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Hypercholesterolemic diet applied to rat dams protects their offspring against cognitive deficits. Simulated neonatal anoxia model

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Abstract

There is accumulating data suggesting a neuroprotective activity of cholesterol, especially in stroke and Alzheimer's disease (AD). In the present study, a protective activity of this lipid in simulated neonatal anoxia was investigated. Rats were subjected to high cholesterol by feeding their dams with a diet enriched with cholesterol. Half of these rats were subjected to anoxia. One and a half months later, the rats were tested for their ability to acquire a spatial memory, one group on the linear maze and the other on the Morris water maze. After these assessments, the level of total plasma cholesterol was measured. Rats from dams subjected to neonatal anoxia on standard diet performed worse than control rats in both types of behavioral experiments, whereas anoxic rats from dams were housed on hypercholesterolemic diet performed as control animals. It suggests that dietetic cholesterol applied by their dams protected rats against cognitive deficits elicited by neonatal anoxia. Furthermore, offspring of anoxic rats housed on standard diet had elevated levels of blood cholesterol in relation to control animals. Generally, anoxia affected the concentration of this lipid much stronger than hypercholesterolemic diet of their dams. It might mean that the anoxia-related rise of cholesterol could be involved in physiological phenomenon being an adaptive response to neurotoxic processes. This concept is discussed in relation to pathological mechanisms in AD.

Keywords: Lipid diet; Cholesterol; Cognitive functions; Spatial memory; Anoxia; Neonatal asphyxia; Morris water maze; Neurodegenerative disorders; Alzheimer's disease; Neuroprotection; Central cholinergic transmission

1. Introduction

The majority of brain mass (about 60%) consists of different lipids. Among them, cholesterol and long-chain polyunsaturated fatty acids (LC-PUFA) have particular significance. The role of LC-PUFA in the ontogeny development of an organism as a whole and particularly of the brain is well established [1–6]. In contrast, high levels of cholesterol are still widely considered as a unanimously negative factor, although there is more and more data indicating the beneficial role of cholesterol for brain activity in many situations. The strongest evidence supporting this point of view comes from work of Vauthey et al. [7]. The authors revealed that a high level of total plasma cholesterol correlates with improved outcome in patients who underwent their first stroke. Moreover, there are publications suggesting the positive role of cholesterol in Alzheimer's disease (AD)—the most frequent neurodegenerative disorders in humans. It has been shown that individuals suffering from this illness have reduced levels of cholesterol measured in cerebrospinal fluid in relation to the level of this lipid determined in age-matched controls [8]. In line with the above findings are results obtained in the study exploring vesicles constructed from lipids coming from brains of postmortem brains of AD patients [9]. It was determined that cholesterol content was reduced in these vesicles and their thickness was diminished. The similar alteration in biophysical properties of artificial membranes was determined in another study [10].

It has also been proven that high cholesterol level diminishes the rate of amyloid precursor protein (APP) metabolism, in vitro [11] as well as in vivo [12]. An experiment revealed that in cell culture supplemented with cholesterol, the amount of α-secretase activity products was diminished. Howland et al. [12] used genetically modified mice with a mutated human gene coding for APP (the

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Swedish mutation). Brains of mice fed with hypercholesterolemic diet were found to have a diminished amount of APP metabolites, including β-amyloids. The accumulation of β-amyloids and resulting formation of senile plaques is regarded as a major harmful factor in AD [13].

The brain is the most susceptible organ to the effects of action of different negative factors. Birth in almost all mammals is potentially one of the most pathological processes. Most frequently, complications are related to exposure to anoxia, hypoxia, ischemia or the combination of these states (for the purpose of this work, only one term—anoxia—will be used, meaning one of the above three mentioned disturbances).

In humans, severe anoxia may cause variable locomotor and mental disturbances under the common syndrome of cerebral palsy. Sometimes, the effects of perinatal complications are revealed with a long delay, even years after birth in the form of different mental disturbances. Attention deficit hyperactivity disorder (ADHD) could be regarded as one of the most common kind of such disturbances [14].

Exposure of newborn rats to an atmosphere of pure nitrogen known as simulated neonatal anoxia is used to mimic some effects of human neonatal anoxia. It was found that rats subjected to this procedure demonstrate cognitive and emotional disturbances in later stages of their development [15–17]. Behavioral tasks devoted to evaluate the efficacy of an acquisition of spatial memory in rats (such as different kinds of maze) are useful tools because this kind of memory reflects some features of human declarative memory. Declarative memory is the cognitive function most vulnerable to neurodegenerative processes taking place in normal ageing and to a higher degree in AD. In addition, similar types of declarative memory deficits may occur as a result of previously underwent neonatal anoxia.

Based on the knowledge of the presumed neuroprotective activity of cholesterol in disorders, such as stroke and AD, on the one hand, and concerning cognitive disturbances due to processes during birth on the other, studies aimed to verify whether cholesterol conveys protective activity in the model of simulated neonatal anoxia were carried out.

2. Materials and methods

2.1. Animals and experimental groups

One hundred and eighteen Wistar rats were housed at a temperature of 22 °C (± 1 °C) in constant humidity conditions with a stable 12:12-h light–dark schedule.

The experimental rats were the offspring of rats fed on the modified diets since their mating until the weaning period (end of the first month of the offspring's postnatal life).

At the weaning period, the offspring were isolated from their dams and maintained on standard diet. The experimental rats were divided into eight subgroups, each of them differing at least by one of three dependent variables, such as kind of experimental diet, perinatal oxygen conditions and type of behavioral test used. Fig. 1 summarises data concerning differences in dependent variables between the groups and subgroups and provides the explanation for symbols defining these subgroups.

Experimental diets applied to dams were as follows:

1. Standard diet (commercial rat pellets); letter "s" denotes this diet in symbolic names.
2. Hypercholesterolemic diet—based on commercial pellets (slightly differing in composition from those described in the above point; for more details, see Table 1), enriched with 15% lard and 0.5% cholesterol (denominated in w/w; SIGMA, Poland); letters "ch" denote this diet in symbolic names.

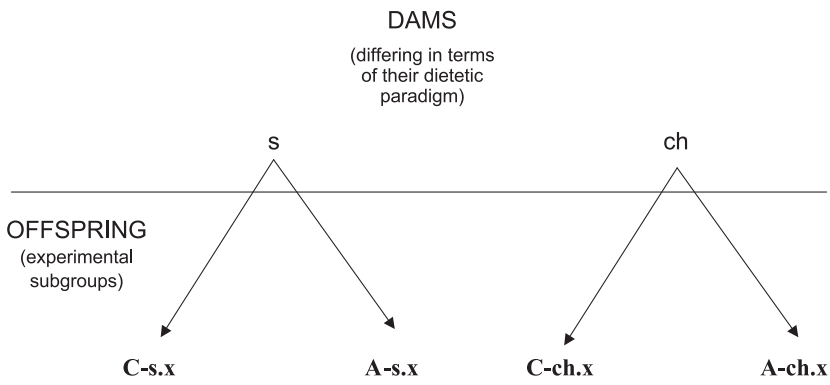

Fig. 1. The origins and characteristics of all experimental groups and definitions of the abbreviations referring to all groups and subgroups. The meaning of presented symbols are as follows: s—offspring of dams housed on standard diet (commercial rat pellets). ch—offspring of dams housed on diet enriched in cholesterol, C—offspring kept in normal atmosphere after birth, A—offspring subjected to neonatal anoxia, *x*—denotes a type of a behavioral test performed by juvenile rats—either the classical maze (substitute by a) or water maze (substitute by b). Two separate lines of dams and their offspring (following the above-shown paradigm) were housed for the purpose of conducting these two tests resulting in an overall number of eight experimental subgroups.

Table 1
Composition of the two types of diet (%)

Substance	Standard diet (s)	Hypercholesterolemic diet (ch)
Lard	0	15
Cholesterol	0.05	0.5
Other lipids	3	3
Casein	20	20
Cornstarch	68	52.5
Cellulose	4	4
Vitamins	1	1
Minerals	4	4

More detailed content of diets is summarised in Table 1.

The weight of experimental animals was measured at the end of the experiments. No statistically significant differences were determined across all subgroups.

2.2. Simulated neonatal anoxia

One half of experimental rats ($n = 59$) were subjected to simulated neonatal anoxia (all "anoxic" subgroups contain a letter "A" in their symbolic names), as described elsewhere [15,16]. Briefly, 2-day-old neonatal rat pups were placed into the hermetic chamber and exposed to an atmosphere of pure nitrogen (Messer, Poland) for 28 min at controlled ambient temperature of 31 °C. This time was previously determined as the minimum time required to elicit critical anoxia (period after which the spontaneous resuscitation is possible) at this temperature [18]. It was done during pilot experiments with the use of a pletysmograph while maintaining this temperature.

The control group (all subgroups contain a letter "C" in their symbolic names) was subjected to similar procedures with the use of atmospheric air in place of nitrogen.

2.3. Behavioral experiments

At the age of 45 ± 2 days, the juvenile rats performed behavioral tests to verify their ability to acquire spatial memory. Half of the animals were tested in a classical linear maze and the remainder in the Morris water maze. Each behavioral group comprised the same four experimental variants of oxygen and diet combinations. Subgroups of offspring of dams housed on the standard diet (C-s.*x* and A-s.*x*) will be called reference subgroups.

2.4. Training procedures in the linear maze

The experimental paradigm was similar to that described by Dell'Anna et al. [15].

The animals were required to remember the way linking the starting point with the aim (the bowl with water). The scheme of the maze is presented in Fig. 2. Twenty-four hours before the experiment, the rats were deprived of water.

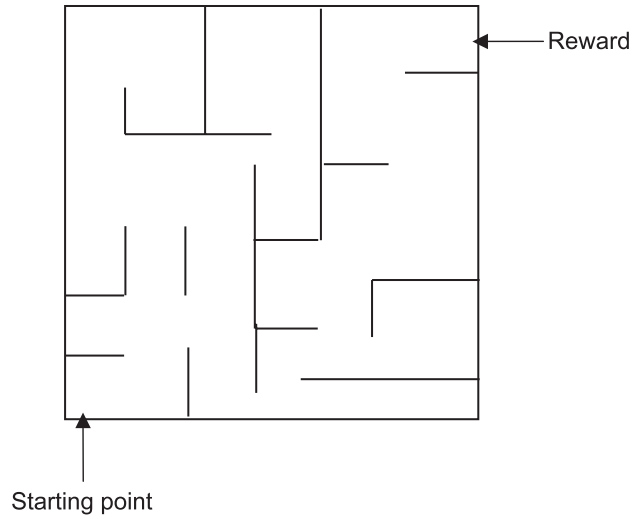

Fig. 2. The scheme of the linear maze.

This kind of deprivation is preferable in the case of dietetic studies [19].

The animals were trained during three sessions on three consecutive days. Each session comprised two trials separated by 4-h intervals, so the total number of trials was six. The time schedule was the same for all experiments. Each trial began at the moment of putting the animal into the starting sector and finished at the moment of reaching the reward.

2.5. Training procedures in the water maze

The procedures for the water maze testing were similar to those described by Cheon et al. [20]. Briefly, the animals were required to remember the position of the platform hidden in the middle of one quadrant of the circular tank (the detailed parameters of the maze are summarised in Table 2). The water was made opaque by means of milk powder and coloured by adding the small amounts of beetroot juice, thus enhancing the contrast between rat and water and enabling the determination of rat position by Ethovision Basic 2.1.6. software (Noldus Information Technology b.v., Holland). The traces were collected by the use of a camera and then digitalised by the software. The rats were trained during four sessions over four consecutive days. Each session comprised four trials. Each trial began at the moment of putting an

Table 2
The most important parameters of the water maze

Parameter	Size (cm)
Inner diameter	138
Height	48
Depth of water	38
Height of water below platform	1
Platform diameter	9

animal into the water and finished at the moment of reaching the aim. The starting points were at the central part of the wall of four quadrants and varied from trial to trial in a pseudorandom order.

Each animal was allowed to search for the aim for not longer than 2 min. In cases when an animal had not found the platform after this time, it was led to the platform by the experimenter. After reaching the platform, rats were allowed to rest there for 20 s.

The latencies of reaching the aims in two behavioral tests have been counted. The latencies were treated as a measure of memory acquisition rate.

2.6. Plasma cholesterol measurements

The plasma cholesterol levels were measured after completing the experiments in the water maze (with overnight fasting) using the enzymatic method with the kit Liquick Cor-CHOL 60 provided by Cormay, Poland. The blood samples were collected from decapitation bleeding with EDTA as an anticoagulant. Because there are no data suggesting the influence of a type of behavioral test on blood cholesterol concentration, it was determined only after one kind of test.

2.7. Statistical evaluation

Results are means (± S.E.M.). The differences between experimental groups and subgroups were evaluated using ANOVA (Minitab Release 13 software). General linear model was applied to estimate significance of differences across many groups and subgroups. In the case of mean serum cholesterol values, post hoc analysis was carried out to compare values obtained for particular subgroups with values obtained for two subgroups of offspring from dams fed on the standard diet (C-s.b and A-s.b).

3. Results

The results of hypercholesterolemic subgroups are always presented together with the results of rats housed on the standard diet (C-s.*x* and A-s.*x*; where *x* could be substituted with "a" as well as with "b", see Materials and methods for more details).

3.1. The results in the linear maze testing

The latencies of reaching the aim by nonanoxic animals in which dams were fed with hypercholesterolemic diet (C-ch.a) in comparison with reference subgroups are illustrated in Fig. 3.

The performance of these rats resembled greatly the performance of the control rats (C-s.a) and no statistical difference between these subgroups was detected. In contrast, the performance of anoxic rats (A-s.a) was significantly worse when compared to control rats [$F(1.4) = 19.08$; $P < .001$]. The effect of perinatal oxygen conditions on performance were much more pronounced than the effect of the time of the experiment (experience) [$F(1,4) = 8.95$; $P < .001$].

The performance of A-ch.a is illustrated in Fig. 4. The hypercholesterolemic rats were significantly better [$F(1,4) = 19.32$; $P < .001$] than anoxic rats wherein dams were not housed on a cholesterol diet (A-s.a). In contrast, no significant difference was detected between the rats of interest and control rats (C-s.a).

3.2. The results of rats in the water maze

Fig. 5 shows the performance of nonanoxic offspring of dams fed on cholesterol-supplemented diet (C-ch.b) together with latencies of reference subgroups.

The latencies of this subgroup were similar to control rats (C-s.b), but less so than the latencies of the corresponding

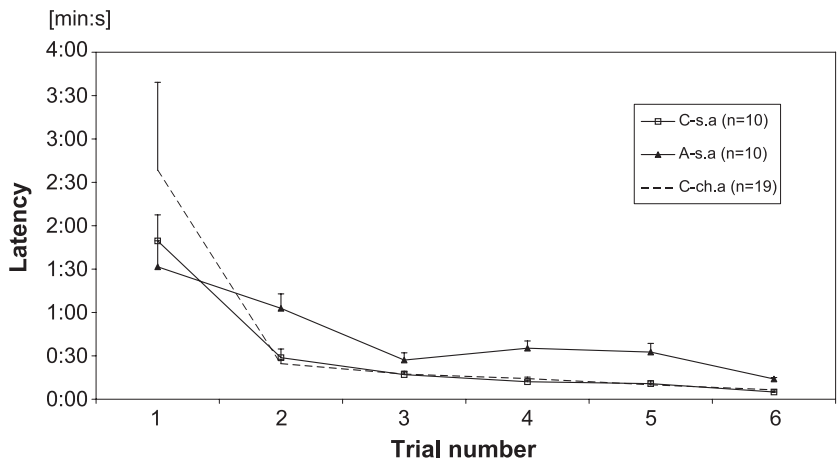

Fig. 3. Mean latencies (± S.E.M.) of reaching the aim on the linear maze illustrating the performance of the offspring rats of dams housed on high-cholesterol diet (C-ch.a) compared to reference subgroups of offspring (C-s.a and A-s.a: see Materials and methods for further details).

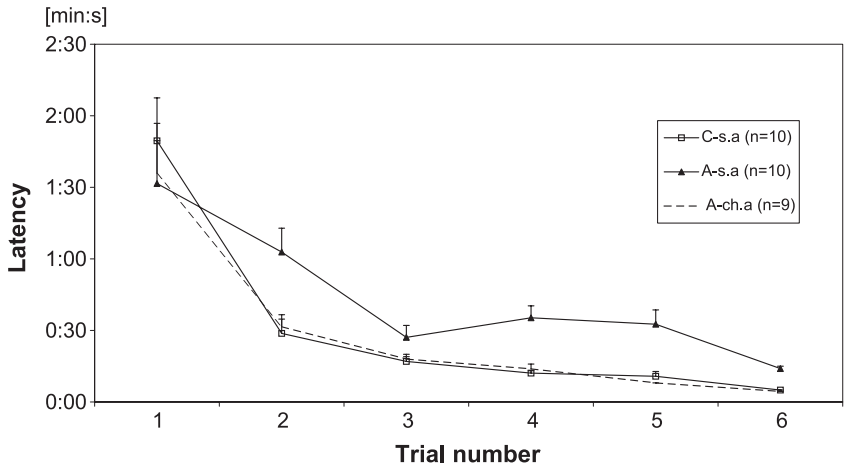

Fig. 4. Mean latencies (± S.E.M.) of reaching the aim on the linear maze illustrating the performance of the offspring rats of dams housed on high-cholesterol diet and subjected to neonatal anoxia (A-ch.a) compared to reference subgroups (C-s.a and A-s.a: see Materials and methods for further details).

subgroup of rats trained in the linear maze. Nevertheless, similarly to the linear maze, the latencies of control rats were significantly better than the anoxic ones [$F(1,14) = 46.70$; $P < .001$] and also the effect of perinatal oxygen conditions was much more pronounced than the effect of experience [$F(1,14) = 9.48$; $P < .001$].

Fig. 6 summarises the results of anoxic rats wherein dams were fed with hypercholesterolemic diet (A.ch.b). They performed better [$F(1,14) = 9.40$; $P < .005$] than anoxic offspring of dams fed on the standard diet (A-s.b). In contrast to animals trained in the linear maze, this difference was not greater than the effect of experience [$F(1,14) = 20.15$; $P < .001$]; moreover, the difference between A-ch.b subgroup and control rats (C-s.a) was statistically significant [$F(1) = 7.51$; $P = .006$].

3.3. Total plasma cholesterol

The results of plasma cholesterol measurements are illustrated in Fig. 7. Statistical comparisons across subgroups revealed a strong influence of perinatal oxygen conditions on this parameter [$F(1,1) = 24.48$; $P < .001$], whereas the effect of diet was not significant [$F(1,1) = 0.22$; $P = .642$].

4. Discussion

In the present work, the protective effect of a maternal hypercholesterolemic diet on the development of cognitive functions of the offspring was demonstrated. To induce cognitive disturbances, a neonatal anoxia model was used and two kinds of mazes were employed to verify the efficacy of acquisition of the spatial memory in a similar way as described in the work [15]. As a result, rats subjected to neonatal anoxia displayed similar cognitive deficits as described in the abovementioned paper; however, offspring of dams fed on hypercholesterolemic diet performed better in both kinds of behavioral tests showing less memory disturbances. Furthermore, higher plasma cholesterol levels

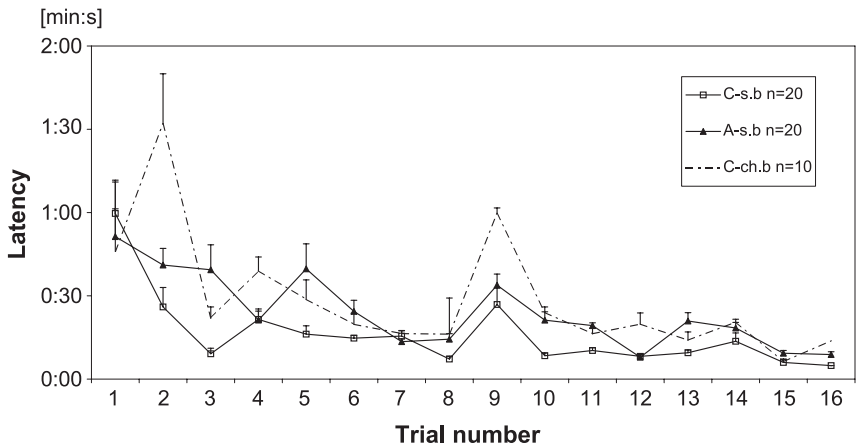

Fig. 5. Mean latencies (± S.E.M.) of reaching the hidden platform in the water maze illustrating the performance of the offspring rats of dams housed on high-cholesterol diet (C-ch.b) compared to reference subgroups (C-s.b and A-s.b: see Materials and methods for further details).

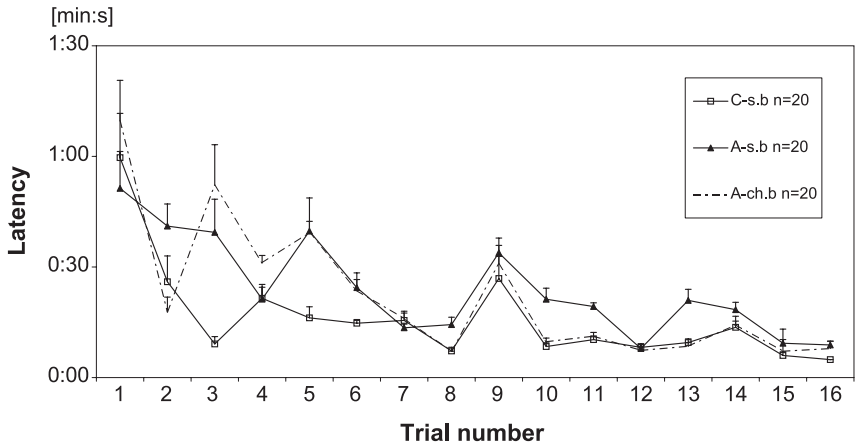

Fig. 6. Mean latencies (± S.E.M.) of reaching the hidden platform in the water maze illustrating the performance of the offspring rats of dams housed on high-cholesterol diet (A-ch.b) compared to reference subgroups (C-s.b and A-s.b: see Materials and methods for further details).

were observed in juvenile rats, which underwent an anoxic event in their neonatal period in comparison to animals subjected to control procedures.

4.1. Dietetic paradigm

The animals were subjected to the effect of hypercholesterolemic diet by feeding their dams during gestation and suckling. It is well established that diet greatly influences the composition of a dam's milk and especially its lipid composition [3,21,22]. There are serious reasons for limiting the time of applying the hypercholesterolemic diet to the abovementioned periods. The late prenatal and early postnatal development is related to high requirement for LC-PUFA [5,6]. Therefore, the direct supplementation of offspring on the diet with lard which contains high amounts of saturated fatty acids might lead to the reduction of unsaturated fatty acids. This phenomenon is due to the well-known competition between these two groups of fatty acids. Application of high cholesterol via mother's milk reduces this competition.

4.2. A comparison of performance of anoxic and nonanoxic rats in the linear maze

From the results obtained, both in the linear and in the water maze testing, the key conclusion could be drawn that in nonanoxic rats (C-ch.*x*), a hypercholesterolemic diet does not influence the rate of acquisition of spatial memory. In contrast, the same diet regime improves the performance in anoxic rats compared to the animals subjected to neonatal anoxia but coming from dams fed with the standard diet (A-ch.*x* vs. A-s.*x*).

Besides these essential similarities, there were some differences between results obtained in these two tests. First, the results obtained in the water maze were characterised by greater values of deviation of the means. This is mainly due to the fact that the water maze is a more difficult task for rats to perform than the linear maze task. It requires memory that is much more precise. However, taking into account only latency values may not be the best way to estimate the cognitive abilities of rats. Very similar values of latencies were obtained for rats displaying two quite different patterns

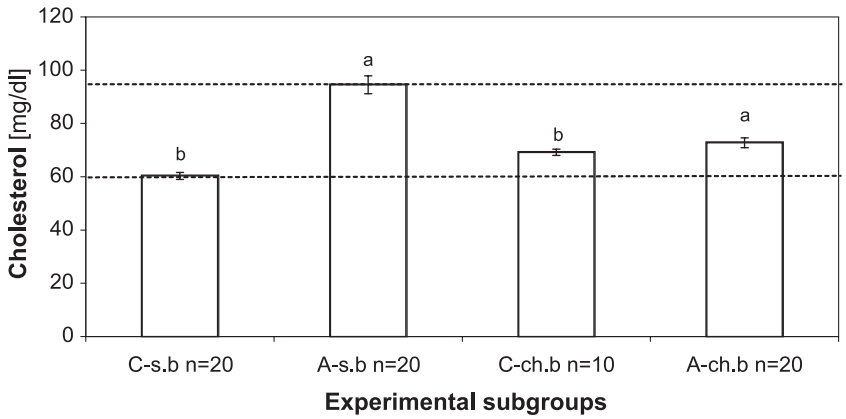

Fig. 7. The mean (± S.E.M.) cholesterol levels measured in all subgroups after experiments in the water maze. The lower dashed line indicates the level of the lowest value across all subgroups and the upper line the highest value. a—significantly different from C-s.a group, b—significantly different from A-s.b group.

of behavior. The first class of them apparently "kept in mind" the approximate position of the platform within a distance as small as 1 cm or less, and explored the area very close to it, but missing it a few times before finally reaching the aim. They even made circular traces surrounding the platform, approaching it to the abovementioned distance. The second class mainly explored the areas much further from the platform.

4.3. Serum cholesterol levels

The values of plasma cholesterol levels measured in control animals (C-s.b) were 60.3 ± 4.6 mg/dl. These were very similar to results obtained by Chiang et al. [23]—60.5 ± 13.7 mg/dl.

There was an interesting relationship between perinatal oxygen conditions (anoxic vs. nonanoxic rats) and the blood concentration of cholesterol measured one and a half months after birth. Anoxia resulted in a major level elevation of this lipid. This effect is even greater than the effect of a hypercholesterolemic diet applied to the dams. Very probably, it is due to a phenomenon that could be called postanoxic hypercholesterolemia. What is striking is that the same effect was noticed in rats of dams fed for a long period with a diet enriched with LC-PUFA. It was provided by feeding dams with 15% fish oil from cod liver; cholesterol was supplemented at a level of 0.05% and all other experimental conditions were the same as described for control rats (C-s.*x*) in the present study. Rats from dams that had been housed on a such dietetic regime and then subjected to neonatal anoxia displayed a higher cholesterol level than the animals not subjected to neonatal anoxia and even higher than the level measured for control animals (C-s.b; Bohr, unpublished observations). In this case, the hypercholesterolemic influence of anoxia even overcame the well-known hypocholesterolemic effect of LC-PUFA-enriched diet. It is probable that this phenomenon has a physiological relevance and that this elevation of cholesterol has a neuroprotective significance. In this context, it is worth stressing that in subjects suffering from AD, the cholesterol level was found to be substantially reduced in ventricular cerebrospinal fluid compared to age-matched controls [8]. This fact is in line with the above suggestions.

In this work, the peripheral levels of cholesterol were measured, and the question might be raised whether this reflects the central cholesterol levels. There are data suggesting that this is the case. In the dietetic experiments conducted by Howland et al. [12], the elevated level of plasma cholesterol paralleled its higher level in the brain.

4.4. Hypothetical mechanism of universal neuroprotective activity of cholesterol

The most intriguing question related to this work is: what mechanisms are of such presumed protective properties of cholesterol? Here, I would like to present a proposition of an answer.

The role of cholesterol in protection of cognitive functions is revealed in the best way in AD. The cognitive deficits occurring in AD are strongly correlated with the progressive decline of cholinergic transmission originating from the forebrain nuclei [24,25] and the relationship between cognitive functions and even consciousness are well established [26].

There are many facts showing relationships between AD and cholesterol metabolism, especially in the brain. It has been proven that there is a strong positive dependence between cholesterol levels in the close membrane environment of cholinergic receptors (muscarinic as well as nicotinic) and their activity [27–30]. In contrast, the high cholesterol level causes a reduction in the activity of other plasmalemmal receptors. It is the case especially in certain monoaminergic receptors, such as beta-adrenergic receptors [31].

It is also very likely that there are the functional relationships between cholinergic receptors and lipid rafts—specialized plasmalemmal structures enriched in cholesterol [32].

It is worth mentioning that overproduction of APP and/or its metabolites is not restricted to AD and could be seen as a universal adaptive response with neuroprotective relevance. There are many data supporting this point of view [33–37]. The increase in APP-originating peptides was observed after different injuries or even after disturbing physiological conditions of brain function in many species and in all filogenetic stages, including newborn babies who underwent an ischemic period [38]. There are data suggesting the involvement of APP/their metabolites in cellular cholesterol uptake [39]. Some indirect evidence supporting this thesis comes from the work of Howland et al. [12] because they found that in mice not expressing apolipoprotein E (apo E), cholesterol did not reduce the rate of APP metabolism. This effect is in line with the concept stating that apo E takes part in cholesterol uptake in the brain. In the light of the above-presented data, the overstimulation of APP metabolism could be regarded as a neuroprotective response leading to the rise in amount of cholesterol uptake by brain cells. Furthermore, this concept provides logic explanation of the abovementioned phenomenon of inverse correlation between cholesterol level and rate of APP metabolism in vitro [11] as well as in vivo [12]. APP metabolism could be cholesterol and acetylcholine dependent in the negative feedback fashion, rising in the situation of hypocholinergic states and leading to elevated, compensatory cholesterol uptake and consequently diminishing in opposite circumstances.

The results of the present work allow to formulate the intriguing hypothesis and show the way for many further investigations, especially dealing with attempts to establish direct links between the level of brain cholesterol and the efficacy of cholinergic transmission (pharmacological and

surgical interventions) and cognitive functions estimated by the use of different behavioral tests.

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