

Effects of new neurotensin analogue on brain activity in rat Parkinson's disease model

A. Popatanasov¹, S. Stoeva¹, M. Lazarova¹, L. Traikov², T. Pajpanova³, R. Kalfin¹, L. Tancheva^{1,4*}

¹*Institute of Neurobiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 23, Sofia, Bulgaria*

²*Faculty of Medicine, Medical University, Zdrave Str. 2, Sofia, Bulgaria*

³*Institute of Molecular Biology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., bl. 21, Sofia, Bulgaria*

⁴*Weston Visiting Professor of Weizmann Institute of Sciences, Herzl Str. 234, Rehovot, Israel*

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Parkinson's disease (PD) results in progressive loss of dopamine (DA) neurons and leads to motor disturbances. The close connection between DA-ergic neurotransmitter system and Neurotensin (NT) mediation was established which suggests that NT is associated with PD. It was reported that NT can modulate the activity of DA neurons. Our previous data demonstrated significant CNS-activity in rodents of some new NT-analogues. The aim of the present study was to evaluate the potential modulating effect of new NT-analogue on the behavior and brain activity in rats with model of PD which was induced in male Wistar rats via unilateral injections of 6-hydroxydopamine (6-OHDA) and verified by apomorphine test. Animals were treated 5 days with new NT analogue in effective doses after the induction of PD. Standard test was used for evaluation of neuro-muscular coordination (Rot-a-Rod). Electroencephalography (EEG) was used also to measure the brain activity in inactive condition. Experimental data were processed by Student-Fisher, or Mann-Whitney or Kruskal–Wallis test. Rot-a-rod test showed gradual improvement in the motor performance of NT-treated animals compared to control PD- rats with saline. In the same time EEG showed differences in spectral composition and patterns above the lesioned areas and their hemispheric counterparts in the PD-animals treated with NT-analogue compared to saline treated PD-rats and similarities with the healthy ones. In conclusion the new NT-analogue is promising anti-PD agent and deserves further investigations.

Keywords: Neurotensin, Parkinson's disease, 6-hydroxydopamine, Neuropeptides, EEG

INTRODUCTION

Parkinson's disease (PD) results in progressive loss of dopaminergic (DA) neurons and leads to motor disturbances. The close connection between DA-ergic neurotransmitter system and Neurotensin (NT) mediation was established which suggests that NT is associated with PD. Moreover, it was reported that NT modulates the activity of DA neurons [1]. NT is a 13-amino-acid peptide and it is demonstrated that in mammals there are NT-containing neurons in some specific central nervous structures as striatum and substantia nigra pars reticulata [2]. The biologic effect of NT results from the specific interaction of the peptide with three different cell-surface receptors referred to as NTS1, NTS2 and NTS3/sortilin [3]. Like many other neuropeptides, NT acts as a neuromodulator in the nervous system and it has been shown to modulate DA release in some brain regions including striatum [4].

However, peptides are rapidly metabolized in plasma by endogenous peptidases, terminating their

biologic effect under physiologic conditions. Therefore, the object of the present study is a promising long-lasting NT analogue - NT2 (Fig. 1) synthesized by Pajpanova et al [5].

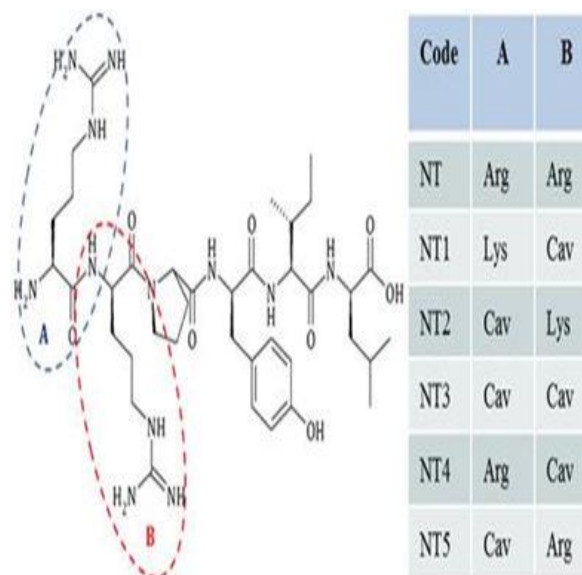


Fig. 1. The amino acid sites replaced with non-proteinogenic amino acids in synthesized NT-analogue [5].

The basic sequence was fragment 8–13 of NT, to which some modifications were introduced in

* To whom all correspondence should be sent:
E-mail: lyubkatancheva@gmail.com; and_atanasov@abv.bg

order to improve its metabolic stability. The terminal Arg unit (position A on Fig. 1) was replaced by canavanine (Cav), which was described as a non-proteinogenic Arg analogue [6]. Our previous unpublished data demonstrated significant CNS-activity in rodents of some NT-analogues.

We aimed to evaluate the potential modulating effect of one new NT-analogue on the brain activity on experimental model of PD in rats.

EXPERIMENTAL

All experiments have been 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).

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 [5].

Surgical procedures

A total of 24 male Wistar rats weighting 220 – 250 g at the beginning of the experiment were used. They were housed four per cage in a temperature-controlled room with a 12 h light-dark cycle, and had free access to food and water. The rats were anaesthetized with chloralhydrate (400 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 unilateral and stereotaxic injection of 2 µl/20 µg 6-hydroxydopamine (6-OHDA, (Sigma-Aldrich, USA)); calculated as free base, dissolved in ice-cold saline with 0.02 % ascorbic acid) in right striatum [7]. The target coordinates for striatum were AP=0; LR=3.5; H=+5 from the bregma, according to the stereotaxic atlas [8]. Sham operated (SO) group received only 2 µl saline. The wound was closed and animals returned to their cages for recovering.

All animals were treated intraperitoneally (i.p.) for 5 days before the induction of PD and were divided into the following groups: SO and 6-OHDA controls were treated daily with 5 ml/kg saline, 6-OHDA + NT and 6-OHDA + NT2 were treated with 5 mg/kg i.p, NT and NT2, respectively.

In postmortem histological analysis it was found that the lesions were located in the striatum.

Electrode implantation

The electrodes were implanted above the lesioned areas with stereotaxic coordinates AP=0; LR=3.5; H=+0.5 from the bregma and symmetrically to the skull midline above their hemispheric counterparts [8]. After the implantation the electrodes were fixed permanently with acrylic to the skull bone. The common ground wire was above the right hemisphere. The position of the electrodes was controlled on custom stereotaxic apparatus with Narishige micromanipulators (Narishige, Japan).

Postmortem histological analysis was performed to validate electrode placement and neurotoxin effects. The placements of the electrodes was in most cases in deep cortical layers AP=0; LR=3.5; H=+0.5-1.5 [8].

Behavioral tests

Rot-a-Rod standard test was used for evaluation of changes in neuro-muscular coordination [9]. On the second week after surgery each animal was placed on a gyrotory (7 rot/min) and the number of falls for 3min was determined.

Electrophysiological methods

Cortical electroencephalography (cortical EEG) was used to measure the brain activity in inactive condition in calm lab environment. The cortical EEG recordings were sampled at 200 Hz with bio-amplifier ETH-255 iWorx (iWorx, USA). To reduce the stress of the new environment the animals were placed in the restrainer for 20 minutes few times in the 2-3 days before the recording sessions on the second week after the surgery.

Immediately after all tests the animals were euthanized via CO₂ inhalation for biochemical and histological studies.

Statistical and data analysis

Results were expressed as means ± SEM. Experimental data were analyzed by Student's *t*-test or Mann-Whitney test, or Kruskal-Wallis test. Differences were considered significant at $P < 0.05$.

Cortical EEG spectral analysis algorithms were used for processing of the data according to Shi *et*

al., 2015 [10]. Schematically the EEG analysis of power spectra was performed in the following order. For a given behavioral recording period, all available EEG recordings were divided in segments of 2 seconds. A second order notch filter at frequency 50 Hz (AC) component was applied to remove power line noise. For every segment estimation of the power spectral density was performed by using the fast Fourier transform. Afterward we normalized the data, using relative power spectra by dividing the power spectral density in the different frequency ranges for each segment to the total power in the segment.

RESULTS AND DISCUSSION

Rot-a-rod test was performed at the second week post lesion (Fig. 2).

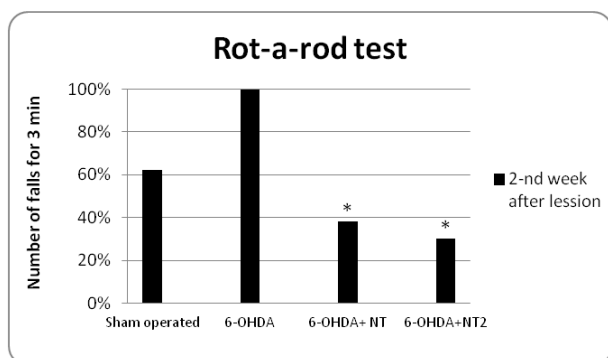


Fig. 2. Effects of new NT analogue and NT as referent on the Rot-a-rod test in rat model of Parkinson's disease; $n = 6$, * $P < 0.05$ vs 6-OHDA group

While the untreated 6-OHDA group showed an average of 1.33 falls/3min, all animals in 6-OHDA group, treated with NT and new NT analogue (NT2) demonstrated a gradual improvement in motor performance. They showed a significant decrease in number of falls for 3 minutes on the second week after lesion (NT by 62, 41% and NT2 by 69, 92%) in compare to saline treated 6-OHDA group.

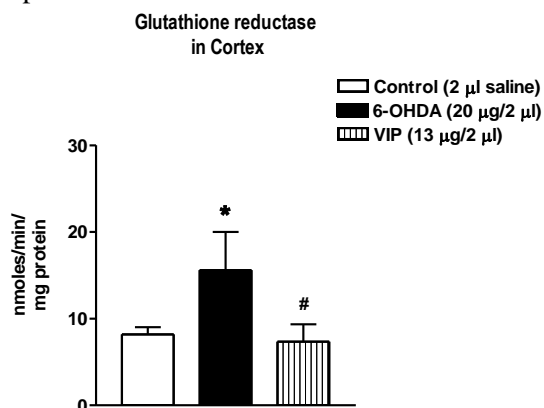


Fig. 3. Levels of glutathione reductase in the cortex of control and Parkinsonian rats. $n = 5$; * $P < 0.05$ vs Control; # $P < 0.05$ vs 6-OHDA-lesioned rats.

EEG analysis: Cortical EEGs showed differences in spectral composition and patterns above the lesioned areas and their hemispheric counterparts in the animals treated with NT-analogue compared to saline treated PD-rats and similarities with the SO ones (Fig. 4 and Fig. 5).

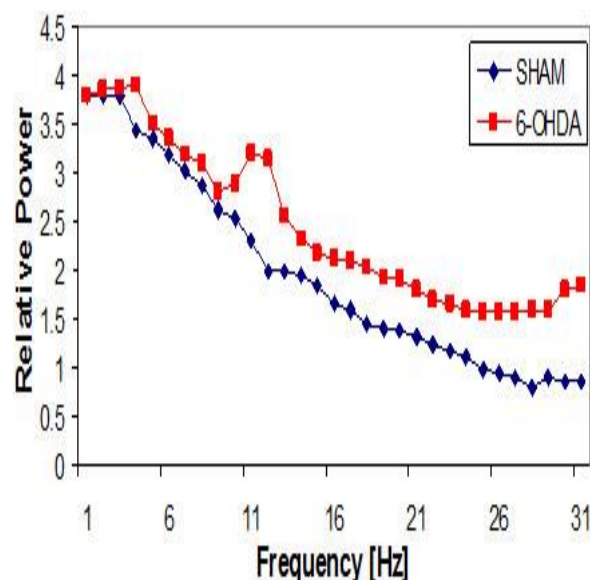


Fig. 4. Power spectra of the sham operated rats vs 6-OHDA (Parkinsonian) group

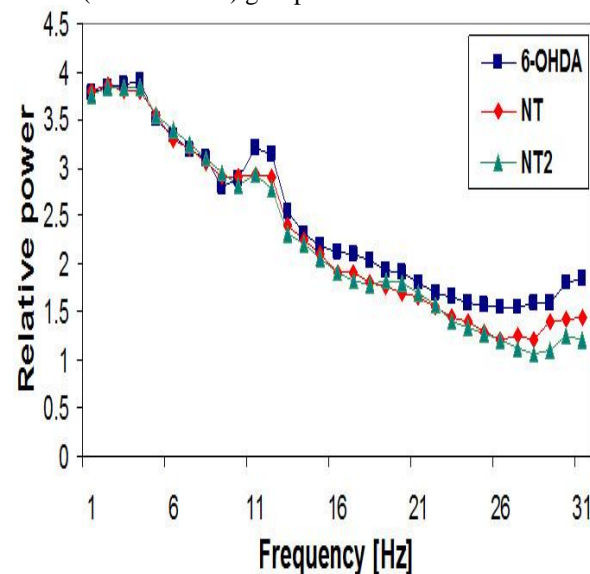


Fig. 5. Power spectra of the NT or NT-analogue treated rats vs 6-OHDA (Parkinsonian) group

The comparison between non-treated 6-OHDA and SO groups showed large increase in the power density through all beta band oscillations range (10-31 Hz) in the former group. Here the beta band oscillations range (10-31 Hz) refers to a new PD specific EEG bands classification proposed by

Gatev et al. in 2006 [12]. These differences were especially well pronounced in the ranges (8-13 Hz) and (26-31 Hz). The averaged values from the sessions for the peak frequency 13 Hz were 3.14 ± 0.16 , 2.1 ± 0.12 and for peak frequency 30 Hz were 1.86 ± 0.17 and 0.85 ± 0.13 for the 6-OHDA and SO group accordingly.

Comparing the NT and NT-analogue treated groups to the non-treated one there was a general decrease in the beta oscillations bandwidth with more pronounced peaks at frequency range of lower (9-13Hz) and upper beta (28-31Hz). The averaged values from the sessions for the peak frequency 13 Hz were 3.14 ± 0.16 , 2.90 ± 0.14 and 2.78 ± 0.15 ; for peak frequency 30 Hz were 1.86 ± 0.17 , 1.44 ± 0.15 and 1.22 ± 0.13 for the 6-OHDA, NT and NT2 group, respectively. According to the Kruskal–Wallis non-parametric test, $P < 0.05$ the differences at these peaks between the OHDA, NT and NT2 groups were significant with $\chi^2=14,02$ and 14.72 , the differences between NT, NT2 and SO groups also followed this trend with $\chi^2=14,92$ and 14.71 .

The curve pattern of the NT and NT2 treated groups (Fig. 5, 6) was relatively similar in the beta band range except at the above mentioned peaks.

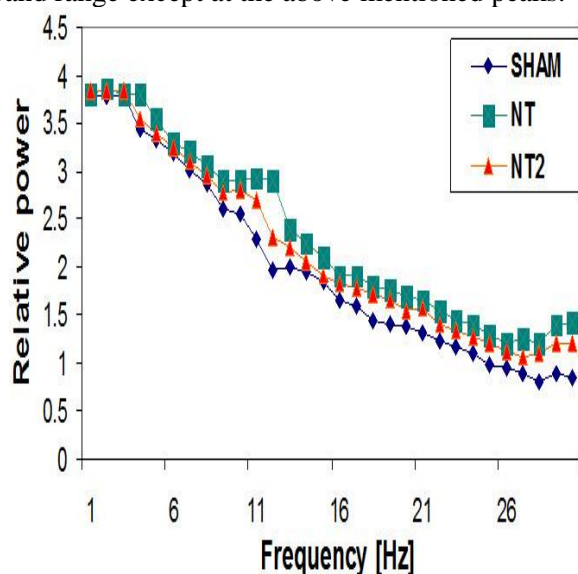


Fig. 6. Power spectra of the NT-analogue treated rats vs sham operated group

The averaged value differences in the frequency range 14-27 Hz were within 0.1 ± 0.05 . Additionally the peaks at 13 Hz and 30 Hz were compared with the Mann-Whitney test and the results showed that the differences were significant at $P < 0.05$ for the former peak.

DISCUSSION

The results of our experiments showed that there was a valuable improvement the motor functions in

both groups of the NT-analogue treated animals over the non-treated 6-OHDA animals which were among the most affected in the PD. The improving effect of NT2 on motor coordination was better than the effect of the referent NT.

Classically EEG oscillations have been grouped into frequency bands as follows: delta (0.5–3.5 Hz), theta (3.5–7.5 Hz), alpha (7.5–12.5 Hz), beta (12.5–30 Hz) and gamma (>30 Hz) [11]. However with the data accumulation from experiments with animal models and patients with PD in 2006 Gatev et al. [12] proposed a new division in the frequency range 3-35 Hz: i). frequencies at the Parkinsonian rest tremor (3-7 Hz) and ii). higher frequencies (10-35 Hz) termed beta band oscillations. Beta band oscillations generally are associated with motor and cognitive functions in normal human and mammal subjects. Our research reconfirms the importance of such specific PD division for EEG since the most pronounced differences in the power spectra were observed in the range of beta band oscillations. In the results of the EEG analysis, the increase in the power density at frequencies 9-13 Hz corresponds to the lower beta bandwidth [12]. Also there's increase in the power density at the frequencies range 26-30 Hz which corresponds to the higher beta-bandwidth. This effect was more pronounced in the NT-treated animals than the NT2-treated ones.

Beta activity is synchronized within and between functionally interconnected circuitries in the basal ganglia, thalamus and cortex. The elevated beta-band activity in the resting brain state showed that the neuromodulatory significance of beta oscillations for information processing in the motor output is impaired in PD rats. Therefore, the dopaminergic medication (or the treatment with NT analogues) reduced the beta-band activity within and between structures of this functional loop in PD rats.

This global increase of absolute power in PD rats is a pathophysiological reaction of dopamine denervation on basal ganglia-thalamo-cortical circuitries. NT analogue-induced modulation of inhibitory basal ganglia output to the thalamus may reduce abnormal thalamo-cortical rhythmicity and normalize high frequency oscillatory power in cortical networks.

The analysis of brain activity recordings in the animal groups treated with NT and NT-analogue showed power spectral pattern closer to the one of the SO or healthy rats and the effects of the NT analogue were better in comparison to the referent NT.

Based on these results we can suggest that the newly synthesized NT-analogue facilitates the survival and compensatory functioning of more DA-neurons in the 6-OHDA affected brain regions and the preservation of the networks in which they are engaged.

CONCLUSION

The new NT-analogue is promising anti-PD agent and deserves further exploration and development.

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ЕФЕКТИ НА НОВ НЕВРОТЕНЗИНОВ АНАЛОГ ВЪРХУ МОЗЪЧНАТА АКТИВНОСТ ПРИ МОДЕЛ НА БОЛЕСТТА НА ПАРКИНСОН ПРИ ПЛЪХОВЕ

А. Попатанасов¹, С. Стоева¹, М. Лазарова¹, Л. Трайков², Т. Пайпанова³, Р. Калфин¹, Л. Танчева^{1,4*}

¹Институт по невробиология, БАН, ул. Акад. Г. Бончев, бл. 23, София 1113, България

²Факултет по медицина, Медицински университет, ул. Здраве 2, София, България

³Институт по молекулярна биология, БАН, ул. Акад. Г. Бончев, бл. 21, София 1113, България

^{4*}Уестон гост-професор във Вайцман научни институти, ул. Херцел 234, Реховот, Израел

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(Резюме)

Болезтта на Паркинсон (БП) причинява прогресивна загуба на допаминергични (DA) неврони и води до двигателни нарушения. Установена е тясна връзка между допаминергичната невротрансмитерна система и невротензиновата (НТ) медиация, което предполага, че НТ е свързан с болестта на Паркинсон. Данните в научната литература показват, че НТ може да модулира активността на DA-неврони. В наши предишни изследвания беше установено, че някои от новосинтезираните от нас НТ-аналози могат да оказват силно влияние върху активността на ЦНС при гризачи.

Целта на настоящото изследване бе да се изследва потенциалния модулаторен ефект на нов НТ-аналог върху поведението и мозъчната активност при плъхове с модел на БП, който бе индуциран при мъжки плъхове от породата Wistar чрез едностранно инжектиране на 6-хидроксидопамин (6-OHDA) и бе верифициран чрез апоморфинов тест. Животните бяха третирани в продължение на 5 дни с НТ или с новия НТ-аналог с ефективни дози след индукция на БП. Стандартен Рот-а-род тест бе използван за оценка на нервно-мускулната координация. Електроенцефалография (ЕЕГ) бе използвана за измерване на мозъчната активност в неподвижно състояние. Експерименталните данни бяха обработени с теста на Стюдънт-Фишер, на Ман-Уитни или на Крускал-Уоллис. Резултатите от Рот-а-род теста показаха постепенно подобрене на двигателната дейност при третираните с НТ-животни в сравнение с контролните животни с БП, третирани с физиологичен разтвор. В същото време ЕЕГ показва наличие на разлики в спектъра и патерните над областите с индуцирани лезии при болните животни третирани с НТ или НТ-аналог в сравнение с плъховете с БП, третирани с физиологичен разтвор, и сходства със здравите опитни животни. В заключение може да се каже, че новия НТ-аналог е обещаващ антипаркинсонов агент и заслужава по-нататъшно изследване.