The role of ventral pallidal neurotensin receptors in the behavioural regulation

Ph.D. thesis

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1. Introduction

The drug addiction and anxiety disorders lead to serious health failures and sociological problems nowadays. The role of various neuromodulators, including neurotensin (NT) [1, 2], has been proven in these processes [3, 4]. NT modulates the effect of numerous other transmitters in the central nervous system, the most important are the dopamine (DA) [5], gamma-amino-butyric-acid (GABA) [6, 7], glutamate [8], serotonin [9, 10] and acetyl-choline [10].

NT can be detected in multiple brain structures, among others, in the terminals of the ventral striatopallidal pathway, that has a key role in motivational and rewarding processes, and projects from the nucleus accumbens (NAC) to the ventral pallidum (VP) [11]. The VP is an important integrating centre of behavioural regulation [12]. It takes part in the processing and integration of the motivational and reward signals [13], and also in the regulation of anxiety [14]. In numerous brain structures, the NT exerts its effects by interaction with the DAergic system [5].

In the VP both NT neuron terminals and NT receptors have been detected: the presence of NT is characteristic to the ventromedial VP subregion (VPvm) only, but it can be barely detected in the dorsolateral part (VPdl) or in other subregions [11, 15] as well. The NT immunoreactivity can be detected mainly on the axon terminals of striatopallidal neurons, but on the perikarya it is not or only minimally detectable [11]. Concerning the NT receptors in the VPvm, the NTS1 receptors are present in the highest concentration [16, 17], typically on the dendrites and on neuronal perikarya [16]. NTS2 receptors can be identified in the VP only to a very low level [17, 18]. There is no evidence of the existence of NTS3 in the VP. The direct electrophysiological effects of NT or NT antagonists after direct microinjections into the VP have not been investigated yet, but it has been shown, that i.p. injection of NTS1 antagonists decreases the firing rate of VPvm neurons, while it has no effect on the VPdl [19]. The NT in the VP increases extracellular GABA level [7]. It is also known, that due to the inhibition of the VPvm the GABAergic efferent pathways are also inhibited, and the ventral tegmental area is disinhibited [20]. Based on these data, the NT injected into the VP can play a role in the positive reinforcing and rewarding processes through influencing the activity of the DAergic neurons of the VTA. Even though, only a

few is known regarding possible behavioural effects of NT microinjected into the VP, but it is certain, that NT plays a role in drug addiction: after cocaine administration and extinction NT(8-13) a in the VP potentiates cocaine-primed, but inhibits cue-primed reinstatement of drug seeking, but has no effect on locomotion [7].

2. Objectives

Based on the literature cited in the introduction, it is reasonable to suppose, that NTS1 receptors is accountable for regulation of the ventral striatopallidal projections, that play an important role in reward and reinforcement [11, 21, 22]. Nevertheless, the direct effects of NT in the VP have not been investigated yet, neither to reinforcement, nor to anxiety. Therefore, the following experiments have been executed:

1. To investigate the possible effects of NT, and the NTS1 receptor antagonist SR 48692 on locomotor activity open field test (OPF) was performed.

2. Another aim of the present experiments was the examination of the possible rewarding effect of NT injected into the VP. Conditioned place preference test (CPP) was employed to answer this question.

3. In case, if the NT tends to be rewarding, a further goal was to reveal, if the NT exerts its effect on the NTS1 receptors, that can be detected in high concentrations in the VP.

4. Another purpose of the present experiments was the examination of the possible anxiolytic effect of NT injected into the VP by means of elevated plus maze (EPM) test.

5. An additional question was, if the NT tends to influence anxiety, whether the NTS1 receptors are involved in this effect.

6. A further aim was, to investigate, whether the inhibition of D2 DA receptors of the VP can modify the effects of NT.

3. Materials and methods

3.1. Subjects

In the experiments 279 male Wistar rats (LATI, Gödöllő) were used, weighing 280–320 g at the beginning of the experiments. One week before the operations the animals have been transported to the temperature- and light-controlled animal room (22 ± 1 °C, air humidity: 55 ± 10%). Artificial illumination was applied according to the natural daylight period, with 12:12 h light-dark cycle. The daylight period started at 7:00 a.m., the dark period started at 7:00 p.m. The animals were housed individually in separated cages, but in the same room. This was necessary, because the headpiece could be damaged or could cause injuries to the other animals by the interactions between the rats. The cages were cleaned daily by skilled staff. Standard laboratory food pellets (CRLT/N Charles River Ltd., Budapest, Hungary) and tap water were available *ad libitum*. Before starting the experiments the animals were handled by the experimenters. The animals were cared in accordance with institutional (BA02/2000-8/2012), national (Hungarian Government Decree, 40/2013. (II. 14.)), and international standards (European Community Council Directive, 86/609/EEC, 1986, 2010).

3.2. Stereotaxic surgery

Operations were carried out under general anaesthesia by intraperitoneal injection of a mixture of ketamine (Calypsol, Richter Gedeon, Hungary, 80 mg/kg body weight) and diazepam (Seduxen, Richter Gedeon, Hungary, 20 mg/kg body weight). By means of the stereotaxic technique, 22 gauge (0.64 mm) stainless steel guide tubes were bilaterally implanted 0.5 mm above the target area. The coordinates of the target area were determined according to the stereotaxic rat brain atlas of Paxinos and Watson [23]. The following coordinates referring to the bregma were used: anteroposterior (AP): -0.26 mm, lateral (ML): 2.2 mm, dorsoventral (DV): 7.1 mm from the surface of the dura. Cannulae were fixed to the skull with self-polymerizing dental acrylic (Duracryl) anchored by 3 stainless steel screws. The guide tubes, when not being used for microinjection, were occluded with

sterile obturators made of 27 gauge (0.36 mm) stainless steel wire, the obturators were removed during microinjections. Antibiotic prophylaxis (penicillin G) was applied during the operations. In the postoperative period, animals were allowed a minimum of 6 days for recovery, before starting the behavioural experiments. Neurological examinations were performed on all animals, to check the intact sensory and motor functions. Behavioural tests were performed during the daylight period between 08:00 and 18:00 h.

3.3. Materials and microinjection procedure

NT (Sigma–Aldrich Co., N 6383) was dissolved in 0.15 M sterile saline solution containing 0.01 M Na-acetate and 0.01 M phosphate buffered saline (PBS, pH 7.4). NT was microinjected in two different doses: 100 ng or 250 ng. The NTS1 antagonist SR 48692 (Tocris Co., Cat. No. 3721) was diluted in 0.15 M saline solution containing 2% dimethyl sulfoxide and 0.01M PBS. SR 48692 was microinjected in a dose of 35 ng. The D2 dopamine receptor antagonist (S)-(-)-sulpiride (Sigma-Aldrich Co., S7771) was dissolved in physiological saline, a dose of 4 μg was applied. All mentioned doses are meant per side.

The doses of NT [24-27], and sulpiride [28] were determined based on pilot experiments, and the effective dose ranges applied by intracerebral microinjections into other brain areas. Dose of SR 48692 was determined to be equimolar with NT, this dose of the antagonist far exceeds the 50% inhibitory concentration [29].

The microinjections were applied on awake, handled rats, gently held by the experimenters. All drugs or vehicles were bilaterally microinjected through 27 gauge stainless steel microinjection tubes extending 0.5 mm below the tips of the implanted guide cannulae. The delivery cannula was attached to a 10 μl Hamilton microsyringe (Hamilton Co., Bonaduz, Switzerland) via polyethylene tubing (PE-10). All injections were delivered by a Cole-Parmer digital infusion pump in the volume of 0.4 μl (Cole Parmer, IITC, Life Sci. Instruments, California) over a 60 sec interval. After accomplishing the microinjection, cannulae were left in place for an additional 60 sec to allow diffusion into the surrounding tissue. Rats were gently held by hand during the injection procedure. After all injections it was verified, that the cannula was not occluded.

The microinjections applied in the different experiments are summarized in the following table (for the details, see below):

OPF	control (veh2 + veh1) (n=6) 100 ng NT (n=8) 250 ng NT (n=6) 35 ng SR 48692 + veh1 (n=6) 35 ng SR 48692 + NT (n=6)
CPP	control (veh1) (n=11) 100 ng NT (n=12) 250 ng NT (n=13)
	control (veh2 + veh1) (n=10) veh2 + 100 ng NT (n=13) 35 ng SR 48692 + veh1 (n=7) 35 ng SR 48692 + 100 ng NT (n=12)
	control (veh3 + veh1) (n=11) veh3 + 100 ng NT (n=6) 4 μg sulpiride + veh1 (n=8) 4 μg sulpiride + 100 ng NT (n=11)
EPM	control (veh1) (n=9) 100 ng NT (n=8) 250 ng NT (n=8)
	control (veh2 + veh1) (n=9) veh2 + 100 ng NT (n=8) 35 ng SR 48692 + veh1 (n=9) 35 ng SR 48692 + 100 ng NT (n=8)
	control (veh3 + veh1) (n=11) veh3 + 100 ng NT (n=6) 4 μg sulpiride + veh1 (n=9) 4 μg sulpiride + 100 ng NT (n=10)

In the OPF experiment, NT was microinjected in 100 ng (n=8) or 250 ng doses (n=6). The antagonist treated group (n=6) received SR 48692 and then 15 min later vehicle of NT (veh1). The NT injected group pretreated with antagonist (n=6) received SR 48692 15 min before being injected with 100 ng NT. Vehicle of SR 48692 (veh2) and then 15 min later veh1 were microinjected in the control group (n=8).

In the first CPP experiment, 100 ng NT (n=12) or 250 ng (n=13) was microinjected bilaterally, control animals received veh1 in equal volume to that used for NT injections. In the second experiment (performed to examine the antagonist), the NT treated group (n=13) received veh2 and then 100 ng NT (the dose, which has been effective in the first experiment, see results). The antagonist treated group (n=7) received SR 48692, then veh1.

The group with combined antagonist and NT treatment (n=12) received SR 48692 15 min prior to being injected with 100 ng NT. Two subsequent vehicle microinjections (veh2 + veh1) were applied in the control group (n=10). The antagonist or veh2 were applied 15 min prior to NT or veh1 injections, respectively. In the third CPP experiment (that was aimed to investigate the interaction with sulpiride) animals treated with NT (n=6) were microinjected with vehicle of sulpiride (veh3), and then 100 ng NT. The sulpiride treated group (n=8) received 4 μg sulpiride, then veh1. The NT injected group pretreated with sulpiride (n=11) received 4 μg sulpiride, then 15 min later 100 ng NT. Animals of the control group (n=11) received two vehicle injections (veh3 + veh1). Sulpiride or veh3 was always microinjected 15 minutes before NT or veh1. In the second and third experiments, NT applied after antagonist or sulpiride pretreatment involved 2 microinjections within 15 min interval, therefore the animals in the other groups received 2-2 microinjections within 15 minutes too, i.e. the results of the different groups were comparable with each other.

In the first EPM experiment animals received 100 ng (n=8) or 250 ng NT (n=8). Control animals (n=9) received veh1 in equal volume. In the second EPM experiment, the NT treated group (n=8) received veh2 and then 100 ng NT. The antagonist treated group (n=9) received SR 48692 and then veh1. The NT injected group pretreated with antagonist (n=8) received SR 48692, then 100 ng NT. Two subsequent vehicle microinjections (veh2 + veh1) were made in the control group (n=9). Similar to the CPP test, The antagonist or veh2 were applied 15 min prior to the NT or veh1 injections, respectively. In the third EPM experiment animals treated with NT (n=6) were microinjected with veh3, and then 100 ng NT. The sulpiride treated group (n=8) received 4 μg sulpiride, then veh1. The NT injected group pretreated with sulpiride (n=10) received 4 μg sulpiride, then 15 min later 100 ng NT. Animals of the control group (n=11) received two vehicle injections (veh3 + veh1). Sulpiride or veh3 were always microinjected 15 minutes before NT or veh1, similarly to the CPP test.

3.4. Behavioural experiments

All experiments were performed in a sound isolated, air-conditioned room. Movement of the animals was recorded by a video camera, data were stored and motion analysis was

made by the Noldus EthoVision Basic software (Noldus Information Technology b.v., Wageningen, The Netherlands).

3.4.1. Open field (OPF) test

The apparatus of OPF consisted of a 50 × 50 × 50 cm grey wooden box. The ground of the arena was divided into 16 identical squares. During observation period the number of crossings and the distance moved were investigated. Each session lasted for 5 minutes. On the first day of the 4 days long experiment a habituation session was accomplished, on the 2nd and the 3rd days basal activity of the animals was studied (without microinjection). On the 4th (test) day animals were placed into the chamber following the bilateral administration of the drugs and the activity of the animals was recorded.

3.4.2. Conditioned place preference (CPP) test

The CPP test can be used to test the rewarding, positive reinforcing or aversive effects of drugs [30, 31]. The CPP apparatus consisted of a circular open field, with a diameter of 85 cm and 40 cm height. The walls and the floor of the apparatus were made of plastic, and grey-coloured. The floor was divided by thin black lines into four quadrants, which could be separated from each other by removable plexiglas barriers during conditioning. Visual cues in the surroundings assisted to distinguish the quadrants and helped the spatial orientation of animals within the apparatus [30]. The room was illuminated dimly by a 40 W bulb.

The place preference procedure consisted of one habituation (1st day), three conditioning (2nd–4th days) and one test (5th day) trials. Each lasted for 900 sec (15 min). In the habituation trial (1st day), animals were placed into the apparatus and had free access to all quadrants for 900 sec. The time that the rats had spent in each of the four quadrants was measured. The treatment quadrant was determined to be one of the four quadrants in which the animal had spent neither the longest nor the shortest time during habituation.

During the conditioning trials (2nd-4th days) the quadrants were physically separated from each other by the plexiglas barriers. Animals were introduced into the treatment quadrant subsequently after the bilateral microinjections. Rats were restricted to the

treatment quadrant for 15 min. On the 5th day (test trial) the separating barriers were removed, i.e. animals had free access to all parts of the apparatus. The time spent in each of the four quadrants and distance moved were measured.

3.4.3. Elevated plus maze (EPM) test

Anxiety was evaluated in the EPM test [32]. The apparatus was constructed of grey coloured wooden planks, i.e. two opposite open arms and two opposite closed arms (50 cm × 10 cm × 40 cm). The area of the arms was 50 cm × 10 cm, the area of the central platform is 10 cm × 10 cm. The closed arms were surrounded with a 40 cm high, grey-coloured wall, the roof was open. The maze was elevated to a height of 100 cm above the floor. After drug or vehicle administrations, animals were placed into the centre of the maze (central platform), facing one of the closed arms. The test was performed once on each animal, the trials lasted for 5 min. The time spent at the different parts of the apparatus (open and closed arms and the end of the open arms) were measured.

3.5. Evaluation of the results

3.5.1. Histology

At the end of all experiments, rats received an overdose of urethane (i.p. injection of 20% urethane solution, in a dose of 1.4 g / kg bw.) and were transcardially perfused with isotonic saline followed by 10% formaldehyde solution.

A week after fixation, brains were frozen, cut into 40 μm serial sections and stained with Cresyl-violet. Cannulae tracks and location of cannulae tips were reconstructed according to the rat brain stereotaxic atlas [23]. Only data from animals with correctly placed cannula positions were analysed.

3.5.2. Statistics

Normal distribution of the data was verified by Shapiro-Wilk-test, then the data were evaluated with two-way or one-way analysis of variance (ANOVA) by means of „SPSS

20.0 for Windows" program. The homogeneity of the sample was examined by F-test. Tukey post hoc test was employed for the comparison of the groups. Statistical significance was established at $p < 0.05$.

4. Results

4.1. Histological results

The cannula tracks and the tip positions were determined on the basis of the stereotaxic atlas of Paxinos and Watson [23]. The histological examination showed that the cannulae were precisely and symmetrically tipped to the target area in 243 of the 279 animals. In case of four rats the acrylate headpiece was damaged or came off, i.e. the microinjections could not be appropriately performed; in the other 32 animals, mistargeted cannula placement was verified, data of these animals were also excluded from subsequent analysis. Among these 32 rats, in 12 cases cannula tips were located laterally or medially from the VP, so on one side injection was made in the interstitial nucleus of the posterior limb of the anterior commissure, on the other side in the fusiform part of the bed nucleus of stria terminalis. In 19 cases, the cannulae tips were 1 mm below the VP, so that the ends of the bilateral microinjection tracks were localized in the nucleus of the horizontal limb of the diagonal band, and the magnocellular preoptic nucleus. In 1 case cannula tips were located above the VP, i.e. microinjections were made to the interstitial nucleus of the posterior limb of the anterior commissure. Due to the great heterogeneity of data and a very low number of cases, these results did not allow to draw far-reaching conclusions about the behavioural effects of the microinjections mistargeted the VP.

4.2. Open field test

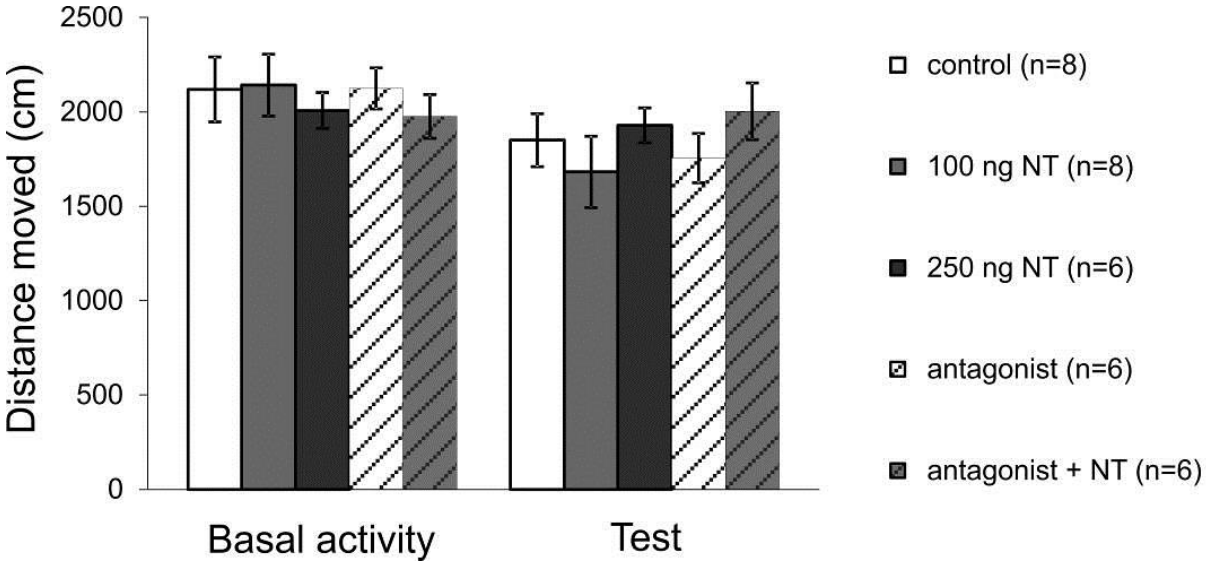

Fig.1: Effects of bilateral NT microinjections and NTS1 antagonist pretreatment in open field (OPF) test. Columns represent distance moved by the animals (± S.E.M.) without microinjection (basal activity), and during the test, respectively. Control: vehicle treated rats (veh2 + veh1; n = 8). 100 ng NT: animals microinjected with 100 ng NT (n = 8). 250 ng NT: animals microinjected with 250 ng NT (n = 6). Antagonist: rats treated with 35 ng NTS1 antagonist + veh1 (n = 6). Antagonist + NT: animals microinjected with 100 ng NT pretreated with 35 ng NTS1 antagonist (n = 6). For more explanation see the text.

The result of the OPF test is presented on Fig. 1. It has been shown by means of two-way ANOVA, that the total distance moved significantly differed between the trials (F [1;58] = 5.838, $p < 0.05$). The most probably reason of this phenomenon is, that all animals moved for shorter distance during the test trial because of the habituation. There was no significant effect for treatment (F [4;58] = 0.104, $p > 0.05$), also without a significant interaction of treatment and trials (F [4;58] = 0.895, $p > 0.05$).

In the number of crossings there was neither significant effect for the trials (F [1;58] = 0.764, $p > 0.05$), nor for treatment (F [4;58] = 0.298, $p > 0.05$), nor for interaction of treatment and trials (F [4;58] = 0.164, $p > 0.05$).

4.3. Conditioned place preference test

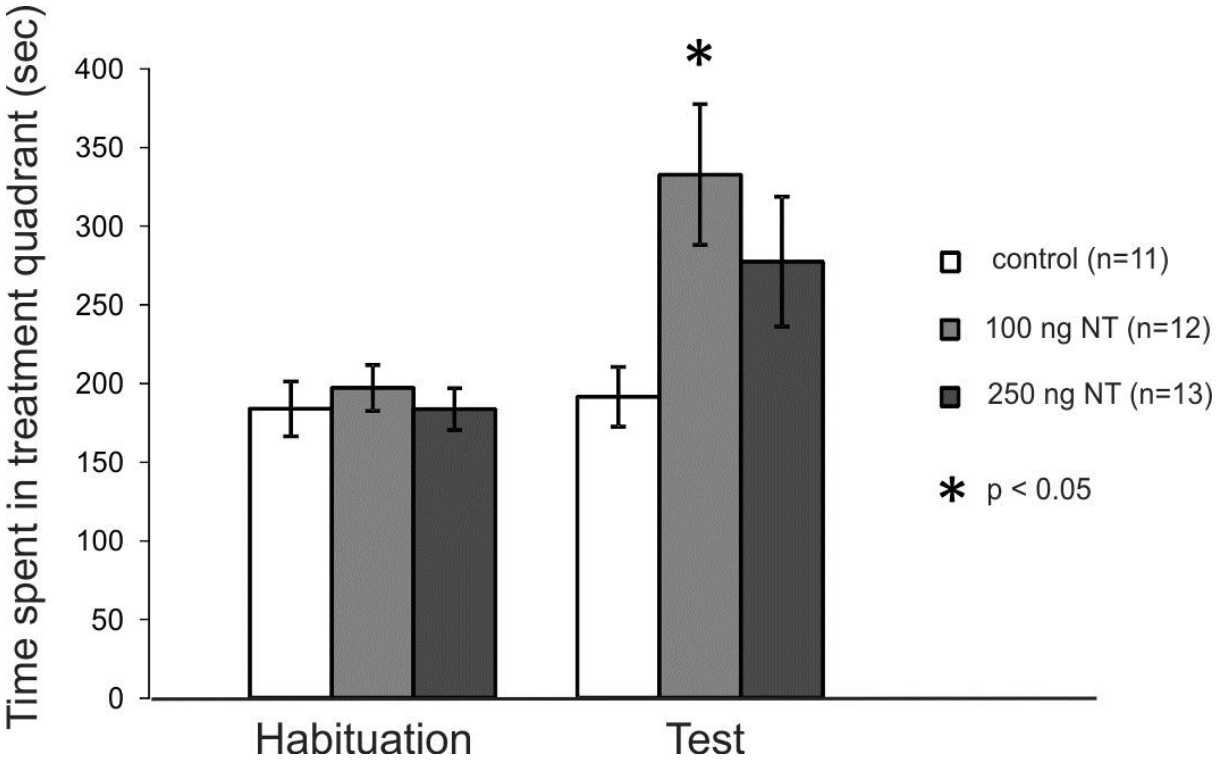

*Fig. 2. Effect of bilateral NT microinjections into the VP in conditioned place preference (CPP) paradigm. Columns represent mean time spent in the treatment quadrant (± S.E.M.) during Habituation and Test sessions, respectively. Control: vehicle treated rats (n = 11). 100 ng NT: animals microinjected with 100 ng NT (n = 12). 250 ng NT: animals microinjected with 250 ng NT (n = 13) *: p < 0.05.*

By means of two-way ANOVA the time spent in the treatment quadrant during the habituation and test trials by the groups were compared (Fig. 2.). There was a significant effect for trials (F [1;66] = 11.189, $p < 0.05$), a significant effect for treatment (F [2;66] = 3.431, $p < 0.05$) without a significant interaction of treatment and trials (F [2;66] = 2.426 $p > 0.05$). Based on the Tukey post hoc test, the time spent in the treatment quadrant was not affected in the control group (n = 11), but it was significantly increased in the 100 ng NT group (n = 12, $p < 0.05$). The mean time spent in the treatment quadrant in the 250 ng NT treated group (n = 13) was elevated, however, this was not significant. Within the trials (habituation, conditionings, test) the distance moved by the animals receiving different treatments (control, 100 ng NT, 250 ng NT) was analyzed by means of one-way ANOVA.

The trials (habituation, conditionings, test) cannot be statistically compared with each other, because during the habituation and test the animals could move within the entire apparatus, however, during the conditioning trial they were restricted to the treatment quadrant. The ANOVA did not show significant difference between the groups, neither during habituation, *(F* [2;33] = 1.736; $p > 0.05$), conditioning trials (*F* [2;33] = 0.842; $p > 0.05$), nor during the test (*F* [2;33] = 0.677; $p > 0.05$). During the conditionings trials animals in all groups moved for a shorter distance, which can be explained with the smaller area.

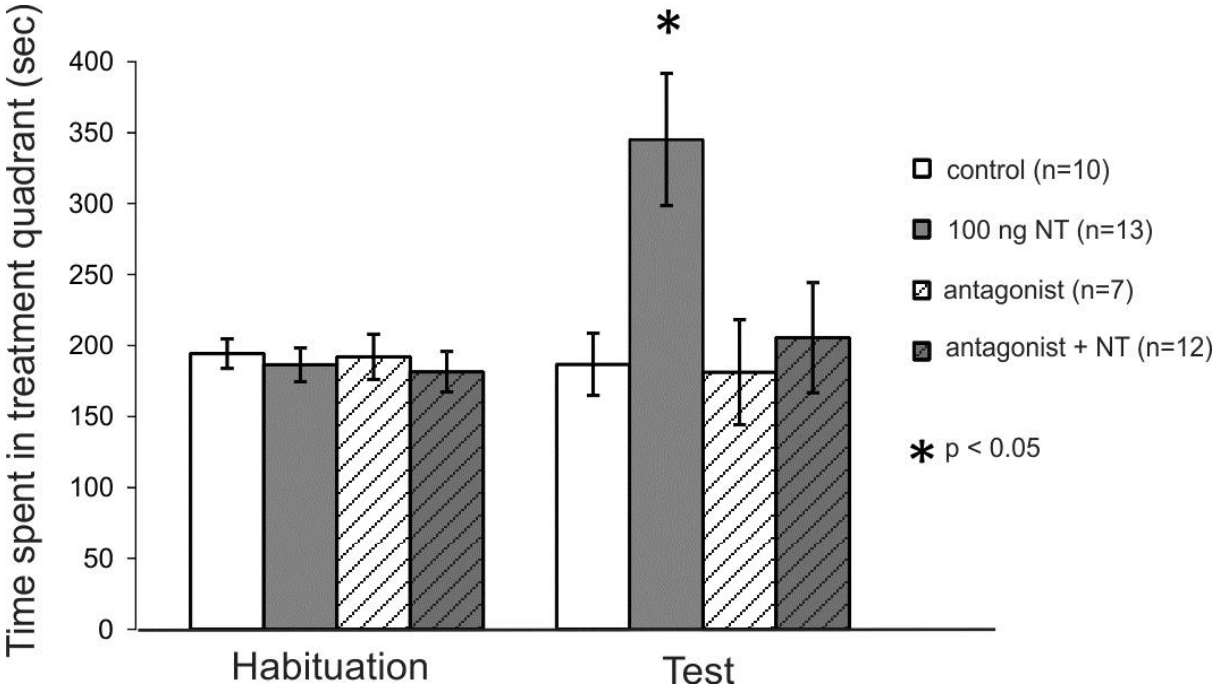

*Fig. 3. Effects of NTS1 antagonist pretreatment in the VP on conditioned place preference. Columns represent mean time spent in the treatment quadrant (± S.E.M.) during Habituation and Test sessions, respectively. Control: vehicle (veh2 + veh1) treated rats (n = 10). 100 ng NT: animals microinjected with veh2 + 100 ng NT (n = 13). Antagonist: rats treated with 35 ng NTS1 antagonist + veh1 (n = 7). Antagonist + NT animals microinjected with 100 ng NT pretreated with 35 ng NTS1 antagonist (n = 12). For more explanation see the text. *: $p < 0.05$.*

In the second experiment, it was examined whether the place preference inducing effect of NT is mediated by NTS1 receptors (Fig. 3.). The animals spent comparable time in the treatment quadrant during the habituation session, similarly to that observed in the previous experiment. Based on two-way ANOVA there was no significant effect for trials (F [1;84] = 3.620, $p > 0.05$), but there was a significant effect for treatment (F [3;84] = 3.637, $p < 0.05$) along with a significant interaction of treatment and trials (F [3;84] = 3.955, $p < 0.05$). Based on Tukey post hoc test, similar to the result observed in the first experiment, 100 ng NT (n=13) increased the time that the animals spent in the treatment quadrant during the test session compared to the control group (n = 10, $p < 0.05$). The pretreatment with NTS1 antagonist SR 48692 effectively blocked the effect of NT (n = 12, $p < 0.05$). The antagonist administered by itself (n = 7) did not influence the time spent in the treatment quadrant: test results did not differ from that of control animals, but significantly deviated from that of the 100 ng NT treated group ($p < 0.05$). The one-way ANOVA did not show a significant difference in the distance moved during the different trials (during habituation: F [3;38] = 0.232; $p > 0.05$; during conditioning trials: F [3;38] = 0.222; $p > 0.05$; during test: F [3;38] = 2.033; $p > 0.05$).

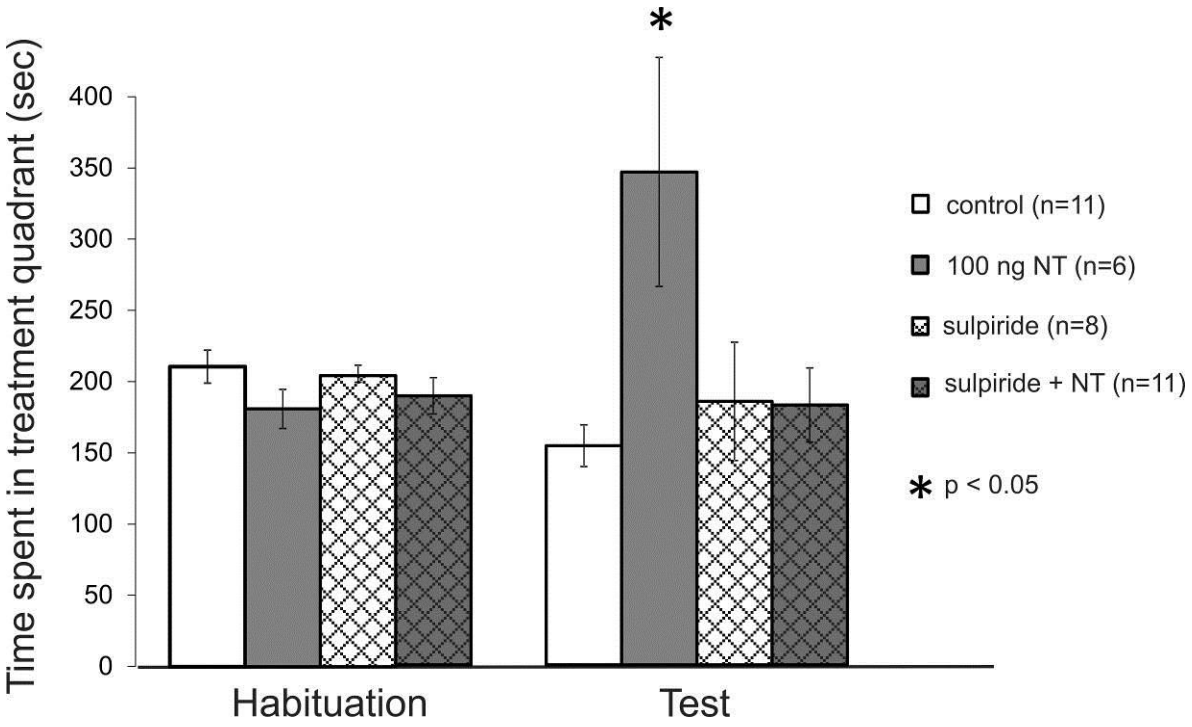

*Fig. 4. Effects of sulpiride pretreatment in the VP on conditioned place preference. Columns represent mean time spent in the treatment quadrant (± S.E.M.) during Habituation and Test sessions, respectively. Control: vehicle treated rats (veh3 + veh1; n = 11). 100 ng NT: animals microinjected with veh3 + 100 ng NT (n = 6). Sulpiride: animals received 4 µg D2 DA receptor antagonist sulpiride + veh1 (n = 8). Sulpiride + NT: effect of microinjections of 100 ng NT after 4 µg sulpiride pretreatment (n = 11). For more explanation see the text. *: p < 0.05.*

In the third CPP experiment it has been investigated, whether in the place preference inducing effect of 100 ng NT an interaction with D2 DA receptors play a role (Fig. 4.). The animals spent a similar time in the treatment quadrant seen in the first two experiments. Two-way ANOVA revealed no significant difference between the trials (F [1;64] = 1.064, $p > 0.05$), but the test showed a significant difference between the treatments (F [3;64] = 3.002, $p < 0.05$), and in the interaction between trials and treatments (F [3;64] = 5.022, $p < 0.05$). Based on Tukey post hoc test, the time spent in the treatment quadrant increased in the 100 ng NT treated group (n=6) relative to the control group (n=11, $p < 0,05$), similarly to the earlier experiments. The sulpiride pretreatment blocked the effect of NT (n = 11, $p < 0,05$). The sulpiride by itself (n = 8) did not influence the time spent in the treatment

quadrant, namely the result did not differ from the control group. Based on one-way ANOVA, in the distance moved there was no significant difference during habituation (F [3;32] = 1.370; $p > 0.05$). However, the analysis showed a significant difference in the mean of the distance moved during conditioning trials (F [3;32] = 12.032; $p < 0.05$), and tests (F [3;32] = 12.848; $p < 0.05$). Based on the Tukey post hoc test, animals treated with sulpiride or with NT after a sulpiride pretreatment, moved for a significantly shorter distance during conditioning trials, than the control group, and the same groups moved a significantly shorter distance during the test trials, compared to the control or the NT treated group.

4.4. Elevated plus maze test

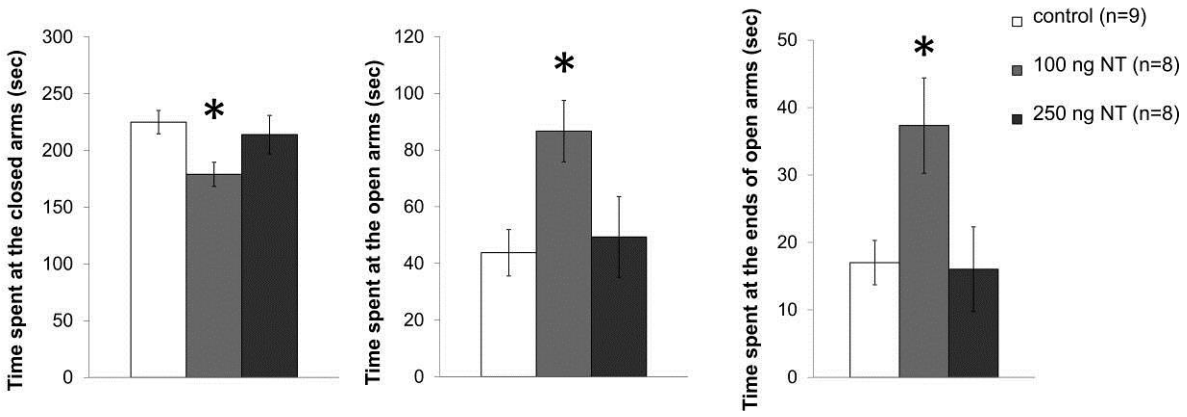

*Fig. 5. Effects of bilateral NT microinjections into the VP in EPM test. Columns represent mean time spent at the closed arms, time spent at the open arms, and time spent at the ends of the open arms, respectively (± S.E.M.). Control: vehicle treated rats (veh1; n = 9). 100 ng NT: animals microinjected with 100 ng NT (n = 8). 250 ng NT: animals microinjected with 250 ng NT (n = 8) *: p < 0.05.*

In the first EPM experiment, effects of bilateral microinjection of 100 ng or 250 ng NT into the VP were investigated (Fig. 5.). Based on one-way ANOVA, there was a significant difference among the groups in the time spent at the closed arms (F [2;22] = 3.513, $p < 0.05$), in the open arms (F [2;22] = 4.329, $p < 0.05$) and also in the ends of the open arms (F [2;22] = 4.479, $p < 0.05$). Tukey post hoc test revealed, that animals treated with 100 ng dose of NT spent significantly longer time at the open arms and at the ends of the open

arms, and shorter time on the closed arms than rats of the control group did. Based on this results 100 ng dose of NT has anxiolytic effect. Result of the 250 ng NT treated group did not differ significantly from the control group in the measured parameters. There was no significant difference between the groups in the distance moved within the entire apparatus, based on one-way ANOVA (F [2;22] = 0.403; $p > 0.05$).

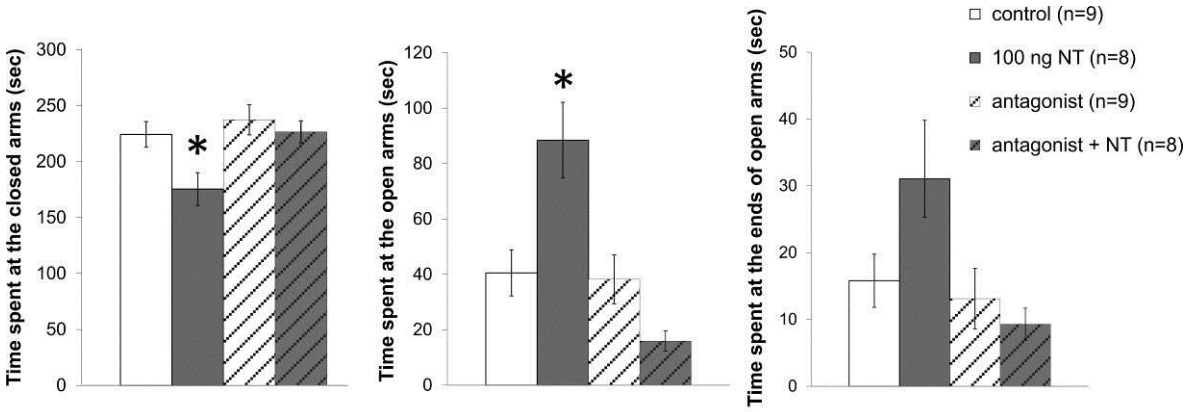

*Fig. 6. Effects of NTS1 antagonist pretreatment in EPM test. Columns represent mean time spent at the closed arms, time spent at the open arms, and time spent at the ends of the open arms, respectively (± S.E.M.). Control: vehicle treated rats (veh2 + veh1; n = 9). 100 ng NT: animals microinjected with veh2 + 100 ng NT (n = 8). Antagonist: rats treated with 35 ng NTS1 antagonist + veh1 (n = 9). Antagonist + NT: animals microinjected with 100 ng NT pretreated with 35 ng NTS1 antagonist (n = 8). For more explanation see the text. *: p < 0.05.*

The aim of the second experiment was to examine, whether the effects of NT in EPM are mediated by NTS1 receptors (Fig. 6.). The one-way ANOVA showed a significant difference among the groups in time spent at the closed arms (F [3;30] = 4.734, $p < 0.05$), in the open arms (F [3;30] = 10.311, $p < 0.05$), and at ends of the open arms (F [3;30] = 3.729, $p < 0.05$). The Tukey post hoc test showed, that the antagonist by itself did not influence the time spent at any part of the arms, the results of the animals were similar to results of the control group. Similarly to the first EPM experiment, the 100 ng NT treated group spent significantly shorter time at the closed arms, than the other groups, and significantly longer time on the open arms, than the control group. The result of antagonist-pretreated animals did not differ from the control group, but they spent significantly longer

time at the closed and significantly shorter time in the open arms, than rats of the 100 ng NT treated group, i.e. the antagonist pretreatment prevented the effects of NT. The one-way ANOVA did not show a significant difference in the distance moved by the different groups (F [3;30] = 1.331; $p > 0.05$).

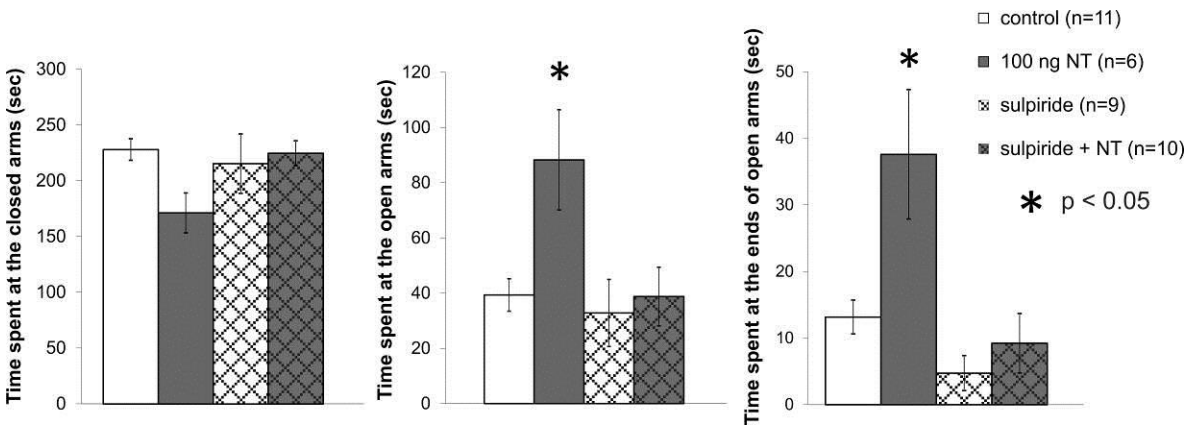

*Fig. 7. Effects of sulpiride pretreatment in the VP in EPM test. Columns represent mean time spent at the closed arms, time spent at the open arms, and time spent at the ends of the open arms, respectively (± S.E.M.). Control: vehicle treated rats (veh3 + veh1; n = 11). 100 ng NT: animals microinjected with veh3 + 100 ng NT (n = 6). Sulpiride: animals received 4 µg D2 DA receptor antagonist sulpiride + veh1 (n = 9). Sulpiride + NT: effect of microinjections of 100 ng NT after 4 µg sulpiride pretreatment (n = 10). For more explanation see the text. *: p < 0.05.*

The aim of the third EPM experiment was to investigate, whether in the anxiolytic effects on NT an interaction with D2 DA receptors plays a role (Fig. 7.). The one-way ANOVA showed no significant difference among the groups in time spent at the closed arms (F [3;32] = 1.864; $p > 0.05$). However, there was a significant difference in time spent at the open arms (F [3;32] = 4.121; $p < 0.05$), and at the ends of the open arms F [3;32] = 7.978; $p < 0.05$). The Tukey post hoc test showed, that sulpiride in the applied dose did not influence the time spent at the different arms. In the sulpiride treated group, although the average time spent at the ends of the open arms revealed a tendency to be shorter, none of the measured times were significantly affected comparing to the control group. The animals treated with 100 ng NT spent significantly more time at the open arms, and at the ends of the open arms, than the control group (similar to the first and second

EPM experiment), and the sulpiride treated group. The results of the NT injected group pretreated with sulpiride did not significantly differ from the results of control group, but they spent significantly shorter time at the open arms, and at the ends of the open arms, than the 100 ng NT treated animals. Consequently the sulpiride pretreatment prevented the effect of NT. The one-way ANOVA showed significant difference in the distance moved by the different groups (F [3;32] = 15.572; $p < 0.05$). Based on Tukey post hoc test, animals treated with sulpiride or with NT after a sulpiride pretreatment, moved a significantly shorter distance during the experiment, than the control or the NT treated group.

5. Discussion

5.1. The role of the NTS1 receptors of the VP in the regulation of locomotor activity

The OPF test revealed, that neither NT, nor the NTS1 antagonist SR 48692 microinjected into the VP influenced locomotor activity significantly in the applied doses. Although, based on the results of all groups, the distance moved by the animals in general was significantly shorter in the test trial compared to the basal activity in drug-free state, and the number of crossings also somewhat decreased (but not significantly), the means of the different groups did not differ from each other neither during drug-free state, nor during the test. This phenomenon can probably be explained by an effect of adaptation (further habituation) to the experimental environment. The results of the OPF test are consistent with the findings of Torregrossa and Kalivas [7] demonstrating that microinjection of NT(8-13) into the VP did not influence spontaneous locomotor activity.

5.2. The role of the NTS1 receptors of the VP in reward

The CPP paradigm has been widely used to test the rewarding-reinforcing effects of different drugs [30, 31]. In the CPP paradigm, conditioned association is formed between the effect of the drug and the “topography” of the treatment area (in case of our apparatus: the treatment quadrant). The formation of place preference has at least two requirements:

the first is the rewarding or positive reinforcing effect of the drug, and the other is the memory acquisition [31]. The animals were tested in drug-free state. This has a major advantage, i.e. the possible acute effects or side-effects of the drugs (on locomotion, pain sensation, anxiety or on other functions) do not influence the experimental results. During the test trial the memory processes related to environmental cues were available to guide or influence the behaviour of animals.

It is known, that the VP plays crucial role in place preference: psychostimulants, such as cocaine and amphetamine induce CPP [33]. Blockade of opioid receptors in the VP causes conditioned place aversion [34], lesions of the VP abolish the acquisition of the amphetamine induced CPP [35-37]. NT was shown to exhibit rewarding effect in other brain structures: it is effective in chemical self-stimulation in the VTA, NAC, subiculum, but not in the medial forebrain bundle [38, 39]. Additionally, NT causes place preference in the VTA [40] and in the central nucleus of amygdala [25].

Our results showed that NT microinjected into the VP has a rewarding and reinforcing effect. Furthermore, our experiments demonstrated, that NT in the CPP paradigm does not influence the locomotor activity of the animals. The results of the CPP test cannot be influenced by the possible acute effects of the drugs on locomotion, because the test was performed in drug-free state. However, if the neurochemical substance would influence the locomotion during the conditioning trials, it could also be visible in the test trials, due to the so called conditioned treatment effect [31, 41], because the locomotor effect during the conditioning trials can be associated with the treatment quadrant. In the present experiments the NT did not influence the distance moved by the animals neither during conditioning, nor during test trials, it coincides with the results of OPF test.

The receptor specificity of the positive reinforcing effect of NT was investigated by NTS1 specific antagonist SR 48692, because only the NTS1 receptors of the three different NT receptors can be identified in the VP in high concentration [17]. In the second experiment the 100 ng NT treated group spent more time in the treatment quadrant, similar to the result of the first experiment, i.e. the place preference inducing effect of 100 ng dose of NT was reproduced. This effect was effectively antagonized by equimolar dose of NTS1 specific antagonist. In the CPP paradigm, the distance moved by the animals was not influenced by NT or SR 48692, the average of the distance moved by the different groups did not differ within the trials.

In the experiments performed with the antagonist during conditioning trials the animals (in all groups) received not one, but two microinjections per side (differently from the first experiment). The other possibility of the experimental design would have been, that the animals treated with one substance (vehicle, NT or SR 48692) received only one, and the animals treated with the antagonist received two microinjections during conditioning trials. One might suppose that in that case the higher microinjection voume could influence the results of the NT group pretreated with the antagonist. To exclude this possibility (and to make it possible, to compare the results of the different groups within the experiment) the animals in the control group received 2-2 microinjections, and the animals treated with 100 ng dose NT were also microinjected with the vehicle of the antagonist 15 minutes before the microinjections. However, the results of the 100 ng NT treated group and of the control group were similar to those seen in the first experiment. Based on this, the result of the experiment was not influenced by the double volume of the microinjections. Additionally, in the second experiment it has been confirmed, that NT in the 100 ng dose has rewarding effect in the VP, because the result of the first experiment was reproduced. The NTS1 antagonist SR 48692 by itself was not effective in the applied dose, however, the antagonist pretreatment prevented the effect of NT, successfully demonstrating the NTS1 receptor specificity of the effect. By means of the CPP test it has been proven, that the NTS1 receptors of the VP play a role in the rewarding processes.

5.3. The role of the NTS1 receptors of the VP in the regulation of anxiety

According to the results of CPP test one may suppose that animals spend more time in the treatment quadrant (motionless) due to an anxiogenic effect of the substance. Furthermore, it has been shown, that some reinforcing drugs may have anxiolytic effect. To clarify these suppositions, the effect of NT on anxiety was also examined. The EPM test is a widely accepted paradigm to test anxiety. It is based on the natural fear of the animals from the open spaces and height [32], which results in an avoidance. So the increased time spent at the open arms and especially at the ends of the open arms indicates the anxiolytic effect.

In our present experiments the 100 ng dose of NT has been shown to produce anxiolytic effect: NT significantly decreased the time spent at the closed arms and increased the time spent at the open arms and in the ends of the open arms.

In the second EPM experiment, the NTS1 antagonist SR 48692 by itself did not influence the experimental results in the applied dose, however, it prevented the anxiolytic effect of NT. Consequently, anxiolytic effects of NT in the VP are mediated via NTS1 receptors. Additionally, in the second experiment the anxiolytic effect of 100 ng dose of NT was successfully reproduced. The importance of NTS1 receptors in the regulation of anxiety had also been proven by systemic administration of selective NTS1 agonist PD 149163, that performed anxiolytic effects [42, 43]. In other brain structures the NTS2 subtype can also be involved in the regulation of anxiety [1]. The double volume of the microinjections did not influence the experimental results in the EPM paradigm, because, similarly to the CPP test, the results of the first experiment were successfully reproduced.

When only the time spent in the different parts of the apparatus is measured, it cannot be excluded, that the results are partially due to the consequences of the potential effect of NT on locomotion, i.e. if NT microinjected into the VP increases locomotor activity, the animals spend longer time in the open arms or at the ends of the open arms during the EPM test. However, the distance moved by the animals during EPM paradigm did not differ in the different groups. Therefore in this experiment it was also successfully demonstrated, that neither NT, nor SR 48692 influences locomotor activity. Additionally, the average of the distance moved by the different groups was similar to the results, that have been recorded in the 5 minutes long OPF test.

Based on our results, it is reasonable to suppose that the anxiolytic effect is due to the activation of NTS1 receptors of the VP evoking a positive motivational-emotional state which was associated to the treatment quadrant in the CPP paradigm. The same phenomenon could be observed in case of substance P, that induces place preference and has anxiolytic effect, when microinjected into nucleus basalis, [14, 44], into the globus pallidus [45, 46] or into the central nucleus of amygdala [46, 47]. While, the CPP inducing effect of NT in the central nucleus of amygdala was similar to that seen in the VP, however, NT microinjections in the amygdala did not influence anxiety [25].

5.4. Possible mechanism of action of NT in the VP

NT receptors can be found almost exclusively in the VPvm subregion, but not in the VPdl or other subregions [11, 15, 48]. Therefore, it is obvious that this subregion is responsible for the positive reinforcing and anxiolytic effects of NT in the VP.

One of the main target of the efferents of VPvm is the VTA [21, 48, 49]. The GABAergic neurons of the VP inhibit the firing of the neurons of the VTA [50], thereby VP influences the population activity of the DAergic neurons of the VTA [51]. It is known, that the microinjection of NT into the VPvm increases extracellular GABA level [7]. Based on the most recent researches the inactivation of the VPvm by artificial (the so called "designer") receptors (during that the VTA is released from the GABAergic inhibition) inhibits the cue-primed drug seeking [20]. Based on these findings, it has a high probability, that the direct microinjection of NT into the VP activates VTA through inactivation of the VPvm, thus inducing the rewarding and anxiolytic effect of NT. The increased activity of the VTA leads to increased extracellular DA level in the NAC [51]. This increased DA level in the VTA and the NAC can be associated with reward and positive reinforcement [52-54].

5.5. Role of DA in the mechanism of action of NT

Based on the literature, in the behavioural effects of NT the modulation of the GABAergic system may play a role [7]. However, it is known, that the $GABA_A$ antagonist picrotoxin in the VP has no effects in CPP paradigm [55]. Therefore the role of GABAergic system in the place preference is probably not remarkable. Based on this, it can be supposed, that the behavioural effects of NT (including the place preference and anxiolytic effects) are mediated at least partially independent from the GABAergic system. One of the most probably mediating neurotransmitters appears to be DA [5, 22]. The concept is supported by the fact, that in the VPvm both NTS1 and DA receptors were identified [11, 48, 56, 57], and that i.v. administered NT receptor antagonists and DA receptor antagonists act similarly to the activity of VP neurons [19]. Based on the aformentioned data, we supposed, that in the behavioural effects of NT in the VP, that is innervated by the fibers of

mesolimbic DAergic system, the modulation of the DAergic system can also play an important role [58].

In our present experiments in the CPP paradigm the sulpiride by itself did not cause place preference or aversion in the applied dose. The place preference inducing effect of 100 ng dose of NT was reproduced again, and this effect was prevented by sulpiride pretreatment. Additionally, the sulpiride by itself and the sulpiride pretreatment significantly reduced the distance moved by the animals in conditioning and test trials. In the test trial that is performed in drug-free state, the result cannot be explained by the acute neurochemical effect. The possible explanation of this phenomenon is the conditioned treatment effect [31], i.e. during conditioning trials the animals move for a shorter distance due to the acute drug effect, and this is associated with the experimental apparatus. There might be an argument against this explanation, that the memory consolidation inhibiting effect of sulpiride [28] should weaken conditioned treatment effect, too. A possible other explanation could be a potential long term motor deficit caused by sulpiride. In Morris water maze test, however, no difference in swimming velocity could be observed in the animals treated by sulpiride earlier, relative to the other groups [28], so the role of motor deficit can be excluded.

In the EPM paradigm the sulpiride pretreatment prevented the anxiolytic effect of NT. Animals treated with sulpiride showed a tendency to spend shorter time (4.73 ± 2.73 sec) at the ends of the open arms than the control group (13.16 ± 2.52 sec), however, this was not significant. Nonetheless, the anxiogenic effect of sulpiride cannot be excluded, i.e. it is more difficult to detect its significant anxiogenic effect relative to controls, because the control animals spend shorter time at the open arms, too.

In our experiments the place preference inducing and anxiolytic effects of NT were detected in three different experiment. It is remarkable, that the averages of the results of the animals treated with 100 ng NT were similar in all three CPP test, independently from the experimental conditions (different groups of animals, in different time, however, in standardized circumstances). In the three EPM tests the results of the control groups and the NT treated groups were also similar. By means of the sulpiride pretreatment it has been verified, that in the VP the NTS1 receptor activation is associated with D2 DA receptors, concerning the rewarding and anxiolytic effect. The activity of the D2 DA receptors is a necessary requirement for evoking the effect of NT in both paradigms, because the

sulpiride pretreatment prevents the effect of NT, but the activity of the D2 DA receptors (at least in the CPP test) by itself is not sufficient to elicit an effect [28]. Additionally, NT receptors of the VP are located postsynaptically [16], and not on the DAergic axon terminals. So the NT cannot modulate the function of DAergic inputs directly (although an interaction between the postsynaptic DA and NT receptors is possible). It is probable, that besides the DA system (via D2 receptors) other neurotransmitter mechanisms are involved in the actions of NT in the VP, but the endogenous DA activity is a necessary requirement of this effect.

6. Summary

The questions in the Objectives can be answered with the followings:

1. NT in the VP does not influence locomotor activity.

2. Microinjection of 100 ng dose of NT into the VP has rewarding effect in CPP test.

3. The rewarding effect of NT is mediated through NTS1 receptors, because it can be prevented by NTS1 specifc antagonist SR 48692.

4. Microinjection of 100 ng dose of NT into the VP has anxiolytic effect.

5. The anxiolytic effect of NT in the VP is mediated through NTS1 receptors, because it can be prevented by NTS1 specifc antagonist SR 48692.

6. The activity of the D2 DA receptors is necessary requirement for both rewarding and anxiolytic effects of NT, because both effects could be prevented by D2 DA receptor antagonist sulpiride.

7. List of publications

The full publication list can be found:
https://vm.mtmt.hu/search/slist.php?lang=0&AuthorID=10020537

7.1. Publications related to the thesis

Ollmann T, Péczely L, László K, Kovács A, Gálosi R, Berente E, Karádi Z, Lénárd L. Positive reinforcing effect of neurotensin microinjection into the ventral pallidum in conditioned place preference test. Behavioural Brain Research. 2015;278:470-5. [IF: 3.028]

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7.2. Other publications with impact factors

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