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Possible role of nitric oxide in the development of L-2-chloropropionic acid-induced cerebellar granule cell necrosis

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1 L-2-Chloropropionic acid (L-CPA) produces selective neuronal cell necrosis in rat cerebellum when administered orally at 750 mg kg^{-1} that is mediated in part through activation of N-methyl-D-aspartate (NMDA) receptors. Cerebellar granule cell death occurs between 30 and 36 h following L-CPA administration exhibiting a number of features in common with excitatory amino acid-induced cell death. We have used this *in vivo* model to examine the neurochemical processes following L-CPA-induced activation of NMDA receptors leading to neuronal cell death in the rat cerebellum.

2 The effects of a number of compounds which potently block nitric oxide synthase *in vitro* were examined on L-CPA-induced neurotoxicity 48 h following L-CPA dosing, to discover whether the neuronal cell death is mediated in part by excessive nitric oxide generation. Four inhibitors were studied, N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-iminoethyl-L-ornithine (L-NIO) and 3-bromo-7-nitroindazole (BrNI).

3 L-NAME (50 mg kg^{-1}, i.p. twice daily) and BrIN (50 mg kg^{-1}, i.p. twice daily) administration prevented the L-CPA-induced loss of granule cells which can reach up to 80-90% of the total cell number in rats treated with L-CPA alone. L-NOARG (50 mg kg^{-1}, i.p. twice daily) and L-NIO administered at either 25 or 100 mg kg^{-1}, twice daily did not produce any significant protection against L-CPA-induced neurotoxicity.

4 Both L-NAME and BrIN also prevented the L-CPA-induced increase in cerebellar water content and sodium concentrations. L-NIO when administered at the highest doses prevented the increase in cerebellar sodium concentration but not water content. L-NIO and L-NOARG were ineffective in preventing the L-CPA-induced increases in cerebellar water and sodium concentrations.

5 L-CPA-induced reductions in cerebellar aspartate and glutamate concentrations and increases in glutamine and GABA concentrations were prevented by L-NAME and BrIN, but not by L-NIO or L-NOARG. Also reductions in L-[^{3}H]-glutamate binding to glutamate ionotrophic and metabotrophic receptors in the granule cell layer of rat cerebellum was prevented by L-NAME and BrIN, but not L-NIO or L-NOARG.

6 In conclusion, the neuroprotection offered by L-NAME and BrIN suggests that L-CPA-induced cerebellar granule cell necrosis is possibly mediated by or associated with excessive generation of nitric oxide. The inability of nitric oxide synthase inhibitors, L-NOARG and L-NIO to afford protection may result from their limited penetration into the brain (L-NIO) or rapid dissociation from the enzyme.

Keywords: L-2-Chloropropionic acid; granule cells; rat cerebellum; nitric oxide synthase; N^G-nitro-L-arginine methyl ester; 3-bromo-7-nitroindazole

Introduction

Substituted propionic acid analogues, 2-chloropropionic acid (CPA) and 3-nitropropionic acid are neurotoxic when administered to rats (Beal *et al.*, 1993; Widdowson *et al.*, 1995a; Simpson *et al.*, 1995) producing damage to the cerebellum and striatum, respectively. Although these substituted propionic acid compounds produce neuronal cell death in different brain regions presumably by affecting different biochemical pathways, their neurotoxicity can be prevented by N-methyl-D-aspartate (NMDA) inhibitors such as the non-competitive antagonist, dizocilpine (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine, MK-801 (Wong & Kemp, 1991; Beal *et al.*, 1993; Widdowson *et al.*, 1995b) suggesting a common role for glutamate receptors in the mechanism of cell death.

We have examined the neurotoxicity induced by L-CPA to explore biochemical events leading to cell death. L-CPA-induced neuronal cell death provides a powerful tool to explore glutamate-mediated cell death (Choi, 1988) since L-CPA can be administered systemically and the neuronal cell loss occurs selectively in one brain region. Moreover, the use of L-CPA neurotoxicity *in vivo* to model glutamate neurotoxicity offers several advantages over isolated neuronal cell cultures allowing the various components of the toxicity, i.e. neurochemical changes and reactive gliosis to be examined together. L-CPA neurotoxicity is characterized by a delayed cerebellar granule cell necrosis affecting up to 90% of the total granule cell population and a small loss in Purkinje cell number at between 36 h following L-CPA administration, becoming more marked by 48 h post dosing (Simpson *et al.*, 1995). Accompanying the loss of cerebellar granule cells, there are time-dependent reductions in concentrations of the excitatory amino acids, glutamic acid and aspartic acid and increases in glutamine and γ-aminobutyric acid (GABA). No changes in amino acid concentrations have been observed in forebrain following L-CPA administration and we have been unable to observe any neuronal cell damage in forebrain regions (Widdowson *et al.*, 1995a; Simpson *et al.*, 1995). Associated with the granule cell loss is a reduction in the specific binding of [^{3}H]-glutamate and [^{3}H]-kainate acid in the cerebellum of L-CPA-treated rats

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(Widdowson *et al.*, 1995a), probably as a result of loss of glutamate receptors associated with granule cell perikarya (Garthwaite & Brodbelt, 1983). Cerebellar oedema occurs in parallel with the onset of cerebellar neuronal cell death (Simpson *et al.*, 1995; Jones *et al.*, 1995). Reactive gliosis, which is confined to the cerebellar cortex and is characterized by astroglial proliferation and migration into the cerebellar granule cell layer occurs only at times beyond 48 h (Simpson *et al.*, 1995).

The exact biochemical events following oral dosing of L-CPA which lead to the loss in cerebellar granule cells are currently unknown. L-CPA does not appear to result in marked disturbance in glucose metabolism in cerebellar tissue following oral dosing to rats (Lock *et al.*, 1995). The neurotoxicity-induced by L-CPA is, however, different from 3-nitropropionic acid which, like malonic acid acts as a succinate dehydrogenase inhibitor (Greene *et al.*, 1993; Henshaw *et al.*, 1994; Greene & Greenamyer, 1995). The formation of nitric oxide has been shown to occur following activation of NMDA receptors suggesting that the formation of nitric oxide may play a role in the cascade of events leading to the L-CPA-mediated neuronal cell death (Dawson *et al.*, 1991). The rat cerebellum contains high concentrations of nitric oxide synthase I (Moncada *et al.*, 1991) which is stimulated following NMDA receptor activation through increases in intracellular calcium concentrations (Bredt & Snyder, 1989; Snyder, 1992). We have therefore performed experiments *in vivo* to discover whether nitric oxide inhibitors could prevent L-CPA-induced granule cell necrosis, cerebellar oedema, reductions in [^{3}H]-glutamate binding to the granule cell layer and changes to cerebellar amino acid concentrations.

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 starved overnight (18 h) and orally dosed with L-CPA (750 mg kg^{-1}; 10 ml kg^{-1}) diluted in deionised water and neutralised to pH 7 with NaOH. The rats were immediately allowed access to food after L-CPA dosing. Control rats received water only. Nitric oxide synthase inhibitors were dissolved in deionised water, sterilised by passing through a 20 μm filter and administered twice daily via the intraperitoneal route (2 ml kg^{-1}). N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), 3-bromo-7-nitroindazole (BrIN) (Dwyer *et al.*, 1991; Moore *et al.*, 1993) were administered at 50 mg kg^{-1} whilst N^G-imino-L-ornithine (L-NIO) (Rees *et al.*, 1990) was administered at 25 or 100 mg kg^{-1}. Rats were monitored twice daily and weighed daily to assess the severity of the neurotoxicity. Forty eight hours after L-CPA dosing, rats were killed by carbon dioxide anaesthesia and their brains removed. Animal care and monitoring were 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.

Experimental procedures

The brains were quickly bisected down the midline. The right half of the brain was divided into forebrain and cerebellum, the medulla oblongata being discarded. The brain pieces were placed in pre-weighed glass scintillation vials and dried to constant weight in an oven (105°C). The water content in the forebrain and cerebellum was expressed as g water g^{-1} dry weight. The dried brain tissue was subsequently homogenized and suspended in 5% trichloroacetic acid and then filtered through 0.2 μm filters. The sodium concentrations in the filtrate were estimated using a Jenway PFP 7 by flame photometry. Sodium concentrations were expressed as μmol g^{-1} dry weight.

The left half of the brain was quickly frozen onto a glass microscope slide over dry ice and stored in a plastic bag at −70°C until analyzed. Twenty micron longitudinal sections were cut from the hemi-brains and thaw mounted on gelatin coated glass slides for use in autoradiography experiments. Also 10 μm sections were cut from the brains, mounted on gelatin coated slides, air dried and then immersion-fixed in 4% buffered paraformaldehyde for between 24 and 48 h. The 10 μm sections were stained with haematoxylin/eosin and examined by light microscopy for the assessment of the degree of granule cell necrosis. Finally, pieces (30 mg) of frozen cerebellum were carefully chipped off the remaining frozen brain tissue, and placed in ice cold 3% perchloric acid and sonicated. Following centrifugation (14 000 *g* for 10 min; 4°C) the amino acid concentrations were estimated in the cerebellum by high performance liquid chromatography as described previously (Widdowson *et al.*, 1995a) using a precolumn derivertization method with *o*-pthaldialdehyde adapted from the method of Lindroth & Mopper (1979). Any remaining cerebellar tissue was assayed for nitric oxide synthase activity as described below. Tissue was not available from L-NAME or L-NOARG-treated animals.

L-[^{3}H]-glutamate (100 nM) binding to glutamate receptors on 20 μm sections was carried out in Tris-acetate buffer (pH 7.1; 4°C) for 40 min using 1 mM L-glutamate to define non-specific binding according to the method of Monaghan *et al.* (1985). The sections were washed twice in Tris buffer (10 s each), rapidly dipped in deionised water and dried under a stream of cold dry air. The brain sections were placed in an X-ray cassette with tritium-sensitive film (Ultrafilm, Amersham) with autoradiography standards (Microscales, Amersham) for 6 weeks. The autoradiographs were developed using Kodak GB/X process and then analyzed by a Kontron VIDAS image analysis system (Imaging Associates, Thame, Bucks).

Measurements of nitric oxide synthase activity

The potency of L-NOARG, L-NAME, L-NIO and BrIN in inhibiting neuronal nitric oxide synthase *in vitro* in cerebellar homogenates was measured by the method of Bredt & Snyder (1989) in which the conversion of [^{3}H]-arginine to [^{3}H]-citrulline is determined.

Chemicals

L-CPA was obtained from ZENECA Specialities, Bioproducts and Fine Chemicals, Billingham, Cleveland, U.K. N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME) were from the Sigma Aldrich Chemical Co, Poole, Dorset. N^G-imino-L-ornithine (L-NIO) and 3-bromo-7-nitroinazole (BrNI) were synthesized in our laboratory according to the methods of Beams *et al.* (1993) and Benchidmi *et al.* (1979) respectively. As the solubility of 3-bromo-7-nitroindazole is poor in aqueous solutions, the sodium salt was prepared according to the method described by Silva *et al.* (1995). Structure and purity of L-NIO and BrNI were confirmed by thin layer chromatography, gas chromatography followed by mass spectroscopy, n.m.r. and elemental analysis. L-[^{3}H]-glutamate (56 Ci mmol^{-1}) was obtained from Amersham, Bucks and [^{3}H]-arginine (36.8 Ci mmol^{-1}) was purchased from NEN. All other chemicals were of the highest purity commercially available.

Results

All four competitive nitric oxide synthase inhibitors potently inhibited the formation of [^{3}H]-citrulline from [^{3}H]-arginine in cerebellar homogenates *in vitro* with IC_{50} values in the high

micromolar range (Table 1). BrNI was the most potent followed by L-NOARG, L-NIO and L-NAME.

Neuropathological examination of the cerebellum confirmed that L-CPA-treated rats showed extensive loss in granule cell number that varied between animals but was typically between 60 and 90% of the total. Granule cells had darkly staining, condensed nuclear material that was typical of karyorhectic cell death and there was a severe oedema throughout the cerebellum. Occasionally darkly staining shrunken Purkinje cells were seen in L-CPA-treated animals, but these damaged and necrotic Purkinje cells represented less than 5% of the total cell number. There was no evidence of cell damage in other forebrain regions. When L-NAME and BrNI were given with L-CPA, the damage to the cerebellar cortex was noticeably reduced. Only small populations of granule cells were damaged with the total number of necrotic cells being approximately 10% of the total number or less in some animals. In contrast, rats which received L-CPA and the nitric oxide synthase inhibitors, L-NOARG and L-NIO administered at both high and low doses did not differ in appearance from rats which had received L-CPA alone. The extent and degree of granule cell damage was approximately equal in the cerebellums obtained from rats treated with L-CPA alone or with L -CPA treated with L-NIO and L-NOARG. Animals which received L-NOARG, L-NAME, L-NIO or BrIN alone did not exhibit any changes in cerebellar or forebrain morphology.

Rats which had been treated with either L-NAME or BrNI in association with L-CPA did not have cerebellar water or sodium concentrations that were significantly different from controls (Figures 1 and 2) but differed significantly from rats receiving L-CPA alone. Rats which had received L-NIO and L-NOARG in association with L-CPA had significantly greater cerebellar water and sodium concentrations as compared to controls. The cerebellar water and sodium concentrations from rats which had received L-NIO or L-NOARG with L-CPA did not significantly differ from rats receiving L-CPA alone (Figures 1 and 2). None of the nitric oxide synthase inhibitors, when administered alone, produced any significant alteration in the cerebellar water or sodium concentration (Figures 1 and 2).

Following L-CPA treatment, cerebellar concentrations of aspartate (44% of controls) and glutamate (72% of controls) significantly decreased whilst concentrations of glutamine (176% of controls) and GABA (259% of controls) significantly increased (Figure 3). Administration of L-NAME or BrNI was able to prevent L-CPA-induced reductions in aspartate and glutamate concentrations and L-NAME prevented L-CPA-induced increases in glutamine and GABA concentrations. BrNI was only able to prevent the L-CPA-induced increases in cerebellar GABA concentrations and did not prevent the L-CPA-induced increases in cerebellar glutamine concentrations (Figure 3). In contrast, neither L-NIO nor L-NOARG was able to alter significantly the L-CPA-induced changes in cerebellar amino acid concentrations (Figure 3) except for the lower concentration of L-NIO (25 mg kg^{-1}) which prevented L-CPA-induced increases in GABA concentrations (Figure 3).

Table 1 Potency of four inhibitors of nitric oxide synthase examined in cerebellar homogenates

	IC_{50} (μM)
BrNI	0.73±0.16
NOARG	0.74±0.04
NIO	1.87±0.26
NAME	2.92±0.52

Data shown as mean±s.e.mean for between 4 and 6 determinations. For key to abbreviations used see text.

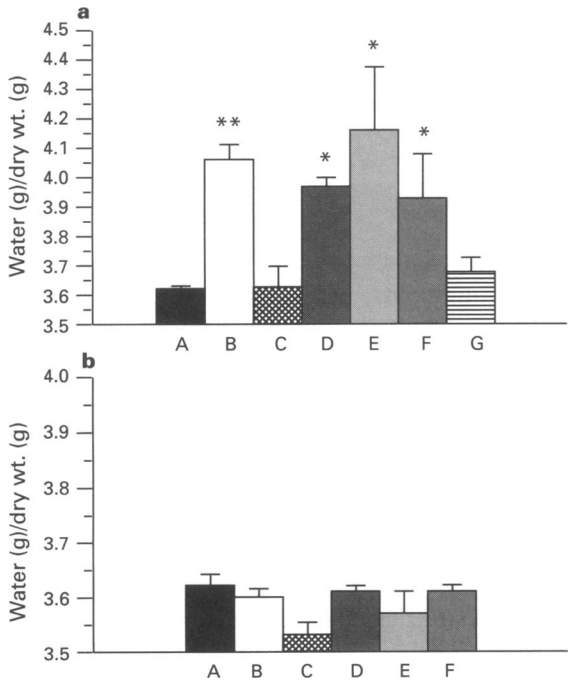

Figure 1 (a) Cerebellar water content from controls (A), L-CPA-treated (B), L-CPA and L-NAME-treated (C), L-CPA and L-NOARG-treated (D), L-CPA and L-NIO (25 mg kg^{-1}) (E), L-CPA and L-NIO (100 mg kg^{-1})-treated (F), L-CPA and BrNI-treated (G). (b) Cerebellar water concentrations in controls (A), and nitric oxide inhibitors when administered alone (L-NAME, B; L-NOARG, C; L-NIO 25 mg kg^{-1}, D; L-NIO, 100 mg kg^{-1}, E; BrNI, F). *$P<0.05$; **$P<0.01$ as compared to saline treated controls.

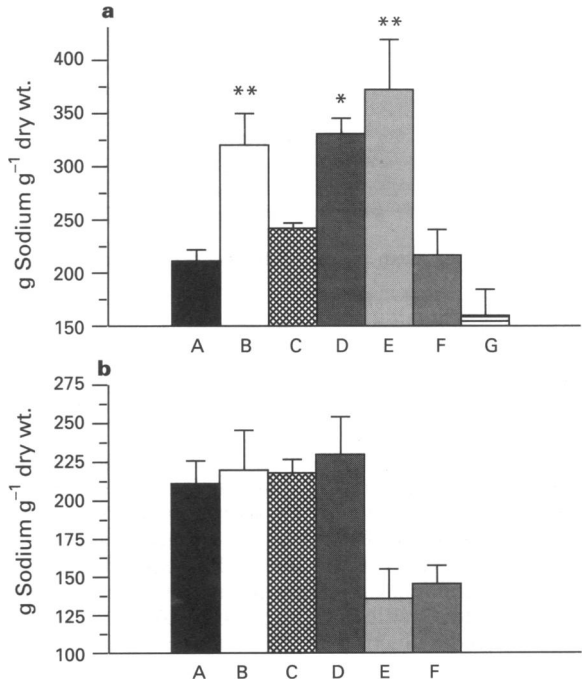

Figure 2 (a) Cerebellar sodium concentrations from controls (A), L-CPA-treated (B), L-CPA and L-NAME-treated (C), L-CPA and L-NOARG-treated (D), L-CPA and L-NIO (25 mg kg^{-1}) (E), L-CPA and L-NIO (100 mg kg^{-1})-treated (F), L-CPA and BrNI-treated (G). (b) Cerebellar sodium concentrations in controls (A), and nitric oxide inhibitors when administered alone (L-NAME, B; L-NOARG, C; L-NIO 25 mg kg^{-1}, D; L-NIO, 100 mg kg^{-1}, E; BrNI, F). *$P<0.05$; **$P<0.01$ as compared to saline-treated controls.

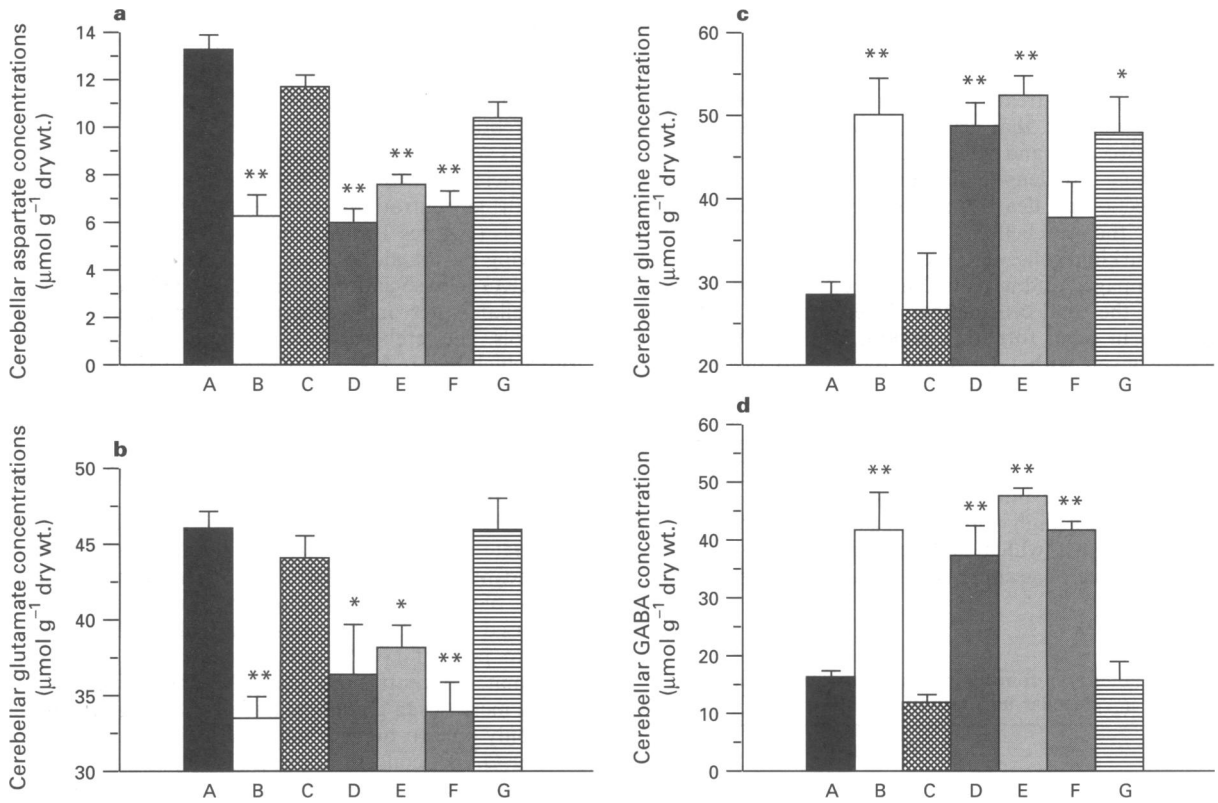

Figure 3 (a) Cerebellar aspartate concentrations, (b) cerebellar glutamate concentrations, (c) cerebellar glutamine concentrations, (d) cerebellar GABA concentrations from controls (A), L-CPA-treated (B), L-CPA and L-NAME-treated (C), L-CPA and L-NOARG-treated (D), L-CPA and L-NIO (25 mg kg^{-1}) (E), L-CPA and L-NIO (100 mg kg^{-1})-treated (F), L-CPA and BrNI-treated (G). *$P<0.05$; **$P<0.01$ as compared to saline-treated controls.

Table 2 Concentrations of amino acids in rat cerebellum following administration of nitric oxide synthase inhibitors alone

	Cerebellar amino acid concentrations (μmol g^{-1} dry weight)			
Inhibitor	*Aspartate*	*Glutamate*	*Glutamine*	*GABA*
Controls	13.3±0.2	46.2±0.5	28.6±0.7	12.1±0.7
Name (50 mg kg^{-1})	12.9±0.2	48.2±1.1	25.1±0.5	10.8±0.9
NOARG (50 mg kg^{-1})	12.5±0.5	45.7±0.8	25.9±0.3	10.3±0.6
NIO (25 mg kg^{-1})	13.2±1.1	44.6±4.0	24.5±2.3	11.6±1.1
NIO (100 mg kg^{-1})	13.3±0.5	46.7±1.2	31.0±0.1	14.7±0.9
BrNI (50 mg kg^{-1})	13.3±0.5	46.5±1.2	30.4±0.9	13.5±0.3

Data shown as mean±s.e.mean for between 3 and 10 animals. For key to abbreviations used see text.

None of the nitric oxide synthase inhibitors significantly altered cerebellar aspartate, glutamine or GABA concentrations when administered alone (Table 2).

Measurements of [^{3}H]-glutamate binding to glutamate receptors in the molecular and granular layer of the cerebellar cortex, demonstrated that L-CPA significantly reduced binding in the granular layer. The nitric oxide synthase inhibitors, L-NAME and BrNI prevented the L-CPA-induced reduction in specific [^{3}H]-glutamate binding in the granular layer of the cerebellar cortex. Neither L-NIO nor L-NOARG prevented the L-CPA-induced reduction in [^{3}H]-glutamate binding in the cerebellar cortex (Figure 4). L-NAME, L-NOARG, L-NIO or BrNI were unable to alter significantly [^{3}H]-glutamate binding in the molecular or granular layers of the cerebellar cortex.

Measurements of nitric oxide synthase activity in the remaining pieces of cerebellar tissue from controls and from tissue obtained from rats which had been treated with L-NOARG, L-NAME, L-NIO (25 and 100 mg kg^{-1}) and BrNI demonstrated that nitric oxide synthase activity was significantly lower in L-NOARG, L-NAME and BrNI-treated rats. L-NOARG and L-NAME treatment inhibited the enzyme activity by approximately 90% whilst BrNI inhibited the nitric oxide synthase by approximately 50% (Figure 5).

Discussion

As reported previously (Widdowson *et al.*, 1995a, b; Simpson *et al.*, 1995; Jones *et al.*, 1995), L-CPA administration culminates in the destruction of granule cells in the cerebellar cortex which is accompanied by cerebellar oedema, as measured by increases in cerebellar water and sodium concentrations. The changes in cerebellar amino acid concentrations are probably secondary to the destruction of granule cells although L-CPA can gain access to forebrain (Wyatt, unpublished observation), there is no change in amino acid concentrations in this brain region which does not show any signs of cellular damage (Widdowson *et al.*, 1995a; Simpson *et al.*, 1995). Granule cells utilise both glutamate and aspartate as excitatory transmitters (Garthwaite & Brodbelt, 1989) and so the massive loss in

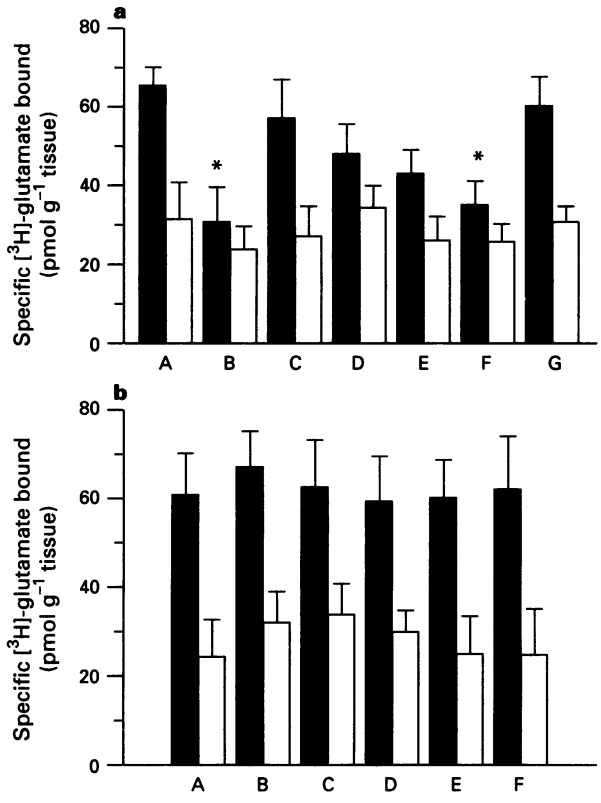

Figure 4 (a) Density of specific L-[^{3}H]-glutamate binding to the glutamate receptors in the granule cell layer (solid columns) and molecular layer (open columns) of the cerebellar cortex of control (A), L-CPA-treated (B), L-CPA and L-NAME-treated (C), L-CPA and L-NOARG-treated (D), L-CPA and L-NIO (25 mg kg^{-1}) (E), L-CPA and L-NIO (100 mg kg^{-1})-treated (F), L-CPA and BrNI-treated (G) rats. (b) Density of specific L-[^{3}H]-glutamate binding to the glutamate receptors in the granule cell layer (solid columns) and molecular layer (open columns) of the cerebellar cortex of control (A), and rats treated with nitric oxide inhibitors alone (L-NAME, B; L-NOARG, C; L-NIO 25 mg kg^{-1}, D; L-NIO, 100 mg kg^{-1}, E; BrNI, F). *$P<0.05$; **$P<0.01$ as compared to saline-treated controls.

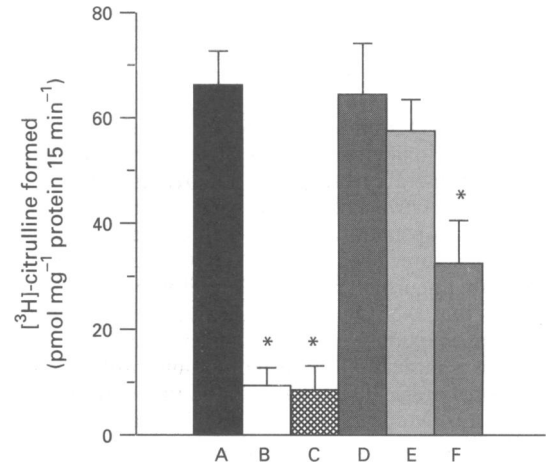

Figure 5 Nitric oxide synthase activity in cerebellar homogenates prepared from control rats (A) and rats treated with L-NAME (B), L-NOARG (C), L-NIO (25 and 100 mg kg^{-1} twice daily, D and E, respectively) and BrNI (F). Results are expressed as mean ± s.e.mean for experiments performed in duplicate on tissue obtained from between 3 and 6 rats. *$P<0.01$ as compared to controls.

granule cells, which constitute approximately 80% of all the cells in the cerebellum, will ultimately result in the loss of some excitatory influence in the cerebellum.

The ability of both L-NAME and BrNI to provide neuroprotection against L-CPA-induced granule cell loss and prevent the associated changes to cerebellar amino acid concentrations and reductions in glutamate receptor density suggests that L-CPA-mediated neurotoxicity is mediated through the formation of excessive nitric oxide concentrations. We and others (Bredt & Snyder, 1989; Dwyer *et al.*, 1991; Moore *et al.*, 1993) have demonstrated that L-NOARG, L-NAME and BrNI are potent inhibitors of the cerebellar nitric oxide synthase enzyme *in vitro*. Furthermore we have demonstrated that L-NAME, L-NOARG and BrnI were able to provide significant blockade of nitric oxide synthase in the cerebellum thus demonstrating that the concentrations administered were active *in vivo*. There are numerous reports that excessive nitric oxide can result in neuronal cell death when added to neuronal cell cultures or brain slices (Garthwaite, 1989; Dawson *et al.*, 1991; Moncada *et al.*, 1991) and excessive nitric oxide production has been suggested to underlie the neuronal cell death in association with ischaemia (Moncada *et al.*, 1991). The report that substituted indazole nitric oxide inhibitors, for example 7-nitroindazole, do not change blood pressure when administered systemically, in contrast to L-NAME, suggests that the neuroprotection produced by both L-NAME and BrNI were not due to changes in the cardiovascular system (Babbedge *et al.*, 1993; Moore *et al.*, 1993).

The lack of change in amino acid concentration or glutamate receptor density when L-NAME or BrNI was administered alone suggests that these compounds are acting to prevent excessive nitric oxide production. Our results obtained with BrNI contrast with the lack of efficacy reported by MacKenzie *et al.* (1995) in their attempts to prevent quinolinic acid-mediated striatal damage with 7-nitroindazole, which is also mediated through NMDA activation (Babbedge *et al.*, 1993). The addition of the bromine atom to 7-nitroindazole increases the potency of nitric oxide synthase blockade (Moore *et al.*, 1993) and may also increase the penetration into the brain. The nitric oxide synthase activity in the cerebellums of rats that had been treated with BrNI was significantly lower than controls showing that BrNI was able to penetrate the brain in sufficient concentrations to provide some inhibition of the enzyme. Another factor which may explain the difference in efficacy between the study using 7-nitroindazole (MacKenzie *et al.*, 1995) and our results obtained with BrNI was that we prepared the sodium salt from the indazole molecule to facilitate brain penetration. The lower inhibition of cerebellar NOS with BrNI as compared to L-NAME may account for the inability of BrNI to prevent L-CPA-induced increases in cerebellar glutamine concentrations. We do not understand why a lower dose of NIO (25 mg kg^{-1}) was able to attenuate the L-CPA-induced loss in [^{3}H]-glutamate binding more than the higher dose. However, there was considerable variation in [^{3}H]-glutamate binding to cerebeller cortex between individual rats and the difference between the doses was not significantly different from each other, or from the L-CPA effect when administered alone.

One interesting feature of L-CPA-mediated neurotoxicity is the delayed granule cell death which occurs between 30 and 36 h following L-CPA administration. Although we do not yet understand the neurochemical and physiological events that are taking place during this 'silent period' and we have been unable to observe any changes in cerebellar biochemistry (Lock *et al.*, 1996) or granule cell morphology (Jones *et al.*, 1995), except for marked depletions in glutathione concentrations (Wyatt *et al.*, 1996), we suggest that it is during this time when excessive nitric oxide is being generated that granule cell biochemistry is being severely compromised. We have previously shown that co-administration of NMDA receptor inhibitors can provide neuroprotection against L-CPA-induced cerebellar neurotoxicity (Widdowson *et al.*, 1995b) in a manner described here for nitric oxide synthase inhibitors. Since NMDA receptor activation results in the activation of nitric

oxide synthase as a result of increased intracellular calcium concentrations (Snyder, 1992), it is probable that activation of NMDA receptors by L-CPA precedes the generation of nitric oxide.

Although L-NIO and L-NOARG are potent inhibitors of cerebellar nitric oxide synthase *in vitro*, these two compounds failed to demonstrate neuroprotection against L-CPA-induced neurotoxicity *in vivo*. Possible reasons for this discrepancy is their poor brain penetration as a result of their highly charged and polar structure. The failure of L-NIO to gain entry into the brain and alter neuronal nitric oxide synthase, *in vivo* has led to suggestions that L-NIO may act selectively on endothelial and inducible nitric oxide synthase (Thiemermann, 1994). This interpretation is misleading as the compound probably does not show pharmacological selectivity at the three nitric oxide synthase isoforms (Moncada *et al.*, 1991), but rather shows functional selectivity based on its pharmacokinetics. We were unable to show significant reductions in brains treated with L-NIO at either 25 or 100 mg kg^{-1}, as compared to controls suggesting that the failure of this compound to cross the blood/brain barrier accounts for its lack of efficacy against L-CPA neurotoxicity. However, the concentration of L-NOARG was demonstrated to block nitric oxide synthase *in vivo* by approximately 90% implying that L-NOARG should be neuroprotective. We do not yet understand why L-NOARG failed to provide neuroprotection against L-CAP-induced neurotoxicity, but the reversibility of L-NOARG at 37°C (Klatt *et al.*, 1994; Salter *et al.*, 1995) may have contributed to the lack of neuroprotection with this nitric oxide synthase inhibitor whereas L-NAME may have acted as a reservoir supplying a steady supply of L-NOARG to constantly block the enzyme.

In conclusion, the ability of two nitric oxide synthase inhibitors, to provide neuroprotection against L-CPA-induced neurotoxicity suggests a role for nitric oxide in the destruction of granule cells. We therefore suggest that following activation of cerebellar NMDA receptors, excessive amounts of nitric oxide are produced which may undergo conversion to cytotoxic free radicals.

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