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

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List of abbreviations

\( \alpha/\beta/\gamma T \) – \( \alpha/\beta/\gamma \)-tocopherol
\( \alpha \text{TTP} \) – \( \alpha \)-tocopherol transfer protein
3-MT – 3-methoxytyramine
5-HIAA – 5-hydroxyindoleacetic acid
5-HT – serotonin or 5-hydroxytryptamine
ACN – acetonitrile
AD – Alzheimer’s disease
ANCOVA – analysis of covariance
ANOVA – analysis of variance
BBB – blood-brain barrier
BCEC – brain capillary endothelial cells
BHT – butylated hydroxyl-toluene
CNS – central nervous system
CSF – cerebrospinal fluid
CV\% – coefficient of variation in percentage
DA – dopamine
DAD – diode-array detector
DATATOP – Deprenyl and tocopherol antioxidative therapy of Parkinsonism
DHBA – 3,4-dihydroxybenzylamine (internal standard)
DOPAC – 3,4-dihydroxyphenylacetic acid
ECD – electrochemical detector
EDTA – ethylenediaminetetraacetic acid
EtOH – ethanol
FLD – fluorescence detector
HDL – high-density lipoprotein
HPLC – high performance liquid chromatography
HVA – homovanillic acid
i.p. – intraperitoneal (injection)
IPA – 2-propanol
IPR – isoproterenol (internal standard)
IS – internal standard
IU – international unit
KO – knock-out (mice)
L-DOPA – levodopa or 3,4-dihydroxyphenylalanine
LOD – limit of detection
LOQ – limit of quantification
MeOH – methanol
MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NA – noradrenaline or norepinephrine
Na₂EDTA – disodium ethylenediaminetetraacetate
NM-5HT – N-methyl-5-hydroxytryptamine or Nω-methylserotonin
NaOS – sodium octyl sulphate
PD – Parkinson’s disease
PLTP – phospholipid transfer protein
RP – reversed-phase
RPM – revolutions per minute
rT – rac-tocol (internal standard)
SNpc – substantia nigra pars compacta
SR-B1 – scavenger receptor class B type 1
THF – tetrahydrofuran
WP – working potential
ww – wet weight
Summary

The aging process clearly increases the demand for antioxidant protection, especially in the brain, involving that provided by α-tocopherol (αT). However, little is known about the age-related changes in brain αT levels and the influencing effect of gender on it, in human or murine samples as well. Besides, several studies supported an increased vulnerability of males regarding Parkinson’s disease (PD) and its animal models, the background of which has not been exactly revealed, yet. In addition to hormonal differences, another possible factor behind that may be a female-predominant increase in endogenous striatal αT level with aging.

Accordingly, our first aim was to detect age-, gender- and region-specific changes in αT concentrations in mouse brain tissues and to assess the influencing effect of plasma αT levels on it. For this purpose, female and male C57Bl/6N mice at the ages of 6, 16 and 66 weeks were applied. αT levels were determined with high performance liquid chromatography (HPLC) from the striatum, cortex, hippocampus, cerebellum, brainstem and from plasma samples. A detailed validation process was carried out for the applied HPLC methods as well. We used a diode-array detector (DAD)-coupled method for plasma αT measurements and two independent methods, namely electrochemical (ECD) and fluorescent (FLD) detections to quantify αT in certain brain regions. The latter two techniques were also compared in terms of applicability.

In the light of the results of our first investigation, on the other hand, our second aim was to assess whether differences in striatal αT concentration may contribute to the above-mentioned distinct vulnerability of genders to nigrostriatal injury. Therefore, female and male C57Bl/6N mice at the age of 16 weeks were injected with 12 mg/kg body weight 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) 5 times at 2 hours intervals or with saline. The levels of αT and some biogenic amines – with a developed ECD method utilizing the simultaneous detection of 8 biogenic compounds and 3 internal standards – were determined from the striatum by validated HPLC methods.

The most sensitive tool for αT analysis is definiteliey the ECD method, but otherwise the run time is significantly longer and its accuracy is lower than that of FLD or DAD measurements. Overall, the obtained validation parameters confirmed that our HPLC methods are suitable for precise, robust αT quantification. Furthermore, another method was successfully developed as well for the quantification of biogenic amines and some of their metabolites. It was
demonstrated by the validated αT methods that brain αT levels significantly increased in the striatum, cortex and hippocampus with aging in both genders, but in a more pronounced way in females with an increasing magnitude of this difference. 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. The results of the MPTP study proved previous findings, i.e., striatal dopamine decrease was less pronounced in females following MPTP treatment, and striatal αT level was significantly higher in female mice, the correlation between these 2 variables was not significant. Surprisingly, MPTP treatment did not affect striatal αT concentrations, but significantly decreased plasma αT levels without differences between genders. Summarily, following the development of validated HPLC methods, age-dependent gender-specific changes in αT level in certain brain regions of the C57Bl/6 mouse strain were demonstrated successfully, yielding meaningful data for future studies targeting aging-related processes. However, in light of the results of the MPTP study, endogenous αT may have less role against the neurotoxic alterations characteristic of PD.
1. **Introduction**

1.1. Biological effects of vitamin E and certain biogenic amines

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 (Binvignat & Olloquequi, 2020). 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 (Szalárdy *et al.*, 2012).

The antioxidant protection is a very complex system that consist of enzymatic (e.g., superoxide dismutase, catalase, glutathione peroxidase (Sies, 1997)) and non-enzymatic processes. The non-enzymatic group involves small molecules, such as β-carotene, coenzyme Q10, vitamin C, vitamin E and flavonoids (Sies, 1993). 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 (Hacquebard & Carpentier, 2005), as it can be seen in [Figure 1](#).

![Figure 1. Structure of Vitamin E compounds.](#)

The deficiency of tocopherols, mainly that of α-tocopherol (αT), can cause several symptoms, such as cerebellar ataxia, peripheral neuropathy, myopathy (Ueda *et al.*, 2009; Muller, 2010),
dementia, visual disturbances, digestive system problems, dry hair or loss of hair, low tissue healing, cardiac arrhythmias and diabetes mellitus (Eggermont, 2006). Accordingly, the deficiency of vitamin E 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 (Lopes da Silva et al., 2014; Raszewski et al., 2015) and in PD (Yang et al., 2017), conditions with an increasing prevalence in the elderly, and in some other neurological diseases as well (Schuelke, 1993; Karg et al., 1999, 2000; Klivényi et al., 2001; Karg et al., 2003).

With regard to the effects of normal aging on serum tocopherol levels, studies have shown a trend of similarly increasing concentrations in both genders (Battisti et al., 1994; Veres et al., 2017), which may be the consequence of the age-related alteration in lipid homeostasis (Rifkind & Segal, 1983). Studies investigated αT levels in the cerebrospinal fluid (CSF) in healthy subjects (Vatassery et al., 2004) and in patients with AD (Tohgi et al., 1994) as well. In addition to the delineation of positive correlations between serum and CSF αT and γ-tocopherol (γT) levels in the healthy ones, significantly decreased CSF αT levels were demonstrated in AD patients compared to controls. 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 (Szalárdy et al., 2015). In relation to human brain tocopherol levels, studies provided contradictory results. Craft et al. (Craft et al., 2004) carried out an examination on the regional distribution of the brain level of αT and γT isomers, demonstrating significant age-related (67-90 years, n = 5, 2 females and 3 males) decline in αT level, more pronounced in females, but due to low case number, statistical comparison could not be performed. With regard to the regional distribution, there were no significant differences between the grey and white matter of frontal and occipital cortices. Later, Johnson et al. (Johnson et al., 2013) demonstrated a significant relationship between cognitive functions and tocopherol levels in the serum, cerebellar, frontal, occipital and temporal cortices of healthy octogenarians and centenarians. In case of serum samples, dramatically lower concentrations were measured in that age groups of interest compared to younger individuals, whereas for given brain regions, significantly higher levels were determined with aging. Accordingly, there was a significantly negative correlation between serum and brain αT levels, except the cerebellum. However, cerebellar αT level, similarly to that of the other brain regions, also
positively correlated with the scores of Mini-Mental State Examination and Severe Impairment Battery, both of which are measures of global cognition.

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. Most of the available rodent tocopherol studies did not separate animals to the necessary groups for later statistical comparisons (Lass et al., 1999; Vécsei et al., 2001; Reboul et al., 2009; Johnson et al., 2012; Hagl et al., 2016). Although Gohil et al. (Gohil et al., 2008) determined αT level in several brain regions (cerebral cortex, hippocampus, cerebellum, midbrain and the remaining part of the brainstem) of 5 months old C57Bl/6 female (n = 5) and male (n = 3) mice and found significantly higher αT concentrations in all the five regions in females compared to their male counterparts, and moreover, significantly lower αT levels were observed in the cerebellum compared to the other examined brain regions, no information was obtained about the effect of aging. With regard to cholesterol levels, there was not any significant difference between genders. There is only one study (Takahashi et al., 2017), which assessed the effect of aging on tocopherol levels of rodents in details and reported a not significant decrease in plasma αT level (from ~ 8 to ~ 6 µM) with aging in C57Bl/6NCr male mice (3, 6, 12, 18 and 24 months old, n = 5) and significantly increasing values only in some brain regions (cerebrum (from 10 to 20 nmol/g tissue), hippocampus (from 12 to 25 nmol/g tissue) and cerebellum (from 11 to 15 nmol/g tissue)). In addition, it was also demonstrated that triglyceride levels were not influenced by aging. 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 (Rodriguez-Oroz et al., 2009). Furthermore, 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) (Barone et al., 2009; Ferrer et al., 2012).

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 (Beal, 2003; Jackson-Lewis & Przedborski, 2007). 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 (Martinovits et al., 1986; Sutphin & Buckman, 1991; Lan & Jiang, 1997; Schwarting et al., 1999; Sedelis et al., 2000b, 2000a; Richardson et al., 2008; Joniec et al., 2009; Ookubo et al., 2009; Antzoulatos et al., 2010; Boyalla et al., 2011; Geldenhuys et al., 2015; Szalárdy et al., 2015; Nakaso et al., 2016; Torok et al., 2017; Schamne et al., 2018). The C57Bl/6 mice serve as one of the most sensitive mouse strains regarding MPTP toxicity (Meredith & Rademacher, 2011; Plangár et al., 2013). 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 (Schwarting et al., 1999; Sedelis et al., 2000b, 2000a; Richardson et al., 2008; Joniec et al., 2009; Ookubo et al., 2009; Geldenhuys et al., 2015; Schamne et al., 2018; Unzeta et al., 1994; Hamre et al., 1999; Xu et al., 2006; Ciesielska et al., 2007; Liu et al., 2008; Ciesielska et al., 2009). Although the obtained results are controversial, the majority of studies demonstrated increased sensitivity in males, especially regarding nigrostriatal injury (Unzeta et al., 1994; Xu et al., 2006; Ciesielska et al., 2007, 2009; Liu et al., 2008; Joniec et al., 2009; Geldenhuys et al., 2015; Schamne et al., 2018). The reason behind this phenomenon has not been exactly revealed, yet.

As oxidative stress is considered to be a key event in the pathogenesis of PD, numerous studies assessed the influencing effect of dietary tocopherol intake on PD-related parameters, including those studies that focused on the achievement of neuroprotection via the administration of exogenous αT (Zádori et al., 2011; Chang et al., 2018; Golbe et al., 1988; The Parkinson Study Group, 1993; Logroscino et al., 1996; Morens et al., 1996; de Rijk et al., 1997; Zhang et al., 2002; Etminan et al., 2005; Potashkin & Seidl, 2011; Schirinzi et al., 2019). However, the results of studies using exogenous tocopherol supplementation are controversial; some demonstrated that vitamin E intake may be beneficial regarding disease evolution (de Rijk et al., 1997; Etminan et al., 2005), whereas others found no effect on it (The Parkinson Study Group, 1993; Logroscino et al., 1996; Zhang et al., 2002; Chang et al., 2018). Long-term αT and ascorbate treatment effectively delayed the need for the use of levodopa (L-DOPA) by an average of 2.5 years (Fahn, 1992) when they were applied in combination at considerably high doses (3200 international unit (IU) αT and 3000 mg ascorbic acid per day) compared to daily
2000 IU αT in the Deprenyl and tocopherol antioxidative therapy of Parkinsonism (DATATOP) trial which could not demonstrate any beneficial effect of daily vitamin E supplementation on delaying the onset of disability in PD (The Parkinson Study Group, 1993). The possible explanations for these findings may be that the ability of dietary αT to enrich cellular membranes, especially in the central nervous system (CNS), is limited and needs the administration of considerably high doses for a long time (Fariss & Zhang, 2003). Furthermore, the form of the administered αT may count as well (Fariss & Zhang, 2003). Accordingly, the achievement of the enrichment of mitochondria with protective levels of αT in the striatum and SNpc is quite challenging.

In line with the human data demonstrated above, 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 (Martinovits et al., 1986; Sutphin & Buckman, 1991; Nakaso et al., 2016; Perry et al., 1985; Yong et al., 1986; Chi et al., 1992; Selley, 1998; Itoh et al., 2006; Ren et al., 2006; Ortiz et al., 2013). In summary, only the administration of considerably high doses of αT provided neuroprotective effects only in a portion of studies (Perry et al., 1985; Yong et al., 1986; Selley, 1998; Ren et al., 2006; Ortiz et al., 2013). In addition to exogenous αT supplementation, another strategy may be the achievement of neuroprotection via the manipulation of endogenous αT homeostasis. The dietary or genetic depletion of brain αT levels yielded conflicting results as well (Itoh et al., 2006; Ren et al., 2006). MPTP intoxication following prolonged dietary vitamin E depletion resulted in increased susceptibility to damage in the substantia nigra, but not in the striatum (Odunze et al., 1990; Itoh et al., 2006). On the contrary, genetic vitamin E deficiency (utilizing αT transfer protein (α-TTP) knock-out (KO) mice) did not influence the striatal DA depletion following MPTP treatment, whereas the number of tyrosine hydroxylase positive neurons of the substantia nigra was not altered at all (Ren et al., 2006). These conflicting findings may be partially explained by the differences in the MPTP treatment regimen as well.

1.2. Analytical methods for the determination of vitamins E and biogenic amines

Due to the chemical structure of vitamin E compounds, almost all existing detection methods are suitable for the quantification of tocopherols and tocotrienols (Lai & Franke, 2013), however, their separation can still be difficult (Grebenstein & Frank, 2012; Saha et al., 2013;
Górnaś et al., 2014). In light of the fact that β-tocopherol (βT) 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\textsubscript{18} stationary phases by reversed-phase high performance liquid chromatography (RP-HPLC) techniques (Veres et al., 2017). However, with the modification of the stationary phase (C\textsubscript{30} (Rimmer et al., 2005; Saha et al., 2013), pentafluorophenyl (Grebenstein & Frank, 2012)), their selective separation may successfully be achieved. Although normal phase HPLC methods were also developed (Saha et al., 2013; Górnaś et al., 2014) and popularly used yielding more selective separation and simpler sample preparation steps compared to RP chromatography techniques, but on the other hand, the used solvents are less environmentally sound. Furthermore, RP mode assures better repeatability of the retention times (Cert et al., 2000), ensures greater stability and durability, and improved compatibility with aqueous biological samples (Grebenstein & Frank, 2012; Górnaś et al., 2014) compared to the normal phase mode.

Vitamin E compounds are mainly assessed by diode-array detector (DAD) (Olmedilla et al., 2001), electrochemical detector (ECD) (Finckh et al., 1995), fluorescence detector (FLD) (Siluk et al., 2007) or mass spectrometry (Johnson et al., 2012). They have absorption maxima in the UV range, which allows the use of DAD, although its selectivity is strongly influenced by the possible UV active interfering compounds of the biological samples (Lai & Franke, 2013). Furthermore, DAD provides low sensitivity compared to the following detection methods. FLD profits from the native fluorescent property of vitamin E and allows very selective and sensitive detection (Lai & Franke, 2013). The aromatic hydroxyl group on the chromanol ring approves the electrochemical measurement, so 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 (Nánási et al., 2018b). For the determination of their concentrations, more sensitive methods are necessary, because with the application of DAD, or even ECD and FLD, usually only αT can be measured reliably (Lai & Franke, 2013).

The measurement of biogenic amines from biological samples requires highly selective and sensitive methods because of their considerably low concentrations in most biological samples (Zhang et al., 2016; Allen et al., 2017). HPLC combined with ECD is one of the best
alternatives for the quantitative detection of monoamines and related compounds (Van Valkenburg et al., 1982; Saller & Salama, 1984) in biological samples because of their electroactive function groups and the exceptional sensitivity of the ECD (Figure 2). The applicability of the method is influenced by many factors, its use and optimization require great expertise. The compositions of the mobile phase can significantly influence the determination of metabolites (Leung & Tsao, 1992). On the other hand, usually the additional volume of acetonitrile is limited to approximately 15% in case of the utilization of the most commonly used glassy carbon working electrode. Moreover, the use of a buffer with adequate buffer capacity is essential to ensure a stable pH value. Even a small change in pH value can have a pronounced effect on the retention time of the compounds. It is advisable to adjust the pH beyond ± 2 units relative to the isoelectric point of the assessed compounds, however, this is difficult to access when a large number of compounds are measured. Furthermore, it is advisable to add some amount of antioxidant to the mobile phase to avoid oxidation of the substances after injection prior reaching the detector (Van Schoors et al., 2015). The additional use of chelators, most commonly ethylenediaminetetraacetic acid (EDTA), should be considered as well to bind contaminants that may interfere with the signal. For ECD, it is also advisable to use a small particle size column and a small injection volume to achieve the best signal-to-noise ratio, however, a low injection volume requires detection with high sensitivity. The signal is also significantly influenced by the applied working potential (WP) (Lim, 1986). As the WP increases, the corresponding signal gradually increases as well until a plateau phase is reached, and the measurement should be performed at the WP value corresponding to the beginning of the plateau phase in order to achieve the most stable signal. At the same time, it is also important to mention that as the WP increases, the signal-to-noise ratio deteriorates, which can significantly limit the feasibility of the measurement with high sensitivity. The quantification of biogenic amines and their metabolites is mostly done by RP ion-pair chromatography, where the amount of ion-pair reagent greatly influences the retention time of each compound as well (Nánási et al., 2018a). Besides, increasing the concentration of ion-pairing agent, the retention time of each compound may increase or decrease depending on its chemical features. Due to the complexity of the system described above, by increasing the number of tested compounds, it is very difficult to develop a method that allows the selective detection of compounds in one run (Zhang et al., 2016). 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 indolamines), for which the applied method should also be selective. Testing multiple brain regions to adjust the selectivity of the method can also be difficult (Allen et al., 2017).

**Figure 2.** Structure and formation of biogenic amines from their precursors and some of their metabolites in living organisms. Arrows do not necessarily represent a stepwise enzymatic transformation. The structural similarity to biogenic amines of the most commonly used ISs (IPR, DHBA and NM-5HT) is demonstrated as well. 3-MT 3-methoxytyramine; 5-HIAA 5-hydroxyindoleacetic acid; 5-HT serotonin; DA dopamine; DHBA 3,4-dihydroxybenzylamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; IPR isoproterenol; IS internal standard; L-DOPA 3,4-dihydroxyphenylalanine; NA norepinephrine; NM-5HT N-methyl-5-hydroxytryptamine; TRP tryptophan; TYR tyrosine.
2. Aims

I., To compare HPLC-FLD and ECD 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 CNS 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 (L-DOPA, 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), DA, 5-hydroxytryptamine (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 MPTP-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**

3.1. **Animals**

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.).

3.1.1. **MPTP treatment**

MPTP hydrochloride was freshly dissolved in physiological saline (0.9 w/w% sodium chloride solution; pH adjusted to 7.4 with 0.1 M sodium hydroxide) and was administered intraperitoneally (i.p.). The prepared solution had a mass concentration of 1.8 mg/mL for MPTP, which is equivalent to 10.4 mM molar concentration. 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. physiological saline injection 5 times at 2 h intervals. After the last MPTP injection, one female and two male mice were found dead. Regarding the control groups, one female mouse was excluded from the study due to unexpected behaviour.

3.2. **Materials**

For tocopherol measurements, αT, ammonium acetate (NH₄CH₃COO), ascorbic acid, butylated hydroxyl-toluene (BHT), 1,4-dioxane, n-hexane, methanol (MeOH), EDTA and sodium-perchlorate (NaClO₄) were purchased from Sigma Aldrich (Saint Louis, MO, USA). Rac-tocol (rT, IS) was acquired from Matreya LLC (Pleasant Gap, PA, USA). HPLC purity acetonitrile (ACN), HPLC purity tetrahydrofuran (THF), HPLC purity absolute ethanol (EtOH), 2-propanol (IPA), sodium chloride (NaCl), disodium hydrogen phosphate dihydrate (Na₂HPO₄*2 H₂O) and
sodium hydrogen carbonate (NaHCO₃) were purchased from VWR International (Radnor, PA, USA). Potassium dihydrogen phosphate (KH₂PO₄), sodium dihydrogen phosphate (NaH₂PO₄), D-glucose monohydrate (D-glucose*H₂O) and calcium chloride dihydrate (CaCl₂*2H₂O) were obtained from Reanal (Budapest, Hungary). Potassium chloride (KCl) and Triton X were purchased from Spektrum 3D (Budapest, Hungary, from 2008: part of VWR), disodium sulphate (Na₂SO₄) from Fine Chemical Co. (Budapest, Hungary) and magnesium chloride hexahydrate (MgCl₂*6 H₂O) from Scharlau (Barcelona, Spain).

We used the following chemicals in the MPTP study beside those, which were mentioned above for αT quantification: disodium ethylenediaminetetraacetate dihydrate (Na₂EDTA*2H₂O; Lach-Ner s.r.o, Neratovice, Czech Republic), sodium metabisulphite (Na₂S₂O₅; Fluka, Budapest, Hungary, Honeywell Group), sodium dihydrogen phosphate (NaH₂PO₄; Reanal Laboratory Chemicals, Budapest, Hungary), MPTP hydrochloride (MedChemExpress, Monmouth Junction, NJ, USA) and the following substances were obtained from Sigma Aldrich (Saint Louis, MO, USA): perchloric acid (HClO₄), phosphoric acid (H₃PO₄), sodium hydroxide, sodium octyl sulphate (NaOS), L-DOPA, DOPAC, NA hydrochloride, 3,4-dihydroxybenzylamine hydrobromide (DHBA*HBr, IS), 5-HIAA, HVA, DA hydrochloride, isoproterenol hydrochloride (IPR*HCl, IS), 5-HT hydrochloride, 3-MT hydrochloride and N-methylserotonin oxalate (NM-5HT*oxalate). Sterile water was purchased from Braun, Zur Universität Pharmacy, Wien, Austria.

3.3. Sample preparation

In the first experiment at the age of 6, 16 and 66 weeks, the animals were deeply anesthetized with isoflurane (Forane®; Abbott Laboratories Hungary Ltd., Budapest, Hungary). After thoracotomy, venous blood was collected from the right ventricle by intracardial puncture into Eppendorf tubes containing Na₂EDTA, followed by perfusion with artificial cerebrospinal fluid (pH = 7.40, composed from the following reagents in mM: 122 NaCl, 3 KCl, 1 Na₂SO₄, 1.25 KH₂PO₄, 10 D-glucose*H₂O, 1 MgCl₂*6 H₂O, 2 CaCl₂*2 H₂O, 6 NaHCO₃) for 5 min by an automatic peristaltic pump.

After centrifugation of blood samples at 4 °C for 5 min at 3500 revolutions 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 BHT in absolute 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 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 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 (UP100H, Hielscher Ultrasound Technology, Germany; amplitude: 100%, cycle: 0.5) in 1,020 μL ice-cooled solution (composition in mM: 75 Na₂HPO₄·2 H₂O, 17 NaH₂PO₄, 121 NaCl, 2 KCl, 1 EDTA, 67 ascorbic acid, 2 BHT and 0.2 v/v% Triton-X, 9.8 v/v% EtOH). 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 (Forane®; Abbott Laboratories Hungary Ltd., Budapest, Hungary). 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 (60 μL/mg striatum) containing 400 μM Na₂S₂O₅, 500 μM Na₂EDTA·2H₂O and ISs (50 ng/mL DHBA, 200 ng/mL IPR and 100 ng/ml NM-5HT in 334 mM HClO₄). 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.
3.4. Methods

3.4.1. Development and validation of HPLC methods for the quantification of αT levels from murine samples

Out of the 3 necessary methods for αT quantification from mouse plasma and brain samples 2 validated analytical methods have been already available, but the validations were carried out on different biological matrices, i.e., on human and mouse serum samples (Veres et al., 2017; Nánási et al., 2018b). Regarding the current samples of interest, the concentrations of β/γT and δ-tocopherol were under the limit of detection in cases of all measurements with all the applied detectors, only αT levels could be quantified, so we focus on this compound in the following part of the methodological descriptions. For all analytical measurements, HPLC grade solvents and reagents of at least analytical grade were used.

3.4.1.1. Validation of αT measurements from mouse plasma samples

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% THF, 6.61 v/v% MeOH, 5.45 v/v% water and 272.4 mg/L NH₄CH₃COO. 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 (FilterBio®, Lab-Ex Kft, Budapest, Hungary). The compounds were simultaneously detected at their wavelengths of maximum absorbance, i.e., 292 and 297 nm for αT and rT, respectively (Veres et al., 2017). Fifty μL aliquots were injected by the autosampler to the analytical column. Run time of a sample with injection took 5 minutes. Chromatograms were evaluated using EZChrome Elite software, αT values of plasma and standard stock solutions were calculated in μM. During validation and measurement processes, calibrators were prepared in 250 mg/L BHT-EtOH, then they were arranged in six different concentration levels with concentration ranges of 1–50 μM and 0.25–12.5 μM for αT and rT, respectively. The peak area responses were plotted as a function of the corresponding concentration and linear regression
computations were evaluated by the least square method with the freely available R software (R Development Core Team, https://www.r-project.org/). Good linearity ($R^2 \geq 0.99$) was detected throughout the concentration ranges for all compounds.

Regarding the validation, pooled plasma samples were obtained from 14 individuals of 4-12 month-old C57BL/6N female and male mice. During validation process we determined the limit of detection (LOD), limit of quantification (LOQ), selectivity, intra- and interday precisions, as well as recovery values. For the calculation of LOD and LOQ values, Equation 1 was applied. Intraday precision (CV%) or predicted standard error is the ratio of the standard deviation and mean value of the test object areas obtained in six parallel measurements, expressed as a percentage. The interday precisions were recorded 3 days apart from intraday precisions. Bias is an estimate of a systematic measurement error and can be calculated as a ratio, whereas the mean of standard deviation of the intra- and interday precision is in the numerator part, and in the denominator part the mean of intra- and interday precision is placed for each subject of analysis. Recovery value means a calculated ratio for a qualified compound, which can be determined from spiked and native samples, whereas a spiked sample is defined as a native sample with the added material under analysis at a known concentration level. Its numerical value indicates how much of this known concentration was determined by the applied method.

The relative recoveries were estimated by measuring spiked samples of αT at two different concentration levels with three replicates of each. No significant differences were observed for the lower and higher concentrations. To analyse the selectivity of the method, we matched the chromatograms of αT and rT for a blank plasma and those for a spiked sample.

\[
\text{LOD} = 3.3 \times \frac{\sigma}{S'} \quad \text{and} \quad \text{LOQ} = 10 \times \frac{\sigma}{S'}
\]

**Equation 1.** The calculation of LOD and LOQ values by formula, where $\sigma$ is the standard error of the intercept and $S'$ is the slope of the calibration curve of the substance. $LOD$ limit of detection; $LOQ$ limit of quantification.

3.4.1.2. Development and validations of αT measurements from mouse brain samples

As previously mentioned, an HPLC-ECD method has already been available for measuring from mouse striatal samples. Although this method allowed sensitive separation, also had some withdraws. Accordingly, besides the further development and validation of this technique, we
aimed at testing another HPLC method utilizing FLD described by Yuan et al. (Yuan et al., 2014) as well. Applying the same samples as in HPLC-ECD, the applicability of FLD and ECD for the measurement of αT from brain samples was compared. 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 an FLD (Agilent Technologies, Santa Clara, CA, USA). Measurements were carried out under isocratic conditions.

The mobile phase consisted of 91.25 v/v% MeOH, 4.25 v/v % water, 4.50 v/v % IPA and 2.81 g/L NaClO₄ and it was delivered at a rate of 1.2 ml/min at 25 °C onto the RP 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 (FilterBio®, Lab-Ex Kft, Budapest, Hungary). Ten μL of aliquots were injected by the autosampler with the cooling module set at 4 °C. Measuring time of a sample with injection took 9.5 minutes. Chromatograms were evaluated using Agilent ChemStation software, and αT content of brain and standard stock solution were calculated in nmol/g wet weight and nM, respectively.

The values of the validation (linearity, LOD, LOQ, intra- and interday precisions, recovery and selectivity) and calibration parameters were determined similarly to the HPLC-DAD method. In case of the calibration stock solutions, the procedure was similar to that of the plasma, with the difference that the mobile phase was used as solvent, and the examined range of linearity was in a narrower concentration range.

Regarding the measurements by FLD, the mobile phase consisted of pure MeOH, applying the slightly modified method of Yuan et al. (Yuan et al., 2014). 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. Since the mobile phase was HPLC grade solvent, no filtration was included in this case. The injection volume was 10 μL, and the samples were thermostated at 4 °C. The sample preparations (liquid-liquid extraction, centrifuging, evaporation and reconstitution with mobile phase, i.e., with pure MeOH) and measurement as well as validation parameters were the same as those have already been described for the DAD and ECD methods.
In the study of possible age- and gender-related effects on αT in CNS samples of mice, 2 parallel measurements were carried out for each sample to be able to compare the appropriateness of FLD and ECD methods as well.

3.4.2. Development and validation of HPLC method for the quantification of the level of some biogenic amines and related compounds from murine samples

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 (DHBA, IPR and NM-5HT) was performed as follows. As a start-up, a previously published method of our research group (Torok et al., 2017) applicable for the measurement of only some metabolites from the above group and utilizing only 1 IS (IPR) was used as following: the pH of the mobile phase containing 75 mM NaH₂PO₄, 2.8 mM NaOS and 100 μM Na₂EDTA was adjusted to 3.0 with 85 w/w% H₃PO₄, and then it was supplemented with 7 v/v% ACN. In the method settings, chromatograms were analyzed using Agilent ChemStation software, and the content of biogenic amines and related compounds of the brain and standard stock solution were calculated in ng/mg wet weight and ng/ml, respectively. 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 WP of the ECD cell. The test solution was the 4th calibrator (test objects and IS concentrations are given in ng/ml units: 40 for DOPAC and NA, 75 for 5-HIAA, HVA and 5-HT, 100 for L-DOPA, DA and 3-MT and 200 for IPR).

The resulting supernatants were pooled and the volume was divided into 4 aliquots and the individual fractions were spiked with pure water (native sample) or with 3 different concentrations of the substances of interest (i.e., spike 1: 5 ng/ml for all test compounds except 10 ng/ml for 3-MT; spike 2: 20 ng/ml for all test compounds except 40 ng/ml for 3-MT and spike 3: 40 ng/ml for all test compounds except 80 ng/ml for 3-MT), complying with international recommendations and guidelines (FDA, 2001; Tsikas, 2018). Completing the validation process, linearity, LOD, LOQ, selectivity, intra- and interday precisions and recovery values were determined as well.
3.5. Statistics

All statistical calculations were performed with the use of R software. We first checked the distribution of data populations with the Shapiro-Wilk test, and we also performed the Levene test to confirm the homogeneity of variances. As the distribution proved to be Gaussian and the variances were equal, two-way analysis of variance (ANOVA) was applied with Tukey HSD post hoc test for pairwise comparisons. We decided a priori that the comparisons of opposite gender and age groups may not yield meaningful information, so they were not implemented, therefore only 9 comparisons were applied between the six groups in the first animal experiment, where the possible age- and gender-related effects were investigated. In the second experiment, this decision making regarding the comparison procedure was similar, i.e., groups that differed in gender and treatment were not taken into account, thus only 4 comparisons were performed. In case of model construction, analysis of covariance (ANCOVA) was applied. We rejected the null hypothesis when the $p$-values were $<0.05$, and in such cases the differences were considered significant. If any significant change was observed, the effect size was calculated (omega square ($\omega^2$) for two-way ANOVA, partial eta square ($\eta^2$) for ANCOVA and Cohen's $d$ for Tukey HSD). Pearson correlation analysis with post hoc Bonferroni correction for the number of analyses was used to investigate the possible relationship between endogenous striatal $\alpha$T content and DA level in each group of the MPTP study. Data were plotted as means ($\pm$ SD). The measured values were presented in ng/mg wet weight (ww) and nmol/g ww regarding catecholamines and $\alpha$T, respectively.
4. Results

4.1. Validation of HPLC methods for the quantification of αT levels from murine samples

The first step of our ECD method development was the determination of the optimal WP for αT and rT, whereas 10 μl of 1.0–1.0 μM of αT and rT was injected onto the analytical column applying different voltage values from +400 to 800 mV and peak areas were measured in each setting. As a result of our measurements (Figure 3) the WP was set at +700 mV. The sensitivity of the ECD was set to 10 mA/V.

Figure 3. Plotted peak areas vs. voltage diagram of αT and rT. αT α-tocopherol; rT rac-tocol.

The validation results of the 3 different applied HPLC methods are shown in the Table 1 and in Figure 4. 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, as shown in Figure 4. Regarding measurements from mouse brain samples, both ECD and FLD methods have strengths and weaknesses. The advantage of ECD is the wider linear range, which also makes the technique more sensitive. With FLD, the αT content of a brain sample can be determined with smaller standard deviation and significantly faster, and the measurement conditions are also much...
simpler than with those used with ECD, since the baseline and signal-to-noise ratio are significantly more stable and better, respectively, with FLD.

**Table 1.** Instrumental settings and validation parameters for mouse plasma (DAD) and brain region (ECD, FLD) measurements.

<table>
<thead>
<tr>
<th>Validation parameters – αT</th>
<th>DAD</th>
<th>ECD</th>
<th>FLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (μM)</td>
<td>1.0 – 50.0</td>
<td>0.05 – 2.0</td>
<td>0.10 – 2.0</td>
</tr>
<tr>
<td>Correlation coefficient (R²)</td>
<td>0.9999</td>
<td>0.9996</td>
<td>0.9996</td>
</tr>
<tr>
<td>LOD (nM)</td>
<td>397</td>
<td>26</td>
<td>41</td>
</tr>
<tr>
<td>LOQ (nM)</td>
<td>1202</td>
<td>77</td>
<td>123</td>
</tr>
<tr>
<td>Intraday precision (CV%)</td>
<td>1.7</td>
<td>2.9</td>
<td>1.1</td>
</tr>
<tr>
<td>Interday precision (bias%)</td>
<td>2.5</td>
<td>3.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>83–95</td>
<td>103–106</td>
<td>87–104</td>
</tr>
<tr>
<td>Run time (with injection, min.)</td>
<td>5.0</td>
<td>9.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Injection volume (μl)</td>
<td>50</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>λ(abs) (nm)</td>
<td>292</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>WP (mV)</td>
<td>-</td>
<td>+700</td>
<td>-</td>
</tr>
<tr>
<td>λ(ex) / λ(em) (nm)</td>
<td>-</td>
<td>-</td>
<td>292 / 330</td>
</tr>
</tbody>
</table>

The validation parameters were calculated as described in the Materials and methods section. αT α-tocopherol; CV% coefficient of variation in percentage value; DAD diode-array detector, ECD electrochemical detector; FLD fluorescence detector; λ(abs) absorption maximum wavelength; λ(em) emission wavelength; λ(ex) excitation wavelength; LOD limit of detection; LOQ limit of quantification; WP working potential of the glassy carbon electrode.
Figure 4. Representative chromatograms of rT and αT. The chromatograms of blank and spiked (with rT and αT) mouse plasma samples measured with DAD (a) and those of blank and spiked (with rT and αT) mouse brain sample homogenizations measured with ECD (b) and FLD (c) are demonstrated. αT α-tocopherol; DAD diode-array detector; ECD electrochemical detector; FLD fluorescence detector, rT rac-tocol.
4.2. Measurements of αT levels from 6, 16 and 66 weeks old female and male mice

4.2.1. Plasma

The results of the measurements of αT concentration from plasma samples are presented in Table 2 and in Figure 5a. The applied two-way ANOVA demonstrated significant difference for age (F = 10.547, df = 2, p < 0.001, ω² = 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, Table 3), with an effect size of 2.234 (Table 4).

4.2.2. Brain regions

The measurements of αT concentration from brain samples are demonstrated in Table 2 and Figure 5b–f. In light of the validation parameters, both ECD and FLD measurements are applicable for the determination of αT from brain samples and accordingly, the results of FLD and ECD measurements were averaged for each individual CNS sample. In the next step, the implementation of two-way ANOVA with Tukey HSD post hoc test yielded the following results.

In the striatum (Figure 5b), there was a significant difference for age (F = 120.019, df = 2, p < 0.001, ω² = 0.0752) and gender (F = 23.062, df = 1, p < 0.001, ω² = 0.0070), and for age vs. gender (F = 3.588, df = 2, p < 0.05, ω² = 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 (Tables 2 and 3).

In the cortex (Figure 5c), there was also a significant difference for age (F = 159.589, df = 2, p < 0.001, ω² = 0.1042), gender (F = 17.377, df = 1, p < 0.001, ω² = 0.0054), and for age vs. gender (F = 5.465, df = 2, p < 0.01, ω² = 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 (Tables 2 and 3).

Furthermore, in the hippocampus (Figure 5d), 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 $\alpha T$ 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 (Tables 2 and 3).

With regard to the cerebellum (Figure 5e) and the brainstem (Figure 5f), 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 $\alpha T$ 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 (Tables 2 and 3).

The corresponding effect size values for the significant differences in brain $\alpha T$ concentrations are presented in Table 4.

**Table 2. $\alpha T$ concentration levels in plasma and CNS of mice.**

<table>
<thead>
<tr>
<th>$\alpha T$ levels</th>
<th>Plasma (μM)</th>
<th>Striatum (nmol/g ww)</th>
<th>Cortex (nmol/g ww)</th>
<th>Hip. (nmol/g ww)</th>
<th>Cerebellum (nmol/g ww)</th>
<th>Brainstem (nmol/g ww)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 w female</strong></td>
<td>6.21 ± 2.53</td>
<td>11.89 ± 2.62</td>
<td>7.96 ± 1.36</td>
<td>9.97 ± 1.63</td>
<td>6.51 ± 1.33</td>
<td>7.95 ± 1.26</td>
</tr>
<tr>
<td><strong>16 w female</strong></td>
<td>4.18 ± 1.04</td>
<td>19.27 ± 3.32</td>
<td>12.15 ± 2.35</td>
<td>17.56 ± 3.16</td>
<td>8.54 ± 2.03</td>
<td>8.39 ± 1.25</td>
</tr>
<tr>
<td><strong>66 w female</strong></td>
<td>5.70 ± 1.56</td>
<td>26.60 ± 3.74</td>
<td>20.31 ± 2.43</td>
<td>26.93 ± 3.37</td>
<td>9.59 ± 1.45</td>
<td>9.27 ± 1.59</td>
</tr>
<tr>
<td><strong>6 w male</strong></td>
<td>5.34 ± 1.01</td>
<td>11.05 ± 1.96</td>
<td>7.63 ± 1.56</td>
<td>9.81 ± 1.96</td>
<td>6.55 ± 1.37</td>
<td>7.35 ± 1.74</td>
</tr>
<tr>
<td><strong>16 w male</strong></td>
<td>3.51 ± 1.27</td>
<td>15.72 ± 3.72</td>
<td>10.60 ± 2.64</td>
<td>14.57 ± 3.28</td>
<td>6.89 ± 1.85</td>
<td>6.84 ± 1.69</td>
</tr>
<tr>
<td><strong>66 w male</strong></td>
<td>6.24 ± 1.18</td>
<td>21.46 ± 3.74</td>
<td>16.14 ± 2.50</td>
<td>21.35 ± 3.45</td>
<td>8.06 ± 1.37</td>
<td>7.83 ± 1.27</td>
</tr>
</tbody>
</table>

Data are presented as mean (± S.D.); *Hip*. Hippocampus; $n = 9$; $\alpha T$ α-tocopherol; $w$ week; $ww$ wet weight.
Figure 5. αT concentration levels in plasma and in different brain regions of mice. We observed significant elevation of αT levels in the plasma (a), striatum (b), cortex (c), hippocampus (d) and cerebellum (e), but not in the brainstem (f) with aging. To avoid the overcomplication of this figure, the results of statistical comparisons are presented elsewhere (Table 2–4), except that for the interaction which may help in the interpretation of the presence or absence of increasing difference between males and females with aging. Data are presented as mean (± S.D.); n = 9; αT α-tocopherol; N.S. not significant.
Table 3. Converted *p*-values of Tukey HSD *post hoc* tests for the measurements of αT concentrations in plasma and brain samples.

<table>
<thead>
<tr>
<th>Compared groups</th>
<th>Plasma</th>
<th>Striatum</th>
<th>Cortex</th>
<th>Hip.</th>
<th>Cer.</th>
<th>Brainstem</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 vs. 16 w female</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>**</td>
<td>-</td>
</tr>
<tr>
<td>6 vs. 16 w male</td>
<td>-</td>
<td>*</td>
<td>*</td>
<td>***</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 vs. 66 w female</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>-</td>
<td>-</td>
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<tr>
<td>16 vs. 66 w male</td>
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<td>***</td>
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<tr>
<td>6 vs. 66 w female</td>
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<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>-</td>
</tr>
<tr>
<td>6 vs. 66 w male</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 w female vs. male</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 w female vs. male</td>
<td>-</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>66 w female vs. male</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n = 9; αT α-tocopherol; Cer. cerebellum; Hip. hippocampus; w week; * p < 0.05; ** p < 0.01; *** p < 0.001.

Table 4. Cohen’s *d* effect size values for αT concentration in plasma and brain samples.

<table>
<thead>
<tr>
<th>Compared groups</th>
<th>Plasma</th>
<th>Striatum</th>
<th>Cortex</th>
<th>Hip.</th>
<th>Cer.</th>
<th>Brainstem</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 vs. 16 w female</td>
<td>-</td>
<td>2.464</td>
<td>2.182</td>
<td>3.018</td>
<td>1.180</td>
<td>-</td>
</tr>
<tr>
<td>6 vs. 16 w male</td>
<td>-</td>
<td>1.571</td>
<td>1.369</td>
<td>1.763</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 vs. 66 w female</td>
<td>-</td>
<td>2.071</td>
<td>3.413</td>
<td>2.868</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 vs. 66 w male</td>
<td>2.234</td>
<td>1.539</td>
<td>2.158</td>
<td>2.012</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 vs. 66 w female</td>
<td>-</td>
<td>4.552</td>
<td>6.268</td>
<td>6.409</td>
<td>2.213</td>
<td>-</td>
</tr>
<tr>
<td>6 vs. 66 w male</td>
<td>-</td>
<td>3.490</td>
<td>4.097</td>
<td>4.107</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 w female vs. male</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 w female vs. male</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>66 w female vs. male</td>
<td>-</td>
<td>-1.006</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n = 9; αT α-tocopherol; Cer. cerebellum; Hip. hippocampus; w week.
4.3. Validation of HPLC-ECD method for the quantification of biogenic amine levels from murine samples

4.3.1. Determination of the optimal pH value of the mobile phase

By changing only the pH of the mobile phase, its effect on the retention time of the tested metabolites and IPR (the other 2 ISs were not available at this stage of the method development) was studied. Increasing the pH from 3.0 to 4.0 reduced the retention time of all compounds (Figure 6), however, it did not improve the efficiency of the separation. Further pH values were not tested because it would have required a change of the applied buffer system and presumably no favorable change in retention times would be obtained.

**Figure 6.** The effect of the pH value of the mobile phase on the retention times of 8 metabolites and IPR. The other 2 parameters were almost the same as in the original settings, i.e.: 2.8 mM NaOS and 5 v/v% ACN. 3-MT 3-methoxytyramine; 5-HIAA 5-hydroxyindoleacetic acid; 5-HT serotonin; ACN acetonitrile; DA dopamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; IPR isoproterenol; L-DOPA 3,4-dihydroxyphenylalanine; NA norepinephrine; NaOS sodium octyl sulphate.
4.3.2. Determination of the optimal concentration of ion pairing component of the mobile phase

During method development, we found that raising the concentration of the ion-pairing reagent (NaOS) affected the retention time of the tested compounds ambivalently: it increased in the case of L-DOPA, DA, NA, 5-HT, 3-MT and IPR, whereas it decreased in case of DOPAC, HVA and 5-HIAA (Figure 7.). In case of the original method settings, i.e., 2.8 mM NaOS, L-DOPA and DOPAC coeluted, but at lower NaOS concentrations, this disadvantage has been resolved. It should be noted that at the highest tested concentration, i.e., at 7.5 mM, perfect separation can be achieved, but the running time was longer than 45 minutes.

In summary, these results showed that the best choice is to keep the pH value at 3.0 and NaOS in the concentration range between 2.10 mM and 2.20 mM with approximately 5–6 v/v% ACN. Increasing the amount of organic solvent (ACN) resulted in a decrease in retention times in all cases, but separation efficiency impaired as well gradually. Regarding our experience, the suggested amount of ACN is between 5 and 6 v/v% (data not shown).

Figure 7. The effect of changing the concentration of the ion pairing compound (NaOS) on the retention times of 8 metabolites and IPR. The other 2 parameters were almost the same as in the original settings, i.e., pH = 3.0 and 5 v/v% ACN. 3-MT 3-methoxytyramine; 5-HIAA 5-hydroxyindoleacetic acid; 5-HT serotonin; ACN acetonitrile; DA dopamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; IPR isoproterenol; L-DOPA 3,4-dihydroxyphenylalanine; NA norepinephrine; NaOS sodium octyl sulphate.
4.3.3. Adaptations of new ISs to the developed method

IPR is an ideal IS for catecholamines, as it is essentially a catecholamine that does not occur in the living organism and can be adapted into the method with adequate selectivity. However, the length of the method as well as the measurement of 5-HT may justify the introduction of new ISs. For catecholamines, DHBA elutes in the first half of the run, whereas another new IS, NM-5HT is ideal regarding indolamine approaching the end of the run (see Figure 8).

**Figure 8.** Representative chromatograms of native and spiked striatal samples of C57Bl/6 mice illustrating the selectivity of the developed and validated HPLC method. Only the concentrations of black-labeled compounds were measured in the MPTP study. 3-MT 3-methoxytyramine; 5-HIAA 5-hydroxyindolacetic acid; 5-HT serotonin; DA dopamine; DHBA 3,4-dihydroxybenzylamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; HPLC high performance liquid chromatography; IPR isoproterenol; L-DOPA levodopa; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NA noradrenaline; NM-5HT N-methyl-5-hydroxytryptamine.
4.3.4. Determining the optimal WP for 11 compounds

As part of method development, the optimal WP was determined for both the 8 test objects (L-DOPA, DOPAC, NA, 5-HIAA, HVA, 3-MT, DA, 5-HT) and the 3 ISs (DHBA, IPR and NM-5HT). The operating potential of the ECD was increased by 50 mV from 400 mV to 800 mV (Figure 9). Although 800 mV would be the ideal WP for HVA and 3-MT, the signal-to-noise ratio has been significantly deteriorated at this voltage, so we decided to use +750 mV.

Summarily, the mobile phase for the improved method consisted of 2.20 mM NaOS, 75 mM NaH₂PO₄, 100 µM Na₂EDTA and 6.25 v/v% ACN. Before adding ACN, the pH value of water phase was set to 3.0 with 85 w/w% H₃PO₄.

Following the method development described above, the applicability of that was tested on pooled brain regions (striatum, cortex and hippocampus), obtained from 9 12-16 week-old C57Bl/6 male and female mice, and the validation process was carried out as well (see Figure 8 and Table 5). Prior to measurements, the brain regions were weighed and then sonicated with an ice-cooled solution as mentioned in Sample preparation section, but without the ISs. The volume-to-weight ratios were the followings: striatum: 60 µl/mg; cortex: 25 µl/mg and hippocampus: 18.75 µl/mg.

![Figure 9. Plotted peak areas vs. voltage diagram of 8 metabolites and 3 ISs. 3-MT 3-methoxytyramine; 5-HIAA 5-hydroxyindoleacetic acid; 5-HT serotonin; DA dopamine; DHBA 3,4-dihydroxybenzylamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; IPR isoproterenol; IS internal standard; L-DOPA 3,4-dihydroxyphenylalanine; NA norepinephrine; NM-5HT N-methyl-5-hydroxytryptamine.](image)
Table 5. Summary of validation parameters of HPLC-ECD method for biogenic amines in mouse striatum.

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>L-DOPA</th>
<th>DOPAC</th>
<th>NA</th>
<th>5-HIAA</th>
<th>HVA</th>
<th>DA</th>
<th>5-HT</th>
<th>3-MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (ng/ml)</td>
<td>5-150</td>
<td>5-80</td>
<td>5-80</td>
<td>10-100</td>
<td>10-100</td>
<td>5-200</td>
<td>5-100</td>
<td>10-200</td>
</tr>
<tr>
<td>Correlation coefficient ($R^2$)</td>
<td>1.000</td>
<td>0.999</td>
<td>0.999</td>
<td>0.998</td>
<td>0.994</td>
<td>0.999</td>
<td>0.999</td>
<td>0.998</td>
</tr>
<tr>
<td>LOD (ng/ml)</td>
<td>1.3</td>
<td>0.5</td>
<td>0.7</td>
<td>5.5</td>
<td>7.4</td>
<td>2.0</td>
<td>3.8</td>
<td>9.8</td>
</tr>
<tr>
<td>LOQ (ng/ml)</td>
<td>3.9</td>
<td>1.5</td>
<td>2.1</td>
<td>16.5</td>
<td>22.3</td>
<td>6.0</td>
<td>11.6</td>
<td>29.7</td>
</tr>
<tr>
<td>Recovery (% mean)</td>
<td>100.7</td>
<td>108.0</td>
<td>110.6</td>
<td>108.9</td>
<td>104.3</td>
<td>109.0</td>
<td>104.4</td>
<td>102.0</td>
</tr>
<tr>
<td>Intraday precision (CV%)</td>
<td>3.19</td>
<td>1.81</td>
<td>3.68</td>
<td>3.82</td>
<td>8.23</td>
<td>1.59</td>
<td>5.36</td>
<td>6.70</td>
</tr>
<tr>
<td>Interday precision (bias%)</td>
<td>3.24</td>
<td>1.83</td>
<td>3.89</td>
<td>3.85</td>
<td>8.59</td>
<td>1.63</td>
<td>5.42</td>
<td>6.85</td>
</tr>
</tbody>
</table>

The validation parameters were determined as illustrated in the Materials and methods section. The applied injection volume was 10 μl. CV% coefficient of variation; 3-MT 3-methoxythyramine; 5-HIAA 5-hydroxyindoleacetic acid and 5-HT serotonin; DA dopamine; DOPAC 3,4- dihydroxyphenylacetic acid; HPLC-ECD high performance liquid chromatography with electrochemical detector; HVA homovanillic acid; L-DOPA levodopa; LOD limit of detection; LOQ limit of quantification; NA noradrenaline.

4.4. Measurements of striatal DA levels and αT content from 16 weeks old female and male mice in the MPTP study

4.4.1. HPLC measurement of DA levels in striatal samples of mice

The measurements of DA, DOPAC and HVA concentration from striatal samples are presented in Table 6 and Figure 10a. The implementation of two-way ANOVA with Tukey HSD post hoc test yielded the following results.

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 \((p < 0.001, \text{Figure 10a})\), with an effect size of \(-2.600\), in MPTP-treated vs. control males \((p < 0.001, \text{Figure 10a})\) with an effect size of \(-5.925\), and in MPTP-treated males vs. females \((p < 0.05, \text{Figure 10a})\) as well with an effect size of \(-1.303\).

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 \((p < 0.001)\), with an effect size of \(-2.309\), in MPTP-treated vs. control males \((p < 0.001)\) with an effect size of \(-4.094\) and in MPTP-treated males vs. females \((p < 0.01)\) with an effect size of \(-1.491\).

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 \((p < 0.01)\) with an effect size of \(-1.629\), and in MPTP-treated vs. control males \((p < 0.001)\) with an effect size of \(-2.464\).

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 \((\text{DOPAC+HVA})/\text{DA}\) 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 \((p < 0.05, \text{Figure 10b})\) with an effect size of \(1.335\), and in MPTP-treated vs. control males \((p < 0.001, \text{Figure 10b})\) with an effect size of \(1.438\).
Figure 10. Measured compound concentrations in mouse striatal and plasma samples. DA levels (a), DA turnover (b), αT levels in the striatum (c) and in the plasma (d) are presented for the demonstration of the effect of MPTP treatment. Data are presented as mean (± S.D.); n (control and MPTP-treated females) = 14; n (control males) = 15; n (MPTP-treated males) = 13; αT α-tocopherol; DA dopamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; n.s. not significant; ww wet weight; * p < 0.05; ** p < 0.01; *** p < 0.001.
Table 6. DA, DOPAC and HVA concentration levels (ng/mg ww) and DA turnover in the striatum of mice.

<table>
<thead>
<tr>
<th>Substances or ratio</th>
<th>Control females(^1)</th>
<th>Control males(^2)</th>
<th>MPTP females(^1)</th>
<th>MPTP males(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>10.255 ± 2.451</td>
<td>10.692 ± 1.983</td>
<td>3.995 ± 2.364</td>
<td>1.651 ± 0.830</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.989 ± 0.170</td>
<td>1.097 ± 0.273</td>
<td>0.496 ± 0.248</td>
<td>0.195 ± 0.135</td>
</tr>
<tr>
<td>HVA</td>
<td>1.781 ± 0.453</td>
<td>1.938 ± 0.696</td>
<td>1.039 ± 0.458</td>
<td>0.604 ± 0.388</td>
</tr>
<tr>
<td>(DOPAC+HVA)/DA</td>
<td>0.276 ± 0.048</td>
<td>0.295 ± 0.062</td>
<td>0.440 ± 0.167</td>
<td>0.527 ± 0.227</td>
</tr>
</tbody>
</table>

Data are presented as mean (± S.D.); \(^1\)n = 14; \(^2\)n = 15; \(^3\)n = 13; DA dopamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ww wet weight.

4.4.2. HPLC measurement of αT levels in plasma and striatal samples of mice

The results of the measurements of αT concentration from plasma with HPLC-DAD and from striatal samples with HPLC-FLD are presented in Table 7, Figure 10c and d. 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 (\(p < 0.05\), Figure 10d), with an effect size of -1.958, and in MPTP-treated vs. control males (\(p < 0.05\), Figure 10d) with an effect size of -0.829.

Table 7. αT concentration levels in the plasma and the striatum of mice.

<table>
<thead>
<tr>
<th>αT levels</th>
<th>Plasma (µM)</th>
<th>Striatum (nmol/g ww)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control females(^1)</td>
<td>5.14 ± 1.32</td>
<td>23.19 ± 1.61</td>
</tr>
<tr>
<td>Control males(^2)</td>
<td>5.03 ± 2.50</td>
<td>20.06 ± 1.83</td>
</tr>
<tr>
<td>MPTP females(^1)</td>
<td>3.11 ± 0.63</td>
<td>22.23 ± 2.24</td>
</tr>
<tr>
<td>MPTP males(^3)</td>
<td>3.30 ± 1.45</td>
<td>18.90 ± 3.02</td>
</tr>
</tbody>
</table>

Data are presented as mean (± S.D.); \(^1\)n = 14; \(^2\)n = 15; \(^3\)n = 13; αT α-tocopherol; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ww wet weight.

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 (\(p < 0.01\), Figure 10c), and in
MPTP-treated female vs. male mice as well ($p < 0.01$, Figure 10c) with effect sizes of 1.811 and 1.261, respectively.

4.4.3. Study of possible relationships between mouse striatal DA and αT levels

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 ($F (1, 52) = 8.689, p < 0.01, \eta^2 = 0.761$), 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 $R^2$s 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; Figure 11).

![Figure 11](image-url)

**Figure 11.** Possible correlations between endogenous αT concentrations and DA levels in the striatum. Following Bonferroni correction, no statistically significant correlations were demonstrated. $n$ (control and MPTP-treated females) = 14; $n$ (control males) = 15; $n$ (MPTP-treated males) = 13; αT α-tocopherol; DA dopamine; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; $ww$ wet weight.
5. Discussion

5.1. The possible mechanisms of αT homeostasis in the CNS

Evidence suggests that tocopherols may have a special role in antioxidant protection in lipid-rich structures, such as the CNS. 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 (Dysken et al., 2014; Grimm et al., 2016; Farina et al., 2017; Zádori et al., 2011; The Parkinson Study Group, 1993). These findings suggest that the pure exogenous administration of αT is not always enough to improve functional outcome, there may be other aspects of brain αT homeostasis, involving the regulation of endogenous αT concentration in the CNS. However, only limited data are available on the changes of endogenous tocopherol levels either in human or murine brain samples with aging (Battisti et al., 1994; Craft et al., 2004; Johnson et al., 2013; Takahashi et al., 2017; Veres et al., 2017). 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.

One of the major challenges during the quantification of tocopherols and tocotrienols from brain and CSF samples is their small concentrations (Craft et al., 2004; Vatassery et al., 2004; Johnson et al., 2013) compared to plasma or serum samples (Veres et al., 2017). This is why that sensitive detection techniques are essential for the reliable measurement of these compounds, especially from rodent samples, where the concentrations are lower by 1-2 orders of magnitude compared to human samples, and therefore, only αT can be quantified in most cases, as it is the most biologically active form and has the highest concentration levels in the vitamin E group (Eggermont, 2006; Nánási et al., 2018b). Two different detection methods (ECD and FLD) were coupled to measure mouse brain αT and their applicability was compared in our first study as well. Although ECD has been shown to be more sensitive than its fluorescent counterpart, unfortunately the run time is approximately twice as long and its
precision is slightly worse than that of FLD. From these considerations, in our second experiment, we decided to use the FLD technique to determine αT levels from brain samples. 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 to 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. These findings are in line with those found by Takahashi (Takahashi et al., 2017) and Gohil (Gohil et al., 2008), i.e., there is a clear elevation of cortical and hippocampal αT levels, but only a moderate increase in cerebellar αT level with aging and significantly higher values in females. 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. Similar to that found by Takahashi (Takahashi et al., 2017) plasma αT level seemingly does not influence the characteristic change in striatal, cortical and hippocampal αT levels.

For better understanding of these results, we looked further into the transport and metabolism of tocopherols via thorough scientific literature review. The transport of vitamin E in the mammalian body is well documented (Spector & Johanson, 2007; Kono & Arai, 2015). Due to the chemical properties of vitamin E compounds, their circulation is associated with lipoproteins, but their transcellular transport is mediated by carrier proteins (Kono & Arai, 2015).

In the brain the CSF has an important role in micronutrient distribution, including vitamins as well (Spector & Johanson, 2007). The transportation of vitamins B₁, B₃ and E from blood to CSF with simple diffusion cannot provide enough concentrations for tissue cells, therefore
several specific systems (facilitated diffusion, active sodium-dependent or independent systems, receptor-related systems and many other mechanisms) are included as well (Spector & Johanson, 2007). Accordingly, it was proposed that brain αT homeostasis has a strict regulation, but the precise description of the pharmacokinetics of vitamin E transport into the CSF and brain is not available yet, although some possible mechanisms were previously described in the literature (Spector & Johanson, 2007).

One of these mechanisms may be connected to the scavenger receptor class B type 1 (SR-B1) (Mardones et al., 2002; Srivastava, 2003; Balázs Z et al., 2004), which is responsible for αT transport across the blood-brain barrier (BBB) by promoting the uptake of high-density lipoprotein (HDL)-associated αT in brain capillary endothelial cells (BCEC). In the study of Srivastava et al. (Srivastava, 2003), C57Bl/6 mice were fed with high-cholesterol and high-fat containing diet and their brain SR-B1 expressions consequently increased, although hepatic SR-B1 expression was not influenced. Furthermore, Mardones (Mardones et al., 2002) demonstrated that SR-B1 KO female and male (n = 3–6, 2–4 months old) mice have significantly higher plasma and lower CNS αT levels, consistent with the postulated role of SR-B1 at the BBB. Surprisingly, the whole brain levels were not decreased enough to cause deficiency symptoms. There are no available data on the influence of aging and gender on SR-B1 expression.

The second possible mechanism may be connected to phospholipid transfer protein (PLTP) (Gander et al., 2002). PLTP is highly expressed in the choroid plexus, which raises the possibility that PLTP is involved in the transfer of αT from plasma into the CSF (Desrumaux et al., 2004; Albers et al., 2012). In PLTP KO female mice (n = 6) Desrumaux (Desrumaux et al., 2004) investigated the brain concentration of αT and found it decreased by 30% compared to controls. Similar to SR-B1, there are also no available data on the influence of aging and gender on PLTP expression.

The α-TTP serves as a third mechanism of αT transport in the mammalian brain (Yokota et al., 2001). The presence of this protein is demonstrated in rodent and human brains as well, although the expression level in normal brain tissue is low (Kaempf-Rotzoll et al., 2003). In α-TTP KO female and male mice (n = 2–2), the plasma and tissue concentrations were less than 0.46 μM and 0.23 nmol/g ww, respectively. Moreover, supplementation with oral vitamin E raised the plasma concentration of these mice close to normal (~ 3.4 μM), but the brain levels
increased only with ~ 20%, however, this small increase in the brain could eliminate the neurological signs (Yokota et al., 2001). Gohil (Gohil et al., 2008) also investigated α-TTP expression in several CNS and liver tissue samples of 5 months old α-TTP KO and control mice with the finding that α-TTP or its mRNA could only be detected from the liver of controls. Later, Takahashi (Takahashi et al., 2017) successfully determined α-TTP expressions from the cerebrum, hippocampus and cerebellum. However, they utilized only 12-month-old male mice for that purpose, therefore the effect of aging and gender could not be analysed.

Another investigated pathway is in relation with afamin, a member of the albumin super family and may serve as a transport protein involved in αT homeostasis as well (Kratzer, I et al., 2008). Afamin is synthesized endogenously by BCEC and assists αT transport to astrocytoma cells, but to a lesser extent than HDL-mediated transport. The expression of afamin was detected by immunohistochemistry in porcine, mouse and post mortem human brains. It was also demonstrated that afamin level correlates with the concentration of αT in the CSF, but not with αT level in the serum (Jerkovic et al., 2005; Kratzer, I et al., 2008). However, there are no available data on the influence of aging and gender on afamin expression.

Although the above-mentioned findings may support the role of SR-B1, PLTP, α-TTP and afamin in the transport and regulation of the αT levels in the CNS, the assessment of the effect of aging and gender on these transport processes warrants further studies.

Probably the major significance of findings of this part of our studies is that the elevations of αT concentrations by aging may serve as to compensate the burden of oxidative stress which increases with aging (Veres et al., 2017; Zádori et al., 2011).

In light of the fact that there are only limited and contradictory data are available on the changes of human brain tocopherol levels with aging, further studies are needed to find the human relevance of the current findings. However, the results may draw attention to the importance of antioxidant protection against oxidative damage, the extent of which clearly increases with aging. With regard to the effect of gender on age-related pathological alterations, several human studies were published (Cowell et al., 1994; Murphy et al., 1996; Malpetti et al., 2017; Király et al., 2016). Király et al. (Király et al., 2016) demonstrated that male brain ages faster, following the assessment of the volume of cortical and subcortical grey matter, including that of the hippocampus, putamen and caudate nucleus, within the age-range of 21 to 58 years. Although the age range is presumably different, this male preponderance was not reassured in
the most common neurodegenerative disorder, AD (Mielke et al., 2014), but was proposed in the second most common neurodegenerative disorder, PD (Moisan et al., 2016). Nevertheless, the fact that there is not enough human data to be able to determine the extent of the influence of the alterations in tocopherol homeostasis on the pathogenetic process in major age-related neurodegenerative disorders, warrants further studies.

5.2. The role of αT in PD

The prevalence of PD was found to be increased in the aging population and in males (Van Den Eeden et al., 2003). 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 (Unzeta et al., 1994; Xu et al., 2006; Ciesielska et al., 2007, 2009; Liu et al., 2008; Joniec et al., 2009; Geldenhuys et al., 2015; Schamne et al., 2018). Although the possible role of sexual hormones behind these findings was proposed by several studies (Unzeta et al., 1994; Xu et al., 2006; Bourque et al., 2011), the exact explanation behind gender differences is still missing.

Amongst strategies of ameliorating disease progression, a popular approach may be the reduction of oxidative injury characteristic of PD (Zádori et al., 2011). Probably the most exhaustive trial to achieve this aim via the administration of αT was the DATATOP study, but the results did not support any neuroprotective effect (The Parkinson Study Group, 1993). Although literature data, coming from preclinical and human studies, are controversial regarding this topic, it can be proposed that the prolonged application of high dose αT initiated in early phases may have beneficial effects on the neurodegenerative processes (Fariss & Zhang, 2003).

Accordingly, 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. Based on the calculated validation parameters, the developed method is reliable and robust with an acceptable running time for the measurement of 8 different substances, using 3 ISs. The method is suitable for the study of biochemical changes in the MPTP model of PD, among others. The results of 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 (Unzeta et al., 1994; Xu et al., 2006; Ciesielska et al., 2007, 2009; Liu et al., 2008; Joniec et al., 2009). It was also assessed whether these findings may be related to differences in DA turnover. Although DA turnover significantly increased in MPTP-treated mice compared to controls, which also corresponds to the results of other studies (Sundström et al., 1990; Vučković et al., 2008), no difference between genders could be demonstrated. Regarding striatal αT levels, the findings of this study confirmed our previous results (Nánási et al., 2018b), i.e., the concentrations were significantly higher in the striata of females compared to males already at 16 weeks of age. Surprisingly, but in line with previous findings (Itoh et al., 2006), 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. Nevertheless, the finding itself that endogenous striatal αT content does not seem to be a major player against MPTP-induced deteriorations may be supported by the data obtained from studies with α-TTP deficient mice or with the application of dietary restriction (Itoh et al., 2006; Ren et al., 2006). Even α-TTP
KO mice with essentially undetectable level of brain αT were not more prone to MPTP-related striatal DA decrease compared to wild-type controls (Ren et al., 2006). Limitations of the current study include the lack of its extension for the assessment of the relationships between striatal DA and αT levels in further age groups. However, the authors presume that although both the sensitivity to MPTP treatment (Xu et al., 2006; Ciesielska et al., 2007, 2009) and the striatal level of αT (Nánási et al., 2018b) increase with aging with the enlargement or at least the persistence of the above-mentioned differences between genders, their relationship does not likely change. Accordingly, keeping in mind the 3R principle (replacement, reduction and refinement) of animal experiments as well, this extension was out of scope of the present work.

6. Conclusion

Our two studies were based on developed HPLC methods, validated according to the guidelines. Shortly, following their comparison from several aspects FLD was selected for further use in the detection of αT from mouse brain samples instead of ECD, although both methods are suitable for robust measurements. Furthermore, a method was also developed for the simultaneous assessment of certain biogenic amines and some of their precursors and metabolites (L-DOPA, DOPAC, NA, 5-HIAA, HVA, DA, 5-HT and 3-MT) from different brain regions of C57Bl/6N mice utilizing 3 ISs. With these techniques we were are able to selectively measure αT and biogenic amines as well as their related compounds from the biological samples of interest. Age-dependent gender-specific changes in αT level have been demonstrated in certain brain regions of the C57Bl/6N mouse strain which finding did not explain the increased vulnerability of male C57Bl/6N mice to MPTP. This latter result 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.
7. Acknowledgement

This work could not have been completed without those researchers, physicians, and friends who had supported and paved my way in the recent years. Furthermore, I would not be here where I am now without the persistent patronage of my family.

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8. References


Grebenstein N & Frank J (2012). Rapid baseline-separation of all eight tocopherols and tocotrienols by reversed-phase liquid-chromatography with a solid-core


APPENDIX
I.
The detection of age-, gender-, and region-specific changes in mouse brain tocopherol levels via the application of different validated HPLC methods

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Abstract
The aging process clearly increases the demand for antioxidant protection, especially in the brain, involving that provided by α-tocopherol (αT). However, little is known about the age-related changes in brain αT levels and the influencing effect of gender on it, in human or murine samples as well. Accordingly, the aim of the current study was to detect age-, gender- and region-specific changes in αT concentrations in mouse brain tissue and to assess the influencing effect of plasma αT levels on it. Female and male C57BL/6 mice at the ages of 6, 16 and 66 weeks (n = 9 in each group) were applied. αT levels were determined with high performance liquid chromatography (HPLC) from the striatum, cortex, hippocampus, cerebellum, brainstem and from plasma samples. A detailed validation process was carried out for the applied HPLC method as well. The results demonstrated that brain αT levels significantly increased in the striatum, cortex, and hippocampus with aging in both genders, but in a more pronounced way in females with an increasing magnitude of this difference. 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. The current study is the first to present age-dependent gender-specific changes in αT level in certain brain regions of the C57Bl/6 mouse strain, and may provide meaningful information for future therapeutic studies targeting aging-related processes.

Keywords Tocopherol · Mouse · Brain · Gender · HPLC · Aging

Introduction
Tocopherols and tocotrienols, also known as the fat-soluble vitamin E, have an outstanding role in antioxidant mechanisms [1]. 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 [2]. Accordingly, the deficiency of vitamin E often causes neurological symptoms and studies have proved significantly lower serum or plasma α-tocopherol (αT) levels in some neurological disorders, such as in Alzheimer’s disease (AD) [3, 4] and in Parkinson’s disease (PD) [5], which have an increasing prevalence in the elderly, and in some other neurological conditions [6–10].

With regard to the effects of normal aging on serum tocopherol levels, studies have shown a trend of similarly increasing concentrations in both gender [11, 12], which may be the consequence of the age-related alteration in lipid homeostasis [13]. Studies investigated αT level in cerebrospinal fluid (CSF) in healthy subjects [14] and in patients with AD [15] as well. In addition to the delineation of positive correlations between serum and CSF αT and γ-tocopherol (γT) levels in the healthy ones, significantly decreased CSF αT level was demonstrated in AD compared to controls. In relation to human brain tocopherol levels, studies provided contradictory results. Craft et al.
[16] carried out an examination on the regional distribution of the level of αT and γT isomers, demonstrating significant age-related (67–90 years, n = 5–2 females and 3 males) decline in αT level, more pronounced in females, but due to low case number, statistical comparison could not be carried out. With regard to the regional distribution, there were no significant differences between the grey and white matter of frontal and occipital cortices. Later, Johnson et al. [17] demonstrated a significant relationship between cognitive functions and tocopherol levels in the serum, cerebellar, frontal, occipital and temporal cortices of healthy octogenarians and centenarians. In case of serum samples, dramatically lower concentrations were measured, whereas for given brain regions, significantly higher levels were determined with aging. There was a significantly negative correlation between serum and brain αT levels, except the cerebellum, although cerebellar αT level, similarly to that of the other brain regions, also positively correlated with the scores of Mini-Mental State Examination (MMSE) and Severe Impairment Battery (SIB), both of which are measures of global cognition.

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. Most of the available rodent tocopherol studies did not separate animals to the necessary groups for later statistical comparisons [18–22]. Although Gohil et al. [23] determined αT level for given brain regions, significantly higher levels were found in males than in females, moreover significantly lower αT level was observed in the cerebellum than those of other examined brain regions, no information was obtained about the effect of aging. With regard to cholesterol levels, there was no any significant difference between genders. There is only one study [24], which assessed the effect of aging on tocopherol levels of rodents in details and reported a not significant decrease in plasma αT level (from ~8 to ~6 µM) with aging in C57Bl/6NCr male mice (3, 6, 12, 18 and 24 months old, n = 5 in each group) and significantly increasing values only in some brain regions [cerebrum (from 10 to 20 nmol/g tissue), hippocampus (from 12 to 25 nmol/g tissue) and cerebellum (from 11 to 15 nmol/g tissue)]. In addition, it was also demonstrated that triglyceride levels were not influenced by aging. However, female mice were not utilized in this study, so the effect of gender cannot be assessed.

There are many available detection methods for the measurement of tocopherol concentrations from plasma or serum and from brain samples [25]. Accordingly, vitamin E compounds are mainly assessed by diode-array detector (DAD) [26], electrochemical detector (ECD) [27], fluorescence detector (FLD) [28] or mass spectrometry [20]. In case of plasma or serum and brain samples of rodents, especially those of mice, the measurements of other tocopherols are challenging because of their small concentration levels. For the determination of their levels, more sensitive methods are necessary, because with the application of ECD, FLD and DAD, usually only αT can be measured reliably [25].

The aim of the current study was the expansion of the research of Takahashi et al. [24] and Gohil et al. [23] to be able to quickly and reliably determine αT concentrations in mouse plasma and brain tissues searching for the influencing effects of gender and aging via the application of robust, reproducible and validated HPLC methods. As a part of this process, the applicability of FLD and ECD for the measurement of αT from brain samples was investigated as well.

Materials and methods

Materials

αT, ammonium acetate, ascorbic acid, butylated hydroxytoluene (BHT), dioxane, n-hexane, methanol (MeOH), ethylenediaminetetraacetic acid (EDTA) and sodium-perchlorate (NaClO4) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Rac-tocol (rT) was acquired from Matreya LLC (Pleasant Gap, PA, USA).

Acetonitrile (ACN), tetrahydrofuran (THF), absolute ethanol (EtOH), isopropanol (IPA), sodium-chloride (NaCl), disodium-hydrogen-phosphate dihydrate (Na2HPO4·2H2O) and sodium-hydrogen-carbonate (NaHCO3) were purchased from VWR International (Radnor, PA, USA).

Potassium-dihydrogen-phosphate (KH2PO4), sodium-dihydrogen-phosphate (NaH2PO4), D-glucose monohydrate (D-glucose·H2O) and calcium-chloride dihydrate (CaCl2·2H2O) were obtained from Reanal (Budapest, Hungary).

Potassium-chloride (KCl) and Triton-X were purchased from Spektrum 3D (Budapest, Hungary, from 2008: part of VWR), disodium-sulfate (Na2SO4) from Fine Chemical Co. (Budapest, Hungary) and magnesium-chloride hexahydrate (MgCl2·6H2O) from Scharlau (Barcelona, Spain).

Animals

For this study C57Bl/6 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. We examined six groups of animals consisting of 6, 16 and 66 weeks old male and female mice (n = 9 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).

Sample preparation

At the age of 6, 16 and 66 weeks, the animals were deeply anesthetized with isoflurane (Forane®, Abott Laboratories Hungary Ltd., Budapest, Hungary). After thoracotomy, venous blood was collected from the right ventricle by intracardial puncture into Eppendorf tubes containing EDTA, followed by perfusion with artificial cerebrospinal fluid (pH 7.4, composition in mM: 122 NaCl, 3 KCl, 1 Na₂SO₄, 1.25 KH₂PO₄, 10 d-glucose*H₂O, 1 MgCl₂*6 H₂O, 2 CaCl₂*2 H₂O, 6 NaHCO₃) for 5 min by an automatic peristaltic pump. After centrifugation of blood samples at 4 °C for 5 min at 3500 RPM, the supernatant plasma (200 µL) were mixed immediately with 200 µL 85 mM ascorbic acid and 400 µL 1.14 mM BHT-EtOH solution and the samples were stored at −80 °C until further use. Before measurements, 600 µL n-hexane containing 1.14 mM BHT and rT, as internal standard (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 75 µL ACN and 50 µL EtOH–dioxane (1:1), then placed into amber-coloured HPLC 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 (UP100H, Hielscher Ultrasound Technology, Germany; amplitude: 100%, cycle: 0.5) in 1020 µL ice-cold solution (composition in mM: 75 Na₂HPO₄*2 H₂O, 17 NaH₂PO₄, 121 NaCl, 2 KCl, 1 EDTA, 67 ascorbic acid, 2 BHT and 0.2 v/v% Triton-X, 9.8 v/v% EtOH). The samples were centrifuged next at 4 °C for 10 min at 12,000 rpm, and the supernatants were collected and stabilized applying the same method as described in case of the plasma.

Chromatographic conditions

The concentrations of the β/γ-tocopherol and δ-tocopherol were under the limit of detection in cases of all measurements with all the applied detectors, only αT levels could be quantified, so we focus on this compound in the following part of the methodological description. For the quantification of αT and the IS (rT) from the mouse plasma samples, a previously published method of the authors was utilized [11], with the slight modification that the chromatographic separations were performed on a Kinetex C18 column, 150×4.6 mm i.d., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA).

Two independent methods, utilizing ECD and FLD, were developed for the quantification of αT from certain different brain regions, also with the application of rT as an IS. 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.

The first step of our ECD method development was the determination of the optimal working potential for αT and rT. As a result of our measurements (Fig. 1) the working potential was set at +700 mV, using a glassy carbon electrode and an Ag/AgCl reference electrode.

The mobile phase consisted of 91.25 v/v% MeOH, 4.25 v/v% distilled water, 4.50 v/v% IPA and 2.81 w/v% NaClO₄ and it was delivered at a rate of 1.2 ml/min at 25 °C onto the reversed-phase column (Luna C18, 75×4.6 mm, 3 µm particle size, Phenomenex Inc., Torrance, CA, USA) after passage through a pre-column (SecurityGuard, 4×3.0 mm i.d., Phenomenex Inc., Torrance, CA, USA). 10 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. [29]. 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.

![Fig. 1 Plotted peak areas vs. voltage diagram of αT and rT. αT α-tocopherol; rT rac-tocotol](image-url)
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. For ECD and FLD, 2 parallel measurements were carried out for each sample.

Validation of the applied methods

Calibration curve and linearity

In case of DAD measurements, calibrators were prepared in 1.14 mM BHT-EtOH, then they were arranged in six different concentration levels with concentration ranges of 1–50 µM and 0.25–12.5 µM for αT and rT, respectively. In case of ECD measurements, these ranges were the following: 0.05–2.0 µM and 0.30–6.0 µM for αT and rT, respectively, whereas in case of FLD measurements, the concentration levels were similarly between 0.10–2.0 µM and 0.30–6.0 µM for αT and rT, respectively. The peak area responses were plotted as a function of the corresponding concentration and linear regression computations were evaluated by the least square method with the freely available R software (R Development Core Team, https://r-project.org/). Good linearity (R² ≥ 0.99) was detected in each method throughout the concentration ranges for all compounds.

Selectivity

To analyse the selectivity of the methods, we matched the chromatograms of αT and rT for a blank plasma or central nervous system (CNS) sample and those for a spiked sample. As shown in Fig. 2, both compounds can be detected without interference from other compounds.

Precision

With regard to the within-run precision, the coefficients of variation of the concentrations were 0.73%, 1.49% and 3.46% for αT and 0.62%, 1.84% and 0.75% for rT in case of DAD, ECD and FLD, respectively.

Recovery

The relative recoveries were estimated by measuring spiked samples of αT at two different concentration levels with three replicates of each. No significant differences were observed for the lower and higher concentrations. The recoveries for the plasma samples ranged from 67 to 71% with DAD, whereas the recoveries for brain samples ranged from 104 to 106% in case of ECD and 87–104% with FLD.

Limit of detection (LOD) and limit of quantification (LOQ)

Regarding the quantitative analysis, both LOD and LOQ are important parameters, LOD showing the smallest concentration that can be detected, but not necessarily quantified, whereas LOQ is the lowest analyte concentration in
a sample that can be measured with an acceptable level of accuracy and precision [30]. In the current study, LOD and LOQ were calculated as shown in Eq. 1. The LOD values for αT were the following: 397 nM, 26 nM and 41 nM for DAD, ECD and FLD, respectively. The LOQ values for αT were 1202 nM, 77 nM and 123 nM for DAD, ECD and FLD, respectively.

\[
\text{LOD} = 3.3 \times \frac{\sigma}{S'} \quad \text{and} \quad \text{LOQ} = 10 \times \frac{\sigma}{S'}
\]

(1)

The calculation of LOD and LOQ values by formula, where \(\sigma\) is the standard error of the intercept and \(S'\) is the slope of the calibration curve of the analyte. LOD limit of detection; LOQ limit of quantification.

Statistical analysis

All statistical calculations were performed with the use of the freely available R software (R Development Core Team, https://r-project.org/). We first checked the distribution of data populations with the Shapiro–Wilk test, and we also performed the Levene test to confirm the homogeneity of variances. As the distribution proved to be Gaussian and the variances were equal, two-way ANOVA was applied with Tukey HSD post hoc test for pairwise comparison. We decided a priori that the comparisons of opposite gender and age groups may not yield meaningful information, so they were not implemented, therefore only 9 comparisons were applied between the six groups. We rejected the null hypothesis when the \(p\) values were < 0.05, and in such cases the differences were considered significant. If any significant change was observed, the effect size was calculated (omega-squared \((\omega^2)\) for two-way ANOVA and Cohen's \(d\) for Tukey HSD). Data were plotted as means (± SD).

Results

Plasma

The results of the measurements of αT concentration from plasma samples are presented in Tables 1 and 2, and in Fig. 3a. The applied two-way ANOVA demonstrated significant difference for age \((F = 10.547, \text{df} = 2, p < 0.001, \omega^2 = 0.0276)\) but no differences for gender \((F = 0.642, \text{df} = 1, p = 0.427)\) or age vs. gender \((F = 1.108, \text{df} = 2, p = 0.339)\). Post hoc analysis with Tukey HSD test yielded a significant increase in αT concentrations with aging only between 16 and 66 weeks old male mice \((p < 0.01; \text{Table 2})\), with a 2.234 effect size (see Supplementary Table 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>αT concentration levels in plasma and CNS of mice</th>
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<tr>
<td>Results of measurements</td>
<td>Plasma (µM)</td>
</tr>
<tr>
<td>6 w female</td>
<td>6.21 ± 2.53</td>
</tr>
<tr>
<td>16 w female</td>
<td>4.18 ± 1.04</td>
</tr>
<tr>
<td>66 w female</td>
<td>5.70 ± 1.56</td>
</tr>
<tr>
<td>6 w male</td>
<td>5.34 ± 1.01</td>
</tr>
<tr>
<td>16 w male</td>
<td>3.51 ± 1.27</td>
</tr>
<tr>
<td>66 w male</td>
<td>6.24 ± 1.18</td>
</tr>
</tbody>
</table>

Data are presented as mean (± SD); \(n = 9\); αT α-tocopherol; \(w\) week; \(ww\) wet weight

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Converted (p) values of Tukey HSD post hoc tests for the measurements of αT concentrations in plasma and brain samples</th>
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<tr>
<td>Compared groups</td>
<td>Plasma</td>
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<tr>
<td>6 vs. 16 w female</td>
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<tr>
<td>6 vs. 16 w male</td>
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<td>16 vs. 66 w female</td>
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<td>6 w female vs. male</td>
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<td>16 w female vs. male</td>
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<tr>
<td>66 w female vs. male</td>
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\(n = 9\); αT α-tocopherol; \(w\) week

\(*p < 0.05 ; **p < 0.01 ; ***p < 0.001\)
Brain regions

The measurements of αT concentration from brain samples are demonstrated in Tables 1 and 2 and Fig. 3b-f. 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 CNS sample. In the next step, the implementation of two-way ANOVA with Tukey HSD post hoc test yielded the following results.

In the striatum (Fig. 3b), there was a significant difference for age (F = 120.019, df = 2, p < 0.001, ω² = 0.0752) and gender (F = 23.062, df = 1, p < 0.001, ω² = 0.0070), and for age vs. gender (F = 3.588, df = 2, p < 0.05, ω² = 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 (Tables 1, 2).

In the cortex (Fig. 3c), there was also a significant difference for age (\( F = 159.589, \text{df} = 2, p < 0.001, \omega^2 = 0.1042 \)), gender (\( F = 17.377, \text{df} = 1, p < 0.001, \omega^2 = 0.0054 \)), and for age vs. gender (\( F = 5.465, \text{df} = 2, p < 0.01, \omega^2 = 0.0029 \)). The Tukey HSD post hoc test revealed significantly elevated \( \alpha T \) 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 (Tables 1, 2).

Furthermore, in the hippocampus (Fig. 3d), similar results were demonstrated for age (\( F = 195.500, \text{df} = 2, p < 0.001, \omega^2 = 0.1056 \)), gender (\( F = 24.343, \text{df} = 1, p < 0.001, \omega^2 = 0.0063 \)), and for age vs. gender (\( F = 7.045, \text{df} = 2, p < 0.01, \omega^2 = 0.0033 \)). The Tukey HSD post hoc test revealed significantly elevated \( \alpha T \) 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 (Tables 1, 2).

With regard to the cerebellum (Fig. 3e) and the brainstem (Fig. 3f), there was a significant difference for age (\( F = 17.091, \text{df} = 2, p < 0.001, \omega^2 = 0.0134 \) and \( F = 3.491, \text{df} = 2, p < 0.05, \omega^2 = 0.0021 \), respectively) and gender (\( F = 10.660, \text{df} = 1, p < 0.01, \omega^2 = 0.0040 \) and \( F = 13.295, \text{df} = 1, p < 0.001, \omega^2 = 0.0051 \), respectively), but not for age vs. gender (\( F = 2.897, \text{df} = 2, p = 0.065 \) and \( F = 0.820, \text{df} = 2, p = 0.446 \), respectively). The Tukey HSD post hoc test revealed significantly elevated \( \alpha T \) 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 (Tables 1, 2).

The corresponding effect size values for the significant differences in brain \( \alpha T \) concentrations are presented in Supplementary Table 1.

Discussion

Evidence suggests that tocopherols may have a special role in antioxidant protection in lipid-rich structures, such as the CNS. 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 \( \alpha T \), but the results are controversial [31–35]. These findings suggest that the pure exogenous administration of \( \alpha T \) is not always enough to improve functional outcome, there may be other aspects of brain \( \alpha T \) homeostasis, involving the regulation of endogenous \( \alpha T \) concentration in the CNS. However, only limited data are available on the changes of endogenous tocopherol levels either in human or murine brain samples with aging [11, 12, 16, 17, 24]. Therefore, there is a special need for the fine assessment of age-related changes in \( \alpha T \) 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.

Accordingly, the aim of the current study was to determine region-, age- and gender-specific changes in brain \( \alpha T \) 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 to judge the possible influence of peripheral changes on brain \( \alpha T \) levels. The results demonstrated that brain \( \alpha T \) 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 \( \alpha T \) 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. These findings are in line with those found by Takahashi et al. [24] and Gohil et al. [23], i.e., there is a clear elevation of cortical and hippocampal \( \alpha T \) levels, but only a moderate increase in cerebellar \( \alpha T \) level with aging and significantly higher values in females. The novelty of the current study is the presentation of such a pronounced elevation in striatal \( \alpha T \) level, while no change in brainstem \( \alpha T \) level, and furthermore, the first delineation that the difference between genders significantly increases with aging in case of the striatum, cortex and hippocampus. Similar to that found by Takahashi et al. [24], plasma \( \alpha T \) level seemingly does not influence the characteristic change in striatal, cortical and hippocampal \( \alpha T \) levels. In addition to the clear and unbiased demonstration of region-, age- and gender-specific changes in mouse brain \( \alpha T \) level via the application of different validated HPLC methods, this article also aims at highlighting the possible underlying factors behind these findings and their hypothetic significance as well.

For better understanding of these results, we looked further into the transport and metabolism of tocopherols. The
transport of vitamin E in the mammalian body is well documented [36, 37]. Due to the chemical properties of vitamin E compounds, their circulation is associated with lipoproteins, but their transcellular transport is mediated by carrier proteins [36].

In the brain the CSF has an important role in micronutrient distribution, including vitamins as well [37]. The transportation of vitamins B₁, B₃ and E from blood to CSF with simple diffusion cannot provide enough concentrations for tissue cells, therefore several specific systems (facilitated diffusion, active sodium-dependent or independent systems, receptor-related systems and many other mechanisms) are included as well [37]. Accordingly, it was proposed that brain αT homeostasis has a strict regulation, but the precise description of the pharmacokinetics of vitamin E transport into the CSF and brain is not available yet, although some possible mechanisms were previously described in the literature [37].

One of these mechanisms may be connected to the scavenger receptor class B type 1 (SR-B1) [38–40], which is responsible for αT transport across the blood–brain barrier (BBB) by promoting the uptake of high-density lipoprotein (HDL)-associated αT in brain capillary endothelial cells (BCEC). In the study of Srivastava et al. [39], C57Bl/6 mice were fed with high-cholesterol and high-fat containing diet and their brain SR-B1 expressions consequently increased, although hepatic SR-B1 expression was not influenced. Furthermore, Mardones et al. [40] demonstrated that SR-B1 knock-out (KO) female and male (n = 3–6, 2–4 months old) mice have significantly higher plasma and lower CNS αT levels, consistent with the postulated role of SR-B1 at the BBB. Surprisingly, the whole brain levels were not decreased enough to cause deficiency symptoms. There are no available data on the influence of aging and gender on SR-B1 expression.

The second possible mechanism may be connected to phospholipid transfer protein (PLTP) [41]. PLTP is highly expressed in the choroid plexus which raises the possibility that PLTP is involved in the transfer of αT from plasma into the CSF [42, 43]. In PLTP KO female mice (n = 6) Desrumaux et al. [42] investigated the brain concentration of αT and found it decreased by 30% compared to controls. Similar to SR-B1, there are also no available data on the influence of aging and gender on PLTP expression.

The αT transfer protein (α-TTP) serves as a third mechanism of αT transport in the mammalian brain [44]. The presence of this protein is demonstrated in rodent and human brains as well, although the expression level in normal brain tissue is low [45]. In α-TTP KO female and male mice (n = 2–2), the plasma and tissue concentrations were less than 0.46 µM and 0.23 nmol/g ww, respectively. Moreover, supplementation with oral vitamin E raised the plasma concentration of these mice close to normal (~3.4 µM), but the brain levels increased only with ~20%, however, this small increase in the brain could eliminate the neurological signs [44]. Gohil et al. [23] also investigated α-TTP expression in several CNS and liver tissue samples of 5 months old α-TTP KO and control mice with the finding that α-TTP protein or its mRNA could only be detected from the liver of controls. Later, Takahashi et al. [24] successfully determined α-TTP expressions from the cerebrum, hippocampus and cerebellum. However, they utilized only 12-month-old male mice for that purpose, therefore the effect of aging and gender could not be analysed.

Another investigated pathway is in relation with afamin, a member of the albumin super family and may serve as a transport protein involved in αT homeostasis, as well [46]. Afamin is synthesized endogenously by BCEC and assists αT transport to astrocytoma cells, but to a lesser extent than HDL-mediated transport. The expression of afamin was detected by immunohistochemistry in porcine, mouse and post mortem human brains. It was also demonstrated that afamin level correlates with the concentration of αT in the CSF, but not with αT level in the serum [46, 47]. However, there are no available data on the influence of aging and gender on afamin expression.

Although the above-mentioned findings may support the role of SR-B1, PLTP, α-TTP and afamin in the transport and regulation of the αT levels in the CNS, the assessment of the effect of aging and gender on these transport processes warrants further studies. Probably the major significance of the current findings is that the elevations of αT concentrations by aging may serve as to compensate the burden of oxidative stress which increases with aging [11, 34]. Furthermore, the demonstrated gender-related differences, i.e., significantly higher αT levels in female striatal, cortical and hippocampal brain regions, may at least partially explain the decreased vulnerability of e.g., C57Bl/6 female mice to certain neurotoxins, e.g., 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [48–51] compared to the male ones.

In light of the fact that only limited and contradictory data are available on the changes of human brain tocopherol levels with aging, further studies are needed to find the human correspondence of the current findings. However, the results may draw attention to the importance of antioxidant protection against oxidative damage, the extent of which clearly increases with aging. With regard to the effect of gender on age-related pathological alterations, several human studies were published [52–55]. Király et al. [55] demonstrated that male brain ages faster, following the assessment of the volume of cortical and subcortical grey matter, including that of the hippocampus, putamen and caudate nucleus, within the age-range of 21–58 years. Although the age range is presumably different, this male preponderance was not reassured in the most common neurodegenerative disorder, AD [56], but
was proposed in the second most common neurodegenerative disorder, PD [57]. Nevertheless, the fact that there is not enough human data to be able to determine the extent of the influence of the alterations in tocopherol homeostasis on the pathogenetic process in major age-related neurodegenerative disorders, warrants further studies.

In conclusion the current study is the first to demonstrate age-dependent gender-specific changes in αT level in certain brain regions of the C57Bl/6 mouse strain, which finding may explain the increased vulnerability of male C57Bl/6 mice to certain neurotoxins targeting these regions, but its verification needs further studies. 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 pathogenic processes, mainly focusing at the restoration of altered brain αT homeostasis.

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Compliance with ethical standards
Conflict of interest The authors declare no conflict of interest.

References


II.
The assessment of possible gender-related effect of endogenous striatal alpha-tocopherol level on MPTP neurotoxicity in mice

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ABSTRACT

Several studies supported an increased vulnerability of males regarding Parkinson's disease (PD) and its animal models, the background of which has not been exactly revealed, yet. In addition to hormonal differences, another possible factor behind that may be a female-predominant increase in endogenous striatal alpha-tocopherol (αT) level with aging, even significant at 16 weeks of age, previously demonstrated by the authors. Accordingly, the aim of the current study was the assessment whether this difference in striatal αT concentration may contribute to the above-mentioned distinct vulnerability of genders to nigrostriatal injury.

Female and male C57Bl/6 mice at the age of 16 weeks were injected with 12 mg/kg body weight 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) 5 times at 2 h intervals or with saline. The levels of some biogenic amines (striatum) and αT (striatum and plasma) were determined by validated high performance liquid chromatography methods.

Although the results proved previous findings, i.e., striatal dopamine decrease was less pronounced in females following MPTP treatment, and striatal αT level was significantly higher in female mice, the correlation between these 2 variables was not significant. Surprisingly, MPTP treatment did not affect striatal αT concentrations, but significantly decreased plasma αT levels without differences between genders.

The current study, examining the possible role of elevated αT in female C57Bl/6 mice behind their decreased sensitivity to MPTP intoxication for the first time, was unable to demonstrate any remarkable connection between these 2 variables. These findings may further confirm that αT does not play a major role against neurotoxicity induced by MPTP.

1. Introduction

Although neurodegenerative disorders, including Parkinson's disease (PD), may differ in clinical and pathological characteristics, their pathomechanisms may involve some common features, such as glutamate excitotoxicity, mitochondrial dysfunction and reduced antioxidant capacity [1]. Regarding the antioxidant aspects, strong evidence suggest that α- and γ-tocopherols have important role in antioxidant protection in the central nervous system (CNS) due to its lipid-rich structure [2, 3, 4, 5]. Numerous studies assessed age- and gender-related differences in α-tocopherol (αT) levels in serum/plasma samples of healthy individuals [6, 7] and furthermore, the influencing effect of dietary tocopherol intake on PD-related parameters, including those studies that focused on the achievement of neuroprotection via the administration of exogenous αT [1, 3, 8, 9, 10, 11, 12, 13, 14, 15, 16]. However, the results of studies using exogenous tocopherol supplementation are controversial; some demonstrated that vitamin E intake may be beneficial regarding disease evolution [12, 14], whereas others found no effect on it [3, 9, 10, 13]. Long-term αT and ascorbate treatment effectively delayed the need for the use of levodopa (L-DOPA) by an average of 2.5 years [17] when they were applied in combination at considerably high doses (3200 IU αT and 3000 mg ascorbic acid per day) compared to daily 2000 IU αT in the DATATOP trial which could not demonstrate any beneficial effect of daily vitamin E supplementation on delaying the onset of disability in PD [9]. The possible explanations for these findings may be that the ability of dietary αT to enrich cellular membranes, especially in the CNS, is limited and needs the administration of considerably high doses for a long time [18]. Furthermore, the form of the administered αT may count as well.

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Accordingly, the achievement of the enrichment of mitochondria with protective levels of αT in the striatum and substantia nigra is quite challenging.

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 [19, 20]. The active metabolite of this toxin, 1-methyl-4-phenylpyridinium ion (MPP+), is capable of selectively damaging dopaminergic neurons of the substantia nigra pars compacta resulting in a decrease of striatal dopamine (DA) level characteristic of PD [21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36]. The C57BL/6 mice serve as one of the most sensitive mouse strains regarding MPTP toxicity [37, 38]. 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 [24, 25, 26, 27, 28, 29, 32, 36, 39, 40, 41, 42, 43, 44]. Although the obtained results are controversial, the majority of studies demonstrated increased sensitivity in males, especially regarding nigrostriatal injury [28, 32, 36, 39, 41, 42, 43, 44]. The reason behind this phenomenon has not been exactly revealed, yet.

In line with the human data demonstrated above, 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 [21, 22, 34, 45, 46, 47, 48, 49, 50, 51]. In summary, only the administration of considerably high doses of αT provided neuroprotective effects only in a portion of studies [45, 46, 48, 50, 51].

In addition to exogenous αT supplementation, another strategy may be the achievement of neuroprotection via the manipulation of endogenous αT homeostasis. The dietary or genetic depletion of brain αT levels yielded conflicting results as well [49, 50]. MPTP intoxication following prolonged dietary vitamin E depletion resulted in increased susceptibility to damage in the substantia nigra, but not in the striatum [49, 52]. On the contrary, genetic vitamin E deficiency (utilizing αT transfer protein (αT-TTP) knockout mice) did not influence the striatal DA depletion following MPTP treatment, whereas the number of tyrosine hydroxylase positive neurons of the substantia nigra was not altered at all [50]. These conflicting findings may be partially explained by the differences in the MPTP treatment regimen as well. Our previous study demonstrated that a female-predominant increase in endogenous striatal αT level evolves with aging, providing significant differences between genders already at 16 weeks of age [2].

In light of the available literature data, the aim of the current study was to further confirm the decreased sensitivity of female C57BL/6 mice to MPTP neurotoxicity and to assess whether this difference is related to elevated endogenous striatal αT content.

2. Materials and methods

2.1. Materials

The reagents for αT high performance liquid chromatography (HPLC) measurement has already been reported [2], and besides those, we used the following chemicals in this study: disodium-ethylenediaminetetraacetate dihydrate (Na₂EDTA·2H₂O; Lach-Ner s.r.o, Neratovice, Czech Republic), following chemicals in this study: disodium-ethylenediaminetetraacetate [21, 22, 34, 45, 46, 47, 48, 49, 50, 51]. In summary, only the administration of considerably high doses of αT provided neuroprotective effects only in a portion of studies [45, 46, 48, 50, 51].

In addition to exogenous αT supplementation, another strategy may be the achievement of neuroprotection via the manipulation of endogenous αT homeostasis. The dietary or genetic depletion of brain αT levels yielded conflicting results as well [49, 50]. MPTP intoxication following prolonged dietary vitamin E depletion resulted in increased susceptibility to damage in the substantia nigra, but not in the striatum [49, 52]. On the contrary, genetic vitamin E deficiency (utilizing αT transfer protein (αT-TTP) knockout mice) did not influence the striatal DA depletion following MPTP treatment, whereas the number of tyrosine hydroxylase positive neurons of the substantia nigra was not altered at all [50]. These conflicting findings may be partially explained by the differences in the MPTP treatment regimen as well. Our previous study demonstrated that a female-predominant increase in endogenous striatal αT level evolves with aging, providing significant differences between genders already at 16 weeks of age [2].

In light of the available literature data, the aim of the current study was to further confirm the decreased sensitivity of female C57BL/6 mice to MPTP neurotoxicity and to assess whether this difference is related to elevated endogenous striatal αT content.

2.2. Animals

For this study, we utilized C57BL/6 mice, housed under standard laboratory conditions with free access to food and water. We examined four groups of animals 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.). The required sample size per groups was determined by power analysis (GPower software) with a result of n = 13 per group. Considering the fact that MPTP treatment may result in death in some proportion of animals [30], we decided to increase the sample size to 15 in each group considering the possible mortality rate.

2.3. Treatment and sample handling

MPTP hydrochloride was freshly dissolved in saline (pH adjusted to 7.4 with 0.1 M NaOH) and was administered intraperitoneally (i.p.). Male and female mice were randomly divided into 2x2 groups. 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 behavior.

One week following the last i.p. injection, all the animals were deeply anesthetized with isoflurane (Forane®; Abbott Laboratories Hungary Ltd., Budapest, Hungary). Sample collection and preparation was similar as described previously [2]. Briefly, plasma and halved striatal samples were collected for the determination of αT and catecholamine concentrations.

Before DA, DOPAC and HVA measurements, the halved striatal samples were weighed and sonicated in ice-cold solution (60 μL/mg striatum) containing 400 μM Na₂S₂O₅, 500 μM Na₂EDTA·2H₂O, and ISs (50 ng/mL DHBA and 200 ng/mL IPR in 334 mM HClO₄). The samples were centrifuged at 4 °C for 30 min at 12000 RPM, and after the supernatants were collected, 10 μL was injected into the HPLC.

2.4. Chromatographic conditions

For the quantification of αT and the IS (rac-tocil) a previously published method was used for both mouse plasma samples (applying diode-array detector (DAD)) [2, 7] and mouse brain samples (fluorescence detector (FLD)) [2]. For the analysis Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) was used under isocratic conditions.

However, for DA, DOPAC and HVA measurements, our previously applied method [35] was modified. Regarding this improved method, the validation process was carried out again on striatal samples. The developed method is applicable for the simultaneous determination of L-DOPA, norepinephrine, 3-methoxytyramine, 5-hydroxyindoleacetic acid, 5-hydroxytryptamine and another IS, 5-hydroxy-N-octyltryptamine as well, but this opportunity was not utilized in the current study.

The mobile phase consisted of 2.20 mM Na₂S, 75 mM Na₂HPO₄, 100 μM Na₂EDTA·2H₂O and 6.25 v/v% ACN. The pH value was set to 3.0 with 85 w/w% H₃PO₄. The mobile phase was delivered at a rate of 1.5 ml/min at 40 °C onto the reversed phase column (Zorbax Eclipse Plus C18, 100 × 4.6 mm i.d., 3.5 μm particle size, Agilent Technologies, Santa Clara, CA, USA) after passage through a precolumn (SecurityGuard, 4 × 3.0 mm i.d., Phenomenex Inc., Torrance, CA, USA). Aliquots were injected with the cooling module set to 4 °C and the working potential to +750 mV, using a glassy carbon electrode and an Ag/AgCl reference electrode.
2.5. Validation of the applied methods

All validation processes were carried out with the guidance of ICH and FDA [53, 54, 55]. The following parameters were determined: linearity ranges, limit of detection, limit of quantification, recoveries at three concentration levels, and intra- and interday precisions.

Calibrators were prepared in acidic solution, containing ascorbic acid and BHT, due to the stability issues of some compounds [35], then they were arranged in five different concentration levels. The peak area response ratios were plotted as a function of the corresponding concentration and linear regression computations were evaluated by the least square method with the freely available R software (R Development Core Team, https://www.r-project.org/). Good linearity ($R^2 \geq 0.999$) was detected throughout the concentration ranges for all compounds.

2.6. Statistical analysis

All statistical calculations were performed with the use of above-mentioned R software. First, we checked the distribution of data populations with the Shapiro-Wilk test and we also performed the Levene test to confirm the homogeneity of variances. As the distribution proved to be Gaussian and the variances were equal, two-way ANOVA was applied with Tukey HSD post hoc test for pairwise comparisons. We decided a priori that the comparisons of control and treatment groups with opposite gender may not yield meaningful information, and accordingly, only four comparisons were implemented regarding the four groups. In case of model construction, ANCOVA was applied. We rejected the null hypothesis when the $p$-values were $<0.05$, and in such cases the differences were considered significant. If any significant change was observed, the effect size was calculated (omega square ($\omega^2$) for two-way ANOVA, partial eta square (p. $\eta^2$) for ANCOVA and Cohen's d for Tukey HSD). Pearson correlation analysis with post hoc Bonferroni correction for the number of analyses was used to investigate the possible relationship between endogenous striatal oT content and DA level in each group. Data were plotted as means (±S.D.). The measured values were presented in ng/mg wet weight (ww) and nmol/g ww regarding catecholamines and oT, respectively, to allow comparison with previous results of the authors [2, 35].

3. Results

3.1. HPLC measurement of DA, DOPAC and HVA

The measurements of DA, DOPAC and HVA concentration from striatal samples are presented in Table 1 and Figure 1a. The implementation of two-way ANOVA with Tukey HSD post hoc test yielded the following results.

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 ($F (1, 52) = 3.627, p = 0.062, \omega^2 = 0.0033$). Post hoc analysis with Tukey HSD test yielded significantly decreased DA concentrations in MPTP-treated vs. control females ($p < 0.001$, Figure 1a), with an effect size of -2.600, in MPTP-treated vs. control males ($p < 0.05$, Figure 1a) with an effect size of -5.925, and in MPTP-treated males vs. females ($p < 0.05$, Figure 1a) as well with an effect size of -1.303.

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 ($F (1, 52) = 12.481, p < 0.001, \omega^2 = 0.0414$), but not for gender ($F (1, 52) = 2.373, p = 0.129, \omega^2 = 0.0022$). Post hoc analysis with Tukey HSD test yielded significantly decreased DOPAC concentrations in MPTP-treated vs. control females ($p < 0.001$), with an effect size of -2.309, in MPTP-treated vs. control males ($p < 0.001$) with an effect size of -4.094 and in MPTP-treated males vs. females ($p < 0.01$) with an effect size of -1.491.

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 ($F (1, 52) = 0.464, p = 0.499, \omega^2 = -0.0008$). Post hoc analysis with Tukey HSD test yielded significantly decreased HVA concentrations in MPTP-treated vs. control females ($p < 0.01$), with an effect size of -1.629, and in MPTP-treated vs. control males ($p < 0.001$) with an effect size of -2.464.

A 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 ($F (1, 52) = 1.842, p = 0.181, \omega^2 = 0.0019$) and treatment vs. gender ($F (1, 52) = 0.779, p = 0.382, \omega^2 = -0.0004$). The Tukey HSD post hoc test yielded significantly increased DA turnover in MPTP-treated vs. control females ($p < 0.05$; Figure 1b) with an effect size of 1.335, and in MPTP-treated vs. control males ($p < 0.001$; Figure 1b) with an effect size of 1.438.

3.2. HPLC measurement of oT

The results of the measurements of oT concentration from plasma and striatal samples are presented in Table 2, Figure 1c and d. The applied two-way ANOVA demonstrated significant difference in oT level of plasma regarding treatment ($F (1, 52) = 18.227, p < 0.001, \omega^2 = 0.0396$), but not for gender ($F (1, 52) = 0.006, p = 0.938, \omega^2 = -0.0023$) and gender vs. treatment ($F (1, 52) = 0.115, p = 0.736, \omega^2 = -0.0020$). Post hoc analysis with Tukey HSD test yielded significantly decreased oT concentrations in MPTP-treated vs. control females ($p < 0.05$, Figure 1d), with an effect size of -1.958, and in MPTP-treated vs. control males ($p < 0.05$, Figure 1d) with an effect size of -0.829.

Regarding the striatum, there was a significant difference for gender ($F (1, 52) = 29.680, p < 0.001, \omega^2 = 0.0555$), but not for treatment ($F (1, 52) = 2.543, p = 0.117, \omega^2 = 0.0044$) and for treatment vs. gender ($F (1, 52) = 0.029, p = 0.865, \omega^2 = -0.0002$). The Tukey HSD post hoc test revealed significantly higher oT concentrations in control female vs. male mice ($p < 0.01$, Figure 1c), and in MPTP-treated female vs. male mice as well ($p < 0.01$, Figure 1c) with effect sizes of 1.811 and 1.261, respectively.

The results of the assessment of the relationship between DA and oT levels in the striatum are presented in the Supplementary Material.

| Table 1. DA, DOPAC and HVA concentration levels (ng/mg ww) and DA turnover in the striatum of mice. |
|---|---|---|---|---|
| **Analytes or ratio** | **Control females** | **Control males** | **MPTP females** | **MPTP males** |
| **DA** | 10.255 ± 2.451 | 10.692 ± 1.983 | 3.995 ± 2.364 | 1.651 ± 0.830 |
| **DOPAC** | 0.989 ± 0.170 | 1.097 ± 0.273 | 0.496 ± 0.248 | 0.195 ± 0.135 |
| **HVA** | 1.781 ± 0.453 | 1.938 ± 0.696 | 1.039 ± 0.458 | 0.604 ± 0.388 |
| **(DOPAC + HVA)/DA** | 0.276 ± 0.048 | 0.295 ± 0.062 | 0.440 ± 0.167 | 0.527 ± 0.227 |

Data are presented as mean (±S.D.); \(n = 14; n = 15; n = 13; DA\) dopamine; **DOPAC** 3,4-dihydroxyphenylacetic acid; **HVA** homovanillic acid; **MPTP** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ww wet weight.
Figure 1. Measured analyte concentrations in mouse striatal and plasma samples. DA levels (a), DA turnover (b), αT levels in the striatum (c) and in the plasma (d) are presented for the demonstration of the effect of MPTP treatment. Data are presented as mean (±S.D.); n (control and MPTP-treated females) = 14; n (control males) = 15; n (MPTP-treated males) = 13; αT α-tocopherol; DA dopamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; n.s. not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

4. Discussion

PD is the second most common neurodegenerative disorder with an increasing prevalence in the aging population and in males [56]. 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 [28, 32, 36, 39, 41, 42, 43, 44]. Although the possible role of sexual hormones behind these findings was proposed by several studies [39, 41, 57], the exact explanation behind gender differences is still missing.

Amongst strategies of ameliorating disease progression, a popular approach may be the reduction of oxidative injury characteristic of PD [1]. Probably the most exhaustive trial to achieve this aim via the administration of αT was the DATATOP study, but the results did not support any neuroprotective effect [9]. Although literature data, coming from preclinical and human studies, are controversial regarding this topic, it can be proposed that the prolonged application of high dose αT initiated in early phases may have beneficial effects on the neurodegenerative processes [18].

The authors demonstrated in one of their previous studies that a significant rise of striatal αT evolved with aging, more pronounced in female mice and already significant at 16 weeks of age [2]. These phenomena may be explained by that the aging brain tries to increase its antioxidant capacity, predominantly in females, which may provide an enhanced protection against neurodegeneration. 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 the current 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. The results 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 [28, 39, 41, 42, 43, 44]. It was also assessed whether these findings may be related to differences in DA turnover. Although DA turnover significantly increased in MPTP-treated mice compared to controls, which also corresponds to the results of other studies [58, 59], no difference between genders could be demonstrated. Regarding striatal αT levels, the findings of the current

Table 2. αT concentration levels in the plasma and the striatum of mice.

<table>
<thead>
<tr>
<th></th>
<th>Plasma (μM)</th>
<th>Striatum (nmol/g ww)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control females¹</td>
<td>5.14 ± 1.32</td>
<td>22.20 ± 1.61</td>
</tr>
<tr>
<td>Control males²</td>
<td>5.03 ± 2.50</td>
<td>20.06 ± 1.83</td>
</tr>
<tr>
<td>MPTP females¹</td>
<td>3.11 ± 0.63</td>
<td>22.23 ± 2.24</td>
</tr>
<tr>
<td>MPTP males²</td>
<td>3.30 ± 1.45</td>
<td>18.90 ± 3.02</td>
</tr>
</tbody>
</table>

Data are presented as mean (±S.D.); ¹n = 14; ²n = 15; ³n = 13; αT α-tocopherol; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; ww wet weight.
study confirmed our previous results [2], i.e., the concentrations were significantly higher in the striata of females compared to males already at 16 weeks of age. Surprisingly, but in line with previous findings [49], 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 the 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 (Supplementary Material), 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. Nevertheless, the finding itself that endogenous striatal αT content does not seem to be a major player against MPTP-induced deteriorations may be supported by the data obtained from studies with α-TTP deficient mice or with the application of dietary restriction [49, 50]. Even α-TTP−/− mice with essentially undetectable level of brain αT were not more prone to MPTP-related striatal DA decrease compared to wild-type controls [50].

Limitations of the current study include the lack of its extension for the assessment of the relationships between striatal DA and αT levels in further age groups. However, the authors presume that although both the sensitivity to MPTP treatment [41, 42, 44] and the striatal level of αT [2] increase with aging with the enlargement or at least the persistence of the above-mentioned differences between genders, their relationship does not likely change. Accordingly, keeping in mind the 3R principle (replacement, reduction and refinement) of animal experiments as well, this extension was out of scope of the present work.

In conclusion, the current study was the first to examine the possible role of elevated αT in female C57BI/6 mice behind their decreased sensitivity to MPTP intoxication. The results, i.e., no significant correlation was found between the above two parameters, 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.

Declarations

Author contribution statement

Nikolett Nánási, Edina K Cseh, Diána Martos, Levente Hadady: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Gabor Veres: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Péter Klívényi, László Vécsei: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dénes Zádorí: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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References

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The assessment of possible gender-related effect of endogenous striatal alpha-tocopherol level on MPTP neurotoxicity in mice

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Study of possible relationships between DA and αT levels in the striatum

Regarding the assessment whether endogenous α-tocopherol (αT) content could affect the change in dopamine (DA) levels following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment, data were analyzed 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 \( \eta^2 \) (p. \( \eta^2 \)) = 0.761), but striatal αT level did not have a significant influence on either striatal DA level (F (1, 52) = 0.487, \( p = 0.488, \) p. \( \eta^2 = 0.007 \)) or on its decrease following MPTP treatment (assessment of interaction; F (1, 52) = 1.879, \( p = 0.176, \) p. \( \eta^2 = 0.035 \)). 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 R²s 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; Supplementary Fig. 1).
Supplementary Fig. 1. Possible correlations between endogenous $\alpha$T concentrations and DA levels in the striatum. Following Bonferroni correction, no statistically significant correlations were demonstrated. $n$ (control and MPTP treated females) = 14; $n$ (control males) = 15; $n$ (MPTP treated males) = 13; $\alpha$T $\alpha$-tocopherol; DA dopamine; MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
III.
PROCEEDINGS OF THE

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E-VITAMIN MÉRÉSRE ALKALMAS NAGYHATÉKONYSÁGÚ FOLYADÉKKROMATOGRÁFIÁS MÓDSZEREK FEJLESZTÉSE ÉS VALIDÁLÁSA

DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODS FOR VITAMIN E MEASUREMENTS

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Abstract

Tocopherols, also known as the ingredients of fat-soluble vitamin E compounds play an important role in antioxidant mechanisms. Their deficiency of tocopherols, mainly that of α-tocopherol, can cause several neurological disorders, such as ataxia, myopathy, retinopathy, peripheral neuropathy. The aim of this study was to validate and establish methods applicable for the detection of α-tocopherol from murine serum and certain brain regions.

In the present study we report 3 different reversed-phase HPLC methods using a single C18 column in isocratic system. The detection was carried out with 3 different detectors. For the detection of murine serum samples, a diode-array detector (DAD) was applied, whereas for murine brain samples electrochemical (ECD) and fluorescence detectors (FLD) were utilized and a detailed validation process was carried out.

The ranges of precision and recovery were the following during the validation processes: 0.61-3.57% and 66.19-105.91%, respectively.

The application of these methods provide a valuable tool for the determination of α-tocopherol from murine serum and brain samples.

Bevezetés

Az E-vitamin csoportjába tartozó 8 vegyület (α-, β-, γ- és δ-tokoferolok, valamint a tokotrienolok) kiemelkedő szereppel bír az oxidatív stressz ellen, közülük is leginkább az aktívakabb és magasabb koncentrációban megtalálható α-tokoferol, mely különös tekintettel az alábbi területeken fejti ki jótékony hatását: keringési- és idegrendszer [1]. Számos neurológiai elváltozás kialakulásában, lefolyásában az antioxidáns rendszer sérült működése állhat. Többek között az ataxia izolált E-vitamin hiány (ataxia with vitamin E deficiency, AVED), mely esetén az α-tokoferol transzporter fehérje (α-tocopherol transport protein, α-TTP) nem expresszálódik a majsejtekben [2], így nincs, ami biztosítja a szervezet számára a megfelelő antioxidáns védelmet.

Célunk egy olyan robosztus, reprodukálható metodika fejlesztése, validálása majd alkalmazása, amely gyors és megbízható α-tokoferol koncentráció méréseket eredményez, úgy az egér szerve, mint az agyi szövetekből is. Egyesek az 1980-as évekre nyúlik vissza az ehhez hasonló kutatások gyökerei [3], azóta több módszert is alkalmaznak találtak E-vitamin és metabolitjainak meghatározására, ám csak csakély számúban talált hiteles adatokat validálás hiányában [4-7]. Nem találtunk olyan közleményt, mely ilyen meghatározást több detektor és metodika összehasonlításával értékelne.
Kísérleti rész

Tanulmányunk során C57BL/6 egerek szérum és egyes agyi régióinak (striatum, cortex, hippocampus, cerebellum és agytörz) α-tokoferol-szintjét határozottuk meg HPLC (Agilent 1100 és 1260 Series) segítségével. Az állatokból nyert mintákat metodikai módszereink segítségével előkészítettük. Az állatok (n = 6) elaltatását követően a bal kamrából vért nyertünk, melyhez meghatározott mennyiségben 50 mg/ml etilén-diamin-tetraacetsav-oldatot (EDTA, Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok) adtunk, 4°C-on tárolás és 30 percnyi alvadás után 3500 fordulat/perc sebesség mellett 5 percig centrífugáltuk a vérmintákat 4°C-on (Hettich Mikro 220, Tuttlingen, Németország). A felfűlűsöket egy 2,2 ml-es Eppendorf csőbe gyűjtöttük össze, majd vortex segítségével 20 másodpercig homogenizáltuk. A homogenizált szérumból 200 μl-nyi mennyiséget azonnal stabilizáltunk 200 μl 85 mM aszkorbinsav-oldattal (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok) és 400 μl 1,14 mM butil-hidroxi-toluol (BHT, Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok) -etanol (VWR International, Radnar, PA, Amerikai Egyesült Államok) oldattal. Az így kapott ~ 800 μl stabilizált és homogenizált szérum mintákat ezt követően -80°C-on tároltuk felhasználásig.

Jelen egerek vérmentes agyi régióit is felhasználtuk, melyeket perfuzionálás utáni kaptunk, melyet perisztaltikus pumpa segítségével a bal kamrába mesterséges agyfolyadékot áramoltattunk 3 percig. Az agyszöveteket jégen választottuk szét, majd 1,5 ml-es Eppendorf csövekbe helyeztük a régiókat és felhasználásig -80°C-on tároltuk őket. A szövetek feldolgozása a következő lépésekkel állt. A fagyott agyi régiókat külön-külön lemértük analitikai mérlegen, majd 1020 μl homogenizáló oldat hozzáadása után UH100 homogenizátorral (amplitúdó 100%, ciklus: 0,5) agytömegtő függően 1-2 perc alatt szétronsoltuk a mintákat. A homogenizált agyi mintákat 10 percig 4°C-on tároltuk, majd 12000 fordulat/perc sebesség mellett 10 percen keresztül ülepítettük őket 4°C-on. A további mintaelőkészítés megegyezett a szérumnál tárgyalatokkal.

Három detektációs módszert alkalmaztunk α-tokoferol meghatározására. A vérből nyert szérumot DAD, az agyi régiókat ECD és FLD detektálás mellett mértük.

Mérés előtt felolvasztott, 4°C-os mintákhoz, mind szérum, mind agyi minta esetén 600 μL 1,13 mM koncentrációban BHT-t és rac-tocolt (Matreya LLC., Pleasant Gap, PA, Amerikai Egyesült Államok) tartalmazó hexán (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok) oldatot adtunk. Pontosan 1 percen keresztül kevertük vortex segítségével az oldatokat. Ezután a hexáns és etanoles fázist elválasztandó 12000 fordulat/perc sebességgel 10 percig centrífugáltuk 4°C-on. A centrífugálást követően a felső, hexáns fázisból 450 μL-t beállítottunk N2 gázzal hozzávetőlegesen 15 perc alatt. Ezután a visszamaradott komponenseket 28,57 V/V% etanol (VWR International, Radnar, PA, Amerikai Egyesült Államok), 28,57 V/V% 1,4-dioxán (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok), 42,86 V/V% acetonitril (ACN, VWR International, Radnar, PA, Amerikai Egyesült Államok) elegyben oldottuk és 10 másodpercig vortexeszel homogenizáltuk, így injekciálásra és elválasztásra kész állapotra hozva a mintákat.

MO, Amerikai Egyesült Államok), 2,72 V/V% ammonium-acétát (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok) 1 vegyes szárazékos oldata, 2,72 V/V% desztillált víz (injekcióhoz való víz). A mobilfázist minden mérés előtt frissen készítettük és az elkészítését követően kerámiaszürűn 0,45 μm-es porúsméretű hidrofób PVDF membránzsürűn vízszugárszavattyú segítségével szőrtük. A mobilfázis áramlása sebessége 2,1 mL/perc volt. Minden mintából 50 μL tétrogatot injektáltunk a mérések során.

Agyminták esetén Agilent 1100 rendszeren Luna C18(2) oszlopot (75x4,6 mm; 3 μm, Phenomenex Inc., Torrance, CA, Amerikai Egyesült Államok) 25°C-os hőmérsékleten termosztálva. A kolonna védelme érdekében szintén előtét kolonnát használtunk: Security Guard C18 (4x3 mm, Phenomenex Inc., Torrance, CA, Amerikai Egyesült Államok). A mobilfázis összetétele ECD méréshez: 91,25 V/V% metanol (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok), 4,25 V/V% desztillált víz, 4,50 V/V% izopropil-alkohol (VWR International, Radnor, PA, Amerikai Egyesült Államok), 0,29% vegyes% nátrium-perklorát (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok). Hasonlóan, mint DAD méréseknél, a mobilfázist minden mérés előtt frissen készítettük és az elkészítését követően kerámiaszürűn 0,45 μm-es porúsméretű hidrofób PVDF membránzsürűn vízszugárszavattyú segítségével szőrtük. A mobilfázis áramlása sebessége 1,2 mL/perc volt. Minden mintából 10 μL tétrogatot injektáltunk a mérések során minttermosztálás mellett (4°C). A mobilfázis összetétele FLD mérés esetén az alábbi volt: 100 V/V% metanol (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok). A mobilfázis áramlása sebessége 1,8 mL/perc volt. Minden mintából 10 μL tétrogatot injektáltunk a mérések során minttermosztálás mellett (4°C)

ECO esetén a beállított munkapotenciál (előzetes feszültség vs. áramerősség görbék felvételével) +700 mV volt, FLD esetén a gerjesztési hullámhossz 292 nm, a mériés hullámhossz pedig 330 nm volt [8]. DAD esetén az α-tokoferol (Sigma-Aldrich, Saint Louis, MO, Amerikai Egyesült Államok) 292 nm-en, míg a belső standardot (rac-tokol, Matreya LLC., Pleasant Gap, PA, Amerikai Egyesült Államok) 297 nm-en regisztráltuk. A metodikákat az ICH által megfogalmazott paraméterek szerint validáltuk, így meghatározuk a metodikák pontosságát, érzékenységét, szelektivitását, lineáritását és visszanyerhetőségét.

**Eredmények**

A validálás során a 1. **Táblázatban** látható eredményeket kaptuk, ahol a variációs együtthatók (coefficient of variance, CV%) értékek láthatók, a mérési variancia és a mérés átlagának hányadosa szárazékos értékbén kifejezve. A visszamérési ténység (recovery factor, RF) kiszámítására 2 olyan oldatot készítünk, mely a natív mintához képest ismert koncentrációban hozzáadott analitot tartalmaz. Az S1 oldat alacsony, míg az S2 oldat magasabb koncentrációban tartalmazza az analitotokat. A mért koncentráció és az ismert koncentráció hanyadosa a RF értékekkel szolgál, szárazékos kifejezve. A lineáritás megállapításához minden esetben 6 tagú kalibráló oldatsorozatot készítettünk. Az integrált csúcsl alatti területeket a kalibráló oldat ismert koncentrációinak függvényében ábrázolva, és alkalmazva a legkisebb négyzetek módszerét, minden analitra igen jó lineáritást mutattunk ki (R² ≥ 0,99, kiértékelő szoftver: R Development Core Team, 2002). A metodikák szelektivitását az 1. **ábra** mutatja be, ahol egy natív és egy S2 mintát mutatunk be. A meghatározás határ (limit of quantification, LOQ) azt a legkisebb koncentrációt jelenti, mely még megfelelő precitizálással és helyességgel meghatározható, míg a kimutatási határ (limit of detection, LOD) az alapjaz háromszorosának megfelelő magasságú jelet adó koncentráció, illetve a következő képletekek határoztuk meg (σ: a vakminta szórása, S': érzékenység).

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LOD = 3.3 \sigma / S^*, illetve LOQ = 10 \sigma / S^*

1. egyenlet. LOD és LOQ számítására alkalmas képletek.

3. Táblázat. HPLC metodikák validálási eredményei.

<table>
<thead>
<tr>
<th>Komponens</th>
<th>α-tokoferol</th>
<th>rac-tokol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metodika</td>
<td>DAD</td>
<td>ECD</td>
</tr>
<tr>
<td>Precizitás</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terület</td>
<td>0,73%</td>
<td>1,46%</td>
</tr>
<tr>
<td>c (\mu M)</td>
<td>0,73%</td>
<td>1,49%</td>
</tr>
<tr>
<td>Visszanyerési</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tényező RF 1</td>
<td>71,00%</td>
<td>103,64%</td>
</tr>
<tr>
<td>RF 2</td>
<td>66,19%</td>
<td>105,91%</td>
</tr>
<tr>
<td>Érzékenység (S')</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5230</td>
<td>0,1086</td>
<td>0,0631</td>
</tr>
<tr>
<td>Kimutatási határ (nM)</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>Meghatározási határ (nM)</td>
<td>69</td>
<td>17</td>
</tr>
</tbody>
</table>

5. ábra. (a) ECD, (b) FLD detektorral regisztrált kromatogramjai ugyanazon agyi szövetek natív és S2 mintáiból, (c) és (d) DAD detektorral regisztrált egérszérum kromatogramjai.
Összefoglalás
Sikerült megfelelő mintaelőkészítést követően α-tокоферolt meghatároznı egér szérum és agyszövetbıl, sikeresen validáltuk a metodikánkat, valamint meghatároztuk, hogy agyszövet esetén a kétféle detektálás (ECD és FLD) hasonlóan robusztus és precíz.

Köszönnetnyilvánítás
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DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE MEASUREMENTS OF BIOGENIC AMINES

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Abstract

Many important biogenic amines (dopamine, noradrenaline and serotonin) are produced from amino acids by enzyme-catalysed processes and play a prominent role in neuronal functions and therefore, they serve as pharmacological target for the treatment of neurological disorders, such as Alzheimer’s disease or Parkinson’s disease.

The aim of the current study was to optimize a high-performance liquid chromatography method that allows selective separation of eight biogenic amines and some of their metabolites (levodopa, 3,4-dihydroxyphenylacetic acid, noradrenaline, 5-hydroxyindoleacetic acid, homovanillic acid, dopamine, serotonin and 3-methoxytyramine) using 3 internal standards with electrochemical detection. During the development of our method, we optimized the amount of ion pairing component, pH and the amount of organic phase. Several selective methods were tested, but the most effective one was used for validation process for mouse and rat brain regions, including the striatum, cortex and hippocampus.

During validation, the limit of detection, the limit of quantification, recovery, intraday and interday precisions were determined for the eight analytes. The ranges of recovery were between 87 and 120%, the intraday and interday precision were < 10% in all cases. The limit of detection and quantification ranged around 2 and 10 ng/ml, respectively.

The developed and optimized method ensures the measurement of the aforementioned biogenic amines from mouse and rat brain regions.

Introduction

Monitoring of the concentration of biogenic amines may have a great importance from several aspects [1]. The measurement of these metabolites from biological samples requires highly selective and sensitive methods because of their considerably low concentrations [1, 2]. High-performance liquid chromatography (HPLC) methods have been widely applied [3] for this purpose. HPLC combined with electrochemical detector (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 aim of the current study was to optimize our latest HPLC-ECD method [3] to be able to determine 8 biogenic amines and some of their metabolites (levodopa (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), noradrenaline (NA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), dopamine (DA), serotonin (5-HT), 3-methoxytyramine (3-MT)) from different biological samples. It is essential to apply internal standard(s) (IS) for HPLC measurements [4, 5], therefore, based on the recommendations [4], we decided to use 3 (3,4-dihydroxybenzylamine (DHBA), isoproterenol (IPR) and N-methyl serotonin (NM-5HT) instead of the previous one. After the successful extension of the method, we applied it to different biological samples, i.e., mice and rat brain regions and the validation process was carried out as well.
Materials and methods

L-DOPA, 5-HT and their metabolites, DA, 3-MT, DOPAC, HVA, NA and 5-HIAA were measured from the striatum, cortex and hippocampus of C57Bl/6 mice and Wistar rat animals. We used an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) combined with a Model 105 ECD (Precision Instruments, Marseille, France) under isocratic conditions. The brain regions were weighed and then homogenized in an ice-cold solution (striatum: 60 μL/mg; cortex: 25 μL/mg and hippocampus: 18.75 μL/mg) containing perchloric acid (3.4 w%, Sigma Aldrich, Saint Louis, MO, USA), sodium-metabisulfite (400 μM, Fluka, Sigma-Aldrich, Saint Louis, MO, USA), ethylenediaminetetraacetic acid disodium salt (Na₂EDTA, 500 μM, Lach-Ner, Neratovice, Czech Republic), distilled water and ISs: 50 ng/ml DHBA, 200 ng/ml IPR and 100 ng/ml NM-5HT (Sigma Aldrich, Saint Louis, MO, USA). The homogenate was centrifuged at 12,000g for 30 min at 4°C. The supernatants of individual brain regions were pooled and spiked with standard solution in three different concentration levels. The working potential of the detector was set at +750 mV, using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase contained sodium-dihydrogenphosphate (NaH₂PO₄; 75 mM, Reanal, Budapest, Hungary), Na-octylsulphate (NaOS, 2.2 mM, Sigma Aldrich, Saint Louis, MO, USA) and Na₂EDTA (50 μM, Lach-Ner, Neratovice, Czech Republic) was supplemented with acetonitrile (ACN; 6.25% v/v, VWR International, Radnor, PA, USA) and the pH was adjusted to 3.0 with phosphoric acid (H₃PO₄; 85% w/w, Sigma Aldrich, Saint Louis, MO, USA). The mobile phase was delivered at a rate of 1.5 ml/min at 40°C onto the column (Zorbax Eclipse Plus C18, 100 x 4.6 mm, 3.5 μm particle size; Agilent Technologies, Santa Clara, CA, USA) after passage through a pre-column (SecurityGuard, 4×3.0 mm i.d., Phenomenex Inc., Torrance, CA, USA)). 10 μL aliquots were injected by the autosampler with the cooling module set at 4°C.

Results

Before validation, the optimal working potential was investigated. The detector was set from 400 mV to 800 mV with 50 mV increments (Fig 1a). Although 800 mV would be the best working potential for HVA and 3-MT, the signal-to-noise ratio also increases with the applied working potential, so we decided to set the detector at +750 mV.

First we determined the effect of the change in NaOS concentration (Fig.1b) with a result that the increasing amount has ambivalent effect on analytes: increased retention times were observed in case of L-DOPA, NA, DA, 5-HT, 3-MT, IPR (IS), whereas in case of DOPAC, 5-HIAA and HVA the retention time decreased. The concentration of 7.5 mM was selective for all the compounds as well, however, the run time was more than 45 min.

Then we checked the influence of the pH value of the mobile phase (Fig.1c). As it can be seen, the increasing value of pH from 3.0 to 4.0 reduced the retention time of all the analytes.

These results showed that the best choice is to keep the pH at 3.0 and NaOS in the concentration range between 2.10 mM and 2.20 mM with 5 or 6 v/v% ACN. With the 2 new internal standards (DHBA and NM-5HT), the optimal mobile phase consists of 2.20 mM NaOS, 75 mM NaH₂PO₄, 50 μM Na₂EDTA and 6.25 v/v% ACN. Before adding ACN, the pH value of water phase was set to 3.0 with 85 w/w% H₃PO₄. Test runs showed that mice and rat brains can be measured well with this method.
Fig. 1 Voltage vs. peak area responses of the analytes and internal standards (a) and the effect of the concentration of NaOS (b) or pH (c) in mobile phase to retention times of analytes and internal standard. The pH was set to 3.0 and ACN was 5 v/v%.

ACN acetonitrile; DA dopamine; DHBA 3,4-dihydroxybenzylamine; DOPAC 3,4-dihydroxyphenylacetic acid; HVA homovanillic acid; IPR isoproterenol; L-DOPA levodopa; NA noradrenaline; NaOS Na-octylsulphate; NM-5HT N-methyl serotonin; 3-MT 3-methoxythramine; 5-HIAA 5-hydroxyindoleacetic acid and 5-HT serotonin.

We only demonstrate the results of the striatum from mice and rats. The results of validations are presented in Table 1. In Fig. 2 the native and the spiked chromatograms are demonstrated. Native sample is from pooled mice or rat striatum, cortex, hippocampus, cerebellum and brainstem regions.
Fig. 2 Native and spiked chromatograms of pooled mice (a) and rat (b) brain regions.

Table 1. Summary of validation parameters of HPLC-ECD method for biogenic amines in mouse and rat striatum.

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>L-DOPA</th>
<th>DOPAC</th>
<th>NA</th>
<th>5-HIAA</th>
<th>HVA</th>
<th>DA</th>
<th>5-HT</th>
<th>3-MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear range (ng/ml)</td>
<td>mouse</td>
<td>5-150</td>
<td>5-80</td>
<td>5-80</td>
<td>10-100</td>
<td>10-100</td>
<td>5-200</td>
<td>5-100</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>mouse</td>
<td>1.000</td>
<td>0.999</td>
<td>0.999</td>
<td>0.998</td>
<td>0.994</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
<td>0.995</td>
<td>0.993</td>
<td>1.000</td>
<td>0.999</td>
</tr>
<tr>
<td>LOD (ng/ml)</td>
<td>mouse</td>
<td>1.3</td>
<td>0.5</td>
<td>0.7</td>
<td>5.5</td>
<td>7.4</td>
<td>2.0</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>3.1</td>
<td>3.6</td>
<td>7.1</td>
<td>7.9</td>
<td>8.6</td>
<td>2.6</td>
<td>3.8</td>
</tr>
<tr>
<td>LOQ (ng/ml)</td>
<td>mouse</td>
<td>3.9</td>
<td>1.5</td>
<td>2.1</td>
<td>16.5</td>
<td>22.3</td>
<td>6.0</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>9.3</td>
<td>10.9</td>
<td>21.7</td>
<td>23.9</td>
<td>26.1</td>
<td>7.9</td>
<td>11.4</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>mouse</td>
<td>100.7</td>
<td>108.0</td>
<td>110.6</td>
<td>108.9</td>
<td>104.3</td>
<td>109.0</td>
<td>104.4</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>108.0</td>
<td>104.9</td>
<td>106.1</td>
<td>109.0</td>
<td>95.3</td>
<td>106.1</td>
<td>103.5</td>
</tr>
<tr>
<td>Intraday precision</td>
<td>mouse</td>
<td>3.19</td>
<td>1.81</td>
<td>3.68</td>
<td>3.82</td>
<td>8.23</td>
<td>1.59</td>
<td>5.36</td>
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<td></td>
<td>rat</td>
<td>1.96</td>
<td>2.57</td>
<td>3.17</td>
<td>2.76</td>
<td>7.28</td>
<td>1.85</td>
<td>3.60</td>
</tr>
</tbody>
</table>

DA dopamine; DHBA 3,4-dihydroxybenzylamine; DOPAC 3,4- dihydroxyphenylacetic acid; HVA homovanillic acid; IPR isoproterenol; L-DOPA levodopa; NA noradrenaline; NM-5HT N-methyl serotonin; 3-MT 3-methoxytyramine; 5-HIAA 5-hydroxyindoleacetic acid and 5-HT serotonin.
**Discussion**

We successfully extended our latest method with 5 new compounds, of which 2 are internal standards. The applied method is suitable for the measurements of brain regions of mice and rats. The validation parameters are in line with international guidelines. Based on our results, this method will be a valuable tool for multiple experiments, such as, rodent toxin models of neurological disorders.

**Acknowledgements**

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**References**


5. ICH (1995) ICH Harmonized tripartite guideline, validation of analytical procedures. Federal Register, 60, 11260