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Microglial Reactivity to N-Methyl-D-Aspartate (NMDA) Induced Excitotoxicity in the Immature Rat Brain

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MICROGLIAL REACTIVITY TO N-METHYL-D-ASPARTATE (NMDA) INDUCED EXCITOTOXICITY IN THE IMMATURE RAT BRAIN

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ABBREVIATIONS

AChE	acetylcholinesterase
AD	anterodorsal thalamic nucleus
AV	anteroventral thalamic nucleus
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
bFGF	basic fibroblast growth factor
CA	cornus ammonis subfield of the hippocampus
CC	corpus callosum
CNS	central nervous system
CSF	colony-stimulating factors
C-P	caudate-putamen
CX	cerebral cortex
DAB	3,3'-diaminobenzidine
GM-CSF	granulocyte-macrophage-colony stimulating factor
GP	globus pallidus
IC	internal capsule
IL-1	interleukin 1
IL-3	interleukin 3
IL-6	interleukin 6

LD	laterodorsal thalamic nucleus
LP	lateroposterior thalamic nucleus
M-CSF	macrophage-colony stimulating factor
MHC II	major histocompatibility complex II
NMDA	*N*-methyl-D-aspartic acid
PO	posterior thalamic nucleus
R	reticular nuclei
R-VA	ventroanterior-reticular nuclei transition
TBS	tris buffered saline
TGFß	transforming growth factor ß
TNF	tumor necrosis factor
VA	ventroanterior thalamic nucleus
VB	ventrobasal complex of the thalamus
VL	ventrolateral thalamic nucleus

INTRODUCTION

Neuronal damage due to an hypoxic-ischemic insult is the major cause of neurological disorder in the developing brain. In providing a useful model to study the degenerative processes associated with hypoxia-ischemia, several reports have demonstrated a similarity between excitotoxin-induced and ischemia-induced brain damage (Ikonomidou et al., 1989a; Ikonomidou et al., 1989b; McDonald and Johnston, 1990; Diemer et al., 1993). A particular advantage to using such models is that the injection of excitotoxins such as *N*-methyl-D-aspartate (NMDA) produce a lesion that is much more consistent in size and location in comparison to an hypoxic-ischemic insult. Accordingly, intraparenchymal injection of the excitotoxin NMDA has been proposed as a model for ischemic-like injury in the developing brain. In this light, the purpose of this research thesis is to study the degenerative process after an injection of NMDA in the immature brain.

Neuronal degeneration induced by excitotoxic lesions, as well as by other types of injury, is associated with marked glial reactivity. While this lesion-induced gliosis may reflect degenerative mechanisms, recent studies provide evidence suggesting that gliosis in some circumstances may represent beneficial, reparative processes. Accordingly, study of the timecourse and intensity of the glial response after excitotoxicity is considered important in understanding the potential relevance of the glial response to neuronal repair in the injured immature and adult brain.

In addition to the well studied astroglial response to injury, several studies since the classical report by Del Rio-Hortega (Del Rio-Hortega, 1920) have demonstrated a prominent reactivity of microglial cells. However, this microglial response, unlike more uniform astrocytic reactivity, depends on the particular characteristics of the inflicted injury. The function of such microglial responses may range from roles in synaptic remodelling to phagocytosis and/or proliferation.

To further understand the lesion-induced microglial response, the present study is based on the hypothesis that an excitotoxic injury in the immature brain causes a marked microglial response which follows a specific pattern and time course related to the degenerative process. In order to examine this hypothesis, stroke-like lesions were induced by injecting NMDA into the cerebral cortex of 6-day old rats. Animals were subsequently sacrificed at various post injection intervals, and parallel sections were stained by the Nissl method and processed histoenzymatically for tomato lectin, a specific method for the visualization of microglial cells. The density and characteristics of the microglial response after each survival time was examined at light microscopic levels.

CHAPTER I

SUMMARY

The intracerebral injection of NMDA has been proposed as a model for hypoxic-ischemic insult in the immature brain. In this light, the aim of the present study was to describe the time course of the microglial reaction in the areas undergoing primary degeneration at the site of intracortical NMDA injection as well as in areas undergoing secondary anterograde and/or retrograde degeneration. Fifty nmols of NMDA were injected in the sensorimotor cortex of 6 day old rats. After survival times ranging from 10 hours to 28 days, cryostat sections were stained for routine histology and for the demonstration of microglial cells by means of tomato lectin histochemistry.

The areas affected by primary degeneration due to the intracortical injection of NMDA were the neocortex, the hippocampus and the rostral thalamus. Secondary degeneration (retrograde and anterograde) was observed in the ventrobasal complex of the thalamus. The cortical lesion also caused anterograde (Wallerian) degeneration of the cortical descending efferents as observed in the basilar pons. Microglial reactivity in all these areas was present at 10 hours post injection and was restricted to the areas undergoing neuronal or axonal degeneration. Reactive microglial cells were stained intensely and showed a round or pseudopodic morphology. At 3 days an apparent increase in the number of tomato lectin positive cells was observed in the areas undergoing neuronal death. By 7 days after the injection, the lesion became non-

progressive, and by 14 and 28 days, microglial cells showed moderate lectin binding and a more ramified morphology.

CHAPTER II

REVIEW OF RELATED LITERATURE

Excitotoxicity in the immature brain

Glutamate is the primary excitatory neurotransmitter in the brain (Fonnum, 1984), and its effects are mediated by several receptor subtypes, commonly classified as *N*-methyl-D-aspartate (NMDA) and non-NMDA type receptors. The latter type includes the quisqualate/AMPA and kainate receptors (McDonald and Johnston, 1990; Pruss, 1993). The excitatory actions of glutamate mediated by NMDA receptors have been linked to important developmental events such as neuronal migration, neurite outgrowth, and synaptic connectivity in the developing brain (McDonald and Johnston, 1990; LoTurco et al., 1991; Komuro and Rakic, 1993; Gould et al., 1994), as well as plasticity, long-term potentiation, and memory processes in the mature brain (Fagg and Foster, 1983; Choi, 1992). In contrast to their involvement in developmental and functional mechanisms, excessive activation of glutamate receptors can initiate a series of intracellular biochemical events that cause neuronal damage, the so called excitotoxic cell death process described more than 20 years ago (Olney, 1969). This process can be divided into two different phases; the first one involves depolarization of neuronal membranes, with Na+ influx followed by passive Cl- and H2O influx, causing neuronal swelling. The second phase implies excessive calcium influx via the NMDA

receptor complex. This increased intracellular calcium concentration causes activation of second messenger systems and mobilization of internal calcium storage, thereby activating lipases, proteases, generating fatty acids and free radicals, depleting energy stores, and ultimately leading to neuronal injury (McDonald and Johnston, 1990; Rothman and Olney, 1987).

Experiments using receptor antagonists suggest that the neuronal death associated with brief and intense glutamate exposure primarily depends on NMDA receptor activation (McDonald et al., 1989; McDonald and Johnston, 1990; Choi, 1992). Accordingly, overactivation of NMDA receptors has been implicated in the pathogenesis of different neurologic disorders such as hypoxia-ischemia, hypoglycemia and seizures (Ikonomidou et al., 1989b; Hattori and Wasterlain, 1990; McDonald and Johnston, 1990; Diemer et al., 1993), as well as neurodegenerative diseases such as Huntington's (Beal et al., 1991) and Alzheimer's diseases (Stewart et al., 1986).

NMDA receptor overactivation has been implicated particularly in the injury seen after hypoxic-ischemic insult in the early postnatal brain, as suggested by the reduction of the ischemia-induced brain damage observed following treatment with the specific NMDA receptor antagonist, MK-801 (Ford et al., 1989; Hattori et al., 1989; Olney et al., 1989). Moreover, the increased susceptibility to hypoxic-ischemic damage seen in one week old rats corresponds to a transient peak of NMDA receptor sensitivity (McDonald et al., 1988; McDonald and Johnston, 1990). The similarity of the histological changes observed in degenerating neurons endangered by ischemic-hypoxic insult or by intraparenchymal injection of NMDA further implicated NMDA receptor involvement in hypoxic-ischemic neuronal death (Ferriero et al., 1988; Ikonomidou et al., 1989a; Ikonomidou et al., 1989b).

Microglial cells

Microglia constitute aproximately 10% of the total glial population and are considered the resident macrophages of the brain (for review see (Thomas, 1992; Nakajima and Kohsaka, 1993b)). Although microglial cell origin was a debated issue in the 1980s, their mesodermal origin is now well established. Microglia arise from monocytes that populate the CNS after it has been vascularized (Boya et al., 1987; Hickey and Kimura, 1988; Ling and Wong, 1993; Ling, 1994). The major morphological subtypes of microglia in normal brain include ameboid, ramified and perivascular microglial cells. *Ameboid* microglial cells are rounded and devoid of any processes. They are found in the embryonic and early postnatal brain, show phagocytic activity, and are believed to contribute to tissue development by removal of dead cells occurring by natural cell death. Ameboid microglial cells transform into ramified microglia during postnatal development (Boya et al., 1991; Wu et al., 1993). *Ramified* microglial cells are found in the adult brain. Characteristically, they have an elliptical or elongated cell body with several processes branching out from the perinuclear cytoplasm. Such processes are usually further divided into spindle-like thinner processes. Distal parts of microglial processes often appear to end in proximity to blood vessel walls or in relation to neurons. *Perivascular* microglial cells are found in the perivascular space and are though to be more closely related to monocytes than to ramified microglia (Honda et al., 1990; Streit and Graeber, 1993).

Microglial cells can be visualized by different techniques, including histoenzymatic demonstration of nucleoside diphosphatase activity on the cell membrane (Castellano et al., 1991a; Castellano et al., 1991b), lectin histochemistry for the demonstration of different membrane sugar residues (Streit and Kreutzberg, 1987; Boya et al., 1991; Acarin et al., 1994), and monoclonal antibodies for the

demonstration of immunological markers such as type 3 complement receptor (OX-42 antibody) (Perry and Gordon, 1987), Fc receptors (Ohno et al., 1992), and specific receptors (MUC 101 and 102 antibodies) (Gehrmann and Kreutzberg, 1991).

The functional role of ramified microglial cells in the normal adult brain is still not known. Their characteristic distribution forming a network of cells covering the entire CNS territory and their reaction to injury by upregulating macrophage characteristics suggests that, in their resting state, they are down-regulated macrophages that form a network of immunocompetent cells in the CNS (Graeber and Streit, 1990; Thomas, 1992; Nakajima and Kohsaka, 1993b). Moreover, the demonstrated pinocytic activity of ramified microglial cells suggests that these cells may contribute to a fluid cleansing system for the extracellular fluid in normal tissue (Thomas, 1990; Glenn et al., 1991).

Microglial response to injury

The neuronal damage produced by the injection of an excitatory neurotoxin, like other types of injury, is accompanied by a marked astroglial reaction and a rapid microglial response (Dusart et al., 1991; Marty et al., 1991; Jorgensen et al., 1993).

In addition to reactive astrogliosis, occurring whenever the integrity of the central nervous system is disturbed, classical studies have shown a prominent reactivity of microglial cells (Del Rio-Hortega, 1920). This activation of microglial cells can lead to a state where reactive microglial cells will only be engaged in "synaptic stripping" and synaptic remodelling (Streit and Kreutzberg, 1987) or to a state where reactive cells will express typical features of macrophages, develop an active role removing cellular debris by phagocytosis (Ludwin, 1990; Svensson et al., 1993), and/or proliferate

(Graeber et al., 1988; Giordana et al., 1994). In addition to their phagocytic role, recent studies have shown that microglial cells are involved in the regulation of the astrocytic response and in the immunological reaction after injury. As indicated by these and other related studies, microglial reactivity can produce both beneficial regenerative and detrimental degenerative effects.

The beneficial effects associated with activated microglial cells include the secretion of basic fibroblast growth factor (bFGF), as demonstrated both *in vitro* (Nakajima and Kohsaka, 1993a) and *in vivo* after injury (Frautschy et al., 1991). Treatment with bFGF increases neuronal resistance to excitotoxic injury (Mattson and Rychlik, 1990; Frim et al., 1993; Nozaki et al., 1993) and induces astrocytic proliferation and angiogenesis (Gomez-Pinilla et al., 1992; Rothwell and Relton, 1993). Neuroprotective effects have also been associated with transforming growth factor ß (TGF-ß) which is expressed by microglial cells after excitotoxic injury and ischemia (Morgan et al., 1993; Wiessner et al., 1993). Recent work indicates that TGF-ß is important in regulating tissue damage, promoting repair, and down-regulating microglial cytotoxicity (Rothwell and Relton, 1993). In addition, activated microglial cells are capable of antigen processing and presentation in the brain by expressing major histocompatibility complex II (MHC II) after injury (Akiyama et al., 1988; Morioka and Streit, 1991; Kaur and Ling, 1992; Morioka et al., 1992; Finsen et al., 1993; Jorgensen et al., 1993). Antigen presentation as well as the secretion of different cytokines suggest that microglial cells represent an important component in mediating inflammation and recruitment after CNS injury.

However, the role of cytokines in the injury response is still unclear. It has been suggested that perturbations leading to the oversecretion of cytokines could be more harmful than the specific actions they exert (Merrill, 1992). Cytokines expressed by

activated microglial cells are mainly interleukin-1 (IL-1) (Woodroofe et al., 1991; Van Dam et al., 1992; Yao et al., 1992; Yabuuchi et al., 1993), tumor necrosis factor (TNF) (Hartung et al., 1992), interleukin-3 (IL-3) (Gebicke-Haerter et al., 1994), and interleukin-6 (IL-6) (Woodroofe et al., 1991; Sawada et al., 1992). These cytokines have multiple and redundant effects on microglial cells themselves as well as on other surrounding cells. IL-1 promotes leukocyte adhesion to endothelial cells (Benveniste, 1993), astrocytic hypertrophy and proliferation (Giulian et al., 1988a; Giulian et al., 1988b; Merrill, 1991; Balasingam et al., 1994). Moreover, IL-1 induces microglial secretion of toxic arachidonic acid and nitric oxide (Hartung et al., 1992), and may have a role in mediating excitotoxic and ischemic cell damage (Relton and Rothwell, 1992). Although TNF has been suggested to mimic some IL-1 effects such as leukocyte adhesion, astrogliosis, or microglial proliferation (Merrill, 1991; Benveniste, 1993; Rothwell and Relton, 1993; Balasingam et al., 1994), recent work has demonstrated its protective actions against NMDA toxicity in hippocampal slices (Cheng et al., 1994). This finding correlates with observations showing that secreted IL-6 can attenuate NMDA excitotoxicity in the adult brain (Toulmond et al., 1992).

Recent *in vitro* studies have demonstrated the cytotoxic effect of microglial-derived products such as glutamate (Piani et al., 1991; Streit, 1993), nitric oxide (Chao et al., 1992; Murphy et al., 1993), arachidonic acid (Hartung et al., 1992), proteases (Nakajima and Kohsaka, 1993a), reactive oxygen species (Colton and Gilbert, 1993; Giulian and Corpuz, 1993; Giulian and Vaca, 1993; Giulian et al., 1993), and non-identified factors acting via a NMDA receptor-mediated mechanism implicated in delayed neuronal death after ischemic injury (Piani et al., 1991; Piani et al., 1992; Giulian et al., 1993; Giulian and Vaca, 1993; Giulian et al., 1993). Detrimental effects have also been attributed to glial scar formation after neurotoxic injury. Although the

glial scar may form a new glia limitans serving to separate damaged from undamaged tissue, and thus prevent extension of the lesion process, these scars may also represent potent inhibitors of axonal regeneration (Bovolenta et al., 1992). Thus, the function of microglial cells after injury is not known, and their beneficial or detrimental role is still controversial. While these seemingly opposing actions may actually represent different aspects of a degenerative/regenerative response, further analysis of the microglial response to different types of injury and in different developmental stages are needed.

CHAPTER III

MATERIALS AND METHODS

NMDA injections

Long Evans black-hooded 6-day old rats of both sexes (day of birth equals day 0) were used in this study. Under ether anesthesia, each pup was placed in a stereotaxic frame adapted for newborns (Kopf). Fifty nmols of NMDA (Sigma, M-3262, St. Louis, MO) diluted in 0.2 µl of saline solution was injected into the right sensorimotor cortex at the level of the coronal suture (2 mm lateral from bregma and at 0.5 mm of depth). After surgery, the pups were placed in an incubator and maintained at 36°C for 2 hours before they were returned to their mothers. The incubator was used to maintain normothermia, because NMDA induced lesions in the immature brain were highly dependent on body temperature (McDonald et al., 1991). In control animals the same procedure was followed except they received an injection of 0.2 µl of saline solution (0.9% NaCl). Eight NMDA injected animals and two saline injected control animals were used for each of six post injection survival times.

Fixation and histology

At 10 hours, 1 day, 3 days, 7 days, 14 days and 28 days after NMDA injection, rats were anesthetized by ether inhalation and perfused intracardially for 10 min. with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were immediately removed, immersed in the same fixative for 4 hours and cryoprotected in a 30% sucrose solution in 0.1M phosphate buffer. Brains were quickly frozen with dry CO_2 and stored at -70°C until sectioned. Coronal sections (30-40 μm thick) were obtained using a Leitz cryostat. Alternate sections were mounted on gelatin coated slides or stored free-floating in antifreeze buffer (Olmos solution).

The mounted series of sections were stained with the Nissl method for routine histological examination or processed histoenzymatically for the demonstration of AChE which facilitates the identification of thalamic nuclei. Free-floating sections were processed for tomato lectin histochemistry for the visualization of microglial cells.

Nissl staining

One series of mounted tissue sections were defatted and stained for 10 min. in a stain solution composed of 15 ml of a 0.1% Toluidine stock solution in 200 ml walpole buffer (3 parts 0.2M acetic acid to 2 parts 0.2M sodium acetate, pH 4.5). The tissue was then rinsed quickly in distilled water and dehydrated before immersion in 2 changes of n-butyl alcohol for 4 min. The sections were then cleared in xylene and coverslipped in DPX.

Acetylcholinesterase histochemistry

One series of mounted tissue sections were processed histochemically for acetylcholinesterase (AChE) (Hedreen et al., 1985). Tissue sections were rinsed in 0.1M acetate buffer twice for 2 min. and incubated for 90 min. in the incubation solution. This solution was made up adding 6.6 mg potassium ferricyanide and 0.1 g acetylthiocholine iodide to 200 ml of a stock solution (1.18 g sodium citrate, 0.75 g cupric sulfate, 665 ml dH_2O, 335 ml 0.2M acetate buffer, pH 6.0) immediately before use. After the incubation, the tissue sections were rinsed five times for 1 min. in 0.1M sodium nitrate, immersed for 1 min. in 1% ammonium sulfide, rinsed again with 0.1M sodium nitrate, submerged for 1 min. in 1% silver nitrate, and finally rinsed in fresh 0.1M sodium nitrate. Before coverslipping with DPX the sections were dehydrated in alcohol.

Tomato lectin histochemistry

Tomato lectin histochemistry for the visualization of ameboid and ramified microglial cells in the rat brain has been described recently (Acarin et al., 1994). Free floating sections were processed as follows: after rinsing for 1 hour in 0.05M tris buffered saline (TBS), endogenous peroxidase was blocked for 10 min. with 2% H_2O_2 in 100% methanol. Sections were then rinsed twice for 10 min. in TBS, once for 10 min. in TBS + 1% Triton X-100, and incubated for 2 hours at room temperature with the biotinylated lectin obtained from *Lycopersicon esculentum* (tomato) (Sigma L-9389; St. Louis, MO, USA) diluted to 6 µg/ml in TBS + 1% Triton X-100. After incubation, sections were washed once for 10 min. in TBS + 1% triton X-100 and twice for 10 min. in TBS, and incubated for 1 hour at room temperature with avidin

peroxidase (Sigma, A-3151, St. Louis, MO, USA) in a 1: 70 dilution in TBS. After two rinses of 10 min. in TBS and one rinse in tris buffer for 10 min., the peroxidase reaction product was visualized using 50 mg of DAB with 33 µl of hydrogen peroxide in 100 ml tris buffer. Finally, the sections were rinsed in tris buffer three times for 10 min., mounted on gelatin coated slides, dehydrated, cleared in xylene, and coverslipped in DPX. Control of tomato lectin specificity was carried out by incubating tomato lectin for 30 min. in a 0.1M solution of N-acetyllactosamine (Sigma, A-7791, St. Louis, MO, USA), sugar to which the lectin binds, and incubating the sections in the lectin solution with the same sugar.

Quantification method

National Institute of Health *Image* software was used to quantify the histochemical labelling of microglial cells. This quantification is presented as a 'reactivity grade' which was defined as a ratio between values of a specific area in the hemisphere ipsilateral to the NMDA injection versus the same area in the contralateral control hemisphere. This ratio method compensates for the variability of lectin staining between sections. This ratio represents: (i) the **intensity of lectin binding**, by measuring the density of the reaction product, and (ii) the **density of cells in that area**, measured as the percentage of the delimited area above the reaction density threshold, which is defined as the mean reaction density of the contralateral control area.

The formula used is summarized as follows:

$$\textbf{REACTIVITY GRADE} = \frac{\text{MEAN REACTION DENSITY}_{\text{Ipsilat.}} \times \frac{\text{AREA ABOVE THRESHOLD}_{\text{Ipsilat.}}}{\text{TOTAL AREA}_{\text{Ipsilat.}}}}{\text{MEAN REACTION DENSITY}_{\text{Contralat.}} \times \frac{\text{AREA ABOVE THRESHOLD}_{\text{Contralat.}}}{\text{TOTAL AREA}_{\text{Contralat.}}}}$$

Considers if cells in the lesioned area are more lectin positive than cells in the control area

Considers if the lesioned area has more space occupied by cells than the control area, either by increase in the number of cells or by enlargement of the cell bodies

A 'reactivity grade' was obtained for each area of study (i.e. cortex, the hippocampus, the rostral thalamus and the VB complex), in 2 sections per animal and 4 animals for each NMDA injected survival group. The same measurements were done in saline injected animals. The measured areas were: (i) a square in the cortex, including the degenerating layers, at the level of the injection site; (ii) the hippocampus; (iii) the rostral thalamus, including all nuclei; and (iv) a rectangle in the caudal thalamus, including the VB complex, reticular nuclei and the internal capsule. Statistical analysis of data was performed using the program Statview 4.0. Analysis of variance (ANOVA) with Fisher's PLSD post-hoc comparisons were used.

CHAPTER IV

RESULTS

The injection of NMDA into the right sensorimotor cortex of six day old rats caused an extensive primary lesion usually involving the cortex, the dorsal striatum, the dorsal hippocampus and the rostral thalamus of the ipsilateral hemisphere. These areas were neuron depleted by one day after the injection. In addition, neuronal swelling and degeneration was also observed in the ventrobasal (VB) complex of the thalamus. This thalamic degeneration was likely due to secondary degeneration caused by loss of the target cortical neurons. Other thalamic nuclei such as the ventrolateral thalamic nucleus (VL) and the posterior thalamic nucleus (Po) were sometimes also affected. Wallerian degeneration of the cortical descending efferents was also caused by neuronal death in the sensorimotor cortex. No neuronal degeneration in the contralateral hemisphere was seen at any survival time. These results are summarized in Figure 1.

Tomato lectin histochemistry allowed the visualization of lesion-induced activated microglial cells (referred to as reactive microglial cells) as well ameboid and ramified microglia observed in control brains. As previously described (Acarin et al., 1994), blood vessels and ependymal cells were also stained.

In gray matter areas of saline injected control animals with short survival times, tomato lectin positive microglial cells were evenly distributed and showed a primary ramified morphology. In some white matter tracts such as the corpus callosum and the

internal capsule, microglial cells appeared ameboid in shape, as a normal feature of development. The adult pattern of ramified microglial cells was first observed in 13 day old control animals. In these animals, microglial reactivity was restricted to the needle track until 3 days after the injection.

After NMDA injection, microglial reaction was generally characterized by a rapid onset already seen at 10 hours after injection. Reactive microglial cells showed a different morphology depending on the area of the brain examined and the post injection time point.

Neocortex and adjacent subcortical areas

Neuronal degeneration in the cerebral cortex followed a very specific pattern, primarily affecting the deep cortical layers while sparing the outer layers (aproximately lamina I-III) in all animals and at all survival times examined. The border between the spared layers and the neuron depleted area was well defined (Fig. 2D). At 10 hours after the NMDA injection, the affected areas displayed neurons with dark round cell bodies. By 1 day postinjection, affected areas were devoid of neurons and cellular debris were observed. The dorsal striatum was not affected as severely as the cerebral cortex, although some neuronal loss was also seen by 1 day. Longer survival times were characterized by shrinkage of the cortex, and the spared outer layers collapsed down to the adjacent white matter. By 7 days the lesion became non-progressive, and a thin layer devoid of cells was seen between the spared layers and the gliotic inner neuron-depleted area (Fig. 3A).

At all survival times examined, microglial reactivity was restricted to areas undergoing neuronal degeneration (Fig. 2E). At 10 hours, microglial cells had lost their

typical uniform distribution and displayed an ameboid or pseudopodic morphology (Figs. 2B,C). They were darkly stained and sometimes were seen in close contact with blood vessels. At 1 and 3 days after the injection, three different microglial morphologies were observed: (i) primary ramified microglial cells (Fig. 2F), which showed moderate lectin staining, were found evenly distributed in the spared outer cortical layers and were comparable to those found in the non-affected contralateral hemisphere; (ii) pseudopodic and intensely stained lectin positive microglial cells (Fig. 2G), with enlarged cell bodies and short thick processes, were observed in the middle cortical layers, which showed extensive neuronal loss with some scattered swollen neurons; and (iii) round and very dark tomato lectin positive cells (Fig. 2H) were seen in the inner cortical layers, which were completely devoid of neurons, and in the adjacent subcortical white matter. At 3 days after the lesion, an apparent increase in the number of tomato lectin positive cells was observed. By 7 days, a prominent tomato lectin positive scar began to emerge between the spared outer layers and the neuron depleted area (Fig. 3B). At this survival time, the more intensely stained microglial cells of the inner cortical layers and the callosal white matter began to change to a bushy-like appearance. This pattern was still obvious at 14 days after the injection. By 28 days, the last survival time analyzed, microglial cells tended to become ramified and to lose lectin binding, but the well established microglial scar was still present (Fig. 3C).

Hippocampus

Nissl stained sections showed that rostral levels of the dorsal hippocampus were most affected. Neuronal loss was seen in rostral pyramidal layers CA1 to CA3 from 1 day post injection, and delayed cell death was seen up to 7 days after the

injection. Medial-caudal hippocampal levels showed selective loss of pyramidal neurons in the dorsal area of CA3. This response was seen in all cases by 1 day after NMDA injection (Figs. 4E, G). Although less affected than at rostral levels, caudal CA1 field showed apparent cytoarchictectural disorganization with some neuronal swelling.

At rostral hippocampal levels, a general microglial reaction was observed by 10 hours after the injection. This microglial reaction was not as evident in caudal hippocampal levels until day 1. At 1 and 3 days after the injection, reactive microglial cells showed an ameboid morphology. These reactive microglial cells were concentrated within the pyramidal layer which showed massive neuronal death (CA1-CA3 at rostral hippocampal levels and dorsal area of CA3 at all levels) (Figs. 4D, F) and in stratum oriens of pyramidal layers which showed neuronal swelling and cytoarchitectural disorganization (CA1 at caudal levels) (Fig. 4B). At 7 days, although remaining in the same location, microglial cells became more ramified, showing moderate staining (Fig. 4H). By 14 days, microglial cells were seen covering neuron depleted areas, but a more ramified morphology was seen.

Thalamus

Thalamic nuclei at early postnatal ages were identified using acetylcholinesterase stained sections and with reference to previous work (Robertson and Mostamand, 1988). Extensive neuronal loss was seen in the anterodorsal (AD), laterodorsal (LD), ventroanterior (VA) and reticular nuclei at 1 day after the cortical injection, while the anteroventral nucleus (AV) was spared (Figs. 5C,E). Microglial reactivity was seen in all neuron depleted areas (Figs. 5B,D). Reactive microglial cells showed a similar

morphology to the reactive microglial found in the neuron depleted areas of the neocortex. An increase in the number of tomato lectin positive reactive cells was also seen 3 days after the lesion (Fig. 5D). At 7 days post injection, microglial cells showed a more ramified morphology (Fig. 5F). No microglial reactivity was found in the AV nucleus in any case.

At more caudal thalamic levels, neuronal swelling and some neuronal loss was observed. This degeneration was restricted to the VB complex but sometimes extended to more medial thalamic nuclei such as VL or Po. Microglial reactivity at this caudal level was evident 1 day after the injection (Figs. 6A,B) and peaked at day 3 (Figs. 6D,E). In general, reactive microglial cells in this location showed a bushy appearance with numerous short thick processes branching out from the enlarged cytoplasm (Fig. 6C). This microglial reactivity disappeared gradually (Fig. 6E) and was nearly absent by 14 days.

Wallerian degeneration of cortical efferents

Wallerian degeneration of cortical efferents was studied at the level of the pons. Microglial reactivity was first seen at 10 hours after the NMDA injection, although sometimes it was difficult to distinguish this response from the normal presence of ameboid cells in white matter tracts during development. Microglial reactivity was most obvious at 1 day post injection (Fig. 7B) and was still present at 3 days, although no obvious increase in the number of tomato lectin positive cells was observed (Figs. 7C, D). In the degenerating tract, reactive microglial cells showed pseudopodic morphology with short spiny processes and an increase in lectin binding (Fig. 7B), losing their

typical orientation following fiber bundles. At 7 days after the injection microglial reactivity was nearly absent (Fig. 7D), although apparent atrophy of the tract remained.

Quantification studies

Quantification data of microglial reactivity is summarized in figure 8 and 9. Saline injected animals (data not shown) showed reactivity grades of 1.02± 0.16 (mean ± S.D), indicating that microglial cell reactivity was equal in the ipsilateral and the contralateral control hemisphere. In NMDA injected animals, a slight increase in the 'reactivity grade' was seen already at 1 day after the injection, especially in the cortex (injection site) and the rostral thalamus, with values of 1.71 and 1.47, respectively. This increase in microglial reactivity did not achieve significance ($p<0.01$) until 3 days after the injection with peak values of 3.5 in the cortex (injection site), 2.32 in the rostral thalamus, 1.85 in the hippocampus, and 1.85 in the VB complex (in this last area, $p<0.05$). The highest 'reactivity grade' of 3.5 was found in the cortex at day 3, when the injected cortex achieved a 'reactivity level' at 3.5 times the 'reactivity level' of the contralateral control hemisphere.

Microglial 'reactivity grades' in the cortex, hippocampus and rostral thalamus decreased steadily by 7 days. However, the high 'reactivity grade' in the VB complex persisted at a level that was statistically higher ($p<0.01$) than the same area in saline injected littermates until 7 days after the injection. This prolonged thalamic microglial reactivity most likely corresponds to the secondary thalamic degeneration that occurs after cortical lesions.

These findings correlate with the morphological observations described in the previous results sections, although morphological characteristics of microglial reactivity

are already obvious at 10 hours and 1 day after the injection, timepoints when there is no statistically significant increase in the 'reactivity grade'.

FIGURE 1.

Camera lucida drawings showing the NMDA injection site (black dot) and the associated neuronal degeneration (stippled) at 1 day post-injection. Abbreviations: AD, anterodorsal thalamic nuclei; AV, anteroventral thalamic nucleus; CA, cornus ammonis; CC, corpus callosum; C-P, caudate-putamen; CX, cerebral cortex; GP, globus pallidus, IC, internal capsule; LD, laterodorsal thalamic nucleus; LP, lateroposterior thalamic nucleus; PO, posterior thalamic nucleus; R, reticular nuclei; R-VA, ventroanterior-reticular nuclei transition; VB, ventrobasal complex of the thalamus.

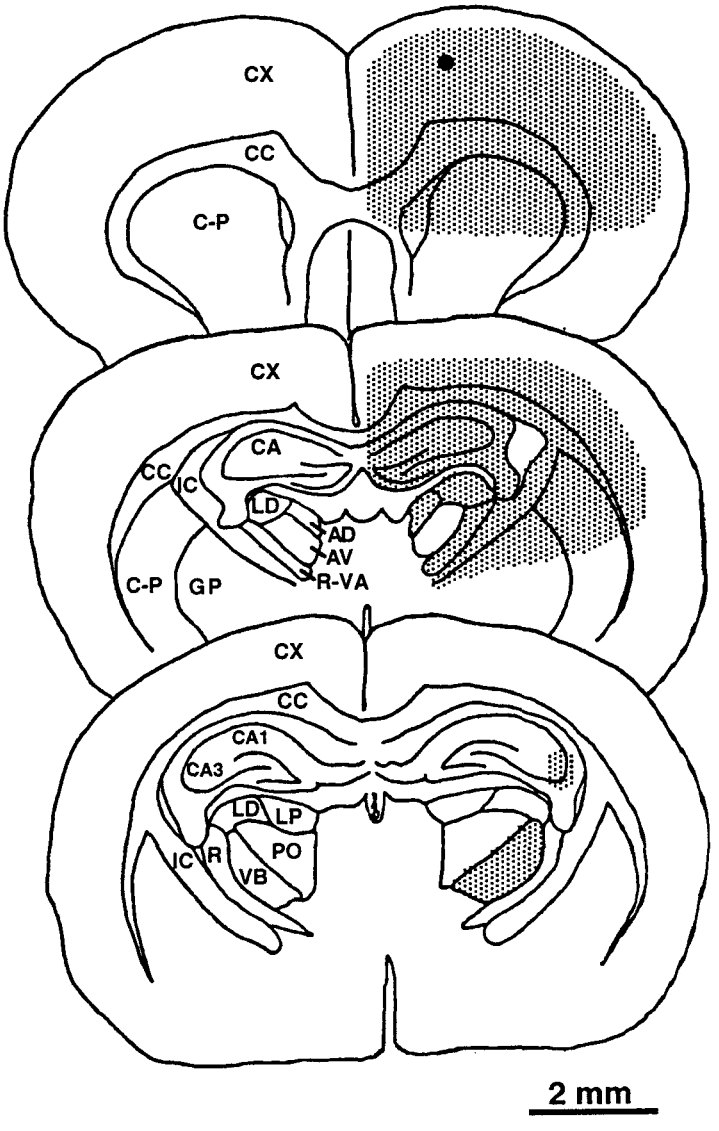

CX
CC
C-P
CX
CA
CC
IC
LD
AD
AV
R-VA
C-P
GP
CX
CC
CA1
CA3
LD
LP
IC
R
PO
VB
2 mm

FIGURE 2.

Photomicrographs demonstrating the contralateral control cerebral cortex (A) and the injected cerebral cortex (B-H) as observed using tomato lectin histochemistry or Nissl stain at 10 hours and 3 days post-injection of NMDA.

A,B. At 10 hours post-injection, the non injected contralateral hemisphere (A) contains primary ramified microglial morphology (arrows) in the cortex and ameboid microglial cells (arrowhead) in the corpus callosum, whereas the injected side (B) shows numerous reactive microglial cells. Note that blood vessels are also lectin positive (bv).

C. A higher magnification of figure B shows the characteristic morphology of reactive microglial cells.

D. At 3 days post injection, the spared external cortical layers (ext) are clearly distinguished from the degenerating internal cortical layers (int). The arrows show the borders between layers. Nissl stain.

E. Tomato lectin histochemistry of a section adjacent to figure D, demonstrates ramified microglial cells in the spared external layers (ext) and the reactive microglial cells found in the degenerating internal cortical layers (int). Note the accumulation of reactive microglial cells in the border (arrows).

F,G,H. At 3 days post-injection, ramified microglial cells (as indicated by the arrow in F) are found throughout the spared external cortical layers, pseudopodic microglial cells (as indicated by the arrow in G) are found in the degenerating internal layers, and intensely stained round microglial cells (as indicated by the arrow in H) are found in the deeper internal layers and the corpus callosum.

Scale bars: A,B,D,E= 100μm; C = 30μm; F,G,H = 40μm. A-C, E-G, Tomato lectin histochemistry; D, Nissl stain.

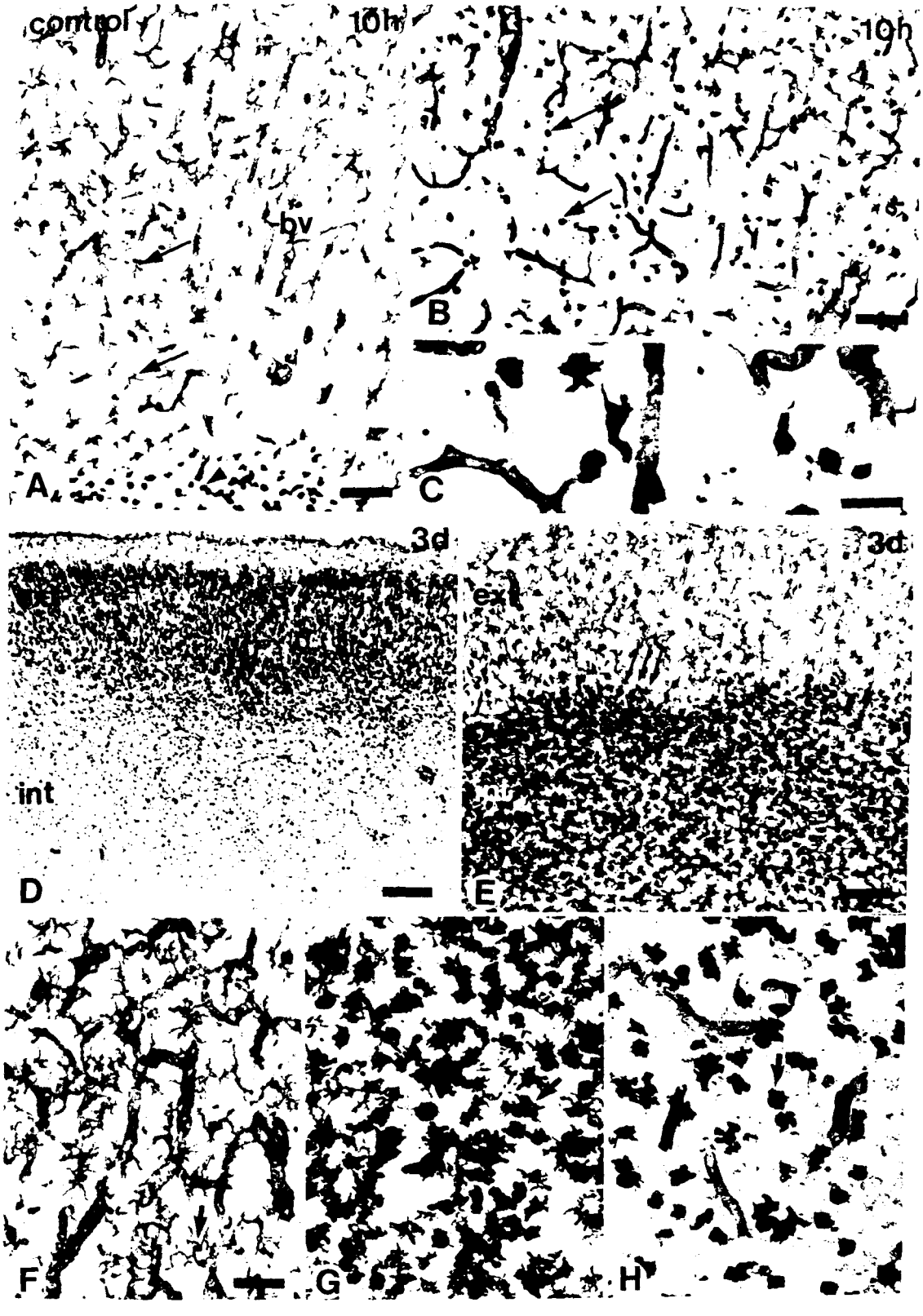

control
10h
bv
A
B
C
10h
3d
int
D
3d
ex
E
F
G
H

FIGURE 3.

Photomicrographs demonstrating the injected cerebral cortex at 7 days and 28 days post-injection of NMDA.

A. At 7 days post-injection, the spared external cortical layers (ext) are clearly distinguished from the degenerating internal cortical layers (int).Nissl stain.

B. Tomato lectin histochemistry of a section adjacent to figure A shows the accumulation of reactive microglial cells in the border between external cortical layers (ext) and internal cortical layers (int).

C. At 28 days post-injection, there is a decrease in lectin binding and microglial cells show a more ramified morphology (arrow).

Scale bars: A,B = 100μm; C = 60μm. B,C, Tomato lectin histochemistry; A, Nissl stain.

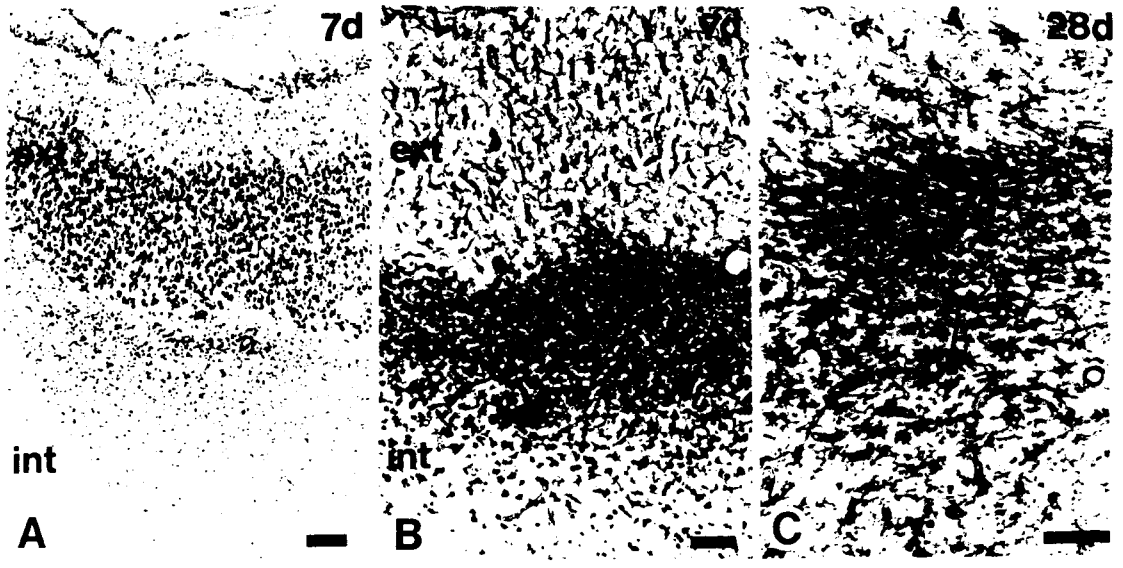

7d
ext
int
A
7d
ext
int
B
28d
C

FIGURE 4.

Photomicrographs demonstrating the hippocampal formation ipsilateral to the injected cerebral cortex at 1 day, 3 days and 7 days after cortical injection of NMDA.

A. At 1 day post-injection, a disorganization of the CA1 layer is observed. Nissl stain.

B. At 1 day, a section adjacent to figure A shows the presence of reactive microglial cells in stratum oriens.

C. At 3 days post-injection, disorganization and neuronal loss is observed in rostral levels of the CA1 pyramidal layer. Nissl stain.

D. At 3 days, a section adjacent to figure C shows the accumulation of reactive microglial cells in the degenerating pyramidal layer.

E. At 3 days post-injection, massive neuronal loss is found in the dorsal zone of CA3 field. Nissl stain.

F. At 3 days, a section adjacent to figure E shows the presence of reactive microglial cells covering the CA3 field.

G. At 7 days post-injection, note the discontinuity of pyramidal CA3 layer. Nissl stain.

H. At 7 days, a section adjacent to figure G demonstrates microglial cells showing moderate lectin binding and a more ramified morphology, covering the CA3 field.

Abbreviations: CA1, CA1 field; CA3, CA3 field; FD, fascia dentata; Or, stratum oriens; Py, pyramidal layer; Ra, stratum radiatum.

Scale bars: A-F = 100μm; G, H = 150μm. A,C,E,G, Nissl stain; B,D,F,H, Tomato lectin histochemistry.

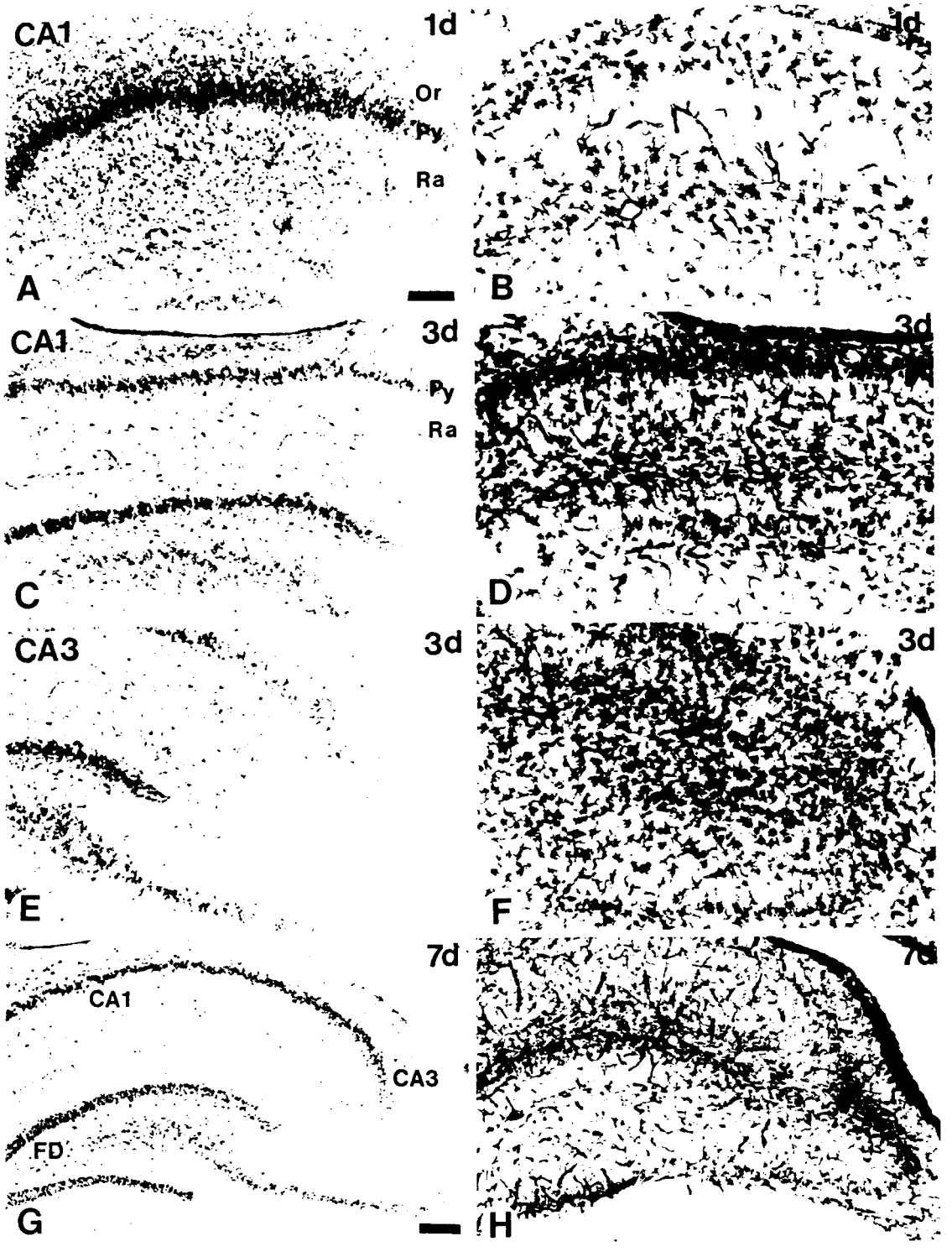

CA1
1d
Or
Py
Ra
A
B
1d
CA1
3d
Py
Ra
C
D
3d
CA3
3d
E
F
3d
7d
CA1
CA3
FD
G
H
7d

FIGURE 5.

Photomicrographs showing the contralateral control rostral thalamus (A) and the ipsilateral lesioned side at 1 day, 3 days and 7 days after cortical injection of NMDA (B-F).

A,B. At 1 day post-injection, contralateral control rostral thalamus contains uniformly distributed microglial cells that show a ramified morphology (A), whereas ipsilateral thalamus contains reactive microglial cells, except in the AV nucleus (B).

C. At 1 day, a section adjacent to figure B shows the observed thalamic neuronal degeneration as well as the spared AV nucleus.

D. At 3 days post-injection, an apparent increase in microglial cells is found in the degenerating nuclei. Note the specific borders between the degenerating nuclei and the AV nucleus.

E. At 3 days, a section adjacent to figure D shows the extensive neuronal loss observed in thalamic nuclei.

F. At 7 days post-injection, reactive microglial cells show moderate lectin binding and a more ramified morphology.

Abbreviations: AD, anterodorsal thalamic nuclei; AV, anteroventral thalamic nuclei; IC, internal capsule; LD, laterodorsal thalamic nuclei; R, reticular nuclei; R-VA, reticular-ventroanterior nuclei transition.

Scale bar A-F = 150μm. A,B,D,F, Tomato lectin histochemistry; C, Nissl stain.

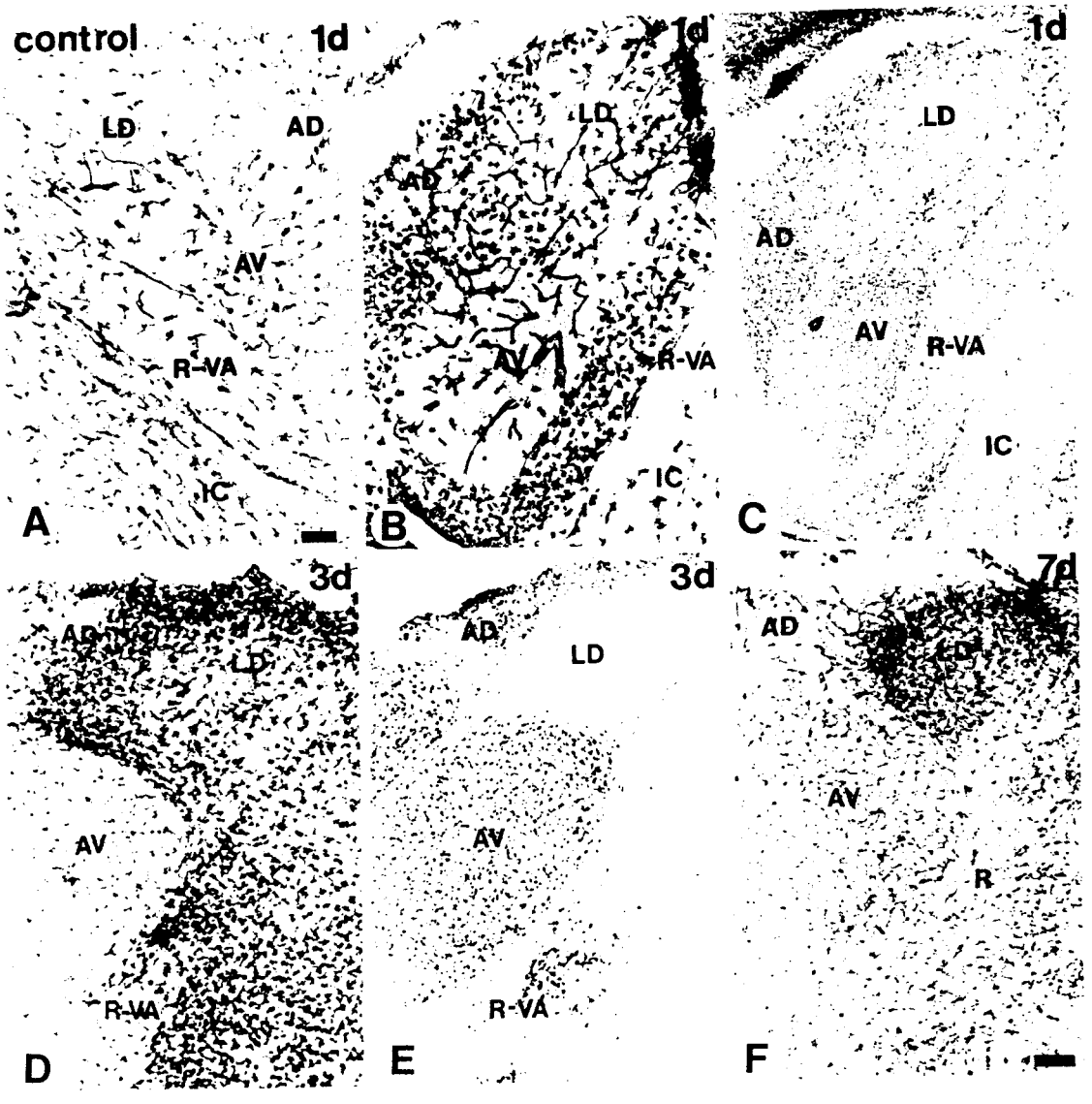

control
1d
LD
AD
AV
R-VA
IC
A
1d
LD
AD
AV
R-VA
IC
B
1d
LD
AD
AV
R-VA
IC
C
3d
AD
LD
AV
R-VA
D
3d
AD
LD
AV
R-VA
E
7d
AD
LD
AV
R
F

FIGURE 6.

Photomicrographs demonstrating the caudal thalamic nuclei in the contralateral control hemisphere (A) and in the ipsilateral lesioned hemisphere at 1 day, 3 days and 7 days after cortical injection of NMDA (B-E).

A,B. At 1 day post-injection, the contralateral control side (A) shows primary ramified microglial cells in the thalamus and ameboid microglial cells in the IC. In the thalamus ipsilateral to the injection site (B), note the accumulation of microglial reactivity at the border between VB and R.

C. A higher magnification of figure B shows the characteristic pseudopodic morphology of reactive microglial cells in the VB complex.

D. At 3 days post-injection, an apparent increase in reactive microglial cells can be observed. Note the lack of reactive microglial cells in R nuclei.

E. At 7 days post-injection, reactive microglial cells show moderate lectin binding and tend to become ramified.

Abbreviations: IC, internal capsule; R, reticular nuclei; VB, ventrobasal complex of the thalamus.

Scale bars: A,B,D,E = 100μm; C = 10μm. A-E, Tomato lectin histochemistry.

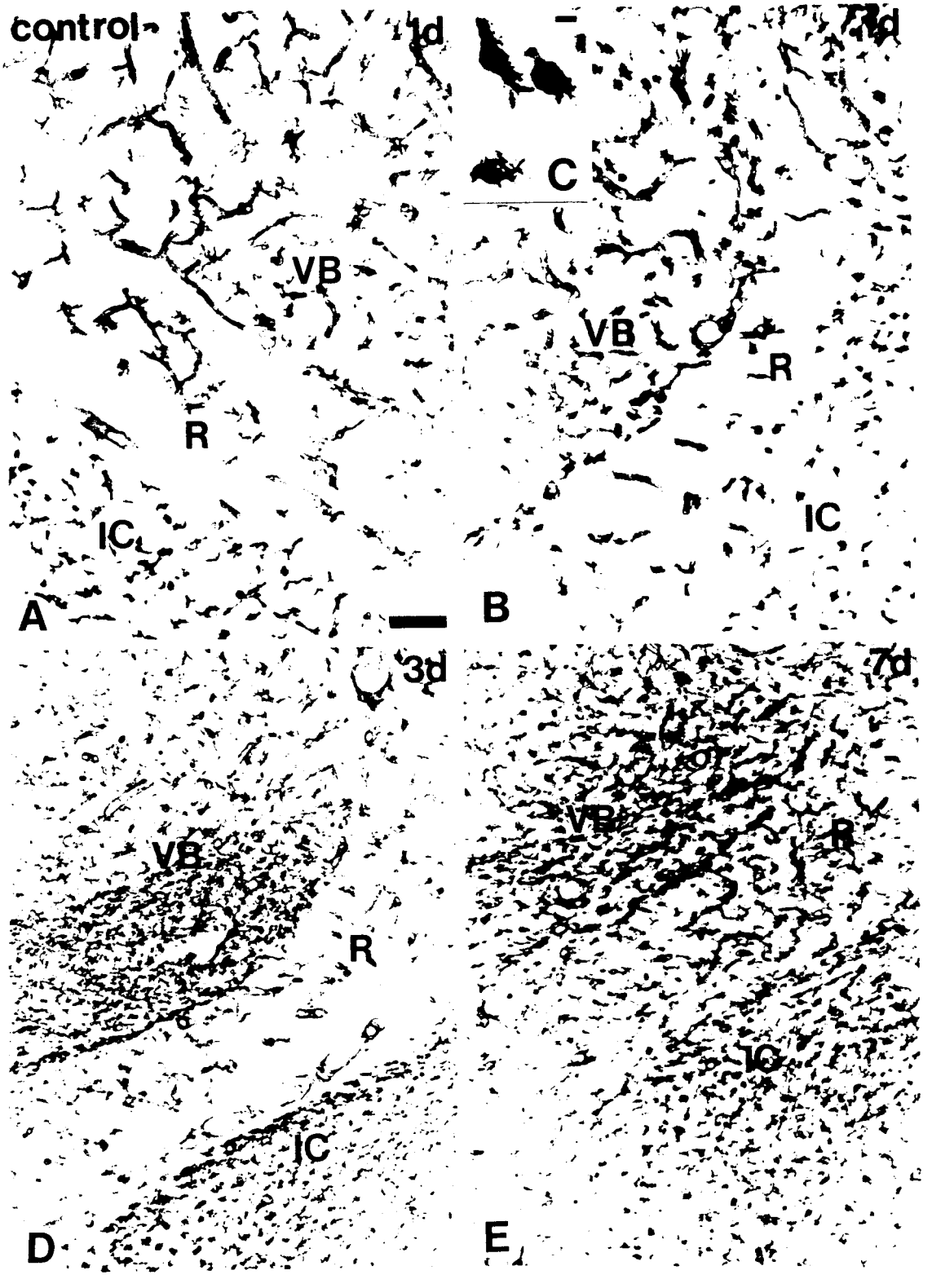

control
1d
VB
R
IC
A
C
1d
VB
R
IC
B
3d
VB
R
IC
D
7d
VB
R
IC
E

FIGURE 7.

Photomicrographs showing area of cortical efferents coursing through the pons, contralateral (A) and ipsilateral to the lesioned neocortex at 1 day, 3 days, and 7 days after NMDA injection (B-D).

A,B. At 1 day post-injection, the contralateral control side (A) shows the primary ramified morphology of microglial cells (arrow) in the non-affected tract, whereas in the tract ipsilateral to the injected neocortex (B) reactive microglial cells show a pseudopodic morphology.

C. At 3 days post-injection, microglial reactivity in the ipsilateral tract is still present.

D. At 7 days post-injection, microglial reactivity in the ipsilateral tract is not as prominent as at earlier time points.

Scale bar = 60μm. A-D, Tomato lectin histochemistry.

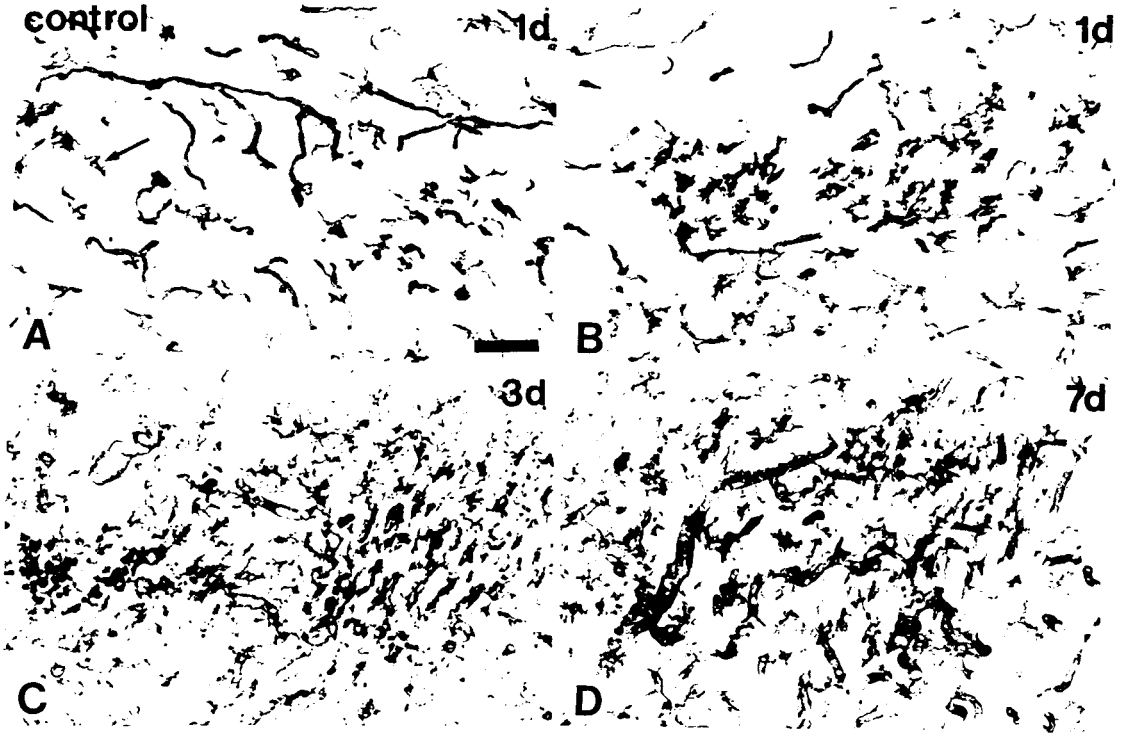
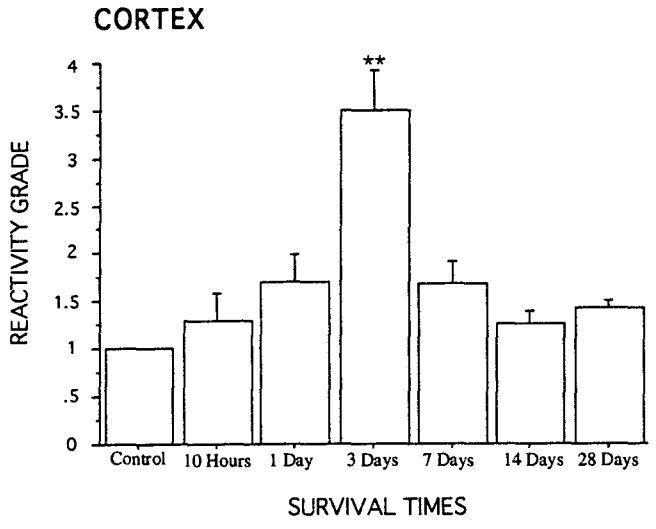

control
1d
1d
A
B
3d
7d
C
D

FIGURE 8.

Quantitative analysis of microglial reactivity in the cortex and the hippocampus and at several survival times after the NMDA injection. Data are presented as the mean values of reactivity grades ± s.e.m. for four animals in each survival time. Statistical analysis was performed using ANOVA (**$p<0.01$) compared to control animals injected with saline at each survival time (data not shown). Significant increase of the reactivity grade was observed in both areas studied at 3 days after the injection.


CORTEX
REACTIVITY GRADE
4
3.5
3
2.5
2
1.5
1
.5
0
**
Control
10 Hours
1 Day
3 Days
7 Days
14 Days
28 Days
SURVIVAL TIMES

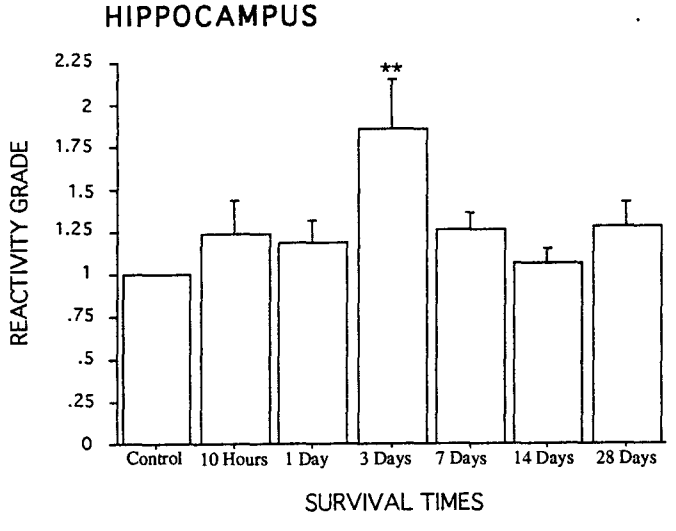
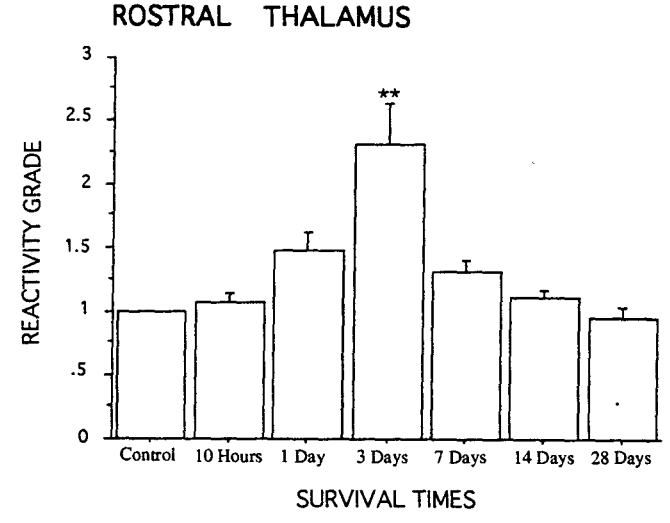
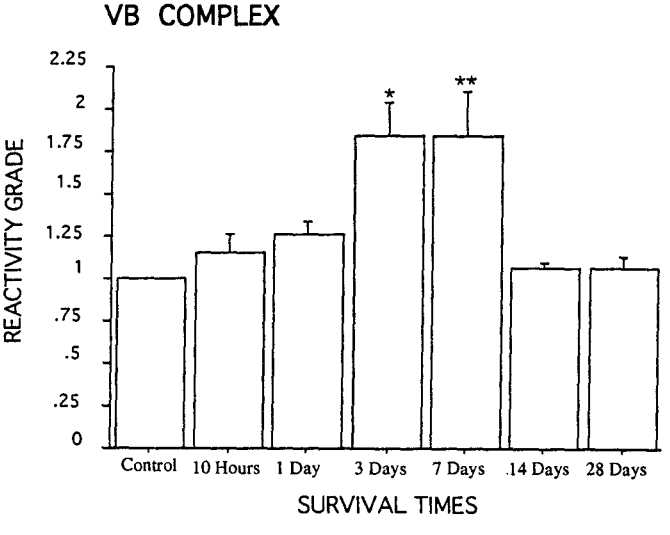

HIPPOCAMPUS
REACTIVITY GRADE
2.25
2
1.75
1.5
1.25
1
.75
.5
.25
0
**
Control
10 Hours
1 Day
3 Days
7 Days
14 Days
28 Days
SURVIVAL TIMES

FIGURE 9.

Quantitative analysis of microglial reactivity in the ristral thalamus and the VB complex at several survival times after the NMDA injection. Data are presented as the mean values of reactivity grades ± s.e.m. for four animals in each survival time. Statistical analysis was performed using ANOVA (**$p<0.01$, *$p<0.05$) compared to control animals injected with saline at each survival time (data not shown). Significant increase of the reactivity grade was observed in all the areas studied at 3 days after the injection and persisted in the ventrobasal complex of the thalamus until day 7.


ROSTRAL THALAMUS
REACTIVITY GRADE
3
2.5
2
1.5
1
.5
0
**
Control
10 Hours
1 Day
3 Days
7 Days
14 Days
28 Days
SURVIVAL TIMES


VB COMPLEX
REACTIVITY GRADE
2.25
2
1.75
1.5
1.25
1
.75
.5
.25
0
*
**
Control
10 Hours
1 Day
3 Days
7 Days
14 Days
28 Days
SURVIVAL TIMES

CHAPTER V

DISCUSSION

Using tomato lectin histochemistry to visualize microglial cells, the present study revealed a rapid and selective microglial response to an NMDA induced lesion in the immature rat brain. Microglial reactivity, seen by 10 hours after NMDA injection, was characterized by changes in microglial cell morphology and an apparent increase in lectin binding. A marked increase in the number of tomato lectin positive cells was observed at 3 days post injection. This response was seen principally in areas showing an extensive loss of neurons, i.e. the deep cortical lamina, hippocampus and thalamus. The increase in lectin positive cells was followed by a progressive decrease in reactivity and the formation of a glial scar seen by 7-14 days after NMDA injection. The selective pattern of neuronal death and microglial reactivity as well as the putative role of the microglial response are discussed.

Selective pattern of neuronal death

The extensive neuronal loss observed after NMDA injection is attributed to the enhanced sensitivity of the immature brain to NMDA excitotoxicity. Indeed, brain injury after NMDA injection into the 7 day old rat brain has been reported to be 60 times more severe than following injections in the adult (McDonald et al., 1988). This

sensitivity does not seem to be related to any reported changes in the expression or regulation of NMDA receptors at early ages but rather is likely to reflect different mechanisms related to events occurring after NMDA receptor activation, calcium buffering, or the developmental stage of inhibitory synapses (McDonald and Johnston, 1990). In this sense, neuronal populations most susceptible to excitotoxic damage would not necessarily correspond to those areas showing the highest concentration of NMDA receptors. The lack of correlation between susceptibility and receptor density becomes especially problematic when considering receptor densities in the mature brain. In this regard, the highest levels of NMDA receptors in the adult neocortex are found in cortical superficial layers (I-III) (Greenamyre et al., 1985; Monaghan and Cotman, 1985), and yet these layers were found in the present study to be resistant to NMDA toxicity. Moreover, superficial cortical lamina also showed a high NMDA receptor density in early development (Insel et al., 1990), although previous work (McDonald et al., 1990) showed that the superficial layers of the immature neocortex are the most resistant to NMDA excitotoxicity. In the same way, the highest levels of NMDA receptors in the adult rat brain are found in the hippocampus, especially the CA1 field (Monaghan and Cotman, 1985; Cotman et al., 1987). However, our observations indicated a marked sensitivity in the dorsal area of CA3 in the immature brain. Although previous work suggested that high levels of NMDA receptors are found equally in all CA fields in the immature brain (Tremblay et al., 1988), our observations would correlate with a more recent study reporting that the highest density of the subunit NMDA R1 is found in CA2-CA3 (Petralia et al., 1994).

Moderate levels of NMDA receptor binding have been found in adult thalamic nuclei, with the anterior thalamic nuclear group showing the highest density (Monaghan and Cotman, 1985). In this regard, NMDA receptor density in the adult brain correlated

with the general pattern of neuronal degeneration seen in the rostral thalamus of our animals. Therefore, neuronal degeneration in the rostral thalamus could be explained by direct spread of the injected excitotoxin, although the selective sparing of the AV nucleus remains unexplained. Spread of the injected NMDA into the anterior thalamus also correlated with the marked neuronal loss seen in the rostral dorsal hippocampus. In contrast, the specific pattern of neuronal cell loss seen in the VB complex of the thalamus is likely due to secondary retrograde degeneration subsequent to target depletion, i.e., the sensorimotor cortical lesion. In addition, transneuronal anterograde degenerative processes may be involved since corticothalamic pathways are glutamatergic (Fonnum et al., 1981; Fagg and Foster, 1983).

The temporal pattern of neuronal cell death induced by overexcitation of glutamate receptors in the immature brain has been studied extensively by Olney and colleagues (Olney, 1971; Olney et al., 1971; Olney et al., 1972; Olney, 1990). According to their work and other related studies, neuronal death after the oral administration of glutamate is characterized by an acute swelling of neuronal cell bodies and dendrites within 30 minutes, and this is followed by degeneration of intracellular organelles and nuclear pyknosis leading to neuronal necrosis in the next several hours (Choi, 1992; Dietrich et al., 1992). Consistent with these findings, we observed dark necrotic neurons at 10 hours post NMDA injection, and neuronal debris was seen in the lesion site at 1 day post injection.

Pattern of microglial response

Although microglial reactivity after an excitotoxin injection into the immature brain has not been described previously, several studies indicate that microglial

activation is the most acute glial response to an excitotoxic lesion in the adult brain (Coffey et al., 1990; Andersson et al., 1991b; Marty et al., 1991; Kaur et al., 1992; Kaur and Ling, 1992; Mitchell et al., 1993). Microglial reactivity observed after NMDA injection into the immature brain of our animals was characterized by a rapid onset and was confined to areas undergoing neuronal degeneration. Accordingly, microglial cells in the spared outer neocortical layers showed a primary ramified morphology, corresponding to the most common microglial morphology found in the normal 7 day old neocortex (Wu et al., 1993). In contrast to this observation, is the previous study that showed that in the outer layers (I-III) of the normal 7 day old rat neocortex there is substantial phagocytosis by macrophages, corresponding to a peak of programmed cell death (Ferrer et al., 1990).

Microglial cells characteristically react to injury in a graded manner. Microglial activation can lead to morphological changes, proliferation, migration and expression of new antigens, or it may involve active phagocytosis when neuronal death occurs (Streit et al., 1988; Thomas, 1992; Streit, 1993). Although morphological distinction between phagocytic and non-phagocytic activated microglial cells is not yet possible at the light microscope level, it is likely that both types of microglial activation are present after NMDA lesions in the immature brain. Within the first few hours, before neuronal death takes place, microglial cells would become activated to remain in an 'alert' state. Subsequently, if neuronal debris are present, microglial cells would probably become further activated to phagocytize. This time course correlates with previous work showing that phagocytosis of dead neurons after glutamate overexcitation in the 10 day old rat brain takes place within the first 24 hours after glutamate ingestion (Olney, 1971).

A most striking characteristic of the observed microglial reaction is the marked increase in tomato lectin positive cells at 3 days after NMDA injection, when neuronal debris have already been removed. Although a shorter time course for these events following an aspiration lesion in the developing brain has been reported (Milligan et al., 1991) our findings correlate with the increased number of microglial-macrophage cells reported in the adult brain at 3 days after excitotoxic injury (Coffey et al., 1990; Andersson et al., 1991b; Andersson et al., 1991a; Marty et al., 1991; Kaur et al., 1992). Whether this increase in the number of activated cells is due to the proliferation of endogenous microglia or to the infiltration of recruited blood monocytes is not known and has been strongly debated in recent years. This issue will remain unsolved until a marker is able to selectively differentiate these two populations of cells.

In our animals, the putative proliferation of endogenous microglial cells could be supported by observations of a dramatic natural increase of microglial cells in the normal rat neocortex between 5 and 20 days of age, which was attributed to the proliferation of resident microglia (Kaur et al., 1989). Moreover, ameboid microglial cells have been demonstrated in the corpus callosum of the normal postnatal brain until 15 days after birth (Ling et al., 1990; Boya et al., 1991; Kaur and Ling, 1991; Wu et al., 1992). These cells, which exhibit features indicative of phagocytosis and share common characteristics with their monocytic precursors (Yee et al., 1990; Ling et al., 1992), would seem likely candidates to become activated and proliferate after injury. However, recent work showed that ameboid cells do not migrate to the site of an stab wound cortical injury in the newborn brain (Leong and Ling, 1992), and that the major cell type responding to neonatal brain injury is the blood-borne phagocyte (Milligan et al., 1991). These findings, together with previous studies showing vascular sprouting in the middle layers of the 7 day old rat neocortex (Hurley and Streit, 1991), the

immaturity of the rat blood-brain barrier until 12 days after birth (Xu et al., 1993) and a blood-brain barrier disruption during the first 24 hours following the injection of an excitotoxin (Andersson et al., 1991a) support the possibility that most of the increase in tomato lectin positive cells, seen at 3 days after the lesion, is due to a peripheral monocytic recruitment. The 3 day delay before massive monocyte infiltration suggests that an early activation of resident microglial cells may be important to trigger the recruitment of blood borne monocytes. Although activation of the resident microglial cells obviously occurs, both proliferation and recruitment from the blood would more likely contribute to the increased number of phagocytic cells. However, the extent of the contribution from each cell population remains speculative.

Wallerian degeneration of the cortical efferent projections coursing through the pons caused an intense microglial response with marked morphological changes. In comparison to the areas where neuronal cell body degeneration takes place, no apparent increase in the number of tomato lectin positive cells was observed. This observation agrees with previous studies which attributed such reactivity to the response of resident microglial cells without participation of blood-borne monocytes (Ludwin, 1990; Perry and Brown, 1992).

Possible activation signals for microglial cells

Several lines of evidence indicate that microglial cell activation precedes the development of neuronal death, suggesting that these glial cells are very sensitive to microenvironmental changes. Factors that induce activation, proliferation or upregulation of macrophage antigens on microglial cells in vitro include IL-1 (Merrill, 1991; Ganter et al., 1992), specific microglial mitogens (Giulian et al., 1991) and a

variety of colony-stimulating factors (CSF) such as macrophage colony stimulating factor (M-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF) and IL-3 (Giulian et al., 1991; Brosnan et al., 1993; Raivich et al., 1993; Streit, 1993). Although microglial cells have been found to express CSF receptors after injury in vivo (Raivich et al., 1991; Streit, 1993) and the release of CSF from neurons are likely to play an important role in microglial activation, the precise role of these factors as well as their specific sources in the brain remains unknown.

Moreover, microglial cells have been shown to be extremely sensitive to changes in potassium concentrations of the extracellular medium, being susceptible to small depolarizing events (Kettenmann et al., 1993; Kettenmann and Ilschner, 1993) that cause complex physiological changes in microglial cells. Such a mechanism could be especially important in relation to the extracellular ionic concentrations that are disrupted by neuronal overexcitation. This possibility is supported by recent work reporting that extracellular ATP (and ADP to a lesser extent), which can accumulate by neuronal leakage after injury, can promote depolarization of microglial cells in culture via a specific membrane diphosphate purinoreceptor (Walz et al., 1993). This mechanism, which could act as an activation signal for microglial cells, corresponds to the increased nucleoside diphosphatase activity seen in the plasma membrane of activated microglial cells under different types of lesions (Jorgensen et al., 1993; Jensen et al., 1994). All these events may be potentially involved in the early activation of microglia, whereas later influences may be associated with the presence of neuronal debris as well as the late astrocytic response.

CHAPTER VI

CONCLUSIONS

The results obtained in this experiment showed that NMDA injection in the somatosensory cortex of a 6 day old rat causes a specific pattern of primary neuronal degeneration in the injected cortex, the ipsilateral hippocampus and the ipsilateral rostral thalamus. The cortical lesion causes neuronal secondary degeneration in the VB complex of the thalamus (retrograde and anterograde) and Wallerian degeneration of cortical efferents.

This excitotoxic lesion causes marked microglial reactivity restricted to the areas undergoing neuronal and/or axonal degeneration. Microglial cells already exhibit morphological features of reactive microglia at 10 hours after the injection. Microglial reactivity peaks at day 3, accompanied by a marked increase in the number of tomato lectin positive cells. In areas undergoing primary degeneration reactivity declines by 7 days, and in areas undergoing neuronal secondary degeneration declines by day 14.

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VITA

Laia Acarin Perez, was born in Barcelona (Spain), on April 6th, 1970, to Montserrat Perez and Nolasc Acarin.

In September, 1987, Laia entered the Universitat Autònoma de Barcelona receiving the degree of Bachelor of Science in Biochemistry in June 1992. From 1989 to 1992 she worked as a research student in the Unit of Medical Histology of the same university, studying glial reactivity in different experimental animal models, under the supervision of Drs. Bernardo Castellano and Berta González. In the fall of 1992 she entered the Neuroscience PhD Program at the Universitat Autònoma Barcelona. Her PhD project is part of a collaboration between Drs. Castellano and Gonzàlez in Barcelona, and Dr. Anthony J. Castro at the Department of Cell Biology, Neurobiology and Anatomy, Loyola University Chicago. She came to the laboratory of Dr. Castro in April 1993, where she began to study the glial reactivity after an excitotoxic lesion in the immature rat brain. In the fall of 1994 she joined the Neuroscience Program, Loyola University Chicago, to obtain a master's degree. She was the recipient of fellowships from the Government of Catalunya, Caixa de Sabadell Foundation and "La Caixa" graduate fellowship program for Spanish students to study abroad.

After finishing her master's at Loyola, she will return to Spain, in order to finish her research towards a PhD in the Neuroscience Program, Universitat Autònoma Barcelona.

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The thesis submitted by Laia Acarin-Perez has been read and approved by the following committee:

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirement for the degree of Master of Science.

31 May 1995
Date

Director's signature