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Biochemical Pharmacology 69 (2005) 1373–1383
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

# The role of NMDA receptor upregulation in phencyclidine-induced cortical apoptosis in organotypic culture

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Received 18 January 2005; accepted 24 February 2005

**Abstract**

Phencyclidine (PCP) is an *N*-methyl-D-aspartate receptor (NMDAR) antagonist known to cause selective neurotoxicity in the cortex following subchronic administration. The purpose of this study was to test the hypothesis that upregulation of the NMDAR plays a role in PCP-induced apoptotic cell death. Corticostriatal slice cultures were used to determine the effects of NMDAR subunit antisense oligodeoxynucleotides (ODNs) on PCP-induced apoptosis and NMDAR upregulation. NR1, NR2A or NR2B antisense ODNs were incubated alone or with PCP for 48 h. One day following washout, it was observed that PCP treatment caused an increase in NR1, NR2A and Bax polypeptides in the cortex, but had no effect on Bcl-xL. These increases were associated with an increase in cortical histone-associated DNA fragments. Co-incubation of PCP with either NR1 or NR2A antisense significantly reduced PCP-induced apoptosis, while neither NR2B antisense ODN nor NR1 sense ODN used as a control had an effect. This effect was exactly correlated with the ability of the antisense ODNs to prevent PCP-induced upregulation of NR subunit proteins and the pro-apoptotic protein, Bax. That is, western analysis showed that antisense ODNs directed against either NR1 or NR2A prevented PCP-induced increases in Bax in addition to preventing the upregulation of the respective receptor proteins. On the other hand, the NR2B antisense ODN had no effect on either NR2B protein or on Bax. These data suggest that NR1 and NR2A antisense ODNs offer neuroprotection from apoptosis, and that upregulation of the NR1 and NR2A subunits following PCP administration is at least partly responsible for the observed apoptotic DNA fragmentation.


*Keywords:* Phencyclidine; Apoptosis; Cortex; *N*-Methyl-D-aspartate receptor; Antisense; Bax

## 1. Introduction

Blockade of *N*-methyl-D-aspartate (NMDA) receptors is known to be neurotoxic, but the underlying mechanism is uncertain. This laboratory has shown that subchronic administration of phencyclidine (PCP) in perinatal rats causes a selective cortical neurotoxicity characterized by DNA fragmentation [1], while acute administration of NMDA antagonists including ketamine, dizocilpine and PCP have been reported to cause a much less selective neurotoxicity characterized by cupric silver staining [2]. This may suggest that acute and chronic blockade of NMDA receptors, particularly during development, may cause different types of cell death, perhaps mediated by different mechanisms. The underlying mechanisms of this neurotoxicity are unknown, though it is possible that reduction of intracellular calcium by blockade of "NMDAergic" tone could be sufficient to trigger cell death [3,4].

An alternative hypothesis is that chronic blockade of NMDA receptors could lead to a compensatory upregulation of NMDA receptors, resulting in cell death by increasing intracellular calcium or by some other unidentified mechanism. Functional native NMDA receptors are believed to be heteromers composed of NR1 and NR2 (A–D) or NR3 subunits [5–11]. The variance in subunit composition results in differential conductance properties, voltage-gating and pharmacological properties. Based on previous experiments that have demonstrated that antisense ODNs specific for NMDA receptor were able to prevent receptor upregulation in response to physiologic or pharmacological challenges [12–14], we used this antisense strategy to test the hypothesis that upregulation of

*Abbreviations:* NMDA, *N*-methyl-D-aspartate; PCP, phencyclidine; ODN, oligodeoxy-nucleotide; NR, *N*-methyl-D-aspartate receptor; TUNEL, terminal d-UTP nick end labeling; DIV, days in vitro

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0006-2952/$ – see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.bcp.2005.02.013

NR1, NR2A and/or NR2B was necessary for PCP-induced cell death.

For this purpose, we utilized organotypic culture of corticostriatal slices under conditions in which PCP has previously been demonstrated to be selectively neurotoxic to the cortex [15]. It was observed that treatment of these slices resulted in a time- and washout-dependent cortical DNA fragmentation (a marker of apoptosis). This was associated with an upregulation of NR1 and NR2A polypeptide, while the NR2B subunit was unaffected. Co-incubation of PCP with an antisense ODN specific to the NR1 mRNA prevented both PCP-induced NR1 upregulation and cortical DNA fragmentation. No effect of either the NR1 sense strand control ODN or an NR2B antisense ODN was observed. However, an NR2A antisense ODN had an effect similar to that observed with the NR1 antisense ODN, in that it prevented both cell death and PCP-induced upregulation of the NR2A protein.

## 2. Methods

### 2.1. Animals

Timed pregnant female Sprague–Dawley rats were obtained on day 14 of pregnancy from Charles River. They were housed individually with a regular 12-h light:12-h dark cycle (lights on at 07:00 h. off at 19:00 h) with food and water available ad libitum. Within 12 h of parturition, the pups from several dams were combined and then randomly cross-fostered to lactating dams in litters consisting of eight pups each. On postnatal day (PN) 7, the pups were killed and their brains removed and processed for slice culture as described below.

In all experimental procedures, all efforts were made to minimize animal suffering. The minimal number of animals necessary to produce reliable scientific results was used. There are no feasible alternatives to the use of live animals in this study.

The protocol under which this study was performed was approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

### 2.2. Drugs and other materials

PCP was acquired from the National Institute on Drug Abuse (Rockville, MD, USA). PCP was dissolved in Dulbecco's Modified Eagle's Medium (DMEM). The medium and horse serum were purchased from Invitrogen (Grand Island, NY). Antisense ODNs specific for NMDA receptor NR1, NR2A and NR2B were obtained from Sigma Genosys Biotechnologies, Inc. (The Woodlands, TX). All other reagents were of the highest chemical purity available from commercial sources.

### 2.3. Organotypic slice culture

Organotypic slice cultures were prepared from 7-days-old neonatal Sprague–Dawley rats essentially according to methods previously described [16]. The brains were sectioned down the midline. The brain tissues were then cut to a thickness of 400 μm by a tissue slicer (BRINKMANN, The Mickle Laboratory Engineering Co.). These slices were taken anterior to a cut made through the frontal cortex (Fr 3) and the striatum-nucleus accumbens complex at the level of the anterior commissure according to "Atlas of the Developing Rat Nervous System" [17]. The slices were maintained for 7–8 days in vitro (DIV) on a porous and translucid membrane (0.4 μm, Culture Plate Insert, MILLIPORE Co, Bedford, MA) at the interface between medium and a $CO_2$-enriched atmosphere as described previously [16,18,19]. The culture medium was a mixture of 50% MEM, 25% horse serum and 25% Hank's solution buffered to pH 7.2 by addition of 5 mM Tris and 4 mM $NaHCO_3$. Penicillin and streptomycin were added to the culture medium. The medium was changed every 2–3 DIV. PCP (3 μM) or PCP plus individual antisense ODNs (2 μM) were added for various times (most typically, 48 h) on DIV 4 and then washed out for 2–24 h (most typically 24 h) with serum-containing medium before harvesting the tissue.

### 2.4. TUNEL assay

This assay is widely used to assess apoptosis in situ. It relies on the detection of fragmented DNA strands, but because fragmentation can occur via non-apoptotic mechanisms, it is not absolutely specific for apoptosis. Following treatment with PCP, the slices were rinsed with PBS, fixed with ice-cold (4 °C) 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, and processed for evaluation of nuclei containing fragmented DNA in situ. Terminal deoxynucleotidyl transferase (TdT), a template-independent polymerase, was used to incorporate biotinylated nucleotides at sites of DNA breaks as previously described [20]. After processing each slide as described, the slides were treated with Hoechst 33258 (bisbenzimide, 0.1 μg/ml) to stain all nuclei of cells that were not TUNEL-positive [21]. The TUNEL- and Hoechst-positive cells were then photographed with the use of an Olympus light microscope equipped with epifluorescence (excitation wavelength of 365 nm for Hoechst 33258). The percentage of TUNEL-positive cells was not estimated in this study as this has been published previously [22]. A single photograph is presented to illustrate the relative distribution and magnitude of the DNA fragmentation caused by PCP in this paradigm.

### 2.5. Fragmented DNA detection by ELISA

Nucleosomal DNA fragmentation is characteristic of apoptotic nuclei [23]. The presence of fragmented DNA

was assessed by measuring DNA associated with nucleosomal histones using a specific two-site ELISA with an antihistone primary antibody and a secondary anti-DNA antibody according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN). Briefly, the dorsal cortical portion of the slice was homogenized in 3 ml of lysis buffer with approximately 15 strokes in a glass/glass tissue homogenizer (Wheaton Tenbroeck, Fisher Scientific, Houston, TX) and incubated for 30 min at room temperature. After centrifugation, the supernatants (cytosol containing low-molecular mass, fragmented DNA) were diluted 1:2 (v/v) with lysis buffer. Protein concentration was determined after dilution of an aliquot of the supernatant using the bicinchoninic/BCA Protein Reagent (Pierce, Rockfrd, IL, USA). Twenty microliters from each sample was transferred to a plate reader well pre-coated with antihistone antibody to which 80 μl of immunoreagent mix, including the secondary antibody, was added. After incubation and washes, the wells were treated with the chromogen 2,2′-azinobis(3-ethylbinzthiazoline) sulfonic acid as a substrate. The intensity of the color that develops was measured at 405 nm, while that at 490 nm was used as a blank (reference wavelength). The optical density of each sample calculated as above was normalized by each sample's protein concentration. This value was taken to represent relative histone-associated DNA fragmentation, a common index of apoptosis.

### 2.6. Western blot analysis

For quantification of protein expression levels, Western blot analysis was used. The frontal cortex and striatum-nucleus accumbens portions of organotypic cultures were quickly dissected out. The tissue was sonicated in 0.5 ml of lysis buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in normal PBS; pH 6.8) containing the protease inhibitors PMSF (phenylmethlylsulfonyl fluoride, 10 μl/ml) and aprotinin (30 μl/ml). The homogenate was centrifuged at 14,000 × *g* for 10 min. The supernatant was stored at −80 °C. The protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (10 μg) from the solubilized fraction (10–20 μg) was loaded on each lane and run on SDS-polyacrylamide gels with a Tris–glycine running buffer system and then transferred to a polyvinylidene difluoride membrane (0.2 μm) in a Mini Electrotransfer Unit (Bio-Rad, Hercules, CA, USA). The blots were probed with an anti-Bcl-xL (1:2000, polyclonal, Santa Cruz), anti-Bax (1:3000, polyclonal, Santa Cruz Biotechnology), anti-NR1 (1:300, monoclonal, PharMingen,), anti-NR2A (1:200, polyclonal, Santa Cruz Biotechnology), anti-NR2B (1:200, polyclonal, Santa Cruz Biotechnology) and anti-actin (1:3000, monoclonal, Chemicon). Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat IgG using the enhanced chemiluminescence Western blotting detection reagents (Amersham Bioscience). The bands corresponding to Bax, Bcl-xL, NR1, NR2A, NR2B and β-actin were scanned and densitometrically analyzed by an automatic image analysis system (Alpha Innotech Corporation, San Leandro, CM). These quantitative analyses were normalized to β-actin (after stripping) and expressed as mean ± S.E. One-way ANOVA was used to compare levels of each protein among different treatment groups.

### 2.7. Application of antisense ODNs

To test whether the administration of antisense ODNs targeted to specific NMDA receptor subunits could block the upregulation of the NMDA receptor subunit synthesis, antisense ODNs targeted to NR1, NR2A and NR2B subunits were used in the corticostriatal organotypic cultures. The ODNs used to alter synthesis of the NR1 subunit were as follows. An 18-mer antisense ODN corresponding to nucleotide 4–21 (5′-CAGCAGGTGCATGGTGCT) of the NR1 subunit mRNA (which directly follows the translation initiation codon) used to prevent synthesis. The sequences of the antisense ODN used for NR2A was 5′-GTAGCTCTTTTAGGTGAGTCC (corresponding to aa 915–921). For NR2B we used 5′-CCTTCAGCTGCAGGTTACCAAATG (corresponding to aa 979–986). As controls, we used the corresponding sense ODN (5′-AGCACCATGCACCTGCTG) for NR1 or a scrambled missense ODN (5′-GCAGGCTAGTGGTGCTCATG) whose AT/GC ratio is similar to that of the NMDAR1, NR2A and NR2B antisense ODNs.

These sequences were chosen to target the 5′ coding region of the NMDAR1, NR2A and NR2B subunit mRNAs, which are highly conserved in mammals (>99%). For all of the ODNs, the GeneBank database was searched to assure that other genes do not share homologous regions. To increase stability, phosphorothioate bonds were incorporated at terminal nucleotides at the 5′ and 3′ ends. The ODNs were synthesized and purified by high-performance liquid chromatography at Sigma Genosys Biotechnologies, Inc. (The Woodlands, TX). The antisense sequences have been proven effective and specific in previous studies [12,24,25].

### 2.8. General experimental protocol

The experimental protocol was as follows: the control cultures (group 1) were maintained in normal culture medium for 6 days. For groups 2–6, organotypic cultures were maintained in normal culture conditions for 3 days. Group 2 was then treated with PCP for 48 h and PCP was washed out and replaced with serum-containing medium for another 24 h. Groups 3, 4, 5 and 6 were treated with PCP plus 2 μM antisense ODN for NR1 (group 3), sense ODN for NR1 (group 4), antisense ODN for NR2A (group

5) and antisense ODN for NR2B (group 6) for 48 h. As with group 2, PCP and the ODNs were washed out and replaced with serum-containing medium for another 24 h before further processing after DIV6.

### 2.9. Statistical analyses

All data was analyzed using a parametric one-way analysis of variance with either the Holm–Slidak method or Tukey's post hoc analysis for individual differences. The null hypothesis was rejected at a probability level of $p < 0.05$.

## 3. Results

Previous research in this laboratory has demonstrated that subchronic administration of PCP on PN 7, 9 and 11 resulted in a selective cortical neurotoxicity as assessed by TUNEL staining on PN 12. In order to facilitate the study of potential mechanisms underlying this toxicity, we established an organotypic culture of corticostriatal slices that could be used to study the mechanism underlying the cortical selectivity of the effects of PCP [22]. An example of the cortical selectivity of PCP neurotoxicity in this model is shown in Fig. 1. Here, DNA fragmentation as assessed by TUNEL staining is shown in the frontal cortex (panels A and B) and striatum (panels C and D) following control (panels A and C) or PCP treatment (3 μM) for 48 h and a 24 h washout (panels B and D). An increase in DNA fragmentation was observed in the frontal cortex (B) but not in the striatum (D) following this PCP treatment regimen. These results are very similar to obtained previously [15,22].

Following these initial experiments, we wanted to optimize the PCP treatment and washout times. Because we had previously observed that treatment of dissociated primary cortical neurons with PCP increased their subsequent sensitivity to NMDA [1], we hypothesized that PCP treatment resulted in upregulation of NMDA receptors and

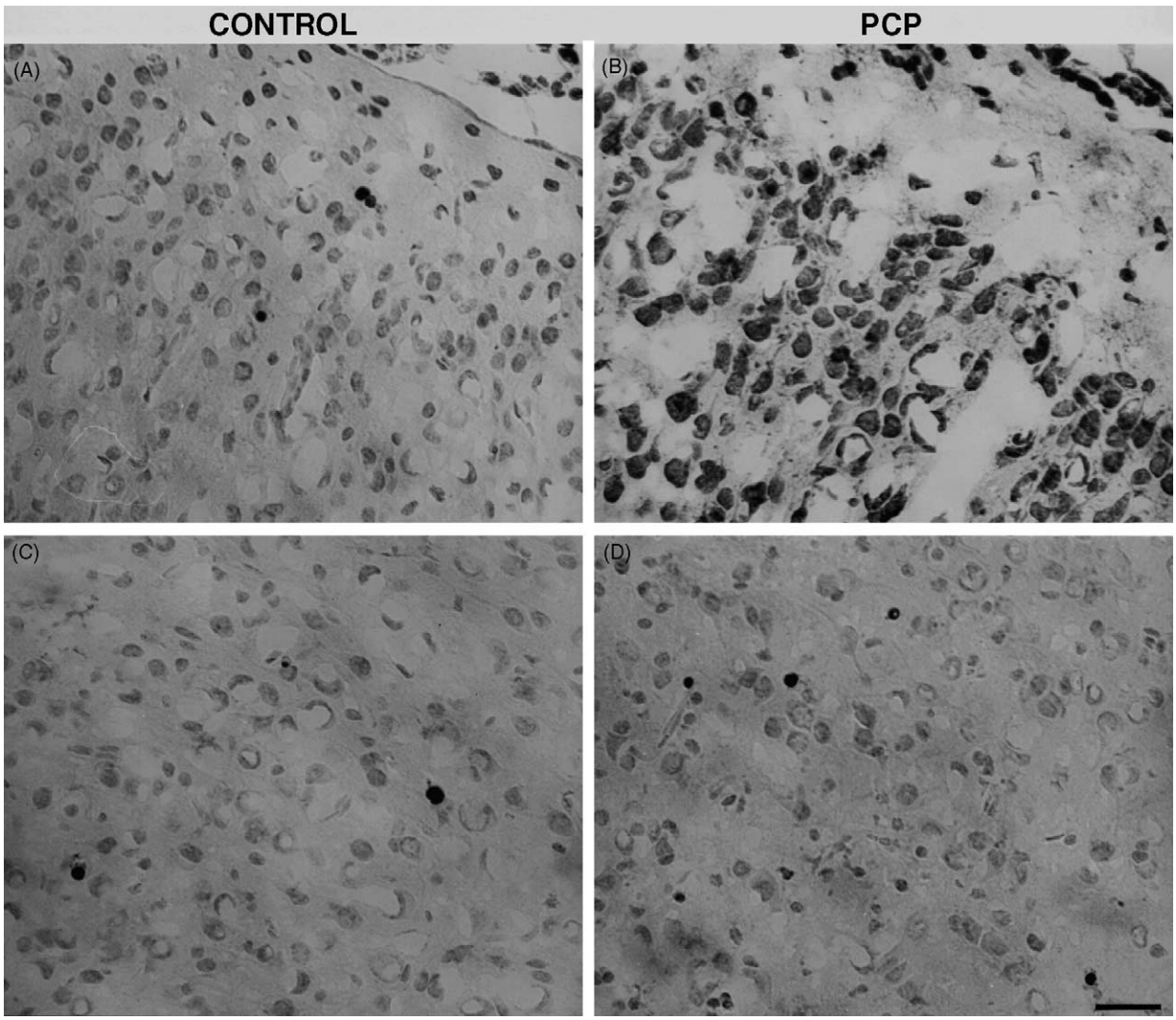


Fig. 1. DNA fragmentation assessed by TUNEL staining in the frontal cortex (panels A and B) and striatum (panels C and D) following control (panels A and C) or PCP treatment (3 μM) for 48 h and a 24 h washout (panels B and D).

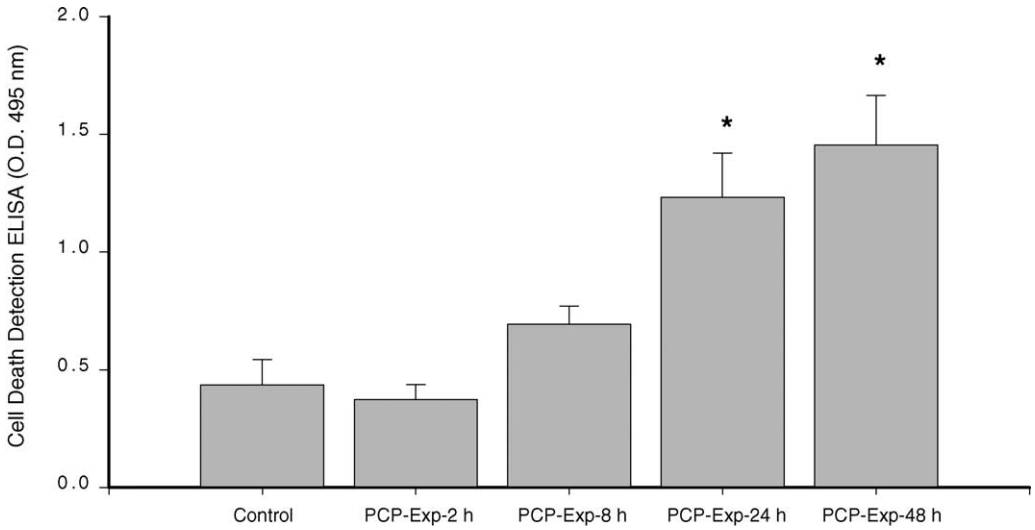


Fig. 2. Induction of PCP-induced cortical cell death as a function of time of exposure. Corticostriatal slices were incubated with 3 μM PCP for the indicated time and apoptotic cell death was measured by a histone-DNA double antibody ELISA (*$p < 0.05$ vs. control).

that removal of PCP would be necessary for neurotoxicity to occur. First, using a 24 h washout period, we examined the PCP treatment time required for apoptotic DNA fragmentation. As illustrated in Fig. 2, PCP treatment of corticostriatal slices for either 2 or 8 h resulted in no histone-associated DNA fragmentation. However, continued treatment for either 24 or 48 h increased this marker of apoptosis in the cortex significantly above control. Fig. 3 shows that following a 48 h treatment period, washout for at least 8 h is necessary for DNA fragmentation and a 24 h washout results in significantly greater neurotoxicity.

In order to test the hypothesis that PCP-induced neurotoxicity involved an upregulation of the NMDA receptor, we used polyacrylamide gel electrophoresis and western analysis to measure the effect of PCP on the NR1 subunit polypeptide and on Bax, a pro-apoptotic protein that we had previously demonstrated to be upregulated by PCP treatment in vivo [26]. We also measured Bcl-xL, an antiapoptotic factor that acts to negate the formation of mitochondrial Bax–Bax homodimers which allow the release of cytochrome *c*, a protein known to activate caspases in many forms of apoptosis. PCP had no affect on Bcl-xL (arbitrary densitometry values: control, $433 \pm 18$; PCP, $451 \pm 18$). However, PCP treatment significantly increased the proapoptotic factor Bax. Fig. 4 shows representative western blots of the effects of PCP in the presence and absence of sense and antisense ODNs on

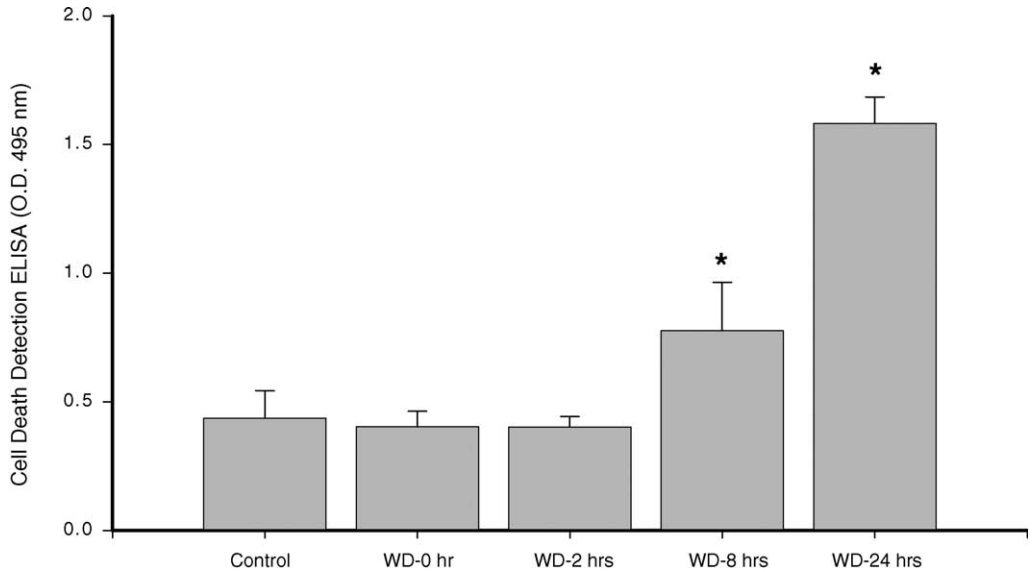


Fig. 3. Induction of PCP-induced cortical cell death as a function of washout time. The slices were exposed to PCP for 48 h. After PCP washout, slices were kept in serum-containing media for 0, 2, 8 or 24 h before being processed for histone-associated DNA fragmentation using the cell death ELISA kit (*$p < 0.05$ vs. control).

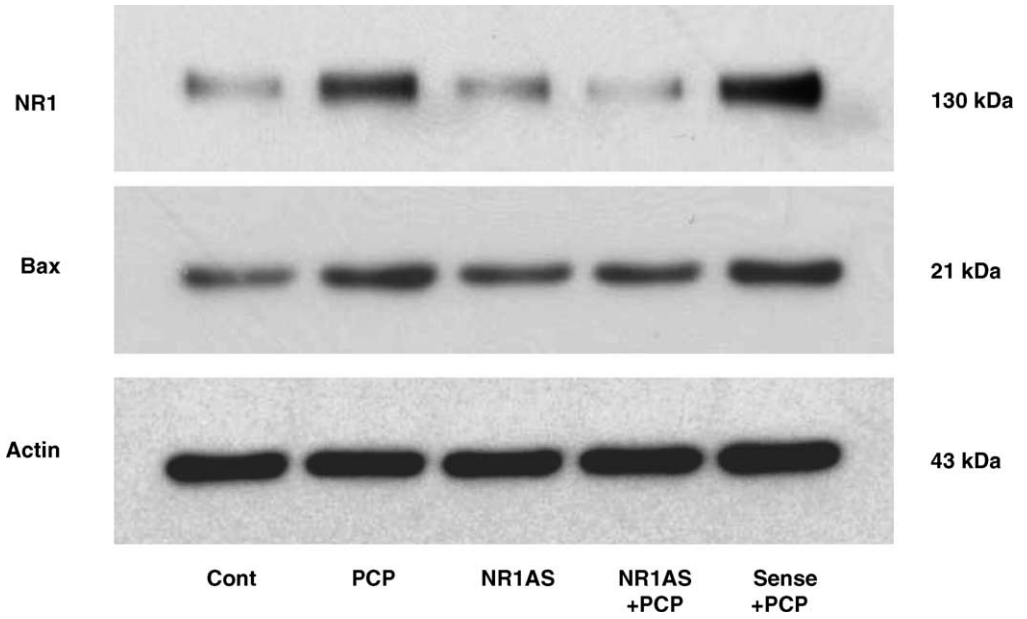


Fig. 4. Representative western blot analysis of NR1 and Bax in cortical tissue taken from organotypic slices following treatment with PCP in the presence or absence of NR1 sense and NR1 antisense oligonucleotides. Actin levels were also measured and used as a loading control.

NR1 and Bax in the cortex. Fig. 5 (left panel) summarizes in graphical form the results of several experiments that analyzed the amount of NR1 protein (relative to actin) in both the cortical and striatal portions of the slice. PCP treatment approximately doubled the relative amount of NR1 polypeptide in both the cortex and striatum when used alone or in combination with the NR1 sense or missense ODNs used as controls. On the other hand, co-incubation with 2 μM NR1 antisense ODN, which had no effect on its own, completely prevented the ability of PCP to increase NR1 in both brain regions. The lack of effect AS treatment on basal polypeptide levels is consistent with a rather slow turnover of basal levels of NR1 protein as well as the stabilization of the NR1 receptor subunits in the membrane by NR2 subunits [27,28].

The sense and antisense ODNs had a similar effect on PCP-induced Bax in the cortex (Fig. 5, right panel), perhaps suggesting that the upregulation of NR1 and Bax by PCP were mechanistically related. Somewhat surprisingly, however, PCP treatment did not cause an increase in striatal Bax protein, despite increasing striatal NR1. Neither the basal levels found under control nor PCP treatment conditions were altered by either sense or antisense ODNs in the striatum.

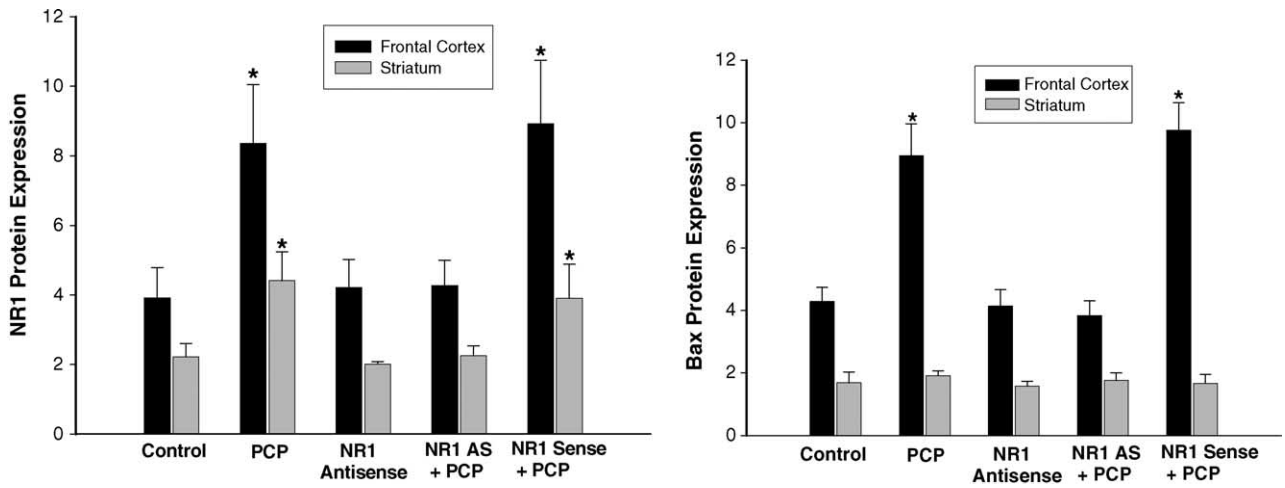


Fig. 5. *Left*: Densitometric analysis of Western blot data measuring the level of NR1 protein expression in the frontal cortex and striatum following treatment with PCP in the presence or absence of NR1 sense or NR1 antisense oligonucleotides. Values given are the relative density of NR1 normalized to actin ($^{\star}p < 0.05$ vs. control). *Right*: Densitometric analysis from Western blot data measuring the level of Bax protein expression (normalized to actin) in the frontal cortex and striatum following treatment with PCP in the presence or absence of NR1 sense or NR1 antisense oligonucleotides. Values given are normalized to actin ($^{\star}p < 0.05$ vs. control).

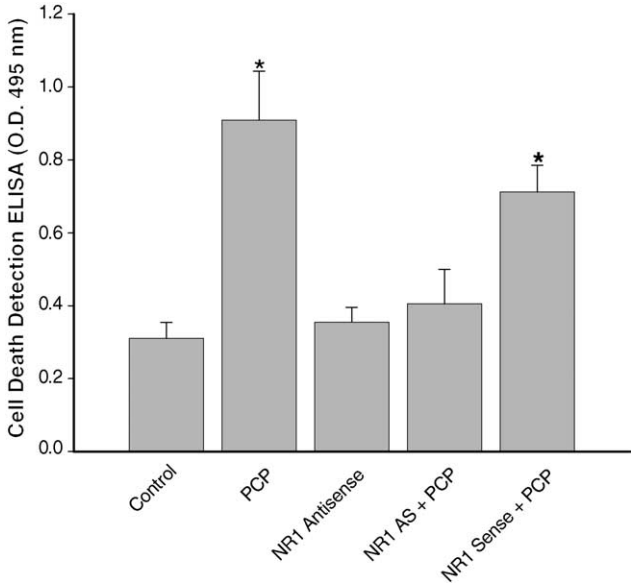


Fig. 6. The effect of PCP treatment in the presence or absence of NR1 sense or antisense oligonucleotides on histone-associated DNA fragmentation in the cortex as assessed by ELISA ($^{*}p < 0.05$ vs. control).

To determine the relationship between NR1, Bax and PCP-induced cortical cell death, we measured the effect of the NR1 ODN on PCP-induced histone-associated DNA fragmentation by a two antibody ELISA (Fig. 6). PCP treatment caused an approximate three-fold increase in this measure of cortical apoptosis and this was completely prevented by co-incubation with the NR1 antisense ODN, which had no effect of its own on this measure. Commiserate with the effects of NR1 sense ODN on NR1 and Bax protein levels, this control ODN did not significantly affect the PCP-induced increase in cell death.

Because functional NMDA receptors are composed of both NR1 and NR2 subunits [7,8], we extended these observations to include both NR2A and 2B, the most prominent forms NR2 subunits in the forebrain of perinatal rat pups [29]. Fig. 7 (left panel) shows that PCP treatment increased the relative level of the NR2A polypeptide in the cortex, but not in the striatum. Further, co-incubation with 2 μM NR2A antisense ODN prevented this effect of PCP, while having no effect of its own. This suggests that the failure of PCP to upregulate the NR2A polypeptide could underlie the relative invulnerability of the striatum to PCP in this model.

As observed in the previous set of experiments, PCP again caused a significant increase in cortical, but not striatal, Bax levels (Fig. 7, right panel). As observed with the NR1 antisense ODN, co-incubation of NR2A antisense ODN PCP prevented the increase of Bax in the cortex caused by PCP. The NR2A antisense ODN, either alone or with PCP, had no effect on striatal Bax. To determine whether the effect of NR2A antisense ODN on the NR2A receptor and Bax was correlated with cell death, we measured the effect of this ODN on PCP-induced DNA fragmentation (Fig. 8). These data demonstrate that the NR2A antisense ODN prevented PCP-induced apoptosis in the cortex, but had no effect on this measure when incubated alone.

To determine whether upregulation of the NR2B protein could play a role in PCP-induced cell death we measured the effect of NR2B antisense ODN on the NR2B polypeptide and on Bax in the presence and absence of PCP. Unlike the effect of PCP on NR1 and 2A, PCP did not affect the relative levels of NR2B protein in the cortex (data not shown). Further, the NR2B antisense had no effect on cortical Bax levels (Fig. 9) or on PCP-induced DNA fragmentation (Fig. 10).

In summary, incubation of corticostriatal slices with PCP for 48 h resulted in an increase in NR1 protein in the cortex and striatum and an increase in NR2A protein in the cortex, but not the striatum. PCP had no effect on the relative amounts of NR2B protein in either area. PCP increased Bax and apoptotic cell death in the cortex only, an effect correlated with the upregulation of NR1 and NR2A. These data are correlated with effects of NMDA

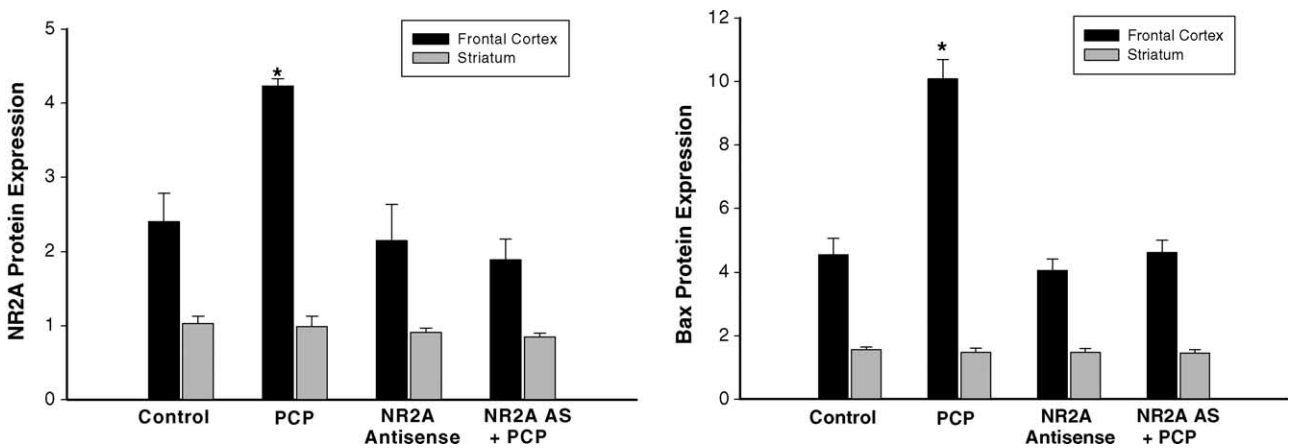


Fig. 7. *Left*: Densitometric analysis of NR2A protein expression in Western blot experiments. The values reported are normalized to actin densities on the same gels, which were used as loading controls. ($^{*}p < 0.05$ vs. control). *Right*: Densitometric analysis of the pro-apoptotic protein Bax (relative to actin) in the frontal cortex and striatum following PCP treatment in the presence and absence of NR2A antisense oligonucleotide ($^{*}p < 0.05$ vs. control).

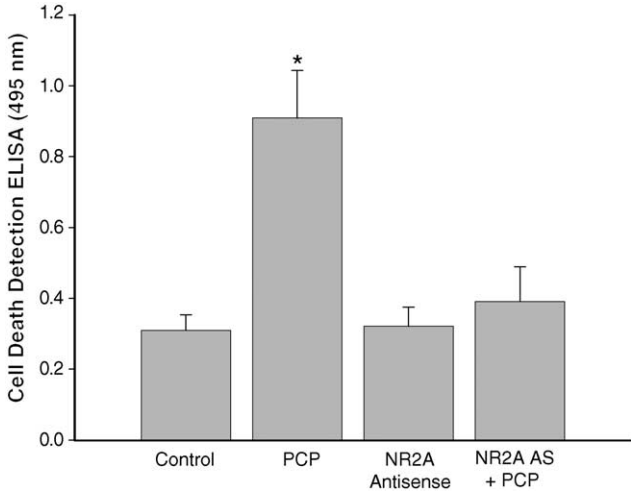


Fig. 8. The effect of PCP treatment in the presence or absence of NR2A antisense oligonucleotides on histone-associated DNA fragmentation in the cortex as assessed by ELISA ($^{*}p < 0.05$ vs. control).

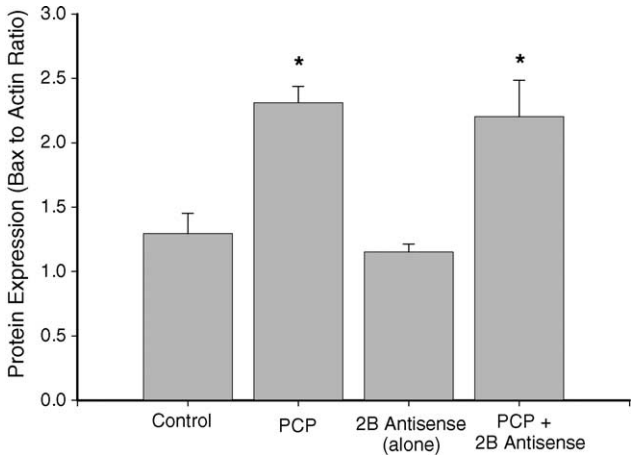


Fig. 9. Densitometric analysis of the pro-apoptotic protein Bax (relative to actin) in the frontal cortex following PCP treatment in the presence and absence of NR2B antisense oligonucleotides ($^{*}p < 0.05$ vs. control).

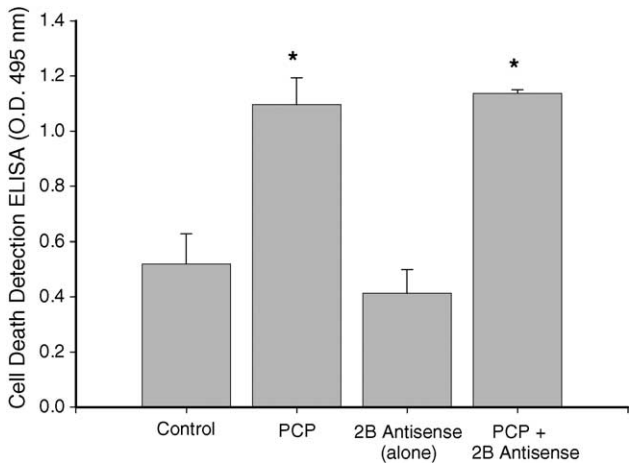


Fig. 10. The effect of PCP treatment in the presence or absence of NR2B antisense oligonucleotides on histone-associated DNA fragmentation in the cortex as assessed by ELISA ($^{*}p < 0.05$ vs. control).

receptor subunit specific antisense ODNs on both PCP-induced increases in Bax and apoptosis.

## 4. Discussion

Together, these data suggest that PCP-induced apoptosis in this preparation requires upregulation of both NR1 and NR2A polypeptides. Although PCP caused an upregulation of the NR1 subunit in the striatum, this was insufficient to cause either an increase in Bax or evidence of apoptosis. We propose that upregulation of both NR1 and NR2A results in increased NMDA receptor function in the cortex, which ultimately leads to apoptotic cell death. It appears that receptor upregulation also triggers an increase in the pro-apoptotic protein Bax, which is probably involved in PCP-induced cell death, though no direct evidence for this was obtained in the present experiments. Receptor upregulation alone may not cause an increase in calcium ion flux through the NMDA channel. Thus, these data also suggest either that there is sufficient glutamatergic tone in the corticostriatal slice to activate these receptors or that PCP increases glutamate release in the cortex as previously observed in vivo [30]. Suppression of spontaneous electrical activity in organotypic cortical slices by NMDA antagonism strongly supports the notion that intrinsic ''NMDAergic'' tone is present in this preparation [31]. Neither the mechanism of receptor upregulation nor the mechanism of PCP-induced cell death is clear at this time. Our previous observation that that treatment of corticostriatal slices with PCP resulted in a partial loss of presynaptic markers in the striatum [22] suggests that the relative loss of striatal glutamatergic tone may also play a role in the relatively protected status of the striatum.

The observation that PCP treatment upregulates NMDA receptors is consistent with previous observations by this laboratory as well as others. For example, PCP administration in vivo results in increased NR1 immunoreactivity in the adult cortex and striatum [32], increased cortical binding of $^{3}$H-MK-801 [33] and increased levels of cortical levels of NR1 mRNA and polypeptide [1]. PCP-induced increases in cortical Bax have also been previously reported [1,26]. That the effect of PCP on apoptosis is at least partially mediated by increased NMDA receptor activity is supported by reports that PCP treatment of dissociated forebrain cultures resulted in an increase in NR1 mRNA (in situ hybridization) and an increased sensitivity to the neurotoxic effects of NMDA itself (following PCP washout, [21]). Additional support for this hypothesis is derived from our previous observation that NMDA caused cell death in a dissociated culture preparation via a mechanism that involved an increase in Bax subsequent to an increase in reactive oxygen species and nuclear transport of nuclear factor-κB (NF-κB) [15].

The mechanism proposed here differs from that previously proposed to account for cell death following treatment with NMDA receptor antagonists. There is substantial data suggesting that intracellular calcium levels are critical for maintaining a host of processes related to cell survival and that either increases or decreases in intracellular calcium concentration can be detrimental [3]. Several laboratories have proposed that NMDA antagonists cause cell death by blocking normal calcium flux through the NMDA receptor, thereby altering a critical calcium set point (e.g. [4]). However, while this hypothesis is very appealing, the direct data supporting this does not rule out other mechanisms. Takadera et al. [4] demonstrated that MK-801, a non-competitive NMDA channel blocker similar to PCP, decreased intracellular calcium concentration from about 100 nM to 50 nM over the course of a 5-min study. This study then demonstrated that MK-801 treatment for 24 h caused an increase in caspase-3 activation (which was used as a marker of apoptosis). Thus, the MK-801-induced reduction in intracellular calcium was postulated to cause apoptosis. Hwang et al. [43] used a similar primary cortical culture in which they demonstrated that treatment with MK-801 for 48 h induced release of the cytosolic enzyme, lactate dehydrogenase. This increase in lactate dehydrogenase release (which is not specific for apoptosis) was antagonized by high $K^+$, which in turn, could be antagonized by cobalt and L-type calcium channel antagonists. While these data do indeed support the notion that blockade of NMDA receptor gated ion channel could alter the intracellular calcium set point, they do not prove that this mechanism is solely responsible for PCP (or MK-801)-induced apoptosis, particularly in vivo or in organotypic slices. On the other hand, recent data (Wang et al. [44]) indicate that treatment of forebrain primary cultures with ketamine (a less, potent, non-competitive NMDA receptor ion channel blocker, used as a general pediatric anesthetic) also results in a dose-related increase in neurotoxicity and an up-regulation of NMDA receptor NR1 subunit. Co-incubation of ketamine with NR1 antisense significantly reduced ketamine-induced apoptosis.

A significant problem in interpreting these experiments is that many markers that are generally considered to be relatively specific for apoptosis, including terminal d-UTP nick end labeling (TUNEL) of DNA fragments, are not completely specific for apoptosis under all conditions. Further, neuronal cell death under certain conditions may consist of both necrosis and apoptosis [34] or of a process that has certain features of both [35]. Nuclear morphology is often said to be the gold-standard for apoptosis. We have used this technique in the past to demonstrate that PCP treatment does cause nuclear condensation and fragmentation in vivo [1] and in vitro using the organotypic slice [22], but this method is not practical for routine quantitation. In the present study we have used the TUNEL method to provide visual evidence for the distribution of DNA fragmentation within the slice and a double antibody ELISA to quantitate histone-associated DNA fragments, thought to represent the internucleosomal cleavage of DNA occurring during apoptosis. As a secondary marker, we measured the pro-apoptotic protein, Bax, by western blotting. Thus, while it is possible that PCP treatment in this paradigm may cause necrosis, it is reasonable to assume that increased histone-associated DNA fragmentation, along with increased Bax levels can be interpreted in the context of apoptosis.

Of particular interest to the data at hand are the possible mechanisms by which PCP treatment could upregulate NMDA receptors. Surprisingly, there is not an abundance of literature concerning this issue, but recently it has been demonstrated that the distal region of the NR1 promoter contains an active NF-κB site, which interestingly is developmentally regulated and appears to bind Sp3/Sp1 somewhat better than NF-κB subunits [36]. NR1 gene expression also appears to be under the control of protein kinase A and the cAMP response element (CRE) [37]. The latter study demonstrated that forskolin increased NR1 mRNA and polypeptide to a similar extent in primary cortical neurons and immunoprecipitation experiments found that CREB was bound to the NR1 gene following activation of adenylyl cyclase. This regulatory pathway could be important here because of the well-known ability of PCP to increase the release of DA along with glutamate in the prefrontal cortex [30]. There is less information on the regulation of NR2 proteins, but a recent study has shown that activation of the extracellular signal-regulated kinase (ERK) 1/2 pathway by neurotrophin-4/5 resulted in increased NR2A protein as well as an increase in phospho-NR2A and NR2B [38]. These authors also showed that overactivation of the trk B pathway by NT-4/5 results in cell death.

In recent years a number of ‘‘physiological’’ regulators have been described that upregulate NMDA receptors. For example, focal ischemic lesion of the visual cortex results in an increase in NR1 protein in the area surrounding the lesion [39]. Other ‘‘physiologic’’ literature suggests that regulation of NMDA receptors is likely to be much more complex that is currently appreciated. For example, knockout of dopamine $D_4$ receptors results in an upregulation of $D_2$ and NMDA receptor binding [40]. Further, knockout of postsynaptic density-93 results in a markedly increased response to hypoxia/ischemia that involves increases in NR2A, nNOS and PSD-95 [41]. Finally, blockade of NMDA receptors is likely to modulate many other systems that could eventually feedback and alter certain aspects of the balance between apoptosis and survival. For example, a recent study found that chronic treatment of rats with MK-801 resulted in marked changes in genes for mitogen activated protein kinase 1, acyl Co-A synthetase, apoliprotein D, protein kinase C β I and II, protein tyrosine phosphatase I β, metabotropic glutamate receptors 2/3, adenyl cyclase II and dopamine $D_{1A}$ and $D_2$ receptors [42].

In summary, this study has demonstrated that incubation of corticostriatal slices with PCP results in a significant increase in histone-associated DNA fragmentation in the cortex, but not in the striatum. Experimental evidence using antisense ODNs for the NR1, 2A and 2B subunits is presented that strongly suggests that PCP-induced apoptosis depends on the upregulation of the NR1 and NR2A subunits in the cortex. The lack of effect of PCP in this paradigm in the striatum may be due to the lack of concomitant upregulation of an NR2 subunit to complement the upregulation of the NR1 subunit.

## Acknowledgment

This work was supported by NIH grant DA-02073 and MH-63871 and by the National Toxicological Program P-00636 and E2155.01.

## References

[1] Wang C, McInnis J, Ross-Sanchez M, Shinnick-Gallagher P, Wiley JL, Johnson KM. Long-term behavioral and neurodegenerative effects of perinatal phencyclidine administration: implications for schizophrenia. Neuroscience 2001;107:535–50.

[2] Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, et al. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. Science 1999;283:70–4.

[3] Koike T, Martin DP, Johnson Jr EM. Role of $Ca^{2+}$ channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal $Ca^{2+}$ determine nerve growth factor dependence of sympathetic ganglion cells. Proc Natl Acad Sci USA 1989;86:6421–5.

[4] Takadera T, Matsuda I, Ohyashiki T. Apoptotic cell death and caspase-3 activation induced by *N*-methyl-D-aspartate receptor antagonists and their prevention by insulin-like growth factor I. J Neurochem 1999; 73:548–56.

[5] Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S. Molecular cloning and characterization of the rat NMDA receptor. Nature 1991;354:31–6.

[6] Kutsuwada T, Kashiwabuchi N, Mori H, Sakimura K, Kushiya E, Araki K, et al. Molecular diversity of the NMDA receptor channel. Nature 1992;358:36–41.

[7] Meguro H, Mori H, Araki K, Kushiya E, Kutsuwada T, Yamazaki M, et al. Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature 1992;357:70–4.

[8] Monyer H, Sprengel R, Schoepfer R, Herb A, Higuchi M, Lomeli H, et al. Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science 1992;256:1217–21.

[9] Nakanishi S. Molecular diversity of glutamate receptors and implications for brain function. Science 1992;258:597–603.

[10] Buller AL, Larson HC, Schneider BE, Beaton JA, Morrisett RA, Monaghan DT. The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. J Neurosci 1994;14:5471–84.

[11] Sun L, Margolis FL, Shipley MT, Lidow MS. Identification of a long variant of mRNA encoding the NR3 subunit of the NMDA receptor: its regional distribution and developmental expression in the rat brain. FEBS Lett 1998;441:392–6.

[12] Roberts EB, Meredith MA, Ramoa AS. Suppression of NMDA receptor function using antisense DNA block ocular dominance plasticity while preserving visual responses. J Neurophysiol 1998; 80:1021–32.

[13] Lai SK, Wong CK, Yang MS, Yung KK. Changes in expression of *N*-methyl-D-aspartate receptor subunits in the rat neostriatum after a single dose of antisense oligonucleotide specific for *N*-methyl-D-aspartate receptor 1 subunit. Neuroscience 2000;98: 493–500.

[14] Lai SK, Ng TK, Lau WK, Yang MS, Wong CK, Chan YS, et al. Selective knockdown of gene expression of *N*-methyl-D-aspartate receptor one ameliorates parkinsonian motor symptom in 6-hydroxydopamine-lesioned rats. Neurochem Int 2004;45:11–22.

[15] McInnis J, Wang C, Anastasio N, Hultman M, Ye Y, Salvemini D, et al. The role of superoxide and nuclear factor-kappaB signaling in *N*-methyl-D-aspartate-induced necrosis and apoptosis. J Pharmacol Exp Ther 2002;301:478–87.

[16] Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of nervous tissue. J Neurosci Methods 1991;37:173–82.

[17] Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates, Second edition. New York: Academic Press, 1986.

[18] Muller D, Stoppini L, Wang C, Kiss JZ. A role for polysialylated neural cell adhesion molecule in lesion-induced sprouting in hippocampal organotypic cultures. Neuroscience 1994;61:441–5.

[19] Muller D, Wang C, Skibo G, Toni N, Cremer H, Calaora V, et al. PSA-NCAM is required for activity-induced synaptic plasticity. Neuron 1996;17:413–22.

[20] Johnson KM, Phillips M, Wang C, Kevetter GA. Chronic phencyclidine induces behavioral sensitization and apoptotic cell death in the olfactory and piriform cortex. J Neurosci Res 1998;52:709–22.

[21] Wang C, Kaufmann JA, Sanchez-Ross MG, Johnson KM. Mechanisms of *N*-methyl-D-aspartate-induced apoptosis in phencyclidine-treated cultured forebrain neurons. J Pharmacol Exp Ther 2000; 294:287–95.

[22] Wang C, Anastasio N, Popov V, Leday A, Johnson KM. Blockade of *N*-methyl-D-aspartate receptors by phencyclidine causes the loss of corticostriatal neurons. Neuroscience 2004;125:473–83.

[23] Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493–501.

[24] Wahlestedt C, Golanov E, Yamamoto S, Yee F, Ericson H, Yoo H, et al. Antisense oligodeoxynucleotides to NMDA-R1 receptor channel protect cortical neurons from excitotoxicity and reduce focal ischaemic infarctions. Nature 1993;363:260–3.

[25] Wang C, Pralong WF, Schulz MF, Rougon G, Aubry JM, Pagliusi S, et al. Functional *N*-methyl-D-aspartate receptors in O-2A glial precursor cells: a critical role in regulating polysialic acid-neural cell adhesion molecule expression and cell migration. J Cell Biol 1996;135:1565–81.

[26] Wang C, McInnis J, West JB, Bao J, Anastasio N, Guidry JA, et al. Blockade of phencyclidine-induced cortical apoptosis and deficits in prepulse inhibition by M40403, a superoxide dismutase mimetic. J Pharmacol Exp Ther 2003;304:266–71.

[27] Abe M, Fukaya M, Yagi T, Mishina M, Watanabe M, Sakimura K. NMDA receptor GluRepsilon/NR2 subunits are essential for postsynaptic localization and protein stability of GluRzeta1/NR1 subunit. J Neurosci 2004;24:9292–304.

[28] Steinmetz RD, Firla B, Steinhilber D. Inhibition of the functional expression of *N*-methyl-D-aspartate receptors in a stably transformed cell line by cyclosporin A. Biochem Pharmacol 2004;68:563–71.

[29] van Zundert B, Yoshii A, Constantine-Paton M. Receptor compartmentalization and trafficking at glutamate synapses: a developmental proposal. Trends Neurosci 2004;27:428–37.

[30] Adams B, Moghaddam B. Corticolimbic dopamine neurotransmission is temporally dissociated from the cognitive and locomotor effects of phencyclidine. J Neurosci 1998;18:5545–54.

[31] Uesaka N, Hirai S, Maruyama T, Ruthazer ES, Yamamoto N. Activity dependence of cortical axon branch formation: a morphological and

electrophysiological study using organotypic slice cultures. J Neurosci 2005;25:1–9.

[32] Hanania T, Hillman GR, Johnson KM. Augmentation of locomotor activity by chronic phencyclidine is associated with an increase in striatal NMDA receptor function and an upregulation of the NR1 receptor subunit. Synapse 1999;31:229–39.

[33] Sircar R. Postnatal phencyclidine-induced deficit in adult water maze performance is associated with *N*-methyl-D-aspartate receptor upregulation. Int J Dev Neurosci 2003;21:159–67.

[34] Lesort M, Esclaire F, Yardin C, Hugon J. NMDA induces apoptosis and necrosis in neuronal cultures. Increased APP immunoreactivity is linked to apoptotic cells. Neurosci Lett 1997;221: 213–6.

[35] Portera-Cailliau C, Price DL, Martin LJ. Non-NMDA and NMDA receptor-mediated excitotoxic neuronal deaths in adult brain are morphologically distinct: further evidence for an apoptosis-necrosis continuum. J Comp Neurol 1997;378:88–104.

[36] Liu A, Hoffman PW, Lu W, Bai G. NF-kappaB site interacts with Sp factors and up-regulates the NR1 promoter during neuronal differentiation. J Biol Chem 2004;279:17449–58.

[37] Lau GC, Saha S, Faris R, Russek SJ. Up-regulation of NMDAR1 subunit gene expression in cortical neurons via a PKA-dependent pathway. J Neurochem 2004;88:564–75.

[38] Choi SY, Hwang JJ, Koh JY. NR2A induction and NMDA receptor-dependent neuronal death by neurotrophin-4/5 in cortical cell culture. J Neurochem 2004;88:708–16.

[39] Zepeda A, Sengpiel F, Guagnelli MA, Vaca L, Arias C. Functional reorganization of visual cortex maps after ischemic lesions is accompanied by changes in expression of cytoskeletal proteins and NMDA and GABA(A) receptor subunits. J Neurosci 2004;24:1812–21.

[40] Gan L, Falzone TL, Zhang K, Rubinstein M, Ldessarini RJ, Tarazi FI. Enhanced expression of dopamine D(1) and glutamate NMDA receptors in dopamine D(4) receptor knockout mice. J Mol Neurosci 2004;22:167–78.

[41] Jiang X, Mu D, Sheldon RA, Glidden DV, Ferriero DM. Neonatal hypoxia-ischemia differentially upregulates MAGUKs and associated proteins in PSD-93-deficient mouse brain. Stroke 2003;34:2958–63.

[42] O'Donnell J, Stemmelin J, Nitta A, Brouillette J, Quirion R. Gene expression profiling following chronic NMDA receptor blockade-induced learning deficits in rats. Synapse 2003;50:171–80.

[43] Hwang JY, Kim YH, Ahn YH, Wie MB, Koh JY. *N*-methyl-D-aspartate receptor blockade induces neuronal apoptosis in cortical culture. Exp Neurol 1999;159:124–30.

[44] Wang C, Sadovova N, Fu X, Schmued L, Scallet A, Hanig J, et al. The role of *N*-methyl-D-aspartate receptor in ketamine-induced apoptosis in rat forebrain culture. Neuroscience 2005; in press.