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Short-Term Exposure to Enriched Environment in Adult Rats Restores MK-801-Induced Cognitive Deficits and GABAergic Interneuron Immunoreactivity Loss

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Abstract Perinatal injections of *N*-methyl-D-aspartate (NMDA) receptor antagonist in rodents emulate some cognitive impairments and neurochemical alterations, such as decreased GABAergic (gamma aminobutyric acid) interneuron immunoreactivity, also found in schizophrenia. These features are pervasive, and developing neuroprotective or neurorestorative strategies is of special interest. In this work, we aimed to investigate if a short exposure to enriched environment (EE) in early adulthood (P55–P73) was an effective strategy to improve cognitive dysfunction and to restore interneuron expression in medial prefrontal cortex (mPFC) and hippocampus (HPC). For that purpose, we administered MK-801 intraperitoneally to Long Evans rats from postnatal days 10 to 20. Twenty-four hours after the last injection, MK-801 produced a transient decrease in spontaneous motor activity and exploration, but those abnormalities were absent at P24 and P55. The open field test on P73 manifested that EE reduced anxiety-like behavior. In addition, MK-801-treated rats showed cognitive impairment in novel object recognition test that was reversed by EE. We quantified different interneuron populations based on their calcium-binding protein expression (parvalbumin, calretinin, and calbindin), glutamic acid decarboxylase 67, and neuronal nuclei-positive cells by means of unbiased stereology and found that EE enhanced interneuron immunoreactivity up to normal values in MK-801-treated rats. Our results demonstrate that a timely intervention with EE is a powerful tool to reverse long-lasting changes in cognition and neurochemical markers of interneurons in an animal model of schizophrenia.

Keywords MK-801 · Interneurons · Cognitive dysfunction · Calcium-binding proteins · Enriched environment

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Introduction

Interneurons are the main source of inhibitory input in the central nervous system, and gamma aminobutyric acid (GABA) is their primary neurotransmitter. Although GABAergic interneurons only account for 10–25% of total cell number, depending on the brain region, they display extremely distinct chemical, morphological, and functional features, making their classification a challenging task. It has been postulated that the rich variety of interneurons is essential for providing constant matched inhibitory input to the remarkably diverse incoming stimuli [1]. In addition, the computational diversity they provide allows proper dynamics for higher cognitive functions. Nevertheless, GABAergic interneurons also play an important role during postnatal development [2]. GABA promotes the migration of glutamatergic and GABAergic neurons and dictates the final location of different subpopulations of interneurons. GABA also

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contributes to the regulation of synapse elimination in the developing brain and optimizes excitatory/inhibitory balance for regulated information transfer in an activity-dependent manner [2].

N-methyl-D-aspartate receptor (NMDAR)-mediated activity is likewise important for the functional development of neural circuits. Neonatal blockade of NMDAR alters gamma oscillations, decreases parvalbumin (PV) and 67 kDa isoform of glutamic acid decarboxylase (GAD67), and induces myriad of behavioral, cellular, and molecular changes that can be traced to a disruption of GABAergic interneurons [3–5]. Since abnormal development of GABAergic system seems to be responsible for some cognitive deficits found in schizophrenia [6], NMDAR hypofunction hypothesis has gained attention to model the mechanism that could explain the symptoms and natural course of the disease. It is now widely accepted that schizophrenia is a neurodevelopmental disorder, and chronic perinatal injections of NMDAR antagonists, like MK-801 (dizolcipine), are needed to model of the disease [7]. The medial prefrontal cortex (mPFC) and hippocampus are critical brain regions involved in the pathophysiology of schizophrenia, and relevant for cognitive functions. Functional remodeling of mPFC occurs during adolescence, when fine tuning of GABAergic activity is integrated [2, 8]. Late-adolescence (P50) onset of hippocampal-dependent input also contributes to the functional maturation of mPFC [8]. Developmental administration of MK-801 alters the mechanisms underlying the protracted maturation of GABAergic system and hinders the establishment of appropriate information processing mechanisms that support complex cognitive functions [7, 9, 10].

Up to date, studies have focused on investigating the deleterious long-term effects of neonatal NMDAR blockade, but little is known about possible interventions that could enhance or restore the pathophysiological findings, such as cognitive impairment and decreased GABAergic marker immunoreactivity. Enriched environment (EE) is an experimental paradigm to study the potential plastic changes induced by increased physical exercise, sensory input, and social stimulation [11]. Environmentally stimulating conditions promote cell survival and neuronal protection through several molecular and cellular changes that are accompanied by improvements in cognitive performance [12–15].

The exposure to an EE has long been considered to have beneficial effects in health and disease. Therefore, animals subjected to perinatal NMDAR antagonism could be benefited from a short intervention with EE in early adulthood that may have important implications for developing future neuroprotective strategies. In the present study, we sought to investigate the behavioral and anatomical consequences of adult exposure to EE in animals subjected to chronic NMDA receptor blockade.

Materials and Methods

Novel object recognition (NOR) task was performed for assessing cognitive function, and locomotor activity was evaluated in open field test. Then, we quantified three subgroups of interneurons in mPFC and hippocampus identified upon their calcium-binding protein expression: PV, calretinin (CR), and calbindin (CB). For further analyzing cellular and structural changes occurring after MK-801 administration and EE, GAD67 and neuronal nuclei (NeuN)-positive cells were quantified, and cortical and hippocampal volumes were estimated.

Animals

Female rats with male litters were purchased from Janvier Labs (France). All animals were maintained at 12-h light/dark cycle (lights on at 08:00 a.m.) with access to food and water ad libitum. All procedures were performed in accordance with the European Recommendation 2007/526/EC and were approved by Ethical Committee and Animal Welfare of the University of the Basque Country.

Pharmacological Procedures and Housing Conditions

MK-801 Administration 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 used a dose of 0.5 mg/kg, which has been shown to be the threshold dose for apoptotic damage [16] and for inducing long-term behavioral alterations [7]. The drug was administered intraperitoneally to rat pups once daily from P10 to P20 diluted in 0.9% NaCl. A final volume of 1 ml/100 g of animal weight was used. Controls received the same volume of saline. Body weights were recorded every day during the treatment period and every 2 weeks from drug cessation to P73.

Housing Conditions

Four different experimental groups were used (Fig. 1):

(a) VH: rats raised under standard conditions that received saline from P10 to P20.
(b) MK-801: rats raised under standard conditions that received MK-801 (0.5 mg/kg) from P10 to P20.
(c) MK-801 + EE: rats raised under standard conditions from P0 to P55 and in an enriched environment from P55 to P73. This group received MK-801 (0.5 mg/kg) from P10 to P20.

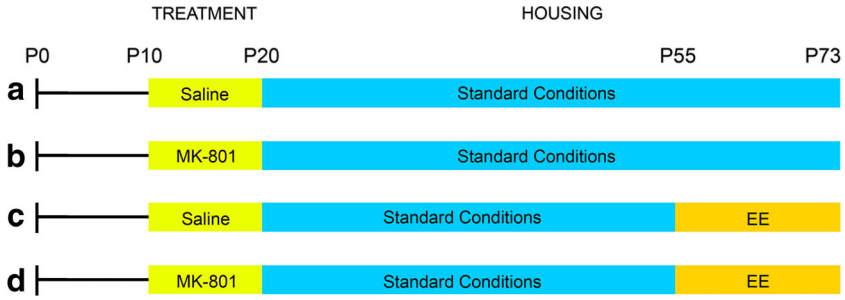

Fig. 1 Timeline of pharmacological treatment and housing conditions. Animals received saline or MK-801 (0.5 mg/kg) treatment from P10 to P20. On P55, one group of saline and one group of MK-801 were changed to enriched cages for 18 days. *P* postnatal days, *SE* standard environment, *EE* enriched environment. **a** Vehicle group. **b** MK-801 group. **c** MK-801 + EE group. **d** Vehicle + EE group

(d) VH + EE: rats raised under standard conditions from P0 to P55 and in an enriched environment from P55 to P73. This group received saline from P10 to P20.

In standard conditions, three animals were housed per cage (500 mm × 280 mm × 140 mm). In EE, six animals were housed per cage promoting social interaction. The EE 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.

Behavioral Tasks

For behavioral tasks, between 10 and 12 animal were used per group. Those animals pertaining to EE groups were maintained in the same environment during test trials. Rats were tested in open field test on P21 and P24 to assess short-term effects of MK-801, and before (P55) and after (P73) EE to assess anxiety-like behavior and locomotion. Novel object recognition test was conducted on P71.

Open Field Test Spontaneous locomotor activity was fully automated using custom-designed software Actitrack (Panlab, Spain). The apparatus consisted of a square arena (44 × 44 × 35 cm) made of plexiglas with parallel frames, few centimeters apart, provided with 16 × 16 infrared beams for optimal animal detection. The floor was divided into two squares that allowed the definition of central and peripheral areas. Each rat was placed in the center of the arena, and its activity was recorded for 10 min. Horizontal displacement in the center and the periphery of the arena was measured. Relative distance in center was defined as the following formula: 100 × [total distance moved in center arena / total distance in periphery].

Novel Object Recognition The NOR task evaluates the ability of the animal to recognize a novel object based on rodent's natural tendency to explore new objects in their environment. It consists of three phases: habituation, familiarization, and probe phase. In the habituation phase, rats were placed individually in an opaque arena (90 × 90 × 40 cm) for 10 min to freely explore the environment without objects. During the familiarization phase, two identical objects made of plastic (lego pieces) were displayed in adjacent quadrants of the arena, and the time exploring each object was measured during a 5-min period. Rodents were released facing the wall to prevent coercion. After a retention interval of 90 min, the rodent was returned to the arena for a 3-min test trial, in which a familiar object was replaced by a novel object. Novel object was made in the same material as familiar object. No visual cues were used in this procedure.

To reduce biases to particular objects or locations, objects and their locations were counterbalanced among animals. A dim light illuminated the arena homogeneously. To avoid the presence of olfactory trails, the arena was thoroughly cleaned with 96% ethanol between trials. Exploration was considered when the animal sniffed, touched the object with forepaws, or looked straightforward at a distance closed than 1 cm. At least 12 s of active exploration during the 5-min period were required to include the animal in the analysis. Discrimination index was expressed by the ratio (TN − TF) / (TN + TF) [TN = time exploring the novel object, TF = time exploring the familiar object].

Immunohistochemistry

The immunohistochemical studies were performed in six animals of each experimental condition. Rats were euthanized by sodium pentobarbital before transcardial perfusion with sodium chloride (0.9% sodium chloride, pH = 7.4) followed by 4% paraformaldehyde (in 0.1 M PBS). Brains were removed, postfixed overnight in the same fixative at 4 °C, and stored in 30% sucrose solution at 4 °C. Serial coronal brain sections (50 μm thick) were cut on a freezing microtome (Leica, Wetzlar, Germany). Free-floating sections were washed two times for 5 min in 0.1 M PBS and incubated for 20 min in a solution of 3% hydrogen peroxide to eliminate endogenous peroxidase activity. After three

washes, free-floating sections were blocked in 5% normal horse serum (NHS) in 0.1 M PBS with 0.5% Triton X-100 (PBS-TX) during 1 h and incubated in blocking solution with the following primary antibodies overnight at 4 °C: mouse anti-parvalbumin (Ref. PV 235; 1:5000; Swant, Switzerland), mouse anti-calretinin (Ref. 6B3; 1:2000; Swant, Switzerland), and mouse anti-GAD67 (Ref. MAB5406; 1:10,000; Merck Millipore, Germany). TX was excluded in all steps of GAD67 immunohistochemistry. The next day, followed by another washing step, sections were incubated with secondary antibodies (horse anti-mouse IgG, Ref. PK-6102 1:200; Vector Laboratories, USA) at room temperature in PBS-TX for 1 h. After washing three times for 5 min in 0.1 M PBS, sections were incubated in avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories) and developed with 3,3′-diaminobenzidine (DAB, Ref. D5637, Sigma-Aldrich, Spain) and H_2O_2 as peroxidase substrate. Finally, sections were mounted, air-dried, cleaned in Xilol for 2 h, and coverslipped with DPX mounting medium (Sigma-Aldrich).

For calbindin and NeuN immunodetection, antigen retrieval was performed in sodium citrate pH = 6.0 for 10 min at 95 °C as the first step. Sections were incubated in blocking solution for 2 h, and primary antibodies applied overnight: mouse anti-calbindin (Ref. CB D-28k 300; 1:1000; Swant, Switzerland) and mouse anti-NeuN (Ref. MAB377; 1:2000; Merck Millipore, Germany). In the next day, sections were washed and incubated in secondary antibody for 2 h (horse anti-mouse IgG, Ref. PK-6102; Vector Laboratories). For CB inmunohistochemistry, a dilution of 1:200 of secondary antibody was used and for NeuN a dilution of 1:1000.

Unbiased Stereology

The number of immunoreactive cells throughout mPFC (prelimbic and anterior cingulate cortex) and dorsal hippocampus (dentate gyrus and cornu ammonis 1) were quantified with unbiased stereology. mPFC was sampled between 4.68 and 1.92 mm from Bregma, whereas in hippocampus, CA1 and dentate gyrus were sampled from − 2.40 to − 5.76 mm. Mercator Image Analysis system (Explora-Nova, La Rochelle, France) was used along with a digital camera attached to Olympus BX51 microscope containing a three-axis motorized stage. Immunopositive cells were counted with 40× objective using optical fractionator approach. The fractionator method estimates the total number of cells from the number of cells sampled with systematic randomly sampled set of unbiased virtual counting spaces that cover the entire region of interest with uniform distances in *X*, *Y* and *Z* directions. For determining total cell number, the following formula is used: $N = \sum Q \times 1/\mathrm{ssf} \times 1/\mathrm{asf} \times 1/\mathrm{hfs}$, where *Q* constitutes the actual number of counted cells in a specimen and *N* the total cell estimate. The section sampling factor (ssf) used in this study was 1/8 for mPFC and 1/10 for hippocampus. Areas of interest were delineated with 4× objective. The grid and counting frame sizes were different depending on the analyzed area (Table 1), and a guard zone of 5% was chosen. Quantification was performed following stereological counting rules. For interneuron estimation in hippocampus, area sampling fraction (asf) and height sampling fraction (hfs) were set to 1. Overall, at least six sections were counted per animal in hippocampus, and eight in mPFC. Volume estimations were obtained using Cavalieri's method.

In this study, pyramidal layer of CA1 and granular layer of dentate gyrus were dismissed from stereological estimations of CB-positive interneurons, because principal cells and mature granule cells of hippocampus express CB. Similarly, pyramidal cells of LII/III of mPFC slightly stained for CB, and therefore, only deep layers of mPFC are represented in CB estimations.

In our preliminary analysis used to define the counting parameters, we determined that the contribution of the coefficient of error (CE) to the total observed variance was lower than 20%, given by the ratio CE^2/CV^2, where CE^2 is the variability of stereological estimates and CV^2 is the biological variability. Usually, with a ratio lower than 0.5, stereological estimates are considered precise enough, meaning that our sampling procedure was correct.

Statistical Analysis and Figure Preparation

Body weight differences and behavior in open field test (OFT) at P21, P24, and P55 were analyzed using Student's *t* test or Mann-Whitney *U* test if data were not normally distributed. Data from volumetry were subjected to two-way ANOVA, being the factors the treatment (saline vs. MK-801) and housing condition (standard vs. EE). All other data, including open field results on P73, discrimination index of novel object recognition test, and stereological estimations were analyzed with one-way ANOVA. Data were first assessed for normality and homogeneity of variances with Shapiro-Wilks test and Levene's test, respectively. Data with equal variances were assessed post hoc using the Bonferroni test, while data that violated Levene's test were assessed using Tamhane's T2 to ensure significance of the ANOVA. The correlation between behavioral data and the number of interneurons was analyzed using simple linear regression analysis. All computations were made using the SPSS software package (version 23.0, IBM), and differences with *p* values less than 0.05 were considered statistically significant. The results are expressed as the mean ± SEM.

Images of tissue sections were taken with Olympus BX41 microscope and prepared for publication with Adobe Photoshop 5.0. Brightness and contrast were the only adjusted parameters. Prism 4 software (GraphPad, La Jolla, CA, USA) was used to create the graphs.

Table 1 Area sampling fraction (asf) values for the fractionator method

		Regions	Counting frame size	Spacing
NeuN	mPFC	PL, AC	25 × 25	350 × 350
	HPC	Molecular layer (DG)	50 × 50	75 × 75
		Polymorphic layer (DG)	25 × 25	75 × 75
		CA1 (except pyramidal layer)	50 × 50	150 × 150
GAD67	mPFC	PL, AC	80 × 80	270 × 270
	HPC	CA1, DG	80 × 80	270 × 270
PV, CR, CB	mPFC	PL, AC	80 × 80	220 × 220

mPFC medial prefrontal cortex, *HPC* hippocampus, *PL* prelimbic region of mPFC, *AC* anterior cingulate cortex, *CA1* cornus ammonis 1 of hippocampus, *DG* dentate gyrus, *GAD67* glutamic acid decarboxylase 67, *PV* parvalbumin, *CR* calretinin, *CB* calbindin

Results

Body Weight Gain Is Affected by MK-801 During Treatment Period

It has been previously described that MK-801 administration decreases body weight gain in rodents. We therefore measured body weight between P10 and P20 on a daily basis and every 2 weeks thereafter. Student's *t* test revealed significantly lower body weight gain during treatment period in MK-801-treated rats that was observed from P12 to treatment cessation (Fig. 2), but no differences were found at P31 or later on.

Volume of Medial Prefrontal Cortex and Hippocampus

mPFC and hippocampal volumes were measured in the same sections used for NeuN quantification. Two-way ANOVA showed a significant decrease in prelimbic ($F(1, 20) = 8.32$, $p = 0.009$) and anterior cingulate ($F(1, 20) = 8.19$, $p = 0.01$) volume associated with early life MK-801 administration (Fig. 3). Similarly, the treatment significantly reduced hippocampal CA1 volume ($F(1, 20) = 9.08$, $p = 0.007$), but not that of dentate gyrus ($F(1, 20) = 1.48$, $p = 0.237$). Contrarily, EE significantly increased mPFC volume (prelimbic cortex $F(1, 20) = 7.02$, $p = 0.015$; anterior cingulate cortex $F(1, 20) = 11.57$, $p = 0.003$), and the volume of DG ($F(1, 29) = 4.99$, $p = 0.04$). No significant changes were detected in CA1 of hippocampus caused by EE (Fig. 3).

Alterations of MK-801 and Beneficial Effects of EE in Locomotor Activity

In the open field test, the total distance traveled was significantly shorter in MK-801-treated rats than in saline controls 24 h after last injection (Mann-Whitney *U* test, $p < 0.001$; Fig. 4a). In addition, the relative distance moved in the center of the arena was also significantly shorter (Mann-Whitney *U* test, $p < 0.001$; Fig. 4b). On P24, these differences were absent ($p = 0.378$), and normal behavior was preserved until P55 ($p = 0.403$).

After a short exposure to EE in early adulthood, rats exhibited significant changes in locomotor activity in terms of total distance moved ($F(3, 35) = 22.31$, $p < 0.0001$) and the relative distance spent in the center ($F(3, 34) = 7.31$, $p < 0.001$). Post hoc analysis revealed that there was no difference between standard

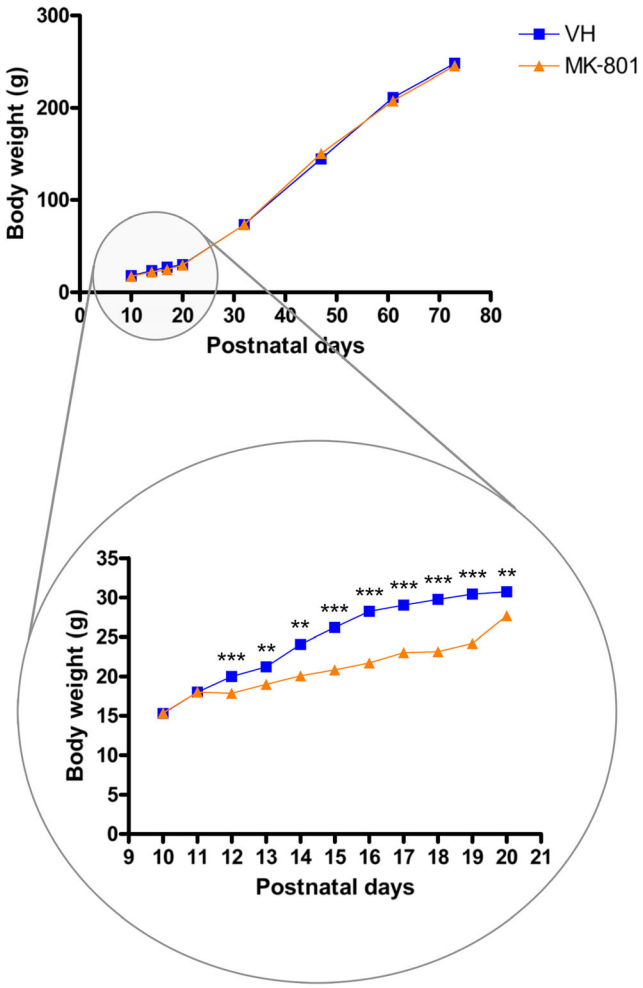

Fig. 2 Effect of neonatal MK-801 administration on body weight gain across development. MK-801 reduces body weight gain during administration period, but no differences are found thereafter. Data are represented as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

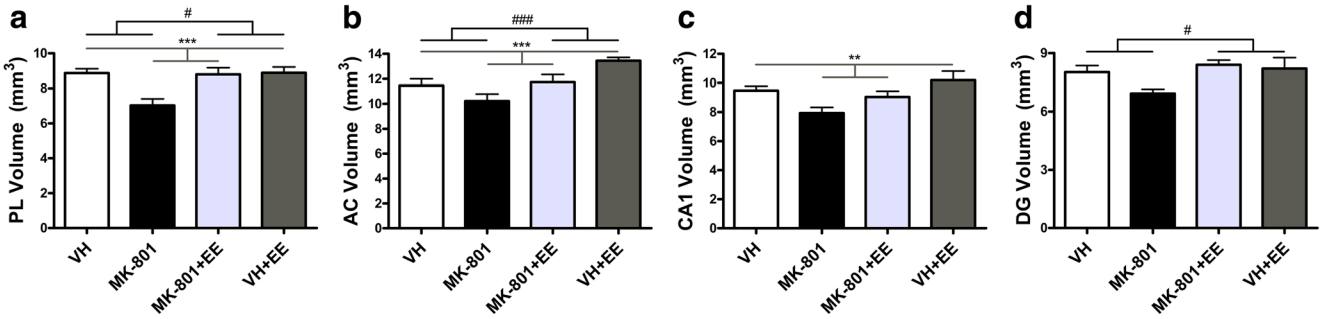

Fig. 3 MK-801 induced a marked reduction in cortical and hippocampal volumes, whereas EE tended to increase it. Note that EE had no significant effect on CA1 volume, and MK-801 did not alter the volume of DG. Histograms show the estimated volumes of the prelimbic cortex (**a**), anterior cingulate cortex (**b**), cornu ammonis 1 (**c**), and dentate gyrus of hippocampus (**d**) in different experimental groups. Estimates of mPFC and hippocampal volumes were obtained by Cavalieri's method. Values represent mean ± SEM. *Significance of treatment (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); #significance of housing (#$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$)

environment raised animals in either parameter on P73. However, MK-801 + EE group showed significantly less activity than MK-801 group ($p < 0.0001$; Fig. 4c). Comparisons of relative distance in center showed that VH + EE traveled about one third as much distance in center compared to the other 3 groups (Fig. 4d). The tendency to avoid the periphery of the OFT is widely used to assess anxiolytic-like effects of treatments or interventions.

EE Restores Long-Lasting Recognition Memory Impairment Induced by MK-801

Novel object recognition test is widely used to assess cognitive function in animal models. There was a significant difference in the mean performance of different groups in NOR test ($F(3, 26) = 15.75$, $p < 0.0001$; Fig. 5). Post hoc analysis showed that early MK-801 treatment led to cognitive dysfunction, as discrimination index was significantly lower in MK-801 group compared to saline controls ($p = 0.04$).

MK-801 + EE group presented normal recognition memory ($p = 0.412$ vs. VH), and discrimination index was significantly augmented when compared to MK-801 group ($p = 0.012$), representing major memory improvement. Rats in VH + EE group showed the greatest discrimination index, but not statistically different from MK-801 + EE group (VH vs. VH + EE $p = 0.001$; MK-801 vs. VH + EE $p < 0.0001$; Fig. 5).

Number of Calcium-Binding Proteins in Hippocampus and Medial Prefrontal Cortex

The total number of different populations of interneurons was estimated by unbiased stereology in prelimbic and anterior cingulate regions of mPFC and in CA1 and dentate gyrus (DG) of hippocampus. Interneurons were identified upon their calcium-binding protein expression, i.e., PV, CR, and CB.

One-way ANOVA showed that there was a significant difference in the number of PV and CR-expressing interneurons, but

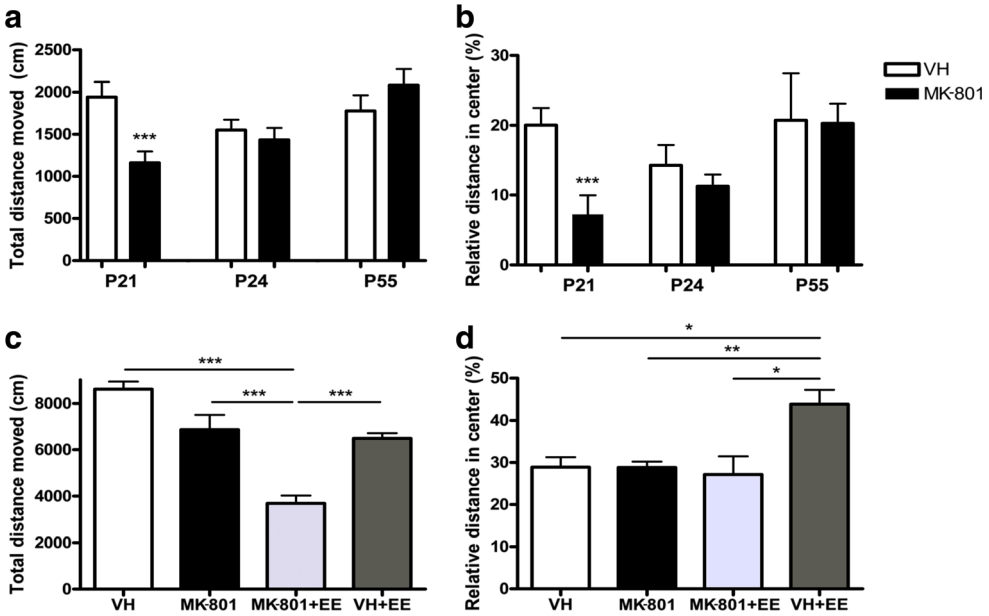

Fig. 4 Chronic MK-801 treatment decreased locomotor activity (**a**) and relative distance spent in the center vs. periphery (**b**) measured in the open field test on P21, but no differences are found on P24 or P55 (**a**, **b**). EE animals showed reduced locomotor activity compared to standard condition animals on P73 (**c**), but EE only promoted anxiolytic-like effect in VH + EE group (**d**). Data are plotted as mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

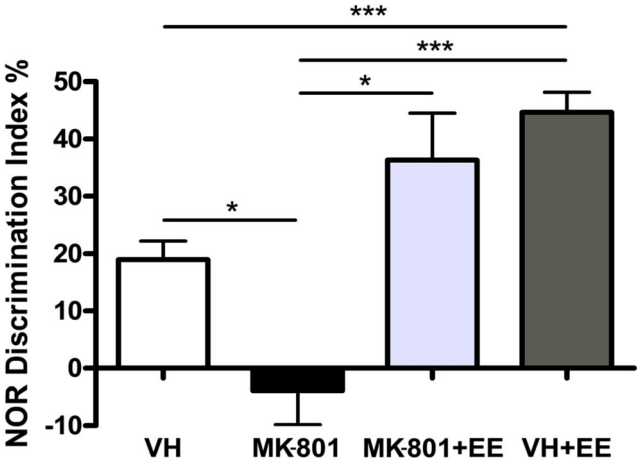

Fig. 5 Neonatal MK-801 administration impaired recognition memory in adult animals that could be restored by late exposure to environmental enrichment (between P55 and P73). Histograms show average discrimination index in novel object recognition task for each group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

not in CB-expressing ones. PV-positive differences were present in all analyzed regions [prelimbic ($F(3, 20) = 11.77, p < 0.0001$), anterior cingulate ($F(3, 20) = 11.66, p < 0.0001$), CA1 ($F(3, 20) = 10.40, p < 0.0001$), and DG ($F(3, 20) = 7.14, p = 0.02$]. Similar differences were found in CR-expressing interneurons [prelimbic ($F(3, 20) = 5.97, p = 0.004$), anterior cingulate ($F(3, 20) = 14.13, p < 0.0001$), and CA1 ($F(3, 20) = 3.64, p = 0.03$)], except for DG ($F(3, 20) = 2.30, p = 0.11$).

In mPFC, MK-801-treated rats showed a dramatic decrease in the number of PV-positive cells in prelimbic ($p = 0.022$) and anterior cingulate ($p = 0.01$) cortices compared to vehicle group (Fig. 6a). Regarding the hippocampal PV-positive cell estimations, a significant decrease was also observed in CA1 ($p < 0.0001$) and DG ($p = 0.024$) (Fig. 6d). These deficits in PV-positive interneuron expression were partially recovered by a short exposure to EE, as the number of PV-positive cells in MK-801 + EE group was not statistically different from the control group in either region, except for CA1 ($p = 0.042$) (Fig. 7). The number of PV-positive interneurons in MK-801 + EE was different from MK-801-treated rats without environmental intervention in anterior cingulate cortex ($p = 0.004$) and DG ($p = 0.023$), but not in prelimbic cortex ($p = 0.081$) or CA1 ($p = 0.244$). VH + EE group showed augmented mean PV-positive interneurons in mPFC compared to all other groups (Fig. 8), but it was only significantly higher than MK-801 + EE group in prelimbic region ($p = 0.027$). Similar to vehicle group, VH + EE was significantly different from MK-801 group in all regions that were analyzed (prelimbic, $p < 0.0001$; anterior cingulate, $p < 0.0001$; CA1, $p = 0.003$; DG, $p = 0.002$) (Fig. 6a, d).

The decrease in CR-expressing interneurons in MK-801 group was less evident, still significantly different from vehicle animals in both regions of mPFC (prelimbic cortex, $p = 0.023$; anterior cingulate cortex, $p = 0.001$) and in CA1 of hippocampus ($p = 0.035$). Similar to what happened with PV-positive interneurons, EE partially reversed the loss of CR immunoreactivity (MK-801 + EE vs. VH: prelimbic $p = 0.59$; anterior cingulate, $p = 1.0$; CA1, $p = 0.1$) (Fig. 6b, e).

No significant differences were found in either region of mPFC or hippocampus of CB-expressing interneurons [prelimbic ($F(3, 20) = 0.87, p = 0.48$), anterior cingulate ($F(3, 20) = 2.23, p < 0.12$), CA1 ($F(3, 20) = 1.52, p = 0.24$),

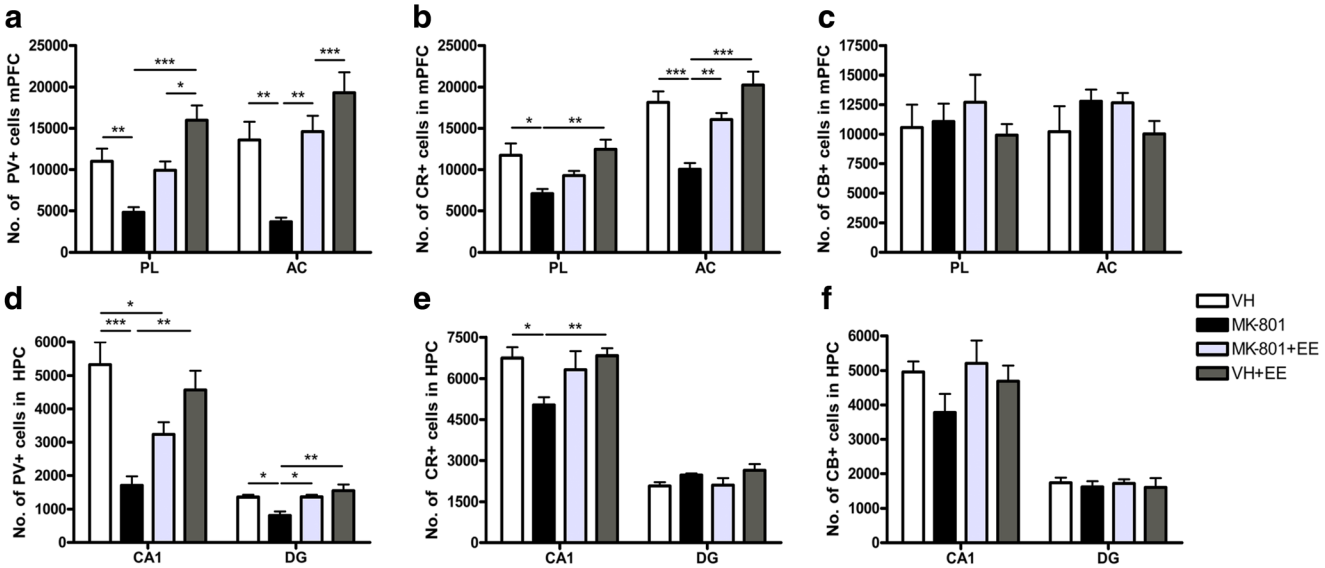

Fig. 6 Neonatal MK-801 administration decreased PV and CR expression in mPFC and hippocampus that could be partially or totally rescued by EE, except for PV immunoreactivity in CA1 region of hippocampus. Graph shows the effects of perinatal exposure to MK-801 and enriched environment on the number of different interneuron immunoreactivity in medial prefrontal cortex (mPFC) (**a**, **b**, **c**) and hippocampus (**d**, **e**, **f**). Interneurons are identified by their calcium-binding protein expression: **a**, **d** parvalbumin, **b**, **e** calretinin, and **c**, **f** calbindin. *PL* prelimbic cortex, *AC* anterior cingulate cortex, *CA1* cornu ammonis 1, *DG* dentate gyrus of hippocampus. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

and DG ($F(3, 20) = 0.14$, $p = 0.94$] (Fig. 6c, f). It is worth to mention that only deep layers of mPFC were considered for CB-positive interneuron estimations to avoid confounding effects of CB-expressing pyramidal cells of LII/III.

Lack of Hippocampal Inhibition in MK-801-Treated Rats Without Overall Cell Loss

One-way ANOVA on NeuN immunoreactivity showed no differences between groups in the total number of cells in mPFC and hippocampus [NeuN: prelimbic ($F(3, 20) = 2.35$, $p = 0.10$), anterior cingulate ($F(3, 20) = 0.38$, $p = 0.77$), CA1 ($F(3, 20) = 0.13$, $p = 0.94$), and DG ($F(3, 20) = 1.63$, $p = 0.21$] (Fig. 9a, b). Contrarily, the number of GAD67-expressing GABAergic interneurons was significantly changed in all regions of mPFC [prelimbic cortex ($F(3, 20) = 4.94$, $p = 0.01$), anterior cingulate cortex ($F(3, 20) = 5.88$, $p < 0.005$)] and hippocampus [CA1 ($F(3, 17) = 28.16$, $p < 0.0001$), and DG ($F(3, 19) = 6.96$, $p = 0.002$)] (Fig. 9c, d). In prelimbic cortex, the number of GAD67 immunoreactive cells in VH + EE group was significantly increased compared to MK-801 ($p = 0.015$) and MK-801 + EE ($p = 0.033$) groups. In anterior cingulate cortex, VH + EE only differed from MK-801 group ($p = 0.015$). In hippocampal CA1, a relevant reduction of GAD67 could be found between VH and MK-801 rats ($p < 0.0001$) that was recovered by EE (MK-801 vs. MK-801 + EE $p = 0.006$). VH + EE group presented the greatest number of GAD67-positive cells in CA1 (VH vs. VH + EE $p = 0.036$, MK-801 vs. VH + EE $p < 0.0001$, MK-801 + EE vs. VH + EE $p = 0.001$). Only the animals exposed to EE (VH + EE and MK-801 + EE) presented significantly increased GAD67 immunoreactivity in DG compared to MK-801-treated rats (MK-801 + EE vs. MK-801 $p = 0.001$, VH + EE vs. MK-801 $p = 0.02$) (Fig. 7).

Relationship Between Cognitive Function and Number of PV-Positive and GAD67-Positive Cells

The observed alterations of PV-positive cells in mPFC and hippocampus, as well as the huge differences in GAD67 expression in hippocampus, could affect behavior in the anxiogenic (open field) and cognitive (NOR) tests. Simple regression analysis revealed that the number of PV-positive interneurons tended to be positively correlated with the performance in NOR. A moderate correlation was also found between NOR performance and the number of GAD67 cells in hippocampus. Table 2 shows the Pearson's correlation

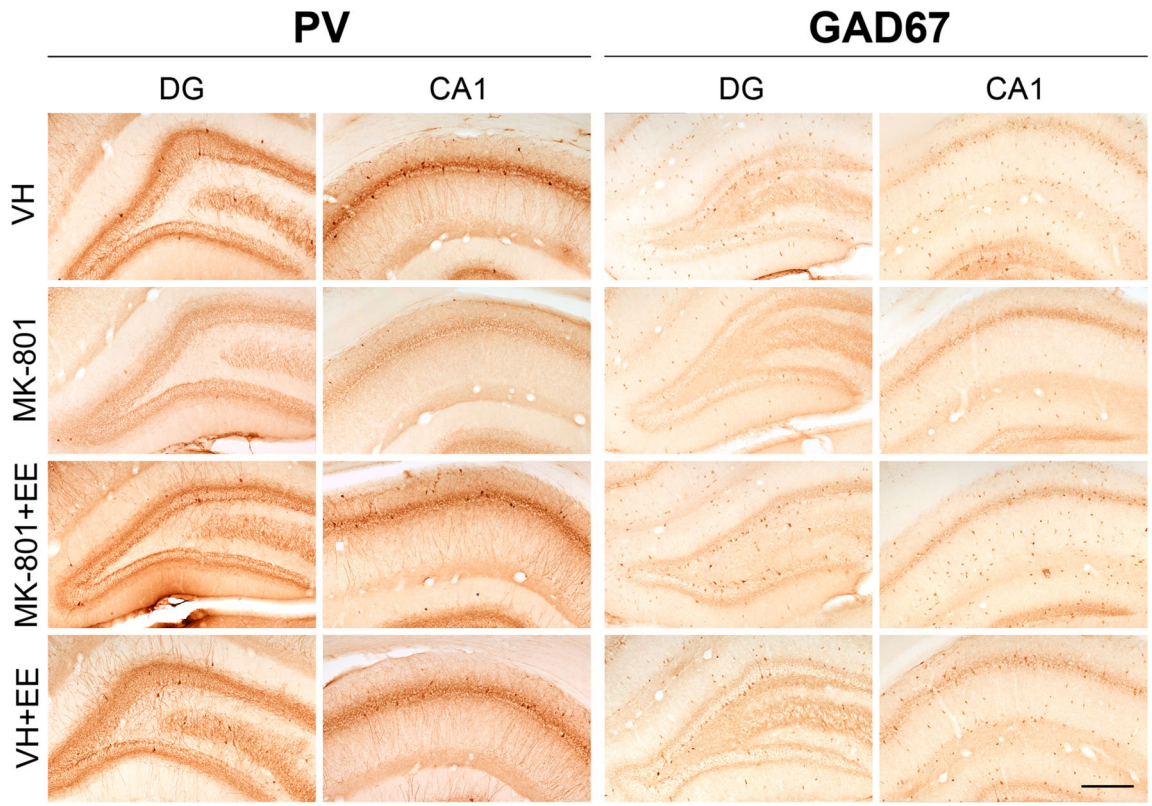

Fig. 7 Glutamic acid decarboxylase 67 (GAD67) expression was upregulated in hippocampus after EE regardless of the neonatal treatment. Contrarily, EE only increased PV expression in MK-801 + EE animals, while it had little effect on PV immunoreactivity of vehicle rats. Images show distinct expression of parvalbumin (PV) and GAD67 somata in hippocampal cornu ammonis 1 (CA1) and dentate gyrus (DG) in hippocampus. *Scale bar* = 300 μm

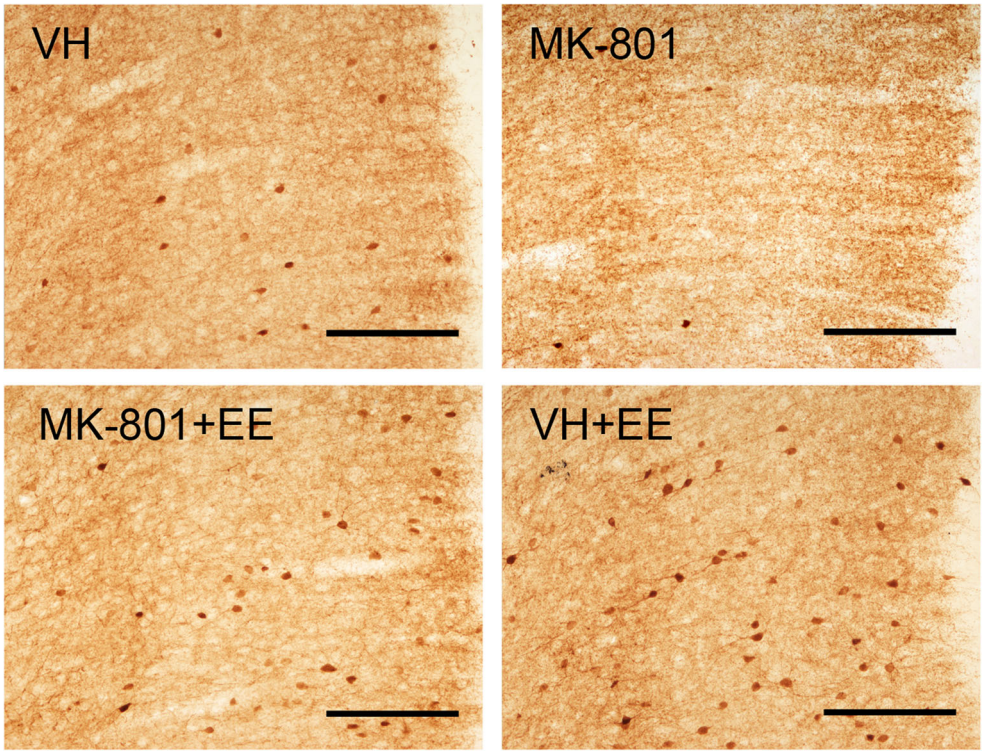

Fig. 8 The depletion of PV-positive interneurons in mPFC secondary to neonatal MK-801 treatment was reversed by early adult life environmental enrichment. Representative images of the number of PV-positive interneurons in the prelimbic region of the mPFC. *Scale bar* = 200 μm

coefficients (*r*) and the significance levels (*p*). Furthermore, the simple regression analysis also manifested that the number of PV-positive cells in prelimbic region tended to be positively correlated with the distance traveled in the center of the arena (Table 2). On the other hand, neither the number of PV-expressing interneurons nor the number of GAD67 in hippocampus was significantly correlated with the total distance traveled.

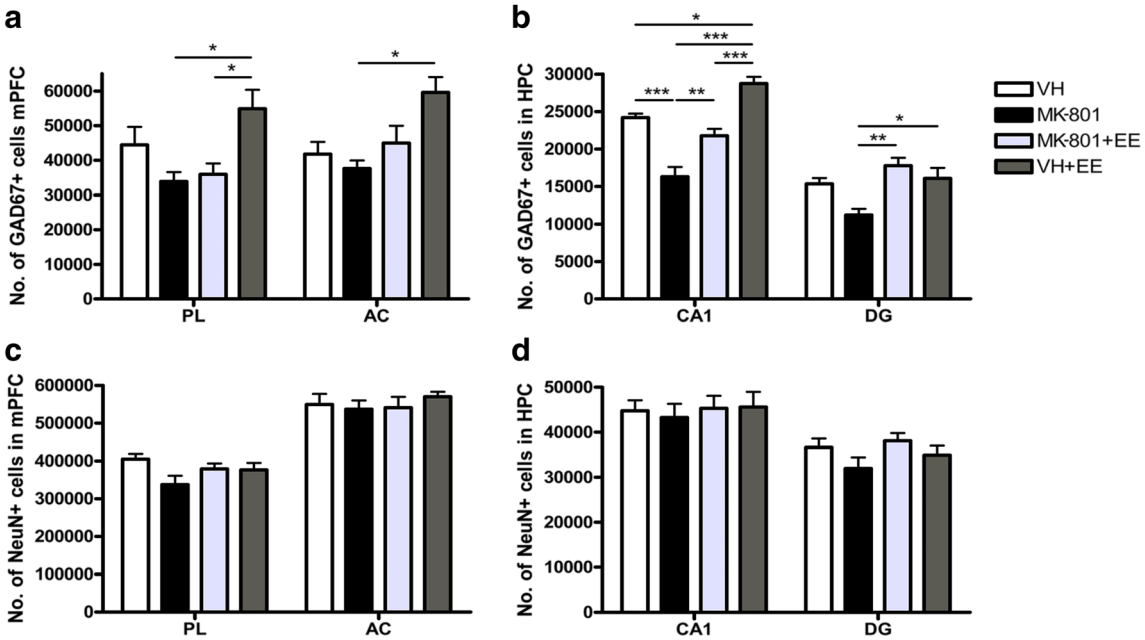

Fig. 9 Early life MK-801 administration tended to decrease GAD67 immunoreactivity in a region-specific manner, and EE had the opposite effect, without overall change in the number of total neurons, suggesting cortical and hippocampal disinhibition. Number of glutamic acid decarboxylase 67 (GAD67) and NeuN immunoreactive cells in different regions of medial prefrontal cortex (mPFC) and hippocampus. *PL* prelimbic cortex, *AC* anterior cingulate cortex, *CA1* cornu ammonis 1, *DG* dentate gyrus of hippocampus. Graphs show mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 2 Correlation between behavioral tasks and PV and GAD67 immunoreactivity

		PV				GAD67	
		PL	AC	CA1	DG	CA1	DG
NOR	*r*	0.515*	0.641**	0.418*	0.636**	0.586**	0.667**
	p	0.010	0.001	0.042	0.001	0.003	0.000
Total distance traveled	*r*	0.124	− 0.014	0.339	0.053	0.017	− 0.361
	p	0.565	0.949	0.105	0.805	0.935	0.083
Relative distance in center	*r*	0.534**	0.327	0.332	0.270	0.240	− 0.043
	p	0.007	0.118	0.113	0.203	0.259	0.842

r Pearson's correlation coefficient, *p* significance of the correlation, *PV* parvalbumin, *GAD67* glutamic acid decarboxylase 67, *PL* prelimbic cortex, *AC* anterior cingulate cortex, *CA1* cornu ammonis 1 of hippocampus, *DG* dentate gyrus of hippocampus

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Increased Number of Immature Neurons in VH + EE Group

The number of CR-expressing cells was estimated in the granule cell layer of dentate gyrus. In adult hippocampal neurogenesis, immature neurons transiently express CR before differentiating into dentate granule cells and fully integrating in the hippocampal circuit. The total number of CR expressing cells was increased in granule cell layer of VH + EE group when compared to all other groups ($F(3, 20) = 4.97$, $p < 0.01$; Fig. 10).

Discussion

The present study demonstrates the beneficial effects of a limited exposure to EE on behavior and the interneuron restoration in an animal model that mimics cognitive features of schizophrenia. We found that early life MK-801 administration impaired recognition memory in adulthood. Moreover, MK-801 treatment reduced the number of PV and CR-expressing interneurons in mPFC and hippocampus. MK-801 also diminished GAD67 immunoreactivity without overall cell loss. According to our results, a short-term exposure (18 days) EE in early adulthood partially restored long-lasting GABAergic marker deficits and improved cognitive dysfunction.

The OFT was used to measure anxiety at different time points (P21, P24, P55) in response to repeated administration of MK-801 during postnatal period (P10–P20). Quantifying the total distance traveled and the relative distance in center in a 10-min period, we found that the locomotor alterations produced by MK-801 did not persist long beyond treatment. The hypoactivity seen 24 h after treatment cessation was absent at 96 h, and normal locomotor activity was conserved until P55. Our results coincide with those of Latysheva and Rayevsky [17], who found a transient decrease in spontaneous locomotor activity 23 h after last injection, but failed to find any difference after 6 days or 4 months. Nevertheless, studies in the literature have mentioned either a robust increase of locomotion or decreased locomotor activity shortly after treatment [10]. Long-term behavioral effects of MK-801 are also conflicting. For instance, some studies have found decreased locomotion in adulthood (P60) [5], but others failed to observe any abnormalities [18, 19]. The variability in results might be attributed to the diversity of dosing regimen. In line with other studies using similar doses [18, 19], we confirmed that MK-801 treatment had no long-term effects in locomotor activity. A number of authors have shown EE to reduce locomotor activity in OFT and habituate more rapidly than controls. This appears to be a relatively consistent finding independently of the employed protocol [20, 21]. Our results are in line with earlier findings regarding decreased traveled distance in OFT after EE [20, 21]. Moreover, after EE, rodents also displayed reduced anxiety-like behavior in OFT indicated by the distance spent in center relative to periphery. Unfortunately, we only observed anxiolytic effects in saline controls housed in EE, and not in neonatally MK-801-injected rats exposed to EE.

EE Improves Cognitive Function

EE is also a widely used paradigm to stimulate cognitive processes, as mice and rats generally show improved performance in learning and memory tasks after EE than standard housed control animals. Many studies initiate EE at relatively early stages. Environmentally stimulating conditions from birth or weaning prevent cognitive and behavioral deficits secondary to chronic MK-801 treatment [22, 23]. However, no single study to date has assessed the potential benefits of EE in adulthood after neonatal NMDA receptor blockade, although studies in which EE has started at adult

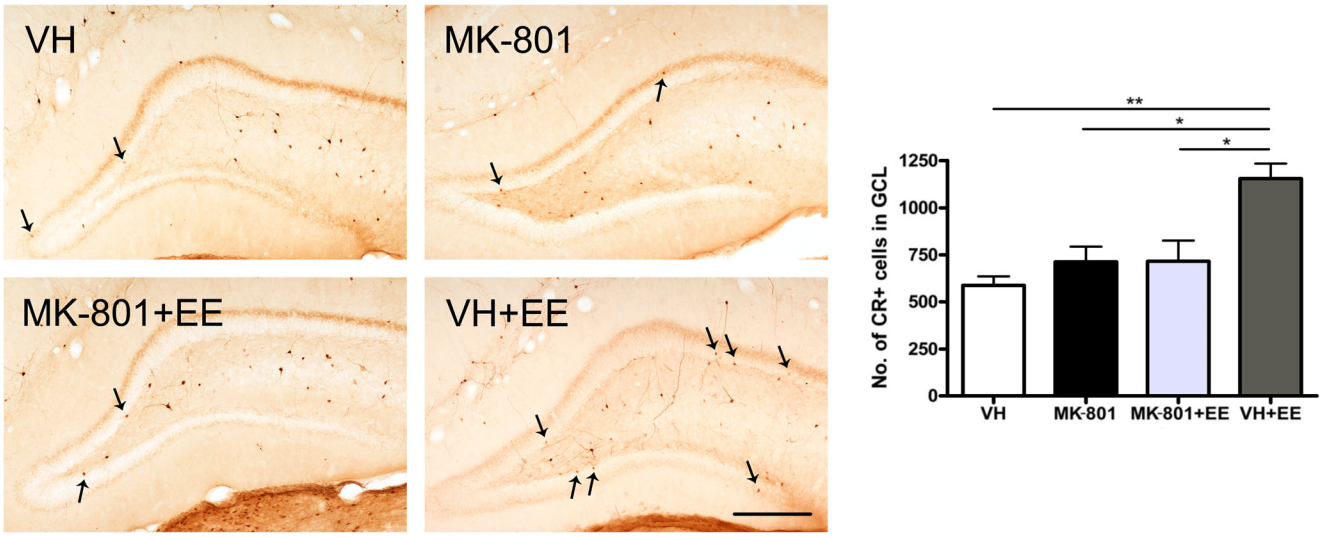

Fig. 10 Early life MK-801 administration did not alter the number of adult immature granule cell in GCL. Only vehicle animals subjected to adult life EE showed significantly increased number of CR-positive granule cells. On the left, calretinin-positive cells in the granular layer of dentate gyrus (*arrows*). On the right, histogram showing the differences in the total number of calretinin-expressing cells between groups. *GCL* granular cell layer of dentate gyrus. Data are represented as mean ± SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. *Scale bar* = 300 μm

stages also exhibited beneficial effects in other conditions [24]. As a matter of fact, short exposure to EE (10 days) in adulthood enhances neurogenesis, vascular network, and dendritic complexity in hippocampus [24]. In this work, we demonstrated that an intervention with EE in early adulthood enhanced cognitive function of neonatally MK-801-treated animals. NOR is a widely used test to assess memory function in schizophrenic humans and animal models. Previous studies have shown that acute administration of MK-801 before NOR impairs acquisition and encoding of object recognition memory [25], and it therefore decreases discrimination index [26–28]. Nevertheless, long-term effects have been somewhat controversial. Adopting a once daily 0.25 mg/kg dose from P6 to P21, Baier et al. [5] concluded that MK-801 did not had long-term adverse consequences on NOR. Likewise, Lim et al. [29] failed to find any impairment in recognition memory in adult rats using a daily dose of 0.2 mg/kg between P7 and P10. It is now known that drug administration schedule highly influences NOR performance in adulthood. Chronic daily doses of 0.5 mg/kg or higher during neurodevelopment seem to be necessary for long-lasting recognition memory impairment in adult animals [7]—the threshold dose for apoptotic injury [16]. On this background, Li et al. [30] administered 0.25 mg/kg MK-801 twice daily from P5 to P14 and demonstrated impaired object recognition memory in adult rats that was already present in juvenile animals. This is consistent with our results, in which a daily dose of 0.5 mg/kg from P10 to P20 impaired recognition memory. The low performance of MK-801-treated rats in NOR test was not attributable to malnutrition or anxiety, in view of normal body weight and locomotor activity in OFT. As stated previously, as far as we know, this is the first study that demonstrates a beneficial effect of EE in cognition when applied in early adulthood in a neurodevelopmental model of schizophrenia.

Early Life MK-801 Administration Reduces the Number of PV and CR-Expressing Interneurons

Deficit in PV immunoreactive interneurons is the most consistent finding in animal models of schizophrenia [3, 4, 9, 30, 31] and human patients [32–34]. Our results showing a reduced number of PV-positive interneurons in CA1 and DG of hippocampus and in PL and AC regions of mPFC are in agreement with previous works. Studies using repeated injections of NMDAR antagonists have demonstrated a reduction PV-positive cell density in hippocampus [35, 36] and mPFC [31, 37], and neurodevelopmental models further support this finding [9, 30, 31]. MK-801 is a non-competitive NMDAR antagonist that selectively disrupts GABAergic cells. A number of hypotheses have been proposed to explain the mechanisms by which PV-positive cells can be selectively susceptible to damage after MK-801 administration. The open probability of NMDAR of PV-positive cells is higher than in other types of interneurons, as most of PV-expressing interneurons are fast-spiking (FS) cells. Thus, the probability of MK-801 for blocking NMDAR of FS-PV-positive cells increases. Moreover, Wang and Gao [38] showed that NMDAR in presynaptic glutamatergic terminals targeting pyramidal cells and FS interneurons was distinctly affected after subchronic MK-

801 exposure. Presynaptic NMDAR is critical to modulate and facilitate neurotransmitter release. The authors demonstrated that MK-801 completely blocked presynaptic NMDA receptors of terminals that targeted FS-PV interneurons, whereas NMDA receptors of glutamatergic terminals that synapsed with pyramidal neurons were upregulated [38], shifting the excitation/inhibition balance towards excitation. Another hypothesis that supports a presumably selective disruption of PV-positive interneurons is that our MK-801 administration schedule coincides with the developmental expression of PV [39, 40]. PV expression is low to absent at P7 and gradually increases until P21 [39, 40]. It has been suggested that calcium-binding proteins, especially PV, play a neuroprotective role when facing dysregulation of calcium homeostasis [41]. On the other hand, PV is the only calcium-binding protein that has been linked to specific brain functions, like attention [42] or cognitive flexibility [43]. Interneurons that express PV are also involved in feedback loops that create gamma oscillation—the physiological correlate of higher cognitive functions [44]. It was therefore expected that our finding of persistent PV deficiency would result in cognitive impairment. In fact, we found a moderate correlation between the number of PV-positive interneurons and NOR performance. However, Bygrave et al. [45] claim that an exclusive NMDA receptor hypofunction of PV interneurons might not be the starting point of schizophrenia, but rather support the idea of NMDA hypofunction in several cell types.

Unlike what has been observed in the present study, CR-expressing interneurons are not altered in schizophrenic brains [9, 46–48], and neither in animal models of MK-801 treatment [30, 49]. One of the reasons might be that usually cell densities are reported instead of absolute cell numbers, although reductions in cortical and hippocampal volume due to MK-801 have been repeatedly documented in the scientific literature [50]. This means that small changes in the number of CR-positive interneurons could be masked by paralleled changes in volume. In fact, Gilabert-Juan et al. [50] found that CR gene expression was reduced in mPFC caused by MK-801 treatment. Differences between humans and animals might arise on this respect, as CR interneurons express acetylcholine transporters in rats, but not in humans [51]. Another plausible explanation could be that CR is downregulated as a compensatory mechanism for brain hyperactivity. Hippocampal hyperactivity is a core feature of schizophrenia that has been replicated in MK-801 animal models. CR-positive interneurons are specialized in innervating other interneurons in mPFC and hippocampus [52, 53]. In mPFC, they usually target somatostatin (SST) and PV-expressing interneurons [54], whereas in hippocampus, they predominantly contact with CB-positive interneurons and other CR-expressing interneurons [52], presumably those that co-express somatostatin [44, 52]. Hence, CR-positive interneurons are part of the disinhibitory circuit, and they are in the position to govern the inhibition carried out by other interneurons [54]. CR could be downregulated in an attempt to compensate for network over-activation triggered by MK-801, but this hypothesis needs to be further confirmed.

Regarding the number of CB-positive cells, conflicting results are found in humans and animal models. Some studies have found increased CB expression in human schizophrenic patients [55, 56], others have found no changes [57], and some have reported reduced CB immunoreactivity [48]. Results from animal studies are equally variable. In this study, we excluded from stereological estimations those layers in which CB expression could be confounded by CB-expressing excitatory cells, like pyramidal layer and granular layer of hippocampus and LII/III of mPFC, and no differences were found. In an attempt to solve this question, Li et al. [30] used double immunohistochemistry with CB and SST and concluded that CB-positive interneurons were decreased in both superficial and deep layer of mPFC as a consequence of MK-801 administration. However, it should be considered that not all CB-positive cells express SST, neither all SST interneurons are CB-positive. Contrarily, Gilabert-Juan et al. [50] found increased CB mRNA in mPFC that was paralleled by an increase in the number of CB-expressing cells. More studies should be conducted to solve this discrepancy.

EE Promotes the Expression of GABAergic Markers

EE intervention increased GABAergic marker immunoreactivity without changing overall cell quantity. This indicates that MK-801 reduced the expression of calcium-binding proteins in interneurons, and consequently their activity, instead of promoting programmed cell death. Nevertheless, the rate of apoptosis that previous studies have reported after MK-801 administration is relatively small compared to the total number of cells [16]. Thus, MK-801 probably augmented programmed cell death perinatally, but this loss could not be detected by stereological estimations in adulthood. In fact, our dose of 0.5 mg/kg has been documented to be the threshold dose for apoptotic damage [16]. It has been shown that hippocampal PV-positive cells are especially sensitive to NMDAR blockade. This could partly explain why PV immunoreactivity in CA1 region of hippocampus is the only region that EE could not restore to normal values. EE completely restored the number of PV-positive and CR-positive interneurons in anterior cingulate and PV-positive interneurons in DG. However, only a partial restoration was found in PV-positive and CR-positive cells in prelimbic region of mPFC, and in CR-positive in CA1. The partial recovery demonstrates the beneficial effects of EE, but it remains to be determined if longer periods in EE could further increase calcium-

binding protein markers up to normal values, or the lack of complete recovery is caused by the perinatal MK-801-induced cell death.

In the mammalian brain, the primary inhibitory neurotransmitter GABA is mainly synthesized by GAD67. This isoform of glutamic acid decarboxylase is the responsible for over 90% of GABA production [58]. Postmortem studies of schizophrenic individuals have revealed reduced levels of GAD67 in the PFC [59–61]. Despite the scarcity of investigations, the available findings thus far suggest that GAD67 expression is also decreased in hippocampus [62]. The present results are partially in accordance with findings in humans, showing reduction in GAD67 expression in hippocampus, albeit mPFC expression was maintained. It is noteworthy that GAD67 deficiency is primarily present in a subset of GABAergic cells, namely PV interneurons [33, 63]. Given that we considered the overall GAD67 expression in mPFC, we were not able to detect significant differences. Interestingly, EE in vehicle rats led to increased number of GAD67 cells. EE enhances glutamatergic neurotransmission [64–66] through BDNF and its receptor TrkB [67, 68], and genetic approaches have demonstrated that GAD67 levels directly contribute to the strength of synaptic inhibition [69]. We speculate that the mild increase in GAD67 cells might occur in an attempt to counterbalance increased excitatory input.

The mechanisms by which EE is able to improve cognition and restore GABAergic cell immunoreactivity are not fully uncovered. Previous studies have reported that exercise alone has also beneficial effects at anatomical and behavioral levels [70], and exposure to novelty improves spatial memory [71], but the combination of this components together with social interaction acts synergistically. EE promotes structural changes in the brain like increased spine density and dendritic branching, induces changes in neurotransmitters, and enhances the gene expression and protein levels of different growth factors [13, 14], such as nerve growth factor (NGF) [12, 72], brain-derived neurotrophic factor (BDNF) [73], glial-derived neurotrophic factor (GDNF) [74], or vascular endothelial growth factor (VEGF) [75]. Accumulating evidence has yielded to the notion that NMDAR hypofunction underlies cognitive dysfunction and anatomical alterations of schizophrenia. Albeit the scarcity of investigations about the pathways that regulate NMDA receptor trafficking and mobility related to EE, it is known that EE increases NR1, NR2A, and NR2B subunits of NMDA receptor in several regions of the brain, including hippocampus, forebrain, and amygdala [76]. It remains to be determined if the release of growth factors promoted by EE directly influence on NMDARs. Future studies could examine whether these and other specific neurochemical changes account for cellular and behavioral differences seen after exposure to EE.

EE Increases Immature Granule Cells in Hippocampus

Mature granule cells of dentate gyrus express CB and NeuN. Heretofore, postmitotic neuronal cells transiently express CR, before being fully integrated in the hippocampal circuitry. Former studies have speculated about the immature dentate gyrus as being a potential endophenotype of neuropsychiatric disorders, including schizophrenia [77]. Postmortem immunohistochemical analysis of schizophrenic brain revealed that they displayed significantly increased CR expression in dentate gyrus compared to normal controls [77, 78]. Considering that glutamate inhibits cell proliferation and NMDA receptor blockade increases it in the subgranular zone of DG [79], it seemed possible that MK-801 administration could increase CR-positive cells in granular cell layer. Nevertheless, we failed to find any difference in CR-expressing cells in granular cell layer, which indicates that chronic MK-801 administration during neurodevelopment does not promote immature phenotype of DG, at least, on a long-term basis. Other studies have demonstrated long-lasting increased cell proliferation and neurogenesis, but when MK-801 was administered in adult rats [80–82]. Contrarily, we observed significantly more CR-positive cells in VH + EE animals compared to all other three groups. Several studies confirm that EE increases adult neurogenesis, and this is often paralleled with improvements in learning and memory tasks [25, 83]. It has been discussed that EE promotes survival of newborn neurons rather than increasing cell proliferation [14]. Independently of the mechanism, any of these options will result in more CR-positive expressing immature neurons in granule cell layer, which coincides with our results. The contribution of immature granule cells to hippocampal function is still under intensive debate. Recent studies support their critical role in enhancing pattern separation [84].

Conclusions

To conclude, the present study revealed that chronic neonatal blockade of NMDAR with MK-801 disrupted recognition memory in adult animals and decreased the number of PV and CR-expressing interneurons in mPFC and hippocampus. This decrease was accompanied by an overall GAD67 loss in hippocampus. These biochemical abnormalities were correlated with disturbances in cognitive function. A brief exposure to complex environmental conditions in early adulthood reversed memory impairment and partially restored GABAergic markers. Taken together, these results lend to support the usefulness of environmental intervention in early adulthood as a neurorestorative paradigm in an animal model of schizophrenia.

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