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Cerebellar Toxicity of Phencyclidine

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Phencyclidine (PCP), dizocilpine maleate (MK801), and other NMDA antagonists are toxic to neurons in the posterior cingulate and retrosplenial cortex. To determine if additional neurons are damaged, the distribution of microglial activation and 70 kDa heat shock protein (HSP70) induction was studied following the administration of PCP and MK801 to rats. PCP (10–50 mg/kg) induced microglial activation and neuronal HSP70 mRNA and protein expression in the posterior cingulate and retrosplenial cortex. In addition, coronal sections of the cerebellar vermis of PCP (50 mg/kg) treated rats contained vertical stripes of activated microglial in the molecular layer. In the sagittal plane, the microglial activation occurred in irregularly shaped patches, suggesting damage to Purkinje cells. In accord with this finding, PCP induced HSP70 protein and mRNA expression in Purkinje cells. Although there were relatively few foci of microglial activation and cells with HSP70 protein induction, HSP70 mRNA was detected in many Purkinje cells located throughout the cerebellar hemispheres as well as the vermis.

MK801, at doses of 5–10 mg/kg, induced microglial activation and neuronal HSP70 mRNA and protein expression in the cingulate and retrosplenial cortex but not in the cerebellum. At the dose of 1 mg/kg MK801 induced HSP70 but did not consistently activate microglia. These data suggest that microglia are activated by MK801 doses that kill or severely damage neurons, whereas HSP70 is induced in "stressed" neurons at MK801 doses well below those that produce severe neurotoxicity.

These observations suggest that PCP, but not MK801, is toxic to Purkinje cells and raise the question of whether NMDA antagonists or sigma ligands other than PCP are toxic to the cerebellum. Moreover, this study illustrates the usefulness of microglial activation and HSP70 induction as markers of neurotoxicity.

[Key words: Purkinje cell, heat shock genes, microglia, NMDA receptors, NMDA antagonists, σ receptor]

Phencyclidine (PCP), dizocilpine maleate (MK801), and other NMDA receptor antagonists have attracted increasing attention because of their therapeutic potential. These drugs have neuroprotective properties in animal studies of focal brain ischemia, where excitotoxicity is proposed to be an important mechanism of neuronal cell death (Dalkara et al., 1990; Martinez-Arizala et al., 1990). Moreover, NMDA antagonists decrease neuronal damage and dysfunction in other pathological conditions, including hypoglycemia (Nellgard and Wieloch, 1992) and prolonged seizures (Church and Lodge, 1990; Faingold et al., 1993).

However, NMDA antagonists are toxic to certain neuronal populations in the brain. Olney et al. (1989) demonstrated that the noncompetitive NMDA antagonists, PCP, MK801, and ketamine, induce morphological damage characterized by cytoplasmic vacuoles in neurons in the posterior cingulate and retrosplenial cortex. Sharp et al. (1991b) and Olney et al. (1991) subsequently showed that these drugs induce the 70 kDa heat shock protein (HSP70) in these damaged neurons and that the HSP70 induction is an excellent light microscopic marker for the injured cells.

The mechanism of toxicity of PCP and MK801 is unknown. The drugs are noncompetitive NMDA receptor antagonists. They bind to a specific site (referred to here as the NMDA/PCP receptor) in the NMDA receptor macromolecular complex located inside the ion channel, but do not block glutamate binding to the NMDA receptor (Reynolds and Miller, 1990). MK801 binds with much higher affinity than PCP to the NMDA/PCP receptor (Wong et al., 1986). Binding to the NMDA/PCP receptor is dependent on the presence of glutamate and glycine and it is also voltage dependent (Reynolds and Miller, 1990). The anatomical distribution of PCP binding sites is virtually identical with NMDA receptors in cerebral cortex, whereas in cerebellum the distribution of the PCP binding is quite different from that of NMDA (Maragos et al., 1988; Jarvis et al., 1987).

PCP also has a modest affinity for the σ-site (also called the σ-receptor), which is distinct from the NMDA receptor complex (Quirion et al., 1987; Contreras et al., 1988). MK801 has little affinity for the σ-site (Heroux et al., 1992). σ-Receptors are thought to mediate the psychotomimetic effects of certain benzomorphan opiates (Su, 1993). It is hypothesized that σ or NMDA/PCP receptors play a role in mental illness (Jansen and Faull, 1991; Javitt and Zukin, 1991), as PCP induced psychosis mimics schizophrenia (Domino and Luby, 1981). In addition to binding to NMDA/PCP and σ-receptors, PCP also affects several other signaling systems. These include muscarinic and nicotinic receptors (Lodge and Johnson, 1990), the transmitters dopamine (Lodge and Johnson, 1990), norepinephrine (Lew, 1989; Lodge and Johnson, 1990; Massarimi and Duckles, 1991), ACh (Palma and Wang, 1991; Lodge and Johnson, 1990), and GABA

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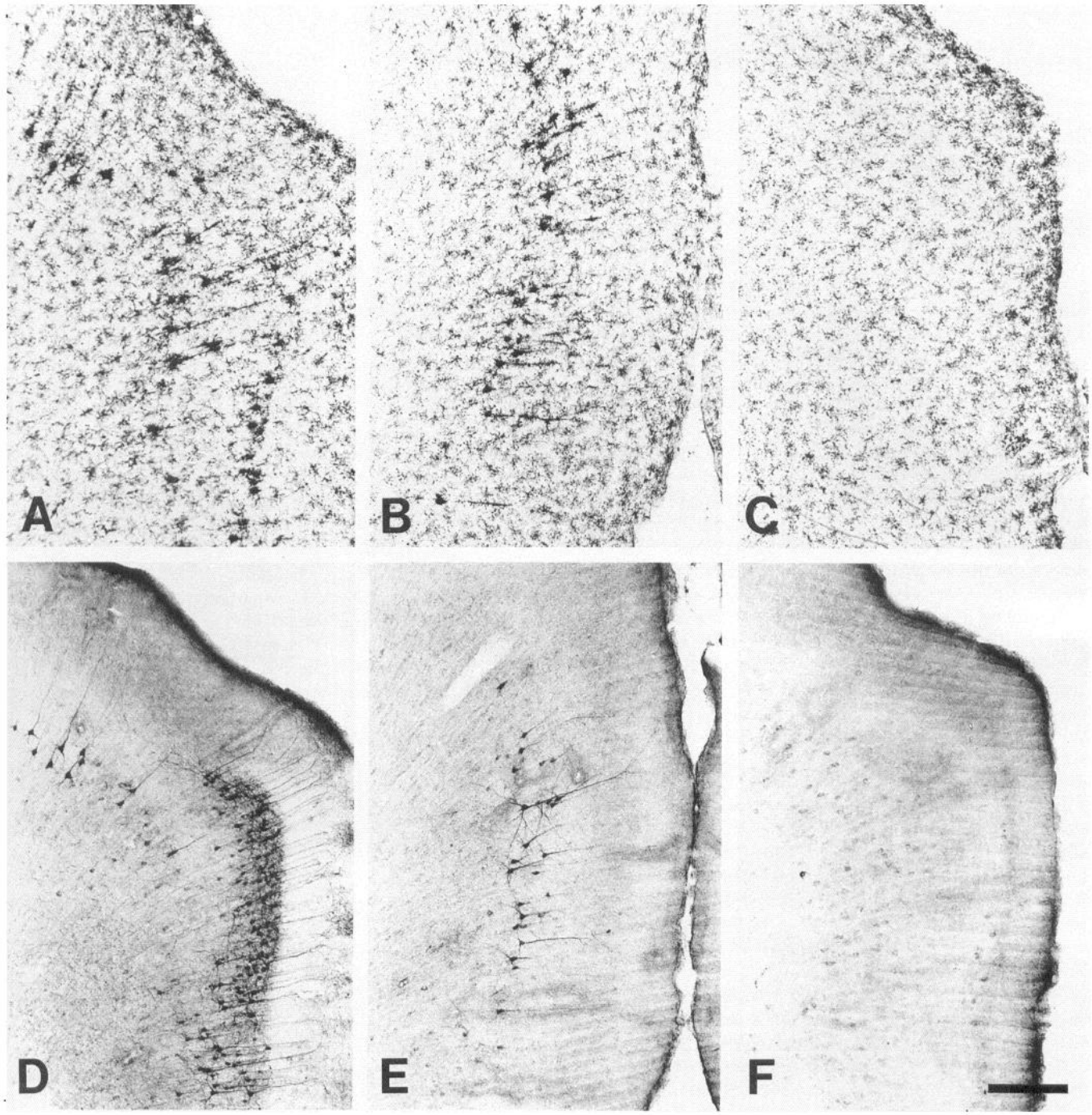


Figure 1. PCP (50 mg/kg i.p.) activates microglia (*A, B*) and induces HSP70 (*D, E*) in the posterior cingulate (*A, D*) and retrosplenial (*B, E*) cortex 3 d after injection. There is no microglial activation or HSP70 induction in saline treated controls (*C, F*). Monoclonal antibodies against C3-complement receptor (OX-42) and heat shock 70 protein (HSP70) were used for microglial and HSP70 staining, respectively. Scale bar, 200 μm.

(Seiler and Grauffel, 1992; Sharp et al., 1994); at least one amine transporter (Rothman et al., 1990, 1992; Akunne et al., 1991, 1992); and a K^+ channel (Lodge and Johnson, 1990).

The purpose of this study was to further examine PCP and MK801 neurotoxicity in the rat brain. Because of the therapeutic potential of NMDA antagonists, it is important to determine the localization, pharmacology, and possible mechanisms of their neurotoxic effects. In addition, understanding the neurotoxicity of these compounds, particularly PCP, could further our understanding of the neurobiological bases of schizophrenia and related mental illnesses.

Materials and Methods

Tissue preparation

Immunocytochemistry. Adult male Sprague–Dawley rats (180–280 gm) received a single intraperitoneal injection of PCP (n = 40) or MK801 (n = 12). The PCP doses were 5 (n = 2), 10 (n = 5), 25 (n = 3), and 50 (n = 30) mg/kg and the MK801 doses were 1 (n = 4), 5 (n = 4), and 10 mg/kg (n = 4). Control animals received an equal volume of

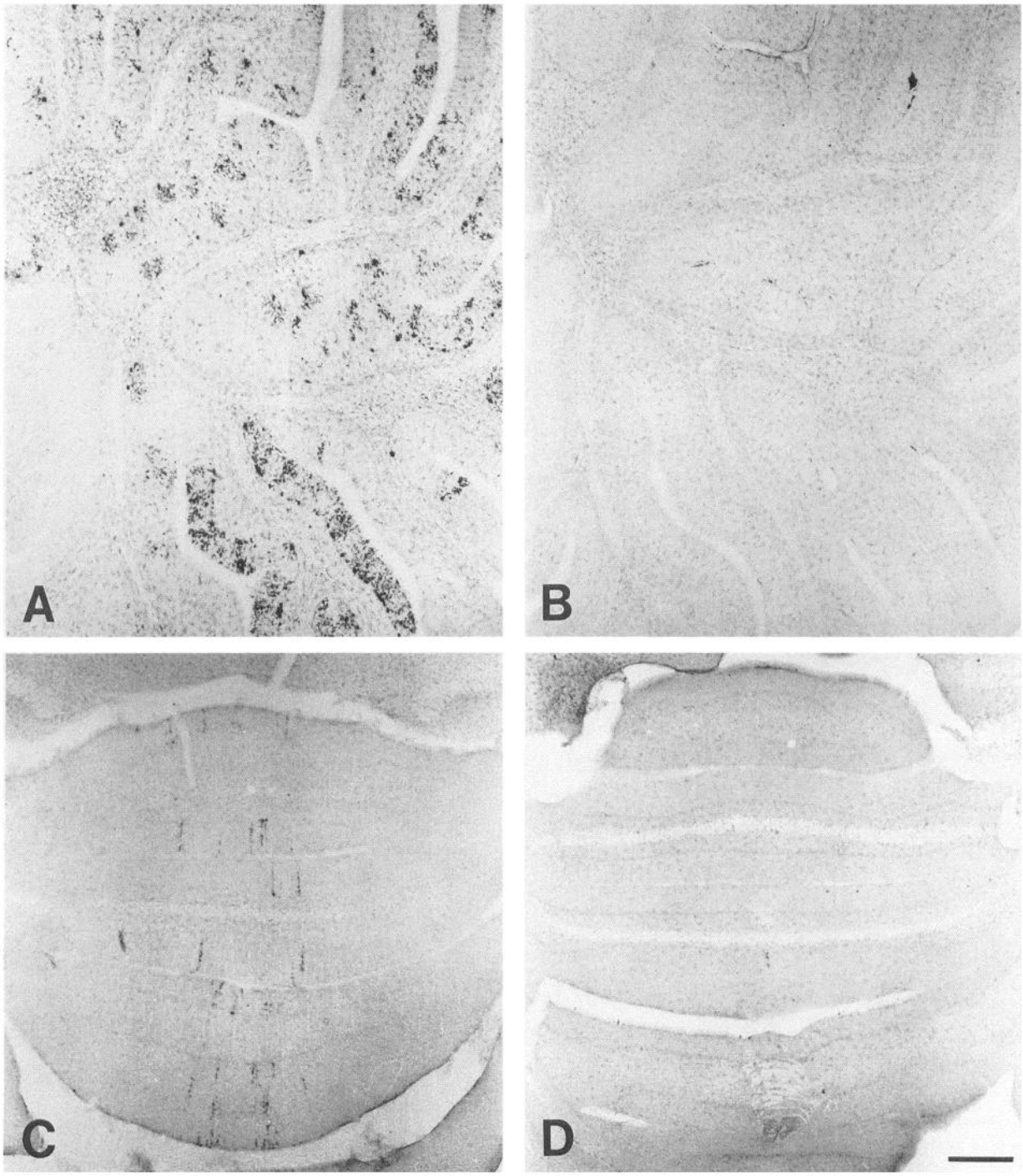


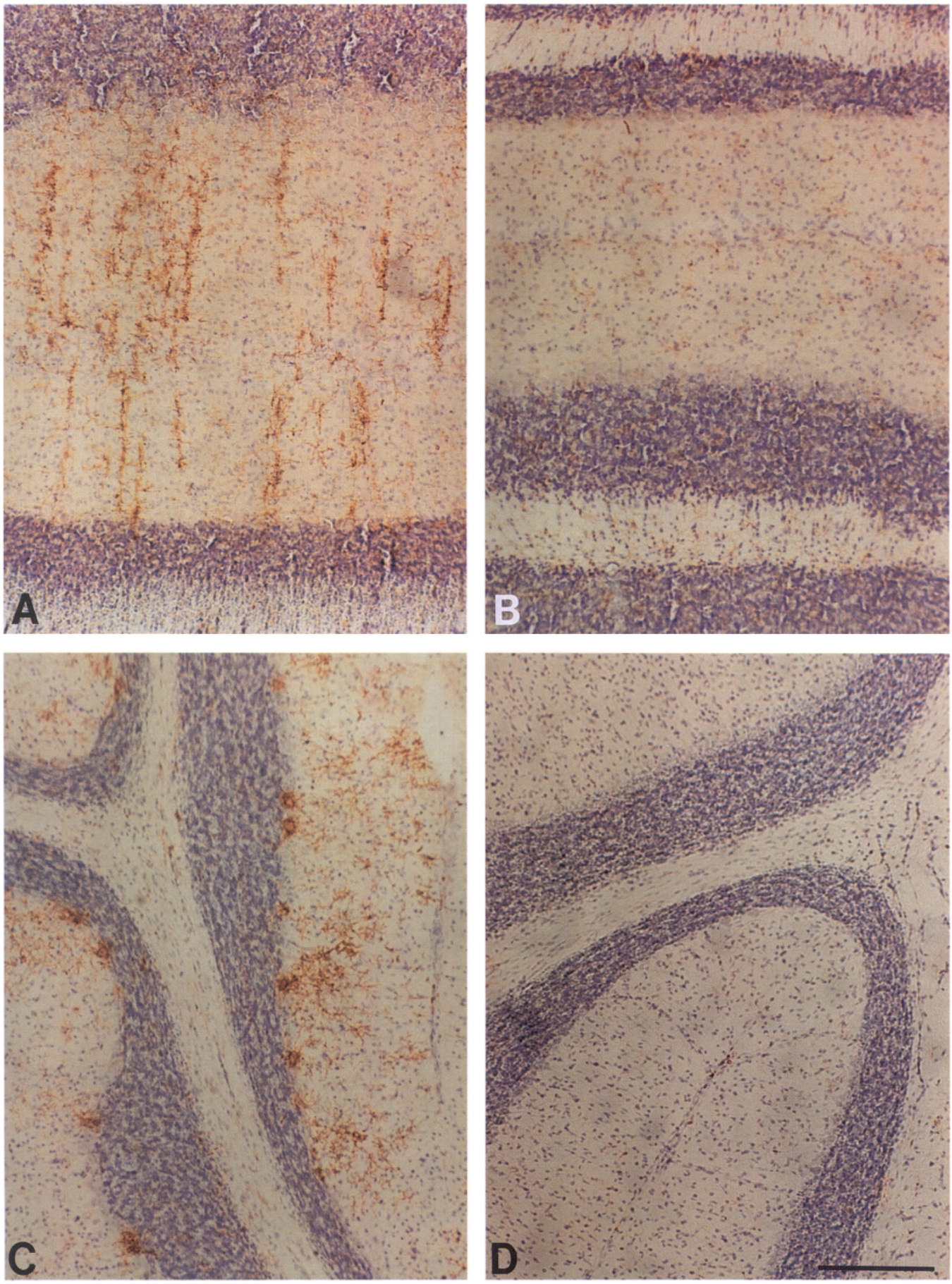
Figure 2. Microglial activation (OX-42) in cerebellar sagittal (*A*) and coronal (*C*) sections 2 d after injection of PCP (50 mg/kg i.p.). Saline treated controls showed no evidence of microglial activation (*B, D*). Scale bar, 500 μm.

0.9% saline (n = 12). After 1–30 d survivals, animals were anesthetized with sodium pentobarbital (125 mg/kg) and perfused through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M, pH 7.4 sodium phosphate buffer (PB). The brains were postfixed in the same fixative for 2–4 hr at 4°C. Fifty micrometer thick vibratome sections were processed for immunocytochemistry.

In situ *hybridization.* Adult rats injected intraperitoneally with PCP (50 mg/kg) (n = 2), MK801 (10 mg/kg) (n = 1), or 0.9% saline (n = 2) were allowed to survive 6 hr. They were narcotized with carbon dioxide and decapitated. Brains were removed, blocked, and frozen in 2-methylbutane at −30°C. Twenty micrometer thick sections were cut on a cryostat and thaw mounted onto Probe-On-Slides (Fischer Biotech). Sections were air dried and kept frozen until used for *in situ* hybridization.

Immunocytochemistry

The monoclonal antibody OX-42 (Serotec, Oxford, England; 1:5000) against the complement C3 receptor was used to detect activated microglia. Additionally, the monoclonal antibodies OX-6 (Serotec, 1:1000) against the major histocompatibility complex Class II antigen, and ED-1 (Serotec, 1:1000) against a cytoplasmic monocyte/macrophage antigen and biotinylated isolectin B4 (IB4) (Sigma Chemical Co., St. Louis, MO; 4 μg/ml) from *Griffonia simplicifolia* were used to detect microg-


A
B
C
D

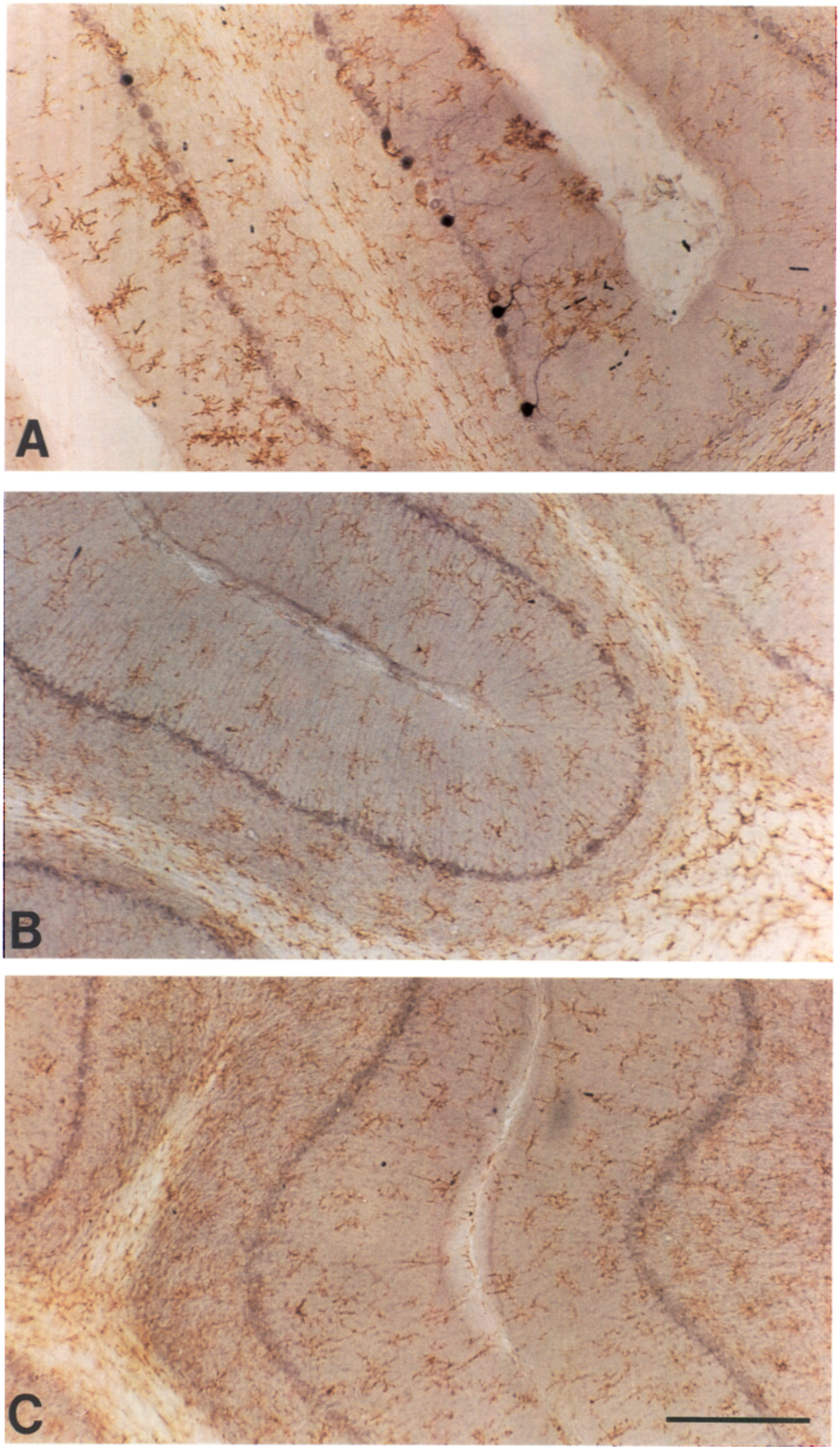


Figure 4. Cerebellar sagittal sections of rats injected with (*A*) PCP (50 mg/kg i.p.), (*B*) MK801 (10 mg/kg i.p.), and (*C*) saline 24 hr previously. Sections are immunostained for HSP70 (*dark blue/black*) and microglia (OX-42, *brown*). PCP treated animals showed activated microglia in the molecular layer and HSP70 immunoreactivity in Purkinje cells (*A*). MK801 (*B*) and saline (*C*) treated animals showed resting microglia and no HSP70 immunostaining. Scale bar, 200 μm.

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Figure 3. Microglial activation (OX-42) in cerebellar coronal (*A*) and sagittal (*C*) sections 4 d after injection of PCP (50 mg/kg i.p.). Saline treated controls showed no evidence of microglial activation (*B, D*). Sections are counterstained with cresyl violet. Scale bar, 200 μm.

lia in selected sections. The monoclonal antibody to HSP70 from Amersham U.K. (1:4000) was used to stain for the inducible heat shock protein 70 (HSP70). This antibody (Welch and Suhan, 1986) produces one major band on Western blots of ischemic gerbil brain (Vass et al., 1988), one band on Western blots of heated rat brain and retina (Barbe et al., 1988), and one band on Western blots of heat shocked rat astrocyte cultures (Swanson et al., personal communication). The antibody recognizes the product of the rat HSP70 gene that we have cloned and expressed in fibroblasts (Longo et al., 1993).

The primary antibody or lectin was diluted in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin, 0.2% Triton X-100, and 2% horse serum. The tissue sections were incubated in the primary antibody for 36–72 hr at 4°C and the bound antibody was visualized with the avidin–biotin–peroxidase (ABC) method (Vectastain Kit, Vector Labs., Burlingame, CA) using 3,3′-diaminobenzidine (DAB) as the peroxidase substrate. For HSP70 staining biotinylated sheep anti-mouse Ig (Amersham) was used as the secondary antibody. Controls for the immunostaining, which included omission of the primary antibody or lectin, demonstrated no microglial or HSP70 staining. Some sections were counterstained with cresyl violet, hematoxylin and eosin, or neutral red.

In situ *hybridization histochemistry*

A 30-mer anti-sense oligonucleotide probe complimentary to the sequence coding for amino acids 122–129 of the inducible HSP70 gene was used (5′-CGATCTCCTTCATCTTGGTCAGCACCATGG-3′). This oligonucleotide distinguishes between the inducible HSP70 (Hunt and Morimoto, 1985) and constitutive HSC73 genes on Northern blots of rat brain (Miller et al., 1991) and produces very little signal on *in situ* hybridization of normal rat brain (Sharp et al., 1994). The probe was 3′ end labeled with ^{35}S-ATP using terminal deoxynucleotidyl transferase (New England Nuclear) and purified over Nuctrap Push Columns (Stratagene). Two hundred microliters of the hybridization solution containing 10×10^6 cpm/ml probe, 40 μl of 5 M dithiothreitol, 50 μl of salmon sperm DNA (10 mg/ml), and 900 μl of hybridization cocktail (50 ml of formamide, 20 ml of 20 × SSC, 2 ml of 50 × Denhardt's reagent, 10 ml of 0.2 M sodium phosphate buffer pH 7.4, 10 gm of dextran, 4 ml of 25% sarcosyl) was placed between two slides which were sandwiched together. After 18 hr at 37°C, sections were washed 2 × 5 sec in 1 × SSC at 55°C, 4 × 15 min in 1 × SSC at 55°C, 60 min in 1 × SSC at room temperature, 2 × 5 min in deionized water at room temperature, 30 sec in 60% ethanol and 30 sec in 95% ethanol at room temperature. The sections were air dried and autoradiographed on Kodak SB5 x-ray film. Slides were dipped in Kodak NTB2 nuclear emulsion and exposed for 30–60 d. After photographic developing, the sections were counterstained with neutral red. Control sections treated with RNase and hybridized with the antisense probe and control sections hybridized with a sense oligonucleotide probe showed no evidence of specific hybridization.

Results

Behavioral observations

Behavioral responses occurred within 1–2 min of PCP and 2–5 min of MK801 administration. At lower doses (PCP 5–25 mg/kg, MK801 1–2 mg/kg) the rats exhibited characteristic hyperactivity, including head waving, circling, and rearing movements together with tremor, truncal ataxia, decreased muscle tone, and extended curved tail position. At higher doses (PCP 50 mg/kg, MK801 5–10 mg/kg) they initially lay in one place with tremulous extremities and then gradually, over 2–4 hr, exhibited increased motor activity similar to that of the low dose animals. The abnormal behavior persisted for 2–12 hr and was gradually followed by normal spontaneous activity. The duration of the symptoms varied with different doses of the drugs from 2–3 hr with low doses of PCP to 7–8 hr with high doses of PCP and 8–12 hr with high doses of MK801. At 24 hr, the drug treated rats appeared behaviorally similar to the saline treated controls. There was little difference between the behavior of PCP and MK801 treated rats.

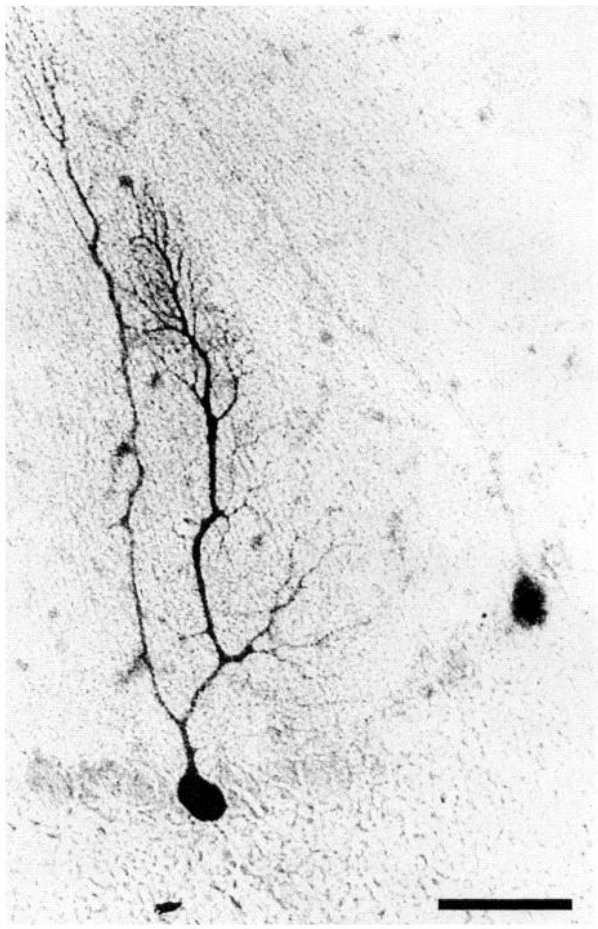

Figure 5. HSP70 immunoreactive Purkinje cell in a sagittal section of cerebellum of a rat injected with PCP (50 mg/kg i.p.) 24 hr previously. Scale bar, 50 μm.

PCP- and MK801-induced microglial activation

Activated microglia were stained with all of the markers used. Though OX-42 stains both activated and resting microglia, the staining of the activated microglia was more intense. OX-6, ED-1, and the lectin IB4 stain the activated microglia intensely but few or none of the resting microglia. Because OX-42 produced the most robust and consistent immunostaining, it was used routinely in these experiments. The activated microglia detected with OX-42 were also detected using OX-6, ED-1, and IB4 in each experiment, confirming that identical patterns of microglial activation were obtained with each of the markers.

PCP produced microglial activation in the posterior cingulate and retrosplenial cortex at doses of 10–50 mg/kg (Fig. 1*A,B*). When studied 1–10 d after injection, the reaction was consistently detected in the rats injected with doses of 25 (n = 3) and 50 mg/kg (n = 24) PCP but only in two of the five rats receiving the 10 mg/kg dose. In the retrosplenial and most posterior cingulate cortex microglial activation was detected in cortical layers 3 and 5 (Fig. 1*A*), whereas in more anterior parts of posterior cingulate only one layer of activated microglia could be visualized (Fig. 1*B*). At least some of the PCP treated animals also had activated microglia in the pyriform cortex and neocortex, but detailed analyses of these areas were not carried out for the present study.

In addition to these areas, microglial activation was consistently observed in the cerebellum (Figs. 2A,C; 3A,C) 1–10 d following the administration of 50 mg/kg of PCP (n = 24). Coronal sections of PCP (50 mg/kg) treated rats contained vertical stripes of activated microglia in the molecular layer of the cerebellum (Figs. 2A, 3A). Almost all of the stripes were in the vermis (Fig. 2C). In sagittal sections the activated microglia appeared in irregularly shaped clusters in the molecular layer following the pattern of Purkinje cell dendrites, the most intense staining being around Purkinje cell bodies (Fig. 3C). Although activation of microglia was seen in the cerebellums of all animals receiving 50 mg/kg PCP, the number of patches of activated microglia varied between individual rats. The 10 (n = 5) and 25 mg/kg (n = 3) doses of PCP produced activated microglia in the cingulate and retrosplenial cortex, but never in the cerebellum. The 5 mg/kg dose of PCP (n = 2) produced patterns of microglial staining that were indistinguishable from the saline treated controls.

Microglial activation in posterior cingulate cortex occurred as early as 1 d after PCP (50 mg/kg) treatment and reached its peak between 3 and 7 d. At 30 d the microglia appeared to have returned to the resting state, since the patterns of OX-42 staining at 30 d were similar to the patterns of OX-42 stained microglia in the saline injected control animals. The time course of microglial activation after PCP injection was similar in the cerebral cortex and in the cerebellum.

MK801, like PCP, induced a dose dependent microglial response in the cingulate, retrosplenial, and pyriform cortex when examined 1–5 d after intraperitoneal injection. Microglial activation was seen in posterior cingulate and retrosplenial cortex in one of the four animals receiving 1 mg/kg dose of MK801 and in all of the animals receiving 5 (n = 4) and 10 mg/kg (n = 4) MK801. The number of activated microglial cells increased at higher doses of MK801. However, MK801 did not induce cerebellar microglial activation at any of the doses used (Fig. 4B).The saline injected controls (n = 12) (Figs. 1C; 2B,D; 3B,D) exhibited only rare, scattered activated microglial cells with no consistent regional localization.

PCP- and MK801-induced HSP70 protein expression

HSP70 immunostaining was detected 12 hr, 1 d, 3 d, and 7 d after PCP (10–50 mg/kg) (n = 20) or MK801 (1–10 mg/kg) (n = 12) treatment. Longer time points were not studied though MK801 has been shown to induce HSP70 protein for up to 2 weeks (Sharp et al., 1991b). HSP70 immunostaining was induced in pyramidal neurons in the same areas as the microglial activation in the cingulate, retrosplenial, pyriform, and neocortex in PCP (Fig. 1D,E) and MK801 treated animals. In PCP (50 mg/kg) (n = 7) treated animals HSP70 immunoreactive Purkinje cells were consistently detected in cerebellum (Figs. 4A, 5), whereas HSP70 immunoreactive Purkinje cells were never detected in cerebellum of MK801 (n = 12) (Fig. 4B) or saline (n = 12) (Fig. 4C) treated animals.

The HSP70 immunoreactive Purkinje cells in PCP treated rats did not consistently co-localize with the activated microglia, even though both of the markers were concentrated in the cerebellar vermis (Figs. 4A, 6A–C). Some Purkinje cells stained for HSP70 without evidence of microglial activation (Fig. 6A), and other Purkinje cells were HSP70 negative but were surrounded by activated microglia (Fig. 6C). There were also instances of HSP70 immunoreactive Purkinje cells that had activated microglia surrounding their perikarya and dendrites (Fig. 6B). No additional HSP70 positive cells, aside from Purkinje cells, were detected in cerebellum. No HSP70 positive staining was detected in any cells of the forebrain or cerebellum of the saline injected control animals.

PCP- and MK801-induced HSP70 mRNA expression

In situ hybridization histochemistry of brain sections from PCP (50 mg/kg) (n = 2) treated rats demonstrated the expression of HSP70 mRNA in Purkinje cells throughout the cerebellum. Cellular resolution autoradiography confirmed that the silver grains were located mainly over Purkinje cell bodies (Fig. 7A,B). The HSP70 mRNA expression varied between individual Purkinje cells (Fig. 7B). The number of HSP70 mRNA positive Purkinje cells was many times larger than the number of activated microglial stripes or HSP70 immunostained Purkinje cells in the cerebellum of rats treated with 50 mg/kg of PCP. HSP70 mRNA was detected not only in vermal Purkinje cells but also in Purkinje cells located throughout the cerebellar hemispheres. PCP (50 mg/kg) also induced HSP70 mRNA in pyramidal neurons located in layers 3 and 5 of posterior cingulate and retrosplenial cortex as previously reported (Sharp et al., 1992).

MK801 (10 mg/kg) (n = 1) also induced HSP70 mRNA in pyramidal neurons in layers 3 and 5 of the cingulate and retrosplenial cortex. There were no HSP70 mRNA positive neurons in the cerebellum of rats injected with MK801 (Fig. 7C). The saline injected animals (n = 2) did not exhibit specific staining for HSP70 mRNA in the cerebrum or cerebellum (Fig. 7D).

Discussion

Previous studies have shown that the NMDA receptor antagonists PCP, MK801, and ketamine produce vacuoles in the cytoplasm of pyramidal neurons in layers 3 and 5 of posterior cingulate and retrosplenial cortex (Olney et al., 1989, 1991; Allen and Iversen, 1990; Sharp et al., 1991b; Fix et al., 1993). Subsequently, the HSP70 heat shock protein was found to be induced in these vacuolated neurons (Olney et al., 1991; Sharp et al., 1991b, 1992). Moreover, MK801 has been demonstrated to cause neuronal death in the retrosplenial cortex (Fix et al., 1993). The present study shows that PCP and MK801 activate microglia around pyramidal neurons in the same areas. Moreover, high doses of PCP (50 mg/kg), but not MK801, activate microglia surrounding Purkinje cells and induce HSP70 mRNA and HSP70 protein in Purkinje cells. These results strongly suggest that PCP, but not MK801, injures Purkinje cells in rodent cerebellum.

Microglial activation has been well characterized in numerous studies as a marker of neural injury (Thomas, 1992). Normal brain microglia, the resident macrophage population in the CNS, have a "resting," ramified morphology. They express some, but not all macrophage markers, including complement C3 receptors (Perry and Gordon, 1988) and carbohydrates that bind to certain lectins (Streit and Kreutzberg, 1987). However, most of their macrophage properties are downregulated. Following brain injury microglia proliferate, collect at the site of injury, undergo morphological alteration, and express or upregulate macrophage associated antigens (Thomas, 1992). This results in more intense staining with antibodies to the complement receptor and lectins and in the expression of macrophage markers such as ED1 and major histocompatibility complex (MHC) Class II antigens. Activated microglia can become phagocytic and participate in removing dead tissue (Thomas, 1992). Microglial activation is a sensitive and relatively nonspecific indicator of cell damage. It

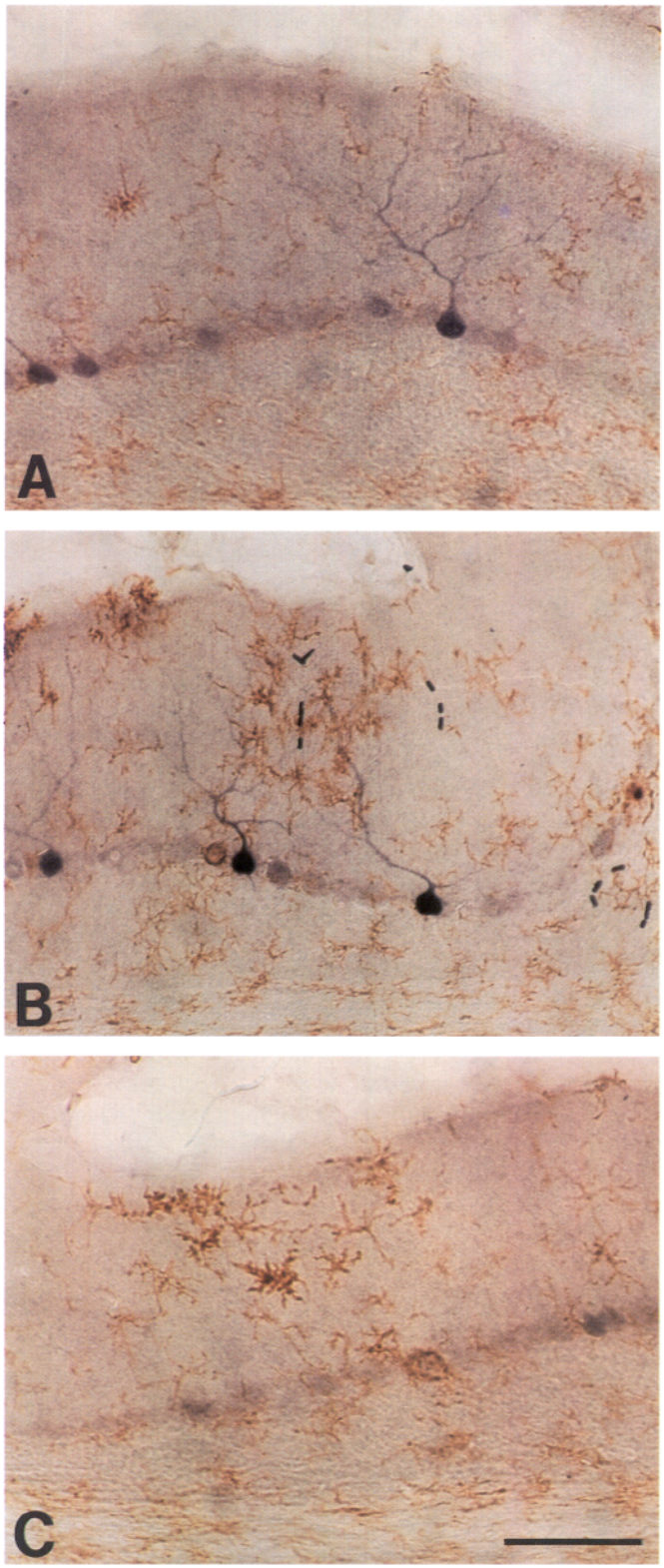

Figure 6. HSP70 positive immunostaining (*dark blue/black*) and OX-42 microglial immunostaining (*brown*) in sagittal sections of a rat injected with PCP (50 mg/kg i.p.) 24 hr previously. Some of the HSP70 positive Purkinje cells had activated microglial surrounding them (*B*) and some did not (*A*). Activated microglia were also detected around Purkinje cells that were not immunoreactive for HSP70 (*C*). Scale bar, 100 μm.

occurs in a variety of injuries, including physical, chemical, ischemic, and infectious insults (Streit et al., 1988; Thomas, 1992). However, the actual molecular mechanism of microglial activation in neuronal injury remains to be defined. Myelin and axonal degeneration have been suggested to induce microglial activation (Finsen et al., 1993). Moreover, cytokines secreted by astrocytes are mitogenic for microglia (Frei et al., 1986). On the other hand, interleukin-1 (IL-1), which is secreted by microglia, is mitogenic to astrocytes (Giulian and Lachman, 1985; Giulian et al., 1986), indicating a reciprocal interaction between microglia and astrocytes in CNS damage and healing. Despite this extensive literature, it remains unknown if microglial activation after neuronal injury requires the death of neurons or can result from sublethal neuronal injury.

HSP70 induction as a marker of neural injury differs from the microglial reaction. HSP70 induction likely indicates cell damage or "stress" rather than cell death (Gonzalez et al., 1989, 1991; Simon et al., 1991). HSP70 synthesis is induced by various stresses, including heat shock, ischemia, prolonged seizures, and toxins (Vass et al., 1988, 1989; Gonzalez et al., 1989, 1991; Brown, 1990; Sharp et al., 1991a). Most of these conditions are thought to denature intracellular proteins and activate heat shock factors that bind to the heat shock elements in the promoters of heat shock genes to initiate heat shock gene transcription (Anathan et al., 1986). HSP70 protein family members have a protective function. They prevent partially denatured proteins from becoming irreversibly denatured (Pelham, 1986), facilitate protein import into cell organelles (Deshaies et al., 1988), and interact transiently with newly synthesized proteins to ensure proper folding (Beckmann et al., 1990). The protective role of HSP70 is supported by the observation that HSP70 protein increases cell survival after heat shock (Johnston and Kucey, 1988; Riabowol et al., 1988) and that HSP70 induction prior to a stressor decreases cell damage (Walsh et al., 1987, 1989; Barbe et al., 1988; Tytell et al., 1989). The preponderance of evidence indicates that HSP70 is produced in cells that will survive the stress (Vass et al., 1988; Sharp et al., 1991a), although HSP70 protein can be induced in cells that are destined to die in global ischemia (Simon et al., 1991). However, following focal ischemia severe enough to produce cell death, the expression of HSP70 mRNA and protein is blocked in most neurons and glia that go on to die from the insult (Li et al., 1992; Kinouchi et al., 1993).

High doses of MK801 (5–10 mg/kg) lead to death of scattered pyramidal neurons in the posterior cingulate and retrosplenial cortex of adult rats (Allen and Iversen, 1990; Fix et al., 1993). These are the same doses of MK801 required to produce microglial activation in posterior cingulate (the present study; Fix et al., 1993). Moreover, Fix et al. (1993) demonstrated that reactive microglial cells contact necrotic neurons after high doses of MK801. MK801 induces HSP70 in posterior cingulate and retrosplenial pyramidal neurons at doses as low as 0.2 mg/kg (Sharp et al., 1991b), doses well below those that lead to cell death. This suggests, but does not prove, that MK801-induced microglial activation reflects neural death while HSP70 expression indicates primarily a sublethal neural injury.

With PCP, the present study did not reveal a dissociation of the doses of drug required to produce HSP70 expression and microglial activation. In the forebrain, both microglial activation and HSP70 expression occurs after PCP over a range of doses from 10 to 50 mg/kg. At 10 mg/kg, PCP produces only occasional activated microglial cells in the forebrain, whereas doses

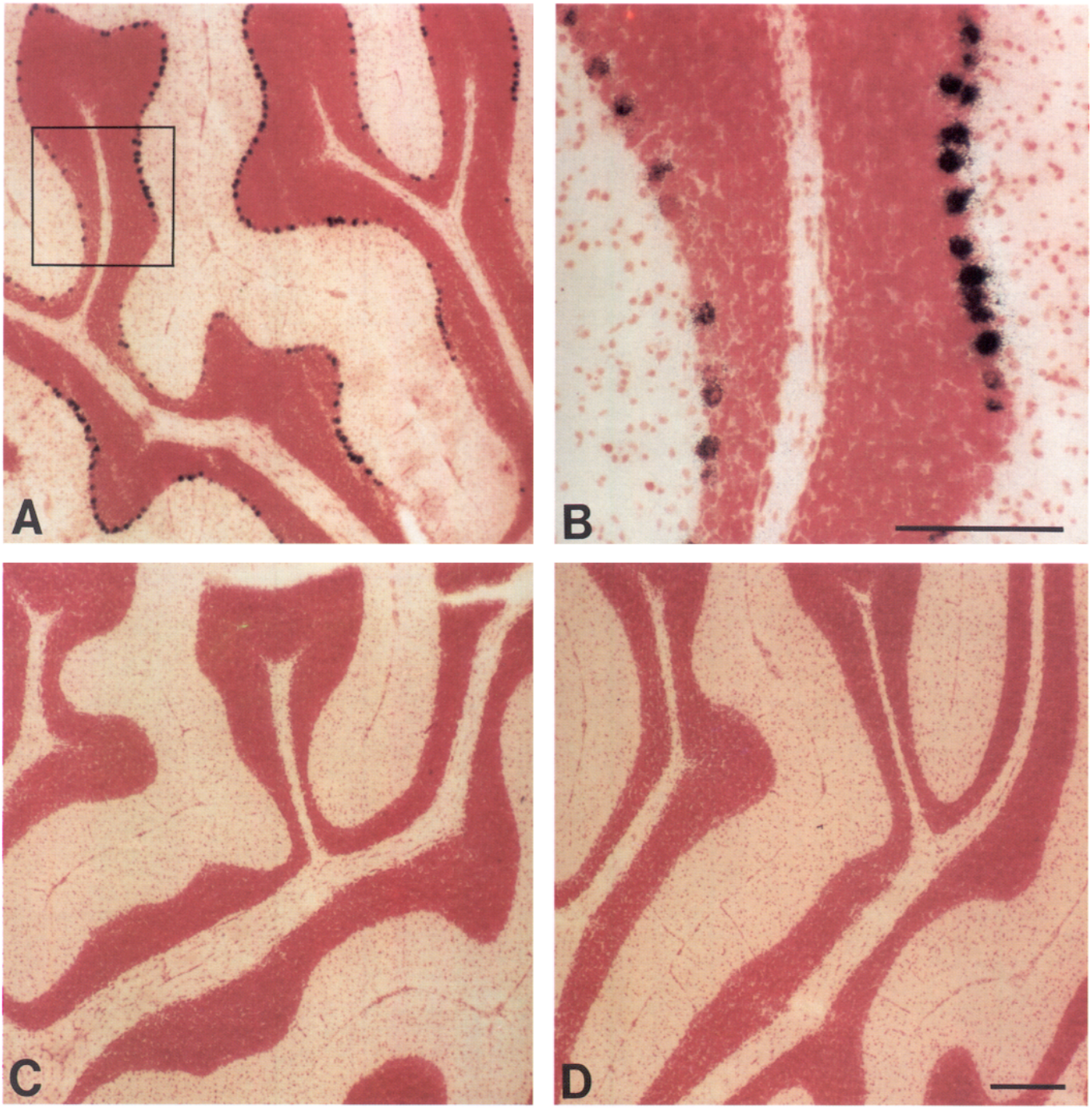

Figure 7. HSP70 mRNA in the Purkinje cells of a rat injected with PCP (50 mg/kg i.p.) 6 hr previously (*A, B*). Rats injected with MK801 (10 mg/kg i.p.) (*C*), and saline (*D*) did not exhibit any specific hybridization signal for HSP70 mRNA in the cerebellum. Sections are counterstained with neutral red. Scale bars, 200 μm.

of 25 and 50 mg/kg consistently produce substantial numbers of activated microglia (Fig. 1*A,B*). In the forebrain, the vast majority of activated microglia and neurons expressing HSP70 are detected in posterior cingulate and retrosplenial cortex. In the cerebellum, PCP, 50 mg/kg, but not lower doses, consistently produced patches of activated microglia surrounding cerebellar Purkinje cell bodies and in the molecular layer. Moreover, HSP70 mRNA and protein were expressed in Purkinje cells. As in the forebrain, it remains to be determined if the activated microglia mark dying or damaged Purkinje cells.

The distribution of microglial activation matches the pattern of HSP70 induction in the posterior cingulate and retrosplenial cortex following the administration of both PCP and MK801. However, the distribution of microglial activation overlaps, but does not match, the pattern of HSP70 induction in cerebellar Purkinje cells after high dose PCP. This discrepancy could reflect either different degrees or different types of neuronal injury. It is therefore possible that HSP70 positive Purkinje cells without surrounding activated microglia are less severely injured than HSP70 positive Purkinje cells which are surrounded by activated

microglia. The Purkinje cells that are HSP70 negative but are surrounded by activated microglia may have sustained an injury so severe that they are unable to synthesize the HSP70 protein. Alternatively, it is possible that PCP damages Purkinje cells through two separate mechanisms, one marked by HSP70 induction and the other by microglial activation. If this explanation is true, our observations suggest that only a minority of Purkinje cells are vulnerable to both mechanisms.

The present observations could be confounded by different time courses in the expression of the markers. The time required for maximal HSP70 protein expression is shorter (12–48 hr) (Sharp et al., 1991b) than the time required for microglial activation (24–96 hr) (Brierley and Brown, 1982; Streit and Kreutzberg, 1987). When examining the rats surviving 24 hr after PCP treatment for HSP70, it is likely that much of the microglial activation had not yet occurred. However, when examined at 3 d, the distribution of HSP70 induction still failed to match the distribution of microglial activation in cerebellum, supporting the idea that there are different degrees or mechanisms of injury in different Purkinje cell neurons.

The possibility that damaged cells other than Purkinje cells contribute to the microglial activation in the molecular layer appears unlikely. The most intense microglial activation occurs surrounding Purkinje cell bodies, and the patches of activated microglia in the molecular layer are confined to the plane of Purkinje cell dendrites. Moreover, HSP70 mRNA and protein are expressed exclusively in Purkinje cells in the cerebellum, suggesting selective damage to these cells.

The anatomical site of predominant cerebellar microglial activation and HSP70 protein induction is the cerebellar vermis. Though the highest density of HSP70 mRNA expression is in Purkinje cells located in the vermis, HSP70 mRNA is also induced in Purkinje cells scattered throughout the intermediate zone and in the cerebellar hemispheres. In the vermis HSP70 mRNA is detected in a larger number of Purkinje cells than HSP70 protein or microglial activation. The relative paucity of HSP70 immunostained Purkinje cells in comparison with the relative abundance of mRNA expression could be explained by several factors. (1) The HSP70 protein concentration is too low to produce detectable immunostaining in Purkinje cells, whereas the *in situ* hybridization histochemistry is relatively more sensitive. (2) The HSP70 protein is not formed in all of the HSP70 mRNA positive cells, either because of severe injury to these cells or because of a specific translation block. (3) The protein is rapidly degraded posttranslationally and therefore cannot be detected immunocytochemically.

The mechanism by which PCP and other NMDA antagonists damage neurons is unknown. Disinhibition of cortical pyramidal cells with consequent overexcitation has been hypothesized to explain the injury to cingulate and retrosplenial cortex (Olney et al., 1991; Sharp et al., 1994). A similar mechanism could apply to PCP injury to the cerebellum, although electrophysiological evidence indicates that systemically administered PCP inhibits rather than excites Purkinje cells in anesthetized rats (Kim and Bickford, 1992).

The possibility that the proposed damage to Purkinje cells is an indirect effect of some systemic response to high dose PCP can not be excluded. However, body temperature measurements indicate that the HSP70 induction and microglial activation is not due to hyperthermia, as PCP (10–50 mg/kg) causes hypothermia (Pechnick and George, 1989; Pechnick et al., 1989, our unpublished observations). Furthermore, the fact that PCP and MK801 induce almost identical behavioral responses but only PCP affects Purkinje cells, argues against a systemic mechanism.

The indole alkaloid ibogaine induces glial activation and Purkinje cell degeneration in the cerebellum (O'Hearn and Molliver, 1993; O'Hearn et al., 1993). Interestingly, the distribution of microglial reaction is similar to that shown here, being confined to the molecular layer and concentrated in the cerebellar vermis. The chemical structure of ibogaine is similar to harmaline, which produces identical cerebellar toxicity. Like PCP, ibogaine induces psychotomimetic effects and tremor in experimental animals (Zetler et al., 1972; Glick et al., 1991). The mechanism of ibogaine and harmaline neurotoxicity is unclear, although excitotoxic mechanisms mediated by the inferior olive might be involved (O'Hearn and Molliver, 1993). The vulnerability of vermal Purkinje cells to PCP as well as ibogaine and other toxins could be a consequence of regional differences in neural circuitry or of intrinsic cellular vulnerability.

The present study suggests that PCP damages rodent Purkinje cells whereas MK801 does not. Both drugs administered at high doses have indistinguishable behavioral effects, including tremor and ataxia. The absence of evidence of MK801 induced cerebellar damage supports differences in the actions of these agents in the cerebellum. Future studies are required to define the morphology, pharmacology, and mechanism of the proposed Purkinje cell damage by PCP.

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