Expression of parvalbumin and glutamic acid decarboxylase-67 after acute administration of MK-801. Implications for the NMDA hypofunction model of schizophrenia

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Abstract

Rationale A reduction of GABAergic markers in postmortem tissue is consistently found in schizophrenia. This is generally mediated by a decreased expression of the calcium-binding protein, parvalbumin (PV), and the 67-kDa isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD_{67}). Similar reductions of PV or GAD_{67} are observed after repeated exposure to *N*-methyl-D-aspartate (NMDA) receptor antagonists but less attention has been paid to what occurs after their acute administration. *Objectives* Here, we have used in situ hybridization to examine the expression of PV and GAD_{67} mRNAs at 4 h and 24 h after an acute administration of MK-801 (1 mg/kg). *Results* Four hours after MK-801, the expression of PV mRNA decreased only in dentate gyrus of the hippocampus. Twenty four hours after this treatment, a reduction of the levels of PV mRNA was found in the medial prefrontal, orbitofrontal and entorhinal cortices, hippocampus and the basolateral nucleus of the amygdala. In contrast, no changes in the expression of GAD_{67} were observed in any of the brain regions examined. Interestingly, the reduction in PV mRNA expression is observed in discrete corticolimbic subregions that have been implicated in schizophrenia, which is coincident with changes observed in postmortem tissue of schizophrenia brain. *Conclusions* These findings indicate that acute administration of a NMDA antagonist delineate a pattern of changes in GABAergic markers different from those observed in postmortem tissue in schizophrenia inasmuch as only deficits in parvalbumin (but not GAD_{67}) were seen.

Keywords

MK-801, NMDA antagonism, Animal Model, Schizophrenia, Prefrontal cortex, Hippocampus, Parvalbumin, GAD_{67}

Introduction

One of the most compelling findings in schizophrenia is a reduction of GABAergic markers in postmortem tissue. Reduced levels of the mRNA for the 67-kDa isoform of glutamic acid decarboxylase (GAD_{67}), the primary synthesizing enzyme for GABA, have been consistently found in neocortical regions of subjects with schizophrenia (Volk et al. 2000; Vawter et al. 2002; Hashimoto et al. 2003; Woo et al. 2004; Akbarian and Huang 2006). Although both GAD_{65} and GAD_{67} isoforms coexist in almost all GABAergic neurons in the brain (Esclapez et al. 1994), only exiguous changes in the expression of GAD_{65} have been found in schizophrenic patients (Todtenkopf and Benes 1998). These changes do not appear to be generalized, but circumscribed to a subset of GABAergic interneurons containing the calcium-binding protein, parvalbumin (PV), i.e. basket and chandelier cells, that endow perisomatic and axo-axonic innervation of pyramidal neurons, respectively (Markram et al. 2004; Lewis et al. 2005). Indeed, neocortical expression of PV is found also to be reduced in individuals with schizophrenia (Benes and Beretta 2001; Beasley et al. 2002; Hashimoto et al. 2003; Lewis et al. 2005). PV interneurons regulate the oscillatory activity in the cerebral cortex (Whittington and Traub 2003; Bartos et al. 2007; Sohal et al. 2009), and disruption of cortical oscillatory dynamics is postulated to underlie many of the neurocognitive deficits of schizophrenia (Cho et al. 2005; Gonzalez-Burgos and Lewis 2008).

In the animal setting, it is generally considered that sustained exposure to NMDA receptor antagonists is needed to produce a robust decrease of the expression of PV in rodents and monkey (Cochran et al. 2003; Rujescu et al. 2006; Braun et al. 2007; Morrow et al. 2007). Acute administration of NMDA receptor antagonists including PCP, ketamine and MK-801 is also used to recapitulate the symptoms of schizophrenia in behavioral assays (Swerdlow et al. 1994; Corbett et al. 1995; Abekawa et al. 2003; Jackson et al. 2004; López-Gil et al. 2007). For this reason it was deemed of importance to determine whether such treatment could also produce pathological alterations similar to those occurring in schizophrenia. In the present study, we have addressed this

issue by measuring the expression of PV and GAD_{67} mRNAs in brain regions presumed to be involved in schizophrenia, at 4 h and 24 h after a single injection of MK-801.

Materials and Methods

Animals and Treatment

Adult male Wistar rats (Charles River Laboratories, Cerdanyola del Vallès, Spain) weighing 275–300 g were used. Animals were maintained on a 12 h light/dark cycle (lights on 07:00) with unrestricted access to food and water. All experimental procedures followed the European Communities Council Directive of November 24, 1986 (86/609/EEC), and were approved by the Institutional Animal Care and Use Committee. MK-801 (Sigma-Aldrich, Tres Cantos, Spain) was dissolved in saline for intraperitoneal (i.p.) administration. The dose of MK-801 (1 mg/kg) was chosen from previous studies that showed robust increase in cortical glutamate release (López-Gil et al. 2007) and injury to pyramidal neurons (Sharp et al. 2001), alterations that are considered to occur in schizophrenia.

Hybridization probes

GABAergic cells were identified by the presence of the GABA synthesizing enzyme, GAD_{67}. The oligonucleotides used were complementary to the bases 1600-1653 (GenBank Accession No NM_017007). PV cells were identified by hybridization with oligonucleotides complementary to the bases 115-155 (GenBank Accession No NM_022499) of PV mRNA. All oligonucleotides were synthesized and purified with HPLC by Isogen Bioscience BV (De Meern, The Netherlands). Each oligonucleotide was labeled at their 3'-end with [^{33}P]α-dATP

(3000 Ci/mmol, New England Nuclear, Boston, MA) and terminal deoxynucleotidyltransferase (Calbiochem, San Diego, CA). Labeled probes were purified through ProbeQuant G-50 microcolumns (GE Healthcare, Little Chalfont, UK). The specificity of the autoradiographic signal obtained in the in situ hybridization histochemistry experiments was confirmed by performing a series of routine controls (Pompeiano et al. 1992). Briefly, for each of the mRNA under study, several oligonucleotide probes complementary to different regions of the same mRNA were used independently as hybridization probes in consecutive tissue sections showing identical pattern of hybridization. For a given oligonucleotide, the addition of an excess of the same unlabeled oligonucleotide to the hybridization solution resulted in the complete abolition of the specific hybridization signal. The thermal stability of the hybrids was also examined by washing at increasing temperatures, which resulted in a sharp decrease in the hybridization signal at a temperature consistent with the Tm of the hybrids.

In Situ Hybridization Histochemistry Procedures

Rats were killed by decapitation at 4 h and 24 h after an intraperitoneal injection of MK-801 (1 mg/kg). The brains were then rapidly removed, frozen on dry ice, and stored at –80 ℃. Tissue sections, 14-µm thick, were cut using a cryostat (HM500 OM; Microm, Walldorf, Germany), thaw mounted onto 3-aminopropyltriethoxysilane (Sigma-Aldrich, Tres Cantos, Spain) coated slides, and kept at –20℃ until use. The protocols for in situ hybridization histochemistry were based on previously described procedures (Tomiyama et al. 1997). Frozen tissues were brought to room temperature, air-dried and fixed for 20 min in 4% paraformaldehyde in phosphate-buffered saline (PBS: 2.6 mM KCl, 1.4 mM KH_2PO_4, 136 mM NaCl, 8 mM Na_2HPO_4). They were then washed once in 3·x PBS, twice in 1·x phosphate-buffered saline (PBS), 5 min each, and incubated in a freshly prepared solution of predigested pronase (Calbiochem, San Diego, CA) at a final concentration of 12 U/mL in 50 mM Tris-HCl pH 7.5 and 5 mM EDTA for 2 min at 20℃. Proteolytic activity was stopped by

immersion for 30 s in 2 mg/mL glycine in 1xPBS. Tissues were rinsed in 1 x PBS and dehydrated in 70% and 100% ethanol for 2 min each. For hybridization, the radioactive probes were diluted in hybridization buffer (50% formamide, 4·x SSC, 1·x Denhardt's solution, 1% sarkosyl, 10% dextran sulfate, 20 mM phosphate buffer, pH 7, 250 µg/mL yeast tRNA and 500 µg/mL salmon sperm DNA) at 1–2 x 10^4 cpm/µL. Tissues were incubated in humid boxes overnight at 42 ℃ and then washed four times (45 min each) in 600 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA at 60℃. Hybridized sections were exposed to Biomax–MR (Kodak, Rochester, NY) films for 3-15 days at -70℃ with intensifying screens.

Analysis of the Results

The relative optical density (ROD) of PV and GAD_{67} mRNA labelings in different brain areas was measured by densitometry of developed films using the computerized image-analysis system AIS (Imaging Research Inc. St. Catharines, Ontario, Canada). ROD measurements were performed in triplicate and the values are expressed as mean ± SEM of six animals per group. Differences between saline- and MK-801-injected animals in the expression of PV and GAD_{67} in each of the brain structures examined were tested by unpaired Student's *t*-test (two-tailed) followed by the Bonferroni correction for multiple comparisons. $P < 0.05$ was considered statistically significant.

Preparation of the figures

Photographs of the film autoradiograms of the hybridized tissue sections were taken with a Wild 420 Leica microscope equipped with a digital camera (DXM1200 F, Nikon, Tokyo, Japan) and ACT-1 Nikon Software. Figures were prepared for publication using Adobe Photoshop CS3 extended software

(Adobe Systems, Inc., San Jose, CA, USA). Contrast and brightness of images were the only variables adjusted digitally.

Results

Distribution of PV and GAD_{67} in the rat brain

The densities and distribution of mRNAs for PV and GAD_{67} are shown in Figure 1. Cells containing PV mRNA can be found along different cortical areas with a preferential distribution within layers III and V (Figure 1, panels A1 to A4). As in a previous study (Tseng et al. 2008), the density of PV in the medial prefrontal cortex (mPFC) appeared to be higher in the dorsal than in the ventral part (ROD values: 0.220 ± 0.005 for prelimbic area vs. 0.170 ± 0.004 for infralimbic area; $P < 0.002$, Student's *t*-test). Therefore, PV mRNA was measured separately in the prelimbic and infralimbic regions. A moderate expression of PV was also found in the basolateral nucleus of the amygdala (Figure 1, panel A3), hippocampal CA1/CA3 and dentate gyrus (Figure 1, panels A3 and A4). The greatest content of PV mRNA was observed in the reticular nucleus of the thalamus (Figure 1, panel A3). GAD_{67} mRNAs is highly expressed in basal ganglia (Figure 1, panels B2 and C2) and in the reticular nucleus of the thalamus (Fig. 1, panels B3 and C3).

Effects of acute MK-801

As depicted in Figure 2A, 4 h after the systemic administration of MK-801 (1 mg/kg) a significant reduction of the concentration of PV mRNA was found only in dentate gyrus ($P < 0.004$). However, the same effect was patent also in other areas of the brain 24 h later (Figure 2B), when similar reductions of PV mRNA were also found in the prelimbic ($P < 0.05$) and infralimbic ($P < 0.02$) areas of

the mPFC, the entorhinal ($P < 0.05$) and orbitofrontal cortices ($P < 0.02$), the dentate gyrus ($P < 0.02$) and CA3/CA1 ($P < 0.005$) subdivisions of the dorsal hippocampus, and the basolateral nucleus of the amygdala ($P < 0.05$). Representative pseudocolored pictures of such reductions in the mPFC, orbitofrontal cortex and dorsal hippocampus are depicted in Figure 3. In contrast, MK-801 did not change the expression of GAD_{67} at any of the time lapses examined (Figure 4).

Discussion

Distribution of PV and GAD_{67} in the rat brain

The distribution of PV and GAD_{67} in the brain of the rat is similar to what had been described in earlier studies (Esclapez et al. 1994; Feldblum et al. 1993; Hof et al. 1999). In the hippocampus, amygdala and the neocortex virtually all PV neurons are also positive for GAD_{67} labeling, which further confirms previous findings showing that neurons containing PV in these regions are commonly GABAergic interneurons (Kawaguchi and Kubota 1993; Freund and Buzsaki 1996; De Felipe 1997; Kempainen and Pitkänen 2000; McDonald and Mascagni 2001; Gritti et al. 2003). This also holds true for the reticular nucleus of the thalamus where all GABAergic neurons appear to contain PV (Celio 1990; Arai et al. 1994; Csillik et al. 2005).

Effects of acute MK-801

The main finding from the present study is that a single injection of MK-801 was able to significantly reduce PV mRNA in discrete brain regions known to play a role in the pathophysiology of schizophrenia. This effect was observable shortly (4 h) after the administration of MK-801 only in the dentate gyrus of the

hippocampus, but was clearly patent in other areas of the brain 24 h later. Although several works have examined the effects of repeated exposure to NMDA antagonists on GABAergic markers in the rodent brain (Cochran et al.,2003; Rujescu et al. 2006; Braun et al. 2007; Morrow et al. 2007), less attention has been paid to such changes following acute NMDA antagonism, which is widely used as a pharmacological model of schizophrenia. Thus, it has been reported a decrease in GAD_{65} (but not GAD_{67}) mRNA levels in a dose-dependent manner in the striatum and frontal cortex of rats 3 h after the administration of MK-801 (Laprade and Soghomonian 1995). The preferential distribution of GAD_{65} in nerve endings suggests that it could be involved predominantly in the synthesis and release of vesicular GABA (Soghomonian and Martin 1998). Therefore, such rapid effect on GAD_{65} suggests that the changes in the expression of this isoform might be one of the earliest indications that the release of GABA is compromised in a subpopulation of GABAergic neurons after the administration of NMDA antagonists. In contrast, other GABAergic markers do not appear to be altered at the same time scale. For instance, Cochran and coworkers failed to reproduce the loss of prefrontal PV (as seen in schizophrenia) after a single dose of PCP (Cochran et al., 2002). This was probably due to the low dose of PCP used (2.58 mg/kg), together with the lower affinity of this antagonist (compared to that of MK-801) for the NMDA channel site (Wong et al., 1986). In fact, this treatment was insufficient to induce the synthesis of heat-shock protein 70 (Hsp 70) (Cochran et al. 2002). The formation of this protein is considered to be suggestive of the cellular effects induced by PCP and ketamine in rodents, and could also occur in schizophrenia (Sharp et al. 2001). With a dose of MK-801 known to induce the formation of Hsp 70 in the cortex of the rat (Tomitaka et al. 2000), in the present study, we have observed that the reduction in PV mRNA expression occurred in a structure-specific manner. Interestingly, this was found in discrete corticolimbic regions (notably in prefrontal cortex, hippocampus and basolateral amygdala) that have been implicated in schizophrenia (Volk et al. 2000; Beasley et al. 2002; Hashimoto et al. 2003; Woo et al. 2004; Akbarian and Huang 2006). It has been previously shown that GABAergic neurons are very sensitive to NMDA antagonists (Grunze et al, 1996; Li et al, 2002) and is also of note from our results that PV cells of the dentate gyrus of the hippocampus appear to be

particularly responsive to the hypofunction of NMDA receptors. Further support for the crucial role of hippocampus in the effects of NMDA antagonists has been evidenced by results showing that the local administration of MK-801 in this structure increased the spontaneous firing rate of hippocampal neurons projecting directly to the mPFC (Jodo et al. 2005). Although the exact mechanism remains unknown, it has been proposed that NMDA antagonists would attenuate the tonic activation of GABA interneurons, which would result in a disinhibition of glutamatergic transmission (Olney and Farber, 1995; Moghaddam et al, 1997; Krystal et al. 2003). In addition, NMDA receptor hypofunction in parvalbumin-positive hippocampal interneurons impairs hippocampal synchrony and working memory (Korotkova et al. 2010), processes that are also impaired in schizophrenia and mediated by bidirectional interactions between the hippocampus and the mPFC (Meyer-Lindenberg et al. 2005). In the conditions of the present study, the reduction of PV mRNA expression is probably a reflection of a decreased activity of such GABAergic interneurons, which results from NMDA blockade by MK-801. It is possible that the lack of any apparent change in the expression of GAD_{67} in the present and another (Laprade and Soghomonian 1995) study is accounted for by the small subset of interneurons affected, i.e. only those containing PV, which represents ~25 and ~35% of total GABA neurons in mPFC and hippocampus of the rat, respectively (T. Romón, G. Mengod and A. Adell, unpublished results). In fact, alterations in GAD_{67} have been observed only when MK-801 was administered at postnatal day 7 (Coleman et al. 2009; Turner et al. 2009) or after repeated exposure (Qin et al. 1994). This suggests that changes in the expression of GAD_{67} could reflect adaptive changes in development and maturation of those cells, and that might be the reason for a single administration of MK-801 does not replicate the loss of GAD_{67} expression observed in schizophrenia.

In summary, acute NMDA blockade would model the decrement in the expression of PV, but not that of GAD_{67}, seen in the schizophrenia brain. Although the changes of PV observed herein and in postmortem human tissue are quantitatively limited, they likely have a significant impact inasmuch as subtle disturbances in GABAergic cells in prefrontal cortex are now supposed to

be a causal part of the pathophysiology of schizophrenia (Lewis and Gonzalez-Burgos 2008).

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FIGURE LEGENDS

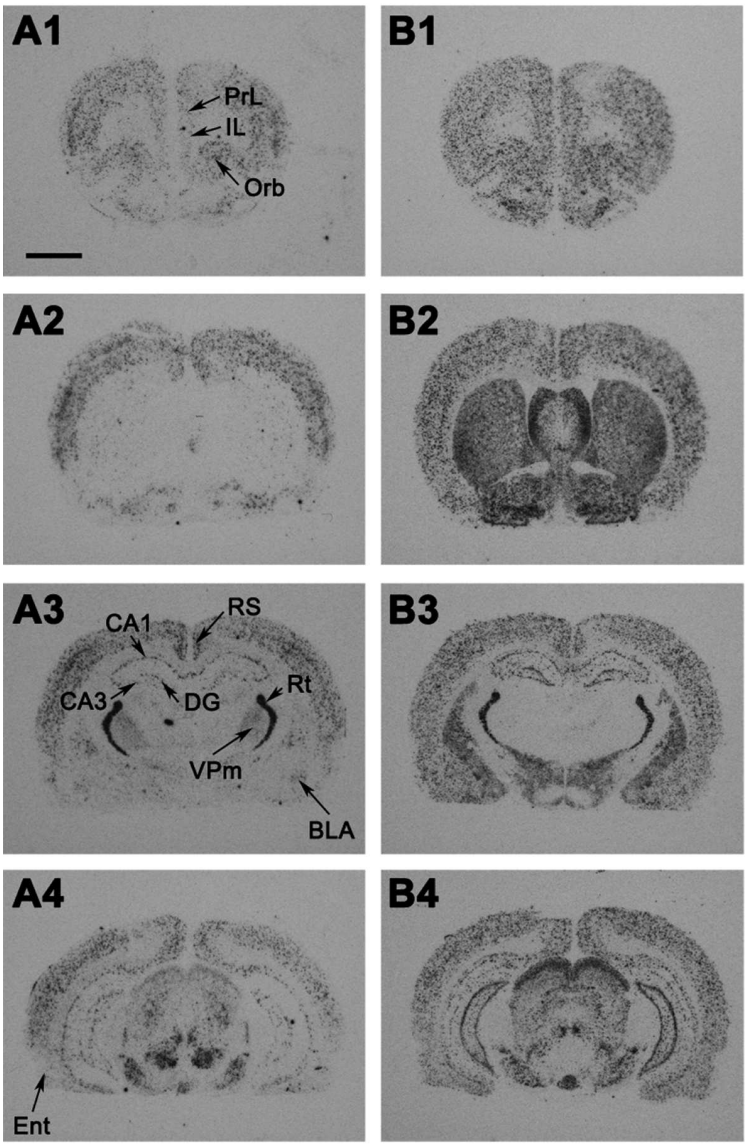
Figure 1: Distribution of the mRNAs for parvalbumin (panels A1 to A4) and GAD_{67} (panels B1 to B4) in the rat brain. Abbreviations: PrL (prelimbic area), IL (infralimbic area), Orb (orbitofrontal cortex), RS (retrosplenial cortex), Ent (entorhinal cortex), VP (ventral pallidum), DG (dentate gyrus), Sub (subiculum), BLA (basolateral nucleus of the amygdala), VPm (ventral posteriomedial thalamic nucleus), and Rt (reticular nucleus of the thalamus). Scale bar, 3 mm.

Figure 2: Relative optical density (ROD) of parvalbumin mRNA in different regions of the rat brain at 4 h (A) and 24 h (B) after the administration of MK-801 (1 mg/kg, i.p.). ROD measurements were calculated in duplicate and the values are expressed as mean ± SEM of six animals per group. $^{*}P < 0.05$, or less, comparing saline- and MK-801-injected animals (unpaired Student's *t*-test). Abbreviations: PrL (prelimbic area), IL (infralimbic area), Orb (orbitofrontal cortex), RS (retrosplenial cortex), Ent (entorhinal cortex), VP (ventral pallidum), DG (dentate gyrus), Sub (subiculum), BLA (basolateral nucleus of the amygdala), and Rt (reticular nucleus of the thalamus).

Figure 3: Representative pseudocolored pictures of the effects of MK-801 (1 mg/kg, i.p.) on the relative optical density (ROD) of parvalbumin (PV) mRNA expression. The acute administration of MK-801 (panels A2 and B2) significantly reduced PV mRNA in comparison with saline-injected animals (panels A1 and B1) in the prelimbic (PrL) and infralimbic (IL) areas of the prefrontal cortex, orbitofrontal cortex (Orb), CA1/CA3, dentate gyrus (DG) and basolateral nucleus of the amygdala (BLA). Scale bar, 3 mm.

Figure 4: Relative optical density (ROD) of GAD_{67} mRNA in different regions of the rat brain at 4 h (A) and 24 h (B) after the administration of MK-801 (1 mg/kg, i.p.). ROD measurements were calculated in duplicate and the values are expressed as mean ± SEM of three animals per group. There is no significant

difference between saline- and MK-801-injected animals. Abbreviations as in Figure 2.



Distribution of the mRNAs for parvalbumin (panels A1 to A4) and GAD_{67} (panels B1 to B4) in the rat brain. Abbreviations: PrL (prelimbic area), IL (infralimbic area), Orb (orbitofrontal cortex), RS (retrosplenial cortex), Ent (entorhinal cortex), VP (ventral pallidum), DG (dentate gyrus), Sub (subiculum), BLA (basolateral nucleus of the amygdala), VPm (ventral posteriomedial thalamic nucleus), and Rt (reticular nucleus of the thalamus). Scale bar, 3 mm
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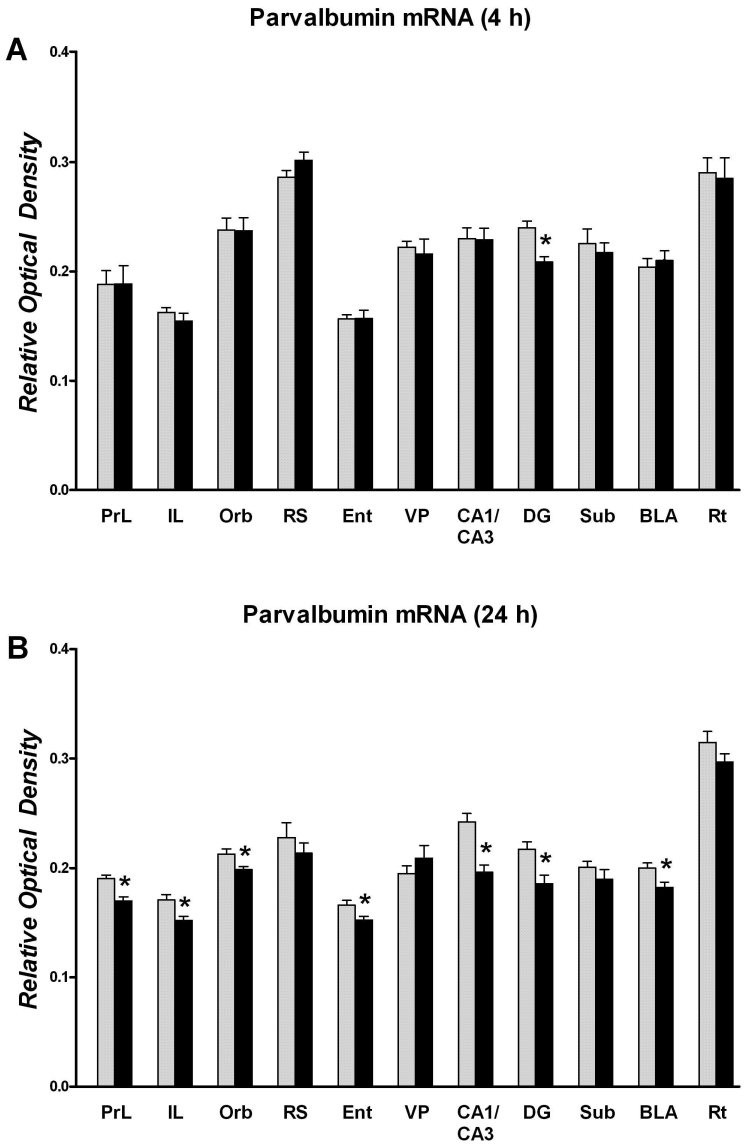

Relative optical density (ROD) of parvalbumin mRNA in different regions of the rat brain at 4 h (A) and 24 h (B) after the administration of MK-801 (1 mg/kg, i.p.). ROD measurements were calculated in duplicate and the values are expressed as mean ± SEM of six animals per group. *P < 0.05, or less, comparing saline- and MK-801-injected animals (unpaired Student's t-test). Abbreviations: PrL (prelimbic area), IL (infralimbic area), Orb (orbitofrontal cortex), RS (retrosplenial cortex), Ent (entorhinal cortex), VP (ventral pallidum), DG (dentate gyrus), Sub (subiculum), BLA (basolateral nucleus of the amygdala), and Rt (reticular nucleus of the thalamus)
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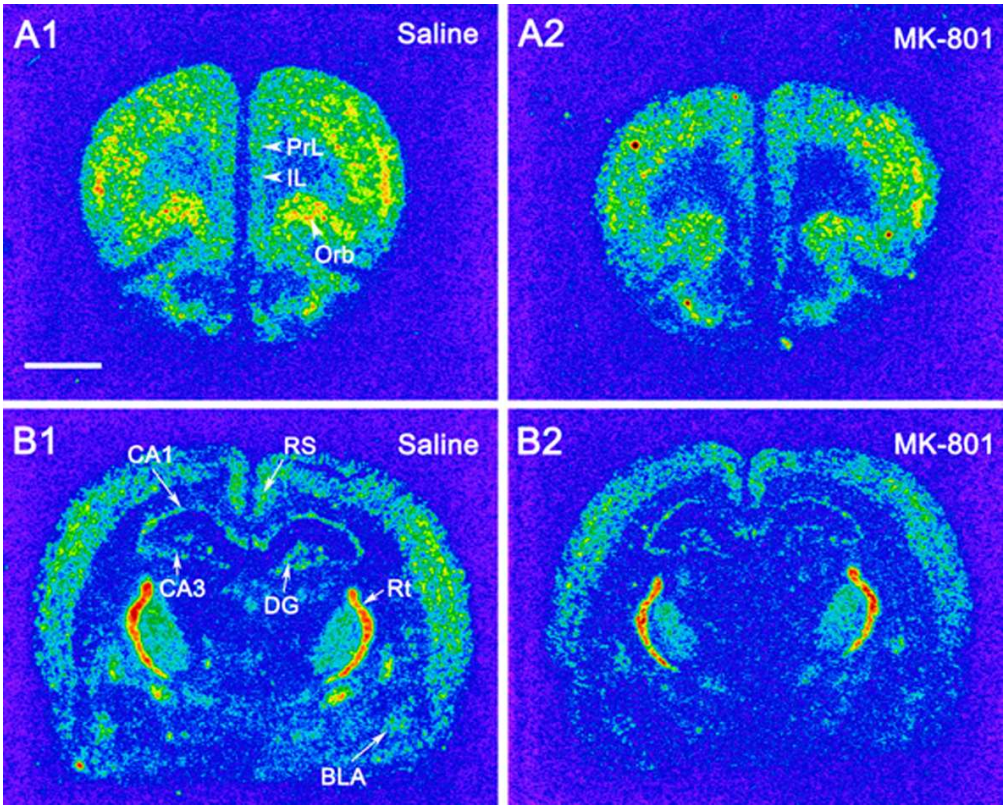

Representative pseudocolored pictures of the effects of MK-801 (1 mg/kg, i.p.) on the relative optical density (ROD) of parvalbumin (PV) mRNA expression. The acute administration of MK-801 (panels A2 and B2) significantly reduced PV mRNA in comparison with saline-injected animals (panels A1 and B1) in the prelimbic (PrL) and infralimbic (IL) areas of the prefrontal cortex, orbitofrontal cortex (Orb), CA1/CA3, dentate gyrus (DG) and basolateral nucleus of the amygdala (BLA). Scale bar, 3 mm
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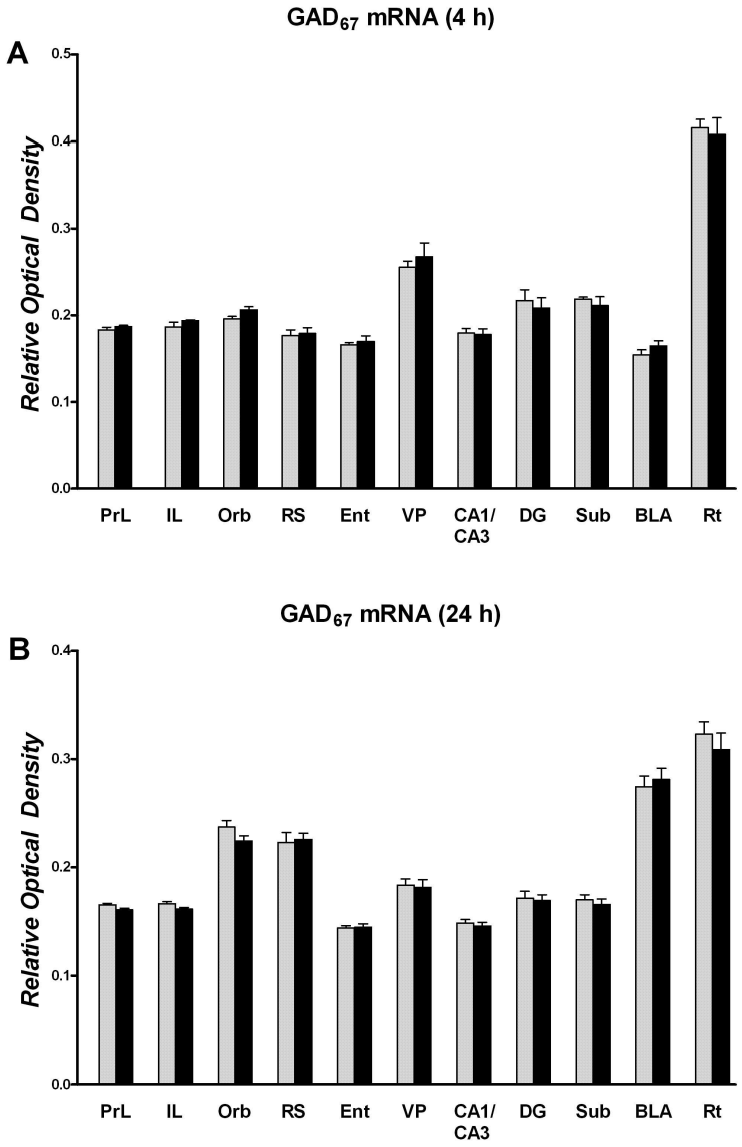

Relative optical density (ROD) of GAD_{67} mRNA in different regions of the rat brain at 4 h (A) and 24 h (B) after the administration of MK-801 (1 mg/kg, i.p.). ROD measurements were calculated in duplicate and the values are expressed as mean ± SEM of three animals per group. There is no significant difference between saline- and MK-801-injected animals. Abbreviations as in Figure 2
88x136mm (600 x 600 DPI)