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New neurotensin analogue with improving effect on some affective symptoms in Parkinson's disease model in rats

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Neurotensin (NT) is a small neuropeptide acting as neurotransmitter and neuromodulator in the nervous system. However, natural neurotensin is rapidly degraded in the body, therefore artificial analogues are needed to prolong its bioavailability and effects. Parkinson's disease (PD) is a neurological disease with specific motor, cognitive and affective disturbances and is associated with high oxidative stress and mitochondrial dysfunction and degeneration of the dopaminergic system. The scope of the study was to evaluate some effects of a new neurotensin analogue (NT2) upon the behavior of rats with a model of PD induced with striatal injection of the neurotoxin 6-hydroxydopamine (6-OHDA). The PD model was produced *via* striatal 6-OHDA (12 μg in 2 μl saline) injection of male Wistar rats. NT2 treatment was with an effective daily dose of 5 mg/kg i.p. for 5 days. NT2 effects were evaluated *via* behavioral tests for locomotor activity and anxiety. Student's t-test was used at $p<0.05$. The PD model was verified by rotarod tests on the 2nd and 3rd week after the operation and was compared to sham operated animals. There was a significant performance decrease in the mood and affective disturbances. The affective disturbances as anxiety in NT2-treated animals were reduced (both on the 2nd and 3rd week) compared to PD-controls.

Key words: neurotensin, affective disorder, anxiety, Parkinson disease.

INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases with specific motor, cognitive and affective disturbances and is associated with high oxidative stress and mitochondrial dysfunction and degeneration of the dopaminergic system. Among the non-motor symptoms, some of the most common ones are depression and anxiety [1]. They may seriously impact the wellbeing and performance of the patients and are an additional source of stress for the care givers [2]. However, the current therapies are mostly focused to combat the motor symptoms, while the non-motor ones often remain neglected [3].

One of the first discovered connections between the receptors of the different neurotransmitters and neuromodulators was the one between the DA-receptors and neurotensin (NT) receptors. Such close connection suggests that NT is associated with PD [4]. Additionally, several years after these findings the researchers gradually started to discover that NT can be related to some affective symptoms in the psychiatric disorders [5], which further stimulated such research and the quest for NT-like agents.

Neurotensin is a tridecapeptide acting as neurotransmitter and neuromodulator in the nervous system. It is secreted in both the central nervous system and the gut.

NT exercises its biologic effect from the specific interaction of the peptide with three different cell-surface receptors referred to as NTS1, NTS2 and NTS3/sortilin [6]. However, natural neurotensin is rapidly degraded in the body, therefore artificial analogues are needed to prolong the bioavailability and effects. Therefore, the object of this study is a promising long-lasting NT analogue with code NT2 (Fig. 1) synthesized by Pajpanova *et al*. [7].

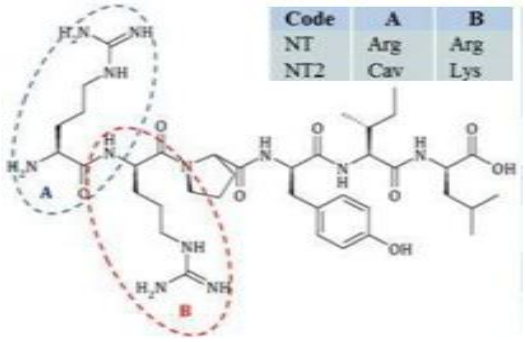

Fig. 1. The amino acid sequence of NT and NT2-analogue.

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Our previous research on this NT-analogue showed that it has better permeability of the blood-brain barrier than the native NT and improved hydrolytic stability [8, 9].

Based on all this the goal of our study is to explore the impact of the new NT2-analogue on some affective disturbances in PD.

EXPERIMENTAL

Synthesis of NT analogue

NT-analogue (with code NT2) was synthesized through standard solid-phase method. The peptide chain was assembled on a Wang resin (0.1 mmol scale) with a Fmoc/Boc strategy. The coupling of each amino acid was performed in the presence of 3 mol excess of Fmoc-amino acid, 3 mol excess of HOBt, 3 mol excess of DIC and 5 mol excess of DIPEA. The cleavage step from the resin and the final deprotection of all remained protecting groups was done in a standard cocktail containing TFA, TIPS, thioanisole, and water [7].

Animal experiments and treatment

A total of 24 male Wistar rats (220 – 250 g) were used. Before the beginning of the experiment the rats were adapted to the new conditions in our vivarium. They were housed in groups of four per cage in a temperature-controlled room with a 12 h light-dark cycle, and had free access to food and water.

The animals were treated intraperitoneally (i.p.) for 5 days as follows: SO and 6-OHDA controls with 5 ml/kg saline; 6-OHDA + NT2 group with 5 mg/kg i.p. NT2.

All experiments were performed according to the "Principles of laboratory animal care" (NIH publication No. 85-23), and the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

PD experimental model and animal surgical procedures

The rats were anaesthetized with chloralhydrate (420 mg/kg, i.p.), their heads were shaved and skin cleaned with 70 % alcohol. Then the rats were positioned in the stereotaxic apparatus. PD model was induced *via* stereotaxic injection of 2 µl/12 µg 6-hydroxydopamine (6-OHDA, (Sigma-Aldrich, USA)); calculated as free base, dissolved in ice-cold saline with 0.02 % ascorbic acid) in striatum [10]. The target coordinates for striatum were AP=0; ML=3.5; H=-5 from the bregma and dura, according to the stereotaxic atlas [11]. Sham operated (SO) group received only 2 µl of saline. The wound was closed and animals returned to their cages for recovering.

Biochemical assay

At the end of the experiments rats were euthanized by CO_2 and decapitated, the brains were quickly removed and placed on ice until further processing. The dopamine determination followed the method of Jacobowitz *et al.* [12]. In brief, the brain tissue samples were homogenized in N-butanol. Dopamine (DA) was extracted in 0.1 M phosphate buffer. After 20 min DA fluorescence was measured at 320 nm activation/385 nm emission wavelengths. The fluorescence readings were converted into µg DA per g of brain tissue.

Rotarod test

Rotarod test was used for assessment of the motor coordination of the animals. The apparatus consisted of a horizontal rod (6 cm diameter) with discs (40 cm diameter). The rotation speed was set to 8 rotations per minute. During the training and testing sessions the animals were placed on the rotating rod with head pointing against the rotational direction so that they had to walk forward to maintain their equilibrium. The animals were trained one day before the surgery. The number of falls during a period of 180 seconds was recorded [13].

Elevated plus maze

The elevated plus maze consisted of two open arms (length 50 cm and width 11 cm) and two enclosed arms (length 50 cm, width 11 cm, and height 40 cm), arranged in cross formation in such way that two pairs of arms with same construction were opposite to each other surrounding a central platform (11×11 cm). The whole apparatus was positioned 50 cm above ground level. Experiments were performed under dim light conditions. Each animal was placed on the central platform with head facing an open arm. The animal activity was video recorded for 5 min. After each session the apparatus was carefully cleaned. As measures of anxiety the following parameters of the behavior were assessed: 1) percent time spent on the open arms (% open time); 2) percent open arm entries from the total arm entries.

As indicators of locomotor activity of the rats tested on the elevated plus maze test were also assessed: closed arm entries and total arm entries, since anxiety-related effects can be confounded by changes in motor activity [14, 15]. An entry in the

arm was considered when the whole body and four paws were placed on the arm.

Open field test

In order to determine the nonspecific motor effects which may have flustered the assessments in the elevated plus maze test, assessment of the locomotor activity was performed in the same animals following the elevated plus maze test procedures. The assessment of the locomotor activity was done in the same room as the elevated plus maze test.

Additionally, the number of entries from the outer zone into the central zone of the arena was measured which was used to estimate the anti-thigmotactic ratio as an indicator of increased or decreased anxiety of the tested animals. This ratio is calculated by the number of entries into the central arena of the open field to total distance travelled, multiplied by 1000. Initially the rats were placed in the center of the open field arena [16]. The open field apparatus comprised a cylinder (diameter 1 m, height 40 cm) on contrasting background. The arena was divided in two zones – central zone and outer zone equidistantly surrounding it. The testing sessions lasted for 20 min and were video recorded for analysis.

Statistical and data analysis

Results were expressed as means ± SEM. Experimental data were analyzed by Student's t-test. Differences were considered significant at $p < 0.05$.

The video materials from open field and elevated plus maze tests were processed with the specialized neurobehavioral and video analysis software Noldus EthoVision [17]; raw data of the spatiotemporal analysis were imported into Microsoft Excel 2007 (Microsoft Corp., USA) for further processing. The total distance traveled, velocities and entrance and time spent at important areas of the apparatus were calculated with custom-built algorithms.

RESULTS

Dopamine levels

The results from the biochemical assay (Fig. 2) showed decreased levels of DA in the brain tissue in the 6-OHDA intoxicated rats in comparison with the sham operated group. However, the rats treated also with NT2 analogue showed a significant improvement in this parameter (by a factor of 9.37) compared to the non-treated animals. In comparison with the control group the NT2 treated one has lower values by a factor of 0.81. So basically the DA levels were closer to the ones of the control group than to the ones of the non-treated PD group.

Rotarod test

After the training and the recovery from the surgery the rats' performance on the rotarod test showed gradual decrease in the motor coordination with the progress of the lesion in the 6-OHDA intoxicated animals (Fig. 3).

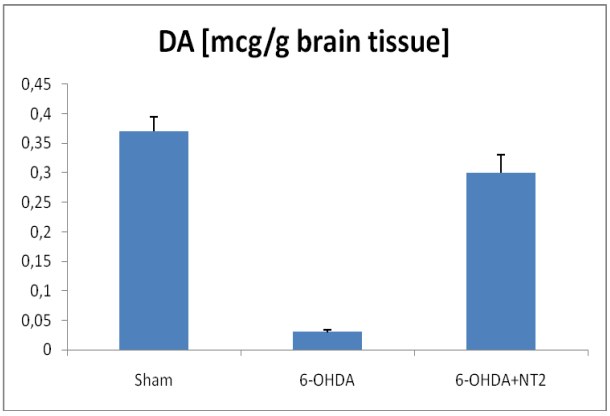

Fig. 2. DA levels in the brain among the groups on the 3rd week after the surgery.

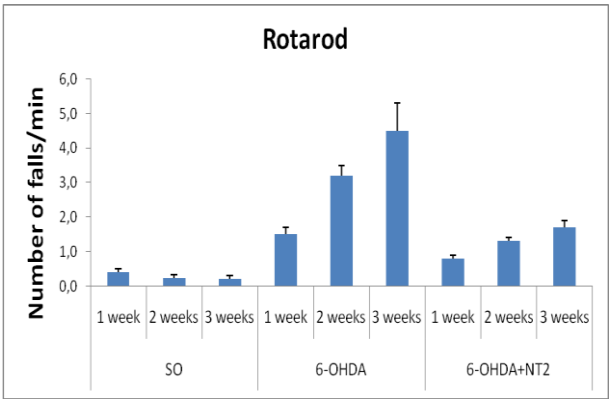

Fig. 3. Rotarod test performance on the 1st, 2nd and 3rd week after the surgery.

The differences in the first week after the lesion were less pronounced: the NT2 treated group showed 30% increase of the number of falls compared to the SO group while this number was increased by 153% in the non-treated 6-OHDA intoxicated group. Two weeks after the lesion the total number of falls in the NT2 treated group was increased by 33% compared to the SO group, while in the non-treated 6-OHDA intoxicated group this number was increased 5 times. On the third week the NT2 treated group showed an increased number of falls by 85% compared to the SO group while in the non-treated 6-OHDA intoxicated group this number was increased by 412%.

Open field test

On the complementary part of the open field test evaluations for possible decrease in the locomotor activity which may confound the results of the other tests, no statistically significant differences were observed among the groups on their respective performance during the first, second and third week after the lesion.

However, on the anxiety related part of the test we see different trends and patterns as shown by the values of the number of entrances in the central area and the anti-thigmotactic ratio (Figs. 4, 5).

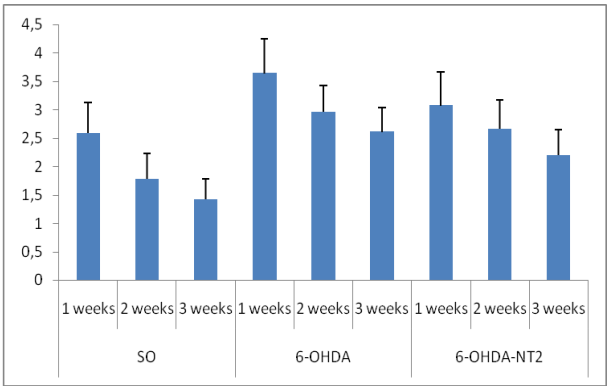

Fig. 4. Open field test number of entrances in the central area from the 1st, 2nd and 3rd week.

In general, these parameters decrease with the longer time from the surgery they were measured. But anti-thigmotactic parameter also differs between the intoxicated groups for the 1st, 2nd and 3rd week by 10.56%, 12.25 and 19.28%, respectively. In comparison with the control group the NT2 treated group has higher values for the different weeks by 22.06%, 37.5% and 42.14%, respectively.

Elevated plus maze

The elevated plus maze test also showed an increase of the measured behavioral parameters related to anxiety in the NT2 treated group, while the trend was opposite in the non-treated 6-OHDA intoxicated group compared to the SO group (Fig. 6).

On the third week the percentage of open arm entries in the NT2 treated group was 12% and in the non-treated 6-OHDA group it was 10%, while in comparison to the SO group the parameter was 21%. The parameters for locomotor activity – closed arm entries and total arm entries showed no statistically significant differences among the groups at $p<0.05$.

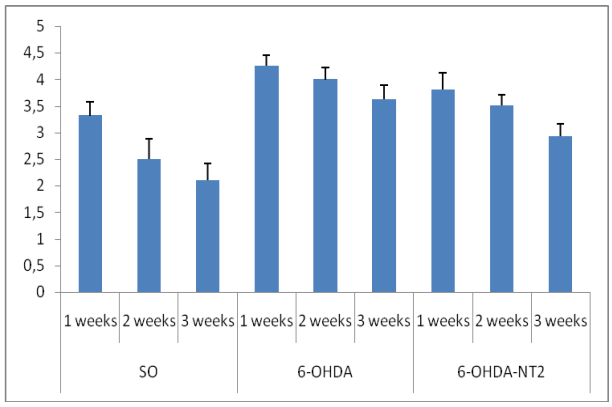

Fig. 5. Open field test anxiety related anti-thigmotactic ratios from the 1st, 2nd and 3rd week.

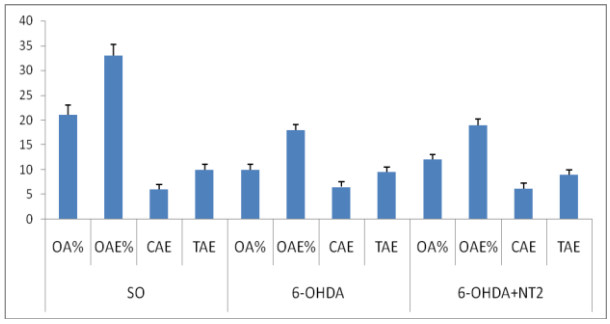

Fig. 6. Elevated plus maze test performance on the 1st, 2nd and 3rd week after the surgery.

DISCUSSION

The worsened locomotor coordination and performance on the Rot-a-rod test verified the chemically induced model of PD. The model was further confirmed by the results of the biochemical assay for the dopamine levels in the brain which show nearly 10-fold decrease in the PD animals. Also, in postmortem histological analysis it was found that the lesions were located in the striatum.

Further, the behavior performance results show that overall, the new NT2 analogue has beneficiary effects on some of the affective symptoms of PD.

Typically, these fearful animals have quite pronounced thigmotaxis when exposed to a new unknown environment especially in open areas without hiding places and objects. Their natural behavioral tendency is to remain as close as possible to the available walls (thigmotaxis) and hiding objects. Although there is little research on these phenomena in relation with DA-ergic neurotransmission, some studies found that some agonists of the D1 and D2 dopamine receptors can have modulatory effects on this parameter, generally by reducing it [18]. Here we see that the damage of DA-ergic system increases it, while treatment with NT2 partly restores it.

In general, the DA-ergic system is less frequently accounted for as engaged in the anxiety and the related disturbances but some studies showed that through D1 and D2 dopamine receptors in mesolimbic circuits the DA-ergic system may also have a modulatory role in this emotional behavior [19]. And here we observe a similar pattern – destruction of a large part of the dopaminergic system leads to affective disturbances as shown in our tests.

The mesolimbic circuits of the dopaminergic system also contain a relatively high level of co-localized NT-receptors as some neuroanatomical studies show [20]. In behavioral concordance with this is the observed significant improvement in the NT2 treated group performance in all the anxiety tests, which can be related to stimulation of compensatory mechanisms by the NT2 in this pathway, such as the fact that NT elicits evoked DA release in the striatum and prefrontal cortex [21]. A clinic research by Ruiz *et al.* from 1992 [22] showed that plasma levels of some neuropeptides are significantly reduced in patients with depression or anxiety disorder, which get restored after recovery. With our study we go further by confirming that there is a functional connection between the NT associated part of the dopaminergic system and some affective disturbances, since by introducing the NT2 analogue into the plasma we partially mitigate some of the affective symptoms incurred by the massive selective neurotoxin destruction of the dopaminergic system.

In our previous studies [9, 10] we showed the beneficiary effects of NT2 on the behavior related to the damaged DA-ergic circuits in PD in charge for the motor and cognitive behavior. In this study it was shown that NT2 impacts positively other DA-ergic neural circuits related to the emotional behavior.

CONCLUSION

The new NT-analogue is a promising agent for the management of some of the non-motor symptoms of PD as anxiety, and deserves further exploration and development.

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