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Manuscript

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Investigation of trace amine-associated receptors as novel targets for the treatment of central nervous system disorders

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Contents

1. Introduction

The relevance of research

Trace amine associated receptors (TAARs) were discovered in 2001 [1,2]. Since then, more and more data on the localization of these receptors in the central nervous system and their functional role in the brain have been accumulated. The most studied receptor at the time of writing is TAAR1: the modulatory effect of TAAR1 on serotonin, dopamine neurons and glutamate neurotransmission in the cerebral cortex has been described in the literature [3,4]. For this reason, compounds with agonistic activity against TAAR1 are being actively studied as anti-addictive, antipsychotic, anxiolytic and antidepressant agents. Two TAAR1 agonists are in the late stages of clinical trials: ulotaront from Sunovion (USA) and ralmitaront from Hoffmann-La Roche (Switzerland). Ulotaront, if it successfully passes the third phase of clinical trials, may become the first antipsychotic drug in clinical practice since the fifties of the last century that does not directly affect D2-like and 5HT2A receptors [5]. The development of new TAAR1 agonists is one of the most promising directions in modern pharmacology. The functions of other receptors of the family remain poorly studied. Recently, data on the localization of the TAAR5 receptor in the central nervous system, as well as the presence of changes in behavior and physiology in TAAR5 gene knockout animals have appeared [6]. These data change current thinking, as all TAARs except subtype 1 receptors were previously thought to play a predominantly olfactory role [7–9]. The study of the physiological significance of activation of other TAAR subtypes in the CNS may become the same fundamental basis as the study of receptors to other monoamine receptors. This activity may result in new knowledge about CNS physiology, pathogenesis of various diseases, as well as theoretical justification of the use of TAARs as targets for drugs with new mechanisms of action, which is an urgent task of modern pharmacology.

The theoretical and practical significance of the work

The study identified two novel, active in vivo compounds, AP163 and LK00764, with agonism against TAAR1. Both compounds showed efficacy in animal models of schizophrenia and mania. LK00764 was also found to have potential anxiolytic properties. These studies may become the beginning of further development and introduction of these pharmacological agents into clinical practice and serve as early stages of preclinical development of antipsychotics and anxiolytics with a new mechanism of action. Also on the basis of the structure of the studied molecules it is possible to create other compounds by their chemical modification. The obtained data on the effect of TAAR2 gene knockout on the motor activity of animals will help in further identification of the functional role of this gene in rodent and human physiology. Identification of changes in behavior and response to

pharmacological drugs caused by TAAR6 gene mutation will be a starting point for further studies of the functions of this gene. Taken together, the presence of changes in behavior, neurochemistry, and response to neurotropic drugs in TAAR2 knockout and TAAR6 mutant animals seems to suggest additional roles for these genes in the central nervous system beyond the already known olfactory functions. The first theoretical justification for the use of TAAR2 and TAAR6 receptors as novel targets for neurotropic pharmacological agents is emerging.

Aims and objectives of the study

Aims of study:

1. To study the profile of pharmacological activity of TAAR1-agonists synthesized at the Institute of Chemistry of SPbU on various rat and mouse models.
2. To study the role of TAAR2 and TAAR6 genes in the regulation of behavior and physiology.

Objectives of study:

1. Evaluation of the effects of TAAR1 agonists AP161, AP162, AP163 and AP164 on the motor activity of rats knocked out by the dopamine transporter gene;
2. Evaluation of the effects of TAAR1 agonist LK00764 on hyperactivity in mice induced by administration of the dopamine transporter blocker GBR 12909
3. Evaluation of the ability of LK00764 to reduce stress-induced hyperthermia
4. Conduct a battery of behavioral tests following acute and subchronic administration of substance LK0074
5. Perform behavioral screening of TAAR2 gene knockout mice and TAAR6 gene mutant mice
6. Evaluate the effect of the dopamine transporter inhibitor GBR 12909 on motor activity of TAAR2 gene knockout mice
7. Study the hypothermic effect of 5HT1A-agonist 8-OH-DPAT on TAAR6 mutant mice
8. Conduct chromatographic analysis of brain structures of TAAR2 knockout mice and TAAR6 mutant mice

The scientific novelty of the study

The in vivo activity of a group of new TAAR1 agonists was investigated for the first time, and several active compounds, code names AP163 and LK00764, were identified. Both compounds were found to have potential antipsychotic and antimanic properties, while the drug LK0074 was also found to have potential anxiolytic properties. TAAR2 gene knockout animals and animals with a mutation in

the TAAR6 gene were characterized for the first time. The effect of TAAR2 gene knockout on the motor activity of mice was found. It was found for the first time that mutation in the TAAR6 gene can influence some parameters of anxious behavior of mice; also, TAAR6-mutant animals showed increased hypothermic response to the administration of 5HT1A-agonist 8-OH-DPAT.

Statements to be defended

1. LK00764 and AP163 as well as their pharmacophores may be useful for the development of novel TAAR1-agonists
2. TAAR6 could be a potential target for neurotropic drugs modulating the serotoninergic system
3. TAAR2 can be used as a target for the development of drugs that modulate the dopaminergic system

Personal contribution of the author

The author was directly involved in the work described in the thesis, namely in the development of protocols, conducting and statistical analysis of experiments, publication, presentation and discussion of the results obtained. The author also participated in the collection of brain and other tissue samples, performed genotyping of several lines of knockout animals, extracted information from RNA expression databases, independently prepared solutions and performed injections/gavage, thermometry, performed method setting and equipment setup, and participated in the organizational part of the work described in the thesis.

Publications

12 papers have been published on the materials of the dissertation: 10 scientific articles in journals indexed in WoS and/or Scopus and RINC, 1 review in the journal included in the list of VAK, 1 chapter in the book. The main provisions and scientific results of the thesis were presented in reports at 4 scientific conferences, including 3 international conferences: 26th Multidisciplinary International Neuroscience and Biological Psychiatry Conference "Stress and Behavior" May 16-19, 2019; 32nd ECNP Congress, September 7-10, 2019, Copenhagen, Denmark; 33rd ECNP Congress Hybrid, Virtual, September 12-15, 2020; 34th ECNP Congress Hybrid, October 2-5, 2021, Lisbon, Portugal; Actual Problems of Translational Biomedicine - 2022, Leningrad Region, Russia, July 25-26, 2022.

2. Literature review

Trace amine-associated receptors

Trace amine associated receptors (TAARs) were discovered in 2001 by two independent groups of researchers [1,2]. TAARs belong to the G-protein-coupled receptor (GPCR) family. GPCRs are transmembrane receptors whose function is to transmit signals across the lipid layer [10]. There are 6 functional receptors, TAAR1, TAAR2, TAAR5, TAAR6, TAAR8 and TAAR9 in humans, 15 in mice and 17 in rats [8]. Trace amines usually include substances such as beta-phenylethylamine, octopamine, tyramine and tryptamine; the term "trace" is used because of the low concentrations of these substances in tissues in mammals [11,12]. The endogenous ligands of TAAR1 are tyramine and beta-phenylethylamine, while the endogenous ligands for the other receptors are currently unknown. All TAARs, except TAAR1, were found to be expressed in the olfactory epithelium, so it was previously thought that their main function was olfactory [8]. A brief description of the currently available knowledge about each receptor in the family will follow.

TAAR1

TAAR1 is the most studied of the receptors associated with trace amines [13]. TAAR1 expression has been shown in various brain regions, such as the ventral covering area (VTA), the suture nuclei (DRN), the substantia nigra, the contiguous septal nucleus, the prefrontal cortex and the hypothalamus. The presence of the receptor has also been described outside the CNS: in cells of the immune system, gastrointestinal tract, pancreas, adipose tissue and testes [12].

Many ligands with agonism to TAAR1 have been identified, including such substances as beta-phenylethylamine, amphetamine, methamphetamine, clonidine, guanfacine, m-CPP and apomorphine. In addition, a number of compounds relatively selective for the receptor have been synthesized: RO5263397, RO5166017, RO5203648 and other experimental drugs, Only one antagonist, EPPTB, has been identified and used in experiments on cell cultures and tissue sections [14].

There is a large amount of data on the interactions between TAAR1 and the dopaminergic system. Possible co-expression of D2 and TAAR1 has been described. Notably, administration of TAAR1 agonists is able to reduce catalepsy induced by the D2-blocker haloperidol, and TAAR1-knockout mice and heterozygotes have reduced severity of haloperidol-induced catalepsy [15,16]. Knockout animals have increased D2-dopamine receptors in the striatum and decreased PPIs [17]. TAAR1-knockout mice show increased locomotion to d-amphetamine administration compared to wild-type animals; they also have increased dopamine and noradrenaline release in the dorsal striatum

to d-amphetamine administration [17]. TAAR1 agonists have been shown to be able to reduce cocaine-induced hyperlocomotion in both mice and rats [18]. Using a model of double knockouts on the dopamine transporter gene and TAAR1, we proved that the reduction of hyperlocomotion in DAT knockouts by RO5166017 is TAAR1-dependent [3]. It has also been described that TAAR1 may participate in the effects of dopaminergic agonists such as apomorphine. Apomorphine is a partial TAAR1 receptor agonist with activity estimated to be about 75% that of beta-phenylethylamine. Thus, TAAR1-knockouts have reduced verticalization time in the apomorphine climbing test compared to wild-type animals and a decrease in stereotyped behavior (licking), while no differences in horizontal activity in the locomotor activity test were found, as well as in chewing and sniffing [19]. Electrophysiologic studies also revealed changes in the functioning of dopaminergic neurons in TAAR1-knockout animals. The frequency of spontaneous discharges of ventral tegmental dopaminergic neurons was increased in TAAR1-knockouts compared to wild-type animals, and in response to the TAAR1 agonist para-tyramine, the frequency of spontaneous discharges could be reduced in wild-type animals, while the same response to para-tyramine was absent in knockout animals [20].

TAAR1 is also involved in interactions with the serotoninergic system. In serotoninergic neurons, the TAAR1 agonist RO5203648 increased the frequency of spontaneous discharges of serotoninergic neurons in the dorsal suture nucleus, with impulse rates returning to control levels after washout [18]. It is known that TAAR1 agonists can reduce stress-induced hyperthermia, which is likely due to TAAR1 expression in serotoninergic and thermoregulatory regions such as the dorsal suture nuclei and hypothalamus [3]. Notably, in contrast to wild-type animals, MDMA at doses of 10-20 mg/kg is not able to induce hypothermia in TAAR1-knockout animals, but instead causes an increase in temperature; also, TAAR1-knockouts have an increased riling effect of MDMA on serotonin levels as measured by microdialysis [21].

Interestingly, TAAR1 can also modulate glutamate neurotransmission. There is evidence that TAAR1-knockout animals have decreased expression of GluN1 and GluN2B subunit and decreased phosphorylation of GluN1 at serine 896 in prefrontal cortex. Knockouts also have increased resting membrane potential in layer V cortical neurons, the amplitude and kinetics of evoked potentials, and the ratio of NMDA/AMPA currents [4]. It is also known that the selective TAAR1 agonist RO5203648 can dose-dependently reduce motor hyperactivity induced by administration of the NMDA antagonist L-687,414 and can also reduce spontaneous hyperactivity in animals with reduced expression of the NR1 subunit of the NMDA receptor [18].

TAAR2

TAAR2 expression has been detected in olfactory epithelium, gastrointestinal tract, heart, lung, leukocytes, and testes [12,22,23]. Ligands to TAAR2 have not yet been identified; however, the receptor is able to be activated by high concentrations of some primary amines. A recent study by our group indirectly showed expression in the pear-shaped cortex, lateral hypothalamus, CA1 field of the hippocampus, lateral habenula, fifth layer of somatosensory cortex and suture nuclei. In addition, increased dopamine levels were found in the substantia nigra and striatum of TAAR2 gene knockouts, and an increased number of TH-positive neurons in the substantia nigra was detected [24].

TAAR5

When the TAAR5 receptor was initially identified, it was given the name PNR - Putative Human Neurotransmitter Receptor - and the researchers described expression in the human brain: amygdala, thalamus, hypothalamus, hippocampus and caudate nucleus [25]. Later, data were published on the low expression in the substantia nigra [26]. In situ hybridization signals specific for TAAR5 RNA were detected in the mouse arcuate nucleus, ventromedial hypothalamus, and amygdaloid body [27]. Similarly, TAAR5 expression has been described in olfactory epithelium and olfactory bulbs [7,28]. Indirectly by immunohistochemical staining, possible expression within the mouse brain was assessed in animals with the LacZ gene inserted in place of the TAAR5 gene: staining was detected in the contiguous septal nucleus, orbitofrontal cortex, ventromedial nucleus of the hypothalamus amygdala, CA1 region of the hippocampus, anterior olfactory nucleus, pear-shaped cortex, thalamic region, entorhinal cortex, amygdala and cerebellum [6,29].

Several substances are currently known to act on the TAAR5 receptor. Trimethylamine has agonistic activity: apparently, the perception of the smell of trimethylamine, which smells like rotten fish, is partly due to TAAR5 expressed in the olfactory epithelium. An alpha-NETA agonist that is also a choline acetyltransferase inhibitor and an antagonist of chemokine-like receptor 1 (CMKLR1) has also been described [30,31]. In an in vivo study, alpha-NETA has neurotropic properties, which may be due in part to TAAR5 activation [32–35].

N-N-dimethylethylamine and N-methylpiperidine have weak agonist properties [7]. Recently, two potential TAAR5 antagonists were identified by screening compounds obtained by in silico modeling [36].

A recent study of TAAR5 gene knockout mice revealed a decrease in several measures of anxiety behavior in the Open Field, Elevated Cross Maze, and Light and Dark Chamber tests. Knockouts also appeared to have reduced levels of serotonin in the striatum and hippocampus, and

reduced amounts of 5-hydroxyindoleacetic acid in the hypothalamus. In addition, TAAR5 knockouts showed an increased hypothermic response to injection of the 5HT1A agonist 8-OH-DPAT [6].

TAAR6.

At the time of the beginning of this work, there was little knowledge about the possible functional role of the TAAR6 receptor. The receptor is known to be expressed in the olfactory epithelium, and expression has also been found in the amygdala and hippocampus [2,37]. In addition, there is evidence for low expression of TAAR6 mRNA in humans in the hippocampus, prefrontal cortex, substantia nigra, amygdala, and basal ganglia [38]. Our group conducted an additional study of TAAR6 expression in the CNS based on the analysis of public open transcriptomic databases. It was found that this receptor is also expressed in the prefrontal cortex and adjacent septal nucleus [39].

The literature has repeatedly described possible associations of single nucleotide polymorphisms in the TAAR6 SNP gene with the risk of psychiatric disorders such as bipolar affective disorder and schizophrenia [38,40–46], however, the data are conflicting, with some more recent studies refuting the association [47–51].

TAAR8, TAAR9

To date, there is little information on the functions of the TAAR8 receptor. TAAR8 expression has been detected in mouse kidneys and amygdala, as well as in rat heart [2,52]. It is worth noting that mice, unlike humans, have three orthologs of the TAAR8 gene - TAAR8a, TAAR8b, TAAR8c. Several ligands to TAAR8, N-methylpiperidine and N,N-dimethylcyclohexylamine, have been identified [7,53,54].

TAAR9 receptor expression has been detected in olfactory epithelium pituitary, spinal cord, duodenum and stomach, spleen, lymphocytes and skeletal muscles [22,55–61]. TAAR9 activation is possible with N-methylpiperidine and N,N-dimethylcyclohexylamine, as well as components of carnivore and noncarnivore urine [7,53,62]. Our group recently found decreased levels of cholesterol and low-density lipoprotein in TAAR9 gene knockout rats [57]. Further work is underway to identify the functions of the receptor both in the CNS and outside the CNS.

Ectopic localization of olfactory receptors

An interesting question is the possible localization of olfactory receptors outside the olfactory epithelium and their performance of functions different from olfactory receptors in the classical sense. Olfactory receptors are the largest gene family in the human genome [63]. Initially, it was assumed that olfactory receptors were highly specialized, expressed only within the olfactory epithelium and performing their functions exclusively in the concept of olfactory chemoreception. However, over the

last decade, exceptions to this rule have been repeatedly found, strongly favored by the advent of high-throughput omics technologies such as tissue microarrays or RNA sequencing [64].

To date, ectopic extraolfactor localization has been demonstrated for many types of olfactory receptors. One example is OR2A4/7, expressed in primary human melanocytes and human skin sections, whose expression has been demonstrated at both the mRNA and protein levels. It has been suggested that this olfactory receptor is involved in the regulation of melanocyte differentiation, melanin synthesis, and melanocyte growth [65]. Another example is Olfr78, an olfactory receptor expressed in the renal juxtaglomerular apparatus, where it mediates renin secretion; it is also expressed in smooth muscle cells of small resistive vessels and has been reported to respond to short-chain fatty acids, which may be produced by gut microflora, among others [66]. The possible involvement of olfactory receptors in metabolism, diabetes and obesity, intestinal function and interaction with intestinal microflora has been the subject of an increasing number of research studies [67], in particular because of their expression in the liver [68,69]. There is also evidence of olfactory receptor expression in CD4+ T-cells, macrophages, and the possible involvement of olfactory receptors in inflammation and various immune responses is discussed [70]. Particular attention should be paid to the presence of olfactory receptors in spermatozoa - one striking example is OR17-4, which reacts to bourgenal (which is a chemoattractant for spermatozoa) and modulates the motility of male germ cells depending on the concentration of this substance, thereby regulating chemotaxis towards the oocyte [71]. Similar functions were also found in OR7A5 and OR4D1 [72]. In addition to functions in the adult organism, there may be a role for olfactory receptors outside of the olfactory organ at the embryonic developmental stage [73].

More and more data on the functions of ectopic olfactory receptors are gradually accumulating and there are suggestions about possible pharmacological modulation of the work of tissues and organs through the effect on this group of receptors, that is, it is quite possible to use them in the future as targets for drugs with completely new mechanisms of action [74,75]. An interesting example would be the activation by a synthetic terpenoid flavoring called sandalore of the OR2AT4 receptor found in keratinocytes, which causes cell proliferation and migration, thereby promoting wound healing [76]. This receptor is also expressed in human hair follicles, and its activation significantly increases the active phase of hair follicle growth by inducing the production of insulin-like growth factor 1 (IGF1), which may contribute to the stimulation of hair growth [77].

The expression of olfactory receptors can be altered in some pathologies, which may facilitate their use as a biomarker during screening and for accurate diagnosis. Thus OR51E2 is a potential

marker for prostate cancer, while OR2B6, OR2C3 and OR10H1 can serve as biomarkers for breast cancer, melanoma and urinary tract cancer [75]. Recently, our group has shown that the expression of receptors associated with trace amines is detected in nevus samples, while their expression is decreased in melanoma samples and its pattern is also altered [78]. In addition, TAAR1 expression correlates with the level of breast cancer differentiation and may be a predictor of patient survival rates [79].

As new data accumulate, it is also possible to rename receptors depending on their localization and new functions discovered [64].

It can be concluded that the extraolfactant localization and function of receptors primary defined by olfactory researchers is widespread. On the other hand, there is nothing extraordinary that the same "building blocks" in the course of evolution are used in completely different organs and tissues of the same organism. In recent years, new data on the localization of trace amine receptors outside the olfactory epithelium have been appearing, and one of the urgent tasks is to study their functions in these tissues and organs.

Investigation of TAAR1 agonists as potential medicines

Since TAAR1 expression is found in dopaminergic neurons, many studies have focused on investigating the possible modulation of dopaminergic neuron function by TAAR1 and the possible use of TAAR1 agonists in the treatment of conditions whose pathophysiology affects dopaminergic signaling. Such disorders are, for example, schizophrenia and chemical addictions.

Schizophrenia is a chronic disabling disorder that on average affects approximately 1% of the world's population. According to data from the United States, the average life loss for patients with schizophrenia is about 28.5 years [80], and the costs associated with disability and medical care cost the government 281.6 billion in 2020 [81]. The symptoms of schizophrenia are conventionally divided into positive symptoms, which include delusions, hallucinations, and disorganization of thought, and negative symptoms, which include poverty of emotional responses and speech, apathy, and abulia [82–84]. The main drug treatment for schizophrenia is antipsychotic medication. Most antipsychotic drugs affect D2-like dopamine receptors in one way or another, being either antagonists (haloperidol, risperidone, odanzapine, etc.) or partial agonists (aripiprazole, brexpiprazole, cariprazine, and lumateperone). The receptor profile of the drugs can vary considerably, but most often 5HT2A serotonin receptors are another target: these drugs are usually classified as second-generation antipsychotics (atypical antipsychotics). One of the antipsychotic drugs that does not act on dopamine

receptors but is an inverse agonist of 5HT2A receptors, pimavanserin, can reduce psychotic manifestations in patients with Parkinson's disease psychosis [85], it is also currently being studied in clinical trials for its potential to reduce negative symptomatology in patients with schizophrenia as an adjunctive therapy [86]. Another antipsychotic drug that does not act on targets such as D2 and 5HT2A receptors is in the final stages of clinical trials for the treatment of schizophrenia from Karuna Therapeutics Inc. - KarXT [87]. KarXT, which is a combination of the M1-, M4-agonist xanomelin and the peripheral nonselective cholinolytic trospium, has shown the ability to reduce positive and negative symptoms of schizophrenia, and possibly cognitive symptoms [88]. Xanomelin, the centrally active component of the drug, can also alleviate cognitive and behavioral symptoms in patients with Alzheimer's disease [89]. The vast majority of antipsychotic drugs are characterized by side effects such as hyperprolactinemia, weight gain, hyperlipidemia and type 2 diabetes mellitus, altered cardiac conduction, and extrapyramidal side effects such as drug-induced parkinsonism, akathisia, and tardive dyskinesia [90]. Second-generation antipsychotics often have a less severe extrapyramidal side-effect profile but are not always significantly superior in efficacy to their earlier counterparts [91]. It should be noted that the division into generations is rather conventional, and the difference in the pharmacological profiles of individual drugs can vary greatly. Unfortunately, not all patients are helped by existing pharmacotherapy, and a significant proportion of patients with symptoms resistant to the therapeutic effects of antipsychotic medications is an urgent problem in modern psychiatry [92]. Due to its increased effectiveness in reducing psychotic symptoms, clozapine can be singled out separately, but its use is limited by the need for regular monitoring of blood parameters due to the risk of agranulocytosis development [93]. Another of the problems of drug therapy for schizophrenia is the insufficiently satisfactory effect on negative and cognitive symptoms [94,95]. Moreover, medications may themselves cause negative symptoms, which certainly worsens the results of therapy [96]. Thus, the direction of further development of antipsychotic drugs is to reduce the side effects of therapy, increase their effectiveness in overcoming therapeutic resistance, as well as to increase the impact on negative and cognitive symptoms of schizophrenia. A possible solution to these problems is the study and introduction into the clinic of drugs acting on new targets and having different mechanisms of action from their predecessors.

One possible new pharmacologic agent acting on the symptoms of schizophrenia is the 5HT1A-, TAAR1-agonist ulotaront [5,97]. Ulotaront (SEP-363856, SEP-856) has been identified as a potential antipsychotic drug through a collaborative effort between Sunovion Pharmaceuticals and PsychoGenics Inc. using in-vivo screening on the SmartCube® high-throughput platform, which processes behavioral tests using artificial intelligence for phenotypic drug discovery [98]. Further

studies have confirmed this hypothesis: the drug has received breakthrough therapy status from the FDA, and phase III clinical trials are currently underway [99]. Another TAAR1 agonist called ralmitarone from Hoffmann-La Roche has reached Phase 2 clinical trials and its effect was studied in schizophrenia and schizoaffective disorder (NCT04512066, NCT03669640). At the time of writing, these studies have not been published.

Pharmacological characterization of ulotaront was performed through studies in cell cultures and animal models. In a cell culture radioligand binding screening assay, ulotaront (at 10 μM) showed > 50% inhibition of specific radioligand binding 5-HT1A, 5-HT1B, 5-HT1D, 5-HT2A, 5-HT2B, 5-HT2, 5-102 HT7, α2A, α2B и D2R receptors. Ki values ranged from 0.031 to 21 μM. In subsequent functional assays it was identified as an agonist of the human receptor TAAR1 with EC50 104 0,14 ± 0,062 μM and Emax = 101,3 ± 1,3% (mean ± S.E.M.) and 5-HT1A receptor with EC50 2,3 ± 1,40 μM and Emax =74,7 ± 19,60%. Weak effect on all other targets (5-HT1B, 106 5-HT1D, 5-HT2A, 5-HT2B, 5-HT2, 5-HT7, α2A, α2B and D2 receptors) was observed only at high micromolar concentrations. In functional assays of D2R receptors, ulotaront exhibited weak partial agonist activity with values of EC50 10,44 ± 4 μM (cAMP, Emax = 23,9% ± 109 7,6%) and 8 μM (β-arrestin, Emax = 27,1%). At 100 μM, 34% ± 1.16% inhibition was observed in the cAMP assay, and no antagonism was observed at concentrations up to 100 μM in the β-arrestin assay. Low-potency partial agonist activities were also detected on 5-HT1B (EC50 = 15,6 ± 11,6 μM, Emax = 22,4% ± 10,9%), 5-HT1D (EC50 = 0,262 ± 113 0,09 μM, Emax = 57,1% ± 6,0%) and 5-HT7 receptors (EC50 = 6,7 ± 1,32 μM, Emax = 41,0% 114 ± 9,5%). In a functional assay of 5-HT2B activity, ulotaront showed no agonism up to concentrations of 100 μM. At the 5-HT2A receptor, activity was virtually undetectable 29.3% agonism was observed only at the highest concentration of 10 μM tested. Ulotharont did not show activity in any of the enzymes tested up to a concentration of 100 μM [100]. Interestingly, a more recent study using an alternative cellular method to determine TAAR1 activity in vitro showed a more potent (EC50 38 ± 11 nM; Emax = 109% ± 3%) agonist effect of ulotaront, suggesting a higher level of selectivity for 5-HT1A activity [101]. A similar difference in activity on TAAR1 compared to 5-HT1A was shown in an independent study [102]. Ulotaront has been shown to be a potent full agonist of TAAR1, acting specifically through Gαs (pEC50: 6.08 ± 0.22; Emax: 96.41% ± 15.26).

Ulotaront showed slightly less activity against TAAR1 than beta-phenylethylamine (pEC50: 6.49 ± 0.23) but more than para-tyramine (pEC50: 5.65 ± 0.06). In contrast, ulotaront demonstrated several orders of magnitude less partial agonist activity against the 5-HT1A receptor. Similar to the endogenous agonist, at extremely high micromolar concentrations ulotaront could induce 5-HT1A-dependent attraction of Gαq, Gαi and to a lesser extent Gαs (Emax: 36-47%) [102]. It has also been

shown that ulotaront can affect 5-HT1A-mediated GIRK activity. The ability of TAAR1 agonists to activate GIRK via TAAR1 has been demonstrated previously [3,14]. Ulotaront induced Gβγ-mediated activation of GIRK (Emax: 55.03% ± 14.09), indicating that the compound at high micromolar concentrations can induce potassium currents of the GIRK channel through the 5-HT1A receptor, presumably by conjugating Gαi/o [102]. However, whether these high concentrations (>100 μM) of ulotaront required for activity on 5HT1A have any physiological significance remains unclear. In addition, the activity of ulotarone against the D2 receptor has been evaluated. Consistent with previous results [100], ulotaront demonstrated only low potency and efficacy in D2R with respect to Gαi engagement and GIRK activation only at very high (up to 1 mM) concentrations.

The pharmacokinetics of ulotaront has been studied in both animals and humans. Pharmacokinetics of intravenous and oral administration of ulotaront was preclinically evaluated in male ICR mice (10 mg/kg, per os), Sprague-Dawley rats (5 and 10 mg/kg, orally and intravenously) and rhesus macaques (10 mg/kg, orally and intravenously) [100]. In mice, Cmax for 10 mg/kg, orally, was 2854 ± 298 ng/mL and 7972 ± 2908 ng/g in plasma and brain, respectively; Tmax was 30 min for plasma and 15 min for brain tissue; T1/2 was 0.847 h and 0.808 h in plasma and brain, respectively.

In rats after administration of 10 mg/kg p/o, Cmax was 1750 ± 369 ng/mL and 3762 ± 1324 ng/g in plasma and brain, respectively; Tmax in plasma and brain tissue was 15 min; T1/2 was 2.1 h and 2.33 h in plasma and brain, respectively. In rats, after administration of 5 mg/kg intravenously and 5 mg/kg orally, Cmax in plasma was 2578 ± 110 ng/mL and 1056 ± 173 ng/g; Tmax was 0.083 and 0.42 ± 0.14 h; T1/2 in plasma was 1.17 ± 0.16 h and 1.24 ± 0.1 h, respectively.

In monkeys after oral administration of 5 mg/kg, Cmax in plasma was 431 ± 104 ng/mL, Tmax was 6.00 ± 2.83 h, and T1/2 was 3.03 h. In monkeys, after administration of 5 mg/kg intravenously, Cmax in plasma was 2191 ± 194 ng/mL, Tmax, 0.083; T1/2, 3.14 ± 1.26 h. The mean residence time was 5.90 h [100].

Thus, in experimental animals, ulotaront is rapidly absorbed, has good bioavailability (~100% in rats, 92% in dogs, and 71% in monkeys), and tends to concentrate in brain tissue (brain concentration and AUC in brain were approximately 3-fold higher than in plasma). Subsequent in vitro ADME and preclinical pharmacokinetics studies also demonstrated high solubility and permeability across the blood-brain barrier [103]. In this study, ulotaront demonstrated low binding to animal and human plasma proteins with an unbound fraction of more than 78% (in both animals and humans). Ulotaront demonstrated low to moderate hepatic clearance in mouse, rat, monkey and human hepatocytes. The formation of hepatic clearance of ulotaront is mainly determined by CYP2D6 and

appears to involve both NADPH-dependent and NADPH-independent pathways involved in its metabolism. The major metabolite detected in the plasma of mice, rats, rabbits, dogs, monkeys, and humans following single or repeated administration of ulotaront is SEP-383103 [103].

The population pharmacokinetics of ulotaront in adults were analyzed using pooled data from seven Phase I studies, one acute Phase II study, and one 6-month extension study. Pharmacokinetic parameters were evaluated in males and females aged 18 to 55 years. Data were obtained from healthy volunteers (n = 99) and patients with schizophrenia (n = 305). 53.7% of subjects were white, 31.4% black, 10.9% Asian, and 3.9% other/mixed race. More than 80% of Asian subjects in the analysis were Japanese [97]. Single and multiple oral doses (5-150 mg/day) were used. According to pharmacokinetic analysis, ulotaront is well absorbed by oral administration. Ulotaront demonstrated dose proportionality at doses ranging from 25 to 100 mg in terms of median maximum concentration, area under the concentration-time curve, and minimum concentration. The estimated median Tmax was 2.8 hours and the mean effective elimination half-life was 7 hours, resulting in an accumulation factor of 1.1 with daily administration. Pharmacokinetic parameters did not change significantly after 12 weeks of daily administration. Pharmacokinetic parameters of ulotaront were independent of sex, race, age, drug composition, or the presence of schizophrenia. Only patients' body weight affected the pharmacokinetics of ulotaront. Taken together, these data suggest that ulotaront has a pharmacokinetic profile that is consistent with once daily administration of the drug [104]. The bioequivalence of the tablet and capsulated forms of ulotaront was compared and found no significant differences. In addition, no effect of food on the pharmacokinetics of the tablet form in humans was detected2 [105].

To assess the mechanisms responsible for the action of ulotaront at the neuronal level, patch-clamp recordings were performed in isolated mouse brain slices from the dorsal suture nuclei (DRN), where the cell bodies of serotonin neurons are located, and the ventral tegmental area (VTA), containing the cell bodies of mesolimbic dopaminergic neurons. Ulotaront induced inhibitory responses in dorsal suture nuclei neurons, and this effect was attenuated by the 5-HT1A antagonist WAY-100635 but not by the TAAR1 antagonist EPPTB. Ventral tegmental neuronal activity was also reduced by ulotaront: inhibitory effects in the VTA were attenuated by the TAAR1 antagonist EPPTB but not by the 5-HT1A antagonist WAY-100635. It appears that the inhibitory effects of ulotaront on the activity of dorsal suture nuclei neurons were mediated through activation of serotonin 5-HT1A receptors, but in VTA neurons they were at least partially dependent on TAAR1 activation [100]. Analysis of the extracellular activity of dorsal suture neurons of DRN of rats confirmed the results obtained by patch-clamp recordings in isolated mouse brain slices. At a dose of 5 mg/kg (intravenously) ulotaront completely inhibited neuronal excitation, and this inhibition was completely

offset by the serotonin 5-HT1A antagonist WAY-100635, indicating that the inhibitory effect of ulotaront on DRN neurons is mediated exclusively through serotonin 5-HT1A receptors [100]. To directly assess the effect of ulotaront on serotonin 5-HT1A receptors in brain tissue in vitro, radiography was performed in rat brain slices. Radioligand binding of the serotonin 5-HT1A agonist 8-OH-DPAT in the absence and presence of ulotaront was quantitatively assessed. Ulotaront displaced 8-OH-DPAT in a concentration-dependent manner, and the greatest receptor binding was observed in the septum and throughout the cortex [100].

To evaluate the binding of ulotaront to D2-receptors, in vivo autoradiography experiments were performed with the D2-receptor radioligand raclopride in Sprague-Dawley rats. Ulotaront did not induce significant D2-receptor binding in the brain at plasma concentrations 200 times higher than behaviorally effective, indicating no significant interaction of the drug with D2-receptors [100]. In addition, positron emission tomographic imaging of the radioligand fallipride in anesthetized baboons was performed to determine binding to D2 receptors in primates. Ulotharont, even at very high concentrations, showed weak levels of binding to D2-receptors (less than 10%) in various brain regions. The lack of direct interaction with dopamine D2-receptors appears to extend to primates as well [100].

Taken together, these data indicate that ulotaront may act predominantly through activation of TAAR1 and serotonin 5-HT1A receptors without significant effects on dopamine D2 receptors and serotonin 5-HT2A receptors, unlike most antipsychotics.

Some of the most recognized pathogenetic hypotheses for schizophrenia are increased dopaminergic and decreased glutamatergic neurotransmission [106,107]. Drugs acting through increased dopamine or decreased glutamate signaling are commonly used to model schizophrenia endophenotypes in rodents and to test potential drugs in these models. Positive symptoms are commonly modeled in tests involving locomotor hyperactivity in mice and rats following the dopaminergic stimulant amphetamine or the glutamate NMDA receptor antagonist phencyclidine (PCP) [108].

Administration of ulotaront effectively reduced hyperactivity in animals under the influence of PCP. Administration of all tested doses (0.3, 1 and 3 mg/kg, orally) was accompanied by a dose-dependent reduction of increased motor activity in mice [100]. Attenuation of PCP-induced hyperlocomotion was also observed in rats after administration of a minimum effective dose of 1 mg/kg (orally). The serotonin 5-HT1A receptor antagonist WAY-100635 partially reduced the ability of ulotaront to attenuate PCP-induced hyperactivity in mice [100]. The potential role of the 5-HT1A

mechanism (as well as other TAAR1-independent mechanisms) of ulotaront in its action on PCP-induced hyperactivity was recently confirmed in TAAR1-knockout mice: pre-administration of the drug (10 mg/kg, orally) reduced MK-801-induced hyperactivity independently of TAAR1 [102].

Interestingly, ulotaront failed to reduce locomotor hyperactivity induced by dopaminergic drugs, indicating a complex mechanism of action of the drug on dopamine neurotransmission and emphasizing that not only D2-receptor mechanisms appear to be involved. Thus, preliminary administration of the drug (dose range: 1-10 mg/kg, orally) did not reduce d-amphetamine-induced hyperlocomotion [102,109]. Similar data were obtained when evaluating the effect of ulotaront in the apomorphine-induced climbing test in mice (apomorphine is an agonist of dopamine D1 and D2 receptors) [110]. At the same time, ulotaront administration potentiated the effects of the antipsychotic drug olanzapine in this test as well as in the MK-801-induced hyperactivity test in mice (MK-801 is an NMDA antagonist) [110].

Ulotaront has also shown results in preclinical models of negative symptoms in schizophrenia. One frequently used model of negative symptoms is the decrease in social interaction in rodents induced by chronic administration of phencyclidine (PCP) [111,112]. Ulotaront was effective against subchronic PCP-induced deficits in the social interaction test in rats. All tested doses of ulotaront increased the social interaction time with a tendency for the magnitude of the effect to decrease with increasing doses from 1 to 10 mg/kg [109]. Ulotaront at a dose of 10 mg/kg also ameliorated cognitive impairment induced by subchronic PCP treatment in a novel object recognition test in rats [109]. In addition, ulotaront somewhat ameliorated MK-801-induced impairments in the Morris water maze test and potentiated the effects of olanzapine in this cognitive test [110]. Sensory information filtering deficit (sensorimotor gating) is present in schizophrenic patients and can be modeled in rodents. Studies of drug effects on the prepulse inhibition (PPI) test, the acoustic startle reflex in rodents have shown good predictive value in identifying potential antipsychotic drugs [113]. Ulotharont at doses of 0.3-30 mg/kg (oral) dose-dependently increased PPI at a minimum effective dose of 3 mg/kg [100]. These observations were further corroborated by an independent group of researchers [102]. Administration of ulotaront at a dose of 10 mg/kg (orally) increased PPI and, importantly, restored PPI reduced by pre-injection of MK-801 to wild-type mice. Notably, a similar effect was absent when ulotaront was administered to TAAR1-knockout mice, confirming the drug's mechanism of action on prepulse inhibition as a model of negative symptoms via agonism to the TAAR1 receptor [102].

Extrapyramidal symptoms and weight gain are among the common adverse reactions caused by antipsychotic therapy. A bar test was performed to evaluate the potential for ulotaront to develop

extrapyramidal symptoms, with haloperidol used as a positive control. Ulotaront at the highest investigated dose of 100 mg/kg (orally) did not induce cataleptic effects in mice, indicating a low potential for the drug to induce cataleptic effects at doses well above the effective doses in mouse models of psychosis [100]. However, ulotaront (10 mg/kg orally) was found to statistically significantly reduce basal locomotor activity in mice [102]. The latter effect appears to be TAAR1-dependent, as the drug did not affect locomotor activity in TAAR1-knockout mice [102]. The effect of ulotaront on animal weight was also evaluated in mice. It was shown that chronic treatment with the drug (in the dose range of 2-3 mg/kg orally) did not lead to weight gain. Moreover, administration of ulotaront (3 mg/kg orally) prevented weight gain in animals chronically treated with olanzapine [110].

Second-generation antipsychotics are well known as adjunctive agents in the treatment of depression [114,115]. Analysis of the effect of ulotaront in the forced swim test (FST), a standard animal test for evaluating the antidepressant-like activity of pharmacological agents, showed that administration of the drug (in the dose range of 1-10 mg/kg orally) resulted in a reduction in immobilization time in mice, indicating that ulotaront may have antidepressant-like effects [100]. These findings have recently been confirmed by another group [116]. In this study, ulotaront reduced immobility time in the forced swimming test and its analog, the tail suspension test, and potentiated the effects of the antidepressant duloxetine. Moreover, ulotaront (15 mg/kg orally for 21 days) alleviated the modeled state of anhedonia induced by chronic unpredictable stress in the sucrose preference test without any effect on non-stressed mice [116].

Ulotaront was tested in rats to determine its effects on sleep architecture. Ulotaront at doses of 1, 3, and 10 mg/kg (per os) caused a dose-dependent decrease in REM sleep, an increase in REM sleep latency, and an increase in cumulative wakefulness time. Ulotaront had no effect on cumulative non-REM sleep time and onset time of non-REM sleep. Taken together, these results suggest that ulotaront may improve alertness/wakefulness when administered in the inactive phase [100].

In both the four-week and six-month studies, ulotaront demonstrated efficacy against symptoms of schizophrenia, as reflected in a reduction in the PANSS and BNSS positive and negative symptom scores. The most frequent adverse events were headaches, insomnia, anxiety and exacerbation of schizophrenia [117]. Notably, the group receiving the drug also had improvements in general functioning as measured by the UPSA-B scale, as well as improvements in cognitive functioning as assessed by the Cogstate questionnaire [118].

Ulotaront in both preclinical and clinical studies has not demonstrated the ability to cause extrapyramidal side effects characteristic of most antipsychotics. Extrapyramidal side effects in clinical trials did not differ between the groups receiving ulotaront and placebo [117].

Regarding to metabolic side effects, preclinical studies have not shown an increase in body weight in subchronic animals treated with ulotaront. On the contrary, there is evidence that co-administration of ulotaront and olanzapine may reduce olanzapine-induced body weight gain [110]. During clinical trials, weight gain was not detected in patients receiving ulotaront, nor were significant changes in triglyceride and glycated hemoglobin levels observed [117]. At therapeutic doses, ulotaront had no effect on the conduction system, as evidenced by the absence of changes in the QT interval [117,119].

Because most antipsychotic drugs affect D2 receptors, it is very common for them to cause an increase in prolactin levels in patients [120]. Ulotaront, due to the different target profiles it is able to target, has not shown an effect on prolactin levels of treated patients in clinical trials [117].

Ulotaront can be effective in patients with Parkinson's disease psychosis. In a pilot clinical trial (NCT02969369), patients treated with ulotaront showed improvements on the SAPS-PD Parkinson's Disease Positive Symptom Scale without deterioration of motor symptoms [121]. But because of small sample size, the difference between the groups did not reach statistical significance. Next studies should clarify the effects of ulotaront in this group of patients.

In addition to studies focused on schizophrenia, the efficacy and safety of ulotaront is being investigated in major depressive disorder (NCT05593029) and generalized anxiety disorder (NCT05729373). One study examined the effect of ulotaront on patients with narcolepsy-cataplexy (NCT05015673). Ulotaront at doses of 25 and 50 mg daily for two weeks reduced the number of short daily episodes of REM sleep in patients as well as the number of episodes of cataplexy, but neither dose had a statistically and clinically significant effect on patients' daytime sleepiness [122]. In earlier studies ulotaront showed the ability to suppress sleep with rapid eye movements [100,123]. The effects of single oral doses (50 and 10 mg) of ulotaront on REM sleep were investigated in healthy men (N = 12 for each dose) in a randomized, double-blind, placebo-controlled crossover study. Plasma concentrations of the drug were also analyzed. Ulotaront at a dose of 50mg had a large effect (>3) on REM sleep parameters after a single dose and plasma concentrations ≥100 ng/mL. Below this effective concentration, the 10mg dose induced a much smaller effect, increasing only the time to REM-sleep onset with an effect size equal to 1, in parallel not changing the time spent in REM-sleep. Treatment with ulotarone at a dose of 50 mg was associated with a small increase in time spent in non-REM sleep

(stages 2 and 3) [123]. Results of post-hoc analysis revealed a significant effect of ulotaront on the amount of REM sleep without atonia in healthy subjects after a dose of 50 but not 10 mg [124].

Summarizing all the above, we can conclude that ulotaront is able to exert antipsychotic action without directly affecting D2 and 5HT2A receptors, using targets different from classical antipsychotics, among which TAAR1 and 5HT1A receptors have been identified. Preclinical data suggest that part of the effects are TAAR1-dependent. The reduction of negative symptoms may be the result of both the lack of direct effects on D2 receptors causing secondary-negative sypmtomatization with classical antipsychotic drugs and other mechanisms. Future studies will demonstrate the comparative efficacy of Ulotaront and other clinically used antipsychotics, and will help to identify patient populations in which Ulotaront could be the drug of choice for exacerbation management and maintenance treatment. Long-term safety and efficacy is another important topic to be studied. Also of interest is the follow-up characterization of patients who respond or do not respond to the new treatment and the possible use of this therapy in resistant patient groups. These studies will be interesting in terms of characterizing the endophenotypes of the disease, as the etiology and pathogenesis of schizophrenia is still not fully understood. If the drug is approved for use in the clinic, it is possible that it could be used not only as monotherapy, but also as drug combinations for various disorders.

TAAR1 agonists are being actively studied as potential antiaddictive drugs [125].

Substances with addictive potential are known to be able to lower the current thresholds at which self-stimulation is maintained in previously trained animals [126]. The TAAR1 agonists RO5256390 and RO5263397 administered to cocaine-treated animals dose-dependently reduced the ability of cocaine to decrease self-stimulation thresholds in rats, with the full agonist RO5256390 having no effect on self-stimulation thresholds in intact animals, whereas the partial agonist RO5263397 at the maximum dose studied increased the available thresholds [127].

One of the critical problems in the treatment of substance abuse is relapse [128,129]. Sustained remission is an ultimate treatment goal in addiction medicine. Craving and subsequent relapse in patients are often caused by administration of the drug (priming effect) [130], environmental contexts previously associated with drug intake [131] or stress [132]. This clinical scenario is often investigated using an animal models of reinstatement of extinguished self administration (ESA) [133]. Different stimuli can induce the reinstatement of ESA in laboratory animals, depending on previous learning experience. This can be an unconditioned stimulus, such as the priming-injection of a drug that the animal has been trained to self-administer, as well a drug of a similar class [134]. In addition to

unconditioned stimuli, a wide range of conditioned stimuli associated with the self-administration can induce the reinstatement of ESA. It is also possible to reactivate the ESA after exposure to certain stressors [135]. By studying the effect of experimental drugs on ESA reinstatement in laboratory animals, it is possible to preclinically evaluate the ability of pharmacologic agents to prevent relapses. The method has good content and construct validity [136], predictive validity has also been proven by numerous studies, although it has some limitations [137]. Various TAAR1 agonists were tested for their ability to prevent both prime-, and cue-induced reinstatement of ESA. Intraperitoneal injection of RO5256390 and RO5203648 to rats inhibited the ESA reinstatement of cocaine, induced by both priming-injection and conditioned stimuli [138]. Similar data were obtained for morphine when examining the effects of RO5263397 [139]. In addition, RO5263397 blocked the methamphetamine ESA reinstatement, induced by both priming-injection [140], and presentation of visual conditioned stimuli [141]. A similar effect was found when testing the ability of this experimental drug to inhibit the ESA reinstatement of nicotine, induced by associated with the drug visual stimuli [142]. One study provided interesting data on which specific CNS regions are involved in the effects of TAAR1 agonists on ESA reinstatement. For this purpose, RO5166017 was injected into different brain regions and its effect on the reinstatement of ESA of cocaine provoked by both priming administration and presentation of conditioned stimuli was evaluated. The results suggest that preventing of ESA reinstatement by RO5166017 is due to activation of TAAR1 receptors in the Nucleus Accumbens and VTA [143]. This finding confirms the role of TAAR1 receptors in modulating the operation of reward and motivation systems. Thus, we can conclude that TAAR1 agonists are effective in several preclinical models of addiction with different classes of addictive drugs.

3. In vivo activity studies of newly synthesized TAAR1-agonists

The Institute of Chemistry of St. Petersburg State University under the direction of Professor Mikhail Yurievich Krasavin synthesized a number of compounds with TAAR1 agonist properties (Figure 1). Later, the synthesized library of compounds was studied by employees of the Institute of Translational Biomedicine of St. Petersburg State University on a cell culture expressing TAAR1 receptors to measure effective concentrations and maximum effect. The ability of the drugs to activate TAAR1 was assessed in vitro by increasing intracellular levels of cyclic adenosine monophosphate (cAMP) using the bioluminescence resonance energy transfer (BRET) technique. BRET is based on the ability of the chimeric Rluc-EPAC-YFP protein to change its conformation upon binding to cAMP. As a result, the energy donor molecule (luciferase of the coral polyp R. reniformis - Rluc) and the acceptor molecule (yellow fluorescent protein - YFP), usually located close to each other in the inactivated state EPAC (exchange protein activated by cAMP), are significantly distanced, which leads to a decrease in resonance non-radiative energy transfer from the donor to the acceptor. As a result, the ratio of acceptor luminescence intensity (525 nm) to donor luminescence intensity (480 nm), or the so-called BRET ratio, changes. Thus, a decrease in the BRET ratio will be observed upon activation of the Gαs-signaling pathway arising from activation of the receptor under study by any ligand. Consequently, the greater the decrease in this ratio from baseline, the stronger the agonist the chemical compound will be considered to be. The most active compounds were selected for further in vivo activity studies in various animal models.

Previously, a team from the Laboratory of Neuroscience and Molecular Pharmacology at the Institute of Translational Biomedicine and colleagues from the Valdman Institute of Pharmacology had identified the ability of LK00764 to suppress hyperlocomotion in rats knocked out for the dopamine transporter (DAT) as well as to suppress motor hyperacivity. The ability of LK00764 to inhibit hyperlocomotion in DAT-knockout rats, as well as to suppress motor hyperactivity in mice induced by administration of the NMDA antagonist dizocilpine (MK-801) was revealed.

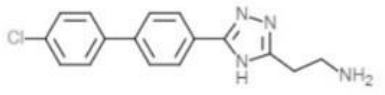
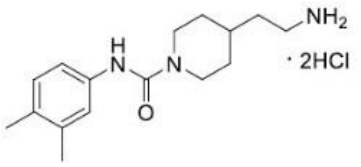
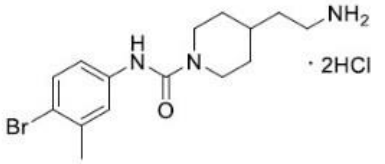
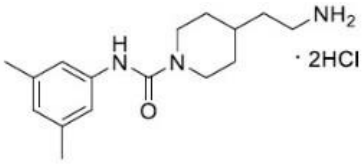
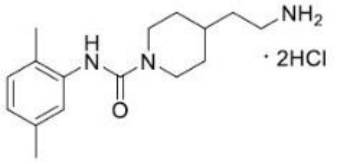
Name	Formula	Emax%	EC50
LK00764		101	4 nM
AP 161		84,39	33 nM
AP 162		104,16	37 nM
AP 163		85,22	112 nM
AP 164		79,71	42 nM

Figure 1. Chemical formulas and characteristics of TAAR1 agonists whose in vivo activity was investigated in this work.

Study of the effect of substance LK00764 on motor hyperactivity of mice induced by administration of dopamine transporter inhibitor GBR 12909

Materials and methods.

Twenty-one adult female Swiss mice were used for the experiment. The animals were kept in groups of 5-6 mice in Plexiglas cages, water and feed were provided to the mice *ad libitum* for the whole time, except for the time of the experiment. The light regime was set as 12 hours of light/12 hours of darkness, with experiments performed during the second half of the light phase. The temperature in the animal housing room and the experimental room ranged between 20-23 degrees Celsius, and the relative humidity in the rooms was maintained between 30-70%.

In this experiment, the potency of the substance LK00764 was investigated by intraperitoneal (i.p.) administration. Animals were randomly divided into 3 experimental groups, the number of animals per group was equal to 7 (Table 1). Animals were injected i.p. with physiologic solution/GBR 12909, followed by intraperitoneal injection with saline/LK00764 immediately before being placed in locomotor boxes, The experimental groups are described in detail in the Table 1. The injection volume was 10 ml/kg. Animals were placed in 40cm*40cm*40cm*40cm locomotor boxes for 1.5 hours, horizontal locomotor activity was recorded using Noldus EthoVision video tracking.

Statistical data analysis was performed using two-way RM ANOVA followed by Bonferroni test for multiple comparisons. The results were considered statistically significant at $p<0.05$. Data processing was performed using GraphPad Prism software.

Table 1. Characterization of animal groups in the experiment to study the effect of intraperitoneal injection of LK00764 in a mouse model of motor hyperactivity induced by administration of the dopamine transporter inhibitor GBR 12909.

Group	Number of animals	i.p. injection 1	i.p. injection 2
Negative control	7	NaCl 0,9%	NaCl 0,9%
GBR-12909	7	GBR 12909 10 mg/kg	NaCl 0,9%
LK00764 i.p. 10 mg/kg	7	GBR 12909 10 mg/kg	LK00764 10 mg/kg

Results.

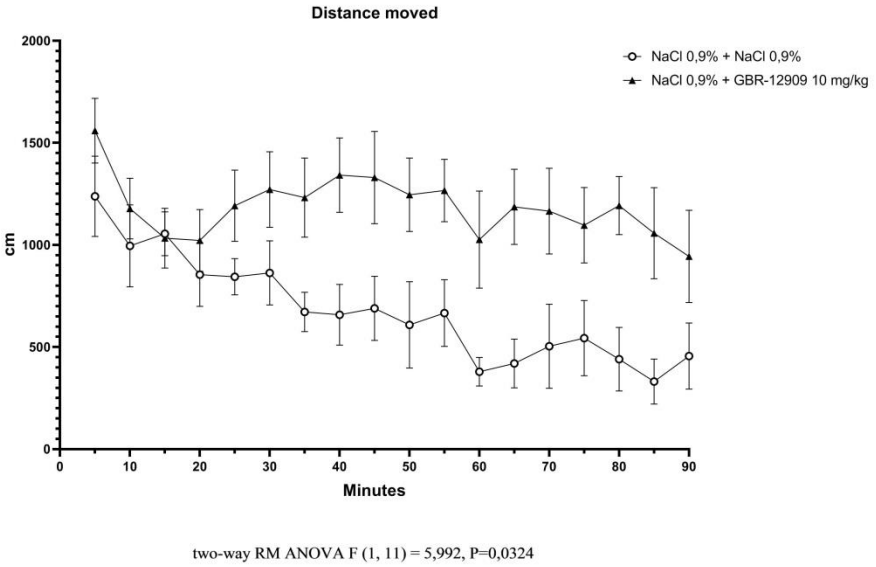


Figure 2. Distance moved in 90 minutes after i.p. injection by the control group and the group receiving GBR 12909. GBR 12909 at a dose of 10 mg/kg statistically significantly increased the locomotor activity of mice, two-way RM ANOVA F (1, 11) = 5.992, p=0.0324. The graph shows mean ± S.E.M.

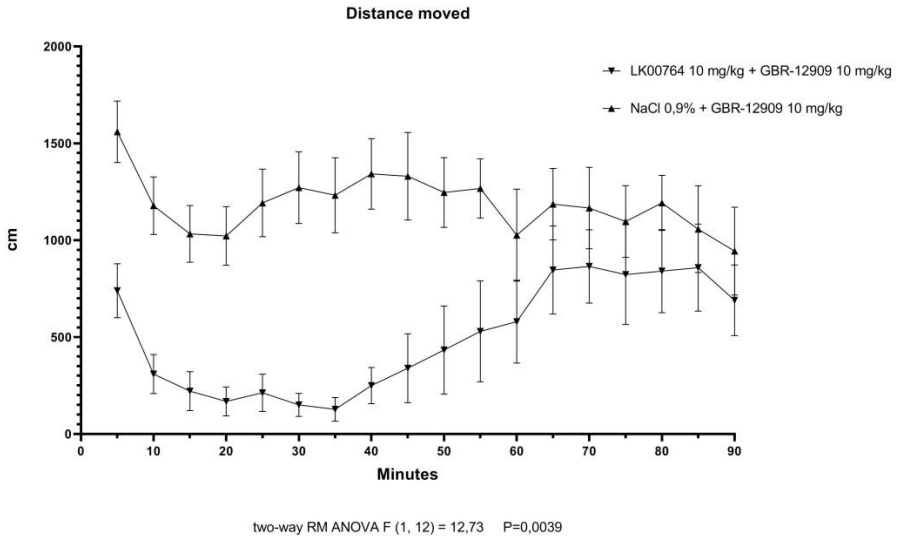


Figure 3. Distance traveled in 90 minutes after injections by the group receiving GBR 12909 and the group receiving GBR 12909 and LK00764 intraperitoneally at a dose of 10 mg/kg. Intraperitoneal administration of 10 mg/kg LK00764 (dissolved in saline) statistically significantly reduced locomotor hyperactivity induced by 10 mg/kg GBR 12909, two-way RM ANOVA F (1, 12) = 12.73, p=0.0039. The graph shows mean ± S.E.M.

As a result of the experiments performed, it was found that substance LK00764 at a dose of 10 mg/kg at intraperitoneal injection is able to reduce hyperlocomotion caused by the administration of DAT-inhibitor GBR 12909. Graphs illustrating the results of this series of experiments are shown in Figures 2-3.

Study of hypothermic effect of LK00764 on rats

Materials and methods.

To study the hypothermic effect of acute administration of LK00764 8 adult male Wistar rats were used. Animals were kept in groups of 4 mice in Plexiglas cages, water and food were provided *ad libitum* for the whole time of keeping, including the time of experiments. The light regime was set as 12 hours of light/12 hours of darkness, with experiments performed during the second half of the light phase. The room temperature ranged between 20-23 degrees Celsius, and the relative humidity in the rooms was maintained between 30-70%.

Thermometry in rats was performed rectally using a BIOSEB thermometer. Eight male Wistar rats were injected intraperitoneally with vehicle (10% polysorbate 80 dissolved in physiologic solution) or LK00764 at a dose of 3 or 5 mg/kg dissolved in 10% polysorbate 80 (in saline). Injections were performed at a volume of 1 ml/kg. The Latin square design was used for the experiment. The first measurement was performed immediately before injection, and the second measurement was performed 15 min later. The difference between the second and first measurements was calculated.

Statistical data analysis was performed using GraphPad Prism software, RM one-way ANOVA test followed by Bonferroni test for multiple comparisons was used. Values of $p<0.05$ were considered statistically significant.

Results.

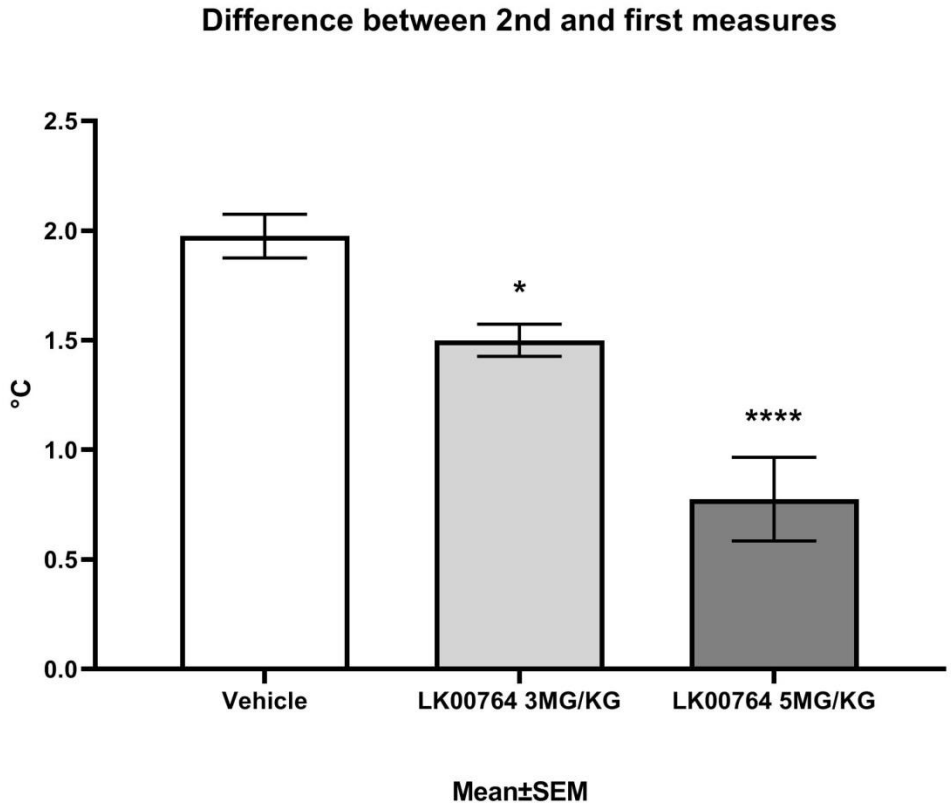


Figure 4. Temperature differences between second and first injections. LK00764 at a dose of 3 mg/kg at intraperitoneal injection statistically significantly reduced manifestations of stress-induced hyperthermia - Bonferroni's multiple comparisons test, p=0.0123, a similar effect was observed at a dose of 5 mg/kg - Bonferroni's multiple comparisons test, p<0.0001.

Thus, LK00764 is dose-dependently able to reduce the manifestations of stress-induced hyperthermia, which is a possible predictor of anxiolytic action [144,145], and this result correlates with previous preclinical studies of TAAR1 agonists [3]. The results of the experiment are shown in Figure 4.

Study of the effect on the behavior of mice of LK00764 during acute administration in Open Field, Elevated Plus Maze and Forced Swim Test

Materials and methods.

24 adult female Swiss mice were used to study the effect of acute administration of LK00764 on behavior. The animals were kept in groups of 4-6 mice in Plexiglas cages, water and food were provided *ad libitum* for the whole time except for the time of experiments. The light regime was set as 12 hours of light/12 hours of darkness, with experiments performed during the second half of the light

phase. The temperature in the animal housing room and in the experimental room varied between 20-23 degrees Celsius, and the relative humidity in the rooms was maintained between 30-70%.

Mice were randomly divided into 3 experimental groups of 8 animals each (Table 2). The drug LK00764 or saline was administered 30 minutes before the experiments in the volume of 10 ml/kg. Then, the tests "Open field" - 5 minutes, "Elevated cruciform maze" - 5 minutes, "Forced swimming test" - 10 minutes were performed in the order indicated. The control group received injections of saline intraperitoneally and subcutaneously, the "LK00764 - subcutaneously" group received an injection of saline intraperitoneally and an injection of 10 mg/kg LK00764 subcutaneously, the "LK00764 - intraperitoneally" group received an injection of 10 mg/kg LK00764 intraperitoneally and an injection of saline subcutaneously.

The open-field circular test was used to analyze exploratory activity. The apparatus consisted of a gray plastic circular arena (63 cm diameter) with 13 holes in the floor (1.6 cm hole diameter) and lighting conditions of 200 lux. Each mouse was placed in the center of the arena and spontaneous exploratory activity was recorded using Noldus Ethovision video tracking software for 5 min. The following behavioral parameters were tracked: total distance moved, cumulative duration of stay in the central zone, the number of rearings, number of holes' explorations ang time of grooming.

The "elevated cruciform maze" test was used to measure the level of anxious behavior. The maze consisted of two opposite open (30 x 5 cm) and two opposite closed arms (30 x 5 x 15 cm) elevated 40 cm from the floor. Mice were placed in the center of the elevated cruciform maze to the closed arm and experiment was recorded for 5 min. The following parameters were analyzed using Noldus Ethovision software: cumulative duration in the open arms, frequency of entries into the open arms, total distance traveled, the number of rearing, and the number of head dipping.

The forced swimming test was used to assess depression-like behavior. Each mouse was separately placed in a Plexiglas cylinder (diameter: 10 cm, height: 21 cm) filled 2/3 with water. The water temperature was within 24°C ± 1°C. The test was carried out for 10 min. The water level was deep enough (18 cm) so that the mouse tail did not touch the bottom. After bathing, mice were removed from the water, wiped dry with a towel, returned to their home cage and left in a warm room. The total time of immobilization, active and passive swimming was evaluated.

One-Way ANOVA test with Bonferroni correction for multiple comparisons was used to process the obtained data. Differences were considered statistically significant when $p<0.05$. Statistical data analysis was performed using the GraphPad Prism.

Table 2. Characterization of animal groups in experiments on the effect of acute administration of LK00764 on the behavior of mice.

Group	Number of animals
Control	8
LK00764 10 mg/kg intraperitoneally (dissolved in saline) + saline subcutaneously	8
LK00764 10 mg/kg subcutaneously (dissolved in saline) + saline intraperitoneally	8

Results.

Open field.

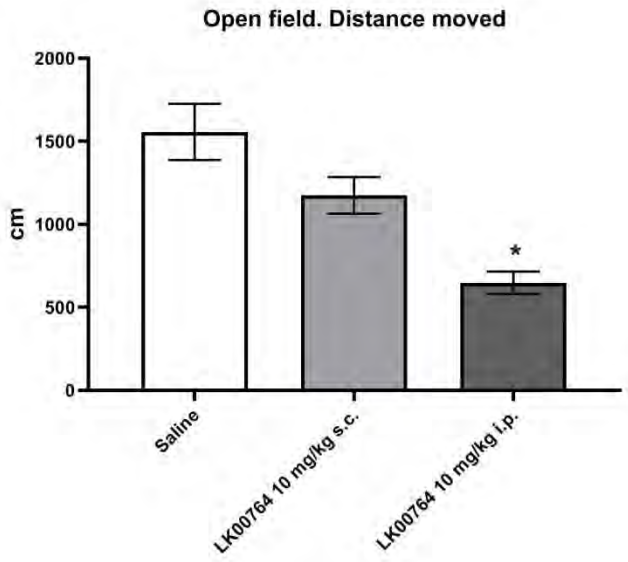


Figure 5. Distance moved by mice in the open field test. LK00764 at a dose of 10 mg/kg administered subcutaneously did not statistically significantly reduce the distance traveled during the test, but there was a tendency for its reduction - Bonferroni's multiple comparisons test, $p=0.0733$. At intraperitoneal injection LK00764 at a dose of 10 mg/kg statistically significantly reduced the distance moved - Bonferroni's multiple comparisons test, $p<0.0001$. The graph displays mean ± S.E.M.

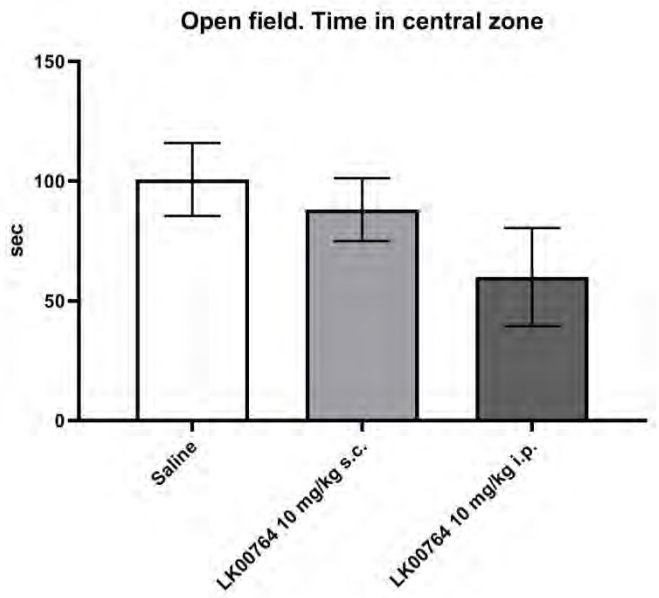


Figure 6. The time spent by mice in the central zone of the open field. LK00764 at a dose of 10 mg/kg by both subcutaneous and intraperitoneal administration had no statistically significant effect on the time spent in the central zone of the open field, Bonferroni's multiple comparisons test, $p>0.05$. The graph displays mean ± S.E.M.

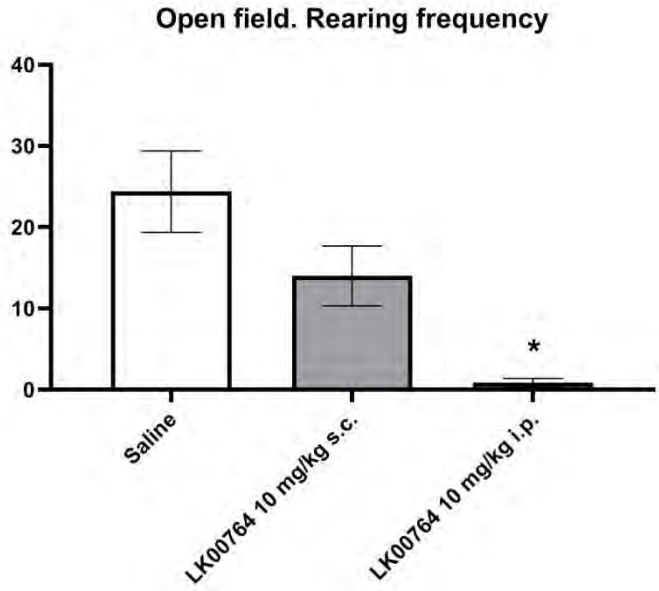


Figure 7. The rearing frequency during the open field test. LK00764 at a dose of 10 mg/kg administered subcutaneously had no statistically significant effect on the number of rearings, Bonferroni's multiple comparisons test, $p>0.05$. When administered intraperitoneally, LK00764 at a dose of 10 mg/kg decreased the number of rearings, Bonferroni's multiple comparisons test, $p= 0.0004$. The graph displays mean ± S.E.M.

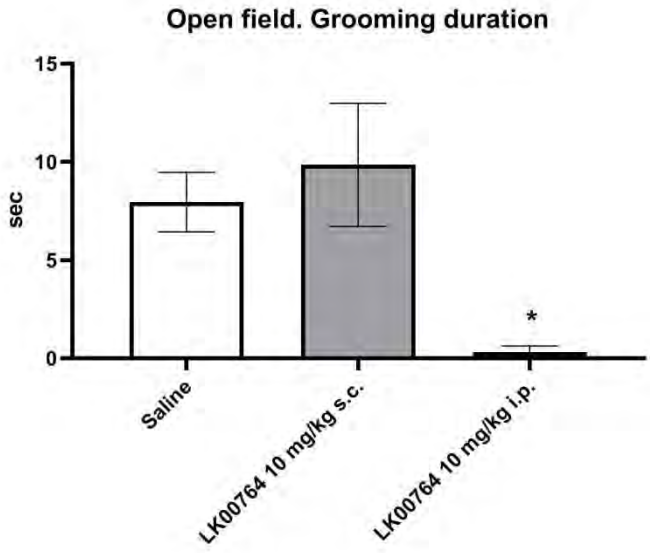


Figure 8. Duration of grooming in open field test. LK00764 at a dose of 10 mg/kg administered subcutaneously did not significantly affect grooming duration, Bonferroni's multiple comparisons test, $p>0.05$. When administered intraperitoneally, LK00764 at a dose of 10 mg/kg statistically significantly decreased grooming duration, Bonferroni's multiple comparisons test, $p=0.0433$. The graph displays mean ± S.E.M.

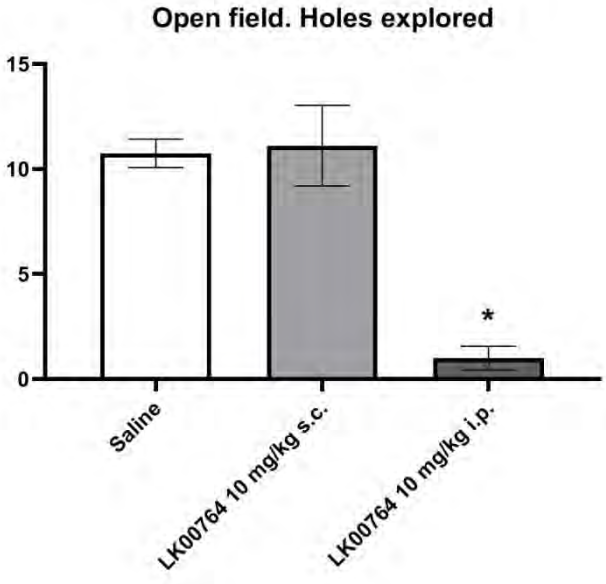


Figure 9. Number of explored holes during the open-field test. LK00764 at a dose of 10 mg/kg administered subcutaneously did not significantly affect the number explored holes, Bonferroni's multiple comparisons test, $p>0.05$. When administered intraperitoneally, LK00764 at a dose of 10 mg/kg statistically significantly decreased the number of explored holes, Bonferroni's multiple comparisons test, $p<0.0001$. The graph displays mean ± S.E.M.

Elevated Plus Maze

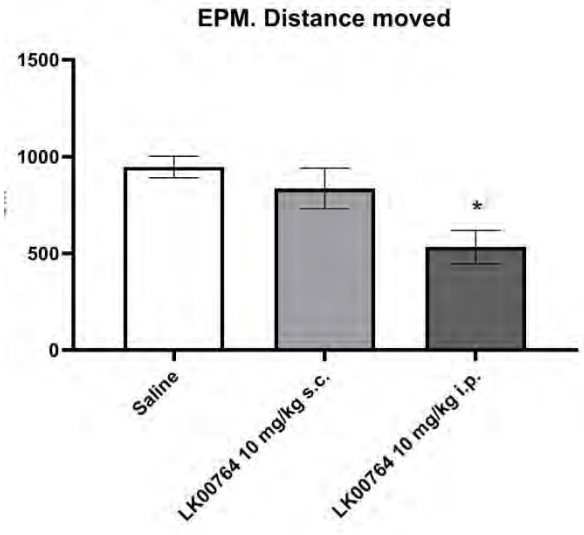


Figure 10. Distance moved by mice during the performance of the elevated plus maze test. LK00764 at a dose of 10 mg/kg administered subcutaneously did not significantly affect the distance moved, Bonferroni's multiple comparisons test, $p>0.05$. When administered intraperitoneally, LK00764 at a dose of 10 mg/kg statistically significantly decreased the distance moved, Bonferroni's multiple comparisons test, $p=0.0065$. The graph displays mean ± S.E.M.

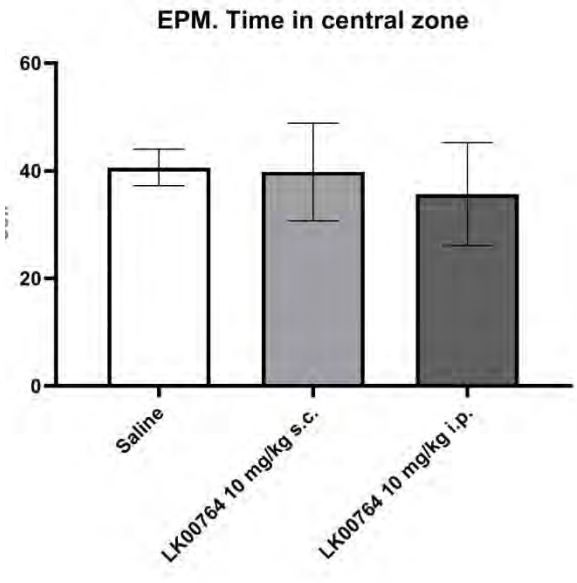


Figure 11. Time spent in the central zone by mice in the elevated plus maze test. LK00764 at a dose of 10 mg/kg both by subcutaneous and intraperitoneal administration did not significantly affect the time spent in the central zone, Bonferroni's multiple comparisons test, $p>0.05$. The graph displays mean ± S.E.M.

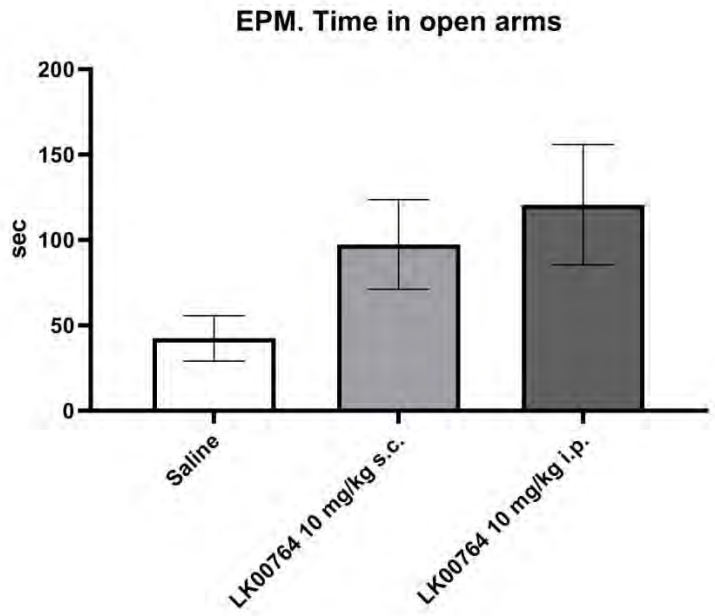


Figure 12. Time spent by mice in the open arms of the elevated plus maze. LK00764 at a dose of 10 mg/kg by both subcutaneous and intraperitoneal administration did not significantly affect the time spent in open arms, Bonferroni's multiple comparisons test, $p>0.05$. The graph displays mean ± S.E.M.

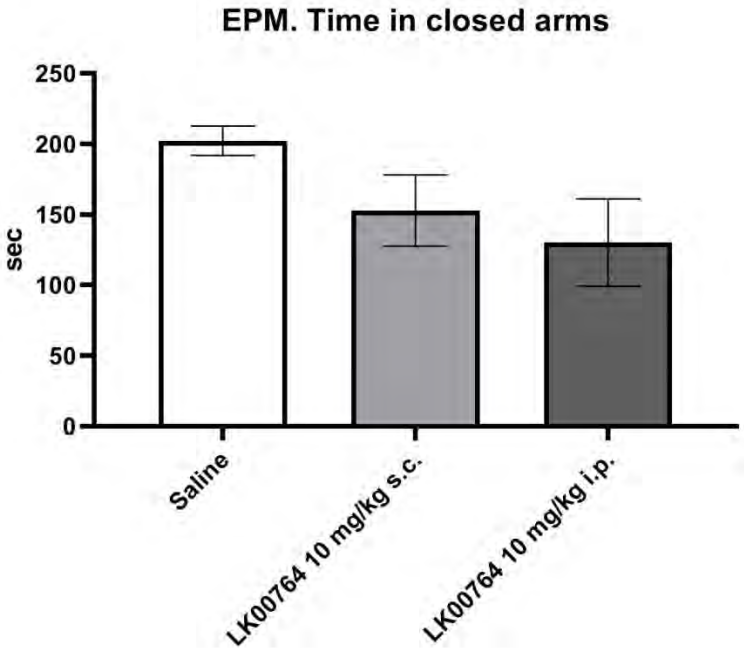


Figure 13. Time spent by mice in the closed arms of the elevated plus maze. LK00764 at a dose of 10 mg/kg by both subcutaneous and intraperitoneal administration did not significantly affect the time spent in closed arms, Bonferroni's multiple comparisons test, $p>0.05$. The graph displays mean ± S.E.M.

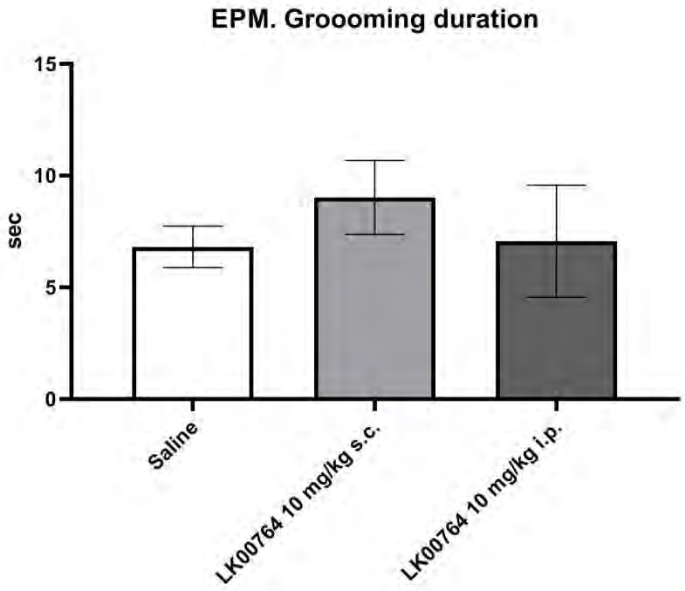


Figure 14. Grooming duration in the elevated plus maze test. LK00764 at a dose of 10 mg/kg for both subcutaneous and intraperitoneal administration did not significantly affect grooming time, Bonferroni's multiple comparisons test, $p>0.05$. The graph displays mean ± S.E.M.

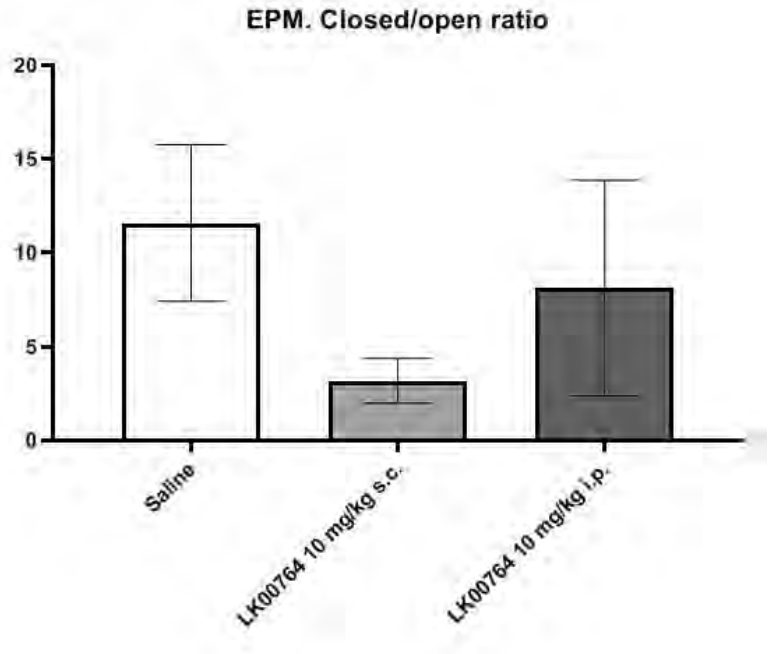


Figure 15. The ratio of time spent in closed and open arms during the elevated plus maze test. LK00764 at a dose of 10 mg/kg for both subcutaneous and intraperitoneal administration did not significantly affect the ratio of time spent in closed and open arms, Bonferroni's multiple comparisons test, $p>0.05$. The graph displays mean ± S.E.M.

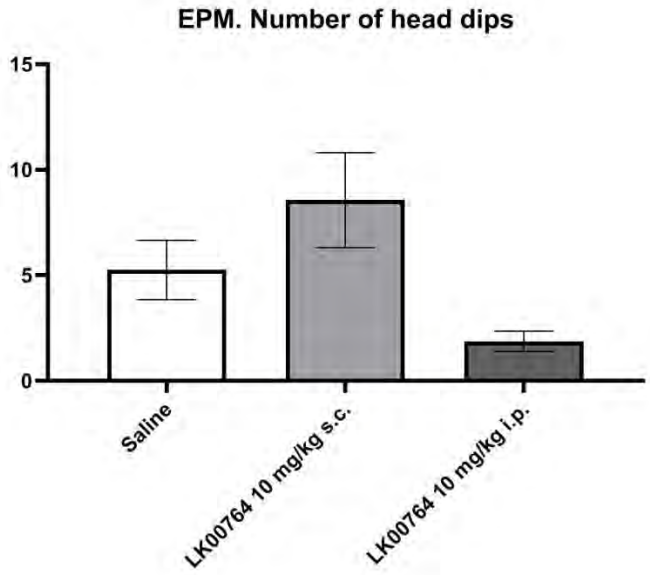


Figure 16. The number of head dips in the elevated plus maze test. LK00764 at a dose of 10 mg/kg for both subcutaneous and intraperitoneal administration did not significantly affect the number of head dips, Bonferroni's multiple comparisons test, $p>0.05$. The graph shows mean ± S.E.M.

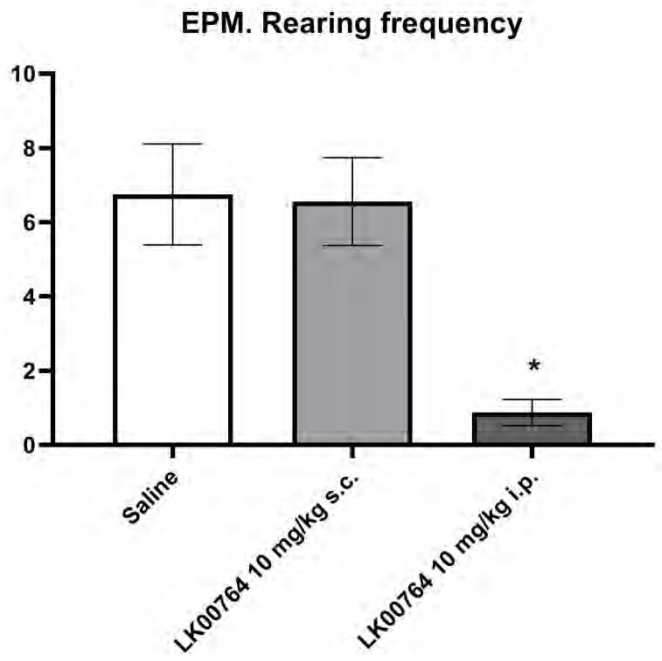


Figure 17. The number of rearings during the elevated plus maze test. LK00764 at a dose of 10 mg/kg administered subcutaneously did not significantly affect the number of rearings, Bonferroni's multiple comparisons test, $p>0.05$. When administered intraperitoneally, LK00764 at a dose of 10 mg/kg statistically significantly reduced the number of rearings, Bonferroni's multiple comparisons test, $p=0.0020$. The graph shows mean ± S.E.M.

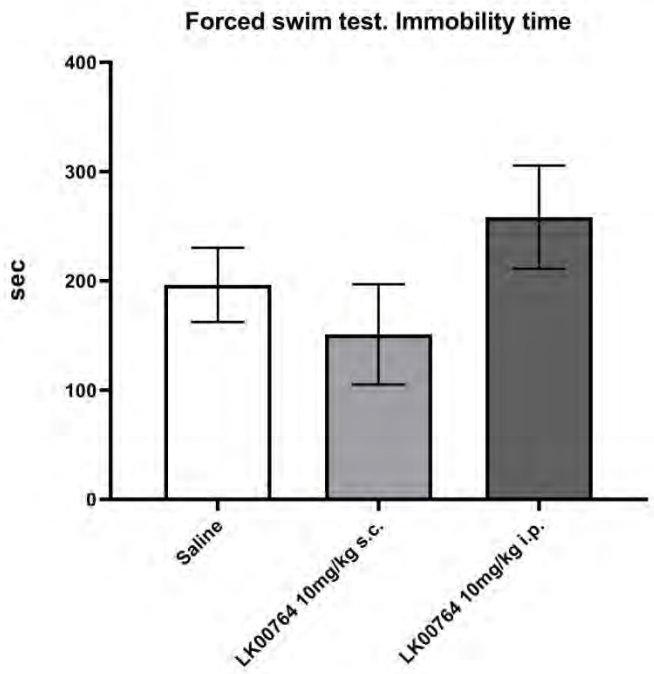


Figure 18. Immobility time during the forced swim test. LK00764 at a dose of 10 mg/kg for both subcutaneous and intraperitoneal administration did not significantly affect immobilization time, Bonferroni's multiple comparisons test, $p>0.05$. The graph displays mean ± S.E.M.

From the obtained results we can make conclusions about the depressing effect of LK00764 on locomotor and exploratory activity. In the test "Open field" LK00764 at a dose of 10 mg/kg at intraperitoneal administration statistically significantly decreased the distance moved by animals during the test, a similar trend was observed at subcutaneous administration (results are shown in Figure 5). The distance traveled also decreased in the test "Elevated plus maze" with intraperitoneal administration of the drug (Figure 10). Vertical locomotor activity was also decreased in the group receiving LK00764 intraperitoneally, as evidenced by the decrease in rearing number in the tests "Open field" and "Elevated plus maze", as shown in Figures 7 and 17. In addition to these results, intraperitoneal administration decreased grooming duration and the number of holes explored during the Open Field test, but not during the Elevated Cruciform Maze test, as shown in Figures 8, 9, and 14. The differences in results between intraperitoneal and subcutaneous administration can be explained by differences in pharmacokinetic parameters such as maximum drug concentration and time to reach it.

The drug did not demonstrate anxiolytic properties during acute administration, as can be seen by the absence of changes in such parameters as time spent in the central zone of the open field, as

well as the time spent in the open, closed arms and the central zone of the elevated plus maze and the number of head dips from the open arms of the maze, as shown in Figures 6 and 11-16..

LK00764 did not demonstrate antidepressant properties during acute administration, as there were no statistically significant differences in immobility time in the forced swim test between the experimental groups and the control group, as demonstrated in Figure 18.

Based on the results obtained, LK00764 shows predominantly sedative properties with acute intraperitoneal administration at a dose of 10 mg/kg, but such effects are almost not observed between 30 and 50 minutes after subcutaneous injection of the drug.

Investigation of the effects on the behavior of mice of LK00764 administered subchronically for 14 days in the Open Field, Elevated Plus Maze and Forced Swim tests

Materials and methods.

To study the effect of subchronic administration of LK00764 on behavior, 20 adult female Swiss mice were used. The animals were kept in groups of 4-6 mice in Plexiglas cages, water and food were provided *ad libitum* except the time of experiments. The light regime was set as 12 hours of light/12 hours of darkness, with experiments performed during the second half of the light phase. The temperature in the animal housing room and in the experimental room varied between 20-23 degrees Celsius, and the relative humidity in the rooms was maintained between 30-70%.

Mice were randomly divided into 2 experimental groups (Table 3). The first group (control) received subcutaneous injections of saline (10 ml/kg). The second group (LK00764) received subcutaneous injections of LK00764 at a dose of 10 mg/kg dissolved in saline in a volume of 10 ml/kg. The procedure was repeated daily for 14 days. The test is performed on the day after the last drug administration (24 hours break). The tests "Open field" - 5 minutes, "Elevated plus maze" - 5 minutes, "Forced swimming test" - 10 minutes were performed alternately.

The open-field circular test was used to analyze exploratory activity. The apparatus consisted of a gray plastic circular arena (63 cm diameter) with 13 holes in the floor (1.6 cm hole diameter) and lighting conditions of 200 lux. Each mouse was placed in the center of the arena and spontaneous exploratory activity was recorded using Noldus Ethovision video tracking software for 5 min. The following behavioral parameters were tracked: total distance traveled, cumulative duration of stay in the central zone, number of rearings, and number of holes' explorations.

The elevated plus maze test was used to measure the of anxiety-like behavior. The maze consisted of two opposite open (30 x 5 cm) and two opposite closed arms (30 x 5 x 15 cm) elevated 40 cm from the floor. Mice were placed in the center of the elevated plus maze to the closed arm and recorded for 5 min. The following parameters were analyzed using Noldus Ethovision software: cumulative duration in the open arms, frequency of entries into the open arms, total distance traveled, number of rearings and number of head dipping.

The forced swimming test was used to assess depression-like behavior. Each mouse was separately placed in a Plexiglas cylinder (diameter: 10 cm, height: 21 cm) filled 2/3 with water. The water temperature was within 24°C ± 1°C. The test was carried out for 10 minutes. The water level was deep enough (18 cm) so that the mouse tail did not touch the bottom. After bathing, mice were removed from the water, wiped dry with a towel, returned to their home cage and left in a warm room. The total time of immobilization, active and passive swimming was estimated.

Unpaired t test was used for statistical analysis in the comparison of groups, statistically significant differences were recognized at values of $p<0.05$. Data processing was performed using the statistical software GraphPad Prism.

Table 3. Characterization of animal groups in experiments to study the effect of subchronic 14-day administration of LK00764 on the behavior of mice.

Group	Number of animals
Saline	10
LK00764 10 mg/kg s.c.	10

Results.

Open field.

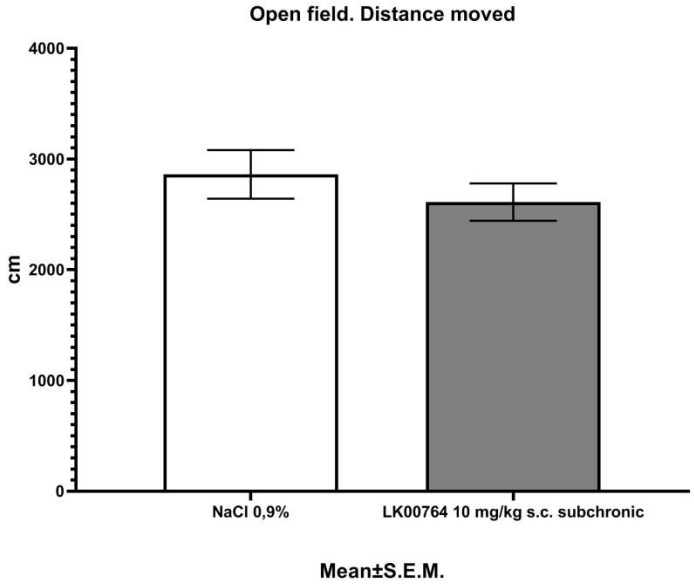


Figure 19. Distance moved during the the open field test. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on the distance traveled, Unpaired t test, p=0.3784.

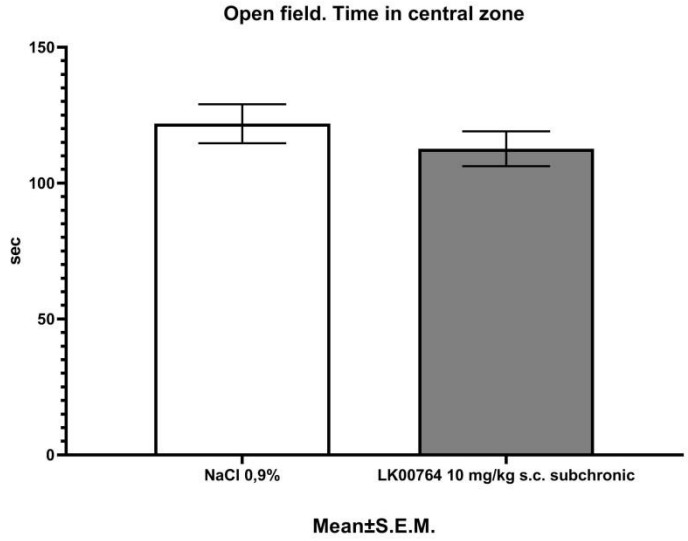


Figure 20. Time spent by mice in the central zone of the open field. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on the time spent in the central zone, Unpaired t test, p=0.3505.

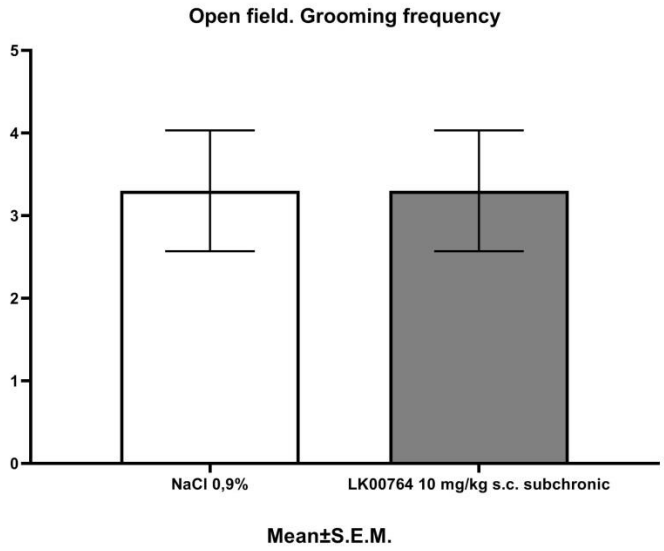


Figure 21. Frequency of grooming during the open field test. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on grooming frequency, Unpaired t test, p>0.9999.

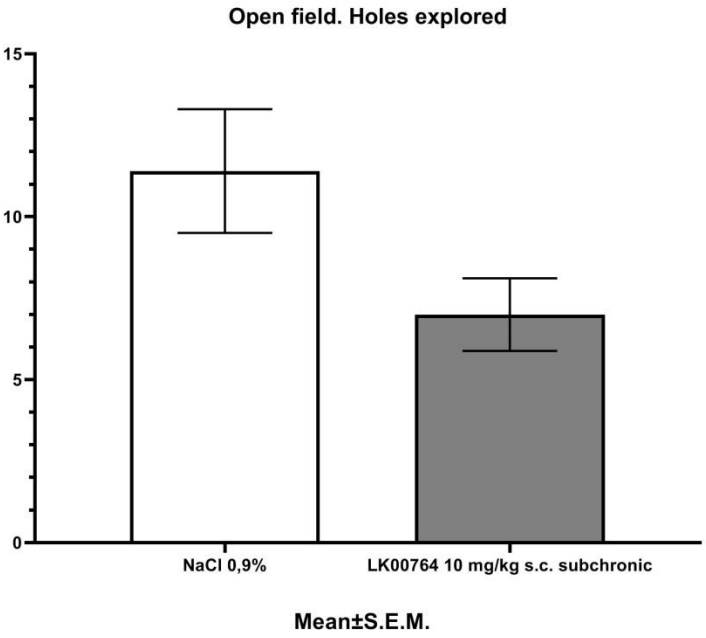


Figure 22. Number explored holes during the open field test. Subchronic subcutaneous administration of the substance LK00764 at a dose of 10 mg/kg had no significant effect on the number of examined holes, Unpaired t test, p=0.0610.

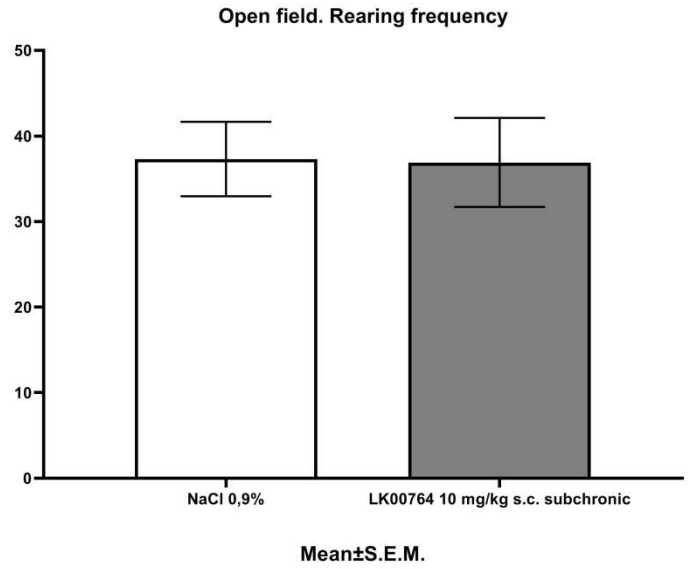


Figure 23. Number of rearings during the open field test. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on the number of hind paw lifts, Unpaired t test, p=0.9536.

Elevated plus maze

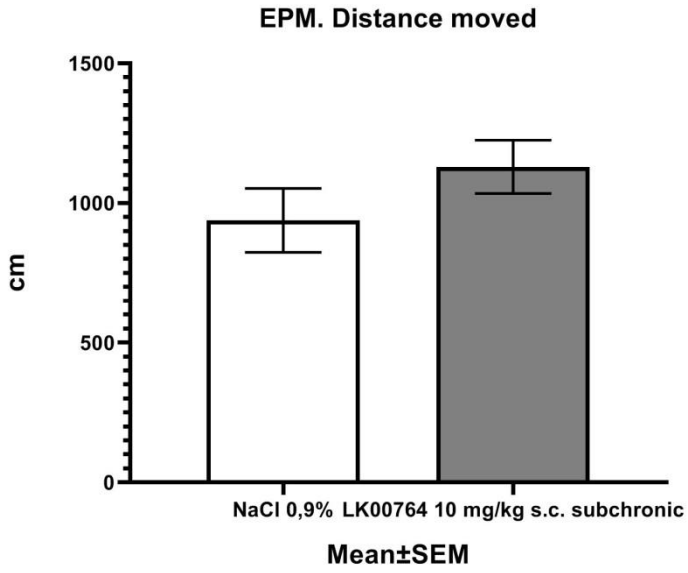


Figure 24. Distance moved in the elevated plus maze test. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on the distance traveled in the test, Unpaired t test, p=0.2159.

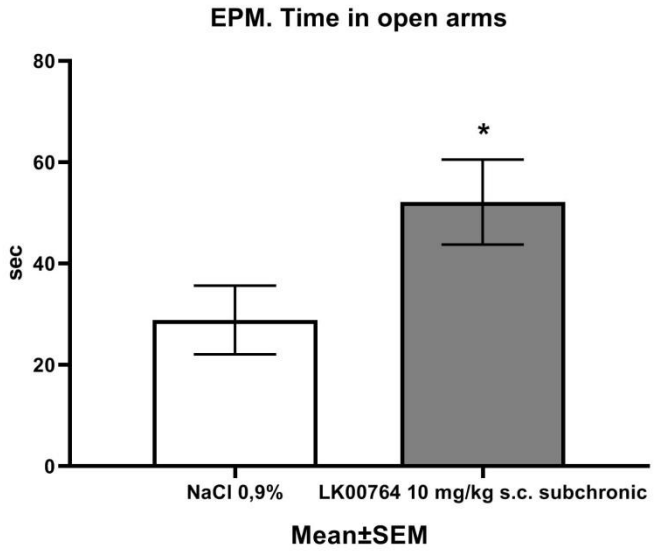


Figure 25. Time spent by mice in open arms of the elevated plus maze. Subchronic subcutaneous administration of substance LK00764 at a dose of 10 mg/kg increased the time spent in open arms, Unpaired t test, p=0.0447.

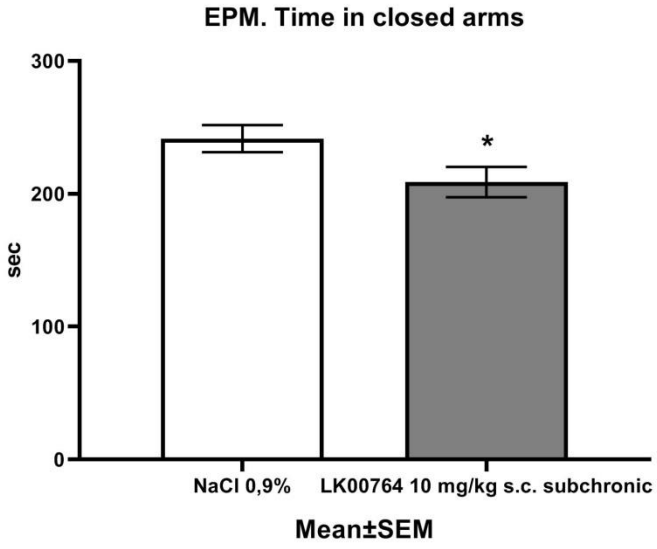


Figure 26. Time spent by mice in closed arms of the elevated plus maze test. Subchronic subcutaneous administration of substance LK00764 at a dose of 10 mg/kg decreased the time spent in closed arms, Unpaired t test, p=0.0462.

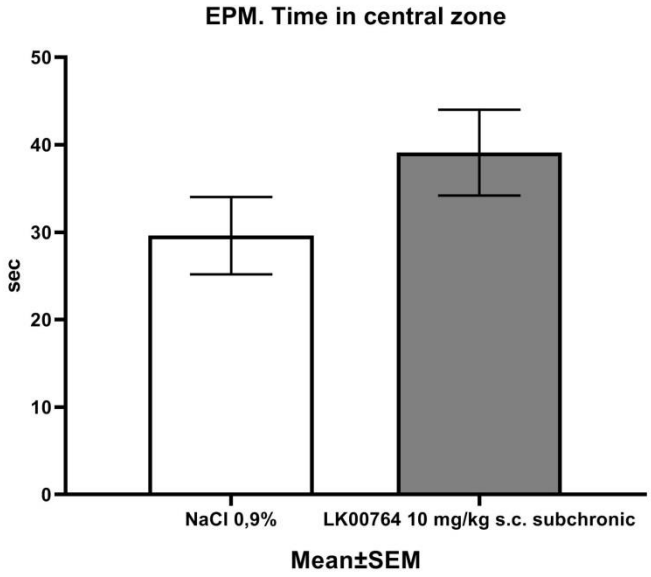


Figure 27. Time spent by mice in the central zone of the elevated plus maze. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on the time spent in the central zone, Unpaired t test, p=0.1675.

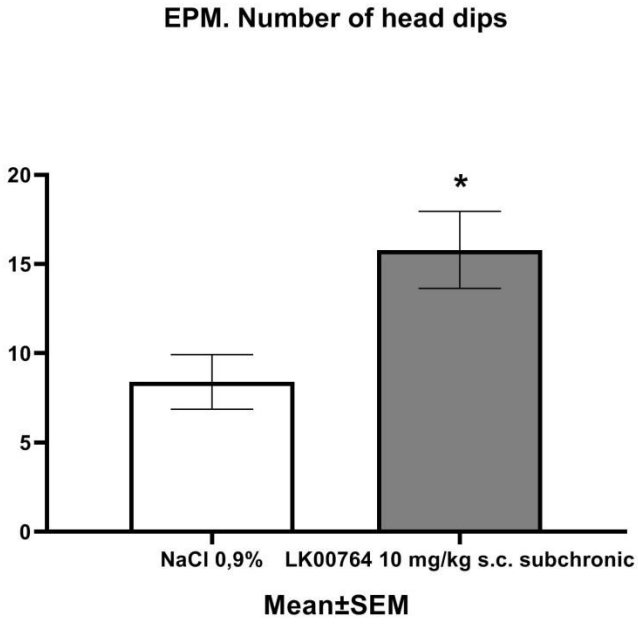


Figure 28. The number of head dipping from open arms of the elevated plus maze. Subchronic subcutaneous administration of the substance LK00764 at a dose of 10 mg/kg increased the number of head dipping, Unpaired t test, p=0.0119.

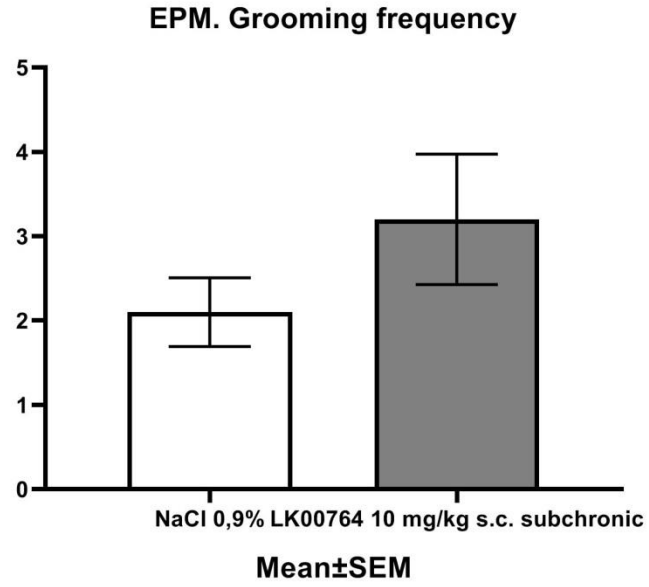


Figure 29. Frequency of grooming during elevated plus maze test. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on grooming frequency, Unpaired t test, p=0.2235.

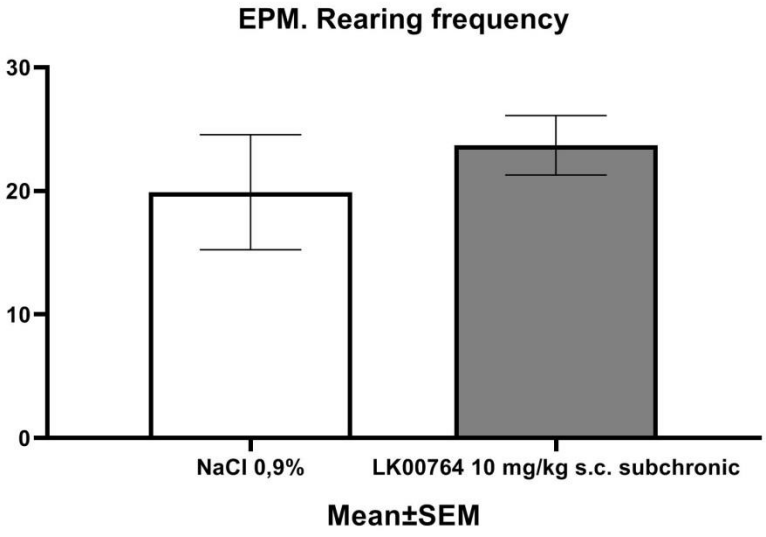


Figure 30. Number of rearings during the elevated plus maze test. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no significant effect on the number of hind paw lifts, Unpaired t test, p=0.4778.

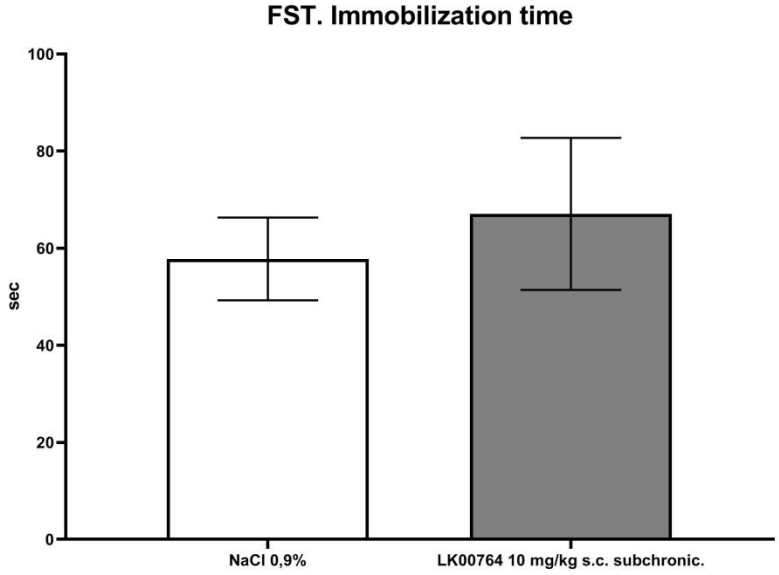


Figure 31. Immobilization time in the forced swim test. Subchronic subcutaneous administration of LK00764 at a dose of 10 mg/kg had no statistically significant effect on immobilization time in the forced swim test, Unpaired t test, $p>0.05$. The graph shows Mean ± S.E.M.

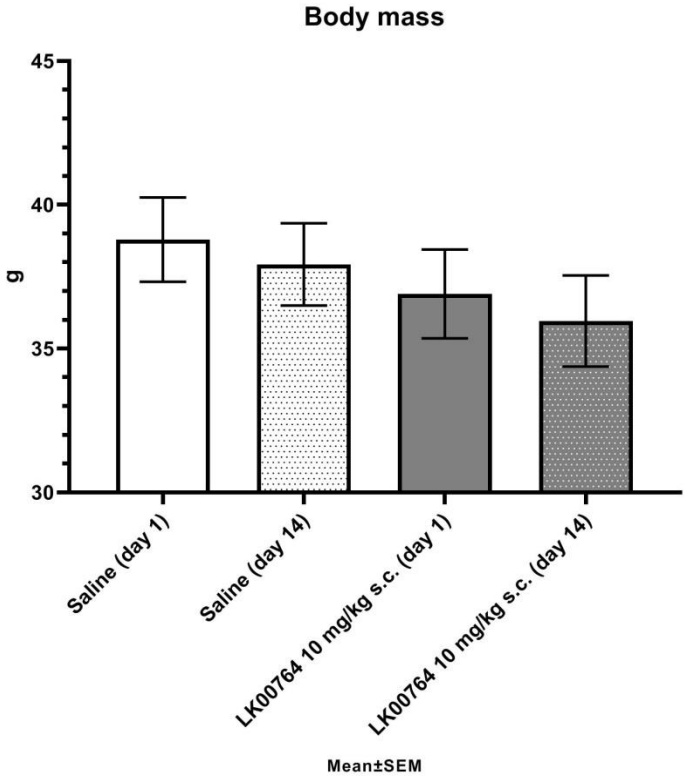


Figure 32. Body weight on the first and fourteenth day of the experiment. Subcutaneous injection of saline as well as subcutaneous injection of LK00764 at a dose of 10 mg/kg had no statistically significant effect on body weight of animals, Bonferroni's multiple comparisons test, $p>0.05$.

According to the results of the experiments, it can be concluded that there is an anxiolytic effect in subchronic 14-day subcutaneous administration of LK00764 at a dose of 10 mg/kg. In the elevated plus maze test a statistically significant increase in the time spent in the open arms of the maze was revealed, as well as an increase in the number of head dipping from the open arms; in addition, the time spent in the closed arms was reduced (Figures 25,26 and 28).

In the presence of anxiolytic effects, no depressant effect on the motor and exploratory activity of the animals was detected: there was no effect on the distance traveled in the Open Field and Elevated Cross Maze tests, no changes in the vertical motor activity of the animals during these tests, no decrease in the number of holes explored, and no effect on the duration and frequency of grooming (Figures 19-24, 27, 29, and 30).

No potential antidepressant activity was detected during subchronic administration of LK00764, as evidenced by the lack of significant differences in the duration of immobilization during the forced swim test (Figure 31).

It should also be noted that subchronic subcutaneous administration of LK00764 had no significant effect on the body weight of animals (Figure 32). It may be a possible predictor of the absence of metabolic side effects in clinical use.

Investigation of AP161-AP164 activity in a rat model of motor hyperactivity induced by dopamine transporter knockout

Materials and methods.

12 adult dopamine transporter (DAT) knockout female rats were used to test the drugs AP161, AP162, AP163 and AP164. The animals were kept in groups of 3-4 rats in Plexiglas cages. Water and food were provided *ad libitum* except the time of the experiments. The light regime was set as 12 hours of light/12 hours of darkness, with experiments performed during the second half of the light phase. The temperature in the animal housing room and experimental room ranged between 20-23 degrees Celsius, and the relative humidity in the rooms was maintained between 30-70%.

All substances were dissolved in saline (NaCl 0.9%) immediately before the experiment. Animals received intraperitoneal injections of 1 ml/kg immediately before boarding in the experimental setup. The animals were randomly divided into groups (6 rats each) before the experiment, receiving either saline or a solution of one of the compounds under study.

The experimental units were transparent Plexiglas boxes without bedding, 40cm*40cm*40cm in size. After injections the animals were placed in the boxes for 90 minutes, immediately video recording of horizontal locomotor activity was switched on by the program Noldus EthoVision, by means of which the distance traveled during the experiment was measured.

Statistical processing of the obtained data was performed using 2way ANOVA followed by Bonferroni test for multiple comparisons, Mann-Whitney and Kruskal-Wallis tests were also used. The results were considered statistically significant at p values < 0.05. Data processing was performed using the GraphPad Prism statistical package.

Results.

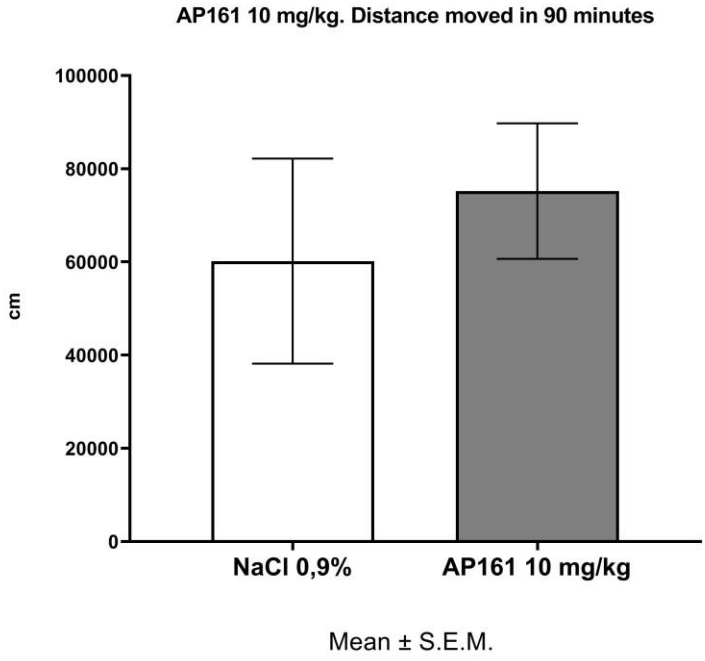


Figure 33. Distance moved by DAT-knockouts after intraperitoneal injection of AP161 at a dose of 10 mg/kg. AP161 at a dose of 10 mg/kg had no statistically significant effect on motor activity of DAT-knockouts. Mann Whitney test, $p>0.05$ ($U=12$).

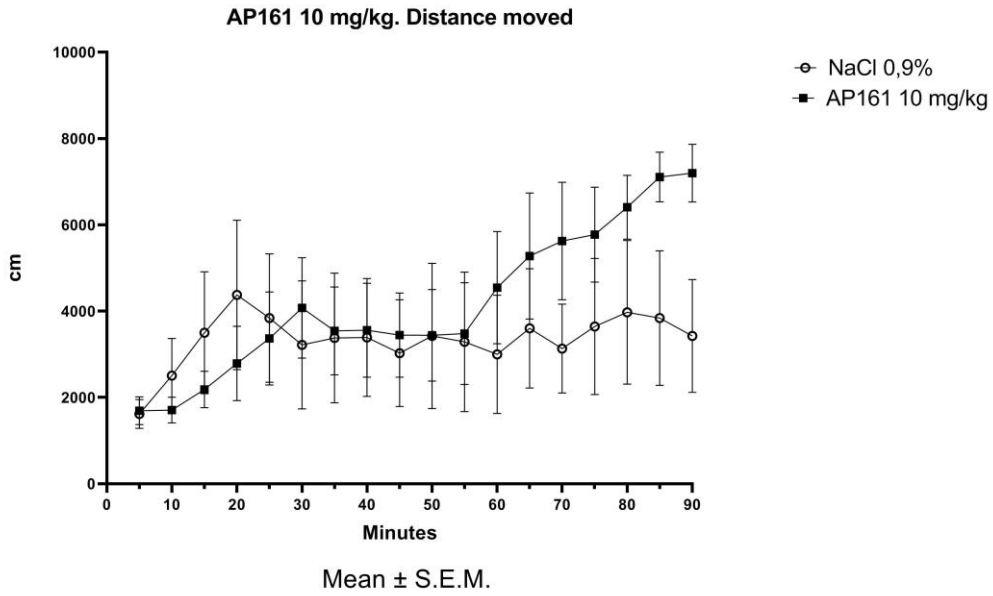


Figure 34. Distance moved by DAT-knockout rats after intraperitoneal injection of AP161 at a dose of 10 mg/kg: five-minute intervals. AP161 at a dose of 10 mg/kg did not demonstrate the ability to reduce locomotor activity of DAT-knockout rats at 90 minutes post-injection. Bonferroni's multiple comparisons test, $p>0.05$ for all time intervals.

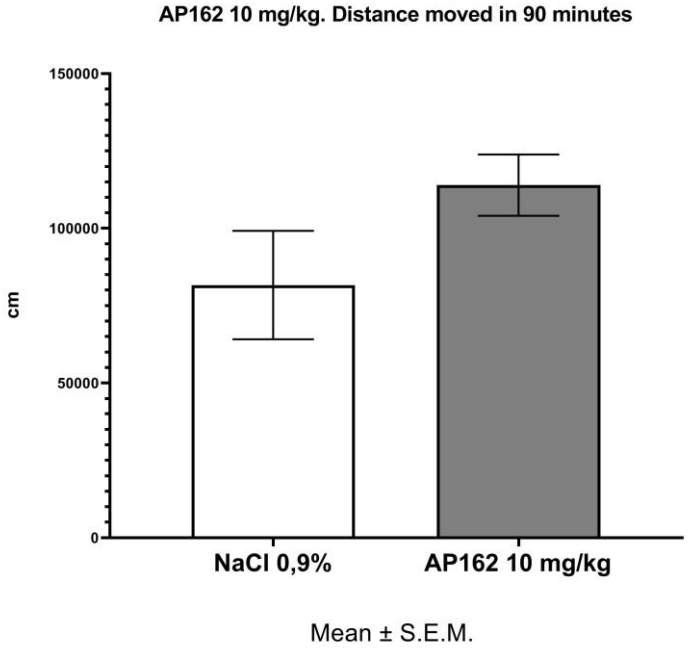


Figure 35. Distance moved by DAT-knockouts after intraperitoneal injection of AP162 at a dose of 10 mg/kg. AP162 at a dose of 10 mg/kg had no statistically significant effect on the locomotor activity of DAT-knockouts. Mann Whitney test, $p>0.05$ ($U=10$).

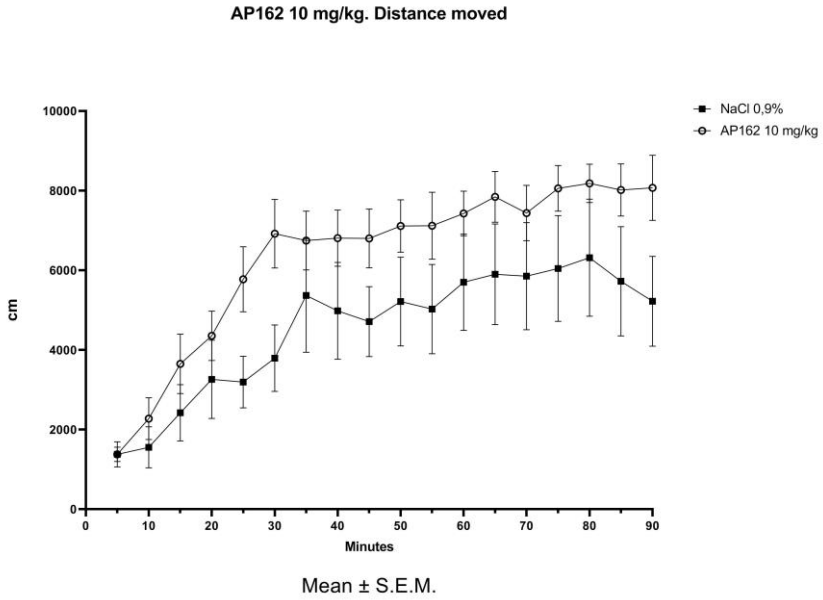


Figure 36. Distance moved by DAT-knockouts after intraperitoneal injection of AP162 at a dose of 10 mg/kg: five-minute intervals. AP162 at a dose of 10 mg/kg had no statistically significant effect on the locomotor activity of DAT-knockouts. Bonferroni's multiple comparisons test, $p>0.05$ for all time intervals.

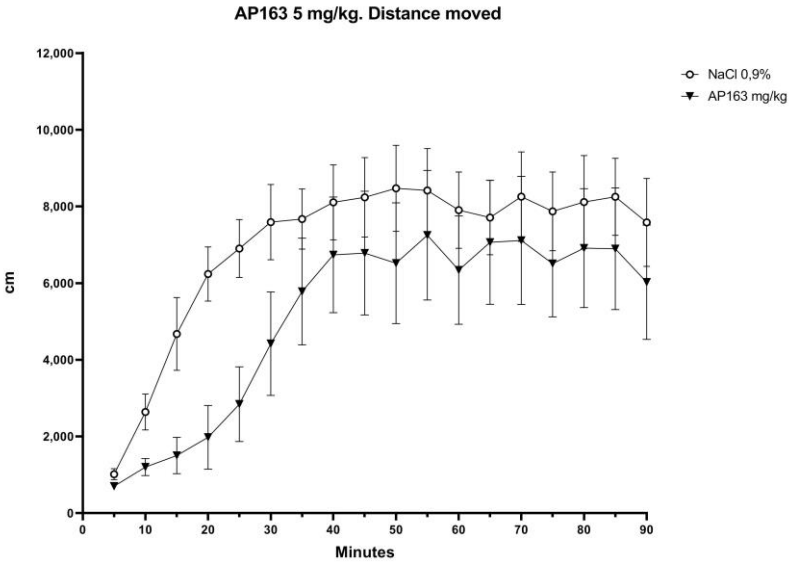


Figure 37. Distance moved by DAT-knockouts after intraperitoneal injection of AP163 at a dose of 5 mg/kg: five-minute intervals. According to the Bonferroni test results, no statistically significant differences were found between groups throughout the test, although there was a tendency to decrease locomotor activity from the tenth to the twentieth minute of the experiment. The graph shows mean ± S.E.M.

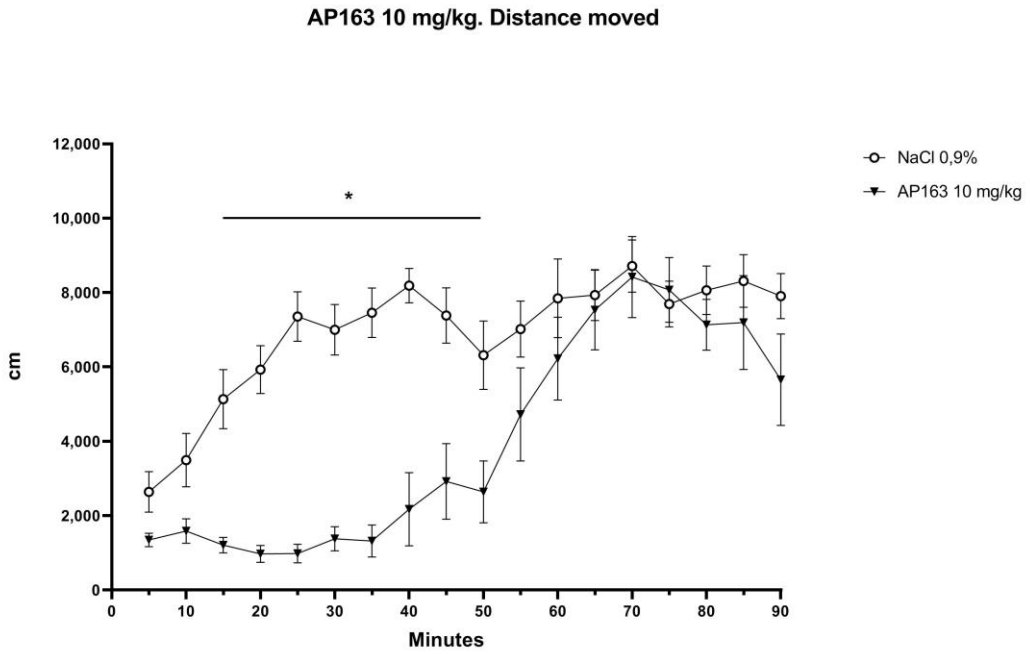


Figure 38. Distance moved by DAT-knockouts after intraperitoneal injection of AP163 at a dose of 10 mg/kg: five-minute intervals. AP163 statistically significantly decreased motor activity of DAT-knockout rats from the tenth to the fiftieth minute of the test. Asterisk indicates intervals where statistically significant differences between groups were detected by Bonferroni test: p value in the indicated interval 0.0001-0.0188. The graph shows mean ± S.E.M.

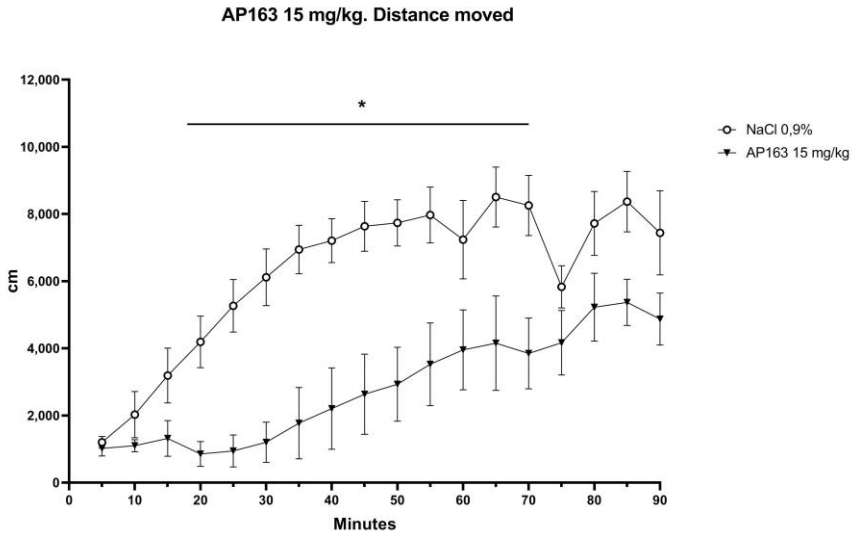


Figure 39. Distance traveled by DAT-knockouts after intraperitoneal injection of AP163 at a dose of 15 mg/kg: five-minute intervals. Asterisk indicates intervals where statistically significant differences between groups were detected by the Bonferroni test: p value in the indicated interval 0.0009-0.0113. The graph shows mean ± S.E.M

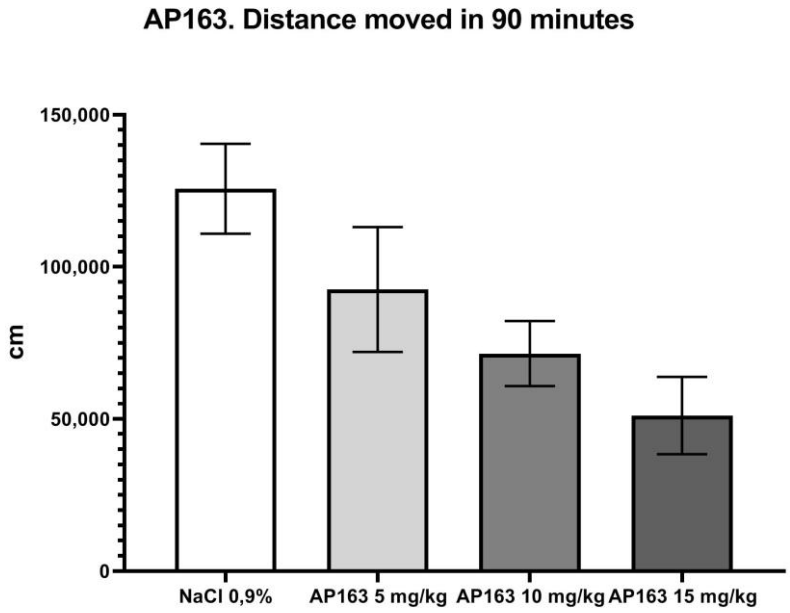


Figure 40. Distance moved by DAT-knockouts after intraperitoneal injection of substance AP163 at doses of 5-15 mg/kg: five-minute intervals. Substance AP163 dose-dependently decreased horizontal locomotor activity of DAT-knockout rats; p=0.0417 (Kruskal - Wallis test). The graph shows mean ± S.E.M.

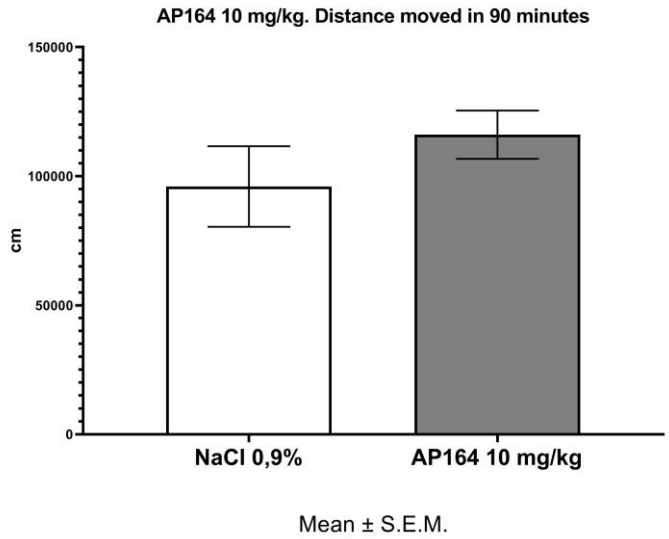


Figure 41. Distance moved by DAT-knockouts after intraperitoneal injection of AP164 at a dose of 10 mg/kg. AP164 at a dose of 10 mg/kg had no statistically significant effect on the locomotor activity of DAT-knockouts. Mann Whitney test, p>0.05 (U=11).

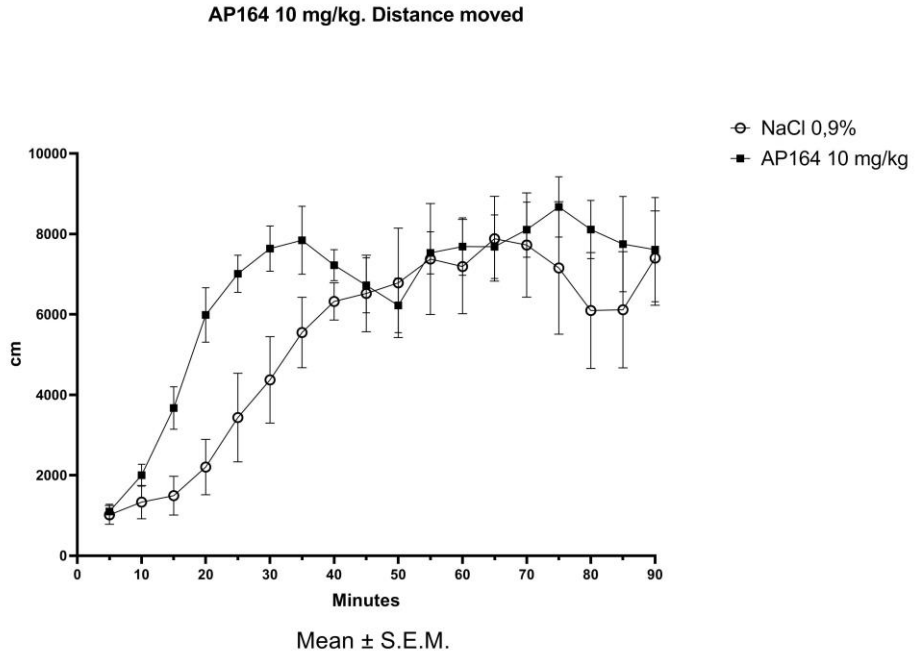


Figure 42. Distance moved by DAT-knockout rats after intraperitoneal injection of AP164 at a dose of 10 mg/kg: five-minute intervals. AP164 at a dose of 10 mg/kg had no statistically significant effect on locomotor activity of DAT-knockout rats. Bonferroni's multiple comparisons test, $p>0.05$ for all time intervals.

As a result of this series of experiments, the ability of AP 163 to dose-dependently reduce motor hyperactivity in dopamine transporter knockout rats in the dose range of 5-15 mg/kg was revealed (Figures 37-40). The potential antipsychotic effects of the compound and other possible therapeutic applications are subject to further investigation. The molecule can be used for further modification and development of more active TAAR1 agonists.

AP 161, AP 162 and AP 164 at a dose of 10 mg/kg did not show any ability to reduce locomotor hyperactivity in dopamine transporter knockout rats (Figures 33-36 and 41-42), which may presumably be the result of poor passage of the drugs across the blood-brain barrier or pharmacodynamic interactions with other possible targets.

4. Behavioral screening of TAAR6-mutant mice

Mice with a mutation in the TAAR6 gene were created at the Institute of Translational Biomedicine of St. Petersburg State University by Elena Leonova. An insertion of about 50 nucleotide pairs was made into the gene - a multiple repeat of (CAGAGG)n.

Materials and methods.

To identify possible differences in the behavior of TAAR6 mutant mice, 10 adult males of the wild-type (C57Black/6j) and 11 TAAR6 mutant littermates with the same age were used. Animals were kept in groups of 3-5 mice in Plexiglas cages, water and food were provided ad libitum for the entire time of confinement except for the time of experiments. The light regime was set as 12 hours of light/12 hours of darkness, with experiments performed during the second half of the light phase. The temperature in the animal housing room and in the experimental room varied between 20-23 degrees Celsius, and the relative humidity in the rooms was maintained between 30-70%.

Mice were divided into 2 experimental groups of 10 and 11 males, respectively. At intervals of 1-3 days, the tests "Open field" - 5 minutes, test of recognition of a new object (5+5 minutes), test of digging - 3 minutes, marble burying - 30 minutes, test of social interaction, (5+5 minutes) test "Light-dark box" - 3 minutes, "Elevated plus maze" - 5 minutes, forced swim test - 10 minutes were performed. After a series of experiments, samples of various brain structures were taken from the animals for subsequent chromatographic evaluation of the content of the main neurotransmitters and their metabolites.

The circular open-field test was used to analyze exploratory activity. The apparatus consisted of a gray plastic circular arena (63 cm diameter) with 13 holes in the floor (1.6 cm hole diameter) and lighting conditions of 200 lux. Each mouse was placed in the center of the arena and spontaneous exploratory activity was recorded using Noldus Ethovision video tracking software for 5 min. The following behavioral parameters were monitored: total distance traveled, cumulative duration in the central area, number of hind paw lifts, and number of burrow explorations. The elevated plus maze test was used to measure the level of anxious behavior. The maze consisted of two opposite open (30 x 5 cm) and two opposite closed arms (30 x 5 x 15 cm) elevated 40 cm from the floor. Mice were placed in the center of the elevated plus maze to the closed arm and recorded for 5 min. The following parameters were analyzed using Noldus Ethovision software: cumulative duration in the open arms, frequency of entries into the open arms, total distance traveled, number of rearings and head dippings.

The dark-light box test was used to analyze the anxious behavior of animals. The apparatus consisted of two chambers [20 cm (L) × 20 cm (W) × 20 cm (H)] each. One of the chambers was brightly illuminated while the other remained dark. The chambers were connected by hole that allowed the mice to move freely between the chambers. Each mouse was placed in the bright part of the apparatus in the direction away from the dark chamber and allowed to explore the apparatus for 3 min. The time of delay in entering the dark chamber and the time of delay in returning to the light chamber were recorded, as well as the total time spent in the light chamber and the number of entries into the light chamber.

In the marble burying test, each mouse was placed for 30 min in a Plexiglas cage of size [35 cm (L) × 19 cm (W) × 14 cm (H)] filled with 5 centimeters of fresh sawdust bedding with five rows of three balls in each. The number of buried balls was counted after 30 min of the experiment. The balls were considered buried if at least two-thirds of their surface was covered by the bedding. The forced swim test was used to assess depression-like behavior. Each mouse was individually placed in a Plexiglas cylinder (diameter: 10 cm, height: 21 cm) filled 2/3 with water. The water temperature was within 24°C ± 1°C. The test was carried out for 10 min. The water level was deep enough (18 cm) so that the mouse tail did not touch the bottom. After bathing, mice were removed from the water, wiped dry with a towel, returned to their home cage and left in a warm room. The total time of immobilization, active and passive swimming was estimated.

Thermometry after 8-OH-DPAT injection. Male TAAR6-mutant mice (n=9) and wild-type (WT) mice (n=10) were used for the experiments. Mice received food and water ad libitum and were maintained under standard laboratory conditions under a 12-hour light/dark cycle. One hour before experiments, mice were habituated to the experimental room. (±)-8-OH-DPAT hydrobromide ((±)-2-Dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene, Sigma-Aldrich) was dissolved in physiological saline. All injections were performed at a volume of 10 mL/kg. Mice were divided into 4 groups: WT mice - saline solution; WT mice - 5 mg/kg of 8-OH-DPAT solution; TAAR6 mutant mice - saline solution; TAAR6 mutant mice - 5 mg/kg of 8-OH-DPAT solution. The researcher was blinded during the experiments. The experiments were performed in the following order: first rectal temperature measurement, intravenous injection of 8-OH-DPAT, and second rectal temperature measurement 30 min later. The temperature difference was calculated by subtracting the result of the first measurement from the result of the second measurement in each group.

The Mann-Whitney test was used to process the obtained data. Differences were considered statistically significant at values $p<0.05$. Statistical processing of data was performed using the GraphPad Prism software.

Results.

Open field.

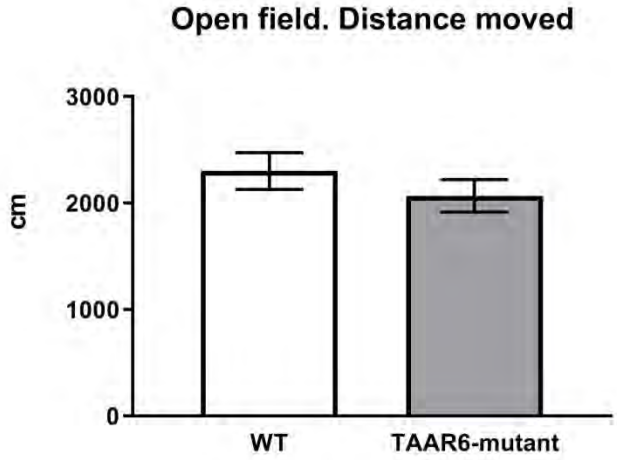


Figure 43. Distance traveled by mice in the open-field test. TAAR6 mutant mice did not differ in the distance traveled in the open field from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=41$). The graph shows mean ± S.E.M.

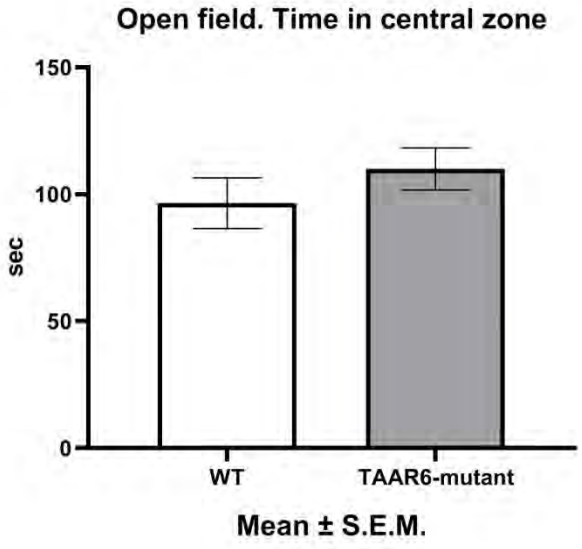


Figure 44. Time spent in the central zone when performing the Open Field test. TAAR6-mutant mice did not differ in the time spent in the central zone from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=39$).

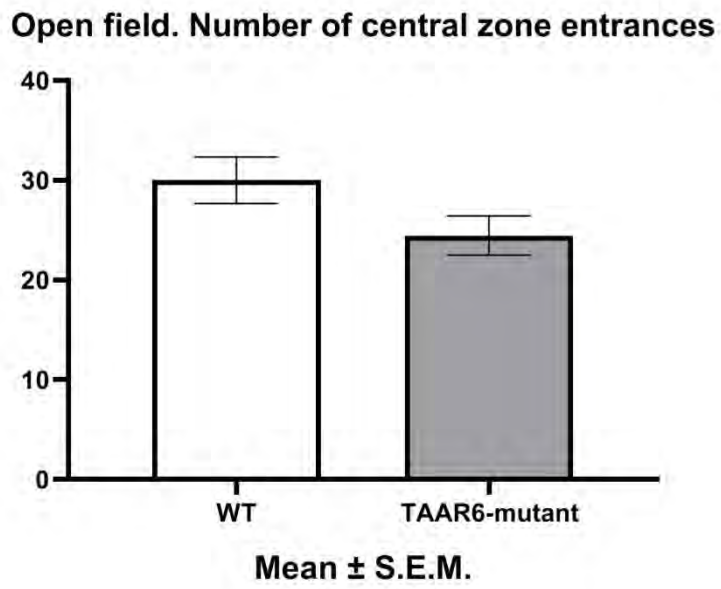


Figure 45. Number of entrances to the central zone during the Open Field test. TAAR6-mutant mice did not differ in the number of entrances to the central zone of the "Open Field" from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=32$).

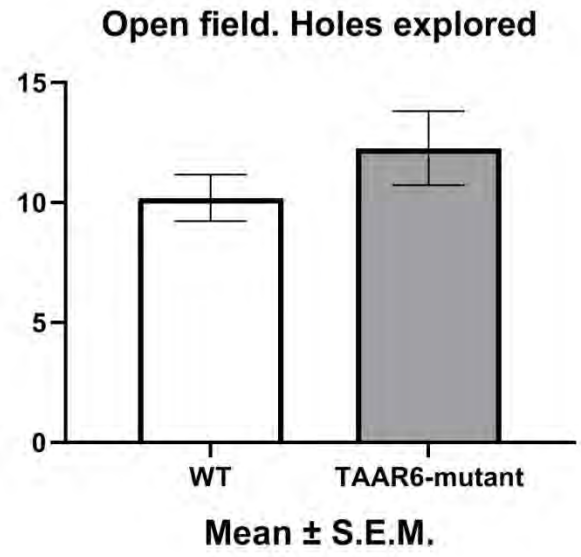


Figure 46. Number of explored holes in the Open Field test. TAAR6-mutant mice did not differ in the number of explored holes from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=41$).

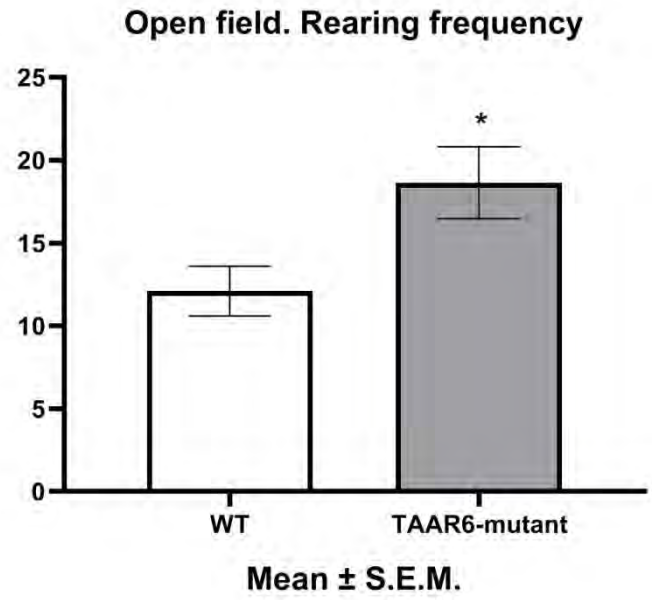


Figure 47. Number of rearings during the open field test. TAAR6 mutant mice lifted their hind legs more often compared to wild-type mice, Mann-Whitney test, p=0.0475 (U=27).

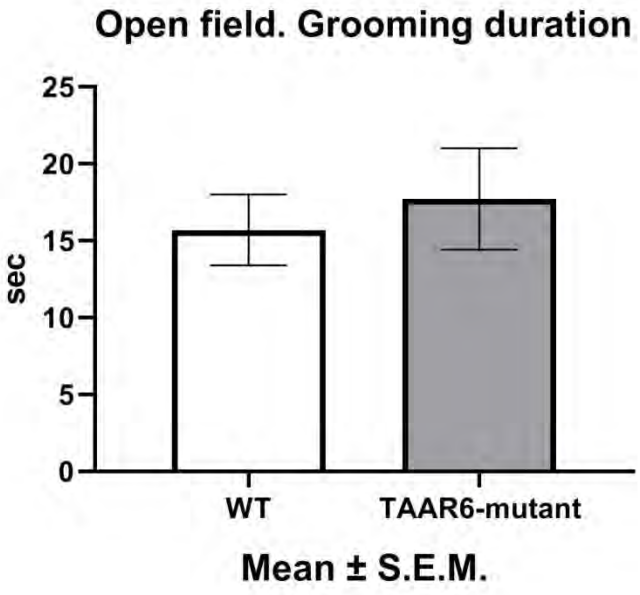


Figure 48. Duration of grooming during performance of the Open Field test. TAAR6-mutant mice did not differ in grooming duration during the "Open Field" test from wild-type mice, Mann-Whitney test, p>0.05 (U=47).

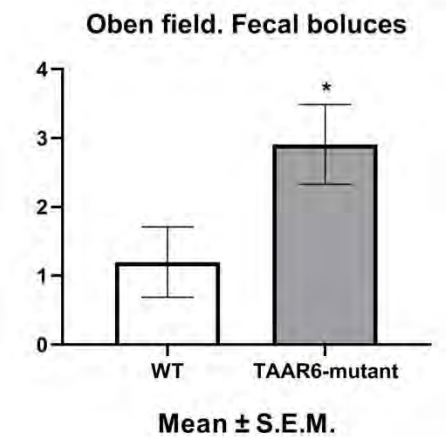


Figure 49. Number of fecal boluses in the open-field test. TAAR6-mutant mice left more fecal boluses compared to wild-type mice, Mann-Whitney test, p=0.0267 (U=25).

In the Open Field test, an increased number of hind leg lifts was detected in mice mutant for the TAAR6 gene, as shown in Figure 47. This indicator is interpreted by researchers as a manifestation of the animals' exploratory activity [146]. At the same time, the groups of animals did not differ in another parameter of exploratory behavior - the number of holes examined, which is shown in Figure 46. Time spent in the central zone and the number of entries, duration of grooming and distance traveled did not differ between groups (Figures 43, 44, 45 and 48). The number of fecal boluses was elevated in TAAR6-mutant mice compared to controls, as shown in Figure 49.

Digging

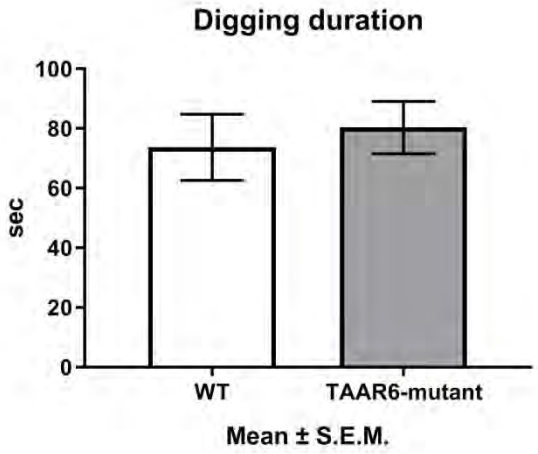


Figure 50. Digging duration in the digging test. TAAR6-mutant mice did not differ in digging duration from wild-type mice, Mann-Whitney test, p>0.05 (U=47).

Figure 50 shows that there are no differences between groups digging duration in digging test.

Marble burying

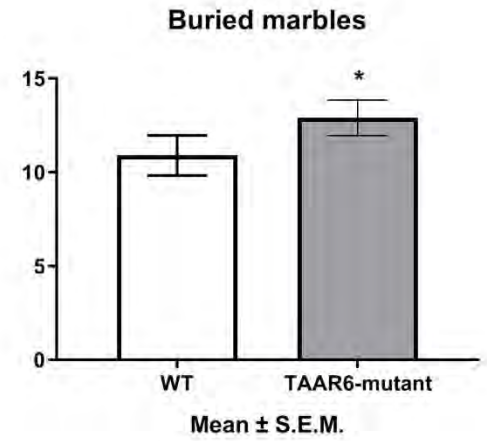


Figure 51. Number of buried marbles. TAAR6 mutant mice buried more beads compared to wild-type mice, Mann-Whitney test, p=0.0141 (U=21.5).

In the TAAR6-mutant animals buried more marbles in the marble burying test compared to the control group (shown in Figure 51). This indicator is often interpreted as a manifestation of anxious or compulsive behavior of animals, but there are also alternative opinions on the interpretation of the test results [147].

Light-dark box

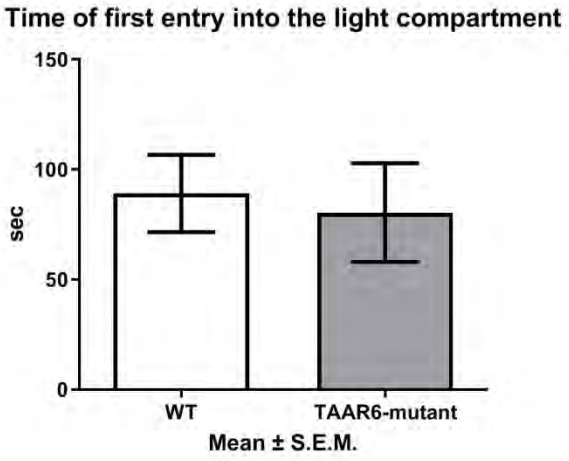


Figure 52. Time of first entry into the light chamber in the light-dark box test. TAAR6 mutant mice did not differ in the time to enter the light compartment from wild-type mice, Mann-Whitney test, p>0.05 (U=41).

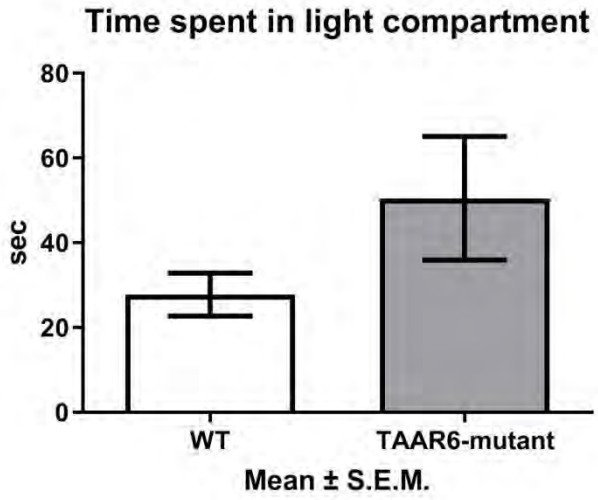


Figure 53. Time spent in the light chamber in the light-dark box test. TAAR6 mutant mice did not differ in time spent in the light chamber from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=32$).

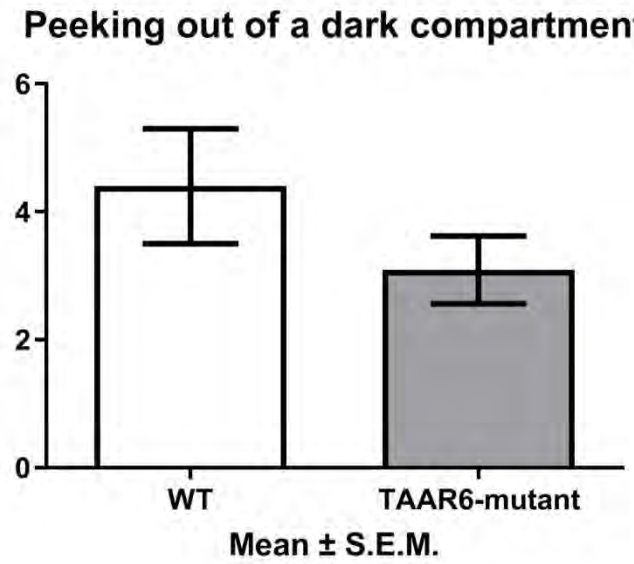


Figure 54. Number of peeking out of the dark compartment in the light-dark box test. TAAR6 mutant mice did not differ in the number of looks out of the dark compartment from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=40.5$).

None of the measured parameters in the Light-Dark Box test were changed in TAAR6-mutant mice, as shown in Figures 52-54.

T-maze

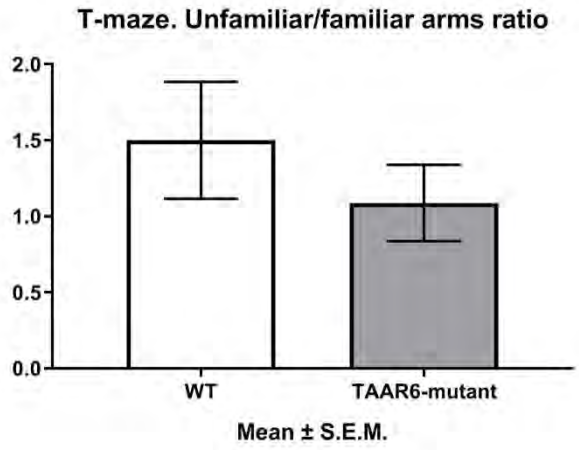


Figure 55. Ratio of time spent in unfamiliar arms of the T-maze to time spent in familiar arms of the maze. TAAR6-mutant mice did not differ in this parameter from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=43$).

In this short-term memory test, there were no differences between the animal groups, graph is shown in Figure 55.

Elevated plus maze

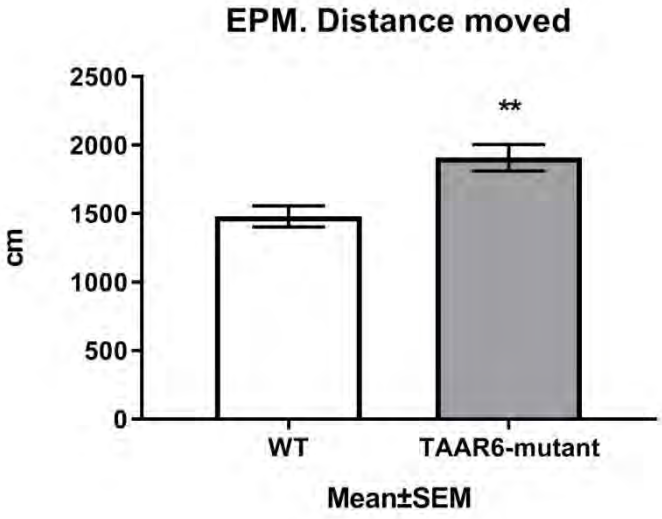


Figure 56. Distance moved by mice in the Elevated Plus Maze test. TAAR6-mutant mice traveled a greater distance compared to wild-type mice, Mann-Whitney test, $p=0.0028$ ($U=14$).

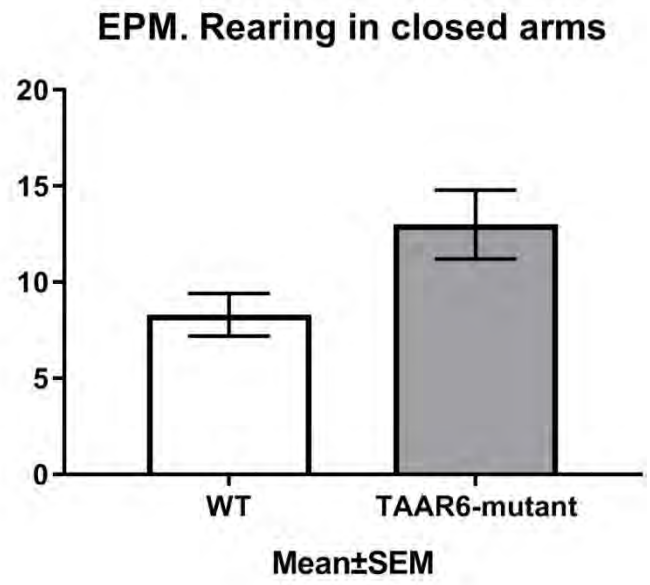


Figure 57. Number of rearings in closed arms during performance of the elevated plus maze test. There were no statistically significant differences in the number of rearings, although there was a tendency to increase this index in TAAR6-mutant mice, Mann-Whitney test, p=0.0847 (U=30.5).

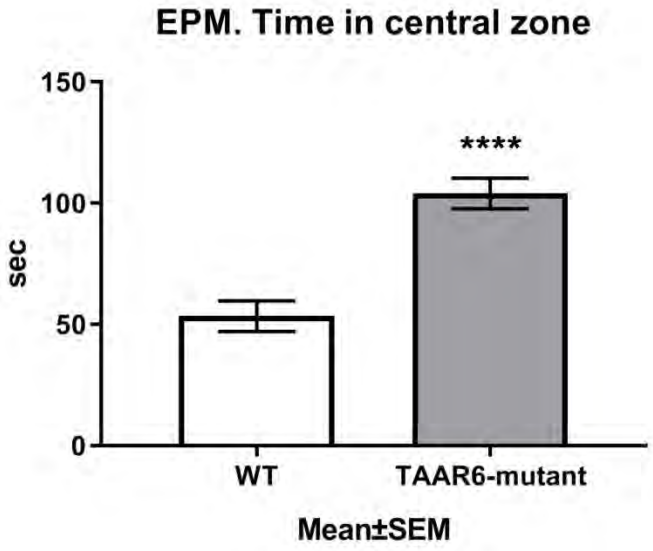


Figure 58. Time spent in the central zone in elevated plus maze test. TAAR6-mutant mice spent more time in the central zone compared to wild-type mice, Mann-Whitney test, p<0.0001 (U=3).

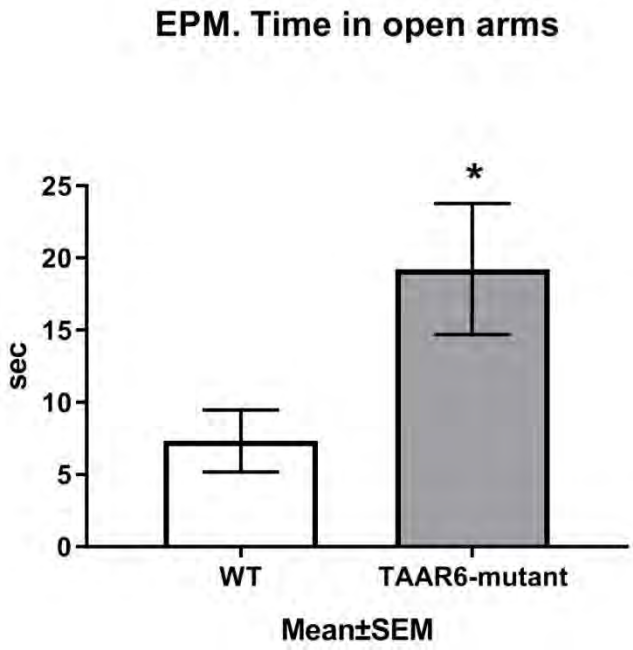


Figure 59. Time spent in the open arms of the Elevated Cruciform Maze. TAAR6-mutant mice spent more time in the open arms of the maze compared to wild-type mice, Mann-Whitney test, p=0.0166 (U=21.5).

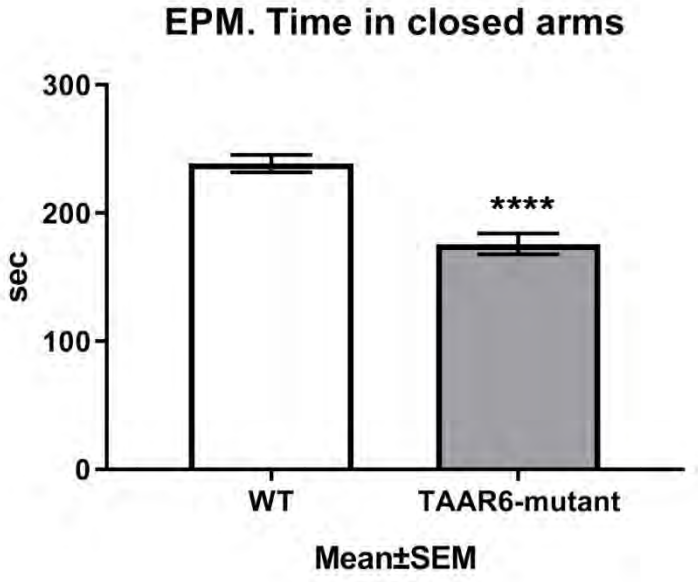


Figure 60. Time spent in the closed beams of the Elevated Plus Maze. TAAR6-mutant mice spent less time in the closed arms of the maze compared to wild-type mice, Mann-Whitney test, p<0.0001 (U=3).

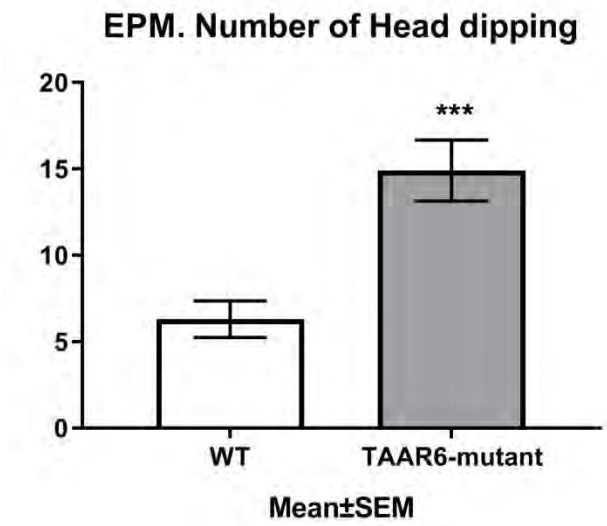


Figure 61. Number of head dippings from open arms of the Elevated Plus Maze. TAAR6-mutant mice made more head dippings compared to wild-type mice, Mann-Whitney test, p=0.0003 (U=7.5).

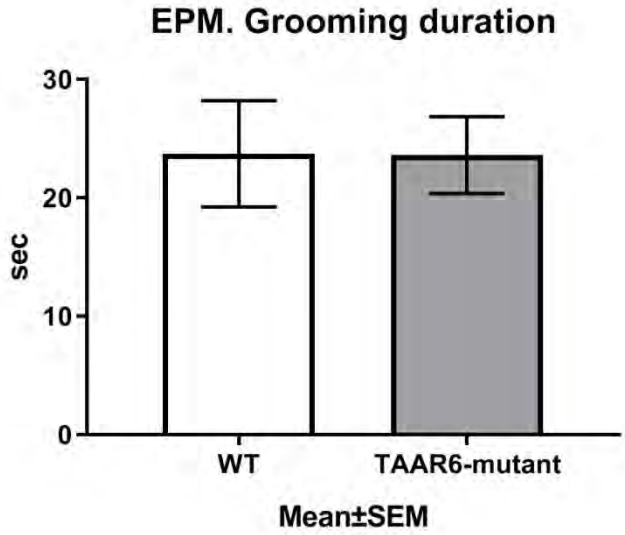


Figure 62. Duration of grooming in the elevated cruciform maze test. TAAR6-mutant mice did not differ in grooming duration from wild-type mice, Mann-Whitney test, p>0.05 (U=54).

TAAR6 mutant mice compared to wild-type mice spent more time in open arms and the central zone of the elevated plus maze, as well as made more head dippings from open arms (Figures 58, 59, and 61). TAAR6-mutants had a increased time spent in opened arms (Figure 60). Interestingly, the distance traveled was increased in TAAR6-mutants, possibly it can be an indicator of increased exploratory activity (Figure 56). There were no differences between the groups in the number of rearings and duration of grooming, as shown in Figures 57 and 62.

Forced swim test

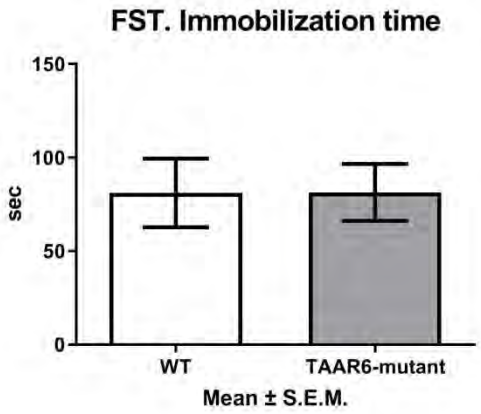


Figure 63. Immobilization time in the forced swim test. TAAR6-mutant mice did not differ in immobilization time in the Porsolt test from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=53$).

According to the results of the Porsolt test, no differences in the duration of immobilization were found between the groups of animals (Figure 63).

Thermometry after 8-OH-DPAT injection

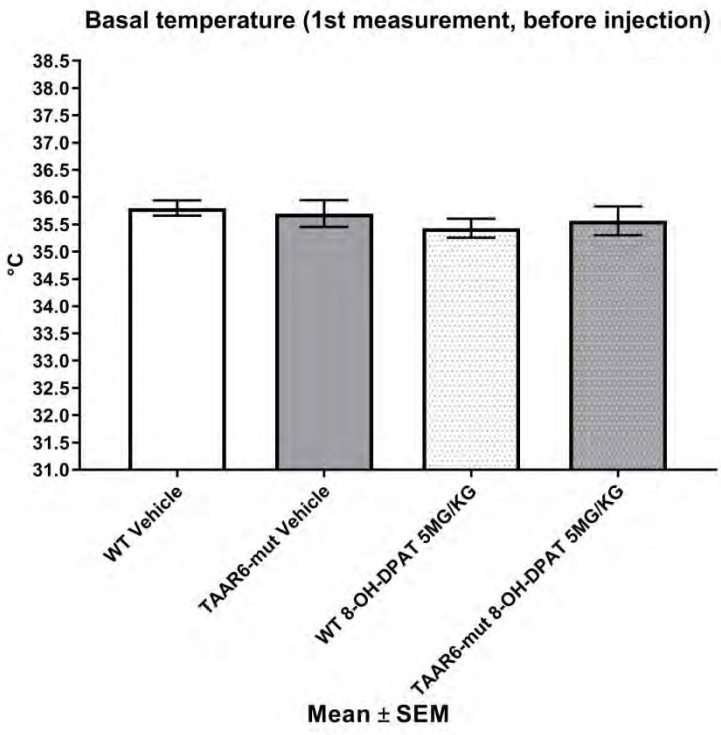


Figure 64. Pre-injection temperature. All groups did not differ in basal temperature, Bonferroni's multiple comparisons test, $p>0.9999$ for all possible comparisons.

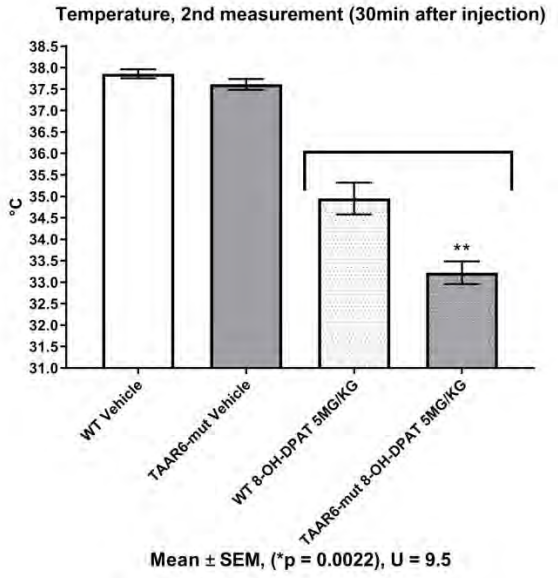


Figure 65. Temperature 30 minutes after injection. TAAR6 mutants had a lower temperature after 8-OH-DPAT injection compared to wild-type mice, Mann-Whitney test, p=0.0022 (U=9.5).

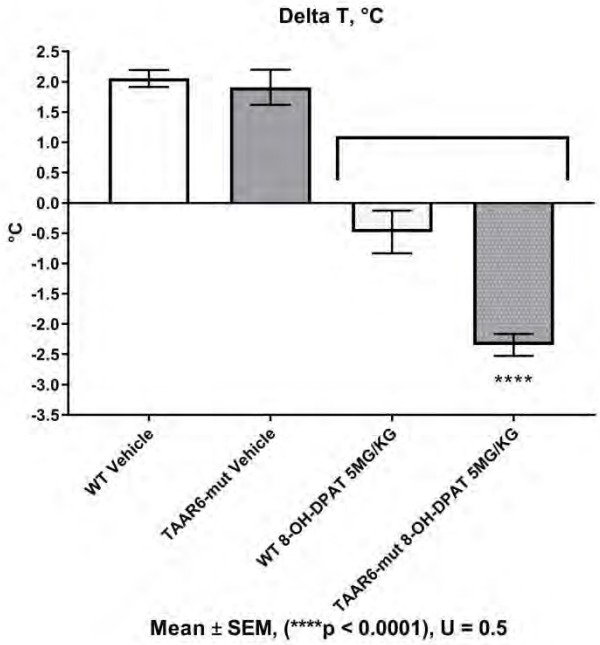


Figure 66. Difference from initial temperature 30 min after injection. TAAR6-mutant mice had a stronger decrease in temperature after 8-OH-DPAT injection compared to wild-type mice, Mann-Whitney test, p<0.0001 (U=0.5).

The study of hypothermic effects of the 5HT1A agonist 8-OH-DPAT revealed an enhanced hypothermic effect of the drug in TAAR6 gene mutant mice (Figures 64-66). However, the temperature increase (stress-induced hyperthermia) did not differ between wild type and mutants. Basal temperature at first measurement was also not different between the groups.

5. Measurement of monoamines in different brain regions of TAAR6-mutant mice

Materials and methods.

High-performance liquid chromatography was performed to investigate the content of serotonin, noradrenaline, dopamine, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, and homovanillic acid in various brain structures. For sampling, was made a cervical dislocation, decapitation, and then the frontal cortex, striatum, hypothalamus, and hippocampus were dissected briefly on ice. Samples were immediately frozen in liquid nitrogen and stored at -80°C. Samples for analysis were homogenized in 0.1 M HClO4, centrifuged (10 min, +4 °C; 14,000 g), and washed using centrifuge flotation devices (PVDF membrane, 0.22 μm pore size, Millipore). Monoamine content of tissue samples was measured using high-performance liquid chromatography with electrochemical detection (Eicom, HTEC-500, Japan) with a WE-3G carbon electrode (Eicom, Japan) at an applied potential of +650 mV. The system was equipped with a CA-50DS reversed-phase column (150 × 2.1 mm, Eicom, Japan) with a flow rate of 200 μL/min. The mobile phase contained 100 mM sodium phosphate buffer, 0.17 mM EDTA, 1.8 mM sodium salt of octanesulfonic acid and 18% (vol/vol) methanol, pH 4.5. All peaks obtained were normalized to the internal standard 3,4-dihydroxybenzylamine, and final values of monoamine and metabolite levels were expressed as nanograms per milligram of wet tissue weight. Statistical processing of data was performed using Mann-Whitney test in GraphPad Prism, significant differences were considered at $p<0.05$. 11 adult wild-type C57Black/6j mice and 14 TAAR6 mutant mice of the same litter and age were used for this study. Animals were kept 3-5 heads in Plexiglas cages, water and food were provided in unlimited amount for the whole time of keeping, light regime was set as 12 hours of light/12 hours of darkness, brain sampling took place in the second half of the light phase. The temperature in the animal housing room and in the experimental room ranged between 20-23 degrees Celsius, the relative humidity in the rooms was maintained between 30-70%. Brain sampling and chromatographic analysis of the content of monoamines and their metabolites were performed jointly with senior researcher of the Laboratory of Neuroscience and Molecular Pharmacology Evgenia Efimova, PhD, and junior researcher Mikael Mor.

Results.

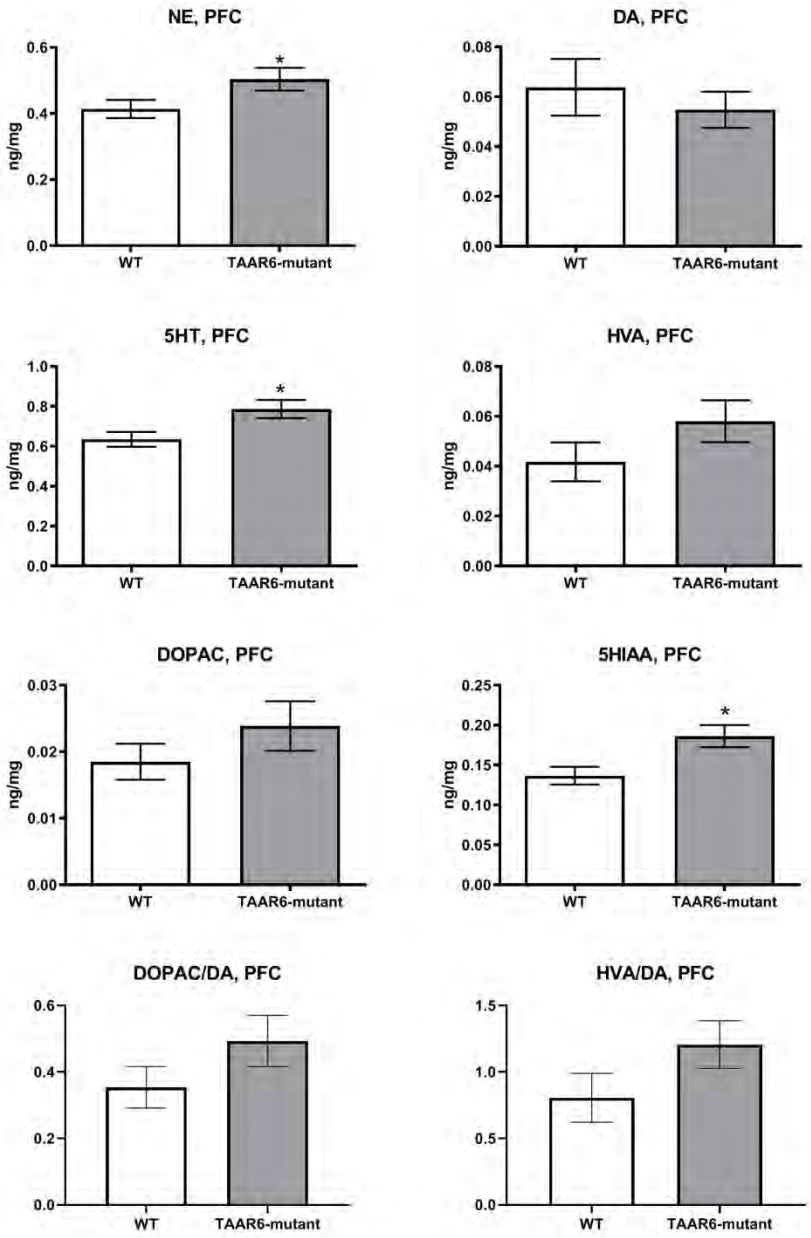


Figure 67. Levels of monoamines and their metabolites in the prefrontal cortex of TAAR6-mutant and wild-type animals. Wild type, n=11; TAAR6-mutant, n=13. The graphs show mean ± S.E.M.

The analysis revealed an increase in norepinephrine (U=37, p=0.0474), serotonin (U=33, p=0.0257), and 5-hydroxyindoleacetic acid (U=33, p=0.0257) in the prefrontal cortex of TAAR6 mutant animals compared to wild-type animals. No statistically significant differences were found for the other measured parameters (p>0.05). The graphs are shown in Figure 67.

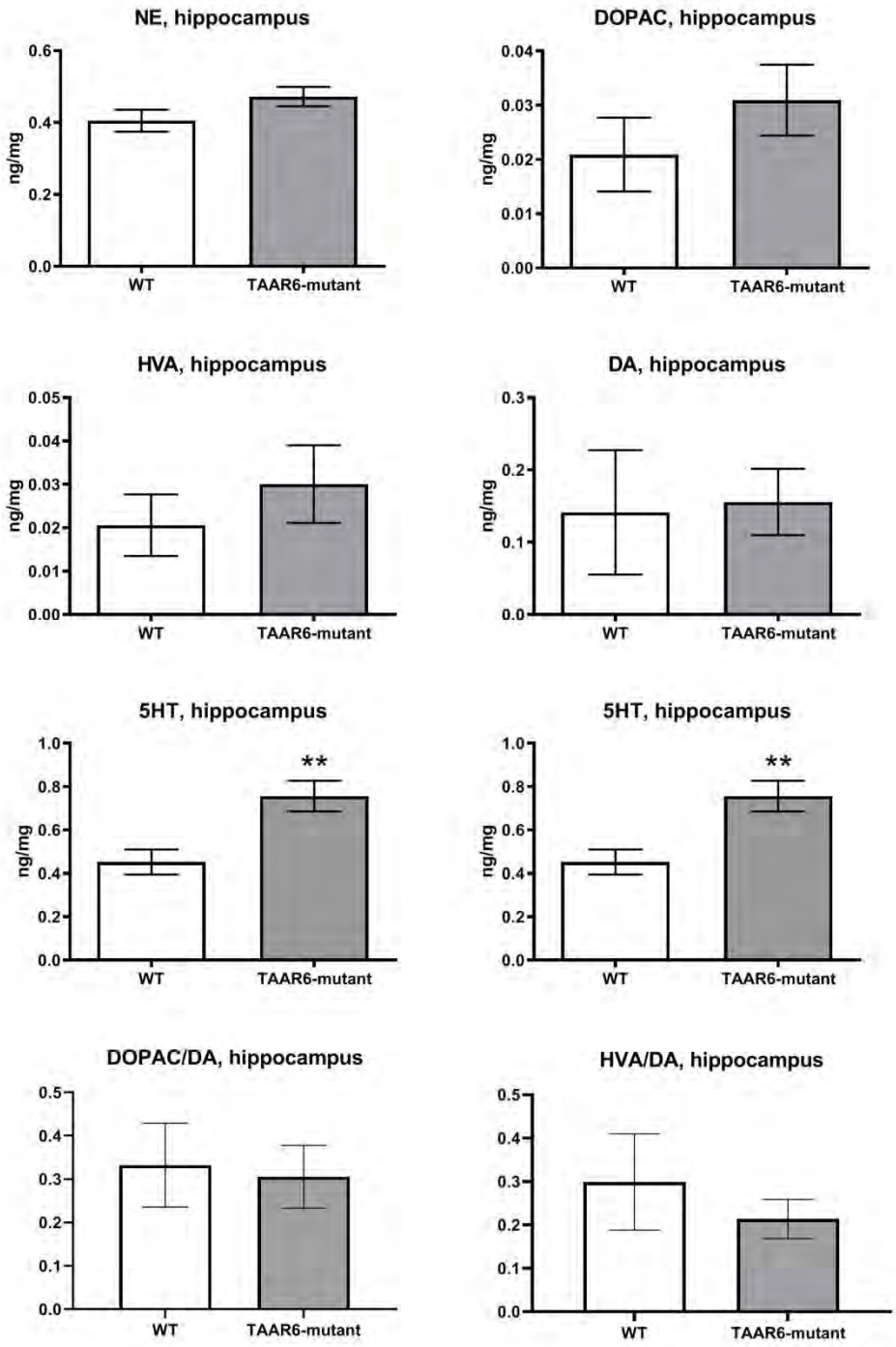


Figure 68. Levels of monoamines and their metabolites in the hippocampus of TAAR6-mutant and wild-type animals. Wild type, n=7; TAAR6-mutant, n=9. The graphs show mean ± S.E.M.

The analysis revealed an increase in serotonin (U=7, p=0.0079) and 5-hydroxyindoleacetic acid (U=1, p=0.0003) in the hippocampus of TAAR6-mutants compared to wild-type animals. No statistically significant differences were found for the other measured parameters (p>0.05). The results are displayed in Figure 68.

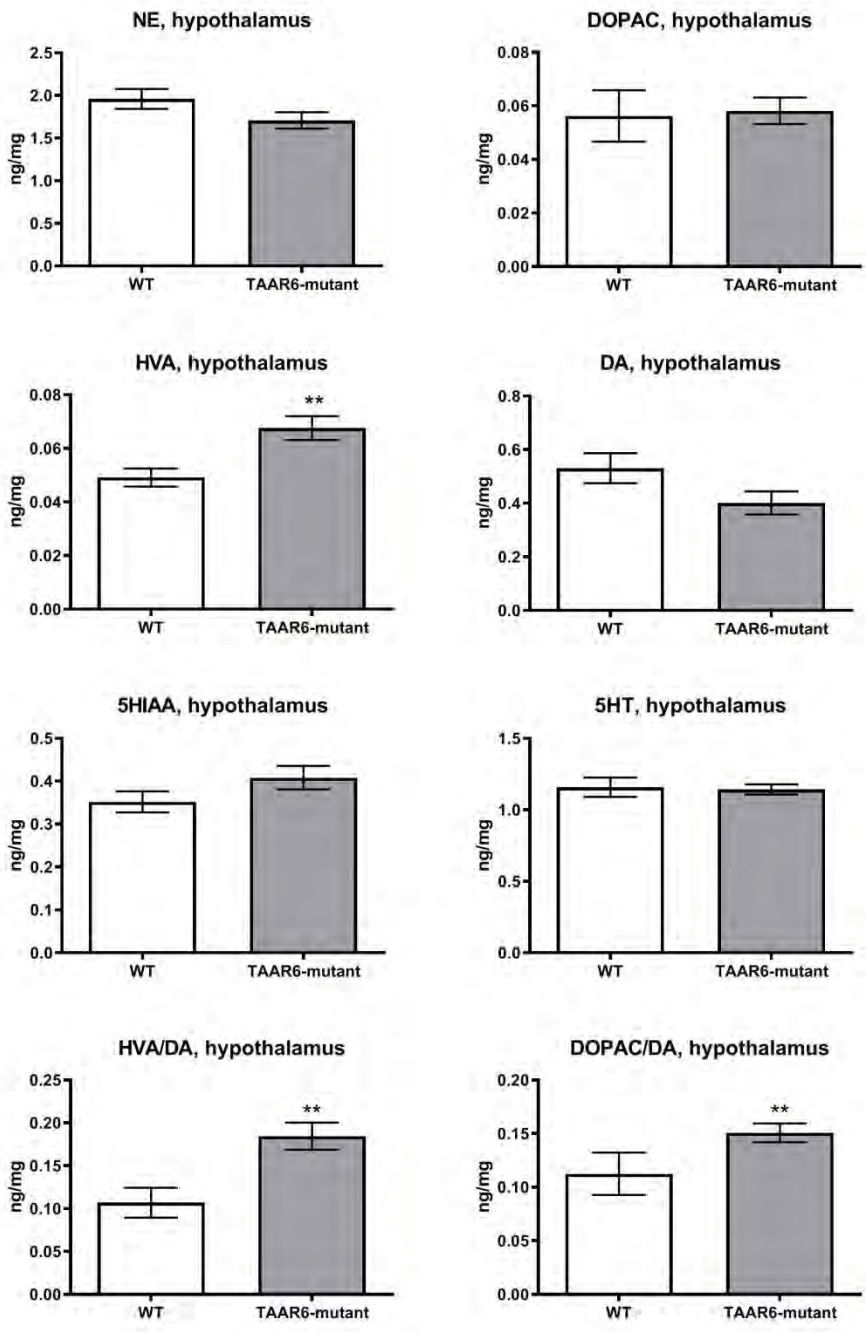


Figure 69. Levels of monoamines and their metabolites in the hypothalamus of TAAR6-mutant and wild-type animals. Wild type, n=11; TAAR6-mutant, n=14. The graphs show mean ± S.E.M.

The analysis revealed an increase in homovanillic acid (U=24, p=0.0028). The ratios of homovanillic acid to dopamine (U=21, p=0.0014) and 3,4-dihydroxyphenylacetic acid to dopamine (U=25, p=0.0034) were also increased in the hypothalamus of TAAR6 mutant animals compared to wild type animals. No statistically significant differences were recorded for the other measured parameters (p>0.05). Graphs with the results of the analysis are shown in Figure 69.

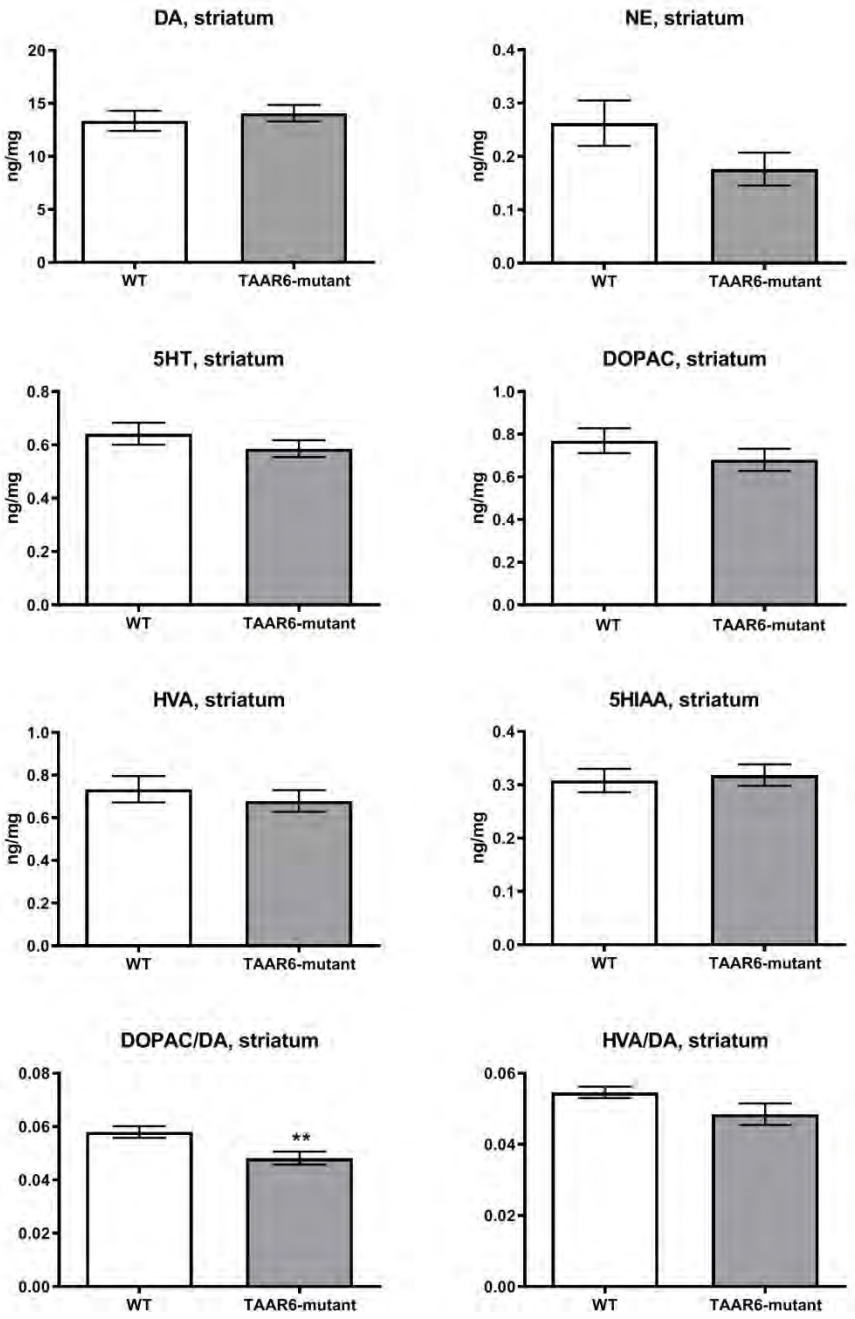


Figure 70. Levels of monoamines and their metabolites in striatum of TAAR6-mutant and wild-type animals. Wild type, n=9; TAAR6-mutant, n=8. The graphs show mean ± S.E.M.

The analysis revealed a decrease in the ratio of 3,4-dihydroxyphenylacetic acid to dopamine ($U=23$, $p=0.0039$) in the striatum of TAAR6 mutant animals compared to wild-type animals. There was also a trend towards a decrease in homovanillic acid to dopamine ($U=41$, $p=0.0821$). No statistically significant differences were recorded for the other measured parameters ($p>0.05$). The results are displayed in Figure 70.

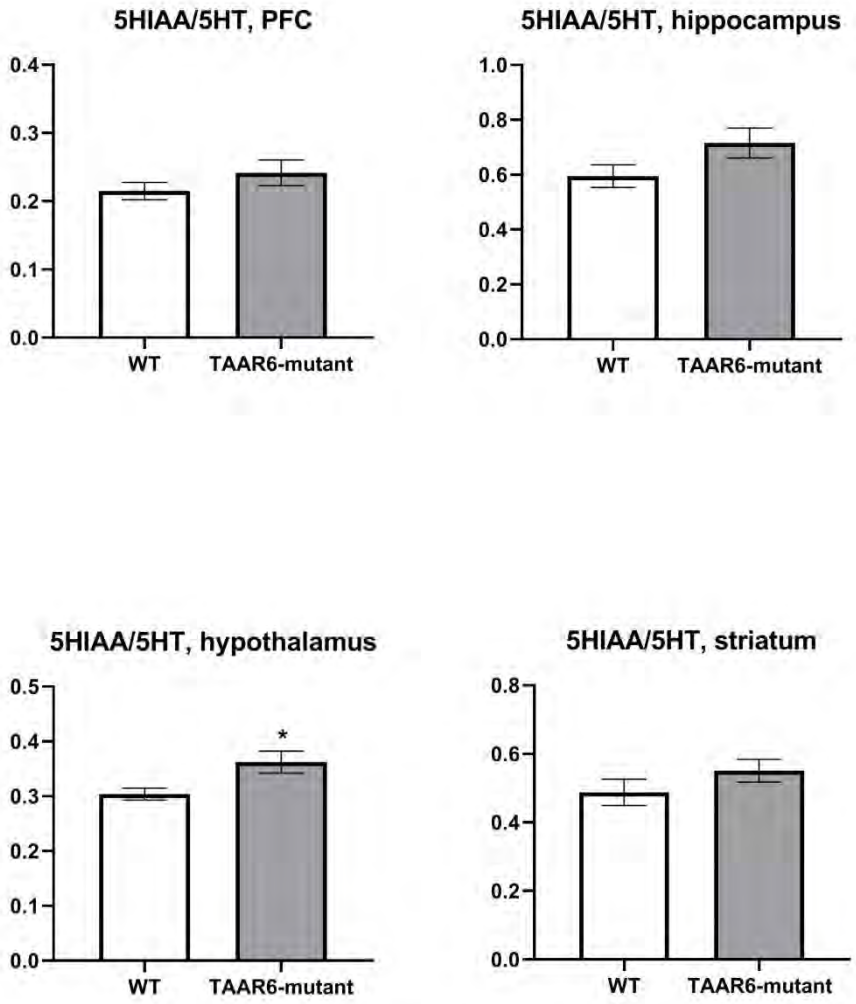


Figure 71. The ratio of 5-hydroxyindoleacetic acid to serotonin in each of the studied structures. The ratio of 5-hydroxyindoleacetic acid to serotonin was increased in the hypothalamus of TAAR6 mutant animals (U=29, p=0.0246). The graphs show mean ± S.E.M.

When analyzing the ratio of 5-hydroxyindoleacetic acid to serotonin, an increase in this parameter was detected in the hypothalamus of TAAR6-mutant mice. In the other structures studied, no differences in the 5HIAA/HT ratio were found between groups. The graphs are shown in Figure 71.

6. Behavioral screening of TAAR2-knockout mice

Materials and methods.

To identify possible differences in the behavior of TAAR2 knockout mice, 10 adult males of the wild-type (C57Black/6n) and 11 TAAR2 knockout males of the same litter and age were used. Animals were housed in groups of 3-5 mice in Plexiglas cages, water and food were provided ad libitum for the whole time except for the time of experiments. The light regime was set as 12 hours of light/12 hours of darkness, with experiments performed during the second half of the light phase. The temperature in the animal housing room and in the experimental room varied between 20-23 degrees Celsius, and the relative humidity in the rooms was maintained between 30-70%.

Mice were divided into 2 experimental groups of 10 and 11 males, respectively. At intervals of 1-3 days, the tests "Open field" - 5 minutes, test of recognition of a new object (5+5 minutes), test of digging - 3 minutes, test of marble burying - 30 minutes, test of social interaction, (5+5 minutes) test "Light-dark chamber" - 3 minutes, "Elevated plus maze" - 5 minutes, forced swimming test - 10 minutes were performed. After a series of experiments, samples of various brain regions were taken from the animals for subsequent chromatographic evaluation of the content of the main neurotransmitters and their metabolites.

The circular open-field test was used to analyze exploratory activity. The apparatus consisted of a gray plastic circular arena (63 cm diameter) with 13 holes in the floor (1.6 cm hole diameter) and lighting conditions of 200 lux. Each mouse was placed in the center of the arena and spontaneous exploratory activity was recorded using Noldus Ethovision video tracking software for 5 min. The following behavioral parameters were monitored: total distance traveled, cumulative duration in the central area, number of rearings, and number of burrow explorations. The elevated plus maze test was used to measure the level of anxious behavior. The maze consisted of two opposite open arms (30 x 5 cm) and two opposite closed arms (30 x 5 x 15 cm) elevated 40 cm from the floor. Mice were placed in the center of the elevated plus maze towards the closed arm and recorded for 5 min. The following parameters were analyzed using Noldus Ethovision software: cumulative duration in the open arms, frequency of entries into the open arms, total distance traveled, number of hind paw lifts, number of hind paw hangs.

The social interaction test was used to quantify the social behavior of mice toward an unfamiliar male of the same strain, age, and weight kept in the group. The experimental setup was a Plexiglas box [35 cm (L) × 48 cm (W) × 19 cm (H)] divided into two parts by a white partition with a

small hole allowed free movement of the mouse between the two parts of the setup. A small perforated cage [11 cm (L) × 10 cm (W) × 10 cm (H)] was placed in each part. Mice were pre-trained to the apparatus with two empty cages for 5 min. The next day, an unfamiliar mouse was placed in one of the wired cages. For the test mice, the time spent in the area near the cages with and without mice and the time spent exploring the cages were recorded. The zone near the cage was marked at a distance of 8 cm from the cage. Cage exploration was recorded manually. Mice were considered to have explored a cage if they sniffed the cage and approached the cage.

The dark-light box test was used to analyze the anxiety-like behavior of animals. The apparatus consisted of two chambers [20 cm (L) × 20 cm (W) × 20 cm (H)] each. One of the chambers was brightly illuminated while the other remained dark. The chambers were connected by a passage that allowed the mice to move freely between the chambers. Each mouse was placed in the bright part of the apparatus in the direction away from the dark chamber and allowed to explore the apparatus for 3 min. The time of delay in entering the dark chamber and the time of delay in returning to the light chamber were recorded, as well as the total time spent in the light chamber and the number of entries into the light chamber.

In the marble burying test, each mouse was placed for 30 min in a Plexiglas cage of size [35 cm (L) × 19 cm (W) × 14 cm (H)] filled with 5 centimeters of fresh sawdust bedding with five rows of three marbles in each. The number of buried marbles was counted after 30 min of the experiment. The balls were considered buried if at least two-thirds of their surface was covered by the bedding. The forced swim test was used to assess depression-like behavior. Each mouse was individually placed in a Plexiglas cylinder (diameter: 10 cm, height: 21 cm) filled 2/3 with water. The water temperature was within 24°C ± 1°C. The test was carried out for 10 min. The water level was deep enough (18 cm) so that the mouse tail did not touch the bottom. After bathing, mice were removed from the water, wiped dry with a towel, returned to their home cage and left in a warm room. The total time of immobilization, active and passive swimming was estimated.

Horizontal locomotor activity of mice was assessed using the Noldus Ethovision program. Fifteen wild-type males and 21 TAAR2 knockout males were used for the test. The test was performed using an open-field square apparatus that was unfamiliar to the animals. Mice were placed in Plexiglas boxes measuring 40 cm (L) × 40 cm (W) × 40 cm (H) and their horizontal activity was recorded for 120 min. Bedding, food, and water were not provided to the animals in this test.

To evaluate the effects of GBR 12909, 8 wild-type and 7 TAAR2 knockout males were used. Injections were given at a volume of 10 ml/kg, and GBR 12909 was administered at a dose of 10

mg/kg. Males were placed in 40 cm (L) × 40 cm (W) × 40 cm (H) Plexiglas boxes, and locomotor activity was recorded using Noldus Ethovision software for 120 min after injection.

Mann-Whitney test or 2-way ANOVA followed by Bonferroni test for multiple comparisons were used to analyse the obtained data. Differences were considered statistically significant when $p<0.05$. Statistical processing of data was performed using the GraphPad Prism software.

Results.

Open field.

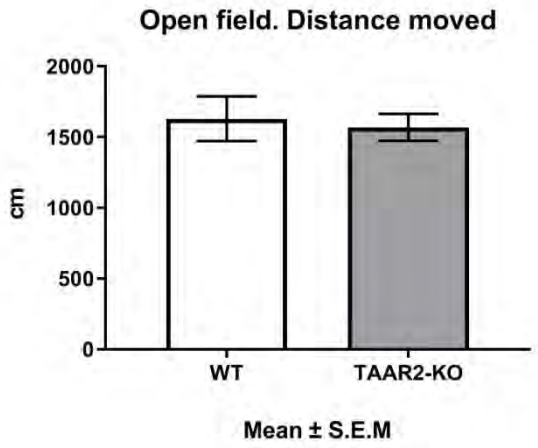


Figure 72. Distance moved in the open-field test. TAAR2-knockout mice did not differ in the distance traveled in the open field from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=45$).

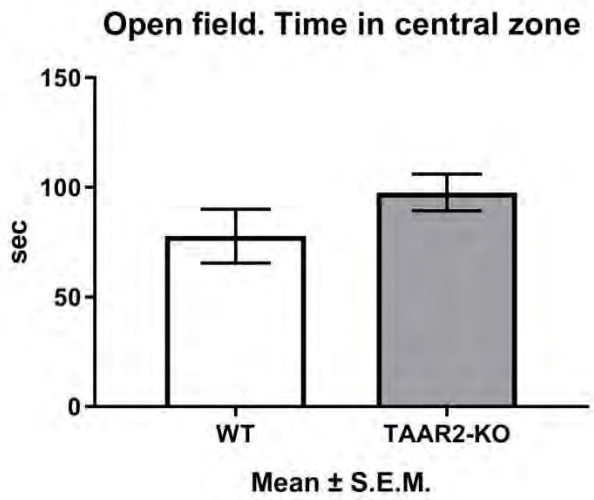


Figure 73. Time spent in the central zone in the open-field test. TAAR2-knockout mice did not differ in time spent in the central zone from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=37$).

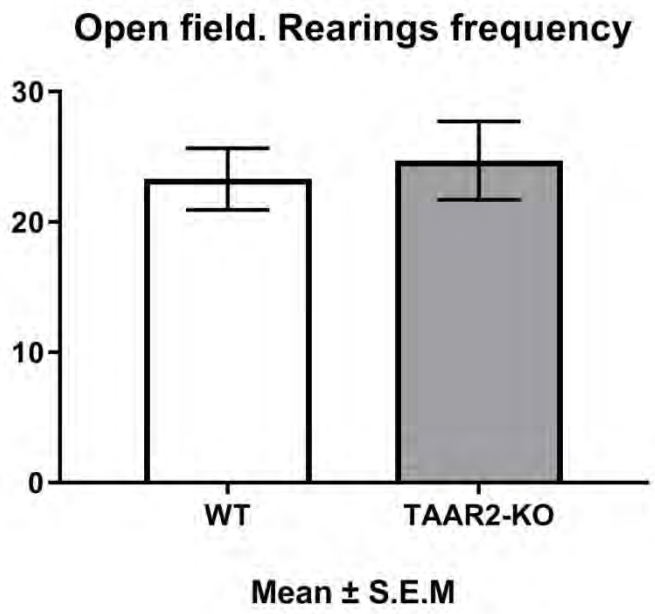


Figure 74. Number of rearings in the open field test. TAAR2-knockout mice did not differ in the number of rearings from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=44$).

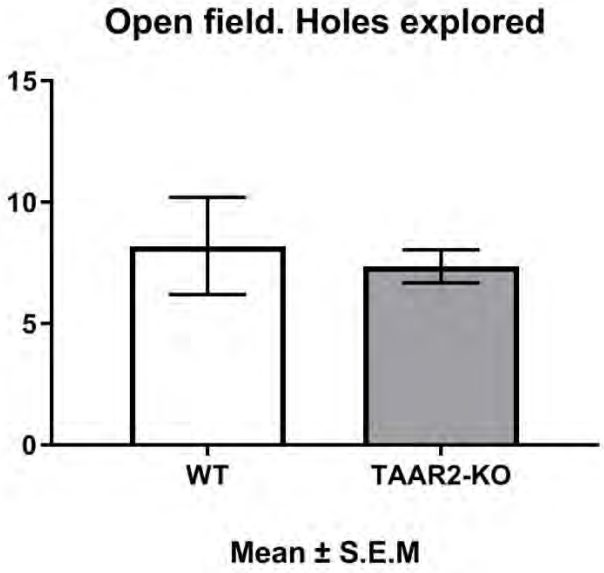


Figure 75. Number of explored holes in the open-field test. TAAR2-knockout mice did not differ in the number of explored holes from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=46$).

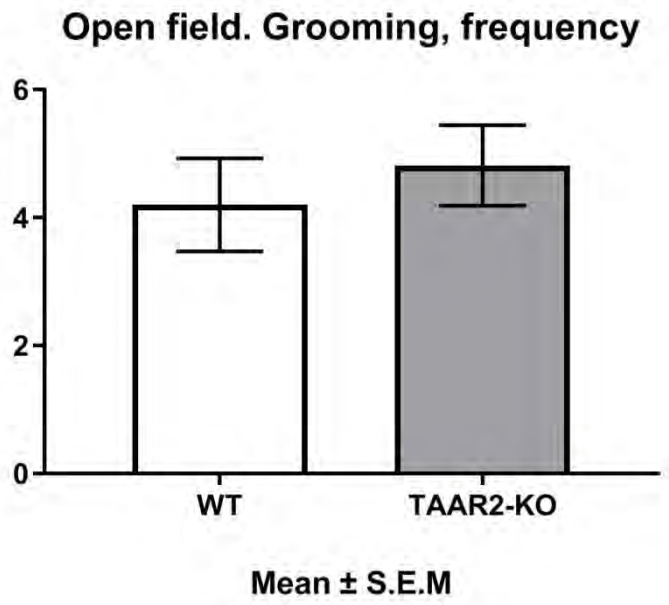


Figure 76. Grooming frequency in the open-field test. TAAR2-knockout mice did not differ in grooming frequency from wild-type mice, Mann-Whitney test, p>0.05 (U=45.5).

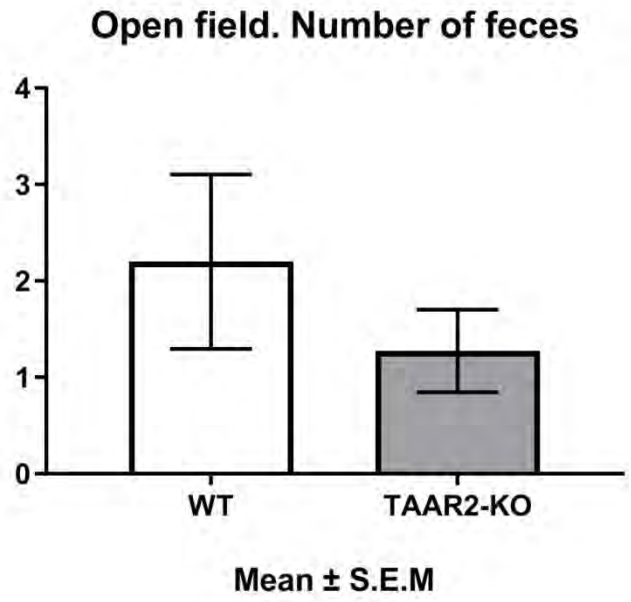


Figure 77. Number of fecal boluses in the open-field test. TAAR2-knockout mice did not differ in the number of fecal boluses from wild-type mice, Mann-Whitney test, p>0.05 (U=47).

TAAR2-knockout animals did not differ from wild-type animals in any of the studied parameters in the "Open Field" test, which is shown in Figures 72-77.

Marble burying test

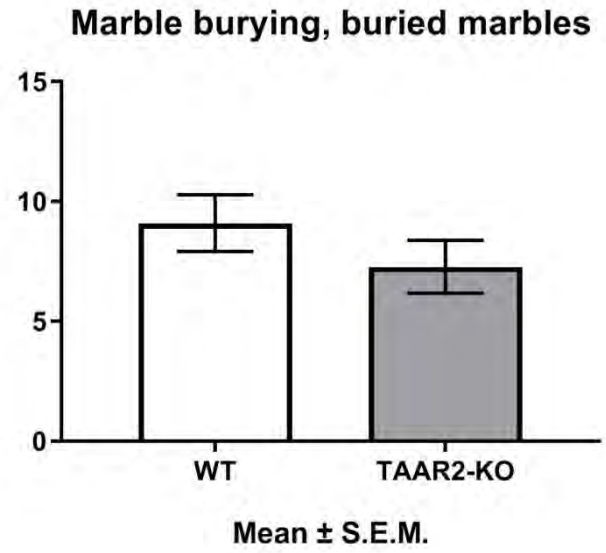


Figure 78. Number of buried marbles in the marble burying test. TAAR2-knockout mice did not differ in the number of buried marbles from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=38.5$).

Social interaction test

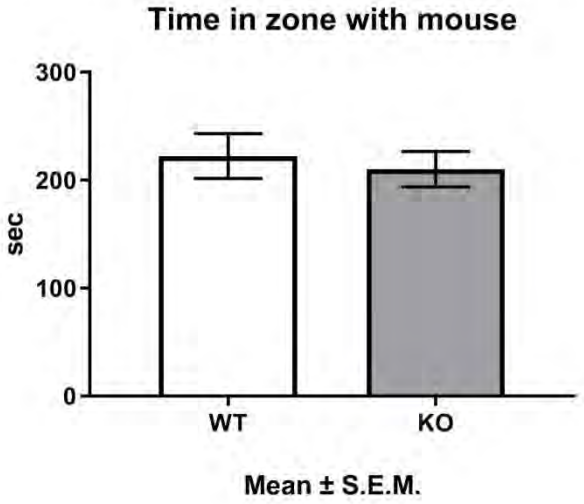


Figure 79. Time spent in the compartment with the mouse in the social interaction test. TAAR2-knockout mice did not differ in time spent in the compartment with the mouse from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=38$).

The marble burying test and the social interaction test showed no differences between groups (Figure 78-79).

Elevated plus maze

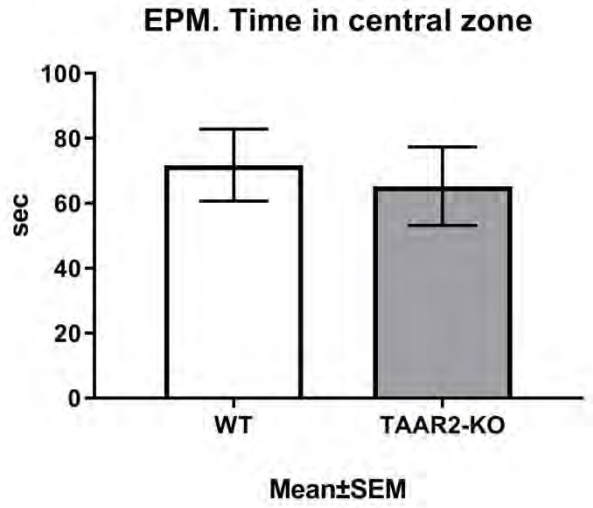


Figure 80. Time spent in the central zone in elevated plus maze test. TAAR2-knockout mice did not differ in time spent in the central zone from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=49$).

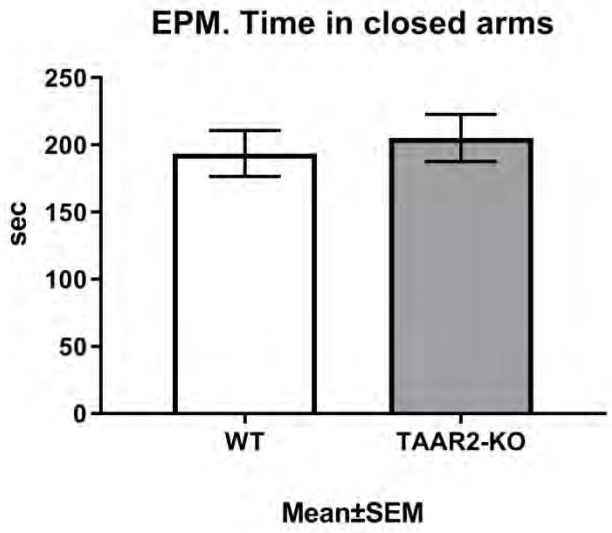


Figure 81. Time spent in closed arms when performing the elevated plus maze test. TAAR2-knockout mice did not differ in time spent in closed arms from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=50$).

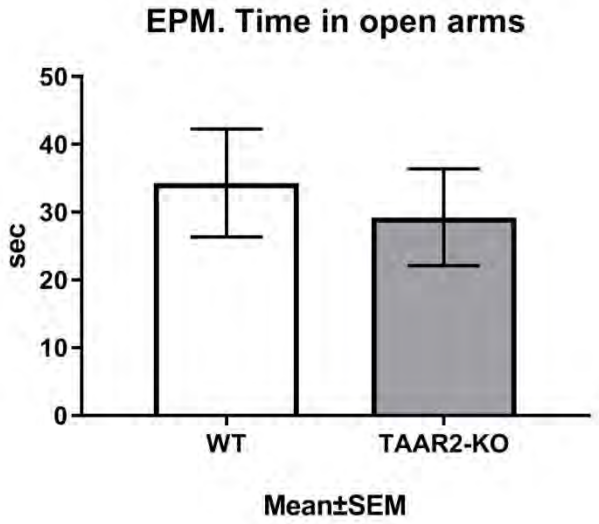


Figure 82. Time spent in open arms in elevated plus maze test. TAAR2-knockout mice did not differ in the time spent in open beams from wild-type mice, Mann-Whitney test, $p>0.05$ ($U=51$).

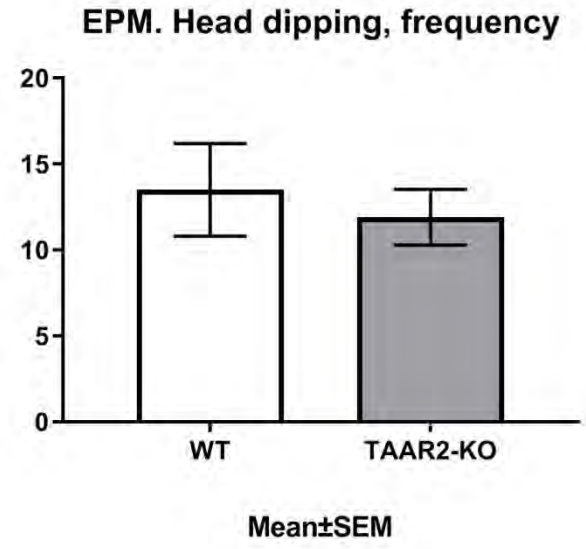


Figure 83. The number of head dipping from open arms in elevated plus maze test. TAAR2-knockout mice did not differ from wild-type mice in the number of open ray retractions, Mann-Whitney test, $p>0.05$ ($U=51$).

In the elevated plus maze test, there were no differences between wild-type and TAAR2 gene knockout animals in any of the parameters examined, as displayed in Figures 80-83.

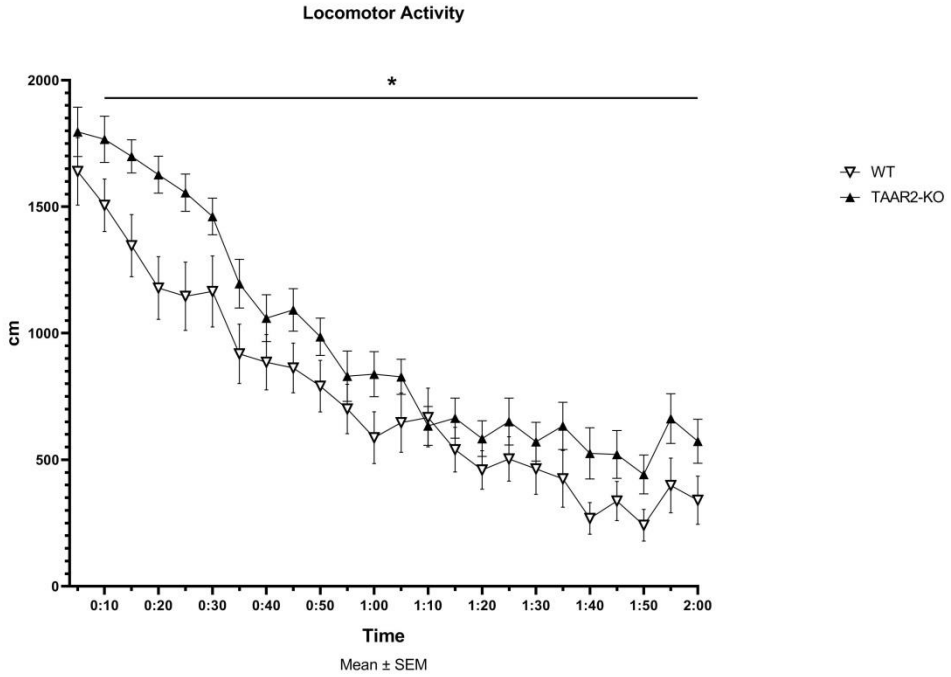


Figure 84. Distance traveled in the locomotor activity test. TAAR2-knockout mice traveled a statistically significant greater distance compared to wild-type mice, Two-way RM ANOVA, p=0.0205.

In the two-hour locomotor activity test, TAAR2-knockouts traveled a greater distance compared to wild-type animals (graph shown in Figure 84).

Evaluation of locomotor activity after injection of the DAT inhibitor GBR 12909

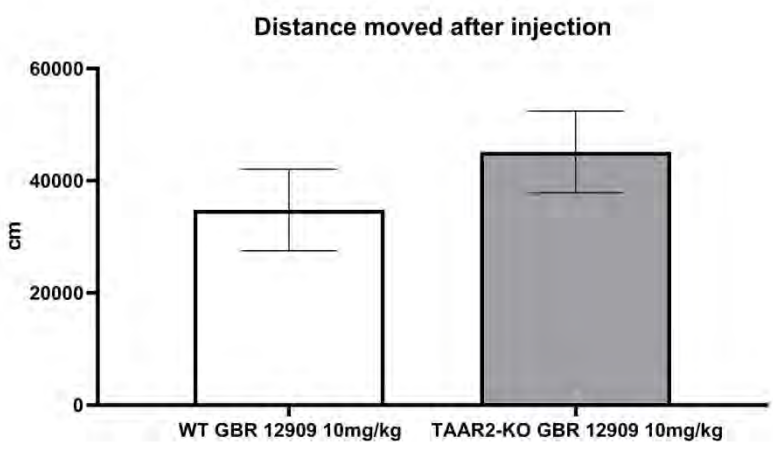


Figure 85. Distance moved in the locomotor activity test after GBR 12909 injection. TAAR2-knockout mice did not differ in distance traveled from wild-type mice, Mann-Whitney test, p=0.1893 (U=16).

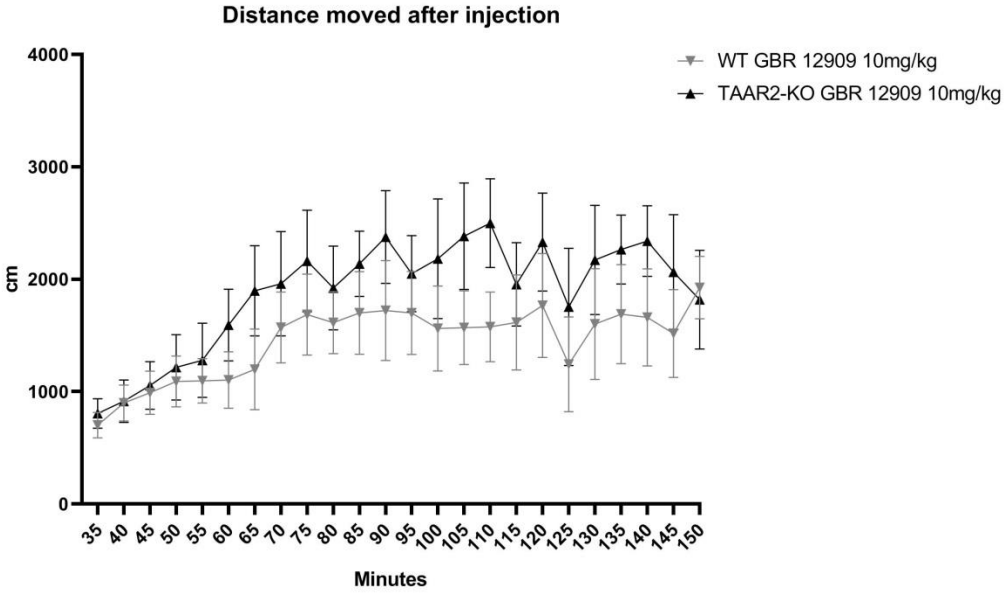


Figure 86. Distance moved after GBR12909 injection. No statistically significant differences were identified between TAAR2-knockout and wild-type mice, Bonferroni's multiple comparisons test, $p>0.05$ for all time intervals.

In the locomotor hyperactivity test induced by administration of the dopamine transporter inhibitor GBR 12909, there were no differences between the knockout group and wild-type animals, as shown in Figures 85-86.

7. Assessment of monoamine levels in the brain of TAAR2-knockout mice

Materials and methods.

High-performance liquid chromatography was used to investigate the content of serotonin, noradrenaline, dopamine, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, and homovanillic acid in various brain structures. For sampling, mice were cervical dislocation, decapitation, and then the frontal cortex, striatum, hypothalamus, and hippocampus were dissected briefly on ice. Samples were immediately frozen in liquid nitrogen and stored at -80°C. Samples for analysis were homogenized in 0.1 M HClO4, centrifuged (10 min, +4 °C; 14,000 g), and washed using centrifuge flotation devices (PVDF membrane, pore size 0.22 μm,Millipore). Monoamine content of tissue samples was measured using high-performance liquid chromatography with electrochemical detection (Eicom, HTEC-500, Japan) with a WE-3G carbon electrode (Eicom, Japan) at an applied potential of +650 mV. The system was equipped with a CA-50DS reversed-phase column (150 × 2.1 mm, Eicom, Japan) with a flow rate of 200 μL/min. The mobile phase contained 100 mM sodium phosphate buffer, 0.17 mM EDTA, 1.8 mM sodium salt of octanesulfonic acid and 18% (vol/vol) methanol, pH 4.5. All peaks obtained were normalized to the internal standard 3,4-dihydroxybenzylamine, and final values of monoamine and metabolite levels were expressed as nanograms per milligram of wet tissue weight. Statistical processing of data was performed by Mann-Whitney test, analysis was performed using GraphPad Prism statistical package, significant differences were considered at $p<0.05$. 9 adult wild-type C57Black/6n mice and 12 TAAR2 gene knockout mice of the same litter and age were used for this study. The animals were kept 3-5 heads each in Plexiglas cages, water and food were provided in unlimited amount for the whole time of keeping, light regime was set as 12 hours of light/12 hours of darkness, brain sampling took place in the second half of the light phase. The temperature in the animal housing room and in the experimental room ranged between 20-23 degrees Celsius, the relative humidity in the rooms was maintained between 30-70%.

Brain sampling and chromatographic analysis of the content of monoamines and their metabolites were performed jointly with senior researcher of the Laboratory of Neuroscience and Molecular Pharmacology Evgenia Efimova, PhD, and junior researcher Mikael Mor.

Results.

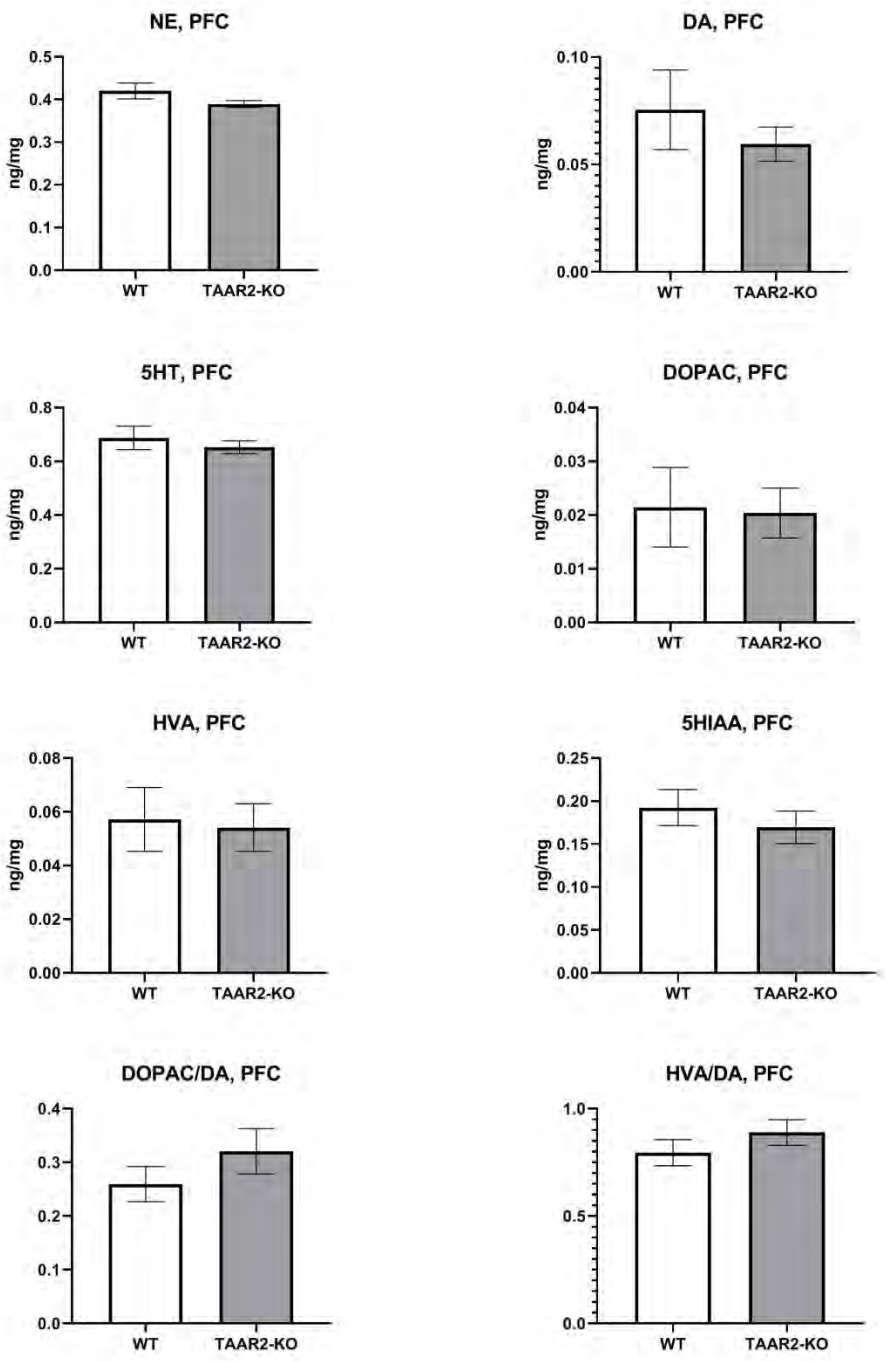


Figure 87. Levels of monoamines and their metabolites in the prefrontal cortex of TAAR2-knockout mice and wild-type mice. Wild type, n=6; TAAR2-KO, n=8. Graphs show mean ± S.E.M.

The analysis revealed no statistically significant differences in the levels of monoamines and their metabolites in the prefrontal cortex between TAAR2-knockout animals and wild-type animals (Mann-Whitney test, $U>11$, $p>0.05$ for all studied parameters), which is displayed in Figure 87.

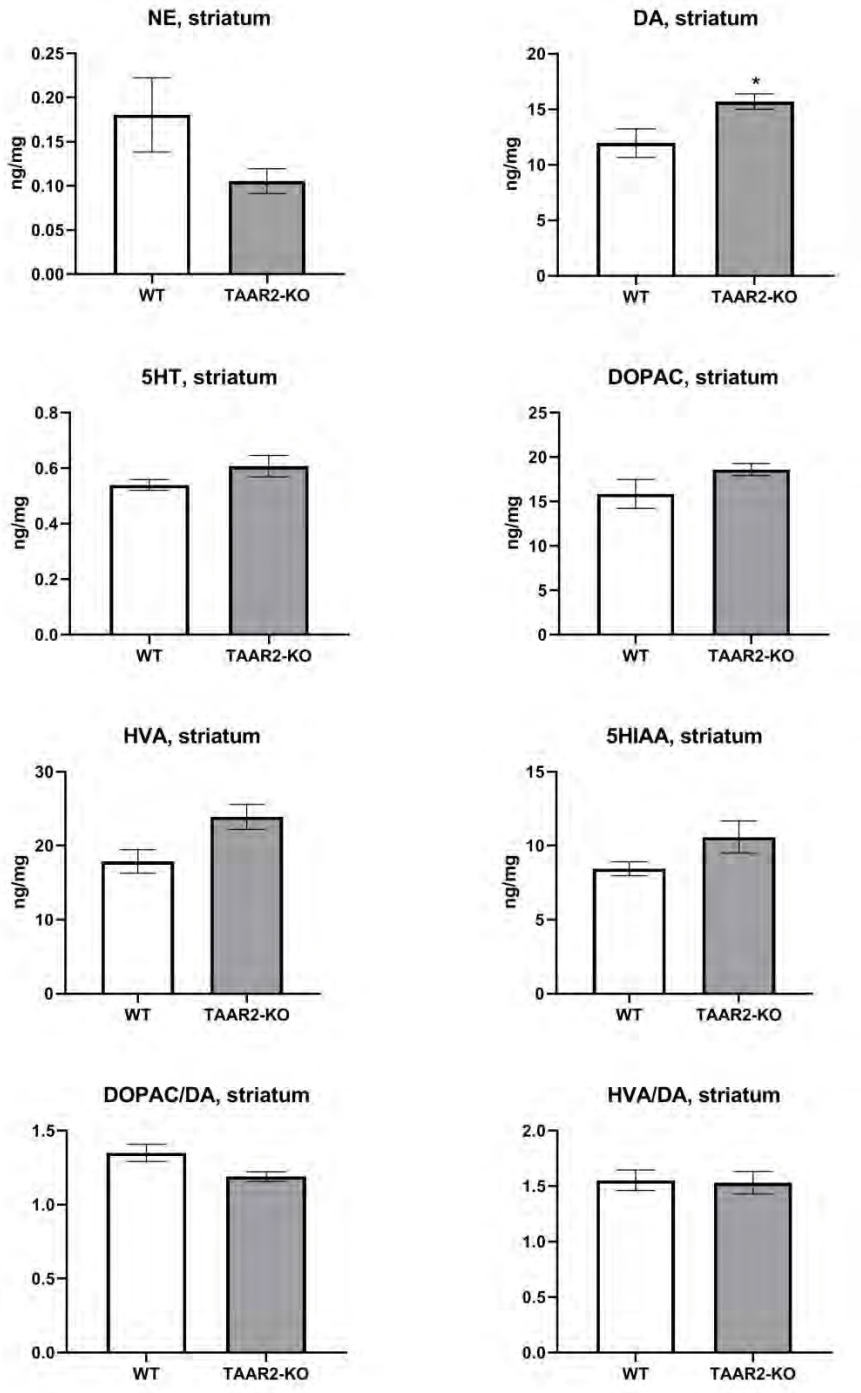


Figure 88. Levels of monoamines and their metabolites in the striatum of TAAR2-knockout mice and wild-type mice. Wild type, n=9; TAAR2-KO, n=8. Graphs show mean ± S.E.M.The analysis revealed an increase of dopamine in the striatum of TAAR2-knockout animals compared to wild-type animals (Mann-Whitney test, U=10, p=0.0111). No statistically significant differences were found between the groups for the other parameters studied (Mann-Whitney test, U>16, p>0.05). The results are shown in Figure 88.

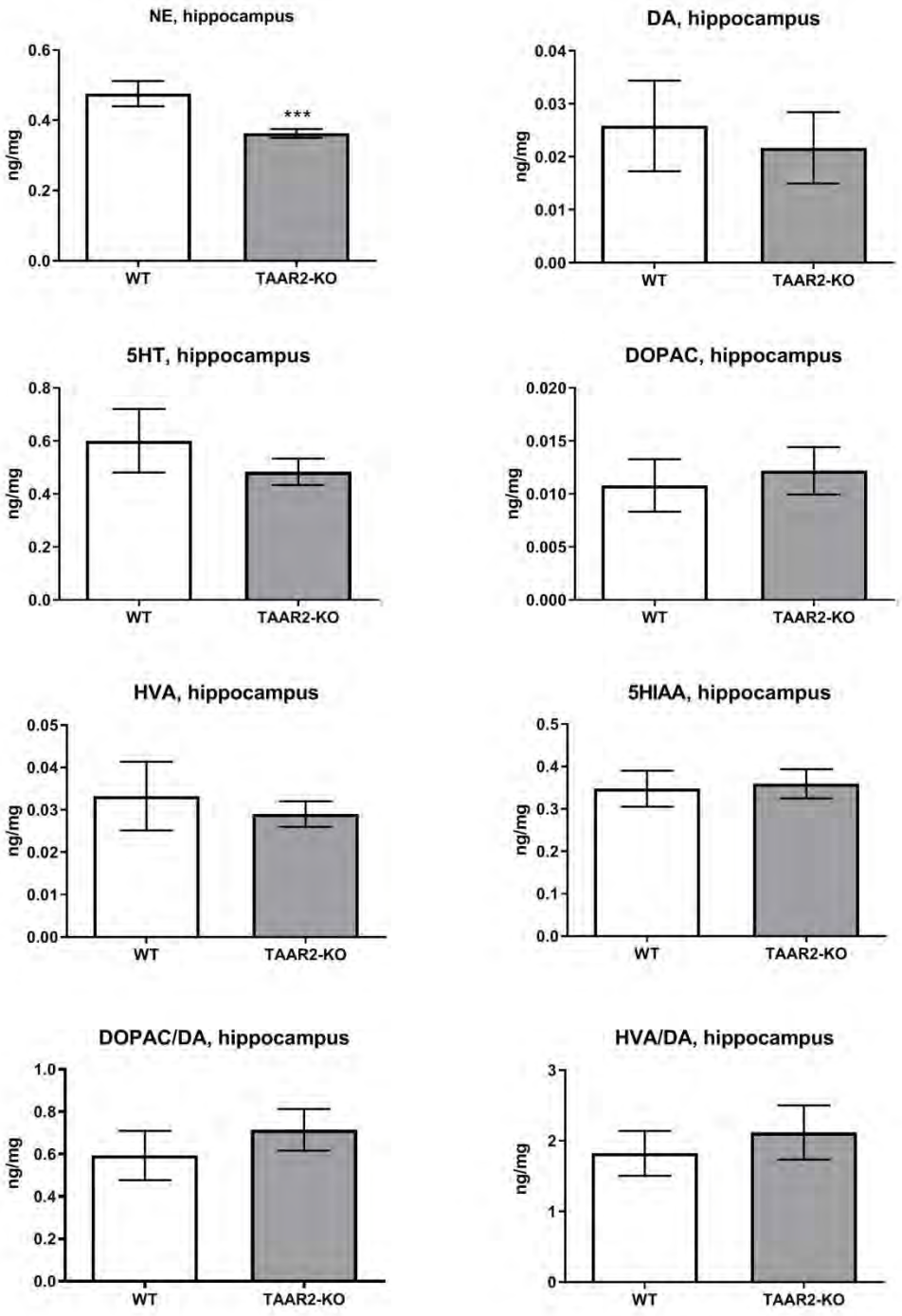


Figure 89. Levels of monoamines and their metabolites in the hippocampus of TAAR2-knockout mice and wild-type mice. Wild type, n=9; TAAR2-KO, n=12. Graphs show mean ± S.E.M.

The analysis revealed a decrease in norepinephrine in the hippocampus of TAAR2-knockout animals compared to wild-type animals (Mann-Whitney test, U=8, p=0.0005). No statistically significant differences were found between the groups for the other parameters studied (Mann-Whitney test, U>28, p>0.05). The graphs are presented in Figure 89.

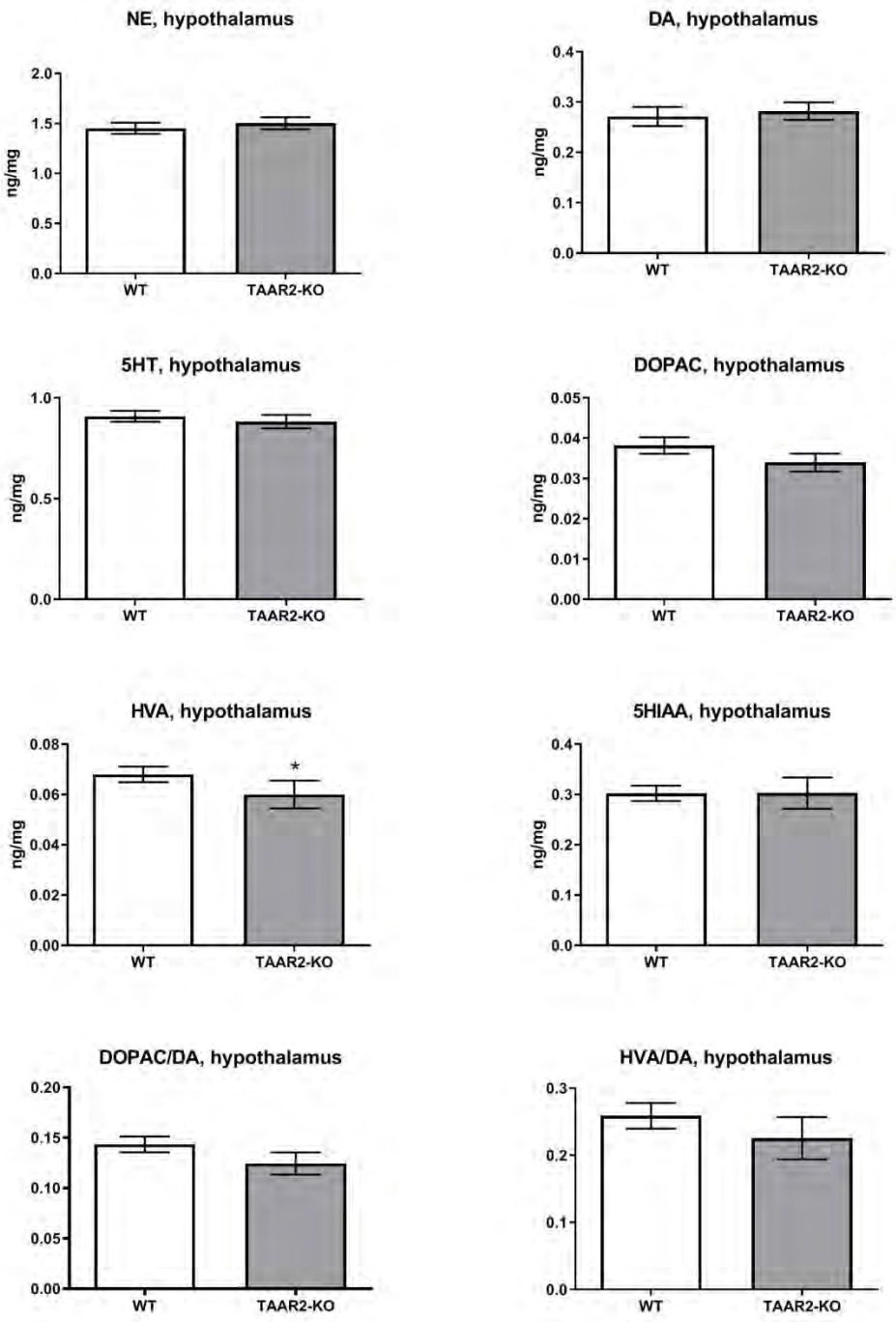


Figure 90. Levels of monoamines and their metabolites in the hypothalamus of TAAR2-knockout mice and wild-type mice. Wild type, n=9; TAAR2-KO, n=12. Graphs show mean ± S.E.M

The analysis revealed a decrease in homovanillic acid in the hypothalamus of TAAR2-knockout animals compared to wild-type animals (Mann-Whitney test, U=26, p=0.0491). For the other parameters studied, no statistically significant differences were found between groups (Mann-Whitney test, U>31, p>0.05). The results are shown in Figure 90.

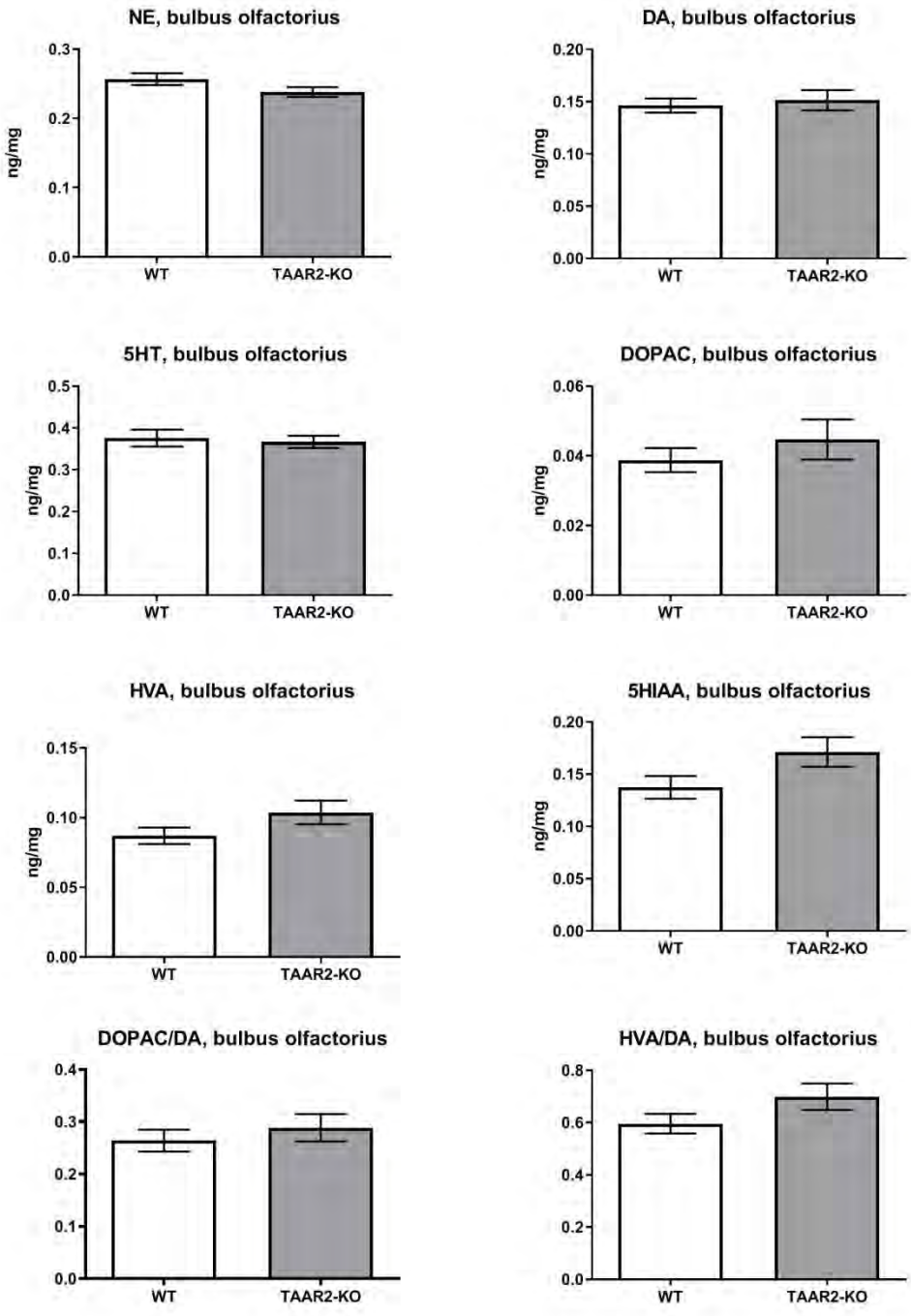


Figure 91. Levels of monoamines and their metabolites in the olfactory bulb of TAAR2-knockout mice and wild-type mice. Wild type, n=9; TAAR2-KO, n=12. Graphs show mean ± S.E.M.

The analysis showed no statistically significant differences in the tissue content of monoamines and their metabolites in the olfactory bulb between TAAR2-knockout animals and wild-type animals (Mann-Whitney test, $U>33$, $p>0.05$ for all parameters studied), graphs with the results are shown in Figure 91.

8. Discussion

In the course of this work, we identified several in vivo active TAAR1 agonists using different animal models. Compound AP163 was effective in suppressing motor hyperactivity in DAT-KO rats [148]. The model of motor hyperactivity caused by knockout of the dopamine transporter gene can be a non-pharmacological model of disorders in the pathophysiology of which dopaminergic neurotransmission is increased for some reason. These include schizophrenia, attention deficit hyperactivity disorder, and the manic phase of bipolar disorder [149–151]. To further study the therapeutic potential of the compound in schizophrenia, it is possible to test its activity in other models, such as PCP- and MK-801-induced hyperactivity, the ability to affect pre-pulse inhibition, and the effect on the decrease in social interaction and memory induced by phencyclidine administration. It also remains an open question how much of the drug's effect in this model is due to agonism to TAAR1 receptors. At the time of the study, our laboratory had the resources to determine the in vitro activity of the substances for TAAR1; potential interactions with other receptors had not been performed. Unfortunately, at the time of making this work, there are no in vivo active TAAR1 antagonists available, the administration of which could be used to assess the contribution of TAAR1 to the effects of experimental TAAR1 agonists. A possible option to test whether the reduction in hyperactivity is TAAR1-dependent could be to compare the effects of the drug in TAAR1-knockout animals and wild-type animals in a model of hyperactivity induced by administration of stimulants, which may be difficult due to the low availability of this group of drugs for research. A pharmacological model with the administration of stimulants could be complementary, but it is not exactly identical to the genetic model used. The closest to the model used (to identify whether the action of AP163 is TAAR1-dependent) would be a genetic model of double knockouts on the TAAR1 gene and the dopamine transporter, on which the contribution of TAAR1 to the effects of the drug could be assessed [3], however, this does not appear to be economically feasible. Other drugs of this series AP161, AP162 and AP164 did not reduce hyperactivity in dopamine transporter knockout rats, which presumably may be a manifestation of their low passage through the blood-brain barrier or be the result of a first-pass effect. Pharmacokinetic studies as well as higher dose studies in animal models could be useful to answer this question. Complex pharmacodynamic drug interactions with many other potential targets are also possible.

An in-depth study of another TAAR1 agonist, LK00764, revealed the experimental drug's ability to reduce motor hyperactivity in mice induced by administration of the dopamine transporter inhibitor GBR 12909. This model is also considered by researchers as a way to study mania, attention deficit hyperactivity disorder and schizophrenia [152–154]. Previously, the drug was found to reduce

locomotor hyperactivity in two other animal models: hyperactivity induced by administration of the NMDA-antagonist MK-801 and a genetic model in animals knocked out for the dopamine transporter gene [155].

When studying the effect of LK00764 on behavior, it was found that at acute intraperitoneal administration at a dose of 10 mg/kg the effect of the drug can be described as sedative, reducing mainly motor and exploratory activity of animals. At subcutaneous administration, apparently due to lower peak concentrations, these effects of the drug are expressed much less significantly. For a detailed study in the future, a series of experiments should be conducted in which a larger range of doses at each route of administration would be studied to better characterize the effects of the drug and their duration. The ability of LK00764 to dose-dependently reduce the stress-induced hyperthermia was also found during acute administration, which may be a predictor of the anxiolytic effect of the drug [144,145]. Interestingly, the findings are consistent with past results from studies of experimental TAAR1 agonists [3].

Notably, potential anxiolytic effects of LK00764 were also identified when its effects on behavior were examined by subchronic subcutaneous injection at a dose of 10 mg/kg. In the elevated plus maze test, animals receiving LK00764 injections spent more time in the open arms and central zone compared to the control group, which is classically interpreted as a decrease in anxiety-like behavior [156,157].

Thus, LK00764 is effective in various animal models of schizophrenia and also demonstrates anxiolytic properties according to the results of several experiments. As in the case with AP163, potential interaction with other targets is subject to further study, and it is also advisable to identify TAAR1-dependent effects of the drug in other experiments.

The increase in locomotor activity in a new environment observed in TAAR2-knockout mice may be an argument in favor of the regulatory functions of this receptor in the central nervous system. The chromatographic study revealed an increase in the level of dopamine in the striatum of knockout animals, which may be one of the reasons for the observed changes. The increase in the level of dopamine may be both a manifestation of an increase in the number of dopaminergic neurons and a decrease in the expression of the monoamine oxidase-B, which was found in the study of TAAR2-knockouts [24]. At the behavioral level, we also revealed a decrease in immobilization time in the forced swim test. Together with an increase in locomotor activity it may be a manifestation of increased dopamine signaling. To test this hypothesis, we compared locomotor activity in wild-type and TAAR2-knockout mice after the injection of the dopamine transporter inhibitor GBR 12909.

However, the locomotor activity of the knockouts was not statistically significantly different from that of wild-type mice according to the results of the test performed. A possible explanation could be the small number of animals in both groups, which made statistical power insufficient to detect small differences in locomotor activity. The possibility that mechanisms other than increased dopamine and adaptive processes may be involved in the increase in locomotion should also be considered. Increased tissue dopamine levels are a potential indicator of changes in signal transduction by dopamine neurons, but for in-depth study of these processes it is necessary to conduct microdialysis of extracellular dopamine and electrophysiological studies of the dopamine neurons. Interestingly, indirectly TAAR2 expression has been shown in other brain structures such as the hippocampus, hypothalamus, and DRN, changes in the functioning of these areas may also have an effect on animal behavior [158].

TAAR6 mutant mice also showed changes in behavioral tests compared to wild-type mice. One of these was the elevated plus maze. The elevated plus maze is a simple method of assessing anxiety responses in rodents. This test is based on rodents' tendency toward dark enclosed spaces and unconditional fear of open spaces and heights. The ratio of being in closed and open arms in this test reflects the conflict between the innate motivation to explore new environments and the rodent's preference for sheltered spaces. This test has predictive validity in the study of anxiogenic and anxiolytic drugs. Anxiolytic drugs increase the time spent in the open arms of the maze, may also increase the time spent in the central zone of the maze and the number of head dipping from the open arms, while anxiogenic drugs have the opposite effect [156]. TAAR6-mutant mice show an increase in the time spent in the open arms and central zone of the elevated plus maze, and a decrease in the time spent by TAAR6-mutant mice in the closed arms of the maze. These parameters are classically interpreted as a reduction in anxiety behavior [157]. Increased time spent in the central zone may also be an indirect indicator of increased impulsivity in rodents [159].

TAAR6-mutant animals left more fecal boluses in the open-field test. There are different opinions regarding the interpretation of this indicator. Some researchers interpret the number of fecal boluses as increased emotionality and anxiety during the test, which may inversely correlate with locomotor activity [160]; this view was subsequently challenged [161]. Another possible indicator of change in anxiety behavior during the Open Field test was not altered, namely the number of entries to the central zone and the time spent in it. Horizontal locomotor activity during the Open Field test did not differ between mutant and wild-type mice. Vertical activity was increased in TAAR6 mutant mice, as evidenced by an increase in rearings during the test. Rearings are considered by researchers as a variant of exploratory activity of animals. An increase in this parameter can also be interpreted as a decrease in anxiety during the test [146].

In the marble burying test, TAAR6-mutant mice buried more marbles than wild-type mice. There are many interpretations of this test [147]. An increase in the number of buried marbles may be the result of anxiety about unknown objects, as well as an increase in stereotypic or compulsive behavior [162]. It is worth noting that digging itself was not altered in TAAR6 mutant mice.

Anxiety can have several different aspects depending on the context, and there are separate tests for different aspects of anxious behavior that focus on a particular factor [163]. These results suggest that the mutation in the TAAR6 gene is likely to have an effect only on anxiety related to open space and height, because in the light-dark box there were no changes in the parameters usually interpreted as indicators of anxiety behavior, while in the open field and elevated plus maze some of these parameters were altered. For in-depth study of changes in the anxiety behavior of mutant animals, it is possible to perform additional tests emphasizing factors not investigated in this screening [164].

The study of hypothermic effects of the 5HT1A receptor agonist 8-OH-DPAT revealed an enhanced hypothermic effect of the drug in TAAR6 mutant mice. Together with the data obtained by our laboratory team on changes in serotonin levels in several brain structures of mutant animals, we can assume that there are some changes in serotonin signaling and functioning of 5HT1A receptors [165,166]. The interaction between 8-OH-DPAT and the TAAR6 receptor needs to be excluded for differentiation in future studies, because it may also play a role in the altered hypothermic response to the drug in TAAR6-mutant mice. Interestingly, the results described in this work partially overlap with previously described changes observed in TAAR5 knockout mice [6]. Further studies of the functioning of different types of serotonin receptors may help answer the question of which signaling processes are altered by the TAAR6 mutation.

9. Conclusions

TAAR1 receptor agonists AP161-AP164 as well as LK00764 synthesized at the Institute of Chemistry of SPbU were studied in various in vivo models. Behavioral and neurochemical characterization of TAAR2 knockout mice and TAAR6 mutant mice was performed, as well as several pharmacological tests.

As a result of this study, in accordance with the set tasks, the following principal findings were made:

Principal findings

1) An active in vivo TAAR1 agonist AP163 has been identified that can dose-dependently reduce motor hyperactivity in dopamine transporter knockout rats, which may be a predictor of both antipsychotic and antimanic activity of this compound

2) The potential anxiolytic properties of the TAAR1 agonist LK00764 were identified, and its ability to attenuate hyperactivity in mice induced by administration of the dopamine transporter inhibitor GBR 12909 was also demonstrated

3) TAAR2-knockout and TAAR6-mutant mice were characterized for the first time. The effect of TAAR2 knockout on the motor activity of animals was found, as well as the effect of mutation in the TAAR6 gene on anxious behavior and increased hypothermic response to the administration of 5HT1A-agonist 8-OH-DPAT. Neurochemical analysis revealed changes in monoamine and metabolite levels in the brains of TAAR2-knockout and TAAR6-mutant mice

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Abbreviations

CNS – central nervous system

ESA – extingusded self-administration

AP 163 – 4-(2-aminoethyl)-N-(3,5-dimethylphenyl)piperidine-1-carboxamide hydrochloride

BNSS – The Brief Negative Symptom Scale

DAT – dopamine transporter

DRN – dorsal raphe nucleus

EPPTB – N-(3-Ethoxy-phenyl)-4-pyrrolidin-1-yl-3-trifluoromethyl-benzamide

GPCR – G-protein-coupled receptors

LK00764 – 2-(5-(4′-Chloro-(1,1′-biphenyl)-4-yl)-4H-1,2,4-triazol-3-yl)ethan-1-amine hydrochloride

NMDA – N-methyl-D-aspartate

PANSS – Positive and Negative Syndrome Scale

PCP – phencyclidine

TAAR – trace amine-associated receptors

VTA – ventral tegmental area

WT – wild type

5HT1A – 5-hydroxytryptamine receptor 1A

8-OH-DPAT – (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene