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NEURODEGENERATION AND PROLONGED IMMEDIATE EARLY GENE EXPRESSION THROUGHOUT CORTICAL AREAS OF THE RAT BRAIN FOLLOWING ACUTE ADMINISTRATION OF DIZOCILPINE

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Abstract—*N*-methyl-D-aspartate receptor antagonist drugs (NMDA-A), such as dizocilpine (MK801), induce long-lasting behavioral disturbances reminiscent to psychotic disorders in humans. To identify cortical structures affected by NMDA-A, we used a single dose of MK801 (10 mg/kg) that caused low and high neurodegeneration in intact and orchiectomized male rats, respectively. Degenerating somas (neuronal death) and axonal/synaptic endings (terminal degeneration) were depicted by a silver technique, and functionally affected cortical neuronal subpopulations by Egr-1, c-Fos, and FosB/ΔFosB-immunolabeling. In intact males, MK801 triggered a c-Fos induction that remained high for more than 24 h in selected layers of the retrosplenial, somatosensory and entorhinal cortices. MK801-induced neurodegeneration reached its peak at 72 h. Degenerating somas were restricted to layer IV of the granular subdivision of the retrosplenial cortex, and were accompanied by suppression of Egr-1 immunolabeling. Terminal degeneration extended to selected layers of the retrosplenial, somatosensory and parahippocampal cortices, which are target areas of retrosplenial cortex. Induction of FosB/ΔFosB by MK801 also extended to the same cortical layers affected by terminal degeneration, likely reflecting the damage of synaptic connectivity. In orchiectomized males, the neurodegenerative and functional effects of MK801 were exacerbated. Degenerative somas in layer IV of the retrosplenial cortex significantly increased, with a parallel enhancement of terminal degeneration and FosB/ΔFosB-expression in the mentioned cortical structures, but no additional areas were affected. These observations reveal that synaptic dysfunction/degeneration in the retrosplenial, somatosensory and parahippocampal cortices might underlie the long-lasting impairments induced by NMDA-A.

Key words: Egr-1, FosB, MK-801, neurodegeneration, retrosplenial cortex, somatosensory cortex.

Phencyclidine, ketamine and dizocilpine (MK801) are dissociative anaesthetics that belong to the family of non-competitive *N*-methyl-D-aspartate receptor-antagonist drugs (NMDA-A). In humans, aside of their anaesthetic properties, these drugs induce behavioural changes reminiscent of schizophrenia symptoms that, in same cases, can persist for weeks (Luby et al., 1959; Jentsch and Roth, 1999). Therefore, NMDA-A are widely used as a pharmacological model of psychosis (Farber, 2003). In addition, NMDA-A are considered potential drugs of abuse because they are illicitly used with recreational purposes due to the psychedelic and/or hallucinogenic effects (Morgan et al., 2009).

In the rat, a single application of MK801 ($\geq$4 mg/kg) induces behavioural alterations that remain even after a week (Wöhrl et al., 2007; Manahan-Vaughan et al., 2008), indicating that functional changes persist long after the exposure to the drug. The neuroanatomical substrate affected by MK801 remains elusive, and might provide insights into psychotic disorders. Low doses of MK801 ($<$3 mg/kg) induce transient vacuolization and induction of heat shock proteins in selected layers of the granular subdivision of the retrosplenial cortex (RSG) (Olney et al., 1989; Sharp et al., 1991), indicating a reversible stress in these neurons. The induction of immediate early gene proteins (IEGs) after low doses of MK801 was also documented in the RSG (Gass et al., 1993; Gao et al., 1998; Zhang et al., 1999), further indicating the functional involvement of this cortical structure. MK801-dependent induction of IEGs has also been evidenced in the somatosensory and parahipppocampal cortices, the hippocampus, and thalamic nuclei (Vaisanen et al., 2004), but whether these changes are independent or linked to the functional alterations in RSG remains unknown.

In the female rat, MK801 ($>$3 mg/kg) causes neuronal death particularly in the RSG, but also in olfactory structures, hippocampus and parahippocampal cortex. In addition, axonal and synaptic degeneration also affects olfactory structures, hippocampus, retrosplenial, parahippocampal and somatosensory cortices (Bueno et al., 2003) indicating that the neurodegenerative effect of the drug expands far beyond the RSG. Interestingly, due to the protective effect of testosterone, male rats are much less vulnerable to MK801-toxicity, and somatodendritic degeneration is confined exclusively to the RSG (de Olmos et al., 2008). Thus, the neurotoxic effect of MK801 in the male rat might offer a unique opportunity to evaluate the impact of the selective damage of RSG neurons

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Abbreviations: A–Cu–Ag, amino–cupric–silver; ENT, entorhinal cortex; FJB, fluoro-jade B; IEGs, immediate early genes; MK801, dizocilpine; NHS, normal horse serum; NMDA-A, *N*-methyl-D-aspartate receptor antagonists; ORC, orchiectomized; PBS, phosphate-buffered saline; RSD, dysgranular retrosplenial cortex; RSG, granular retrosplenial cortex; V1, primary visual cortex; V2L, secondary visual cortex, lateral area; V2MM, secondary visual cortex, mediomedial area.

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on the anatomofunctional alterations in target cortical structures.

IEGs are transcription factors widely used for the anatomofunctional identification of the substrates that respond to a stimulus. Combining the analysis of different IEGs might provide a better strategy to identify neuronal subpopulations that are affected by conditions that induce both, transient and prolonged functional alterations. For example, due to its quick and transitory expression, c-Fos allows the identification of neurons that respond to an acute stimulus, but is less useful for persistent or chronic conditions (Kovács, 1998). On the contrary, FosB, and particularly ΔFosB proteins, are appropriate IEGs to track neuronal populations reacting to chronic conditions or to neuroplastic changes that persist long-after the exposure to the stimulus (Chen et al., 1997; Nestler et al., 1999; McClung et al., 2004; Carle et al., 2007; Perrotti et al., 2008). On the other hand, Egr-1 (also known as zif 268, Krox-24, NGFI-A), which exhibits a high basal expression, can be a marker of abnormal synaptic activity or neurodegeneration (Beckmann and Wilce, 1997; Knapska and Kaczmarek, 2004). Curiously, there are no studies analysing the expression of IEGs after a neurotoxic dose of MK801 that induces long-lasting changes.

The aim of our study was to link MK801-induced neuronal death in the retrosplenial cortex with the anatomofunctional changes in target cortical areas. We used a dose of MK801 that induces death of RSG-neurons that was sparse in intact males but profuse in orchidectomized (ORC) animals and, concomitantly, low and high levels of terminal degeneration aroused in the retrosplenial cortex and target cortical areas. To analyse neurodegeneration, we employed a silver technique that depicted both, somatodendritic and terminal degeneration. Additionally, anatomofunctional changes were evidenced by immunostaining of three different IEGs, c-Fos, FosB/ΔFosB and Egr-1.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on male Wistar rats from the Instituto de Investigación Médica Mercedes y Martín Ferreyra vivarium, housed with food and water *ad libitum*. At 45 days of age, a group of male rats were anaesthetized i.p. with 5% chloral hydrate (0.5 ml/100 g body weight) and bilaterally orchiectomized, and returned to their cages until use. Experiments were carried out with intact and ORC males of 70 to 76 days, weighing 315–350 g. All experiments conformed to named local and international guidelines on the ethical use of animals. Every effort was made to minimize discomfort of the animals and the overall number of animals used.

MK801 treatment

Intact and ORC males received either a single i.p. injection of vehicle, 0.9% of sodium chloride (NaCl) (control), or 10 mg/kg of dizocilpine hydrogen maleate, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK801; Sigma-Aldrich, St. Louis, MO, USA). This dose of MK801 reliably produces irreversible neurodegeneration in adult male rats (Fix et al., 1993, 1995). Behavioural signs of MK801 treatment appeared 2–4 min after the injection, which included increased locomotion, stereotyped head weaving, backward and forward movements, ataxia and recumbency. After 24 h of MK801 treatment, animals had completely recovered, moving around in their cages and did not present any significant weight loss. Two animals per group were used for the study of the time course (3, 8, 24, 48, 72 and 120 h) of the neurodegeneration and c-Fos expression induced by MK801 in intact and ORC rats. The survival time for the following set of experiments analysing FosB/FosB and Egr-1 expression was carried out at the peak of the neurodegeneration, which was at 72 h post-treatment, with a minimum of four animals per group.

Perfusion and histological procedure

For brain fixation, all animals were anaesthetized i.p. with 30% chloral hydrate; perfused transcardially and fixed with 4% paraformaldehyde in 0.2 M borate buffer (pH 7.4). Brains were left overnight in the skull and afterward removed and placed in 30% sucrose. Once the brains sank in the sucrose, serial sagittal sections of 40 μm were obtained in a freezing microtome and stored at 4 °C until processing. Four series of sister sections were either stored in phosphate-buffered saline (PBS) 0.01 M in order to immediately process for immunohistochemistry or fluoro-jade B (FJB) technique (Schmued and Hopkins, 2000) and the fifth series was stored in 4× % paraformaldehyde in order to asses neurotoxicity using the amino-cupric-silver technique (A–Cu–Ag) (de Olmos et al., 1994).

Detection of neuronal degeneration

Neuronal degeneration was analysed by two different methods, the A–Cu–Ag, and the FJB techniques. The A–Cu–Ag is a more recent version of the cupric–silver method (de Olmos et al., 1981) which is suitable for staining degenerating perikarya, dendrites, stem axons and their terminal ramifications in brain tissue subjected to different experimental and neuropathological conditions. The procedure was carried out following the protocol previously described (de Olmos et al., 1994). Briefly, sections were rinsed in double-distilled water and incubated in a pre-impregnating solution of silver nitrate at 50 °C. After cooling to room temperature, sections were rinsed with acetone and transferred to a concentrated impregnating silver diamine solution for 40 min. Sections were then immersed in a reducing formaldehyde/citric acid solution for 25 min and then the reaction was stopped in 0.5% acetic acid. Bleaching was done in two steps to eliminate the non-specific deposits of silver on the tissue, first in 6% potassium ferricyanide, washed in double-distilled water, then transferred to 0.06% potassium permanganate for 20 s. After washing sections again, stabilization was done in 2% sodium thiosulfate, washed, placed in a fixer solution for 1 min, and then the sections were mounted and placed on a slide warmer (30 °C) until they were fully dry. The dry slides were cleared by immersion in xylene for 10 min before coverslipping.

For FJB staining we followed the protocol described by Schmued and Hopkins (2000), in which mounted brain sections on slides were immersed in a 1% sodium hydroxide solution (80% ethanol) for 5 min. Slides were then placed in 70% alcohol for 2 min and then rinsed in distilled water for 2 min. Afterwards slides were transferred to a solution of 0.06% potassium permanganate for 10 min and then rinsed in distilled water for 2 min. The staining solution was prepared from a 0.01% stock solution of FJB (Chemicon International, Temecula, CA, USA) that was prepared by the manufacturer's instructions. The stock solution was diluted by adding 0.1% acetic acid, resulting in a final dye concentration of 0.0004%. The working stain solution was prepared within 10 min of use and was not reused. Slides were stained for 20 min and then rinsed in distilled water (3×1 min). The slides were then placed on a slide warmer (50 °C) until they were fully dry. The dry slides were cleared by immersion in xylene for 2 min before coverslipping.

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Immunohistochemistry and antibodies

Sections were first incubated for 1 h at room temperature in a solution of 3% hydrogen peroxide and 10% methanol in PBS 0.01 M to quench endogenous peroxidase. Thereafter, the sections were washed three times in 0.01 M PBS and incubated in a blocking solution of 5% normal horse serum (NHS) for 1 h. After blocking, sections were directly incubated for 48 h at 4 °C with a polyclonal antibody against, c-Fos (s.c.-52, Santa Cruz Biotechnology, CA, USA, 1:1000); Egr-1 (s.c.-110, Santa Cruz Biotechnology, CA, USA, 1:1500) or FosB/FosB (s.c.-48, Santa Cruz Biotechnology, CA, USA, 1:1500) diluted in 0.01 M PBS containing 1% NHS. The FosB/FosB antibody was raised against the residues 75–150 of the FosB molecule and has been demonstrated, by Western blotting analyses, that proteins with a molecular weight corresponding to ΔFosB can be recognized. Because the drug was administered 72 h before analysis, we considered all FosB-like immunoreactivity, detected with the pan-FosB antibody, to reflect FosB/FosB (Perrotti et al., 2004, 2005). Following incubation in the primary antibody, sections were washed three times in 0.01 M PBS and incubated for 2 h in biotinylated secondary antibody (Vector Laboratories, CA, USA); diluted 1:200 in 0.01 M PBS containing 1% NHS, washed three times in 0.01 M PBS, and followed by an avidin–biotin–peroxidase complex (Vectastain ABC Kit, Vector Laboratories, CA, USA) for 1 h at room temperature. Finally, sections were incubated for 5 min with a solution containing 0.05% 3–3′-diamino-benzidine tetra hydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) and 0.01% hydrogen peroxidase. Sections were mounted onto gelatine-coated slides, dehydrated and cover slipped prior to viewing with a light microscope. For double staining of FosB/ΔFosB and A–Cu–Ag technique, sections were first stained with the A–Cu–AG technique and immediately after stabilizing in thiosulfate, sections were placed in 5% NHS for blocking, followed by the protocol described above for immunohistochemistry.

Image analysis and quantitative assessment of neuronal number

Argyrophilic somas revealed by the A–Cu–Ag technique were assessed at 10× and 20× objective lens. At these magnifications intense silver stain of the cell body and processes of degenerating neurons were easily identified, as well as the terminal degeneration in several cortical areas. FJB positive cells were visualized in a fluorescent microscope (Carl Zeiss, Jena, Germany) and corresponding sections of A–Cu–Ag presented approximately the same number of cells. A previous study (Fix et al., 1995; Auer, 1996; de Olmos et al., 2008) showed that neuropathological changes in layer IV of the RSG induced by MK801 increases caudally, with an approximate peak between −5.3 and 7.3 mm respect to Bregma. Therefore, serial sagittal sections were considered optimum for revealing the extent of degeneration induced by MK801 within the RSG and other cortical areas.

Quantitative assessment was accomplished with a light microscope at 20× objective lens, equipped with a Leica LC200 video camera, which acquired and saved images with a Photoshop 7.0 program. Afterwards, positive immunoreactive cells were counted in selected cortical brain areas using the SCION program from the NIH. The counting was carried out by a treatment blind investigator and performed using a predefined area of an identical size (0.16 mm^2) and shape for each brain region. Automated counts of IEG positive nuclei were obtained from each area of interest, maintaining constant background intensity across different areas. Counts from the left and right hemisphere were obtained from three sections within layer IV of the RSG, dysgranular retrosplenial cortex (RSD), secondary visual cortex, mediomedial and lateral areas, (V2MM and V2L), and layer III of the entorhinal cortex (ENT). The lateral medial (coordinates from Paxinos and Watson, 2007) sections included for detailed analysis were lateral 0.9; 1.13 and 1.4 (RSG and RSD); lateral 1.55, 1.9 and 2.10 mm (V2MM); lateral 4.20, 4.32, 4.50 mm (V2L and ENT). The mean number of IEG positive cells per animal was used for statistical analysis.

Statistical analysis

Counts obtained from the left and right hemisphere in different brain sections throughout an area of interest were averaged to generate one value of IEG positive nuclei per region. Data from each region were analysed using a three-way ANOVA (treatment× area×orchiectomy) or two-way ANOVA (layer×treatment). Results showing significant overall changes were subjected to post hoc Newman–Keuls test with values of $P<0.05$ being considered as statistically significant.

RESULTS

Neurotoxic effect of MK801 in cortical areas of intact and ORC male rats

Intact and ORC male rats were treated with a single i.p. of 0.9% NaCl (control) or 10 mg/kg of MK801 and the time-course of neurodegeneration was evaluated at 24, 48, 72 and 120 h post-treatment. In adjacent sections, neurotoxicity was examined with the A–Cu–Ag and FJB techniques. In control animals, no FJB- or A–Cu–Ag-positive neurons were observed in any cortical area at any time point. On the contrary, systematic neuronal degeneration was observed in MK801-treated animals, in which dying somas were confined mainly to layer IV of the RSG, with a similar pattern of degenerating somas depicted with both FJB and A–Cu–Ag techniques. However, degenerating dendrites, axons and synaptic terminals were only revealed by using the A–Cu–Ag method, and therefore this technique was selected for further description of the neurodegenerative effect of MK801.

In the RSG, somatic and argyrophilic-terminal degeneration was observed in MK801-treated animals at 24 h, and increased throughout the course of the 72 h post-treatment in both intact and ORC rats, although degeneration was more conspicuous in the later (Fig. 1). Signs of neurotoxicity were greatly reduced at 120 h post-treatment in both intact and ORC rats, and this was accompanied by an advanced profile of neuronal disintegration, with shrunken argyrophilic cell bodies, and fragmented dendrites and axons, indicating that neurodegeneration reached its peak around 72 h post-treatment in both intact and ORC rats (Fig. 1). In ORC males, in addition to the dying somas described in layer IV of RSG, few and sparse degenerating neuronal somas were also found in layer III of ENT and in the posterolateral cortical amygdaloid nucleus. In both, intact and ORC rats, argyrophilic-terminal degeneration reached its peak at 72 h, affecting layers I, IV–V of RSG (Fig. 1), layers I and IV of RSD, areas of the secondary visual cortex, including mediomedial (V2MM), mediolateral (V2ML) and lateral (V2L) parts, and primary visual cortex (V1). Argyrophilic-terminal degeneration was also observed in the temporal cortex, association area (TeA), perirhinal cortex (PRh), ENT, postsubiculum (Post) and in the subiculum (S) (Fig. 2). The pattern of the argyrophilic-terminal degeneration was similar in intact and ORC males, although the intensity was much greater in the latter. Altogether, these

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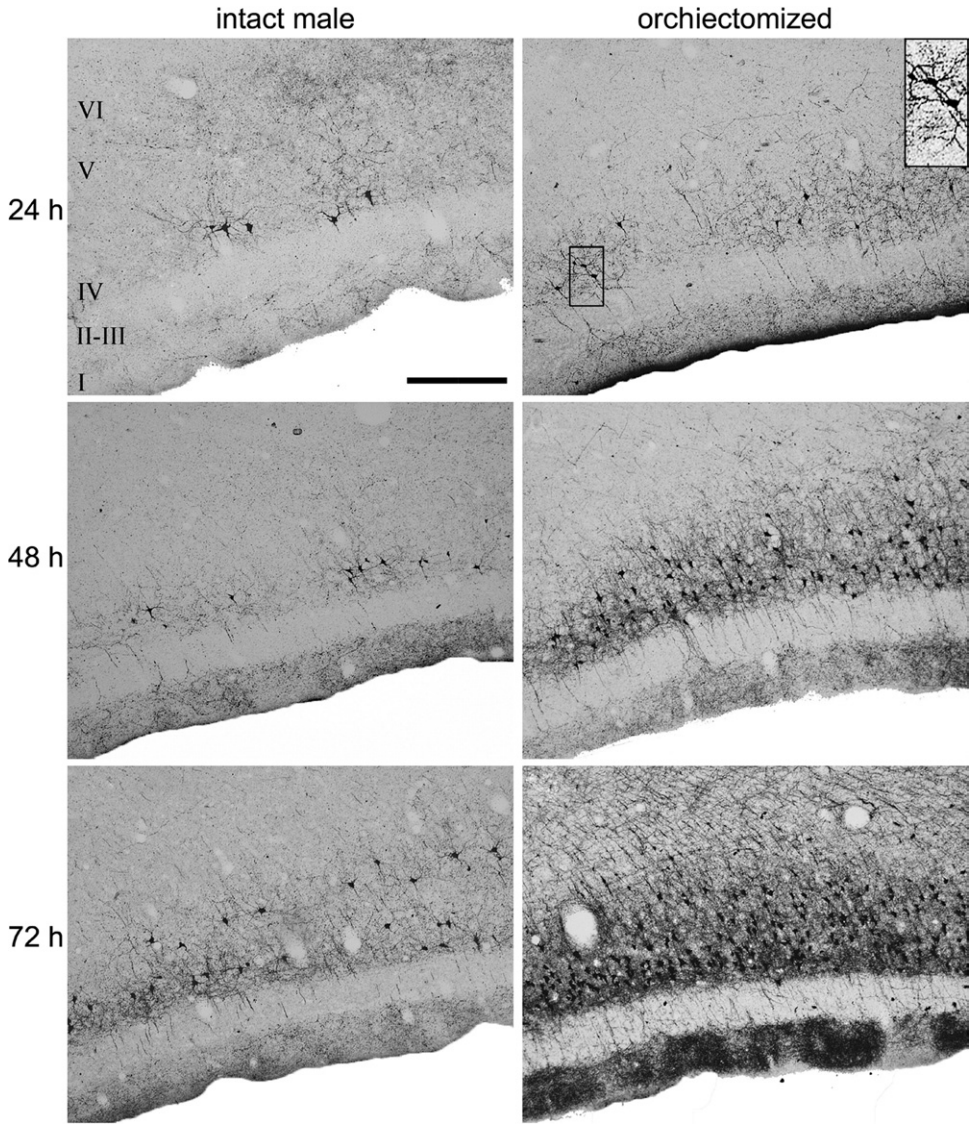

Fig. 1. Time-course of MK801-induced neurodegeneration in the RSG of intact and ORC male rats. Intact and ORC male rats were treated with a single i.p. dose of 0.9% NaCl (control) or MK801 (10 mg/kg), and neuronal degeneration was analyzed with the A–Cu–Ag technique at 24, 48, 72 and 120 h post-treatment. Control animals (not shown) have no signs of neurodegeneration. Shown are representative sagittal sections of the RSG (lat. 1.2 mm) of intact and ORC males at the indicated time-points. At 24 h post-treatment early signs of neurodegeneration are observed, evidenced by argyrophilic cell bodies with corkscrew dendrites (insert). At 72 h post-treatment neurodegeneration reaches the peak, accompanied by signs of advanced cell death, with shrunken cells bodies and fragmented dendrites. Note that somatic degeneration is confined to layer IV of RSG, while terminal degeneration expands to layers I, IV and V. Increased intensity of neuronal degeneration is observed in ORC males. Cortical layers are indicated in the upper-left panel. Scale bar=200 μm.

results show that, in male rats, MK801-induced somatic degeneration was almost exclusively confined to neurons in layer IV of RSG, and was accompanied by concomitant terminal degeneration in several cortical areas that are connected to the retrosplenial cortex.

Effect of an acute neurotoxic dose of MK801 on the expression of Egr-1 in different cortical brain structures in intact and ORC male rats

We analyzed the effect of a single neurotoxic dose of MK801 (10 mg/kg) on the expression of Egr-1, which is an IEG that has been implicated in normal synaptic activity (Beckmann and Wilce, 1997; Knapska and Kaczmarek, 2004), and is altered in processes of neuroplasticity and/or neurodegeneration (Thiriet et al., 2001; Slattery et al., 2005). The expression of Egr-1 was conspicuous in most brain structures, with no apparent differences in intact and ORC males treated with saline (control), indicating that orchiectomy per se had no significant effect on the basal expression of Egr-1. In control animals (intact and ORC), strong immunoreactivity of Egr-1 was observed in layers II, III, IV and VI of RSG (Fig. 3A), RSD, V2MM and V2L. The ENT also revealed strong basal expression of Egr-1 in layers III, V and VI. At 72 h post MK801-treatment, in which

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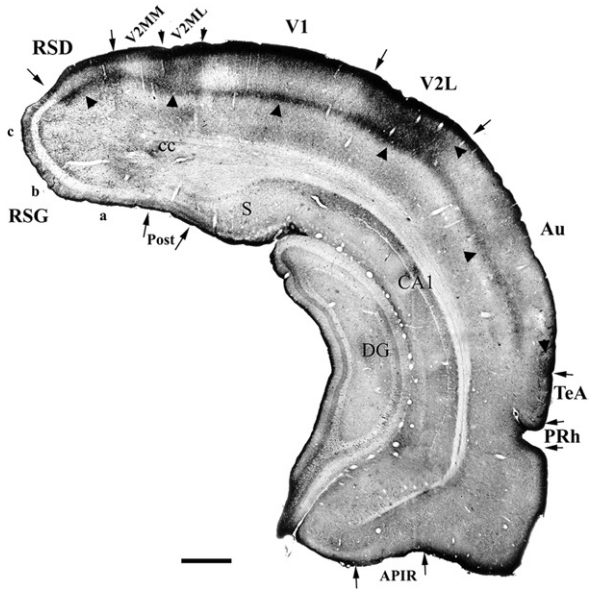

Fig. 2. Terminal neurodegeneration induced by MK801 extends to several cortical areas. Low-power magnification microphotograph showing a frontal section (Bregma −6.24 mm) of an MK801-treated (10 mg/kg) ORC rat at 72 h post-treatment, evaluated with the A–Cu–Ag technique. Note that terminal neurodegeneration (arrowheads) mainly affects layers I and IV of the RSD, V2MM, V1, and V2L. The auditory cortex (Au) presented a lighter pattern of terminal degeneration also confined to layers IV and I. In TeA and PRh terminal degeneration is observed in layer I. Arrows indicate the limits of cortical areas. Scale bar=1 mm. Abbreviations: APIR, Amygdalopiriform transition area; Au, Auditory cortex; CA1, CA1 field of the hippocampus; cc, corpus callosum; DG, Dentate gyrus; Post, Postsubiculum; PRh, Perirhinal cortex; TeA, Temporal cortex, association area; V1, Primary visual cortex, V2L, Secondary visual cortex, lateral part; V2ML, Secondary visual cortex, mediolateral part; V2MM, Secondary visual cortex, mediomedial part.

the peak of the neurotoxic effect is observed, the expression of Egr-1 in intact male rats was reduced in layer IV of the RSG, with noticeable anatomical correspondence with the degenerating somas detected by the A–Cu–Ag method. No appreciable changes in Egr-1 expression were apparent in other cortical layers. To further analyze the relation of Egr-1-expression with neuronal death, Egr-1 immunoreactivity was examined in ORC rats. Similar to intact males, MK801-treated ORC animals also exhibited a striking inhibition of Egr-1 in layer IV of RSG (Fig. 3A, B), which was anatomically coincident with the presence of dying cell bodies (Fig. 3B, D). Egr-1-positive nuclei were scored in control and MK801-treated males, and statistical analysis confirmed a significant reduction of Egr-1 in layer IV of RSG, but not in the other cortical areas analyzed (Fig. 3C). The three-way ANOVA (treatment×area×ORC) showed a significant effect of treatment ($F_{1,12}$=36.20; P<0.00006), area ($F_{4,48}$=4.12; P<0.005), treatment×area ($F_{4,48}$=6.46, P<0.0003) and ORC×area ($F_{4,48}$=7.71; P<0.00008). Other comparisons were not significant. Newman–Keuls post hoc test showed that MK801-treated intact and ORC animals differed from the respective saline groups in RSG, P<0.0001. The effect of MK801 treatment was significantly higher in ORC than intact males, P<0.0007. The tight anatomical juxtaposition of the suppression of Egr-1 expression with the somatic neuronal death, suggests that MK801-induced neuronal degeneration is linked to a concomitant inhibition of Egr-1 expression in layer IV of RSG.

Effect of an acute neurotoxic dose of MK801 on c-Fos and FosB/Δ-FosB expression in different cortical brain structures of intact and ORC male rats

In order to further identify neuronal populations that might be functionally affected by a neurotoxic dose of MK801 (10 mg/kg), c-Fos immunoreactivity was examined at different survival times: 3, 8, 24 and 72 h. In intact and ORC males treated with saline (control) background levels of c-Fos immunoreactivity was observed. Few scattered c-Fos-positive cells in different layers of the visual cortex were evidenced, but other cortical areas were devoid of staining, indicating a faint basal level of c-Fos expression in control animals (Fig. 4). In both, intact and ORC rats, a robust c-Fos-induction was detected 3 h after MK801-treatment in layers IV and VI of RSG, RSD, V2MM, V2L and layer III and VI of ENT. Surprisingly, c-Fos immunolabeling remained high at 8 h post-treatment in all these cortical areas (Fig. 4), and in the ORC rat persisted even after 24 h post-treatment (Fig. 4). At 72 h post-treatment, c-Fos levels had practically returned to saline-control levels (Fig. 4). These results show that a single toxic dose of MK801 promoted an unusually prolonged expression of c-Fos protein in RSG, RSD, V2MM, V2L, and ENT, which are cortical areas that coincidently evidenced argyrophilic-terminal degeneration revealed by the A–Cu–Ag.

The prolonged expression of c-Fos suggests that functional activity remains altered in the retrosplenial cortex and its projection-areas long after the exposure of MK801. Thus, we analysed the expression of FosB/ΔFosB, since its expression has been linked with neuroplastic changes that persist long after the exposure to the stimuli (McClung and Nestler, 2008). FosB/ΔFosB immunoreactivity was analysed at the peak of MK801-induced neurodegeneration (72 h post-treatment) in different cortical areas. In intact males treated with saline (control), the basal level of FosB/ΔFosB expression was appreciable in layers II–III, and V–VI of RSG, RSD, V2MM and V2L (Fig. 5A). In the ENT the basal expression was less prominent, presenting sparse FosB/ΔFosB-positive nuclei in layers II, IV, V and VI (Fig. 5A). In intact males treated with MK801, a strong induction of FosB/ΔFosB immunoreactivity was observed in cortical layer IV of the RSG, RSD, V2MM, V2ML, V2L and V1; and also in the parahippocampal cortex, including the S and the ENT. In the ENT, induction of FosB/ΔFosB immunoreactivity was confined to layer III (Fig. 5B). The tight anatomical correspondence of FosB/ΔFosB induction with the argyrophilic-terminal degeneration triggered by MK801-treatment suggests that both are causally related.

To further analyse the relationship of the terminal degeneration and the induction of FosB/ΔFosB immunoreactivity, we examined the effect of MK801 on the expression of FosB/ΔFosB in ORC males. Basal expression of FosB/ΔFosB in saline-treated ORC animals (control) was similar to that of control intact males, indicating that orchiectomy

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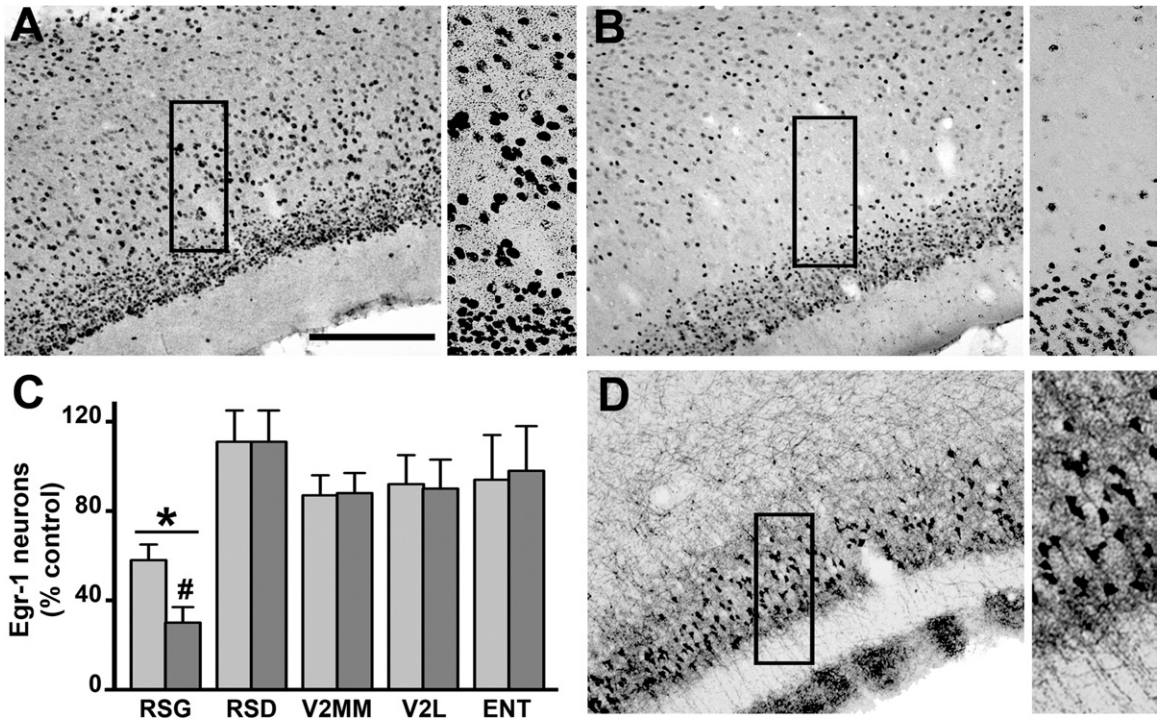

Fig. 3. MK801 induces somatic neurodegeneration and suppression of Egr-1 expression in RSG. Intact and ORC male rats were treated with saline (control) or MK801 (10 mg/kg) and neurodegeneration and Egr-1 expression was analysed in the RSG (lat. 1.4 mm) at 72 h post-treatment. Shown are immunocytochemical staining of Egr-1 expression in control (A) and MK801-treated ORC males (B), and neuronal degeneration as evidenced by the A–Cu–Ag staining in an adjacent section of an ORC male (D). The indicated area is enlarged at the right of the corresponding image. Note that somatic degeneration in layer IV of RSG coincides with suppression of Egr-1 expression. (C) Quantitative assessment of Egr-1 positive neurons in layer IV of RSG of control and MK801-treated intact (light gray) and ORC males (dark grey) in different cortical areas. Bars represent the mean and SEM expressed as percentage of the saline-treated control animal. * $P<0.0001$ vs. saline-treated control animal, # $P<00007$ ORC vs. intact males (three-way ANOVA, treatment×area×ORC), showed significant effect of treatment ($F_{1,12}=36.20$; $P<0.000\ 06$), area ($F_{4,48}=4.12$; $P<0.005$), treatment×area ($F_{4,48}=6.46$, $P<0.0003$) and ORC×area ($F_{4,48}=7.71$ $P<0.00008$). Other comparisons were not significant. Scale bar=200 μm.

per se did not alter FosB/ΔFosB expression (Fig. 6A, C). However, similar to intact males, in ORC males, treatment with MK801 (Fig. 6D, E) triggered a dramatic increase of FosB/ΔFosB immunoreactivity in those cortical areas where terminal degeneration was detected by A–Cu–Ag staining. To obtain a quantitative assessment, FosB/ΔFosB-positive nuclei were scored in layer IV of RSG, RSD, V2MM, V2L and layer III of ENT in control and MK801-treated intact and ORC males. Statistical comparisons indicated that the number of FosB/ΔFosB-positive nuclei significantly increases in MK801-treated animals in all cortical areas analysed, confirming the significant effect of MK801-treatment (Fig. 6E). The three-way ANOVA (treatment×area×ORC) confirmed the significant effect of the treatment ($F_{1,12}=681$; $P<0.000001$), ORC ($F_{1,12}=5.22$; $P<0.041$), area ($F_{4,48}=53.81$; $P<0.000000$), with a significant interaction between the three variables ($F_{4,48}=2.65$; $P<0.044$). Newman–Keuls post hoc comparisons showed no differences between saline-treated intact and ORC rats, confirming that orchiectomy per se does not affect the basal expression of FosB/ΔFosB immunoreactivity. Both, intact and ORC animals presented significant differences with their respective saline controls in the RSG ($P<0.0001$), RSD ($P<0.0001$), V2MM ($P<0.0001$), V2L ($P<0.0001$), ENT ($P<0.0001$). Interestingly, the number of FosB/ΔFosB-positive nuclei induced by MK801-treatment was significantly higher in ORC than intact males in RSG ($P<0.01$), V2L ($P<0.0009$) and ENT ($P<0.006$) (Fig. 6E), which was also coincident with the higher density of terminal degeneration detected in ORC males with the A–Cu–Ag technique.

To confirm that the induction of FosB/ΔFosB after MK801 treatment is restricted to the cortical layer affected by terminal degeneration, the number of FosB/ΔFosB immunoreactive nuclei in all cortical layers of the V2L was scored in control and MK801-treated ORC males. The data indicate that, similar to terminal degeneration, MK801-treatment induces a selective and significant increase in FosB/ΔFosB immunoreactivity in layer IV, but not in layers I, II, III, V or VI (Fig. 6F). The two-way ANOVA (treatment×layer) confirmed the significant interaction between treatment and layer ($F_{4,48}=11.33$; $P<0.00002$). Post hoc Newman–Keuls test indicated a significant difference only in layer IV ($P<0.0001$). To further reveal the anatomical overlap of terminal degeneration and the FosB/ΔFosB-induction, double staining of FosB/ΔFosB and A–Cu–Ag technique were performed. Complete anatomical juxtaposition of silver-positive degenerative terminals and FosB/ΔFosB-positive immunoreactive somas in defined layers of the mentioned cortical areas was observed, as shown for layer IV of RSG and V2L (Fig. 7). Altogether, these experiments provide compelling support to the interpretation that enhanced ex-

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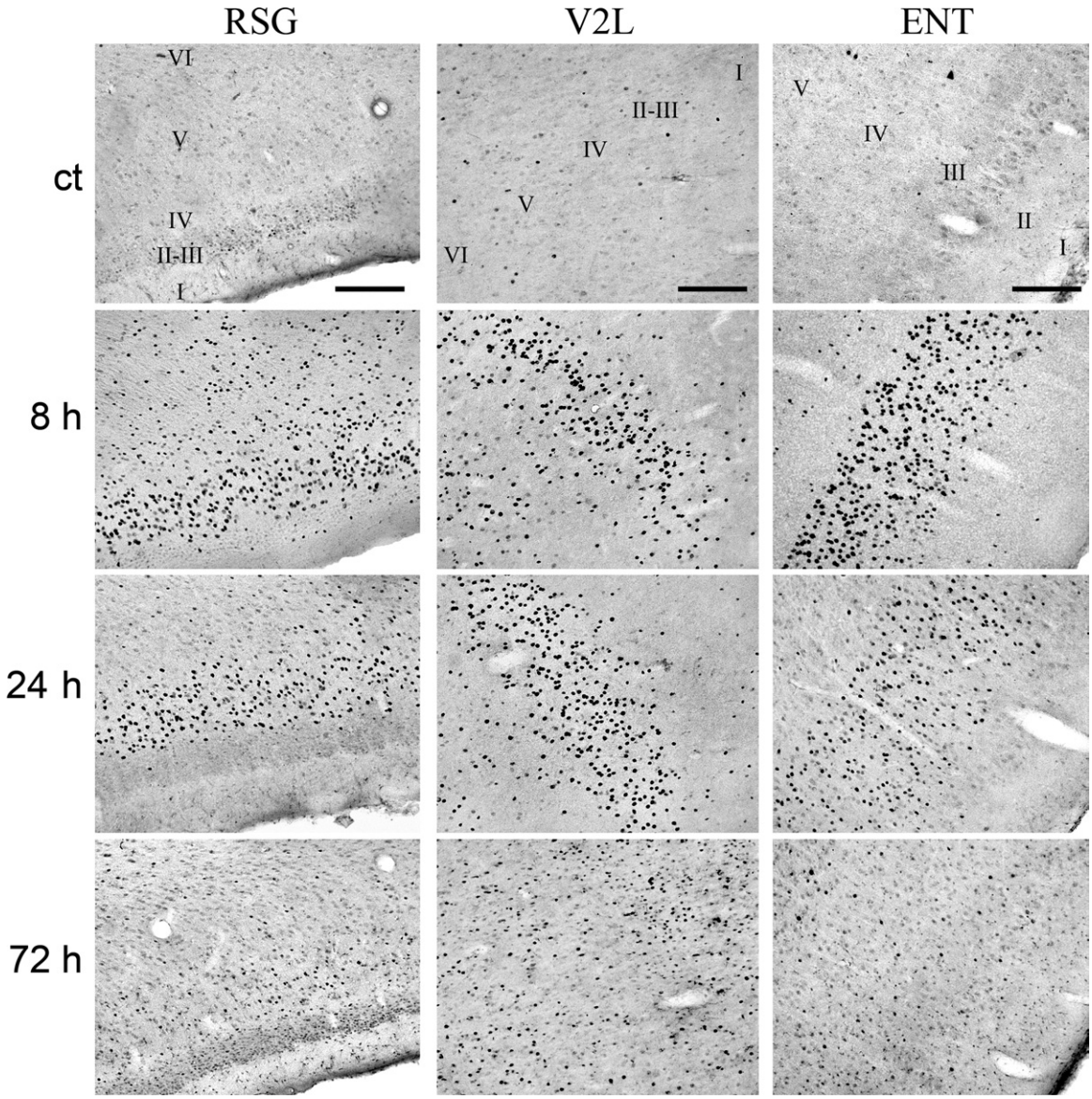

Fig. 4. Time course of c-Fos expression after MK801-treatment in the RSG, V2L and ENT. Intact and ORC male rats were treated with 0.9% NaCl (control) or MK801 (10 mg/kg) and the expression of c-Fos was analysed in different cortical areas. Shown are representative images of c-Fos immunoreactivity in the indicated structures of saline-treated ORC (ct) and MK801-treated ORC at 8, 24 and 72 h post-treatment. Note that MK801-treatment induces a robust and sustained induction in c-Fos in defined layers of RSG (lat. 1.4 mm), V2L (lat. 4.6 mm) and ENT (lat. 4.8 mm). Roman numbers indicates cortical layers. Scale bar=200 μm.

pression of FosB/ΔFosB after MK801 treatment is caused by neuronal deafferentation, likely as a consequence of the degeneration of RSG neurons.

DISCUSSION

In this study we show that a single neurotoxic dose of MK801 induces prolonged morpho-functional alterations in the retrosplenial (RSG and RSD), sensory visual (V2MM and V2L), and entorhinal cortices, which were evidenced by changes in the expression of c-Fos, FosB/ΔFosB, and Egr-1 in distinct cortical layers. Interestingly, the induction of c-Fos immunoreactivity was unusually prolonged, remaining high even after 24 h post-treatment, and was followed by a robust FosB/ΔFosB-expression in the cortical structures, which coincidently depicted axonal and synaptic terminal degeneration. Contrarily, Egr-1 expression was significantly suppressed, accompanying the somatic neurodegeneration in layer IV of RSG.

In rats, neurotoxicity induced by MK801 is sexually dimorphic, females being much more vulnerable than males (Hönack and Löscher, 1993; Fix et al., 1995; Auer, 1996; Wozniak et al., 1998; Andiné et al., 1999; de Olmos et al., 2008). Recently we showed that MK801-toxicity is enhanced by estrogen and reduced by testosterone (de Olmos et al., 2008), indicating that the sexual dimorphism is strongly modulated by gonadal hormones. Here we con-

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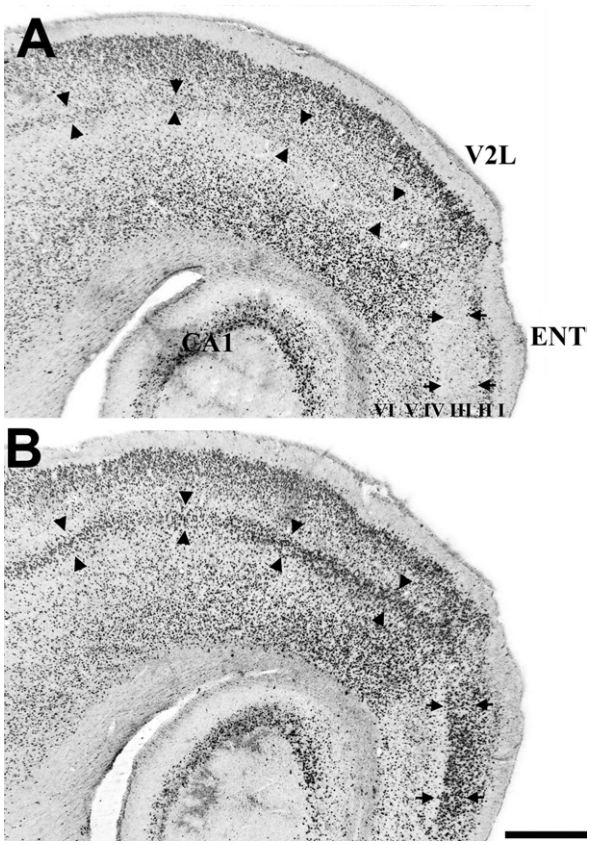

Fig. 5. Expression of FosB/ΔFosB induced by MK801. Intact and ORC male rats were treated with 0.9% NaCl (control) (A) or 10 mg/kg of MK801 (B) and the expression of FosB/ΔFosB was analysed at 72 h post-treatment. Shown are low power photomicrographs of sagittal sections (lat. 3.8 mm) depicting FosB/ΔFosB expression in V2L and ENT. Note that in the control animal some cortical layers show a moderate basal expression of FosB/ΔFosB, and MK801-treatment promotes a selective induction of FosB/ΔFosB only in layer IV of V2L and V1 (arrow heads), and layer III of ENT (arrows). Roman numbers indicates cortical layers of ENT. Scale bar=1 mm.

firmed that orchiectomy sensitizes male rats to MK801-toxicity, increasing somatic and terminal degeneration with a pattern that resembles that of female rats. Thus, to study the morpho-functional impact of MK801 in the expression of IEGs, without the potentially confounding effect of the estrous cycle, we have taken advantage of the comparative analysis of MK801-toxicity in intact and ORC males, that display low and high degeneration, respectively. Our data indicate that paralleling the increase in neurodegeneration, the expression of c-Fos, FosB/ΔFosB, and Egr-1 were more robustly affected by MK801 in ORC than in intact males.

Our observations suggest that expression of IEGs remains affected far beyond the half-life of the pharmacological action of MK801. We observed that the sedative effect that begins minutes after MK801-application has completely disappeared by 24 h post-treatment with no appreciable difference between intact and ORC animals (see materials and methods). The half-life of MK801 in the brain of male rats was estimated in only 2 h (Andiné et al., 1999), and we observed that the time-course of the morpho-functional changes induced by MK801 persist for at least 72 h post-treatment in both, ORC and intact males. These observations indicate that the sustained expression of c-Fos and FosB/ΔFosB could not be explained by the direct pharmacological blockade of NMDA receptors by MK801. Alternatively, neuronal cell death induced by MK801, which progresses over a 3-day period, might be responsible for the prolonged expression of IEGs. A number of different reports have associated the continuous expression of c-Fos with degenerative or adaptive conditions induced by different neurotoxic insults (Smeyne et al., 1993; Dragunow et al., 1995; Shan et al., 1997; Pennypacker et al., 2002; Wu and Liu, 2003). However, in this study we observed no signs of somatic degeneration in MK801-treated animals in cortical structures other than RSG (and very few neurons in RSD and ENT in ORC animals), indicating that the sustained expression of c-Fos or FosB/ΔFosB was not a predictor of neuronal death. On the contrary, we found a striking suppression of Egr-1 immunolabeling restricted exclusively to layer IV of the RSG, tightly overlapping the extension and intensity of somatic degeneration in intact and ORC animals treated with MK801. This observation suggests that changes in Egr-1 expression, but not c-Fos or FosB/ΔFosB, accompany MK801-induced cell death. The fact that we were unable to detect a significant reduction of Egr-1 in RSD or ENT in ORC animals treated with MK801 likely reflects the very limited number of degenerating somas in these structures, and suggests that, in our experimental conditions, Egr-1 suppression is confined to dying neurons. Contrarily to somatic degeneration, the degenerating axonal endings and synaptic contacts (terminal degeneration) anatomically matched the increased expression of c-Fos and particularly that of FosB/ΔFosB. We observed a sustained increase in c-Fos expression that preceded and accompanied the initial stages of neurodegeneration induced by MK801. Thereafter, a detailed analysis at the peak of MK801-induced degeneration revealed a remarkable neuroanatomical overlap of terminal degeneration with the induction of FosB/ΔFosB-immunoreactivity in cortical layer IV of the retrosplenial (RSG and RSD), visual sensory (V1MM, V2MM and V2L), and layer III of ENT cortices. Double staining confirmed the layer-specific superposition of FosB/ΔFosB-induction and the damaged axonal endings and synaptic contacts induced by MK801 in these cortical structures. Thus, it is likely that the long-lasting expression of c-Fos and FosB/ΔFosB induced by MK801 reflects the synaptic dysfunction/deafferentation of these cortical neurons. In agreement with this possibility, it was shown that different electrophysiological parameters that account for the functional connectivity of the hippocampal formation are altered in acutely MK801-treated animals (Dugladze et al., 2004; Rujescu et al., 2006), and these perturbations remained even a week after a single neurotoxic dose of MK801 (Wöhrl et al., 2007; Manahan-Vaughan et al., 2008). These observations indicate that the altered IEGs expression that we report here might be linked to a dysfunctional neuronal circuit. For example, in other experimental conditions such as the destruction of the nigrostriatal pathway by 6-hydroxydopamine, sus-

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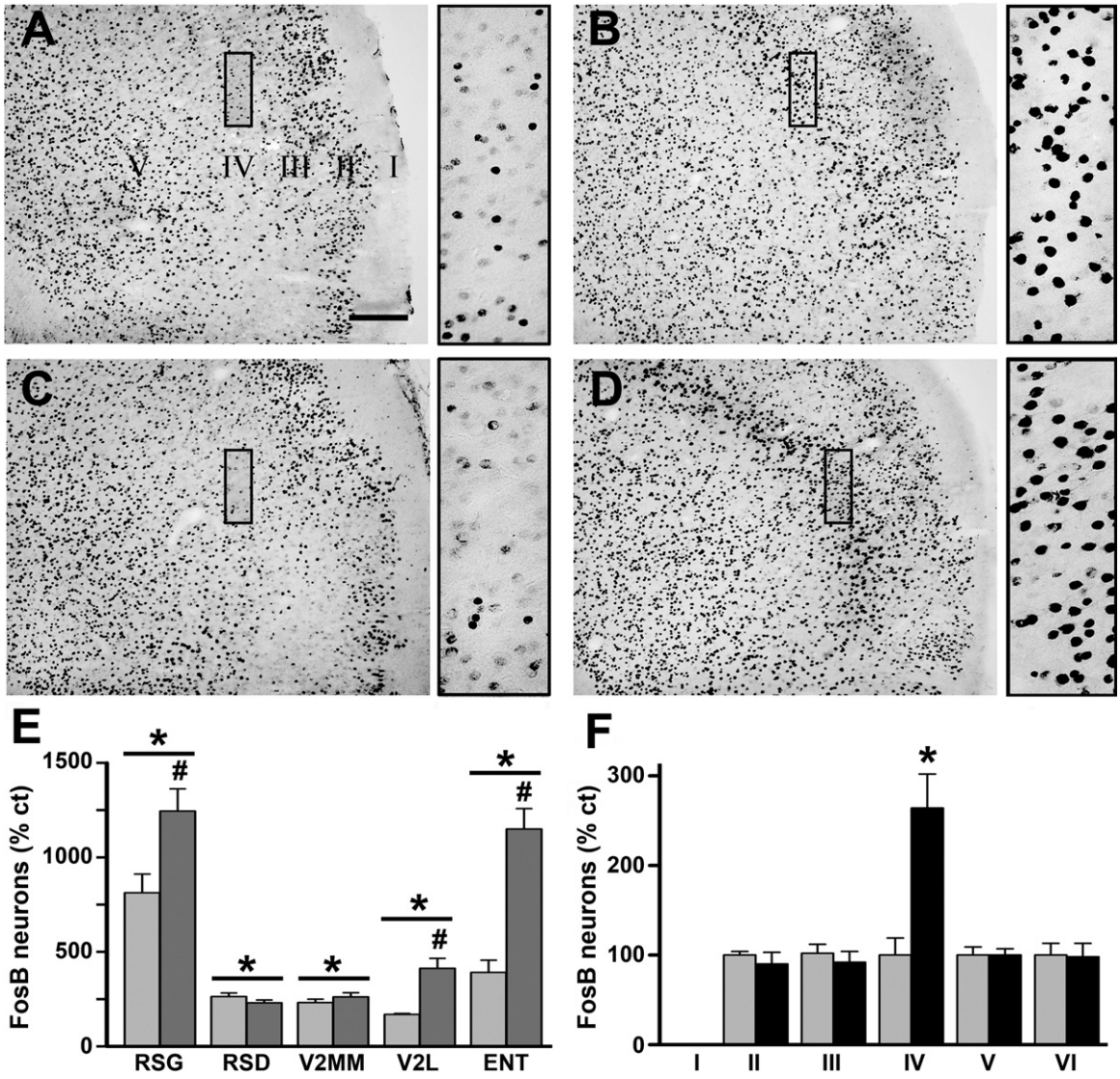

Fig. 6. Comparative analysis of the FosB/ΔFosB expression after MK801-treatment in intact and ORC male rats. Intact and ORC male rats were treated with 0.9% NaCl (control) or 10 mg/kg of MK801, and the expression of FosB/ΔFosB was analysed at 72 h post-treatment in the indicated cortical structures. Shown are representative microphotographs of V2L (lat. 4.6 mm) of saline-treated intact (A), and ORC (C) male rat, and MK801-treated intact (B), and ORC males (D). The inserts are enlarged at the right of the corresponding image, and are representative of the area where FosB/ΔFosB-positive neurons were scored. Roman numbers indicates cortical layers. Scale bar=200 μm. (E) Quantitative analysis of the number of FosB/ΔFosB-positive neurons scored in saline treated control (intact and ORC males) and MK801-treated intact (light grey) and ORC (dark grey) male rats in the indicated cortical areas. Bars represent the mean and SEM expressed as percentage of corresponding saline-treated animal. * $P<0{,}0001$ MK801-treated vs. saline-treated animals. # $P<0{,}01$ ORC vs. intact males (three-way ANOVA, treatment×area×ORC), confirmed the significant effect of treatment ($F_{1,12}=681$; $P<0.000001$), ORC ($F_{1,12}=5.22$; $P<0.041$), area ($F_{4,48}=53.81$; $P<0.000000$), with a significant interaction between the three variables ($F_{4,48}=2.65$; $P<0.044$). (F) Quantitative analysis of the number of FosB/ΔFosB-positive neurons scored in ORC males treated with saline (light grey) and MK801 (black) in the different layers of the V2L. Note that FosB/ΔFos is significantly induced by MK801 only in layer IV. * $P<0{,}0001$ (two-way ANOVA (treatment×layer) confirmed the significant interaction between treatment and layer ($F_{4,48}=11.33$; $P<0.00002$).

tained expression of FosB-like proteins was observed in the deafferentiated striatal neurons (Doucet et al., 1996). It is interesting to note that increased expression of FosB protein and its truncated splice variant, ΔFosB, has also been associated with persistent neuroplastic/adaptive changes elicited by diverse conditions including the administration of antipsychotics, drugs of abuse, or by stress (Atkins et al., 1999; Nestler, 2001; Perrotti et al., 2004, 2005), which are conditions that promote synaptic remodeling. It remains to be determined whether the expression of FosB/ΔFosB after MK801 reflects a permanent altered functionality of the differentiated cortical neurons, or a local remodeling as an attempt to compensate the loss of synaptic inputs in the cortical circuitry. Regardless of that, the dysfunction/degeneration of synaptic contacts in cortical areas that are not directly affected by neuronal death might explain the long-lasting expression of IEGs elicited by MK801 and other NMDA-A.

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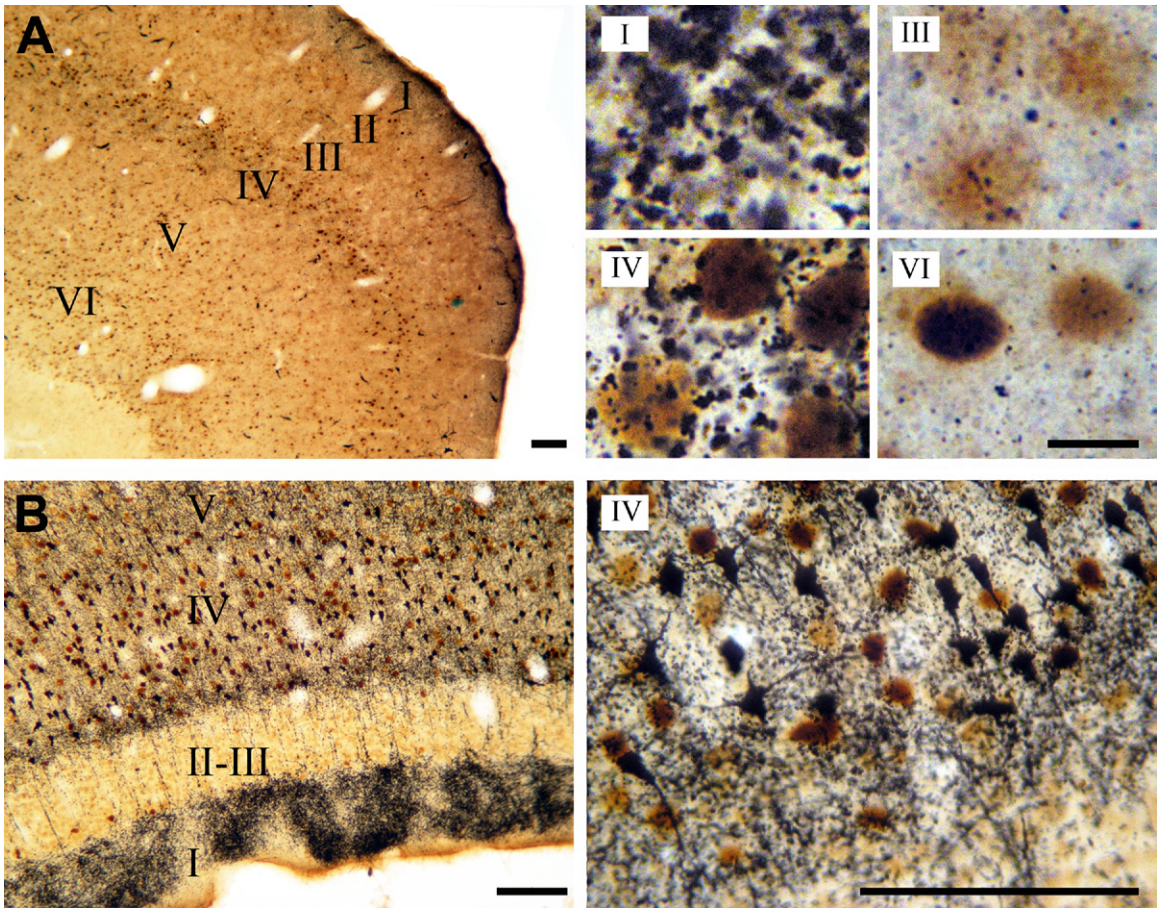

Fig. 7. Terminal degeneration and FosB/ΔFosB-induction by MK801 restricted to specific cortical layers. ORC animals were treated with 10 mg/kg of MK801, after 72 h post-treatment terminal degeneration and FosB/ΔFosB-expression were analysed by the A–Cu–Ag technique and immunohistochemistry, respectively. (A) Low power photomicrograph of a sagittal section (lat 4.6 mm) showing the terminal degeneration (black), and the induction of FosB/ΔFosB (brown) that superimpose in layer IV of the V2L. Images at the right upper corner are representative high power magnification images of the indicated cortical layer. Note that terminal degeneration superposes with MK801-induced-FosB/ΔFosB-positive neurons in layer IV; the basal expression FosB/ΔFosB is high in layer VI but is not altered by MK801-treatment (see Figs. 5, 6). (B) Sagittal section of the RSG (lat 1.4 mm) showing argyrophilic cells and degenerative terminals in layer IV together with FosB/ΔFosB-positive cells in layer (IV) lower-right panel. Magnification of layer IV of RSG is shown in the lower-right panel. Note that FosB/ΔFosB-positive cells are surrounded by silver stained terminals. Scale bar=100 μm for (A), (B), and lower-right panel. Scale bar=10 μm upper-right panels.

The complexity of the cortical circuits makes it difficult to ascertain the identity of the neuronal bodies that originate the degenerating terminals that arises in different cortical areas after MK801-treatment. In our experiments, somatic degeneration in MK801-treated intact males was confined to layer IV of the RSG, suggesting that terminal degeneration detected in cortical areas correspond to axonal and synaptic endings of degenerating RSG neurons. In agreement with this interpretation we found that the number of degenerating somas significantly augmented in MK801-treated ORC males, which was accompanied by a concomitant increase in the density of terminal degeneration in cortical structures. Importantly, all cortical areas where we found terminal degeneration after MK801-treatment have been described as target-projection areas of the retrosplenial cortex (Vogt et al., 2004). Thus, the most parsimonious explanation is that degenerating terminals in somatosensory and parahippocampal cortices that arise after MK801-treatment correspond to degenerating axons of dying RSG neurons. The concomitant induction of FosB/ΔFosB that accompanies terminal degeneration might indicate that loss of RSG-inputs (deafferentiation) functionally affects these cortical areas. In agreement with this interpretation, it was shown that low doses of MK801, which promote behavioral alterations and reversible stress in RSG neurons, induce fast and transitory changes in the expression of IEGs or their mRNA in similar neuroanatomical structures to those described in our study (Dragunow and Faull, 1990; Gass et al., 1993; Gao et al., 1998; Zhang et al., 1999; Vaisanen et al., 2004), suggesting that a transitory stress induced by MK801 also affects the function of the connectivity of the RSG with their target cortical neurons. Although low doses of NMDA-A promote reversible dysfunction, neurodegenerative doses might induce more permanent damage of the connectivity. Therefore, the behavioral disturbances elicited by NMDA-A, either transitory or permanent, appear to result from the dysfunction/damage of the connectivity of layer IV RSG neurons

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with their target neurons in the somatosensory and parahippocampal cortices.

It was proposed that the mechanism of MK801-induced toxicity is mediated by a complex imbalance of inhibitory/excitatory inputs that triggers excitotoxicity in RSG neurons (Olney and Farber, 1995; Rujescu et al., 2006). The particularly high vulnerability of RSG neurons to MK801-induced excitotoxic damage remains unsolved, and might depend on particular intrinsic features of these neurons. Previously, other authors observed that MK801 and phencyclidine induce an initial activation, followed by a suppression of Egr-1 mRNA (Gass et al., 1993; Gao et al., 1998). We observed that suppression of Egr-1 accompanied neuronal death of RSG neurons, suggesting that its expression might be required for the survival of this neuronal population. Although our experiments were not designed to solve this issue, the absence of Egr-1 could indicate that transcriptional activation of proteins downstream of Egr-1 is severely depressed by the excitotoxic damage triggered by MK801. It is noteworthy that the RSG is particularly rich in Zn^{+2} fibers (Casanovas-Aguilar et al., 1998; Miró-Bernié et al., 2006), and Egr-1 activity depends on the cellular levels of Zn^{+2} (Park and Koh, 1999). Therefore, it is possible to speculate that MK801 might trigger an excitotoxic process that induces an AMPA/Kainate-mediated imbalance of Zn^{+2} in RSG neurons, affecting Egr-1 activity and down-stream protein-expression, compromising neuronal viability. Further experiments will be required to add support to this possibility.

In the rat, the retrosplenial cortex is a nodal point for the integration and the subsequent distribution of the reciprocal information between the limbic and visual cortices, the hippocampal formation, and the thalamus (Van Groen and Wyss, 1990, 1992, 2003). Lesions of the retrosplenial cortex in rats produce deficits in the spatial performance and memory (Harker and Whishaw, 2002; Aggleton and Vann, 2004; Van Groen et al., 2004), which are similar to some of the behavioural alterations induced by NMDA-A (Morgan et al., 2004, 2009). In humans, functional neuroimaging studies suggest that the retrosplenial cortex participate in the integration of emotions and episodic memory (Maddock, 1999; Maguire, 2001; von Zerssen et al., 2001). Interestingly, a number of reports described the similarities of the behavioural disturbances elicited by NMDA-A in humans with psychotic syndromes such as schizophrenia (Javitt and Zukin, 1991; Olney and Farber, 1995; Jentsch and Roth, 1999; Farber, 2003). Thus, the dysfunction/damage of the connectivity of RSG neurons with their target somatosensory and parahippocampal cortices, rather than the stress of RSG neurons per se, might underlie the behavioural abnormalities elicited by NMDA-A in animals and humans, and might also play a key role in psychotic disorders.

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