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Perinatal hypoxic ischemic encephalopathy affects the proportion of GABA-immunoreactive neurons in the cerebral cortex of the rat

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The hypothesis was tested whether perinatal hypoxic ischemia leads to a preferential degeneration of the GABAergic inhibitory neurons in the cerebral cortex which, in turn, could account for the reported higher risk of developing epilepsy later in life. To that end rat pups, aged 12–13 days, were made hypoxic by employing a combination of unilateral ligation of one of the carotid arteries and a 90-min exposure to 8% O₂. After recovery periods of 3, 7, 35 and 150 days, the animals were sacrificed by perfusion fixation and the brains embedded in Epon. Transverse semi-thin sections were alternately stained with an antibody against GABA and with Toluidine blue. By using an unbiased morphometric method (the disector) the number of GABA-immunoreactive (GABA-IR) neurons and the total number of nerve cells per unit volume of tissue were estimated in corresponding neocortical areas in the ipsilateral (damaged) and contralateral ('control') hemisphere. For all animals with post-ischemic survival times of 3 and 7 days GABA-IR cells constituted a lower proportion of the total number of nerve cells in the damaged than in the 'control' cortical areas. This finding was consistent with the outcome of an earlier *in vitro* study. By contrast, in all animals with a survival time of 35 and 150 days, the proportion of GABA-IR neurons was higher on the damaged than on the 'control' side. This switch in the direction of the left/right differences, apparently depending on the length of the post-ischemic survival time, was statistically significant. No lateralization in the proportion of GABA-IR cells was detected in the cerebral cortex of the control rats. These observations, therefore, do not support the hypothesis that perinatal hypoxic ischemia ultimately leads to a preferential loss of GABAergic neurons in the cerebral cortex.

INTRODUCTION

Epidemiological data show that perinatal hypoxic-ischemic encephalopathy (HIE) is still a serious health problem. It occurs in about 1.1% of all babies born alive and although the absolute numbers are similar, the incidence is higher in preterm (about 10%) than in full-term babies (about 0.6%)²⁷. One of the consequences of perinatal HIE is that it increases the risk of developing epilepsy later in life at least five times according to a clinical study restricted to full-term births². The nature of the functional disturbance underlying such a secondarily acquired epilepsy is not yet understood.

During the past few years we have been engaged in experiments aimed at testing the hypothesis (based on the study of Sloper et al.³³) that perinatal hypoxic ischemia preferentially destroys the GABAergic (inhibitory) interneurons in the cerebral cortex and that

this GABAergic deficit, in turn, contributes to the higher incidence of epilepsy later in life. At first an *in vitro* model was used involving transient hypoxia in rat neocortex slab cultures. After a 3-day recovery period a significant reduction in the proportion of GABA-immunoreactive (GABA-IR) neurons was observed, a finding supporting the hypothesis (refs. 23, 25; see also ref. 24).

In the present study we tested the hypothesis *in vivo* by examining the effect of transient hypoxia on the proportion of GABA-IR neurons in the cerebral cortex of rat pups after four different recovery periods. The pups were 12–13 days old because at that age the degree of maturation of the cerebral cortex seems to correspond best with that of the human baby at term²⁶. The question whether this is a proper model of hypoxia-induced epilepsy has already been considered in other laboratories^{12,19}. Jensen et al.¹² in particular showed that the epileptogenic effect of transient hy-

Fig. 1. Schematic drawing showing the way of sampling in the cortical region of both hemispheres. Four randomly chosen areas in the penumbra around the core of a cortical infarct zone and their topographically corresponding areas on the contralateral "control" side are depicted by a square counting frame.

poxic ischemia, measured 1–7 days after the treatment, is most pronounced in immature animals treated between 5 and 12 days of age.

MATERIALS AND METHODS

Rat pups (Wistar WU strain, F_1 generation) of 12–13 days old were used. Cerebral hypoxia was induced using the procedure of Levine¹⁵, as modified by Rice et al.²² (see also ref. 32), involving a combination of unilateral ligation of one of the common carotid arteries and subsequent exposure to 8% O_2 . This procedure maintains a proper blood supply to the brain stem via the intact vertebral arteries and therefore has the advantage of a relatively low mortality rate. In addition, only one hemisphere ipsilateral to the ligated carotid artery becomes visibly affected by the treatment as judged by routine histology (refs. 15 and 22; and own observations) so that the contralateral hemisphere (remaining free of cell degeneration) can serve as a 'control'. Carotid artery ligation or hypoxia separately did not cause any cell damage in the ipsilateral hemisphere (unpublished pilot study).

Usually groups of eight animals were treated in one session. The right carotid artery was ligated under ether anesthesia (maximum operation time per animal 15 min), after which the animals were allowed to recover. Ninety minutes after the last operation, all animals were simultaneously exposed to hypoxic conditions (8% O_2 /92% N_2) for 90 min which, in unpublished pilot studies, was found to cause maximally dispersed nerve cell degeneration in the ipsilateral hemisphere together with minimal infarction and mortality. The ambient temperature was kept at 30°C and the relative humidity at 60–90%. In the present study, four groups received such a treatment. The animals were allowed to survive for 3, 7, 35 and 150 days and then sacrificed by perfusion fixation with 5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. In addition, three control rats, aged 15, 49 and 90 days, were studied that had neither been operated upon nor exposed to 8% oxygen. After fixation the brains were processed for Epon embedding. For each brain, four semi-thin (3 μ m thickness) transverse sections were cut approximately 3–5 mm

posterior to the bregma in order to study the parietal cerebral cortex where cell damage was found to be preferentially localized (unpublished pilot study). Moreover, at this level the cerebral cortex shows the lowest threshold and variability for convulsions evoked by direct electrical stimulation⁴⁰. Alternate sections were stained with either Toluidine blue (revealing all neurons and glial cells) or an antibody against GABA (established to selectively stain GABA-containing neurons^{3,31} using the conventional 3-step peroxidase-antiperoxidase method³⁷ followed by intensification with silver methenamine and gold chloride (unpublished method developed in our laboratory). Thus for each animal we obtained one pair of Toluidine blue and one pair of GABA-stained sections that dovetailed each other.

We identified and delineated in the hypoxia-affected cortical side of a Toluidine blue-stained section the area characterized by dispersed, selective neuronal death (i.e. the area showing a mixed population of healthy and abnormal neurons amidst an abundance of reactive glial cells, macrophages and debris). When surrounding an infarct zone such an area is called penumbra (Fig. 1). This area was then copied on a paper sheet with the aid of a drawing-tube attached to the light microscope. By using the second Toluidine blue-stained section and an unbiased square counting frame, we then copied and counted nuclear profiles belonging to neurons with a profile in only one of the two sections (the disector principle³⁶; see also refs. 9, 10), and estimated the number of neurons per unit volume of tissue ($270 \times 270 \times 6 \mu\text{m}^3$) which was unbiased for shape, shrinkage and swelling. Thereafter, the corresponding area in both GABA-immunostained sections was sampled in the same way (although now on the basis of perikarya) so that the number of GABA-immunoreactive (GABA-IR) neurons per unit volume of tissue could be established. From these two estimates, the percentage of GABA-IR neurons relative to the total number of neurons could be calculated. Finally, the analysis was repeated for the topographically corresponding area in the contralateral 'control' side. In this manner 11–25 areas in each cortical side were analyzed until at least 100 GABA-IR neurons per cortical side (and consequently about 5–6 times more Toluidine blue-stained neurons) had been counted in order to reach a coefficient of error of 10% or less (see refs. 8,14). Glial cell profiles and macrophages were only estimated and expressed per test area ($270 \times 270 \mu\text{m}^2$).

Fig. 2. A: hypoxia-damaged cortical area in the penumbra of an animal 3 days after the hypoxic insult (Toluidine blue-stained, 3- μ m-thick plastic section). Observe the degenerating neurons surrounded by dark glial cells and/or macrophages suggesting neurophagia (arrows). B: the corresponding area in an adjacent section stained for GABA by immunocytochemistry. Note in addition to the black neuronal cell bodies the numerous small GABA-immunoreactive nerve endings and/or transected fibres partly lining GABA-positive and GABA-negative cell perikarya. Clear and translucent profiles (arrows) correspond with the degenerating neurons in the Toluidine blue-stained section. Bar = 20 μ m.

TABLE I

Effect of hypoxic ischemia on the percentage of GABA-immunoreactive neurons in the cerebral cortex

GABA-immunoreactive neurons are expressed as a percentage of total nerve cells in a test volume ($270 \times 270 \times 6 \mu\text{m}^3$) for the ipsilateral hypoxia-affected cortical side vs. the matched contralateral 'control' cortical side; Δ stands for the difference between estimates on the two sides. In addition, the number of profiles of (reactive) glial cells, macrophages, and degenerating neurons per test area ($270 \times 270 \mu\text{m}^2$) are given.

Animal number	Survival time after hypoxic insult	GABA-IR neurons as a percentage of total number of neurons per unit volume of tissue		Δ	Number of profiles of (reactive) glial cells plus macrophages per test area		Number of profiles of degenerating neurons per test area	
		'Control' side	Hypoxic side		'Control' side	Hypoxic side	'Control' side	Hypoxic side
Experimental animals								
73	3 days	21.3	19.0	- 2.3	37	73	0	5.3
74	3 days	16.7	16.1	-0.6	9.2	32	0	9
76	3 days	20.1	10.5	-9.6	24	64	0	15
78	3 days	18.8	16.6	-2.2	30	43	0	11
102	7 days	17.0	16.5	-0.5	27	56	0	2
105	7 days	15.5	13.9	-1.6	18	69	0	5
108	7 days	15.3	13.3	-2.0	34	129	0	4
82	35 days	14.2	18.1	+3.9	20	38	0	0
84	35 days	12.6	13.5	+0.9	27	40	0	0
85	35 days	18.9	23.3	+4.4	19	61	0	0.5
87	35 days	16.1	18.6	+2.5	33	79	0	2
54	150 days	14.5	19.0	+4.5	23	57	0	0
Control animals								
		left	right		left	right	left	right
212	15 days	18.6	19.8	+1.2	28	31	0	0
174	49 days	17.8	17.9	+0.1	32	31	0	0
202	2 months	18.2	17.4	-0.8	25	28	0	0

RESULTS

Qualitative analysis

The localization, size and shape of the regions showing dispersed, selective neuronal death (penumbra if surrounding an infarct zone) in the neocortex ipsilateral to the ligated artery showed considerable variation from animal to animal. In a few cases cell death was not detectable at all, although it might have been present at a cortical level more anterior or posterior than that studied. These animals were not examined any further. In one case cortical tissue was reduced to a small rim over an extensive cavity (porencephaly); this animal was also omitted from further evaluation. Cortical damage often coincided with cell death in the ipsilateral hippocampus, striatum, thalamus and habenular nuclei.

In general, cortical cell death was abundant in layers III, IV and V, but also detectable as finger-like exten-

sions into layer II and/or layer VI (Fig. 1). The affected areas were characterized by a mixed population of healthy and abnormal nerve cell bodies amidst an abundance of macrophages, reactive glial cells and debris together with a deficit of small capillaries (Table I). During the early stages, i.e. at 3 and 7 days after hypoxia, such areas showed, in addition, signs of neurophagia (Fig. 2). Striking at later stages, i.e. 35 and 150 days after hypoxia, were the massive aggregates of clear and translucent profiles reflecting degenerated neurons. These translucent profiles were interspersed with healthy looking neurons (both GABA-positive and GABA-negative), small glial cells and/or macrophages, and numerous GABA-immunoreactive nerve endings and/or transected fibres. The translucent profiles did not show any positive staining in the adjacent Toluidine blue-stained sections (Figs. 3 and 5, compare with Fig. 4). And when observed by phase-contrast microscopy, their content was of the same optical density

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Fig. 3. A: hypoxia-damaged cortical area in the penumbra of an animal 35 days after the hypoxic insult (Toluidine blue-stained, $3\text{-}\mu\text{m}$ -thick plastic section). Note the small, dark glial cells scattered between the large neuronal cell bodies. B: the corresponding area in a consecutive section stained for GABA by immunocytochemistry. Conspicuous are the massive aggregates of clear, translucent profiles reflecting degenerated neurons of which no remnants are visible anymore in the Toluidine blue-stained section. When comparing both pictures, it is evident that more than 50% of the neuronal profiles in this particular affected area show a positive reaction for GABA. Some GABA-negative perikarya are indicated by arrows. Bar = $20 \mu\text{m}$.

as that of the extracellular space (not documented). Surprisingly, furthermore, was that even 35 days after the hypoxic insult, the sampled areas in the hypoxia-affected cortical side occasionally showed neurons surrounded by glial cells and/or macrophages and being evidently in an acute state of degeneration (Fig. 6, see also Table I). Degenerating neurons were never observed in the contralateral 'control' cortex.

The immunocytochemically stained sections showed an uneven distribution of GABA-IR profiles throughout the cerebral cortex on the 'control' side of the experimental animals as well as on both cortical sides of the control animals; they were more or less clustered in irregularly shaped patches. Such a pattern of areas either rich or poor in GABA-IR profiles was also seen in the penumbra directly bordering an infarct zone (Fig. 3). These uneven patterns were not due to local differences in the cell diameter, but reflected real differences in the proportions of GABA-IR neurons per test volume of tissue in such areas, as was shown by our quantitative estimations (data not given).

Quantitative analysis

Our cell counts revealed that the percentage of GABA-IR neurons was lower in the hypoxia-damaged cortex on the ipsilateral side than in the 'control' cortex on the contralateral side in each animal after a survival time of 3 ($n = 4$) or 7 days ($n = 3$) following the hypoxic insult (Table I). By contrast, in all the animals with longer survival times, i.e. 35 ($n = 4$) and 150 days ($n = 1$), the percentage of GABA-IR neurons was higher on the hypoxia-damaged cortical side than on the contralateral side. The control animals ($n = 3$) did not show such left/right differences in the percentages of GABA-IR neurons.

When the individual GABA percentages of the hypoxic vs. the 'control' cortical sides were averaged per survival time and subsequently analyzed (Fig. 7), the difference between the cortical sides was not statistically significant for any survival time. As already shown in Table I, the trend towards a lower proportion of GABA-IR cells in the hypoxia-affected cortical sides at 3 and 7 days after the insult became reversed from 35 days onwards. This conspicuous switch in the direction of the left-right differences (occurring between 7 and 35 days) turned out to be highly significant when the left/right differences in the proportion of GABA-IR cells for the individual animals (Δ , see Table I) per age group were mutually compared (Table II).

TABLE II

Statistical evaluation of the differences in the proportion of GABA-IR cells between the hypoxia-damaged and 'control' cortical sides

This table gives the P -values for the Wilcoxon-Mann-Whitney test (two-tailed) after comparison of groups with respect to the left/right differences in GABA percentages for the individual animals per survival time (Δ , Table I). As an analysis of variance between the four groups, the Kruskal-Wallis test indicated a significant effect ($P < 0.02$).

	Survival time after hypoxia exposure (days)			Control animals
	3	7	35	
3	—			
7	0.078	—		
35	$P \ll 0.001$	$P \ll 0.001$	—	
Control animals	0.048	0.063	0.048	—

In order to arrive at a better understanding of the significance of this switch we examined whether it resulted from changes throughout the entire cortical depth or it was restricted to the upper or deeper layers. To this end, we recalculated the proportion of GABA-IR cells for the upper (layers II–IV) and deeper part (layers V and VI) of the cerebral cortex separately on the basis of the respective cell numbers. However, this subdivision into upper and deeper cortical layers often led to a reduction of the counted GABA-IR cells per test volume far below 100, viz. in case the test volume happened to contain part of layer IV and V. As a result, the aimed coefficient of error of 10% or less was no longer reached. We therefore had to sum up the cell number estimates per cortical side per age group (Fig. 8).

DISCUSSION

Qualitative analysis

The observed relatively large variation in localization, size and shape of the hypoxia-damaged areas in the ipsilateral cortices of the experimental animals is in agreement with other experimental reports^{15,22,29,35} and with clinical observations^{6,16,18,38}. It was striking that even 35 days after the hypoxic insult degenerating neurons were occasionally detected in the ipsilateral cortex. It seems that limited cell injury becomes lethal either after a long latency or as a result of ongoing transneuronal degeneration. Long-term neuronal degeneration has also been described in the mature rat cerebral cortex 4 weeks after 30 min of middle cerebral

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Fig. 4. A: cortical area in the contralateral "control" side of an animal 35 days after the hypoxic insult (Toluidine blue-stained, 3- μ m-thick plastic section). B: the corresponding area in a consecutive section stained for GABA by immunocytochemistry. Bar = 20 μ m.

Fig. 6. Ipsilateral hypoxia-affected cortical area of an animal 35 days after the hypoxic insult (Toluidine blue-stained, 3- μ m-thick plastic section). Observe the couple of neurons surrounded by glial cells and/or macrophages that are evidently in an acute state of degeneration. Bar = 12.5 μ m.

artery occlusion²⁰. If such a protracted neuronal degeneration also occurs in the human brain after an hypoxic insult, therapeutic intervention could be effective even if implemented with some delay. Another observation worth mentioning was the occurrence of massive aggregates of translucent profiles (cavities) reflecting degenerated neurons in hypoxia-damaged cortical areas 35 and 150 days after the hypoxic insult. These cavities were interspersed with GABA-positive and GABA-negative neurons (apparently having survived the hypoxic insult), glial cells and/or macrophages, and numerous GABA-positive nerve endings and/or transected fibres. These findings suggest that a stabilized situation had developed.

The patchy distribution of the GABA-IR neurons in the cerebral cortex may reflect a columnar organization, which has been reported for the distribution of this cell type in the prefrontal cortex of the macaque monkey³⁰. It is not surprising, therefore, that as a result of the hypoxic insult areas either rich or poor in

GABA-IR neurons were also seen in the penumbra directly bordering an infarct zone. This observation may imply that the areas in the penumbra containing a low proportion of GABA-IR neurons lose part of their inhibitory input originally formed by afferents arising from other GABAergic neurons located in the adjacent infarct zone. Such a situation could then give rise to hyperexcitability. However, a penumbra area showing dispersed neuronal death but still containing a high proportion of GABA-IR neurons (see the present quantitative results) does not necessarily exclude the possibility of hyperexcitability either. The rationale based on the hypothesis of Babb et al.¹ is that both the GABAergic and non-GABAergic neurons in such areas are deprived of part of their targets originally located in the infarct zone. By a process of re-growth and sprouting this would lead to recurrent hyperinnervation of the neurons in such penumbra areas. In case the recurrent hyperinhibition is weaker than the recurrent hyperexcitation, seizure activity may occur. How-

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Fig. 5. A: hypoxia-damaged cortical area in the penumbra of an animal 150 days after the hypoxic insult (Toluidine blue-stained, 3- μ m-thick plastic section). B: the corresponding area in an adjacent section stained for GABA by immunocytochemistry. Note that the histological picture does not differ in essence from that observed after a survival time of 35 days (see Fig. 3A and B). Bar = 20 μ m.

Fig. 7. Mean percentage of GABA-immunoreactive neurons ($\pm$ S.E.M.) in the hypoxia-damaged cortical side (hatched bar) vs. the matched 'control' cortical side (dotted bar) for the various survival times after exposure to hypoxia. Numbers above the bars represent the number of animals examined. For the unoperated control animals, both cortical sides were sampled in the same way as was done for the experimental animals. The Wilcoxon signed ranks test (two-tailed) gave for the difference between the cortical sides for each of the first three survival times a *P*-value of 0.125 (applied on the left/right differences in GABA percentages on the individual animals, Δ , Table I).

ever, in case hyperinhibition is stronger, but in functional terms badly tuned to the excitatory activity, seizure activity may occur as well, since GABAergic hyperinhibition may lead to disinhibition. Moreover, GABAergic inhibition is believed to function, among

Fig. 8. Percentage of GABA-immunoreactive neurons calculated on the basis of the summed cell numbers of the individual animals per group broken down according to upper (II, III, IV) and deeper (V, VI) cortical layers. The dotted and hatched bars represent, respectively, the "control" and the hypoxia-damaged cortical sides. Numbers above the bars represent numbers of animals examined. For the unoperated animals the same procedure as for the experimental group was performed.

others, as an important drive for synchronizing spatially dispersed electrical activity^{1,7}, but over-abundant synchronization of electric activity is one of the main features of epileptiform activity⁴.

Quantitative analysis

With respect to Fig. 7, the low number of animals probably precluded reaching statistical significance for the relatively small differences in the proportion of GABA-IR cells between the hypoxia-damaged and 'control' cortical sides at the different survival times. Nevertheless, the trend at 3 days after the hypoxic insult was in keeping with the outcome of our previous in vitro studies, in which a statistically significant preferential decrease of GABA-IR neurons was detected in cortical slab cultures 3 days after hypoxia^{23,25}. However, the trend changed significantly after postischemic times longer than 7 days (which was not investigated in the previous in vitro study), namely the proportion of GABA-IR neurons was found to be higher in the damaged cortical side than in the 'control' cortical side from 35 days onwards. It seems, therefore, that in the long run there is no preferential degeneration of GABAergic neurons in the hypoxia-damaged neocortex, the trend being rather the opposite. Thus, our original hypothesis that cerebral hypoxia in early life ultimately leads to a preferential degeneration of GABAergic (inhibitory) neurons in the cerebral cortex, which could then account for the reported increased risk of developing epilepsy later in life, is not supported by the present observations.

The present findings are in keeping with reports of Johansen et al.¹³, Nitsch et al.²¹ and Schlatter et al.²⁸, who found no preferential loss of GABAergic neurons in the relatively vulnerable hippocampus of animals previously exposed to hypoxia. Sloviter³⁴ and Babb et al.¹, studying kindled and human epileptic hippocampus, respectively, observed not only a substantial cell loss, but also a relatively high resistance of the GABAergic neurons against the adverse effect of repetitive seizure activity. These findings and our observations suggest, therefore, that perinatal hypoxia-induced epilepsy originating in the hippocampus or neocortex – brain structures generally known to represent important sites of epileptiform activity – is not the functional consequence of a preferential loss of GABAergic interneurons; the defect must involve other components of the neural network. Two possibilities were already mentioned before under the heading 'Qualitative analysis'.

With regard to the significant switch in the direction of the left-right differences in the proportions of GABA-IR neurons between 7 and 35 days after the

hypoxic insult (Δ , Table II), the following should be considered. First of all, neuronal cell migration in the developing rat neocortex is completed before the end of the first postnatal week³⁸, while naturally occurring cell death in the neocortex has already diminished to about one degenerating cell per 1000 live neurons at 12 days⁵. In the present work we did not find any degenerating neurons in the 'control' cortical side of the experimental animals. Secondly, the GABA content and thus the GABA immunoreactivity of GABAergic neurons may substantially change according to the functional, metabolic state of the cells. As such their reactivity can drop below or rise above the immunocytochemical detection level (see, e.g. ref. 11). The switch in the direction of the differences in the proportion of GABA-IR cells between the hypoxia-damaged and 'control' cortical sides may reflect, therefore, the operation (separately or in combination) of the following processes: (a) a preferential degeneration of GABAergic neurons and/or a loss of GABA-immunoreactivity in the hypoxia-damaged cortical side during the first week after the hypoxic insult; (b) a decrease in GABA-immunoreactivity in the 'control' cortical side, only beginning after 3 days, (c) a return of GABA-immunoreactivity to the control level in the hypoxia-damaged cortical side after 7 days, and/or a long-term ongoing degeneration of pyramidal (non-GABAergic¹⁷) cells. An implication of option (b) is that the 'control' cortical side of the experimental animals is also affected after the hypoxic insult, probably as a result of cell damage in other parts of the brain.

Fig. 8 suggests that the alterations in the proportion of GABA-IR cells in the hypoxia-damaged cortical side mainly occur in the deep cortical layers, whereas those in the 'control' cortical side take place in the upper layers. Thus, in the developing rat cerebral cortex the alterations induced by perinatal hypoxic ischemia, which presumably depend on the properties of the neurons and their role in the neuronal circuits: (i) show a complex picture; (ii) range over a relatively long period; and (iii) extend towards remote areas that were not directly damaged by the hypoxic insult such as the contralateral neocortex. The latter may result in additional loss of function.

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