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ORIGINAL PAPER

Regulation of Neprilysin Activity and Cognitive Functions in Rats After Prenatal Hypoxia

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Abstract

The amyloid-degrading enzyme neprilysin (NEP) is one of the therapeutic targets in prevention and treatment of Alzheimer's disease (AD). As we have shown previously NEP expression in rat parietal cortex (Cx) and hippocampus (Hip) decreases with age and is also significantly reduced after prenatal hypoxia. Following the paradigms for enhancement of NEP expression and activity developed in cell culture, we analysed the efficacy of various compounds able to upregulate NEP using our model of prenatal hypoxia in rats. In addition to the previous data demonstrating that valproic acid can upregulate NEP expression both in neuroblastoma cells and in rat Cx and Hip we have further confirmed that caspase inhibitors can also restore NEP expression in rat Cx reduced after prenatal hypoxia. Here we also report that administration of a green tea catechin epigallocatechin-3-gallate (EGCG) to adult rats subjected to prenatal hypoxia increased NEP activity in blood plasma, Cx and Hip as well as improved memory performance in the 8-arm maze and novel object recognition tests. Moreover, EGCG administration led to an increased number of dendritic spines in the hippocampal CA1 area which correlated with memory enhancement. The data obtained allowed us to conclude that the decrease in the activity of the amyloid-degrading enzyme NEP, as well as a reduction in the number of labile interneuronal contacts in the hippocampus, contribute to early cognitive deficits caused by prenatal hypoxia and that there are therapeutic avenues to restore these deficits via NEP activation which could also be used for designing preventive strategies in AD.

Keywords Alzheimer's disease · Neprilysin · Novel object recognition test · Prenatal hypoxia · Epigallocatechin gallate (EGCG) · Dendritic spines

Introduction

The excessive production and accumulation of amyloid β peptide (Aβ) in the brain in the form of toxic oligomers and later on as plaques, is the major culprit leading to the pathological features of Alzheimer's disease (AD) [1, 2]. Steady state Aβ levels in the brain represent a balance between its production from the amyloid precursor protein (APP) via its sequential cleavage by two membrane bound enzymes, named β-and γ-secretases, and its proteolytic degradation by a cohort of amyloid-degrading enzymes [3–5]. While familial early onset forms of AD are clearly linked to the excessive production of Aβ, the late onset forms of AD more likely result from its insufficient clearance [6, 7].

A neuropeptidase, neprilysin (NEP), intensively studied since the 1980s [8–10] is now generally accepted as the major contributor to amyloid degradation in the brain and periphery [11–13]. In fact, decreased levels of NEP have

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been observed both in normally aging brain and under some pathological conditions, including AD [14, 15]. As such, upregulation of NEP activity is considered as a therapeutic target in AD and development of animal models for assessing the efficacy of various drugs are of particular importance. Our studies have clearly demonstrated that hypoxia results in reduced NEP levels both in cell cultures and in animal models [16, 17]. On the other hand, inhibition of NEP in the brain leads to memory impairment [18, 19]. Using our model of prenatal hypoxia in rats, which produces offspring with early cognitive deficits [20], we have shown that administration of sodium valproate results in upregulation of NEP levels and activity and memory improvement [21]. This study was a follow up of work in cell culture addressing epigenetic mechanisms of NEP regulation [10, 16, 22, 23].

In neuronal cells NEP expression is regulated by competitive binding of repressor histone-deacetylases or the transcriptional activator AICD (the APP intracellular domain) to its promoter [10]. AICD, produced alongside Aβ in the process of APP cleavage by β- and γ-secretases, provides a physiological feedback mechanism for controlling Aβ levels [10, 24] (Fig. 1). Although AICD is also formed in the non-amyloidogenic APP processing pathway initiated by α-secretase cleavage, it does not participate in regulation of gene expression because it is most likely cleaved in the cytoplasm by insulin-degrading enzyme and other peptidases [25, 26]. AICD regulation of NEP expression is neuronal cell and APP_{695} isoform-specific and the transcriptionally active AICD is produced in the β-secretase APP processing pathway [25]. However, under hypoxic conditions activation of caspases, which can cleave AICD, leads to a reduction of its levels and a decrease in expression and activity of NEP. Treatment of hypoxic NB7 cells with a caspase inhibitor Z-DEVD-FMK prevented AICD degradation and the decrease in NEP activity [23]. Following this observation we have performed experiments in rats subjected to prenatal hypoxia and found that in the neocortex of these animals there was indeed a significant increase in caspase-3 levels and activity correlated with a reduction of AICD and NEP levels and activity [27, 28]. Intraventricular administration of a caspase-3 inhibitor Ac-DEVD-CHO on postnatal day 20 to hypoxic rats not only resulted in a decrease in caspase-3 activity but also prevented the decrease in AICD levels and resulted in higher NEP protein levels and activity compared to untreated hypoxic animals [28]. Our new data on NEP mRNA levels in the parietal cortex and hippocampus confirmed that treatment of rats subjected to prenatal hypoxia with the caspase inhibitor increased NEP expression in both brain structures. Administration of the caspase inhibitor also improved the memory of hypoxic animals [29]. Schematically the results of our previous experiments are summarized in Fig. 1. Following the studies demonstrating that green tea catechins, and especially epigallocatechin-3-gallate (EGCG), can activate NEP [30, 31], we have also hypothesized that treatment of rats subjected to prenatal hypoxia with EGCG might be beneficial for improving their memory and restoring NEP levels in the brain.

Materials and Methods

Animals

Wistar rats from the vivarium of the I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences (St. Petersburg, Russia) were used. All experiments have been performed in accordance with the international guidelines for work with experimental animals [32] and the guidelines of the Russian Academy of Sciences (RAS) and approved by the Scientific Council of the Institute.

Model of Prenatal Hypoxia

Pregnant female rats were subjected to normobaric hypoxia on the 14th day of gestation (E14). Hypoxic conditions were mimicked in a special chamber (100 L in volume) equipped with the systems of thermoregulation, ventilation, CO_2 absorption and gas analysis. To create hypoxia the content of oxygen in the chamber was decreased linearly from 21 to 7% during the first 10 min by substitution with nitrogen and then was maintained at this level (7%) for 3 h. Concentration of CO_2 in the chamber was not higher than 0.2% and the temperature was kept at 22 °C. Female rats from the control group were kept during the same period of time in the same room in a cage under normal oxygen content. After birth, the litters of experimental and control groups were kept as described below until adult age (3–4 months old) and then were used for experiments.

Intracortical Injection of EGCG

Chronic intracortical (*i.c.*) injection of 10^{-3} M EGCG (Sigma, USA) solution in saline was performed into the parietal cortex area (Bregma = + 0.20; L = 3; H = 1 mm [33]) of adult rats (4 months old) from the offspring submitted to prenatal hypoxia, using osmotic mini-pumps (Alzet, USA) during 4 weeks with a constant rate of 0.25 µL/h. Control rats received the same amount of saline.

Oral Administration of EGCG

In another group of 4.5 months old control rats and rats submitted to prenatal hypoxia received EGCG solution (5 mg/kg of body weight) in drinking water (per os, p.o.) available ad lib during 10 days. After both treatments rats underwent

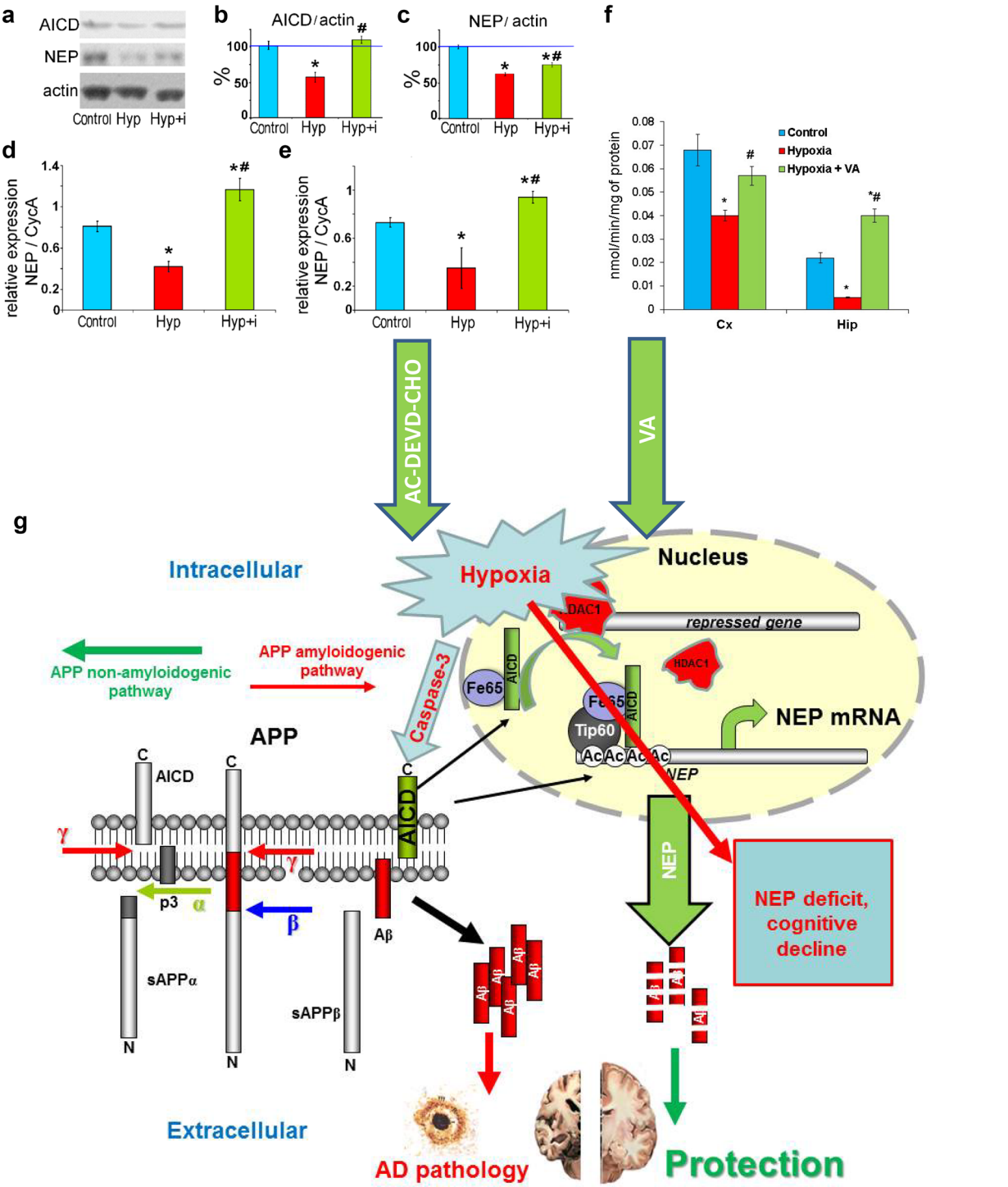


Fig. 1 Effects of hypoxia and pharmacological treatments on NEP regulation in rat brain. Bottom **g** NEP regulation in neuronal cells involves amyloid precursor protein (APP) intracellular domain (AICD) which is formed along with Aβ amyloid peptide via proteolytic β- and γ-secretase cleavage of APP. AICD together with a stabilizing protein Fe65 translocates to the nucleus where it engages a histone acetylase Tip60 and binds to the NEP promoter replacing HDAC and resulting in up-regulation of NEP gene expression [10]. This provides neuronal cells with a feedback mechanism allowing them to control Aβ levels. Under hypoxic conditions increased expression of caspases, which cleave AICD, leads to reduced NEP expression and activity. Administration of a caspase inhibitor Ac-DEVD-CHO to adult rats submitted to prenatal hypoxia on E14, which are characterized by reduced NEP activity, resulted in restoration of AICD levels in rat cortex and increased NEP expression at the mRNA and protein levels (**a**–**e**, adapted from [27]). Administration to adult rats subjected to prenatal hypoxia on E14 of an HDAC inhibitor valproic acid (VA) also led to an increase of NEP activity in the Cx and Hip (**f**) (adapted from [21]). These treatments along with enhanced NEP gene expression and increased enzyme activity also improved the cognitive deficit observed in adult rats subjected to prenatal hypoxia, suggesting neuroprotective properties both of the caspase inhibitor and VA

behavioral tests and then were sacrificed, decapitated, and their cortex or hippocampus from both hemispheres were taken for biochemical and morphological analysis. All efforts were made to minimize animal suffering and to reduce the number of animals used in each experimental group.

Short-term Memory Analysis in the 8-Arm Radial Maze

Prior to the assessment of short-term memory, male rats were grouped and housed, with food and water available ad lib, under the standard conditions of a 12-h light/dark cycle. Following 24 h of food deprivation, animals were taken for 14 daily trials of memory test in a two-level 8-arm maze as described in detail previously [34]. Briefly, the rats were placed in a platform of the two-level eight-arm maze from which they could enter any of the eight arms of the maze. Each arm had a feeder with food pellets (35 mg, containing sugar and starch). Entrances to the arms on the second level and exits from the arm on the first level were equipped with light-weight self-shutting doors opening only in one direction (inside or outside). Rats were allowed to perform eight visits of the arms, and any repeat visit to the same arm was counted as an error. The ability of rats to perform and remember the task was evaluated and expressed as percent of erroneous visits out of 8. The same scheme of experiments was used for testing animals from each of the experimental groups: control rats (n = 14), rats subjected to prenatal hypoxia (n = 9), rats subjected to prenatal hypoxia and *i.c.* administration with EGCG (n = 9).

Novel Object Recognition Test

Prior to assessment of the short (STM) and long-term memory (LTM) in the novel object recognition test [35] animals were grouped and housed as above. At the beginning of the test, an experimental animal was placed in a 100×100 cm^2 box with non-transparent 20 cm high walls for 5 min adaptation in the absence of any specific behavioural stimuli. In the first training session, after 2 h acclimatization to the experimental area, the animal was presented with two novel objects (1 and 2) and left to explore them for 5 min. The test was repeated 10 min later to analyse STM and 60 and 24 h later to analyse LTM. In these tests, which lasted 5 min each, one of the objects (object 2) was changed for a new object (numbered 3 for testing STM, and 4 and 5 for LTM). Object 1 stayed unchanged in all tests. The time spent to explore each object was recorded by an observer blind to the treatment and expressed as a percentage of the total exploration time of both objects computed in seconds. The same scheme of experiments was employed for testing animals from each of the experimental groups: control rats (n = 20), control rats *p.o.* treated with EGCG (n = 10), rats subjected to prenatal hypoxia (n = 20), rats subjected to prenatal hypoxia and *p.o.* treated with EGCG (n = 19) and rats subjected to prenatal hypoxia and *i.c.* treated with EGCG (n = 9).

Immunohistochemistry

The brains, immediately after decapitation of rats, were fixed in 10% formalin in PBS (pH 7.4) for a week, frozen and sectioned in the coronal plane using a cryostat Leica CM 1510S (Leica Microsystems, Germany). Some brain slices (20 µm) were Nissl stained. The structure of the cortical (Bregma = + 0.20 mm) and the hippocampal (Bregma = − 3.30 mm, [33]) sections of the brain was analysed either by light microscopy after Nissl staining (using an ImagerA microscope (Zeiss, Germany) or immunohistochemically to investigate the number and positioning of labile synaptopodin-positive dendritic spines. The analysis of synaptopodin distribution was performed using rabbit monoclonal antibody S9567 (SIGMA, dilution 1:1000) with subsequent visualization by a FITC-conjugated monoclonal secondary antibody against rabbit IgG (SIGMA, dilution 1:500). Immunofluorescent microscopy was performed using a Leica DMR microscope connected to a confocal scanner Leica TCS SL (Leica Microsystems, Germany). The fluorescence of FITC was observed in the 496–537 nm wavelength range. The same scheme of experiments was employed for testing animals from each of the experimental groups: control rats (n = 10), rats subjected to prenatal hypoxia (n = 10), rats subjected to prenatal hypoxia and *i.c.* administration of EGCG (n = 9) or saline (n = 10).

NB7 Cells

The NB7 (SJ-N-CG) neuroblastoma cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum, 50 units/mL penicillin, 50 µg/mL streptomycin and 2 mM glutamine (all from Cambrex Bio Science Ltd., Wokingham, Berkshire, UK) at 37 °C in 5% (v/v) CO^2 and sub-cultured every 7 days. After reaching the confluent stage cells were washed with serum-free medium and control cells in serum-free medium were returned to the same incubator. Two other groups of cells were transferred to an O_2/CO_2 incubator (MC0-175 M, Sanyo, Japan) where they were kept for 24 h under 1% O_2. EGCG stock solution in phosphate buffer (20 mM, pH 7.4) was added to the serum-free culture medium to a final concentration of 50 µg/mL and a similar volume of the buffer was added to the control hypoxic cells. Cells were collected 24 h later and NEP mRNA expression was assessed as described in [16].

Collection of Blood Serum

To obtain blood serum plasma, animals were decapitated and the blood was collected in vials without anticoagulant. After formation of a clot the serum was separated by centrifugation at 20,000×*g*. Serum not containing blood cell elements was aliquoted into Eppendorf tubes, frozen and kept at − 80 °C for not longer than 2 months to avoid loss of enzyme activity. NEP activity in the samples was assayed as described below.

Fluorimetric Assay of NEP Activity

NEP activity was assayed with a fluorogenic substrate Suc–Ala–Ala–Phe–7–AMC (50 µM; Bachem, UK) in the presence or absence of the inhibitor, thiorphan (10 µM, Sigma), as described previously [16]. For preparation of the samples, parietal cortex or hippocampus (at least n = 5 for each experimental group), tissue was homogenised in 50 mM HEPES buffer (pH 7.2) and centrifuged for 20 min at 20,000×g. The pellets were re-suspended in HEPES buffer (pH 7.2) to a final volume of 1 mL. The assay was monitored using an Ascent Fluoroscan (Thermo Scientific, Finland) plate reader. Each sample was tested at least in quadruplicate. The same scheme of experiments was employed for testing animals from each of the experimental groups: control rats (n = 5), rats subjected to prenatal hypoxia (n = 5), rats subjected to prenatal hypoxia and *p.o.* treated with EGCG (n = 10).

mRNA Analysis

Total RNA was isolated using the RNeasy kit (Qiagen, Crawley, W.Sussex, UK) or TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad, UK). cDNA was amplified by real-time PCR performed in an iCycler thermal cycler with multicolour PCR detection system (Bio-Rad, UK) analysing SYBR Green (Bio-Rad, UK) incorporation. Expression was reported relative to CycA mRNA. Primers used in the experiments with rats were as follows: NEP-F: 5′-GGA TCTTGTAAGCAGCCTCAGC-3′; NEP-R: 5′-AGTTGG CACACCGTCTCCAG-3′. CycA-F: 5′-AGGATTCATGTG CCAGGGTG-3′; CycA-R: 5′-CTCAGTCTTGGCAGT GCAGA-3′. For human NB7 cells the primers used as follows: NEP-F: 5′-TAAGCAGCCTCAGCCGAACCTACA -3′; NEP-R: 5′-GACTACAGCTGCTCCACTTATCCA CTC-3′; actin-F: 5′-CTCACCCTGAAGTACCCCATCG-3′; actin-R: 5′-CTTGCTGATCCACATCTGCTGG-3′.

Protein Analysis

Protein concentration was measured by the routine Lowry method. Bovine serum albumin (BSA, 1 mg/mL) was used as a standard. Aliquots of 10 µL of the sample of unknown concentration (two different dilutions) were then analysed in triplicate.

Statistical Analysis

Statistical data analysis was performed using the Graph Pad Instat and the SigmaStat 3.0 software package using the one-way ANOVA with Dunnet or Tukey–Kramer post hoc, two-tailed *t* test, one sample t-test and the non-parametric Mann–Whitney test (Mann–Whitney U-test). All results are presented as mean ± S.E.M. from *n* experiments.

Results

Effect of EGCG on Neprilysin Expression in NB7 Cells Under Hypoxia

Since we have previously shown that incubation of NB7 cells under 1% O_2 resulted in decreased NEP mRNA expression [16] it was important, prior to animal experiments, to evaluate which effect EGCG has on NEP expression in these cells under hypoxic conditions. We have found that incubation of NB7 cells in the presence of 50 µg/ml EGCG for 24 h not only had a protective effect on NEP mRNA levels against hypoxic insult but also led to a higher expression of this gene (Fig. 2a). Incubation of cells with EGCG for 24 h under normal conditions did not affect NEP mRNA levels.

NEP Activity in Brain Structures and Blood Plasma of Rats Subjected to Prenatal Hypoxia

As we have shown previously, prenatal hypoxia resulted in reduced NEP expression at the protein level both in the Cx and Hip when tested in adult animals [17, 21]. Reduced NEP protein levels correlated with its decreased enzyme activity both in the Cx and Hip. In this series of experiments we have also observed roughly a 20% decrease of NEP activity in 4.5 month old rats submitted to prenatal hypoxia (from 3.10 ± 0.11 to 2.58 ± 0.04 nmol AMC/min per 1 mg protein in the Cx, and 3.66 ± 0.04 to 3.19 ± 0.06 in the Hip (Fig. 2b). Administration of EGCG in drinking water during 10 days resulted in restoration of NEP activity reduced by prenatal hypoxia and even led to its two-fold increase both in the Cx and Hip. Treatment of control animals for the same duration of time with EGCG in drinking water also resulted in increased NEP activity in the Cx and Hip.

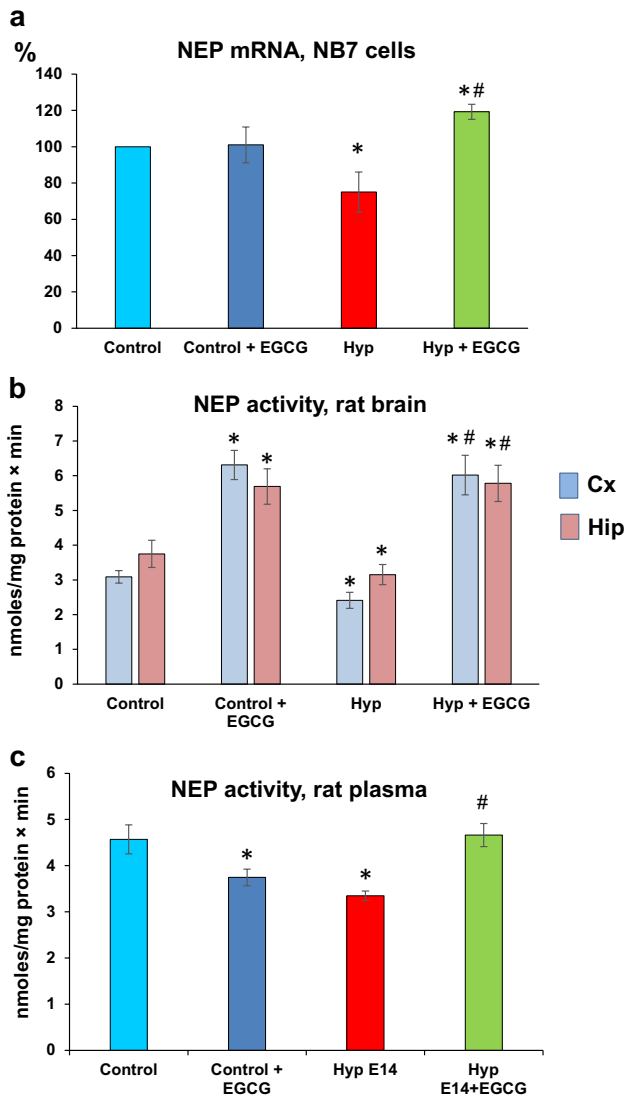


Fig. 2 Effect of prenatal hypoxia and EGCG treatment on NEP activity. **a** Treatment of cells incubated under hypoxic conditions (1% O_2, 24 h) with 50 µg/ml of EGCG in culture medium prevented the hypoxic decrease in NEP expression at mRNA levels. Data are presented as % of control cells (taken as 100%) incubated in serum free medium under standard conditions). *$p < 0.05$ (n = 5). **b** NEP activity in adult rat Cx and Hip and **c** in blood serum after prenatal hypoxia (n = 5) and treatment with EGCG in drinking water (n = 10) compared to controls (n = 5) and controls treated with EGCG (n = 5) as described in Methods. *$p < 0.05$ compared to control, #compared to hypoxia

Analysis of NEP activity in blood of adult rats submitted to prenatal hypoxia has revealed that, as in the brain structures, NEP activity in blood serum was also decreased in these animals by 25% compared to controls. Treatment of hypoxic rats with EGCG in drinking water for 10 days resulted in a significant increase of NEP activity up to the control values while in control rats this treatment resulted in a significant decrease (18%) in NEP activity (Fig. 2c).

Effect of EGCG on Memory of Rats Subjected to Prenatal Hypoxia

Prenatal hypoxia on E14 according to our data disturbs various types of memory in adult rats as assessed either in a radial maze [36] or in the novel object recognition test [21] which was also confirmed in this study. At the age of 4 months the rats subjected to prenatal hypoxia performed 153% more errors in the 8-arm radial maze than the control animals (taken as 100%) (Fig. 3a). Chronic *i.c.* treatment of these rats for 4 weeks with EGCG resulted in improvement of their working memory. As a result, their performance was not different from the control group.

In the novel object recognition test naive animals prefer to explore new objects and spend more time investigating them (Fig. 3b, Control). At the age of 4 months rats subjected to prenatal hypoxia were not able to discriminate between the old and new objects and spent more or less equal time exploring them at all intervals tested (10, 60 min and 24 h) (Fig. 3d) which testifies to memory disruption. After *i.c.* administration of EGCG during 4 weeks these rats demonstrated improved performance 60 min and 24 h after training (Fig. 3e), which suggested that EGCG improved their long-term memory. Oral administration of EGCG was also beneficial and improved rat memory at all tested time points (Fig. 3f). In control rats administration of EGCG did not affect animal performance in the novel object recognition test (Fig. 3c). Comparing the outcomes of the *i.c.* and *p.o.* EGCG modes of treatment we can conclude that EGCG improves animal memory independently of the route of its administration although in *p.o.* EGCG treated rats we have also observed improvement of short-term memory at the 10 min interval.

Analysis of the Number of Synaptopodin-Positive Dendritic Spines

Using the immunofluorescent method we have also performed an analysis of the distribution and number of synaptopodin-positive dendritic spines in the neuropil of rat parietal Cx and different areas of the Hip (Fig. 4). The number of the labile spines in the parietal Cx was maximal in the molecular layer. It is known that the majority of the axon-spine synapses in the molecular cortical layer are formed on the apical dendrites of the pyramidal neurons of II–III and V–VI layers. The number of the labile spines in the CA1 area of the Hip was maximal in the *stratum radiatum-moleculare* and *stratum oriens*, where they are known to be placed on the apical and basal dendrites of the pyramidal neurons. After prenatal hypoxia there was a decrease in the number of labile synaptopodin-positive dendritic spines in the molecular layer of the parietal Cx and in the *stratum radiatum-moleculare* of the CA1 layer of the Hip ($p < 0.01$, one-way

ANOVA, Dunnet post hoc, see Fig. 4, b, e) compared to the naïve control. In rats subjected to prenatal hypoxia and treated with *i.c.* or *p.o.* administration of EGCG we have observed a statistically significant increase (in case of *i.c.* EGCG injections up to the level of the naïve control) in the number of synaptopodin-positive labile spines in the *stratum radiatum-moleculare* of the CA1 layer compared to the animals without EGCG treatment (Fig. 4e, $p = 0.035$, one-way ANOVA, Tukey–Kramer post hoc). However, treatment with EGCG had no effect on the number of synaptopodin-positive dendritic spines in the analysed area of the Cx. Also treatment of control animals with EGCG had no effect on the number of dendritic synaptopodin-positive labile spines.

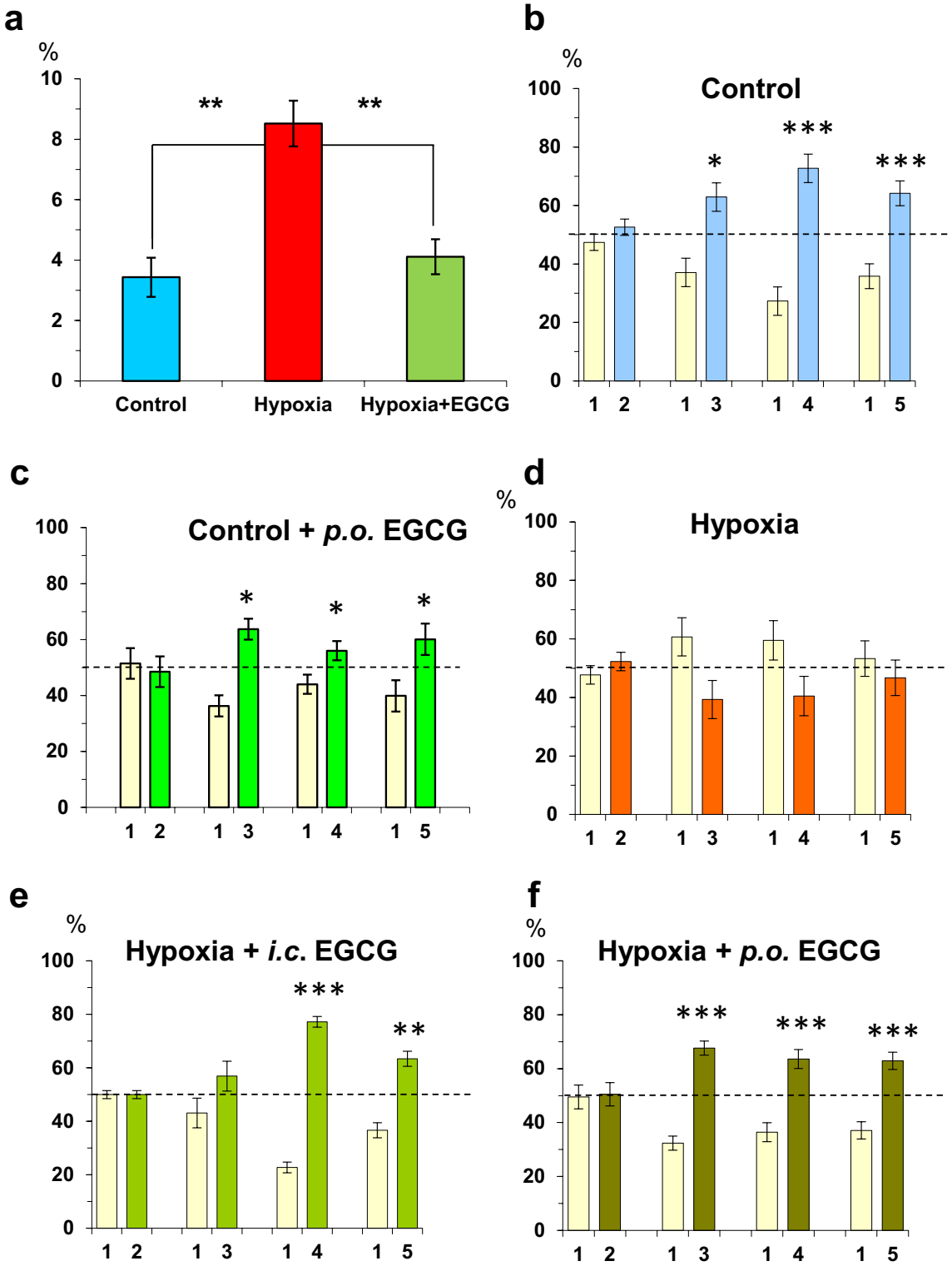
Discussion

The proteolytic enzyme NEP can be found in all animal species from *Caenorhabditis elegans* to mammals [37, 38]. In mammals, including humans, the highest levels of its expression and activity can be found in the kidney where it constitutes about 4% of all protein in the brush border membrane [39]. It is also abundant in the intestine, placenta and in the cells of the immune system [40]. In the brain NEP is expressed in the majority of structures where it regulates activity of various neuropeptides such as substance P [8]. In the developing rat brain NEP mRNA expression is the highest in the olfactory bulbs and choroid plexus although in the adult brain it can be detected in various brain areas and cell types [41]. Although NEP is primarily located on neuronal cells, especially in the nigrostriatal pathway, it is also present in the cortex [17], in the hippocampus, where it inactivates somatostatin [9], and in Schwann cells in the peripheral nervous system [42].

Because NEP has quite a wide range of substrate specificity [43] it is involved in various brain functions including learning and memory. Administration of NEP inhibitors was shown to impair working memory in rats [18]. The ability of NEP to catabolize Aβ peptide, reported in numerous studies, allows it to be considered as the major amyloid-degrading enzyme in the brain and periphery [44, 45]. Indeed, inhibition of NEP activity leads to accumulation of Aβ in the brain [46] and there are reports that NEP levels are reduced in AD brains [14, 15, 47]. Our studies suggest that NEP expression in rat Cx and Hip both at the mRNA and protein levels as well as its activity dramatically decrease with age while it stays relatively high in the striatum [17, 21]. Moreover, we have demonstrated that NEP expression significantly decreases under hypoxic and ischemic conditions both in cell and animal models which are well-known risk factors for AD [16, 17]. Because NEP was shown to prevent accumulation of Aβ in various cell and animal models of AD its pharmacological up-regulation is considered as a valuable therapeutic approach for prevention of late-onset AD [48, 49].

Prenatal hypoxia is among the factors affecting brain development and functions leading to increased risk of neurodegeneration in later life [49, 50]. According to our data, maternal hypoxia on E14 in the period of intensive formation of the major brain structures results not only in delayed formation of motor functions in early postnatal development and in cognitive deficit in later life [36, 51] but it also affects various aspects of amyloid metabolism leading to increased APP levels and reduced expression and activity of such amyloid-degrading enzymes as NEP and endothelin-converting enzyme (ECE) [17, 21]. This makes the model of prenatal hypoxia in rats a convenient instrument for analyzing mechanisms of NEP regulation and testing various compounds for its up-regulation. As we have shown administration of valproic acid and a caspase inhibitor Ac-DEVD-CHO to adult rats subjected to prenatal hypoxia restored NEP expression and activity and improved their cognitive functions [21, 26–28, 51]. In both cases NEP up-regulation involved an AICD-dependent mechanism [10, 24, 25], either via inhibiting histone deacetylases and increasing AICD binding to the NEP promoter or via reducing caspase levels which degrade AICD and are increased in rat brain after prenatal hypoxia [23, 25, 26].

Among the compounds which were shown to up-regulate NEP activity in neuronal cell cultures are green tea catechins, in particular EGCG [29, 30, 52]. In AD mice EGCG administration was shown to decrease Aβ accumulation and improve cognitive decline, both of which were abolished by silencing of the NEP gene [53]. Moreover, recently it was shown that the anti-obesity effect of green tea to a great extent depends on its ability to upregulate peripheral (kidney and intestine) NEP which degrades such orexigens as galanin and neuropeptide Y [54]. The data of our present study confirm that EGCG can also increase NEP mRNA expression in human neuroblastoma NB7 cells incubated under reduced oxygen levels (1%) as well as in the Cx and Hip of rats subjected to prenatal hypoxia (Fig. 2). Moreover, we have found that oral EGCG administration to rats with their drinking water was more beneficial than intracranial injection resulting in better cognitive outcome (Fig. 3). Treatment of control rats with EGCG in drinking water also increased NEP activity in the Cx and Hip although did not affect their cognitive functions and the number of labile dendritic spines in the Cx and Hip. However, taking into account that NEP mRNA and protein levels and its activity reduce significantly during the lifespan of rats [17, 21], upregulation of NEP activity in the brain might testify to a potentially beneficial effect of EGCG not only in hypoxic but ageing animals preventing long term accumulation of the amyloid peptide. We have also for the first time observed that prenatal hypoxia resulted in reduced blood serum NEP activity which was



increased after oral EGCG administration. This suggests that blood NEP activity can be a physiologically relevant marker for evaluating therapeutic effects of biologically active compounds. The decrease in NEP activity in blood plasma of control rats treated with EGCG in drinking water also suggests that the blood pressure decreasing effect of EGCG observed in hypertensive rats and humans [55, 56] might be due to its ability to reduce blood plasma activity of NEP which plays an important role in the renin-angiotensin system [57]. Although such an effect of EGCG on NEP levels

◂**Fig. 3** Effect of EGCG administration on rat memory. **a** Eight-arm radial maze test. Rats subjected to prenatal hypoxia on E14 were treated with EGCG or saline as described in "Materials and Methods". The ordinate shows the average percent of erroneous runs for 14 days of testing for each rat group (1-way ANOVA: $F_{2,29} = 15.717$, $P < 0.0001$). ***$p < 0.001$ (Tukey–Kramer post hoc test). For control rats n = 14, for rats subjected to prenatal hypoxia n = 9, and for rats subjected to prenatal hypoxia and treated with *i.c.* EGCG, n = 9. **b-f** Novel object recognition test. Rats (control or subjected to prenatal hypoxia on E14) were treated at the age of 4.5 months with *p.o.* EGCG or *i.c.* EGCG as described in "Materials and Methods". Memory was assessed 10 min (short-term memory) and 60 min and 24 h (long-term memory) after the initial training session. Data are presented as mean ± SEM of the percentage of the time spent exploring the familiar (1) or new (3–4) objects to the total time of exploration. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$, one sample t-test). Horizontal broken lines indicate 50% value which suggest absence of preferences to the novel and familiar objects. For control rats n = 20, for control rats treated with *p.o.* EGCG n = 10, for rats subjected to prenatal hypoxia n = 20, for rats subjected to prenatal hypoxia and treated with *i.c.* EGCG n = 9, and for rats subjected to prenatal hypoxia and treated with *p.o.* EGCG n = 19

and activity in the blood has to be further investigated, dual beneficial properties of green tea catechins for the brain and cardiovascular system should not be underestimated.

Although therapeutic properties of EGCG have been widely investigated and confirmed that it possesses antioxidant [58], anticancer [59], anti-inflammatory [60] and neuroprotective [61] effects, the exact molecular mechanisms of its action in each particular case are still not clear. Data from computational molecular docking suggest that EGCG can prevent accumulation of fibrous proteins such as Aβ and hyperphosphorylated tau due to catechin-protein binding interaction [62]. On the other hand EGCG is capable of inhibiting the activity of various histone deacetylases (for review see [63]) and DNA methyltransferases [64] suggesting that its effects can be attributed to epigenetic mechanisms. However, these data are mostly obtained in cancer cells, and there are very limited data on epigenetic effects of EGCG in neuronal cells or in the brain.

The data of our study have clearly shown that EGCG can restore brain functions impaired by prenatal hypoxic insult in rats. The decreased ability of rats to remember visited arms in the 8-arm maze test or the objects in the novel object recognition tests was restored to its control values after EGCG treatment. To assess how EGCG administration affects neuronal cells in the Cx and Hip we have performed morphological and immunocytochemical analysis which revealed that indeed, in the Hip, the number of synaptopodin-positive labile dendritic spines in the CA1 layer was increased up to the control levels compared to untreated animals subjected to prenatal hypoxia. These data agree with the observations that EGCG can protect hippocampal formation and spatial memory in aged rats [65]. Although we have not observed significant changes in the number of synaptopodin-positive dendritic spines in the parietal cortex of rats subjected to prenatal hypoxia and treated with EGCG, beneficial effects of this compound might be observed at other levels of Aβ metabolism and cortical synaptic plasticity as shown in AD mice [66] or at the balance of excitatory and inhibitory neurotransmission in the Down syndrome mouse [67].

The outcome of EGCG treatment of rats subjected to prenatal hypoxia is similar to the effects of the HDAC inhibitor valproic acid [21]. However, taking into account potential side effects of this non-specific HDAC inhibitor it seems that treatment with a natural green tea compound is more beneficial in the long term providing upregulation of NEP activity and improving cognitive functions impaired by prenatal insults. Because complications during pregnancy become more frequent with increased air pollution, rate of infectious diseases, stress of life and other unfavorable conditions it is important to design a treatment which will be beneficial both to the mothers and the offspring. Because green tea extracts and EGCG possess a variety of protective properties their administration both to women during pregnancy and to new-born babies can be an effective and safe preventive treatment against a number of neurological disorders. According to recent studies EGCG reverses behavioral and memory deficits in rats caused by traumatic stress [68] and can be used for treatment of posttraumatic disorders. The efficacy of EGCG has also been proven in the maternal and neonatal treatment of gestational diabetes [69] and was even proposed as a potential natural antimicrobial compound in reconstituted baby food [70].

Conclusions

Our work demonstrates that prenatal hypoxia leads to a decrease in the activity of the major amyloid-degrading enzyme NEP in rat brain Cx and Hip as well as in the blood serum in adulthood. In adult rats this was accompanied by memory deficit and a reduced number of labile dendritic spines in the hippocampal CA1 area. EGCG administration to animals, subjected to prenatal hypoxia, resulted in an increase in NEP activity in blood plasma, Cx and Hip as well as improved memory deficit which was paralleled by an increased number of labile dendritic spines in their hippocampal CA1 area. As such, EGCG administration similar to other compounds capable of restoring NEP activity after prenatal hypoxia (e.g. valproic acid and caspase inhibitors), can be considered as a beneficial strategy towards restoration of brain metabolism disturbed after prenatal hypoxia and also to slow development of late onset Alzheimers' disease.

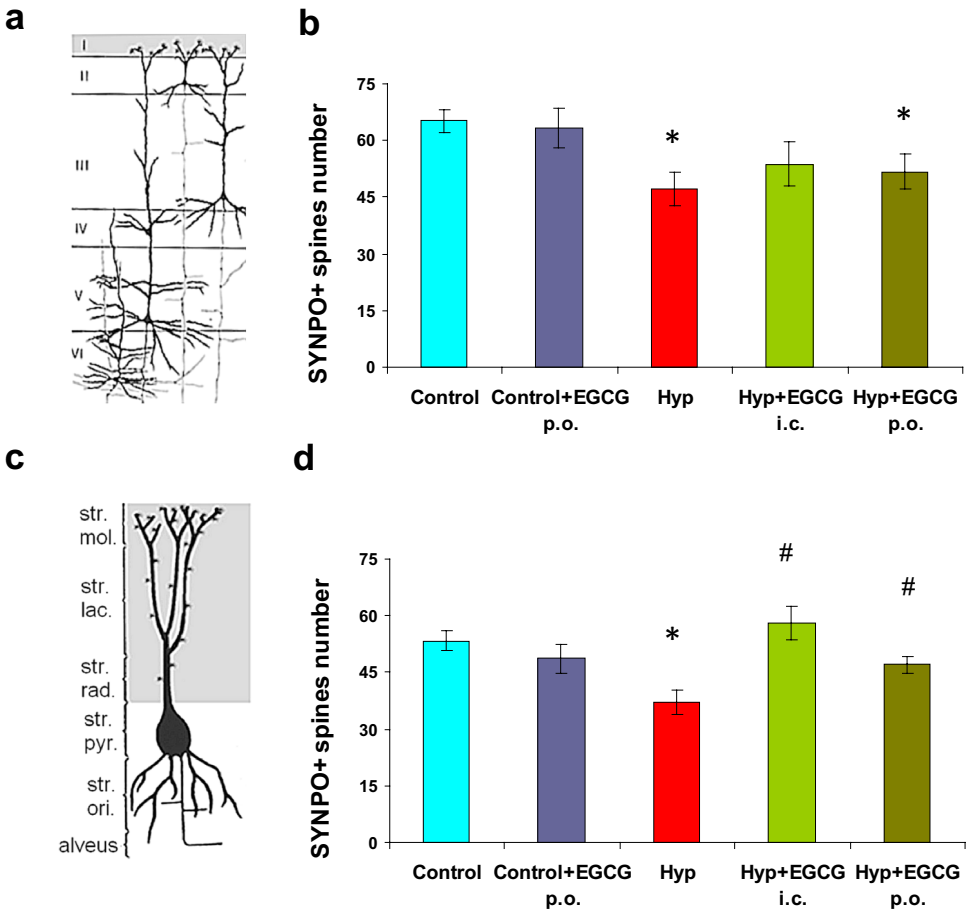


Fig. 4 Analysis of synaptopodin-positive dendritic spines in rat Cx and Hip after prenatal hypoxia and administration of EGCG. **a**, **c** Schematic presentation of cell layers (I–VI) in the parietal cortex (**a**) and of the CA1 layer of the dorsal hippocampus *stratum moleculare* (str.mol.), *tratum lacunosum* (str.lac.) and *stratum radiale* (str.rad.). **c** Grey color highlights the molecular layers in which the number of synaptopodin-positive spines have been evaluated. **b**, **d** Number of labile synaptopodin-positive spines in the molecular layer of the parietal cortex (**b**) and in the CA1 *stratum radiatum-moleculare* of the Hip (D) in naïve adult control rats (Control; n = 10), adult control rats treated *p.o.* with EGCG (Control + EGCG *p.o.*; n = 5), adult rats, subjected to prenatal hypoxia on E14 (Hyp; n = 10) and in adult rats, subjected to prenatal hypoxia on E14 and treated (*i.c.* or *p.o.*) at the age of 4.5 months with EGCG as described in "Materials and Methods" (Hyp + EGCG *i.c.* n = 9 and Hyp + EGCG *p.o.* n = 7, respectively). The ordinate shows the mean number of synaptopodin-positive spines in the area of 10,000 μm^2. Data are presented as mean ± SEM. *statistically significant differences from the control ($p \leq 0.05$; one-way ANOVA, Dunnet post hoc). #statistically significant differences from hypoxic animals without EGCG treatment ($p \leq 0.05$; one-way ANOVA, Tukey post hoc)

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Compliance with Ethical Standards

Conflict of interest Authors declare no conflict of interest.

Ethical Approval All current international, national, and institutional guidelines for the care and use of experimental animals were followed.

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