Journal of Neurochemistry
Lippincott Williams & Wilkins, Philadelphia

Transient Inhibition of Glutamate Uptake In Vivo Induces Neurodegeneration when Energy Metabolism Is Impaired

Ma. del Rayo Sánchez-Carbente and Lourdes Massieu

Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autonoma de México, México D.F., México

Abstract: Impairment of glutamate transport during ischemia might be related to the elevation of the extracellular concentration of glutamate and ischemic neuronal damage. Additionally, impairment of energy metabolism in vivo leads to neurodegeneration apparently mediated by a secondary excitotoxic mechanism. In vitro observations show that glucose deprivation and inhibition of energy metabolism exacerbate the toxic effects of glutamate. We have previously shown that glutamate uptake inhibition in vivo by L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) leads to a substantial elevation in the extracellular concentration of excitatory amino acids that is not associated with cell death. These observations suggest that energy depletion during ischemia might be determinant of ischemic neuronal damage. To investigate whether impairment of energy metabolism in vivo increases neuronal susceptibility to glutamate uptake inhibition, we studied the effect of glutamate accumulation induced by the intrahippocampal or intrastriatal administration of PDC in energy-deficient rats chronically treated with 3-nitropropionic acid (3-NP), which irreversibly inhibits the tricarboxylic acid cycle and electron transport chain. Extracellular glutamate levels were monitored by HPLC from fractions collected from microdialysis probes, and neuronal damage was evaluated by histological analysis. Our results show that glutamate uptake inhibition leads to marked neuronal damage in energy-deficient rats but not in intact animals, which apparently is not related to an additional elevation of glutamate levels induced by 3-NP.
Key Words: Energy depletion—Glutamate transport—Excitotoxicity—L-*trans*-Pyrrolidine-2,4-dicarboxylate.
J. Neurochem. **72,** 129–138 (1999).

Glutamate neurotoxicity has been implicated in acute neurological disorders such as ischemia and in progressive neurological diseases such as Huntington's disease and Alzheimer's disease. The neurotoxic properties of glutamate have been known for >40 years since Lucas and Newhouse (1957) showed that the systemic injection of glutamate induced retinal neurodegeneration in the immature rat. More than 10 years later, studies by Olney (1969) confirmed the neurotoxic potential of glutamate and the term "excitotoxicity" was coined to define neuronal damage induced by excitatory amino acids. It was suggested that neuronal death occurred due to energy depletion after prolonged excitation. During the last decade, many in vitro studies have suggested that calcium influx through glutamate receptors was a determinant step leading to glutamate-induced cell death (Choi et al., 1987; Michaels and Rothman, 1990; Randall and Thayer, 1992; Hartley et al., 1993). Recently, a link between excitotoxicity and impairment of energy metabolism has been suggested, and mitochondrial failure appears to play a major role in glutamate-induced cell death (Novelli et al., 1988; Henneberry, 1989; Beal, 1992; Greene and Greenamyre, 1996; Schinder et al., 1996).

It has been shown in cultured neurons that glutamate excitotoxic damage is exacerbated in the absence of glucose or when oxidative phosphorylation is inhibited (Novelli et al., 1988; Henneberry, 1989). In chick retina, the simultaneous inhibition of glycolysis and the electron transport chain leads to neuronal damage, which is prevented by NMDA receptor antagonists (Zeevalk and Nicklas, 1990). During energy depletion, disruption of the ionic gradients across neuronal plasma membrane will occur due to inhibition of Na^+,K^+-ATPase. As a consequence, the plasma membrane will depolarize and the voltage-dependent Mg^{2+} blockade of the NMDA receptors will be lost, leading to the activation of these receptors in the presence of low concentrations of glutamate (Novelli et al., 1988; Zeevalk and Nicklas, 1992).

Interestingly, in vivo inhibition of the tricarboxylic acid cycle and the electron transport chain by 3-nitropropionic acid (3-NP) and malonic acid (irreversible and reversible inhibitors of succinate dehydrogenase, respectively) reproduces many of the histopathological and neurochemical characteristics of Huntington's disease (Beal et al., 1993*a*,*b*). It is suggested that the neuronal

Received May 19, 1998; revised manuscript received August 12, 1998; accepted August 25, 1998.

Address correspondence and reprint requests to Dr. L. Massieu at Departamento de Neurociencias, Instituto de Fisiología Celular, Universidad Nacional Autonoma de México, México D.F., México AP 70-253.

Abbreviations used: DHK, dihydrokainic acid; 3-NP, 3-nitropropionic acid; PDC, L-*trans*-pyrrolidine-2,4-dicarboxylate.

damage induced by these metabolic inhibitors involves a secondary excitotoxic mechanism, as it is attenuated by prior removal of the corticostriatal glutamatergic afferents and prevented by the administration of glutamate receptor antagonists (Ludolph et al., 1992; Beal et al., 1993*a*; Greene et al., 1993).

During cerebral ischemia, the extracellular concentration of glutamate increases substantially, probably due to impairment of its high-affinity uptake system (Benveniste et al., 1984; Attwell et al., 1993). It has been suggested that glutamate might be responsible for the subsequent neurodegeneration associated with this disorder because glutamate receptor antagonists are good protectants against ischemic damage (Benveniste et al., 1984; Simon et al., 1984; Gill et al., 1987).

We have previously demonstrated that pharmacological inhibition of glutamate uptake in vivo results in substantial elevation of extracellular glutamate levels, but no neuronal damage is observed (Massieu et al., 1995; Massieu and Tapia, 1997). It therefore appears that additional factors such as the obvious energy depletion occurring during ischemia may facilitate neuronal damage. To investigate whether impairment of energy metabolism in vivo increases neuronal vulnerability to glutamate uptake inhibition, we studied the effects of L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC), a potent and selective inhibitor of glutamate uptake, on glutamate levels and neurotoxicity in rats chronically treated with 3-NP. Our results show that when the tricarboxylic acid cycle and the electron transport chain are chronically inhibited with 3-NP, PDC-induced extracellular glutamate accumulation results in marked neuronal damage.

MATERIALS AND METHODS

Animal treatment

Male Wistar rats (200–250 g) were used throughout the study and were handled according to the Rules for Research in Health Matters (Mexico). 3-NP (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in 0.9% saline, and animals received a daily intraperitoneal injection of 20 mg/kg 3-NP over 4 days. They were housed in polypropylene cages with four animals per cage and received food and water ad libitum until they became recumbent from 3-NP intoxication. The term "recumbency" refers to the loss of the normal weight-bearing posture and adoption of severe dystonic ventral or lateral posturing with hindlimbs rigidly extended (Hamilton and Gould, 1987; Guyot et al., 1997). Recumbent animals were almost unable to move although not completely paralyzed. 3-NP administration produces highly predictable brain lesions that correlate with the onset of animal recumbency (Hamilton and Gould, 1987; Guyot et al., 1997). From 25 to 30% of the treated rats showed between the second and third day of injection uncoordinated gait and slowness that evolved to lateral or ventral recumbency by the third or the fourth day of treatment. The animals showing these behavioral patterns were anesthetized and intracardially perfused for subsequent histological analysis of their brains the same day they became recumbent. All recumbent animals showed bilateral and symmetrical damage in the striatum and the hippocampus and were left as positive controls of 3-NP intoxication. The rest of the animals, which were not recumbent at day 4 and thus considered not severely clinically affected by 3-NP, received 1 h after the last intraperitoneal injection of 3-NP an intrastriatal or an intrahippocampal injection of either one of two inhibitors of glutamate uptake, PDC (500 nmol/μl) or dihydrokainic acid (DHK; 50 nmol/μl for the striatum and 5 nmol/μl for the hippocampus). Additionally, some animals were injected with NMDA (15 nmol/μl for the striatum and 7.5 nmol/μl for the hippocampus) or with 1 μl of 0.9% saline. Control animals received four injections of 0.9% saline, and 1 h after the fourth administration, they were injected either in the striatum or in the hippocampus with PDC, DHK, or NMDA at the concentrations mentioned above. Animals were killed 24 h after the surgery. Drugs (obtained from Tocris, Ballwin, MO, U.S.A.) were dissolved in 1 *M* NaOH; the pH was adjusted to 7.0–7.4 and brought to the desired volume with phosphate buffer (10 m*M*, pH 7.0).

For intracerebral injections, animals were anesthetized with 4–5% halothane in a 95% O_2/5% CO_2 mixture and placed in a stereotaxic frame with the nose bar positioned at -3.3. The skin was cut and the skull was exposed. A 1- to 2-mm hole was drilled, and a Hamilton needle was positioned in the striatum or the hippocampus [coordinates: A $+0.7$ mm from bregma, L $+2.8$ from midline, and V 4.0 mm from dura for the striatum and A -3.6, L $+2.0$, and V 2.0 for the hippocampus, according to Paxinos and Watson (1986)]. A 1-μl volume was unilaterally injected at a rate of 0.5 μl/min via a 27-gauge stainless-steel needle connected to a Hamilton syringe with the aid of microinjection pump (Carnegie CMA100). Rats were maintained under low anesthesia (0.5% halothane) throughout the injection, and 2 min after the injection was completed, the needle was withdrawn and the skin was sutured.

Histologic evaluation

Twenty-four hours after the surgery, rats were anesthetized with pentobarbital and transcardially perfused with 250 ml of 0.9% saline followed by 250 ml of 0.4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.3). Brains were removed and left in fixative for an additional 24 h. They were transferred successively to 10, 20, and 30% sucrose (24 h each), and 30-μm coronal sections were cut in a cryostat and stained with cresyl violet. Ten to 12 sections from each animal were examined under light microscopy, and the lesioned area was quantified with the aid of an image analyzer system (Macintosh Image 1.6). The tissue sections used flanked the needle tract, and it was visible in all of them. In the case of the injured animals the area measured included all the damaged region and the needle tract. In noninjured animals in which no damage was found beyond the needle tract, the measured area included only the tract. The average damage per tissue section was calculated in each animal, and results are expressed as means of the damaged area per each treated group.

Statistical analysis was performed by a one-way ANOVA, followed by a Fisher's least significant difference multiple comparison test.

Microdialysis experiments

Recovery of amino acids from standard solutions. Microdialysis cannulas 2 mm long and 0.5 mm in diameter (CMA12; Bioanalytical Systems, West Lafayette, IN, U.S.A.) were used. Before implantation, each probe was flushed with distilled water for 5 min at a flow rate of 40 μl/min. To assess the recovery of the amino acids from the dialysis membrane, some dialysis probes were immersed in 1.5 ml of a standard amino acid mixture (1 nmol/10 μl) of aspartate, glutamate, glutamine,

glycine, taurine, alanine, and GABA dissolved in a Krebs–Ringer medium containing (in m*M*) NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10 (pH 7.4). Probes were perfused with Krebs–Ringer medium at a rate of 2 μl/min, and 25-μl fractions of perfusate were collected and analyzed by HPLC as described below. The calculated recovery of amino acids was as follows (%): alanine, 6.9 ± 1.1; aspartate, 6.2 ± 0.85; glutamate, 6.1 ± 0.46; glutamine, 7.7 ± 0.10; glycine, 8.8 ± 0.8; taurine, 9.1 ± 0.31; and GABA 7.2 ± 0.33 (n = 4).

Implantation of dialysis probes. Male Wistar rats (220–250 g) were anesthetized and placed in a stereotaxic frame as described above. Animals were implanted unilaterally in either the left striatum or the hippocampus with a microdialysis cannula [coordinates: A +0.7 mm from bregma, L +2.8 mm from midline, and V 6.0 mm from dura for the striatum and A −3.6 mm, L +2.0 mm, and V 4.0 mm for the hippocampus, according to Paxinos and Watson (1986)] and maintained under low anesthesia (0.5% halothane) throughout the experiment. The probes were perfused continuously with the Krebs–Ringer solution at a flux rate of 2 μl/min using a microinjection pump (Carnegie CMA100). After a 60-min equilibration period, 25-μl (12.5-min) consecutive fractions of perfusate were continuously collected. After the first three fractions (basal level of amino acids) were collected, one of the following solutions was perfused, PDC (25 m*M*), 3-NP (5 m*M*), or a solution of PDC (25 m*M*) +3-NP (5 m*M*), during seven additional fractions. At the end of the experiment, the skin was sutured and the animals were maintained for 1 week for further histological observation as described above. Stock solutions of PDC and 3-NP were prepared as described above and added directly to the Krebs–Ringer perfusion medium to give the desired concentration. The osmolarity of the Krebs–Ringer medium was adjusted by replacing NaCl with the drugs used.

Seven days after the microdialysis experiments, the animals were transcardially perfused and prepared for histological analysis as already described.

Amino acid analysis of striatal and hippocampal dialysates. Amino acids were measured in dialysates by HPLC as previously described (Salazar et al., 1994). In brief, the 25-μl collected fractions were derivatized with the same volume of *o*-phthalaldehyde and 3 min later 20 μl was injected into an HPLC system (Beckman). An ODS column (25 cm × 4 mm internal diameter) was used. The mobile phase was methanol/potassium acetate (0.1 *M*, pH 5.5) and was run at a rate of 1.5 ml/min in a linear gradient (15-min duration) from 25 to 75% methanol. The results obtained were compared with a standard mixture of amino acids (Sigma) equally processed.

RESULTS

Chronic 3-NP treatment

3-NP effects per se. Of the animals chronically treated with 3-NP, 25–30% showed lateral or ventral recumbency 2–4 days after the onset of the treatment. Six of 23 rats were bilaterally lesioned in both the striatum and the hippocampus, and 1 of 23 showed bilateral damage in the hippocampus but not in the striatum (Fig. 1). Of the seven injured animals, five were recumbent after the fourth injection of 3-NP, one after the third injection, and one after the second injection. In the lesioned animals, most of the striatum was damaged, enclosing the caudate–putamen (Fig. 2F). In the hippocampus, a bilateral

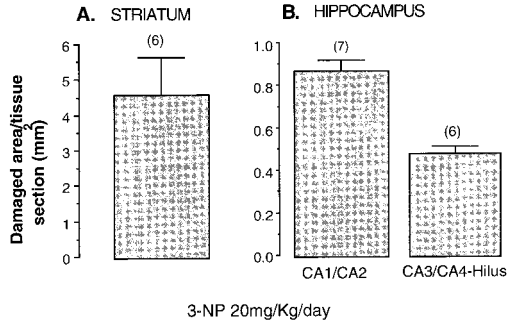

FIG. 1. Damaged area/tissue section (mm^2) of striatum (**A**) or hippocampus (**B**) of recumbent rats chronically treated with 3-NP. Animals received a daily injection of 3-NP (20 mg/kg) and were killed under anesthesia the same day they showed ventral or lateral recumbency (see Materials and Methods). Brains were prepared for histology and the damaged areas quantified as described. From a total of 23 treated animals, 7 were recumbent. Six recumbent rats showed bilateral and symmetrical damage in all hippocampal regions and in the striatum, and one recumbent rat was lesioned only in the CA1 hippocampal subfield and showed no damage in the striatum. The lesioned areas shown correspond to the left striatum or hippocampus, but very similar data were observed when the lesioned areas were quantified in the right side: 5.42 ± 1.56 mm^2 for the striatum, 0.89 ± 0.04 mm^2 for the CA1/CA2 hippocampal regions, and 0.43 ± 0.03 mm^2 for the CA3/CA4 hilar hippocampal regions. The numbers in parentheses indicate the numbers of injured animals.

lesion extending throughout the CA1 subfield was present in all lesioned animals (Fig. 3G). These findings are consistent with those previously described by others (Hamilton and Gould, 1987; Beal et al., 1993*a*; Guyot et al., 1997).

Effects of PDC in chronically 3-NP-treated rats. As previously reported (Massieu et al., 1995; Massieu and Tapia, 1997), administration of PDC (500 nmol/μl) directly into the striatum (Fig. 2A) or the hippocampus (Fig. 3A) did not produce any damage beyond the needle tract. The mean damaged area was not statistically different from that observed when 0.9% saline was administered (Fig. 4). However, when PDC was administered to rats chronically treated with 3-NP but that showed no recumbency at day 4, a clear lesion was observed in either the striatum (Fig. 2B) or the hippocampus (Fig. 3C). In the striatum, 60% of the animals chronically treated with 3-NP showed an extensive lesion 24 h after PDC administration, with a mean damaged area 3.4-fold larger than that present in the untreated animals (Fig. 4A). No damage was observed in the contralateral uninjected striatum (Fig. 2B). In the hippocampus, PDC induced a clear lesion in the CA1 subfield in 77% of the animals chronically treated with 3-NP. These animals showed a mean damaged area 8.8-fold larger than that present in those animals that received a PDC injection but were not treated with 3-NP (Fig. 4B). No damage was observed in the contralateral uninjected hippocampus (Fig. 3D).

We previously showed that the glutamate uptake inhibitor DHK is toxic to striatal and hippocampal neurons when directly administered at high doses (200–400

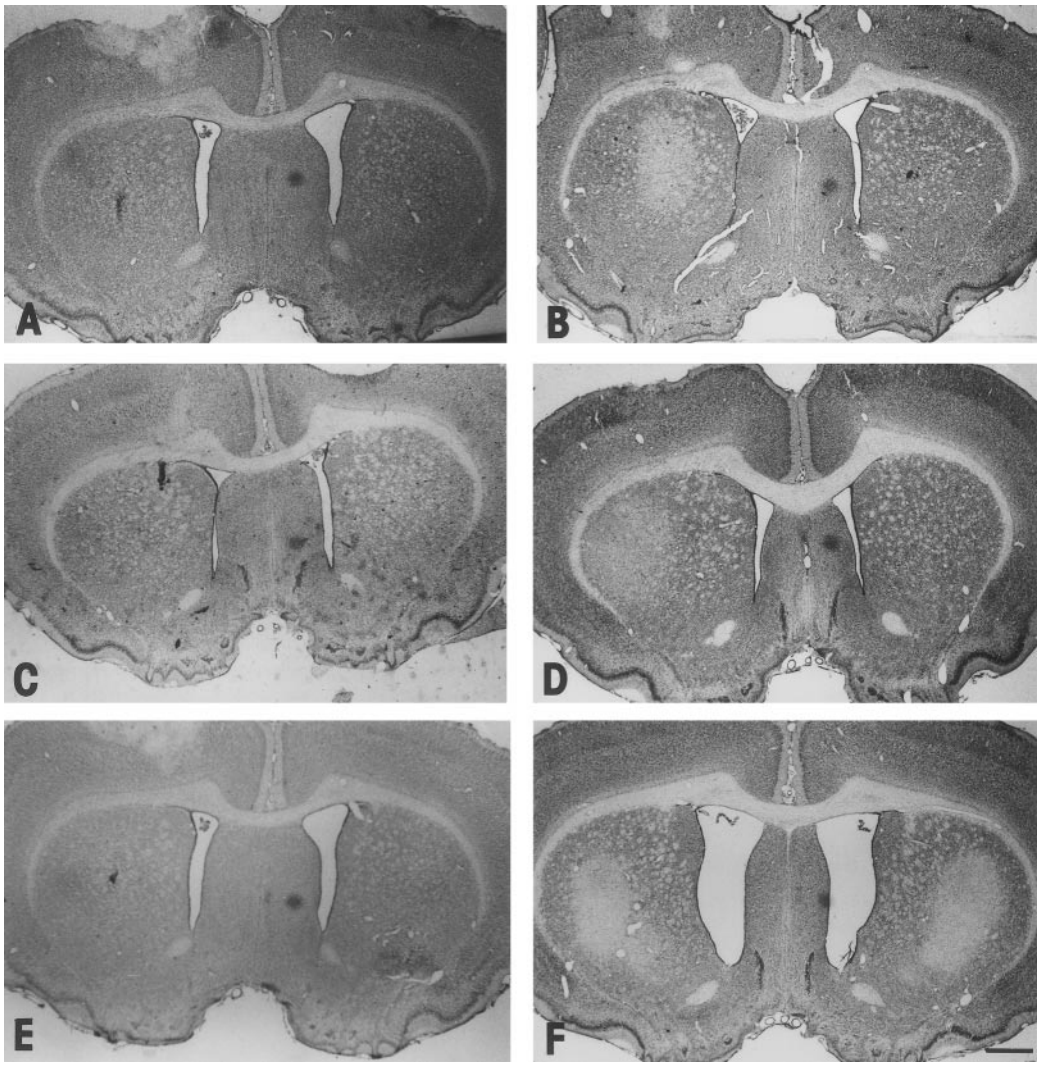

FIG. 2. Micrographs showing the effect of intrastriatal PDC and DHK administration in normal rats and rats chronically treated with 3-NP. Rats received a daily injection of 3-NP (20 mg/kg) during 4 days, and 1 h after the fourth administration, they were intrastriatally injected with PDC (500 nmol/μl) or DHK (50 nmol/μl). In control animals not treated with 3-NP, intrastriatal administration of either PDC (500 nmol/μl) or a low dose of DHK (50 nmol/μl) produced only a small lesion in the center of the striatum (**A** and **C**, respectively). In contrast, in 3-NP-treated rats intrastriatally injected either with PDC (**B**) or DHK (**D**), a much larger lesion was observed, as indicated by the pale zone in the ipsilateral striatum, which represents cell loss. Note the lack of tissue damage in the contralateral uninjected striata. Bilateral and symmetrical damage in the striatum of a representative recumbent rat treated with 3-NP is also shown in (**F**). The rat was recumbent at day 4 of treatment and showed bilateral damage additionally in the hippocampus. A representative control animal chronically treated with 3-NP and injected with 0.9% saline is presented (**E**). Bar = 1 mm.

nmol/μl) (Massieu et al., 1995; Massieu and Tapia, 1997). To investigate if DHK-induced lesions are exacerbated in energy-deficient rats, a low dose (50 nmol for the striatum and 5 nmol/μl for the hippocampus) of DHK was chosen. At this concentration, DHK administered into the striatum injured only a small area surrounding the needle tract (Fig. 2C), and in the hippocampus a small lesion was observed in the CA1 subfield (Fig. 3B). In neither case was the mean damaged area statistically different from that produced by a saline injection (Fig. 4). When DHK was administered to nonrecumbent chronically 3-NP-treated rats, a clear potentiation of the lesion was observed. In the striatum, seven of nine rats showed a larger lesion (Fig. 2D) with a mean area 3.9-fold larger than that present in the non-energy-deficient animals (Fig. 4A). No damage was observed in the contralateral uninjected striatum (Fig. 2D). In the hippocampus, only four of 11 animals showed an exacerbated lesion in the CA1 subfield (Fig. 3E), with a mean damaged area fivefold larger than that present in the DHK-injected animals not treated with 3-NP (Fig. 4B). No damage was observed in the contralateral uninjected hippocampus (Fig. 3F).

To test the putative potentiating effect of energy depletion on neuronal damage induced by direct activation of NMDA receptors, some animals chronically treated

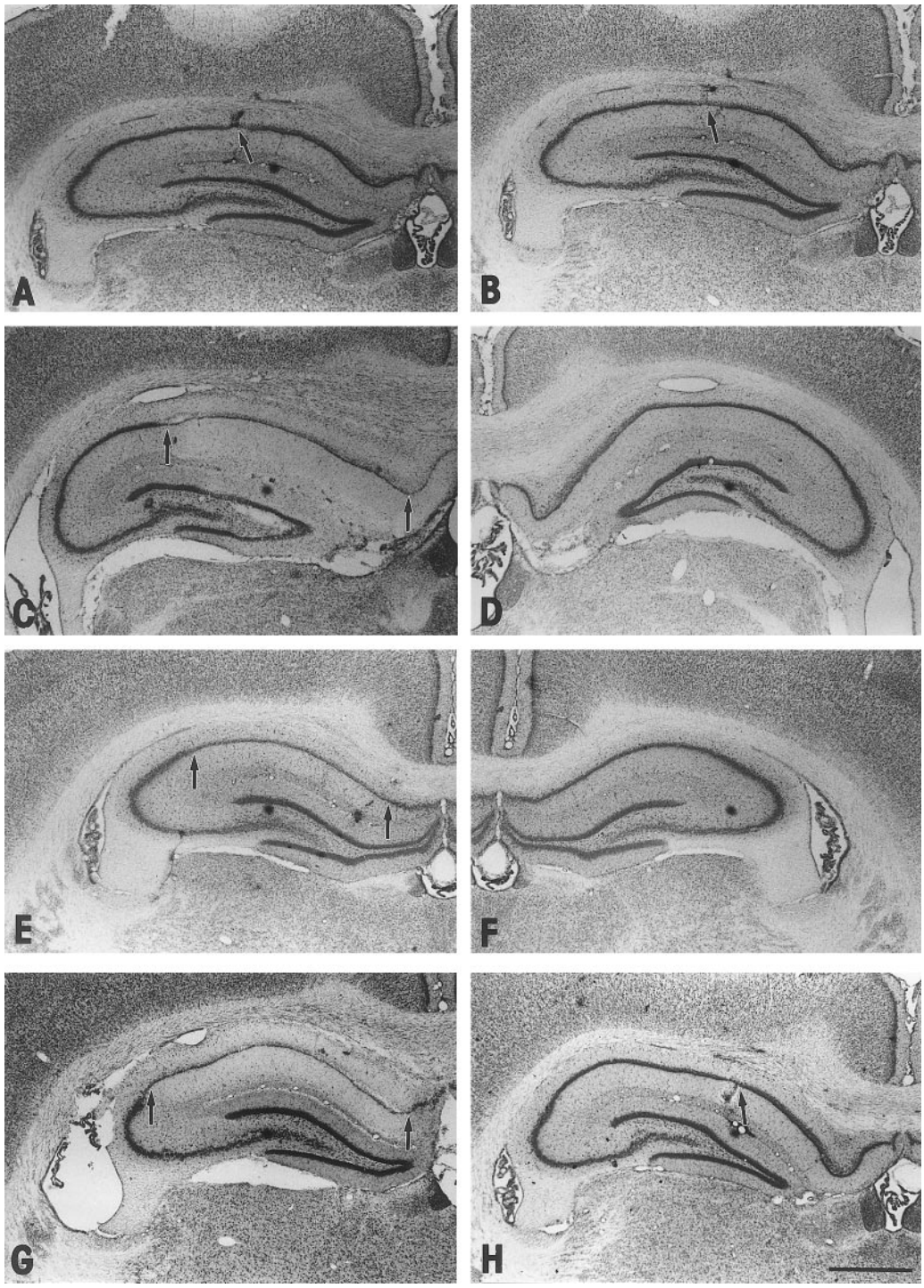

FIG. 3. Micrographs showing the effect of intrahippocampal administration of PDC (500 nmol/μl) or DHK (5 nmol/μl) in normal and chronically 3-NP-treated rats. Animals were treated as described in Fig. 2. The administration of PDC (**A**) or DHK (**B**) in the hippocampus of normal rats not treated with 3-NP produced only a small lesion in the CA1 region of the hippocampus. The site of injection is indicated by arrows. When the compounds were administered at the same doses in 3-NP-treated rats, a much larger lesion extending throughout the CA1 hippocampal subfield (area between the arrows) was observed in animals injected with PDC (**C**) or DHK (**E**) but not in saline-injected animals (**H**). The arrow in H indicates the site of saline injection. Note the lack of tissue damage in the contralateral hippocampus of 3-NP-treated rats injected with PDC (**D**) or DHK (**F**). Bilateral and symmetrical damage in the hippocampus of a representative recumbent rat treated with 3-NP is also shown (**G**). The rat was recumbent at day 4 of treatment and showed bilateral damage additionally in the striatum. Bar = 1 mm.

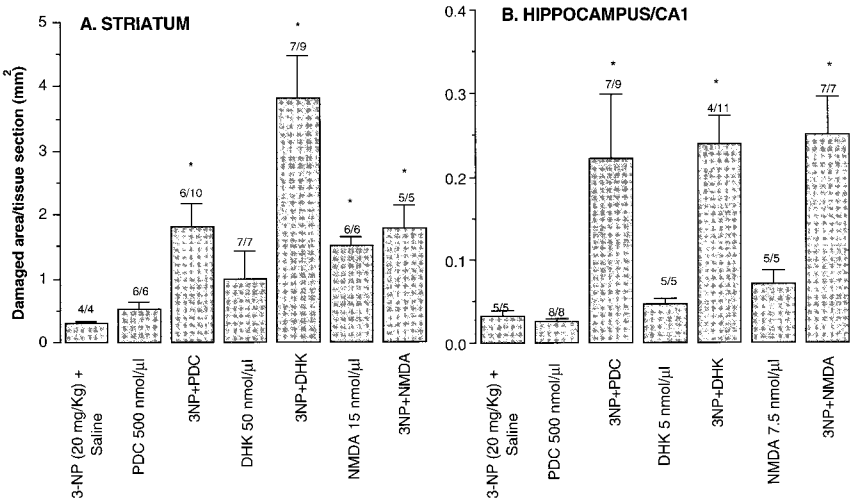

FIG. 4. Damaged area/tissue section (mm^2) observed in the striatum (**A**) or the hippocampus (**B**) in intact rats or in rats chronically treated with 3-NP (20 mg/kg) during 4 days and intracerebrally injected with either 0.9% saline, PDC, DHK, or NMDA at the doses indicated below the columns. Animals were treated as described in Fig. 2. The numbers at the top of each column represent the numbers of injured animals relative to the total numbers of animals intracerebrally injected. $^*p < 0.05$ as compared with the control (non-3-NP-treated group).

with 3-NP were injected with a low dose of NMDA in either the striatum (15 nmol/μl) or the hippocampus (7.5 nmol/μl). The lesion observed in these experimental conditions was compared with that induced by injection of the same doses of NMDA in non-energy-deficient animals. As shown in Fig. 4A, administration of 15 nmol of NMDA injured the striatum per se, producing a lesion with a mean damaged area larger than and statistically different from that induced by saline injection. This lesion was not potentiated by chronic 3-NP treatment. In contrast, in the hippocampus, 7.5 nmol of NMDA injured only a small area of the CA1 subfield, not statistically different from that produced by saline injection (Fig. 4B). All nonrecumbent animals treated with 3-NP that received 7.5 nmol of NMDA in the hippocampus showed a lesion extending throughout the CA1 subfield (not shown) that was 3.6-fold larger than that present in the animals not treated with 3-NP (Fig. 4B). No damage was observed in the contralateral uninjected striatum or hippocampus (not shown).

Microdialysis experiments

As we have previously shown, perfusion of 25 m*M* PDC through a dialysis probe induces substantial elevations in the extracellular concentration of glutamate and aspartate (Massieu et al., 1995; Massieu and Tapia, 1997). In the striatum, PDC perfusion increased by 6.4-fold the extracellular concentration of glutamate and by 4.7-fold the extracellular concentration of aspartate. In contrast to PDC, perfusion of 3-NP at a concentration of 5 m*M* did not induce any change in the extracellular concentration of either glutamate or aspartate. When the two compounds were perfused in the same solution, the peak concentration of glutamate was close to 40% lower than that reached in the absence of 3-NP; however, this difference was not statistically significant. The PDC-induced increase in aspartate levels was not modified in the presence of 3-NP (Fig. 5A).

Consistent with what we previously reported (Massieu and Tapia, 1997), PDC perfusion in the hippocampus induced a 7.0-fold increase in the extracellular levels of glutamate and a 4.8-fold increase in aspartate levels. Perfusion of 5 m*M* 3-NP induced only a small increase in glutamate levels (1.98-fold) and no change in aspartate levels. In contrast to the striatum, perfusion of PDC and 3-NP in the same solution caused a larger increase in glutamate and aspartate levels and the peak concentration of both amino acids was reached in fraction 5 instead of fraction 6 (Fig. 5).

Changes in the extracellular concentration of other amino acids were less notable. In the striatum, nonsignificant changes in the extracellular levels of alanine, glutamine, and glycine were observed in the presence of PDC, and only a statistically significant threefold increase in taurine concentration was observed (Table 1). Perfusion of 3-NP induced a significant threefold increase in the extracellular concentration of glycine and nonsignificant small changes in the concentration of the other amino acids tested. When PDC and 3-NP were co-perfused, only the extracellular concentration of taurine showed a statistically significant increase relative to basal levels, but less than that induced by PDC perfusion (Table 1).

In the hippocampus, PDC induced statistically significant small increases in the extracellular levels of glycine (2.1-fold) and alanine (2.6-fold). Statistically nonsignificant changes in the concentrations of alanine, glutamine, glycine, and taurine were observed in the presence of 3-NP. When PDC and 3-NP were perfused in the same solution, significant increases in the concentrations of alanine (2.7-fold), glutamine (2.3-fold), glycine (1.77-fold), and taurine (2.9-fold) relative to their basal levels were observed (Table 1). The extracellular concentration of GABA was too small to be reliably detected with the methodology used.

No neuronal damage was found in the animals perfused with 3-NP, PDC, or 3-NP + PDC through the dialysis probe in either the hippocampus or the striatum as revealed by the histological analysis of brain sections.

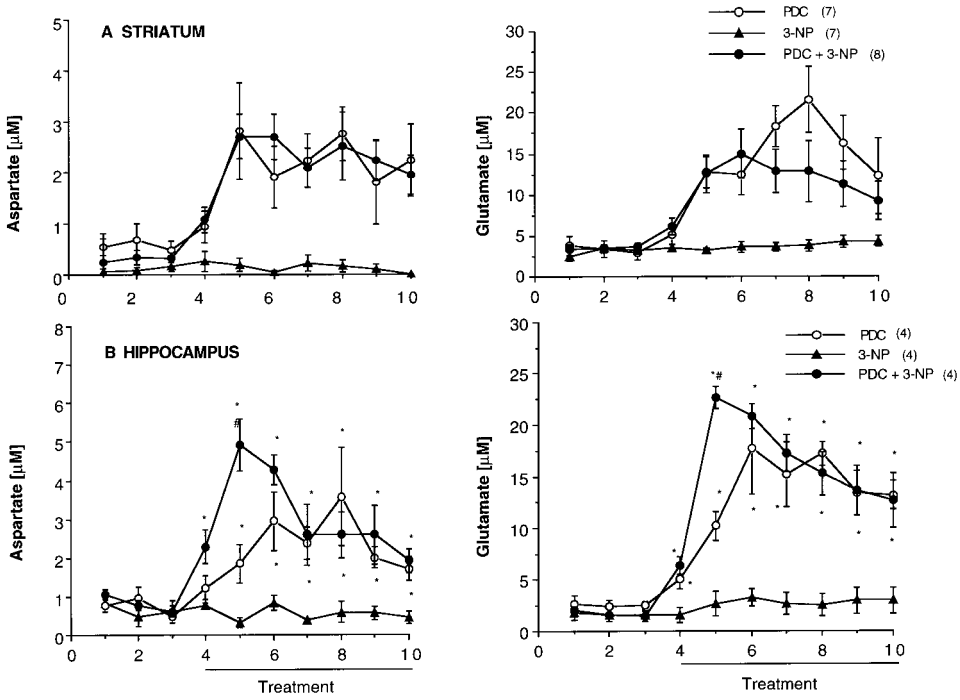

FIG. 5. Effect of PDC (25 m*M*), 3-NP (5 m*M*), or PDC + 3-NP on the extracellular concentration of aspartate and glutamate in the striatum (**A**) or the hippocampus (**B**). Microdialysis was carried out as described in Materials and Methods. The first three fractions correspond to the basal levels of amino acids, and at the end of the third fraction, PDC, 3-NP, or PDC + 3-NP was perfused for the next seven fractions. Data represent means ± SEM of the number of animals indicated in parentheses. $^{*}p < 0.05$ relative to 3-NP; $^{\#}p < 0.05$ relative to PDC.

DISCUSSION

It is believed that neuronal degeneration associated with brain ischemia might be a consequence of the excessive activation of glutamate receptors. This hypothesis is supported by the observations that glutamate receptor antagonists efficiently protect against ischemic damage and that important elevations in the extracellular concentration of excitatory amino acids occur during the ischemic insult in brain regions where neuronal degeneration will finally take place (Benveniste et al., 1984; Gill et al., 1987). The mechanism leading to the accumulation of excitatory amino acids in the extracellular space during ischemia is not clear, but it is suggested that it may result from the

TABLE 1. *Basal extracellular levels of amino acids and their changes induced by perfusion of PDC, 3-NP, or 3-NP + PDC in striatum and hippocampus of rat*

	PDC (25 m*M*)		3-NP (5 m*M*)		3-NP (5 m*M*) + PDC (25 m*M*)	
Amino acid	Basal	Peak	Basal	Peak	Basal	Peak
Striatum						
Alanine	2.0 ± 0.3	3.2 ± 1.2 (159)	1.6 ± 0.3	2.5 ± 1.0 (155)	0.9 ± 0.1	1.2 ± 0.3 (183)
Glutamine	6.8 ± 1.0	5.5 ± 2.3	14.4 ± 3.7	20.1 ± 9.1 (134)	4.9 ± 1.0	10.0 ± 2.7 (202)
Glycine	2.6 ± 0.4	4.2 ± 0.8 (159)	2.2 ± 0.4	6.8 ± 3.4[a] (305)	1.7 ± 0.2	2.9 ± 0.4 (173)
Taurine	4.4 ± 0.8	13.4 ± 3.0[a] (300)	3.5 ± 0.4	3.2 ± 1.3	2.9 ± 0.3	8.0 ± 1.8[a] (273)
Hippocampus						
Alanine	1.7 ± 0.21	4.5 ± 1.5[a] (164)	1.5 ± 0.1	2.0 ± 0.4 (33)	1.9 ± 0.26	5.2 ± 1.8[a] (170)
Glutamine	13.3 ± 3.6	17.9 ± 9.7 (34)	13.1 ± 1.8	16.8 ± 6.6 (28)	7.4 ± 1.0	17.1 ± 3.2[a] (130)
Glycine	2.9 ± 0.3	6.2 ± 2.3[a] (111)	3.2 ± 0.2	3.5 ± 0.2 (8)	3.8 ± 0.2	6.8 ± 0.4[a] (77)
Taurine	4.5 ± 0.3	6.4 ± 0.9 (41)	3.8 ± 0.2	1.6 ± 0.3 (42)	4.9 ± 0.3	14.3 ± 1.9[a] (192)

Amino acid levels were determined by HPLC as described in Materials and Methods. Basal levels correspond to the pooled data of the first three fractions collected, and peak levels correspond to concentrations determined in fractions 5, 6, or 7. PDC concentration was 25 m*M* and 3-NP concentration was 5 m*M*. Values are means ± SEM of seven or eight rats. The numbers in parentheses are the percent increases relative to the corresponding basal levels.

[a] $p < 0.05$ as compared with basal levels.

deficient or the inverse functioning of glutamate transporters (Attwell et al., 1993).

In previous studies, we have shown that pharmacological inhibition of glutamate uptake in vivo clearly leads to an important increase in the extracellular concentration of glutamate and aspartate. However, no neuronal damage is observed even after prolonged (2–4 h) brain perfusion with the glutamate uptake inhibitor PDC (Massieu et al., 1995; Massieu and Tapia, 1997). Other in vivo studies have shown that continuous pharmacological inhibition of glutamate transport by PDC during 14 days elicits neuronal damage in the rat striatum (Liévens et al., 1997) and that interruption of the synthesis of the glial glutamate transporters GLAST and GLT-1 for 7 days leads to increases in glutamate levels and neuronal damage (Rothstein et al., 1996). Similarly, Tanaka et al. (1997) have shown that mice lacking the glial glutamate transporter GLT-1 are more prone to pharmacologically induced epileptic seizures and to edema formation after cold-induced injury than the wild-type mice. Additionally, these mice show selective neuronal damage in the CA1 hippocampal subfield. In contrast to the in vivo situation, several studies in cortical cultures have demonstrated that pharmacological inhibition of glutamate transport induces neuronal damage that correlates with a rise in the extracellular concentration of glutamate (Blitzblau et al., 1996; Velasco et al., 1996; Volterra et al., 1996). These studies suggest that in vivo, sustained removal of glutamate transporter proteins or persistent inhibition of their action is necessary to induce neurodegeneration, whereas in vitro temporary inhibition of glutamate uptake appears to be sufficient to induce neuronal damage.

Several in vitro studies have shown that metabolic disturbances increase neuronal vulnerability to glutamate-mediated neurotoxicity (Novelli et al., 1988; Henneberry, 1989; Zeevalk and Nicklas, 1990; Greene et al. 1997), and in vivo it was demonstrated that NMDA-, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)-, glutamate-, and quinolinic acid-induced excitotoxic lesions are exacerbated when mitochondrial function is disrupted (Greene and Greenamyre, 1995; Bazzett et al., 1996). During hypoxia/ischemia, neuronal cells might become vulnerable to the transient elevation in extracellular glutamate levels due to the prompt decline in energy metabolism prevailing during such conditions. Our present results favor this hypothesis because we demonstrate that temporary inhibition of glutamate uptake in vivo leads to neuronal damage in energy-deficient rats chronically treated with 3-NP but not in intact animals.

As previously shown, the brain regions mostly affected by 3-NP intoxication are the striatum and the hippocampus (Hamilton and Gould, 1987; Beal et al., 1993*a*). It is interesting that only 20–30% of the animals intoxicated with 3-NP became recumbent 2–4 days after the treatment and showed bilateral degeneration in the striatum and the hippocampus. Therefore, a good correlation was found between the behavioral manifestations of 3-NP poisoning, which might reflect a severe metabolic deficit in these animals, and brain injury. Despite the metabolic impairment caused by 3-NP intoxication in the animals that were not recumbent after 3-NP treatment, they did not show apparent neuronal damage, as indicated by the normal histological appearance of the contralateral uninjected tissue of the rats unilaterally injected with the different compounds.

Similarly to PDC, subtoxic concentrations of DHK and NMDA induced substantial neuronal damage when administered in the hippocampus of 3-NP-treated rats. In contrast, NMDA-induced neuronal damage in the striatum was not potentiated by 3-NP treatment. In a previous study, neuronal injury resulting from the intrastriatal injection of NMDA was exacerbated by systemic 3-NP administration (Simpson and Isacson, 1993). This discrepancy might be explained by differences in the experimental protocol as in contrast to our study, older animals were used and a single but higher dose of 3-NP was administered. Additionally, in the present study, NMDA was injected 1 h (instead of 12 h) after 3-NP administration, which is expected to correspond to a lower degree of 3-NP-induced mitochondrial dysfunction as compared with a more delayed administration (Brouillet et al., 1998).

The variability in the size of the lesions and the number of animals injured with the different compounds might reflect variations in the susceptibility of the animals to the 3-NP-induced metabolic impairment.

The present results indicate that the neuronal degeneration observed after PDC administration in energy-deficient animals is not related to a further increase in the PDC-induced elevation of the extracellular levels of glutamate. In fact, microdialysis experiments showed lower concentrations of glutamate after co-perfusion of PDC and 3-NP in the striatum. In the hippocampus, glutamate and aspartate levels rose more steeply when PDC and 3-NP were co-administered; however, very similar peak concentrations of glutamate and aspartate were reached when PDC was perfused in either the presence or the absence of 3-NP. Furthermore, it is unlikely that the neuronal damage observed in recumbent animals chronically treated with 3-NP is due to an increase in extracellular glutamate levels, as direct 3-NP infusion did not produce significant changes in the concentration of this amino acid. Similar results were found by Beal et al. (1993*a*) after systemic injection of 3-NP. In contrast, direct intrastriatal injection of malonate causes a delayed increase in the extracellular concentration of glutamate (Messam et al., 1995). However, as this increase was blocked by the NMDA antagonist MK-801, it apparently results from the previous activation of NMDA receptors, consistent with a secondary excitotoxic mechanism.

The lack of neuronal damage in the animals co-perfused with 3-NP + PDC through the dialysis probe may indicate that neuronal vulnerability to glutamate transport inhibition will be apparent only after chronic metabolic impairment. 3-NP infusion through the dialysis membrane did not induce neuronal damage probably due

to the low dose of 3-NP used in these experiments, as we have observed notable tissue injury after acute injections of high doses of 3-NP (50–500 nmol/μl) in both the hippocampus and the striatum (not published).

Neuronal damage observed in our experimental conditions might result from a secondary excitotoxic damage due to the persistent activation of glutamate receptors. As has been suggested in chick retina, neuronal damage induced by metabolic inhibition is not related to an increase in the extracellular concentration of excitatory amino acids, but it is attenuated by high concentrations of Mg^{2+} in the extracellular medium (Zeevalk and Nicklas, 1992). This result suggests that a loss of the voltage-dependent Mg^{2+} blockade of NMDA receptors, and thus their persistent activation, may occur even in the presence of low levels of glutamate when energy metabolism is impaired. Alternatively, neuronal damage might result from the loss of the mitochondrial capacity to remove the elevated cytosolic calcium concentrations induced by the transient exposure of neurons to high concentrations of glutamate.

The present results suggest that increases in the extracellular concentration of glutamate induced by temporary inhibition of glutamate uptake can lead to neurodegeneration when mitochondrial function is impaired. We believe that the experimental conditions described in this study approximate events occurring during ischemia/hypoxia and may provide a useful model with which to study ischemic neuronal damage.

Acknowledgment: The authors thank Dr. Ricardo Tapia for his kind review of and useful comments on the manuscript and Federico Jandete for his help in histological techniques. This work was supported by DGAPA (project IN205095) of the Universidad Nacional Autónoma de México.

REFERENCES

Attwell D., Barbour B., and Szatkowski M. (1993) Nonvesicular release of neurotransmitter. *Neuron* **11,** 401–407.

Bazzett T. J., Falik R. C., Becker J. B., and Albin R. L. (1996) Synergistic effects of chronic exposure to subthreshold concentrations of quinolinic acid and malonate in the rat striatum. *Brain Res.* **718,** 228–232.

Beal M. F. (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann. Neurol.* **32,** 119–130.

Beal M. F., Brouillet E., Jenkins B. G., Ferrante R. J., Kowall N. W., Miller J. M., Storey E., Srivastava R., Rosen B. R., and Hyman B. T. (1993*a*) Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *J. Neurosci.* **13,** 4181–4192.

Beal M. F., Brouillet E., Jenkins B., Henshaw R., Rosen B., and Hyman B. T. (1993*b*) Age-dependent striatal lesions produced by the endogenous mitochondrial inhibitor malonate. *J. Neurochem.* **61,** 1147–1150.

Benveniste H., Drejer J., Schousboe A., and Diemer N. H. (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* **43,** 1369–1374.

Blitzblau R., Gupta S., Djali S., Robinson M. B., and Rosenberg P. A. (1996) The glutamate transport inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate indirectly evokes NMDA receptor mediated neurotoxicity in rat cortical cultures. *Eur. J. Neurosci.* **8,** 1840–1852.

Brouillet E., Guyot M.-C., Mittoux V., Altairae S., Condé F., Palfi S., and Hantraye P. (1998) Partial inhibition of brain succinate dehydrogenase by 3-nitropropionic acid is sufficient to initiate striatal degeneration in rat. *J. Neurochem.* **70,** 794–805.

Choi D. W., Maulucci-Gedde M. A., and Kriegstein A. R. (1987) Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* **7,** 357–368.

Gill R., Foster A. C., and Woodruff G. N. (1987) Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. *J. Neurosci.* **7,** 3343–3349.

Greene J. G. and Greenamyre J. T. (1995) Exacerbation of NMDA, AMPA, and L-glutamate excitotoxicity by the succinate dehydrogenase inhibitor malonate. *J. Neurochem.* **64,** 2332–2338.

Greene J. G. and Greenamyre J. T. (1996) Bioenergetics and glutamate excitotoxicity. *Prog. Neurobiol.* **48,** 613–634.

Greene J. G., Porter R. H. P., Eller R. V., and Greenamyre J. T. (1993) Inhibition of succinate dehydrogenase by malonic acid produces an "excitotoxic" lesion in rat striatum. *J. Neurochem.* **61,** 1151–1154.

Greene J. G., Shieu S. S., Gross R. A., and Greenamyre J. T. (1997) 3-Nitropropionic acid exacerbates *N*-methyl-D-aspartate toxicity in striatal culture by multiple mechanisms. *Neuroscience* **84,** 503–510.

Guyot M. C., Hantraye P., Dolan R., Palfi S., Maziére M., and Brouillet E. (1997) Quantifiable bradykinesia, gait abnormalities and Huntington's disease-like striatal lesions in rats chronically treated with 3-nitropropionic acid. *Neuroscience* **79,** 45–56.

Hamilton B. F. and Gould D. H. (1987) Nature and distribution of brain lesions in rats intoxicated with 3-nitropropionic acid: a type of hypoxic (energy deficient) brain damage. *Acta Neuropathol.* **72,** 286–297.

Hartley D. M., Kurth M. C., Bjerkness L., Weiss J. H., and Choi D. W. (1993) Glutamate receptor-induced $^{45}Ca^{2+}$ accumulation in cortical cell culture correlates with subsequent neuronal degeneration. *J. Neurosci.* **13,** 1993–2000.

Henneberry R. C. (1989) The role of neuronal energy in the neurotoxicity of excitatory amino acids. *Neurobiol. Aging* **10,** 611–613.

Liévens J. C., Duterte M., Forni C., Salin P., and Kerkerian-Le Goff L. (1997) Continuous administration of the glutamate uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate produces striatal lesion. *Mol. Brain Res.* **50,** 181–189.

Lucas D. R. and Newhouse J. P. (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina. *Arch. Ophthalmol.* **58,** 193–201.

Ludolph A. C., Seelig M., Ludolph A. G., Sabri M. I., and Spencer P. S. (1992) ATP deficits and neuronal degeneration induced by 3-nitropropionic acid. *Ann. NY Acad. Sci.* **648,** 300–302.

Massieu L. and Tapia R. (1997) Glutamate uptake impairment and neuronal damage in young and aged rats in vivo. *J. Neurochem.* **69,** 1151–1160.

Massieu L., Morales-Villagrán A., and Tapia R. (1995) Accumulation of extracellular glutamate by inhibition of its uptake is not sufficient for inducing neuronal damage: an in vivo microdialysis study. *J. Neurochem.* **64,** 2262–2272.

Messam C. A., Greene J. C., Greenamyre J. T., and Robinson M. B. (1995) Intrastriatal injections of the succinate dehydrogenase inhibitor, malonate, cause a rise in extracellular amino acids. *Brain Res.* **684,** 221–224.

Michaels R. L. and Rothman S. M. (1990) Glutamate neurotoxicity in vitro: antagonists, pharmacology and intracellular calcium concentrations. *J. Neurosci.* **10,** 283–292.

Novelli A., Reilly J. A., Lysko P. G., and Henneberry R. C. (1988) Glutamate becomes neurotoxic via the NMDA receptor when intracellular energy levels are reduced. *Brain Res.* **451,** 205–212.

Olney J. W. (1969) Brain lesions, obesity and other disturbances in mice treated with monosodium glutamate. *Science* **164,** 719–721.

Paxinos G. and Watson C. (1986) *The Rat Brain in Stereotaxic Coordinates.* Academic Press, Sydney.

Randall R. D. and Thayer S. A. (1992) Glutamate-induced calcium transients trigger delayed calcium overload and neurotoxicity in rat hippocampal neurons. *J. Neurosci.* **12,** 1882–1895.

Rothstein J. D., Dykes-Hoberg M., Pardo C. A., Bristol L. A., Jin L., Kuncl R. W., Kanai Y., Hediger M. A., Wang Y., Schielke J. P., and Welty D. F. (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* **16,** 675–686.

Salazar P., Montiel T., Brailowsky S., and Tapia R. (1994) Decrease of glutamate decarboxylase activity after in vivo cortical infusion of γ-aminobutyric acid. *Neurochem. Int.* **24,** 363–368.

Schinder A. F., Olson E. C., Spitzer N. C., and Montal M. (1996) Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *J. Neurosci.* **16,** 6125–6133.

Simon R. P., Swan J. H., Griffiths T., and Meldrum B. S. (1984) Blockade of *N*-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* **226,** 850–852.

Simpson J. R. and Isacson O. (1993) Mitochondrial impairment reduces the threshold for in vivo NMDA-mediated neuronal death in the striatum. *Exp. Neurol.* **121,** 57–64.

Tanaka K., Watase K., Manabe T., Yamada K., Watanabe M., Takahashi K., Iwama H., Nishikawa T., Ichihara N., Kikuchi T., Okuyama S., Kawashima N., Hori S., Takimoto M., and Wada K. (1997) Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* **276,** 1699–1702.

Velasco I., Tapia R., and Massieu L. (1996) Inhibition of glutamate uptake induces progressive accumulation of extracellular glutamate and neuronal damage in rat cortical cultures. *J. Neurosci. Res.* **44,** 551–561.

Volterra A., Bezzi P., Rizzini B. L., Trotti D., Ullensvang K., Danbolt N. C., and Racagni G. (1996) The competitive transport inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate triggers excitotoxicity in rat cortical neuron-astrocyte co-cultures via glutamate release rather than uptake inhibition. *Eur. J. Neurosci.* **8,** 2019–2028.

Zeevalk G. D. and Nicklas W. J. (1990) Chemically induced hypoglycemia and anoxia: relationship to glutamate receptor-mediated toxicity in retina. *J. Pharmacol. Exp. Ther.* **253,** 1285–1292.

Zeevalk G. D. and Nicklas W. J. (1992) Evidence that the loss of the voltage-dependent Mg^{2+} block at the *N*-methyl-D-aspartate receptor underlies receptor activation during inhibition of neuronal metabolism. *J. Neurochem.* **59,** 1211–1220.