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 NMDA receptor antagonist in rodents emulate some cognitive impairments and neurochemical alterations such as deficits in GABAergic interneuron immunoreactivity also found in schizophrenia. These features are pervasive, and developing neuroprotective or neurorestorative strategies are of special interest.

In this work, we aimed to investigate if a short exposure to Enriched Environment (EE) in early adulthood (P55-73) was an effective strategy to improve cognitive dysfunction and restored 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 not detected 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.

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 diversity of interneurons is essential for providing constant matched inhibitory input to the variety of stimulus, regardless of intensity and complex dynamics (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 contributes to the regulation of synapse elimination in the developing brain, and in an activity-dependent manner, optimizes excitatory-inhibitory balance for regulated information transfer (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) (4) (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 the disease (7). Critical brain regions involved in the pathophysiology of schizophrenia, and relevant for cognitive functions, are the medial prefrontal cortex (mPFC) and hippocampus. Functional remodeling of mPFC occurs during adolescence, when fine tuning of GABAergic activity is integrated (2) (8). Late-adolescence (P50) onset of hippocamapal-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 prevents establishing appropriate information processing mechanisms that support complex cognitive functions (9) (7) (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) (13) (14) (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 would have important implications for developing future neuroprotective strategies.

In the present study, we sought to investigate the consequences of adult exposure to EE in animals subjected to chronic NMDA receptor blockade. For that, we examined recognition memory as surrogate of cognitive functions, and the number of GABAergic cells in mPFC and hippocampus.

MATERIALS AND METHODS

We quantified three subgroups of interneurons identified upon their calcium-binding protein expression: parvalbumin (PV), calretinin (CR), and calbindin (CB), and GAD67 and NeuN immunoreactivity in mPFC and hippocampus. For further assessing structural and behavioral changes occurring after MK-801 administration and EE, cortical and hippocampal volumes were estimated and locomotor activity evaluated in Open-Field Test.

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 am) 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 adminsitration: 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 (St. Louis, MO, USA; Ref: M107). 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 1ml/100g of animal weight was used. Controls received the same volume of saline. Body weights were recorded every day during the treatment period and every two 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 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 from P10 to P20.

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 3 animals were housed per cage (500 mm x 280 mm x 140 mm). In EE 6 animals were housed per cage promoting social interaction. The enriched environment (EE) consists 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 (OFT): Spontaneous locomotor activity was fully automated using custom designed software Actitrack (Panlab, Spain). The apparatus consisted of a square arena (44x44x35 cm) made of Plexiglas with parallel frames, few centimeters apart, provided with 16x16 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 minutes. Horizontal displacement in the center and the periphery of the arena was measured. Relative distance in center was defined as the following formula: 100 x [total distance moved in center arena / total distance in periphery].

Novel Object Recognition (NOR): 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 (90x90x40 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. Rodents were released facing the wall to prevent coercion. After a retention interval of 90 min, the rodent was returned to the arena for 3 minute 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 seconds of active exploration during the 5

minute 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 6 animals of each experimental condition. Rats were euthanized by sodium pentaorbital before transcardial perfusion with Sodium Chloride (0.9 % sodium chloride, pH=7.4) followed by 4% paraformaldehyde (in 0.1M PBS). Brains were removed, post-fixed overnight in the same fixative at 4° C, and stored in 30% sucrose solution at 4° C. Serial coronal brain sections (50μm thick) were cut on a freezing microtome (Leica, Wetzlar, Germany). Free-floating sections were washed two times for 5 min in 0.1M PBS, and incubated for 20 min in a solution of 3% hydrogen peroxide to eliminate endogenous peroxidise activity. After three washes, free-floating sections were blocked in 5% normal horse serum (NHS) in 0.1M PBS with 0.5% Triton X-100 (PBS-TX) during 1h, 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), mouse anti-GAD67 (Ref: MAB5406; 1:10000; Merck Millipore, Germany). TX was excluded in all steps of GAD67 immunohistochemistry. In 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 1h. After washing three times for 5 min in 0.1M PBS, sections were incubated in Avidin-Biotin Complex (Vectastain Elite ABC kit, Vector Laboratories), and developed with DAB (Ref: D5637, Sigma-Aldrich, Spain) and H_2O_2 as peroxidise substrate. Finally, sections were mounted, air-dried, cleaned in Xilol for 2 hours, and coversliped 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 95ºC as a first step. Sections were incubated in blocking solution for 2h and primary antibodies applied overnight: mouse anti-calbindin (Ref: CB D-28k 300; 1:1000; Swant, Switzerland), mouse anti-NeuN (Ref: MAB377; 1:2000; Merck Millipore, Germany). The next day, sections were washed and incubated in secondary antibody for 2h (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

Estimates of immunoreactive cells throughout mPFC (prelimbic and anterior cingulate cortex) and dorsal hippocampus (dentate gyrus and Cornus 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 monitorized stage. Immunopositive cells were counted with 40x 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: $N = \sum Q \times 1/ssf \times 1/asf \times 1/hsf$, 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 4x 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, asf and hfs were set to 1. Overall, at least 6 sections were counted per animal in hippocampus, and 8 in mPFC. Volume estimates 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 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 behaviour in 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 estimates 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.

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 two 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

Medial prefrontal cortex (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-hours 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, $p < 0.001$; Figure 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 not any difference between standard environment raised animals in either parameter on P73. However, MK-801+EE 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 (NOR) $(F_{(3,26)}=15.75, p<0.0001$; 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 cortex and anterior cingulate cortex of mPFC, and in CA1 and dentate gyrus of hippocampus. Interneurons were identified upon their calcium-binding protein expression, i.e. parvalbumin (PV), calretinin (CR), and calbindin (CB).

One-way ANOVA showed that there was a significant difference in the number of PV and CR-expressing interneurons, but 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 estimates, 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). 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 prelimibic 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, 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 and 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) (Fig.6b and e). 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 and 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 ($F_{(3,20)}=1.52$, $p=0.24$), and DG ($F_{(3,20)}=0.14$, $p=0.94$)] (Fig.6c and 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 and b). Contrarily, the number of GAD67-expressing GABAergic interneurons were 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 ($F_{(3,17)}=28.16$, $p<0.0001$); DG ($F_{(3,19)}=6.96$, $p=0.002$)] (Fig.9c and 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 vehicle 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 $p=0.02$)

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 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.

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 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 enriched environment (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-801during postnatal period (P10-P20). Quantifying the total distance traveled and the relative distance in center in a 10 minute period, we found that the locomotor alterations produced by MK-801 did not persist long beyond treatment. The hypoactivity seen 24-hours after treatment cessation was absent at 96 hours and normal locomotor activity was conserved until P55. Our results coincide with those of Latysheva & Rayevsky (17), who found a transient decrease in spontaneous locomotor activity 23-hours 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 not 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 with 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 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) (27) (28). Nevertheless, long-term effects have been somewhat controversial. Adopting a once daily 0,25mg/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 0.2mg/kg between P7 and P10. It is now known that drug administration schedule highly influences NOR performance in adulthood. Chronic daily doses of 0.5mg/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 0.25mg/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 0.5mg/kg from P10 to P20 impaired recognition memory. The low performance of MK-801-treated rats in Novel Object Recognition test (NOR) was not attributable to malnutrition or anxiety, in view of normal body weight and locomotor activity in Open Field Test (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) (33) (34). Our results showing a reduced number of PV-positive interneurons in CA1 and DG of hippocampus, and PL and AC cortices in mPFC are in agreement with previous works. Studies using repeated injections of NMDAR antagonists have demonstrated a reduction PV density in hippocampus (35) (36) and mPFC (31) (37), and neurodevelopmental models further support this finding (9) (31) (30). 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 can be selectively susceptible to damage after MK-801 administration. The open-probability of NMDAR of PV 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 cells increases. Moreover, Wang & Gao (38) showed that NMDAR in presynaptic glutamatergic terminals targeting pyramidal cells and FS interneurons were distinctly affected after subchronic MK-801 exposure. Presynaptic NMDAR are 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 feed-back 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 results 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 cells types.

Unlike what has been observed in the present study, CR-expressing interneurons are not altered in schizophrenic brains (46) (47) (48) (31), neither in animal models of MK-801 treatment (49) (30). 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 (52) (44). Hence, CR-positive interneurons are part of the dishinhibitory 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 overactivation 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 (58). Results from animal studies are equally variable. In this study, we excluded from stereological estimates 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 found no differences. 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 0.5mg/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 (59). Post-mortem studies of schizophrenic individuals have revealed reduced levels of GAD67 in the PFC (60) (61) (62). Despite the scarcity of investigations, the available findings thus far suggest that GAD67 expression is also decreased in hippocampus (63). 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) (64). 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 neurotramission (65) (66) (67) through BDNF and its receptor TrkB (68) (69), and genetic approaches have demonstrated that GAD67 levels directly contribute to the strength of synaptic inhibition (70). 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 (71), and exposure to novelty improves spatial

memory (72), but the combination of this components together with social interaction act 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 (14) (13), such as nerve growth factor (NGF) (12) (73), brain-derived neurotrophic factor (BDNF) (74), glial-derived neurotrophic factor (GDNF) (75) or vascular endothelial growth factor (VEGF) (76). 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 (77). It remains to be determined if the release of growth factors promoted by EE directly influences on NMDAR. 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 post-mitotic 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 (78). Postmortem immunohistochemical analysis of schizophrenic brain revealed that they displayed significantly increased CR expression in dentate gyrus compared to normal controls (78) (79). Considering that glutamate inhibits cell proliferation and NMDA receptor blockade increases it in the subgranular zone of DG (80), 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 after MK-801 administration, but in adult rats (81) (82) (83). Contrarily, we observed significantly more CR-positive cells in VH+EE animals compared to all other 3 groups. Several studies confirm that EE increases adult neurogenesis, and this is often paralleled with improvements in learning and memory tasks (25) (84). 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.

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. Accompanied by an overall GAD67 loss in hippocampus, these

biochemical abnormalities were correlated with disturbances in cognitive functions. A brief exposure to complex environmental conditions in early adulthood reversed memory impairment and partially restored GABAegic 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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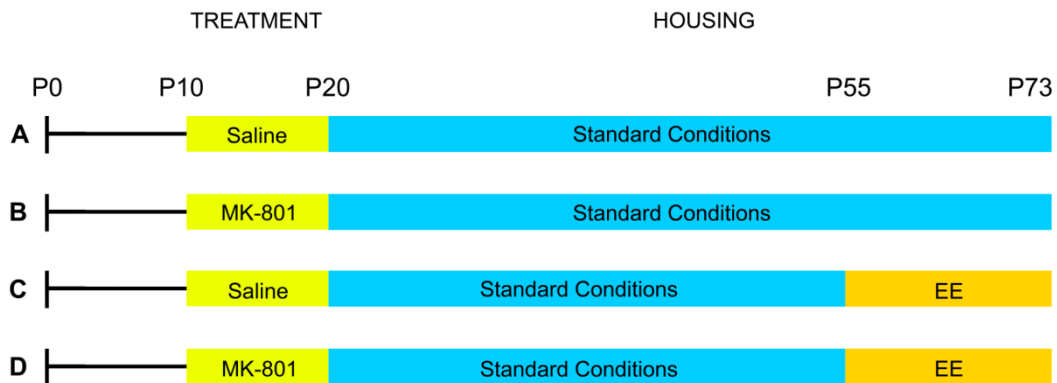
FIGURES AND FIGURE LEGENDS

Fig. 1 Experimental timelines of pharmacological treatment and housing conditions in the four groups. P, postnatal days; SE, standard environment; EE, enriched environment.
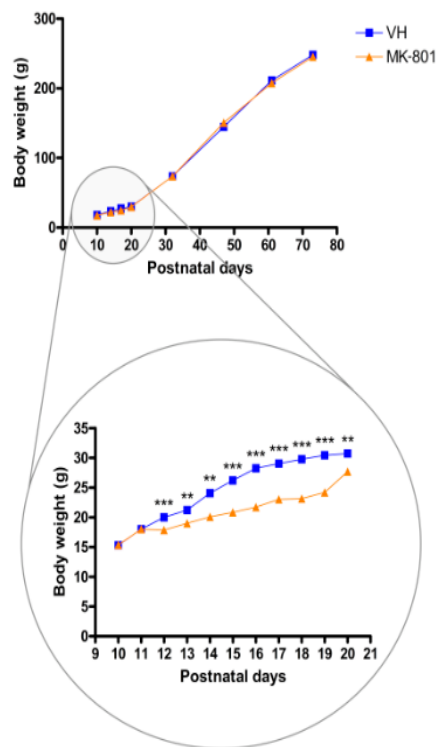

Fig. 2 Effect of neonatal MK-801 administration on body weight gain across development. Data are represented as mean ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

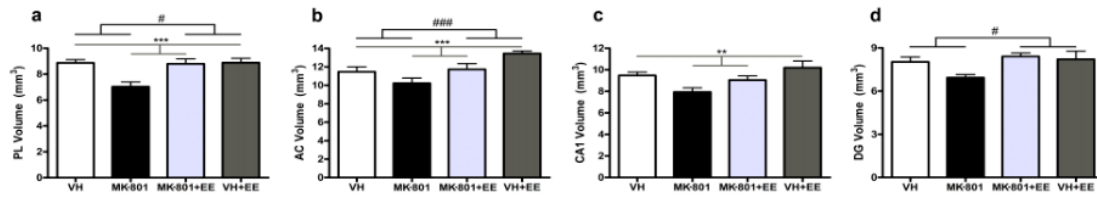**Fig. 3**. Estimates of mPFC and hippocampal volumes by Cavalieri's method. Histograms show the estimated volumes of the prelimbic cortex (a), anterior cingulate cortex (b), Cornus Ammonis 1 (c), and dentate gyrus of hippocampus (d) in different experimental groups. Values represent mean ± SEM. * Significance of treatment (*p<0.05; **p<0.01; ***p<0.001), # significance of housing (#p<0.05; ##p<0.01; ###p<0.001).
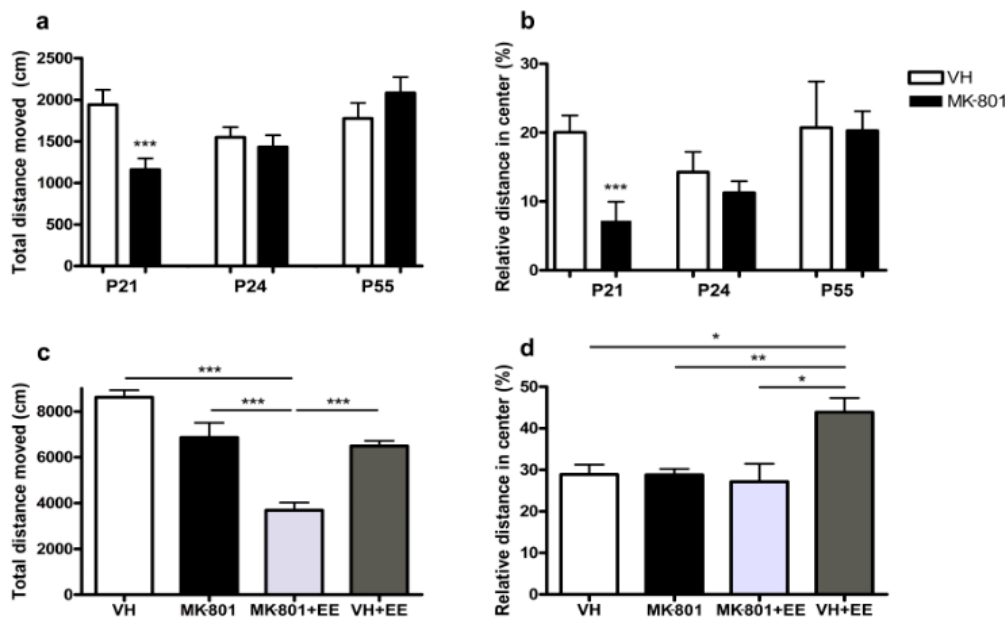
Fig. 4. Effects of chronic MK-801 treatment on locomotor activity in the Open Field Test at postnatal days (P) 21, 24 and 55 (a,b) and after exposure to enriched environment on P73 (c,d). Data in a, c represent the total distance traveled whereas b, d show the percentage of distance moved in center vs. periphery. Data are plotted as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001.
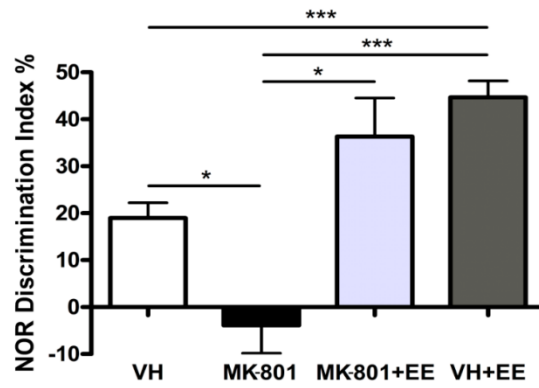
Fig. 5. Discrimination Index in Novel Object Recognition task. *p<0.05; **p<0.01; ***p<0.001.
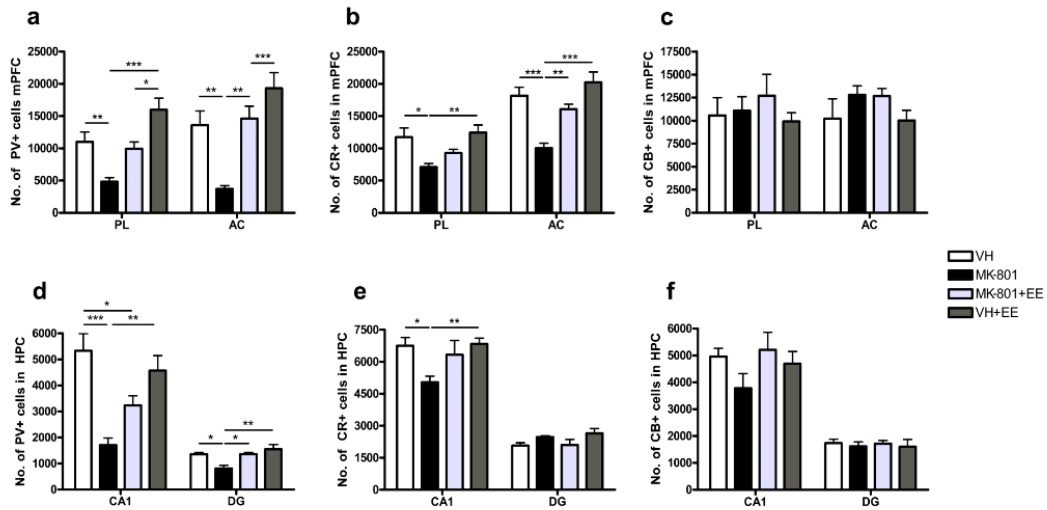

Fig. 6. Effects of prenatal exposure to MK-801 and enriched environment on the number of different interneuron immunoreactivity in medial prefrontal cortex (mPFC) (a,b,c) and hippocampus (d,e,f). Interneurons are identified by their calcium-binding protein expression: (a,d) parvalbumin, (b,e) calretinin, and (c,f) calbindin. PL, prelimbic cortex; AC, anterior cingulated cortex; CA1, Cornus Ammonis 1; DG, dentate gyrus of hippocampus. *p<0.05; **p<0.01; ***p<0.001.
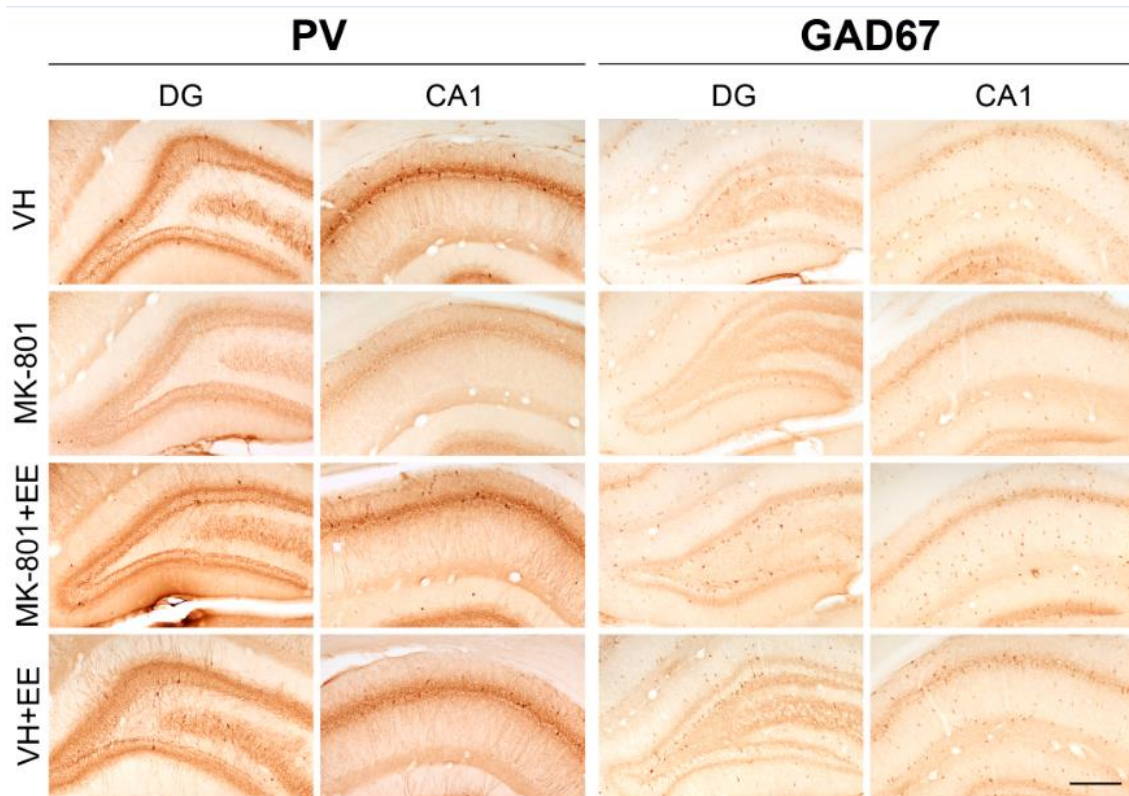
Fig. 7. Representative images showing distinct expression of parvalbumin (PV) and glutamic acid decarboxylase-67 (GAD67) somata in hippocampal *Cornus Ammonis* 1 (CA1) and dentate gyrus (DG) of different experimental groups. Scale bar= 300 μm.
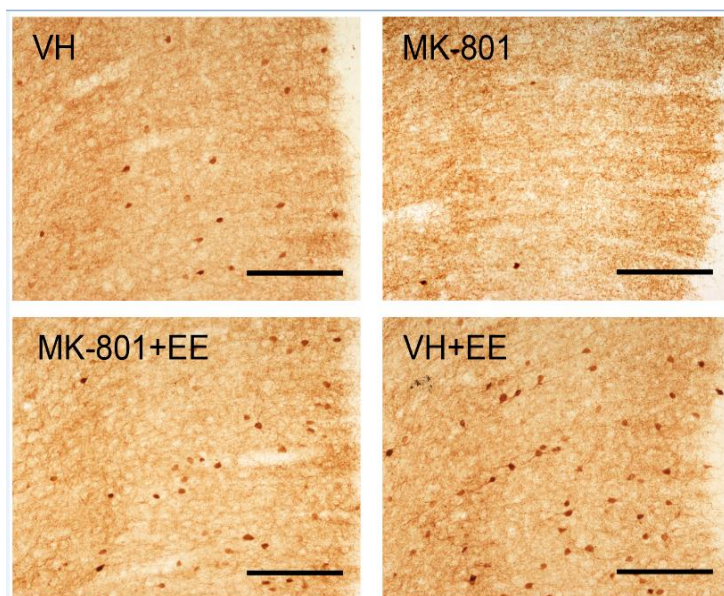

Fig. 8 Representative images of the number of PV-positive interneurons in the prelimbic region of the mPFC. Scale bar= 200 μm
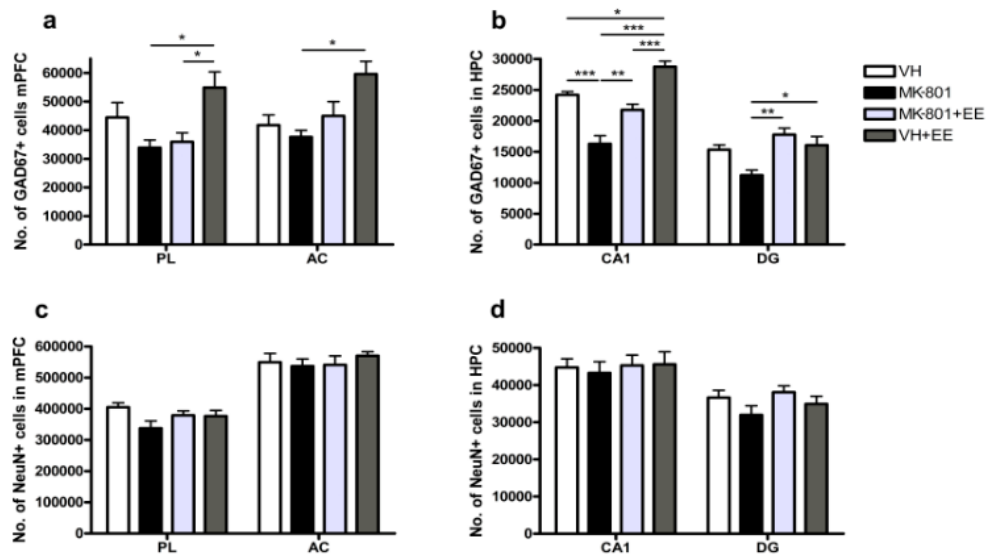
Fig. 9 Number of glutamic acid decarboxylase 67 (GAD67) and NeuN immunoreactive cells in different regions of medial prefrontal cortex (mPFC) and hippocampus. PL: prelimbic cortex; AC: anterior cingulate cortex; CA1: Cornus Ammonis 1; DG: dentate gyrus of hippocampus. Graphs show mean ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$.
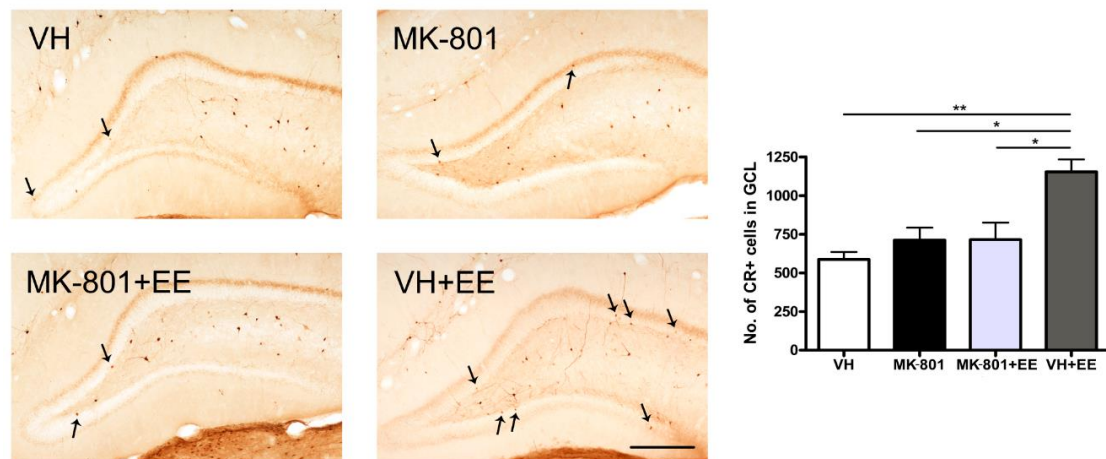

Fig. 10 The arrows indicate calretinin-positive (CR-positive) cells in the granular layer of dentate gyrus. Histogram shows the differences in the total number of calretinin-expressing cells between groups. GCL: granular cell layer of dentate gyrus. Data are represented as mean ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$. Scale bar= 300 μm.

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

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

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.

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 (*p<0.05; **p<0.01; ***p<0.001). PV: parvalbumin; GAD67: glutamic acid decarboxylase-67; PL: prelimbic cortex; AC: anterior cingulate cortex; CA1: Cornus Ammonis 1 of hippocampus; DG: dentate gyrus of hippocampus.