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Prenatal Hypoxia Predisposes to Impaired Expression of the *chrna4* and *chrna7* Genes in Adult Rats without Affecting Acetylcholine Metabolism during Embryonic Development

Oleg V. Vetrovoy[1,a]*, Sofiia S. Potapova[1], Viktor A. Stratilov[1], and Ekaterina I. Tyulkova[1]

[1]*Laboratory of Regulation of Brain Neuronal Functions, Pavlov Institute of Physiology, Russian Academy of Sciences, 199034 Saint Petersburg, Russia*

[a]*e-mail: vov210292@yandex.ru*

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Abstract—Previous studies have shown that the combined effect of fetal hypoxia and maternal stress hormones predetermines tendency to nicotine addiction in adulthood. This study in rats aimed to investigate the effect of prenatal severe hypoxia (PSH) on acetylcholine metabolism in the developing brain, as well as on expression of acetylcholine receptors *chrna4* and *chrna7* in both the developing brain and adult brain structures following nicotine consumption. In the developing brain of PSH rats, no changes were found in the activity of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) or disturbances in the acetylcholine levels. However, decreased *chrna4* expression was detected on the day 15 of pregnancy, while elevation in the *chrna7* expression was observed on the days 15 and 16 of embryogenesis. In adulthood, the consequences of PSH were manifested as decreased expression of *chrna4* in the medial prefrontal cortex (PFC), nucleus accumbens (NAacc), and hypothalamus (HT), decreased expression of *chrna7* in the PFC and hippocampus (HPC). Whereas, nicotine consumption did not decrease the expression levels of *chrna4* and *chrna7* compared to the control group in the adult PSH rats. Thus, prenatal hypoxia predisposes to impaired expression of the *chrna4* and *chrna7* genes in adult rats without affecting acetylcholine metabolism during embryonic development.

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Keywords: rat, brain development, prenatal hypoxia, acetylcholine metabolism, *chrna4*, *chrna7*

INTRODUCTION

Nicotine addiction is a serious public threat, with a death toll of up to 8 million people every year [1]. In contemporary understanding, alongside with the genetic predisposition to nicotine addiction [2, 3], epigenetic changes influenced by environmental factors, which occur especially during embryonic brain development, are also of great importance [4, 5]. Thus, exposure to environmental stressors during the prenatal period, mediated by the maternal endocrine system or changes in fetal oxygen availability, increases the risk of substance use disorders [6, 7], especially nicotine dependence, in offsprings [8, 9].

Fetal hypoxia, often accompanied by the response of the mother's glucocorticoid system to impaired oxygen delivery, is one of the most significant factors that predetermines disturbances in the development

Abbreviations: AMG, amygdala; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; *chrna4*, acetylcholine receptor subunit alpha-4 mRNA; *chrna7* (CHRNA7), acetylcholine receptor subunit alpha-7 mRNA (protein); HPC, hippocampus; HT, hypothalamus; NAcc, nucleus accumbens; PFC, medial prefrontal cortex; PSH, prenatal severe hypoxia; PVN, thalamic paraventricular nucleus; VTA, ventral tegmental area.

* To whom correspondence should be addressed.

of the mesolimbic system of the brain. This condition leads to heightened nicotine consumption in an adult offspring [9]. In particular, our previous studies on rats have highlighted that the increased risk of nicotine addiction in adult rats might be associated with the episodes of severe hypoxia during 14th-16th days of embryogenesis [10, 11], equivalent to 5th-7th weeks of pregnancy in humans [12]. During this period, dopaminergic neurons of the ventral tegmental area (VTA) complete their axonal guidance to the nucleus accumbens (NAcc) [13], while hippocampus and other cortical structures innervating the NAcc begin to form [14]. In our previous investigation, we additionally compared the effects of fetal intrauterine ischemia [11, 15] and prenatal hypoxia induced within a pressure chamber, combined with the maternal stress response. We found that the key role in predisposing individuals to nicotine addiction lies not solely in hypoxia itself, but also in the excessive supply of glucocorticoids to the developing brain [11]. This led to the aberrant expression profile of glucocorticoid receptors in the extrahypothalamic brain structures, causing disruption of the circadian dynamics of glucocorticoids, impaired glucocorticoid-dependent expression, and, consequently, resulting in malfunctioning of the glucocorticoid-dependent processes throughout later life [16-18]. Hypoxia and stress-related processes could influence maturation of the cholinergic mediator system, both by altering expression of the individual elements [19] and by requiring a sufficient level of aerobic oxidative metabolism for the effective acetylcholine synthesis [20-22].

Primary mechanisms underlying impact of prenatal hypoxia and maternal glucocorticoid stress hormones on fetal brain development, crucial for nicotine addiction, still remain unclear. Therefore, in this study, we studied activity dynamics of the key enzymes involved in acetylcholine synthesis and degradation, namely choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), as well as fluctuations in acetylcholine concentration over the third week of embryonic development (e15, e16, e17, e20), as well as on the day 1 after their birth (p1) in the control rats and the rats exposed to prenatal severe hypoxia (PSH). Given the potential for disruptions in the expression of acetylcholine receptors, we also investigated transcription dynamics of acetylcholine receptor subunits alpha-4 (*chrna4*) and alpha-7 (*chrna7*) in both developing and adult brains, as well as under the influence of prenatal hypoxia. Chronic nicotine dependence originates from the reduced transcription of acetylcholine receptors due to external nicotine intake. We have previously shown that the adult PSH rats exhibit an increased propensity to consume nicotine under free-choice conditions [10], while chronic nicotine intake through osmotic pumps causes more pronounced behavioral signs of nicotine dependence in these rats compared to the control animals [10, 11]. In this study, we also used osmotic minipumps to provide a continuous supply of nicotine over two weeks, comparing effects of nicotine on the *chrna4* and *chrna7* transcription in the brain structures of adult rats in the control and PSH groups.

MATERIALS AND METHODS

Animals. The study was carried out using animals from the CCU "Biocollection of laboratory mammals of different taxonomic affiliation" of the Pavlov Institute of Physiology of the Russian Academy of Sciences. Adult pregnant female Wistar rats, aged 12-13 weeks and weighing 220-250 g, along with their embryonic (e15, e16, e17, e20), newborn (p1) progeny without sex definition and adult male offspring, aged 12 weeks and weighing 320-350 g, were utilized. All experimental procedures were performed in compliance with The Guidelines for Reporting Animal Research [23] and were approved by the Ethical Committee for the Use of Animal Subjects at the Pavlov Institute of Physiology (protocol no. 08/02 of 02.08.2022).

Prenatal severe hypoxia. Model of prenatal severe hypoxia (PSH) described in our previous studies was used as a reliable model of fetal hypoxia [10, 11, 16-18]. To model PSH, we used a flow-type hypobaric chamber at a temperature of 20°C to 25°C in which atmospheric pressure was gradually reduced to 180 Torr reaching 5% of oxygen content (equivalent to 11,000 m above sea level) during 20 min. After 3 h of treatment the oxygen content was returned to normal within 20 min. Pregnant dams were treated under such conditions for 3 consecutive days (14th, 15th, and 16th days of pregnancy) with an interval of 24 h between the sessions. Mortality rate in the hypobaric chamber was around 15%. Intact control females were also placed in the hypobaric chamber for 3 h on the 14th, 15th, and 16th days of pregnancy without being subjected to hypoxic or hypobaric exposure. Gestation period was 22-23 days.

Colorimetric methods. To collect brain samples for colorimetric analysis, tissues from embryonic (e15, e16, e17, e20) and newborn (p1) brains of both control and PSH rats were dissected and frozen in liquid nitrogen. Each rat group consisted of randomly selected embryos or pups from different dams to minimize litter bias.

Measurement of choline acetyltransferase activity. ChAT activity was analyzed using a colorimetric assay kit (E-BC-K125-M; Elabscience, USA). Dissected brain samples were washed and homogenized in PBS (0.01 M, pH 7.4) at 4°C and centrifuged at 10,000*g* for 10 min to isolate cytosolic proteins. The assay procedures were

conducted following the manufacturer's protocol, and absorbance was measured at 324 nm using a microplate reader (CLARIOstar PLUS, BMG Labtech, Germany). Amount of coenzyme A generated during the reaction was determined using a standard curve. ChAT activity was calculated as nanomoles of coenzyme A generated per minute per milligram of total protein. Both here and in the other biochemical tests below, total protein in the samples was measured using a Pierce Rapid Gold Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

Measurement of acetylcholinesterase activity. AChE activity was analyzed using a colorimetric assay kit (E-BC-K052-S; Elabscience). Dissected brain samples were washed and homogenized in 0.9% NaCl at 4°C. The assay procedures were conducted following the manufacturer's protocol, and absorbance was measured at 520 nm using a microplate reader (CLARIOstar PLUS, BMG Labtech). Amount of acetylcholine remaining after the reaction was determined using a standard curve. AChE activity was calculated as nanomoles of acetylcholine hydrolyzed per minute per milligram of total protein.

Measurement of acetylcholine levels. Acetylcholine levels were analyzed using a colorimetric ELISA kit (E-EL-0081; Elabscience). Dissected brain samples were washed and homogenized in PBS (0.01 M, pH 7.4) at 4°C and centrifuged at 5000*g* for 10 min to isolate supernatant containing acetylcholine. The assay procedures were conducted following the manufacturer's protocol, and absorbance was measured at 450 nm using a microplate reader (CLARIOstar PLUS, BMG Labtech). Amount of acetylcholine was quantified using a standard curve, calculated and expressed as picomoles per milligram of total protein.

Chronic treatment with nicotine in adult rats. Rat pups were weaned at 30 days of age, a time when dams spent no more than 2 h nursing [24]. This postpartum day aligns with our prior studies on prenatal pathologies, mitigating the stress typically associated with weaning. After weaning, the rats were housed in cages 60 × 30 × 20 cm in size, with 5-6 animals in each. Each rat group consisted of randomly selected rats born from different dams to minimize litter bias. The rats received food and water *ad libitum* and were kept on a 12 : 12-h dark-light cycle at room temperature with a constant humidity of approximately 60%. For the experimental procedures, we used adult male offspring with active spermatogenesis from the control and PSH groups at the age of 3 months. On day 1, osmotic minipumps (2002W, RWD Systems, China) were subcutaneously implanted into the rats under isoflurane anesthesia and a nicotine tartrate solution was pumped at a flow rate of 0.5 µl/h. Nicotine concentration in the pumps was adjusted for the differences in rat body weight, resulting in a continuous subcutaneous infusion of nicotine tartrate at a rate of 9 mg/kg per day. In the nicotine-naive control and PSH rats minipumps were filled with saline (vehicle). After two weeks of nicotine or vehicle consumption, the rats were scarified by guillotine, and samples of brain structures were collected for PCR analysis.

Quantitative RT PCR. Total RNA from embryonic (e15, e16, e17, e20) and newborn (p1) brain samples of the control and PSH rats, as well as from the samples of hippocampus (HPC), medial prefrontal cortex (PFC), amygdala (AMG), nucleus accumbens (NAcc), thalamic paraventricular nucleus (PVN), hypothalamus (HT), and VTA of adult rats (two weeks after nicotine or vehicle consumption) was isolated using an ExtractRNA Kit (BC032, Evrogen, Russia) and purified using DNAseI (SB-G3342, Servicebio, China) according to the manufacturers' instructions. Quality and concentration of total RNA were determined by measuring optical density at 260 nm and 280 nm using a microplate reader (CLARIOstar PLUS, BMG Labtech). cDNA templates were synthesized from 2 µg of total RNA using a MMLV Reverse Transcription Kit (SK021, Evrogen). Quantitative reverse transcription polymerase chain reaction (RT PCR) was carried out with a qPCRmix-HS SYBR+LowROX kit (Evrogen) using a Gentier 96E Thermal Cycler (Tianlong, China). Expression levels of the target acetylcholine receptor subunit alpha-4 (*chrna4*) and acetylcholine receptor subunit alpha-7 (*chrna7*) genes were estimated using the ΔΔCt method with normalization to *β-tubulin* mRNA content as a reference gene. We used the following primer sequences: *chrna4*, forward: GGTGAAGGAGG ACTGGAA, reverse: AAGGCAGACAATGATGAACA (annealing temperature 58°C, 78 bp product); *chrna7*, forward: CTCTTGGAATAACTGTCTT, reverse: CGAAGT ATTGTGCTATCA (annealing temperature 58°C, 105 bp product); β-tubulin, forward: TAGAGGAGATGCTACTTA, reverse: AATGGTGATAATACTGTTAA (annealing temperature 58°C, 147 bp product).

Western blotting. To confirm the effect of changes in *chrna7* mRNA expression on the CHRNA7 protein levels in the brain structures of adult control and PSH rats we used Western blotting. To obtain total protein extracts for Western blotting, samples of HPC, PFC, AMG, NAcc, PVN, HT, and VTA were homogenized in a 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 1% Triton X100 and a cocktail of protease and phosphatase inhibitors (SB-G2006, SB-G2007, Servicebio). Homogenates were incubated on a shaker for 30 min at 4°C, centrifuged for 10 min at 14,000*g*, and supernatants were collected. The samples containing equal amounts of total protein were boiled for 10 min at 70°C with a 3x Laemmli buffer.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

and next transferred to PVDF membranes (Thermo Scientific, USA). After blocking for 1 h in PBS containing 5% skim milk, the membranes were incubated in PBS with rabbit anti-CHRNA7 (1 : 2000, DF13247, Affinity Biosciences, USA) and anti-β-Tubulin (1 : 5000, ab179513, Abcam, UK) primary antibodies for 2 h at room temperature.

The membranes were then washed thrice with PBST (PBS with 0.1% Tween 20) and incubated in PBS with HRP-conjugated anti-rabbit secondary antibodies (1 : 5000, E-AB-1003, Elabscience) for 1 h at room temperature. The membranes were next washed twice with PBST. Immunoreactive protein bands were visualized using a Clarity ECL chemiluminescence kit (Bio-Rad, USA) with a ChemiScope 6000 Imaging System (Clinx Science Instruments, China). Protein levels were quantified using ImageJ software (NIH, USA) and normalized to β-Tubulin. Full images of the Western blots are presented in the Online Resource 1 (Fig. S1).

Statistical analysis. Statistical analysis was performed using Prism 10 (GraphPad, Inc.). All samples were assessed for normal distribution using the Shapiro–Wilk test ($p > 0.05$) and QQ-plot. One- or two-way ANOVA was used as a parametric test. *Post hoc* comparisons were performed using Tukey's honest significance test. Statistical significance was set at $p < 0.05$. The results are expressed as a mean ± standard error of the mean (SEM). For RT PCR and Western blotting, the mean and SEM were recalculated as % of a respective age control, taken as 100%.

RESULTS

Impact of prenatal hypoxia on acetylcholine metabolism in the rat brain during prenatal and early postnatal development. To investigate the effect of prenatal hypoxia on acetylcholine metabolism, we measured activity of choline acetyltransferase (ChAT), an enzyme that synthesizes this neurotransmitter, as well as activity of acetylcholinesterase (AChE), an enzyme that degrades acetylcholine, in the developing brain of rat embryos during the third week of pregnancy (e15, e16, e17, e20) and in the newborn rats (p1) (Fig. 1, a, b). We did not detect any changes in the activity of these enzymes in the brain of PSH rats compared to the controls. In the brain of PSH rats, we observed no alterations in the activity of ChAT and AChE. Likewise, there were no significant changes in the concentration of acetylcholine across all periods studied (Fig. 1c).

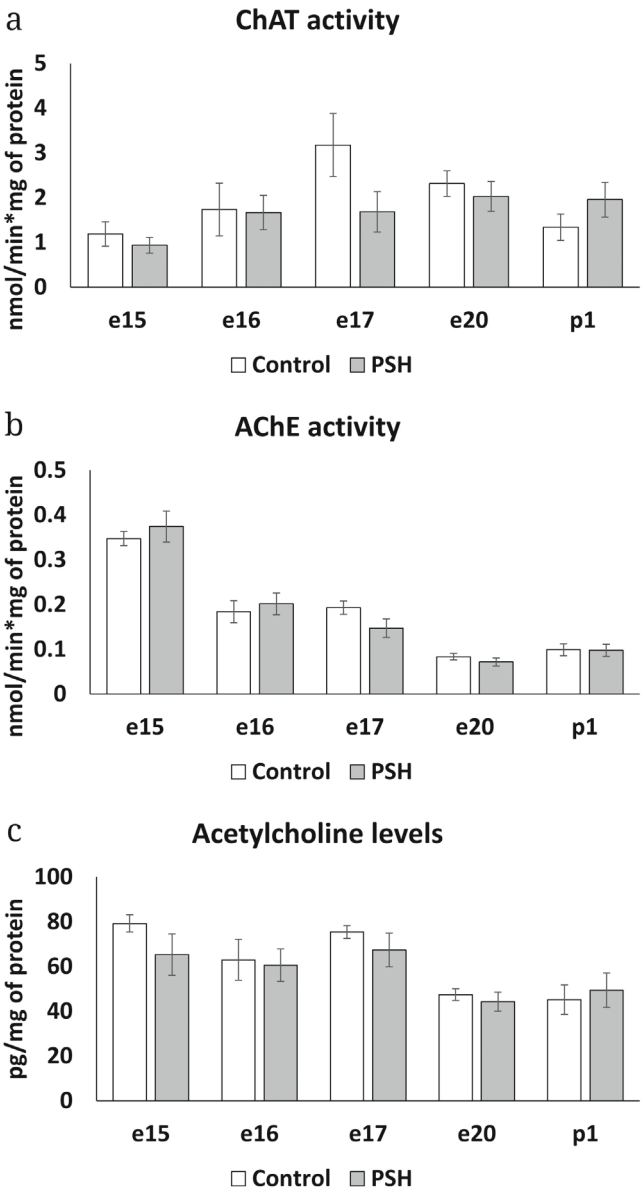

Fig. 1. Effect of PSH on ChAT (a) and AChE (b) activity and acetylcholine (c) levels in the rat brain during prenatal (embryonic days e15, e16, e17, e20) and early postnatal (postnatal day p1) development, detected by colorimetric tests. $n = 5$.

Impact of prenatal hypoxia on expression of acetylcholine receptors mRNA in the rat brain during prenatal and early postnatal development. To evaluate the effect of prenatal hypoxia on the receptor part of acetylcholine neurotransmitter system, we measured relative content of the acetylcholine receptor subunit alpha-4 (*chrna4*) and acetylcholine receptor subunit alpha-7 (*chrna7*) mRNA in the developing brain during the third week of pregnancy (e15, e16, e17, e20) and in the brains of newborn rats (p1) (Fig. 2, a, b). Decrease in the relative amount of *chrna4* mRNA was found in the rat brain one day after the first PSH session (Fig. 2a, e15, ANOVA F (1, 9) = 10.2212, $p = 0.0127$; control vs. PSH, $p = 0.0126685$, Tukey's HST). During further prenatal development and in the newborn PSH rats, no significant changes in the *chrna4* expression in the brain

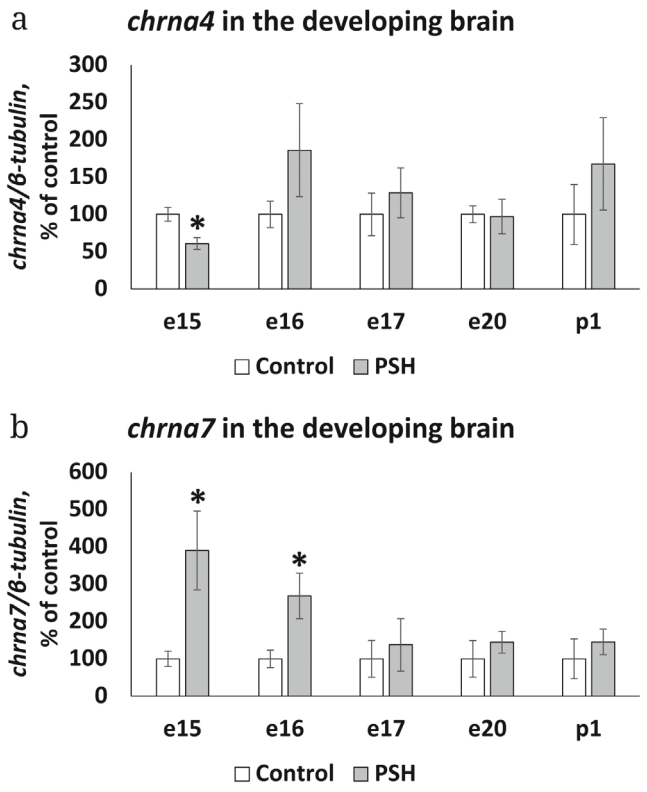

Fig. 2. Effect of PSH on *chrna4* (a) and *chrna7* (b) mRNA expression levels in the rat brain during prenatal (embryonic days e15, e16, e17, e20) and early postnatal (postnatal day p1) development, detected by RT PCR. * Significant differences vs. relative control, $p < 0.05$ (one-way ANOVA, Tukey HST). $n = 5$.

were detected. In addition, we found increase in the relative amount of the *chrna7* mRNA both 24 h after the first PSH session (Fig. 2b, e15, ANOVA F (1, 9) = = 7.2211, $p = 0.0276$; control vs. PSH, $p = 0.0276166$, Tukey's HST) and one day after the second PSH session (Fig. 2b, e16, ANOVA F (1, 9) = 6.6590, $p = 0.0326$; control vs. PSH, $p = 0.0325905$, Tukey's HST). Throughout further prenatal development and in the newborn PSH rats, no significant changes in the *chrna7* expression in the brain were detected. Thus, despite the lack of effect on acetylcholine metabolism, prenatal hypoxia causes changes in the expression of acetylcholine receptors in the developing brain.

Impact of prenatal hypoxia on the expression of acetylcholine receptors mRNA in the rat brain structures in normal adult animals and in animals exposed to nicotine. To study long-term consequences of prenatal hypoxia on the expression of acetylcholine receptors in the rat brain, we measured relative content of *chrna4* and *chrna7* mRNA in the limbic system structures (HPC, PFC, AMG, NAcc, PVN, HT, VTA) of the adult (3-month-old) control and PSH rats, both before and after two weeks of stable nicotine consumption via osmotic pumps.

When assessing relative amount of mRNA, significant decreases in the expression of *chrna4* gene caused by PSH was observed in the PFC (Fig. 3b, two-way ANOVA Group × Nicotine F (1, 16) = 9.872, $p = 0.0063$; control vs. PSH, $p = 0.0399817$, Tukey's HST), NAcc (Fig. 3d, ANOVA Group × Nicotine F (1, 16) = = 5.644, $p = 0.0304$; control vs. PSH, $p = 0.0190$, Tukey's HST), and HT (Fig. 3f, two-way ANOVA Group × Nicotine F (1, 16) = 9.673, $p = 0.0067$; control vs. PSH, $p = = 0.0061796$, Tukey's HST), with no effect on HPC (Fig. 3a), AMG (Fig. 3c), PVN (Fig. 3e), and VTA (Fig. 3g). Moreover, following two weeks of nicotine consumption, the amount of *chrna4* mRNA in the brains of control rats significantly decreased to the values comparable to those of the PSH rats in HPC (Fig. 3a, two-way ANOVA Nicotine F (1, 17) = 9.989, $p = 0.0057$; control vs. control + nicotine, $p = 0.0057263$, Tukey's HST), PFC (Fig. 3b, two-way ANOVA Group × Nicotine F (1, 16) = 9.872, $p = 0.0063$, $p = 0.0234$; control vs. control + nicotine, $p = 0.0022758$, Tukey's HST), NAcc (Fig. 3d, two-way ANOVA Group × Nicotine F (1, 16) = = 5.644, $p = 0.0304$; control vs. control + nicotine, $p = = 0.0421275$, Tukey's HST) and HT (Fig. 3f, two-way ANOVA Group × Nicotine F (1, 16) = 9.673, $p = 0.0067$; control vs. control + nicotine, $p = 0.0157127$, Tukey's HST). In contrast, nicotine consumption by the PSH rats did not induce alterations in the *chrna4* mRNA content across all brain structures when compared to the intact PSH rats (Fig. 3).

When assessing relative amount of *chrna7* mRNA in the brains of adult rats, PSH was found to cause significant decrease of the gene expression in the HPC (Fig. 3h, two-way ANOVA Group × Nicotine F (1, 16) = = 7.945, $p = 0.0124$; control vs. PSH, $p = 0.038$ Tukey's HST) and PFC (Fig. 3i, two-way ANOVA Group × Nicotine F (1, 16) = 9.55, $p = 0.00702$; control vs. PSH, $p = = 0.0157$, Tukey's HST), while leaving the AMG (Fig. 3j), PVN (Fig. 3k), NAcc (Fig. 3l), HT (Fig. 3m) and VTA (Fig. 3n) unaffected. Association between the changes in *chrna7* mRNA levels and the patterns of CHRNA7 protein expression in the brain structures of adult control and PSH rats was subsequently tested by Western blotting (Fig. 4). Similar to the changes in relative mRNA levels, PSH was found to cause decrease in the CHRNA7 protein levels in the HPC (Fig. 4a, ANOVA F (1, 9) = 5.6864, $p = 0.0442$; control vs. PSH, $p = 0.0442243$, Tukey's HST) and PFC (Fig. 4b, ANOVA F (1, 9) = 11.9926, $p = 0.0085$; control vs. PSH, $p = = 0.0085305$, Tukey's HST), but not in AMG (Fig. 4c), PVN (Fig. 4d), NAcc (Fig. 4e), HT (Fig. 4f) and VTA (Fig. 4g).

Furthermore, following two weeks of nicotine consumption, the amount of *chrna7* mRNA in the brains of control rats significantly decreased to the levels observed in the HPC of the PSH rats (Fig. 3h, two-way ANOVA Group × Nicotine F (1, 16) = 7.945, $p = 0.0124$; control vs. control + nicotine, $p = 0.0103$,

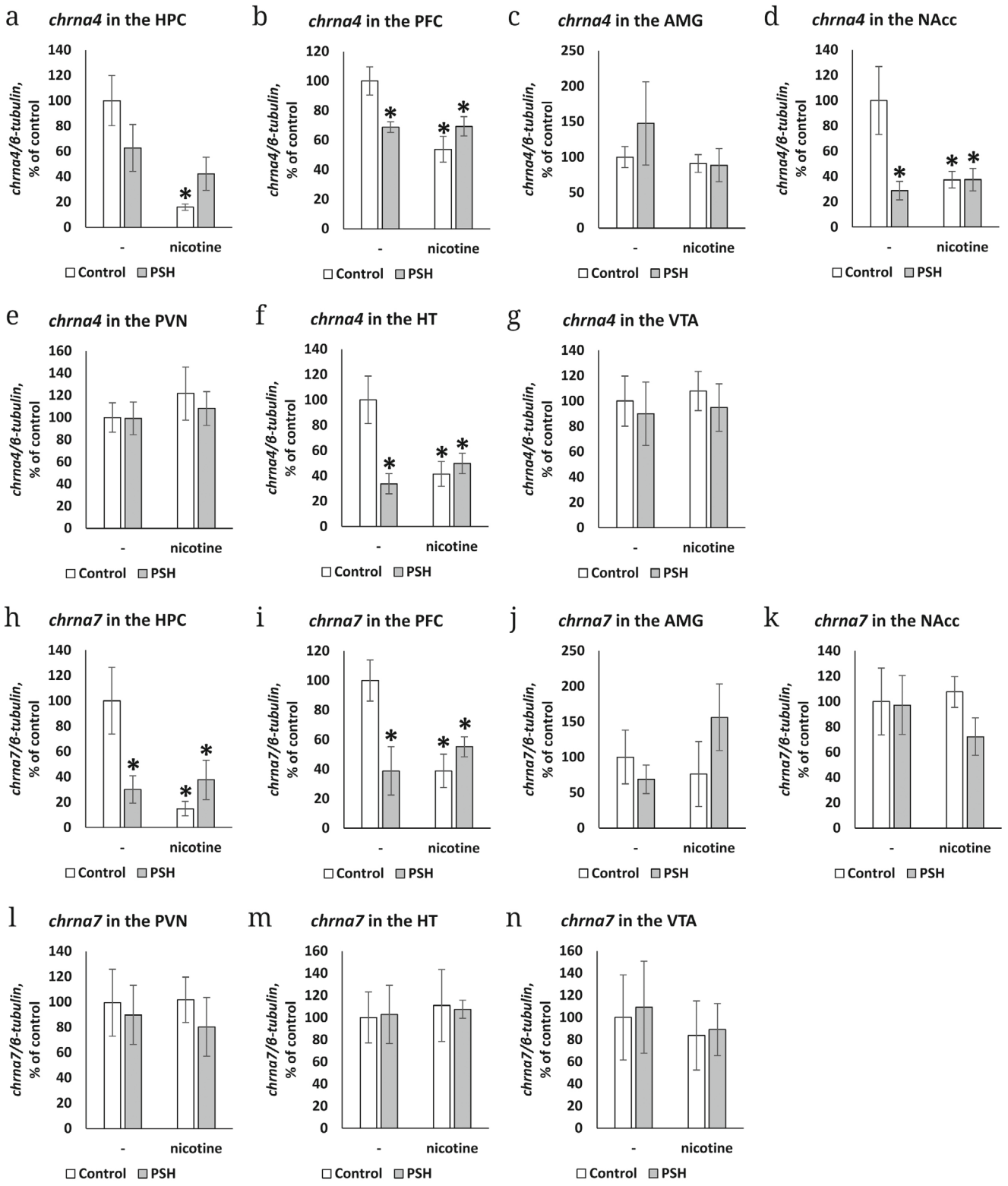

Fig. 3. Effects of PSH and nicotine consumption on *chrna4* (a-g) and *chrna7* (h-n) mRNA expression levels in the HPC (a, h), PFC (b, i), AMG (c, j), NAcc (d, k), PVN (e, l), HT (f, m), VTA (g, n) of adult rats, detected by RT PCR. * Significant differences vs. control, $p < 0.05$ (two-way ANOVA, Tukey HST). $n = 5$.

Tukey's HST) and PFC (Fig. 3i, two-way ANOVA Group × Nicotine F (1, 16) = 9.55, $p = 0.00702$; control vs. control + nicotine, $p = 0.0156$, Tukey's HST). Meanwhile, in the brains of PSH rats, nicotine consumption did not cause changes in the *chrna7* mRNA levels compared to the intact PSH rats.

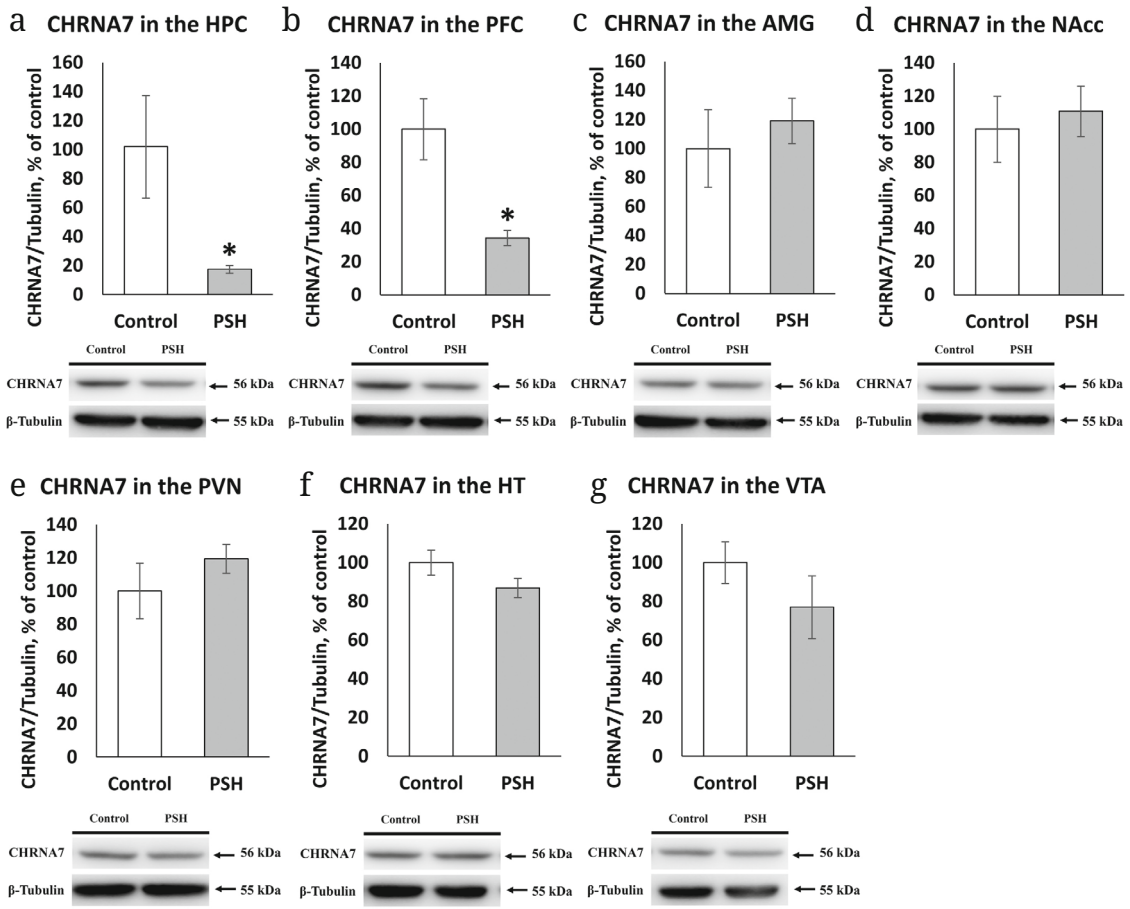

Fig. 4. Effects of PSH on CHRNA7 protein expression levels in the HPC (a), PFC (b), AMG (c), NAcc (d), PVN (e), HT (f), VTA (g) of adult rats, detected by western blotting. * Significant differences vs. control, $p < 0.05$ (one-way ANOVA, Tukey HST). $n = 5$.

DISCUSSION

Environmental factors exert significant impacts on the developing organism. Conditions for formation of individual organs and tissues of the fetus predetermine their subsequent life activities [25-27]. During a normal pregnancy, there is high efficiency in providing energy substrates essential for intensive processes of cell proliferation, migration, and establishment of connections between the brain cells [28]. Moreover, steroid hormone supply is restricted until the later stages of pregnancy, when glucocorticoids are involved in the processes such as terminal differentiation of neuronal cells and lung maturation [29-32]. Restriction of oxygen supply to an embryo causes significant metabolic disorder, resulting in the developmental deceleration [25-27, 33, 34], whereas excessive infiltration of glucocorticoids may disrupt formation of an adequate tissue-specific expression profile, which could remain throughout the life at epigenetic level [16, 17, 35, 36].

Hypoxia is inevitably accompanied by the shift of cellular metabolism towards anaerobic metabolism regulated by hypoxia-inducible factors (HIF) [37], and decline in aerobic energy metabolism, resulting in the reduced production of ATP [38, 39], which is required, in particular, for the synthesis of acetylcholine [40]. However, severity of the effects of hypoxia and maternal stress can vary significantly depending on the time of the developing brain exposure [12]. Thus, when prenatal hypoxia was induced on the days 14-16 of embryonic development, no significant changes were observed in the activity of both choline acetyltransferase and acetylcholinesterase. Consequently, this lack of change did not affect concentration of acetylcholine in the developing embryonic and early postnatal rat brain. This finding contrasts with the prior studies, including our own, which demonstrated intensification of the hypoxia-dependent signaling during both embryogenesis and the postnatal period [26, 41-45]. At the same time, at the early stages of hypoxic episodes, decrease in the transcription of *chrna4* (e15) and, potentially, glucocorticoid-dependent increase in the transcription of *chrna7* (e15, e16) was shown [11, 19]. During this period of brain development, cholinergic brain structures have been already formed [12, 46], which could help to increase resistance of the acetylcholine-producing neurons to external stressors.

However, at this moment formation of the acetylcholine-receptive GABAergic neurons within the regions such as ventral striatum, hippocampus, prefrontal cortex, and amygdala is just beginning [12, 14, 47]. As a consequence, these brain structures exhibit the most striking disturbances in the expression of acetylcholine receptors in adulthood. These disturbances may be considered either as a cause of, or as a compensatory response to the increased excitability of the brain cells reported by other authors [48]. We have previously shown that the prenatal hypoxia causes lifelong changes in the expression of glucocorticoid receptors in the hippocampus and prefrontal cortex, which is accompanied by the decrease in glucocorticoid-dependent transcription and disruption of glucocorticoid negative feedback [11, 16, 17]. Moreover, the alpha-7 subunit of acetylcholine receptor has been identified as a target of transcriptional activity of glucocorticoid receptors [19, 49]. Taking into consideration this information, we detected decrease in the expression of *chrna7* mRNA and protein specifically in the hippocampus and prefrontal cortex caused by prenatal hypoxia. These glutamatergic brain structures activate GABAergic neurons in the striatum, which is accompanied by the increased dopamine release from the neurons in the ventral tegmental area, inducing a phenomenon known as intrinsic reinforcement [50-52]. Decline in the efficiency of glutamate release, attributed to the reduced expression of *chrna4* and *chrna7* in the prefrontal cortex, *chrna7* in the hippocampus, and *chrna4* in the striatum of the adult rats due to prenatal hypoxia, could explain the previously described tendency of these animals towards nicotine addiction and pronounced withdrawal syndrome [10, 11]. Moreover, amygdalar neurons are known to indirectly inhibit action potentials in the cells of the ventral striatum [53, 54]. Concurrently, our findings did not reveal changes in the expression of *chrna4* and *chrna7* in the amygdala, which could also contribute to the disruption of the limbic system as a result of prenatal hypoxia. Finally, hypothalamic neurons exhibit capacity for the increased stimulation of dopamine neurons in the ventral tegmental area [55], while concurrently exhibit reduction in the relative *chrna4* expression.

The process of developing resistance to nicotine, which determines development of the dependence on further consumption, is widely acknowledged in clinical practice. It is known that one of the mechanisms of such resistance is the decrease in expression of acetylcholine receptors, including alpha-4 and alpha-7 subunits [56-61]. Interestingly, after one week of nicotine administration, decrease in the expression of *chrna4* (HPC, PFC, NAcc, HT) and *chrna7* (HPC, PFC) was observed solely in the control group. Conversely, the initially low expression of these genes in the brains of rats exposed to prenatal hypoxia remained unchanged at the low level.

Therefore, prenatal hypoxia does not affect activity of acetylcholine synthesis and degradation in the developing brain. However, it does causes significant disturbances in the expression of acetylcholine receptors alpha-4 and alpha-7 within the limbic structures of rats. These disturbances may underlie the previously observed tendency to nicotine consumption and manifestation of severe withdrawal syndrome upon cessation.

Supplementary information. The online version contains supplementary material available at https://doi.org/10.1134/S0006297924110099.

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Contributions. Conceptualization, O.V.V.; methodology, O.V.V., V.A.S., and E.I.T.; formal analysis, O.V.V. and V.A.S.; investigation, O.V.V., V.A.S., S.S.P., and E.I.T.; writing – original draft preparation, O.V.V.; writing – review and editing, O.V.V., V.A.S., and E.I.T.; visualization, O.V.V. and V.A.S.; supervision, O.V.V.; project administration, O.V.V.; funding acquisition, O.V.V. All authors have read and agreed to the published version of the manuscript.

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Ethics declarations. Animal experiments were performed in accordance with The Guidelines for Reporting Animal Research. This study protocol was reviewed and approved by the local ethics committee of the Pavlov Institute of Physiology (protocol no. 08/02 of 02.08.2022). The authors declare that they have no conflicts of interest.

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