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ORIGINAL ARTICLE

Developing brain as a source of circulating norepinephrine in rats during the critical period of morphogenesis

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Abstract

The development of individual organs and the whole organism is under the control by morphogenetic factors over the critical period of morphogenesis. This study was aimed to test our hypothesis that the developing brain operates as an endocrine organ during morphogenesis, in rats during the perinatal period (Ugrumov in Neuro Chem 35:837–850, 2010). Norepinephrine, which is a morphogenetic factor, was used as a marker of the endocrine activity of the developing brain, although it is also secreted by peripheral organs. In this study, it was first shown that the concentration of norepinephrine in the peripheral blood of neonatal rats is sufficient to ensure the morphogenetic effect on the peripheral organs and the brain itself. Using pharmacological suppression of norepinephrine production in the brain, but not in peripheral organs, it was shown that norepinephrine is delivered from the brain to the general circulation in neonatal rats, that is, during morphogenesis. In fact, even partial suppression of norepinephrine production in the brain of neonatal rats led to a significant decrease of norepinephrine concentration in plasma, suggesting that at this time the brain is an important source of circulating norepinephrine. Conversely, the suppression of the production of norepinephrine in the brain of prepubertal rats did not cause a change in its concentration in plasma, showing no secretion of brain-derived norepinephrine to the bloodstream after morphogenesis. The above data support our hypothesis that morphogenetic factors, including norepinephrine, are delivered from the developing brain to the bloodstream, which occurs only during the critical period of morphogenesis.

Keywords General · Circulation · Developing brain · Norepinephrine · Morphogenesis · Rat

Abbreviations

DHBA	Dihydroxybenzylamine
DBH	Dopamine ß-hydroxylase
E	Embryonic day
HPLC-ED	High performance liquid chromatography with electrochemical detection
6-OHDA	6-Hydroxydopamine
ICH	Immunocytochemistry
NE	Norepinephrine
PBS	Phosphate buffer saline
P	Postnatal day

Introduction

The development of individual organs and the whole organism occurs during so-called critical period of morphogenesis. Despite the fact that this period is slightly different for the development of various organs, in rodents it corresponds mainly to the perinatal period. At this time, the developmental processes are under the control by intercellular chemical signals, so-called morphogenetic factors (transcription factors, programming factors or inductors of development). They provide an irreversible morphogenetic action on target cells via specific receptors (Lauder 1993; Nguyen et al. 2001; Pathania et al. 2010; Sullivan and Levin 2016; Ugrumov 1997). A well-known example of morphogenesis is the masculinization of the brain under the influence of circulating testosterone, which is converted to estrogen in the brain. In rats, this process begins in late prenatal life with a sharp increase in the testosterone concentration in the blood, and ends in the neonatal period with the manifestation of sex differences in the brain, particularly in the preoptic area of the

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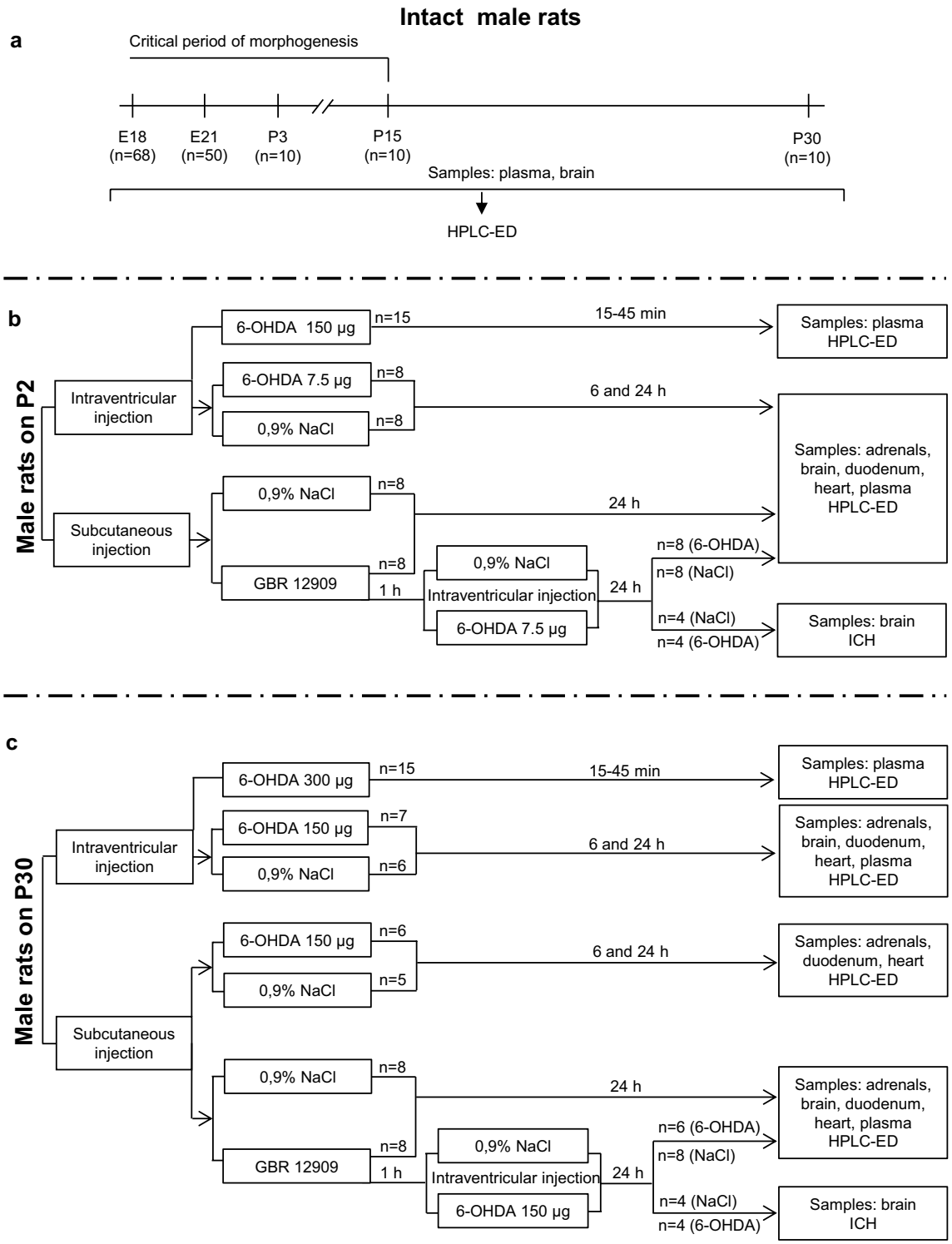

hypothalamus (Gorski 1985; Rhees et al. 1990a, b; Simerly et al. 1985).

Norepinephrine (NE) is one of the potent morphogenetic factors. It is produced in the brain, the sympathetic nervous system, adrenals and extra-adrenal chromaffin tissue (Goldstein et al. 2003; Huber et al. 2009; Moore and Bloom 1979; Tank and Wong 2015). NE, produced in the brain is believed to provide only autocrine regulation of the differentiation of norepinephrine-ergic neurons (autoregulation) and paracrine regulation of the development of some other neurons and glial cells within the brain. Namely, NE is involved in the regulation of apoptosis, the guided growth of neuronal

◂**Fig. 1** Design of experiments. **a** Collecting plasma and brain tissue samples from intact male rats on the 18th embryonic day (E18), E21, 3rd postnatal day (P3), P15 and P30 for high-performance liquid chromatography with electrochemical detection (HPLC-ED) assay. **b** Manipulations with rats on P2, which include: (1) Intraventricular injection of 6-OHDA at the dose of 150 µg, followed by collecting plasma 15, 30, and 45 min later for HPLC-ED assay of 6-OHDA; (2) Intraventricular injection of 6-hydroxydopamine (6-OHDA) at the dose of 7.5 µg or 0.9% NaCl (control) and after 24 h, collecting adrenals, brain, duodenum, heart and plasma for HPLC-ED assay; (3) Subcutaneous injection of GBR 12,909 (40 mg/kg) or 0.9% NaCl (control) and after 24 h, collecting brain, plasma and peripheral organs (adrenals, duodenum, heart) for HPLC-ED assay; (4) Subcutaneous injection of GBR 12,909 (40 mg/kg) and after 1 h intraventricular injection of 6-OHDA at the dose of 7.5 µg or 0.9% NaCl (control) and after 24 h, collecting peripheral organs, plasma and brain for HPLC-ED and immunocytochemistry, respectively. **c** Manipulations with rats on P30, which include: (1) Intraventricular injection of 6-OHDA at the dose of 300 µg, followed by collecting plasma 15, 30, and 45 min later for HPLC-ED assay of 6-OHDA; (2) Subcutaneous injection of -OHDA at the dose of 150 µg or 0.9% NaCl (control) and 24 h later collecting peripheral organs (adrenals, duodenum, heart) for HPLC-ED assay; (3) intraventricular injection of 6-hydroxydopamine (6-OHDA) at the dose of 150 µg or 0.9% NaCl (control) and 24 h later collecting brain, plasma and peripheral organs (adrenals, duodenum, heart) for HPLC-ED assay; (4) Subcutaneous injection of GBR 12,909 (40 mg/kg) or 0.9% NaCl (control) and 24 h later collecting brain, plasma and peripheral organs for HPLC-ED assay; (5) Subcutaneous injection of GBR 12,909 (40 mg/kg) and after 1 h intraventricular injection of 6-OHDA at the dose of 150 µg or 0.9% NaCl (control) and 24 h later collecting peripheral organs, plasma and brain for HPLC-ED and immunocytochemistry, respectively

processes, synaptogenesis and gliogenesis (Berger-Sweeney and Hohmann 1997; Blanchi and Sieweke 2008; Felten et al. 1982; Kreider et al. 2004; Viemari et al. 2004). It is believed that, unlike NE of cerebral origin, NE secreted by peripheral organs into the bloodstream exerts endocrine action on the development of peripheral cells by modulating the gene expression of certain proteins, e.g., thermogenin, lipoprotein lipase, transcription factors in brown adipose tissue (Nedergaard et al. 1995). The lack of NE in the developing organism is incompatible with life. Indeed, the mice with a knockout for the gene of dopamine ß-hydroxylase (DBH), the last enzyme of NE synthesis, die in utero due to maldevelopment of the cardiovascular system (Thomas et al. 1995). This pathology is also characteristic of humans with the abnormal development of the sympathetic nervous system, which is considered as a risk factor for sudden infant death syndrome (Hildreth et al. 2009; Lavezzi et al. 2013; Viemari et al. 2004).

Until recently, it was believed that NE, as other substances which do not penetrate through the brain-blood barrier, is delivered to the general circulation only from peripheral organs producing NE. However, we have shown that a number of biologically active substances, such as dopamine, serotonin, and gonadotropin-releasing hormone, are delivered from the brain to the general circulation in perinatal rats (Ugrumov et al. 2005, 2012; Zubova et al. 2014), although they do not pass, at least in adulthood, through the blood–brain barrier (Kostrzewa 2007; Sachs 1973; Singh and Champlain 1972). Based on these data, we hypothesized that the developing brain, before the establishment of the brain-blood barrier, operates as a multipotent endocrine organ, providing endocrine control of the development of peripheral target organs and the brain itself (Ugrumov 2010). The aim of this study was to continue testing our hypothesis that morphogenetic factors synthesized in the brain are delivered to the bloodstream during the critical period of morphogenesis, using NE as a marker of the brain endocrine activity. The objectives were to assess the level of NE: (1) in the general circulation and in the brain in rats in ontogenesis; (2) in the peripheral blood of neonatal rats after chronic (irreversible) suppression of NE production in the brain; (3) in the peripheral blood of prepubertal rats after chronic (irreversible) suppression of NE production in the brain.

Materials and methods

Animals

Wistar rats—pregnant females, male fetuses on the 18th embryonic day (E18) and E21, as well as male pups on the 2nd postnatal day (P2), P3, P15, P30 and P31 were used in this study. Animals were maintained at 21–23 °C in a light–dark 12-h cycle with free access to food and tap water. To obtain dated pregnancy, adult males were placed to a cage with adult females in the evening, and the next morning vaginal smears were examined. The day of sperm finding in a smear was considered as the first day of pregnancy or E1. The birthday of rats was considered as P1. The sex of animals was determined by a revision of gonads in fetuses or estimation of the anogenital distance in postnatal animals.

All experimental procedures were carried out in the period from 2016 to 2019, in different seasons (summer, autumn, winter and spring). Materials for analysis were obtained always at the same time from 14.00 to 18.00 in order to avoid differences in circadian rhythmic activity. Indeed, it has previously been shown that circadian rhythms do not affect the content of NE in the brain, at least in rats aged 8–66 days (Asano 1971). When manipulating with animals we strictly followed the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC) for care and use of laboratory

animals and were approved by the Animal Care and Use Committee of the Institute of Developmental Biology of the Russian Academy of Sciences.

Collecting plasma and brain tissue in intact rats

The blood was obtained from the heart of fetuses on E18 ($n=68$) and E21 ($n=50$), as well as pups on P3 ($n=10$), P15 ($n=10$) and P30 ($n=10$) under anesthesia with chloral hydrate (400 mg/kg) of pregnant mothers or pups, respectively (Fig. 1a). The blood was pooled as a single sample from three fetuses on E18 and two fetuses on E21. After birth, the blood from individual animals served as a single sample. Plasma was separated from the blood cells by centrifugation at 1350 g for 10 min, and 250 pmol/ml 3,4-dihydroxybenzylamine (DHBA) hydrobromide (internal standard, Sigma, St. Louis, USA) in 0.1 N $HClO_4$ was added to supernatant (plasma). This solution was cleared from high molecular weight proteins by centrifugation at 16,500 g for 20 min, and the supernatant was frozen and stored at − 70 °C until high-performance liquid chromatography with electrochemical detection (HPLC-ED) assay of NE.

After blood collection, the animals were decapitated, and the brains were removed. The entire brain on E18 and one hemisphere of the brain from older fetuses and postnatal rats were used as single samples. The brain tissue was weighed, frozen and maintained at − 70 °C until HPLC-ED assay of NE.

Experiments: rats on postnatal day 2

Experiment 1

Rats on P2 were anesthetized with isoflurane using the SomnoSuite Small Animal Anesthesia System (Kent Scientific, USA), followed by stereotactic intraventricular administration of 150 µg 6-hydroxydopamine (6-OHDA) (Sigma, St. Louis, USA), neurotoxin of catecholaminergic neurons (Blum et al. 2001), in 2 µl of 0,9% NaCl and 0.1% ascorbic acid ($n=15$). The injection was made through a glass microcannula (tip diameter—50 µm), which was stereotactically inserted into the lateral cerebral ventricle at 1.2 mm lateral to the bregma and 2.0–2.4 mm deep into the brain. Plasma was collected 15, 30 and 45 min after 6-OHDA injection, frozen, and stored at − 70 °C until HPLC-ED assay of 6-OHDA (Fig. 1b).

Experiment 2

Rats on P2 were anesthetized with isoflurane, followed by intraventricular administration of 7.5 µg 6-OHDA in 2 µl of 0.9% NaCl and 0.1% ascorbic acid ($n=8$) or 2 µl of 0.9% NaCl and 0.1% ascorbic acid ($n=8$) (control). Considering that the blood–brain barrier in neonatal rats is permeable to monoamines (Loizou 1970; Kostrzewa 2007), we preliminary selected the maximum dose of 6-OHDA—7.5 µg, at which, after intraventricular administration, it does not affect the peripheral NE-containing organs. This was proven by the absence of a change in the content of NE and dopamine in these organs 6 and 24 h after intraventricular injection of 6-OHDA, i.e. during the period of neurotoxin-induced degeneration of catecholaminergic neurons (Kolacheva et al. 2014; Yokoyama et al. 1993). Brain, plasma and peripheral catecholamine-containing organs (adrenals, duodenum, heart) were collected under anesthesia with chloral hydrate (400 mg/kg), frozen and stored at − 70 °C until HPLC-ED assay of NE and dopamine (Fig. 1b).

Experiment 3

Rats on P2 received subcutaneously 40 mg/kg GBR 12,909 in 25 µl 0.9% NaCl ($n=8$) or 25 µl of 0.9% NaCl (control) ($n=8$). Twenty-four hours later, animals were anesthetized with chloral hydrate (400 mg/kg), and the brain, plasma and peripheral catecholamine-containing organs (adrenals, duodenum, heart) were dissected, frozen, and stored at − 70 °C until HPLC-ED assay of NE (Fig. 1b).

Experiment 4

Rats on P2, which received subcutaneously 40 mg/kg GBR 12,909 (Sigma, St. Louis, USA), an inhibitor of dopamine uptake (Teicher et al. 1986), an hour later were anesthetized with isoflurane, followed by intraventricular injection of 7.5 µg 6-OHDA in 2 µl of 0.9% NaCl and 0.1% ascorbic acid ($n=12$) or 2 µl of 0.9% NaCl and 0.1% ascorbic acid ($n=12$) (control). Twenty-four hours after intraventricular injection of 6-OHDA, animals were anesthetized with chloral hydrate (400 mg/kg). Brain, plasma and peripheral catecholamine-containing organs (adrenals, duodenum, heart) were obtained, frozen, and stored at − 70 °C until HPLC-ED assay of NE and dopamine. Some animals from the experimental group ($n=4$) and control group ($n=4$) were anesthetized with chloral hydrate (400 mg/kg) and perfused via the heart with first 0.02 M sodium phosphate buffer saline (PBS) (pH 7.2–7.4) for 10 min, and then 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2–7.4) for 10 min. Then, the animals were decapitated, and the brain was removed and post-fixed by immersion in the same fixative for 12 h at 4 °C, rinsed in PBS three times for 10 min each, immersed in 20% sucrose in PBS for 24 h at 4 °C, frozen in hexane at − 40 °C, and stored at − 70 °C until immunocytochemistry for DBH (Fig. 1b).

Experiments: rats on postnatal day 30

Experiment 5

Animals were anesthetized with isoflurane, followed by stereotactic intraventricular administration of 300 µg 6-OHDA in 10 µl of 0.9% NaCl and 0.1% ascorbic acid ($n = 15$). Plasma was collected 15, 30 and 45 min after 6-OHDA injection, frozen, and stored at − 70 °C until HPLC-ED assay of 6-OHDA (Fig. 1c).

Experiment 6

Considering that the blood–brain barrier is impermeable to monoamines in adult rats (Loizou 1970; Kostrzewa 2007), we selected in a preliminary study the maximum dose of 6-OHDA—150 µg, at which, after subcutaneous injection, it does not affect the peripheral NE-containing organs (Fig. 1c). This was proven by the absence of a change in the content of NE in these organs 6 and 24 h after administration of 6-OHDA, i.e. during the period of neurotoxin-induced degeneration of catecholaminergic neurons (Kolacheva et al. 2014; Yokoyama et al. 1993). Twenty-four hours after administration of 6-OHDA, animals were anesthetized with chloral hydrate (400 mg/kg), and the peripheral organs (adrenals, duodenum, heart) were obtained, frozen and stored at − 70 °C until HPLC-ED assay of NE and dopamine (Fig. 1c). Based on these data, in subsequent experiments, we used only materials obtained from animals 24 h after the administration of 6-OHDA.

Experiment 7

Rats on P30 were anesthetized with isoflurane, followed by intraventricular administration of 6-OHDA at a preselected dose of 150 µg in 10 µl of 0.9% NaCl and 0.1% ascorbic acid ($n = 7$) or 10 µl of 0.9% NaCl and 0.1% ascorbic acid ($n = 6$) (control). The stereotactic injection was made into the lateral ventricle of the brain at coordinates: 0.4 mm caudal to the bregma, 1.3 mm lateral to the bregma and 2.5 mm deep into the brain. Twenty-four hours after injection, animals were anesthetized with chloral hydrate (400 mg/kg), and the brain, plasma and peripheral organs (adrenals, duodenum, heart) were obtained, frozen and stored at − 70 °C until HPLC-ED assay of NE and dopamine (Fig. 1c).

Experiment 8

Rats on P30 received subcutaneously 40 mg/kg GBR 12,909 (Sigma, St. Louis, USA) in 100 µl 0.9% NaCl ($n = 8$) or 100 µl 0.9% NaCl (control) ($n = 8$). Twenty-four hours later, animals were anesthetized with chloral hydrate (400 mg/kg), and the brain, plasma and peripheral organs (adrenals, duodenum, heart) were dissected, frozen and stored at − 70 °C until HPLC-ED assay of NE (Fig. 1c).

Experiment 9

Rats on P30, which received subcutaneously 40 mg/kg GBR 12,909, an hour later were anesthetized with isoflurane, followed by intraventricular injection of 150 µg 6-OHDA in 10 µl of 0.9% NaCl ($n = 10$) or 10 µl of 0.9% NaCl and 0.1% ascorbic acid ($n = 12$) (control). Twenty-four hours later, the animals were anesthetized with chloral hydrate (400 mg/kg), and the brain, plasma and peripheral organs (adrenals, duodenum, heart) were obtained, frozen, and stored at − 70 °C until HPLC-ED assay of NE and dopamine (Fig. 1c). Some animals from the experimental group ($n = 4$) and control group ($n = 4$) were anesthetized with chloral hydrate (400 mg/kg) and perfused via the heart with first PBS for 10 min, and then 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2–7.4) for 10 min. Then the animals were decapitated, and the brains were removed and post-fixed by immersion in the same fixative for 12 h at 4 °C, rinsed in PBS three times for 10 min each, immersed in 20% sucrose in PBS for 24 h at 4 °C, frozen in hexane at − 40 °C and maintained at − 70 °C until immunocytochemistry for DBH (Fig. 1c).

High-performance liquid chromatography with electrochemical detection

NE and dopamine were measured in the brain, peripheral organs (adrenals, duodenum, heart) and plasma with the reverse phase HPLC-ED LC-20ADsp (Shimadzu, Japan). Electrochemical detector DECADE II (Antec, the Netherlands) was equipped with a glass carbon flow cell and salt-bridge Ag/AgCl reference electrode with the potential set at + 0.85 V. Collected tissues were homogenized in 10 volumes of 0.1 N $HClO_4$ containing DHBA hydrobromide (50–500 pmol/ml) by an ultrasonic homogenizer (Sartorius AG, Germany), centrifuged at 16,500 g for 20 min, and catecholamines in the supernatant were measured. Separation of catecholamines was performed on a 10 cm C18 (3.3 µm) column with an internal diameter of 4 mm (Dr. Maisch GmbH, Germany). The mobile phase consisted of 0.1 M citrate–phosphate buffer containing 0.3 mM sodium octanesulfonate (Sigma, St. Louis, USA), 0.1 mM EDTA and 8% acetonitrile (Sigma, St. Louis, USA) (pH 2.54). A flow rate of mobile phase was 0.8 ml/min. Peaks corresponding to NE and dopamine were identified according to elution time of the standard solution, and the contents of substances were estimated as a ratio of the peak area of the internal standard solution to that of the specimen using software LabSolutions (Shimadzu, Japan). The concentration of NE and dopamine in plasma was calculated as the

amount of NE or dopamine per unit of plasma volume (ng/ml). The NE content in plasma was calculated in our previous study as a product of the NE concentration on the total plasma volume, which was accounted for 0.197 ml at E18, 0.38 ml at E21, 0.52 ml at P3, 1.1 ml at P15 and 3.7 ml at P30 (Ugrumov et al. 2005).

In experiments 1 and 5, 6-OHDA was measured in plasma after precipitation on aluminum. The minimum volume of eluate used for HPLC-ED assay was 120 µl. The mobile phase consisted of 0.1 M citrate–phosphate buffer containing 0.3 mM sodium octanesulfonate (Sigma, St. Louis, USA), 0.1 mM EDTA and 6% acetonitrile (Sigma, St. Louis, USA) (pH 2,7). A flow rate of mobile phase was 0.6 ml/min. Column temperature was 25 °C.

Immunocytochemistry and image analysis

Frontal serial sections, 14 µm thick, of the locus coeruleus (bregma: − 5.20 to − 6.00 at P3 and − 9.48 to − 10.32 at P31) (Ashwell and Paxinos 2008; Paxinos and Watson 2009; Khazipov et al. 2015) were prepared with a cryostat (Leica CM1950, GmbH, Germany) and mounted on slides. Each slide contained the sections of the locus coeruleus from one rat on P3 or P31 treated with GBR 12,909 and 6-OHDA and from one control rat of the same age. Sections were immunostained by successive incubations with (a) 3% bovine serum albumin (Sigma, St. Louis, MO, USA) and 0.1% Triton X100 (Sigma, St. Louis, MO, USA) for 30 min at 20 °C; (b) monoclonal mouse antibodies to DBH (1:300) (Millipore, USA) with 3% bovine serum albumin and 0.1% Triton X-100 for 36 h at 4 °C; (c) biotinylated goat antibodies to mouse γ-globulins (1:200) (Vector Laboratories, Burlingame, CA, USA) for 2 h at 20 °C; and (d) avidin–biotin complex linked to peroxidase (Vector Laboratories, Burlingame, CA, USA) for 1 h at 20 °C. All solutions were prepared with PBS, which was also used for rinsing the sections after each step of incubation. Peroxidase of the avidin–biotin complex was revealed by simultaneous incubation of all sections with 0.05% 3,3′-diamino-benzidinetetrahydrochlorid (Sigma, St. Louis, MO, USA) and 0.02% H_2O_2 in PBS at 20 °C. Finally, the sections were dehydrated, mounted in DPX Mountant for hystology (Sigma, St. Louis, MO, USA) and coverslipped.

To avoid double counting of neurons, every second slice among serial slices of locus coeruleus was examined in a conventional microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan) under 20× magnification and recorded with a digital camera (Olympus DP70, Olympus Corporation, Tokyo, Japan). The recorded images were subsequently used for counting DBH-immunoreactive neuronal cell bodies with only a clear outline and visible nucleus to avoid counting of degrading neurons. Considering our preliminary data that the number of DBH-immunoreactive neurons was the same in the left and right hemisphere of the brain (the locus coeruleus) in control rats and 6-OHDA-treated rats, we counted only the neurons in the left hemisphere. Finally, the total number of DBH-immunoreactive neurons was counted in the entire locus coeruleus and compared in rats on P3 and P31 treated with GBR 12,909 and 6-OHDA, and control animals received GBR 12,909 and 0.9% NaCl. Data are presented as a percentage of control.

Statistics

The data on the NE content in the brain and the NE concentration in the plasma in rats from E18 to P15 were treated using one way ANOVA, whereas in rats on P30 the same data were treated with Mann–Whitney *U* test. Other data were processed first with F-test to assess the homogeneity of test samples and then with Student's test to determine statistically significant differences. All tests are included in GraphPad Prism Version 6.0, software package for Windows (GraphPad Software, USA). All results are represented as the mean ± SEM.

Results

Norepinephrine in the plasma and brain in intact rats in ontogenesis

The concentration of NE in the plasma of rat fetuses on E18 was 6.9 ± 0.97 ng/ml (Fig. 2a). It decreased abruptly from E18 to E21, and then gradually increased from E21 to P15, being on P15 2.5 times higher than on E21. From P15 to P30, the NE concentration reduced by twofold. The content

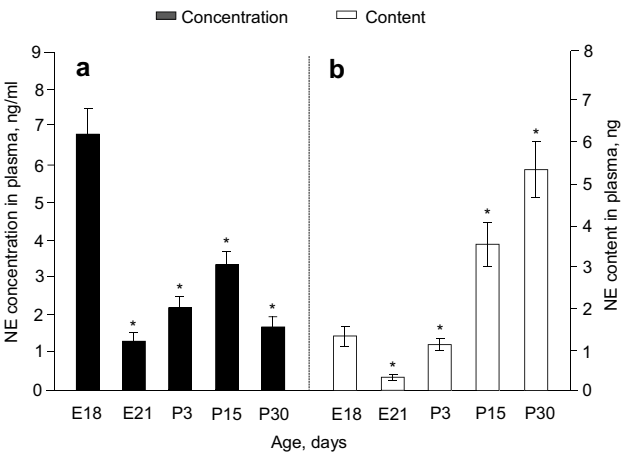

Fig. 2 The concentration (**a**) and content (**b**) of norepinephrine (NE) in plasma in intact rats on the 18th embryonic day (E18), E21, 3rd postnatal day (P3), P15 and P30. *$p < 0.05$ compared to the previous age group

of NE first decreased several times from E18 to E21, but then gradually increased up to P30 (Fig. 2b).

The content of NE in the brain of rat fetuses on E18 was 0.69 ± 0.04 ng (Fig. 3). The content of NE in the brain increased 6.5 times from E18 to E21 and 8 times from E21 to P3. It continued to increase postnatally, from P3 to P15 at almost the same rate as in the previous period, and from P15 to P30 at a lower rate (Fig. 3).

Norepinephrine in the brain, peripheral organs and plasma in 6-hydroxydopamine-treated rats on postnatal day 3

Six and 24 h after intraventricular injection of 7.5 µg of 6-OHDA, there was a decrease in the levels of dopamine and NE in the brain. After 6 h, data are not presented, and after 24 h, the content of NE and dopamine decreased by 57.4% and 25%, respectively (Fig. 4). In the same experiment, the concentration of NE in the plasma decreased by 36.3% compared with control, whereas the concentration of dopamine did not change (Fig. 4). At the same time, no changes in the content of NE and dopamine in peripheral organs were detected as compared with the control (Appendix 1).

In subsequent experiments, we used only materials obtained from animals 24 h after the administration of 6-OHDA. So, 24 h after intraventricular injection of 7.5 µg 6-OHDA to rats on P2, which were previously subcutaneously injected with GBR 12,909, the NE content in the brain and the NE concentration in plasma decreased almost the same as after intraventricular injection of 6-OHDA without pretreatment with GBR 12,909 (Fig. 5). However, in this experiment, the content of dopamine in the brain and the concentration of dopamine in the plasma did not change (Fig. 5) compared with the control. There was also no change in the content of NE in the adrenals, duodenum and heart (Appendix 2). Additional control with subcutaneous administration of GBR 12,909 showed no change in the content of NE in the brain and peripheral organs compared with the subcutaneous administration of 0.9% NaCl (Appendix 3). According to our quantitative immunohistochemical study, intraventricular injection of 7.5 µg 6-OHDA to rats pretreated with GBR 12,909 caused a loss of 46.51% of DBH-immunoreactive neurons (cell bodies) in the locus coeruleus compared to the control (Fig. 6 a, b, c).

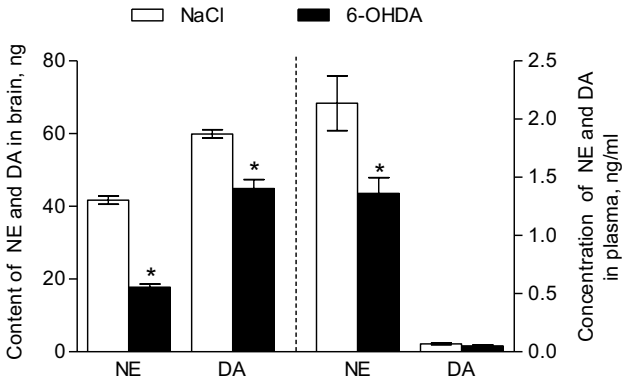

Fig. 4 The content of norepinephrine (NE) and dopamine (DA) in the brain (left side) and the concentration of NE and DA in plasma (right side) in rats on postnatal day 3, 24 h after intraventricular injection of 7.5 µg 6-hydroxydopamine (6-OHDA) or 0.9% NaCl (control). *$p < 0.05$ compared to the control

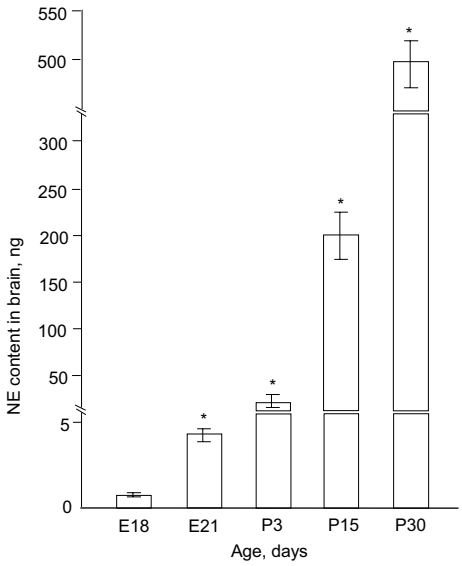

Fig. 3 The content of norepinephrine (NE) in the brain of intact rats on the 18th embryonic day (E18), E21, 3rd postnatal day (P3), P15 and P30. *$p < 0.05$ compared to the previous age group

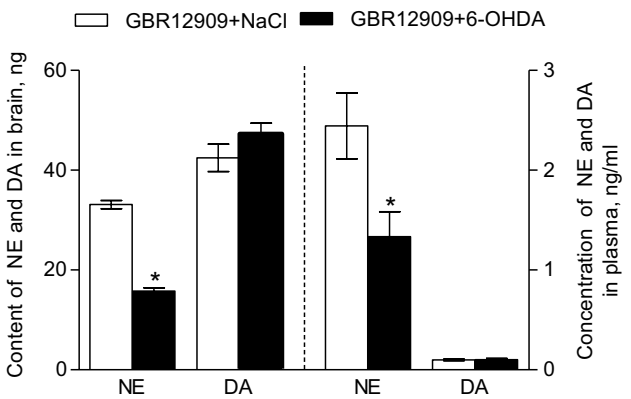

Fig. 5 The content of norepinephrine (NE) and dopamine (DA) in the brain (left side) and the concentration of NE and DA in plasma (right side) in rats on postnatal day 3 after subcutaneous injection of GBR 12,909 (40 mg/kg) and 1 h later intraventricular injection of 6-OHDA at the dose of 7.5 µg or 0.9% NaCl (control), followed by 24 h later collecting materials. *$p < 0.05$ compared to the control

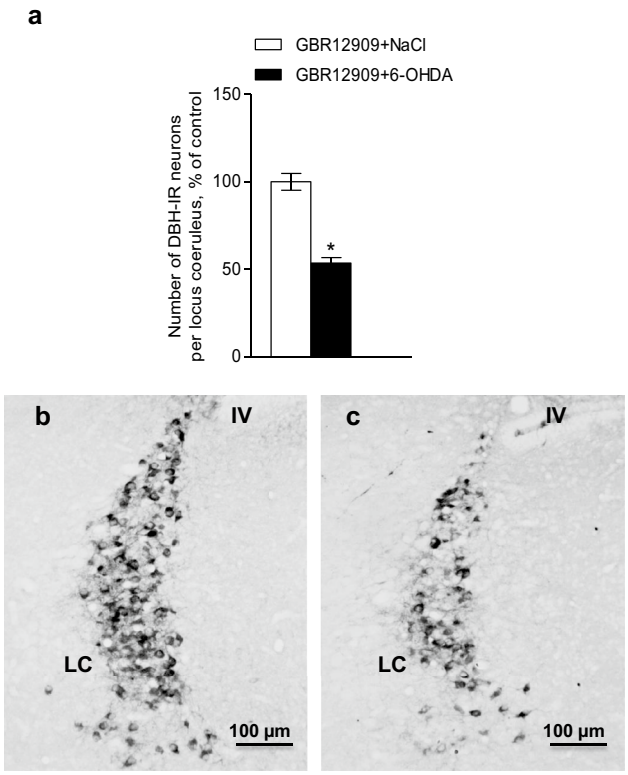

Fig. 6 Dopamine ß-hydroxylase-immunoreactive (DBH-IR) neurons in the locus coeruleus in rats on postnatal day 3 (P3) after subcutaneous injection of GBR 12,909 (40 mg/kg) on P2 and 1 h later intraventricular injection of 6-OHDA at the dose of 7.5 µg or 0.9% NaCl and 24 h later collecting materials. **a** DBH-IR neurons percentage of control; **b** GBR 12,909 + NaCl; **c** GBR 12,909 + 6-OHDA. *IV* fourth cerebral ventricle, *LC* locus coeruleus. Bar = 100 µm. *$p < 0.05$ compared to the control

Intraventricular administration of 6-OHDA was used not only to induce the degeneration of catecholaminergic neurons in the brain but also at a considerably higher dose (150 µg) as a marker of permeability of the brain-blood barrier. In this study, 6-OHDA was found with HPLC-ED in the plasma over the entire studied period at a concentration about 4 ng/ml.

Norepinephrine in the brain, peripheral organs and plasma in rats treated with 6-hydroxydopamine on postnatal day 31

In a preliminary screening study on rats on P30, it was shown that 150 µg is the maximum dose of 6-OHDA, in which the content of NE and dopamine does not change in catecholamine-containing peripheral organs (adrenals, duodenum, heart) after 6 h (data not shown) and 24 h after at subcutaneous administration of this neurotoxin compared to control (subcutaneous administration of 0.9% NaCl) (Appendix 1). However, 24 h after intraventricular injection of 150 µg 6-OHDA, there was a decrease in the NE content in the brain by 46.1%, whereas only a tendency to a decrease in the dopamine content was noted ($p = 0.09$) (Fig. 7). In the same experiment, the concentration of NE and dopamine in the blood, as well as the content of NE and dopamine in the peripheral organs did not change (Fig. 7, Appendix 1).

In the experiment with successive administration, first, GBR 12,909 subcutaneously, and then intraventricularly 6-OHDA, the content of NE in the brain decreased by about 2.5 times, whereas the content of dopamine in the brain and the concentration of NE and dopamine in the plasma did not change (Fig. 8). In the same experiment, 30% DBH-immunoreactive neurons were lost in the locus coeruleus. In an additional control, there was no change in the content of NE in the brain, peripheral organs and in the plasma 24 h

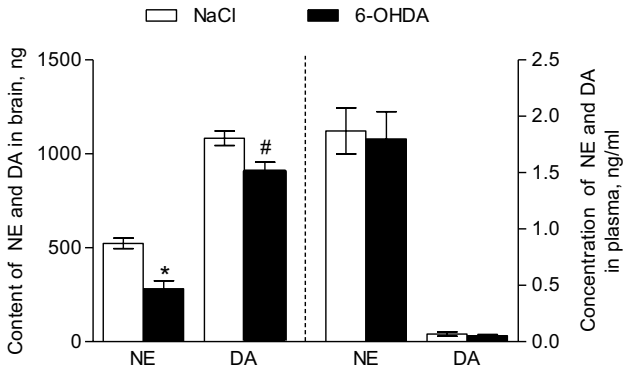

Fig. 7 The content of norepinephrine (NE) and dopamine (DA) in the brain (left side) and the concentration of NE and DA in plasma (right side) of rats on postnatal day 31, 24 h after intraventricular administration of 150 µg 6-hydroxydopamine (6-OHDA) or 0.9% NaCl (control). *$p < 0.05$ compared to the control, #$p = 0.09$

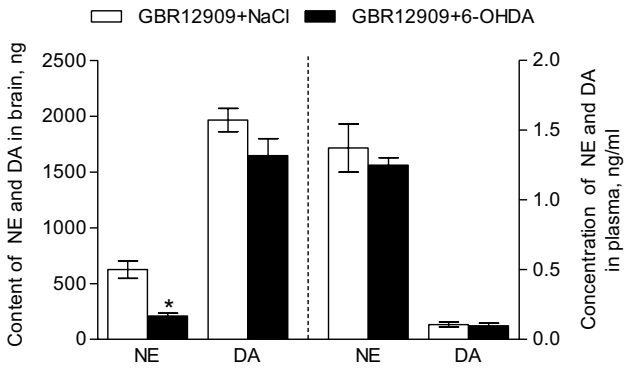

Fig. 8 The content of norepinephrine (NE) and dopamine (DA) in the brain (left side) and the concentration of NE and DA in plasma (right side) in rats on postnatal day 31 (P31) after subcutaneous injection of GBR 12,909 (40 mg/kg) on P30 and 1 h later intraventricular injection of 6-OHDA at the dose of 150 µg or 0.9% NaCl (control) and 24 h later collecting materials. *$p < 0.05$ compared to the control

after subcutaneous administration of only GBR 12,909 to rats on P30 (Appendix 3).

To assess the permeability of the blood–brain barrier on P30, 6-OHDA was used at a dose of 300 µg that was 2 times higher than the dose used on P2 for the same purpose. The dose of 6-OHDA was increased in proportion to the increase in the CSF volume from P2 to P30. In this experiment, we did not find 6-OHDA in the plasma within 15-45 min after intraventricular injection.

Discussion

The results of this study support our hypothesis that during the critical period of morphogenesis the brain operates as an endocrine organ, secreting morphogenetic factors in the general circulation (Ugrumov 2010). First, direct evidence was obtained that the brain-blood barrier is permeable to monoamines in the neonatal period of ontogenesis, but not in adult rats. Then, it was shown that NE, which is a potent morphogenetic factor, is delivered from the brain to the general circulation in neonatal rats, but not in prepubertal rats after cessation of morphogenesis. It means that the developing brain is a source of circulating NE in neonatal rats, although it is also secreted by the peripheral NE-producing organs of the sympatho-adrenal system. This finding expands the list of morphogenetic factors, such as dopamine, serotonin, gonadotropin-releasing hormone, which are secreted by the brain into the bloodstream in perinatal rats (Ugrumov et al. 2005, 2012; Zubova et al. 2014).

In the initial study, we showed that 6-OHDA, administered into the ventricles, is delivered to the general circulation in neonatal rats (P2), but its concentration in the blood is not high. This is probably due to the uptake of 6-OHDA on the way to the blood vessels and in the blood by the cells that have a catecholamine membrane transporter (catecholaminergic neurons, lymphocytes, etc.). These data are the first direct evidence of the permeability of the brain-blood barrier to monoamine-like substances during the critical period of morphogenesis, and they are in good agreement with the data on the permeability of the blood–brain barrier for monoamines in the same period of ontogenesis (Loizou 1970; Kostrzewa 2007).

In the next study, we estimated changes in the concentration of NE and dopamine in plasma after suppressing catecholamine production in the brain of neonatal rats (P2), which is considered as an indicator of the endocrine function of the brain. Catecholamine production was irreversibly suppressed by intraventricular injection of 6-OHDA, neurotoxin of catecholaminergic neurons (Blum et al. 2001). Since there is no brain-blood barrier in neonatal rats (see Results; Loizou 1970; Kostrzewa 2007), 6-OHDA can diffuse from the cerebral ventricles into the bloodstream, providing a direct toxic effect on peripheral catecholamine-containing organs. To avoid this effect on the periphery, we selected a dose of 7.5 µg per animal in a preliminary screening study, at which 6-OHDA causes degradation of the catecholaminergic system in the brain, but does not affect peripheral organs. NE and dopamine were measured in the brain and peripheral organs 6 and 24 h after intraventricular administration of 6-OHDA, which is the period of neurodegeneration caused by specific toxins of catecholaminergic neurons (Kolacheva et al. 2014; Yokoyama et al. 1993). The content of NE and dopamine in the brain decreased significantly but did not change in peripheral organs. This is accompanied by a decrease in the concentration of circulating NE. Interestingly, dopamine is reduced in the brain in the 6-OHDA-treated animals to a lesser extent than NE, which is consistent with earlier studies (Smith et al. 1973; Uretsky and Iversen 1970). Taken together, our data for the first time showed that in neonatal rats, NE is delivered from the brain to the general circulation (Fig. 9).

In the next experiment, we managed to cause the degeneration mainly of norepinephrine-ergic neurons due to the use, in addition to 6-OHDA, of GBR 12,909, a dopamine membrane transporter that plays the role of a neuroprotector of dopaminergic neurons (Teicher et al. 1986; Davids et al. 2002). In this experiment, it was shown that intraventricular administration of 7.5 µg 6-OHDA to rats on P2 after prior subcutaneous administration of GBR 12,909 causes a depletion in the brain of NE, but not of dopamine. An immunocytochemical study of DBH-immunoreactive neurons in the locus coeruleus confirmed biochemical data that the decrease in the content of NE in the brain is due to the degeneration of norepinephrine-ergic neurons, whose processes are distributed throughout the brain. Taken together, our biochemical and morphological data show that a decrease in plasma NE concentration is the result of a decrease in its production and release in the brain, but not in peripheral organs. The latter is confirmed by no change in the content of NE and dopamine in the studied peripheral organs. The use of intraventricular administration of

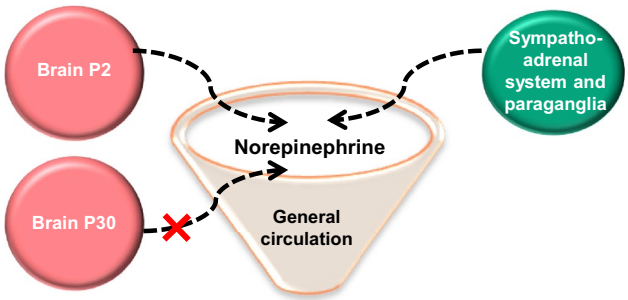

Fig. 9 Main sources of circulating norepinephrine in rats in ontogenesis. *P* postnatal day

6-OHDA would allow not only to show that NE is delivered from the developing brain to the general circulation during the critical period of morphogenesis but also to test our hypothesis about the morphogenetic action of NE. This can be done by evaluating the effect of irreversible 6-OHDA-induced inhibition of NE production in the neonatal period on gene expression and the phenotype of cells of peripheral organs in adulthood, especially of the cardiovascular system.

Although the barrier between the brain and blood vessels is considered asymmetric (Terasaki and Ohtsuki 2005; Pardridge 2016) there is little, if any, evidence of permeability of the brain-blood barrier in ontogenesis. Therefore, when discussing our results, we are obliged to refer to extensive studies of the permeability of the blood–brain barrier in ontogenesis. Most of these studies are consistent with our findings, claiming that the blood–brain barrier remains open for monoamines in rats until the end of the 2nd postnatal week. In fact, it was shown that in neonatal rats: (1) monoamines after systemic administration are captured by monoaminergic neurons in the brain (Loizou 1970); (2) dopamine after systemic injection is delivered into the cerebral ventricles (Miyaguchi et al. 1999); (3) 6-OHDA after systemic injection penetrates into the brain, causing degeneration of catecholaminergic neurons (Sachs 1973; Kostrzewa 2007); (4) [^{3}H]metaraminol, an analogue of NE, after systemic injection is delivered to the brain (Kostrzewa 2007).

Our data on the delivery of NE from the brain to the general circulation and the literature data on the permeability of the blood–brain barrier to monoamines in neonatal rats contradict the statement of some authors that this barrier is already closed in rodents in the prenatal period. This statement is based mainly on electron microscopic observations of tight junctions between the endothelial cells of the cerebral vessels and the expression of specific proteins such as occludin and claudin-5 (Bauer et al. 2014; Daneman 2012; Guerra et al. 2011; Saunders et al. 2012). However, these data are not functional and therefore cannot serve as evidence of impermeability of tight junctions. Indeed, in an electron microscope on single ultrathin sections, it is impossible to distinguish point-like permeable tight junctions from impermeable tight junctions encircling endothelial cells (Ugrumov et al. 1983; Schulze and Firth 1992). Recent findings on the impermeability of the blood–brain barrier to macromolecular markers in perinatal rodents (Fernandez-Lopez et al. 2012; Ben-Zvi et al. 2014) also cannot be considered as an argument against the permeability of this barrier to low-molecular weight substances, such as monoamines.

The next objective of this study was to test our hypothesis that NE is delivered from the brain into the bloodstream only during the period of morphogenesis, but not later. To solve this problem, we suppressed NE production in the brain of rats on P30 by intraventricular administration of 6-OHDA, i.e., after cessation of morphogenesis. Considering that the blood–brain barrier is impermeable to monoamines in adult rats (Loizou 1970; Kostrzewa 2007), we selected the maximum dose of 6-OHDA, 150 µg, at which this toxin under subcutaneous administration does not affect peripheral catecholamine-containing organs. As in neonatal rats, intraventricular administration of 6-OHDA to rats on P30 after 24 h resulted in a similar decrease in the content of NE in the brain. However, unlike rats on P2, when dopamine decreases in the brain after intraventricular administration of 6-OHDA, in a similar experiment on P30 there is only a tendency to a decrease in dopamine.

In the following experiment, in which 6-OHDA (150 µg) was injected into the cerebral ventricles in rats on P30, but after systemic administration of GBR 12,909, the content of NE in the brain decreased to the same extent as after administration of only 6-OHDA (see above). Moreover, in rats on P30, as on P2, treatment with 6-OHDA or 6-OHDA and GBR 12,909 did not lead to a change in the content of catecholamines in the peripheral organs. It should be emphasized that, unlike neontal rats, there were no changes in plasma concentration of NE in the prepubertal rats after their treatment with GBR and 6-OHDA, showing that there is no delivery of NE from the brain into the general circulation after cessation of morphogenesis. Apparently, this is due to the establishment of the brain-blood barrier in rats by age P30. The fact that 6-OHDA was not detected in the blood after its intraventricular administration at the dose of 300 µg is considered as direct evidence of the closure of the brain-blood barrier. Altogether, the above data show that the brain-derived NE does not reach the general circulation in rats on P30 either through the blood–brain barrier or with a CSF flow towards the venous sinus (Fig. 9).

Although it was shown that in neonatal rats, NE was secreted by the brain into the general circulation, and therefore the brain is a source of circulating NE (Fig. 9), it was necessary to test whether the concentration of NE in the blood is sufficient to affect the peripheral organs. According to our data, the concentration of NE in peripheral blood in intact fetuses and young rats is comparable to that in the hypophysial portal circulation and the general circulation in adult rats (Thomas et al. 1989). These data suggest that NE circulating in neonatal rats is able to exert morphogenetic effect on peripheral organs and the brain (self-regulation). This suggestion is supported by the detection of adrenoreceptors in organs such as the brain, heart, lungs and brown adipose tissue in perinatal rats (Berger-Sweeney and Hohmann 1997; Espinasse et al. 1995; Kitamura et al. 1989; Marunaka et al. 1999; Nedergaard et al. 1995). Interestingly, the number of adrenoreceptors per cell (density of receptors) gradually increases during the perinatal period, while their affinity for NE remains unchanged. In particular, the density of α2-adrenoreceptors continuously increases in almost all

areas of the developing brain in rats in the early postnatal period, reaching an adult level by P15 (Happe et al. 2004). In turn, the density of β-adrenoreceptors and adenylate cyclase activity reach a maximum level in the rat brain by P8 (Keshles and Levitzki 1984). A significant increase of adrenoceptor density and hence the cell sensitivity to NE in ontogenesis, apparently, should promote morphogenetic action of NE.

Our observation that the concentration of NE in the plasma of rats on E18 many times exceeds that in the next period of ontogenesis and in adulthood is of particular interest. Plasma concentrations of markers of the brain endocrine activity, dopamine and gonadotropin-releasing hormone studied so far, also decreased from E18 to E21, but to a lesser extent than NE (Ugrumov et al. 2005, 2012). Interestingly that similarly to NE, there is a surge in testosterone secretion in male rats on E18, when its plasma concentration was two times higher than in neonates (Weisz and Ward 1980). Such a temporary surge of secretion of signaling molecules in the period of morphogenesis, apparently, is crucial for the morphogenetic action. While an increase in the testosterone concentration in the blood in rats on E18 is associated with masculinization of the brain (Weisz and Ward 1980), the functional significance of the high concentration of NE in plasma at this age remains uncertain.

Interestingly, according to our data, the concentration of NE in the plasma gradually increases from E21 to P15 due to a gradual increase in the NE secretion by the brain and/or peripheral organs, despite a simultaneous increase in blood volume (Ugrumov et al. 2012). To evaluate an endocrine capacity of the brain in ontogenesis, we assessed the content of NE in the developing brain. It was shown that the content of NE and, apparently, NE synthesis in the brain sharply increases during the perinatal period, being comparable to that in the adrenals. The correlation observed in this study between the gradual increase in the NE content in the plasma and in the brain during the perinatal period (E21 to P15) suggests a causal link between both events. Unexpectedly, the content of NE in the brain in rats only on P30, measured in this study, is almost two times higher than the content of NE, measured 3 years ago in our previous study (Murtazina et al. 2016). This is probably due to the fact that during this time the nuclear stock of animals has changed, and new animals are somewhat different from previous animals in phenotype. This assumption is partly confirmed by the fact that the weight of rats, at least in the prepubertal period, can vary significantly, from 60 to 110 g (Breese and Traylor 1972; Kvetnanský et al. 1978).

Thus, the data obtained in this study confirm our hypothesis that the developing brain functions as a multipotent endocrine organ during the critical period of morphogenesis.

Authors contributions MVU created the concept and design of the study, interpreted the experimental data; ARM, YON, NSB, AYS performed experiments, analyzed and interpreted biochemical data; LKD carried out immunohistochemistry and image analysis, prepared figures. All authors have approved the final manuscript and agree to be accountable for all aspects of the work.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Appendix 1

The content of norepinephrine (NE) and dopamine (DA) in peripheral organs in rats on postnatal days 3 (P3) and P31 24 h after: (1) intraventricular injection of 6-hydroxydopamine (6-OHDA) at the dose of 7.5 µg on P2 and 150 µg on P30, or 0.9% NaCl in both age groups (control); (2) subcutaneous injection of 6-OHDA at the dose of 150 µg, or 0.9% NaCl (control) on P30

		Intraventricular injection				Subcutaneous injection	
Age		Postnatal day 3		Postnatal day 31		Postnatal day 31	
Substance/Organ	NE (ng), DA (ng)	Control (NaCl)	6-OHDA (7.5 µg)	Control (NaCl)	6-OHDA (150 µg)	Control (NaCl)	6-OHDA (150 µg)
Adrenals	NE	126.62 ± 11.3	114.91 ± 9.51	1323.3 ± 98.41	1513.82 ± 122.54	1765.7 ± 236.33	1828.43 ± 127.46
	DA	5.38 ± 0.36	4.15 ± 0.47	74.9 ± 10.93	64.22 ± 4.14	65.42 ± 10.07	95.79 ± 10.76
Duodenum	NE	4.86 ± 0.68	5.19 ± 0.85	187.84 ± 13.32	162.75 ± 12.82	218.41 ± 16.55	207.95 ± 14.47
	DA	0.23 ± 0.05	0.23 ± 0.08	4.99 ± 0.63	6.46 ± 0.69	6.54 ± 0.2	8 ± 0.16
Heart	NE	3.98 ± 0.4	3.25 ± 0.15	288.56 ± 20.44	277.82 ± 20.66	177.36 ± 7.62	149.02 ± 21.41
	DA	0.2 ± 0.07	0.15 ± 0.06	5.23 ± 0.66	4.97 ± 0.59	2.42 ± 0.25	3.46 ± 0.59

Values are mean ± SEM. There are no differences between experiments (6-OHDA) and controls (NaCl)

Appendix 2

The content of norepinephrine (NE) and dopamine (DA) in peripheral organs in rats on postnatal days 3 (P3) and P31, 25 h after the administration, first subcutaneously GBR 12,909 (40 mg/kg), and then after 1 h, of 6-hydroxydopamine (6-OHDA) at the dose of 7.5 µg on P2 or 150 µg on P30 intraventricularly; control animals received 0.9% NaCl in both age groups

Age		Postnatal day 3		Postnatal day 31	
Substance/Organ	NE, DA (ng)	Control (Nacl)	GBR and 6-OHDA	Control (Nacl)	GBR and 6-OHDA
Adrenals	NE	128.04 ± 15.46	122.5 ± 14.61	1909.9 ± 149.85	1808.54 ± 238.12
	DA	6.09 ± 0.84	3.89 ± 0.35	97.27 ± 20.89	76.4 ± 14.89
Duodenum	NE	3.33 ± 0.32	2.8 ± 0.3	197.94 ± 10.31	175.6 ± 27.96
	DA	0.41 ± 0.05	0.38 ± 0.05	4.21 ± 0.57	3.59 ± 0.74
Heart	NE	2.81 ± 0.3	3.17 ± 0.3	339.92 ± 23.65	284.26 ± 37.57
	DA	0.18 ± 0.05	0.17 ± 0.06	5.99 ± 1.24	7.53 ± 0.6

Values are mean ± SEM. There are no differences between experiments (GBR and 6-OHDA) and controls (NaCl)

Appendix 3

The content of norepinephrine (NE) in the brain, peripheral organs, and the concentration of NE in plasma in rats on postnatal days 3 (P3) and P31, 24 h after a subcutaneous injection of GBR 12,909 or 0.9% NaCl (control)

Age		Postnatal day 3		Postnatal day 31	
Substance/Organ	NE	Control (NaCl)	GBR 12,909	Control (NaCl)	GBR 12,909
Brain	NE (ng)	58.94 ± 2.17	63.51 ± 3.19	521.37 ± 28.4	436.99 ± 22.68
Adrenals	NE (ng)	118.85 ± 6.5	124.14 ± 17.49	1483.29 ± 285.21	1757.96 ± 277.55
Duodenum	NE (ng)	6.04 ± 1.26	4.74 ± 1.06	115.44 ± 2.86	122.05 ± 10.95
Heart	NE (ng)	7.04 ± 0.6	5.34 ± 0.54	210.47 ± 8.73	249.53 ± 25.53
Plasma	NE (ng/ml)	3.21 ± 0.76	4.71 ± 0.97	3.04 ± 0.72	3.58 ± 0.4

Values are mean ± SEM. There are no differences between experiments (GBR 12,909) and controls (NaCl)

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