

# Comparative protective effects of nicardipine, flunarizine, lidoflazine and nimodipine against ischaemic injury in the hippocampus of the Mongolian gerbil

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1 Morphological changes characterizing delayed neuronal death (DND) of selectively vulnerable CA<sub>1</sub> pyramidal cells in the hippocampus of the Mongolian gerbil brain occurred 72 h after transient (5 min) bilateral occlusion of the common carotid arteries.

2 Different groups of animals were treated 15 min before carotid artery occlusion and twice daily during the 72 h post-ischaemia period with either saline alone, nicardipine, flunarizine, lidoflazine or nimodipine at doses of 500 µg kg<sup>-1</sup> intraperitoneally.

3 At 72 h the animals were killed and their brains examined histologically. Absolute cell counts were made from 5 sites distributed linearly throughout the hippocampal CA<sub>1</sub> subfield in each hemisphere to determine the percentage DND in each group. Normal brains and those of sham-operated animals were included in the study for comparison.

4 Features of DND were distributed evenly throughout the CA<sub>1</sub> subfield in both hemispheres in all groups of gerbils. Nicardipine, lidoflazine and flunarizine, but not nimodipine, were protective. This protection extended linearly throughout the hippocampus without altering the pattern of neuronal damage.

5 Compared to saline-treated (78.3 ± 2.9% DND) and nimodipine-treated (76.5 ± 3.4% DND) gerbils, the overall protection afforded by nicardipine (41.8 ± 3.8% DND) was statistically significant. The effects of lidoflazine (53.6 ± 7.1%) and flunarizine (55.8 ± 3.9% DND) were of borderline significance.

6 Abnormal neurones appeared in normal and sham-operated brains to the extent of 4.5 ± 1.0% and 4.6 ± 0.4%, respectively. Such changes can be attributed to fixation artefacts.

7 The results demonstrate that overall protection is conferred on ischaemic hippocampal CA<sub>1</sub> neurones by nicardipine and to a lesser extent by flunarizine and lidoflazine, but not by nimodipine.

## Introduction

The use of the Mongolian gerbil (*Meriones unguiculatus*) as a model in studies of cerebral ischaemia and infarction is well documented in the literature (Levine & Payan, 1966; Kahn, 1972). Kirino (1982) described a gerbil model of forebrain ischaemia, induced by transient occlusion of both common carotid arteries, which results in a reproducible discrete lesion featuring extensive neuronal degeneration in the CA<sub>1</sub> subfield of the hippocampus

in 90% of experiments. Although the ischaemic damage initially progresses slowly, by 2 to 4 days it is predictive of an irreversible change which has been termed 'delayed neuronal death' (DND) (Kirino, 1982; Kirino & Sano, 1984). A similar temporal profile of neuronal ischaemic damage in rats subjected to transient forebrain ischaemia has been described by Pulsinelli *et al.* (1982) and Kirino *et al.* (1984).

Hass (1981) hypothesized that neuronal damage resulting from cerebral ischaemia was a direct result

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of an imbalance in the normal calcium extracellular-intracellular homeostasis. Ischaemia-induced dysfunction of high threshold calcium conductance channels found in the CA<sub>1</sub> neurones (Siesjo, 1981) may, therefore, account in part for the high calcium concentrations found within the mitochondria of these neurones immediately before and following restitution of the cerebral circulation (Simon *et al.*, 1984).

Nicardipine is a Class I calcium entry blocking agent (Spedding, 1984), which appears effectively to cross the blood brain barrier of normal and cerebrally ischaemic rats on first pass extraction following intracarotid injection, an appreciable amount of unmetabolized compound being located in the hippocampus (Grotta *et al.*, 1986). The drug has been shown to afford good cytoprotection in acute and chronic models of myocardial infarction (Alps *et al.*, 1983a,b).

This paper enlarges upon preliminary data presented for nicardipine (Alps *et al.*, 1986) and compares its neurocytoprotective effects with those of the calcium channel blocking agents, flunarizine, lidoflazine and nimodipine, in the forebrain ischaemia model of Kirino (1982).

## Methods

Mongolian gerbils of either sex weighing 50–80 g were anaesthetized with 5% halothane (Halothane, BP, May & Baker, Dagenham, U.K.) in a 30% oxygen:70% nitrous oxide mixture delivered directly via a face mask. Following a rapid induction the halothane concentration was decreased to 1.5–2.0% and maintained at this level throughout the remainder of the surgical procedure. The left and right common carotid arteries were exposed in the paratracheal region through a ventral midline cervical skin incision and freed from the accompanying vagosympathetic nerve trunks. A loose ligature was placed around the arteries to facilitate identification at the time of occlusion.

Sterile drug solutions (500 µg kg<sup>-1</sup>, i.p.) or sterile saline (0.9% w/v NaCl) were injected 15 min before artery occlusion. All groups received repeat doses of their respective test drug (500 µg kg<sup>-1</sup>, i.p.) twice daily during a 72 h post-ischaemia survival period.

Following 5 min bilateral carotid artery occlusion by use of microvascular clips (Ackland C2V, Weiss, London, U.K.) blood flow was restored, the wound dusted with antibiotic powder (Cicatrín, Calmic Medical Division, The Wellcome Foundation Limited, London, U.K.) and repaired. The animals were caged singly and allowed free access to food and water.

Seventy-two hours after carotid artery occlusion

the gerbils were anaesthetized with pentobarbitone sodium (4 mg i.p.) and their brains perfuse-fixed by intracardiac injection of 10% buffered formol saline solution. The heads were stored overnight at 4°C in fresh formalin. The brains were then removed from the calvarium and fixed for 1 week, before being embedded in paraffin wax, sectioned at 8 µm and stained with cresyl fast violet and haematoxylin-eosin. The co-ordinates for selecting the coronal section including the hippocampal structure have been given by Kirino (1982).

## Quantification and statistical evaluation of data

Only one section per brain was taken for histological analysis, since we have confirmed that the hippocampal CA<sub>1</sub> damage observed was representative of similar changes occurring throughout the dorsal hippocampus as described by Kirino & Sano (1984). The interpretation of neuronal abnormality was strict as this was a comparative study. Blind assessment of all material was carried out independently by two of the authors with a high degree of concordance. Any cells giving the appearance of abnormality, however slight, were counted as damaged. The following criteria were observed in judging overt or pending neuronal death: retraction of the cell body with eosinophilia of the cytoplasm, disappearance of Nissl bodies, pyknosis and hyperchromasia of the nucleus. The differentiation of potential artefactual changes affecting the morphological appearance of neurones was allowed for by including sections of normal gerbil brain among the various groups. Neurones were classified as normal if they exhibited perfect nuclear, nucleolar and cytoplasmic preservation with the presence of Nissl bodies. Seven sham-operated gerbils were subjected to the anaesthetic procedure and surgical exposure of the carotid arteries but without the ischaemic insult.

The extent of neuronal damage was determined in three ways: (a) counts were pooled for all animals in a group, (b) individual animal mean counts were pooled for a group value, (c) the differences between the five positions in each hemisphere were examined. A repeated measures analysis of variance (Fleiss, 1986) was performed with grouping factor treatment and the repeated measures factors hemisphere and position. The differences between the five positions were broken down into orthogonal polynomials. Initially, the hypothesis that the treatment did not affect the differences between the ten subfields was tested by F-tests. These were not statistically significant and therefore the active treatments were compared to control using animal means by *t* tests (Snedecor & Cochran, 1976) with standard errors derived from the analysis of variance. These were one-tailed since *a priori* it was believed that the

treatments would, if anything, reduce percentage CA<sub>1</sub> neuronal death. A Bonferroni adjustment was made by multiplying the *P* values by the number of comparisons (i.e. four). All computations were carried out with the programme BMDP 2V (Dixon *et al.*, 1985). With these statistical adjustments the criterion of significance was taken where *P* < 0.05.

### Drugs

The following drugs were used: nicardipine HCl (Cardene, Rydene, Rycardene, Syntex); nimodipine (synthesized by Dr R. Clark, Institute of Organic Chemistry, Syntex); flunarizine (HCl)<sub>2</sub>, lidoflazine (Janssen Pharmaceutica). Doses of nicardipine and flunarizine are expressed in terms of base substance (conversion factors 1.07 and 1.18, respectively). All drugs were prepared as 100 µg ml<sup>-1</sup> solutions. Initially drugs were dissolved in a minimal quantity (0.2–0.8%) of solvent (citric acid for lidoflazine, ethanol for nimodipine, flunarizine and nicardipine) and then made up to a volume of 50 ml with sterile saline (0.9% w/v NaCl) solution.

### Results

All animals recovered quickly from anaesthesia and the ischaemic insult. They did not exhibit signs of locomotor disturbance, obvious suppression of spontaneous motor activity, seizures, rolling or circling behaviour.

More than 78% of CA<sub>1</sub> neurones in the sections taken from the saline control group showed structural abnormality. The pattern of cellular involvement in all groups was essentially bilaterally symmetrical. The greatest changes were observed in the paramedian region, both for saline treated control animals and for the distribution of the small number of abnormally appearing neurones in normal (4.5 ± 1.0%) and sham-operated (4.6 ± 0.4%) brains.

Significant protective effects were found for nicardipine with all field counts pooled (*P* < 0.002), as shown in Table 1, and there was a reduction in the degree of damage to a similar extent linearly throughout the whole CA<sub>1</sub> subfield in each hemisphere (Figure 1). There was still significant overall protection (*P* < 0.02) when single animal brain values were used. Flunarizine and lidoflazine had significant protective effects (*P* < 0.002 and *P* < 0.02, respectively) when all fields were included in the count but not when single brain values were used, emphasising that the protective effects of these agents were less than that of nicardipine. Flunarizine and lidoflazine also appeared to reduce neuronal damage linearly, if not significantly at the dose

**Table 1** The effect of drug treatment on the percentage delayed neuronal death in the hippocampal CA<sub>1</sub> subfield of the gerbil brain

Treatment	Percentage abnormal neurone count	Number of animals
Saline	78.3 ± 2.9	12
Nicardipine	41.8 ± 3.8**	12
Nimodipine	76.5 ± 3.4 <sup>NS</sup>	12
Flunarizine	55.8 ± 3.9**	13
Lidoflazine	53.6 ± 7.1*	5

Compared to saline-treated group: \**P* < 0.02, \*\**P* < 0.002, NS not significant. One-tailed *t* test with Bonferroni adjustment for multiple comparisons.

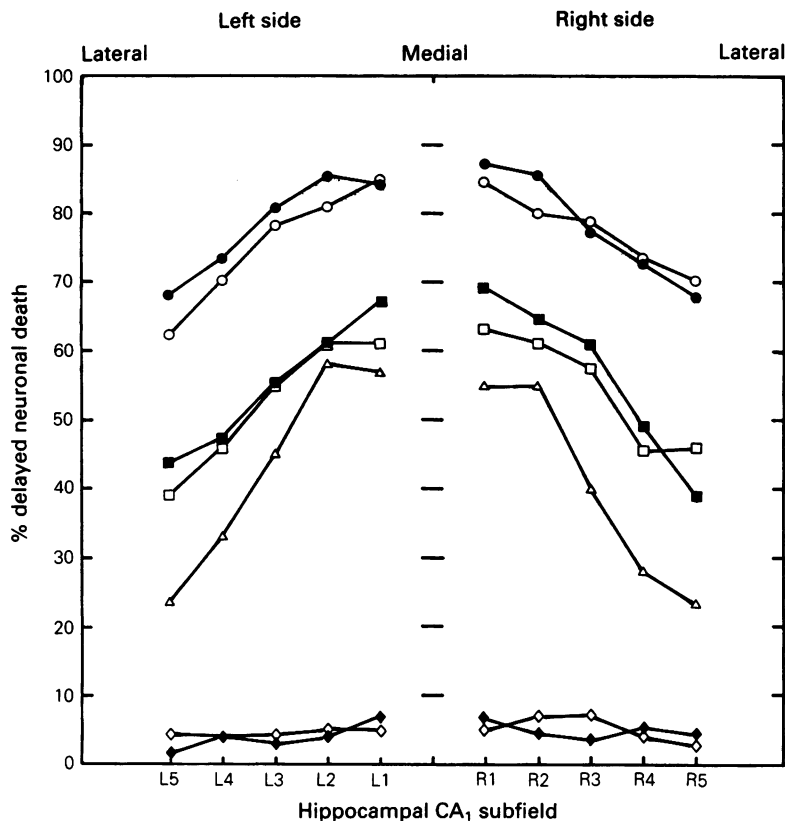
tested, throughout the CA<sub>1</sub> subfield. Nimodipine was not protective.

The representative light microscopical appearance of normal pyramidal cells in the paramedian region is shown in Figure 2a, which contrasts markedly with the gross degenerative changes observed 72 h following the ischaemic insult in saline-treated animals (Figure 2b). Again, in contrast to the untreated situation, the partial protection afforded by nicardipine in this region is shown in Figure 2c.

### Discussion

The present results demonstrate the ability of nicardipine and, to a lesser extent, flunarizine and lidoflazine, to reduce the incidence of delayed neuronal death in the CA<sub>1</sub> subfield of the gerbil hippocampus following transient bilateral carotid artery occlusion. Nimodipine was inactive in this model. The small number of abnormally appearing neurones observed in sham-operated gerbils excludes the possibility of the anaesthetic procedure exacerbating CA<sub>1</sub> damage above the artefact level. It is also interesting to note that in gerbil brains showing severe CA<sub>1</sub> damage there appeared to be other areas (in the hippocampus, thalamus and neocortex) which were suggestive of intracellular oedema. Where there was therapeutic benefit with a reduction in DND these changes were much less obvious.

Combined pre- and post-ischaemia treatment with nicardipine at i.p. doses identical to those used in the present study has previously been shown to protect the CA<sub>1</sub> neurones in a rat 72 h survival model of forebrain ischaemia (Alps & Hass, 1985; 1987). The question of treatment with nicardipine during the post-ischaemic period has not yet been addressed in the gerbil model, but it is known that nicardipine can protect the CA<sub>1</sub> neurones in the rat when given solely during this time (Alps *et al.*, 1987). The protective effect of flunarizine against cerebral cortical

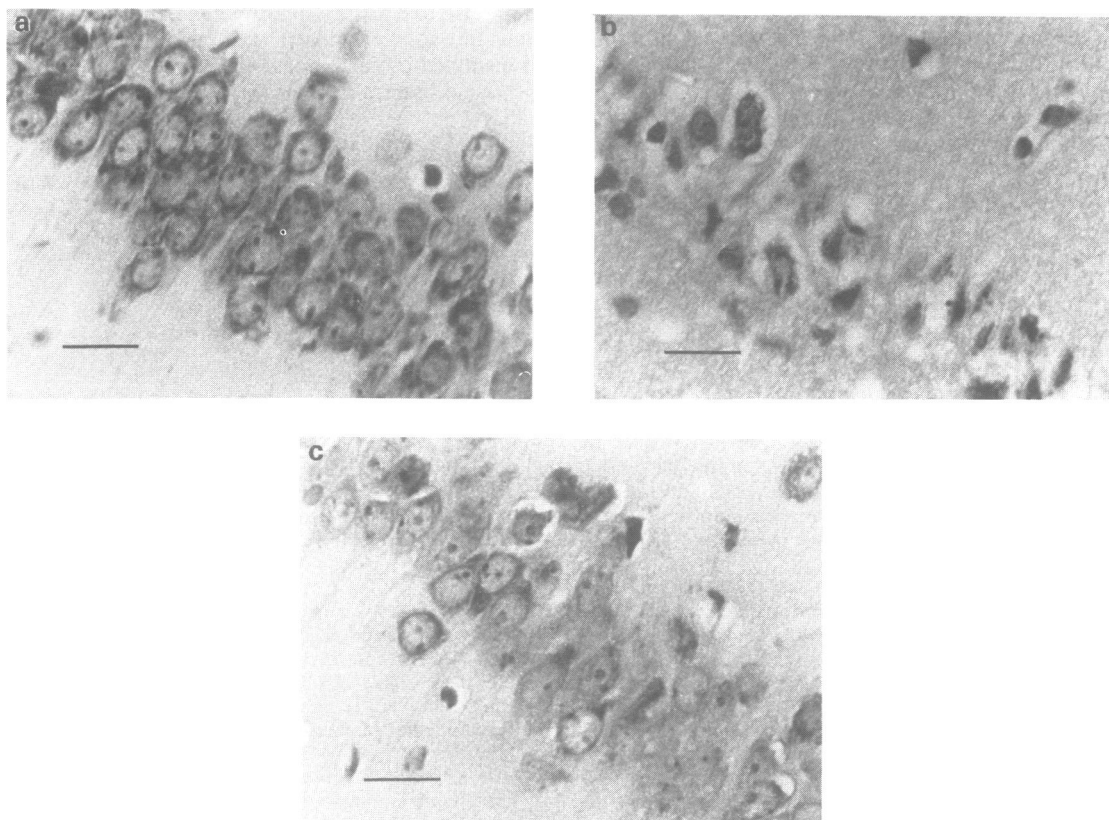


**Figure 1** The 72 h protective effect of nicardipine ( $\Delta$ ,  $n = 12$ ), flunarizine ( $\blacksquare$ ,  $n = 13$ ), lidoflazine ( $\square$ ,  $n = 5$ ), nimodipine ( $\circ$ ,  $n = 12$ ) and saline ( $\bullet$ ,  $n = 12$ ) against 5 min ischaemic damage in 5 areas throughout the hippocampal CA<sub>1</sub> subfield in each hemisphere of the gerbil brain. The incidence of staining artefacts in brains from normal ( $\diamond$ ,  $n = 10$ ) and sham-operated ( $\blacklozenge$ ,  $n = 7$ ) animals is shown for comparison. Drugs were injected at  $500 \mu\text{g kg}^{-1}$  i.p. 15 min before occlusion of both carotids and repeated twice daily. Mean % values are plotted but  $\pm$  s.e. bars are omitted for clarity.

injury induced by a combined hypoxic-ischaemic insult in rats has been described by Van Reempts *et al.* (1983); they attributed the beneficial effect to be most probably due to calcium entry block. Further supporting evidence for flunarizine's pre-ischaemia protective action in rats has been found by Deshpande & Wieloch (1985). These workers additionally demonstrated that the drug reduced neuronal necrosis when administered early in the post-ischaemic period.

Observations have also been reported (Vaagenes *et al.*, 1984) on lidoflazine's action in ameliorating late post-ischaemic neurological deficit in dogs subjected to 10 min of cerebral ischaemia induced by ventricular fibrillation. Vaagenes *et al.* (1984) proposed that lidoflazine exerted its beneficial effect by a direct action on neurones, possibly by reducing reperfusion calcium loading of mitochondria. It is

evident in experimental models of forebrain ischaemia that a marked decrease of extracellular calcium activity occurs during ischaemia and that selectively vulnerable neurones show accumulation of intracellular calcium early during the reperfusion phase. This calcium accumulates principally within mitochondria with a diffuse non-selective pattern in the hippocampus (Meldrum *et al.*, 1985). However, from the lack of effect seen with nimodipine, it must be suspected that the potent binding *per se* of dihydropyridine structures to voltage-dependent calcium channels in brain membranes (Heffez *et al.*, 1985) may not necessarily impart a direct neurocytoprotective action to this class of drug. Vibulresth *et al.* (1986) have shown that nimodipine was unable to protect rats subjected to 20 min of four-vessel occlusion, but we would consider this a rather excessive insult in this type of model. The suggestion



**Figure 2** Representative photomicrographs from the worst affected paramedian area of the hippocampal CA<sub>1</sub> subfield: (a) In a gerbil brain showing pyramidal cells from a non-ischaemic animal. A few cells (7.5%) showed abnormal features characteristic of 'dark neurones'. Such artefactual changes can be attributed to the fixation process. (b) Shows complete loss of structural integrity and complete (100%) neuronal death in an untreated animal subjected to 5 min bilateral carotid artery occlusion with 72 h survival. 'Ghost' cell spaces, darkly stained shrunken dead cells and cellular debris are characteristic features of delayed neuronal death. (c) Shows partial protection of pyramidal cells in a nicardipine-treated animal subjected to the procedure outlined in (b). Cells of normal appearance which have survived the ischaemic insult stain lightly. In the slide of the brain represented here 25% cells showed morphological damage when viewed by light microscopy. Sections stained with Cresyl fast violet. Bar represents 20 μm.

has been made that nimodipine improves post-ischaemic hypoperfusion (Hideo *et al.*, 1985), but brief carotid occlusion in gerbils does not apparently evoke the 'no reflow phenomenon' (Levy *et al.*, 1975; Kirino, 1982). However, the potent cerebral vasodilator properties of nimodipine are probably responsible for its beneficial effect in rat models of acute focal ischaemia (Hakim, 1986; Germano *et al.*, 1986), where tissue at risk in the penumbra of the infarct can be salvaged by improved local blood flow.

Meldrum *et al.* (1985) advocated two general approaches to the treatment of cerebral ischaemia which might indirectly help to restore normal neuro-

nal activity, either by enhancing  $\gamma$ -aminobutyric acid (GABA)-ergic inhibition of hippocampal neuronal firing, or by blockade of N-methyl-D-aspartate (NMDA) receptors to inhibit the postsynaptic action of excitatory amino acid neurotransmitters. Both procedures will prevent the paroxysmal depolarization shift associated with burst firing and the subsequent voltage-dependent entry of calcium. There is reason to believe that, apart from any mechanistic consideration arising from its use as a calcium entry blocker, nicardipine may additionally promote the GABA-ergic effect at proven anti-ischaemic doses. In this respect nicardipine, but not nimodipine, has been shown significantly to inhibit tonic seizures

induced by pentylentetrazole in mice and reduce the associated mortality (Allely, unpublished observations). The potential beneficial effects of antagonizing NMDA receptors to give protection against ischaemia-induced hippocampal degeneration in the gerbil has been well demonstrated by the use of MK-801 (Foster *et al.*, 1987).

In conclusion, within the experimental conditions described for the gerbil model of transient forebrain ischaemia, it would appear that overall protection is conferred on hippocampal CA<sub>1</sub> neurones by nicardi-

pine, and to a lesser extent by flunarizine and lidoflazine, but not by nimodipine. Whether this protection is mediated by calcium channel blockade or by some other mechanism remains to be determined.

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