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Evidence for the Involvement of Central Serotonin in Mechanism of Domestication of Silver Foxes

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POPOVA, N. K., N. N. VOITENKO, A. V. KULIKOV AND D. F. AVGUSTINOVICH. *Evidence for the involvement of central serotonin in mechanism of domestication of silver foxes.* PHARMACOL BIOCHEM BEHAV **40**(4) 751–756, 1991.—Silver foxes selected for more than 30 years for tame behavior and displaying no defensive reaction to human contact were shown to have a higher serotonin level in midbrain and hypothalamus, and a higher 5-hydroxyindole acetic acid (5-HIAA) content in midbrain, hypothalamus and hippocampus in comparison to nonselected wild silver foxes bred in captivity over the same time span. Tryptophan hydroxylase (TPH) activity in midbrain and hypothalamus in domesticated foxes was increased as compared with their aggressive/defensive counterparts. Monoamine oxidase type A (MAO A) activity was decreased with an increased K_m and unchanged V_{max} in domesticated foxes. No changes in specific [^{3}H]ketanserin or [^{3}H]8-OH-DPAT binding in frontal cortex was revealed. A reduced density (B_{max}) of $5HT_{1A}$ receptors in hypothalamic membranes in domesticated foxes was shown. It is suggested that the brain serotonergic system is involved in the mechanism of domestication converting wild aggressive/defensive animals into tame ones.

Defensive behavior Domestication Tryptophan hydroxylase Monoamine oxidase
$5\text{-}HT_{1A}$ serotonergic receptors

ONE of the main differences of domesticated animals from their wild ancestors is very low, or even the lack of, fear-induced defensive response to man. The mechanism of these drastic inherited changes in behavior of domesticated animals presents a dilemma. However, this investigation was complicated by the absence of a meaningful control, since domesticated animals have diverged from their wild ancestors after thousands years of domestication.

It was hypothesized by Belyaev (2) that at the earliest stages of animal domestication a selection was unconsciously carried out by man for nonaggressive behavior of captured wild animals, since a high defensive response made their coexistence with man impossible. To test this hypothesis, an experiment was started more than 30 years ago at the Institute of Cytology and Genetics, Novosibirsk, on the selection of silver foxes for their nonaggressive behavior towards man. As a result of this long-term selection, a population of silver foxes quite different in their behavior from foxes of initial unselected farm population was obtained. The foxes selected for tame behavior are not afraid of people and resemble domestic dogs in behavior characteristics (2,31).

Animals selected for nonaggressive behavior represent a promising model for study of neural mechanisms of defensive reactions. The neurotransmitter serotonin (5-HT) attracts special attention due to the data indicating the involvement of brain serotonergic system in the regulation of some types of defensive response (4,24). Significant changes in 5-HT level, tryptophan hydroxylase (TPH) activity and serotonergic $5\text{-}HT_2$-receptors were found in Norway rats selected for reduced aggressiveness towards man (23). In our previous experiments it was shown that 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA) levels in some brain regions of domesticated silver foxes were increased (27).

To elucidate in more detail the changes in brain serotonergic system, the activity of the key enzyme in 5-HT biosynthesis (TPH), the main enzyme of 5-HT degradation monoamine oxidase type A (MAO A) and serotonergic $5\text{-}HT_{1A}$ and $5\text{-}HT_2$ receptors in the brain of silver foxes bred for the lack of defensive behavior with respect to man were studied.

METHOD

Experiments were performed on males of silver foxes (*Vulpus fulvus*, D.) at the age of one year weighing 4.5–5 kg. Animals were kept on the experimental farm of the Institute of Cytology and Genetics, Novosibirsk. Two groups of silver foxes were compared: silver foxes selected for about 20 generations for tame behavior towards man ($n=34$) and unselected silver foxes from a farm population which preserved behavior characteristics of wild animals and clearly manifested defensive response to man ($n=29$). In the TPH study only, a third group selected for the high levels of defensive attack was also used ($n=10$).

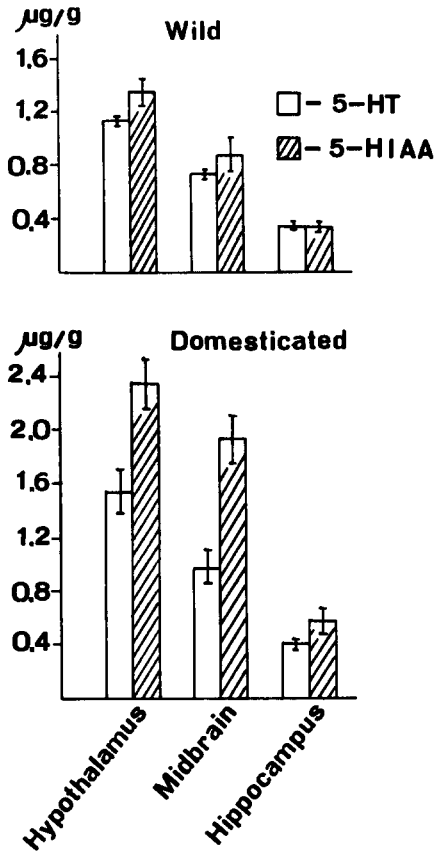

FIG. 1. Concentrations of 5-HT and 5-HIAA in the brain regions of domesticated and nonselected (wild) silver foxes. The difference between domesticated and wild animals in 5-HT: in hypothalamus, $t(10)=2.40$, $p<0.05$, in midbrain, $t(11)=2.46$, $p<0.05$, in hippocampus, $t(11)=1.4$, $p>0.05$. The difference between domesticated and wild foxes in 5-HIAA: in hypothalamus, $t(9)=6.68$, $p<0.001$, in midbrain, $t(11)=6.40$, $p<0.001$, in hippocampus, $t(11)=2.27$, $p=0.05$.

Defensive attack occurred in situations involving fear and was elicited by the approach of a person. The intensity of an animal's reaction to the attempt to touch it and to give food was evaluated by scores and described elsewhere (31). All subjects from the population selected for the lack of defensive aggression to man were characterized by absence of any defensive reactions to a person's approach. They could be handled without special precautions against bites. Moreover, they displayed obvious enjoyment of human contact.

The control animals had been bred on the farm without any special selection and displayed clear defensive reaction, such as attack, although only about 30% of them were extremely aggressive towards man (3). In one series of experiments, silver foxes selected for 8 generations for high defensive reaction to man were used. These animals were extremely aggressive and received the highest score in aggressiveness.

Food and water were given ad lib. All experiments were performed at 10.00–12.00 in November when silver foxes were terminated for commercial uses.

Biochemical Procedures

After a quick decapitation, the brains were immediately removed in the cold. The hypothalamus, hippocampus and midbrain for 5-HT and 5-HIAA determination or brainstem for MAO A activity assays were rapidly excised, weighed, placed into test-tubes and stored at −20°C until use. To determine the activity of TPH, hypothalamus and midbrain were isolated, immediately frozen with liquid nitrogen and stored at −190° until use.

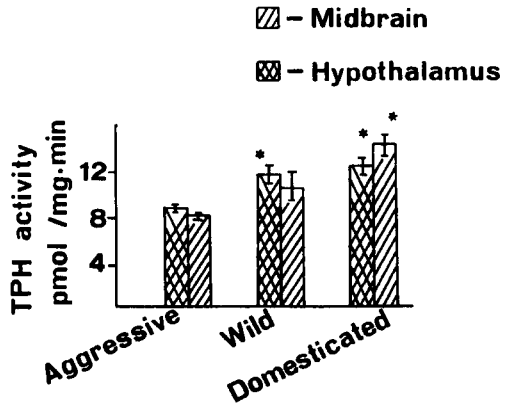

FIG. 2. The activity of TPH in the brain of domesticated, nonselected (wild) and selected for high defensive aggressiveness (aggressive) silver foxes. The difference in TPH activity between selected for high levels of defensive attack (aggressive) and domesticated animals: in hypothalamus, $t(11)=3.6$, $p<0.01$, in midbrain, $t(9)=4.4$, $p<0.01$. The difference in TPH activity in hypothalamus between aggressive and nonselected (wild) animals: $t(10)=2.5$, $p<0.05$.

TPH activity was determined by fluorometric assay (15). The samples were homogenized with 5 volumes of 50 mM tris acetate (pH 7.5) containing 1 mM dithiothreitol. The homogenate was centrifuged at 18,000 × g for 30 min at 4°C. The supernatant (0.5–2 mg of protein) was incubated for 15 min at 37°C with 0.8 mM l-tryptophan, 0.5 mM 6,7-dimethyl-5,6,7,8-tetrahydropteridin and 10 μg catalase in 200 μl of 0.05 M Tris-acetate buffer containing 1 mM dithiothreitol. The reaction was stopped by placing the samples into boiling water for 3 min. After cooling 15 μg pyridoxal-5-phosphate and 5 U decarboxylase (50 μl) were added, and the samples were incubated for another 60 min at 37°C for complete conversion of 5-hydroxytryptophan (5-HTP) into 5-HT. The reaction of decarboxylation was stopped with 20 μl 50% three-chloroacetic acid and samples were centrifuged to remove protein. The supernatant was neutralized with K_2CO_3 and 5-HT was extracted with benzene/butanol (1:1), then reextracted from organic phase with 0.1% l-cystein in 0.1 N HCl and after condensation with o-phthalaldehyde the amount of 5-HT corresponding to the amount of 5-HTP was determined fluorometrically on an spectrofluorometer Hitachi MF-4. The protein was determined by Lowry. The standard and blank tubes containing 0.5 nmoles 5-HTP and buffer instead of supernatant were carried through the entire procedure. TPH activity was expressed in pmoles of 5-HTP formed per mg of protein/min.

To determine type A MAO activity brainstem was homogenized with 9 volumes of ice-cold 0.32 sucrose and a crude mitochondrial fraction (P_2) was prepared (13). The activity of type A MAO was assayed (12) using 1.0 mM 5-HT as a substrate and was expressed in nmol NH_3/ mg protein min. The kinetic of 5-HT deamination by MAO A has been calculated by Cornish-Bowden (6) using concentrations of 0.0625, 0.125, 0.250, 0.5 and 1.0 mM of 5-HT.

5-HT and 5-HIAA levels were determined fluorometrically (28). Receptor binding assays were performed according to the

TABLE 1

KINETIC PROPERTIES OF TRYPTOPHAN HYDROXYLASE FROM MIDBRAINS OF ANIMALS SELECTED FOR TAME BEHAVIOR, FOR AGGRESSIVE REACTION TO MAN AND NONSELECTED SILVER FOXES

Groups of Animals	V_{max} (pmol/mg/min)	K_m (μM)
Tame	8.08 ± 0.40	10.0 ± 2.0*
Aggressive	9.73 ± 1.05	84.0 ± 24.0
Nonselected	11.81 ± 0.88	72.0 ± 21.0

Fifteen concentrations of l-tryptophan varied from 0.01 to 0.80 mM in the presence of 0.5 mM 6,7-dimethyl-5,6,7,8-tetrahydropteridine were used.

*$p<0.01$ vs. aggressive, $t(26)=3.1$, and nonselected, $t(26)=3.0$, animals.

method of Peroutka and Snyder (21) with minor modifications. Brain tissues were homogenized in 20–30 volumes of 50 mM Tris-HCl (pH 7.6) at 25° using glass homogenizers. For 15 min homogenates were put into snow to complete cell lysis and then centrifuged at 20,000 × g for 25 min. The supernatant was discarded and the pellets were resuspended in the same volume of buffer and centrifuged at 25,000 × g for 25 min. The final pellet was resuspended in Tris-HCl buffer only (for 5-HT_2 receptors) or in Tris-HCl buffer containing 10 μM pargyline, 4 mM $CaCl_2$, and 0.1% ascorbic acid (for 5-HT_{1A} receptors). Binding assays consisted of 0.05 ml solution of 0.125–4 nM [^{3}H]ketanserin or 0.0625–4 nM [^{3}H]8-OH-DPAT, 0.05 ml of buffer or displacing drug and 0.9 ml of tissue suspension. Following incubation at 37°C for 10 min (5-HT_{1A} receptor binding) or 15 min (5-HT_2 receptor binding) probes were rapidly filtered through Whatman CF/B filter with three washes with 50 mM Tris-HCl buffer. Filters were then placed in 4 ml of dioxan scintillator. Radioactivity was measured by a Delta-300 liquid scintillation counter at 40–50% efficiency. Specific binding was defined using 10 μM serotonin or 1 μM ritanserin. To evaluate B_{max} and K_D, binding studies were carried out at 9 different concentrations of the radioligands.

Drugs were obtained from the following sources: [^{3}H]8-OH-DPAT (183 Ci/mmol) from Amersham (UK), [^{3}H]ketanserin (64.1 Ci/mmol) from New England Nuclear (Germany), 5-HT creatinine sulphate fron Reanal (Hungary), ritanserin from Janssen Pharmaceutica (Belgium).

Statistics

The statistical evaluation of the results was made by the use of Student's *t*-test. In studies of receptor characteristics, linear regression analysis (6) was used to obtain all values of dissociation constant (K_D) and maximum numbers of binding sites (B_{max}).

TABLE 2

MAO A ACTIVITY AND KINETIC OR OXIDATIVE DEAMINATION OF 5-HT IN WILD AND DOMESTICATED SILVER FOXES

	Wild	Domesticated
MAO A activity[a]	2.86 ± 0.12	2.36 ± 0.12*
K_m (nmol/ml)	103.62 ± 16.92	226.82 ± 20.60†
V_{max} (nmol NH_3/min·mg protein)	3.45 ± 0.17	3.32 ± 0.18

*$p<0.05$; †$p<0.001$

[a]MAO A activity was evaluated in nmol NH_3/mg protein·min after incubation for 30 min at 37°C, in 0.1 M Na,K-phosphate buffer pH 7.4. The kinetic of 5-HT deamination by MAO A was determined using concentrations of 0.0625, 0.125, 0.25, 0.5 and 1.0 mM of 5-HT.

TABLE 3

CHARACTERISTICS OF [^{3}H]8-OH-DPAT SPECIFIC BINDING IN HYPOTHALAMUS AND FRONTAL CORTEX OF DOMESTICATED AND WILD SILVER FOXES

	Wild	Domesticated
Hypothalamus		
K_d (nM)	0.85 ± 0.12	0.94 ± 0.08
B_{max}[a]	99.50 ± 9.19	73.20 ± 4.24*
Frontal cortex		
K_d (nM)	1.17 ± 0.14	1.06 ± 0.18
B_{max}[a]	76.79 ± 6.54	75.93 ± 9.03

Nonspecific binding was determined in the presence of 10 μM nonlabelled serotonin. Specific binding was the difference between total and nonspecific bindings.

[a]Unit: fmol/mg protein. *$p<0.05$ vs. B_{max} in wild foxes.

The values represent mean ± S.E.M. Six determinations each were performed on wild and domestic animals.

RESULTS

5-HT and 5-HIAA levels in hypothalamus, and midbrain of domesticated silver foxes were significantly higher than in nonselected "wild" animals (Fig. 1). There were no considerable changes in 5-HT in hippocampus of silver foxes selected for the absence of defensive response to man, although 5-HIAA levels in hippocampus of tame animals were increased.

A significant difference in TPH activity was found between foxes selected for low defensive response to man and those selected for high aggressive reaction. TPH activity in midbrain was by 27 percent higher in nonaggressive foxes and by 34 percent lower in the aggressive population than in nonselected silver foxes. The difference between nonaggressive and highly aggressive animals was significant, $t(9)=4.4$, $p<0.01$, and was associated with a decreased K_m with unchanged V in tame animals (Table 1). An increase in TPH activity was also found in hypothalamus of domesticated foxes as compared with the aggressive animals, $t(11)=3.6$, $p<0.01$, Fig. 2.

Selection of silver foxes for tame behavior was followed by a moderate decrease in type A MAO activity in their brainstem, $t(8)=3.12$, $p<0.05$, and by an increase in K_m of the oxidative deamination of 5-HT, $t(8)=4.37$, $p<0.001$. At the same time, V was not different in domesticated and nondomesticated silver foxes, $t(8)=0.54$, $p<0.05$, Table 2.

Scatchard plots for specific binding of [^{3}H]ketanserin in frontal cortex (Fig. 4), as well as [^{3}H]8-OH-DPAT in frontal cortex and hypothalamus (Fig. 3) are linear, thus corresponding to the single-site model of receptor-ligand interactions. No differences in either B_{max} or K_D values of 8-OH-DPAT binding were noted in cortical membranes. However, significantly lower density (B_{max}) of 5-HT_{1A} receptors was found in hypothalamic membranes in domesticated silver foxes than that in their wild counterparts (Table 3). No significant changes in 5-HT_2 receptors in frontal cortex of domesticated foxes were observed (Fig. 4) and no differences between wild and domesticated animals in B_{max} or K_D values of [^{3}H]ketanserin binding were apparent (Table 4).

DISCUSSION

Long-term selection of silver foxes for reduced defensive re-

TABLE 4

CHARACTERISTICS OF [^{3}H]KETANSERIN SPECIFIC BINDING IN FRONTAL CORTEX OF DOMESTICATED AND WILD SILVER FOXES

Groups of Animals	[^{3}H]Ketanserin Specific Binding	
	K_d (nM)	B_{max} (fmol/mg protein)
Wild	2.17 ± 0.36	280.5 ± 33.9
Domesticated	2.82 ± 0.63	284.5 ± 48.7

Nonspecific binding was determined in the presence of 1 μM nonlabelled ritanserin. The values represent mean ± S.E.M. Six determinations each were performed on wild and on domestic animals.

sponse to man was associated with consistent changes in serotonergic brain system. It should be emphasized that such foxes are quite tame not as a result of training or taming but due to a prolonged breeding for nonaggressive genotype and hereditary reorganization of behavior (2). Thus the changes which involve 5-HT and its main metabolite 5-HIAA level, key enzymes of 5-HT metabolism, TPH and MAO A, and serotonergic 5-HT_{1A} receptors seem to have a hereditary basis.

It is relevant to note that these changes were specifically related to the decrease of defensive aggression of animals, since in silver foxes selected for high aggressive reaction to man TPH activity in midbrain and hypothalamus was decreased in contrast to animals selected for tame behavior. The decreased Michaelis constant (K_m) without notable changes in V found in domesticated silver foxes shows the increased affinity of TPH to substrate rather than increased synthesis of TPH de novo.

The increased 5-HT level in midbrain and hypothalamus of domesticated animals corresponds to a moderately increased TPH activity and decreased MAO A activity with decreased affinity of this enzyme to 5-HT. The combination of higher K_m with unchanged V values suggests changes in the efficiency of substrate-enzyme complex formation or the influence of an endogenous inhibitor. Since MAO A represents the principal enzyme in 5-HT degradation, it is somewhat puzzling that notwithstanding a decreased MAO A activity, the level of deaminated metabolite of 5-HT, 5-HIAA, was increased. The increase of 5-HIAA level was found also in hypothalamus of Norway rats selected for tame behavior (20). It suggests an increased 5-HT neuronal firing and, possibly, a slowed egress of 5-HIAA from the brain in domesticated animals.

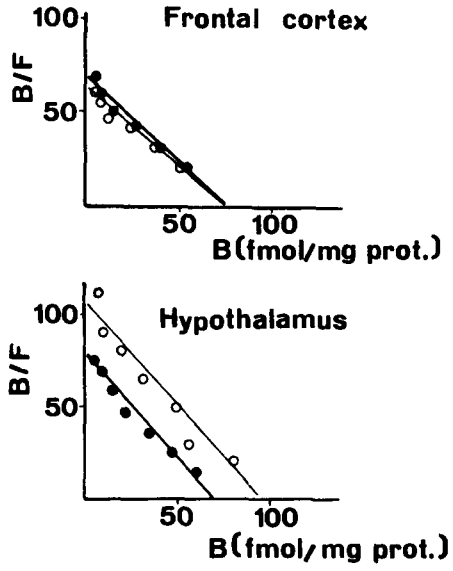

FIG. 3. Scatchard plots for specific [^{3}H]8-OH-DPAT binding in the frontal cortex and hypothalamus of domesticated (solid circles) and wild (open circles) silver foxes. Specific binding/free concentration (B/F) is on the vertical line, and specific binding (B) is on the horizontal line. Standard procedure used was described in the Method section.

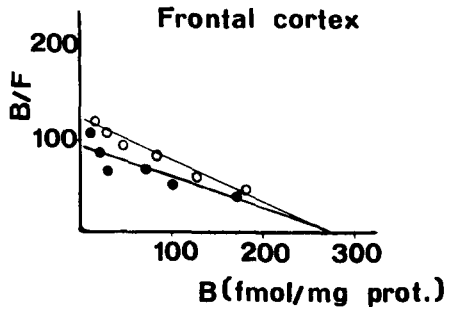

FIG. 4. Scatchard plots for specific [^{3}H]ketanserin binding in the frontal cortex of wild (open circles) and domesticated (solid circles) silver foxes. Specific binding/free concentration (B/F) is on the vertical line, and specific binding (B) is on the horizontal line.

The increased 5-HT level in midbrain and hypothalamus of domesticated foxes along with increased 5-HIAA level in midbrain, hypothalamus and hippocampus are in a good agreement with the numerous data implicating 5-HT as an inhibitory factor in the mechanism of fear-induced defensive aggression (4, 5, 9, 10, 24, 29).

It is essential to note that the changes in 5-HT brain system found in the silver foxes bred for low defensive behavior are not species specific. As it was shown earlier (23), the selection of Norway rats for tame behavior with respect to man was also accompanied by (a) the increase in TPH activity and 5-HT level in midbrain and hypothalamus; (b) the increase in 5-HIAA level in hypothalamus; and (c) the increase in 5-HT_2 receptor density in frontal cortex. These findings were interpreted as an indication to an increased functional activity of the brain serotonergic system.

Although there are some variations in the types of 5-HT receptors affected by selection in these different species of animals, the coincidence of the main patterns in 5-HT metabolism changes is remarkable. The consistent effect of selection for tame behavior on 5-HT metabolism observed in two diverse species provides further evidence supporting our idea (27) that the brain 5-HT system is involved in the crucial mechanism converting a wild aggressive animal into domestic one.

An increase in 5-HT and 5-HIAA levels along with the changes in TPH activity indicating an increased neuronal firing was found in the midbrain where 5-HT neuron perycarions are mainly located in median and dorsal raphe nuclei. In this respect, the character of changes in serotonergic system in animals selected for nonaggressive behavior is quite different from those found in rats selected for another pattern of defensive behavior—freezing. An increased TPH activity in striatum, i.e., the brain region involved in the control of muscular tonus, but not in midbrain was found in rats selected for 15 generations for predisposition to catalepsy [excessive freezing (14,22)]. A similar increase in TPH activity in striatum without any significant changes in midbrain was shown in mice with genetic predisposition to catalepsy (16). Thus selection for these two behaviors appears to involve different hereditary changes in brain 5-HT system. It is hypothesized that the involvement of midbrain will

lead to rather extensive changes in serotonergic innervation of different brain regions and thus affect the regulation of 5-HT-dependent physiologic systems in domesticated animals. Taking into consideration the variety of physiologic systems under control of brain 5-HT, it appears (26) that the activity of brain 5-HT systems increased during selection for tame behavior could also be the cause of the so-called correlative effects of selection. Such correlative effects of domestication are seen in some patterns peculiar to domestic animals, e.g., polyestrous and decreased stress response (2). Selection of the silver foxes for tame behavior resulted in a changes in pituitary-adrenocortical system as well as gonadal hormone regulation (18). At the same time, it is well known that 5-HT plays an important role in the regulation of the pituitary-adrenal system (17,25) and pituitary-gonadal system (19). A hypothesis implicating the changes in 5-HT brain system occurring in the process of domestication in the mechanism of some correlative traits seems to be corroborated by the positive intergroup correlations found between the stress-induced elevation of plasma corticosteroids and the increase of 5-HIAA in hypothalamus of wild and domesticated silver foxes (26).

The effect on some 5-HT-dependent physiologic functions can be elicited not only by increased 5-HT metabolism but also by changed density of 5-HT receptors. There were no significant differences between domesticated and wild foxes in either receptor density or ligand affinity at 5-HT_2 and 5-HT_{1A} binding sites in frontal cortex. At the same time, a significantly lower density of 5-HT_{1A} receptor binding sites was observed in hypothalamus of domesticated animals. This finding is of considerable interest in view of the data showing that 5-HT_{1A} receptor agonists exhibit an anxiolytic activity (8) and reduce defensive reactivity (4). There is also some evidence for the effect of the 5-HT_{1A} agonist 8-OH-DPAT on male sexual behavior (1,30) and stimulatory action on pituitary-adrenocortical system (7,11). Thus hypothalamic 5-HT_{1A} receptors may be involved not only in the taming of animals, but also in the changes of gonadal hormone regulation and of stress response found in domesticated silver foxes.

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