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Acute Sublethal Global Hypoxia Induces Transient Increase of GAP-43 Immunoreactivity in the Striatum of Neonatal Rats

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KEY WORDS GAP-43; SNARE; striatum; hypoxia; development; anxiety

ABSTRACT We assessed immunoreactivity (IR) in the cerebral cortex (CC), hippocampus (Hipp), and striatum (ST) of a growth-associated protein, GAP-43, and of proteins of the synaptic vesicle fusion complex: VAMP-2, Syntaxin-1, and SNAP-25 (SNARE proteins) throughout postnatal development of rats after submitting the animals to acute global postnatal hypoxia (6.5% O_2, 70 min) at postnatal day 4 (PND4). In the CC only the IR of the SNARE protein SNAP-25 increased significantly with age. The hypoxic animals showed the same pattern of IR for SNAP-25, although with lower levels at PND11, and also a significant increase of VAMP-2. SNAP-25 (control): PND11 $P < 0.001$ vs. PND18, 25, and 40, SNAP-25 (hypoxic): $P < 0.001$ vs. PND18, 25, and 40; VAMP-2 (hypoxic): $P < 0.05$ PND11 vs. PND18, and $P < 0.01$ vs. PND25 and PND40; one-way ANOVA and Bonferroni post-test. In the Hipp, SNAP-25 and syntaxin-1 increased significantly with age, reaching a plateau at PND25 through PND40 in control animals (one-way ANOVA: syntaxin-1: $P = 0.043$; Bonferroni: NS; SNAP-25: $P = 0.013$; Bonferroni: $P < 0.01$ PND11 vs. PND40). Hypoxic rats showed higher levels of significance in the one-way ANOVA than controls (syntaxin-1: $P = 0.009$; Bonferroni: $P < 0.05$ PND11 vs. PND25 and $P < 0.001$ PND11 vs. PND40). In the ST, GAP-43 differed significantly among hypoxic and control animals and the two-way ANOVA revealed significant differences with age ($F = 3.23$; $P = 0.037$) and treatment ($F = 4.84$; $P = 0.036$). VAMP-2 expression also reached statistical significance when comparing control and treated animals ($F = 6.25$, $P = 0.018$) without changes regarding to age. Elevated plus maze test performed at PND40 indicated a lower level of anxiety in the hypoxic animals. At adulthood (12 weeks) learning, memory and locomotor abilities were identical in both groups of animals. With these results, we demonstrate that proteins of the presynaptic structures of the ST are sensitive to acute disruption of homeostatic conditions, such as a temporary decrease of the O_2 concentration. Modifications in the activity of these proteins could contribute to the long term altered responses to stress due to acute hypoxic insult in the neonatal period. **Synapse 61:124–137, 2007.**

INTRODUCTION

Acute global hypoxia in the neonatal rat has been used as a model of the stress suffered by the human fetus during gestation and at the moment of birth. Prenatal hypoxia, at an early embryonic period, was found to lead to delayed neurogenesis (delayed establishment of elements of the neuropil and differentiation of cells) and abnormalities in the structure of the

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striatum (ST) (Zhuravin et al., 2006). By submitting the rat pups to systemic hypoxia near the period of birth, researchers in this field have reported short (Berger et al., 2000) and long-term effects such as stress susceptivity (El-Khodor and Boksa, 2000), and modifications in dopamine (DA) related behaviors (Decker et al., 2003). At postnatal days 10–12 global hypoxia has a more marked effect, inducing behavioral changes, memory impairment, spontaneous seizures, and chronic brain injury, mimicking clinical aspects of neonatal hypoxia in the human (Jensen, 1995). Perinatal asphyxia (at birth) was found to cause apoptosis and delayed neuronal death in the CA1 hippocampus (Hipp), accompanied by specific upregulation of proliferation in the germinative subventricular zone (Daval et al., 2004; Scheepens et al., 2003). However, there are few reports on the effects of global systemic hypoxia during the first week of the life of the rat, equivalent to the late gestational period of the human fetus.

By means of massive transcriptome analysis it was shown that sublethal chronic postnatal hypoxia accentuates genes subserving presynaptic function and suppresses genes involved in synaptic maturation, post synaptic function, and neurotransmission (Curristin et al., 2002). The expression of synaptic proteins in the neonatal cerebral cortex (CC) is different than in the adult, an observation likely related to their specific roles during the growth cone to synapse transition and the subsequent critical period of synaptic consolidation (Nikonenko et al., 2003; Patterson and Skene, 1999; Shimohama et al., 1998). The core components of the neuronal SNARE complex (syntaxin-1, SNAP-25, and VAMP-2) accumulate before postnatal day 7, when synaptic vesicles start to appear in greater numbers. Besides their role in transmitter release, many of these synaptic proteins are also known for their participation in the complex processes of axonal growth and neosynaptogenesis. When overexpressed in PC12 cells, VAMP-2, for example, promotes neurite elongation, whereas SNAP-25 stimulates elongation and neurite sprouting (Kimura et al., 2003). Furthermore, elevation of SNAP-25 in the Hipp has been observed following unilateral transection of hippocampal afferent fibers, indicating a possible role for this protein in repair responses of the CNS (Patanow et al., 1997). Rab 3A and VAMP-2 transiently increased 1 h after 6.5% O_2 hypoxia (Manzur et al., 2001) and there are reports indicating that SNARE-mediated mechanisms operating in the growth cone are involved in the membrane expansion underlying axon growth (Futerman and Banker, 1996). These apparent changes were transient and detected during stage II, described as a transition stage that occurs between PND 7 and 14, when the growth cones mature into fully functional presynaptic terminals (Igarashi et al., 1997). A lesion close to the critical periods of development could cause the loss of synaptic integrity, involving synaptic proteins attached to small vesicles and plasma membrane of the presynaptic nerve terminals.

While the molecular mechanisms through which these proteins act are still a matter of debate, few studies have addressed the question of whether they are affected by neurotoxic events during the critical period of synaptic organization and whether synaptic vesicle proteins and synaptic membrane proteins are equally or differentially affected in each brain region. Synaptic plasticity could either have beneficial effects—favoring the repair mechanisms—or, on the contrary, could have adverse effects by promoting the formation of anomalous connections. In this report, we describe the normal pattern of immunoreactivity (IR) of the growth associated protein, GAP-43 and of the core proteins of the SNARE complex, measured by Western blot in the second post nuclear pellet (fraction P_2) of three different brain regions from rats throughout their postnatal development. We also used quantitative immunoblotting to evaluate changes in the level of expression of these proteins after submitting the 4-day old pups to a low concentration of O_2 (hypoxia) during a period of time considered to be critical, beyond which the mortality rate increases markedly. In addition, we evaluated behavioral parameters, such as memory and locomotor activity at the age of 12 weeks, to assess possible long-term effects of the lesion, and anxiety levels in the hypoxic animals at PND40, in coincidence with the end of the experimental period.

MATERIALS AND METHODS

Animals and tissue processing

Neonatal Sprague–Dawley rats, from our local breeding facility were used. Animals were kept in a controlled environment, with food and water ad libitum. We followed procedures in accordance with CEEA (Comité Ético de Experimentación Animal, Argentina) guidelines. All animals were kept in a room with controlled lights (lights on from 6 a.m. to 8 p.m.) and temperature (22–24°C). After sacrifice by decapitation, the brains were rapidly removed. The whole cerebral cortex (CC), corpus striatum (ST), and hippocampus (Hipp) were dissected out on a cold plate according to Heffner et al., 1980, and placed in tubes containing homogenization buffer. A group of animals was sacrificed immediately after hypoxia at PND4 and trunk blood samples were collected for lactic acid measurement.

Hypoxia

Acute hypoxia was induced by placing the rat pups (4-days-old) in a chamber with controlled humidity and temperature (36.5°C), flushed with a continuous stream of 93.5% nitrogen and 6.5% oxygen for 70 min. In each experiment 4–6 pups were removed from their

cages 15 min prior to hypoxia to allow their body temperature to adjust to the chamber environment. The surviving pups (death rate 10%) were allowed to recover in room air for 30 min, then returned to their dams until sacrifice at postnatal days 5, 7, 11, 18, 25, and 40. Animals were weaned at day 21 after birth. Control animals were placed in a chamber and exposed to room air for 70 min and sacrificed at the same times as the experimental group.

Materials

A monoclonal antibody to GAP-43 (9-1E12, routinely used at 1/5000 dilution) was donated by Dr. Pate Skene (Duke University, Durham, NC). Monoclonal antibodies to VAMP-2 (1/5000) and syntaxin-1 (1/4000) were obtained from Synaptic Systems GmbH (Göttingen, Germany). Monoclonal antibody to SNAP-25 (SMI-81, 1/5000) was obtained from Sternberger Monoclonals. Horseradish peroxidase-conjugated goat antimouse (1/5000) was obtained from Caltag Company (Burlingame, CA). Prestained protein molecular weight standards were from BioRad (Hercules, CA).

Western blot

The P_2 subcellular fraction was prepared according to the techniques of Gordon-Weeks modified by Patterson and Skene, 1999. Briefly, Sprague–Dawley rats were sacrificed by decapitation and the CC, ST, and Hipp were removed and placed in ice-cold STE buffer (0.32 M sucrose, 5 mM Tris·HCl, 1 mM EDTA, pH 7.4) with protease inhibitors (100 mM AEBST, 10 mM leupeptin, and 10 mM bestatin). All subsequent steps were carried out on ice. For each assay, cerebral areas from four animals were pooled to prepare the P_2 fraction. This number of animals is the minimum necessary to obtain sufficient protein for assay. Each assay was repeated 4–5 times. The tissues were minced with a clean razor blade, homogenized in a glass/Teflon homogenizer in STE, and centrifuged at 1000*g* for 5 min in a swinging bucket rotor. The supernatants were transferred to fresh tubes and pooled with a further wash of the low speed pellet. The combined supernatants were centrifuged at 19,000*g* for 15 min, the supernatants were discarded and the upper fluffy portion of the pellet was resuspended in STE and transferred, leaving behind the darker mitochondrial pellet. The homogenates were repelleted, and collected as before in STE, with trituration through a 16-g needle. This suspension was the origin of P_2 fraction. The protein concentration was determined by Bradford assay on triplicate aliquots.

Immunodetection of proteins

Ten micrograms of proteins from each homogenate were solubilized by boiling in sample buffer. The samples were analyzed for the expression of growth-associated (GAP-43) and synaptic SNARE proteins (syntaxin–1, SNAP-25, VAMP-2) after separation by 12% SDS-PAGE on minigels in parallel with prestained protein molecular weight standards. Proteins were electrotransferred onto 0.2 μm pore diameter nitrocellulose (Hybond C, Amershan Life Science) in a semidry blotter at 1 mA/cm^2 overnight in 20% methanol/40 mM Tris-glycine buffer (pH 9.0). Protein transfer was assessed by staining with red Ponceau S. The membranes were blocked with 0.5% BSA/2% horse serum/0.05% Triton X-100/PBS for 30 min at room temperature, followed by 1.5 h incubation in primary antibody diluted in blocking solution at room temperature. Membranes were then washed five times in large volumes of 0.05% Triton X-100/PBS over 45 min, incubated with horseradish peroxidase-conjugated goat antimouse in blocking solution at 1/5000 dilution for 60 min. Secondary antibody was removed by washing five times in 0.05% Triton X-100/PBS over 45 min, then for 15 min in 10 mM phosphate buffer (pH 7.4). Specific protein bands were detected using a chemiluminescence kit (Amersham or NEN) according to the manufacturer's instruction. Multiple exposures of different times were made, to bring the exposures within the linear response range of the film (X-OMAT-AR, Kodak).

The images on the films were scanned and the optical density of the immunoreactive bands was derived using the program NIH Image 1.6/ppc (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Samples from control and hypoxic animals at PND 11, 18, 25, and 40 were run and developed on the same film. The data from the different experiments were pooled after initially normalizing to the highest expression of each protein (= 100% MPE: percentage of maximal protein expression within each experiment). Figures were assembled from representative scans belonging to one film.

Immunohistochemistry for light microscopy

At postnatal days 11, 18, 25, and 40 the animals were deeply anesthetized with chloral hydrate, perfused intracardially with saline, and then with 4% paraformaldehyde in 0.01 M borate buffer, pH 7.4. The whole heads, wrapped in aluminum foil, were stored at 4°C overnight for complete fixation. The next day the brains were removed from the skull and cryoprotected with 30% (w/v) sucrose in buffered paraformaldehyde and kept refrigerated until they sunk in the solution. After that the brains were rapidly frozen at −20°C in isopentane and serially sectioned (40 μm slices) using a cryostat. Sections displaying the anterior ST were selected for immunostaining. The locations of the brain areas were determined according to coordinates described in the Atlas

of Paxinos and Watson, second edition. Briefly, the free floating slices were thoroughly washed in 50 mM Tris-HCl, NaCl (0.9%), pH 7.4, reacted with 3% hydrogen peroxide/methanol to quench endogenous peroxidase, incubated in 10% goat serum in blocking solution (saline/Tris buffer with 0.1% Triton X-100, 1% goat serum, and 1% bovine serum albumin), followed by incubation for 72 h at 4°C with anti GAP-43 (1:500) monoclonal antibodies in the same blocking solution. The secondary antibody was goat antimouse IgG (biotin conjugate) and was detected by means of the Vectastain avidin-biotin-peroxidase system (Vector Laboratories). Diaminobenzidine was used as chromagen with glucose oxidase (Sigma) plus nickel ammonium sulfate as color enhancer. The stained slides were then mounted, dehydrated, and coverslipped with synthetic Permount®.

Enzymatic determination of lactate

The concentration of lactate in plasma was determined by a colorimetric assay (Biomeriux Kit, Marcy-L'Etoile, France) according to manufacturer's instructions. Trunk blood was collected after sacrifice and plasma (using sodium fluoride as anticoagulant) was separated by centrifugation within 15 min. The assay was carried out immediately, and for the hypoxic group the plasma was diluted 1/5.

Behavior

Passive avoidance test

We conducted the step-down passive avoidance test when the animals were 12 weeks of age (body weight: 290–320 g). The test was carried out during the light phase (13:00–17:00 h), and each animal was housed individually during the test and for 1 h prior to starting. Male rats were subjected to one-trial, step-down as described previously, (Martinez et al., 2002). The training apparatus was a 30 × 30 × 40 cm white acrylic box, the floor of which was a series of 1-mm-caliber bronze bars spaced 1 cm apart. The left end of the floor was covered by a 13-cm-wide, 20-cm-long, and 3-cm-high wooden platform. Animals were gently placed on the platform facing the left rear corner at that moment room-lights are turned off and internal box-light turns on. When they stepped down onto the grid and had placed their four paws on it, they received a 3 s, 1.0 mA scrambled shock to the foot and were then immediately removed from the training box and placed in their home cages. The long-term memory associated with the learning of this task persisted for at least 31 days after the training session. Rats were tested for retention and latency (this latter considered as the time that the animal remains in the platform until placing the four paws on the grid) at 24 h and 7 days after training. A ceiling of 300 s was imposed on retention test measures.

Elevated plus maze

Forty-day-old and three-month-old rats were tested on an elevated plus-maze (EPM). The open to closed arm entry ratio in an EPM is considered a reliable measure of anxiety in the rat (Pellow et al., 1985; Pellow and File, 1986). Briefly, the plus-maze consists of two wooden, opposite facing, open arms (45 × 10 cm) and two opposite facing closed arms (45 × 10 × 10 cm, length × width × height). The whole plus-maze was mounted on a basement stand 45 cm above the floor. Rats were placed singly in the plus-maze. The test was begun by placing the rat in the end of one of the closed arms.

The number of entries into arms, the latency of the first entry into an open arm, and the time spent in the open and closed arms was recorded over a 5-min period. Arm entry was registered when all the four paws were placed in the arm. The maze was thoroughly cleaned after each test.

Each rat was used only once. The main measures of plus-maze behavior calculated were: the percentage of entries into the open arms (100 × open/total) and the percentage of time spent in the open arms (100 × open/total). The percentage of time spent in the open arms was interpreted as an index of fear and anxiety, while the number of closed arms entries was an index of general activity. Since the measurement of anxiety may be influenced by locomotor activity, we also examined the absolute number of closed arm entries considered as a clear index of general motor activity (Cruz et al., 1994). We also recorded a number of other behavioral parameters: grooming, rearing (partial or complete standing on the hind limbs), peeps, defecation (number of fecal boli expelled), and freezing (as seconds of immobility).

Spontaneous motor activity

A group of male rats 12 weeks of age (body weight: 290–320 g) were divided into two groups, naive animals reared in normoxic conditions (controls) and animals that were submitted to acute hypoxia at PND4 for 70 min (see Hypoxia section). The animals were placed individually in a Plexiglas cage (30 × 30 × 30 cm) inside a soundproof room. Spontaneous locomotor activity was monitored through an Opto-Varimex Mini (Columbus Instruments, Columbus, OH) and the following responses were recorded simultaneously: number of displacement movements (horizontal and vertical activity) and number of rearings. Each animal was observed for a total of 5 min.

Statistical analysis

The total number of animals used for each determination is shown in the legend to each figure. For the analysis of protein IR at the different developmental

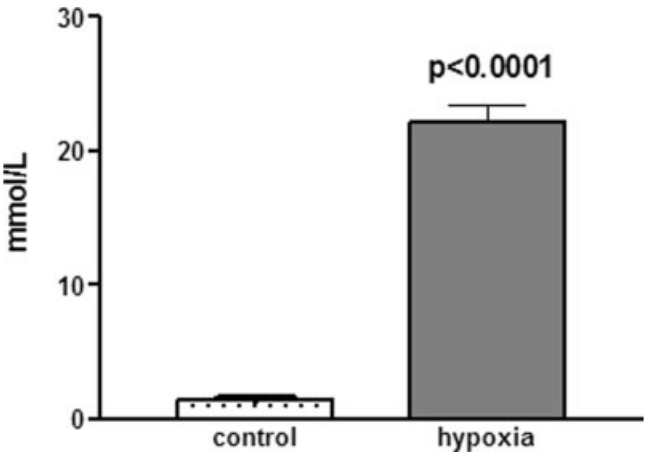

Fig. 1. Lactate levels in plasma. Blood samples were obtained at PND4, immediately after removing the rat pups from the hypoxic chamber. After 70 min oxygen deprivation, blood lactate levels had increased significantly ($P < 0.0001$ unpaired Student's t-test vs. controls, each column, $n = 6$).

stages, one-way ANOVA followed by the Bonferroni post-test analysis was applied for comparisons within the same group. Two-way analysis of variance was used to make comparisons between normoxic and hypoxic animals ("treatment" and "age" comparisons). When only two groups were compared, such as, for example, in the behavior experiments, Student's t-test was used, and the level of significance was set at $P < 0.05$. GraphPad Prism 2.01 program was used to perform statistical analysis and graphing.

RESULTS

Blood lactate

To corroborate the effectiveness of the hypoxia, blood samples for measuring lactate levels were obtained at PND4 right after removing the pups from the hypoxic chamber. The values from the animals submitted to oxygen deprivation were significantly different ($P < 0.0001$ unpaired Student's t-test) (Fig. 1) from those of the control group in every single animal tested. This parameter was important because any reduction in the chamber temperature would immediately be reflected in a decrease in lactate levels, thus controlling for the known protective effect of temperature on hypoxic injury (Yager et al., 1993).

Immunoblots for GAP-43, VAMP-2, SNAP-25, and syntaxin-1 in the P_2 fraction of rat brain

We quantified variations in protein levels in the P_2 fraction as described in the methods section. This material comprises all the major cellular components except nuclei and mitochondria, and contains both growth cones and synaptosomes. We chose to work with this unfractionated preparation because it contains mature as well as immature synaptic structures and axonal fragments. A more purified subcellular fraction, such as isolated synaptosomes, might not include immature or transitional presynaptic structures (Patterson and Skene, 1999). Since the proteins analyzed are highly enriched in nerve terminals we consider it unlikely that nonneuronal elements in the preparation would interfere with the axon-derived signal. Thus, use of the P_2 fraction avoided assumptions about possible effects of hypoxia on the maturation or dedifferentiation of the synaptic terminals. The relative expression of proteins in P_2 from PND11 to PND40 was quantified from the Western blots by densitometry and were normalized to %MPE, the highest expression of each protein seen in each experiment (= 100%), prior to pooling the data. Since the measurements were done at several postnatal ages, it was expected that the relative amount of immature structures in the pellet would differ according to the age of the animals. To ensure that changes in IR levels detected among samples were due to actual differences in protein content, and not to differences in protein loading, samples were additionally normalized by densitometry of a parallel gel stained with Coomasie Blue.

Cerebral cortex

GAP-43 levels were low during the first week of life (24 h after hypoxia at PND5 and 72 h after hypoxia at PND7). Differences for GAP-43 IR between control and hypoxic groups of the same age reached statistical significance at PND7 (unpaired Student's t-test $P < 0.05$, Fig. 2a). Syntaxin-1 and VAMP-2 levels, also were low at PND5 and increased significantly at PND7 ($P < 0.01$ and 0.001 respectively, unpaired t-test) in both, control and treated animals (Figs. 2b and 2c).

During the developmental period, from PND11 to PND 40, the IR of GAP-43 and of the synaptic proteins, in the control groups showed different patterns of expression. Syntaxin-1 and VAMP-2 varied between 65 and 85% of the maximal protein expression (%MPE) with a tendency to increase during the earlier period and to plateau at the end of the fourth week of age. SNAP-25 showed lower levels at the beginning of the second week of age, and increases significantly by the third week of age. The hypoxic animals showed a different pattern of expression comparing to normal animals. For the SNARE proteins we observed that although the % MPE levels were reached at about 25/40 days of age, the initial levels at PND11 were significantly lower than at more advanced age (VAMP-2: $P < 0.05$ vs. PND18 and $P < 0.01$ vs. PND-25 and PND40; SNAP-25: $P < 0.001$ vs. PND18, 25 and 40 (Fig. 3: one-way ANOVA; within the same experimental group). In the case of

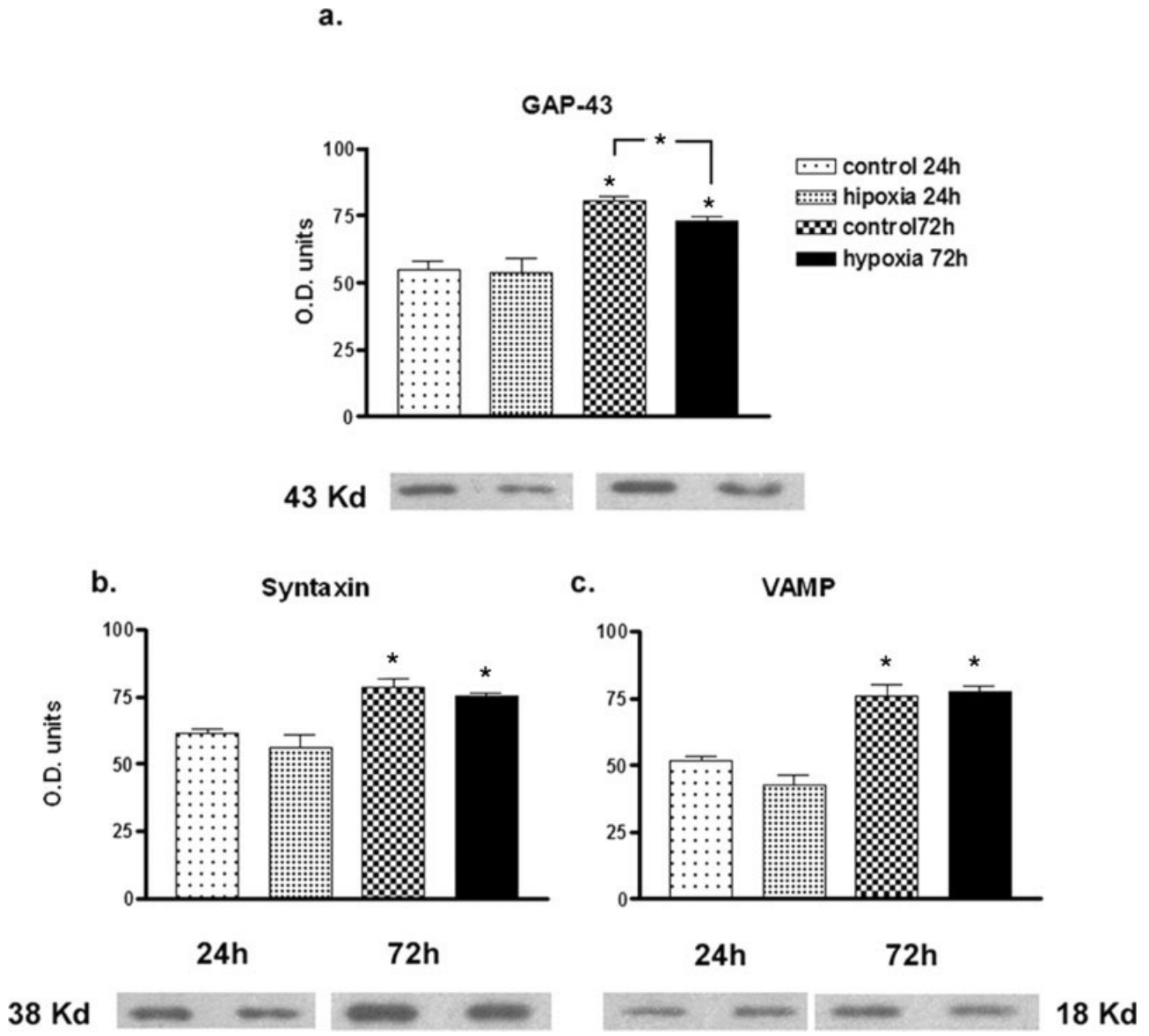

Fig. 2. Western blot of the subcellular fractions (P2) of neonatal rat brain cortex. Homogenates from control animals during the first week of age and 24 and 72 h after an acute global hypoxia at PND4. Specific monoclonal antibodies against GAP-43 (**a**), syntaxin-1 (**b**), and VAMP-2 (**c**) were used. For each protein we show the quantification of protein levels of the nerve terminal fractions as arbitrary optic densities units (ODs) determined by densitometry as described in the text. At the bottom of each figure, the representative Western Blot lanes illustrate the specificity of the antibodies used in this study. The results are the mean ± SEM for three independent experiments. Each sample is a pool of whole cerebral cortex from four neonatal rats of both sexes. For GAP-43, $*P < 0.05$ vs. control 24 h and vs. control 72 h, respectively for syntaxin-1, and VAMP-2 $*P < 0.05$ and $P < 0.001$ respectively vs. control 24 h, (unpaired Student's *t*-test).

syntaxin-1, no significant changes were observed with one-way ANOVA analysis, and GAP-43 levels in the animals submitted to hypoxia remained over 75% of MEP throughout the measured period. Two-way ANOVA analysis of the four proteins indicated notable differences for SNAP-25 ($F = 26.69$; $P < 0.0001$); syntaxin-1 ($F = 4.16$; $P = 0.007$), and VAMP-2 ($F = 10.41$; $P < 0.001$) with regards to the "age" parameter, but no significant differences due to "treatment." Thus, CC SNARE protein expression in both control and hypoxic animals seems to run in parallel throughout development, although in some cases animals that suffered hypoxia started from lower values. In contrast, GAP-43 did not show significant differences due to "treatment" nor "age" when analyzed by two-way ANOVA (Fig. 3).

Hippocampus

During normal development, the expression of the SNARE protein SNAP-25 increased significantly with age, reaching a plateau at PND25 through PND40;

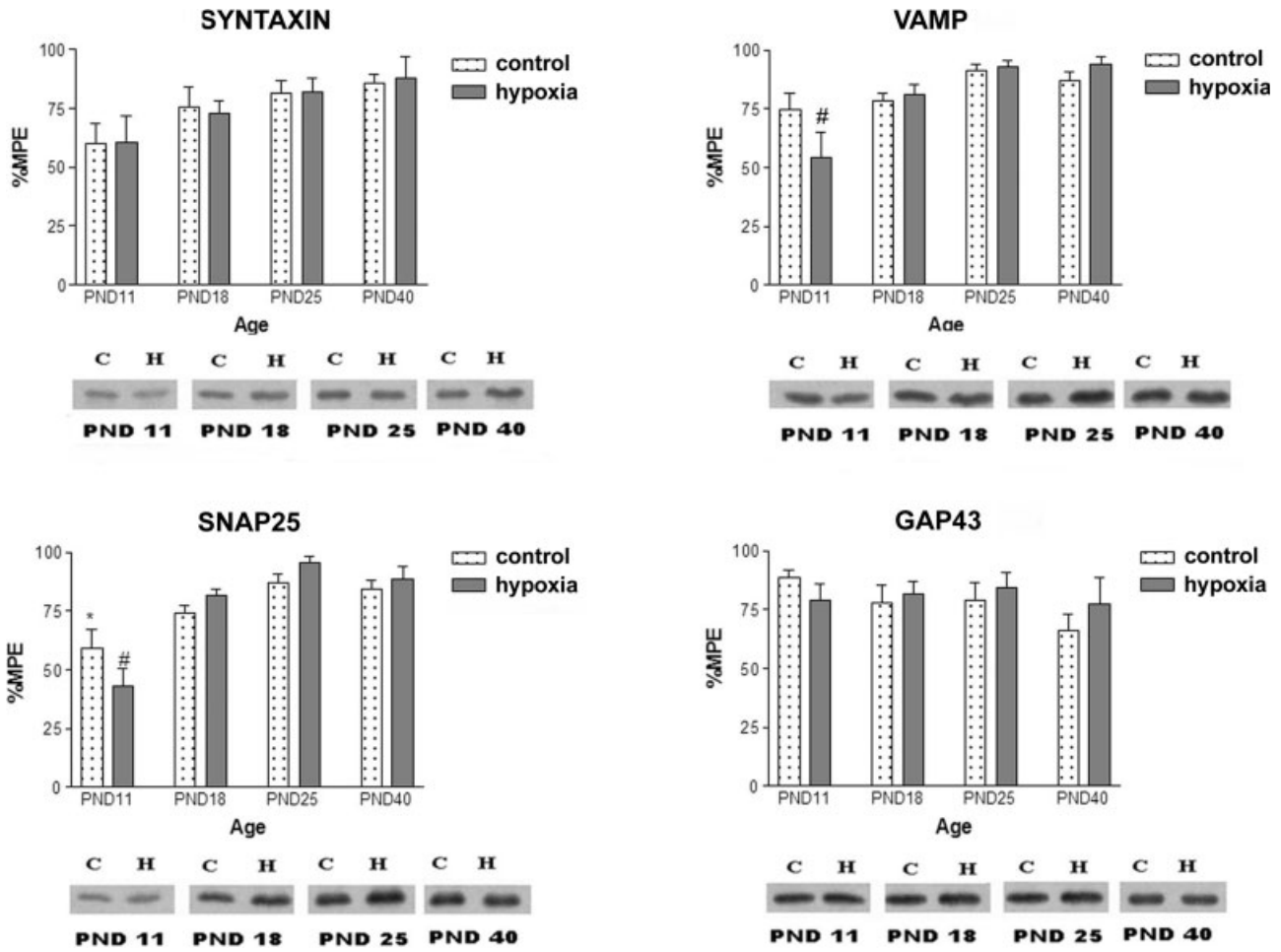

Fig. 3. Western blot of rat cerebral cortex: Immunoreactivity for syntaxin-1, SNAP-25, VAMP-2, and a growth-associated protein, GAP-43 in the P_2 fraction of rat cerebral cortex during postnatal development (between PND11 and PND 40) and after an acute mild hypoxia at PND 4. The bars represent the mean ± SEM for 5–6 independent experiments. The data from the different experiments were pooled after initially normalizing to the highest expression of each protein (=100% MPE: percentage of maximal protein expression). At the bottom of each figure, representative Western blot lanes illustrate the specificity of the antibodies used in this study. For VAMP-2: # $P < 0.05$ vs. PND18 and $P < 0.01$ vs. PND-25 and PND40 and for SNAP25: # $P < 0.001$ vs. PND18, 25 and 40 (hypoxic rats) and *$P < 0.001$ vs. PND18, 25 and 40 (control rats); (one-way ANOVA and Bonferroni post test, within the same experimental group).

syntaxin-1 showed the same tendency, but not statistically significant (one-way ANOVA: SNAP-25: $P = 0.013$; Bonferroni: $P < 0.01$ PND11 vs. PND40; syntaxin-1: $P = 0.043$; Bonferroni: NS). Hypoxic rats showed higher levels of significance in the one-way ANOVA than controls (syntaxin-1: $P = 0.009$; Bonferroni: $P < 0.05$ PND11 vs. PND25 and $P < 0.001$ PND11 vs. PND40). VAMP-2 showed no differences in the pattern of IR respect to age(one-way ANOVA: controls: $P = 0.6267$ Bonferroni: NS; hypoxic: $P = 0.0443$; Bonferroni: NS). On the other hand, in this brain region GAP-43 values remained steady at over 75% MPE during the developmental period in both control and hypoxic animals, consistent with the protein's postulated role in plasticity. Two-way ANOVA analysis was significant for the "age" parameter for syntaxin-1 ($F = 10.78$; $P < 0.0001$) and SNAP-25 ($F = 10.57$; $P < 0.0001$), (Fig. 4). These results suggest that the lesioned animals may have an increased rate of neurite outgrowth in the developing axons (Igarashi et al., 1997).

Striatum

This brain area evinces the most substantial effects of hypoxia on synaptic proteins. The pattern of expression of GAP-43 differed significantly among hypoxic and control animals and the two-way ANOVA revealed highly significant differences with age ($F = 3.23$; $P = 0.037$) and treatment ($F = 4.84$; $P = 0.036$). At PND 11, a significant increase in GAP-43 IR in the hypoxic animals was observed compared to controls ($P < 0.009$; Student's t-test). Similarly, VAMP-2 expression in the P_2 fraction of ST homogenates was also sig-

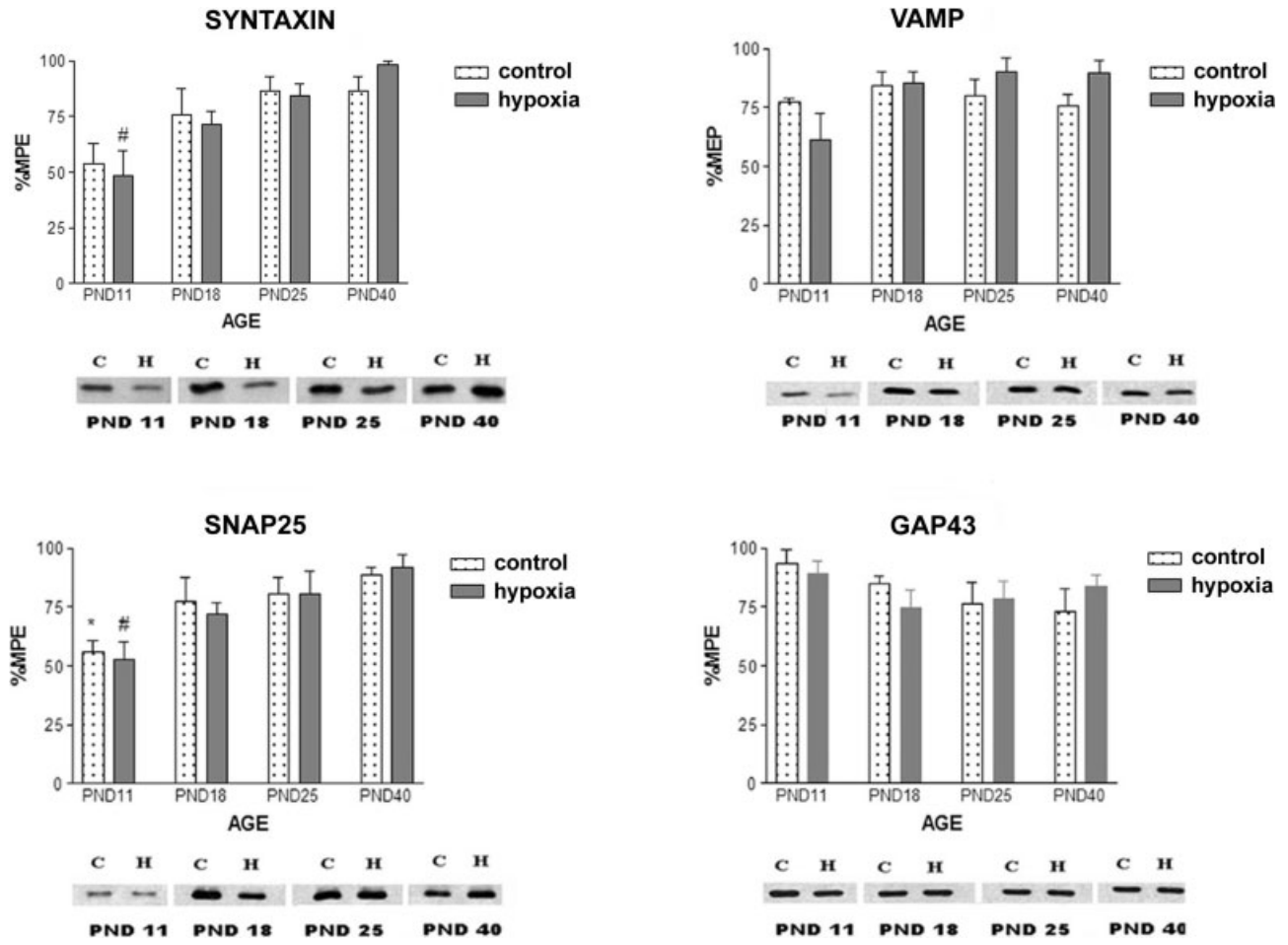

Fig. 4. Western blot of rat hippocampus. SNARE proteins (syntaxin-1, SNAP-25, VAMP-2) and GAP-43 in the P_2 fraction of rat hippocampus during postnatal development and after an acute mild hypoxia at PND 4. For details read in the legend of Figure 3 SNAP-25: * $P < 0.01$ PND11 vs. PND40; syntaxin-1 and SNAP-25: # $P < 0.05$ PND11 vs. PND25 and $P < 0.001$ PND11 vs. PND40, respectively one-way ANOVA and Bonferroni post-test.

nificantly different between control and treated animals ($F = 6.25$, $P = 0.018$), but not with regards to age.

Conversely, by two-way ANOVA, syntaxin-1 differed with age ($F = 3.16$; $P = 0.038$) but not by "treatment" with hypoxia, and SNAP-25, contrary to what was observed in CC and Hippo, remained around 75% MPE in both control and experimental groups in this area. One-way ANOVA found no significant differences within each group at different ages for any of the proteins (Fig. 5).

Immunohistochemistry

Immunostaining using the GAP-43 antibody showed an intense positive signal for fibers throughout the marginal layer and layer I of the CC. In agreement with other authors (Dani et al., 1991), we observed a punctate staining of the cortical neuropil concentrated mostly in the marginal layer and negative staining of the neuronal somata. The protein seems to be distributed in patches along the layer, and at some points there are bundles of fibers projecting towards the cortical plate. This pattern tends to disappear in the more mature animals (Figs. 6e–6h). In the ST of control and hypoxic animals at PND11, an intensely stained pack of fibers is observed in the lining of the lateral ventricle, and this is more notorious in some hypoxic animals. The rest of the ST displays the typical image of stained neuropil around bundles of unstained fibers and neuronal cell bodies. Except for the fibers mentioned before, control and hypoxic rats show similar pattern of immunostaining for GAP-43. (Fig. 6).

Behavior

Passive avoidance

The results of the passive avoidance behavioral test are shown in Table I. The data demonstrate that both the hypoxic animals and the controls were successful in acquisition of passive avoidance (learning) and in

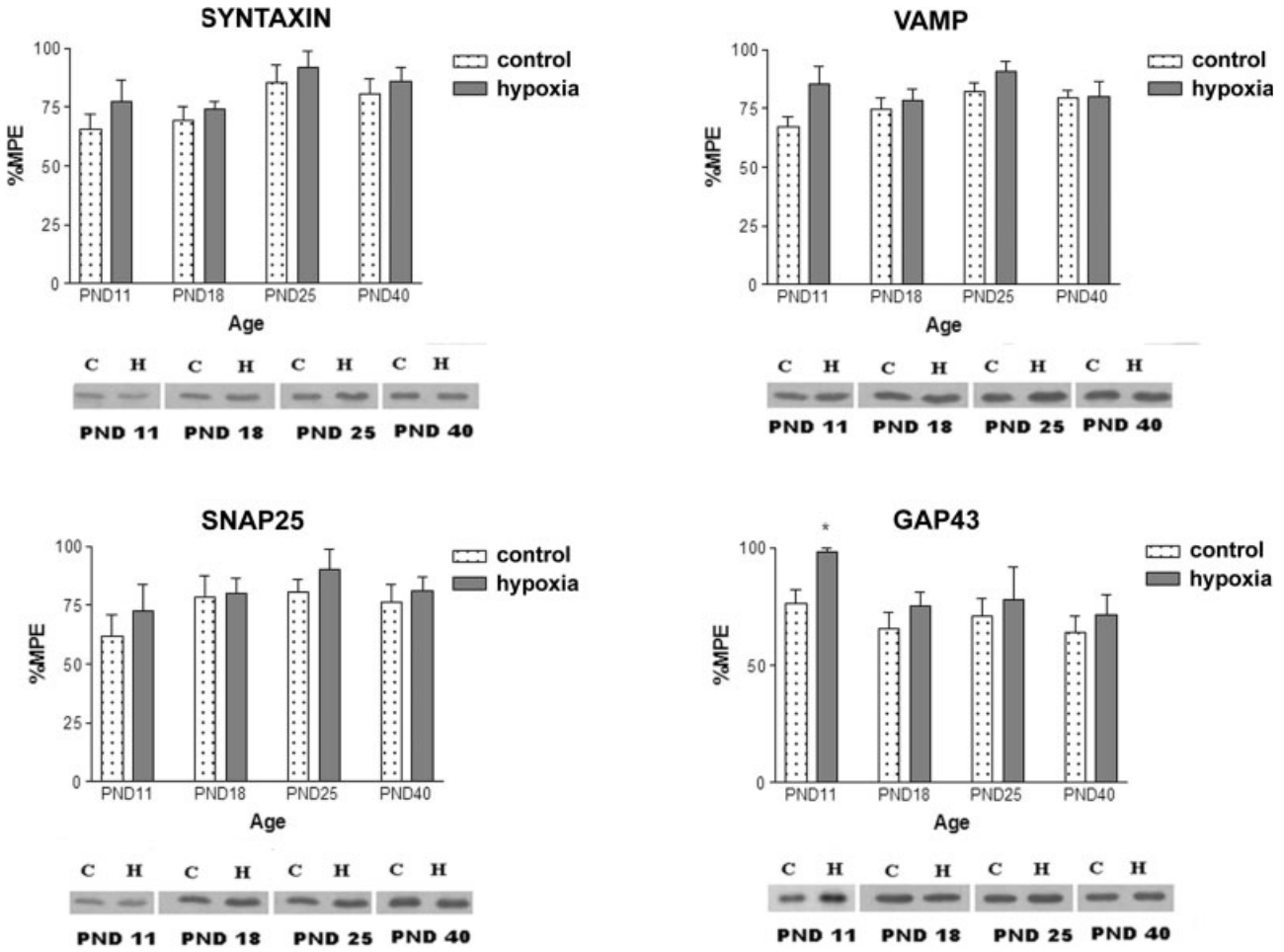

Fig. 5. Western blot of rat striatum. SNARE proteins (syntaxin-1, SNAP-25, VAMP-2) and GAP-43 in the P_2 fraction of rat striatum during postnatal development and after an acute mild hypoxia at PND4. At the bottom of each figure, representative Western blot lanes from one of the experiments are illustrated. GAP-43 was significantly augmented at PND11 in the hypoxic animals with respect to controls (*$P < 0.009$; Student's *t*-test).

retention (memory), and there were no significant differences between the groups.

Elevated plus maze

The percentage of entries and number of entries into the open arm were significantly higher ($P < 0.002$ Student's *t*-test) in neonatal hypoxic animals with respect to controls at PND40 (Fig. 7a). The percentage of time spent in the open arm was higher in the hypoxic animals than in the controls ($P < 0.006$ Student's *t*-test) (Fig. 7b). The latency until the first open arm entry was significantly higher ($P < 0.01$ Student's *t*-test) in the normoxic group compared with the hypoxic animals (Fig. 7c). Furthermore, we observed that hypoxic rats spent more time ($P < 0.001$ Student's *t*-test) in each exploration of the open arm (Fig. 7d). The number of closed arm entries did not differ significantly between groups indicating a similar general locomotor activity of the animals tested by EPM. The other behavioral parameters, grooming (including face and head grooming), peeps, rearings, freezing, and defecation were not modified by hypoxia (Table II).

Spontaneous motor behavior

Locomotor activity (OVM test). There were no statistical differences between the two groups (Student's *t*-test) in either horizontal or vertical activity or in the number of rears during the test period of 5 min (Fig. 8).

DISCUSSION

Global hypoxia in the newborn rat has been proposed as a model for the premature human infant. Rats, after mild postnatal hypoxia, show short-term loss of cortical thickness, behavioral alterations, and long-term modifications in the pattern of neurotrans-

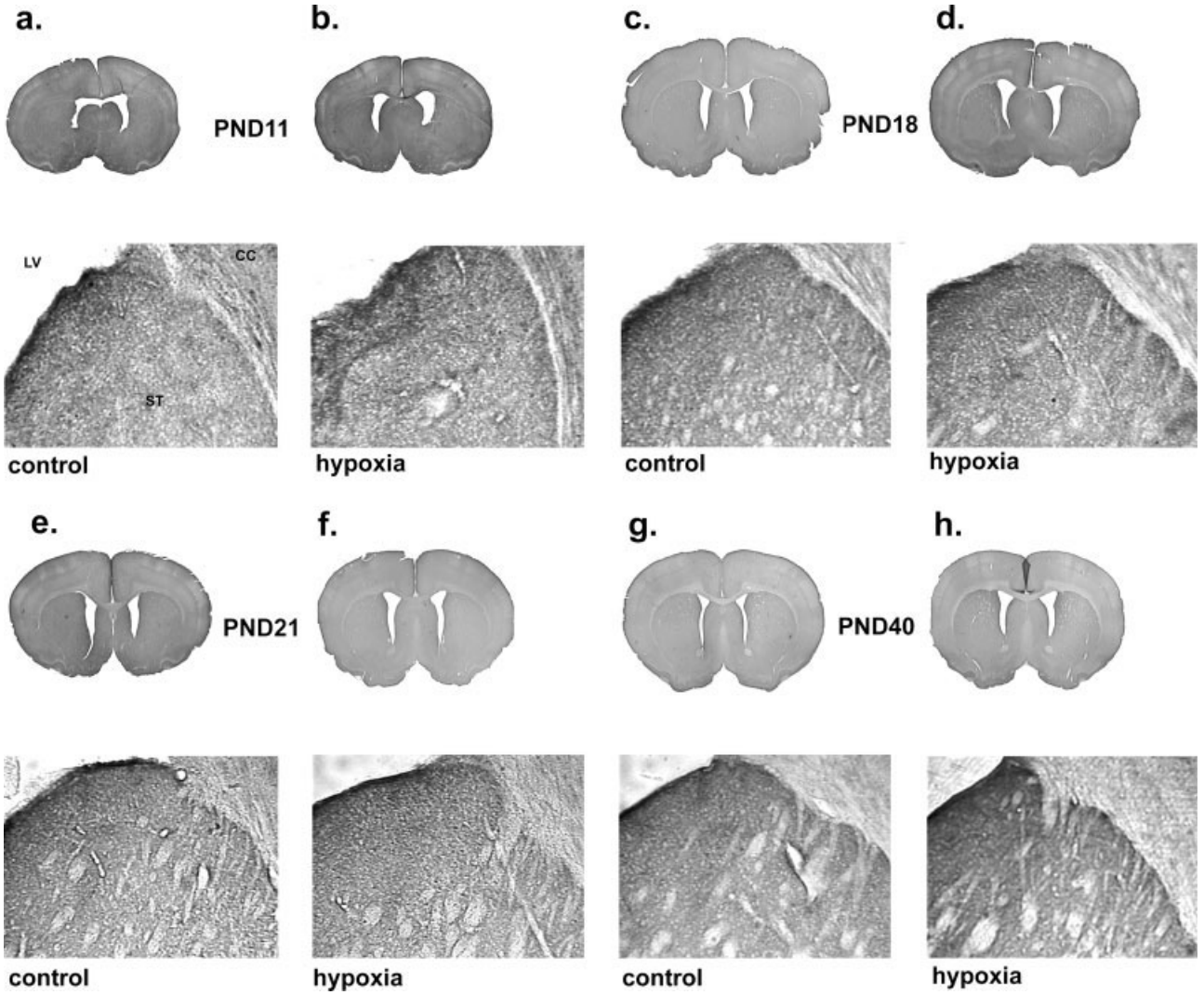

Fig. 6. GAP-43 immunostaining. Low magnification images of representative coronal sections (40 μm) of control and hypoxic rat brains at PND 11,18, 21 and 40 (**a**–**h**) (upper panels). Free floating sections were incubated with anti-GAP-43 antibody and visualized using biotinilated-conjugated secondary antibody and nickel-enhancement technique. Sections are samples from the brain areas depicted in plates between Bregma 1.00 and Bregma 0.48 of the Atlas of Paxinos & Watson, 2nd Edition. A higher magnification showing the marginal zone of the lateral ventricle (LV) and a portion of the ST and CC of the same sections (lower panels). Control rats (left panels) and hypoxia rats (right panels). A total of five animals per group were examined.

TABLE I. Test of retention

	Latency (s)	
Trial time	Control ($n = 31$)	Hypoxia ($n = 30$)
24 h	197 ± 23	221 ± 21 (NS)
7 days	235 ± 20	261 ± 16 (NS)

Values are expressed as mean ± SEM; NS, not significant vs. control, unpaired Student's *t*-test with Welch's correction; $P = 0.1385$; *n*, number of animals.

mission (Gonzalez de Dios and Moya, 1996; Gross et al., 1993).

The SNARE proteins participate in membrane fusion mechanisms underpinning synaptic vesicle exocytosis (Sudhof, 1995), and their decline could affect neurotransmitter release. We chose to measure these proteins based on the information stated above and the concepts reviewed by Masliah and Terry, 1993, that pointed out the importance of presynaptic markers of dendritic damage and disrupted synaptogenesis under diverse pathological conditions.

In a previous paper (Manzur et al., 2001) we reported an early and transient increase in the expression of the v-SNARE protein VAMP-2 and of the vesicle-associated rab3A protein subsequent to hypoxia. The present study was carried out during the second week of life, and a week after the hypoxia, a time corresponding with the transition stage II, when the growth cones mature into fully functional presynaptic terminals. By means of transcriptome analysis applied to a model of global hypoxia, it was observed that the loss of transcription of postsynaptic and syn-

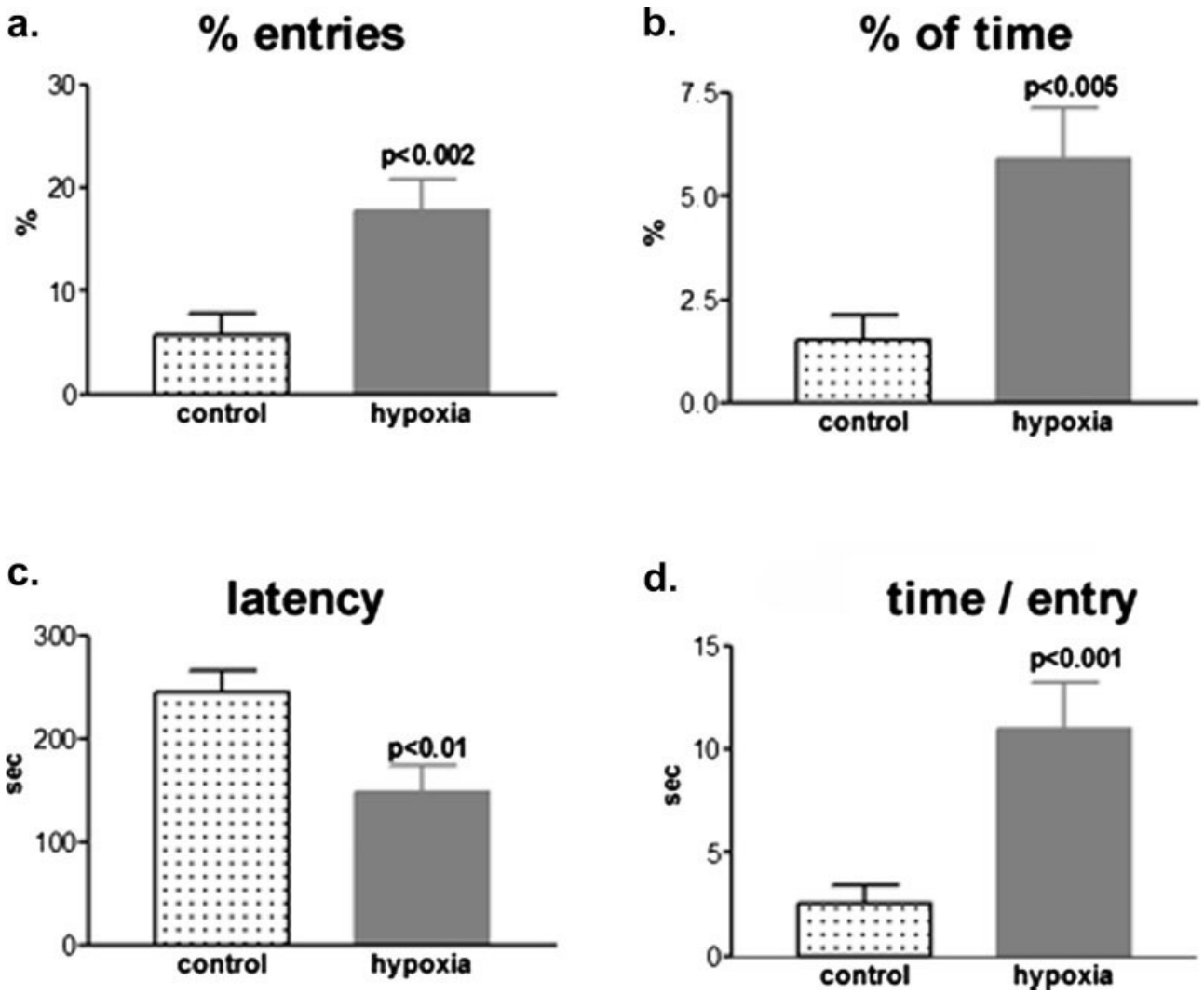

Fig. 7. Elevated Plus Maze. The bars represent the performance of the control and hypoxic animals at PND40 in the maze expressed as (**a**) percentage of entries into the open arm (100 × open/total number of entries); (**b**) percentage of time spent in the open arm (100 × open/total time); (**c**) time in seconds spent by the animal before the first entry into the open arm (latency); and (**d**) time spent in the open arm at each entry. Number of entries were calculated in a period of 300 s per animal. $P < 0.05$ or less is considered significant.

TABLE II. Other behavioral parameters at PND40

	Control ($n = 22$)	Hypoxia ($n = 15$)
Closed arm entries	6.13 ± 0.62	6.40 ± 0.55 (NS)
Grooming	5.73 ± 0.85	5.00 ± 1.03 (NS)
Rearings	2.95 ± 0.50	3.07 ± 0.75 (NS)
Peeps	9.37 ± 0.74	10.60 ± 0.80 (NS)
Fecal boli	0.91 ± 0.40	0.80 ± 0.34 (NS)
Freezing (s)	18.50 ± 7.02	34.00 ± 10.51 (NS)

Values are expressed as mean ± SEM; NS, not significant vs. control, unpaired Student's *t*-test with Welch's correction; *n*, number of animals.

aptic maturation genes typically occurred in two waves, an initial fall at PND4, followed by recovery at PND6, and a more profound fall at PND11. Conversely, the bulk of the up-regulation of presynaptic genes occurred late in the time course of the hypoxic period (PND11) (Curristin et al., 2002). Accordingly, although our model of hypoxia is different to the one applied in the mentioned work, we observed a fall of presynaptic protein expression during the first week of life, a rebound during the second week, and normalization by the third and fourth weeks.

In this work we also show independent regional variability in the normal pattern of IR of the three SNARE proteins during the developmental period and in their response to acute hypoxia. We observed that in the CC of the normal rat, SNAP-25 was the protein that demonstrated the most significant increases with age and that hypoxia did not interfere with this increase. VAMP-2 expression demonstrated a scant sensitivity to hypoxia after the second week, and normalized by PND18. These results agree with those of Patterson and Skene (1999) who also ob-

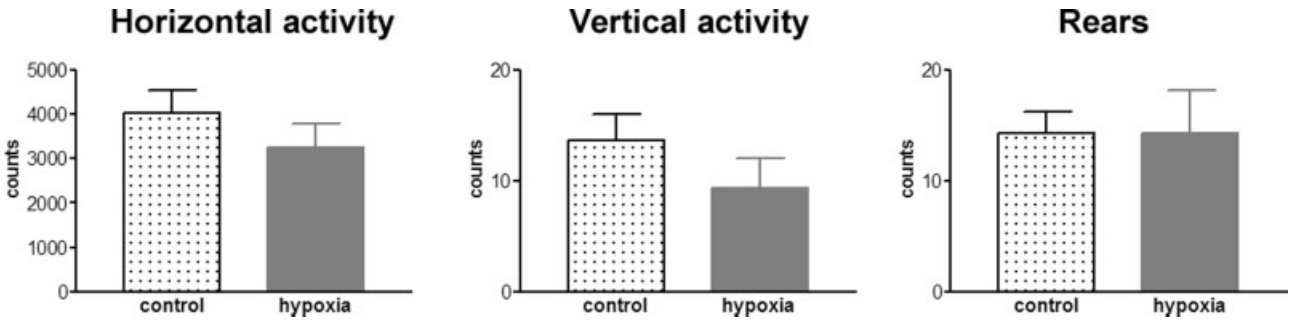

Fig. 8. Locomotor activity. The number of crossings (horizontal activity), bar approaches (vertical activity), and rears in the OVM are represented by the dotted columns for the control group, and filled columns for the animals that were submitted to hypoxia. Columns represent the mean ± SEM for 8–10 animals. Results are expressed in counts during a period of 5 min. NS - not significant vs. control (Student's *t*-test).

served that maximal expression of syntaxin-1 and VAMP-2 in synaptosomes of the CC of normal animals occurred between PND28-45.

In the Hipp of normal rats we observed a similar pattern of changes for the CC, that is the three SNARE proteins tend to increase with age, with SNAP-25 showing the most significant increase and acute hypoxia had slight impact on the SNARE proteins in this brain region after the second week of age.

In the ST, contrary to the pattern shown in the other two regions, the three SNARE proteins remained comparatively steady during normal growth. Previous reports indicate that maximal expression of synaptic GAP-43 can be observed in the CC between PND14-28 (Patterson and Skene, 1999). Hypoxia did not modify the expression of this protein in the CC and in the Hippo, but caused a remarkable increase by PND11 in the ST, for GAP-43 and also for VAMP-2.

Several years ago, Jackson and Nurse (1995) reported that hypoxia stimulates GAP-43 IR in the catecholamine-containing glomus cells, neurons with the capability to act as oxygen chemoreceptors in the carotid body. This effect was dose-dependent and peaked around an oxygen tension of 6%. Carotid body glomus cells are physiologic arterial oxygen sensors that release large amounts of DA in response to hypoxia, and had been widely used in cell grafts as DA-cell replacement in animal models of Parkinson's disease (Espejo et al., 1998). GAP-43 is considered among the early responsive genes in oxidative stress-mediated dopaminergic cell death by 6-OHDA, an operative mechanism in the development of Parkinson's disease, suggesting an important role of this protein on the ST and brain related areas. There have been found other similarities between the carotid bodies and the nigrostriatal pathway, as for example, both mediate baroreflex sensitivity (Lu et al., 1995), so our results suggest that in striatal neurons could operate common second messenger pathways as those proposed to explain the sensitivity of glomus cells to hypoxia. Increases in GAP-43 are usually associated with regeneration (Jacobson et al., 1986) and neurite outgrowth (Meiri et al., 1998) but also to other functions e.g., enhancing neurotransmitter release or sensitization of G-protein-coupled receptor transduction. We leave open the question about the roles of this protein in the developing ST.

Altogether, our results suggest that, at the second week of age, the early hypoxic insult induces an activation of synaptic protein expression rather than a decline in their synthesis. Nonetheless, we cannot discard the possibility of alterations in IR resulting from other mechanisms besides stimulation of protein synthesis, for example, changes in subcellular localization due to changes in phosphorylation (Moretto et al., 1999) or palmitoylation (Patterson and Skene, 1999).

The lack of a parallel increase of the GAP-43 immunostaining compared to the observed peak at PND11 in the immunoblotings of the STs of hypoxied animals could have several explanations: One of them would be differences in the tissues handling for both techniques. The time consuming fixation could be hampering rapid molecular events. This disadvantage is more likely to be corrected by the rapid freezing of tissues destinated to be the samples for WB. Perhaps a time course immunohistochemical study of this event may help to find out the exact anatomical location of the hypoxia sensitive neurons in the ST. On the other side, we could have lost detail using the technique with nickel enhancement obtaining higher backgrounds in detriment of contrast. In spite of these drawbacks and lack of additional data, we speculate that this might be a transient event, lasting a few hours during PND 11 as a result of the hypoxic stimulus performed a week before. In a previous work we detected an "early wave" of activation, of a synaptic protein after hypoxia (Manzur et al., 2001). We still don't know if these are genomic or posttransductional events and further work needs to be done to understand better the nature of this observation.

After intermittent hypoxia, Simonova (Simonova et al., 2003) found that rats learn a task more slowly than control animals tested in the Morris water maze. Our procedure seems to be innocuous with respect to learning and memory processes in young adult animals submitted to acute hypoxia during the early postnatal period. Nonetheless, it is reasonable to assume that other behavioral systems may be disturbed, based on previous data on effects of postnatal anoxia on the locomotor system (Buwalda et al., 1995). We evaluated locomotor activity by the OVM test and anxiety by means of the EPM paradigm. The higher percentage of time spent in the open arms of the plus maze by the hypoxic rats means that they display a significantly lower level of anxiety than their normoxic counterparts in the 40-days old animals and this effect persisted over time, as we were able to observe the same results in another group of 3-month-old male rats (data not shown). The lack of differences in the number of entries to the closed arm among the hypoxic and control rats indicates that the locomotor activity was not significantly altered by the neonatal hypoxia. This interpretation was supported by the spontaneous activity test, which showed no major changes with respect to normal animals. These results suggest that there may be an anxiolytic component unmasked by hypoxia. These results are in agreement with previous observations after acute neonatal asphyxia; young adult animals showed reduced anxiety-related behavior evaluated by the EPM (Hoeger et al., 2000), but it is puzzling to explain a relationship between this kind of behavioral response and a molecular change in the ST, a brain area more related to motor responses and less likely to be involved in emotional stress.

We are still far from being able to establish the exact consequences of perinatal hypoxia, whether detrimental, through formation of anomalous connections, or beneficial, by compensating for neuronal disruption as it has been observed when applied as a preconditioning stimulus (Vanucci et al., 1998). The critical interface between normal mechanisms of development and postinjury adaptive processes in the immature brain remains poorly defined, or whether or not these adaptative mechanisms are able to induce behavior modifications. This study supports the concept that subtle changes at the molecular level, during the development of the synaptic system, could be associated to mild behavioral disruption at later stages of life. On the other hand, the lack of more sustained damage or permanent disruption of the synaptic protein expression is showing us the potency of self-repair ability mechanisms operating after birth, even long after many neural pathways have yet been fully consolidated. Additional efforts should be focused on this point to elucidate protective therapies that could be applied to the human infant at risk of perinatal asphyxia.

In conclusion, we report that acute global neonatal hypoxia induces subtle transient modifications of SNARE protein expression in specific areas of the brain. We found that the protein GAP-43 is sensitive to hypoxia in the ST during the first and second week of life of the rat. These changes might be functionally related to altered long-term behavioral responses, such as a decrease in anxiety.

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