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Effects of postnatal hypoxia–ischemia on cholinergic neurons in the developing rat forebrain: choline acetyltransferase immunocytochemistry

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We studied the effect of early postnatal hypoxia–ischemia on cholinergic neurons in the developing rat forebrain using immunohistochemistry for choline acetyltransferase (ChAT). In 7-day-old rat pups, hypoxia–ischemia was induced in one cerebral hemisphere by combining unilateral carotid ligation with exposure to 8% oxygen for 2.5 h. This procedure caused brain injury in the hemisphere ipsilateral to ligation, most prominent in the corpus striatum, hippocampus and overlying cortex. In animals sacrificed 2–3 weeks after the insult, at approximately 3 weeks of age, the density of cholinergic cell bodies was slightly higher in the lesioned rostral caudate-putamen than the opposite side (+12%, $P < 0.05$). In the more caudal portion of caudate-putamen, this effect was greater. In contrast, the size of the cholinergic perikarya in the injured striatum was significantly reduced. Cholinergic neurons in the septum (Ch1, Ch2), globus pallidus and nucleus basalis (Ch4) were relatively unaffected. Considered together with previously reported neurochemical data, these observations suggest that the immature cholinergic neurons are less vulnerable to death from hypoxia–ischemia than other components of the striatum. However, differentiation of surviving cholinergic perikarya and possibly their axonodendritic processes may be disrupted by the early insult.

INTRODUCTION

Cholinergic neurons mature later than many other neurotransmitter-specific neuronal groups (e.g. catecholamine projections) in the mammalian forebrain[4,5,11,14,18,21]. In rodent caudate-putamen and cerebral cortex, activity of the acetylcholine synthetic enzyme, choline acetyltransferase (ChAT), remains low during the first postnatal week, and rises rapidly over the following two weeks. However, several more weeks are required for adult levels of enzyme activity to be reached. This development curve is paralleled by an increase in activity of the synaptosomal uptake mechanism for [^{3}H]choline, another biochemical marker for cholinergic nerve endings[14]. ChAT activity in human neocortex appears to follow a similar protracted maturational pattern during childhood[6]. This data indicates that cholinergic neurons differentiate predominantly in the postnatal period.

Since cholinergic neurons are relatively immature at birth, it seems possible that certain environmental factors might disrupt their development. Although the physiology of perinatal asphyxial brain injury has been described, little information is available about its effects on differentiation of specific neuronal groups[13]. We studied an experimental model which combines hypoxia with unilateral cerebral ischemia in 7-day-old rat pups[23].

Previous studies with this model indicated that the caudate-putamen, hippocampus and neocortex in the hypoxic–ischemic hemisphere are injured frequently while the brainstem and cerebellum are relatively spared[13]. Neurochemical analysis of markers for sev-

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eral neurotransmitter systems in the model suggested that hypoxic–ischemic injury disrupts development of cholinergic innervation. At two weeks after the injury, [^{3}H]choline synaptosomal uptake and ChAT activity were both reduced in the damaged caudate-putamen compared to the contralateral hemisphere in the same animals or in age matched controls. In contrast, biochemical markers for dopaminergic nerve terminals, nerve endings of GABAergic interneurons in the caudate putamen and GABAergic neurons projecting out of the caudate-putamen were less affected.

These results suggested that cholinergic interneurons in the caudate-putamen are relatively sensitive to early hypoxia–ischemia, either because they are more vulnerable to destruction than other surrounding neurons or because their development is disrupted. We examined this issue by immunostaining cholinergic neurons for ChAT several weeks after the injury.

MATERIALS AND METHODS

Pregnant Sprague–Dawley rats (Charles River) were housed in separate cages after 17 days of gestation, and pups were born on day 21 or 22 of gestation. To produce hypoxic–ischemic injury in one cerebral hemisphere, 7-day-old rat pups were briefly anesthetized with ether and the right common carotid artery was ligated using microsurgical technique[13,26]. After 1 h, in which the pups were allowed to suckle with the dam, they were placed in a Plexiglas chamber warmed to 37 °C and supplied with humidified 8% oxygen, 92% nitrogen. The pups were removed to room air 2.5 h later and were returned to the dam. Nineteen hypoxic–ischemic pups were studied and 13 littermates were used as controls.

At 19–28 days of age, experimental and control animals were anesthetized with 20% chloral hydrate, and then were perfused through the heart with: (1) 20–40 ml of cold 0.9% saline over 2–3 min; (2) 100–150 ml of 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer pH 7.4; (3) 100 ml of 10% sucrose in cold 0.1 M phosphate buffer. The descending aorta was clamped shut to avoid perfusion of the lower extremities. The brains were removed after perfusion and placed in 30% sucrose with 0.1 M phosphate buffer at 4 °C overnight. Coronal sections of 40–50 μm thickness were cut on a sliding microtome, and temporarily placed in cold 0.1 M Tris buffer, pH 7.6, until immunohistochemical staining.

Immunostaining for ChAT was performed using a rat immunoglobulin (product of rat/mouse fusion, Ab8) directed against bovine ChAT, the production and specificity of which have been described[17,19,20]. Free floating sections were placed in cold 0.4% Triton X-100 in 0.1 M Tris, pH 7.6 (20 min) and after 3 changes of cold Tris-buffered salt rinse (0.85% NaCl, 0.01 M Tris, pH 7.6, 5 min each), sections were placed in incubation media (0.02% Triton X-100, 3% normal rabbit serum, 0.1 M Tris) for 30 min. Sections were rinsed with 3 changes of Tris buffered salt rinse and incubated overnight at 4 °C with a primary antibody dilution of 1/500 with shaking. Sections were then rinsed and incubated with rabbit anti-rat second antibody (Pel Freeze, 1/150) for 1 h at 4 °C. After rinsing, sections were incubated in a solution containing rat derived peroxidase–antiperoxidase (PAP, Sternberger-Meyer, 1/300) for 1 h at 4 °C. Then, after rinsing, the sections, were re-incubated in rabbit anti-rat immunoglobulin and rat peroxidase–antiperoxidase for 1 h each. Sections were then rinsed and incubated for 10 min in 0.05% 3, 3-diaminobenzidine in 0.1 M Tris buffer, containing 0.01% hydrogen peroxide to form a dark reaction product as previously described[17].

The total number and density per unit area of immunostained neurons were determined in the caudate-putamen in serial coronal sections from the forebrains of lesioned and control animals. The sections from separate animals were matched using anatomical landmarks and the atlas of König and Klippel[16]. The location of the sections used for quantitation is described in the tables and figure legends. Additional immunostained sections were examined to verify the uniformity of the changes and representative examples are shown in the figures. Montages were constructed from enlarged photomicrographs and the cell bodies were counted directly. Cross-sectional areas of the caudate-putamen were measured using a digitizing pad (Summa graphics). Densities of ChAT-containing neurons were determined in at least two sections from each animal. The Abercrombie method was used to correct for the difference in neuronal size in lesioned and control hemispheres[1].

To determine average size of cholinergic neurons 10 randomly selected ChAT positive neurons from each of 5 distinct regions of the striatum (dorsolateral, dorsomedial, center, ventrolateral, ventromedial) were measured in 2 matched sections from each animal (4 experimental, 3 controls, 24–26 days old). This provided measurements of 100 neurons for each side of each brain. The dimensions of neurons were measured at a magnification of ×250 using an eyepiece micrometer.

RESULTS

In rat pups older than 24 days, the immunostaining method for visualizing ChAT revealed intensely reactive cholinergic neurons in caudate-putamen, globus pallidus, substantia innominata/nucleus basalis (Ch4, ref. 20), septum and nucleus of the diagonal band (Ch1–Ch3; Figs. 1–5). Control sections prepared by omitting the first monoclonal antibody or substituting non-immune rat IgG did not produce neuronal staining. We did observe occasional faintly outlined large pyramidal neurons in deep layers of cerebral cortex which were easily distinguishable from specifically stained ChAT-positive neurons. Despite the excellent staining we obtained in the 3-week-old animals, we could not obtain reproducible staining in younger animals, after numerous attempts.

Reactive neurons in the caudate-putamen in control animals had predominantly large (average 35 μm) oval or multipolar cell bodies (Fig. 1). Although relatively evenly distributed, they were sometimes arranged in short chains or clumps around myelinating fasicles (Figs. 3–5). Background staining was very low in these fasicles, but intervening areas showed a diffuse staining pattern containing many reactive fibers. ChAT-containing neurons in the globus pallidus and in the basal forebrain were similarly large and heavily stained (Figs. 2, 4–6). Many neurons were visualized with Golgi-like images of the entire cell body along with axons and branching den-

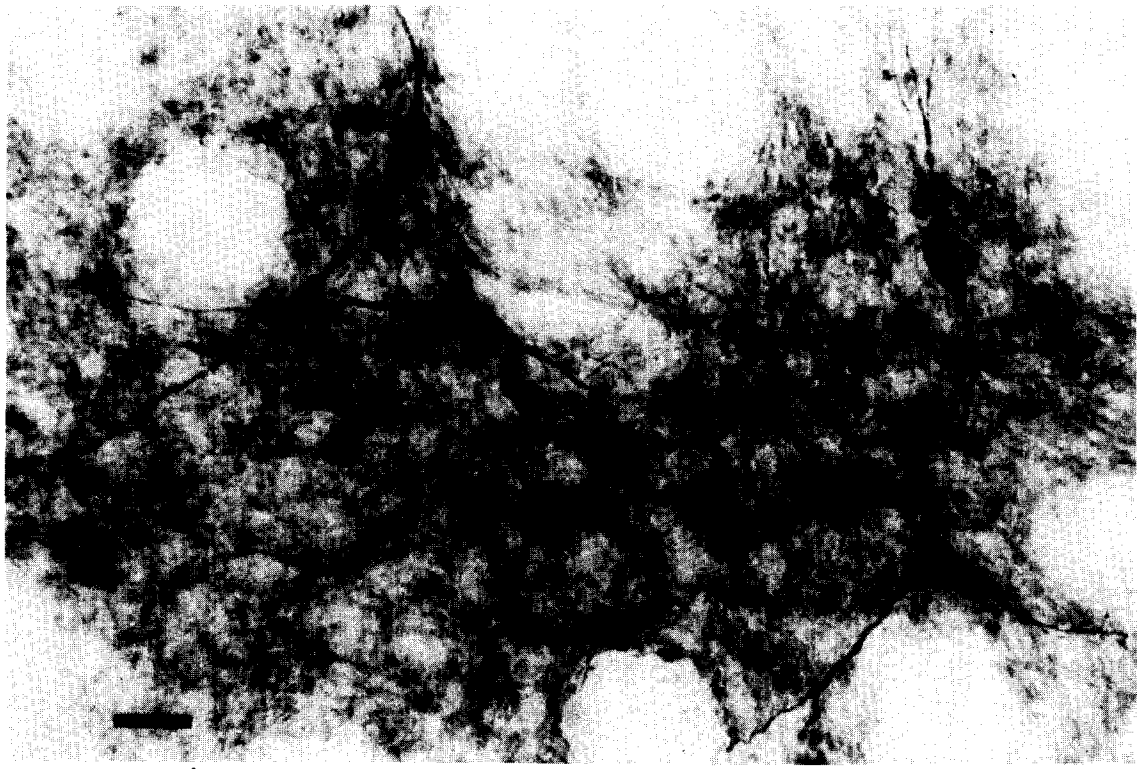

Fig. 1. Neurons in the caudate-putamen immunostained for ChAT from a 26-day-old control rat. Multiple branching dendrites emanate from predominantly oval neurons. The darkly stained background appears under the microscope as a ChAT-containing plexus of neuronal processes. Scale bar = 25 μm.

drites (Fig. 2).

The unilateral hypoxic–ischemic injury at one week of age reduced the right (ligated) hemisphere size in the animals we studied approximately two weeks after hypoxia–ischemia (Figs. 3–5). The caudate-putamen was more than 30% smaller than the opposite (unligated) side. The size of the opposite side was relatively unchanged compared with untreated age matched controls (Table I). The general

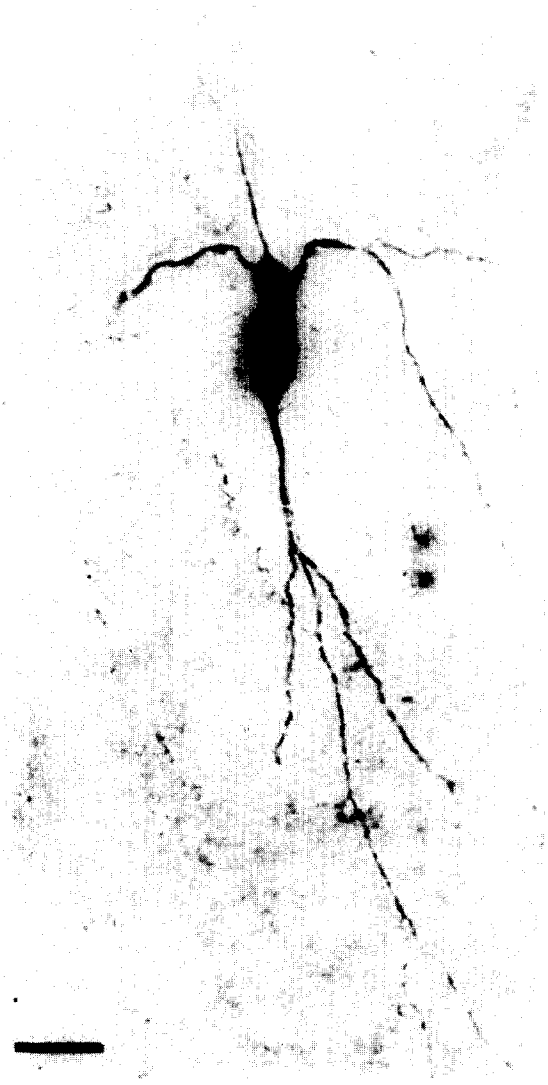

Fig. 2. An immunostained neuron from the globus pallidus of a 28-day-old control rat showing a Golgi-like image. Note the axon projecting from the hillock at the top of the neuron and multiple branching on dendritic processes. Scale bar = 25 μm.

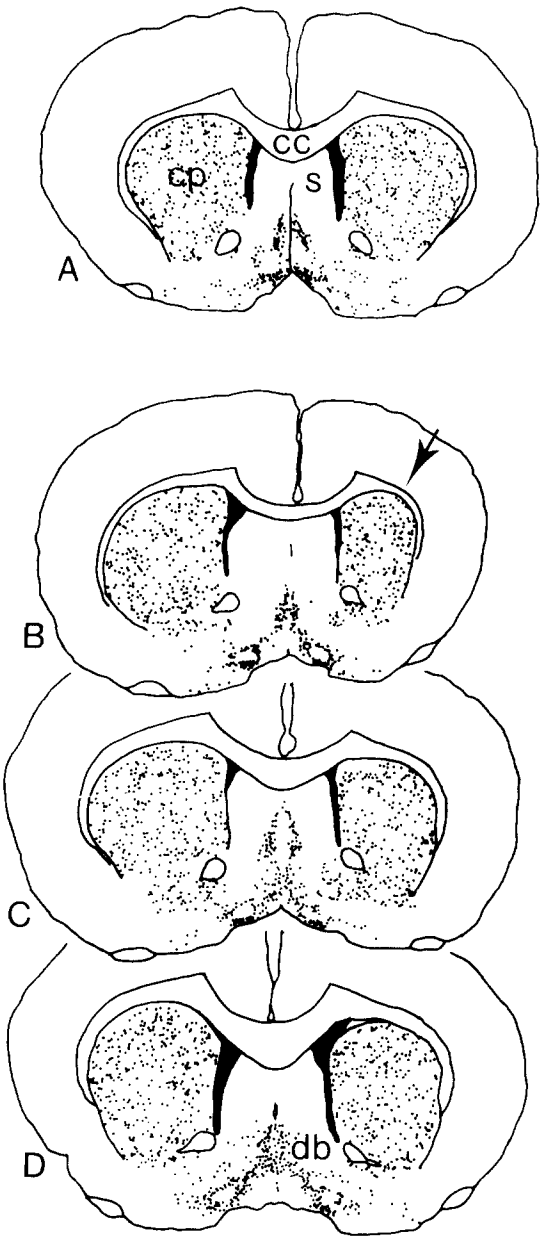

Fig. 3. Drawings based on tracings of photomontages of the ChAT staining neurons in coronal sections of a control and hypoxic–ischemic lesioned 26-day-old rat brain. Panel A shows a section through a level at approximately 8620 μm anterior to the interaural line in the atlas of König and Klippel[16] in a control rat pup and panels B–D show sections at increasingly more caudal levels from 8380 to 7500 μm. The arrow points to the hypoxic–ischemic lesion on the right side of the brain on the side of carotid ligation. The right hemisphere and caudate putamen are smaller but the density and distribution of cholinergic neurons in this structure as well as in the septum and diagonal band appear similar on the two sides. As described in the text, the actual density of cholinergic perikarya on the right side at this level was slightly higher. Abbreviations: cc, corpus callosum; cp, caudate-putamen; db, diagonal band, vertical and horizontal limbs; s, septum.

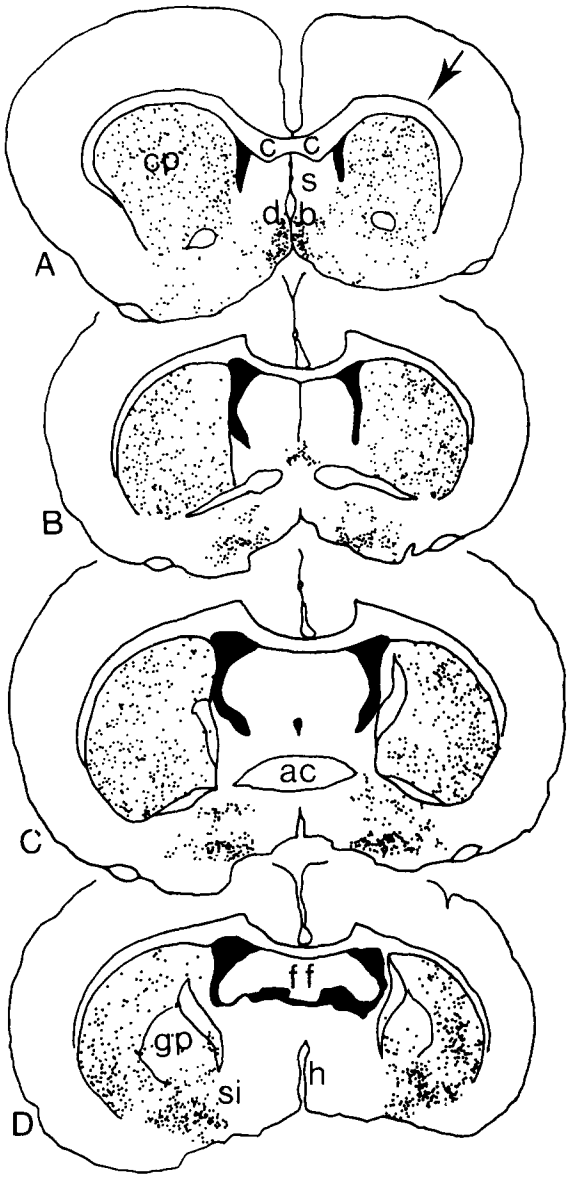

Fig. 4. Coronal sections from another representative rat brain at 24 days of age exposed to right sided carotid ligation plus hypoxia at one week of age. The lesioned side is indicated by the arrow. The most rostral section, in panel A, is approximately at the level of 8380 μm and the most caudal section in panel D is taken from approximately level 6280. The relative density of cholinergic perikarya in the rostral caudate putamen (sections A, B) is approximately the same on the lesion as on the operated side but the density is obviously higher in the most caudal sections (panel C and D). The distribution and number of basal forebrain cholinergic neurons in the globus pallidus and substantia innominata is approximately equal on both sides. Abbreviations: ac, anterior commissure; cc, corpus callosum; cp, caudate putamen; db, diagonal band; ff, fimbria fornix; gp, globus pallidus; h, hypothalamus; si, substantia innominata.

topography of ChAT-containing neurons within the injured caudate-putamen resembled the distribution in the opposite, uninjured side and the distribution in controls. Injury reduced the total number of cholinergic perikarya on the lesioned side by 25% (Table I). The loss of mass in the striatum was greater than the reduction in neuronal number and the density of cholinergic perikarya remaining at 3 weeks was slightly but significantly higher on the lesioned side (+12%). The higher density of cholinergic neurons in the caudate-putamen was most obvious in caudal sections which included the globus pallidus (Fig. 5). In two animals with sufficient sections for analysis, the density of cholinergic neurons was elevated by approximately 70% (Table II) and many neurons were arranged in clumps. In these caudal sections, the total number of cholinergic neurons per section was not diminished by the hypoxic–ischemic injury as was the case at more rostral levels.

While the density of cholinergic neurons in the injured caudate-putamen was increased, their size was reduced by the early injury. The mean length of ChAT-stained cell bodies was reduced slightly but

TABLE I

Number and density of neurons immunostained for ChAT in caudate-putamen of 24 to 28-day-old rats lesioned at 7 days

ChAT-positive cell bodies in the caudate-putamen were identified on montages of enlarged photomicrographs and counted. Controls were unoperated non-hypoxic littermate pups. Lesioned animals were exposed to 8% oxygen for 2.5 h after right carotid artery ligation at 1 week of age. Frozen coronal sections of 40 μm thickness are from levels A8620-A7890 of the atlas of König and Klippel (Figs. 3 and 4). The Abercrombie correction was used to correct for the difference in neuronal size (Table III). Values are mean ± S.E.M. Abbreviations: R, right; L, left; CR, right hemisphere from control animals, right hemisphere.

Hemisphere	*Total cell bodies*	*Area (mm^2)*	*Density (No./mm^2)*
Control (n = 3)			
Left	146 ± 0.2	7.9 ± 0.3	18.5 ± 1
Right	148 ± 7	8.0 ± 0.3	18.6 ± 2
% (R vs L)	+1	+1	+1
Lesioned (n = 5)			
Left	147 ± 8	7.7 ± 0.2	19.1 ± 2
Right (lesion)	111 ± 13	5.2 ± 0.5	21.4 ± 2
% (R vs L)	−24**	−32**	+12*
% (R vs CR)	−25**	−35**	+15

*$P < 0.05$, Student's t-test for paired values.
**$P < 0.01$, Student's t-test.

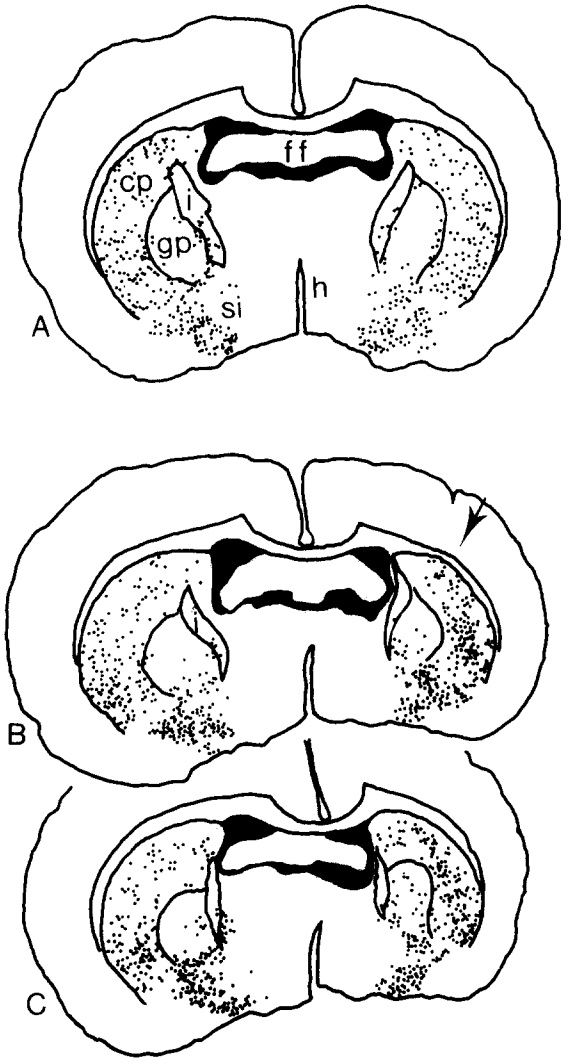

Fig. 5. Comparison of the location of cholinergic perikarya in a coronal section through caudate-putamen and globus pallidus approximately at level 6280 μm in a control 24-day-old rat pup (section A) compared with an animal which was made hypoxic–ischemic on the right side (indicated by the arrow) at one week of age (sections B, C). The two lesioned sections in panels b and c are from level 6280 and 5910 respectively. The density of cholinergic perikarya in the caudate-putamen on the injured side is higher than on the opposite side although the distribution of neurons in the basal forebrain on the two sides is approximately equal.

significantly by 9% compared with the opposite hemisphere ($P < 0.001$, Table III). The reduction in neuronal size is illustrated by comparison of representative immunostained neurons on each side of a lesioned animal in Fig. 6. The decrease in neuronal size produced by the injury was even greater when compared with the analogous hemisphere of non-hypoxic control animals (−17%, $P < 0.001$, Table III). ChAT-containing neurons in the hemisphere subjected to hypoxia alone (contralateral to ligation) were smaller by 11% ($P < 0.001$) compared with the corresponding hemisphere in controls. In controls, the average length of cholinergic neurons in the caudate putamen was approximately twice their width and it is noteworthy that there was no apparent

TABLE II

Density of immunostained neurons in caudal portions of caudate-putamen of 24 to 28-day-old rats

Coronal sections were made at approximately level A6280 (atlas of König and Klippel) to examine the caudate-putamen and globus pallidus at levels behind sections described in Table I (shown in Figs. 4 and 5). Sections stained for ChAT were photographed and analyzed as described in Table I and Methods. Values are mean ± S.D.

Hemisphere	*Total cell bodies per section*	*Area (mm^2)*	*Density ($No./mm^2$)*
Control (n = 2)			
Left	70.7 ± 17	5.2 ± 1.4	13.6 ± 4
Right	64.2 ± 18	4.6 ± 0.7	13.9 ± 4
% (R vs L)	−9	−11	+2
Lesioned (n = 2)			
Left	79 ± 9	5.9 ± 0.7	13.3 ± 1.4
Right (lesion)	86 ± 7	3.7 ± 0.3	23.4 ± 3
% (R vs L)	+10	−37**	+76*
% (R vs CR)	+35	−20	+68*

*$P < 0.05$, Student's t-test.
**$P < 0.01$.

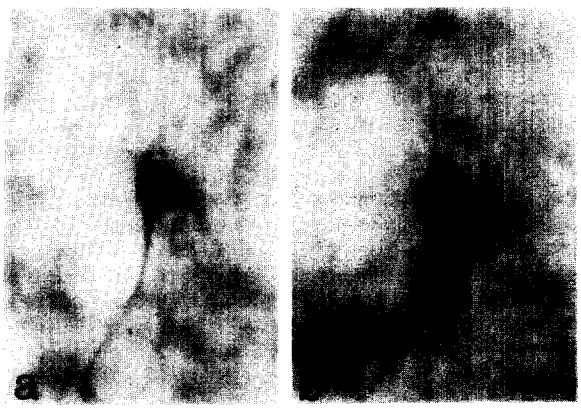

Fig. 6. High power photomicrograph of striatal neurons immunostained for ChAT in a 26-day-old rat with a unilateral hypoxic–ischemic insult produced at 7 days of age. Panel a is a representative neuron from the smaller lesioned striatum while b shows a neuron from the corpus striatum on the opposite side. The scale bar is equal to 25 μm.

TABLE III

Size of striatal perikarya containing choline acetyltransferase in 24- to 28-day-old rat pups lesioned at 7 days

The size of ChAT-positive neurons was compared in topographically matched coronal section from age matched control untreated (n = 3) and hypoxic–ischemic 24- to 28-day-old rat pups (n = 4) which had undergone right common carotid artery ligation and exposure to 2.5 hours of 8% oxygen at 7 days of age. Sections of 40 μm thickness were analyzed from levels A8620 to A7890 in the atlas of König and Klippel[16]. Values are mean ± S.E.M.

Hemisphere	*Neurons counted*	*Length (μm)*	*Δ% (vs contra-lateral)*	*Δ% (vs same side of control)*
Left Control	300	35.8 ± 0.4	–	–
Right Control	300	35.4 ± 0.4	−1	–
Left Hypoxic	395	32.0 ± 0.3	–	−11*
Right (Ischemic Hypoxic)	395	29.2 ± 0.3	−9*	−17*
Left Control	300	14.9 ± 0.2	–	–
Right Control	300	14.4 ± 0.2	−3	–
Left Hypoxic	395	15.1 ± 0.2	–	+1
Right (Ischemic Hypoxic)	395	14.4 ± 0.2	−5	−0

*$P < 0.001$, Student's t-test.

change in this dimension after hypoxia–ischemia. The size of cholinergic neurons in control animals at approximately 3 weeks of age was similar to neurons measured in adult rats.

In contrast to the changes we observed in the number, density and size of cholinergic neurons in the caudate-putamen after hypoxia–ischemia, we found no alterations in neurons in the septum, diagonal band, basal forebrain/nucleus basalis or globus pallidus (Fig. 7). Lightly stained fibers could be seen in some control and hypoxic–ischemic animals in the external capsule and deep layers of cortex but we could not detect disruptions in these processes.

DISCUSSION

The morphology of cholinergic neurons in the rodent forebrain has been described in a number of studies utilizing specific antibodies against ChAT[2,9,12,20,27,30]. We found that immunostained cholinergic neurons in brains from 3-week-old control animals were generally similar to those found in adults and ChAT-containing fiber networks were well developed in the corpus striatum, globus pallidus and basal forebrain. Although other studies have shown that histochemical staining for acetylcholinesterase (ACHE) is detectable in individual cell bodies within the caudate-putamen soon after postnatal day 3, we could not immunostain cholinergic neurons in the caudate-putamen until after 3 weeks of age[3]. This may have been related to the relatively small amount of ChAT protein in the immature brain. ChAT activity reaches approximately 50% of adult levels at 3 weeks of age[4,5].

Unilateral carotid artery occlusion followed by exposure to a reduced oxygen atmosphere for 2.5 h causes focal ischemia and extensive neuronal injury in the ipsilateral cerebral hemisphere[25]. The lesion in the caudate putamen visualized in Nissl-stained sections typically includes an oval area of brain infarction in the rostral, dorsal caudate-putamen and shrinkage in the surrounding more normal appearing striatum[13]. The volume of the more caudal portion of caudate-putamen and the globus pallidus is also reduced compared with the opposite side but obvious foci of infarction are rare at this level.

Hypoxia–ischemia altered the density of cholinergic neurons differently in the rostral compared with the more caudal striatum. At rostral levels, the total number of cholinergic neurons within the striatum was reduced by approximately 25% while in the caudal portion the total number of ChAT-staining neurons was slightly increased. The resulting density of cholinergic neurons in the corpus striatum was elevated modestly in the rostral striatum two weeks after the injury and much more so in the caudal portion. These results suggest that the developing cholinergic cell bodies are relatively less vulnerable than surrounding neurons and other structures to hypoxic–ischemic injury. In the rostral caudate-putamen cholinergic neurons are probably depleted within foci of infarction but survive in the regions exposed to less severe hypoxia–ischemia. The relatively high density of cholinergic neurons in the caudal caudate-putamen may have been due to death of adjacent intrinsic striatal neurons or loss of axonodendritic connections projected from surrounding regions of basal ganglia or cerebral cortex. Also, it is possible that some cholinergic neurons were prevented from undergoing programmed cell death, which occurs in the immature striatum during the postnatal period[8]. Our data are consistent with a previous report showing

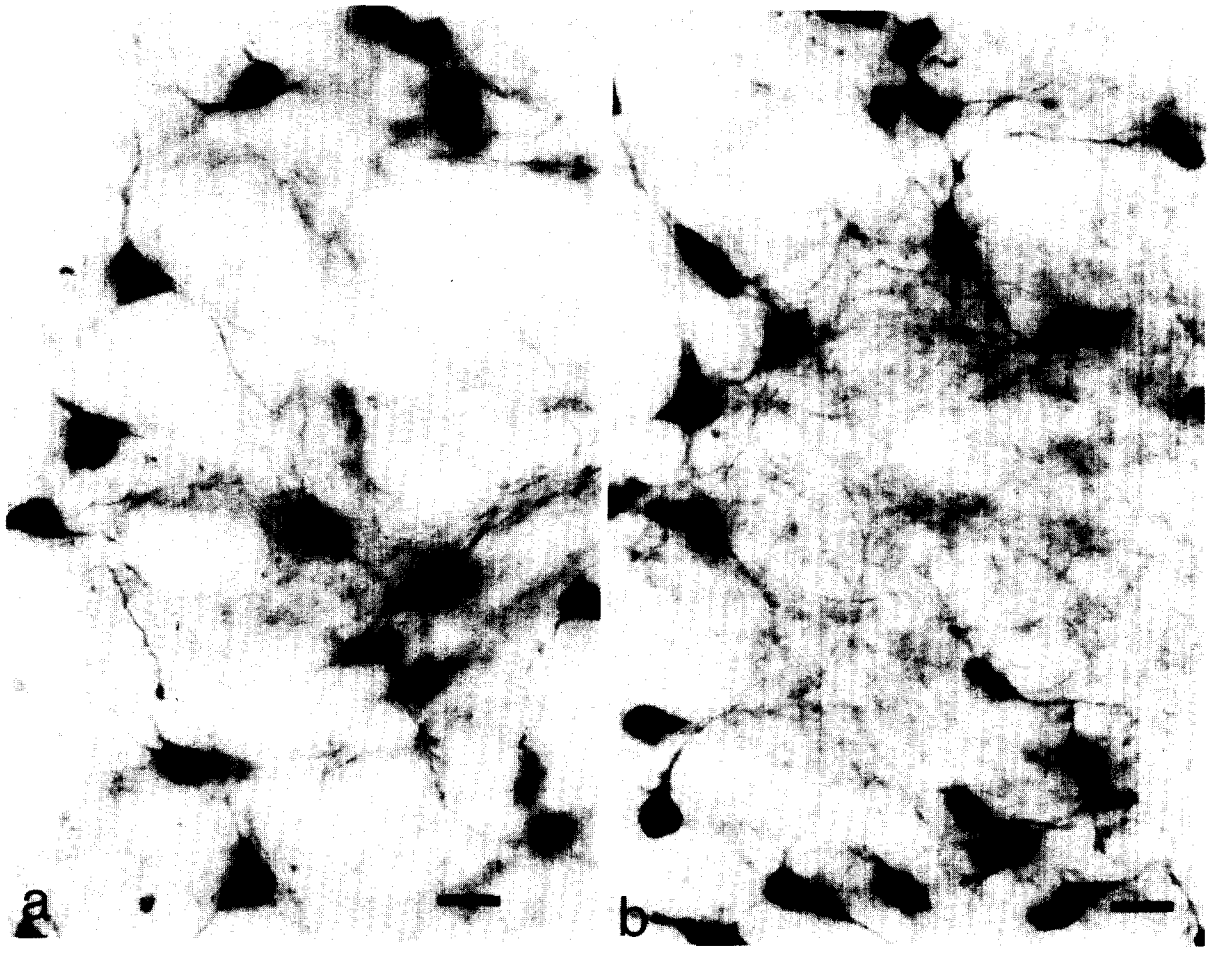

Fig. 7. Immunostained cholinergic neurons from the portion of the nucleus basalis within the globus pallidus (Ch4) in a 26-day-old rat pup with a unilateral hypoxic–ischemic lesion produced as described in the text. The cell bodies are densely stained with areas of nuclear paller and are surrounded by a dense fiber plexus containing ChAT. Scale bar = 25 μm.

that cholinergic neurons are relatively less vulnerable than GABAergic neurons to ischemia in adult rats[7].

Although the density of cholinergic neurons was increased in the caudate-putamen, their size was significantly reduced. This may reflect direct injury as well as an effect on the axonodendritic arbors connecting these cell bodies with other neurons in the shrunken striatum. Shrinkage of cholinergic neurons has been reported after axonal damage in adult animals[28,29]. Unlike the change in neuronal density, the size of cholinergic neurons was reduced in both hemispheres, more in the hypoxic–ischemic side than the opposite one. Hypoxia alone may have reduced neuronal size in the unoperated striatum or it may have been exposed to a modest amount of ischemia at some time during the hypoxic exposure. Previous morphologic studies suggested that neonatal hypoxia stunts the development of the neuropil in the cortex and caudate-putamen[10,22].

Our previous neurochemical analysis of markers for cholinergic neurons in the injured striatum at three weeks of age suggested that their nerve terminals were reduced relative to dopaminergic and GABAergic nerve terminals. The concentration of ChAT and activity of [^{3}H]choline uptake, a marker for cholinergic nerve terminals, were reduced at two weeks after injury but returned to control levels when the animals grew to maturity. It is noteworthy that in these experiments there was also a modest reduction in [^{3}H]choline uptake on the non-operated hypoxic side, possibly correlating with the reduction in size of those neurons we observed[13].

Considered together, the morphological and neu-

rochemical data suggest that cholinergic neuronal cell bodies in the caudate-putamen are relatively resistant to death from hypoxia–ischemia. This result is consistent with the principle that relatively immature neurons are resistant to hypoxic injury[24]. The reduction in cholinergic neurochemical markers is probably correlated with the reduction in neuronal size and possibly to disruption in the development of their axon dendritic arbors.

In contrast to the caudate-putamen, which is heavily damaged by hypoxia–ischemia in this model, we observed no obvious change in the appearance or number of cholinergic neurons in the septum, diagonal band, nucleus basalis/basal forebrain or adjacent globus pallidus. These regions may become less ischemic than the striatum during hypoxic exposure. Although they project axons to ischemic areas which are injured (e.g. hippocampus and cerebral cortex), these projections undergo virtually all of their biochemical maturation after the first postnatal week[9,15,18,21]. This suggests that early injury to their terminal fields may not disrupt the development of these projections[28,29].

Our results show that cerebral hypoxia–ischemia causes regionally selective effects on the density and size of cholinergic neurons in the developing basal ganglia. Hypoxic–ischemic brain injury is a relatively common cause of motor disorders in humans. Some of the functional abnormalities which result may be related to reorganizational changes in cholinergic neuronal circuitry.

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