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Research Article

Long-term sequelae of perinatal asphyxia in the aging rat

R. Weitzdoerfer[a], N. Gerstl[a], H. Hoeger[b], W. Mosgoeller[c], W. Dreher[d], E. Engidawork[a], J. Overgaard-Larsen[e] and B. Lubec[a,*]

[a] Department of Neonatology, AKH, University of Vienna, 1090 Vienna (Austria), Fax: + 43 1 40400 3194, e-mail: gert.lubec@akh-wien-ac.at
[b] Institute of Animal Breeding, Himberg (Austria)
[c] Department of Tumor Biology, AKH, University of Vienna, Vienna (Austria)
[d] Fachbereich 2 (Biologie/Chemie), University of Bremen, Bremen (Germany)
[e] Stereological Research Laboratory, University of Aarhus, Aarhus (Denmark)

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Abstract. Information on the consequences of perinatal asphyxia (PA) on brain morphology and function in the aging rat is missing although several groups have hypothesized that PA may be responsible for neurological and psychiatric deficits in the adult. We therefore decided to study the effects of PA on the central nervous system (CNS) in terms of morphology, immunohistochemistry, neurology and behavior in the aging animal. Hippocampus and cerebellum were evaluated morphologically by histological, immunohistochemical and magnetic resonance imaging and cerebellum also by stereological tests. Neurological function was tested by an observational test battery and rota rod test. Cognitive functions were examined by multiple-T-maze and the Morris water maze (MWM). Increased serotonin transporter (SERT) immunoreactivity in the CA2 region of the hippocampus and a significant difference in the escape latency, when the platform of the MWM was moved to a new location, were observed in asphyxiated rats. We showed that deteriorated cognitive functions accompanied by aberrant expression of hippocampal SERT and impaired relearning are long-term sequelae of perinatal asphyxia, a finding that may form the basis for understanding CNS pathology in the aging subject, animal or human.

Key words. Perinatal asphyxia; aging; magnetic resonance imaging; stereology; serotonin; Morris water maze.

Introduction

Perinatal asphixia (PA) is a major determinant of neurological morbidity and mortality in the neonatal period, causing long-term neurological complications such as motor deficits including cerebral palsy, seizure disorders and mental retardation [1].

Information on the morphological and cognitive long-term consequences in the brain of rats with graded PA is limited to a couple of reports addressing these questions. Delayed neuronal death in the cerebellum of rat pups with PA at the 8th day postpartum has been described previously [2]. In the identical rat model of PA, 3 months following the asphyxiating insult, neuronal loss was found in area CA1 of the hippocampus [3], myelination deficits were observed in the cerebellum of animals that had been subjected to 20 min of PA: astrocytosis/glial proliferation, reduced staining for myelin basic protein and CNPase, a marker for myelination and oligodendroglial density, were found [4]. For 20 min PA, no neuronal loss was found in the cerebellum, however, the number of nuclei with chromatin changes was significantly and remarkably elevated [3].

* Corresponding author.

Immunohistochemical investigations at the same time point in the identical animal model of PA revealed decreased tyrosine hydroxylase staining of fibers in the white matter of the cerebellum [5]. Neurologically, motor deficits possibly linked to cerebellar lesions were observed in rat PA at very early stages ranging from days to several weeks [6], which seemed to be compensated 3 months after PA [7].

However, no single comprehensive study has been carried out and therefore the objective of this work was to show whether PA affects the hippocampus and/or cerebellum in rat aged 2 years, at the end of its life span. We decided to evaluate hippocampal morphology and functions by magnetic resonance imaging (MRI), by histological techniques such as Nissl staining for morphometrical analysis of neuronal density, and including stereological measurements and Kluever-Barrera-myelin staining to visualize white matter defects. For evaluation of gliosis and markers for neurotransmission, immunohistochemical methods were employed. Functionally, animals were tested for cognitive functions including learning and memory and motor functions were examined by neurological and rota rod experiments.

We found a statistically significant increase in the time needed to find a hidden platform in Morris water maze (MWM) in rats with 20 min PA indicating impaired relearning and increased expression of serotonin transporter (SERT) in CA2, indicating involvement of serotonergic innervation in long-term complications of PA in the aging rat.

We showed that deteriorated cognitive functions accompanied by aberrant expression of hippocampal SERT and impaired relearning are long-term sequelae of PA, a finding that may form the basis for understanding central nervous system (CNS) pathology in the aging subject, animal or human.

Materials and methods

Experimental design

PA was induced in pups delivered by Cesarean section on pregnant Sprague-Dawley rats. Within the last day of gestation as evaluated by estabularium protocols, animals were sacrificed by neck dislocation and hysterectomized. The uterus horns, still containing the fetuses, were extirpated and placed into a water bath at 37 °C for periods of 10 and 20 min, the longest period a sufficient number of animals survive in our system. In the groups with normoxia and 10 min of asphyxia 100% survival is found; in the group with 20 min of asphyxia, 74% survive (at 21 min of asphyxia, only 10% of the pups survive; 22 min of asphyxia are inevitably associated with death).

Asphyxiated pups and controls were obtained from the same mother, since each rat delivered approximately 10–14 pups. Following the asphyxiating period, uterus horns were rapidly opened and pups removed. Pups were cleaned, the umbilical cord was ligated and animals were allowed to recover in a hood. Only litters with pups weighing more than 4.5 g at the time of delivery were used in the study. After adaptation in the hood, rat pups were given to surrogate mothers until they were about 3 months old. Female rats were then housed in cages of three to five animals, while male rats had to be kept individually to prevent fighting and cannibalism. After 2 years, animals were either sacrificed for morphological studies or tested in neurological and behavioral assays with subsequent MRI. Animal experiments performed on 2-year-old rats were carried out according to the rules of the American Physiology Society.

Cognitive tests

1) Morris water maze. On 3 consecutive days, 27 female control and asphyxiated animals were tested in a circular pool (140 cm diameter, walls 50 cm high). The pool was filled with water (21 °C) and contained a small platform made of plexiglas to render it invisible to the rats with its top 1–1.5 cm beneath the water surface. Rats were released into the water, head facing the wall and away from the platform and then were allowed to swim until they found the target and stayed on it for 5 s or until 2 min elapsed. If the rat was not able to find the target within 2 min, it was placed manually on the platform and stayed there for 10 s. On 3 consecutive days, the platform remained in the same place and rats were released into the water from a fixed starting position.

The time needed to find the platform (escape latency) was measured. To test memory ability, 10–14 days later, rats were again released into the water under the same conditions. Ten to 14 days later, the platform was moved to a new location (in the opposite quadrant), and the ability of relearning to find the moved platform was evaluated by measuring the escape latency.

2) Multiple-T-maze. Thirty-three female animals of control and asphyxiated groups were tested for 10 min in a multiple-T-maze with seven choice points and dimensions of 150 cm × 130 cm, 15-cm-high walls and a width of 8 cm. Before the test started, rats were deprived of food in order to motivate them. Rats were tested on 4 consecutive days by placing them into the start box and being offered food in the goal box. Ten to 14 days later, their memory was tested. The number of correct and wrong decisions as well as the time needed to find the goal box were measured.

Neurological evaluation of motor functions

Thirty-one female Sprague-Dawley rats of control and asphyxiated groups were examined in the following tests.

1) Locomotor activity. Activity and speed of movements

were scored for 4 s (duration: 0–4 s; speed of movement: slow or inactive = 1, active = 1.5, rapid = 2).
2) Alley traverse. One rat at a time was placed on the middle of an elevated plywood bridge 60 cm long and 3 cm wide with a platform on each end. The distance covered within 15 s was scored (distance: 0–60 cm).
3) Wire maneuver. The animal was lifted by its tail and allowed to grasp the horizontal wire with its forepaws, and was then rotated partially downward and released. The ability of the animal to grasp the wire also with its hind limbs was scored (0 = grasps actively with hind limbs, 2 = moderate difficulties to grasp with hind limbs, 4 = unable to grasp with hind limbs, 6 = falls within 6–10 s, 8 = falls immediately).
4) Grip strength. The animal was allowed to grasp a wire-mesh grid and was then horizontally pulled backwards. The Grip resistance of the animal to this backward pulling was scored (0 = no resistance; 2 = slight grip, semi-effective; 4 = moderate grip, effective; 6 = active grip, effective; 8 = unusually effective).

Rota rod

Animals were placed on a rod (Rota Rod 'Economex'; Columbus Instruments, Ohio) and allowed to acclimatize to the situation. The rota rod was then turned on at a setting such that the majority of the animals could stay on the rod for at least 10 s in the test session which took place before the experiment started (6 rpm and 0.58 rpm/s acceleration speed). The rats were examined in four sessions. The time spent on the rotating rod was measured [8].

Magnetic resonance imaging

Sixteen female control rats and 21 female rats with an asphyxia time of 20 min were used for the experiment. The MRI experiment was performed on a 4.7 T/40 cm Biospec system (Bruker, Karlsruhe, Germany) equipped with actively shielded magnetic field gradients capable of switching 170 mT/m in 0.45 ms.

Stereology

At the age of 2 years, 20 male animals of control and asphyxiated groups were anesthetized and killed by an intraperitoneal overdose of ketamin and xylazin. They were subsequently perfused through the left ventricle with 4% paraformaldehyde in phosphate-bufferd saline (PBS). Brains were removed from the skull, post-fixed and kept at 4 °C until stereological experiments started [3]. One half of the cerebellum was selected randomly for stereological analysis [9]. The total number of granular and Purkinje cells in the cerebellum was estimated using the optical fractionator technique [10]. Total volumes of the molecular layer, granular layer and white matter were estimated according to Cavalieri's principle [11].

Histology and immunohistochemistry

Animals at the age of 2 years were anesthetized with Membumal 50 mg/kg body weight intraperitoneally and perfused transcardially with 30–50 ml of 0.1 M PBS pH 7.4 containing 4% paraformaldehyde, and then transferred to 4 °C for 60 min. Brains were removed from the skull, post-fixed in the same solution for 12–18 h and then rinsed in PBS containing 20% sucrose at 4 °C. Paraffin embedding and sectioning were described previously [2, 12].
Slides were dewaxed in three changes of xylene, rehydrated in descending series of ethanol, and briefly rinsed in distilled water and PBS. For antigen retrieval, slides were incubated in 5 mM EDTA in PBS (pH 8.0) at 95 °C for 60 min, allowed to cool down to room temperature, rinsed in PBS three times, incubated in PBS containing 0.3% Triton-X for 25 min and incubated in PBS containing 5% bovine serum albumin and 0.1% Tween-20 for 20 min to block unspecific binding sites. All primary antibodies were incubated overnight in commercially available antibody diluens (Dako). The following primary antibodies were used: anti-glial fibrillary acidic protein (GFAP) to label astrocytes (Dako, Z0334) diluted 1 : 500; anti-vesicular monoamine transporter (vMAT) (Chemicon, AB1767) diluted 1 : 500; anti-vesicular acetylcholine transporter (vAChT) (Chemicon, AB1578) diluted 1 : 1000; anti-SERT (Oncogene Research Products, PC177L) diluted 1:400. Following incubation with primary antibody, slides were again washed three times in PBS, and incubated with biotin-labeled species-specific secondary antibodies (all from Dako) for 60 min. Non-specific endogeneous peroxidase was blocked in 1% H_2O_2 for 15 min, followed by a rinse with PBS and incubation with streptavidine-labeled with horseradish peroxidase (HRP) for 30 min. Following three rinses, we used DAB as substrate for HRP (Linaris staining kit); the developing time was 14 min. Following three rinses with PBS, slides were counterstained with 0.02 % hematoxylin (Sigma) for 2 min, rinsed in distilled water and tap water. Slides were then dehydrated in ethanol and xylene and mounted with Eukitt.
For generating negative controls, first antibodies were omitted. Slides were examined on a Nikon Microphot equipped with a Nikon digital Coolpix 900 camera. Morphometric anaylsis was done as previously described [3]. Immunoreactive neuronal fibers or perikarya were counted on photographs taken at a magnification ×10 for GFAP in cerebellum and vAChT and SERT in hippocampus, at a magnification ×20 for SERT in cerebellum, at a magnification ×40 for vMAT in cerebellum and hippocampus and at a magnification ×100 for vAChT in cerebellum. The density values obtained were converted to immunoreactivity units mm^2.

Statistics

To compare the results of motor and cognitive functions between groups, Mann-Whitney U tests and χ^2 test were used. In the MWM, the value 120 s was inserted if a rat failed to reach the platform.
Stereological results were statistically evaluated using ANOVA.

Results

Cognitive functions

1) Morris water maze. During learning as well as during the memory test, the time needed to find the hidden platform was comparable between all groups. The relearning test showed a statistically significant elongation in the time needed to find the new location of the platform in rats with 20 minutes of PA compared to control animals ($p = 0.0017$) (table 1).
2) Multiple-T-maze. During all sessions, most of the control as well as asphyxiated animals failed to reach the goal box within 10 min but a Fisher exact test did not reveal any difference between the number of animals that succeeded and those that failed between groups (table 2). The number of correct and wrong decisions could not be used for evaluation, because the group of animals to reaching the goal box was too small.

Motor functions

In the locomotor activity test, rats were active and showed normal motor activity during the testing time of 4 s. No statistically significant difference was found between groups. The covered distance on the elevated alley did not show any difference between asphyxiated and control groups. In the wire maneuver test, most of the rats showed moderate difficulties to grasp the wire with their hind limbs but results were comparable between groups. Though grip strength increased with the length of asphyxia, there was no statistical difference between control and asphyxiated rats. Time rats spent on the rota rod was comparable between groups.

Magnetic resonance imaging

MRI of 9 control and 10 animals with 20 min of PA were evaluated; the other samples could not be used due to hypophyseal tumors leading to brain compression. A Fisher test did not reveal a difference in the number of animals with or without tumorous changes between control and asphyxiated rats.
In 2 out of 9 control and in 2 out of 10 asphyxiated animals, the hippocampus showed hyperintense areas in the subiculum which may have been due to expanded perivascular tissue caused by neuronal loss in this area. No hypointense pathologies, indicating hippocampal apoplexy, were found. All other samples were free of any morphological changes (fig. 1 a).
In sagittal sections, the configuration of the cerebellum in the skull as well as the anatomical structure of the cerebellum appeared to be normal within all groups, and the fourth ventricle in the asphyxiated rats did not show any morphological changes.
In coronal sections, cerebella of asphyxiated rats were free of hyperintense areas, ruling out edema, infarction, scarring and demyelination. No hypointense areas were found (fig. 1 b).

Stereology

Medians of the cerebellar weight and total volumes of the molecular layer, granular layer and white matter were comparable between groups. Estimation of total granular cell number in the cerebellum did not show a significant difference between groups of various asphyxia times. To-

Table 1. Results of Morris water maze.

	Control (n = 11) median (minimum–maximum)	10 min asphyxia (n = 10) median (minimum–maximum)	20 min asphyxia (n = 6) median (minimum–maximum)	Statistical significance (comparison of asphyxiated groups to normoxia group)
Time to reach the platform (first trial)	38.0 (2–88)	44.5 (4–120)	54.5 (12–120)	n. s.
Time to reach the platform (second trial)	43.0 (5–120)	49.5 (5–120)	14.0 (5–95) (n = 5)	n. s.
Time to reach the platform (third trial)	69.0 (19–120)	55 (12–120)	89.0 (11–120)	n. s.
Time to reach the platform (memory)	29 (14–91)	49.5 (20–120)	58.5 (4–120)	n. s.
Time to reach the platform (relearning)	7.5 (4–36) (n = 10)	26.5 (3–120)	74** (22–120)	** $p < 0.01$ between control and 20-min-asphyxia-group

n. s., not significant.

Table 2. Results of multiple-T-maze.

	Control (n = 12)	10 min asphyxia (n = 12)	20 min asphyxia (n = 9)	Statistical significance (comparison of asphyxiated groups to normoxia group)
Succeeded first trial	4 (33.3%)	5 (41.7%)	2 (22.2%)	n.s.
Succeeded second trial	5 (41.7%)	6 (50%)	5 (55.6%)	n.s.
Succeeded third trial	6 (50%)	7 (58.3%)	5 (55.6%)	n.s.
Succeeded fourth trial	2 (16.7%)	6 (50%)	3 (33.3%)	n.s.
Succeeded memory test	3 (25%)	4 (33.3%)	3 (33.3%)	n.s.

n.s., not significant.

tal number of Purkinje cells, volume of Purkinje cells as well as volume of Purkinje cell nuclei were unaffected. The relationship of Purkinje cell nuclei to soma was not statistically different between groups (table 3).

Histology and immunohistochemistry

1) Hippocampus. Morphological evaluation showed cells with nuclei surrounded by well-contrasted cytoplasm containing Nissl substance. An occasional few cells showed signs of degeneration but there was no recognizable difference in cell form, size or degeneration between control and asphyxiated animals. Neuronal density and white matter structure in the hippocampus were similar in control and asphyxiated rats. No difference in GFAP immunoreactivity, as a marker for astrocytic gliosis, was found between groups. Immunostaining with vAChT and vMAT did not show differences between asphyxiated and control groups (fig. 2).

Table 3. Cerebellar weight and volume of the cerebellar layers and stereological results on Purkinje cells and granular cells.

	Control (n = 7) median (minimum–maximum)	10 min asphyxia (n = 6) median (minimum–maximum)	20 min asphyxia (n = 7) median (minimum–maximum)	Statistical significance (comparison of asphyxiated groups to normoxia group)
Cerebellar weight (mg)	273.40 (255.80–296.80)	283.75 (234.90–300.70)	288.70 (253.90–298.40)	n.s.
Molecular layer (mm^3)	56.27 (46.70–75.05)	67.56 (47.82–72.56)	62.02 (43.44–83.15)	n.s.
Granular layer (mm^3)	42.94 (31.03–50.52)	42.90 (31.23–51.90)	48.59 (31.97–52.31)	n.s.
White matter (mm^3)	29.46 (24.22–30.58)	31.26 (18.67–32.47)	30.19 (23.34–35.94)	n.s.
Total Purkinje cell number/cerebellum	380,439 (244,568–468,012)	372,497 (348,083–463,979)	416,424 (217,121–464,227)	n.s.
Total granular cell number/cerebellum	1.613000E+08 (1.054000E+08–2.001000E+08)	1.552000E+08 (1.286000E+08–1.754000E+08)	1.731000E+08 (1.042000E+08–2.239000E+08)	n.s.
Geometric mean volume of the Purkinje cell soma (μm^3)	4361.3 (4286.5–4976.7)	4660.3 (4358.3–5419.5)	4481.4 (3874.9–5363.0)	n.s.
Geometric mean volume of the Purkinje cell nucleus (μm^3)	914.0 (737.8–1031.9)	1007.7 (855.3–1178.7)	886.5 (742.5–1149.9)	n.s.
Nucelus-soma relationship (%)	28	29	27	n.s.

n.s., not significant.

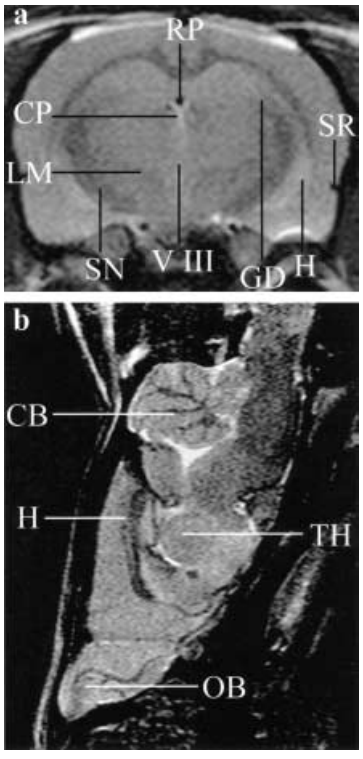

Figure 1. (*a*) MRI in the coronal plane of a rat with 20 min PA (CP, comissura posterior; GD, gyrus dentatus; LM, lemniscus medialis; H, hippocampus; RP, recessus pinealis; SN, substantia nigra; V III, 3rd ventricle). No hypointense areas were found in the hippocampal area. Two control and two animals with PA showed hyperintense areas, maybe due to edema, infarctions, scarring or demyelination. Nevertheless, no significant difference in the hippocampus was found between control animals and animals with PA. (*b*) MRI in the sagittal plane performed on the brain of a rat exposed to 20 min of PA (CB, cerebellum; TH, thalamus; H, hippocampus; OB, olfactory bulb). The anatomical structure of the cerebellum is normal and no hypo- or hyperintense areas are visible, indicating that edema, infarction, demyelination and apoplexy can be ruled out.

SERT immunoreactivity showed a statistically significant increase in CA2 of rats with 20 min of PA compared to control animals (means ± SD; controls, 15.07 ± 6.44; 10 min of asphyxia, 18.20 ± 5.13; 20 min of asphyxia, 29.63 ± 11.61; p = 0.0175, fig 3).

2) Cerebellum. vAChT, vMAT and SERT immunostaining did not show differences between control and asphyxiated rats.

Discussion

PA has been postulated to lead to neurological and psychiatric deficits in adult life [13]. We were challenged by this hypothesis and focused on the effects of PA on the hippocampus and cerebellum as hypoxia-sensitive structures in aged rats. We decided to investigate the effect of PA on the aging rat, expecting aggravation of long-term

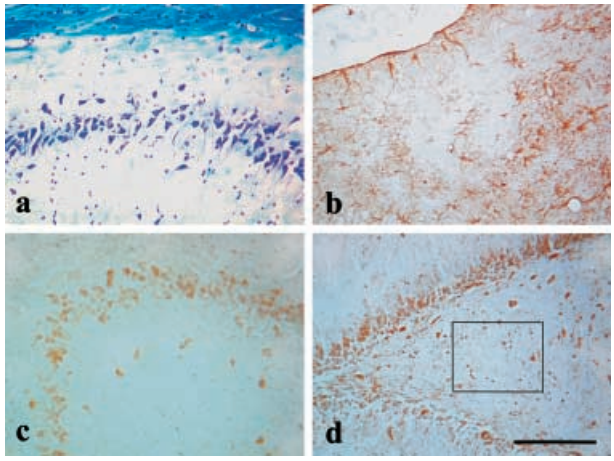

Figure 2. One representative picture of each staining method investigated (×10). Evaluation did not reveal any difference between control animals and the groups with PA. Bar = 150 µm. (*a*) Kluever-Barrera staining (CA2 region of hippocampus). (*b*) Anti-GFAP staining (fimbriae of hippocampus). (*c*) Anti-vAChT staining (CA2 region of hippocampus). (*d*) Anti-vMAT staining (gyrus dentatus: the square indicates the investigated area).

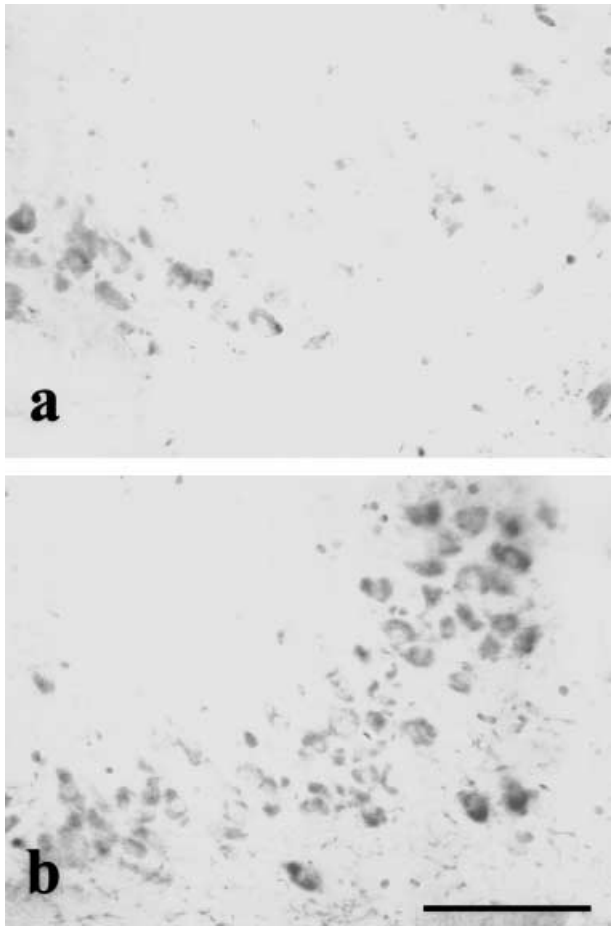

Figure 3. Comparison between area CA2 of a control animal (*a*) and of an animal with 20 min of PA (*b*), with a significant increase in immunoreactivity of serotonin transporter (SERT) in the latter (×20 bar = 75 µm).

complications by the aging process per se. At the age of about 2 years, however, neither morphological changes in the hippocampus and cerebellum nor functional motor impairment were found in the 2-year-old animals asphyxiated for as long as of 20 min (most rat pups do not survive 21 min of PA) [14] but immunostaining with SERT revealed significantly higher immunoreactivity in the hippocampus of rats with 20 min of PA and the MWM test as a cognitive assay revealed a statistically significant reduction in relearning ability in the same group of rats. Impairment in relearning was reflected by swimming in the original training quadrant for a longer period than control animals, remaining in the latter localization of the platform rather than looking for a new possibility to escape. Thus they showed less flexibility in solving a new task leading to significantly longer escape latency (time until the rat mounted the platform). Differences in swimming ability and motivation could be ruled out because escape latencies in all groups during learning and the memory test of the MWM were comparable. Therefore, the aging rat with 20 min of PA showed impaired cognitive flexibility. This finding of deranged hippocampal function as revealed by the MWM test was accompanied by parallel findings of increased SERT immunoreactivity, indicative of aberrant serotonergic innervation in the CA2 region of the hippocampus in aging rats of the same asphyxia group. As shown in the literature, changes in the serotonergic system lead to changes in the ability to perform the MWM test and, indeed, overexpression of SERT modulates the serotonergic function by changing serotonin transport. Malleret et al. [15] showed that knock-out mice of the serotonin receptor type 5-HT1B had reduced escape latency during the transfer task [15]. Furthermore, mice lacking serotonin-1A-receptors improved more slowly during the learning sessions and did not show a preference for the quadrant originally containing the platform during a task when the platform was removed [16]. Whereas serotonin 5-HT-2C receptor mutant mice were shown to have comparable latencies as controls during learning, they did not show a preference for the trained site when the platform was removed [17]. A 5-HT3 receptor antagonist [18] as well as a 5-HT4 receptor agonist [19] were shown to lead to improved acquisition of the MWM task in rats. Therefore our findings of impaired performance in this test in aging rats with 20 min of PA might, at least partially, be explained by increased immunoreactivity of SERT, a marker for the serotonergic system and reflecting deranged serotonin transport in the hippocampal CA2 region in the same asphyxia group.

We conclude that PA is responsible for impaired cognitive function in terms of relearning and a deranged serotonergic system (transport) in the aging rat at the end of its life span in the absence of neurological and morphological changes. This is the first study showing a long-term complication of PA in an aging animal model which may well be relevant and of interest for understanding brain disease in the aging mammalian. We are now about to work on the link between aberrant SERT expression and additional parameters for further evaluation of the serotonergic system and correlation with cognitive tests.

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