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Malonate-Induced Degeneration of Basal Forebrain Cholinergic Neurons: Attenuation by Lamotrigine, MK-801, and 7-Nitroindazole

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Abstract: Previously, we have reported that intranigral infusions of malonate, an inhibitor of mitochondrial function, lead to the degeneration of the dopaminergic neurons of the nigrostriatal pathway that is mediated, at least in part, through NMDA receptor activation and nitric oxide formation. In the present study, unilateral focal infusions of malonate into the nucleus basalis magnocellularis (nbM) of male Sprague–Dawley rats (weighing 250–300 g) resulted in a dose-related depletion in ipsilateral cortical and amygdaloid choline acetyltransferase (ChAT) activity. Infusion of a 3 μmol dose of malonate into the nbM of vehicle-treated animals resulted in a 41 and 54% decrease in cortical and amygdaloid ChAT activity, respectively. Systemic pretreatment with lamotrigine (16 mg/kg, i.p.) and MK-801 (5 mg/kg, i.p.) attenuated the depletions in cortical and amygdaloid ChAT activity that resulted from an infusion of this dose of malonate into the nbM. Acetylcholinesterase (AChE) histochemistry of the nbM following focal infusion of malonate (3 μmol) showed a marked decrease in the number of AChE-positive neurons that was partially prevented by MK-801 pretreatment. Before examining the role of nitric oxide formation in malonate-induced toxicity, the ability of systemic administration of N^{ω}-nitro-L-arginine (L-NA) to inhibit nitric oxide synthase (NOS) activity in the nbM and cerebellum was investigated. L-NA (2, 10, and 20 mg/kg, i.p.) produced a dose-related inhibition of nbM and cerebellar NOS activity that was maximal following a dose of 10 mg/kg L-NA. This level of NOS inhibition persisted for at least 13 h following L-NA (10 mg/kg) administration. Subsequently, the effect of L-NA pretreatment on malonate toxicity was evaluated. Following pretreatment with L-NA (2 and 10 mg/kg, i.p.), the toxic action of malonate on cortical and amygdaloid ChAT activity was not altered. In addition, infusion of a lower dose of malonate (2 μmol) into the nbM resulted in decreases in cortical and amygdaloid ChAT activity that were not altered by pretreatment with L-NA (2 and 10 mg/kg, i.p.). In 7-nitroindazole (7-NI; 25 and 50 mg/kg, i.p.)-pretreated animals, malonate (3 μmol) produced decreases in cortical and amygdaloid ChAT activity that were attenuated by both doses of 7-NI. Thus, malonate-induced destruction of the basal forebrain cholinergic neurons was attenuated by systemic pretreatment with lamotrigine, MK-801, and 7-NI but not by L-NA. **Key Words:** Malonate—Cholinergic toxicity—Lamotrigine—Nitric oxide synthase—7-Nitroindazole—N^{ω}-Nitro-L-arginine.
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Recently, deficits in energy metabolism have been implicated in the pathophysiology of various neurodegenerative disorders (Albin and Greenamyre, 1992; Beal, 1992, 1993). It has consistently been reported that there are impairments in cerebral blood flow, oxygen utilization, glucose metabolism, and cytochrome oxidase activity in Alzheimer's disease (AD) (for review, see Beal, 1994). Recently, a positron emission tomography study by Kennedy et al. (1995) reported that there are decreases in cerebral glucose metabolism in individuals who are at risk of developing AD. It has also been shown that individuals homozygous for the ε4 allele of apolipoprotein E have decreased glucose metabolism in the same regions of the brain as patients with probable AD (Reiman et al., 1996). In addition, the β-amyloid peptide, which has been implicated in the pathophysiology of AD, has been reported to suppress selectively mitochondrial succinate dehydrogenase activity (Kaneko et al., 1995). Thus, there is growing evidence to suggest a role of impaired energy metabolism in the pathology of AD.

Metabolic compromise can lead to indirect *N*-methyl-D-aspartate (NMDA) receptor-mediated cell death, which has been referred to as "weak" or "secondary" excitotoxicity (for reviews, see Lees, 1991;

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Abbreviations used: AChE, acetylcholinesterase; AD, Alzheimer's disease; ChAT, choline acetyltransferase; L-NA, N^{ω}-nitro-L-arginine; nbM, nucleus basalis magnocellularis; 7-NI, 7-nitroindazole; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase.

Albin and Greenamyre, 1992; Beal, 1992, 1993). It has been demonstrated that glutamate becomes neurotoxic to primary cultures of rat cerebellar neurons via the NMDA receptor when intracellular energy levels are reduced (Novelli et al., 1988). It was suggested that reduced intracellular ATP levels lead to the inability of the Na^+,K^+-ATPase to maintain resting membrane potential. Subsequent depolarization of neurons removes the voltage-dependent Mg^{2+} blockade from the NMDA receptor and enables endogenous glutamate to become neurotoxic (Novelli et al., 1988). Similarly, Zeevalk and Nicklas (1990) demonstrated that mild metabolic compromise of the chick retina leads to neurotoxicity that, in the initial stages, is mediated by activation of NMDA receptors. In agreement with the "weak" excitotoxic hypothesis, it has been shown that various mitochondrial inhibitors can produce "excitotoxic" lesions both in vitro and in vivo. When applied in vivo, the mitochondrial succinate dehydrogenase inhibitors malonate and 3-nitropropionic acid have been reported to produce striatal lesions that are attenuated by antagonists of the NMDA receptor (Beal et al., 1993*a,b*; Greene et al., 1993; Bazzett et al., 1995; Greene and Greenamyre, 1995). In addition, malonate and 3-nitropropionic acid produce degeneration of cultured mesencephalic dopaminergic neurons that is NMDA receptor-mediated (Zeevalk et al., 1995*a,b*).

Activation of NMDA receptors on nitric oxide (NO) synthase (NOS; EC 1.14.13.39)-containing neurons leads to the formation of the gaseous messenger, NO (Garthwaite, 1991). Numerous studies have implicated NO as an intermediary in NMDA receptor-mediated toxicity (Dawson et al., 1991); however, conflicting findings have also been reported (Demerlé-Pallardy et al., 1991). Previously, we have demonstrated that inhibition of NO synthesis can *potentiate* direct NMDA receptor-mediated toxicity to nigral dopaminergic neurons in vivo (Connop et al., 1994, 1995). In contrast, a recent report from our laboratory and those of others have found that inhibition of NO formation can *attenuate* the neurotoxicity induced by mitochondrial toxins that is thought to involve indirect NMDA receptor-mediated toxicity (Maragos and Silverstein, 1995; Schulz et al., 1995*a,b*; Connop et al., 1996).

A consistent characteristic of AD pathology is the degeneration of the cholinergic neurons of the nucleus basalis of Meynert leading to reductions in levels of cholinergic markers in cortical regions (Coyle et al., 1983). In experimental animals, depletions in cortical and amygdaloid choline acetyltransferase (ChAT) activity are induced by the injection of excitotoxins into the nucleus basalis magnocellularis (nbM), a homologue of the nucleus basalis of Meynert (Boegman et al., 1992). As metabolic deficits have been implicated in the pathophysiology of AD, the present study examined whether the basal forebrain cholinergic neurons are sensitive to the toxicity associated with focal infusions of the mitochondrial succinate dehydrogenase inhibitor malonate. Because it has been reported that intrastriatal infusions of malonate result in (a) increased glutamate release (Messam et al., 1995) and (b) NMDA receptor-mediated toxicity (Beal et al., 1993*b*; Greene et al., 1993), the neuroprotective potential of lamotrigine, an inhibitor of glutamate release, and of the NMDA antagonist MK-801 was investigated. In addition, recent studies have suggested that NO formation may be involved in malonate-induced toxicity (Maragos and Silverstein, 1995; Schulz et al., 1995*a*; Connop et al., 1996), and it has been reported that there is an increase in the number of NOS-containing neurons within the substantia innominata of the AD brain (Benzing and Mufson, 1995). Thus, the role of NO formation in malonate toxicity was examined in animals treated with the NOS inhibitors N^{ω}-nitro-L-arginine (L-NA) and 7-nitroindazole (7-NI).

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats, obtained from Charles River (St. Constant, Quebec, Canada), weighing between 275 and 300 g were group-housed under temperature- and light-controlled conditions with food (Purina Rodent Chow 5001; Ralston Purina, Toronto, Ontario, Canada) and water provided ad libitum. Animals were used in accordance with the *Canadian Council on Animal Care Guidelines,* and the protocol was approved by the University Committee on Animal Care.

Surgical procedures and ChAT assay

Rats were anesthetized with 4% halothane and maintained with 2% halothane during surgery. Animals were placed in a Narashige stereotaxic apparatus with the incisor bar set at −3.3 mm. Saline (0.9% NaCl; pH 7.4), hypertonic saline (3 μmol of NaCl; pH 7.4), or malonate (1.33–4.4 μmol; pH 7.4) was unilaterally infused through a cannula (28 gauge) into the nbM according to the following coordinates: 1.3 mm posterior to bregma, 2.6 mm lateral to the midline, and 7.5 mm ventral to bregma (Paxinos and Watson, 1982). Malonate was dissolved in 0.9% NaCl and titrated to pH 7.4 with NaOH. Each solution was infused for 146 s through a cannula connected to a Hamilton syringe to deliver a total volume of 1.0 μl. The cannula was left in place for an additional 2 min and then withdrawn, bone wax was applied, and the incision was closed. At 4 or 7 days postinfusion, animals were decapitated, and the brain was rapidly removed and placed in ice-cold saline. Cortical samples were obtained from a coronal section taken 4 mm rostral to the posterior aspect of the optic chiasm. The basolateral amygdala was prepared from a 2-mm coronal slice taken caudal to the optic chiasm by freehand dissection of the tissue lateral to the optic tract, medial to the extension of the corpus callosum, ventral to the rhinal fissure, and dorsal to the periform cortex. The ipsilateral and contralateral cortex and amygdala were subsequently homogenized separately in 1 ml and 300 μl of homogenizing buffer (0.5% Triton X-100 and 1.0 m*M* EDTA, pH 7.3), respectively. Samples were then frozen at −70°C until assayed for ChAT activity. ChAT activity was determined according to the procedure of Fonnum (1975) using [^{14}C]acetyl-CoA as a substrate and 5-min incubation periods. Values for the enzyme activity were expressed as dpm per milligram of protein (Lowry et al., 1951) and calcu-

lated as specific enzyme activity (in units per milligram of protein per hour). Values for the ipsilateral (injected) side were calculated as a percent change compared with the contralateral (uninjected) side.

Pretreatment protocols

In lamotrigine pretreatment experiments, animals were given a single intraperitoneal injection of lamotrigine (16 mg/kg) 1 h before stereotaxic infusion of malonate (3.0 μmol) into the nbM. Animals pretreated with dizocilpine (MK-801) were injected with this agent (5 mg/kg, i.p.) 30 min before stereotaxic infusion of malonate and again 4 h after the initial injection of MK-801. In the L-NA pretreatment experiments, L-NA (2 or 10 mg/kg, i.p.) was given 1 h before infusion of malonate into the nbM. In the 7-NI pretreatment experiments, animals were injected with 7-NI (25 or 50 mg/kg, i.p.) 1 h before stereotaxic infusion of malonate and again 6.5 h following the first injection. In experiments using lamotrigine, MK-801, and L-NA, saline was used as the vehicle control, whereas arachis oil was used as the vehicle for 7-NI.

NOS assay

Before determining the role of NOS inhibition on malonate toxicity, the ability of systemic administration of L-NA to inhibit NOS activity in the nbM and cerebellum was investigated. To determine the relationship between L-NA and NOS activity, animals were given a single intraperitoneal injection of the vehicle (saline) or L-NA (2, 10, or 20 mg/kg). One hour postinjection, animals were killed, and the nbM and cerebellum were removed for the assay of NOS activity. The duration of L-NA-induced inhibition of brain NOS activity was examined 13 h following L-NA (10 mg/kg, i.p.) administration. The nbM from each hemisphere was dissected from a 2-mm-thick coronal section that was removed 1 mm posterior to the optic chiasm, and the entire cerebellum was removed. The nbM from both hemispheres was pooled. Each pooled nbM sample and the cerebellum were homogenized with a Teflon pestle in 120 μl and 1 ml of homogenizing buffer, respectively. The homogenizing buffer consisted of a solution containing 50 m*M* Tris (pH 7.0), 250 m*M* sucrose, 1 m*M* dithiothreitol, 1 m*M* EDTA, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor (0–4°C). The homogenates were centrifuged at 12,000 *g* for 6 min at 4°C, and the supernatant was retained for the assay of NOS activity.

NOS activity was assayed using the method described by Salter et al. (1995), which is a spectrophotometric assay based on the measurement of oxyhemoglobin oxidation to methemoglobin by NO. In brief, tissue samples (50 μl) were incubated at 37°C with 450 μl of reaction buffer consisting of 50 m*M* potassium phosphate (pH 7.2), 278 μ*M* $CaCl_2$, 1.6 μ*M* oxyhemoglobin, 22.2 μ*M* L-arginine, and 111 μ*M* NADPH. The absorbance difference between 421 and 401 nm (ϵ = 77,200) was continuously monitored for 10 min using a multiple-wavelength diode array spectrophotometer (Hewlett-Packard model 8451A). NOS activity was calculated from the first 1 min of assay that displayed a linear oxidation profile. Protein content was determined by the method of Lowry et al. (1951), and the enzyme activity was expressed as nanomoles of oxyhemoglobin oxidized per milligram of protein per minute.

Acetylcholinesterase (AChE) histochemistry

Animals that had received intra-nbM infusions of vehicle (3 μmol NaCl; pH 7.4), malonate (3 μmol), and malonate (3 μmol) in the presence of systemic pretreatment with MK-801 (5 mg/kg, i.p.) were used for morphological analysis of cholinergic neurons. Twenty-four hours following focal infusions of vehicle or malonate into the nbM, the AChE histochemical procedure was used. Animals were pretreated with the irreversible AChE inhibitor diisopropylfluorophosphate 6 h before they were killed to facilitate visualization of AChE-producing neurons in the nbM (Butcher and Bilezikjian, 1975). Rats were killed under deep ketamine anesthesia and transcardially perfused through the ascending aorta with 100 ml of ice-cold 0.9% NaCl followed by 250 ml of ice-cold 4% paraformaldehyde in 5 m*M* sodium phosphate buffer (pH 7.4). After removal, the brain was placed in the same fixative for 48 h at 6°C and then 0.1 *M* sodium phosphate buffer (pH 7.4) containing 30% sucrose for 48 h. Sections were cut 40 μm thick in the transverse plane on a freezing microtome. AChE was visualized according to the method of Karnovsky and Roots (1964) as modified by Butcher and Bilezikjian (1975).

Drugs and chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) except MK-801, which was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.), and lamotrigine isethionate, which was generously donated by the Wellcome Research Laboratories (Beckenham, Kent, U.K.). 7-NI was suspended in arachis oil by heating and sonication.

Statistical analysis

For statistical analysis, four to 10 animals were used to determine each point, and data were expressed as mean ± SEM values. Statistical analysis was performed using a randomized one-way ANOVA followed by Tukey–Kramer post hoc testing to determine statistical significance at $p < 0.05$.

RESULTS

Infusions of malonate into the nbM

Basal ChAT activity in the cortex and amygdala of naive animals was 25.8 ± 1.0 and 89.9 ± 3.9 nmol/μg/h (n = 6), respectively. No difference in contralateral cortical and amygdaloid ChAT activity was observed between any of the animal treatment groups. Figure 1 represents the percent decrease in ipsilateral ChAT activity in the cortex and amygdala measured 4 days following infusions of saline (0.9% NaCl) or malonate (1.33–4.4 μmol) into the nbM. Saline infusion into the nbM produced an 8.3 ± 4.9 and 3.4 ± 2.1% decrease in ipsilateral cortical and amygdaloid ChAT activity, respectively. Infusion of malonate into the nbM resulted in a dose-dependent depletion of cortical and amygdaloid ChAT activity, which was maximal following a dose of 4.4 μmol of malonate (51.0 ± 8.0 and 64.5 ± 10.0%, respectively). As an added control, it was observed that infusion of hypertonic saline (3 μmol of NaCl; pH 7.4) into the nbM did not produce a significant decrease in cortical and amygdaloid ChAT activity [5.0 ± 3.6 and 2.3 ± 3.2% (n = 5), respectively]. This indicates that malonate-induced cholinergic toxicity was not due to the hypertonic nature of the malonate solution. Decreases in cortical ChAT activity

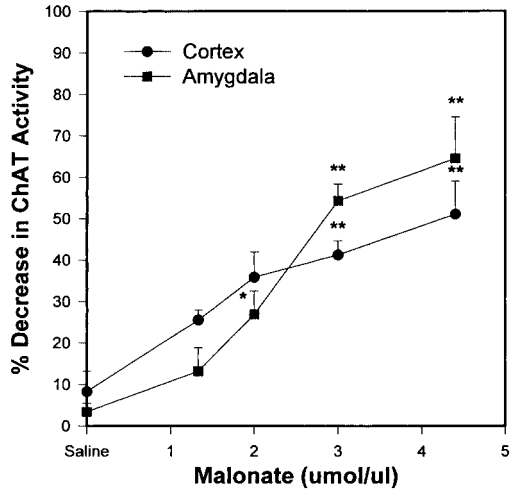

FIG. 1. Decreases in cortical and amygdaloid ChAT activity measured 4 days following infusion of saline (0.9% NaCl) or varying doses of malonate (1.33–4.4 μmol) into the nbM. Values are expressed as percent decrease in cortical and amygdaloid ChAT activity compared with the contralateral (unlesioned) side. Data are mean ± SEM (bars) values (n = 4–7 for each point). Values significantly different from those of the saline group are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$.

following malonate infusions were not different from those observed in the amygdala, indicating that malonate is not preferentially toxic to either of these neuronal pathways. The reductions in ChAT activity in the cortex and amygdala, measured 4 and 7 days postinfusion of malonate (3.0 μmol), were not significantly different.

Effect of lamotrigine and MK-801 on malonate toxicity

In the present study, lamotrigine (16 mg/kg, i.p.) and MK-801 (2 × 5 mg/kg, i.p.) pretreatment regimens that were found to be neuroprotective against striatal malonate infusions (Greene et al., 1993; Henshaw et al., 1994; Schulz et al., 1996*a*) were used to evaluate the role of glutamate release and NMDA receptor involvement in malonate toxicity to cholinergic neurons. Saline infusion into the nbM of vehicle-pretreated animals resulted in a 3.3 ± 3.2 and 1.7 ± 1.6% decrease in cortical and amygdaloid ChAT activity, respectively. These values were not significantly different from values in naive animals receiving saline infusion into the nbM, indicating that vehicle pretreatment does not alter ChAT activity. The effect of lamotrigine and MK-801 pretreatment on the toxicity associated with malonate (3 μmol) infusion into the nbM is shown in Fig. 2. Infusion of a 3 μmol dose of malonate into the nbM of vehicle-treated animals resulted in a 41.3 ± 3.4 and 54.3 ± 4.0% decrease in cortical and amygdaloid ChAT activity, respectively. Following systemic pretreatment with lamotrigine, this dose of malonate produced a 15.0 ± 4.0 and 20.6 ± 6.3% decrease in cortical and amygdaloid ChAT activity, respectively. In MK-801-pretreated animals the corresponding decreases in cortical and amygdaloid ChAT activity were 12.6 ± 3.5 and 12.4 ± 4.2%, respectively. Thus, both lamotrigine and MK-801 significantly attenuated the neurotoxic action of malonate on the cholinergic neurons of the nbM.

The diisopropylfluorophosphate pharmacohistochemical procedure allowed a clear visualization of cholinergic AChE-producing neurons in the nbM (Fig. 3). Focal infusion of the vehicle (3 μmol NaCl) did not appear to alter the number of AChE-positive neurons in the nbM. In contrast, infusion of malonate (3 μmol) into the nbM produced a marked decrease in the number of AChE-positive neurons that was partially prevented following pretreatment with MK-801.

Inhibition of NOS by L-NA

Basal NOS activity in the nbM and cerebellum was 13.3 ± 1.4 and 24.4 ± 2.1 nmol/mg/h, respectively. Figure 4 depicts the dose-dependent effect of systemic L-NA (2, 10, and 20 mg/kg, i.p.) on NOS activity in the nbM and cerebellum measured 1 h postinjection. Intraperitoneal injection of L-NA resulted in potent in-

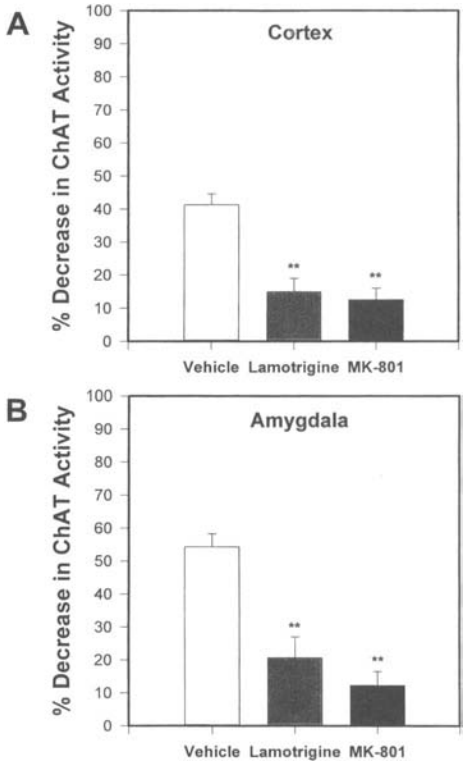

FIG. 2. Decreases in (**A**) cortical and (**B**) amygdaloid ChAT activity measured 4 days following infusion of malonate (3 μmol) into the nbM of vehicle-, lamotrigine (16 mg/kg)-, or MK-801 (5 mg/kg)-pretreated animals. Values are expressed as percent decrease in cortical and amygdaloid ChAT activity compared with the contralateral (unlesioned) side. Data are mean ± SEM (bars) values (n = 5 for malonate + vehicle and n = 6–8 for all other points). Values significantly different from those of malonate + vehicle-treated animals are indicated: $^{**}p < 0.01$.

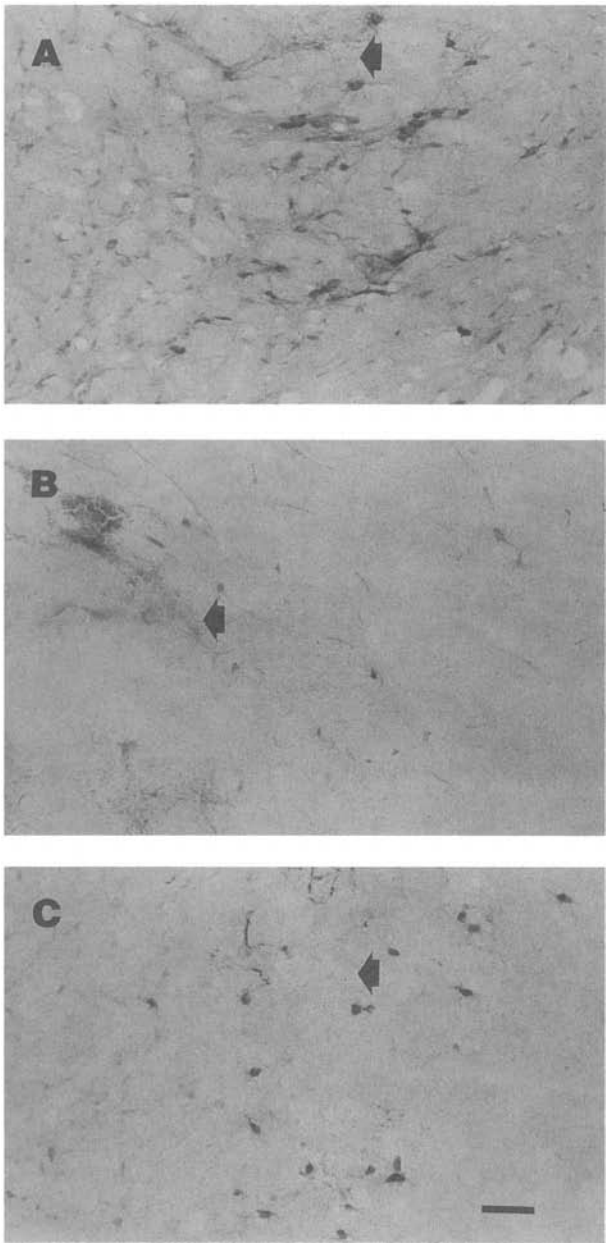

FIG. 3. Photomicrographs of AChE-positive neurons of the nbM following focal infusion of (**A**) vehicle (3 μmol of NaCl), (**B**) malonate (3 μmol), and (**C**) malonate (3 μmol) in the presence of MK-801 (5 mg/kg, i.p.). Arrows indicate the site of the cannula tract. Bar = 50 μm.

hibition of NOS activty in the nbM and cerebellum, which was maximal at a dose of 10 mg/kg (73.2 ± 6.0 and 88.2 ± 6.3%, respectively). This level of L-NA-induced inhibition of cerebellar NOS activity is consistent with that previously reported by Salter et al. (1995). Based on this dose–response relationship, the effect of both a submaximal (2 mg/kg) and maximal (10 mg/kg) dose of L-NA was tested on the action of malonate. To determine the duration of NOS inhibition after L-NA treatment (Salter et al., 1995), the enzyme activity in the nbM and cerebellum was measured 13 h postinjection of L-NA (10 mg/kg). At this time point, L-NA (10 mg/kg) resulted in a 59.5 ± 5.4 and 51.6 ± 9.6% decrease in basal nbM and cerebellar NOS activity, respectively. The level of NOS inhibition in these two brain regions was not significantly different at 1 or 13 h postinjection of L-NA (10 mg/kg), indicating a sustained duration of action. In view of these results and those of Salter et al. (1995), a dosing regimen for L-NA of 2 or 10 mg/kg (i.p.) given 1 h before malonate infusion into the nbM was used in the following experiments. The dosing regimen for 7-NI was based on a previous study from this laboratory (Connop et al., 1996) and those of others (MacKenzie et al., 1994; Salter et al., 1996).

Effect of NOS inhibitors on malonate toxicity

To determine the role of NO formation in malonate-induced degeneration of the basal forebrain cholinergic neurons, animals were pretreated with L-NA (2 or 10 mg/kg, i.p.) and 7-NI (25 or 50 mg/kg, i.p.) 1 h before malonate infusion into the nbM. The effects of both agents on malonate-induced reductions in cortical and amygdaloid ChAT activity are shown in Fig. 5. As previously observed, infusion of a 3 μmol dose of malonate into the nbM resulted in a 41.3 ± 3.4 and 54.3 ± 4.0% decrease in cortical and amygdaloid ChAT activity, respectively. Following pretreatment with L-NA (2 or 10 mg/kg), malonate-induced depletions in cortical and amygdaloid ChAT activity were not altered. Pretreatment with the 25 mg/kg dose of 7-NI before malonate infusion into the nbM resulted in a 23.8 ± 1.8 and 31.9 ± 6.6% depletion of cortical and

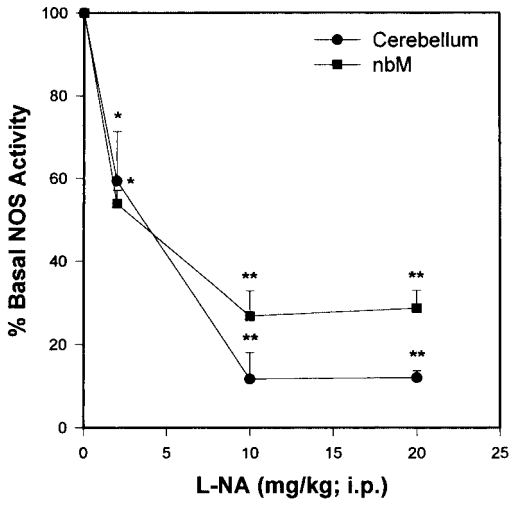

FIG. 4. NOS activity in the nbM and cerebellum following a single intraperitoneal injection of vehicle or varying doses of L-NA (2, 10, and 20 mg/kg). Values are expressed as percent basal NOS activity compared with vehicle-treated animals. Data are mean ± SEM (bars) values (n = 4–6 for each point). Values significantly different from those of vehicle-treated animals are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$.

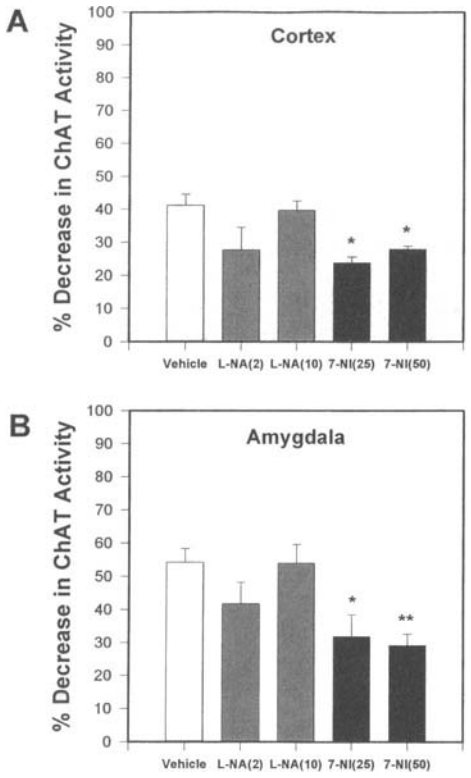

FIG. 5. Decreases in (**A**) cortical and (**B**) amygdaloid ChAT activity measured 4 days following infusion of malonate (3 μmol) into the nbM of vehicle-, L-NA (2 or 10 mg/kg)-, or 7-NI (25 or 50 mg/kg)-pretreated animals. Values are expressed as percent decrease in cortical and amygdaloid ChAT activity compared with the contralateral (unlesioned) side. Data are mean ± SEM (bars) values (n = 5 for malonate + vehicle and n = 6–9 for all other points). Values significantly different from those of malonate + vehicle-treated animals are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$.

amygdaloid ChAT activity, respectively. Similarly, pretreatment with a higher dose of 7-NI (50 mg/kg) resulted in a 27.9 ± 1.0 and 29.3 ± 3.4% decrease in cortical and amygdaloid ChAT activity, respectively. Thus, the toxicity produced by focal infusions of this dose of malonate into the nbM, as assessed by percent decrease in cortical and amygdaloid ChAT activity, was not altered by a submaximal or maximal dose of L-NA (2 and 10 mg/kg) but was partially attenuated by 7-NI (25 and 50 mg/kg).

Because L-NA did not alter the toxicity that resulted from an infusion of a high dose of malonate (3 μmol) into the nbM, the ability of the same doses of L-NA (2 and 10 mg/kg) to alter the toxicity associated with a lower dose of malonate (2 μmol) was examined. The effect of L-NA pretreatment on depletions of cortical and amygdaloid ChAT activity resulting from intra-nbM infusions of malonate (2 μmol) is shown in Table 1. Infusion of malonate (2 μmol) into the nbM resulted in depletions of cortical and amygdaloid ChAT activity that were not altered following pretreatment with either dose of L-NA (2 or 10 mg/kg).

DISCUSSION

Bierer et al. (1995) reported that cholinergic deficits in AD have the highest correlation with the severity of clinical dementia when compared with several other neurochemical markers. Because deficits in energy metabolism have been implicated in the etiology of AD and other neurodegenerative disorders, the present study examined the sensitivity of the basal forebrain cholinergic neurons to the toxic action of the competitive succinate dehydrogenase inhibitor malonate. We report that unilateral focal infusions of malonate (1.33–4.4 μmol/μl) into the nbM of the rat produces a dose-related loss of cholinergic neurons as assessed by depletion of ChAT activity in the ipsilateral cortex and amygdala. From the present study, it appears that both cortical and amygdaloid cholinergic pathways projecting from the nbM are equally sensitive to the neurotoxic action of malonate.

Differential sensitivity of striatal neurons to the toxic actions of malonate and 3-nitropropionic acid has been reported (Beal et al., 1993*b*; Wüllner et al., 1994; Bazzett et al., 1995). In these studies, many of the pathological features of Huntington's disease could be replicated, including the relative sparing of striatal NADPH diaphorase/somatostatin neurons and large cholinergic neurons with loss of spiny GABAergic projection neurons. In addition, it has been reported that cultured mesencephalic dopaminergic neurons are more vulnerable than GABAergic neurons to mild metabolic compromise (Zeevalk et al., 1995*a*) as well as to the synergistic toxic effects of rotenone and NMDA (Marey-Semper et al., 1995). Similarly, we have recently reported that the nigral dopaminergic neurons are sensitive to malonate-induced degeneration in vivo (Connop et al., 1996). In that study, intranigral infusion of 0.5 μmol of malonate resulted in a 60% depletion of striatal tyrosine hydroxylase activity measured 4 days postinfusion. In contrast, in the present study, a

TABLE 1. *Effect of L-NA on cortical and amygdaloid ChAT activity following malonate (2 μmol) infusion into the nbM*

Tissue, pretreatment	% decrease in ChAT activity after malonate (2 μmol)
Cortex	
+Vehicle	34.6 ± 4.9
+L-NA (2 mg/kg)	23.6 ± 3.4
+L-NA (10 mg/kg)	30.3 ± 4.9
Amygdala	
+Vehicle	34.5 ± 8.7
+L-NA (2 mg/kg)	26.9 ± 8.0
+L-NA (10 mg/kg)	30.2 ± 6.7

Decreases in cortical and amygdaloid ChAT activity were measured 4 days following infusion of malonate (2 μmol) into the nbM of vehicle- or L-NA (2 or 10 mg/kg)-pretreated animals. Values are expressed as percent decrease in cortical and amygdaloid ChAT activity as compared with the contralateral (unlesioned) side. Data are mean ± SEM values (n = 4–6 for each group).

significant depletion of cortical and amygdaloid ChAT activity was observed following a dose of 3 μmol of malonate. This suggests that the cholinergic neurons of the nbM are less sensitive to the toxic action of malonate than are the nigral dopaminergic neurons.

To investigate if glutamate release plays a role in malonate-induced degeneration of basal forebrain cholinergic neurons, a lamotrigine pretreatment protocol that previously had been shown to be neuroprotective in the striatum was used (Henshaw et al., 1994; Schulz et al., 1996*a*). Lamotrigine attenuated the toxic action of malonate in the nbM. As lamotrigine is known to block sodium channels (Meldrum, 1994), the neuroprotective effect observed could have been due to reduced presynaptic glutamate release or possibly blockade of postsynaptic sodium channels. To determine the role of NMDA receptor activation in malonate toxicity, an MK-801 pretreatment regimen was based on a protocol that had been found to protect striatal neurons against malonate toxicity independent of changes in core body temperature (Greene and Greenamyre, 1995). As with lamotrigine, MK-801 pretreatment effectively attenuated decreases in cortical and amygdaloid ChAT activity resulting from focal infusions of malonate into the nbM. Thus, malonate toxicity in the nbM appears to be mediated, at least in part, by endogenous glutamate release and NMDA receptor activation. To visualize malonate-induced destruction of the cholinergic neurons of the nbM and the neuroprotective effect of MK-801, AChE histochemistry was used. A marked depletion in the number of AChE-positive neurons was observed following infusion of malonate into the nbM that was partially prevented by systemic pretreatment with MK-801. This observation supports the finding that malonate is neurotoxic to basal forebrain cholinergic neurons and that MK-801 can attenuate this toxicity.

Although some studies using cultured neurons, brain slices, and models of focal ischemia have implicated NO as an intermediary in NMDA receptor-mediated toxicity (Dawson et al., 1991), other reports have suggested that NO does not play a role (Demerlé-Pallardy et al., 1991). In the present study, we investigated the role of NO formation in malonate-induced toxicity to the basal forebrain cholinergic neurons. This was motivated by our previous observation that NOS inhibitors protected the dopaminergic neurons of the nigrostriatal pathway against malonate toxicity (Connop et al., 1996). Because malonate infusion results in a decrease in ATP level, which returns to basal values within 12 h (Beal et al., 1993*b*), NOS inhibitor pretreatment protocols that would maintain reduced NOS activity for at least 12 h were used. A spectrophotometric NOS assay was used to determine an optimal dosing regimen that would provide submaximal and maximal levels of NOS inhibition in the nbM following systemic L-NA pretreatment. This assay was used based on the finding that inhibition of brain NOS activity produced by systemic administration of L-NA is more accurately measured ex vivo using the oxyhemoglobin spectrophotometric assay as opposed to the radiolabeled arginine to citrulline conversion assay (Salter et al., 1995, 1996). A dose of 10 mg/kg L-NA was found to produce maximal and sustained inhibition of NOS activity in the nbM. Pretreatment with a submaximal (2 mg/kg) or maximal (10 mg/kg) dose of L-NA did not reverse the depletions in cortical or amygdaloid ChAT activity that resulted from malonate infusion into the nbM. In addition, the ability of the selective neuronal NOS inhibitor 7-NI to modulate malonate toxicity to the basal forebrain cholinergic neurons was examined. The 7-NI pretreatment regimen used was based on our previous work and that of others demonstrating 7-NI-induced inhibition of brain NOS (MacKenzie et al., 1994; Connop et al., 1996; Salter et al., 1996). In contrast to L-NA, pretreatment with 7-NI attenuated the decreases in cortical and amygdaloid ChAT activity that resulted from infusion of malonate into the nbM.

The lack of effect of L-NA pretreatment on the action of malonate implies that NO formation is not an intermediary in malonate-induced cholinergic damage. In contrast, experiments with 7-NI appear to implicate NO formation in malonate-induced degeneration in the nbM. Previously, we have reported that systemic administration of 7-NI can produce sedative effects that are independent of NOS inhibition (Connop et al., 1994). Thus, it is possible that the modest neuroprotective effect of 7-NI was due to nonspecific sedative effects and that NO formation does not play a major role in the toxicity of malonate in the nbM.

Alternatively, the neuroprotective effect of 7-NI may be due to its selectivity for the neuronal isozyme of NOS. Schulz et al. (1995*a*,*b*) have demonstrated that 7-NI is neuroprotective against the toxicity produced by the mitochondrial toxins malonate and MPTP. In addition, several recent studies have reported that neuronal NOS knockout mice are resistant to the toxicity produced by NMDA, malonate, and ischemic damage (Dawson et al., 1996; Huang et al., 1996; Schulz et al., 1996*b*). Thus, it is possible that the modest neuroprotective effect of 7-NI against malonate toxicity in the nbM may be due to its selective inhibitory action on the neuronal isoform of NOS. However, previous results from our laboratory and those of others have shown that the nonselective NOS inhibitors L-NA and L-NA methyl ester are also neuroprotective against malonate toxicity (Maragos and Silverstein, 1995; Connop et al., 1996). Further investigation into the complex role that different NOS isozymes play in the toxicity produced by mitochondrial toxins appears to be warranted.

In summary, the present study reports that the basal forebrain cholinergic neurons are destroyed by focal infusions of malonate but appear to be less sensitive to malonate toxicity than are the nigral dopaminergic neurons. In addition, malonate toxicity to these cholinergic neurons is mediated, at least in part, by glutamate release and NMDA receptor activation; however, NO

formation does not appear to play a major role in this paradigm.

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