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POSTNATAL DEVELOPMENT OF HIPPOCAMPAL AND NEOCORTICAL CHOLINERGIC AND SEROTONERGIC INNERVATION IN RAT: EFFECTS OF NITRITE-INDUCED PRENATAL HYPOXIA AND NIMODIPINE TREATMENT

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Abstract—Postnatal development of ingrowing cholinergic and serotonergic fiber patterns were studied in the rat hippocampus and parietal cortex employing a histochemical procedure for acetylcholinesterase as a cholinergic fiber marker, and immunocytochemistry of serotonin for serotonergic fiber staining. The rat pups were killed at postnatal days 1, 3, 5, 7, 10, and 20. The development of cholinergic and serotonergic innervation was described and the fiber density quantified under normal conditions and after long-term prenatal anemic hypoxia induced by chronic exposure to sodium nitrite. Furthermore, a third group was studied in which the nitrite hypoxia was combined with a simultaneous treatment with the Ca^{2+} -entry blocker nimodipine to test the neuroprotective potential of this drug. Quantitative measurement of fiber density from postnatal day 1 to day 20 yielded the following results: (i) both neurotransmitter systems revealed an age-dependent and an anatomically-organized developmental pattern; (ii) the serotonergic innervation of the dorsal hippocampus preceded that of cholinergic afferentation in postnatal days 1–3; (iii) prenatal hypoxia induced a transient delay in the innervation of parietal neocortex and dentate gyrus for both neurotransmitter systems, but left the innervation of the cornu ammonis unaffected; and (iv) the hypoxia-induced retardation of cholinergic and serotonergic fiber development was prevented by concomitant application of the Ca^{2+} -antagonist nimodipine during the hypoxia.

The results indicate that prenatal hypoxia evokes a temporary delay in the cholinergic and serotonergic fiber outgrowth in cortical target areas in a region-specific manner. The hypoxia-induced growth inhibition is prevented by the calcium antagonist nimodipine, which supports the importance of the intracellular Ca^{2+} homeostasis of cells and growth cones in regulating axonal proliferation.

During postnatal development of the brain the timely arrival of axonal terminations originating from subcortical projection neurons to their intracortical target cells may be regarded as being of key importance in the establishment of cell to cell connections including synaptogenesis. The ingrowth and local arborization of extrinsic fibers during early postnatal development are determined not only by genetic factors but also by environmental neurochemical influences. Neuroteratological research has produced ample evidence on the vulnerability of the developing brain to hazard and toxic factors in the perinatal age. Among them sodium nitrite (NaNO_2), also as a contaminant in food and drinking water, is regarded as a toxin which induces methemoglobinemia and thereby leads to a chronic type of anemic hypoxia in both humans^{9,76} and in experimental animals like

rats.^{25,33} Previous studies in our laboratory show that prenatal nitrite exposure affects motor activity⁵⁶ and influences cognitive and novelty-induced behaviors⁵⁵ in the offspring.

In the adult brain the hippocampus and cerebral cortex are extensively innervated by cholinergic (ACh)^{15,18,32,44} and serotonergic (5-HT)^{52,75} fiber systems from subcortical origin, both of which were shown to be intimately involved in mechanisms of learning and memory^{6,47,58,76,79} and pathogenesis of depression and anxiety.^{8,35} The forebrain cholinergic neurons are susceptible to even mild hypoxic insult in adult age.^{21,22,70} Interestingly, the developing cholinergic neurons, at least at the level of the cell body, are relatively resistant to hypoxia as was shown *in vivo* and *in vitro* conditions.^{17,34} It remains unclear, however, how perinatal hypoxia and other hazard factors may influence the axodendritic development of cholinergic neurons.

There are several histochemical^{23,45,50,63} and biochemical^{53,77} reports on the postnatal development of septohippocampal cholinergic projection based on the analysis of cholinergic marker enzymes acetylcholinesterase (AChE) and choline acetyltransferase

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Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CA1, cornu ammonis field 1; ChAT, choline acetyltransferase; DG, dentate gyrus; 5-HT, serotonin; 5-hydroxytryptamine; PAP, peroxidase-antiperoxidase; PBS, phosphate-buffered saline; PD, postnatal day.

(ChAT). These studies and the results obtained with high-affinity choline uptake in the developing hippocampus⁶⁸ led to the conclusion that cholinergic afferentation to the hippocampus in rat is only minimal prior to postnatal age of three days (PD3). The development of neocortical cholinergic innervation has also been studied by AChE histochemistry and ChAT immunocytochemistry.^{13,23,37,64} The observations in these studies, however, were restricted to a few selected postnatal ages, dealt with limited parts of the forebrain or answered specific developmental questions. Since the rat brain develops very rapidly in the early postnatal period⁶⁶ and the number of synapses in dentate gyrus (DG) is doubled each day from the period of PD4 to PD11,¹⁰ we investigated a more graded time-course of development of innervation patterns. The density of AChE-positive fibers was quantified in the parietal cortex and in the dorsal hippocampus at PD1, 3, 5, 7, 10, and 20 under control and nitrite-induced hypoxic conditions.

A preliminary study from this laboratory⁵⁶ demonstrated that prenatal hypoxia in rat not only affects the cholinergic system, but also disturbs the normal development of serotonin fiber proliferation. 5-HT, apart from its function as a neurotransmitter, is considered as a differentiating^{39,48} and a trophic^{12,38} chemical signal during early neurogenesis. It has also been shown that 5-HT, and also ACh, is a potent regulator of growth cone behavior.^{46,85} The postnatal development of serotonergic ascending pathways and terminal fields in the forebrain was previously studied with immunohistochemical methods in the rat^{41,82} and various other mammalian species.^{19,78,81} In the present experiment we investigated the normal and the prenatal hypoxia-modulated development of 5-HT innervation in several hippocampal regions and neocortical areas. Furthermore, the 5-HT innervation was compared with the development of AChE-positive afferentation within the same experiment allowing us to study the associative aspects of the two neurotransmitter systems during normal development and after prenatal hypoxia. Concurrent study of ACh and 5-HT in early neurogenesis is of particular interest because of the reported neurotrophic interaction between both transmitter systems in brain growth.^{38,39}

Several lines of evidence point to the specific role of intracellular free calcium ion in the growth process of the developing nerve cell.^{36,46,48} It is well documented that hypoxia and ischemia lead to an intracellular Ca^{2+} overload, which correlates to neuronal injury and degeneration.^{27,40,49,71} Drugs preventing the excessive influx of Ca^{2+} may have a beneficial effect on preventing or attenuating brain damage caused by hypoxia or ischemia.^{40,71} Calcium antagonists of the dihydropyridine class such as the L-type channel blocker nimodipine have received attention recently as potent neuroprotective and anti-ischemic drugs.^{31,55-57,65,69,71} Therefore, in the present study a

third group of animals was simultaneously treated both with nitrite and nimodipine in the prenatal period to test the effects of nimodipine in hypoxia-induced developmental brain damage.

In summary, the present investigation addresses the following questions: (i) how do AChE positive and 5-HT immunoreactive fiber systems develop in selective forebrain regions that participate in cognition, i.e. the dorsal hippocampus and parietal cortex? (ii) how is this developmental process affected by prenatal anemic hypoxia? and (iii) can the hypoxic effects on these developing forebrain systems be antagonized by a potential anti-ischemic calcium-entry blocker? In order to assess the possible effects of treatments on the general growth of the hippocampus, the thickness of cell and fiber layers of the hippocampus in the various experimental groups was measured by image analysis.

EXPERIMENTAL PROCEDURES

Animals and treatments

Pregnant Wistar rats, assessed by vaginal smears, were group-housed until the 11th postconceptional day. From this day on individual animals were kept isolated and randomly assigned to one of three groups. The first group was treated with nimodipine (BAY E 9736) at a concentration of 1000 p.p.m. suspended in standard food pellets. The other two groups received placebo rat chow. Two days later, so beginning on day 13 of gestation until delivery, sodium nitrite (NaNO_2) was offered in the drinking water *ad libitum* in a dose of 2 g/l to one of the placebo-fed groups and to the nimodipine-treated rats. Consequently, the three groups were as follows: (i) a control group receiving placebo food and normal drinking water, (ii) a hypoxic group provided with placebo food and drinking water containing sodium nitrite, and (iii) a hypoxic group treated with nimodipine via nitrite-containing water and nimodipine-containing food pellet. The nitrite solution was refreshed daily. The nitrite treatment induced a methemoglobinemia in the dams as was previously documented.⁵⁶ Near the end of gestation the animals were checked twice daily for recording the time of birth with a precision of 12 h. All treatments were terminated after parturition after which the litters were culled to eight pups and raised under normal routine laboratory conditions including a fixed light-dark cycle (light on from 07.00 to 19.00 h). The first 24 h after delivery were considered as PD1. Male offspring of different ages (PD1, 3, 5, 7, 10, and 20) were used in the experiments. From each litter group only a single animal was assigned to one of the age-groups to avoid the interfering effect of differences among litters. The brains of pups of different ages (from PD1 to PD20) were processed for staining of AChE- and 5-HT-positive fibers.

Acetylcholinesterase fiber staining

Pups at ages of PD1–20 were deeply anesthetized with pentobarbital and transcardially perfused with a buffered mixture of 2.5% glutaraldehyde, 0.5% paraformaldehyde, and 4% sucrose in 0.05 M phosphate buffer pH 7.4. After postfixation of 8–12 h and dehydration in a 30% phosphate-buffered sucrose solution, 20- μm (PD1, 3, 5, and 7) or 10- μm (PD10, and 20) coronal sections were cut on a cryostat microtome. The sections were collected serially and stained for AChE according to the AChE-histochemical procedure of Hedreen *et al.*,²⁹ yielding a clear staining pattern of AChE-positive fibers against a light background.

Briefly, sections were rinsed in five changes of 0.1 M acetate buffer pH 6.0 and incubated for 1 h in 0.05% acetylthiocholine-iodide, 4 mM sodium citrate, 3 mM cupric sulfate, and 0.1 mM potassium ferricyanide in 0.065 M acetate buffer pH 6.0. The initial staining was intensified by subsequent processing with a 1% sodium sulfide and 0.1% silver nitrate solution.

Serotonin immunohistochemistry

To enhance immunostaining from serotonin the pups were drug pretreated before transcardiac perfusion as described by Zhou *et al.*⁸⁷ Namely, a monoamine oxidase inhibitor pargyline (100 mg/kg) and L-tryptophan (100 mg/kg), a 5-HT biosynthesis precursor were injected i.p. 1.5 and 1 h, respectively, prior to fixation of the brain. Under deep pentobarbital anesthesia the pups were quickly perfused transcardially with cold heparinized saline containing 0.1% MgSO_4 ⁸⁷ for 12–15 s. The saline pre-rinse, depending on the age, at a perfusion rate of 15–35 ml/min was followed by a high-speed perfusion with 4% paraformaldehyde and 15% saturated solution of picric acid in 0.05 M phosphate buffer pH 7.4 at 4°C. The brains were postfixed 6–8 h, dehydrated in a 30% sucrose solution and sectioned at the cryostat microtome at a thickness of 30 μm . The sections were rinsed in several washes of phosphate-buffered saline (PBS) and incubated in 0.25% H_2O_2 -PBS for 30 min to exhaust endogenous peroxidase. The immunostaining procedure consisted of subsequent exposures to 5% normal goat serum, polyclonal rabbit anti-serotonin (Sanbio) diluted at 1:1500 with 0.1% azide PBS for 72 h at 4°C, goat anti-rabbit IgG (Tago) 1:50 for 24 h, 4°C, and finally to rabbit peroxidase-antiperoxidase complex (PAP, DAKO) 1:300 for 4 h. The PAP labels were stained with 0.05% diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer pH 7.6.

Fiber morphometry

The relative density of AChE- and 5-HT-positive fibers was measured in the dorsal hippocampus and in a strip of parietal cortex perpendicular to the lateral ventricle. Sections equivalent to the adult brain at the anterior-posterior level of -3.3 to -3.8 mm relative to Bregma³⁹ were selected for measurement. For quantification of fiber density an ocular with a built-in counting grid was applied at $10 \times 40 \times 1.25$ magnification. At this magnification one grid square measured $40 \times 40 \mu\text{m}$. A row of squares was radially positioned over the entire thickness of hippocampus CA1 and inner blade of DG, or over the parietal cortex (inserts in Figs 7–10, and 12, 13). The mediolateral position of the grid chain in dorsal hippocampus measurements was adjusted to the middle of the inner blade of DG. The relative fiber density was established by counting the crossings of fibers with the longitudinal and intersecting transversal grid lines at both sides of the selected brain section.

The size of perfused brains was measured in three dimensions (length, width, and height) before postfixation with an accuracy of 0.1 mm in order to correct fiber density measurements to brain growth. For the expression of relative brain size the volume measures of $l \times w \times h$ obtained at 10 days old animal were taken as a 100% value (factor 1.00). In the other age groups to correct for the dimensions of the sections for brain growth, the following correction factors were applied calculated from the $l \times w \times h$ values of the respective ages: 0.43 (PD1), 0.55 (PD3), 0.68 (PD5), 0.84 (PD7), and 1.31 (PD20).

Morphometry of cell layers

Adjacent sections, stained with Cresyl Violet, at the same anterior-posterior level used for assessment of fiber density were selected to measure the surface areas of pyramidal and

Fig. 1. Camera lucida drawings showing the development of AChE-reactive fiber patterns in the dorsal hippocampus of rats at PD3–10. The fissura hippocampi, dividing the hippocampus into CA1 and DG, is indicated by asterisks. The section thickness is indicated at the bottom right of each panel. Note that from PD3 to PD7 the drawing is made of 20- μm -thick sections. For PD10 the section thickness is 10 μm because of the very high density of the fibers.

Fig. 2. Series of camera lucida drawings of AChE-positive fiber patterns in the developing parietal cortex. The arrows indicate the border between superficial layers I–IV and the deeper layers V–VI. The inserts at the bottom left of the figures indicate the selected cortical region (stippled area) and the animal number.

granule cells and their apical dendrites. At a magnification of $105\times$, a columnar strip of $550\mu\text{m}$ width of hippocampal tissue including the CA1 and DG was measured by using a Zeiss image analysis system (IBAS). Within the hippocampal strip the surface area of the pyramidal cell layer, the DG inner blade granule cell layer, and the corresponding apical dendritic fields of these principal neurons, i.e. molecular layers were determined and expressed as $\mu\text{m}^2 \times 10^3/\text{mm}$ column width. Two sections were selected and from each animal two sections were measured bilaterally. The mean values were processed per group for statistical analysis.

The statistical evaluation of morphometric data was carried out with analysis of variance (ANOVA) followed by a *post hoc t*-test for comparison between two groups according to the STATS statistical package.

RESULTS

Development of hippocampal and neocortical cholinergic innervation

The AChE cytochemical staining of Hedreen *et al.*²⁹ proved to be a reproducible method to visualize the patterns of ingrowing cholinergic fibers and their

discrete regional and laminar distribution in the developing hippocampus (Figs 1, 10) and in parietal neocortex (Figs 2, 3). In both brain areas the fiber density increased markedly with age as it can be observed in the series of camera lucida drawings (Figs 1, 2). For the sake of comparison between drawn and calculated fiber densities, the numerical data of AChE fiber countings for the untreated controls from PD1 to PD20 have been summarized in Figs 7, 8, 9, 11. The camera lucida drawings were made from those control individuals which we considered as typical representatives of their age-groups. At PD1 the cholinergic fibers as demonstrated by AChE histochemistry are extremely sparse in the dorsal hippocampus and for this reason were not included in Fig. 1. At PD3 all CA regions became diffusely innervated by cholinergic fibers except CA4 in the hilus of DG. The inner blade of DG also received a dense innervation, which at this age was almost exclusively confined to the supragranular region. This pattern was followed by the appearance of

Fig. 3. Darkfield photomicrographs of the development of AChE-positive fiber patterns in the parietal neocortex of rats at PD1 (A), PD3 (B), PD7 (C), and PD10 (D). MZ, marginal zone; CP, cortical plate; SP, subplate. Arrowheads show the border between cortical plate and subplate (at PD1 and PD3) or layers IV and V (at PD7 and PD10). Arrows in panels A and B point to AChE-positive cell bodies. Scale bar = $50\mu\text{m}$; all panels have the same magnification. Section thickness in A–C is $20\mu\text{m}$, and in D is $10\mu\text{m}$.

Fig. 3.

a dense plexus in other sublayers like infra- and suprapyramidal regions at PD3 to 5 and stratum lacunosum-moleculare at PD5–7, sublayers known to contain characteristically dense cholinergic fiber networks in the mature hippocampus. The density of afferent fibers showed an increment in the infragranular as well as in the hilar regions from PD3 to PD5 (Fig. 10A). Fiber proliferation in the apical dendritic fields of principal neurons (pyramidal and granule cells) followed a typical developmental pattern. The supragranular network, which could be clearly distinguished at PD3 and 5, became more widespread in seven- and 10-day-old animals which coincides with the rapid growth of apical dendrites of the granule cells in this postnatal period. By PD10 an advanced fiber pattern resembling the adult age condition has practically been reached.

Contrary to the dorsal hippocampus, in the parietal neocortex a considerable fiber ingrowth could be observed in the deeper layers even in newborn rats (PD1; Figs 2, 3A). This initial proliferation was most dense in the subplate region. Only few, radially oriented fibers infiltrated the cortical plate, the direction of axonal growth being predominantly from deep to superficial layers. At PD1 no fibers of extrinsic origin were identified in the marginal zone under the pial surface. From PD3 on the marginal zone received a gradually increasing AChE-positive innervation by axons spreading under the pial surface and leading to the formation of a cholinergic fiber network in molecular layer I (Fig. 3B). At PD1 and PD3 a number of AChE-positive cell bodies could be distinguished in the marginal zone but also in other layers of the cortex (Fig. 3A, B). The number of these cells decreased from PD5 on. After PD5 by the age of PD7 the fiber density dramatically increased (Figs 2, 3C). Due to the rapid development and displacement of neurons from the cortical plate, a radial arrangement of cholinergic fibers parallel to the apical dendrites of developing pyramidal neurons became more and more apparent. In the neocortex, rather than in the hippocampus, it was easier to recognize that the AChE fiber ingrowth appeared in a patchy-like pattern, probably due to locally isolated proliferation of a single or few axons, as can be seen in the deeper layers of the parietal cortex at PD1 and PD3 (Fig. 2). After PD5 the ramifications became more intense in the deeper layers and the fiber patches were solved into a massive innervation network.

In general, the innervation of hippocampus and neocortex by afferent cholinergic fibers proceeded from the anterior to more posterior regions. Therefore, the ventral hippocampus or the occipital cortex became innervated later than the currently described structures that were subject to quantitative analysis, i.e. the dorsal hippocampus and the parietal cortex. With respect to the neocortex, the direction of cholinergic fiber development proceeded not only from rostral to caudal but also from deeper to more superficial layers.

Development of hippocampal and neocortical serotonergic innervation

Similar to the cholinergic fibers the serotonergic axons exhibited a marked increase in their proliferation in the course of the PD1 to PD20 period. However, contrary to the cholinergic system numerous 5-HT-immunoreactive fibers were found to invade both the CA and DG areas of the hippocampus of newborn rats at PD1 (Figs 4, 5A). With regard to the developing cytoarchitectonic hippocampal lamination the 5-HT immunoreactive structures were more evenly distributed within the hippocampus as compared to the cholinergic system development (cf. Figs 1 and 4), especially in the early postnatal period (PD1–5). The hilar region received a considerable number of 5-HT fibers in the early postnatal ages, but the rest of DG inner blade and CA1 were relatively more densely innervated. In newborn rats (PD1), due to the underdeveloped apical dendrites of principal neurons, 5-HT fibers were grouped around the hippocampal fissura (Fig. 5A). From PD3 to PD7 the distance between the layers of pyramidal and granule cell bodies increased and the apical dendritic fields were filled up with an increasing amount of 5-HT positive fibers. At PD7 a dense network located at the level of the stratum lacunosum-moleculare started to emerge (Figs 4, 5D). At PD10 the fiber pattern was essentially indistinguishable from that of 20-day-old or adult rats.

Unlike the development of innervation patterns seen in the hippocampus, the innervation of parietal cortex by 5-HT fibers followed a developmental pattern more similar to the AChE-positive system outgrowth (cf. Figs 5 and 2). At PD1, the superficial layers (cortical plate and marginal zone) received only few 5-HT positive fibers. From PD3 on, proliferation in all layers advanced intensively. The innervation pattern in superficial layers at PD7 and 10 was different from that of AChE fibers. The 5-HT axons in layers IV to II displayed more diffuse randomly organized arborization networks, in contrast with the mostly radially oriented cholinergic fibers. The local ramifications of axons, both in the hippocampus and even more so in the parietal cortex, resulted in the already mentioned patchy-like fiber proliferations. This phenomenon could be distinguished at all ages shown in Figs 4–6.

A second structural formation which is typical for neuritic development, the growth cone, was frequently seen in the 5-HT-immunostained material. Considerable numbers of growth cones were observed in virtually all areas and layers in the hippocampus and parietal cortex, and were especially noticeable at PD1 and PD3 (Fig. 5).

Comparison of development of the two neurotransmitter systems

Since the camera lucida drawings (Figs 1, 2, 4, 6) and the numerical data of fiber-crossing measure-

Fig. 4. Postnatal development of serotonergic fiber proliferation in the dorsal hippocampus shown by camera lucida drawings of sections from PD1 to PD10 animals. Asterisks point to the fissura hippocampi. Pyramidal (Pyr) and granular (Gr) cell layers are marked. The section thickness for each age was 30 μ m. Inserts show the selected areas of CA1 and DG (stippled) and the serial number of animals.

ments (Figs 9, 11–13) were made from sections of standard thickness, corrections for brain growth were necessarily applied in order to obtain a more accurate quantitative description of age-dependent changes in fiber density. Moreover, this way a comparison between the dynamics of fiber proliferations in the two investigated neurotransmitter systems also became available. The corrected numbers of fiber density in the dorsal hippocampus and in the parietal cortex were expressed as a percentage relative to the values obtained in the CA1 and in the deep cortical layers of PD10 animals, respectively (Figs 7, 8). The data were analysed with ANOVA using repeated measures for regions, and two independent variables such as neurotransmitter system and age. An age-dependent increase in density was clearly established both for neurotransmitter systems and for brain regions ($P < 0.001$). The increment of cholinergic fiber density was steeper than that of serotonin in the hippocampus, due to a low density in the early postnatal days and a marked fiber proliferation from PD10 to PD20 (significant interaction between age and neurotransmitter system, $P < 0.001$). In the latter two ages the proliferation of serotonergic fibers appeared to slow down. In the parietal cortex the same tendencies, although less pronounced, were also present. The delayed innervation of superficial layers by both transmitter systems was typical for the parietal cortex

($P < 0.001$). This delayed innervation proved to be age-dependent in the case of cholinergic fibers ($P < 0.001$) but was not statistically significant in the case of serotonergic fibers ($P = 0.09$).

Treatment effects on cholinergic fiber development

The effects of anemic hypoxia imposed by nitrite exposure with and without nimodipine treatment on AChE fiber density in the two investigated brain areas are shown in Figs 9–11. For statistical evaluation of treatment effects the two hippocampal regions (CA1 and DG) were separately subjected to analysis (Fig. 9). PD20 data were left out from the ANOVA test because of the insignificant differences between the various groups. Both in CA1 and in DG there were overall differences between groups due to the effect of treatments (CA1: $F_{2,61} = 3.94$, $P < 0.05$, DG: $F_{2,61} = 5.49$, $P < 0.01$). The nitrite treatment influenced the fiber density significantly only in DG ($F_{1,39} = 3.92$, $P < 0.05$), while its effect was insignificant in the CA1 region ($P = 0.29$). This result points to a region-selective action of nitrite treatment. Hypoxic animals which were concomitantly treated with nimodipine showed a consistently higher density of AChE fibers in both hippocampal subdivisions as compared to nitrite treatment alone (CA1: $P < 0.05$, DG: $P < 0.002$). Therefore, the nimodipine treatment prevented the growth inhibiting action of nitrite. The

Fig. 5. 5-HT-immunoreactive fibers in the dorsal hippocampus of one, three, five and seven days old rats (A, B, C, and D, respectively) innervating CA1 and DG. The distance between pyramidal (p) and granule (g) cell layers increases with age and gives an idea on the growth of apical dendritic extent of these principal neurons. Asterisks mark the fissura hippocampi. Arrows in panels A and B point to growth cones. Calibration bar is 50 μ m. Section thickness in each case is 30 μ m.

Fig. 6. Developmental profile of serotonergic fiber patterns in the course of PD1 to PD10 in the parietal cortex of rats. Arrows indicate the border between superficial and deep layers of the neocortex. The thickness of sections is 30 μ m. The insets show the cortical strips selected for presentation just above the lateral ventricle.

Fig. 7. Dynamics of cholinergic (AChE) and serotonergic (5-HT) fiber ingrowth into the developing hippocampus between PD1 and PD20. Values are expressed as percentages of fiber density obtained in CA1 area of PD10 animals (100%) and are corrected for brain growth (see text). Means $\pm$ S.E.M.s are shown. The number of animals in the age-groups are indicated under the columns.

Fig. 8. Comparison of relative density of cholinergic (AChE) and serotonergic (5-HT) fiber proliferations in superficial and deeper layers of the parietal cortex during postnatal development from PD1 to PD20. All values are corrected for the increase of brain size and section thickness and expressed as percentage of mean values obtained in PD10 deep layer. Means $\pm$ S.E.M.s are shown from three to nine animals per group as indicated under the columns.

results of contrast analysis following ANOVA using *t*-test with pooled variance between two groups are shown above the columns in the figure and marked by symbols. The number of fiber crossings was decreased in PD5 and PD7 animals after hypoxic treatment in DG. At both ages nimodipine prevented this effect since the effect of hypoxia against the control group was not present with simultaneous nimodipine administration (Fig. 10). Furthermore, at PD10 nimodipine-treated animals displayed an even higher AChE positive fiber density than did controls in both regions of the hippocampus.

The prenatal treatments exerted similar influences on the development of cholinergic fiber arborization in the parietal cortex (Fig. 11). The AChE positive fibers in the deeper layers V and VI were more numerous than in the superficial layers I–IV, especially in the early postnatal period of PD1–5. The statistical analysis with ANOVA was carried out for ages PD3–10. It revealed a treatment effect which was restricted to the deeper layers ($F_{2,66} = 3.71$, $P < 0.05$). The nitrite treatment resulted in a lower fiber density ($F_{1,45} = 5.67$, $P = 0.02$). The nimodipine-treated hypoxic animals were not different from controls, but showed significantly more fibers as compared to the nitrite plus vehicle-fed animals ($F_{1,45} = 4.53$, $P < 0.05$). The nitrite treatment was most effective at PD3 and PD5 as marked with asterisks above the columns in Fig. 11.

Treatment effects on serotonergic fiber development

The effects of prenatal nitrite and nimodipine treatments on the postnatal increment of 5-HT fiber density in hippocampus and parietal cortex are shown in Figs 12–14. An overall effect of treatment was found in DG (ANOVA, PD3–PD20, $F_{2,67} = 5.29$, $P < 0.01$, Fig. 12), but not in CA1. Comparing

control and nitrite-treated groups revealed that the latter group displayed a less dense postnatal fiber ingrowth ($F_{1,44} = 5.97$, $P < 0.02$). The nimodipine treatment prevented the growth retarding effect of nitrite since no statistical difference was found between control and nitrite-nimodipine groups ($P = 0.35$), while there was a marked difference between the two nitrite-treated groups as a result of nimodipine treatment ($F_{1,44} = 9.09$, $P < 0.005$). The developmental inhibition of nitrite in DG was most pronounced at PD7 indicated by the result of the *t*-test ($P < 0.05$).

The different treatments altered the development of the 5-HT fiber density in both deep and superficial divisions of the parietal cortex (layers I–IV: $F_{2,67} = 6.11$, $P < 0.005$; layers V–VI: $F_{2,67} = 3.56$, $P < 0.05$). Comparisons between groups were made after ANOVA with *post hoc t*-test, and the results are indicated in Fig. 13. At PD7 5-HT fiber density was significantly lower in the hypoxic animals in both layers of the parietal cortex. This was also the age which showed nitrite treatment effect in the hippocampal DG (Fig. 12). Interestingly, at PD10 a higher fiber density could be observed in the nitrite-nimodipine group as compared to the other groups including controls. This result indicates that nimodipine not only antagonized the action of hypoxia, but also induced an advanced development of 5-HT fiber ingrowth (Fig. 14).

Treatment effects on neuronal development in the hippocampus

The anatomical areas occupied by the cell bodies and apical dendrites of pyramidal cells in CA1 and granule cells in DG were measured in order to observe or exclude a possible cell loss after prenatal hypoxia and nimodipine treatments. The size of cell

Fig. 9. Effect of prenatal nitrite (hypoxia) and nitrite + nimodipine treatments on cholinergic (AChE) fiber density in the dorsal hippocampus measured in the course of PD3 to PD20. Measurements were taken from CA1 and from the inner blade of DG, as indicated by the insert at top left. Means $\pm$ S.E.M.s are shown from four to nine animals per group. PD1 values were left out because in a large number of animals only a very few AChE-positive fibers could be identified. ANOVA revealed a significant effect of prenatal hypoxia in the dentate gyrus between PD3 and PD10: $F_{1,39} = 3.92$, $P < 0.05$. Results of *post hoc* two-tailed *t*-test: * $P < 0.05$, ** $P < 0.01$ vs control group, ° $P < 0.05$ vs nitrite-treated group).

and dendrite layers were measured in those postnatal days in which changes in ACh and 5-HT afferent fiber density could be observed. The results are summarized in Table 1. The thickness of the pyramidal cell layer did not change between PD5 and PD7 but increased at PD10, while the dendritic molecular layer showed a consistent growth at all three ages. Increase in thickness of both cell bodies and dendrites

were also measured between all ages in the DG. The statistical analysis with ANOVA revealed a significant effect of age in all areas as follows: CA1 pyramidal cell bodies: $F_{2,54} = 45.55$, $P < 0.001$; CA1 molecular layer: $F_{2,54} = 56.68$, $P < 0.001$; DG granule cell bodies: $F_{2,54} = 19.90$, $P < 0.001$; DG molecular layer: $F_{2,54} = 155.51$, $P < 0.001$. However, the ANOVA test revealed no treatment effect in any one of the four regions investigated. This excludes the fact that the prenatal hypoxic treatment led to a significant loss of principal neurons or their dendrites in the hippocampus, the same areas which were quantified for ingrowing cholinergic and serotonergic fiber densities. The combined treatment of nitrite and nimodipine was also ineffective to influence the size of cell and dendritic layers.

DISCUSSION

Qualitative and quantitative aspects of developing cholinergic and serotonergic fiber patterns

The present study showed that the intensive proliferation of ingrowing cholinergic and serotonergic fibers in the hippocampus as well as in the parietal cortex in the rat is almost exclusively confined to the early postnatal period. The maturation of fiber patterns of both neurotransmitter systems showed rapid changes in the course of the first postnatal week. The hippocampal and cortical innervation densities revealed regional differences with regard to layers and subfields even in newborn rats at PD1 and 3. During the following postnatal days the innervation patterns evolved in relation to the cytoarchitectonic development, then became more clearly laminated and gradually reached the adult condition around PD10. From this age on fine fiber ramifications took place, especially in the case of cholinergic afferentation, which was reflected by an additional increase in fiber density between PD10 and 20.

Employing quantitative measurement of density of ingrowing fibers, we quantitatively assessed the developmental dynamics of cholinergic and serotonergic afferentation in the course of first postnatal days as well as the plastic changes due to exogenous factors like prenatal hypoxia and Ca^{2+} -antagonist treatments. It became evident that the innervation of the dorsal hippocampus by 5-HT fibers preceded the exogenous cholinergic afferentation. The cholinergic innervation in the parietal cortex was also somewhat delayed although not so markedly as in the hippocampus. Therefore, the arriving 5-HT fibers potentially could influence the development of intrinsic target neurons and that of cholinergic fiber ingrowth based on their known neurotrophic and growth regulating properties.^{12,38,39,48,85} After PD10 the increment in arborization of 5-HT fibers slowed down markedly, while the cholinergic fibers exhibited a continuing and intensive ramification up to PD20, the highest age investigated in this study.

Fig. 10. The effects of prenatal treatments on the pattern and density of ingrowing cholinergic fibers in the dorsal hippocampus of five-day-old rats presented by darkfield photomicrographs. A strip from CA1 and the inner blade of dentate gyrus is shown comparable to Fig. 9. Pyramidal cell layer and the dorsal part of granule cell layer are indicated by 'p' and 'g', respectively. Groups are: control (A), nitrite-treated (B), nitrite- and nimodipine-treated (C). Note the less dense fiber arborizations in the hypoxic rat (B) in the infra- and suprapyramidal and in the supragranular layers as compared to either other two cases. The 50- μ m scale bar applied to all three panels.

Fig. 11. Development of AChE fiber density in the parietal cortex in control, hypoxic (nitrite) and nitrite + nimodipine-treated groups. Number of fiber crossings was measured separately in the deep (V-VI) and superficial (I-IV) layers as shown in the insert at top left. Means $\pm$ S.E.M.s are shown. Note that the fiber development in the deeper layers precedes the ingrowth in the superficial layers. The nitrite treatment reduced the fiber density which was only significant in the deep layers: $F_{1,45} = 5.67$, $P < 0.05$. *Post hoc* *t*-test: * $P < 0.05$ vs control, ° $P < 0.05$ vs nitrite group.

With regard to the currently used methods, several questions need to be discussed. First, which information is provided by AChE or 5-HT fiber identification, and second, to what extent can fiber density measurements be considered as being conclusive? These questions are especially relevant to AChE staining, since this metabolizing marker enzyme is not representative of the synthesis of the neurotransmitter acetylcholine itself. This question can be approached from both an anatomical and a functional point of view.

Anatomically, it was shown with retrograde transport methods, that septal axons are present in the hippocampal formation as early as the day of birth.^{11,66} However, it may well be that such retrograde labeling results from wide spread of tracer common in the neonatal brain. ChAT positive neurons in rat can be detected at fetal age of 17 days in the septum/diagonal band complex.² Since at birth AChE positive fibers in the dorsal hippocampus were only scarcely present several alternatives may be taken into consideration. Firstly, the retrograde labeling of septal afferents by fluorescent dyes at PD1¹¹

Fig. 12. Postnatal development of 5-HT fiber arborization in CA1 and DG in three groups of animals; controls, hypoxic (nitrite), and hypoxic but treated with nimodipine. Columns are means $\pm$ S.E.M.s from four to seven rats per group. The hypoxic treatment reduced 5-HT fiber growth, but this effect was significant only in DG (ANOVA for PD3-20: $F_{1,44} = 5.97$, $P < 0.02$). Results of *post hoc* paired comparisons with *t*-test: * $P < 0.05$ vs control, ° $P < 0.05$ vs nitrite.

Fig. 13. 5-HT fiber density in the parietal cortex during postnatal development in controls, hypoxic (nitrite) and hypoxic + nimodipine treated rats. The insert shows the two cortical regions (layers I-IV and V-VI), divided by an arrow, which were quantified separately. Columns represent means $\pm$ S.E.M.s. Numbers under the columns indicate group size. * $P < 0.05$, ** $P < 0.01$ vs control; ° $P < 0.05$, °° $P < 0.01$ vs nitrite group (*t*-test using pooled variance after ANOVA).

visualized mainly GABAergic septal neurons that are known to comprise half of the cells in the septum/diagonal band complex. Secondly, the filling of the already existing putative cholinergic axons with the AChE enzyme molecules requires a few days to reach a histochemically detectable enzyme level. The problem of a detection threshold for AChE appeared to be even larger for ChAT as a cholinergic marker, since this enzyme with immunochemical methods was not detected before PD7 in the forebrain cortex.^{13,23}

In contrast, biochemical studies clearly indicate that the hippocampus and the neocortex do contain

active ChAT enzyme at early postnatal ages, albeit with low concentrations.^{16,42,63,77} Thus, it may be envisaged that improvement of detection sensitivity may reveal immunoreactive neuronal elements of the extrinsic cholinergic projection in the early postnatal period. To date, however, it appears that AChE fiber staining is an acceptable alternative to visualize the maturation of cholinergic fibers during the early postnatal development, provided we take its limitation into consideration.

Prenatal hypoxia, calcium antagonists and the modulation of fiber pattern development

Exogenous toxic factors like prenatal nitrite exposure were shown to modulate the development of AChE and 5-HT positive fiber ingrowth into the hippocampal DG and parietal neocortex in the course of the first postnatal week. The hypoxia effect, i.e. the suppression on both ingrowing pathways, was region-selective in the hippocampus and restricted to the dentate gyrus. In the parietal cortex the delay of cholinergic fiber ingrowth was more pronounced in the deeper than in the superficial layers, while the serotonergic innervation was influenced more evenly. By PD10 the differences between nitrite-treated and control rats were no longer present and, therefore, the effects of prenatal hypoxia are regarded as being transient. Simultaneous treatment with nimodipine, an L-type Ca^{2+} -channel blocker,⁶⁵ provided a protection from the suppressing effect of nitrite. It is well documented that nitrite passes the placenta²⁵ and causes a mixed, anaemic-cytotoxic type of hypoxia.^{25,33,56} Nimodipine also penetrates the placenta,⁷² and consequently can exert a pharmacological action directly in the fetus. The effect of nitrite is considered as a chronic hypoxia and the interfering action of nimodipine can be due, first of all, to its neuroprotective or antihypoxic action as previously discussed in detail.^{55,56,57,69}

The impact of prenatal nitrite exposure was detectable during the postnatal period in which a rapid increment of fiber proliferation takes place from PD3 to 7. The growth delaying effects of the hypoxia on cholinergic innervation was highest between PD3 and 7, while the serotonergic fiber development was mostly affected at PD7. The same prenatal nitrite treatment as was used in the present study, was reported to induce a discrimination learning deficit and a long-term memory disturbance in rats, while avoidance behavior was not influenced.^{55,56} The profile of behavioral changes point to a site of action in the neocortex and hippocampus, this assumption is supported by the present findings showing a disturbed development of neurotransmitter fiber patterns in these forebrain areas. The effect of prenatal hypoxia was transient, at least by the age of PD10 to 20 it disappeared.

The hypoxic and concurrent nimodipine treatments obviously did not affect the general developmental neuronal growth in the forebrain as we

Fig. 14. Darkfield photomicrographs of serotonin immunoreactive fiber patterns in the parietal neocortex at PD7 in representative individuals of control (A), nitrite (B), and nitrite + nimodipine (C) groups. A relatively low fiber density is observed in B panel (hypoxic rat) compared to the other two cases (control and hypoxic but treated with nimodipine). The arrowheads are between layers IV and V. The lower part of layer V and layer VI are not shown at this level of magnification. Scale bar = 50 μ m.

Table 1. Quantified areas of somatal and apical dendritic layers of principal neurons in the hippocampus of control, hypoxic and nimodipine-treated hypoxic rats in different postnatal ages

Age	Treatment	CA1 pyramidal cell layers		DG granule cell layers	
		Cell body	Dendrites	Cell body	Dendrites
PD5	C + Veh	95.7 ± 10.7	265 ± 27	78.6 ± 5.6	76.6 ± 11.3
	N + Veh	94.1 ± 6.4	267 ± 15	77.2 ± 8.0	68.9 ± 13.2
	N + Nim	90.2 ± 5.3	279 ± 33	75.0 ± 6.8	70.5 ± 8.0
PD7	C + Veh	94.9 ± 3.4	347 ± 26	84.9 ± 5.5	117 ± 19
	N + Veh	92.0 ± 10.3	326 ± 52	87.0 ± 11.3	107 ± 14
	N + Nim	95.9 ± 9.2	322 ± 45	89.0 ± 12.2	116 ± 13
PD10	C + Veh	105 ± 12	369 ± 55	90.0 ± 13.9	139 ± 12
	N + Veh	108 ± 14	361 ± 45	93.4 ± 9.6	136 ± 23
	N + Nim	112 ± 12	383 ± 41	93.5 ± 13.4	142 ± 17

Areas are expressed in $\mu^2 \times 10^3/\text{mm}$ column width. Values are means ± standard deviations (S.D.); each group contained seven animals. C + Veh: vehicle-treated controls, N + Veh: nitrite + vehicle, N + Nim: nitrite + nimodipine. In the dentate gyrus (DG), the measurements were made in the inner blade.

measured in the various hippocampal layers. This absence of hypoxic effects on neuronal damage is consistent with the notion that neurons in the developmental stages are relatively resistant to hypoxic conditions compared to adult neuronal tissue.¹⁷ It appears much more likely that the damaging mechanisms of perinatal hypoxia relate to subtle derangements during synaptogenesis, however, with profound functional and behavioral consequences. The transient retarded fiber growth probably results from inhibitory processes, e.g. at growth cone levels, and could well lead to an abnormal synapse formation. The sensitivity of cholinergic neurons to hypoxia is well documented in adult rats both *in vivo*^{21,22,70} and *in vitro*.⁵¹ Neonatal anoxia in rats causes cholinergic dysfunction with a concomitant change in the development of adrenergic and dopaminergic neurotransmitter systems.^{24,28,30,73} Both hypoxia⁵¹ and an increase in the intracellular concentration of Ca^{2+} ⁵ attenuate the release of acetylcholine. These findings point to an inhibited function of cholinergic neurons after hypoxia combined with an increased intracellular Ca^{2+} concentration, which is commonly associated with hypoxia and ischemia.^{74,80} Next to these biochemical findings the present results indicate that prenatal hypoxia may lead to a retarded development of some neurotransmitter pathways such as cholinergic and serotonergic systems, resulting in long-lasting dysfunctions in the developing rat brain. The recent finding that early postnatal cholinergic lesion results in a permanent decline in postsynaptic muscarinic receptor expression, lends further support to a persistent effect of retarded growth on cholinergic synaptic transduction.⁴³

Perinatal brain damage of hypoxic/ischemic origin results in an increased intracellular concentration of Ca^{2+} in newborn rats.⁷⁴ The antihypoxic effect of nimodipine, an L-type calcium channel antagonist, may be due to the prevention of high intracellular Ca^{2+} -induced cellular chain reactions leading to neuronal damage. Nimodipine acts on dihydropyridine receptors of the central type found in highest

concentration in the hippocampal DG and in the neocortex.⁶¹ A direct regulatory effect on developing neurites and growth cones by chronic nimodipine exposure may be anticipated for the following reasons: (i) the voltage sensitive Ca^{2+} -channels are localized in high numbers on the developing neurites,³ and (ii) the mobility of growth cones depends on a narrow range of intracellular Ca^{2+} concentrations.^{36,48} Hypoxic animals treated with nimodipine exhibited a higher level of fiber arborization at PD10 even when compared with control pups. This effect of nimodipine on developing cholinergic and serotonergic neurons may be considered as a neurotrophic action of this compound. The neuronal growth promoting effect of nimodipine is not restricted to these neurotransmitter systems. Perinatal treatment with nimodipine also potentially accelerates the developmental expression of Ca^{2+} -binding proteins calbindin-28k, parvalbumin and S-100.^{7,54}

General aspects of fiber pattern development

The developmental profile of fiber patterns depends not only on the afferentation side but also on the postsynaptic neuronal recipients.^{14,26,67} Among the prerequisites of proper synaptogenesis the timely arrival of projectional axons and the maturational stages of recipient neurons are both important factors. The anatomical and chemical properties of developing intrinsic cells can also be targets of modulating factors like hypoxia or drug treatments. Theoretically an alternative indirect route of action might go through other neurotransmitter systems, like somatostatinergic, catecholaminergic or GABAergic neurons,³⁸ which also form synaptic contacts with the developing principal neurons in the hippocampus and neocortex.⁶⁷ In the present study it was clearly visible that the premature cholinergic lacunosum-molecular layer in CA1 or the molecular layer in DG followed the development of apical dendrites of principal neurons. Dendritic branches and spines increase dramatically early in development of principal neurons in the neocortex⁶⁰ and in the hippocampal DG¹⁷ and CA1 area.⁶¹ It has also been known for

a long time that the development of DG lags behind that of other regions of the hippocampal formation, and that the granule cell neurogenesis is confined to the late embryonic and early postnatal periods.^{1,86} As was shown by the present findings nitrite treatment delayed the development of fiber patterns of both neurotransmitter systems only in DG but not in CA1. Therefore, it may be reasoned that the prenatal hypoxia primarily delayed the development of DG intrinsic neurons and this way inhibited the fiber density of arriving afferents. Besides, it appears that the dentate granule cells in newborn rats, unlike in adults, are more sensitive to hypoxia than the CA1 pyramidal cells.⁸⁴ These authors associated the increased vulnerability of developing granule cells with their lower content of a Ca^{2+} -binding protein calbindin-28k. As mentioned above, nimodipine increased the expression of immunoreactive calbindin-28k in the granule cells of pups' brain at ages of PD5–10.^{7,54} Therefore, the beneficial action of nimodipine treatment may well be due to the increased Ca^{2+} -buffering capacity of hippocampal granule cells, which assures resistance against hypoxic insult.

Based on these data we assume that the neuroprotective action of nimodipine includes both antihypoxic and growth regulating mechanisms. Since at PD10 the nimodipine-treated rats displayed even higher cholinergic and serotonergic fiber densities than found in control animals, a neuronal growth promoting action of nimodipine is strongly suggested. Finally, with regard to the growth suppressing effect of nitrite treatment, further studies should address the question as to whether primarily pre- or postsynaptic elements and mechanisms are involved. The lack of differences in the thickness of cellular and molecular layers between the different treatment groups at various postnatal ages points to a predominantly presynaptic influence of the hypoxic treatment.

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