis or degradation. We measured the glutamatergic changes only at 2 hours after the end of the brief EE exposure: we are aware that a series of timepoint assessments may provide a better understanding. We are also aware that only detecting protein expression cannot establish a causal relationship and that protein expression alone may not reflect functional mechanisms. Our rats were kept isolated thus raising the possibility that housing conditions may indeed influence the response of the glutamatergic synapse. For instance, we recently demonstrated in adult male mice that living environment (i.e., social vs. isolation housing) influences the efficacy of EE/fluoxetine interaction in inhibiting relapse to sucrose (Pintori et al., 2022). Moreover, it is well known that novelty aspects (i.e., novel environment) play an important contribution in the higher sensitivity to drug effects (e.g., psychostimulants) induced by EE (Ball et al., 2006; Rebec et al., 1997). Although our study was designed to control for these novelty effects exposing the NoEE group to a novel housing cage, we are aware that we cannot discern the influence of novel environment from EE components in our

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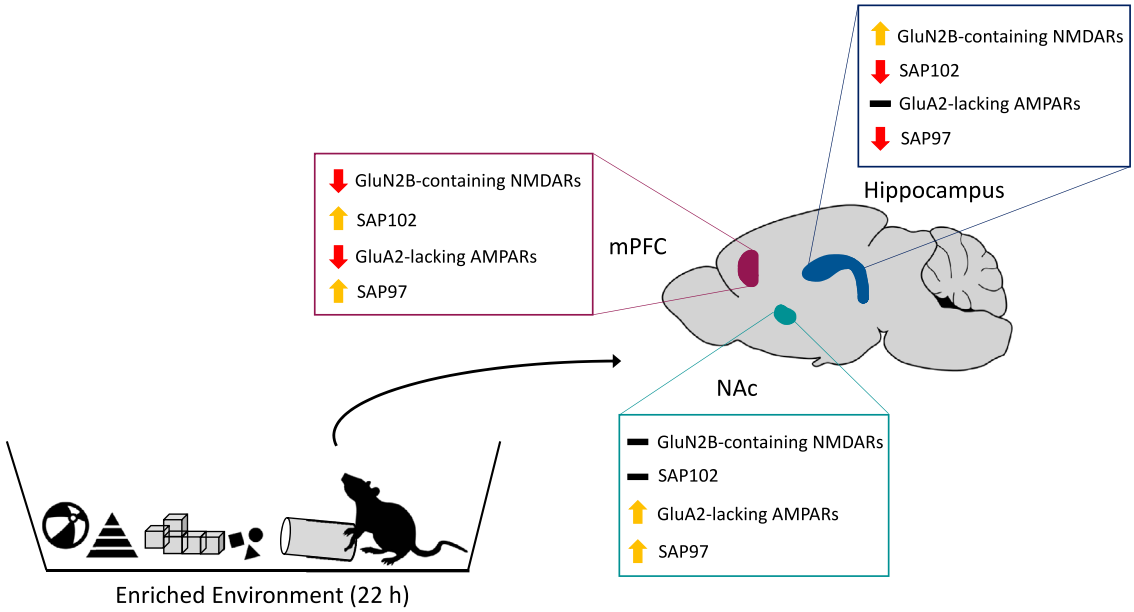

FIGURE 4 Graphical representation of the main metaplastic changes on glutamatergic mPFC-NAc-Hipp brain areas induced by brief EE exposure. A brief EE exposure was able to alter postsynaptic NMDA/AMPA receptor configurations, through changes in the subunit composition of such receptors, as well as changes in the expression of their main scaffolding proteins (i.e., SAP102, SAP97). Importantly, the modulation profile of glutamatergic synapses induced by brief EE exposure was unique for each brain region investigated (mPFC, NAc, Hipp). Abbreviations: ↑: increased expression; ↓: decreased expression; −: no change of expression; GluA2, glutamate AMPA receptor subunit 2; GluN2B, glutamate NMDA receptor subunit 2B; Hipp, hippocampus; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; SAP102, synapse-associated protein 102; SAP97, synapse-associated protein 97.

findings. Finally, we used only male rats in our study and, therefore, we do not know if our findings can be extended to female rats.

6 | CONCLUSIONS

Our findings demonstrate that a brief EE exposure dynamically modulates the glutamatergic synapses in mPFC-NAc-Hipp circuitry, which play an important role in reward and reward-related processes. Particularly, brief EE exposure was able to alter postsynaptic NMDA/AMPA receptor configurations, through changes in the subunit composition of such receptors, as well as changes in the expression of their main scaffolding proteins.

An added value of our study derives from the fact that each investigated region showed its specific modulation profile of the glutamate synapse. Consistently with our previous behavioural findings (Pintori et al., 2022), this metaplastic fingerprint induced by EE supports the hypothesis that brief EE exposure potentiates sucrose-seeking behaviours affecting subsequent learning processes, such as renewal or Cx-memory reactivation and reconsolidation. In line with this speculation, the reduction of cortical excitability induced by EE experience, along with a concomitant increase of Ca^{2+} permeability in the NAc and the Hipp, may alter salience attribution and behavioural response to reward-associated stimuli, thus enhancing sucrose-seeking behaviours when re-exposed to sucrose-paired context (Figure 4).

In conclusion, our data confirm the modulatory activity of brief EE manipulation on glutamatergic synapses, which could pave the way to develop innovative configurations of EE - with greater translational value feasibility - to improve existing therapeutic approaches against addiction and addiction-related CNS disorders.

AUTHOR CONTRIBUTIONS

Cristiano Chiamulera: Conceptualization; methodology; project administration; founding acquisition, resources; writing - original draft. Nicholas Pintori: Conceptualization; methodology; investigation; data curation; formal analysis; visualization; writing- original draft; writing - review & editing. Fabio Fumagalli: Conceptualization, methodology; writing- original draft. Alessandro Piva: investigation; data curation; visualization. Francesca Mottarlini: Investigation; data curation; formal analysis; visualization. Coralie Maggi: Investigation; data curation; visualization. Lucia Caffino: Investigation; data curation; formal analysis; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL

This study was approved by the ethical committee (OPBA) of the University of Verona and by the Ministry of Health (authorization n. 780/2019-PR) in compliance with the European Union regulations and Directive 2010/63/EU.

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