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Dipeptidyl Peptidase 4 Inhibitors Diprotin A and Sitagliptin Administered on Weeks 2-3 of Postnatal Development Modulate Monoamine Metabolism in the Striatum of Adult Rats

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The levels of monoamines and their metabolites in brain structures of adult (3-month-old) rats with emotional and motivational disorders induced by inhibitors of dipeptidyl peptidase 4 (DPP-4; EC 3.4.14.5) diprotin A and sitagliptin on weeks 2-3 of postnatal development (postnatal days 5-18) were studied by HPLC with electrochemical detection. A significant decrease in the level of serotonin metabolite, 5-hydroxyindoleacetic acid, and a pronounced tendency towards reduced serotonin level were detected in the striatum of rats in both study groups. In adult rats treated with diprotin A during the neonatal period, a tendency towards activation of dopamine metabolism was observed (judging from DOPAC/DA ratio). The levels of monoamines and their metabolites in the frontal cortex, hypothalamus, and amygdala remained unchanged. The findings suggest that administration of DPP-4 inhibitors during the neonatal period induces long-term dysfunction of the serotonergic and dopaminergic systems of the brain.

Key Words: *dipeptidyl peptidase 4 inhibitors diprotin A and sitagliptin; emotional and motivational disorders; monoamines; rat brain structures*

Our previous studies have demonstrated that inhibitors of dipeptidyl peptidase 4 (DPP-4; EC 3.4.14.5) with different mechanisms of action, in particular, noncompetitive irreversible synthetic inhibitor methyonyl-2-(*S*)-cyanopyrrolidine and competitive selective inhibitors sitagliptin and tripeptide diprotin A administered systemically to rat pups on postnatal weeks 2-3 (postnatal days 5-18) induced the development of mixed emotional and motivational disorders that were fundamentally similar although differed by their intensity in juvenile (1-month-old) and adult rats. These disorders included depression-like behavior and signs of increased anxiety combined with increased aggressiveness that manifested after 2- or 3-day isolation of the animals [2,11].

According to current views, the development of emotional and motivational disorders of different genesis is related to dysfunction and disturbed interaction of the major neurotransmitter systems in the CNS, primarily the monoaminergic system [10,13,15]. The commonly observed combination of symptoms of depression, anxiety disorder, and increased aggression [8] suggests that the pathophysiological mechanisms of the development of these psychoemotional disorders share elements in the neural pathways whose function is determined, among other factors, by the status of the monoaminergic systems of the brain. Using the model of experimentally induced anxiety-depressive

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disorder in rats exposed to DPP-4 inhibitor methionyl-2-(*S*)-cyanopyrrolidine during weeks 2-3 of postnatal development, we detected changes in the central catecholaminergic (mostly dopaminergic) systems in the emotiogenic brain structures of adult male rats [4,6].

Here we analyzed the levels of monoamines and their metabolites in the frontal cortex, striatum, hypothalamus, and amygdala of adult male rats with emotional and motivational disorders induced by DPP-4 inhibitors sitagliptin and diprotin A administered on weeks 2-3 of postnatal development.

MATERIALS AND METHODS

Male Wistar rats from the vivarium of the Research Institute of General Pathology and Pathophysiology were used for the experiments. All the procedures and experiments with animals were conducted in compliance with the Guidelines for Good Laboratory Practice in the Russian Federation adopted by the Order No. 708n of the Ministry of Health of the Russian Federation (August 23, 2010) and approved by the Ethics Committee of the Research Institute of General Pathology and Pathophysiology. The animals were kept in groups under standard vivarium conditions under natural light with unrestricted access to food, except for the time when the animals were subjected to water drinking and sucrose preference tests.

The day of birth was considered as postnatal day (PD) 0. On the next day, the litters were regrouped so that each included 5 male pups. DPP-4 inhibitors, diprotin A (H-Ile-Pro-Ile-OH, MB 341.45, 2 mg/kg; Sigma-Aldrich) and sitagliptin (phosphate monohydrate, MB 523.32, 4 mg/kg; Sigma-Aldrich) were dissolved in normal saline and injected intraperitoneally in approximately equimolar doses (0.1 ml/10 g body weight) daily on PD 5-18. Control animals received normal saline according to the same scheme.

The pups were separated from their mothers at the age of 1 month and the study groups consisting of the pups from 3-4 litters were formed. Behavior of rats in anxiety, depression, and aggression tests was assessed in dynamics (1-3 months of life). The behavioral tests and results of assessment were described previously [11].

At the age of 3 months (body weight 340-400 g), some animals exposed to neonatal treatment with diprotin A (n=8), sitagliptin (n=7), or saline (n=8) were decapitated; the frontal cortex, striatum, hypothalamus, and amygdala were separated on ice under visual control. Tissue samples were divided into two portions and used for analysis of gene expression (data not shown) and quantitative assay of monoamines and their metabolites. Immediately after isolation, the samples were placed into liquid nitrogen and stored in a Sanio MDF-193 deep-freeze refrigerator at -83°C for further analysis.

For neurotransmitter assay, the samples were homogenized in a Teflon-glass homogenizer in a 20-fold volume of 0.1 N $HClO_4$; 3,4-dioxybenzylamine (500 pmol/ml) was added as an internal standard. The samples were centrifuged at 10,000*g* for 10 min. The content of monoamines and their metabolites were determined by HPLC/ED on an LC-304T chromatograph (BAS) with a ReptroSil-Pur ODS analytical column (C18, 100×4 mm, 3 μ; Dr Maisch). Mobile phase consisted of 0.1 M citrate-phosphate buffer with 1.85 mM sodium octyl sulfonate, 0.27 mM EDTA, and 9% acetonitrile (pH 3.0); elution rate 1 ml/min. The measurements were performed on an LC-4B electrochemical detector (BAS) at 850 mV using glass-carbon electrode and Ag/AgCl reference electrode. A solution containing dioxybenzylamine (internal standard), norepinephrine (NE), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 3-methoxytyramine (3-MT), 5-hydroxytryptamine (5-HT, serotonin), and 5-hydroxyindolacetic acid (5-HIAA) in concentration of 50 pmol/ml were used to measure the monoamine concentrations in the rat brain structures. The samples were analyzed using a Multichrome 1.5 software and hardware complex (Ampersend). All the reagents used for analysis were of high purity grade (special purity and reagent grades). Monoamine concentrations in the study samples were calculated using the internal standard method on the basis of the ratio between peak areas in the standard mixture and in the sample and expressed in nmol/g of tissue.

The results were statistically processed using the algorithms of the Statistica 6.0 software. Taking into account that preliminary verification using the Kolmogorov—Smirnov test did not reject the agreement between the distribution of the empirical data and the normal probability law, we used parametric one-way ANOVA followed by post hoc analysis using the LSD test. The data are presented as $M \pm SEM$. The assumed significance level was 5%.

RESULTS

The characteristics of emotional and motivational disorders in rats treated to diprotin A and sitagliptin on PD 5-18 are summarized in Table 1 (the results were presented in more detail in [11]). In rats treated with diprotin A during the neonatal period, depression-like behavior at the age of 1 and 2 months was revealed in three tests and at the age of 3 months in two tests. Increased anxiety was revealed in two tests in adolescent rats, while increased aggressiveness was observed throughout 3-month experiment. In rats treated with sitagliptin during the neonatal period, the symptoms

of depression-like behavior and increased anxiety were revealed only in one test used to assess these disorders, and only at adolescent age. Only adult 2-month-old rats exhibited increased aggressiveness. Hence, emotional and motivational disorders caused by exposure to diprotin A during the neonatal period were more severe at the age of 3 months (when the levels of monoamines and their metabolites in the rat brain structures were analyzed) and persisted as the increased levels of depression and aggressiveness. No motor disorders were observed in study groups during the entire investigation.

Changes in monoamine metabolism were revealed only in the striatum (Table 2). The rats treated with diprotin A and sitagliptin had significantly reduced level of serotonin metabolite 5-HIAA: $F_{(2,18)}$=4.280, p=0.030. Moreover, a tendency towards reduced 5-HT level ($F_{(2,18)}$=3.080, p=0.071) was revealed in both study groups. In animals treated with sitagliptin, 5-HIAA/5-HT ratio tended to decrease ($F_{(2,18)}$=2.926, p=0.079), while in animals treated with diprotin A, DOPAC/DA ratio tended to increase ($F_{(2,16)}$=2.976, p=0.080).

The findings suggest that during the postnatal age, DPP-4 inhibitors, either directly or indirectly, disturb the formation of 5-HT-ergic and, apparently, DA-ergic brain systems involved in the development of mixed anxiety-depressive disorder accompanied by increased aggressiveness leading to the development of emotional and motivational disorders in animals. The results of our study are consistent with the current views on the role of the brain monoaminergic systems in the development of emotional and motivational disorders [10] and confirm the adequacy of the developed models at the neurochemical level.

The results of the study draw attention to the role played by the striatal structures in the genesis and maintenance of emotional and motivational disorders. The hypothesis that hyperactivation of the striatal structures plays a special role in the formation of pathological systems mediating the development of emotional and behavioral disorders has been earlier formulated in our studies from the results of neurophysiological and neurochemical experiments on other models of emotional and motivational disorders [1,5,6]. We suppose that the neuroplastic changes in the striatum induced by aversive exposure during the early postnatal period can determine the special role played by this structure as a pathological determinant that organizes the formation of a pathological system in the CNS manifesting as emotional and motivational disorders at the behavioral level. Striatal changes can maintain the existence of this pathological system during various periods of its activity, when there are no clinical signs of the disorders. This assumption is confirmed by the data on reduced 5-HT metabolism in the striatum in both rats with depression-like behavior and increased aggressiveness that persisted at the age of 3 months (the group treated with diprotin A) and animals without signs of behavioral disorders detected (the group treated with sitagliptin). The assumption about the special role played by disturbed striatal functions in the development of emotional and motivational disorders, mostly the depressive states, and in the main-

TABLE 1. Emotional and Motivational Disorders in Rats Aged 1-3 Months Treated with DPP-4 Inhibitors Diprotin A (D) and Sitagliptin (S) on PD 5-18

Parameter (tests used)	Age					
	1 month		2 months		3 months	
	D	S	D	S	D	S
Motor activity (open field and elevated plus-maze tests)	= =	= =	= =	= =	= =	= =
Depression-like behavior (forced swim, preference of sucrose, and zoosocial interaction tests)	+ + +	= + =	+ + +	= = =	+ = +	= = =
Anxiety-like behavior (elevated plus-maze test, method for integrated assessment rat anxiety-phobic status)	+ +	+ =	= =	= =	= =	= =
Aggression (zoosocial interactions)	+	=	+	+	+	=

Note. "+", an increase in anxiety/depression/aggression level; ""=", no significant differences compared to the control (administration of normal saline) was revealed. Signs of depression-like behavior: increased immobility duration ("behavioral despair") and reduced duration of active swimming in the forced swim test, reduction of sucrose preference (hedonic disorders), the reduced level of drinking motivation (reduction of one of the essential motivations), and reduction of the number and duration of nonaggressive social contacts in the zoosocial interaction test. Signs of anxiety-like behavior include reduced preference of open arms and an increased number of entries into closed arms in the elevated plus-maze test, an increased latency time of hanging from the open arms; and an increased anxiety score according to assessment of the anxiety-phobic level in rats based on the ranged scale. Signs of increased aggression in the zoosocial interaction test (after isolation for two days) include the increased number and duration of aggressive social contacts.

TABLE 2. Levels of Monoamines and Their Metabolites (nmol/g tissue) in Brain Structures of the Rats That Received DPP-4 Inhibitors Diprotin A and Sitagliptin or Normal Saline Solution on Weeks 2-3 of Postnatal Development (PD 5-18)

Groups	NE	DA	DOPAC	3-MT	HVA	DOPAC/DA	HVA/DA	5-HT	5-HIAA	5-HIAA/5-HT
Frontal cortex										
Diprotin A	1.20±0.13	0.72±0.06	0.42±0.04	—	0.24±0.05	0.64±0.10	0.32±0.05	3.00±0.46	1.96±0.35	0.80±0.27
Sitagliptin	1.35±0.26	0.82±0.09	0.40±0.06	—	0.39±0.06	0.50±0.07	0.49±0.07	3.10±0.27	1.72±0.15	0.57±0.05
Control (normal saline)	1.55±0.32	0.96±0.20	0.49±0.17	—	0.46±0.16	0.47±0.08	0.44±0.05	3.87±0.66	1.92±0.26	0.53±0.05
Striatum										
Diprotin A	0.62±0.21	51.55±3.36	4.95±0.24	0.46±0.03	2.69±0.12	0.10±0.00^{+}	0.05±0.00	2.47±0.11^{+}	2.10±0.16*	0.84±0.04
Sitagliptin	0.32±0.03	52.28±1.78	4.66±0.25	0.51±0.05	2.55±0.12	0.09±0.00	0.05±0.00	2.55±0.13^{+}	2.04±0.19*	0.79±0.05^{+}
Control (normal saline)	0.53±0.11	51.57±3.33	4.30±0.39	0.62±0.13	2.98±0.23	0.08±0.00	0.06±0.01	2.95±0.19	2.75±0.22	0.93±0.04
Hypothalamus										
Diprotin A	6.49±0.69	2.24±0.31	0.62±0.11	0.52±0.17	0.23±0.11	0.28±0.04	0.10±0.04	4.45±0.28	3.00±0.13	0.68±0.03
Sitagliptin	6.18±0.54	2.39±0.34	0.54±0.10	0.31±0.04	0.16±0.05	0.22±0.01	0.07±0.02	4.64±0.28	3.12±0.14	0.68±0.04
Control (normal saline)	7.14±0.50	2.41±0.20	0.61±0.09	0.41±0.05	0.14±0.05	0.25±0.03	0.06±0.02	4.88±0.17	3.06±0.12	0.63±0.03
Amygdala										
Diprotin A	1.79±0.08	1.83±0.23	0.31±0.04	—	0.30±0.06	0.18±0.03	0.19±0.06	3.24±0.19	1.90±0.08	0.60±0.04
Sitagliptin	1.62±0.13	1.65±0.16	0.33±0.06	—	0.43±0.08	0.20±0.03	0.29±0.07	3.22±0.30	1.61±0.21	0.49±0.05
Control (normal saline)	1.78±0.08	1.56±0.14	0.37±0.07	—	0.40±0.05	0.23±0.03	0.27±0.04	3.07±0.16	1.77±0.13	0.57±0.03

Note. $^{*}p<0.05$, $^{+}p<0.07$ in comparison with the control (LSD post hoc analysis after one-way ANOVA). "—" — the substance cannot be detected by this method.

tenance of predisposition for them agrees well with the currently developed views that striatal abnormalities play a role in emotional disorders associated with the major depressive disorder [9].

Abnormal content and disturbed metabolism of 5-HT in the striatum can result from reduced activity of tryptophan hydroxylase-2, the key serotonin biosynthesis enzyme in the brain [12]. However, changes in activity of monoamine oxidase (MAO) catalyzing cleavage of 5-HT to 5-HIAA, which is linked to the depressive symptoms or predisposition to it, should not be ruled out [14]. Signs of increased DA metabolism were also observed in the striatum of the rats exposed to diprotin A in the neonatal age, which can be related to persistent aggressiveness in the animals [7]. The tendency towards enhanced DA conversion into to DOPAC catalyzed by MAO in the absence of differences in the content of DA and its metabolites, 3-MT and HVA, from the control values allows one to expect changes in the rate of biosynthesis of the newly synthesized DA and impaired (increased?) activity of MAO, but not catechol-O-methyltransferase (COMT) involved in the generation of 3-MT from DA. We have earlier detected reduced DA metabolism compared to the HVA/DA ratio in the striatum of 3-month-old rats in the model of the anxiety-depressive state induced by administration of a synthetic DPP-4 inhibitor at week 2-3 of postnatal development; the reduced metabolism persisted in 7-month-old animals [4], thus giving grounds for suggesting that COMT activity has changed. It should be mentioned that an increased DOPAC/DA ratio was also observed in the striatum of 7-month-old animals. In this study, we detected no reduction in metabolism of DA *vs*. HVA/DA but noticed an increased metabolism of DA with respect to DOPAC/DA in 3-month-old rats. Comparison of the data obtained using the models of emotional and motivational disorders induced by the effect of DPP-4 inhibitors with different mechanisms of action during the neonatal period (the chronic mild stress model [3] and the model of MPTP-induced depressive syndrome [1])

indicates, on the one hand, that DA metabolism in the striatum is qualitatively different in these models, while revealing the general regularity: involvement of the nigrostriatal DA-ergic system in the development of mixed anxiety-depressive disorders.

Taking into account the fact that changes in the monoamine metabolism in brain were revealed in adult rats with emotional and motivational disorders induced by administration of DPP-4 inhibitors during weeks 2-3 of postnatal development and the fact that these disorders are associated with increased activity of proline-specific peptides in the CNS [4], it is fair to expect that the genes of proline-specific peptidases, primarily DPP-4, are involved in regulation of activity of monoaminergic systems (probably by influencing the expression of the genes of tryptophan hydroxylase-2 and MAO). Testing this hypothesis is subject to further research.

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