The bioanalytical assessment of brain alpha-tocopherol homeostasis in normal aging and in MPTP-induced neurotoxicity in mice

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Summary of Ph.D. Thesis

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I. **Nánási N.**, Veres G., Cseh E.K., Szentirmai M., Martos D., Sümegi E., Hadady L., Klivényi P., Vécsei L., Zádori D. The detection of age-, gender-, and region-specific changes in mouse brain tocopherol levels via the application of different validated HPLC methods. Neurochem. Res. 2018, 43:2081–2091 (original paper, IF: 2.782, **Q2**)

II. **Nánási N.**, Veres G., Cseh E.K., Martos D., Hadady L., Klivényi P., Vécsei L., Zádori D. The assessment of possible gender-related effect of endogenous striatal alpha-tocopherol level on MPTP neurotoxicity in mice. Heliyon 2020, 6:e04425. (original paper, IF: -, **Q1**)

III. **Nánási N.**, Cseh E.K., Szentirmai M., Veres G., Klivényi P., Vécsei L., Zádori D. Development and validation of high performance liquid chromatography methods for vitamin E measurements. *Proceeding of the 23rd International Symposium on Analytical and Environmental Problems.* 2017. pp. 469-473. Alapi T., Ilisz I. (eds). University of Szeged, Department of Inorganic and Analytical Chemistry, H-6720 Szeged, Dóm tér 7, Hungary. **ISBN 978-963-306-563-1** *(conference proceeding).*

IV. **Nánási N.**, Hadady L., Cseh E., Veres G., Klivényi P., Vécsei L., Zádori D. Development and validation of high performance liquid chromatography method for the measurements of biogenic amines. *Proceeding of the 24th International Symposium on Analytical and Environmental Problems.* 2018. pp. 368-372. Alapi T., Ilisz I. (eds). University of Szeged, H-6720 Szeged, Dugonics tér 13, Hungary. **ISBN 978-963-306-623-2** *(conference proceeding).*

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II. Cseh, E.K., **Nánási N.**, Veres G., Polyák H., Körtési T., Vécsei L., Zádori D. Development, validation and application of a HPLC method for the assessment of some tryptophan metabolites from murine samples. *Analysis of Carboxylic Acids with Ion Chromatography L36.* 2019. **ISBN: 978-615-527-057-4** *(conference proceeding).*

III. Cseh E.K., Veres G., Danics K., Szalárdy L., **Nánási N.**, Klivényi P., Vécsei L., Zádori D. Additional value of tau protein measurement in the diagnosis of Creutzfeldt-Jakob disease. Ideggy. Sz. 2019, 72: 39 (original paper IF: 0.337, **Q4**)

IV. Cseh E.K., Veres G., Szentirmai M., **Nánási N.**, Szatmári I., Fülöp F., Vécsei L., Zádori D. HPLC method for the assessment of tryptophan metabolism utilizing separate internal standard for each detector. Anal. Biochem. 2019, 574:7 (original paper, IF: 2.877, **Q3**)

V. Cseh E.K., Veres G., Körtési T., Polyák H., **Nánási N**., Tajti J., Klivényi P., Vécsei L., Zádori D. Neurotransmitter and tryptophan metabolite concentration changes in the Complete Freund's adjuvant model of orofacial pain. J. Head. Pain. 2020, 21:35 (original paper, IF: 4.797, **Q1**)

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1. Introduction

The main characteristic of neurodegeneration is the progressive injury of neurons and the decrease in their number in some regions of the brain. This process results in the loss of functions in these regions which leads to the development of various diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease. Although these disorders differ regarding many clinical, biochemical, and histopathological aspects, they may have common features as well, such as mitochondrial dysfunction, glutamatergic excitotoxicity, decreased antioxidant capacity, or abnormalities in the tryptophan metabolism.

The antioxidant protection is a very complex system that consist of enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic processes. The non-enzymatic group involves small molecules, such as β-carotene, coenzyme Q10, vitamin C, vitamin E and flavonoids. The lipophilic vitamin E group includes 4 – 4 tocopherols and tocotrienols and their structure comprises a chromanol ring with an aliphatic side chain, saturated for tocopherols and unsaturated for tocotrienols. The different forms of tocopherols and tocotrienols (namely α, β, γ and δ forms) can be identified by the number and position of methyl groups on the chromanol ring. This antioxidant protection may have a special relevance with regard to the brain, which organ is especially prone to oxidative injuries due to its high energy demand and elevated lipid content. The deficiency of vitamin E, mainly that of α-tocopherol (αT) is often accompanied by neurological symptoms and studies have proved significantly lower serum or plasma αT levels in some neurological disorders, such as in AD and in PD, conditions with an increasing prevalence in the elderly, and in some other neurological diseases as well.

With regard to rodent studies on tocopherol homeostasis, only limited data are available about the effect of aging and gender on plasma or serum and brain tocopherol levels. Although Gohil et al. determined αT level in several brain regions of 5 months old C57Bl/6 female and male mice and found significantly higher αT concentrations in all invegstigated regions in females compared to their male counterparts, however, no information was obtained about the effect of aging. There is only one study, which assessed the effect of aging on tocopherol levels of rodents in details and reported a not significant decrease in plasma αT level with aging in C57Bl/6NCr male mice and significantly increasing values only in some brain regions. However, female mice were not utilized in this study, so the effect of gender cannot be assessed.

PD is an incurable progressive neurodegenerative disease that can only be treated symptomatically. The destruction of dopaminergic neurons of the substantia nigra pars compacta (SNpc) in the midbrain leads to striatal dopamine (DA) loss resulting in basal ganglia dysfunction responsible for the development of main motor symptoms (bradykinesia, rigidity and tremor) of PD. The deficiency of DA as well as that of other biogenic amines in other brain areas results in the development of specific non-motor symptoms (e.g.: sleep disorders, psychiatric and cognitive abnormalities). Moreover, it has been demonstrated that, among many other factors, oxidative stress and mitochondrial respiratory chain dysfunctions eventually lead to neuronal apoptosis in PD.

Regarding experimental models of PD, probably the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin with mitochondrial respiratory chain complex I inhibitory properties is the most widely applied. The active metabolite of this toxin, 1-methyl-4-phenylpyridinium ion, is capable of selectively damaging dopaminergic neurons of the SNpc resulting in a decrease of striatal DA levels characteristic of PD. The C57Bl/6 mice serve as one of the most sensitive mouse strains regarding MPTP toxicity. In addition to the demonstration of increased sensitivity to neurotoxicity with aging, several studies assessed gender differences in C57Bl/6 mice following MPTP intoxication as well. Although the obtained results are controversial, the majority of studies demonstrated increased sensitivity in males, especially regarding nigrostriatal injury. The reason behind this phenomenon has not been exactly revealed, yet. The assessment of neuroprotection in C57Bl/6 male or female mice applying αT supplementation in the MPTP model of PD yields controversial results as well. In summary, only the administration of considerably high doses of αT provided neuroprotective effects only in a portion of studies.

In light of the fact that β-tocopherol and γT are constitutive isomers, as well as β- and γ-tocotrienols are, their analytical separation from each other is quite challenging on the most commonly applied C_{18} stationary phases in reversed-phase high performance liquid chromatography (RP-HPLC) techniques. However, with the modification of the stationary phase (C_{30}, pentafluorophenyl), their selective separation may successfully be achieved. Vitamin E compounds have absorption maxima in the UV range, which allows the use of diode-array detector (DAD), although its selectivity is strongly influenced by the possible UV active interfering compounds of the biological samples. Furthermore, DAD provides low sensitivity compared to the following detection methods. Fluorescence detection (FLD) profits from the

native fluorescence of vitamin E and allows very selective and sensitive detection. The aromatic hydroxyl group on the chromanol ring approves the electrochemical measurement, so electrochemical detection (ECD) is also popular in addition to FLD because similar if not better sensitivity can be achieved with its application. In case of plasma or serum and brain samples of rodents, especially those of mice, the measurements of tocopherols are challenging, except αT, because of their small concentration levels.

The measurement of biogenic amines from biological samples requires highly selective and sensitive methods because of their considerably low concentrations. HPLC combined with ECD is one of the best alternatives for the quantitative detection of monoamines and related compounds in biological samples because of their electroactive function groups and the exceptional sensitivity of the ECD. The applicability of the method is influenced by many factors, its use and optimization requires great expertise. The compositions of the mobile phase can significantly influence the determination of metabolites. Regarding the complex process of sample management, especially for brain samples, the application of internal standards (ISs) is essential to assess sample loss during sample preparation and injection and to make the necessary corrections. The measurement of a large number of biogenic amines requires the simultaneous application of several ISs due to the long run time and different chemical structures of the measured compounds (catecholamines and serotonin (5-HT)), for which the applied method should also be selective. Testing multiple brain regions to adjust the selectivity of the method can also be difficult.

2. Aims

I., To compare HPLC-FLD and HPLC-ECD methods regarding their applicability for the measurement of αT levels from C57Bl/6N mouse brain samples.

II., To investigate the possible age- and gender-related effects on αT status in the central nervous system and in the blood of C57Bl/6N female and male mice using the developed and validated HPLC methods.

III., To develop an HPLC-ECD method for the simultaneous assessment of certain biogenic amines and some of their related compounds (levodopa (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), noradrenaline (NA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid

(HVA), DA, 5-HT and 3-methoxytyramine (3-MT)) from different brain regions of C57Bl/6N mice utilizing 3 ISs.

IV., To determine the possible gender-related relationship between striatal αT content and striatal DA level in the MPT-induced neurotoxicity animal model of PD using C57Bl/6N female and male mice and utilizing the developed and validated HPLC methods.

3. Materials and methods

For both studies C57Bl/6N female and male mice were used. The animals were housed under standard laboratory conditions (50% ± 2% humidity, 22 °C ± 1 °C temperature range and 12 h–12 h light–dark cycle) in cages (max. 4 per cage) with free access to food (standard rodent diet) and drinking water. To examine gender- and age-related differences, animals were divided into six groups consisting of 6, 16, and 66 weeks old male and female mice (n = 9 in each group).

In the next experiment, animals were separated into four groups consisting of control and MPTP-treated 16 weeks old male and female mice (initially n = 15 in each group). All animal experiments were carried out in accordance with the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV./352/2012.) and were approved by the Committee of Animal Research at the University of Szeged (XI./243/2019.).

In the MPTP study MPTP hydrochloride was freshly dissolved in saline (pH adjusted to 7.4 with 0.1 M NaOH) and was administered intraperitoneally (i.p.). Two groups received i.p. injection of 12 mg/kg body weight MPTP 5 times at 2 h intervals. The other 2 groups served as controls and received i.p. saline injection 5 times at 2 h intervals. After the last MPTP injection, two male and one female mice were found dead. Regarding the control groups, one female mouse was excluded from the study due to unexpected behaviour.

In the first experiment at the age of 6, 16 and 66 weeks, the animals were deeply anesthetized with isoflurane. After thoracotomy, venous blood was collected from the right ventricle by intracardial puncture into Eppendorf tubes containing Na_2EDTA, followed by perfusion with artificial cerebrospinal fluid for 5 min by an automatic peristaltic pump. After centrifugation of blood samples at 4°C for 5 min at 3500 resolutions per minute (RPM), the supernatant plasma (200 μL) were mixed immediately with 200 μL 15 mg/ml ascorbic acid and 400 μL 250 mg/L butylated hydroxyl-toluene (BHT) in absolute ethanol (EtOH) solutions and the samples were stored at −80 °C until further use. Before measurements, 600 μL *n*-hexane containing 250 mg/L BHT and rac-tocol (rT), as IS, was added to the stabilized and freshly thawed plasma samples.

After an intensive 1 min long vortex, the samples were centrifuged at 4 °C for 10 min at 12,000 RPM. In the next step, 450 µL of the hexane layer was evaporated under nitrogen flow. The residue was resolved with the mix of 75 µL acetonitrile (ACN) and 50 µL EtOH:1,4-dioxane (1:1), then placed into amber-coloured vials for measurements.

The anatomical borders of five different brain regions (striatum, cortex, hippocampus, cerebellum and brainstem) were determined with the aid of the online-available Allen Brain Atlas: Mouse Brain (Allen Institute for Brain Science, Seattle, WA, USA; http://mouse .brain - map.org/static/atlas), and they were rapidly removed on ice and stored at −80 °C until further use. Before measurements, the samples were weighed and sonicated in 1,020 µL ice-cooled solution. The samples were centrifuged next at 4 °C for 10 min at 12,000 RPM, and the supernatants were collected then stabilized and measured applying the same method as described in case of the plasma, except that the residue of evaporated brain region samples was dissolved in the applied mobile phase as described in the following subsections.

In the MPTP study, one week following the last i.p. injection, all the animals were deeply anesthetized with isoflurane. Sample collection and the stabilisation process were similar as described above. Briefly, plasma and medially halved striatal samples were collected for the determination of αT and catecholamine concentrations. The measurement of αT (including sample preparation steps) was the same, as previously mentioned. Before DA, DOPAC and HVA measurements, the halved striatal samples were weighed and sonicated in ice-cooled solution. The samples were centrifuged at 4°C for 30 min at 12,000 RPM, and after the supernatants were collected, 10 µL was injected into the HPLC.

An HPLC method with DAD was utilized for mouse plasma samples. The separation was performed using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The mobile phase contained 66.54 v/v% ACN, 21.40 v/v% tetrahydrofuran, 6.61 v/v% methanol, 5.45 v/v% water and 272.4 mg/L NH_4CH_3COO. The mobile phase was delivered at a rate of 2.1 ml/min onto a Kinetex C18 column (150 x 4.6 mm, 5 µm particle size; Phenomenex Inc., Torrance, CA, USA) thermostated at 25 °C after passage through a pre-column of the same phase (SecurityGuard, 4×3.0 mm i.d., Phenomenex Inc., Torrance, CA, USA). Before use, the mobile phase was filtered through a polyvinylidene difluoride membrane with 0.45 µm pore size. The compounds were simultaneously detected at their wavelengths of maximum absorbance, i.e., 292 and 297 nm for αT and rT, respectively. Fifty µL aliquots were injected by the autosampler to the analytical column.

For mouse brain sample measurement HPLC-ECD and FLD methods were applied. Both methods involved the utilization of an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Model 105 ECD (Precision Instruments, Marseille, France) and a FLD (Agilent Technologies, Santa Clara, CA, USA). Measurements were carried out under isocratic conditions. In case of ECD, the mobile phase consisted of 91.25 v/v% methanol, 4.25 v/v % water, 4.50 v/v % isopropanol and 2.81 g/L $NaClO_4$ and it was delivered at a rate of 1.2 ml/min at 25 °C onto the column (Luna C18, 75 × 4.6 mm, 3 μm particle size, Phenomenex Inc., Torrance, CA, USA) after passage through a pre-column of the same phase (SecurityGuard, 4 × 3.0 mm i.d., Phenomenex Inc., Torrance, CA, USA). Before mobile phase application, the solution was filtered through a polyvinylidene difluoride membrane with 0.45 μm pore size. Ten μL of aliquots were injected by the autosampler with the cooling module set at 4 °C. For the method, using FLD, the mobile phase consisted of pure methanol, applying the slightly modified method of Yuan et al. The flow rate was 1.8 ml/min at 25 °C, using the same column as in the ECD method. The excitation and emission wavelengths were set at 292 nm and 330 nm, respectively, for the determination of both αT and rT based on spectral analysis. The injection volume was 10 μL, and the samples were thermostated at 4 °C. During validation process we determined the limit of detection (LOD), limit of quantification (LOQ), selectivity, intra- and interday precisions, as well as recovery values in all cases. In the study of possible age- and gender-related effects on αT in brain samples of mice, 2 parallel measurements were carried out for each sample to compare the appropriateness of FLD and ECD methods as well.

The measurement of the 3 biogenic amines (DA, NA, 5-HT) and their precursors and metabolites (L-DOPA, DOPAC, HVA, 3-MT, 5-HIAA) from mouse brain samples using 3 ISs (3,4-dihydroxybenzylamine, isoproterenol and *N*ω-methylserotonin) was performed as follows. For method development, the initial mobile phase consisted of 2.80 mM sodium octyl sulphate (NaOS), 75 mM NaH_2PO_4, 100 μM Na_2EDTA and 7.0 v/v% ACN. Before adding ACN, the pH value of water phase was set to 3.0 with 85 w/w% H_3PO_4. During the development of the current method, the effect of four parameters on the mentioned 11 components was investigated, the first of which was the pH value, followed by the concentration of the ion-pairing material (NaOS), the amount of the organic solvent (ACN) and finally the working potential of the ECD cell. Completing the validation process, linearity, LOD, LOQ, sensitivity, intra- and interday precisions and recovery values were determined as well.

4. Results

After proper sample preparation, we were able to determine αT concentrations from mouse plasma and brain tissue in a robust and precise manner. The selectivity of the utilized methods was also tested demonstrating that both compounds (αT and rT (IS)) can be detected without interference from the other compounds.

In our first study, regarding the plasma samples, the applied two-way ANOVA demonstrated significant difference for age ($F = 10.547$, $df = 2$, $p < 0.001$, $\omega^2 = 0.0276$) but no differences for gender ($F = 0.642$, $df = 1$, $p = 0.427$) or age *vs.* gender ($F = 1.108$, $df = 2$, $p = 0.339$). *Post hoc* analysis with Tukey HSD test showed a significant increase in αT concentrations with aging only between 16 and 66 weeks old male mice ($p < 0.01$).

In case of brain samples, both methods have strengths and weaknesses. With the application of ECD, this positive property is the wider linear range, which also makes the technique more sensitive. With FLD, the αT content of a brain samples can be determined with smaller standard deviation and significantly faster, and the measurement conditions are also much simpler than with those of ECD, since the baseline and signal-to-noise ratio are significantly better with FLD. In light of the validation parameters, both ECD and FLD measurement are applicable for the determination of αT from brain samples and accordingly, the results of FLD and ECD measurements were averaged for each individual brain sample.

In the next step, the implementation of two-way ANOVA with Tukey HSD *post hoc* test yielded the following results. In the striatum there was a significant difference for age ($F = 120.019$, $df = 2$, $p < 0.001$, $\omega^2 = 0.0752$) and gender ($F = 23.062$, $df = 1$, $p < 0.001$, $\omega^2 = 0.0070$), and for age *vs.* gender ($F = 3.588$, $df = 2$, $p < 0.05$, $\omega^2 = 0.0016$) as well. The Tukey HSD *post hoc* test revealed significantly elevated αT concentrations in the latter groups in the following pairwise comparisons from those of *a priori* decided: $p < 0.001$ for 6 *vs.* 16 weeks old females and $p < 0.01$ for males; $p < 0.001$ for both 16 *vs.* 66 weeks old females and males; $p < 0.001$ for both 6 *vs.* 66 weeks old females and males; $p < 0.05$ for 16 weeks old males *vs.* females, and $p < 0.001$ for 66 weeks old males *vs.* females. In the cortex there was also a significant difference for age ($F = 159.589$, $df = 2$, $p < 0.001$, $\omega^2 = 0.1042$), gender ($F = 17.377$, $df = 1$, $p < 0.001$, $\omega^2 = 0.0054$), and for age *vs.* gender ($F = 5.465$, $df = 2$, $p < 0.01$, $\omega^2 = 0.0029$). The Tukey HSD *post hoc* test revealed significantly elevated αT concentrations in the latter groups in the following pairwise comparisons from those of *a priori* decided: $p < 0.001$ for 6 *vs.* 16 weeks old females and $p <$

0.05 for males; $p < 0.001$ for both 16 *vs.* 66 weeks old females and males; $p < 0.001$ for both 6 *vs.* 66 weeks old females and males and $p < 0.001$ for 66 weeks old males *vs.* females. Furthermore, in the hippocampus, similar results were demonstrated for age ($F = 195.500$, $df = 2$, $p < 0.001$, $\omega^2 = 0.1056$), gender ($F = 24.343$, $df = 1$, $p < 0.001$, $\omega^2 = 0.0063$), and for age *vs.* gender ($F = 7.045$, $df = 2$, $p < 0.01$, $\omega^2 = 0.0033$). The Tukey HSD *post hoc* test revealed significantly elevated αT concentrations in the latter groups in the following pairwise comparisons from those of *a priori* decided: $p < 0.001$ for both 6 *vs.* 16 weeks old females and males; $p < 0.001$ for both 16 *vs.* 66 weeks old females and males; $p < 0.001$ for both 6 *vs.* 66 weeks old females and males and $p < 0.001$ for 66 weeks old males *vs.* females. With regard to the cerebellum and the brainstem, there was a significant difference for age ($F = 17.091$, $df = 2$, $p < 0.001$, $\omega^2 = 0.0134$ and $F = 3.491$, $df = 2$, $p < 0.05$, $\omega^2 = 0.0021$, respectively) and gender ($F = 10.660$, $df = 1$, $p < 0.01$, $\omega^2 = 0.0040$ and $F = 13.295$, $df = 1$, $p < 0.001$, $\omega^2 = 0.0051$, respectively), but not for age *vs.* gender ($F = 2.897$, $df = 2$, $p = 0.065$ and $F = 0.820$, $df = 2$, $p = 0.446$, respectively). The Tukey HSD *post hoc* test revealed significantly elevated αT concentrations only in case of the cerebellum in the latter groups in the following pairwise comparisons from those of *a priori* decided: $p < 0.01$ for 6 *vs.* 16 weeks old females and $p < 0.001$ for 6 *vs.* 66 weeks old females.

To perform our second study, we also developed and validated an HPLC-ECD method for biogenic amines measurement. During the development, we successfully optimized the amount of ion-pairing reagent and the amount of organic solvent in the mobile phase, the working potential of the ECD cell and we were able to add two new ISs to the method.

In the halved striatal samples of the MPTP study, significant differences were observed in DA levels regarding treatment ($F (1, 52) = 196.355$, $p < 0.001$, $\omega^2 = 0.2201$) and regarding treatment *vs.* gender as well ($F (1, 52) = 5.703$, $p < 0.05$, $\omega^2 = 0.0053$), but not for gender ($F (1, 52) = 3.627$, $p = 0.062$). *Post hoc* analysis with Tukey HSD test yielded significantly decreased DA concentrations in MPTP-treated *vs.* control females ($p < 0.001$), in MPTP-treated *vs.* control males ($p < 0.001$), and in MPTP-treated males *vs.* females ($p < 0.05$). Similar to the above-mentioned changes in DA levels, significant differences were observed in DOPAC levels as well, regarding treatment ($F (1, 52) = 143.741$, $p < 0.001$, $\omega^2 = 0.1769$) and regarding treatment *vs.* gender ($F (1, 52) = 12.481$, $p < 0.001$, $\omega^2 = 0.0141$), but not for gender ($F (1, 52) = 2.373$, $p = 0.129$). *Post hoc* analysis with Tukey HSD test yielded significantly decreased DOPAC concentrations in MPTP-treated *vs.* control females ($p < 0.001$), in MPTP-treated *vs.* control

males ($p < 0.001$) and in MPTP-treated males *vs.* females ($p < 0.01$). Significant differences were observed in HVA levels as well regarding treatment (F (1, 52) = 59.920, $p < 0.001$, $\omega^2 = 0.1137$) and regarding treatment *vs.* gender (F (1, 52) = 5.704, $p < 0.05$, $\omega^2 = 0.0090$), but not for gender (F (1, 52) = 0.464, $p = 0.499$). *Post hoc* analysis with Tukey HSD test yielded significantly decreased HVA concentrations in MPTP-treated *vs.* control females ($p < 0.01$) and in MPTP-treated *vs.* control males ($p < 0.001$). The metabolite rate was also determined in all the four groups and the values were compared with two-way ANOVA and Tukey HSD *post hoc* test. There was a significant increase in the calculated (DOPAC+HVA)/DA ratio (DA turnover) regarding the treatment (F (1, 52) = 26.129, $p < 0.001$, $\omega^2 = 0.0538$), but not for gender (F (1, 52) = 1.842, $p = 0.181$) and treatment *vs.* gender (F (1, 52) = 0.779, $p = 0.382$). The Tukey HSD *post hoc* test yielded significantly increased DA turnover in MPTP-treated *vs.* control females ($p < 0.05$), and in MPTP-treated *vs.* control males ($p < 0.001$).

The applied two-way ANOVA demonstrated significant difference in αT level of plasma regarding treatment (F (1, 52) = 18.227, $p < 0.001$, $\omega^2 = 0.0396$), but not for gender (F (1, 52) = 0.006, $p = 0.938$) and gender *vs.* treatment (F (1, 52) = 0.115, $p = 0.736$). *Post hoc* analysis with Tukey HSD test yielded significantly decreased αT concentrations in MPTP-treated *vs.* control females ($p < 0.05$), and in MPTP-treated *vs.* control males ($p < 0.05$). Regarding the striatum, there was a significant difference for gender (F (1, 52) = 29.680, $p < 0.001$, $\omega^2 = 0.0055$), but not for treatment (F (1, 52) = 2.543, $p = 0.117$) and for treatment *vs.* gender (F (1, 52) = 0.029, $p = 0.865$). The Tukey HSD *post hoc* test revealed significantly higher αT concentrations in control female *vs.* male mice ($p < 0.01$), and in MPTP-treated female *vs.* male mice as well ($p < 0.01$).

Regarding the assessment whether endogenous αT content could affect the change in DA levels following MPTP treatment, data were analysed by ANCOVA. The results of this complex statistical analysis demonstrated that MPTP treatment significantly influence striatal DA level (F (1, 52) = 8.689, $p < 0.01$, partial $\eta^2 = 0.761$), but striatal αT level did not have a significant influence on either striatal DA level (F (1, 52) = 0.487, $p = 0.488$) or on its decrease following MPTP treatment (assessment of interaction; F (1, 52) = 1.879, $p = 0.176$). For the confirmation of these findings in each group, correlation analyses were carried out, similarly presenting no significant correlation between striatal DA and αT levels (Pearson's R^2s and p values prior to Bonferroni correction: control females: 0.0069 and 0.779, respectively; control males: 0.036 and 0.495, respectively; MPTP-treated females: 0.023 and 0.603, respectively; MPTP-treated males: 0.369 and 0.028, respectively).

5. Discussion

Evidence suggests that tocopherols may have a special role in antioxidant protection in lipid-rich structures, such as the central nervous system. As there is a clear worsening of brain functioning with aging, there were several approaches which aimed at the achievement of neuroprotection via the administration of exogenous αT, but the results are controversial. However, only limited data are available on the changes of endogenous tocopherol levels either in human or murine brain samples with aging. Therefore, there is a special need for the fine assessment of age-related changes in αT levels. The identification of clear trends with regard to either certain brain regions or genders may help to understand the differences in the sensitivity to oxidative damage and to work out the therapeutic strategies to devastating neurodegenerative disorders. Although the main focus may be paid on human studies, the assessment of rodents from this point of view may also yield relevant information in light of the fact that most preclinical research on neurodegeneration is carried out in animals belonging to this subfamily.

Following the HPLC method development for the determination of αT concentrations, our next aim was to assess region-, age- and gender-specific changes in brain αT level in the C57Bl/6 mouse strain, which is one of the most commonly applied strains in the research on neurodegeneration. Furthermore, our study was supplemented with the assessment of plasma samples as well to be able the judge the possible influence of peripheral changes on brain αT levels. The results demonstrated that brain αT levels significantly increased in the striatum, cortex, and hippocampus with aging in both genders. This increase was more pronounced in females and the magnitude of this difference also rose with aging in case of all the above-mentioned brain regions. However, in case of the cerebellum, a moderate elevation could be detected only in females, whereas in case of the brainstem there was no significant change in αT level. With regard to plasma samples, no clear trend could be identified, a significant difference was found only between 16 and 66 weeks old males. The novelty of the current study is the presentation of such a pronounced elevation in striatal αT level, while no change in brainstem αT level, and furthermore, the first delineation that the difference between genders significantly increases with aging in case of the striatum, cortex, and hippocampus.

PD is the second most common neurodegenerative disorder with an increasing prevalence in the aging population and in males. These phenomena, i.e., increasing sensitivity to nigrostriatal injury with aging and in males have been considerably well represented in the MPTP mouse

model of PD as well. Although the possible role of sexual hormones behind these findings was proposed by several studies, the exact explanation behind gender differences is still missing. The assessment whether higher αT levels in female striatum may at least partially explain the decreased vulnerability of C57Bl/6 female mice in the MPTP model of PD may have a special interest. However, this hypothesis, i.e., whether higher striatal αT level in females correlates with less reduction in striatal DA level following MPTP intoxication, has never been tested before. Accordingly, the aim of our next study was to examine whether gender-related difference in endogenous striatal αT level has an influence on the distinctly decreased DA levels in MPTP-treated C57Bl/6 female and male mice.

However, before that an HPLC-ECD method for the simultaneous assessment of certain biogenic amines and some of their precursors and metabolites with 3 ISs was developed and the method was optimized to reach appropriate sensitivity and selectivity for our research purposes. Following that the results from the MPTP study demonstrated that striatal DA levels of MPTP-treated female and male mice were significantly decreased to 39% and to 15.4%, respectively, compared to the corresponding control groups. The significantly decreased sensitivity to MPTP intoxication in female C57Bl/6 mice compared to their male counterparts is in line with the majority of literature data. Although DA turnover significantly increased in MPTP-treated mice compared to controls, which also corresponds to the results of other studies, no difference between genders could be demonstrated. Regarding striatal αT levels, the findings of this study confirmed our previous results. Surprisingly, but in line with previous findings, these striatal αT levels were not influenced by MPTP treatment, however, plasma αT levels significantly decreased in both genders. Keeping in mind that the samples for bioanalytical studies were obtained 7 days following acute MPTP intoxication, a peripheral to central redistribution might took place as an effort to prevent brain injury. In the next part of this study the possible relationship between the above-detailed 2 parameters were assessed, i.e., whether higher striatal αT content is capable of exerting protection against MPTP-induced neurotoxicity. However, the applied statistical analyses could not demonstrate any significant correlation between striatal DA and αT levels following MPTP treatment, and therefore, the hypothesis that higher striatal αT concentration in females may be responsible for the less reduction in striatal DA level following MPTP intoxication at 16 weeks of age could not be proved. This latter finding may further confirm that αT does not play a major role against neurotoxicity induced by MPTP. Anyway, the assessment of factors behind the decreased sensitivity of female mice to nigrostriatal MPTP

toxicity may warrant further studies to explore novel possible therapeutic targets. Furthermore, a deeper insight into this aspect of antioxidant protection may have clinical relevance as well, i.e., it may help in the development of therapeutic strategies against age-related pathogenetic processes, mainly focusing at the restoration of altered brain antioxidant homeostasis.

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