British Journal of Pharmacology (1996) 119, 374-378

Evidence for mediation of L-2-chloropropionic acid-induced delayed neuronal cell death by activation of a constitutive nitric oxide synthase

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1 Delayed neuronal cell death elicited by excess excitatory amino acid concentrations has been strongly implicated in many neurological disorders including head trauma, stroke, motor neurone disease and Huntington's disease. We have used the neurotoxin, L-2-chloropropionic acid (L-CPA) to model cellular events *in vivo* leading to delayed neuronal cell loss which is confined to the cerebellar cortex and can be prevented by inhibitors of nitric oxide synthase such as N^G-nitro-L-arginine methyl ester.

2 Experiments were performed to determine whether the constitutive nitric oxide synthase (NOS) or inducible form of NOS (iNOS) was responsible for the neuronal cell death. Activation of NOS was confirmed by a 39% increase in cerebellar total nitrate and nitrite concentrations in L-CPA-treated brains, as compared to controls (controls $= 2.53 \pm 0.10$; L-CPA treated $= 3.51 \pm 0.31$ nmol mg^{-1} protein, $P < 0.01$ Student's t tests, $n = 6$, mean $\pm$ s.e.mean). Biochemical measurements of total NOS activity were made in homogenates of cerebellum 6 h and 48 h following L-CPA administration, times at which L-CPA concentrations are maximal in brain and a time when there is a high proportion of cerebellar granule cell death, respectively. NOS activity as measured by the amount of [^{3}H]-arginine converted to [^{3}H]-citrulline, did not reveal any difference between controls (rats dosed with water) and animals dosed with L-CPA at either 6 or 48 h following dosing. Furthermore the ability of three NOS inhibitors, N^G-nitro-L-arginine, 7-bromo-3-nitroindazole and S-methylisothiourea to block the conversion of [^{3}H]-citrulline to [^{3}H]-arginine was identical at 6 and 48 h time points in control and L-CPA treated rats.

3 Quantitative autoradiography using [^{3}H]-N^G-nitro-L-arginine was used to measure the relative anatomical distribution and amount of NOS enzyme in the cerebellum of controls and L-CPA-treated rats 48 h following dosing. There was no significant alteration in the binding of [^{3}H]-N^G-nitro-L-arginine to granular and molecular layers of the cerebellum of control and L-CPA-treated rat brains.

4 Western blotting using antibodies against the inducible NOS enzyme failed to detect the protein in cerebellums of L-CPA-treated rats when measured 48 h after L-CPA dosing.

5 In conclusion, the increase in cerebellar nitrate/nitrite concentrations in L-CPA-treated rats provides further evidence for activation of NOS in the cerebellum following administration of L-CPA. The failure to demonstrate an increase in NOS activity at 6 or 48 h in L-CPA-treated rats as compared to controls suggests that the source of nitric oxide responsible for the granule cell death must originate from the constitutive NOS enzyme, probably the neuronal form which is highly enriched in the cerebellum. This hypothesis was further substantiated by Western blotting and quantitative autoradiography.

Keywords: L-2-Chloropropionic acid; nitric oxide synthase; neuronal cell death; cerebellum; granule cells

Introduction

Neuronal cell death resulting from excess extracellular concentrations of excitatory amino acids, such as aspartate and glutamate and the resultant over activation of glutamate receptors has now been implicated in a number of neurological disorders including stroke, Alzheimer's disease, Huntington's disease, motor neurone disease and the neural cell death resulting from head trauma (Simon *et al.*, 1984; Choi, 1988; Garthwaite, 1989). The biochemical events surrounding delayed excitatory amino acid-induced neuronal cell death have become ever more complex. Recently the pathways implicated in the neuronal cell loss following activation of glutamate receptors have expanded to include the generation of excess quantities of nitric oxide (Moncada *et al.*, 1991; Zhang & Snyder, 1995), activation of calpains (Siman & Noszek, 1988; Croall & DeMartino, 1991), and the generation of excess amounts of cytotoxic free radicals (Lafon-Cazal *et al.*, 1993). Some of the results obtained using isolated cultured cells, for studying the biochemical events leading to excitatory amino acid-induced neuronal cell death have been recently challenged with the apparent lack of consistency between data reported from *in vivo* animal models of cytotoxicity and those obtained *in vitro*. For example, prevention of calcium-activated neutral proteases (calpains) activation (Croall & DeMartino, 1991) by leupeptin has been demonstrated to be cytoprotective *in vivo* (Lee *et al.*, 1991) against focal ischaemia in the rat, but not against glutamate excitotoxicity using cultured granule cells (Manev *et al.*, 1991). Concurrently, the failure to detect hydroxyl radicals in rodent brains following bilateral carotid occlusion (Patthy *et al.*, 1995) or measure increased amounts of lipid peroxidation as a result of excess free radical production (Eggett & Reynolds, 1995) in animal models of excitotoxicity has raised questions as to the data obtained using isolated cells and tissue slices. We have therefore chosen to study the biochemical events surrounding neuronal excitotoxicity by employing an animal model based on the neurotoxin, L-2-chloropropionic acid which produces a selective lesion in the cerebellar cortex, mostly involving the death of granule cells (Simpson *et al.*, 1995; Jones *et al.*, 1995). Following oral administration of L-CPA to rats as a neutral salt at high concentrations (750 mg kg^{-1}), there is a progressive loss of cerebellar granule cells beginning 30 and 36 h post dosing following a 'silent period' when there is no evidence of neuronal or astroglial cell morphological changes (Simpson *et al.*,

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1995; Jones *et al.*, 1995). By 54 h post dosing, the granule cell necrosis typically encompasses up to 70–80% of the total cerebellar granule cell number resulting in a severe disruption of normal locomotor activity. Rats display a flat body posture, which is noticeable at 36–48 h post dosing and locomotion is laboured with a typical swinging gait. No other abnormal behavioural patterns are observed following L-CPA administration.

The involvement of excitatory amino acids in the L-CPA neurotoxicity has been demonstrated with the prevention of the granule cell necrosis and resultant oedema by co-administration of N-methyl-D-aspartate receptor antagonist, MK-801 (Widdowson *et al.*, 1996b). We have further explored L-CPA neurotoxicity by examining the role of nitric oxide which may lead to neuronal cell death as glutamate excitotoxicity has been shown to be mediated by nitric oxide production in primary cortical cultures (Dawson *et al.*, 1991). We have recently demonstrated that blockade of nitric oxide synthase (NOS) with N^G-nitro-L-arginine or 7-bromo-3-nitroindazole can partially prevent the development of the L-CPA-induced granule cell necrosis and cerebellar oedema suggesting a central role of nitric oxide in the cell death (Widdowson *et al.*, 1996a). Mammalian cells are endowed with at least three genes encoding distinct isoforms of NOS, one in endothelial cells (eNOS), a second in neuronal and certain other cell types including kidney, β-pancreatic cells, skeletal muscle and uterus (nNOS) and a third which is induced by immunological stimuli (iNOS) (Moncada *et al.*, 1991). Under normal physiological conditions, both eNOS and nNOS produce only small pulses of NO which is under the control of intracellular calcium concentrations. However, iNOS is capable of producing large and continuous elevations in NO until substrates (e.g. arginine) become limiting (Moncada *et al.*, 1991). Consequently iNOS, rather than constitutive eNOS or nNOS, is thought to be the isoform that produces large quantities of NO that can result in tissue damage or death. However in certain pathophysiological circumstances, such as tissue ischaemia/reperfusion (Patel *et al.*, 1993) or stoke (Nowicki *et al.*, 1991) a sufficiently sustained elevation in intracellular calcium may cause the constitutive NOS isoforms to produce cytotoxic quantities of NO. We performed experiments to discover whether there was any alteration in NOS activity in rat cerebellum during the development of L-CPA-induced neurotoxicity that may be indicative of expression of the iNOS isoform or whether the neurotoxicity was due to over activation of the constitutive NOS isoform.

Methods

Animal treatments

Male Alderley Park rats (Alpk: APfSD, 200–220 g) were housed in groups of 5 animals in an air conditioned environment (temperature = 22 ± 1°C; relative humidity = 50–70%) with a 12 h light/dark cycle (lights on 06 h 00 min) and allowed free access to food (Porton combined diet) and tap water *ad libitum*. Prior to dosing with L-CPA, animals were deprived of food overnight (18 h) and dosed orally between 09 h 00 min and 10 h 00 min with L-CPA (750 mg kg^{-1}; 10 ml kg^{-1}) diluted in deionised water and nautralised to pH 7 with NaOH. The rats were immediately allowed access to food after L-CPA dosing. Control rats received water only. Six and 48 h following L-CPA dosing, rats were killed by carbon dioxide anaesthesia. The brains were quickly removed, bisected down the midline and frozen over dry ice onto glass microscope slides for measurements of the nitrate and nitrite concentrations and for autoradiographic experiments. The frozen hemi brains were then stored at −70°C. Alternatively fresh cerebellums were used for biochemical measurements of NOS activity and Western blots for the inducible NOS enzyme. Animal care and monitoring was carried out in strict accordance to guidelines approved by government animal experimentation licences. Animals were killed when deemed to be under moderate stress or discomfort.

Assay of nitrate and nitrite (NOx)

Pieces of frozen cerebellum (approximately 10 mg) were chipped off the frozen brain and placed in 50 mM potassium phosphate buffer (pH 7.4; 20°C). The tissue was disrupted by sonication (10 s) and 100 μl removed for estimation of the protein. The proteins were precipitated with 5% TCA and pelleted by centrifugation (14,000 *g* for 10 min). The proteins were resuspended in 1 M NaOH and the protein concentrations measured by the method of Lowry *et al.* (1951). The remaining tissue homogenate was centrifuged (14,000 *g*) and 100 μl of supernatant incubated with 20 μl, 20 mu nitrate reductase in 50 mM potassium phosphate buffer containing 5 mM FAD and 1 mM NADH for 1 h at 37°C. The nitrite concentrations were measured in a 96-well microplate by the addition of 100 μl of supernatant and the addition of 100 μl of Griess reagent (0.1% naphthylethylenediamine and 1% sulphanilamide in 5% phosphoric acid) as described by Szabo *et al.* (1993). Optical density at 550 nm (OD_{550}) was measured using a microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared with the nitrate reductase medium containing NADH and FAD.

Biochemical evaluation of nitric oxide synthase activity

The nitric oxide synthase activity was examined *ex vivo* according to the method of Bredt & Snyder (1989) by measuring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline. Basically fresh rat cerebellums obtained from rats killed by CO_2 anaesthesia were homogenized in 20 vol 20 mM Tris HCl buffer containing 2 mM disodium EDTA, pH 7.4 at 4°C using a glass Teflon motorised homogenizer and then subject to centrifugation at 10,000 *g* for 15 min at 4°C. Twenty five microlitres of the supernatant containing the nitric oxide synthase enzyme was added to 50 μl of 50 mM Tris HCl buffer containing 3 mM $CaCl_2$, 2 mM NADPH and 0.5 μCi [^{3}H]-arginine. Finally, either 25 μl of water or NOS inhibitors (N^G-nitro-L-arginine, 3-bromo-7-nitroindazole and S-methylisothiourea, 10 μM, 10 μM and 1 μM final concentrations, respectively) in water were added to make a final volume of 100 μl and then the mixture was incubated at 0°C for 15 min and transferred to a water bath at 37°C for a further 15 min. The incubation was stopped by the addition of 3 ml ice-cold 20 mM HEPES buffer containing 2 mM disodium EDTA, pH 5.5 and the total volume added to mini columns containing Dowex AG-50WR-8 resin (sodium form) which had been previously equilibrated with the 20 mM HEPES/2 mM EDTA buffer (pH 5.5). The [^{3}H]-citrulline in the elutant was collected into 10 ml scintillation tubes and the radioactivity estimated by liquid scintillation.

Quantitative autoradiography

The distribution and density of NOS was measured by quantitative autoradiography as described by Kidd *et al.* (1995); 20 μm sections of the frozen hemi-brains were cut using a cryostat at −20°C, approximately 0.5 mm from the midline. The sections were thaw mounted on chrome-alum coated glass slides, dehydrated overnight at 4°C and then stored frozen at −70°C until used. The sections were subsequently brought to room temperature and pre-incubated in 50 mM Tris-HCl buffer containing 3 mM $CaCl_2$ and 0.025% Triton-X100; pH 7.4 for 30 min at 22°C. The sections were then incubated with 20 nmM [^{3}H]-N^G-nitro-L-arginine for 60 min at 4°C in 50 mM Tris buffer containing 3 mM $CaCl_2$. Non specific binding was estimated with 1 μM N^G-nitro-L-arginine. Following the incubation, the slides were washed three times for 10 min each in ice cold 50 mM Tris buffer/3 mM $CaCl_2$ buffer and then rapidly dipped in deionised water. The sections were rapidly dried

under a stream of cold, dry air and placed in an X-ray cassette with tritium autoradiography standards (Microscales; Amersham International, U.K.). The slides were exposed to tritium-sensitive film (Ultrafilm, Amersham) for 8 weeks. The autoradiographs were developed by the Kodak GB/X process and the autoradiographs analyzed with a Kontron VIDAS image analysis system (Imaging Associates, Thame, Bucks, U.K.). The amount of [^{3}H]-N^G-nitro-L-arginine binding to the sections was expressed as fmol mg^{-1} tissue.

Western blots

Brains from control and L-CPA-treated rats were removed from fresh tissue and the cerebellums separated. The cerebellum was homogenized in phosphate buffered saline (pH 7.4) containing 2% SDS, 5% glycerol and then boiled for 3 min. Particulate matter was removed by centrifugation (14,000 *g* for 5 min) and the proteins separated on a 7.5% SDS-polyacrylamide gel. After transfer to Hybond-C membranes (Amersham), the proteins were blotted using a commercially available polyclonal anti-macrophage (inducible) NOS antibodies (Affinity Research Products Ltd) according to protocols indicated by the supplier. The blot was compared to a positive control obtained from Affiniti Research Products Ltd consisting of homogenates of mouse macrophage cells from the RAW 264.7 cell line stimulated with interferon-γ.

Drugs and chemicals

L-2-Chloropropionic acid (92.5% purity) was obtained from ZENECA Specialities, Bioproducts and Fine Chemicals, Billingham, Cleveland, U.K. [^{3}H]-arginine 36.8 (Ci mmol^{-1}) was purchased from NEN/DuPont, Stevenage, U.K., and [^{3}H]-N^G-nitro-L-arginine (58 Ci mmol^{-1}) was from Amersham International. 7-Bromo-3-nitroindazole (sodium salt) was synthesized at the ZENECA Central Toxicology Laboratory (D.D.) according to the method described by Benchidimi *et al.* (1979). All other reagents, including N^G-nitro-L-arginine and S-methylisothiourea were purchased from the Sigma/Aldrich Chemical Company, Poole, Dorset, U.K. and were of the highest purity commercially available. All other chemicals and reagents were purchased from Sigma and were of the highest purity commercially available.

Results

The cerebellar concentration of NOx (nitrate and nitrite concentrations) was significantly increased in L-CPA-treated rats as compared to controls (controls = 2.53 ± 0.10; L-CPA-treated = 3.51 ± 0.31 nmol mg^{-1} protein, $P < 0.02$ Student's *t* tests, $n = 6$, mean ± s.e.mean) when measured 48 h after dosing. The concentration of [^{3}H]-citrulline formed from rat cerebellums of vehicle and L-CPA-treated rats 6 and 48 h following the L-CPA dosing did not reveal a statistical difference between the experiment and control groups (Student's *t* tests) (Figure 1). There was also no change in the total amount of [^{3}H]-citrulline produced between the 6 h and 48 h groups of either the control or L-CPA groups (Student's *t* tests). The inhibition of [^{3}H]-citrulline production by 10 μM N^G-nitro-L-arginine, 10 μM 7-bromo-3-nitroindazole or 1 μM S-methylisothiourea was not significantly different between controls of L-CPA-treated rats at either time point following L-CPA oral dosing (6 h, ANOVA $F_{(5,35)} = 1.12$; not significant; 48 h, ANOVA $F_{(5,35)} = 0.87$; not significant) (Figure 1).

Autoradiographical analysis of [^{3}H]-N^G-nitro-L-arginine binding to nitric oxide synthase in the cerebellum did not reveal any differences in brains obtained from rats dosed with water and those dosed with L-CPA, despite the severe oedema and neuronal cell loss in the granule layer (Figure 2; Table 1). [^{3}H]-N^G-nitro-L-arginine binding was highest in the cerebellum and olfactory bulb, as compared to other brain structures. In the cerebellar cortex, specific [^{3}H]-N^G-nitro-L-arginine binding

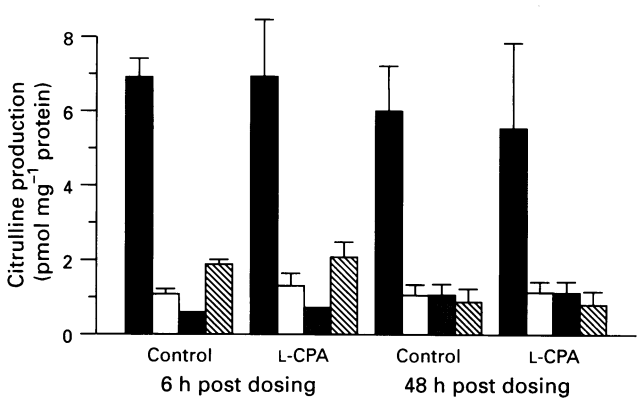

Figure 1 NOS activity as expressed as the amount of conversion of [^{3}H]-arginine to [^{3}H]-citrulline in cerebellar homogenates from controls or rats treated with L-CPA and killed at 6 h or 48 h post dosing (solid columns). The NOS activity in the presence of 10 μM N^G-nitro-L-arginine (open columns), 10 μM 3-bromo-7-nitroindazole (stippled columns) and 1 μM S-methylisothiourea (hatched columns) is also shown. Data are expressed as mean ± s.e.mean for 6 rats per group.

was higher in the molecular layer than in the granular layer (Table 1). [^{3}H]-N^G-nitro-L-arginine binding to white matter was low whilst there was a moderate amount of binding to the cerebral cortex (Table 1).

Finally, Western blotting for the inducible NOS enzyme in cerebellar homogenates did not show staining for this 130 kDa protein in homogenates of either control or L-CPA-treated rats (data not shown).

Discussion

The role of the inducible isoform of NOS in inflammation and neuronal cell death following traumatic and ischaemic insults is still unknown. Although there is now emerging evidence for a pivotal role of cytokines in the development of neuronal cell death and brain oedema (Rothwell & Hopkins, 1995), whether the cytokine subtypes or their concentrations are sufficient to induce the iNOS (Moncada *et al.*, 1991) is uncertain. We have used the L-CPA model to examine these questions and to try to determine whether the cerebellar granule cell death is due to excessive activity of the constitutive NOS, mainly the nNOS in the cerebellum or may be due to the expression of iNOS.

We have previously reported that L-CPA-induced neurotoxicity can be significantly reduced by blocking NOS with L-NAME or 3-bromo-7-nitroindazole (Widdowson *et al.*, 1996a) with significantly fewer numbers of dead granule cells observed in the cerebellar cortex and a much reduced cerebellar oedematous reaction. Measurements of the NOx concentrations in rat cerebellum 48 h following L-CPA treatment demonstrated a significant increase in nitrate and nitrite production providing further evidence in support of NOS activation by L-CPA, as has been demonstrated in other systems (Szabo *et al.*, 1993). Preliminary pharmokinetic studies with [^{14}C]-L-CPA to examine the time course, distribution and concentrations of L-CPA in the blood and brain following oral dosing demonstrated a high concentration of L-CPA remaining in the brain for up to 8 h post-dosing (Wyatt, unpublished results). Thus L-CPA may activate NMDA receptors and liberate NO for up to 8 h. Biochemical experiments failed to detect a change in the total NOS in the cerebellum nor was there a change in the pharmacological profile of the NOS inhibitors at an early time point when NMDA receptors are being activated by L-CPA (6 h) or at a later time when there is considerable neuronal cell damage (48 h). We have used the ability of 10 μM N^G-nitro-L-arginine to block nNOS activity, but not iNOS in the cerebellar homogenates to determine whether there may have been a change in the iNOS or nNOS activity. N^G-nitro-L-arginine

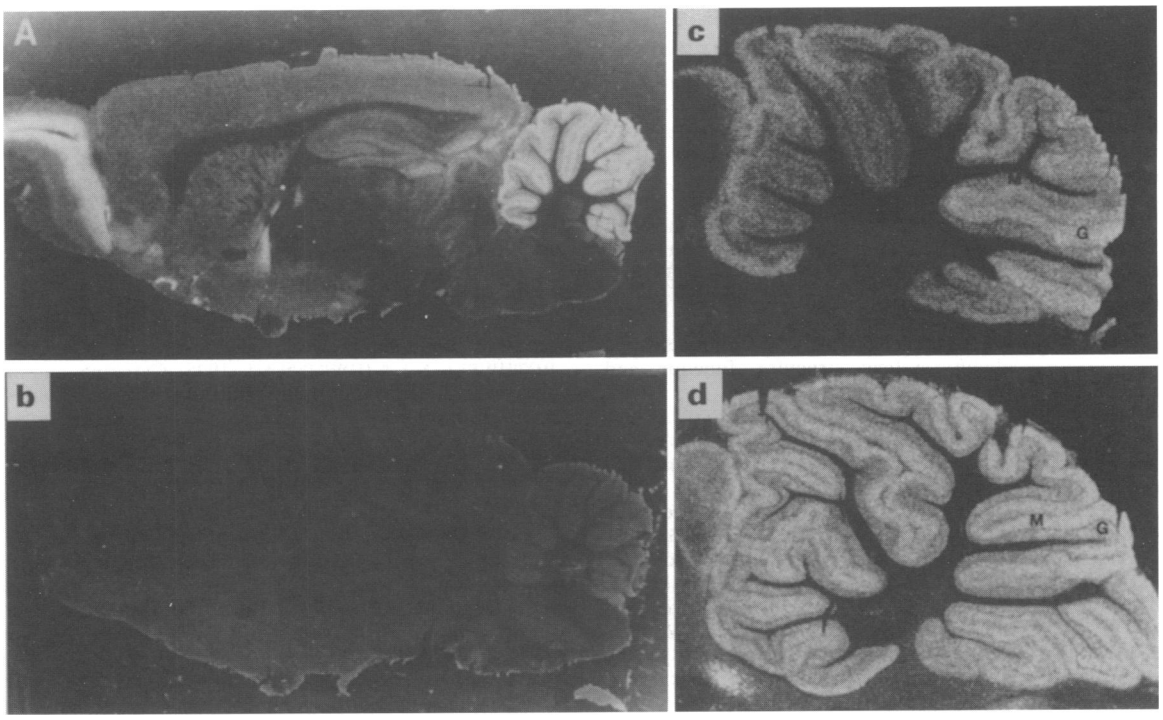

Figure 2 Representative autoradiograms of [^{3}H]-N^G-nitro-L-arginine binding to NOS in rat brain. (a) Total [^{3}H]-N^G-nitro-L-arginine binding; (b) non-specific binding in the presence of 1 μM N^G-nitro-L-arginine; (c) total binding in a cerebellum from a control rat and (d) total binding in the cerebellum of an L-CPA-treated rat killed 48 h after dosing.

Table 1 [^{3}H]-N^G-nitroarginine binding to sections of rat brain

	Specific binding (fmol mg^{-1} tissue)	
	controls	*L-CPA-treated*
Cerebellar granular level	39.7±4.4	38.4±2.2
Cerebellar molecular layer	56.9±7.7	52.1±4.0
Cerebellar white matter	6.0±1.0	5.7±0.8
Occipital cortex, layers I-VI	11.2±1.2	12.7±1.6

Data shown as mean±s.e.mean for $n=5$ brains per group.

has been reported to be over 25–100 fold selective for the nNOS over the iNOS (Bland-Ward *et al.*, 1993; Southan *et al.*, 1995) whereas 3-bromo-7-nitroindazole is non selective for isoforms of NOS (Bland-Ward *et al.*, 1993) and S-methylisothiourea is 5 fold more potent at the iNOS than at eNOS (Southan *et al.*, 1995) but is lower at nNOS ($IC_{50}=2.6$ μM; Widdowson, unpublished results) as compared with 3-bromo-7-nitroindazole ($IC_{50}=0.7$ μM; Widdowson *et al.*, 1996a). Thus at 10 μM concentration of N^G-nitro-L-arginine, all of the nNOS activity will be inhibited ($IC_{50}=0.7$ μM; Widdowson *et al.*, 1996a), but only approximately 25% of the iNOS activity would be inhibited whereas 10 μM of both 3-bromo-7-nitroindazole and S-methylisothiourea would be capable of inhibiting all of the NOS activity. Therefore an increase in iNOS activity produced by an increased expression of iNOS protein would be expected to lead to NOS activity that was not blocked by N^G-nitro-L-arginine, but sensitive to 3-bromo-7-nitroindazole and S-methylisothiourea. The proportion of NOS activity that was inhibited by 10 mM of N^G-nitro-L-arginine was not significantly different between homogenates prepared from controls and L-CPA-treated rats, demonstrating that there was no evidence for an increase in iNOS activity. Western blotting for the iNOS protein failed to produce any staining for the enzyme in homogenates of L-CPA or control cerebellum suggesting that this enzyme is not expressed under basal or L-CPA-stimulated conditions.

As reported previously (Kidd *et al.*, 1995), [^{3}H]-N^G-nitro-L-arginine binding to NOS is highly enriched in the cerebellum with slightly more binding found in the molecular layers of the cerebellar cortex than in the granule layer. There were no changes in [^{3}H]-N^G-nitro-L-arginine binding in either granular or molecular layers, 48 h following L-CPA dosing, a time point when there is extensive granule cell loss. We can find no evidence for a reduction in nNOS in the granule cell layer as might have been expected with the loss of granule cells and a possible compensatory increase in NOS in the molecular layer as a result of iNOS expression. Thus there is no evidence of a change in either total NOS, total NOS activity or a change in the anatomical distribution of NOS in the brain. A neuropathological examination of L-CPA-treated brains did not reveal an accumulation of microglia over the damaged portion of the granule cell layer (Simpson *et al.*, 1995) which may be responsible for an induction of iNOS. We therefore suggest that the constitutive NOS is responsible for the granule cell necrosis which is most probably the nNOS subtype since immunocytochemistry has demonstrated the cerebellum to be enriched in this type (Moncada *et al.*, 1991). The link between NO and L-CPA granule cell death model is further evidence that this model may provide vital clues as to the biochemical events surrounding excitatory amino acid-induced cell death.

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(Received January 24, 1996
Revised May 15, 1996
Accepted June 24, 1996)