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Chronic Phencyclidine Induces Behavioral Sensitization and Apoptotic Cell Death in the Olfactory and Piriform Cortex

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In this study, we tested the hypothesis that chronic administration of phencyclidine (PCP), an N-methyl-D-aspartate (NMDA) receptor antagonist, would cause a long-lasting behavioral sensitization associated with neuronal toxicity. Female Sprague-Dawley rats were administered PCP (20 mg/kg, i.p.) once a day for 5 days, withdrawn for 72 hr, placed in locomotor activity chambers, and challenged with 3.2 mg/kg PCP. Following assessment of locomotor activity, the rats were killed and their brains processed for analysis of apoptosis by either electron microscopy or terminal dUTP nick-end labeling (TUNEL). In study I, PCP challenge produced a much more robust and long-lasting increase in locomotor activity in rats chronically treated with PCP than in those chronically treated with saline. In study II, clozapine pretreatment blunted the degree of sensitization caused by PCP. In study I, a marked increase in TUNEL-positive neurons was found in layer II of the olfactory tubercle and piriform cortex of rats chronically treated with PCP. Many of these neurons had crescent-shaped nuclei consistent with apoptotic condensation and margination of nuclear chromatin under the nuclear membrane. Acute PCP had no effect. Electron microscopy revealed that PCP caused nuclear condensation and neuronal degeneration consistent with apoptosis. Cell counts in layer II of the piriform cortex revealed that chronic PCP treatment resulted in the loss of almost 25% of the cells in this region. However, an increase in glial fibrillary acidic protein (GFAP)-positive cells in the molecular layer suggests that this neurotoxicity also may involve necrosis. In study II, the PCP-induced neuronal degeneration was essentially completely abolished by clozapine pretreatment. This pattern of degeneration was found to coincide with the distribution of the mRNA of the NR1 subunit of the NMDA receptor. The relevance of these data to a PCP model of chronic NMDA receptor hypofunction is discussed. J. Neurosci. Res. 52:709–722, 1998.

Key words: phencyclidine; sensitization; neurotoxicity; apoptosis; programmed cell death; olfactory tubercle; piriform cortex; NMDA receptors; dopamine

INTRODUCTION

Phencyclidine (PCP) is a drug of abuse that is known to cause schizophrenia-like symptoms in naïve individuals and to markedly worsen psychosis in schizophrenics (Allen and Young, 1978; Ban et al., 1961; Cohen et al., 1962; Lahti et al., 1995, Luby et al., 1959). PCP and other noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonists such as ketamine have the ability to partially mimic both positive and negative symptoms of schizophrenia (Javitt and Zukin, 1991; Lahti et al., 1995). In recent years a substantial amount of evidence has accumulated that implicates structural and/or developmental abnormalities in the etiology of schizophrenia (e.g., Andreasen et al., 1994; Brown et al., 1986; Benes et al., 1986, 1991; Shenton et al., 1992; Olney and Farber, 1995). Also in recent years, it has been demonstrated that NMDA antagonists such as PCP and MK-801 cause neurodegeneration in corticolimbic regions of the rat brain (Hargreaves et al., 1993; Fix et al., 1993; Olney et al., 1989, 1991; Sharp et al., 1991, 1994). Thus, the psychotomimetic effects of PCP along with the similarity between the brain regions affected by PCP and those altered morphologically in schizophrenia have prompted the formulation of a hypothesis that suggests that reduced glutamatergic neurotransmission through the NMDA receptor may be important in schizophrenia (Olney 1989; Olney and Farber 1995). Reduced NMDA-mediated synaptic transmission also plays a prominent role in the

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thalamic filter dysfunction hypothesis of schizophrenia (Carlsson, 1988).

Unlike the acute behavioral effects of PCP in man and rats, schizophrenia is a chronic illness. Therefore, we sought a model whereby PCP administration produces a long-lasting behavioral disturbance that could be used to further characterize the relationship between PCP administration and behavioral and morphological alterations in the brain. Recently, Xu and Domino (1994) reported that a 4-day treatment of female rats with low-dose PCP resulted in a modest but significant enhancement of locomotor activity. Therefore, we postulated that chronic treatment with high-dose PCP would reveal a more robust sensitization to low-dose PCP challenge. Further, we felt that this sensitization could provide a reasonable model of chronically reduced NMDA function that might have some relevance to schizophrenia, as well as chronic PCP abuse. We also postulated that chronically reduced NMDA receptor function would result in regionally specific cell death and that clozapine, an atypical antipsychotic noted for its effects on the negative as well as positive symptoms of schizophrenia, would prevent the effects of PCP on both sensitization and neuronal viability.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Harlan) weighing 250–275 g were housed in groups of three under standard laboratory conditions which included a 12–12 hr light-dark cycle (lights on at 0700 hr) and free access to food and water. Experiments were conducted between 0930 and 1500 hr.

Drugs

PCP was obtained from the NIDA, Rockville, MD, while clozapine was a gift from Sandoz Pharmaceuticals (East Hanover, NJ). PCP was dissolved in 0.9% NaCl; clozapine was dissolved in 1% L-lactic acid before dissolution in 0.9% NaCl (0.1% L-lactic acid, final concentration). Either 0.9% NaCl or 0.1% L-lactic acid was injected i.p. where appropriate as vehicle control (1 cc/kg).

Experimental Design of Behavioral Studies

The effect of chronic PCP treatment on PCP-induced locomotor activity was determined in study I. In this study, 12 rats were randomly divided into two groups. One group was administered 20 mg/kg PCP (i.p.) once per day for 5 days; the other was similarly treated with saline vehicle. Seventy-two hours following the last injection of either PCP or saline, the rats were placed individually into Plexiglas activity chambers where locomotor activity was automatically monitored (see below). After a 1-hour habituation period, half of each group was challenged with 3.2 mg/kg PCP (i.p.), while the other half was injected with saline. Locomotor activity was monitored for an additional 90 min. Thus, the four groups consisted of chronic saline/acute saline, chronic saline/acute PCP, chronic PCP/acute saline, and chronic PCP/acute PCP (n = 3 for each group).

The ability of clozapine to alter the effect of chronic PCP observed in study I was tested in study II. Thirteen rats were randomly divided into four groups. One group (n = 3) received chronic saline for 5 days; another (n = 3), chronic clozapine (10 mg/kg) for 5 days; a third group (n = 4) received chronic PCP (20 mg/kg); a final group (n = 3) received 10 mg/kg clozapine 1 hour prior to chronic administration of 20 mg/kg PCP. The first three groups also received an additional injection of either lactic acid or saline at the appropriate time as a control for either clozapine or PCP, respectively. Again, after 72 hr, the rats were placed in activity cages for a 1-hour habituation period and all rats were then injected with 3.2 mg/kg PCP. Locomotor activity was measured for an additional 90 minutes as in study I.

Locomotor Activity Assay

Locomotor activity was measured by using an open field activity system. Rats were placed individually in a Plexiglas activity chamber (40 × 40 × 40 cm) and photobeam interruptions were recorded. After 1 hour of habituation, all rats were challenged with PCP or saline. Locomotor activity records included central and peripheral activity measured by a 4 × 4 photobeam array located 4 cm above the cage floor. Data were collected during the 60 minutes of habituation and 90 minutes after PCP challenge in 5-minute intervals. The locomotor activity following the PCP challenge was analyzed statistically using Kruskal-Wallis one-way analysis of variance with Dunn's method for pairwise post-hoc comparisons.

In Situ Apoptosis Assay

Thirty to 180 minutes after the locomotor activity assay, the rats were anesthetized with sodium pentobarbital (50 mg/kg), perfused with saline, and then fixed by transaortic perfusion of ice-cold (4°C) 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brains were removed, postfixed in the same fixative overnight, and then washed in phosphate buffer and immersed in 20% sucrose solution in 0.9% NaCl solution. Coronal sections (10 µm) through the forebrain, cingulate cortex, retrosplenial cortex, hippocampus, and cerebellum were cut with a cryostat and thawed onto gelatin-coated slides and processed for assessment of apoptosis in situ. These regions were located with a reference atlas (Paxinos and Watson, 1986). Three additional rats that were only given a single injection of 20 mg/kg PCP (i.p.) were also included. These rats were killed 24 hr after PCP treatment without measuring locomotor activity.

The apoptosis assay (TUNEL technique) relies on the detection of broken DNA strands resulting from the nucleosomal DNA fragmentation characteristic of apoptotic nuclei (Gabacchi et al., 1992; Rabacchi et al., 1994). Terminal deoxynucleotidyl transferase (TdT), a template-independent polymerase, is used to incorporate biotinylated nucleotides at sites of DNA breaks. The signal was amplified by avidin-biotin peroxidase, enabling conventional histochemical identification by light microscopy. Briefly, the brain sections were treated with proteinase K (20 µg/ml; Sigma Chemical Co., St. Louis, MO) to permeabilize cellular membranes and to dissociate proteins from DNA for 15 minutes at room temperature (RT); the sections were then washed three times in ice-cold phosphate-buffered saline (PBS) for 5 minutes. Endogenous peroxidase was inactivated by covering the sections with 2% H_2O_2 for 5 minutes at RT. The sections were rinsed with cold PBS solution and immersed in TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). This buffer was then replaced by one containing TdT (0.3 U/µl; Boehringer Mannheim, Indianapolis, IN) and biotinylated dUTP (0.2 nM/10 U TdT; Boehringer Mannheim) and then incubated in a humid atmosphere at 37°C for 90 minutes. The reaction was terminated by transferring the sections to cold buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes. The sections were rinsed with cold PBS, covered with 2% bovine serum albumin (BSA) for 10 minutes, and rinsed again in cold PBS solution. The sections were covered with the Biotin/Avidin peroxidase complex (1:50 in PBS solution; Vectastain ABC kit), incubated for 30 minutes at 37°C, and immersed in 0.05 M Tris-HCl (pH 7.4). The reaction product was visualized with 3,3′-diaminobenzidine (Sigma). For negative controls, TdT was omitted from the reaction mixture. (As a positive control, the brain sections were treated with 1 N HCl for 20 minutes prior to the terminal transferase.) The sections were photographed with the use of an Olympus light microscope.

In order to measure the density of TUNEL-positive neural cells, the following procedure was followed. After reviewing the 10-µm coronal sections, it was determined that the effect of PCP was restricted to the olfactory tubercle and piriform cortex (see Results). Therefore, several (2–5) photographs (50× magnification) were taken of different regions within both the olfactory tubercle and piriform cortex. Although TUNEL experiments were performed on sections from all rats, not all sections were suitable for further photographic analysis. In the final analysis, photographs were used from four saline-treated and five PCP-treated rats from experiment I and from four saline-, three clozapine-, two PCP-, and three PCP + clozapine-treated rats from experiment II. These photographs (180 total) were randomly assembled and scored by four blind raters using a semiquantitative rating score that ranged from 0 to 3, where 0 was used to indicate no TUNEL-positive cells and 3 indicated intense labeling of almost all nuclei in the field. The photographs were then grouped by treatment and brain region and the mean score of each rater per region was taken for each section. The data were analyzed with Sigma Stat Statistical software using a two-way analysis of variance on one factor with Tukey’s post-hoc test. Significance was established by a $P < 0.05$.

In Situ Hybridization

An oligonucleotide probe complementary to the mRNA encoding the NMDA glutamate receptor subunit NR1 was selected on the basis of the cloned cDNA sequence. The NR1 probe is complementary to a sequence encoding amino acid residues 566–580 of the mature NR1 polypeptide. It was 3’-end-labeled by incubation with ^{35}S deoxy-ATP (New England Nuclear, Boston, MA) and terminal deoxynucleotidyl transferase (Boehringer) to attain specific activities of about $5–8 \times 10^8$ cpm/µg. The specificity of the probe has been previously described (Monyer et al., 1992).

Coronal sections (10 µm) through the forebrain were cut with a cryostat, rinsed in PBS, fixed in 4% paraformaldehyde, and processed for in situ hybridization as described previously (Bartanusz et al., 1993). After an overnight hybridization at 41°C, slides were washed successively in 4×, 1× and 0.1× SSC, quickly dehydrated in ethanol (70%) and air-dried. For autoradiography, slides were dipped in Kodak NTB3 emulsion and exposed 3 weeks at 4°C. Analysis of in situ hybridization autoradiographs was accomplished on hematoxylin-eosin-counterstained sections. The negative control was performed by adding an excess amount (50-fold) of unlabeled probe.

Electron Microscopy

For electron microscopic analysis, two rats were treated chronically with either saline or PCP (20 mg/kg); after 72 hr these rats were anesthetized and fixed by transaortic perfusion of ice-cold (4°C) 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. Rat brains were removed and coronal sections (100 µm) were cut with a vibratome (Campden Instruments Ltd. 752M Vibroslice, Loughborough, UK). Piriform cortex and olfactory tubercle regions were microdissected, postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Thin sections were counterstained with uranyl acetate and lead citrate. The sections were examined at 60 KV using a Philips 301 transmission electron microscope.

Light Microscopic Immunocytochemistry

In order to evaluate the astrocytic response in PCP-treated and saline-treated rats, glia fibrillary acidic protein (GFAP) antigenicity was used as a marker. Sections adjacent to those used in the TUNEL assay were taken from two saline- and two PCP-treated rats. A rabbit polyclonal antibody (Dakopatts, Denmark) to GFAP was used (1:300 dilution) and the indirect immunofluorescence technique was used to visualize immunoreactivities. The sections were permeabilized with a solution of PBS/0.5%BSA/0.3%Triton X-100, and incubated with the primary antibody at 4°C overnight. Bound antibodies were revealed with rhodamine-conjugated sheep anti-rabbit IgG (diluted 1:40; Boehringer) secondary antibody (diluted in PBS/0.5%BSA solution). The sections were examined with an Olympus light microscope equipped with epifluorescence.

Nuclear Staining

In order to assess the extent of cell death and removal prior to examination by TUNEL, sections adjacent to those used in apoptosis assay and immunocytochemistry were taken and stained with bisbenzimide solution (Hoechst 33258, Sigma). Bisbenzimide (0.1 µg/ml) was dissolved in PBS glycerol (1:1) solution. The sections were dehydrated and rehydrated in a series of graded ethanol (50%, 70%, 90%, and 100%), then washed three times in PBS. After putting two drops of bisbenzimide solution on the section, the coverslips were mounted onto microscope slides and observed under a fluorescence microscope at 365-nm wavelength excitation light. The number of Hoechst-stained nuclei was counted in a 0.33 mm^2 area (0.466 mm x 0.7 mm) of the piriform cortex located near the lateral olfactory tract. This area was selected by centering it over layer II. Included in this analysis are nine sections from three saline-treated rats and nine sections from three PCP-treated rats.

Statistical Analysis

Analysis of TUNEL assays was carried out by one-way analysis of variance (ANOVA) on ranks across treatment group with Dunn's post-doc test for significance. ANOVA with Dunn's post-hoc test was also used to assess the effect of drug treatment on locomotor activity in either 5-minute or 30-minute time bins. The effect of chronic PCP treatment on cell number in layer II of the piriform cortex was assessed by comparison to saline treatment using Student's *t*-test. In all cases, $P < 0.05$ was considered to be significant.

RESULTS

In our initial attempts to produce long-lasting behavioral effects with PCP, we treated both male and female rats for 5 days with 10 mg/kg PCP (i.p.) and then challenged them 3 days later with 3.2 mg/kg PCP. We found minimal evidence of behavioral sensitization to the locomotor activating effects of PCP challenge (data not shown).

Because female rats are known to be more sensitive to the acute neurotoxic effects of PCP than male rats, we tested the effects of a higher dose of PCP (20 mg/kg) in females. Acutely, this dose produces an increase in locomotor activity and stereotypic behavior that is confounded by moderate to severe ataxia. These behavioral alterations were not routinely quantitated, but were similar to those previously reported by others (Chen et al., 1959; Murray and Horita, 1979; Sturgeon et al., 1979). In one preliminary experiment in which the rating scale of Sturgeon et al. (1979) was used, we observed that the most prominent effect after a single injection was ataxia. This effect persisted for about 6 hr. However, after the fifth injection, the most prominent effect was an increase in forward locomotion, particularly later in the rating period, after the ataxia subsided (data not shown). Despite a tendency for the rats to lose weight in the 24-hr period following the initial dose, there was no significant difference in weight between the PCP and control groups after 5 days of administration. There also was no weight difference between treatment groups following 3 days of withdrawal.

Administration of 3.2 mg/kg PCP to rats treated chronically with saline produced a very modest increase in locomotor activity that remained above baseline for only 10 minutes (Fig. 1). This response was essentially identical to saline challenge of either PCP- or saline-treated rats. On the other hand, a 3.2-mg/kg PCP challenge of rats 72 hr following a 5-day regimen of 20 mg/kg PCP each day resulted in a remarkably robust increase in locomotor activity that lasted longer than 90 min. The magnitude of the sensitization observed in this experiment is much greater than that observed by Nabeshima et al. (1987) who used a 5-day protocol of 10 mg/kg PCP in male rats, followed 24 hr later by a challenge of either 5 or 10 mg/kg PCP. Further, the sensitization observed with this protocol is even larger still than that observed by Xu and Domino (1994), who treated female rats with 3.2 mg/kg PCP for 4 days. We have also observed that this protocol induces a profound sensitization of rats of either gender, though females are significantly more sensitive (unpublished observations).

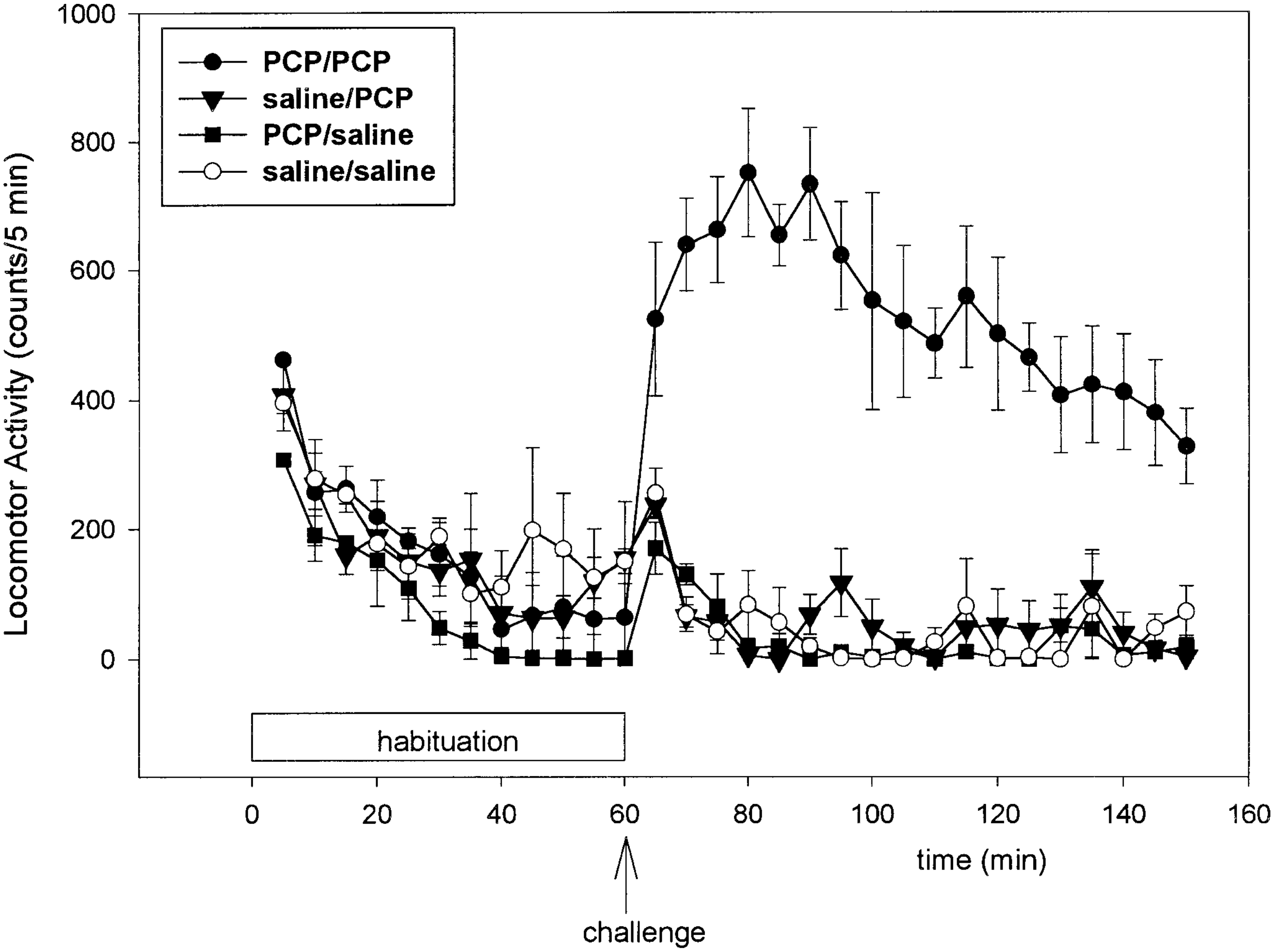

Fig. 1. The effect of chronic treatment with saline or phencyclidine (PCP; 20 mg/kg) on the locomotor activity produced by administration of saline or 3.2 mg/kg PCP following 3 days withdrawal. The locomotor activity in the PCP/PCP group was significantly greater than the activity of all other groups from 65 to 150 minutes; $P < 0.05$.

Because clozapine is an antischizophrenic drug that is effective against both positive and negative symptoms of schizophrenia and has also been demonstrated to reduce the acute neurotoxicity of MK-801, the effect of a 1-hr pretreatment with 10 mg/kg clozapine on PCP-induced sensitization was investigated. In this particular experiment, the challenge with 3.2 mg/kg PCP in the saline-pretreated rats produced a larger increase in locomotor activity than was observed in study I. Although PCP challenge resulted in a somewhat greater effect in the chronic PCP pretreatment group, this was significant only in the second and third 30-minute bins following challenge (Fig. 2). The effect of clozapine pretreatment was complex. While it reduced the effect of chronic PCP treatment on PCP challenge, it also sensitized the rats to PCP challenge when administered alone (Fig. 2). Thus, the reduction in sensitization to PCP was observed in the face of an increased response to PCP caused by clozapine administration.

In parallel with locomotor activity assays, we monitored neurotoxicity. We found that within 72 hr of drug withdrawal following 5 days of chronic PCP treatment, a population of cells in piriform cortex and olfactory tubercle undergoes degeneration (Fig. 3A). In this study, we used the TUNEL technique to assess nuclear damage after chronic PCP treatment. Although all parts of the forebrain including the cingulate cortex, retrosplenial cortex, and hippocampus were examined in this study, TUNEL-positive cells were prominent only in the piriform cortex and olfactory tubercle in the chronic PCP treatment group. PCP also had no effect in the cerebellum.

The piriform cortex and olfactory tubercle have a trilaminar structure characteristic of most of the olfactory

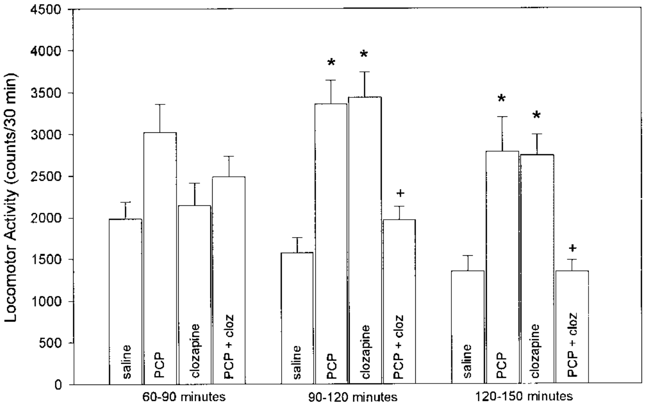

Fig. 2. The effect of clozapine pretreatment on PCP-induced behavioral sensitization. The experiment was carried out as depicted in Figure 1, but the data were analyzed in 30-minute bins following challenge with 3.2 mg/kg PCP. The pretreatment for each group is indicated within each bar. *Significantly different from the saline pretreatment group, $P < 0.05$; $^{+}$significantly different from the PCP pretreatment group. $P < 0.05$.

cortex, consisting of a superficial molecular layer, layer I, and two deep cellular layers, layer II and layer III. Layer II in the olfactory cortex has tightly packed neurons, most of which have a single (although short) apical dendrite that branches in layer I and a well-developed dendritic tree (Friedman and Price, 1986a). The topographic and laminar distribution of TUNEL-positive cells is quite consistent in different rats. The most severe cell degeneration is found in layer II, although there are also labeled nuclei in layers I and III (Fig. 3C,F). This pattern of neuronal degeneration is seen only after chronic PCP treatment. Most of the stained cells in piriform cortex and olfactory tubercle were located around the lateral olfactory tract (LO). In contrast, TUNEL-labeled cells were almost absent in other cortical regions in the same section (Fig. 3A).

Examination of high-magnification photomicrographs revealed an advanced state of degeneration, with distorted cell bodies, chromatin condensation at the nuclear periphery, and very fragmented nuclei (Fig. 3B). In PCP-treated rats, many cells were characterized by an intense TUNEL-labeled nucleus with irregular nuclear shape. Such dark nuclei were not seen in control rats (Fig. 3D,G) or PCP-clozapine-treated rats (Fig. 3E,H). Thus, the attenuation of degeneration observed in this study by clozapine is significant. Note that some meningeal cells are TUNEL-labeled in control and PCP-clozapine-treated rats (Fig. 3D–G), indicating that the lack of TUNEL-positive nuclei in control or PCP-clozapine-treated rats is not an assay artifact. Quantitation of the density of TUNEL-positive cells in study II is shown in Figure 4.

Although the TUNEL technique is widely regarded as being specific for apoptosis, there are examples of TUNEL-positive necrotic cell damage. In order to qualitatively confirm the presence of apoptosis, electron microscopic studies were performed in layer II of piriform cortex and olfactory tubercle sections from saline- and PCP-treated rats. Electron micrographs showed tightly packed nuerons in the deep zone of layer II of both piriform cortex and olfactory tubercle in a saline-treated rat. Figure 5A shows an example of a piriform cortex neuron from a saline-treated rat with normal ultrastructure and nucleus. This neuron has a large nucleus with well-defined plasma and nuclear membranes and an intact nucleus. In the PCP-treated rats, electron microscopy revealed that many neurons in layer II were morphologically apoptotic. Figure 5B shows an example of a piriform neuron in what appears to be an intermediate stage of apoptosis. The nuclear membrane and nucleolus are still intact, but the nucleus is extremely shrunken relative to the normal nucleus in Figure 5A. Intact mitochondria are also present in this cell (Fig. 5B). An example of a clearly apoptotic neuron in layer II of the piriform cortex is shown in Figure 5C. The cytoplasmic membrane is still intact, but the cytoplasm is extremely shrunken and the nucleus shows advanced condensation of chromatin material and decomposition of the nuclear membrane. Again, an intact mitochrondrion can be seen just to the right of the nucleus. Around these advanced apoptotic neurons were many neurons with mildly condensed nuclei with a dispersion of the nucleolus in the center (data not shown). Thus, although these data are not quantitative, these electron micrographs support the hypothesis that the PCP-induced increase in TUNEL staining is associated with apoptosis. However, these data do not rule out the possibility that necrotic cell death could

Fig. 3. **A:** General view of the ventral part of forebrain showing the distribution of apoptotic cells detected by the dUTP nick-end labeling (TUNEL) assay in a PCP-treated rat. Numerous stained cells were found predominately in piriform cortex and olfactory tubercle located around the lateral olfactory tract (LO). In contrast, TUNEL-labeled apoptotic cells are almost absent in other cortical regions at the same section. **B:** High-magnification photomicrograph shows an advanced state of degeneration, with distorted cell body, margination of chromatin, and very fragmented nuclei (arrowheads). C–E show coronal sections of piriform cortex in different treatment groups. In PCP-reated rats (**C**), apoptotic cells are characterized by a large number of TUNEL-labeled dark nuclei with irregular nuclear shape. Such dark apoptotic nuclei are not seen in control rats (**D**) or PCP-clozapine-reated rats (**E**). Note that some meningeal cells are TUNEL-labeled (arrows) in D and E. **F–H** show sections of olfactory tubercle that are treated analogously to those shown in C–E. Bars = 500 μm (A); 35 μm (B); 100 μm (C–H).

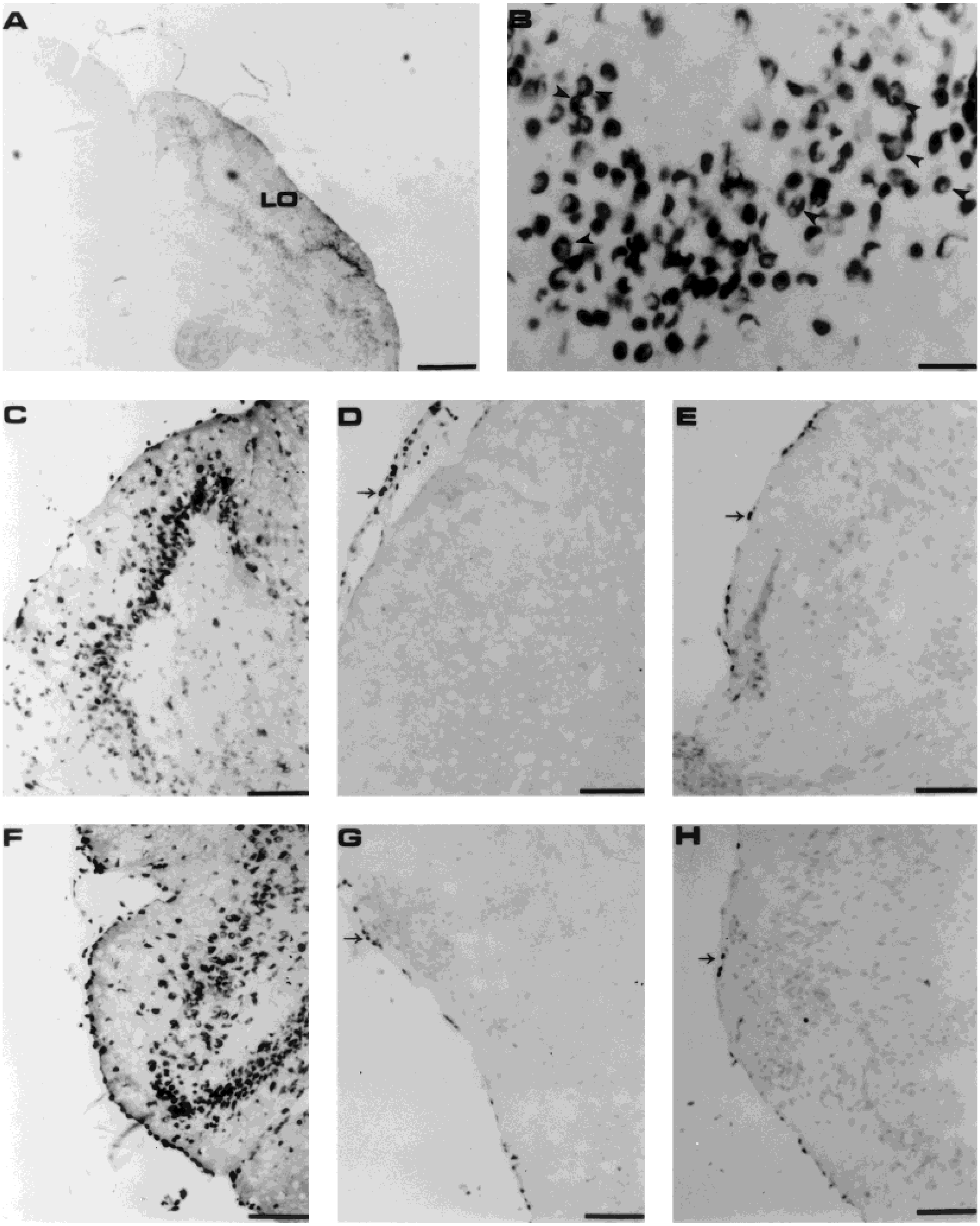

Figure 3.

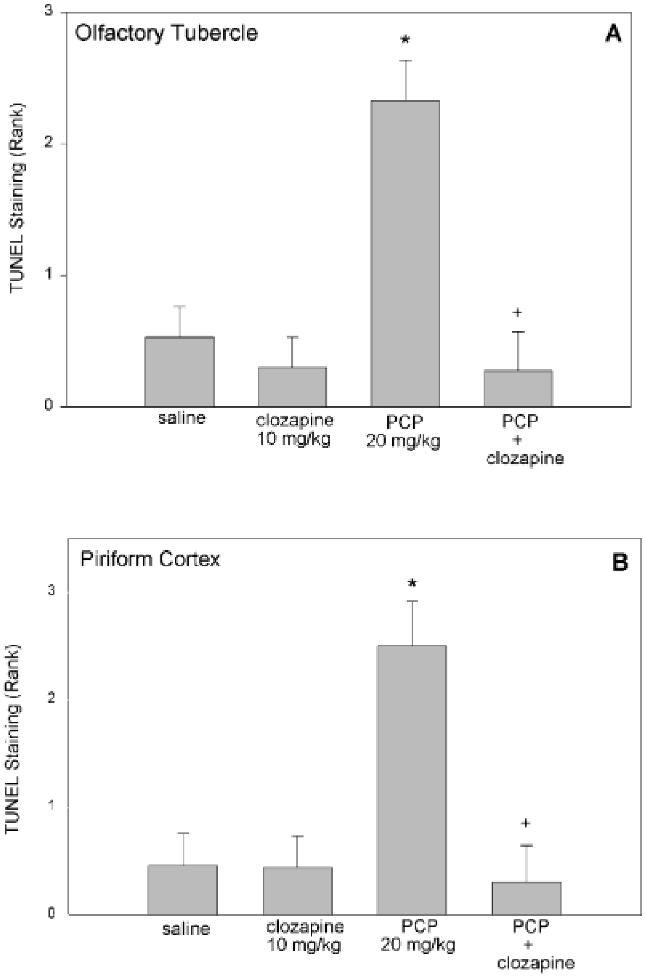

Fig. 4. Semiquantitative analysis of the density of TUNEL staining of olfactory tubercle (**A**) and piriform cortex (**B**) in rats from study II. The rats were treated and processed as described in Materials and Methods. $*P < 0.05$ vs. control; $^{+}P < 0.05$ vs. PCP.

occur simultaneously in other, nearby neurons or in neurons in other brain regions.

The previously mentioned neurodegeneration in corticolimbic regions caused by acute PCP and MK-801 has been reported to be associated with reactive gliosis, as assessed by an increase in immunoreactivity to glial fibrillary acidic protein GFAP (Fix et al., 1995). To assess the possibility that the PCP-induced increased in TUNEL-positive staining was associated with reactive gliosis, we measured GFAP immunoreactivity in two olfactory/piriform sections taken from each of four rats treated with either chronic PCP (n = 2) or chronic saline (n = 2). These sections, taken adjacent to the sections used for the TUNEL assay, revealed a substantial increase in GFAP-labeled cells in the region surrounding the lateral olfactory tract (Fig. 6). As an example of a chronic saline-treated rat, Figure 6 (left) shows dense GFAP labeling of cells in a layer bordering the ventral meningeal membranes below the lateral olfactory tract. Also shown are several clearly defined astrocytes dispersed sporadically throughout layers II and III. Three days after chronic PCP treatment, there is a marked increase in the number of GFAP-labeled cells, consistent with a proliferation of astrocytes, particularly in the ~200-μm-thick molecular layer between the meningeal membranes and layer II.

In order to assess the potential loss of cells in these rats, the number of cells in layer II of the olfactory tubercle/piriform cortex of saline- (n = 3) and PCP-treated (n = 3) rats were counted. In these experiments, three sections adjacent to those used for TUNEL analysis were taken from each rat for analysis of Hoescht 33258 staining. Figure 7 shows that PCP treatment significantly reduced the number of fluorescent nuclei in layer II by about 25%. The rationale for using Hoescht 33258 over a more conventional method such as hematoxylin and eosin staining is that we thought we might also be able to gain additional insight into the effect of PCP on nuclear morphology. However, there was no clear-cut difference in the nuclear morphology of cells from PCP- or saline-treated rats. Although there appeared to be condensed nuclei, it was not possible to discriminate condensed neuronal nuclei from the smaller astrocytic nuclei. The fact that this nuclear dye does not discriminate between neurons and neuroglia suggests that when considered in the light of the increased number of GFAP-positive astrocytes (Fig. 6), the reduced number of Hoescht 33258-positive cells may even be an underestimate of the neuronal degeneration in layer II of this region.

Finally, because PCP is an NMDA antagonist, it seemed possible that the localization of apoptotic neurons in the olfactory cortex might correspond to the location of NMDA receptors. In a preliminary experiment, we used in situ hybridization of an NR1 probe to localize the mRNA for this obligatory subunit of the NMDA receptor. The emulsion autoradiograph shown in Figure 8 indicates a high concentration of silver grains over most cells in layer II of the olfactory tubercle and piriform cortex of a control rat. The concentration of NR1 mRNA in layer II appears to correspond to the region of the olfactory cortex from PCP-treated rats that was most heavily stained in the TUNEL assay. However, there are areas in which the NR1 mRNA is less concentrated where TUNEL-positive neurons were also observed.

DISCUSSION

The potential use of NMDA antagonists in the therapy of excitotoxicity associated with ischemia, neurotrauma, Alzheimer's, and Parkinson's disease initially led Olney's laboratory to investigate the possible neurotoxic effects of PCP, ketamine, and MK-801 (Olney et al., 1989). The finding that acute administration of these

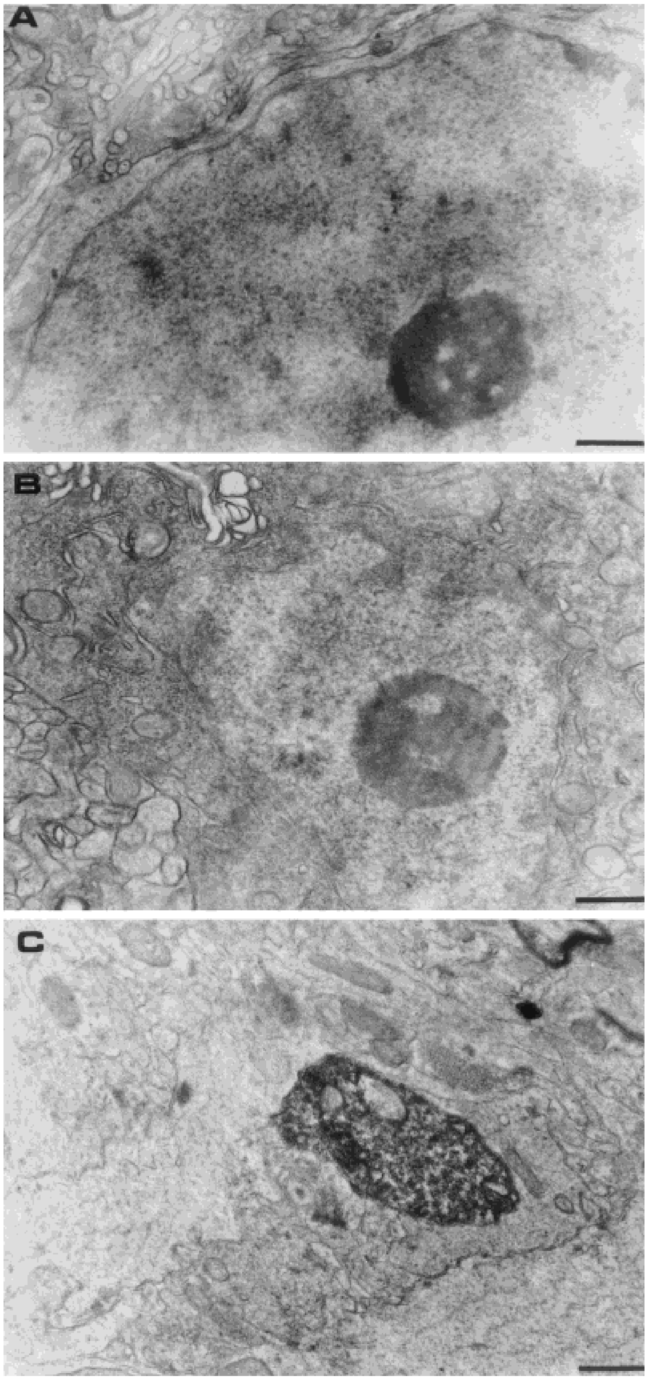

Fig. 5. Electron micrographs of a normal neuron from a saline-treated rat (**A**) and of neurons from a PCP-treated rat in intermediate (**B**) and advanced (**C**) stages of nuclear condensation. A shows a normal neuron with normal large nucleus and nucleolus. Both plasma membrane and nuclear membrane are clearly visible (upper left). B shows an irregularly shaped, shrunken nucleus in an irregularly shaped neuron with several intact mitochondria. The nuclear membrane bilayer can also be seen. C shows an extremely condensed nucleus. The nucleolus and nuclear membrane are indistinguishable. However, the plasma membrane is still intact and some mitochondria are visible. Bars = 0.54 μm (A and B); 0.64 μm (C).

drugs produces a long-lasting, perhaps irreversible necrotic toxicity has sharply curtailed the development of NMDA antagonists as therapeutic agents. However, at the same time, this observation has supported the development of NMDA antagonists as both behavioral and anatomical models of schizophrenia (Olney and Farber, 1995) and the dementias (Ellison, 1995).

Although appealing, this model of schizophrenia presented two issues of concern to us. The first was that although PCP exacerbates psychosis in schizophrenic patients and produces symptoms of schizophrenia in normals, acute PCP does not generally result in long-lived alterations in behavior, in either humans or rats. The second is that acute administration of PCP-like compounds produces vacuolization and reactive gliosis suggestive of a necrotic mechanism (Fix et al., 1995; O'Callaghan, 1994), while there is little evidence of gliosis in postmortem samples of schizophrenic brain (Roberts, 1990).

Regarding the first issue, some evidence does exist that suggests that repeated PCP abuse causes longer-lived symptoms of schizophrenia (Carlin et al., 1979; Cosgrove and Newell, 1991). Furthermore, others have reported that chronic PCP or MK-801 administration in rats resulted in a modest behavioral sensitization (Nabeshima et al., 1987; Wolf and Khansa, 1991; Xu and Domino, 1994). The regimen that we settled on (20 mg/kg PCP for 5 days, 3 days of withdrawal, and challenge with 3.2 mg/kg) produced an easily measured, robust sensitization that lasted at least 3 days. Other, subsequent experiments in this laboratory have shown that this dosage regimen produces a sensitization that continues largely unabated for at least 8 days (Hanania and Johnson, unpublished observation). Therefore, we believe that this PCP treatment protocol produces a long-lasting stable behavioral alteration that is suitable for experimentation over this time frame. This model has the obvious advantage that the altered state underlying the enhanced responsiveness to PCP is independent of the acute presence of the drug.

With regard to the issue of a relative lack of glial scarring in damaged regions of the schizophrenic brain, it has been argued that if reduced NMDA function were responsible for neurodegeneration in an on-going process, then it might not elicit a conspicuous glial reaction and the evidence of a necrotic mechanism would be missed (Olney and Farber, 1995). Alternatively, it is also possible that the degeneration observed in schizophrenic brains is a result of apoptosis, a process classically thought not to involve reactive gliosis (Bredesen, 1995; Gordon, 1995). Although, it is certain that NMDA antagonists produce necrotic degeneration along with a long-lasting increase in GFAP-positive cells (Fix et al., 1995), our data suggest that chronic PCP also initiates apoptosis.

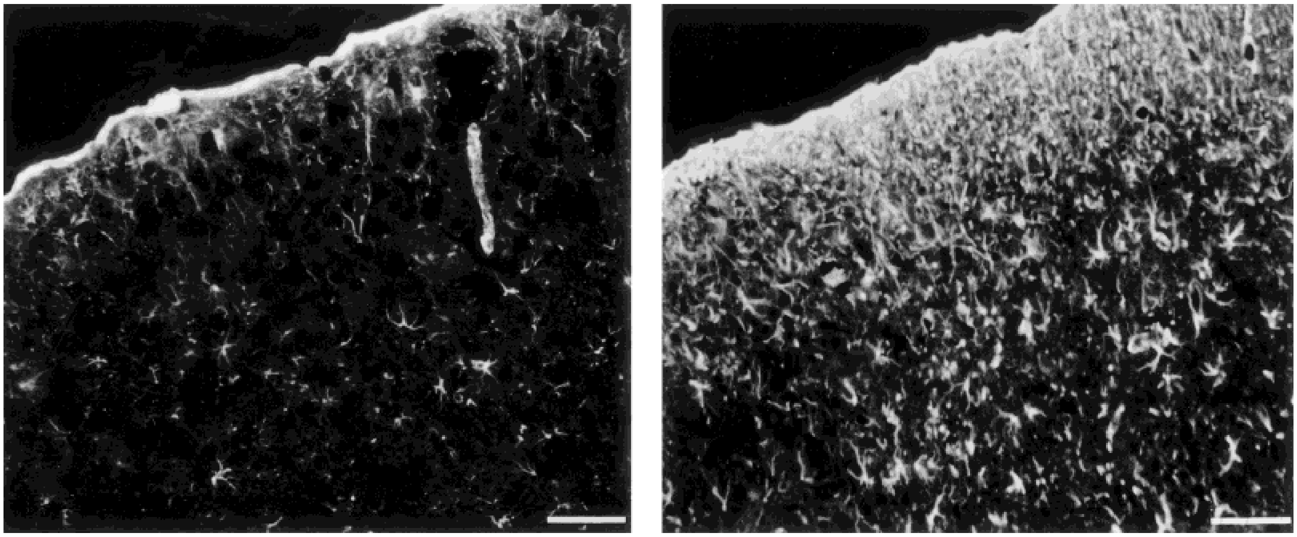

Fig. 6. Glial fibrillary acidic protein (GFAP) immunoreactivity in the region of the lateral olfactory tract of a saline-treated rat (**left**) and a PCP-treated rat (**right**). A dense layer of labeling of astrocytes is seen abutted to the pia mater of the ventral surface of the brain (top) in both pictures. Astrocytes appear sporadically in the adjacent molecular layer (~200-µm-thick) and in layer II of the saline control rat (left). The density of GFAP-positive neuroglia is dramatically increased by chronic PCP treatment, particularly in the molecular layer (right). Bars = 90 µm.

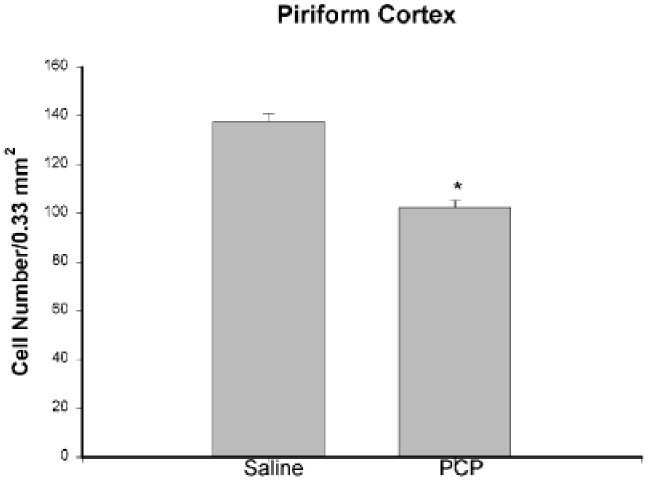

Fig. 7. The effect of chronic PCP treatment on cell number in layer II of the piriform cortex. Hoescht 33258-positive cells in three fields were counted manually and averaged for each rat. n = 3 for saline and n = 3 for chronic PCP. $*P < 0.05$, Student's *t*-test.

Whether the apoptosis observed here following a chronic PCP regimen is related to the acute vacuolization observed by others is not certain. Although the brain regions found to be affected here are quite different from the retrosplenial and posterior cingulate areas initially reported to be affected by MK-801 and PCP (Olney et al., 1989, 1991), more recent experiments have shown that either continuous administration or high doses of NMDA antagonists induce degeneration or HSP 70 in other limbic regions of the brain including the amygdala, dentate gyrus, entorhinal cortex olfactory tubercle and piriform cortex (Ellison, 1994; Ellison and Switzer, 1993; Sharp et al., 1992, 1994). Although the induction of HSP 70 is normally thought of as a protective mechanism, its induction in these areas does suggest that an insult has occurred. Whether this protective mechanism was successful is unknown, but these data do suggest a precedent for PCP-induced neurotoxicity in the piriform cortex. Another point here is that at any given time only a relatively small number of cells may be undergoing apoptosis. Thus, it is possible that if we were to have examined the brain for TUNEL-positive neurons at another time, either earlier or later, we may have caught an entirely different population of neurons undergoing apoptotic cell death. Since the HSP 70 response has been shown to last about 2 weeks following acute administration, it is difficult to say that the acute vacuolization/necrosis and the apoptosis observed following chronic PCP administration are not related (Portera-Cailliau et al., 1997).

Another way to determine the possible relationship between apoptosis and vacuolization is to compare the pharmacology of the two responses. It has been demonstrated that pretreatment with either muscarinic antagonists, gamma aminobutyric acid $(GABA)_A$ facilitators such as diazepam and pentobarbitol, non-NMDA glutamate receptor antagonists, α_2-adrenergic agonists, typical antipsychotic agents such as haloperidol, and atypical agents such as clozapine block either the neurotoxicity itself or the HSP 70 response to acute PCP or MK-801

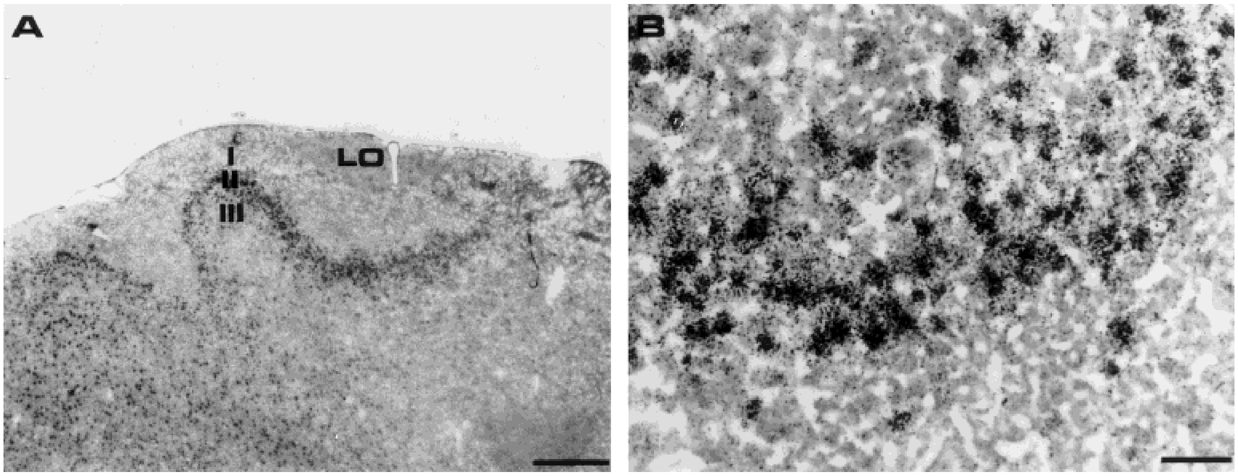

Fig. 8. In situ hybridization of NR1 in ventral cortex near the lateral olfactory tract (LO). **A** shows a high concentration of the mRNA probe for this subunit in layer II. **B** shows a high magnification view of neurons in layer II. The distribution of grains in the autoradiograph indicates that the NR1 mRNA is localized to individual neurons, sometimes found in clusters. Bars = 500 μm (A); 100 μm (B).

administration (Olney et al., 1991; Olney and Farber, 1995; Sharp et al., 1992). The complete blockade of the appearance of TUNEL-positive cells in both the olfactory tubercle and piriform cortex by clozapine suggests that PCP-induced apoptosis in these areas may be related mechanistically to PCP-induced vacuolization in the retrosplenial and cingulate cortex. However, additional experiments with drugs such as atropine, pentobarbitol, haloperidol, and 6,7-dinitroquinoxaline-2,3-dione (DNQX) are necessary to either firmly support or refute this hypothesis.

The possibility that behavioral sensitization and apoptosis induced by PCP could be used as a model of chronic NMDA receptor hypofunction seems to be compromised by the inability of clozapine to completely prevent the development of the sensitized response to PCP. Although the response was significantly blunted by clozapine at the later time intervals, its effectiveness was clearly less than it was in preventing the development of apoptosis. This could be related to clozapine kinetics or, as mentioned above, this may be because chronic clozapine alone significantly increased the locomotor response to the acute PCP challenge. This effect of chronic clozapine may be related either to its ability to upregulate D_1 receptor density in various basal ganglia and mesolimbic areas or to down-regulate 5-HT_2 receptors in several cortical regions and the ventral striatum (Coward, 1992). In recent experiments, we have shown that pretreatment with 1 mg/kg haloperidol, a more selective D_2 antagonist than clozapine, completely prevented the development of sensitization to PCP (Phillips et al., 1997) Unlike clozapine, chronic haloperidol treatment of saline-treated rats did not influence the effect of PCP challenge. Thus, it may be that one property of clozapine may be responsible for its ability to block apoptosis, and another responsible for its ability to sensitize rats to PCP. In this context, it should be pointed out that we do not wish to imply that neuronal degeneration in the olfactory tubercle/piriform cortex alone is responsible for behavioral sensitization to PCP. Our hypothesis is that apoptosis in this region of the brain is a corollary of the mechanism underlying sensitization. That is, apoptosis is a subset of the many factors, possibly also including necrosis (Portera-Cailliau et al., 1997), that probably underlie the development of sensitization to PCP.

The mechanisms that underlie the selective vulnerability of the neurons in the olfactory cortex to the apoptotic process are largely unknown. The pharmacology of clozapine suggests that dopamine, serotonin, norepinephrine, and acetylcholine could be involved. PCP is known to interact with each of these systems (Johnson and Jones, 1990), but its effect on dopaminergic systems is most dramatic. Acute PCP or MK-801 increases dopamine (DA) turnover and release in the striatum (Miller and Abercrombie, 1996; Rao et al., 1990), nucleus accumbens (Hertel et al., 1996; Rao et al., 1990; Jentsch et al., 1997), and prefrontal cortex (Jentsch et al., 1997; Rao et al., 1990; Hertel et al., 1996). Consistent with these reports, PCP and MK-801 have been shown to increase, and at higher doses decrease, the firing of dopaminergic cells in the substantia nigra and ventral tegmental area (Freeman and Bunney, 1994; French, 1986; French and Ceci, 1990). In addition, one study showed that MK-801 increased DA turnover to a greater extent and at lower doses in the olfactory tubercle and piriform cortex than in the striatum and nucleus accumbens (Rao et al., 1990). Thus, it seems plausible that activation of dopaminergic systems may play a role in both the behavioral sensitization and neuronal degeneration caused by PCP. On the other hand, the failure to see evidence of apoptosis in the striatum, nucleus accumbens, and prefrontal cortex suggests that dopamine is not the only player.

The NMDA receptor itself is the other obvious potential player in that it could participate in at least two ways. First, with chronic blockade by PCP, it might be upregulated in a way that normal glutamatergic transmission might lead to excitoxicity. Second, as proposed by Olney et al. (1991), blockade of NMDA receptors on GABAergic interneurons could lead to disinhibition and overexcitation via a number of pathways. However, there are many areas of the brain with a high density of NMDA receptors that did not undergo apparent apoptotic degeneration. Further, in contrast to the recent report of acute MK-801 producing apoptosis in the retrosplenial and cingulate cortices (Zhang et al., 1996), we did not observe TUNEL-positive cells in these areas following chronic PCP. This suggests that Olney's model may not be sufficient to account for our observations. One interesting hypothesis is that apoptosis occurs in regions where there is both a high density of NMDA receptors and an excessive amount of dopamine. Based on our in situ hybridization experiment, the mRNA for the obligatory NR1 subunit is as dense in layer II of the olfactory tubercle and piriform cortex as it is anywhere in the brain. Further, this layer also receives a moderately dense dopaminergic input from the ventral tegmental area (VTA) (Bjorklund and Lindvall, 1984). Finally, dopamine has been shown to be neurotoxic in culture by both receptor and nonreceptor mechanisms (Alagarsamy et al., 1997; Hoyt et al., 1997; Shinkai et al., 1997). Interestingly, nontoxic concentrations of dopamine and glutamate are toxic when added together (Hoyt et al., 1997). Thus, it seems possible that a PCP-induced increase in DA levels along with either an upregulation of NMDA receptors or a chronic disinhibition could act in concert to produce the selective injury of cortical neurons observed in this study. Experiments using rats with discrete lesions of the VTA should be able to provide evidence relevant to this hypothesis.

In summary, we have provided evidence that a relatively short period of daily PCP administration produces a long-lasting behavioral sensitization that is revealed by a low-dose PCP challenge. This dosage regimen results in a regionally selective death of a subset of neurons that receive mesolimbic dopaminergic input. Morphological assessment of TUNEL-stained cells and electron microscopy suggests that some of these neurons die by an apoptotic mechanism, but an increase in GFAP-positive astrocytes suggests that a necrotic mechanism also may be involved. It is not yet clear whether this phenomenon is related to the process of vacuolization that is initiated by acute administration of NMDA antagonists, though both are prevented by the atypical antischizophrenic drug, clozapine. Based on the pharmacology of PCP and clozapine, we propose that the death of cells in the olfactory tubercle and piriform cortex is the result of a convergence of increased levels of dopamine and increased NMDA receptor activity, either through NMDA receptor upregulation or chronic disinhibition. Finally, we propose that the chronic administration of PCP produces a stable animal model suitable for investigating behavioral, cellular, and molecular mechanisms underlying changes associated with chronic NMDA receptor hypofunction.

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