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INVOLVEMENT OF AMPA/KAINATE-EXCITOTOXICITY IN MK801-INDUCED NEURONAL DEATH IN THE RETROSPLENIAL CORTEX

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Abstract—MK801 is a prototypical non-competitive NMDA receptor-antagonist that induces behavioural changes and reversible toxicity at low doses, while at higher doses triggers neuronal death that mainly affects the retrosplenial cortex (RSC) and to a lesser extent other structures such as the posterolateral cortical amygdaloid nucleus (PLCo). The mechanism of MK801-induced neurodegeneration remains poorly understood. In this study we analysed the participation of GABA-ergic and glutamatergic neurotransmission in MK801-induced neuronal death. We used a single i.p. injection of MK801 (2.5 mg/kg) that induced moderate neuronal death in the RSC and PLCo of female rats, and combined this treatment with the i.p., i.c.v., or intra-RSC infusion of drugs that are selective agonists or antagonists of the GABA-ergic or glutamatergic neurotransmission. We found that neuronal death in the RSC, but not the PLCo, was significantly reduced by the i.p. injection of thiopental, and the i.c.v. application of muscimol, both GABA-A agonists. MK801-toxicity in RSC was abrogated by intra-RSC infusion of muscimol, but the GABA antagonist picrotoxin had no effect. HPLC-analysis showed that levels of glutamate, but not GABA, in the RSC decreased after i.p. treatment with MK801. Intra-RSC infusion of MK801 did not enhance toxicity triggered by the i.p. injection of MK801, indicating that toxicity is not due to direct blockade of NMDA receptors in RSC neurons. MK801-toxicity in the RSC was abrogated by i.c.v. and intra-RSC infusions of the AMPA/kainate antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX). Interestingly, i.c.v. application of neither muscimol or DNQX inhibited MK801-toxicity in the PLCo, suggesting that the mechanism of neuronal death in the RSC and the PLCo might be different. 1-naphthylacetyl spermine trihydrochloride (NASPM), which blocks Ca2+ permeable AMPA/kainate receptors, also reduced MK801-induced toxicity in the RSC. Intra-RSC infusion of AMPA or kainic acid alone promoted death of RSC neurons and was reminiscent of the degeneration induced by the i.p. treatment with MK801. Collectively, these experiments provide evidence for an AMPA/kainate-dependent mechanism of excitotoxicity in the death of RSC neurons after i.p. treatment with MK801.

Key words: NMDA receptor antagonist, dizocilpine, neurodegeneration, retrosplenial cortex, AMPA/kainate, excitotoxicity.

Glutamate is the most common excitatory neurotransmitter in the brain, and N-methyl-D-aspartate (NMDA) receptor is the most widely distributed ionotropic receptor for glutamate (Ozawa et al., 1998). Concomitantly, NMDA receptor-antagonists (NMDA-A) such as phencyclidine, ketamine, nitrous oxide, dextrometorphan, memantine and dizocilpine (MK801) have several applications in neurobiology. For example, memantine is used for the treatment of Alzheimer's disease (Bassil and Grossberg, 2009), while nitrous oxide and ketamine are employed as anesthetics for both humans and animals (Becker and Rosenberg, 2008; Sinner and Graf, 2008). However, in humans, aside of their anaesthetic properties, NMDA-A induce behavioural changes which are reminiscent of schizophrenia symptoms that, in some cases, can persist for weeks (Luby et al., 1959; Jentsch and Roth, 1999; Krystal et al., 2003), therefore, NMDA-A such as phencyclidine, ketamine and MK801 are also widely used as a pharmacological model of psychosis (Farber, 2003; Mouri et al., 2007). In addition, NMDA-A are considered potential drugs of abuse because they are illicitly used for recreational purposes as they also produce psychedelic and/or rewarding effects (Morgan et al., 2009). The discovery that, in experimental animals, NMDA-A induced neurodegeneration in the retrosplenial cortex (RSC) and other cortico-limbic areas (Olney et al., 1989; Jevtović-Todorović et al., 1998a; Bueno et al., 2003a,b) caused alarm in the scientific community as this toxic effect was reported even for NMDA-A that have clinical use such as nitrous oxide (Jevtovic-Todorovic et al., 2003), memantine (Creeley et al., 2008) and ketamine (Olney et al., 1989). Therefore, addressing the mechanisms of neurotoxicity of NMDA-A is important to avoid the detrimental and/or undesired side effects of this type of drugs.

MK801 is a prototypical non-competitive NMDA-A. Like other NMDA-A, low doses ($<$0.5 mg/kg) of MK801 induce behavioural changes and reversible neurotoxicity in rats (Olney and Farber, 1995). This toxicity affects neuronal populations of layers IV-V of the RSC, and includes the

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Abbreviations: A-Cu-Ag, amino-cupric-silver; DMSO, dimethyl sulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EDTA, ethylenediaminetetraacetic acid; FJ-B, Fluoro-Jade B; HPLC, high pressure liquid chromatography; HSP70, heat shock proteins 70; MK801, dizocilpine; NaCl, sodium chloride; NASPM, 1-Naphthylacetyl spermine trihydrochloride; NMDA-A, N-methyl-D-aspartate receptor antagonists; PBS, phosphate buffer saline; PLCo, posterolateral cortical amygdaloid nucleus; RSC, retrosplenial cortex.

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transient expression of heat shock proteins 70 (HSP70) and cytoplasmic vacuolization (Olney et al., 1989; Sharp et al., 1991). Electron microscope analysis revealed that neuronal vacuolization corresponds to swollen mitochondria and cisternae of the endoplasmic reticulum, which disappears soon after treatment (Olney and Farber, 1995), indicating that it represents a reversible stress elicited by the NMDA-A. Several studies indicate that reversible toxicity induced by NMDA-A appears not to be caused by the direct blockade of NMDA receptors in RSC neurons, but rather by blockade of NMDA receptors in neurons that project to RSC and promote an imbalance of neurotransmission. Several neurotransmitter systems, including the dopaminergic (Fujimura et al., 2000; Dickerson and Sharp, 2006; Arif et al., 2007), serotoninergic (Farber et al., 1998; Tomitaka et al., 2000a), cholinergic (Olney et al., 1991; Farber et al., 2002), GABA-ergic (Olney et al., 1991; Tomitaka et al., 2000b; Farber et al., 2003), adrenergic (Farber et al., 1995; Jevtovic-Todorovic et al., 1998b) and glutamatergic (Sharp et al., 1995; Farber et al., 2002) have been implicated in the reversible stress promoted by NMDA-A in the RSC, but the resultant effect appears to be an imbalance of the inhibitory/excitatory inputs in the RSC, generating a local excitotoxic stress (Olney et al., 1999).

In rats, higher doses (2–10 mg/kg) or sub-chronic treatments (2–5 days) with moderate doses of MK801 or other NMDA-A induce irreversible neurodegeneration (neuronal death) affecting layers IV-V of the RSC and, to a lesser extent, other structures including the posterolateral cortical amygdaloid nucleus (PLCo), hippocampus, olfactory cortex, olfactory bulb, entorhinal cortex and other cortical-limbic nuclei (Olney et al., 1989; Ellison, 1994; Fix et al., 1996; Horváth et al., 1997; Bueno et al., 2003a, b; de Olmos et al., 2009). The mechanism of irreversible neurodegeneration has not been addressed. Because i.p. treatment with GABA agonists effectively inhibits both reversible and irreversible toxicity (Olney et al., 1991; Sharp et al., 1994; Jevtovic-Todorovic et al., 2001), it is generally assumed that a similar mechanism of toxicity underlies both events, but systematic studies aimed to address this important aspect of NMDA-A action are still lacking. Interestingly, we recently showed that estrogen enhanced neuronal death induced by MK801 (de Olmos et al., 2008), while it was previously shown that reversible stress is reduced by the hormone (Dribben et al., 2003). These observations indicate that reversible and irreversible toxicity induced by NMDA-A might not necessarily operate by identical mechanisms.

In this study we performed a comprehensive analysis of the local participation of the GABA-ergic and glutamatergic neurotransmission in the permanent neurodegenerative changes (neuronal death) induced by MK801 in the RSC. To that end, we used a dose of MK801 that induces moderate neuronal death of RSC neurons and combined this treatment with the systemic or local infusion of drugs that selectively modulate GABA-ergic and glutamatergic neurotransmission.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed on 3-month-old (210–250 g) female Wistar rats from the Instituto de Investigación Médica Mercedes y Martín Ferreyra vivarium. Female rats were used because of their high sensitivity to MK801-toxicity, which make them a suitable model for studying mechanisms of degeneration induced by NMDA-A (Creeley et al., 2008; de Olmos et al., 2008; Fix et al., 1996; Farber et al., 2002; Horváth et al., 1997; Tomitaka et al., 2000b; Jevtovic-Todorovic et al., 2003; Willis et al., 2006). All procedures were carried out in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised in 1996) and local guidelines on the ethical use of animals. Groups of six rats were housed in large cages ($22\times40\times60$ cm^3) under a controlled environment with a temperature at 20 °C, and light-dark cycle of 12/12-h, which are conditions that cannot be considered neither a socially stressful (no overcrowdness) nor a socially enriched environment. Water and food pellets for laboratory animals not containing soy (GepsaFeeds-Cargill, Grupo Pilar SA) were available *ad libitum* for the duration of the experiment. Efforts were made to minimize the number of animals used and their suffering.

Treatments and drug administrations

MK801 (dizocilpine maleate, (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate, Sigma-Aldrich) was used as the prototypical NMDA-A because of its high affinity and specificity (Ozawa et al., 1998). A single intraperitoneal (i.p.) injection of MK801 2.5 mg/kg was used because it consistently induces death of RSC neurons at a level below maximal (Fix et al., 1995), and therefore it allows to observe either inhibition or enhancement of neurotoxicity by the application of different drugs. Previous studies have shown that MK801-treatment begins to show signs irreversible neurotoxicity approximately at 12–24 h, reaching its plateau at 48 h (Olney et al., 1989; Horváth et al., 1997; Jevtovic-Todorovic et al., 2001; Bueno et al., 2003a; de Olmos et al., 2009). Also, inhibition of MK801-toxicity by drug-treatments can be observed between 0 and 8 h post MK801-treatment (Colbourne et al., 1999). Based on these observations, GABA and glutamate agonists and antagonists were applied 0–24 h post-MK801, and neuronal degeneration was analyzed 48 h after MK801-treatment.

Thiopental (Sigma-Aldrich) at 5 or 10 mg/kg was applied in three i.p. injections at 0, 4 and 8 h post-MK801 (Jevtovic-Todorovic et al., 2001; Liu and Yao, 2005). Muscimol (Flucka) at 0.4 μg was applied in two intracerebroventricular (i.c.v.) injections at 0 and 4 h post MK801 injection (Farber et al., 2003; Tomitaka et al., 2000b). In another set of experiments a single intra-RSC muscimol infusion of muscimol at 0.01, 0.1 or 1 μg was applied 30 min after MK801 (Farber et al., 2003; Tomitaka et al., 2000b). Picrotoxin (Sigma-Aldrich) at 5 mg/kg was used in a single i.p. injection (Turski et al., 1985), or infused intra-RSC at 0.3 or 1.2 μg (Turski et al., 1985) 30 min after MK801. 6,7-Dinitroquinoxaline-2,3-dione (DNQX) (Sigma-Aldrich) at 15 μg was applied in 3 i.c.v. injection at 0, 4 and 8 h post MK801 administration (Sharp et al., 1995; Young and Dragunow, 1993). For intra-RSC a single infusion of DNQX at 0.5, 5 and 10 μg was used (Sharp et al., 1995; Young and Dragunow, 1993) 30 min after MK801. In another set of experiments, a single intra-RSC infusion of musimol (100 ng), DNQX (5 μg), MK801 (3 μg), or 1-naphthylacetyl spermine trihydrochloride (NASPM; Sigma Aldrich) (10 μg) (Noh et al., 2005), was administered at 0.5, 5, 10 or 24 h after i.p. MK801-treatment to analyze the local protection interval of each drug. Kainic acid (Sigma-Aldrich) 0.5 and 1 μg (Farber et al., 2002; Nadler, 1981), or AMPA 0.45 and 0.90 μg (Farber et al., 2002; Fowler et al., 2003) were infused intra-RSC and neurodegeneration was ana-

lyzed 48 h later. Drugs administered i.p. were dissolved in 0.9% saline, and those infused i.c.v. or intra-RSC were dissolved in phosphate buffer saline (PBS), except for DNQX that was dissolved in pure dimethyl sulfoxide (DMSO; Merck), control animals received the respective vehicle (saline, PBS or DMSO).

Final volumes of drugs administered i.c.v. was 2 μl. When drugs were infused intra-RSC to test their local effect on MK801-toxicity, 0.5 μl was applied to restrain diffusion. To analyze the local excitotoxic damage, kainic acid and AMPA were infused intra-RSC in 1 μl to increase the ratio of drug diffusion.

Surgery

Rats were anaesthetized i.p. with 40/10 mg/kg of sodium ketamine/xylazine (Sigma-Aldrich), and securely placed into a stereotaxic device with bregma and lambda at horizontal levels. For i.c.v. and intra-RSC microinjections, 22-gauge stainless steel guide cannulas were bilaterally implanted at AP−0.8 mm, LM±1.5 mm, VD−1.8 and AP−6.00 mm, LM±1.6 mm, VD=−0.3 mm from bregma, respectively, according to the atlas of Paxinos and Watson (2007). Guide cannulas were secured to the skull with dental cement and stainless steel screws. After surgery the animals were allowed to recover for 7 days before drug-treatments. Infusions of drugs were done in freely moving animals with the use of a micro-syringe (Hamilton, Reno, NV, USA) connected to 30-gauge injecting-cannula that was inserted into the guide cannula. Injecting cannulas exceeded the length that of the guide cannulas in 1.5 and 2 mm for intra-RSC and i.c.v. infusions, respectively. Infusions were performed at a rate of 0.1 μl/min, and the injecting cannula was left in place for additional 3 min before removal. Both, guide and injecting cannulas were hand-made from stainless-steel sterile needles (Deltajet) and the appropriate length of each one was carefully controlled under a microscope.

Perfusion and histological procedure

For brain fixation, all animals were anaesthetized i.p. with 30% chloral hydrate (Socram, Switzerland) diluted in saline, then perfused transcardially and fixed with 4% paraformaldehyde (Sigma Aldrich) in 0.2 M borate buffer (pH 7.4). Brains were left overnight in the skull and afterwards removed and placed in 30% sucrose diluted in water, until they sank. Serial frontal sections of 40 μm were obtained in a freezing microtome (Leica). Serial sections were stored at 4 °C in either 0.01 M PBS or 4% paraformaldehyde, for Fluoro-Jade B (Chemicon) (Schmued and Hopkins, 2000) or for the amino-cupric-silver (A-Cu-Ag) technique (de Olmos et al., 1994) processing, respectively.

Detection of neuronal degeneration

Neuronal degeneration was analysed by two different methods, the (A-Cu-Ag), and the Fluoro-Jade B (FJ-B) techniques that specifically stain dying neurons. The A-Cu-Ag technique used (de Olmos et al., 1994) is the most recent version of the previous one (de Olmos et al., 1981), and is highly sensitive for staining degenerating perikarya, dendrites, axons and terminal ramifications in brain tissue subjected to different toxic conditions, including excitotoxicity (de Olmos et al., 1994). 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 for 2 h in a pre-impregnating solution that consisted of: $AgNO_3$ (Sigma-Aldrich) 100 mg; α–amino-n-butyric acid (Sigma-Aldrich) 53 mg; alanine (Sigma-Aldrich) 46 mg; 2 ml of 0.5% $CuNO_3$ (Sigma-Aldrich); 0.2 ml of 0.5% $CdNO_3$ (Sigma-Aldrich); 1.5 ml of 0.5% $LaNO_3$ (Sigma-Aldrich); 0.5 ml of 0.5% neutral red (Sigma-Aldrich); 1 ml piridine (Merck); 1 ml triethanolamine (Merck); 2 ml isopropanol (Merck); 100 ml double distilled water at 50 °C. After cooling to room temperature, sections were rinsed with acetone and transferred to a concentrated impregnating silver diamine solution: 412 mg $AgNO_3$ (Sigma-Aldrich); 4 ml ethanol (Merck), 0.05 ml acetone (Merck); 3 ml of 0.4% LiOH (Sigma-Aldrich), 0.65 ml NH_4OH (Merck); 5 ml double distilled water for 50 min at room temperature. Sections were then immersed in a reducing formaldehyde/citric acid (Sigma-Aldrich) solution for 25 min and then the reaction was stopped in 0.5% acetic acid (Merck). Bleaching was done in two steps to eliminate the non-specific deposits of silver on the tissue, first in 6% potassium ferricyanide (Sigma-Aldrich), washed in double distilled water, then transferred to 0.06% potassium permanganate (Sigma-Aldrich) for 20 s. After washing sections again, stabilization was done in 2% sodium thiosulfate (Sigma-Aldrich), washed, placed in a rapid fixer solution (Kodak), diluted 1:6 in distilled water 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 being coverslipped with DPX (Flucka).

For FJ-B (Chemicon) staining we followed the protocol described by Schmued and Hopkins (2000), in which mounted brain sections on slides were immersed in xylene 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 within 10 min of use from a 0.01% stock solution of FJ-B (Chemicon) that was prepared following the manufacturer's instructions. The stock solution was diluted by adding 0.1% acetic acid, resulting in a final dye concentration of 0.0004%. Slides were stained for 20 min and then rinsed in distilled water (3×1 min). The slides were then placed on a slide warmer (30°C) until they were fully dry. The dry slides were cleared by immersion in xylene (Merck) for 2 min before being coverslipped with DPX (Flucka).

Quantitative assessment of neuronal degeneration

Degenerating neurons were easily identified using a 20× and 40× objectives since both the A-Cu-Ag and FJ-B techniques only stain dying neurons. Quantitative assessment was carried out in a treatment-blind manner. As in previous studies (Bueno et al., 2003a, b; de Olmos et al., 2008, 2009) the precise layer location of degenerating neurons revealed by the A-Cu-Ag or FJ-B techniques were confirmed by counterstaining adjacent sections with neutral red or Hoechst, respectively. In animals that were infused either i.p. or i.c.v. neurodegeneration was analysed in the entire RSC and PLCo, and quantitative assessment was performed by counting the number of degenerating neurons in three sections at −6.7±0.2 mm and −2.8±0.2 mm for the RSC and the PLCo, respectively, because it accurately reflects the overall situation of these structures (Colbourne et al., 1999; de Olmos et al., 2008, 2009; Fix et al., 1995; Noguchi et al., 2005). In animals that received intra-RSC infusion of drugs neurodegeneration was also analysed in the entire RSC, allowing us to control the overall effect of the drug and to precisely locate the injection site. To assess neurotoxicity within and outside the radius of the diffusion of the drug, the number of degenerating neurons were scored in both hemispheres in three sections located near (at 0.7 mm) or far (3.5 mm) from the injection site.

GABA and glutamate determination

In each animal, vaginal desquamation was analysed daily to determine the day of the estrous cycle. Female rats in the first day of the diestrus were treated with a single i.p. dose of saline or 2.5 mg/kg of MK801 and decapitated 8 or 24 h later, which are time points that precedes the beginning and the plateau of neuronal degeneration, respectively (Bueno et al., 2003a; Fix et al., 1995; Horváth et al., 1997). The brains were quickly removed and placed on an ice cold brain matrix (RBM-40000; ASI Instruments, Houston, TX, USA) to dissected the olfactory bulbs at AP+5 mm from

bregma, and the posterior portion of the brain between −2.5 and −7,5 from bregma. The posterior portion of the brain was placed under the microscope in an ice cold glass to dissect the RSC, and a tissue portion containing the entire PLCo, a fraction of the piriform and entorhinal cortices, which for simplicity we designated as the PLCo-region. The RSC was dissected following the procedure described by Zhang et al. (1999). The PLCo-region was obtained by sequential cuts: (1) a sagittal cut to separate the hemispheres, (2) a horizontal cut at the level of the rhinal scissure, (3) the remaining tissue was dissected eliminating the diencephalon, the mesencephalon, the ventral hippocampus and the basolateral amygdala. Dissected tissue was rapidly preserved at −70 °C until use. Samples were homogenized mechanically with perchloric acid 0.1 M (10 μl of acid/mg of tissue) and were centrifuged at 30.000 × g during 5 min at 4 °C. Afterwards, 50 μl buffer borate 0.1 M, pH 10.4, 13 μl of 0.1% 2-mercaptoetanol in methanol, and 2.5 μl of 0.1% ortho-Phthalaldehyde (OPA) in methanol was added to 65 μl of supernatants. These homogenates were incubated at 37 °C for 10 min and, finally, 50 μl of derivatized solution was used to determine GABA and glutamate levels. GABA and glutamate levels were quantified by high pressure liquid chromatography (HPLC) (Waters 1515, Isocratic HPLC Pump) coupled to an electrochemical detector (Waters 2465) with an ODS column (150×4.6 mm, Symmetry; Waters Corp.). Data were obtained and processed by Empower Pro (Waters corporation. 2002). The mobile phase consisted of a solution (pH 3.5) containing 0.5 M acetic acid, 0.05 mM EDTA, 2 mM NaCl, 5% methanol and 12% acetonitrile. The electrode potential was fixed at 1000 mV against a working vitreous-carbon electrode. The sensitivity of electrochemical detection was adjusted to 50 nA. The column and detector were thermostatized at 45 °C. The mobile phase flow rate was 1.6 ml/min during the first 12 min and then was changed to 2.5 ml/min until the final step (40 min).

Statistical analysis

In each experiment between four to six animals per condition were used. Statistical comparisons of neuronal degeneration were performed by a two-way analysis of variance (ANOVA) (treatment×area or treatment×time, depending on experiment) using the software Statistica 6.0. Results showing significant overall changes were subjected to post hoc LSD Fisher tests with values of $P<0.05$ being considered statistically significant. In treatments performed i.p. or i.c.v., neuronal degeneration was expressed as percent of saline-treated animals (controls); in treatments performed intra-RSC neuronal degeneration was expressed as the percentage of the ipsi versus the contralateral site.

RESULTS

Effect of GABAergic drugs on MK801 induced neurodegeneration

In the initial series of experiments we tested whether the disruption of the inhibitory GABA-ergic neurotransmission mediates MK801-induced neurodegeneration in the RSC. Rats were treated with a single i.p. dose of 2.5 mg/kg MK801, which systematically induced moderate (but not maximal) neurodegeneration in several brain structures. After MK801, the animals were infused i.c.v. (lateral ventricle) with two applications (at 0 and 4 h post-MK801) of saline or 0.4 μg of the GABA-A agonist muscimol. This procedure prolonged the effect muscimol without the necessity of using higher anesthetic doses that, when combined with MK801, are lethal (data not shown). Two days after treatment the animals were sacrificed and MK801-induced irreversible neurotoxicity (cell death) was evaluated in the RSC and the PLCo, two cortical areas that are sensitive to MK801-toxicity. Animals that were treated with saline and muscimol showed no signs of neurodegeneration, and therefore were not included in further comparisons. In MK801/saline-treated (control) animals neuronal death was clearly observed in both RSC and PLCo. We found that argyrophilic degenerating neurites spread through most layers of both the RSC and the PLCo, while the somas of dying neurons were confined to layers IV-Va of RSC and II-III of PLCo, in agreement with several previous reports that showed that MK801-sensitive neuronal populations are restricted to these cortical layers (Bueno et al., 2003a, b; Horváth et al., 1997; Noguchi et al., 2005). Application of muscimol dramatically reduced neuronal degeneration in the RSC (Fig. 1A–D), but had no apparent protective effect in the PLCo (Fig. 1E, F). Quantitative assessment of degenerated somas in control and MK801/muscimol-treated animals confirmed a significant 90.6%± 2.7 protection in RSC but not in PLCo, in which a trend to an exacerbated toxicity was apparent (two way ANOVA: treatment [$F_{1,20}=17.35$ $P<0.0001$], brain structure [$F_{1,20}=12.34$ $P<0.01$] and treatment×brain structure [$F_{1,20}=13.04$ $P<0.01$]; followed by LSD Fisher, $P<0.0001$: muscimol vs. PBS in RSC) (Fig. 1G). To confirm and extend this observation, we analyzed the effect of thiopental, another GABA-A agonist previously shown to protect from MK801-toxicity (Jevtovic-Todorovic et al., 2001), by using a systemic application to ensure the cerebral distribution of the drug. After MK801, the animals received three i.p. injections (at 0 h 4 h and 8 h post MK801) of saline (control), or different doses of thiopental, to ensure a prolonged inhibition of GABA transmission avoiding the use of a single higher dose that would have anaesthetic effects. Thiopental inhibited MK801-toxicity in RSC in a dose-dependent manner. On the contrary, in PLCo, the low dose of thiopental increased MK801-toxicity while the high dose showed no significant effect (two way ANOVA: treatment [$F_{2,21}=14.42$ $P<0.001$], brain structure [$F_{1,21}=43.93$ $P<0.0000$] and treatment×brain structure [$F_{2,21}=13.33$ $P<0.001$]. LSD Fisher post hoc: $P<0.01$: thiopental 15 mg vs. saline in RSC; $P<0.0001$: thiopental 30 mg vs. saline in RSC; and $P<0.001$: thiopental 15 mg vs. saline in PLCo) (Fig. 1H).

We then analyzed the effect of local GABA-ergic neurotransmission on MK801-toxicity in RSC by the local infusion of muscimol near the callosal splenium. Animals were treated with MK801 and 30 min later were unilaterally infused intra-RSC with PBS (control) or different doses (10, 100 and 1000 ng) of muscimol. Neurotoxicity was evaluated by scoring the number of degenerated somas in the RSC in sections located near (AP −6.7 mm) or far (AP −2.5 mm) of the infusion site in both, ipsi and contralateral hemispheres (Fig. 2A, B). Infusion of muscimol reduced MK801-induced neuronal death near the infusion site, but did not have an appreciable effect in distant areas, or in the contralateral hemispheres (Fig. 2A, B). Quantitative analysis showed that infusion of PBS had no effect on MK801-toxicity, while 10 and 100 ng of muscimol significantly

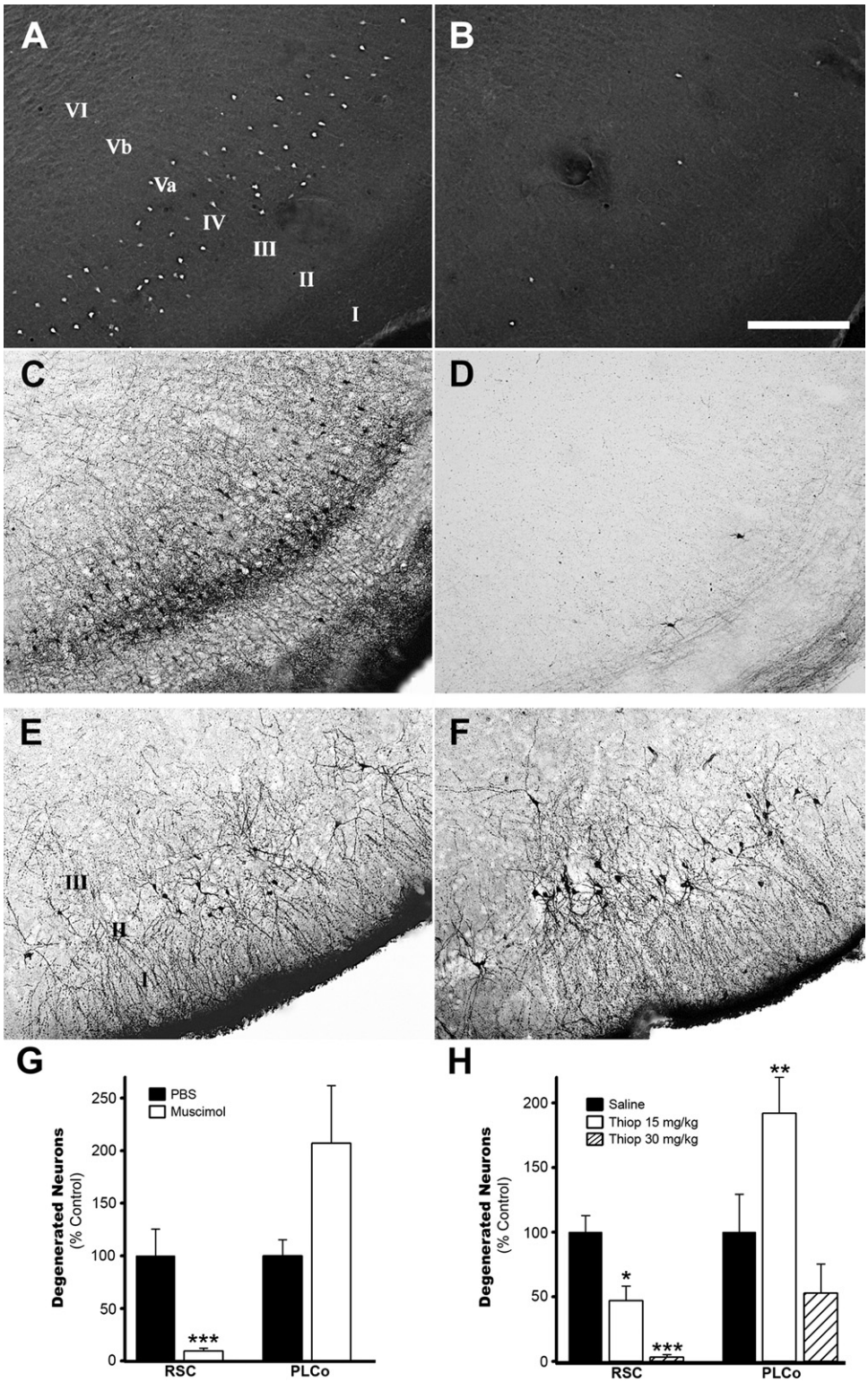

Fig. 1. GABA agonists protect RSC but not PLCo neurons from MK801-induced neurodegeneration. Animals were treated with an i.p. dose of MK801 (2.5 mg/kg), 30 min later were infused i.c.v. with saline (A, C, E, G) or 0.8 μg muscimol (B, D, F, G), or 15 or 30 mg/kg thiopental (H). Neuronal degeneration was analyzed with the FJ-B (A, B) and A-Cu-Ag techniques (C–F) in the RSC (A–D) and the PLCo (E, F). (A–F) Shown are representative coronal brain sections of animals treated with MK801 and saline or MK801 and muscimol. Cortical layers are indicated in roman numbers in (A) and (E). Scale bar 200 μm. (G) Effect of muscimol (white bars) on MK801-induced neurodegeneration. Degeneration is expressed as percent of saline (control, black bars) *** $P<0.0001$ versus saline (control). (H) Effect of thiopental, 15 mg/kg (white bars) and 30 mg/kg (striped bars) * $P<0.01$, ** $P<0.001$, *** $P<0.0001$.

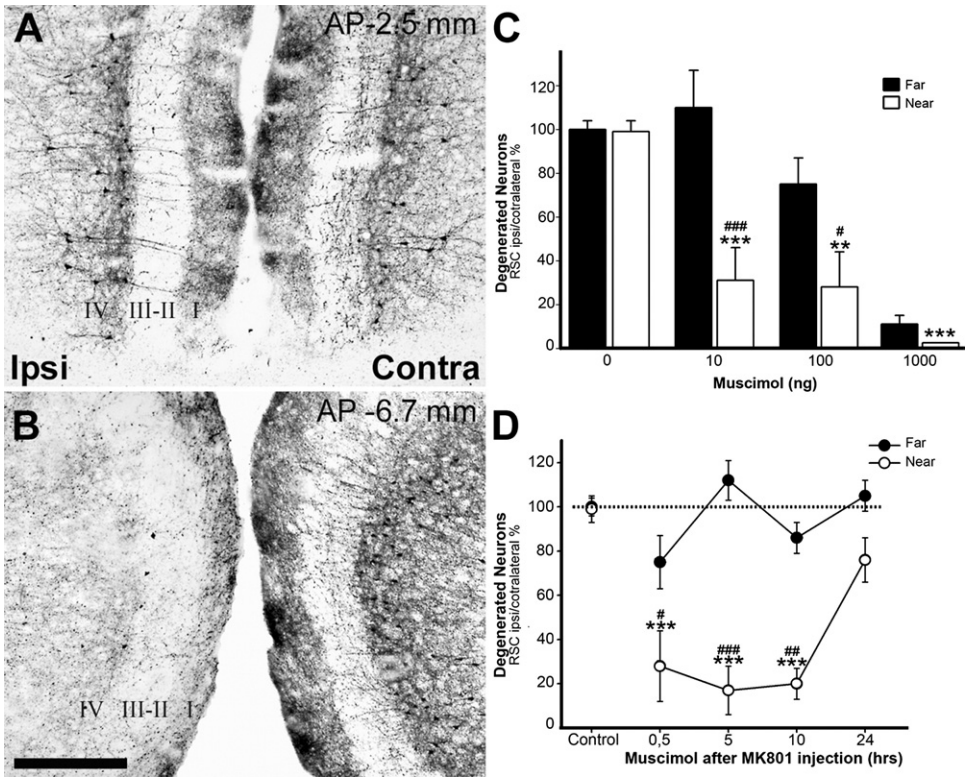

Fig. 2. Local infusion of muscimol protects RSC neurons from MK801-induced toxicity. Rats were treated with MK801 (2.5 mg/kg), and infused unilaterally in the RSC with vehicle or different doses of muscimol. (A, B) Infusion of muscimol locally inhibits MK801-tocixity. Shown are coronal sections of the RSC at different anteroposterior levels (AP −2.5 and −6.7 mm) corresponding to an animal infused unilaterally with 10 ng of muscimol 30 min after MK801. Cortical layers are indicated with roman numbers. Scale bar 200 μm. (C) Dose-response of muscimol on MK801-toxicity in the RSC. Degenerated neurons were expressed as the percentage of the ipsi versus contralateral site. ** $P<0.001$, *** $P<0.0001$ versus saline (control, muscimol 0); $^{\#}$ $P<0.05$, $^{\#\#\#}$ $P<0.0001$ versus the respective far injection-site. (D) Time interval of muscimol protection against MK801-induced toxicity in the RSC. Muscimol (100 ng) was unilaterally infused in the RSC at different times after MK801. *** $P<0.0001$ versus saline (control); $^{\#}$ $P<0.01$ $^{\#\#}$ $P<0.001$ $^{\#\#\#}$ $P<0.0001$ versus the respective far injection-site.

protected RSC neurons only in areas near the infusion site. However protection induced by 1 μg of muscimol reached also to distant areas (two way ANOVA: treatment [$F_{3,18}=40.97$ $P<0.0000$], distance [$F_{1,18}=21.61$ $P<0.001$], and treatment×distance [$F_{3,18}=6.34$ $P<0.01$]. LSD Fisher post hoc between near scores $P<0.0001$: muscimol at 0 vs 10 and 1000 ng; $P<0.001$: muscimol at 100 ng; and when compared near vs. far scores $P<0.0001$: muscimol at 10 ng; $P<0.01$: muscimol at 100 ng) (Fig. 2C).

To analyze the protection-interval, animals were treated i.p. with MK801 and 0.5, 5, 10 or 24 h later were unilaterally infused intra-RSC with a single dose of 100 ng muscimol, and degenerated neurons in the RSC were scored. Muscimol significantly reduced neurodegeneration near the infusion site up to 10 h after MK801-treatment, but was ineffective after 24 h (two way ANOVA: time [$F_{4,20}=11.55$ $P<0.0000$], distance [$F_{1,20}=45.45$ $P<0.0000$] and time×distance [$F_{4,20}=6.92$ $P<0.01$]. LSD Fisher post hoc between near scores: $P<0.0001$: control vs. muscimol at 0.5, 5 and 10 h; and when compared near vs. far scores $P<0.0001$ at 0.5 h, $P<0.01$ at 5 h and $P<0.001$ at 10 h) (Fig. 2D). This observation opens the possibility that MK801 promoted a local down-regulation of GABA-ergic neurotransmission causing degeneration of RSC neurons. Therefore, we analyzed the effect of the GABA-A antagonist picrotoxin. We first observed that i.p. application of picrotoxin induced seizures and eventually the death of the animal, but the intra-RSC infusion (1200 ng) alone neither induced seizures nor promoted toxicity (data not shown). Afterwards rats were treated with MK801 and 30 min later were unilaterally infused intra-RSC with saline (control) or picrotoxin (300 and 1200 ng). Results showed that MK801-toxicity was not significantly affected even when 1.2 μg picrotoxin was applied (Fig. 3).

Levels of GABA and glutamate in MK801-treated animals

To further explore the potential involvement of the GABA-ergic and glutamatergic neurotransmission on MK801 toxicity we measured the levels of GABA and glutamate in the RSC, the PLCo-region (a portion of tissue containing the entire PLCo, and a fraction of the piriform and entorhinal cortices), and the olfactory bulb (included as an additional control area). Rats were injected i.p. with saline (control) or MK801, and the levels of GABA and glutamate were determined by HPLC at 8 and 24 h post-treatment, because these time points precedes the beginning and the plateau

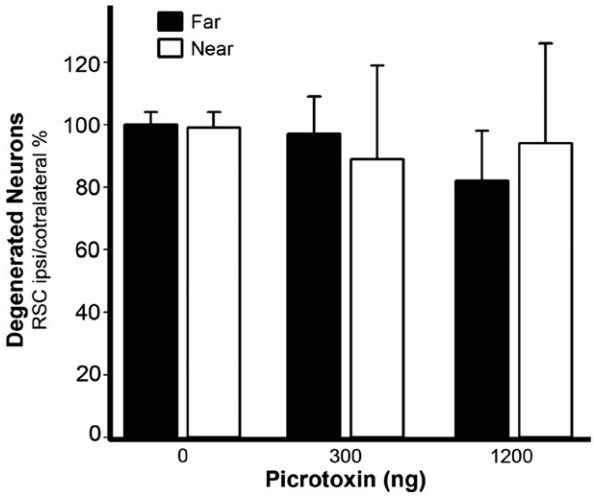

Fig. 3. MK801-induced neurotoxicity in RSC is not altered by local infusion of picrotoxin. Rats were treated with MK801 (2.5 mg/kg) and 30 min later infused intra-RSC with picrotoxin (300 and 1200 ng) or saline. Degenerated neurons in the RSC were scored near (−6.7 bregma) and far (−2.5 bregma) from the injection site and expressed as the percentage of ipsi versus contralateral side.

of neuronal degeneration, respectively (Bueno et al., 2003a; Fix et al., 1995; Horváth et al., 1997). The data showed that MK801-treatment did not significantly alter the levels of GABA in these structures (Fig. 4). On the contrary, after 8 h of MK801-treatment, glutamate levels in the RSC were significantly decreased to approximately 84% of saline (one way ANOVA: $[F_{2,21}=3.72\ P<0.05]$, followed by LSD Fisher, $P<0.05$: saline vs. MK801 at 8 h) and 73.3%±6.11 in the PLCo-region (one way ANOVA: $[F_{2,21}=3.57\ P<0.05]$, followed by LSD Fisher, $P<0.05$: saline vs. MK801 at 8 h), but not in the olfactory bulb (Fig. 4), suggesting that MK801-treatment alters local glutamatergic neurotransmission in the RSC. After 24 h of MK801 the levels of glutamate were not significantly different to control animals.

Effect of glutamatergic drugs on MK801 induced neurodegeneration

In the next series of experiments we tested whether an altered glutamatergic neurotransmission mediates MK801-induced neurodegeneration in the RSC. We initially analysed if the direct blockade of NMDA receptors caused neuronal degeneration in the RSC. Animals were treated i.p. with MK801 and 0.5, 5, 10, and 24 h later were unilaterally infused intra-RSC with a single dose of 3 μg MK801. Quantitative analysis showed that application of MK801 in the RSC at 0.5, 10 or 24 h had no significant effect while a modest but significant protection near the infusion site was observed when MK801 was infused after 5 h (two way ANOVA: time $[F_{4,24}=2.92\ P<0.05]$ and time×distance $[F_{4,24}=2.89\ P<0.05]$. LSD Fisher post hoc between near scores, and near vs. far scores $P<0.01$ control vs. MK801 at 5 h) (Fig. 5).

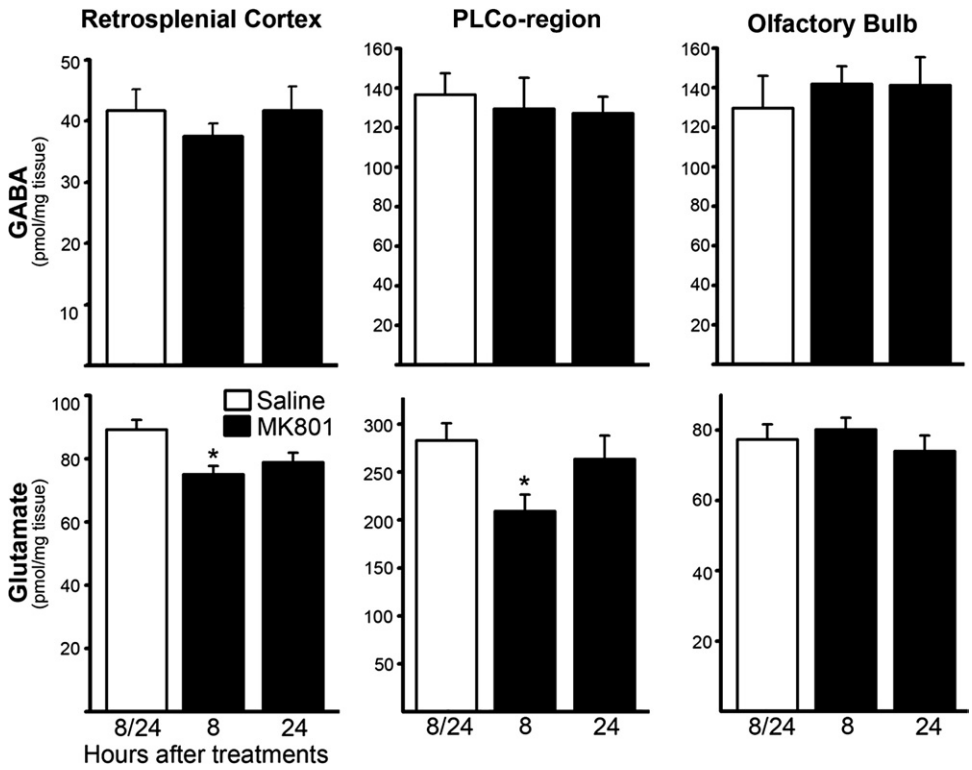

Fig. 4. Treatment with MK801 alters the levels of glutamate in RSC. Rats were treated with saline (white bars) or 2.5 mg/kg of MK801 (black bars), 8 or 24 h after treatment the RSC, the PLCo-region (which contains the PLCo and a portion of the piriform and entorhinal cortices) and the olfactory bulb were dissected and the levels of GABA and glutamate were determined in each sample by HPLC. * $P<0.05$ versus control.

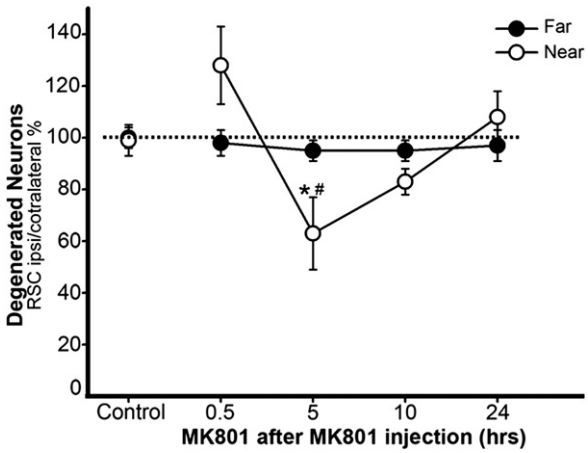

Fig. 5. Local application of MK801 did not enhance neurotoxicity induced by i.p. treatment with MK801. Rats were treated i.p. with MK801 (2.5 mg/kg) and later unilaterally infused with 3 μg MK801 in the RSC at different times. Neurotoxicity was evaluated near (AP −6.7) and far (AP −2.5) of the infusion site, and expressed as the percentage of ipsi versus contralateral site. * $P<0.01$ versus control; # $P<0.01$ versus the respective far injection-site.

Afterward we analysed the effect of the AMPA/kainate antagonist DNQX on MK801-toxicity. Rats were treated with MK801 and thereafter infused i.c.v. with three applications (at 0 h 4 h and 8 h post MK801) of vehicle or 15 μg DNQX, and MK801-induced neurotoxicity was evaluated in the RSC and the PLCo. Application of DNQX significantly reduced neuronal degeneration in the RSC to 30%±11.82, but had no protective effect in the PLCo (two way ANOVA: treatment [$F_{1,18}=10.82$ $P<0.01$], brain structure [$F_{1,18}=22.71$ $P<0.001$] and treatment×brain structure [$F_{1,18}=8.51$ $P<0.01$]; followed by LSD Fisher post hoc, $P<0.001$: DNQX vs. DMSO in RSC) (Fig. 6A–C).

We further analysed the effect of local glutamatergic neurotransmission on MK801-toxicity by the single intra-RSC application of different doses of DNQX (0.5, 5 and 10 μg). Quantitative analysis showed that infusion of DNQX reduced MK801-induced neuronal death near the infusion site, but not in distant areas (two way ANOVA: treatment [$F_{3,15}=40.97$ $P<0.001$], distance [$F_{1,15}=21.61$ $P<0.0000$] and treatment×distance [$F_{3,15}=6.34$ $P<0.001$]. LSD Fisher post hoc between near scores, $P<0.0001$: DNQX at 0 vs 0.5, 5 and 10 μg; when compared near vs. far scores, $P<0.0001$ at 0.5 μg, $P<0.01$ at 5 μg, and $P<0.001$ at 10 μg) (Fig. 6D).

To analyse the protection-interval, we analysed the effect of a single intra-RSC application of 5 μg of DNQX at different time after an i.p. injection of MK801. DNQX almost completely abrogated degeneration near the infusion site even 10 h after MK801-treatment, but was ineffective after 24 h (two way ANOVA: time [$F_{4,17}=10.73$ $P<0.001$], distance [$F_{1,17}=61.72$ $P<0.0000$] and time×distance [$F_{4,17}=11.79$ $P<0.0001$]. LSD Fisher post hoc between near scores: $P<0.0001$: control vs. DNQX at 0.5, 5 and 10 h; and when compared near vs. far scores: $P<0.01$ at 0.5 h, $P<0.0001$ at 5 and 10 h) (Fig. 7A).

The local effect of NASPM, a selective antagonist of the Ca^{2+}-permeable AMPA/kainate receptors, was also evaluated at various times after MK801-treatment. Application of NASPM short-time after MK801 had no appreciable effect, while at 5 and 10 h its protective effect near the infusion site was significant (two way ANOVA: time [$F_{4,24}=4.64$ $P<0.01$], distance [$F_{1,24}=7.79$ $P<0.05$] and time×distance [$F_{4,24}=5.96$ $P<0.01$]. LSD Fisher post hoc between near scores, and near vs. far scores, $P<0.001$: control vs. NASPM at 5 h and $P<0.0001$ at 10 h) (Fig. 7B). Collectively, these experiments suggest that excitotoxic damage of RSC neurons induced by MK801 involves the activation of different glutamatergic receptor-subtypes, mainly the AMPA/kainate.

To further test if the local activation of AMPA/kainate receptors in the RSC is sufficient to cause the selective degeneration of layers IV-Va neurons, 2.5 or 5 nmol of AMPA or kainic acid were unilaterally infused in the RSC and toxicity was analysed 48 h later. Application of AMPA promoted a sparse neuronal death affecting neurons on layers IV-Va and deeper layers, but preserving those on layers II-III, resulting in a pattern of neurodegeneration that partially overlapped MK801-toxicity (Fig. 8A, B). Kainic acid-induced neuritic and terminal degeneration was conspicuous, and affected layers IV, V-ab, VI and I, while layers II-III were notably free of degeneration except for some fibres that emerged from layer IV and crossed to reach layer I depicting dome-like structures. Although more conspicuous, this pattern is reminiscent to that induced by MK801 (Fig. 8D, E). In addition, somatic neurodegeneration induced by kainic acid mainly affected layers IV-Va, but largely preserved neurons on layers II-III, and deeper layers of the RSC (Fig. 8A, C, see also Suppl. Fig.). Collectively, these observations suggest that neuronal populations of the RSC that are sensitive to MK801 toxicity are also vulnerable to excitotoxic damage induced by AMPA/kainate agonists.

DISCUSSION

In this study we provide evidence that an i.p. application of MK801 induces neuronal death of RSC neurons by a mechanism that is largely dependent on AMPA/kainate-receptor mediated neurotoxicity, which is consistent with the excitotoxic mechanism previously proposed (Olney et al., 1999). We found that local infusion of different AMPA/kainate antagonists effectively inhibited MK801-induced neuronal death. In addition, the intra-RSC application of AMPA and kainic promoted degeneration of layer IV-Va neurons, which are the ones affected by MK801-toxicity, indicating that these neurons are vulnerable to AMPA/kainate excitotoxicity. Importantly, intra-RSC infusion of MK801 did not enhance toxicity induced by systemic application of MK801 neurodegeneration of RSC neurons, indicating that death of RSC neurons was not caused by the blockade of NMDA receptors in the RSC. This interpretation is reinforced by previous studies showing that the infusion of MK801 in the RSC also failed to trigger HSP70 induction and neuronal vacuolization (Tomitaka et al.,

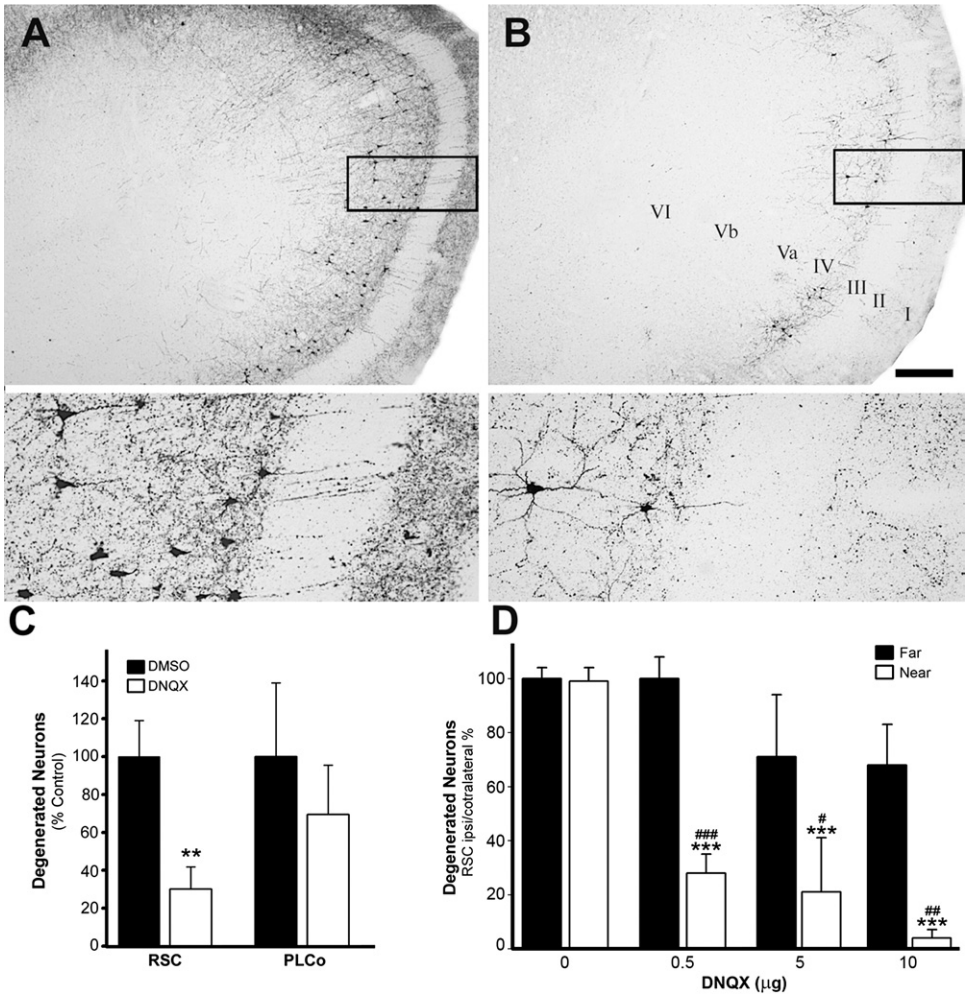

Fig. 6. DNQX protects RSC but not PLCo neurons from MK801-induced neurodegeneration. Rats were treated with MK801 (2.5 mg/kg) and i.c.v. with vehicle (DMSO) (A) or DNQX (B) and neurodegeneration in the RSC and the PLCo analyzed. (A, B), Shown are representative coronal section through the RSC of animals treated with MK801/vehicle (A) or MK801/DNQX (B). Depicted areas are enlarged at the bottom. Cortical layers are indicated in roman numbers in B. Scale bar 200 μm. (C) Effect of DNQX on MK801-induced neurodegeneration. Degeneration was expressed as percent of vehicle (control, black bars). ** $P<0.001$ versus vehicle (DMSO, control). (D) Dose-response of local application of DNQX on MK801-induced neurodegeneration in the RSC. Degeneration was expressed as the percentage of the ipsi versus contralateral site. *** $P<0.0001$ versus vehicle (DMSO; DNQX 0); # $P<0.01$ ## $P<0.001$ ### $P<0.0001$.

2000b; Farber et al., 2002), which are signs of reversible stress typically elicited by i.p. treatment with low doses of NMDA-A (Olney and Farber, 1995; Sharp et al., 2001). Together, these observations strongly indicate that both transient and permanent neurotoxic effects of NMDA-A must depend on the blockade of NMDA receptors in neuronal populations located outside the RSC. This interpretation adds further support to the hypothesis proposed by Olney, in which the blockade of NMDA receptors in inhibitory neurons enhances the excitatory output from brain regions that project to RSC (such as the anterior thalamus), triggering excitotoxic stress in selected neuronal subpopulations of the RSC (Olney et al., 1999).

We observed that intra-RSC application of the AMPA/kainate receptor-antagonists (DNQX and to a lesser extent NASPM) almost completely abrogated MK801 toxicity. Similarly, it was previously shown that intra-RSC infusion of NBQX substantially reduced the vacuolization produced by low doses of MK801 (Farber et al., 2002). Concomitantly, the intra-RSC infusion of AMPA/kainate receptor-agonists kainic acid and, to a lesser extent, AMPA promoted neuronal death of layers IV-Va neurons in the RSC in a pattern that was reminiscent of MK801-induced death, indicating that over-stimulation of AMPA/kainate receptors in the RSC would be sufficient to trigger neuronal death. Curiously, the intra-RSC infusion of kainic acid alone did not induce vacuolization of RSC neurons, which was readily produced by the simultaneous infusion of the cholinergic agonist carbachol together with low doses of kainic acid. Moreover, the muscarinic antagonist scopolamine strongly prevented vacuolization in the RSC (Farber et al., 2002), and the destruction of cholinergic populations that project to RSC reduced reversible toxicity but had no impact on irreversible toxicity induced by MK801 (Willis et al., 2006). These observations suggest that enhanced cholinergic neurotransmission is involved in reversible neurotox-

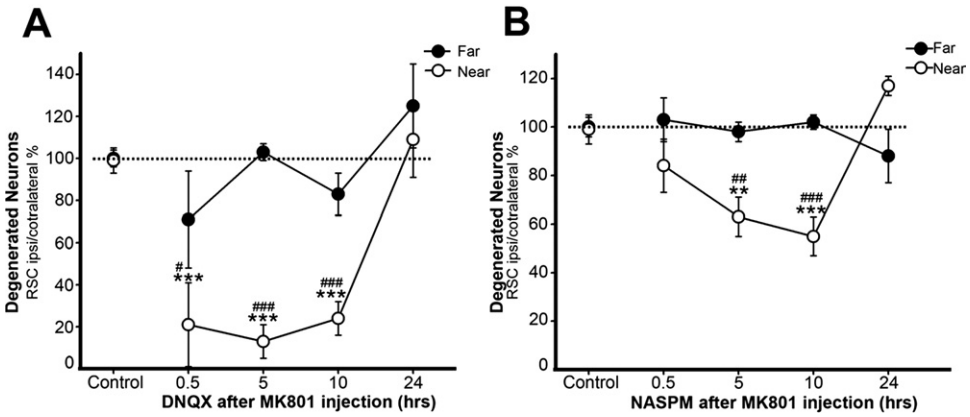

Fig. 7. Protection interval of local application of AMPA/kainate antagonists on MK801-induced neurodegeneration in the RSC. Rats were treated with MK801 (2.5 mg/kg) and after different times glutamatergic antagonists were unilaterally infused in the RSC. Neurotoxicity was expressed as the percentage of degenerated neurons in the ipsi versus contralateral site. (A) Protection interval of 5 μg DNQX. *** $P<0.0001$ versus control; # $P<0.01$, ### $P<0.0001$ versus the respective far injection-site. (B) Protection interval of 10 μg NASPM. ** $P<0.001$ *** $P<0.0001$ versus control; ## $P<0.001$ ### $P<0.0001$ versus the respective far injection-site.

icity but might not significantly participate in neuronal death induced by NMDA-A. Although a comprehensive analysis of the cholinergic neurotransmission in the RSC after higher doses of MK801 is required to completely rule out its potential participation in neuronal death, the evidence presented above suggests that vacuolization and neuronal death are events that occur by different mechanisms. In fact, neuronal vacuolization is transiently elicited soon after the application of low doses of MK801, and is reduced by estrogens (Dribben et al., 2003), while neuronal death requires higher doses of the NMDA antagonist, becomes evident several hours after treatment, and is enhanced by estrogens (de Olmos et al., 2008). These findings further suggest that the reversible stress (vacuolization) and the permanent damage (neuronal death) induced by low and high doses of MK801, respectively, occur by different mechanisms. Thus, it is likely that the imbalance of excitatory/inhibitory neurotransmission induced by low doses of NMDA-antagonists might be quantitatively and qualitatively different to that elicited by higher doses.

Interestingly, DNQX effectively protected neuronal death even when applied several hours after MK801, sug-

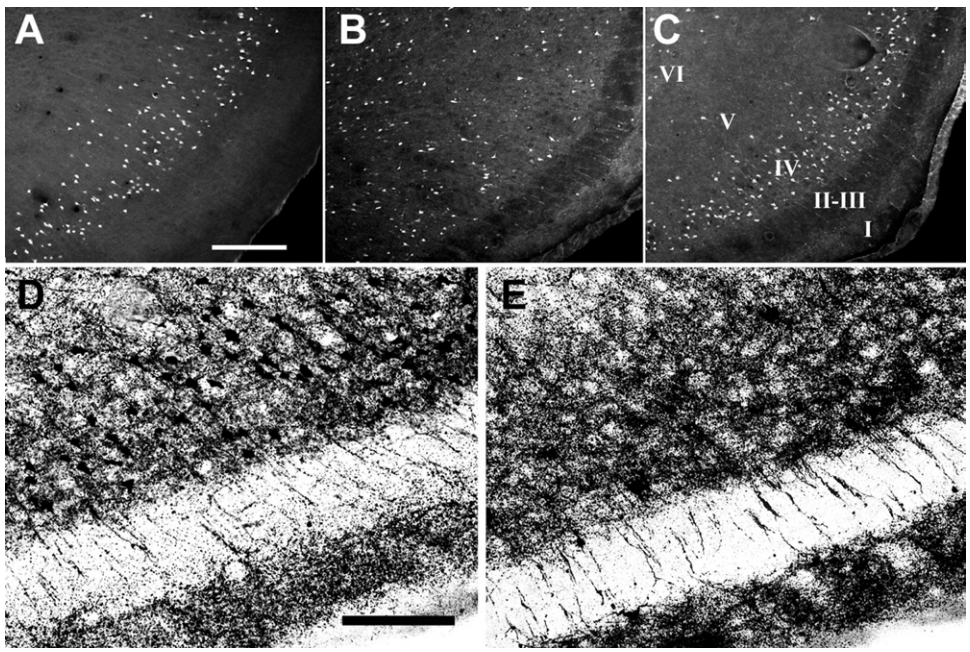

Fig. 8. Comparative analysis of neuronal degeneration in the RSC induced by the i.p. application of MK801 or the local infusion of AMPA/kainate agonists. Rats were treated with a single i.p. dose of 5 mg/kg MK801 (A, D) or unilaterally infused in the RSC with a single dose of 2.5 nmol AMPA (B) or 2.5 nmol kainic acid (C, E). Neurotoxicity was evaluated 2 days later with the FJ-B (A–C) and A-Cu-Ag (D, E) techniques. Note the similarities in the pattern of neurodegeneration induced by MK801 and kainic acid. Cortical layers are indicated in roman numbers in C. Scale bar 200 μm (A–C) and 100 μm (D, E).

gesting that an enhanced AMPA/kainate glutamatergic neurotransmission in the RSC remains long after the i.p. application of the NMDA-A. We found that the levels of glutamate in the RSC and the PLCo-region, but not the olfactory bulb, were significantly reduced 8 h after MK801-treatment, a time point when signs of neuronal death begins. Previously, by using microdialysis it was reported an enhanced release of glutamate in the RSC soon after low doses of MK801 (Noguchi et al., 1998). Thus, it is tempting to speculate that the reduced levels of glutamate reflect a partial depletion of the neurotransmitter as a result of its enhanced intra-RSC liberation due to MK801-treatment. For example, in an experimental model of hypoxia-ischemia an increased liberation of glutamate was followed by a significant depletion of the neurotransmitter at later times (Benveniste et al., 1984; Obrenovitch and Urenjak, 1997). Nevertheless, further experiments will be required to directly address whether in our experimental conditions the reduced glutamate levels in the RSC is directly caused by its enhanced and prolonged release due to MK801 treatment.

We were unable to detect significant changes in the levels of GABA in the RSC after MK801, which could suggest that GABA-ergic neurotransmission in the RSC was not dramatically affected by the i.p. injection of the NMDA-A. Previous reports (Jevtovic-Todorovic et al., 2001; Farber et al., 2003) and our data show that GABA-A agonists strongly prevented MK801-induced both vacuolization and cell death in the RSC. These data per se do not indicate that MK801-toxicity is due to reduced GABA-ergic neurotransmission in the RSC, since GABA receptor-agonist will induce an inhibitory response (hiperpolarization) even in the absence of a basal or tonic endogenous GABA-ergic activity. To show that a reduction in GABA-ergic neurotransmission mediates MK801 toxicity it must be shown that blocking GABA receptors enhances toxicity. We found that local application of picrotoxin alone (without MK801) in the RSC did not induce neurodegeneration, suggesting that the survival of RSC neurons is not affected by the blockade of endogenous GABA transmission. Moreover, the intra-RSC application of the picrotoxin did not enhance MK801 toxicity, showing that the contribution of endogenous GABA-ergic neurotransmission to MK801-induced neuronal death is negligible. Thus, the most straightforward interpretation to these observations is that GABA agonists effectively protect RSC neurons from MK801-toxicity, because they reduce neuronal excitability of RSC neurons counteracting the excitotoxic damage induced by enhanced AMPA/kainate activity.

The fact that GABA agonists effectively protect from MK801-toxicity even when administered several hours after the NMDA-A, provide rational support for the potential benefit of using GABA agonists in conditions that require the clinical use of NMDA-A or for the treatment of individuals affected by an intoxication with NMDA-A (Petersen and Stillman, 1978; Domino et al., 1984; Sinner and Graf, 2008; Strayer and Nelson, 2008). However, our study indicates that a more detailed analysis in other brain structures (e.g., the PLCo) will be required to exclude potential deleterious actions of the combined effect of NMDA-A and GABA agonists.

Increasing evidence indicates that the mechanism of neuronal death is not the same for all neuronal populations that are sensitive to the toxic effect of NMDA-A. It was shown that neuronal death induced by NMDA-A in the piriform cortex is apoptotic (Zhou et al., 2007), while excitotoxic neuronal death occurs in both, the RSC and the PLCo (Bueno et al., 2003a, b). Moreover, we found that GABA-A agonists or AMPA/kainate antagonists abrogate MK801-toxicity in the RSC, but the same treatments were ineffective in the PLCo. On the contrary, haloperidol significantly inhibited MK801 toxicity in the PLCo, but had no effect in the RSC (Bender and Lorenzo, unpublished data). Thus, even the excitotoxic mechanism of death appears to be distinct for neuronal subpopulations in different brain structures that are sensitive to MK801 toxicity. Collectively, these observations further support the interpretation that MK801 is not toxic per se, but rather initiates toxicity by a mechanism that involves altered neurotransmission that damages different neuronal populations according to their particular vulnerability. The molecular determinants that characterize each neuronal population and confer selective vulnerability to a particular insult remain as a conundrum in neurobiology. In this regard, we showed that infusion of kainic acid in the RSC promoted neuronal death that was restricted to layer IV-Va neurons, demonstrating its particular vulnerability to AMPA/kainate excitotoxicity. It is interesting to note that we have recently reported that i.p. application of toxic doses of MK801 induced the selective inhibition of the expression of the early growth response 1 (EGR-1, also known as zif 268, Krox-24, NGFI-A) in neurons of layer IV-Va in the RSC, but not in any other cortical structure analyzed (de Olmos et al., 2009). This observation suggests that a particular mechanism of control of EGR-1 expression operates in these neurons. Therefore, addressing if EGR-1 expression in layer IV-Va neurons in the RSC is controlled by AMPA/kainate receptor-activity, and whether the expression of EGR-1 and down-stream proteins participate in the control of the viability of this neuronal population could lead to the elucidation of the molecular particularities of these neurons that make them sensible to NMDA-A. Moreover, considering that EGR-1 has been implicated in the molecular mechanism of memory reconsolidation, (Lee et al., 2004; Lee, 2008) it will be important to explore whether the psychotic syndromes induced by NMDA-A are linked to altered AMPA/kainate neurotransmission and EGR-1 expression in RSC neurons.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2010.05.007.

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