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EXPERIMENTAL ARTICLES

The Changes in Exploratory Behavior and *Fgf2* Gene Expression in Cells of the Rat Brain after the Early Postnatal Administration of Bacterial Lipopolysaccharide

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Abstract—Infectious diseases at early ages are often followed by impairments in cognitive functions in children and adolescents. A possible mechanism of these impairments is an altered production of proteins that are involved in the regulation of neuroplasticity in brain cells. One of these proteins is the basic fibroblast growth factor (FGF2). In the present study, we examined the behavior in the "open field" test and FGF2 mRNA production in the medial prefrontal cortex, ventral and dorsal hippocampus, and amygdala of 22- and 23-day-old rats that were injected with bacterial lipopolysaccharide at a dose of 25 μg/kg on days 14, 16, and 18 after birth. We observed decreased exploratory activity and increased static movement compared to the control animals that were injected with a pyrogen-free physiological solution. Impaired behavior was associated with the lower expression of the *Fgf2* gene in cells of the medial prefrontal cortex compared to that observed in the intact rats.

Keywords: bacterial lipopolysaccharide, exploratory behavior, basic fibroblast growth factor, mRNA, early postnatal ontogeny

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INTRODUCTION

Cognitive impairments, including changes in attention, memory, and other types, are the important problems of the neurology and psychiatry of childhood due to their wide distribution, involving up to 20% of all children and adolescents [1]. They are also very difficult to treat, probably because of insufficient study of their pathophysiological mechanisms.

One of the main causes of the development of cognitive impairments in children and adolescents is prenatal or early postnatal infection diseases [1]. Under experimental conditions, these pathologies may be modeled using the element of a cell membrane of gram-negative bacteria-lipopolysaccharide (LPS, endotoxin). Administration of LPS to rats or mice during the first postnatal days results in the impaired differentiation of hippocampal cells [2] and delayed disturbances of memory and exploratory activity that are observed in adolescents and adults [3–5]. The impairments of long-term potentiation and neurogenesis in the hippocampus were observed in the offspring of rats that were injected with endotoxin during pregnancy [6, 7].

The pattern of LPS-induced impairments allows us to suggest that one of their causes is an alteration of the production of proteins that, on the one hand, influence the development of neurons in early ontogenesis and, on the other hand, are involved in the regulation of neuroplasticity processes in the adult brain.

Here, we revised this hypothesis with reference to the basic fibroblast growth factor-2 (FGF2). This protein is produced in the brain mainly by astrocytes. FGF2 is a neurotrophic factor that regulates the development of the nervous system, regenerative plasticity, and adult neurogenesis (see review [8]). Altered production of FGF2 is considered as a key mechanism that mediates the effects of negative environmental factors that affect the development of the brain in early ontogenesis [9]. FGF2 influenced behavioral activity in a novel environment [10]. It is involved in the regulation of mechanisms of cerebral neuroplasticity, learning, and memory [11–13].

In the present study, we examined behavior in a novel environment and expression of the *Fgf2* gene in cells of brain structures that are involved in cognitive functions, including the medial prefrontal cortex, basolateral amygdalar nucleus, and dorsal and ventral hippocampus, in rats injected with LPS in early postnatal ontogenesis. It was important to differentiate the mRNA FGF2 production in two subdivisions of the hippocampus because of their specific roles in some forms of learning [14] and higher sensitivity of the cells of the dorsal hippocampus to neonatal LPS treatment than of the ventral hippocampus [15].

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Table 1. The nucleotide sequences of primers and probes that were used for genetic studies

Gapdh	Forward primer	5'-TGCACCACCAACTGCTTAG-3'
	Reverse primer	5'-GGATGCAGGGATGATGTTC-3'
	Probe	5'-HEX-ATCACGCCACAGCTTTCCAGA-BHQ1-3'
Fgf2	Forward primer	5'-TCAAGGATCCCAAGCGGCTCTACT-3'
	Reverse primer	5'-CACTCCCTTGATGGACACAAC-3'

MATERIALS AND METHODS

Forty-four male pups of Wistar rats were used for the experiment in accordance with the humanity principles of the European Community Directive no. 86/609 EC and the protocol approved by the Commission on Biomedical Ethics of the Institute of Experimental Medicine of the Northwest Branch of the Russian Academy of Medical Sciences. The rat pups were housed under the standard conditions with their dams (one rat with a litter per cage) with free access to water and food. The number of pups in each litter was made equal. Some newborn females were left but not used for the experiments. Males of each litter were divided into three groups, which were injected with bacterial LPS from *Escherichia coli*, serotype O55:B5 (Sigma, United States) at a dose of 25 μg/kg (experimental group), pyrogen-free saline (control group), or were not injected (intact group). The injections were performed on postnatal days 14, 16, and 18. These days were selected based on data of the critical days for the effects of IL-1β on developing cognitive functions [16]. The level of development of the central nervous system in rats of this age is similar to that in the human perinatal period [17]. The dose of LPS (25 μg/kg) was chosen in a pilot experiment as a dose with a moderate pyrogenic effect.

In order to study the delayed effect of LPS injection on exploratory behavior, the animals were evaluated in the "open-field" test at the age of 22 days. We used a round arena, with a floor divided into 20 × 20 cm squares and holes made at the intersections. The test duration was 3 min. Behavior in the test was recorded and analyzed using "Field 4" software that was developed at the Department of Physiology named after I.P. Pavlov of the Institute of Experimental Medicine of the Northwest Branch of the Russian Academy of Medical Sciences. We evaluated the locomotor activity (ambulations and static movements), anxiety (grooming, rearing, and freezing), and exploratory activity (sniffing, hole exploration, and climbing).

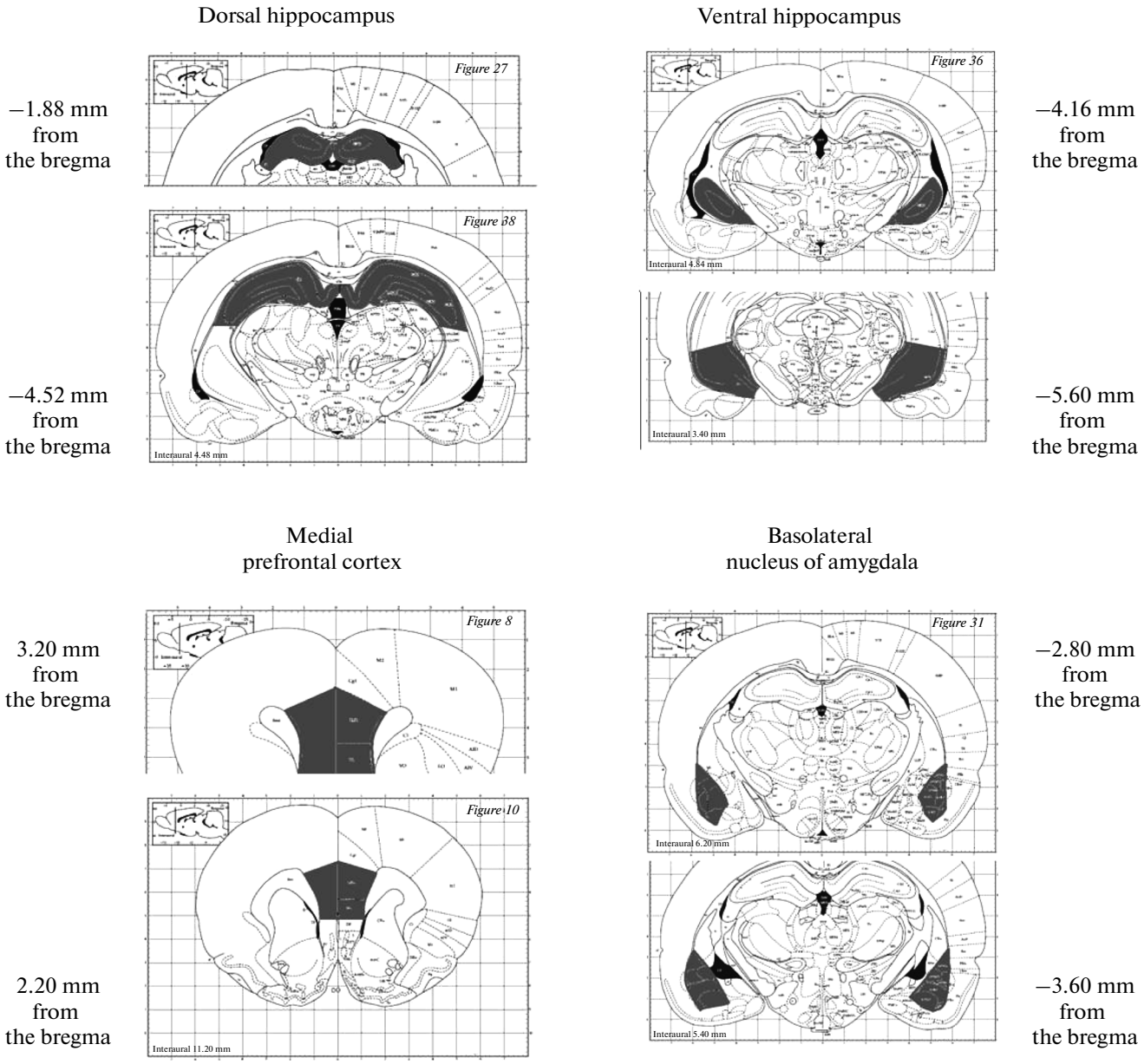
At 1 day after the open-field test, some rats were randomly selected for brain sampling. The brains were removed, immediately frozen, and stored at –70°C. Brain sections were made using a cryostat; the medial prefrontal cortex, dorsal and ventral hippocampi, and amygdala were dissected from the sections in accordance with Paxinos and Watson's atlas [18]. The scheme of brain dissection is presented in the figure. Total RNA was extracted with the acidic guanidine isocyanate–phenol–chloroform method using TRI reagent (Molecular Research Center, United States) according to the manufacturer's protocol. Reverse transcription was performed using oligo dT-primers (Medigen, Russia) and MMLV reverse transcriptase (Promega, United States). Gene expression was assayed by the real time PCR method using a C1000 Touch™ Thermal Cycler (Bio-Rad, United States). We used the *Gapdh* gene as a reference gene. The SYBR Green technique was used to study the expression of the *Fgf2* gene (GenBank NM_019305) and the TaqMan technique was used to study the level of mRNA of housekeeping *Gapdh* gene (GenBank NM_017008). Sequences of the primers and probes that were used are presented in Table 1. They were synthesized by the Bigl' company (St. Petersburg, Russia). Each sample was analyzed in duplicate. Using the SYBR Green technique we analyzed the melting curves. The relative level of FGF2 mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method [19].

Statistical analysis was performed using Statistica 5.0. and SPSS 16.0. software. The distribution normality was evaluated using the Lilliefors test. The distributions of some behavioral and real time PCR data did not correspond to the normal distribution, therefore, we applied non-parametrical statistical methods, such as the Kruskal–Wallis *H*-test followed by the Mann–Whitney paired group comparison with the Bonferroni correction for multiple comparisons. The differences were considered as statistically significant at $p \leq 0.05$. The data in the tables are presented as the median and quartiles.

RESULTS

LPS administration did not significantly affect the general development of the rat pups. Body weight gain was similar after the drug administration; specifically, on day 23, the body weights in the control group, which were injected with saline, and in the experimental group were 39.0 ± 2.3 and 38.6 ± 2.4 g, respectively. More than half of the intact, control, and experimental rats exhibited eye opening by day 16 and all animals had opened their eyes by day 18. Administration of the drugs did not influence the time of eye opening.

The open-field test was performed on day 22; we observed substantial alterations in hole-exploratory behavior in the rats that were subjected to administration of bacterial LPS during the third postnatal week,



The scheme of brain-structure dissection according to Paxinos and Watson [18]. The dissected areas are in gray.

which indicates impaired exploratory behavior. Using the Kruskal–Wallis *H*-test, we revealed significant differences in both the total time of hole exploration and single hole exploratory episodes (Table 2). Paired comparison of group data using the Mann–Whitney *U*-test demonstrated a significant decrease in these indices in the experimental animals compared to both intact and control rats injected with physiological saline (for the total time of hole exploration $U = 24$, $p = 0.001$ and $U = 38$, $p = 0.006$, respectively, and for single hole exploratory episode $U = 47$, $p = 0.011$ and $U = 54$, $p = 0.015$, respectively). A higher level of the fussy behavior of the rats that were injected with LPS resulted in an increased duration of static movements (Table 2). A few animals exhibited rearings and freezing; therefore, we do not present these data. We did not observe any significant differences between the behavioral indices in the control and intact rats.

The contents of FGF2 mRNA tended to decline in all brain structures that were studied in the experimental rats although a significant decrease was only observed in the medial prefrontal cortex ($H = 6.14$; $p = 0.046$ according to the Kruskal–Wallis *H*-test) (Table 3). A paired comparison revealed a significant decrease in the FGF2 mRNA level in the experimental animals compared to the intact rats ($U = 0$, $p = 0.009$ according to the Mann–Whitney *U*-test with Bonferroni correction) but not the control animals

Table 2. The behavioral indices of the experimental and control animals in the open-field test

Patterns	Indices	Groups of animals			Kruskal–Wallis test	
		intact ($n = 14$)	control, saline ($n = 15$)	experimental, LPS, 25 μg/kg ($n = 15$)	$H =$	$p =$
Hole exploration	Number of episodes	9.0 (7.0; 13.3)	7.0 (7.0; 9.0)	8.0 (5.0; 10.0)	2.8	0.245
	Total duration, s	12.4 (8.7; 3.2)	9.1 (8.0; 14.5)	5.7 (3.7; 8.3)*#	**13.1**	**0.001**
	Mean duration of hole exploration, s	1.5 (0.9; 2.2)	1.2 (0.9;1.8)	0.8 (0.6; 1.1)*#	**8.4**	**0.015**
Climbing	Number of episodes	3.0 (1.0; 4.5)	1.0 (1.0;2.0)	1.0 (0.0; 5.0)	1.3	0.533
	Total duration, s	1.8 (0.5; 2.8)	1.1 (0.6; 1.8)	0.9 (0.0; 2.4)	1.0	0.604
Grooming	Number of episodes	4.0 (2.0; 5.0)	3.0 (3.0;4.0)	3.0 (2.0; 6.0)	0.8	0.684
	Total duration, s	15.3 (9.6; 23.2)	11.6 (8.7; 33.3)	15.1 (5.9; 23.8)	0.2	0.896
Locomotion	Total duration, s	12.0 (7.0; 25.1)	17.6 (9.3; 26.7)	15.9 (11.7; 23.4)	1.2	0.556
Static movements	Total duration, s	17.6 (15.3; 20.0)	17.0 (15.0; 19.7)	22.0 (18.3; 24.8)*#	**8.3**	**0.016**
Sniffing	Total duration, s	110.8 (100.6; 115.7)	112.5 (106.1; 118.3)	116.1 (106.1; 132.2)	1.7	0.422

Significant differences according to the Kruskal–Wallis H-test are in bold. * Significant differences compared to intact rats; # significant differences compared to control rats injected with apyrogenic physiological saline solution, according to Mann–Whitney U-test with the Bonferroni correction; n, the number of animals in the groups.

Table 3. The relative levels of FGF2 mRNA in the brain structures

Brain structure	Groups of animals			Kruskal–Wallis test	
	intact ($n = 5$)	control, saline ($n = 7$)	experimental, LPS, 25 μg/kg ($n = 6$)	$H =$	$p =$
Dorsal hippocampus	1.78 (0.24; 12.92)	1.92 (0.01; 6.71)	0.14 (0.01; 1.29)	2.37	0.305
Ventral hippocampus	0.85 (0.73; 1.71)	0.83 (0.02; 1.79)	0.01 (0.01; 1.109)	2.04	0.362
Medial prefrontal cortex	1.22 (0.24; 5.77)	0.04 (0.02; 3.17)	0.03 (0.01; 0.06)*	**6.14**	**0.046**
Basolateral nucleus of amygdala	1.85 (0.18; 8.96)	0.17 (0.00; 1.41)	0.02 (0.00; 0.15)	5.12	0.077

Significant differences according to the Kruskal–Wallis H-test are in bold. * Significant differences compared to intact rats, according to the Mann–Whitney U-test with the Bonferroni correction; n, the number of animals in the groups.

that were injected with saline ($U = 8$, $p = 0.347$). We did not observe any differences between the control and intact groups ($U = 7$, $p = 0.251$).

Although the differences in the contents of FGF2 mRNA in the amygdala of the intact and experimental rats were close to significant ($U = 2$, $p = 0.028$), we could not consider them as significant when taking the Bonferroni correction into account. We did not find any differences in the expression of the *Fgf2* gene in the dorsal or ventral hippocampus of the experimental, control, and intact rats.

DISCUSSION

Our studies demonstrated impaired exploratory behavior and *Fgf2* gene expression in cells of the medial prefrontal cortex of rats that were treated with bacterial LPS during the third postnatal week.

The decreased mRNA content does not allow us to conclude that a similar decrease will be observed for the protein; however, a strong correlation between the levels of protein and mRNA of FGF2, which was demonstrated by Zhao et al. [20] and several other authors in the cells of the rat brain under various experimental conditions, allows us to hypothesize that the alterations found in the present study may be functionally important.

Rico et al. [21] reported the changes in the open-field behavior of 40–46-day-old adolescent rats that were injected with LPS on postnatal days 3 and 5. In contrast to our data, these authors demonstrated an higher exploratory activity, including hole exploration. This contradiction may be due to the different time points that were chosen for LPS injections and the time delay between the injections and behavioral testing.

In the present study, we observed significant changes in FGF2 mRNA production in the medial prefrontal cortex. It has been shown previously that an FGF2 level in this brain region influences anxiety in a novel environment and that an increased FGF2 content decreases the fear of a new space [22]. It is possible to assume that the lower level of FGF2 results in neophobia and, thus, inhibition of exploratory behavior. However, we did not observe any substantial increase in the intensity of grooming, freezing, and rearing in the experimental animals, as well as inhibition of locomotor activity, which taken together might indicate increased anxiety.

We revealed a significant decrease in the FGF2 mRNA level in the experimental animals compared to the intact but not the control rats. In the animals that were injected with saline on the third postnatal week the expression of the *Fgf2* gene in cells of the medial prefrontal cortex was also lower than in the intact rats, although this decrease was insignificant. These data suggest the involvement of stress hormones in the impaired FGF2 production. In rats, the third week of life is known to be one of the "critical" periods of early postnatal development that are related to enhanced vulnerability of the developing brain to stress [23], even including negligible experimental manipulations. In its turn, LPS administration substantially increases the activity of the hypothalamus–pituitary–adrenal axis [24 and other] and thus, the effect of stress becomes significant. This hypothesis is supported by the data of Fumagalli et al. [25] who reported the decreased FGF2 mRNA level in the prefrontal cortex of rats subjected to prenatal stress.

One more cause of the inhibition of FGF2 synthesis and impaired exploratory behavior in the experimental rats is probably the modification of the functional activity of the dopaminergic system of the medial prefrontal cortex. First, the involvement of this system in the brain response to stimulus novelty has been repeatedly reported [26, 27]. Secondly, administration of LPS to pregnant females and several-day-old rat pups results in a decreased number of tyrosine hydroxylase-positive cells and lower dopamine content in the frontal cortex [28]. Finally, it is evident that dopamine stimulates FGF2 production [29, 30].

Impairments of the formation of the neocortex and other integrative brain regions may be a result of decreased production of FGF2 in the early age. Specifically, impairments of the normal architectonics of the cerebral cortex and the development of cortical pyramidal neurons have been revealed in mice that are deficient in the *Fgf2* gene [31]. In adult FGF2-deficient mice, the processes of hippocampal neurogenesis are impaired [32].

The opportunity for the correction of a decreased FGF2 level using the administration of exogenous protein is very limited because of its low permeability via the blood–brain barrier [33] and the probable stimulatory effect on the development of some forms of brain cancer [34]. Therefore, the finding of natural stimulators of endogenous FGF2 production with no side effects is very significant. In this context, an interesting study by Seo et al. [35] demonstrated that the maintenance of rat pups in an enriched environment recovered FGF2 production in the frontal cortex and cerebellum, where it was impaired due to chronic hypoxia. Taking the fact into account that the damaging effects of hypoxia and LPS on brain cells are based on similar pathogenetic mechanisms, such as increased production of proinflammatory cytokines, we hypothesized that an enhanced cognitive load in early ontogenesis, including raising under the developmental conditions, may compensate to some extent for the negative effects of neonatal infections on FGF2 production in the brain. Studies on this issue seem promising.

CONCLUSIONS

Our study demonstrated that the course of treatment with bacterial lipopolysaccharide during the third week of postnatal ontogeny resulted in impaired exploratory behavior and lower production of FGF2 mRNA in cells of the medial prefrontal cortex in 22–23-day-old rats.

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