SUMMARY We examined the impact of pre-treatment with the calcium antagonist flunarizine on the development of hypoxic-ischemic brain injury in the immature rat. Unilateral carotid artery ligation and subsequent exposure to 2 hours of 8% oxygen in 7-day-old rats was used as a model for perinatal hypoxic-ischemic encephalopathy. This procedure leads to atrophy in the cerebral hemisphere ipsilateral to carotid occlusion, with prominent foci of neuronal infarction in the caudate-putamen (striatum). The morphologic injury develops after 1½ hours of hypoxia; and there is an equivalent time threshold for duration of hypoxic exposure needed to acutely stimulate dopamine release in the ipsilateral striatum. Parenteral administration of 30 mg/kg of flunarizine before hypoxic exposure limited both the release of dopamine acutely and the extent of morphologic damage observed two weeks after the insult. Oral administration of 30 mg/kg of flunarizine in a different vehicle prevented morphologic damage but had no effect on stimulated dopamine release. The drug vehicle for the parenteral preparation also prevented tissue injury, but to a lesser degree than flunarizine. However the parenteral vehicle was equipotent with parenteral flunarizine in limiting acute stimulation of dopamine release. The results demonstrate that flunarizine has potent neuroprotective properties against morphologic brain injury from hypoxia-ischemia, acting by a mechanism which is independent of effects on dopamine release.

IN THE CENTRAL NERVOUS SYSTEM (CNS), hypoxia-ischemia initiates a series of closely interconnected biochemical reactions that may lead to irreversible cell damage.1 The intracellular events that lead to neuronal death are not well-defined nor are the mechanisms that account for variation in susceptibility to injury of specific groups of neurons. Recent studies suggest that increased free intracellular calcium accumulation may play a central role in the pathogenesis of neuronal damage, as it does in other cells.2,3 Several investigators have demonstrated apparent calcium overload in dilated mitochondria and/or edematous dendritic processes of selectively vulnerable CNS neurons.4,5 Transient increases in cytosolc calcium activate degradative enzymes and, in specific nerve terminals, may stimulate release of excitotoxic neurotransmitters; both of these mechanisms could contribute to on-going neuronal damage. If pathological calcium entry into injured cells could be limited, the adverse effects of ischemia might be reduced.

Organic calcium antagonists include a large group of drugs that block calcium influx at voltage gated calcium channels. There is conflicting evidence about the functional activity of calcium antagonists in the CNS but recent studies have demonstrated limitation of depolarization evoked calcium influx in vitro,6 inhibition of ischemic calcium uptake in vivo7 and inhibition of neurotransmitter release8 by calcium antagonists in specific experimental settings. Several studies have suggested that some of these agents have neuroprotective properties.9-11

In this study we examined the impact of pre-treatment with the calcium antagonists flunarizine on the development of hypoxic-ischemic neuronal injury in the immature brain. Flunarizine, which is selectively concentrated in brain, also has other pharmacologic properties, e.g. free radical scavenging,12 which could contribute to neuroprotection.

Unilateral carotid ligation and subsequent exposure to 2 hours of 8% oxygen in 7-day-old rats is a well-characterized small animal model for perinatal hypoxic-ischemic encephalopathy.13 The age was chosen because the development of rat brain at this stage resembles the maturational level of the full term human neonate.14 The pathogenesis of neuronal injury appears to be related to progressive ischemia that develops as the duration of hypoxic exposure lengthens.15 The combination of moderate hypoxemia and ischemia causes a reproducible pattern of acute and chronic ischemic neuronal changes in the forebrain ipsilateral to carotid occlusion. Foci of infarction in the corpus striatum, hippocampus and cortex occur commonly, surrounded by areas of disrupted neuronal organization.16 The infarctions resemble the pattern of injury now being recognized more frequently in human infants.17

Progressive hypoxia-ischemia in the animal model is also accompanied by marked, acute changes in neurotransmitter metabolism in specific brain regions.18 In striatum on the side of ligation, after 1½ hours of hypoxia, dopamine metabolism increases abruptly. The duration of hypoxic exposure leading to stimulation of dopamine release coincides with the time threshold necessary for production of obvious morphologic injury in animals raised to maturity before sacrifice. Although the relationship between stimulation of
dopamine metabolism and tissue damage is uncertain, previous studies suggest that catecholamine release might reflect injury severe enough to cause permanent damage. We examined the influence of flunarizine pretreatment on the extent of morphologic damage and the acute stimulation of dopamine release to test the drug’s effects and to learn more about its mechanism of action.

**Methods**

**Model of Unilateral Hypoxic-Ischemic Injury**

Seven-day-old rat pups (Sprague Dawley) underwent right carotid ligation under ether anesthesia followed, after a two hour recovery period with the dam, by timed exposure to humidified 8% oxygen in a closed chamber warmed to 37° C. In each experiment, half the litter received drug in the dosage and route being tested and the other half received an equal volume of drug vehicle or saline (see details below) before exposure to hypoxia. At the end of the hypoxic interval, pups were either returned to the dam for 2 weeks prior to sacrifice or were immediately decapitated. Each experiment was repeated on at least 3 occasions.

**Drug Administration**

Flunarizine was donated by Janssen Pharmaceutica, Beerse Belgium. Initial experiments were performed using a parenteral solution of flunarizine (2 mg/ml). Two doses of the drug were used. Ten mg/kg was administered in a single 50 microlitre injection volume intraperitoneally immediately before exposure to hypoxia. Thirty mg/kg was administered in two divided doses (75 microlitres/injection). To administer 30 mg/kg, based on the results of pilot studies using a variety of dosage schedules, in these experiments, 15 mg/kg was given as soon as the animals recovered from anesthesia, and another 15 mg/kg was injected 1½ hours later, just before exposure to hypoxia. When these experiments were performed, we did not have access to the commercially prepared drug vehicle. Therefore, litter-mate controls were injected with equal volumes of 0.9% NaCl solution.

When a commercial preparation of the diluent became available (Janssen Pharmaceutica), experiments were done in which the effects of pretreatment with 150 μl of diluent or an equal volume of saline were compared. The water-based diluent contained 25 mg/ml of tartaric acid and 0.2 mg/ml of chlorhexidine digluconate.

To evaluate flunarizine without the vehicle, purified powder was dissolved in 0.5% methylcellulose containing 5% 0.1 N HCl. The drug was administered orally in a total volume of 50 microliters (10 mg/kg) or 100 microliters (30 mg/kg) through a 1 cm plastic catheter attached to a 1 ml tuberculin syringe. Litter-mate controls were given equal volumes of the methylcellulose solution.

**Analysis of Morphologic Changes**

Comparisons of bilateral cerebral hemisphere weights two weeks after hypoxia-ischemia (21 days of age) were used to quantitate the extent of unilateral morphologic damage resulting from the lesion. The brain was removed from the skull after sacrifice and the forebrain was separated from the brainstem/cerebellum by a coronal cut just caudal to the occipital cortex. Then the hemispheres were separated by a mid-sagittal cut and weighed separately on an electronic microbalance (Cahn). The injury occurs during a phase of rapid brain growth, and the early injury limits the potential development of hemisphere mass. With maturation, cerebral hemisphere asymmetry evolves. When animals are sacrificed at 21 days of age, and the cerebral hemispheres are separated and weighed, an 18 ± 3% reduction in the weight of the hemisphere ipsilateral to ligation is observed. Any intervention that limits neuronal damage could be predicted to minimize this asymmetry.

In addition, in separate experiments, in which the same protocol was used to administer 30 mg/kg of parenteral flunarizine, brains were removed intact, and immersed in 10% phosphate buffered formalin for 1 week, followed by immersion in 10% formalin plus 30% sucrose for another week. Frozen coronal sections (30 μm) were cut from the forebrain on a frozen stage microtome and stained for Nissl substance with cresyl violet. In selected stained sections, changes in cross-sectional area of the entire hemispheres and caudate-putamen were quantified using a computerized digitizing pad (Summagraphics).

**Neurochemical Analysis of Striatal Dopamine Turnover**

To examine dopamine release acutely, groups of animals were killed at the end of hypoxic exposure, the corpus striatum was dissected out at 4° C and frozen at −70° C. Striatal extracts were prepared in 0.4 M perchloric acid with 10% methanol and dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured simultaneously using HPLC with electrochemical detection. HVA is the major extraneuronal metabolite of dopamine and we have previously found that dopamine depletion and HVA accumulation occur concurrently in hypoxic-ischemic striatum. As a measure of dopamine turnover, the HVA/dopamine ratio for each striatum was calculated.

**Data Analysis**

In both the morphologic and neurochemical studies, results in experimental groups were always compared with litter-mate controls. Group means for values ipsilateral and contralateral to ligation were calculated and values for treated and control pups were compared using one-tailed Student’s t-test.

**Results**

Three sets of experiments were done to assess flunarizine’s ability to limit hypoxia-ischemia induced tissue loss caused by unilateral carotid ligation plus hypoxia (fig. 1). In the first group, parenteral flunarizine pretreatment was compared with saline treatment for its ability to prevent hemisphere asymmetry 2
When the commercially prepared vehicle for flunarizine was obtained (Janssen Pharmaceutica), the experiments were repeated comparing the outcome at 21 days in diluent and saline treated ligates. The volume of diluent was equal to that administered with 30 mg/kg of parenteral flunarizine (150 μl). This pretreatment (fig. 1C) also modified outcome, but to a lesser degree: 21 ± 4.5% reduction in brain mass ipsilateral to ligation in controls vs. 9.8 ± 3.4% reduction in the vehicle-treated group (p < .05, Student’s t-test).

Separate groups of rat pups were prepared as described and used for histologic analysis. Figure 2 demonstrates typical patterns of histologic changes in saline and flunarizine treated animals at 21 days. Figure 2A is a Nissl stained coronal section at the level of striatum in a control ligate and illustrates the typical distribution of histologic injury at this level 2 weeks after the hypoxic-ischemic insult. Unilateral loss of volume in striatum and cortex and clumps of abnormally staining neurons in areas of infarction, especially in dorsomedial striatum, were evident. Although not shown in the figure, areas of infarction and focal neuronal loss were also seen frequently in the hippocampus and lateral neocortex. In contrast, Figure 2B, a corresponding Nissl-stained section from a representative ligate pretreated with 30 mg/kg of parenteral flunarizine shows no obvious lesion. Microscopic examination (N = 5 saline treated hypoxic-ischemic pups and N = 4 with flunarizine) verified that brain mass was relatively preserved on the side of ligation. Occasional small areas of neuronal loss were seen in flunarizine treated pups. Measurements of cross-sectional areas using a computerized digitizing pad enabled quantitation of these results. In coronal sections all cut at the level of globus pallidus (A6570-A5910, atlas of Konig and Klippel20) from the 5 saline-treated ligates, the mean cross-sectional area of the hemisphere ipsilateral to ligation was 42.92 ± 3.77 mm² and was 50.71 ± .39 on the opposite side (−15%, p < .01). In contrast, in sections from 4 animals pretreated with 30 mg/kg of intraperitoneal flunarizine, cross-sectional areas were 49.06 ± 0.25 ipsilaterally and 48.77 ± .2 mm² on the opposite side (+1%). Similarly in saline ligates, mean cross sectional area of caudate-putamen-globus pallidus was 8.43 ± 1.08 mm² on the side of ligation and 11.41 ± 3 mm² on the opposite side (−26%, p < .001). In contrast, in the flunarizine group, the area on the right side was 10.73 ± .61 and on the left was 10.54 ± .57 mm² (+2%, NS).

Previous work suggested that in this animal preparation, an abrupt, marked increase in striatal dopamine metabolism is temporally associated with morphologic neuronal injury.18 To determine if flunarizine pretreatment limited acute stimulation of dopamine release as it reduced histologic injury, 3 sets of experiments corresponding with the first group were done. Again, in each experiment, all animals were subject to unilateral carotid artery ligation and subsequent exposure to 2 hours of 8% oxygen. Half the animals received drug treatment before hypoxic exposure and half received an equal volume of saline or methylcellulose solution.

### Figure 1. Cerebral hemisphere weight changes in 3-week-old animals that underwent right carotid ligation and exposure to 2 hours of 8% O₂ at 7 days. Each pair of bars represents left (open bar) and right (closed bar) hemisphere weights (mean ± SEM); comparisons are made with the left hemisphere of control ligates. Panel A compares values in saline (0.9% NaCl) treated controls and in animals pre-treated with 2 doses of flunarizine (FLU). Parenteral FLU was injected i.p. in 50 μl preparation, 150 μl was injected i.p.; controls received an equal volume of saline. Panel B compares values in methylcellulose (MC) treated controls and in animals pre-treated with FLU orally, by syringe (30 mg/kg in 100 μl); controls received an equal volume of MC. Panel C compares values in saline and drug vehicle treated animals. To test the drug vehicle administered orally, by syringe (30 mg/kg in 100 μl); controls FLU orally. FLU, dissolved in 0.5% MC with 0.1 N HCl, was p < .02, t • = p < .05, * = p < .005, † = p < .05 for Student’s t test comparison of right hemisphere weights in drug-treated and control ligates. i.p. = intraperitoneal, μl = microlitre.

weeks later. Each pair of bars represents mean percentage change in left and right hemisphere weights compared to mean left hemisphere weight in control ligates for that experiment. In the first group of saline treated ligates (fig. 1A), right hemisphere weight was reduced by 15 ± 3.5% (mean ± SEM) and results were similar in litter-mates treated with 10 mg/kg of flunarizine: 14 ± 5% reduction on the side of ligation and no change on the opposite side. At a higher (30 mg/kg) dose, control ligates again had marked hemisphere asymmetry (right side, 19 ± 5% reduction) but pre-treatment with flunarizine virtually eliminated this effect (1.5 ± 2% reduction in right hemisphere weights, p < .005, right hemisphere weight of flunarizine-treated vs. right hemisphere of saline-treated ligate controls).

Pretreatment with 30 mg/kg of oral flunarizine yielded similar results (fig. 1B). In control ligates, pre-treated with 100 microlitres of 0.5% methylcellulose diluent, mean right hemisphere weight was reduced by 19 ± 4% compared with the opposite side of brain. In the experimental group, tissue loss was diminished significantly (right side, 5.6 ± 2% reduction, p < .02, comparison with right hemisphere weight of control ligates).
In addition, experiments were done in which treatment with the commercial drug vehicle and saline were compared.

Homovanillic acid (HVA) to dopamine ratio was calculated as a measure of dopamine metabolism and an estimate of turnover. In normal 7-day-old rat pups the striatal HVA: dopamine ratio is relatively constant (0.09 ± 0.01, N = 20). In hypoxic ligates, after 1½ hours of hypoxic exposure, HVA levels increase and dopamine is depleted so that the HVA: dopamine ratio rises. Since the extent varies among experiments (range: 0.77 ± 0.12 to 2.15 ± 0.5 in 4 different groups, 8 to 20 fold increase), results from ischemic striatum in drug-treated animals are compared only with corresponding values in litter-mate ligate controls in the same experiment.

In animals pretreated with 10 mg/kg of intraperitoneal flunarizine (fig. 3A), striatal HVA: dopamine did not differ from values in control hypoxic ligates (0.76 ± 0.27, N = 11, vs. 0.93 ± 0.27, N = 12). In contrast, 30 mg/kg of parenteral flunarizine, the dose that afforded protection from morphologic damage, markedly inhibited dopamine release (0.37 ± 0.17, N = 14 flunarizine treated, vs. 2.15 ± 0.5, N = 16 control ligates, p < .005). However, injection of an equal volume of the drug vehicle (fig. 3C) reproduced this effect (0.52 ± 0.2, N = 20, vs. 1.5 ± 0.44, p < .025). In contrast, administration of 30 mg/kg of flunarizine orally, dissolved in 0.5% methylcellulose, did not prevent stimulated dopamine release when given before exposure to hypoxia (fig. 1B) or up to 2.5 hours earlier (data not shown).

Discussion

In contrast to the adult animal, in seven-day-old rat pups the cerebral hemispheres are expanding rapidly. The phase of high velocity brain growth at this time in rodents resembles the stage of human central nervous system maturation at the end of a full term pregnancy. Unilateral carotid ligation combined with exposure of 8% oxygen for two hours disrupts this rapid growth on one side by infarcting discrete foci of brain (e.g. in dorsal striatum, lateral cortex and dorsal hippocampus) and restricting subsequent differentiation of neurons and myelinating neuronal processes. Although edema distorts the injured hemisphere within

FIGURE 2. A. Photograph of coronal Nissl-stained section from a 3-week-old animal that underwent right carotid artery ligation and exposure to 2 hours of 8% oxygen at 7 days of age. There is unilateral loss of tissue in striatum and cortex and there are clumps of abnormally staining pyknotic neurons and gliosis, especially in dorsal striatum where areas of infarction and scarring frequently persist in older animals. Bar = 1 mm. B. Photograph of corresponding Nissl-stained section from a ligate pretreated with 30 mg/kg of parenteral flunarizine showing no obvious lesion. In coronal sections from 5 saline-treated ligates, the mean hemisphere cross-sectional area was 15% smaller on the right than on the left side, but there was no difference between sides in four flunarizine-treated animals (see text). Bar = 1 mm.

FIGURE 3. Comparisons of ratio of homovanillic acid (HVA): dopamine (DOP) content in striatum ipsilateral to carotid ligation after 2 hours of exposure to 8% oxygen in 7-day-old animals. Each pair of bars compare values (mean ± SEM) in drug-treated animals (open bars) and controls (closed bars). Each pair includes values from 3 separate experiments (n ≥ 11 in each group). Panel A compares striatal HVA/DOP in animals treated with 2 doses of parenteral flunarizine (FLU) and littermate controls treated with equal volumes of saline. Panel B compares HVA/DOP in animals pre-treated with 30 mg/kg FLU dissolved in 0.5% methylcellulose (MC) administered orally, and in littermate controls pretreated with equal volumes of MC. Panel C compares HVA/DOP in animals pretreated with a 150 μl injection of the drug vehicle and an equal volume of saline. * indicates p < .025, Student's t-test.
hours to days after the insult, this acute effect and other transient histopathologic changes have subsided two weeks later when our studies were done.\textsuperscript{13, 16} Therefore, measurement of hemisphere weight in the 21-day-old rat provides a simple, accurate means of quantitating the extent of morphological injury.

Previous studies with this animal preparation suggest that the morphologic injury is caused by cerebral ischemia superimposed on hypoxemia. Examination of the brain at intervals after intracardiac injection of India ink demonstrated discrete areas of hypoperfusion in vulnerable regions of the hemisphere ipsilateral to carotid ligation.\textsuperscript{15} Quantitation of perfusion in the corpus striatum by measuring the extraction of 14C-iodoantipyrine and 3H-flunitrazepam suggested that blood flow in the injured hemisphere dropped by almost 70\% within a half hour of starting the hypoxic exposure and then continued to decrease more gradually.\textsuperscript{15} The perfusion deficit preceded the time necessary to cause readily detectable morphologic injury in the model (1 \frac{1}{2} hours of hypoxia). These data indicate that this immature rodent preparation can best be described as a model of moderate hypoxemia combined with incomplete cerebral ischemia during the initial insult.

Pretreatment with flunarizine, an organic calcium antagonist, limits the extent of hypoxia-ischemia induced morphologic brain injury in this setting. The drug is effective both orally and parenterally in preventing the unilateral reduction in hemisphere mass and markedly lessens the severity of histopathologic changes. The effective dose is in the range reported to be neuroprotective in several models of anoxic-ischemic injury in older animals.\textsuperscript{11, 12} At this dose, the drug had no evident toxicity and the survival rate was the same in saline and flunarizine treated pups.

It has been much easier to demonstrate a neuroprotective effect for flunarizine than for other calcium antagonist compounds. Other drugs including verapamil, diltiazem, nicardipine and nifedipine have not been effective in models of cerebral anoxia-ischemia\textsuperscript{26} (Johnston, M.V. and Silverstein, F.S., unpublished data). This may be because flunarizine's difluoropiperazine structure allows it to penetrate the blood brain barrier easily and reach a relatively high level in the brain compared with serum over a prolonged period.\textsuperscript{20}

Two major hypotheses have been proposed to rationalize the use of calcium antagonist drugs for brain anoxia-ischemia. First, they might augment the blood supply to ischemic brain. They might act to improve perfusion during the acute episode, in a fashion analogous to their role of relieving vascular spasm in ischemic heart disease. Also, they could enhance blood flow in the post-ischemic recovery period. With ischemic neuronal injury, characteristic cellular changes and intracellular calcium overload in vulnerable neurons are most prominent during the reperfusion period after ischemia.\textsuperscript{4} Flunarizine's beneficial effect could result from normalization of perfusion in the post-hypoxia period. Studies of flunarizine's impact on cerebral blood flow and neurologic recovery after cerebral ischemia have yielded conflicting results. Dramatic benefits\textsuperscript{6} and no improvement in cerebral perfusion or neurologic outcome have been observed.\textsuperscript{21, 22} However, in these studies, flunarizine was administered after episodes of "complete" cerebral ischemia. The pathogenesis of neuronal injury in such settings may differ significantly from that in our experiments in which flunarizine was administered prophylactically and ischemia was not complete.

Alternatively, flunarizine could more directly reduce calcium entry into neurons and other cells and enhance their resistance to ischemic injury. Neurons have specific recognition sites for calcium antagonist drugs,\textsuperscript{23} but their functional activity has been questioned. In initial studies, calcium antagonists did not prevent depolarization induced calcium fluxes into synaptosomes,\textsuperscript{24} or depolarization evoked neurotransmitter release in vitro.\textsuperscript{25} However, recent reports demonstrated that the calcium antagonist nitrendipine could limit depolarization evoked calcium influx\textsuperscript{6} and could inhibit neurotransmitter release in specific experimental settings.\textsuperscript{2} These conflicting results are both consistent with the hypothesis that the dihydropyridine calcium antagonist sites do not alter normal neurotransmitter release but that sites at which these drugs are active may be unmasked under certain circumstances (e.g. ischemia or seizures).

The results of our experiments examining dopamine turnover provide some mechanistic information about flunarizine's neuroprotective effect. We demonstrated previously in this model that over the period of hypoxia, dopamine turnover in the corpus striatum is biphasic: it drops slightly over the first hour and then increases abruptly during the next hour to 10-20 fold higher levels.\textsuperscript{18} This major shift, which may reflect a threshold for neurotransmitter release from hypoxia-ischemia, occurs at approximately the same time as that required to produce morphologic injury. Since depolarization-induced transmitter release is generally calcium dependent, we determined whether flunarizine would block this response to hypoxia-ischemia. The parenteral form of flunarizine in a solution containing tartaric acid did block the rise in dopamine turnover in a dose dependent fashion and also prevent morphologic injury. However, since stimulated catecholamine release was still observed after pretreatment with 30 mg/kg orally, it is likely that flunarizine itself does not prevent the initial development of ischemia. In contrast, the vehicle, which influences both the acute neurochemical events and the development of tissue damage, may limit the initial progression of ischemia. The data demonstrate a clear distinction between the effects of the drug and its diluent and suggest that flunarizine acts by a mechanism independent of neurotransmitter release. The possibility remains that the drug limits calcium overload into neurons postsynaptic to certain stimulated axons.\textsuperscript{5, 20} Ischemic changes at postsynaptic sites, perhaps related to release of excitotoxic transmitters, might lead to increased membrane calcium conductance and might concurrently expose new calcium antagonist sites.
In addition to these mechanisms, several other pharmacologic properties could contribute to neuroprotection. Flunarizine is a potent inhibitor of adenine uptake and may enhance reactive hyperemia in the cerebral vasculature during the post-hypoxic recovery period. Flunarizine is a free radical scavenger in myocardial tissue. If it has this role in brain, this may contribute to modulation of the impact of ischemic injury. As well, flunarizine’s anticonvulsant properties might be beneficial, although we have not detected acute seizures in the pups. Further studies are required to define flunarizine’s mechanisms of action in this model, and to delineate features which are specific to the immature brain. The observation that the vehicle has a potent but distinct effect also deserves further attention since the mechanisms for this action remain obscure.

These observations in an animal model suggest that flunarizine or a similar compound might be clinically useful to reduce brain injury from hypoxic-ischemic encephalopathy, the most common cause of chronic neurologic disability in infants. In the immature rat it is unknown whether flunarizine will protect the brain if given during or after the hypoxic-ischemic period. Nevertheless, even if the drug is not found to be effective at a later time, pretreatment might be clinically feasible by administration of the drug to mothers of infants at high risk for perinatal hypoxia-ischemia.

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