Effects of adult enriched environment on cognition, hippocampal-prefrontal plasticity and NMDAR subunit expression in MK-801-induced schizophrenia model

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ABSTRACT

Schizophrenia is a mental disorder characterized by psychosis, negative symptoms and cognitive impairment. Cognitive deficits are enduring and represent the most disabling symptom but are currently poorly treated. N-methyl D-aspartate receptor (NMDAR) hypofunction hypothesis has been notably successful in explaining the pathophysiological findings and symptomatology of schizophrenia. Thereby, NMDAR blockade in rodents represents a useful tool to identify new therapeutic approaches. In this regard, enriched environment (EE) could play an essential role. Using amultilevel approach of behavior, electrophysiology and protein analysis, we showed that a short-term exposure to EE in adulthood ameliorated spatial learning and object-place associative memory impairment observed in postnatally MK-801-treated Long Evans rats. Moreover, EE in adult life restored long-term potentiation (LTP) in hippocampal-medial prefrontal pathway abolished by MK-801 treatment. EE in adulthood also induced a set of modifications in the expression of proteins related to glutamatergic neurotransmission. Taken together, these findings shed new light on the neurobiological effects of EE to reverse the actions of MK-801 and offer a preclinical testing of a therapeutic strategy that may be remarkably effective for managing cognitive symptoms of schizophrenia.

Keywords: neurodevelopmental disorder, spatial learning, associative memory, electrophysiology, glutamate receptor.

1. INTRODUCTION

Schizophrenia is a disabling psychiatric disorder that affects approximately 1% of population worldwide (McGrath et al., 2008). Psychotic symptoms usually emerge during late adolescence/early adulthood and have been the hallmark of the disease. However, current trends point towards cognitive impairment as a core feature of schizophrenia (Kahn and Keefe, 2013). According to several epidemiological studies, cognitive deficits are present as early as age 4 (Agnew-Blais et al., 2015), and continue to progress before the onset of adolescence (van Oel et al., 2002; Woodberry et al., 2008) and beyond (Ullman et al., 2012). Cognitive symptoms in schizophrenia are irresponsive to antipsychotic treatment and are still largely untreated. Nevertheless, they markedly influence on daily functioning, social relationships and in the ability to sustain an employment, and thus represent the most debilitating symptom. Therefore, it is imperative to develop effective interventions for cognitive impairment and to identify the biological underpinnings of cognitive-enhancing strategies with the hope of finding novel therapeutic approaches.

Symptoms of schizophrenia emerge as a result of altered neurobiological cascades, although the precise etiopathogenesis remains to be elucidated. N-methyl-D-aspartate receptor (NMDAR) hypofunction has been proposed as the possible neuropathological substrate of schizophrenia (Nakazawa et al., 2017; Snyder and Gao, 2013; Weickert et al., 2013). This hypothesis arose from the observation that the administration of drugs that antagonize NMDAR, such as phencyclidine, ketamine or dizocilpine (MK-801), confer a pattern of behavioral, network, and neurochemical disturbances in animal models that resembled those seen in schizophrenic subjects (Bubenikova-Valesova et al., 2008; Lim et al., 2012). Moreover, NMDAR hypofunction hypothesis of schizophrenia is supported by *post mortem* studies, in which the obligatory NR1 subunit of NMDARs is found to be significantly reduced in prefrontal cortex (Catts et al., 2015) and hippocampus of schizophrenic individuals (Law and Deakin, 2001). Similarly, mRNA and protein expression of postsynaptic density 95 (PSD-95), a scaffolding protein required for NMDAR-mediated neurotransmission, is also diminished (Catts et al., 2015; Funk et al., 2017). PSD-95 associates with NR2 subunits of NMDARs to drive activity-dependent synapse stabilization, and regulates synaptic plasticity, further suggesting disturbed NMDAR signaling in schizophrenia.

Animal models of neurodevelopmental NMDAR hypofunction faithfully represent the symptom clusters and neurochemical alterations of schizophrenia. Although the neurobiological mechanisms of NMDAR antagonists to produce these alterations are not fully understood, compelling evidence suggests that patients with schizophrenia show aberrant functional coupling between hippocampus (HPC) and prefrontal cortex that might be crucial for cognitive dysfunction (Molina et al., 2017; Wolf et al., 2009; Zhou et al., 2016). The interaction of frontotemporal networks support navigation skills and the formation of associative memories (Sigurdsson and Duvarci, 2015). Some authors have termed the hippocampal-prefrontal pathway as the weak link in psychiatric disorders (Godsil et al., 2013), and it has been related to negative and cognitive symptoms of the disease (Ghoshal and Conn, 2015). Animal studies further support this, as the administration of MK-801 alters hippocampal-prefrontal connectivity and related cognitive functions (Blot et al., 2015).

Several findings indicate that enriched environment (EE), a paradigm that combines sensory, motor and social stimulation, is remarkably efficient in improving cognitive functions in health and disease (van Praag et al., 2000). One intriguing possibility is that EE may be successful in managing cognitive symptoms of schizophrenia, as adult rodents exposed to complex environment for short periods display a myriad of cellular and molecular changes that lead to improved cognition (Beauquis et al., 2010). This might be particularly useful in schizophrenia, because cognitive symptoms are pervasive and the diagnosis is usually protracted until the onset of psychotic symptoms in adolescence, although disturbances in neurodevelopmental processes are pivotal in dictating the occurrence of the illness (Fatemi and Folsom, 2009). Accumulating evidences suggest that EE upregulates the expression of neurotrophic factors and neurotransmitter receptors, increases dendritic branching, and enhances learning and memory processes, all of which are disturbed in schizophrenia (Ickes et al., 2000; Liu et al., 2017; Mohammed et al., 2002; Nithianantharajah et al., 2008; Uttl et al., 2018; Uylings et al., 1978; van Praag et al., 2000). Previous studies have provided EE from birth in genetic and pharmacological animal models of schizophrenia, concluding that EE is able to prevent sensorimotor gating and social interaction deficits (Bator et al., 2018; Burrows et al., 2015; Kentner et al., 2016), but the outcomes of rearing animals in EE from early life might considerably differ from a short exposure to EE during adolescence (Nozari et al., 2014) or adulthood. We have

previously reported that EE during adulthood improved recognition memory and GABAergic markers in animals that were postnatally administered MK-801 (Murueta-Goyena et al., 2018). As far as we know, how adult life EE intervention could reverse MK-801-induced deficits when administered in early life is still unknown. Thus, we aimed to further extend our previous results. We conducted a preclinical testing in MK-801 neurodevelopmental model by briefly exposing the animals to EE in adulthood, which offers a treatment with translational interest for managing cognitive deficits of schizophrenia. In the present study, we combined pharmacological and environmental strategies with behavioral, electrophysiological and neurochemical analyses to shed new light on the neurobiological underpinnings used by EE to reverse the actions of MK-801.

2. EXPERIMENTAL PROCEDURES

Animals

Long Evans rats were purchased from Janvier Labs (France). The day of birth was considered as postnatal day (P) 0, and pups were weaned on P21. All animals were maintained at 12-h light/dark cycle (lights on at 08:00 am) with access to food and water *ad libitum*. All procedures were performed in accordance with the European Recommendation 2010/63/EU and with Spanish Law (RD 53/2013) for the care and use of laboratory animals and were approved by Ethical Committee and Animal Welfare of the University of the Basque Country. A total of 83 animals were used in this study: 46 for behavioral and neurochemical analyses and 37 for electrophysiological analyses.

MK-801 administration and housing conditions

MK-801 [(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate, Dizocilpine hydrogen maleate] was purchased from Sigma-Aldrich (Ref: M107, St. Louis, MO, USA). Based on previous studies, we administered a dose of 0.5 mg/kg, which confers long-term behavioral and structural changes that recapitulate schizophrenia phenotype (van der Staay et al., 2011). The drug was administered intraperitoneally to rat pups once daily from P10 to P20 diluted in 0.9% NaCl. This dosing schedule coincides with the developmental expression of

parvalbumin, and its alteration is a major neuropathological hallmark of schizophrenia (Jaaro-Peled H Fau - Jaaro-Peled et al., 2010). Controls received the same volume of saline.

All animals were housed in standard conditions (SC) from P0 to P55. At this postnatal day, rats were housed in standard conditions or enriched environment (EE) until sacrifice in (P73), thus forming 4 groups: vehicle injected and standard housed (VH), MK-801 injected and standard housed (MK-801), MK-801 injected and housed in EE (MK-801+EE), and vehicle injected and housed in EE (VH+EE). The exposure to enriched environment (EE) lasted for 18 days during adult life (P55-P73) and consisted of a large cage (720 mm × 550 mm × 300 mm) with free access to wheel-runners (voluntary exercise) and differently shaped objects (e.g., shelters, tunnels) that were changed every 2 days. In standard conditions (SC), 2-3 animals were housed per cage (500 mm x 280 mm x 140 mm), whereas in EE 6 animals were housed per cage to promote social interaction. Figure 1A shows the timeline of different procedures. Animals were maintained in the same housing conditions during behavioral task period.

Cognitive tasks

Spatial learning deficits (Enomoto et al., 2008; Gorter and de Bruin, 1992; Nemeth et al., 2002) and object-in-place performance (Howland Jg Fau - Howland et al., 2012) are known to be impaired in animal models and are schizophrenia-relevant behavioral tasks (Powell and Miyakawa, 2006). However, the effect of EE in these tests has been largely unexplored.

Morris Water Maze

Morris Water Maze (MWM) was performed as previously described with minor modifications (Ortuzar et al., 2013). Rats (n=10-12 per group) were released from four different cardinal points with alternate starting points every day and in a random order for 5 days (P65-P69). On the sixth day (P70) the platform was removed to assess spatial reference memory by means of Mean Distance to Target (MDT). This parameter is a measure of proximity that is calculated as the average distance in centimeters between

the rodent and the center of the previous platform location during the 120s trial. A final trial with visible platform (cue) was conducted to ensure proper visual and motor abilities.

Object-in-place task

Object-in-place is an associative test in which the animal is supposed to link an object to a specific place where it has been previously encountered. After completion of MWM test, rats were habituated for 10 min in the opaque arena (90 x 90 x 40 cm) 24h prior to object-in-place test. During the familiarization phase on P72, animals were placed in the center of arena and allowed to investigate four objects made of plastic (Lego pieces) for 5 minutes before being removed and placed in the home cage for a retention interval of 90 min. Two visual cues were available on the walls for spatial orientation. In the test phase, two of the objects were relocated, and rats were allowed to explore the objects for 3 minutes. At least 12 seconds of active exploration (sniffing, touching the object with forepaws or looking straightforward at a distance closer than 1 cm) during the 5-minute period were required to include the animal in the analysis. To reduce biases to objects or locations, objects and their locations were counterbalanced among animals. To avoid the presence of olfactory trails, the arena was thoroughly cleaned with 30% ethanol between trials. The discrimination index (DI) was calculated as the ratio between the difference in time spent exploring the relocated objects (TR – TU) over the total amount of exploration time: DI = (TR – TU) / (TR + TU) [TR = time exploring relocated objects, TU = time exploring unmoved objects].

Electrophysiological recordings

A separate cohort of animals (n=7-11 per group) was used for electrophysiological recordings. Animals were anesthetized by intraperitoneally (i.p.) injected urethane (1.2 g/kg) and placed in a stereotaxic frame (Kopf, CA, USA). The body temperature was maintained at 37 ºC for the entire experiment by a rectal thermometer connected to a homeothermic blanket control unit. For all recordings, the head was oriented in the horizontal plane. A bur hole (3 mm diameter approximately) was drilled and a glass electrode was placed in the following coordinates in the mPFC (relative to Bregma): AP, 3.3 mm; ML, 0.8 mm; DV, - 3.5 mm (Paxinos and Watson, 1997). For the ventral HPC stimulation, a hole was drilled and a bipolar coaxial

stainless steel electrode (external diameter, 250 µm; tip diameter, 100 µm; tip-to-barrel distance, 300 µm) (Cibertec S.A.) was positioned in the following coordinates (relative to bregma) AP, 5.8-6.2 mm; ML, 5.3-5.6 mm; DV, -4 to -6 mm (Paxinos and Watson, 1997).

Long-term potentiation (LTP) was induced in the HPC-mPFC pathway following the procedure described in (Blot et al., 2015). Ventral HPC-mPFC postsynaptic potentials (PSPs) were evoked at 0.033 Hz by delivering constant current, monophasic square pulses of 250 µs width (Cibertec, S.A.). The stimulus intensity was adjusted to elicit a PSP amplitude that was of 60% of the maximum amplitude obtained in the test. After a stable baseline recording of ventral HPC-mPFC PSPs for at least 30 min, the LTP induction protocol was applied. This protocol consisted in a tetanic stimulation: a train of 50 pulses delivered at 250 Hz, repeated 10 times at 0.1 Hz, 2 such episodes in 6-min interval. PSPs were recorded for 2 hours after the LTP protocol induction. Long-term synaptic efficacy changes were measured after these 2 hours. After LTP induction protocol, PSPs were again recorded with ventral hippocampal stimuli delivered at 0.033 Hz (same conditions that in the baseline) for 2 hours.

Off-line analysis was performed using Spike2 software (Cambridge Electronic Design, Cambridge, UK). The strength of the synaptic transmission was measured as the amplitude from the peak of the first positive deflection of the evoked PSP to the peak of the following negative potential. Fifteen successive PSP amplitudes (corresponding to 10 min recording) were averaged and expressed as mean ± SEM. To study changes of the response, mean PSP values obtained after the induction protocol (corresponding to the last 10 min of the post-protocol recording) were compared to the mean basal values (corresponding to the last 10 min of the pre-protocol recording). Synaptic efficacy changes were classified as LTP when the mean of normalized PSP amplitudes before and after the induction protocol was significantly different.

Western Blot

Animals that performed cognitive tasks were sacrificed on P73 (n=6 per group) for molecular analysis. Relative expression levels of the following proteins were quantified in mPFC and HPC: NMDAR subunit NR1 (mouse anti-NR1, 1:1000,

Ref:05432, Merck-Millipore, USA), NR2A (mouse anti-NR2A, 1:1000, Ref: MAB5216, Merck-Millipore, USA), NR2B (rabbit anti-NR2B, 1:1000, Ref: AB1557, Merck-Millipore, USA), and the postsynaptic density-95 (rabbit anti-PSD-95, 1:1000, Ref: Af628, Frontier Institute, USA).

Tissue was homogenized in RIPA lysis buffer, and equal amounts of proteins were separated on SDS-polyacrylamide gels. For NR1 and PSD-95 immunodetection, proteins were transferred onto PVDF membranes by semi-dry transfer in Trans-Blot® Turbo™ Transfer System (Bio-Rad, Hercules, CA, USA). For NR2A and NR2B subunit detection, overnight wet transfer was performed onto nitrocellulose membranes. Membranes were incubated for 1 h in TBS buffer (100 mM Tris-HCl; 0.9% NaCl, 1% Tween 20, pH 7.4) containing 5% non-fat dry milk to block nonspecific binding sites. Blots were then incubated in primary antibodies overnight. Actin was used as a loading control (1:2000; Ref: A-2066, Sigma-Aldrich, St. Louis, USA). The following day, HRP conjugated secondary antibody (1:20.000, anti-rabbit IgG [Ref:A-6164] or anti-mouse IgG [Ref:A-9044], Sigma-Aldrich, Spain) was added for 1h at room temperature, and a chemiluminescent detection system (Ref: 34076, SuperSignal® West Dura Extended Duration Substrate, Fisher Scientific, Spain) was used to visualize the immunoreactive proteins. Images were acquired using ChemiDoc™ XRS+ Imaging System (Bio-Rad, Hercules, CA, USA) and optical densities were quantified with Image Studio Digits 3.1 (LI-COR BioScience Biotechnology, Germany). The values for NR1, NR2A, NR2B, and PSD95 are expressed as the relative change from the vehicle group.

Statistical analysis

Learning phase in MWM task was analyzed with two-way ANOVA for repeated-measures. All other data were analyzed with two-way ANOVA with MK-801 and housing conditions as factors, followed by post hoc tests as appropriate. Welch's ANOVA with Games-Howell post hoc test was used for specific planned comparisons between groups when homogeneity of variances was violated. All computations were made using the SPSS software package (version 23.0, IBM, Spain), and differences with $p<0.05$ were considered significant.

3. RESULTS

Environmental enrichment improves spatial learning and associative memory impairment after postnatal MK-801 treatment

To determine the presence of cognitive deficits after neonatal MK-801 treatment, and to evaluate the potential therapeutic effects of EE, we tested the animals in MWM and in object-in-place task. Repeated-measures ANOVA revealed a significant main effect of trial day during the acquisition phase in MWM on latency ($F_{(4,39)}$=43.838, p<0.0001) and distance ($F_{(4,39)}$=26.908, p<0.0001), meaning that all groups learned significantly over trials. However, we found a significant main effect of MK-801 ($F_{(1,42)}$=10.601, p=0.002) and drug x housing interaction ($F_{(1,42)}$=11.350, p=0.002, Figure 1B) on latency during the learning phase of MWM (Figure 1, B.1). MK-801 ($F_{(1,42)}$=16.368, p<0.0001) and EE ($F_{(1,42)}$=7.271, p=0.01) also had a significant effect on the distance (Figure 1, B.2). Post hoc comparisons showed that MK-801-treated rats spent more time and swam longer distances to find the hidden platform when compared to VH (latency, p<0.0001; distance, p=0.003) and VH+EE rats (latency, p=0.005; distance, p<0.0001). This impairment in spatial learning of MK-801-treated rats allowed dissecting the role of EE in cognitive enhancement. Indeed, MK-801+EE group spent less time to locate the platform compared to MK-801 rats in SC (p=0.004), and traveled less distance (p=0.035), which strongly suggests improvement in spatial learning abilities. After 5 days of training, spatial reference memory was evaluated. Two-way ANOVA showed a significant effect of EE ($F_{(1,35)}$=32.563, p<0.0001) on Mean Distance to Target, but no effect of MK-801 ($F_{(1,35)}$=0.569, p=0.456). These results indicate that animals housed in EE during adulthood for a short period swam closer to the previous platform location than SC animals, which is a readout of improved reference memory (Figure 1, B.3).

We next tested the animals for object-place associative memory using object-in-place task (Figure 1, C.1). During the sample and test trials, the interaction time with objects was comparable across groups [test trial: main effect of MK-801 ($F_{(1,35)}$=2.970, p=0.92), main effect of EE ($F_{(1,35)}$=2.637, p=0.113), MK-801 x EE interaction ($F_{(1,35)}$=0.613, p=0.439), Figure 1, C.2]. In the test trial, two-way ANOVA showed significant effect of MK-801 ($F_{(1,35)}$=14.714, p=0.001) and EE ($F_{(1,35)}$=4.921, p=0.033)

on discrimination index (Figure 1, C.3). Rats neonatally injected with MK-801 displayed significantly reduced discrimination index compared to vehicles (p=0.038) that was increased by 3-fold in MK-801+EE animals (15.2%), showing a partial recovery of associative memory. VH+EE group showed the highest discrimination index (30.5%).

Long-term potentiation in hippocampal-prefrontal pathway in MK-801-treated rats is rescued by adult exposure to environmental enrichment

The electrical stimulations of the ventral HPC region evoked PSP responses in the prelimbic mPFC of anaesthetized rats constituted by two components (a small positive deflection and a following negative deflection) in which latency and amplitude parameters were analyzed (Figure 2A). Neither MK-801 nor EE changed baseline recording (30 min) response parameters (PSP latency: VH: 30 ± 3 ms, MK-801: 25 ± 3 ms, MK-801+EE: 33 ± 2 ms, VH+EE: 27 ± 4 ms; PSP amplitude: VH: 0.67 ± 0.23 mV, MK-801: 0.90 ± 0.34 mV, MK-801+EE: 0.62 ± 0.25 mV, VH+EE: 0.88 ± 0.31 mV; p>0.05, two-way ANOVA).

Tetanic stimulation induced LTP of HPC-mPFC responses leading to an increment of baseline PSP amplitude 2 hours after induction protocol (Figure 2B and C). A robust test for equality means was run (Welch's ANOVA) followed by the Games-Howell post hoc test for comparing LTP induction between groups, as the assumption of homogeneity of variances was violated (p=0.001, Levene's test) and the effect on MK-801, EE and their interaction could be invalid using two-way ANOVA. Welch's ANOVA revealed a significant group effect ($F_{(3,14.4)}$=4.798, p=0.016).We observed that in the MK-801 group, the PSP amplitude increase from baseline was significantly lower than for VH group (p=0.033) and this impairment was reversed in MK-801+EE animals (p=0.041, compared to MK-801; Figure 2C). Therefore, these results indicate that MK-801 abolished LTP induction and adult exposure to EE restored it.

Environmental enrichment reverts MK-801-induced downregulation of NR1 in hippocampus and increases NR2A, NR2B and PSD-95 expression

Given that EE promotes the synthesis and surface expression of NMDARs, we compared the amount of the obligatory NR1 subunit and facultative NR2A and NR2B subunits in HPC and mPFC. Western Blot analysis showed a significant effect of MK-801 in NR1 levels in mPFC ($F_{(1,14)}$=35.617, p<0.0001; Figure 3A) and HPC ($F_{(1,15)}$=39.037, p<0.0001; Figure 3B). There was also a significant effect of EE on this subunit (mPFC: $F_{(1,14)}$=16.123, p=0.001, HPC: $F_{(1,15)}$=102.745, p<0.0001), and a significant interaction of drug x housing for NR1 in HPC ($F_{(1,15)}$=86.592, p<0.0001). Post hoc analyses showed that the increase in NR1 subunit was significant in MK-801+EE animals compared to MK-801 group in HPC (p<0.0001), but not in mPFC (p=0.131).

Regarding NMDAR 2A and 2B subunits, two-way ANOVA yielded MK-801 main effect for NR2A subunit in mPFC ($F_{(1,14)}$=57.942, p<0.0001; Figure 3A) and NR2B subunit in HPC ($F_{(1,15)}$=35.038, p<0.0001; Figure 3B). On the other hand, the effect of EE was significant for NR2A and NR2B in both regions (p<0.0001). It should be noted that Post hoc analyses revealed significant differences between MK-801 and MK-801+EE groups in NR2B levels of mPFC (p=0.003). Likewise, the upregulation of NR2A and NR2B subunits in HPC was particularly noticeable in MK-801+EE group, being significantly higher than in MK-801 group in standard conditions [NR2A (p=0.022); NRB2 (p<0.0001)]. The effect of MK-801 on PSD-95 levels did not reach significance in mPFC ($F_{(1,14)}$=4.123, p=0.062), nor in hippocampus ($F_{(1,15)}$=0.002, p=0.969). Nonetheless, the effect of EE was significant in both regions (mPFC: $F_{(1,14)}$=44.588, p<0.0001; $F_{(1,15)}$=0.244, p=0.628; Figure 3C).

Taken together, adult environmental intervention triggered an upregulation of the components of glutamatergic neurotransmission in MK-801-injected animals, including the increase in the expression of NR1 subunit that was deficient in MK-801 animals, with a parallel increase of NR2B subunit, the scaffolding protein PSD-95 and hippocampal NR2A subunit expression.

4. DISCUSSION

The present study reveals new insights into the beneficial effects of EE exposure in adulthood of animals treated postnatally with MK-801 and renders EE a useful strategy to improve cognitive impairment, network disturbances and neurochemical alterations relevant to schizophrenia. We previously reported that exposing Long Evans rats to EE for a short period during adult life restored recognition memory and GABAergic markers in postnatally MK-801-injected animals (Murueta-Goyena et al., 2018). The present results further extend those observations and propose that EE-mediated regulation of NMDARs might have crucial implications in synaptic plasticity and improvement of cognitive alterations emerging upon NMDAR blockade. The changes in NMDAR subunit levels after pharmacological and environmental interventions could also explain some modifications in GABAergic markers that we found in our earlier work, as parvalbumin and GAD67 are NMDAR-dependent (Kinney et al., 2006).

For assessing the powerfulness of enrichment in reversing cognitive impairment, we tested the animals in MWM and object-in-place task. A number of studies in rodents have already demonstrated that early-life MK-801 administration resulted in impaired spatial learning in MWM, which was predominantly manifested after maturity and without alterations in reference memory, consistent with the present findings (Enomoto et al., 2008; Gorter and de Bruin, 1992; Nemeth et al., 2002). The delay in the acquisition phase of MWM with preserved reference memory in MK-801 group led us to question whether difficulties in locating visual cues in space could be the underlying factor contributing to slower learning rate. Consistent with this, the discrimination index in object-in-place task was significantly affected by MK-801 administration, suggesting that MK-801-injected animals could not properly identify or associate objects to a specific spatial location. This result resembled the impaired performance in working memory binding task (Burglen et al., 2004) and pattern-location association (Wood et al., 2002) of human patients with schizophrenia. However, we should not exclude the possibility that impaired object recognition might have contributed to decreased discrimination index in object-in-place task, as postnatal injections of MK-801 perturb object recognition memory in adulthood (Murueta-Goyena et al., 2018). On the other hand, few studies have addressed the potential benefits of EE on MK-801-induced spatial learning deficits. According to Nozariet al.,(Nozari et al., 2014), EE prevented

spatial learning and memory deficits in Morris Water Maze in juvenile rats after chronic high doses of MK-801 (1mg/kg) from P6-P10. Our results indicate that adult-life intervention with EE reversed spatial learning deficit and increased the discrimination index on object-in-place task in MK-801-treated animals. As far as we know, this is the first study demonstrating that late exposure to EE is effective in reverting spatial learning impairment in MWM and improving deficient associative memory after transient NMDAR blockade during the postnatal period. We previously reported that adult life EE was able to reverse recognition memory to normal levels in MK-801-injected animals (Murueta-Goyena et al., 2018), but object-in-place performance was only partially rescued. In contrast to novel object recognition task, object-in-place requires four items to be remembered instead of one, and temporal and spatial components of objects need to be represented, increasing task difficulty. Moreover, the neural networks involved in each task differ. Whereas perirhinal cortex is essential for object recognition, object-in-place memory also depends on hippocampus and medial prefrontal cortex (Warburton and Brown, 2015).

Afferents from HPC to mPFC are essential for encoding spatial cues in rodents, (Spellman et al., 2015) and play an essential role in the formation of associative memories. In line with previous results, postnatal MK-801 intraperitoneal injections produced an enduring disruption in long-term synaptic plasticity in the HPC-mPFC pathway that could be responsible of the observed cognitive impairment (Blot et al., 2015). Animal studies have shown uncoupling of the HPC from mPFC circuitry that results from an overactivation of pyramidal neurons after systemic injections of MK-801 (Grüter et al., 2015), similar to that found in schizophrenia (Heckers and Konradi, 2015). Baseline brain excitability has important implications in cortical activity (Chattopadhyaya et al., 2007). As shown by Blot et al., (Blot et al., 2015) shortly after MK-801 administration, increased excitability of mPFC neurons hindered LTP induction in HPC-mPFC pathway. According to these authors, the overly active mPFC led to an aberrant form of LTP, which shared common mechanisms with tetanus-induced LTP, and impaired mPFC-dependent behavior. In a similar vein, excessive brain activity has also been related to decreased spine density and cognitive decline (Lesh et al., 2011). Interestingly, EE enhances synaptic potentiation, at least in hippocampus (Buschler and Manahan-Vaughan, 2017). Here we demonstrated that hippocampal-prefrontal synaptic plasticity abolished by postnatal MK-801 was rescued

by adult-life experience in EE. Although the neurobiological underpinnings of this improvement need to be elucidated, it could be a consequence of EE-mediated modifications in the glutamatergic neurotransmission.

NMDAR hypofunction is one of the leading hypotheses to explain the neurochemical, metabolic, functional and behavioral alterations of schizophrenia (Nakazawa et al., 2017; Snyder and Gao, 2013; Weickert et al., 2013). In this study, we observed that neonatal MK-801 administration in rats downregulated the obligatory NR1 subunit of NMDAR in HPC and mPFC that further supports NMDAR hypofunction and its relation to the pathophysiology of schizophrenia. Weickert et al., (Weickert et al., 2013) provided evidences of reduced mRNA and protein levels of NR1 subunit in dorsolateral prefrontal cortex of schizophrenic patients, and more recently, Catts et al., (Catts et al., 2015) have corroborated these results. Perinatal NMDAR antagonism instills disturbances in the GABAergic system like reduced parvalbumin and glutamic-acid decarboxylase 67 (GAD67) expression that are consistently reported in postmortem brain of schizophrenic patients (Bubenikova-Valesova et al., 2008; Lim et al., 2012) that we also observed in MK-801-administered animals (Murueta-Goyena et al., 2018).According to the current literature, NMDAR hypofunction in GABAergic cells would alter excitation/inhibition balance and elicit a generalized NMDAR internalization to compensate for glutamate spillover (Nakazawa et al., 2017).

After EE exposure in adulthood, NR1 subunit increased in the HPC with a parallel increase in NR2A and NR2B subunits that was more evident in MK-801+EE group. Based on these results, we suggest that EE promoted the synthesis and expression of NMDAR subunits in HPC in rats neonatally treated with MK-801, perhaps as a compensatory mechanism for the induced NMDAR blockade. Moreover, PSD-95, a synaptic protein that regulates the channel gating and surface expression of NMDARs (Lin et al., 2004), was also significantly increased in MK-801+EE animals, further suggesting increased NMDAR-mediated neurotransmission after EE intervention. The effect of EE in the expression of NR1, PSD-95 and other components of glutamatergic system has been previously observed (Burrows et al., 2015; Nithianantharajah et al., 2008; Tang et al., 2001). However, in mPFC, EE-mediated NR1 and NR2A protein level enhancement was minimal in MK-801+EE rats, but we found a major increase in NR2B subunit expression. In fact, enhanced NR2B levels in mPFC of MK-801+EE animals could partially explain the rescue of synaptic plasticity

in the HPC-mPFC pathway. NR2B-containing NMDARs reveal long currents and carry more charge for a single synaptic event than NR2A-containg NMDARs (Erreger et al., 2005). NR2B-containing NMDARs have an essential role in prefrontal cortex-dependent cognitive functions (Monaco et al., 2015), as they couple to intracellular signaling cascades, like Ras-GRF1 and RasGAP and CaMKII (Barria and Malinow, 2005; Zhu et al., 2002), which might favor LTP induction. Therefore, we propose that the enhancement in memory functions of MK-801+EE rats could be the consequence of upregulated NMDAR glutamatergic transmission with paralleled rescue of synaptic potentiation in HPC-mPFC pathway. Nevertheless, we should be cautious in interpreting the mechanism by which EE produced all these modifications, as the present results are only correlational. Previous studies have reported changes in NMDAR subunit mRNA (Oh et al., 2001; Wilson et al., 1998) and protein levels (Lindahl and Keifer, 2004) in response to NMDAR antagonists or EE, separately. In a recent study by Liu et al. (Liu et al., 2017) the authors showed that chronic administration of MK-801 in adult rats also reduced NR1 subunit in mPFC, but not in hippocampus. Similar to our results, they did not find significant differences in NR2A and NR2B subunits compared to control animals, but PSD-95 in mPFC was downregulated, which differs from our results. Contrarily, Uttl et al. (Uttl et al., 2018) reported that subcutaneous chronic injections of MK-801 during adolescence or adulthood did not induce a decrease of NMDAR subunits. These differences might have been a result of age-dependent effects of MK-801. The pattern of NMDAR subunit expression changes dramatically during development, and probably NMDARs are most sensitive to MK-801 in early life, a period that corresponds with the maximum expression of these receptors (Monyer et al., 1994). Here we show that adult experience in EE was capable of upregulating NMDARs in postnatally MK-801-treated rats, suggesting that environmental intervention might be remarkably successful in managing core pathophysiological findings of schizophrenia.

Despite these evidences linking adult intervention with EE to improved cognitive performance in a neurodevelopmental animal model of schizophrenia, several aspects need to be further investigated. Firstly, we focused this study in adult animals and we used short intervention with EE to study its preclinical validity as a therapeutic approach for first-episode of schizophrenia. However, it would be important to explore the temporal boundaries of MK-801 and EE-induced changes, like when the functional

downregulation of NR1 occurs or determine if earlier and/or longer environmental intervention would result in different functional outcomes. Although it is well accepted that NMDAR hypofunction contributes to the manifestation of schizophrenia, the complex combination of genetic, epigenetic and environmental factors rendering NMDARs hypoactive remain unexplained. Alterations in synaptic plasticity also represent a major hallmark of the disease that EE is able to restore, but the extent of the impact of LTP recovery in cognition needs to be more precisely determined. Therefore, a better understanding of the neurobiological basis of schizophrenia and cognition are required to further support of EE as a possible intervention. While future studies are necessary to address these issues, our results establish the foundation for future research and open the possibility of environmental interventions as a therapeutic approach for cognitive symptoms of schizophrenia.

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FIGURES AND FIGURE LEGENDS

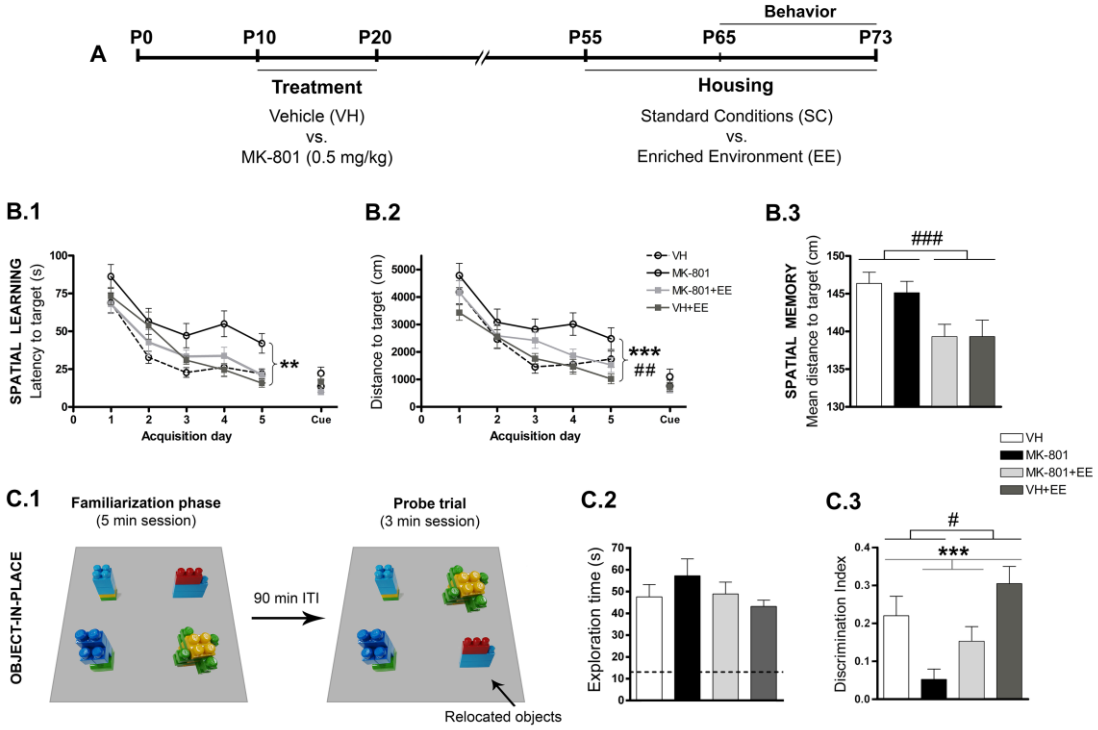

Figure 1. Effects of environmental enrichment (EE) on cognitive functions afterNMDA receptor (NMDAR) blockade in early neurodevelopment. (A) Timeline of experimental procedures. MK-801-treated rats showed impaired spatial learning in terms of (B.1) latency and (B.2) distance to reach the hidden-platform without differences in cued trial that was reversed by EE. (B.3) Mean distance to target (MDT) during Morris Water Maze (MWM) probe trial showed no significant effect of MK-801, but improved spatial reference memory after EE. (C.1) Experimental protocol of object-in-place task. (C.2) Graph shows the mean exploration time during object-in-place test trial. (C.3) Object-in-place discrimination index of each group expressed as the ratio (TR – TU) / (TR + TU) [TR = time exploring relocated objects, TU = time exploring unmoved objects]. VH, n=10, MK-801, n=10, MK-801+EE, n=11, VH+EE, n=12. ITI, intertrial interval; EE, enriched environment. Data are represented as mean ± SEM. *Effect of MK-801 (**p < 0.01, ***p < 0.001); #Effect of EE (#p < 0.05, ##p < 0.01, ###p < 0.001).

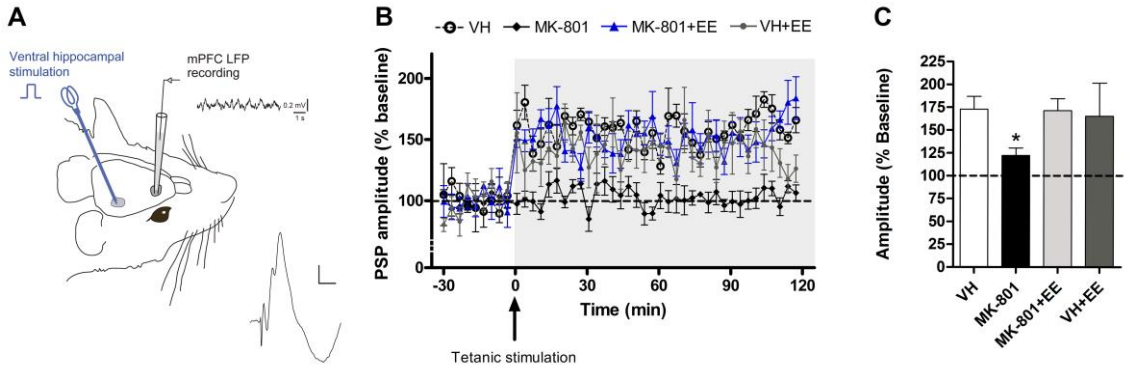

Figure 2. Effects of adult life exposure to environmental enrichment (EE) after neonatal MK-801 treatment on hippocampal-medial prefrontal cortical (HPC-mPFC) synaptic plasticity. (A) Upper: Schematic representation of the in vivo experimental set-up for local field potential (LFP) recording of mPFC and electrical stimulation of the ipsilateral ventral HPC. Lower: Example of mPFC postsynaptic potential (PSP) evoked by a single HPC stimuli delivered at 0.033 Hz (scale bar: 20 ms (horizontal) 2 mV (vertical). (B) Representative experiments illustrating the time courses of synaptic efficacy changes induced by the induction protocol. Each time point corresponds to the average of five consecutive PSPs. (C) Mean amplitude of PSPs calculated the last 10 min of the two-hour recording after LTP induction. Data are represented as mean ± SEM. *p< 0.05 compared to VH and MK-801+EE.

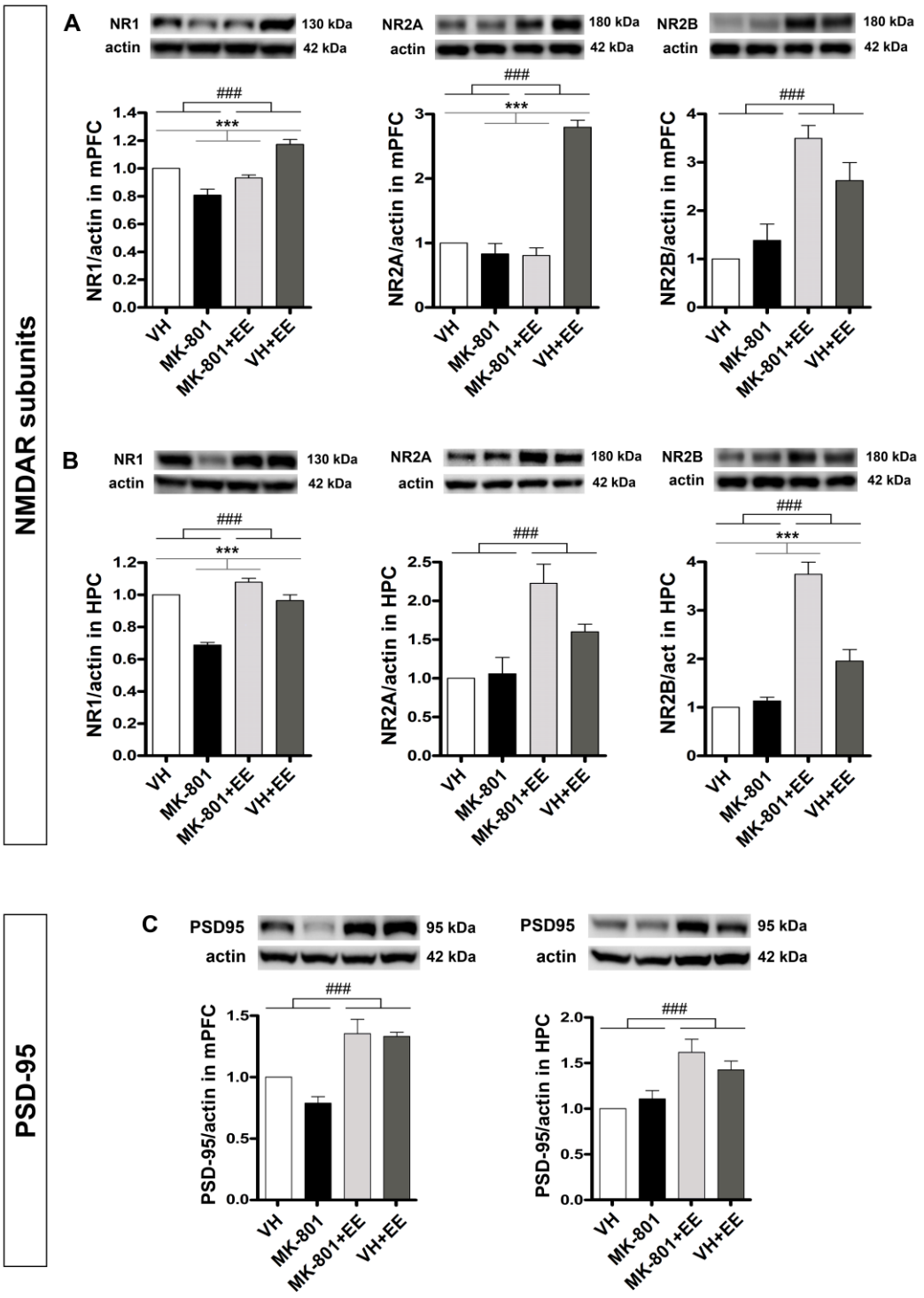

Figure 3. Effects of adult life exposure to environmental enrichment (EE) after neonatal MK-801 treatment on NMDAR subunit expression and its scaffolding protein PSD-95. (A) Expression levels of NMDAR subunits (NR1, NR2A and NR2B) in medial prefrontal cortex, (B) Expression levels of NMDAR subunits (NR1, NR2A and NR2B)

in hippocampus, (C) PSD-95 protein levels in medial prefrontal cortex and hippocampus. Histograms represent optical densities obtained for each primary antibody after protein normalization with actin, expressed as the ratio relative to VH-injected animals. Data are represented as group mean ± SEM. ***Effect of MK-801, $p < 0.001$; ### Effect of EE, $p < 0.001$. mPFC, medial prefrontal cortex: HPC, hippocampus; PSD-95, postsynaptic density-95.