Article

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A novel small molecule supports the survival of cultured dopamine neurons and may restore the dopaminergic innervation of the brain in the MPTP mouse model of Parkinson's disease

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Abstract

We previously showed that monoterpenoid (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol **1** alleviates motor manifestations of Parkinson's disease in animal models. In the present study, we designed and synthesized monoepoxides of (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol **1** and evaluated their biological activity in the MPTP mouse model of Parkinson's disease. We also assessed the ability of these compounds to penetrate the blood–brain barrier (BBB). According to these data, we chose epoxide **4**, which potently restored the locomotor activity in MPTP-treated mice and efficiently penetrated the BBB, to further explore its potential mechanism of action. Epoxide **4** was found to robustly promote the survival of cultured dopamine neurons, protect dopamine neurons against toxin-induced degeneration, and

trigger the mitogen-activated protein kinase (MAPK) signaling cascade in cells of neuronal origin. Meanwhile, neither survival-promoting effect nor MAPK activation was observed in non-neuronal cells treated with epoxide **4**. In the MPTP mouse model of Parkinson's disease, compound **4** increased the density of dopamine neuron fibers in the striatum, which can highlight its potential to stimulate striatal reinnervation and thus to halt disease progression. Taken together, these data indicate that epoxide **4** can be a promising compound for further development, not only as a symptomatic, but also as a neuroprotective and neurorestorative drug for Parkinson's disease.

Keywords: Parkinson's disease, neurorestoration, MAPK signaling, ERK pathway, dopamine neurons, tyrosine hydroxylase.

1. INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide. PD affects about 1–1.5% of the population over 60 years of age[1]; the lifetime risk of developing the disease is 1.5%.[2] The economic burden of PD in the United States exceeded $14.4 billion in 2010.[3] With the global phenomenon of population ageing, the number of PD cases is likely to grow significantly in the future. The major clinical diagnostic criteria for PD include tremor, rigidity, akinesia, postural instability, and bradyphrenia; depression is the most frequent psychopathological disorder.[4] The diagnostic motor symptoms of PD are caused by progressive degeneration and death of dopamine neurons in the brain of affected people.[5] There is no cure for PD. In other words, none of the existing medications can prevent, stop or slow down the underlying neuronal degeneration. Current management of PD is limited to supportive care that partially alleviates disease signs and symptoms. The dopamine precursor levodopa in combination with peripheral decarboxylase inhibitor is still the most available effective medication. The main adverse effects of levodopa

therapy include nausea, motor complications, dyskinesias and the on–off phenomenon, confusion, hallucinations, orthostatic hypotension, and sleep disturbances.[6-8] Importantly, levodopa only supplements endogenous dopamine but does not prevent progressive degeneration of dopamine neurons and may actually even promote it.[9] Therefore, studying the alternative strategies for PD treatment, especially the ones targeting neurodegeneration, is rather relevant today.

We have earlier[10] discovered a small low-toxicity molecule (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol **1** (Fig. 1) that can potently alleviate motor symptoms in rodent models of PD.[10-12] We synthesized and tested *in vivo* a number of derivatives, including all eight stereoisomers of compound **1.**[10] It was found that all of the four functional groups of **1** are needed for the compound to exhibit anti-PD activity.[13] Among N-, O-, S- and C-derivatives at the C9 position of diol **1**, compounds **2** and **3** with butyl or propylthio substituents exhibited a high activity.[14]

When exploring the chemical space around diol, we have synthesized all four isomeric monoepoxides **4-7** in the present study (Fig. 1) and investigated their ability to influence the locomotor activity in the MPTP-induced model of PD. The ability of selected epoxides to penetrate the blood–brain barrier was also evaluated. Since the mode of action and the molecular target(s) of diol and its derivatives were still to be elucidated, we attempted to address this question. We also evaluated the ability of the most promising diol epoxide to promote the survival of dopamine neurons *in vitro* and *in vivo* in the MPTP-induced model of PD.

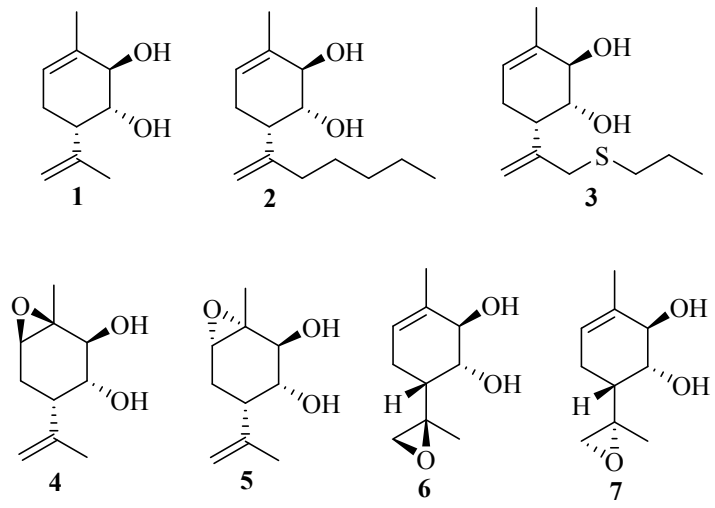


Figure 1. (1*R*,2*R*,6*S*)-3-Methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol **1** and its derivatives **2**-**7**

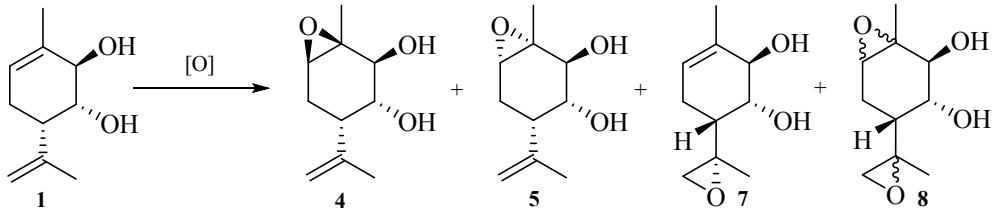
2. RESULTS AND DISCUSSION

2.1. Synthesis of epoxides 4 and 5

First, we focused on epoxidation of the 3,4 double bond in compound **1**. The attempts to epoxidize compound **1** with *t*-BuOOH in the presence of Ti(*i*-PrO)$_4$ (the Sharpless system[15]) or VO(acac)$_2$ in accordance with the method described by Brown S. et al [16] have failed: the starting compound **1** was obtained again or a complex mixture of products was formed. The probable reason for that phenomenon involved secondary transformations of the epoxide group.

Next, we attempted to use non-catalytic methods to oxidize compound **1** with $CH_3CO_3H/NaHCO_3$ or *m*-CPBA in CH_2Cl_2 (Scheme 1). Treatment with $CH_3CO_3H/NaHCO_3$ in CH_2Cl_2 for 3 h at room temperature gave rise to epoxides **4** and **5** with the yields of 16% and 4%, respectively; the degree of conversion of diol **1** was 94%. Oxidation of compound **1** with *m*-CPBA (1.45 eq.) in CH_2Cl_2 at 0 °C for one week led to formation of epoxides **4** and **5** with acceptable yields. The stereoisomeric epoxides **4** and **5** were isolated in their pure forms by column chromatography in silica gel; the yields of the products were 26% and 11%, respectively; the degree of conversion of the starting compound **1** was 91%. In this case, we also detected 2% of epoxide **7** in

the reaction mixture but failed to isolate it. Finally, when compound **1** was treated with *m*-CPBA (1.66 eq.) in Et_2O for 3 weeks, the yields of products **4** and **5** were 32% and 10%, respectively; full conversion of diol **1** was achieved. Moreover, a mixture of four stereoisomeric diepoxides **8** was isolated with a 42% yield.



1 **4** **5** **7** **8**

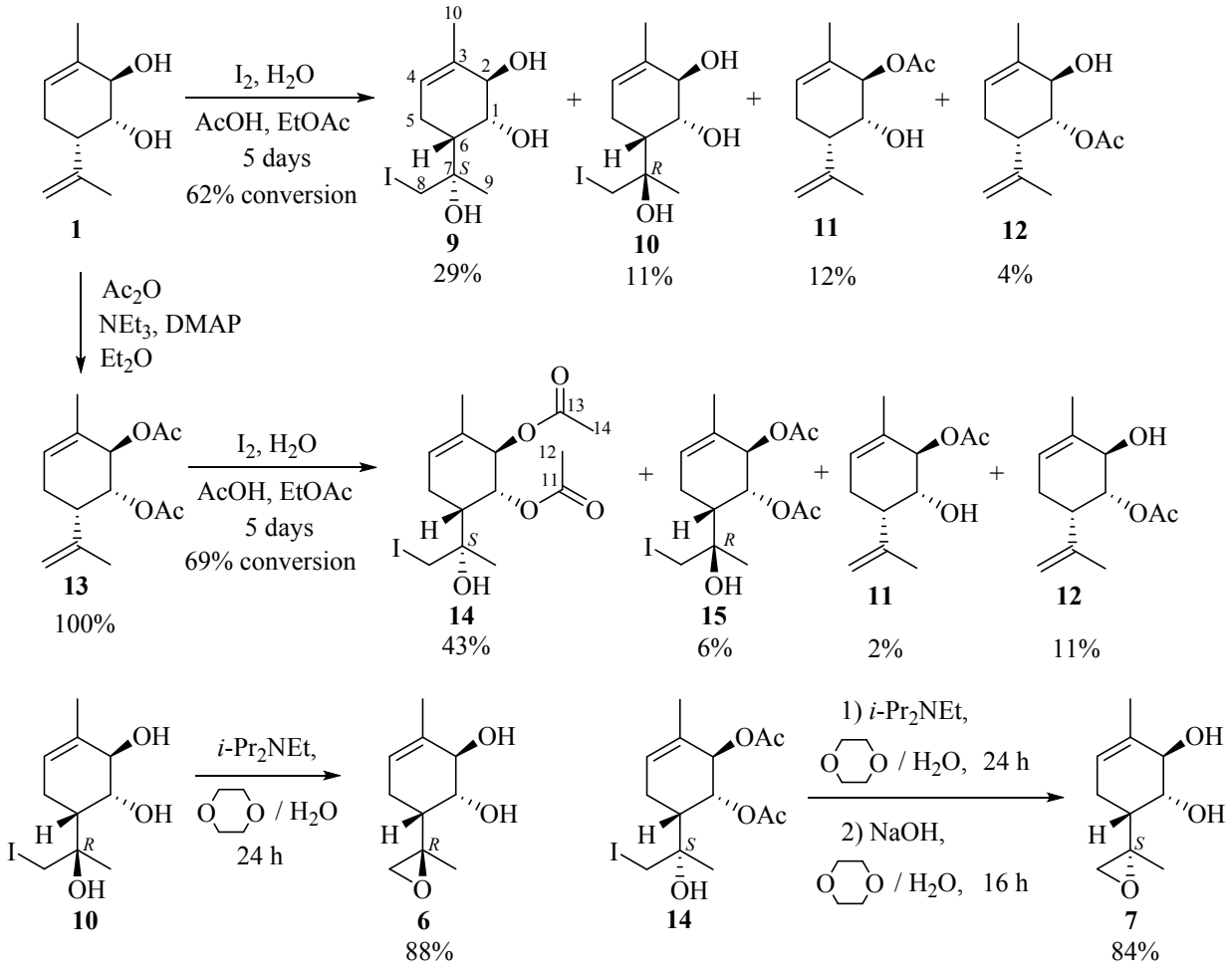
a) CH_3CO_3H, $NaHCO_3$, CH_2Cl_2, 3 h	16%	4%	-	-
b) *m*-CPBA (1.45 eq), CH_2Cl_2, 1 week	26%	11%	(2 %)	-
c) *m*-CPBA (1.66 eq), Et_2O, 3 week	32%	10%	-	42%

Scheme 1. Epoxidation of compound **1**.

2.2 Synthesis of epoxides 6 and 7

Since direct epoxidation of diol **1** proceeds predominantly at the endocyclic double bond, the iodohydrin approach was used to obtain stereoisomeric compounds **6** and **7**. The interaction between diol **1** and iodine in the presence of water and AcOH in EtOAc led to 62% conversion of **1** within 5 days. 7*S*- and 7*R*-epimeric iodohydrins **9** and **10** were obtained as products; the preparative yields calculated with respect to the consumed diol **1** were 29% and 11%, respectively. Esterification proceeded as a side process and gave rise to monoacetates **11** and **12** with the yields of 12% and 4%, respectively. In order to improve this yield, we conducted a similar reaction with diacetate **13**, which was obtained from diol **1** with a quantitative yield as described earlier by Ardashov O. et al.[17] The interaction between diacetate **13** and iodine was much more selective than it was for diol **1** and predominantly gave rise to 7*S*-epimer **14** (43% yield), whereas the content of minor 7*R*-stereoisomer

15 was only 6%. Moreover, the products of partial hydrolysis **11** and **12** were isolated with the yields of 2% and 11%, respectively (Scheme 2).



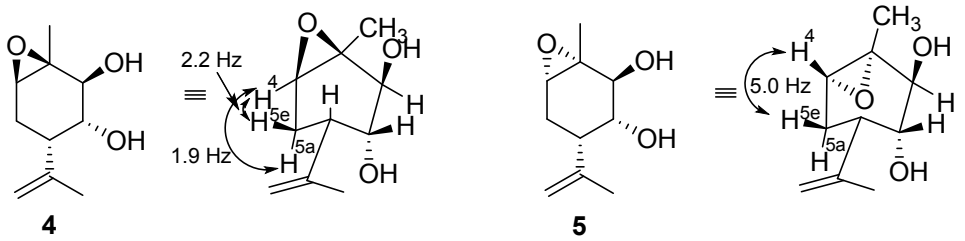
Scheme 2. Synthesis of epoxides **6** and **7**.

Iodohydrin **10** was converted to the respective epoxide **6** (with a 88% yield) using *i*-Pr_2NEt as a base in aqueous dioxane. A similar process for compound **14** included epoxide formation in the presence of *i*-Pr_2NEt and further saponification by NaOH. The epimeric epoxide **7** was formed with an 84% yield (Scheme 2).

2.3. Determination of the configuration of epoxide groups in compounds 4-7

The configuration of epoxide groups in compounds **4** and **5** was determined using the ^{1}H NMR data by analyzing the vicinal ^{1}H-^{1}H constants of the C^4H-C^5H_2-C^6H chain. Proton H^4 in compound **4** takes a position that allows overlapping with both H^{5e} and H^{5a} protons with the vicinal constants of 2.2 and 1.9 Hz, respectively (Fig. 2). On the contrary, proton H^4 in compound **5** interacts only with proton H^{5e} with the vicinal constant of 5.0 Hz.

Determining the configuration of epoxide groups in compounds **6** and **7** was a challenging task. A conformational analysis of epoxides **6** and **7** predicts that the oxygen atom in the epoxide ring in the most energetically favorable forms of these epoxides is oriented towards the hydroxyl proton OH^1 (Fig. 2). This specific conformation is fixed by a strong intramolecular hydrogen bond. The presence of this intramolecular H-bond is confirmed by ^{1}H NMR: the OH^1 signal lies downfield of the OH^2 signal and has a much narrower lineshape. Due to the H-bond fixation of the epoxide conformation, the *R*/*S*-configuration of the C^7 atom in both epoxides **6** and **7** can be reliably determined by NOESY. Indeed, strong cross-peaks are observed in the 2D NOESY spectrum of epoxide **6**. These peaks correspond to NOE between protons H^{1e} and H^{8en}, H^{5e} and $CH_3{}^9$, H^{5a} and $CH_3{}^9$, being indicative of the *R*-configuration of C^7. On the contrary, NOE is observed between protons H^{5e} and H^{8en}, H^{5a} and H^{8en}, H^{1e} and $CH_3{}^9$ in the 2D NOESY spectrum of epoxide **7**, corresponding to the C^7 *S*-configuration in **7** (Fig. 2).



4 **5**

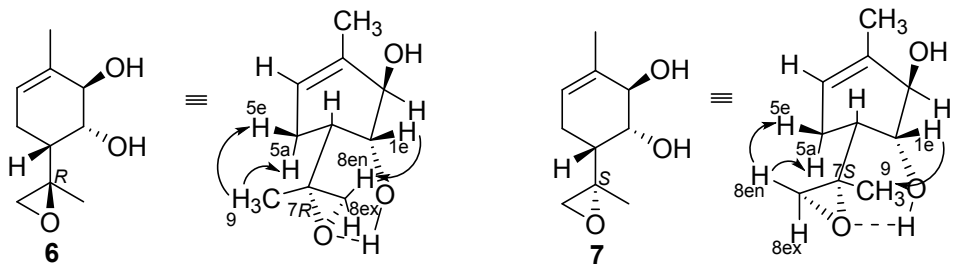


Figure 2. Configuration of epoxide groups in compounds **4**-**7**.

Thus, all four isomeric epoxides **4**-**7** were successfully synthesized in their pure forms. Interestingly, when we compared the mass spectra and retention times in GC-MS for epoxides **4**-**7** with the data on metabolic profiling of diol **1** *in vivo,* it turned out that epoxide **4** is a metabolite of diol **1** present in the blood of experimental animals.

It is important that, unlike epoxides **5**-**7** and other epoxides of similar structure,[18,19] compound **4** is unusually stable in acidic conditions such as 10% H_2SO_4 or montmorillonite clay K10.

2.4. Studying the antiparkinsonian activity of epoxides 4-7

We studied the biological activity of epoxide **4** (identified as a metabolite of compound **1)** and isomeric compounds **5-7** in the MPTP mouse model of PD.

The MPTP-induced model is one of the most commonly used animal models of PD.[5] In the brain, MPTP is converted to MPP^+, which is selectively uptaken by the dopamine transporter into dopaminergic neurons.[20] In dopaminergic neurons, it blocks the electron transport chain in mitochondria and reduces the cellular ATP levels, which leads to formation of toxic reactive oxygen species and causes neuronal death. Systemic administration of MPTP causes reproducible lesions in the nigrostriatal dopaminergic pathway. Although this model has some limitations, MPTP is the

only known dopaminergic neurotoxin capable of causing clinical presentation indistinguishable from idiopathic PD in humans.[21]

Parkinson's disease-like motor symptoms were induced in C57Bl/6 mice by four injections of MPTP (0.12 mmol/kg (20 mg/kg) per dose, every 2 h) in accordance with the procedure described earlier.[21] The studied agents were administered per os 24 h after the last injection of MPTP at a dose of 20 mg/kg. The main markers of locomotor activity were measured 2 h after administration of the agent using the open field test for 2 min. The effectiveness of the studied medication was evaluated according to its ability to reduce the symptoms of hypokinesia induced by MPTP.

Administration of MPTP to mice reduced their locomotor activity, in particular, in movement distance and duration of locomotor activity parameters (Fig. 3).

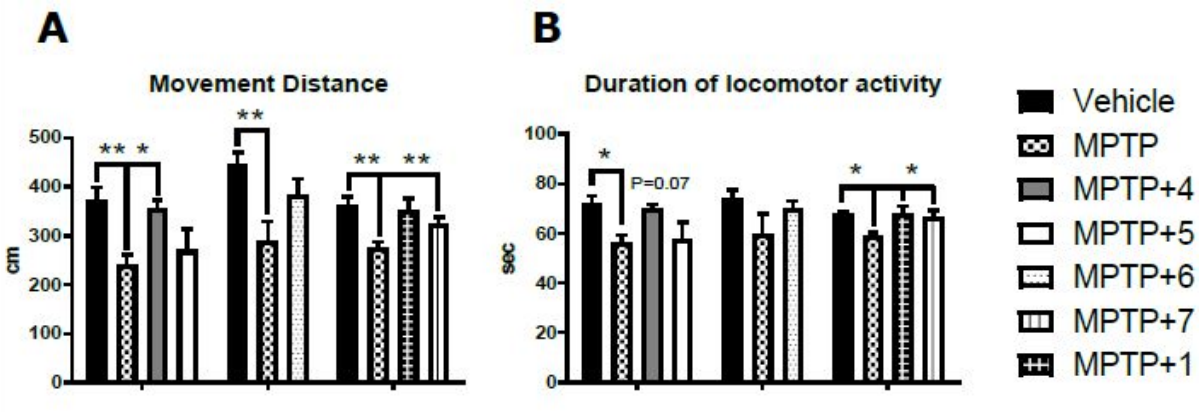


Figure 3. The influence of compounds **1**, **4**-**7** (0.12 mmol/kg (20 mg/kg)) on locomotor activity of MPTP-treated (4×0.12 mmol/kg (4×20 mg/kg)) male C57Bl/6 mice: movement distance (**A**) and duration of locomotor activity (**B**). Mean ± SEM. N = 8 – 10 mice per group. * $P < 0.05$, ** $P < 0.01$ compared to the MPTP group, ANOVA with Dunnett's post-hoc test.

Administration of *trans*-epoxide **4** after MPTP neurotoxin injections led to a virtually complete recovery in movement distance and a trend towards increasing duration of locomotor

activity in animals (Fig. 3, A and B). It should be noted that compound **4** given to naïve mice at the same dose had no effect on animals' locomotor activity (Fig. 4).

Unlike *trans*-epoxide **4**, *cis*-isomer **5** did not affect the locomotor activity in mice treated with MPTP (Fig. 3). Epoxide **6** also failed to influence the locomotor activity. Meanwhile, isomeric epoxide **7** restored the locomotor activity of mice, which was previously reduced by MPTP.

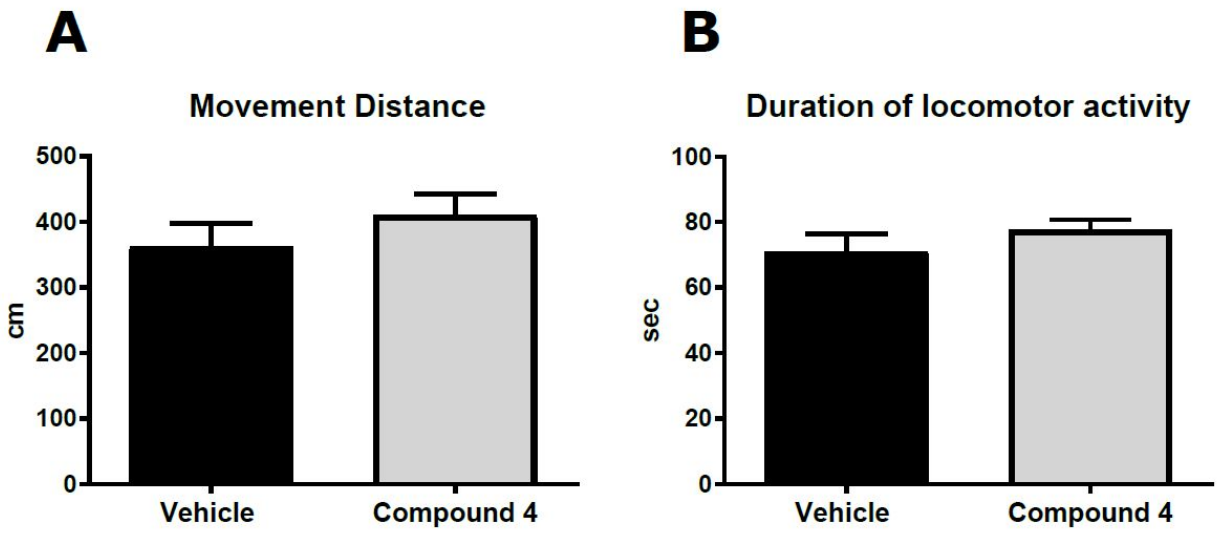


Figure 4. The influence of compound **4** (0.12 mmol/kg (20 mg/kg)) on locomotor activity of naïve male C57Bl/6 mice. Mean ± SEM. N = 10 mice per group.

Thus, we found that two out of the four studied epoxides improved motor performance in the MPTP mouse model of PD. One of these compounds, epoxide **4**, is a product of epoxidation of 3,4-double bound in compound **1**, while another one, compound **7**, is 7,8-epoxide of **1**. It was demonstrated that the configuration of epoxides played a crucial role in their ability to improve motor performance in the MPTP mouse model of PD.

Dose reduction of epoxide **4** to 5 mg/kg led to a less pronounced but statistically significant improvement of locomotor activity in MPTP-treated mice (Fig. 5). Unlike for the results obtained

for compound **4** at a dose of 20 mg/kg (Fig. 3), restoration of locomotor activity (movement distance) was incomplete in this case. However, a statistically significant increase in duration of locomotor activity was observed.

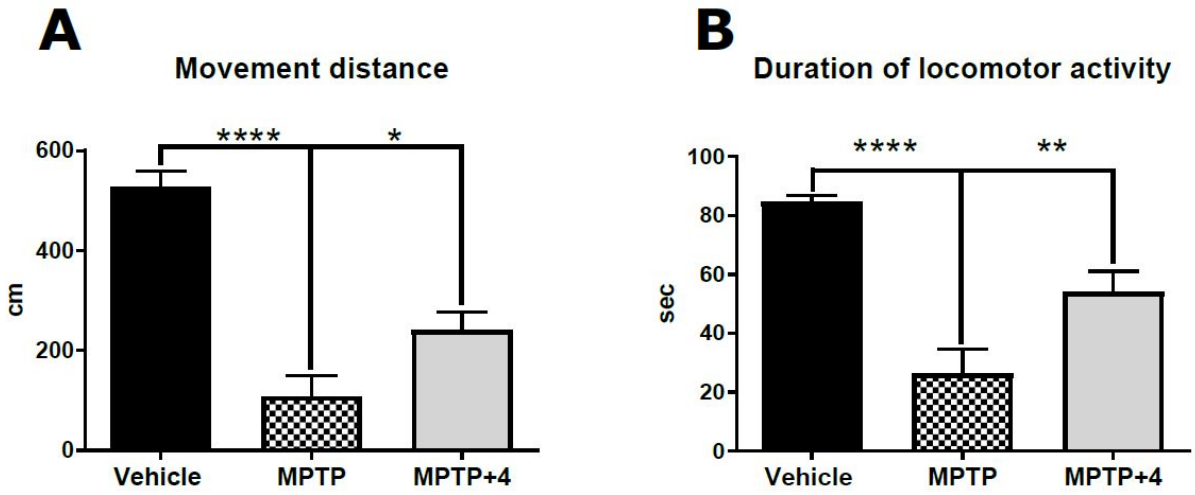


Figure 5. The influence of compound **4** (0.03 mmol/kg (5 mg/kg)) on locomotor activity of MPTP-treated (4×0.12 mmol/kg (4×20 mg/kg)) male C57Bl/6 mice. **A** – movement distance, **B** – duration of locomotor activity. Mean ± SEM. N = 10 mice per group.* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared to the MPTP group, ANOVA with Dunnett's post-hoc test.

We studied the safety of active epoxides **4** and **7**. Neither toxic nor adverse effects were observed in response to a single oral 1000 mg/kg dose of these compounds.[22] After 10 days, all animals were still alive and no abnormalities in general condition were revealed. Hence, these compounds exhibited low acute toxicity.

The ability of epoxide **4** to penetrate the blood–brain barrier was demonstrated by analyzing the brain and plasma extracts 1.5 h after oral administration of the epoxide at a 10 mg/kg dose to rats. Compound **4** was found both in plasma (5.3 μg/mL) and in the brain (3.5 μg/g). These data

indicate that compound **4** readily penetrates the blood–brain barrier and remains in the bloodstream for a relatively long time.

It is interesting to note that we also detected a small amount of epoxide **4** in blood of experimental animals after administration of diol **1**. These data indicate that epoxide **4** is a metabolite of compound **1**. In the present study, we showed that it exhibits a biological activity in the MPTP mouse model of PD.

According to the results of biological experiments and taking into account the good chemical stability of epoxide **4**, this epoxide was chosen for further studies.

2.5. Insights into the molecular mechanism of action of compound 4

Since the diagnostic symptoms of PD are caused by progressive degeneration of dopamine neurons, drugs protecting and/or restoring these neurons can significantly improve the management of PD.[23-25] Therefore, we tested the ability of 0.01–10 μM compound **4** to promote the survival of cultured embryonic dopamine neurons. Midbrain neurons were isolated from mouse embryos (E13.5) and cultured for five days in the presence of compound **4** or a vehicle. Dopamine neurons were labelled with antibodies specific to the key enzyme of dopamine synthesis, tyrosine hydroxylase (TH), imaged using the CellInsight system, and counted using the Cell Profiler software. Glial cell line-derived neurotrophic factor (GDNF), the protein capable of supporting the survival of dopamine neurons,[26,27] was used as a positive control. Compound **4** promoted the survival of naïve TH-positive neurons in a dose-dependent manner. In the wells treated with 0.1 μM and 1 μM compound **4**, the number of TH-positive neurons was 2.1- ($P < 0.05$, ANOVA with Dunnett's post-hoc test) and 2.3-fold ($P < 0.01$, ANOVA with Dunnett's post-hoc test) , respectively, greater than that in vehicle-treated wells ($P = 0.028$, one-way ANOVA) (Fig. 6A).

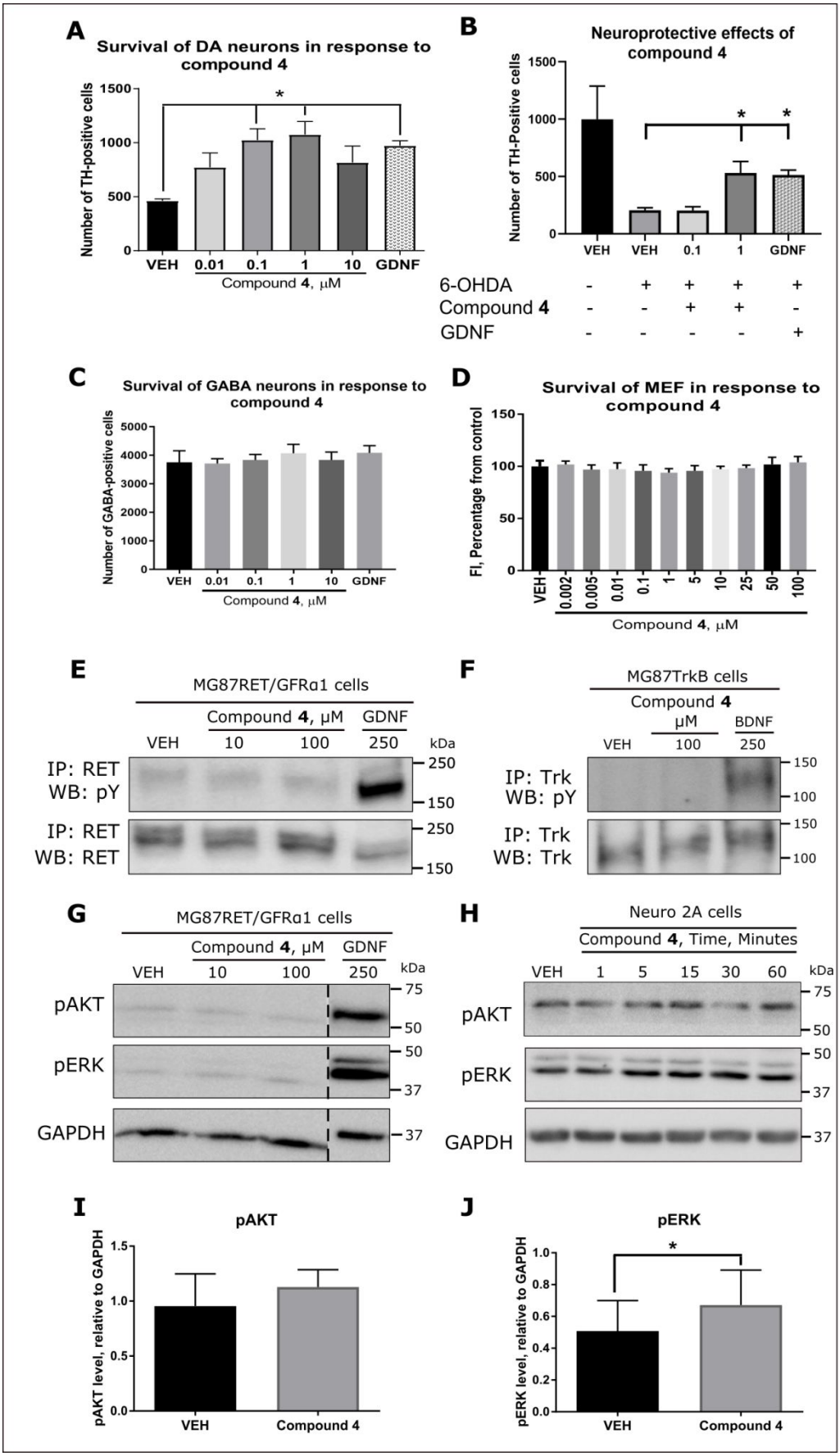
We also studied the effect of compound **4** on the survival of TH-positive neurons in midbrain cultures treated with 6-hydroxydopamine (6-OHDA), a toxin causing selective death of dopamine neurons.[23] Compound **4** protected cultured dopamine neurons against 6-OHDA-induced degeneration (Fig. 6B).

Midbrain cell cultures contain other neuronal types in addition to dopamine neurons (in particular, a significant proportion of neurons producing neurotransmitter gamma-aminobutyric acid (GABA)).[28] Therefore, the effect of compound **4** on the survival of these neurons was investigated. GABA-positive neurons were detected immuocytochemically using anti-GABA antibodies against GABA. We found that compound **4** failed to influence the survival of GABA-positive neurons (Fig. 6C). Compound **4** was also unable to promote the proliferation of primary mesencephalic embryonic fibroblasts (Fig. 6D). These data indicate that the survival-promoting properties of epoxide **4** are restricted to the cells of neuronal origin and especially to dopamine neurons.

We further attempted to address the possible mechanisms of neuroprotective effect of epoxide **4** observed in dopamine neurons. It is known that the survival of cultured dopamine neurons can be positively affected by neurotrophic factors GDNF[27] and brain-derived neurotrophic factor (BDNF).[29] Hence, we accessed the ability of epoxide **4** to activate the receptors of these neurotrophic factors: receptor tyrosine kinases RET[30] and TrkB,[31] respectively. However, epoxide **4** activated neither RET (Fig. 6E), nor TrkB (Fig. 6F) in cultured murine fibroblasts MG87 overexpressing these receptors. In line with these data, epoxide **4** failed to activate Akt and mitogene-activated protein kinase (MAPK/ERK) downstream signaling cascades that are known[32] to be important for the survival and functioning of mammalian cells in MG87RET fibroblasts (Fig. 6G). Based on these results, we hypothesized that the neuroprotective effect of epoxide **4** can be mediated by a receptor or receptors other than RET or TrkB expressed in neuronal cells. Therefore, we assessed the ability of compound **4** to activate the MAPK and AKT signaling cascades in

immortalized cells of neuronal origin. Indeed, the level of phosphorylated ERK in Neuro2A neuroblastoma cells was by 32% higher increased in the cells treated with compound **4** compared to the vehicle-treated cells ($P < 0.05$, paired t-test), whereas no changes were observed in the level of pAKT (Fig. 6 H-J). The levels of pERK and pAKT normalized to GAPDH were calculated separately for each experimental time point and averaged afterwards, because the peaks of pERK and pAKT activation varied in time between the experiments, making direct comparison complicated.

Taken together, our results suggest that compound **4** may be promising both for treatment of disease progression in PD and alleviation of PD symptoms. The receptor of compound **4** is yet to be determined. Furthermore, we cannot rule out the possibility that epoxide **4** has no neuroprotective properties; instead, it might influence the secretion of various neurotrophic factors supporting the survival of dopamine neurons as it has been shown for selegiline.[33,34]


A
Survival of DA neurons in response to compound 4
Number of TH-positive cells
1500
1000
500
0
*
VEH
0.01
0.1
1
10
GDNF
Compound 4, μM
B
Neuroprotective effects of compound 4
Number of TH-Positive cells
1500
1000
500
0
* *
VEH
VEH
0.1
1
GDNF
6-OHDA - + + + +
Compound 4 - - + + -
GDNF - - - - +
C
Survival of GABA neurons in response to compound 4
Number of GABA-positive cells
5000
4000
3000
2000
1000
0
VEH
0.01
0.1
1
10
GDNF
Compound 4, μM
D
Survival of MEF in response to compound 4
FI, Percentage from control
150
100
50
0
VEH
0.002
0.005
0.01
0.1
1
5
10
25
50
100
Compound 4, μM
E
MG87RET/GFRα1 cells
Compound 4, μM
GDNF
VEH
10
100
250
kDa
IP: RET
WB: pY
250
150
IP: RET
WB: RET
250
150
F
MG87TrkB cells
Compound 4
μM
BDNF
VEH
100
250
IP: Trk
WB: pY
150
100
IP: Trk
WB: Trk
150
100
G
MG87RET/GFRα1 cells
Compound 4, μM
GDNF
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10
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250
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pAKT
75
50
pERK
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GAPDH
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Neuro 2A cells
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GAPDH
37
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pAKT
pAKT level, relative to GAPDH
1.5
1.0
0.5
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VEH
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J
pERK
*
pERK level, relative to GAPDH
1.0
0.8
0.6
0.4
0.2
0.0
VEH
Compound 4

Figure 6. The biological effects of compound **4** in cultured cells. The influence of compound **4** on the survival of cultured naïve dopamine neurons (A), on the cultured dopamine neurons treated with 6-OHDA (B), on the survival of GABA neurons (C) and murine mesencephalic embryonic fibroblasts (D). The influence of compound **4** on phosphorylation of receptors of neurotrophic factors GDNF (E) and BDNF (F). The influence of compound **4** on MAPK/ERK and AKT signaling cascades in murine fibroblasts MG87RET transfected with GDNF family receptor alpha 1 (GFRα1) (G) or in neuroblastoma Neuro 2A cells (H) shown by Western blotting. The concentration of compound **4** applied to Neuro 2A cells (H) was 100 μM. Quantitative analysis of pAKT (I) and pERK (J) levels in Neuro2A cells treated with vehicle or compound **4** (100 μM) for 1–60 min. The quantitative data are presented as Mean ± SEM. IP – immunoprecipitation; WB – Western blotting; pY – phosphotyrosine; GAPDH – glyceraldehyde 3-phosphate dehydrogenase, loading control; VEH – vehicle. A – * $P < 0.05$ ANOVA with Dunnett's post-hoc test compared to vehicle, N = 4; H – * $P < 0.05$, paired t-test, compared to vehicle, N = 5.

2.6. Evaluation of the neuroprotective/neurorestorative properties of epoxide 4 in the nigrostriatal dopamine system

According to the *in vitro* data showing the positive influence of compound **4** on the survival of cultured dopamine neurons, we decided to evaluate the neurorestorative properties of diol epoxide in the nigrostriatal dopamine system *in vivo* in the MPTP-induced mouse model of PD. The bodies of nigrostriatal dopamine neurons are located in the brain region known as *substantia nigra pars compacta* (SNpc), while their axons project to the brain region called the striatum. Therefore, we quantified the density of TH-positive fibers in the striatum and the number of TH-positive cells in SNpc. The mice were treated with 4×20 mg of MPTP to induce degeneration of dopamine neurons. Two different doses (5 and 20 mg/kg) were used to study the effect of epoxide **4** in this

model. Epoxide **4** was administered orally to mice 24 h after the last MPTP injection and then for additional 14 days (5 daily administrations per week for 3 weeks). The murine brains were collected 30 days after the lesion. Administration of MPTP reduced the density of TH-positive fibers in the striatum twofold as compared to that in vehicle-treated mice (Fig. 7A). Treatment of MPTP mice with both 5 and 20 mg/kg of compound **4** significantly increased the density of TH-positive fibers in the striatum as compared to that in MPTP-treated mice ($P = 0.0053$ and $P = 0.0329$, respectively, ANOVA with Dunnett's post-hoc test) (Fig. 7A).

We also studied the effect of epoxide **4** on the number of TH-positive neurons in the SNpc. Significant changes in the number of TH-positive neurons were observed in none of treatment groups.

The data presented in Fig. 6 suggest that compound **4** can either stimulate the outgrowth and arborization of neurites of dopamine neurons *in vivo*, which is expected to increase the number of dopaminergic synapses in the striatum and halt the disease progression, or at least stimulate the expression of TH, which will likely elevate dopamine content in the striatum and alleviate symptoms of PD. It is unclear whether compound **4** can also stimulate regeneration of dopamine cell bodies in SNpc, since their loss induced by MPTP in this particular experiment was mild, if any at all. However, we noticed in the *in vitro* experiments that epoxide **4** can activate ERK (Fig 6H, 6J), but not AKT (Fig 6I). In neurons, AKT is the major regulator of neuronal survival,[35,36] while ERK1/2 is important for neurite growth[37] rather than for survival.[38] Thus, the *in vitro* data showing pERK activation in cultured cells taken together with the results of analyzing the TH-positive fiber density may indicate that the mechanism of neurorestoration induced by epoxide **4** is mediated by ERK and includes stimulation of striatal reinnervation by dopamine neurons.

It is important to mention that neurodegeneration in Parkinson's disease starts from axons. The motor symptoms of PD appear when dopamine concentration in the putamen drops by 70%–

80%. Meanwhile, the loss of dopamine neuron bodies in the SNpc at disease onset is only 30%[3]. Moreover, some bodies of dopamine neurons remain in the brain of PD patients even at the very late stage (> 20 years after the diagnosis), while dopamine axons in the putamen disappear almost completely within 5 years after diagnosis[4]. Therefore, restoration of axons of dopamine neurons in the putamen can be a valuable therapeutic strategy in PD.

Our results do not give grounds for drawing a conclusion whether epoxide **4** is able to protect the bodies of dopamine neurons in SNpc. However, it might protect or stimulate axonal regrowth into the striatum. Therefore, our data and the features of PD progression in patients support that epoxide **4** should be further developed not only as symptomatic treatment but also as a potential neuroprotective/neurorestorative therapeutic agent against PD. It will also be interesting to test epoxide **4** in other models of neurodegenerative diseases (e.g., Alzheimer's and Huntington's diseases), as well as in other nervous system disorders associated with neuronal loss (e.g., neuropathic pain). The cellular receptor of epoxide **4** seems to be expressed in the cells of neuronal origin, but its molecular identity remains to be determined. It is possible that other types of neurons than dopamine neurons can also respond positively to compound **4**, thus providing grounds for developing disease-modifying treatment for multiple conditions caused by neuronal cell death.

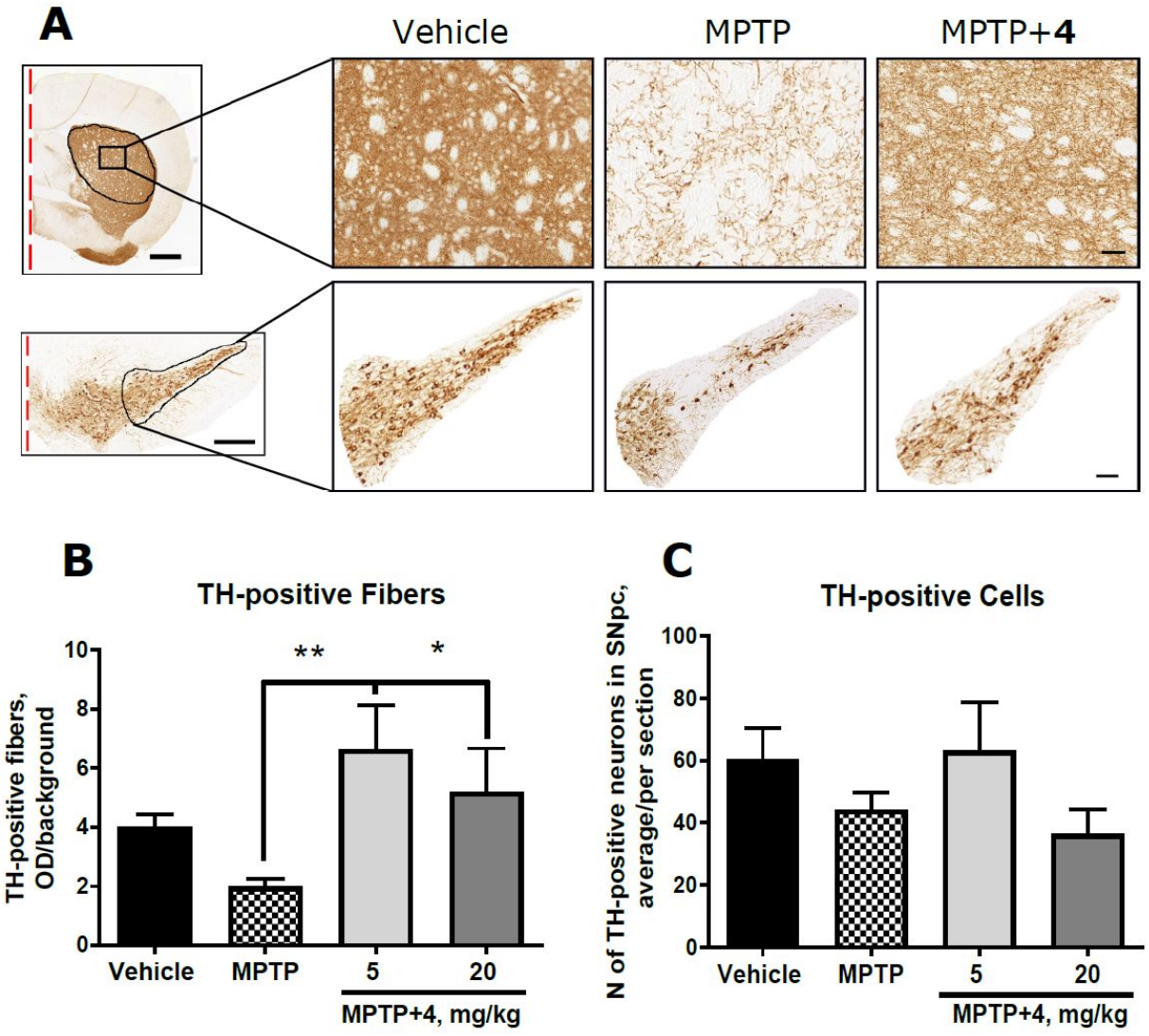

Figure 7. The influence of compound **4** (5 and 20 mg/kg) on the density of TH-positive fibers in the striatum (**A, B**) and the number of TH-positive cells (**C**) in SNpc of MPTP-treated (4×0.12 mmol/kg (4×20 mg/kg)) male C57Bl/6 mice. The data are presented as Mean ± SEM, N = 3–6 per group. * $P < 0.05$, ** $P < 0.01$ compared to the MPTP group, ANOVA with Dunnett's post-hoc test. Scale bar: 40 μM.

2.7. Conclusions

All four isomeric epoxides of (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol **1** have been synthesized. Epoxidation of double bonds at positions 3,4 (epoxide **4**) and 7,8

(epoxide **7**) was found to give rise to biologically active compounds capable of restoring the locomotor activity in the MPTP-induced mouse model of PD. Epoxide **4** was identified to be an active metabolite of **1**. Both compounds were able to penetrate the blood–brain barrier, but epoxide **4** did so with higher efficacy. Moreover, epoxide **4** remained in the bloodstream longer than epoxide **7** did. Interestingly, epoxide **4** is also a metabolite of diol **1** found *in vivo*. Based on all these data, we selected epoxide **4** to study the possible mechanism of its biological activity in the PD model. We found that epoxide **4** protects cultured dopamine neurons against spontaneous and toxin-induced death and selectively activates ERK in the cells of neuronal origin. In animals treated with MPTP, epoxide **4** increases the density of TH-positive fibers in the striatum. Our data indicate that epoxide **4** may produce positive immediate and long-term effects in the PD model; the latter ones might be related to neurorestoration. Hence, epoxide **4** may encourage the development of disease-modifying treatment against PD.

3. Methods

3.1. General methods and materials: Reagents and solvents were purchased from commercial suppliers and used as received. Dry solvents were obtained according to the standard procedures. (1*R*,2*R*,6*S*)-3-Methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol (**1**) ($[\alpha]_D^{31}$ -49.1 (*c* 2.6, $CHCl_3$)) was synthesized from (-)-verbenone (Aldrich) ($[\alpha]_D^{25}$ -210.5 (*c* 0.77, $CHCl_3$)) according to previously described procedure.[39,40]

Spectral and analytical investigations were carried out at Collective Chemical Service center of Siberian Branch of Russian Academy of Sciences. ^{1}H- and ^{13}C-NMR Spectra: *Bruker DRX-500* apparatus at 500.13 MHz (^{1}H) and 125.76 MHz (^{13}C) in $CDCl_3$, $CDCl_3$ / CCl_4 1 : 1 (v/v)or $CDCl_3$ / CD_3OD 10 : 1 (v/v); chemical shifts δ in ppm rel. to residual $CHCl_3$ [δ(H) 7.24, δ(C) 76.90 ppm], *J* in Hz. The structure of the products was determined by analyzing the ^{1}H- and ^{13}C-NMR spectra,

$^{1}H,^{1}H$ double-resonance spectra and $^{13}C,^{1}H$-type 2D-COSY (J(C,H) = 135 Hz). NMR spectra for compounds **6** and **7**: Bruker AV-600 spectrometer (resonance frequency for ^{1}H 600.30 MHz, for ^{13}C 150.95 MHz). Chemical shifts for ^{1}H and ^{13}C measured relative to the internal standard $CDCl_3$ (δ 7.24 ppm for ^{1}H, 76.90 ppm for ^{13}C). For structure determination and NMR signal assignment in **6** and **7** 2D correlation spectra ^{1}H-^{1}H (COSY, NOESY) and ^{1}H-^{13}C (HSQC, HMBC) were used. GC: 7820A gas chromatograph (Agilent Tech., USA); flame-ionization detector; HP-5 capillary column (0.25 mmol Ø × 30 m × 0.25 μm), He as carrier gas (flow rate 2 ml/min, flow division 99 : 1). GC/MS: Agilent 7890A with a quadrupole mass spectrometer Agilent 5975C as a detector, HP-5MS quartz column, 30000x0.25 mm, He (1 atm) as carrier gas. Optical rotation: polAAr 3005 spectrometer; $CHCl_3$ soln. HR-MS: DFS Thermo Scientific spectrometer in a full scan mode (15-500 m/z, 70 eV electron impact ionization, direct sample administration).

Conformational analysis of the epoxides **6** and **7** was performed with the computer program packages ChemAxon Marvin,[41] VeraChem Vconf[42] and Tinker.[43] The geometries of all the conformers were optimized by DFT (Priroda program,[44,45] PBE functional,[46] L1 basis (Λ01,[47] cc-pVDZ analog). Quantum chemical calculations were carried out on the computational cluster at the Information Computation Center, Novosibirsk State University. The purity of the target compounds was determined by gas chromatography methods. All of the target compounds reported in this paper have a purity of no less than 95% (GC-MS data). Target compounds successfully passed the virtual filter for known classes of assay interference compounds (PAINS).[48]

3.2. *(1S,2S,3R,4S,6R)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptane-2,3-diol (4) and (1R,2S,3R,4S,6S)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptane-2,3-diol (5).*

*3.2.1. Epoxidation of (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol (1) with peracetic acid. Method a (Scheme 1).* A mixture of AcOH (200 ml), 35% H_2O_2 (200 ml) and

H_2SO_4 (5 ml) was stored for 5 days. Then a part of this mixture (100 ml) was extracted with CH_2Cl_2 (2 x 50 ml) and combined extract was dried over $MgSO_4$. The concentration of AcOOH was 0.0925 moles per 100 ml of the solution. Grinded Na_2CO_3 (2 g) and then the AcOOH solution (7 ml, 0.648 mmol) were added under stirring to the solution of diol **1** (1.00 g, 5.95 mmol) in CH_2Cl_2 (4 ml). The reaction mixture was stirred for 3 h, diluted with water (40 ml), separated, and the water phase was extracted with CH_2Cl_2 (3 x 20 ml) and EtOAc (3 x 30 ml). The combined organic layers were desiccated over Na_2CO_3. The desiccant was filtered off, the solvent was evaporated. The residue (762 mg) was separated by CC on silica gel (17 g) using 0-100% EtOAc gradient in hexane as eluent. This procedure gave starting diol 1 (64 mg, 0.38 mmol, conv. 94%), (1*S*,2*S*,3*R*,4*S*,6*R*)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptane-2,3-diol (**4**, 179 mg, 0.973 mmol, 16% calculated on reacted diol **1**) and (1*R*,2*S*,3*R*,4*S*,6*S*)-1-methyl-4-(prop-1-en-2-yl)-7-oxabicyclo[4.1.0]heptane-2,3-diol (**5**, 40 mg, 0.217 mmol, 4%)

Compound **4.** $[\alpha]_D^{24}$ -19.8 (c 1.06, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3/CCl_4$ 1 : 1 (v/v)): δ 1.44 (s, $C^{10}H_3$), 1.77 (s, C^9H_3), 1.88 (ddd, H^{5e}, $J_{5e,5a}$ = 14.7, $J_{5e,6a}$ = 4.5, $J_{5e,4}$ = 2.2 Hz), 2.07 (ddd, H^{5a}, $J_{5a,5e}$ = 14.7, $J_{5a,6a}$ = 12.0, $J_{5a,4}$ = 1.9 Hz), 2.21 (dd, H^{6a}, $J_{6a,5a}$ = 12.0, $J_{6a,5e}$ = 4.5 Hz), 2.29 (d, C^1-OH, $J_{OH,1e}$ = 10.0 Hz), 3.31 (dd, H^4, $J_{4,5e}$ = 2.2, $J_{4,5a}$ = 1.9 Hz), 3.58 (s, H^{2e}), 3.76 (dd, H^{1e}, $J_{1e,OH}$ = 10.0, $J_{1e,2e}$ = 2.5 Hz), 4.79 (br. s, H^8), 4.94 (br. s, $H^{8'}$). ^{13}C NMR (125 MHz, $CDCl_3/CCl_4$ 1 : 1 (v/v)): δ 71.32 (d, C^1), 69.43 (d, C^2), 58.44 (s, C^3), 63.32 (d, C^4), 23.12 (t, C^5), 35.95 (d, C^6), 144.98 (s, C^7), 111.78 (t, C^8), 22.92 (q, C^9), 22.17 (q, C^{10}). HR-MS: 166.1000 (M^+-H_2O, $C_{10}H_{14}O_2$; calc. 166.0988).

Compound **5.** $[\alpha]_D^{26}$ -24.1 (c 0.64, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3/CCl_4$ 1 : 1 (v/v)): δ 1.38 (s, $C^{10}H_3$), 1.75 (s, C^9H_3), 2.02 (dddd, H^{5e}, $J_{5e,5a}$ = 15.8, $J_{5e,6a}$ = 7.5, $J_{5e,4}$ = 5.0, $J_{5e,1e}$ = 0.6 Hz), 2.07 (dd, H^{5a}, $J_{5a,5e}$ = 15.8, $J_{5a,6a}$ = 12.0 Hz), 2.37 (dd, H^{6a}, $J_{6a,5a}$ = 12.0, $J_{6a,5e}$ = 7.5 Hz), 3.09 (d, C^1-OH, $J_{OH,1e}$ = 11.3 Hz), 3.16 (d, H^4, $J_{4,5e}$ = 5.0 Hz), 3.32 (br. s, C^2-OH), 3.62 (dm, H^{3e}, $J_{3e,OH}$ = 11.3 Hz), 3.93 (br. s, H^{2e}), 4.78 (dd, H^8, $J_{8,8'}$ = 1.7, $J_{8,6a}$ = 0.7 Hz), 4.84 (dd, $H^{8'}$, $J_{8',8}$ = 1.7, $J_{8',6a}$ = 1.5

Hz). ^{13}C NMR (125 MHz, $CDCl_3/CCl_4$ 1 : 1 (v/v)): δ 73.39 (d, C^1), 70.19 (d, C^2), 60.83 (s, C^3), 60.43 (d, C^4), 23.67 (t, C^5), 37.42 (d, C^6), 145.24 (s, C^7), 111.74 (t, C^8), 22.01 (q, C^9), 19.85 (q, C^{10}). HR-MS: 184.1100 (M^+, $C_{10}H_{16}O_3$; calc. 184.1094).

*3.2.2. Epoxidation of (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol (***1***) with m-CPBA.*

Method b (Scheme 1). A solution of diol **1** (0.312 g, 1.86 mmol) in CH_2Cl_2 (40 ml) was cooled to 0°C. $NaHCO_3$ (1.55 g, 18.5 mmol) and m-CPBA (m-Cl-C_6H_4-CO_3H, "Acros Organics" 0.589 g, 70-75% of content, ~ 2.5 mmol) were added and the reaction mixture was stored in refrigerator (2 - 5°C) for 1 week. Then water (30 ml) was added and the resulting mixture was filtered and separated. The water phase was extracted with EtOAc (3 x 15 ml) Combined organic layers were washed with saturated $Na_2S_2O_3$ (15 ml) and saturated NaCl (20 ml) and dessicated over Na_2SO_4. The desiccant was filtered off, the solvent was evaporated. The residue (293 mg) was separated by CC on silica gel (9 g) using 0-100% EtOAc gradient in hexane as eluent. This procedure gave starting diol **1** (28 mg, 0.17 mmol, conv. 91%), the epoxide **4** (82 mg, 0.446 mmol, 26% calculated on reacted diol **1**) the epoxide **5** (34 mg, 0.185 mmol, 11%) and the epoxide **7** (10 mg with impurities, 0.04-0.05 mmol, 2-3%).

Method c (Scheme 1). A solution of diol **1** (458 mg, 2.73 mmol) in Et_2O (30 ml) was cooled to 0°C. Then m-CPBA ("Acros Organics" 982 mg, 70-75% of content, ~ 4.2 mmol) was added and the reaction mixture was stored in refrigerator (2 - 5°C) for 3 week. The solvent was evaporated and the residue was separated by CC on silica gel (17 g) using 0-100% EtOAc gradient in hexane as eluent. This procedure gave the epoxide **4** (161 mg, 0.875 mmol, 32%) epoxide **5** (49 mg, 0.266 mmol, 10%) and 230 mg (1.15 mmol, 42%) of the mixture of four stereoisomeric diepoxides **8**. The formation of diepoxides is confirmed by the signals of oxirane cycle at 2.5 – 3.5 ppm and by the absence of signals related to double bounds at 4.6 – 6 ppm in ^1H NMR spectra.

3.3. (1R,2R,6R)-3-methyl-6-((R)-2-methyloxiran-2-yl)cyclohex-3-ene-1,2-diol (6) and (1R,2R,6R)-3-methyl-6-((S)-2-methyloxiran-2-yl)cyclohex-3-ene-1,2-diol (7).

*3.3.1. Interaction of diol **1** with iodine.* Iodine (811.4 mg, 3.197 mmol) was grinded in a mortar and dissolved in EtOAc (5 ml). Acetic acid (1.0 ml) and water (3 ml) were added. Then a solution of (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diol (**1**, 523.6 mg, 3.113 mmol) in 10 ml EtOAc was added. The reaction mixture was stirred for 5 days at r.t. Then it was washed with saturated solutions of $NaHCO_3$ (2x20 ml, add slowly) and $Na_2S_2O_3$ (10 ml).The organic phase was desiccated over Na_2SO_4. The desiccant was filtered off, the solvent was evaporated. The residue (682.8 mg) was separated by CC on silica gel (20 g) using 0-100% EtOAc gradient in hexane as eluent. This procedure gave starting diol **1** (326.3 mg, 1.940 mmol, conv. 62%), (1*R*,2*R*,6*R*)-6-((*S*)-2-hydroxy-1-iodopropan-2-yl)-3-methylcyclohex-3-ene-1,2-diol (**9**, 176.1 mg, 0.564 mmol, 29% calculated on consumed diol **1**), (1*R*,2*R*,6*R*)-6-((*R*)-2-hydroxy-1-iodopropan-2-yl)-3-methylcyclohex-3-ene-1,2-diol (**10**, 69.4 mg, 0.208 mmol, 11% calculated on reacted diol **1**), (1*R*,5*S*,6*R*)-6-hydroxy-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-enyl acetate (**11**, 48.2 mg, 0.229 mmol, 12%) and (1*R*,2*R*,6*S*)-2-hydroxy-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-enyl acetate (**12**, 17.9 mg, 0.085 mmol, 4%).

Compound **9.** ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.52 (s, C^9H_3), 1.73 (br. d, C^2-OH, $J_{OH,2e}$ = 5.0 Hz), 1.82 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 2.00 – 2.09 (m, H^{5e}, H^{6a}), 2.25 – 2.35 (m, H^{5a}), 2.90 (br. s, C^7-OH), 3.01 (br. d, C^1-OH, $J_{OH,1e}$ = 3.4 Hz), 3.24 (d, H^8, ^2J= 10.1 Hz), 3.42 (d, $H^{8'}$, ^2J= 10.1 Hz), 3.81 (br. dd, H^{2e}, $J_{2e,OH}$ = 5.0, $J_{2e,1e}$ = 3.0 Hz), 4.30 (ddd, H^{1e}, $J_{1e,OH}$ = 3.4, $J_{1e,2e}$ = 3.0, $J_{1e,6a}$ = 1.5 Hz), 5.65 – 5.69 (m, H^4). ^{13}C NMR (125 MHz, $CDCl_3$): δ 71.26 (d, C^1), 72.08 (d, C^2), 131.14 (s, C^3), 125.16 (d, C^4), 20.71 (t, C^5), 37.37 (d, C^6), 73.44 (s, C^7), 18.39 (t, C^8), 27.15 (q, C^9), 20.87 (q, C^{10}). HR-MS: 294.0109 (M^+-H_2O, $C_{10}H_{15}O_2I$; calc. 294.0111).

Compound **10.** $[\alpha]_D^{26}$ -36.7 (c 0.463, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3/CD_3OD$): δ 1.30 (s, C^9H_3), 1.75 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 2.01 (dddq, H^{5e}, ^2J = 17.6, $J_{5e,6a}$ 5.6, $J_{5e,4}$ = 5.3, $J_{5e,10}$ = 1.6 Hz), 2.12 (ddd, H^{6a}, $J_{6a,5a}$ = 11.3, $J_{6a,5e}$ = 5.6, $J_{6a,1e}$ = 1.8 Hz), 2.26 (dddqd, H^{5a}, ^2J = 17.6, $J_{5a,6a}$ = 11.3, $J_{5a,4} = J_{5a,10}$ = 2.5, $J_{5a,2e}$ = 1.3 Hz), 3.35 (dd, H^8, ^2J = 10.2, $J_{8,9}$ = 0.6 Hz), 3.39 (d, $H^{8'}$, ^2J = 10.2 Hz), 3.64 (br. d, H^{2e}, $J_{2e,1e}$ = 2.7 Hz), 4.10 (dd, H^{1e}, $J_{1e,2e}$ = 2.7, $J_{1e,6a}$ = 1.8 Hz), 5.62 (ddq, H^4, $J_{4,5e}$ = 5.3, $J_{4,5a}$ = 2.5, $J_{4,10}$ = 1.5 Hz). ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$): δ 70.40 (d, C^1), 71.99 (d, C^2), 130.85 (s, C^3), 125.18 (d, C^4), 21.66 (t, C^5), 36.17 (d, C^6), 73.04 (s, C^7), 17.79 (t, C^8), 25.19 (q, C^9), 20.73 (q, C^{10}). HR-MS: 294.0110 (M^+-H_2O, $C_{10}H_{15}O_2I$; calc. 294.0111).

Compound **11.** $[\alpha]_D^{26}$ -65.3 (c 1.28, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 1.67 (d, C^1-OH, $J_{OH,1e}$ = 4.0 Hz), 1.69 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 1.79 (m, C^9H_3, all J < 2.0 Hz), 2.03 (dddq, H^{5e}, ^2J = 17.3, $J_{5e,4}$ = 5.3, $J_{5e,6a}$ = 4.6, $J_{5e,10}$ = 1.5 Hz), 2.08 (s, $C^{12}H_3$), 2.26 (dddqd, H^{5a}, ^2J = 17.3, $J_{5a,6a}$ = 11.4, $J_{5a,4} = J_{5a,10}$ = 2.5, $J_{5a,2e}$ = 1.5 Hz), 2.33 (br. dd, H^{6a}, $J_{6a,5a}$ = 11.4, $J_{6a,5e}$ = 4.6 Hz), 3.91 (ddd, H^{1e}, $J_{1e,OH}$ = 4.0, $J_{1e,2e}$ = 2.6, $J_{1e,6a}$ = 1.7 Hz), 4.85 (m, H^8, all J < 2.0 Hz), 4.96 (m, $H^{8'}$, all J < 2.0 Hz), 5.10 – 5.12 (m, H^{2e}, all J < 3.0 Hz), 5.76 – 5.79 (m, H^4). ^{13}C NMR (125 MHz, $CDCl_3$): δ 68.57 (d, C^1), 73.00 (d, C^2), 128.32 (s, C^3), 127.57 (d, C^4), 24.28 (t, C^5), 40.50 (d, C^6), 145.17 (s, C^7), 111.59 (t, C^8), 22.36 (q, C^9), 20.52 (q, C^{10}), 170.45 (s, C^{11}), 21.01 (q, C^{12}). HR-MS: 168.1149 (M^+, $C_{10}H_{16}O_2$; calc. 168.1145). HR-MS: 168.1145 (M^+-C_2H_2O, $C_{10}H_{16}O_2$; calc. 168.1145).

Compound **12.** $[\alpha]_D^{26}$ -49.4 (c 1.04, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$): δ 1.77 (m, C^9H_3, all J < 2.0 Hz), 1.78 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 1.98 (s, $C^{12}H_3$), 2.03 (dddq, H^{5e}, ^2J = 17.6, $J_{5e,6a}$ = $J_{5e,4}$ = 5.3, $J_{5e,10}$ = 1.5 Hz), 2.27 (dddqd, H^{5a}, ^2J = 17.6, $J_{5a,6a}$ = 11.2, $J_{5a,4} = J_{5a,10}$ = 2.5, $J_{5a,2e}$ = 1.4 Hz), 2.35 (br. d, C^2-OH, $J_{OH,2e} \approx$ 5.0 Hz), 2.47 (br. dd, H^{6a}, $J_{6a,5a}$ = 11.2, $J_{6a,5e}$ = 5.3 Hz), 3.80 – 3.84 (m, H^{2e}), 4.72 (m, H^8, all J < 2.0 Hz), 4.83 (m, $H^{8'}$, all J < 2.0 Hz), 5.12 (dd, H^{1e}, $J_{1e,2e}$ = 2.8, $J_{1e,6a}$ = 1.7 Hz), 5.65 – 5.69 (m, H^4). ^{13}C NMR (125 MHz, $CDCl_3$): δ 73.50 (d, C^1), 69.79 (d, C^2), 131.26 (s,

C^3), 125.61 (d, C^4), 25.35 (t, C^5), 38.08 (d, C^6), 144.81 (s, C^7), 111.12 (t, C^8), 22.19 (q, C^9), 20.54 (q, C^{10}), 170.70 (s, C^{11}), 20.88 (q, C^{12}). HR-MS: 193.1223 (M^+-OH, $C_{12}H_{17}O_2$; calc. 193.1223).

*3.3.2. Interaction of diacetate **13** with iodine.* Iodine (247.8 mg, 0.976 mmol) was grinded in a mortar and dissolved in EtOAc (3 ml). Acetic acid (0.50 ml) and water (1.5 ml) were added. Then a solution of (1*R*,2*R*,6*S*)-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-ene-1,2-diyl diacetate (**13**, 248.7 mg, 0.986 mmol) in 5 ml EtOAc was added. The reaction mixture was stirred for 5 days at r.t. Then it was washed with saturated solutions of $NaHCO_3$ (2x10 ml, add slowly) and $Na_2S_2O_3$ (3 ml).The organic phase was desiccated over Na_2SO_4. The desiccant was filtered off, the solvent was evaporated. The residue (320.9 mg) was separated by CC on silica gel (9 g) using 0-100% EtOAc gradient in hexane as eluent. This procedure gave starting diacetate **13** (77.4 mg, 0.307 mmol, conv. 69%), (1*R*,2*R*,6*R*)-6-((*S*)-2-hydroxy-1-iodopropan-2-yl)-3-methylcyclohex-3-ene-1,2-diyl diacetate (**14**, 108.7 mg, summary formed 116.0 mg, 0.293 mmol, 43% calculated on reacted diacetate **13**), mixture of (1*R*,2*R*,6*R*)-6-((*R*)-2-hydroxy-1-iodopropan-2-yl)-3-methylcyclohex-3-ene-1,2-diol (**14**) and epimeric compound **15** (24.1 mg, **15** : **13** = 2.3 : 1, the content of **15** was 16.8 mg, 0.0424 mmol, 6% calculated on reacted diol **1**), (1*R*,2*R*,6*S*)-2-hydroxy-3-methyl-6-(prop-1-en-2-yl)cyclohex-3-enyl acetate (**12**, 16.0 mg, 0.0761 mmol, 11%) and (1*R*,5*S*,6*R*)-6-hydroxy-2-methyl-5-(prop-1-en-2-yl)cyclohex-2-enyl acetate (**11**, 3.3 mg, 0.0157 mmol, 2%).

Compound **14.** ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.27 (s, C^9H_3), 1.65 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 1.93 (br. s, C^7-OH), 2.00 (s, $C^{12}H_3$), 2.08 (s, $C^{14}H_3$), 2.15 – 2.28 (m, $2H^5$, H^{6a}), 3.36 (d, H^8, ^2J= 10.5 Hz), 3.46 (d, $H^{8'}$, ^2J= 10.5 Hz), 4.96 (br. d, H^{2e}, $J_{2e,1e}$ = 2.7 Hz), 5.17 (dd, H^{1e}, $J_{1e,2e}$ = 2.7, $J_{1e,6a}$ = 1.0 Hz), 5.79 – 5.82 (m, H^4). ^{13}C NMR (125 MHz, $CDCl_3$): δ 68.61 (d, C^1), 70.50 (d, C^2), 128.20 (s, C^3), 127.82 (d, C^4), 21.69 (t, C^5), 40.48 (d, C^6), 70.94 (s, C^7), 21.82 (t, C^8), 23.53 (q, C^9), 20.35 (q, C^{10}), 169.89 (s, C^{11}), 21.01 (q, C^{12}), 169.73 (s, C^{13}), 20.90 (q, C^{14}). HR-MS: 336.2016 (M^+, $C_{14}H_{21}O_5I$; calc. 336.2017).

Compound **15**. The NMR spectra of compound **15** were recorded for the mixture of isomers **15** and **14** in a ratio 2.3 : 1. Data for **15**: ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.30 (s, $C^{9}H_3$), 1.65 – 1.69 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 2.02 (s, $C^{12}H_3$), 2.09(s, $C^{14}H_3$), 2.07 – 2.14 (m, H^{5e}), 2.18 – 2.23 (m, H^{5a}), 2.27 (ddd, H^{6a}, $J_{6a,5a}$ = 11.6, $J_{6a,5e}$ = 4.7, $J_{6a,1e}$ = 1.2 Hz), 3.35 (d, H^{8}, ^{2}J= 10.3 Hz), 3.38 (d, $H^{8'}$, ^{2}J= 10.3 Hz), 5.05 (br. d, H^{2e}, $J_{2e,1e}$ = 2.7 Hz), 5.30 (ddd, H^{1e}, $J_{1e,2e}$ = 2.7, $J_{1e,6a}$ = 1.2, $J_{1e,5e}$ = 0.7 Hz), 5.78 – 5.81 (m, H^{4}). ^{13}C NMR (125 MHz, $CDCl_3$): δ 68.76 (d, C^{1}), 70.22 (d, C^{2}), 128.48 (s, C^{3}), 127.54 (d, C^{4}), 22.49 (t, C^{5}), 39.27 (d, C^{6}), 71.31 (s, C^{7}), 19.71 (t, C^{8}), 24.85 (q, C^{9}), 20.45 (q, C^{10}), 170.18 (s, C^{11}), 21.15 (q, C^{12}), 169.76 (s, C^{13}), 20.94 (q, C^{14}).

3.3.3. Epoxide ***6***. A solution of (1*R*,2*R*,6*R*)-6-((*R*)-2-hydroxy-1-iodopropan-2-yl)-3-methylcyclohex-3-ene-1,2-diol (**10**, 19.3 mg, 0.0618 mmol) in dioxane (5 ml) was added to water (3 ml). Then *N*,*N*-diisopropylethylamine (57 μL, 0.327 mmol) was added. The reaction mixture was stirred for 24 h, then concentrated to 1 ml and diluted with 5 ml saturated NaCl. It was extracted with ethylacetate (3 x 3 ml). The combined organic phase was desiccated over Na_2SO_4. The desiccant was filtered off, the solvent was evaporated. The residue (15.5 mg) was purified by CC on silica gel (2.5 g) using 0-100% EtOAc gradient in hexane as eluent. This procedure gave (1*R*,2*R*,6*R*)-3-methyl-6-((*R*)-2-methyloxiran-2-yl)cyclohex-3-ene-1,2-diol (**6**, 10.0 mg, 0.0543 mmol, 88%).

Epoxide (*R*)-**6.** $[\alpha]_D^{29}$ -74.4 (c 0.597, $CHCl_3$). ^{1}H NMR: δ 1.38 (s, $C^{9}H_3$), 1.80 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 2.11 (ddd, H^{6a}, $J_{6a,5a}$ = 10.0 Hz, $J_{6a,5e}$ = 6.7 Hz, $J_{6a,1e}$ = 1.6 Hz), 2.16 – 2.21 (m, $2H^{5}$), 2.66 (d, H^{8ex}, $J_{8ex,8en}$ = 3.9 Hz), 3.16 (br.d, H^{8en}, $J_{8en,8ex}$ = 3.9 Hz), 3.79 (br.d, H^{2e}, $J_{2e,1e}$ = 2.8 Hz), 3.90 (br. m, H^{1e}, all ≤ 2.8 Hz), 5.65 – 5.68 (m, H^{4}). ^{13}C NMR: δ 70.74 (d, C^{1}), 71.68 (d, C^{2}), 131.76 (s, C^{3}), 124.87 (d, C^{4}), 22.82 (t, C^{5}), 34.98 (d, C^{6}), 60.05 (s, C^{7}), 52.46 (t, C^{8}), 20.87 (q, C^{9}), 20.80 (q, C^{10}). HR-MS: 166.0982 (M^{+}-C_2H_2O, $C_{10}H_{14}O_2$; calc. 166.0988).

3.3.4. Epoxide 7. A solution of (1*R*,2*R*,6*R*)-6-((*S*)-2-hydroxy-1-iodopropan-2-yl)-3-methylcyclohex-3-ene-1,2-diyl diacetate (**14**, 108.3 mg, 0.273 mmol) in dioxane (10 ml) was added to water (6 ml). Then *N*,*N*-diisopropylethylamine (0.200 ml, 1.148 mmol) was added. The reaction mixture was stirred for 24 h, then NaOH (51.3 mg, 1.128 mmol) was added. The mixture was stirred 16 h more, then concentrated to 1 ml and diluted with 5 ml saturated NaCl. It was extracted with ethylacetate (6 x 3 ml). The combined organic phase was desiccated over Na_2SO_4. The desiccant was filtered off, the solvent was evaporated. The residue (57.1 mg) was purified by CC on silica gel (4.5 g) using 0-100% EtOAc gradient in hexane as eluent. This procedure gave (1*R*,2*R*,6*R*)-3-methyl-6-((*S*)-2-methyloxiran-2-yl)cyclohex-3-ene-1,2-diol (**7**, 42.4 mg, 0.230 mmol, 84%).

Epoxide (*S*)-**7.** $[\alpha]_D^{26}$ -76.6 (c 0.950, $CHCl_3$). ^{1}H NMR: δ 1.42 (d, C^9H_3, $J_{9,8en}$ = 0.6 Hz), 1.79 (m, $C^{10}H_3$, all J ≤ 2.5 Hz), 1.87 (dddq, H^{5e}, $J_{5e,5a}$ = 16.8 Hz, $J_{5e,4}$ = $J_{5e,6a}$ = 5.2 Hz, $J_{5e,10\text{-}Me}$ = 1.6 Hz), 1.92 (br.s, OH-C^2), 1.96 (ddm, H^{5a}, $J_{5a,5e}$ = 16.8 Hz, $J_{5a,6a}$ = 11.1 Hz), 2.01 (ddd, H^{6a}, $J_{6a,5a}$ = 11.1 Hz, $J_{6a,5e}$ = 5.2 Hz, $J_{6a,1e}$ = 1.6 Hz), 2.47 (br.d, OH-C^1, $J_{OH,1e}$ = 3.4 Hz), 2.51 (d, H^{8ex}, $J_{8ex,8en}$ = 4.4 Hz), 2.81 (dq, H^{8en}, $J_{8en,8ex}$ = 4.4 Hz, $J_{8en,9\text{-}Me}$ = 0.6 Hz), 3.81 (br.s, H^{2e}), 4.17 (ddd, H^{1e}, $J_{1e,OH}$ = 3.4 Hz, $J_{1e,2e}$ = 2.8 Hz, $J_{1e,6a}$ = 1.6 Hz), 5.59 (ddq, H^4, $J_{4,5e}$ = 5.2 Hz, $J_{4,5a}$ = 2.2 Hz, $J_{4,10}$ = 1.5 Hz). ^{13}C NMR: δ 71.16 (d, C^1), 71.85 (d, C^2), 131.85 (s, C^3), 124.49 (d, C^4), 22.24 (t, C^5), 35.88 (d, C^6), 58.92 (s, C^7), 51.30 (t, C^8), 21.19 (q, C^9), 20.82 (q, C^{10}). HR-MS: 166.0986 (M^+-C_2H_2O, $C_{10}H_{14}O_2$; calc. 166.0988).

3.5. Animals. The experiments were performed on C57BL/6 mice (male) weighing 25-30 g and Wistar rats (male) weighing 200-220 g purcahsed from SPF-vivarium of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. The animals were maintained at 22-25 °C on a 12 h light-dark cycle with food and water available *ad libitum*. All work with animals was guided by 3R principles and performed in strict accordance with the legislation of

the Russian Federation, the regulations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the requirements and recommendations of the Guide for the Care and Use of Laboratory Animals. The use of experimental animals for isolation of primary cell cultures was approved by the Committee for Animal Experiments of the University of Helsinki (License number: KEK15-022) and these animals were treated according European guidelines and regulations of the State of Finland.

3.6. The MPTP mouse model of Parkinson's disease induced by the fourfold administration of the MPTP neurotoxin.

MPTP was injected intraperitoneally to mice of C57Bl/6 line every 2 h in 8 h period in one day in a dose of 0.12 mmol/kg (20 mg/kg) for a total of four doses. The agents were dissolved in saline containing 0.5% Tween 80 just before use and were administered per os, 1 hour before testing. The studied agent or saline was administrated per os 24 h after the last injection of MPTP in a dose of 0.12 mmol/kg (20 mg/kg). The effectiveness of the studied medications was evaluated according to their ability to reduce the symptoms of hypokinesia induced by MPTP, 2 h after the administration of the agents. Hypokinesia caused by neurotoxin administration was evaluated with the "Open field" test performed for 2 min using Tru Scan (USA) 2 h after the administration of the studied agent, registering the main markers of the locomotor activity: movement distance (cm) and duration of locomotor activity (s). The data were analyzed using ANOVA with Dunnett's *posthoc* test in GraphPad Prism 6.0 software

3.7. Influence of compound 4 on naïve mice

The studied agent in a dose of 0.12 mmol/kg (20 mg/kg) or saline was administrated per os to 8 mice of C57Bl/6 line. The main markers of the locomotor activity (movement distance (cm) and

duration of locomotor activity (s)) were registered with the "Open field" test performed for 2 min using Tru Scan (USA), 2 h after the administration of the studied agent.

3.8. Acute toxicity of compound 4.[22]

Acute toxicity studies were performed on non-breeding albino mice weighting 20–25 g (six animals in the group). The agents were dissolved in saline containing 0.5% Tween 80 just before use and administered per os in single dose of 1000 mg/kg. The toxicity was evaluated from the clinical picture of poisoning and survival of animals for 10 days. The acute toxicity studies of **4** in the stated doses did not reveal the clinical picture of poisoning in animals; all animals survived.

3.9. Determination of diol 1 and epoxide 4 in plasma and brain

To identify possible metabolites, diol **1** was administred *p.o*. to Wistar rat in 1200 mg/kg dose. Blood sample was collected over 1 h period post administration. Plasma was isolated and, after protein precipitation with methanol and extraction with ethylacetate, was analyzed using GC/MS. Compound **1** (695 μg/mL) and epoxide **4** (3.2 μg/mL) were identified.

To determine the ability of epoxide **4** to penetrate blood brain barrier, it was administred *p.o.* to Wistar rats (10 mg/kg). Blood samples were collected over a 1.5 h period post administration. Plasma was isolated and the concentration of compounds in plasma was determined by GC/MS after protein precipitation with methanol followed by extraction with ethylacetate. Brain was homogenized in 40% ethanol, filtred and then treated as it was for plasma. Ethylacetate extracts were analyzed using GC/MS. Compound **4** was found both in plasma (5.3 μg/mL) and in brain (3.5 μg/g).

3.10. Preparation of the cultures of primary dopamine neurons.

Cultures of primary dopamine (DA) neurons were prepared from E13.5 embryos of NMR1 mice.[48,50] The embryonic midbrain floors were dissected in Dulbecco's media (0.1 g/l $MgCl_2.6H_2O$, 0.1 g/l $CaCl_2$, 8 g/l NaCl, 0.2 g/l KCl, 1.4 g/l $Na_2HPO_4.2H_2O$, 0.2 g/l KH_2PO_4) containing 2% BSA under dissection microscope (Olympus SZX10 Stereo Microscope), washed three times with Calcium and Magnesium-free Hank's Balanced Salt Solution (HBSS: Gibco, Life Technologies) and incubated in 5 mg/ml trypsin solution in HBSS for 20 minutes at 37°C. Enzymatic activity of trypsin was blocked by 50% Fetal Bovine Serum. To reduce the viscosity, the solution was also treated with DNase I (10 mg/ml). Cells were dissociated by trituration with siliconized glass Pasteur pipette and washed 3 times with cell culture media [(Dulbecco's MEM/Nut mix F12 (Invitrogen/Gibco 21331-020), 1xN_2 serum supplement (Invitrogen/ Gibco 17502-048), 33 mM D-Glucose (Sigma G-8769), 0.5 mM L-Glutamine (Invitrogen/Gibco 25030-032), and 100 μg/ml Primocin (InvivoGen)]. Resulting pellets were resuspended in culture media and the number of alive cells (detected by trypan-blue staining) was counted using TC20 automated cell counter (BIO-RAD). About 30,000 cells were plated per well of 96-well Costar plate coated with Poly-L-ornithine (0.5 mg/ml in 0.15M borate buffer pH 8.7, overnight at 4°C). Different concentrations of compound **4** (0.01, 0.1, 1, 10 uM) and GDNF (Icosagen, 10 ng/ml) were dissolved in culture media containing 1% Dimethyl Sulfoxide (DMSO) and applied to the wells (in triplicates) within 1 hour post-plating. The cells were incubated for 5 days in cell culture incubator at 37°C. Half of the cell culture media was replaced with fresh portion 2.5 days post-plating.

To evaluate neuroprotective properties of epoxide **4** in the presence of dopamine neuron toxin (6-OHDA), dopamine neurons were cultured for 5 days in cell incubator at 37°C and treated with 6-OHDA (10 μM) along with compound **4** (0.1 μM and 1 μM) for another 3 days. GDNF (50 ng/ml) was used as a positive control. In these experiments midbrain neurons were plated in higher density (40,000 cells per well of 96-well Costar plate) to prevent spontaneous death of TH-positive

neurons in culture[48] and ensure that there is enough dopaminergic neurons for reliable statistical analysis on 8 DIV.

3.11. Tyrosine Hydoxylase and gamma-Aminobutyric acid Immunocytochemistry

Cells were fixed with 4% PFA for 20 minutes and washed three times with PBS followed by permeabilization with 0.2% Triton X-100 in PBS for 15 minutes. The cells were blocked by 5% horse serum in 0.2% Triton X-100 in PBS for 1 hour and incubated overnight at 4°C with anti-tyrosine hydroxylase (TH) antibody (1:500 in blocking solution,[50] Millipore Cat# MAB318 Lot# RRID:AB_2201528) and anti-γ-aminobutyric acid (GABA) antibody (Catalogue number: ABN131 Chemicon/Merck., diluted 1:500 in blocking solution). Cells were washed three times with PBS and incubated with secondary antibody conjugated with Alexa Fluor 647 (diluted 1:500 in blocking solution) for 1 hour at room temperature. Unbound antibody was washed out with PBS and nuclei were stained with 0.2 μg/ml DAPI (4',6-diamidino-2-phenylindole) in PBS for 10 minutes at room temperature. Finally, cells were washed three times with PBS and kept in PBS until imaging.

3.12. Imaging and Statistical Analysis

Cells were imaged by CellInsight CX5 High Content Screening (HCS) equipment (Life Technologies). Images were analysed using CellProfiler image analysis software (BROAD Institute). Compound was analysed in triplicates in four independent experiments.[50]

3.13. Primary Mesencephalic Embryonic Fibroblasts (MEF) and analysis of cell viability by Alamar blue assay

Cultures of primary mesencephalic embryonic fibroblasts (MEF) were prepared from E13.5 embryos of NMR1 mice as described previously with slight modifications.[51] Mouse was killed by

exposure to CO_2 and subsequent cervical dislocation. Embryos were dissected from uterus. Head and red organs were discarded, and remaining portions of embryos were placed in separate clean dish containing PBS. The tissues were chopped into small pieces with the help of sterile razor blade. Tissues were washed three times with Calcium and Magnesium-free Hank's Balanced Salt Solution (HBSS: Gibco, Life Technologies) and incubated in 5 mg/ml trypsin solution in HBSS for 20 minutes at 37^{o}C. About two volumes of MEF medium [DMEM (Cat N 21063-029, Gibco), 10% FBS, 1 % L-glutamine (Cat N 25030-032, Invitrogen/ Gibco), 0.2 % Normacin)] was added and then the tissues were triturated three times using Pasteur pipettes. The cells were pelleted by centrifugation, resuspended in warm fresh MEF medium and plated in 10 cm dish. Upon reaching confluency, the cells were collected, distributed to several cryogenic vials and frozen for further experiments.

Analysis of cell viability in MEFs was carried out using method described by Nakayama and co-authors.[52] For each experiment a new vial of MEFs was melted and cells were plated on 96 well (OptiPlate 96 F HB, Wallac) plate. Next day, various concentration of epoxide **4** were applied in media containing DMEM, 2 % DMSO and 30 mM HEPES. After three days of culturing in presence of epoxide **4**, Alamar blue dissolved in cell culture media in ratio of 1:10 was added to the cultured cells. The fluorescence was measured using FLUOstar Omega Multi-Mode Microplate Reader (BMG LABTECH) four hours post treatment with Alamar Blue. Experiments were performed in quadruplicates and repeated 3 times. The data presented in Fig. 6B are from the single experiment, but were reproduced 3 times.

3.14. Analysis of RET, TrkB, ERK and Akt activation by compound 4.

The level of phosphorylated RET, TrkB, Erk and Akt was evaluated using methods described previously.[53,54]

3.14.1. Cell lines

MG87RET murine fibroblasts stably transfected with RET oncogene,[55] MG87TrkB murine fibroblasts stably transfected with TrkB receptor tyrosine kinase, [56,57] and Neuro2A cells (ATCC) were used to study the intracellular signaling in response to compound **4**.

MG87RET cells were transfected with hGFRα1 (Full-length human GFRα1 cDNA subcloned in pCDNA6 (Invitrogen, USA))[58] using Lipofectamine 2000 (Invitrogen), as described by the manufacturer.

3.14.2. Sample preparation

To assess the levels of RET and TrkB phosphorylation MG87RET/GFRα1 or MG87TrkB cells were plated into 6-well plates in DMEM and cultured overnight in DMEM, 10% FBS, normocin. Next day cells were starved for 4 hours in DMEM, 1% DMSO, 15 mM Hepes pH = 7.2, treated with compound **4** or positive control proteins GDNF and BDNF for 15 min, washed with ice-cold PBS (phosphate buffered saline, pH 7.4; 137 mM NaCl, 2.7 mM KCl, 10 mM PO_4^{3-}) containing 1 mM Na_3VO_4 and lysed in RIPA-modified buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% TX-100, 10% glycerol, EDTA-free protease inhibitor cocktail (Roche, Switzerland), 1 mM Na_3VO_4, 6 mM sodium deoxycholate, 1 mM PMSF). To assess the levels phosphorylated Erk and Akt, MG87RET/GFRα1 or Neuro2A cells were plated into 48 –well plates, cultured and starved as described above. Then, the cells were treated with compound **4** (100 μM) or GDNF (MG87RET/GFRα1) and washed with ice-cold PBS containing 1 mM Na_3VO_4 and 1 mM NaF. Cells were lysed by adding Laemmli loading buffer.

3.14.3. Analysis of RET phosphorylation by Western blotting

The level of RET phosphorylation in the cells was analyzed by Western blotting of immunoprecipitated RET with phospho-tyrosine-specific antibody (1:1500, Merck Millipore Cat# 05-321, RRID:AB_309678) as described previously.[58] Equal loading was confirmed by re-probing

the membrane with anti-RET C-20 antibody (1:500, Santa Cruz Biotechnology Cat# sc-1290, RRID:AB_631316).

3.14.4. Western blotting-based ERK and AKT phosphorylation assay

Levels of pERK and pAKT in immortalized cells were analyzed by Western blotting using E4 pERK (1:1000, Santa Cruz Biotechnology Cat# sc-7383, RRID:AB_627545) and pAKT (1:500, Cell Signaling Technology Cat# 9271, RRID:AB_329825) antibodies as described previously.[53] Membranes were probed with GAPDH antibody (1:4000, Millipore Cat# MAB374, RRID:AB_2107445) to confirm equal loading. Western blotting images of phosphorylated ERK and AKT from Neuro2A cells were quantified using Image studio-lite (LI-COR Biosciences, UK) software. The intensity of staining of each band (normalized to area) was divided by the intensity of GAPDH band (normalized to area) for each individual lane on the gel. The data for different time points were averaged and subjected to statistical analysis using paired t-test in GraphPad Prism v6.

3.15. Assessment of neurorestorative properties of epoxide 4 *in vivo*

3.15.1. Sample collection and Tissue Processing.

MPTP was injected intraperitoneally to mice of C57Bl/6 line every 2 h an 8 h period in one day in a dose of 0.12 mmol/kg (20 mg/kg) for a total of four doses. Epoxide **4** or saline were administrated per os 24 h after the last injection of MPTP in doses of 0.12 mmol/kg (20 mg/kg) or 0.03 mmol/kg (5 mg/kg). Then, epoxide **4** or saline were injected for 14 days in 5 day per week regime, for a total of fifteen doses. The samples were collected 7 days after the last administration of epoxide **4**. Mice were perfused trans-cardially with PBS and then 4% paraformaldehyde in PBS in accordance with,[59] the brains were collected, treated and embedded into paraffin blocks using histological complex Microm (Germany).

3.15.2. Immunohistochemistry.

To analyze the effect of epoxide **4** in dopamine neurons *in vivo*, tyrosine hydroxylase immunohistochemistry was performed as described previously.[60] The PFA-fixed and paraffin-embedded brains were cut into 5 μM thick sections (3 sections per slide) and every 5th slide was taken for TH staining. The sections were deparaffinized followed by citrate antigen retrieval procedure. Endogenous peroxidase was inactivated by 30 min incubation with 3% H_2O_2. The sections were blocked with 5% normal goat serum and then probed with monoclonal TH antibody (1:2,000, Millipore Cat# MAB318 Lot# RRID:AB_2201528) overnight at +4°C. The sections were washed, and biotinylated horse antimouse secondary antibody (1:200, Cat# BA-2001, Vector Laboratories, Burlingame, CA, USA) was applied for 1 hour followed by washing. The sections were treated with ABC and DAB staining kits (Vector Laboratories, CA, USA) according to manufacturer's instructions to visualize bound antibodies.

3.16. Optical density and TH positive neurons

The sections were scanned with an automated scanner (3DHistech, Budapest, Hungary; http://www.biocenter.helsinki.fi/bi/histoscanner/index.html), and the images were converted to 16-bit gray scale. The signal from cortical staining was used to measure nonspecific background staining. The integrated optical densities in images were measured in ImageJ (NIH) and divided by area in pixels.

3.17. Statistical Analysis. Statistical analysis of the data was performed using one-way ANOVA with a Dunnett's posthoc test for the comparison of multiple treatment groups or paired t-test in Graphpad Prism v6 software (La Jolla, CA, USA). All data are presented as mean ± SEM. P – values below 0.05 were considered to indicate statistically significant differences between groups. No exclusions were made from the data from behavioral tests. One outlier was excluded from the vehicle-treated group in the experiments assessing the survival of dopamine neurons in response to compound **4** using Grubb's test. No other exclusion were made in any of the experiments.

Supporting information: ^{1}H and ^{13}C NMR spectra for epoxides **4-7**.

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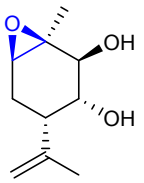

High anti-Parkinsonian activity *in vivo*

Protection of cultured dopamine neurons *in vitro*

Restoration the dopamine innervation in striatum of MPTP-treated mice

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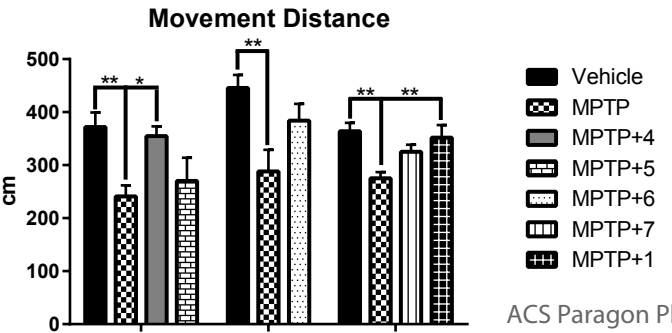

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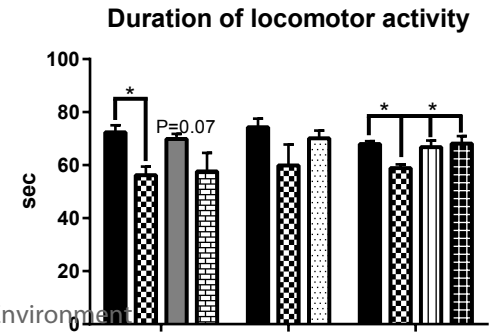
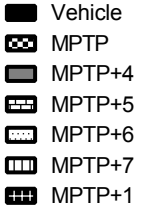
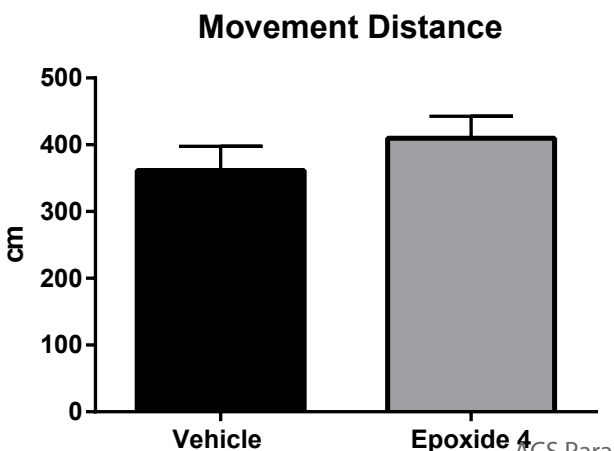
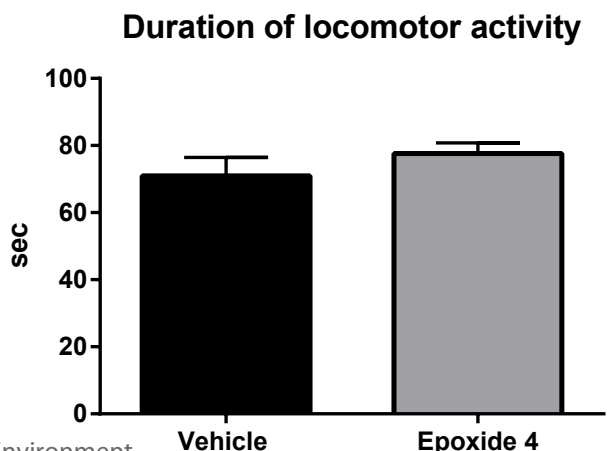
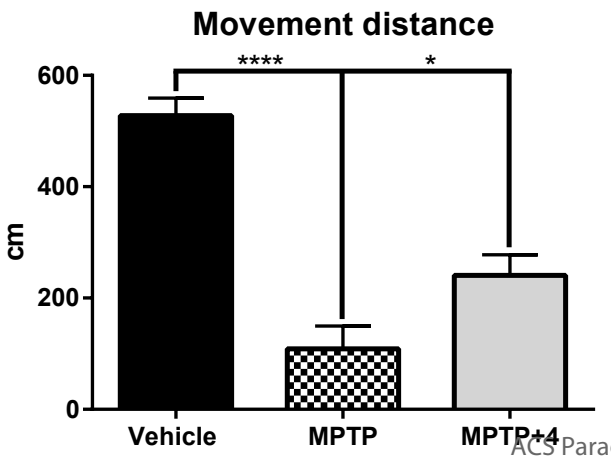
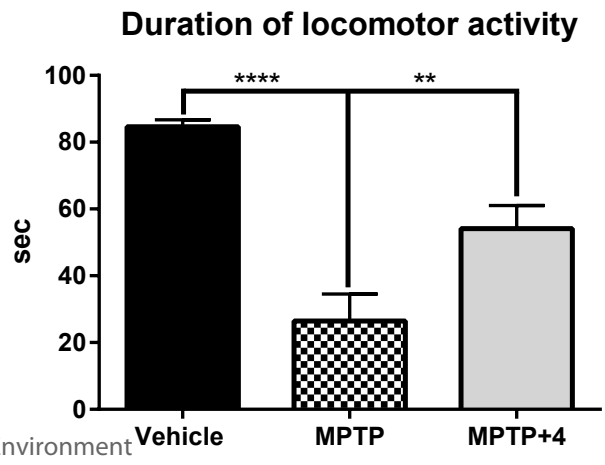


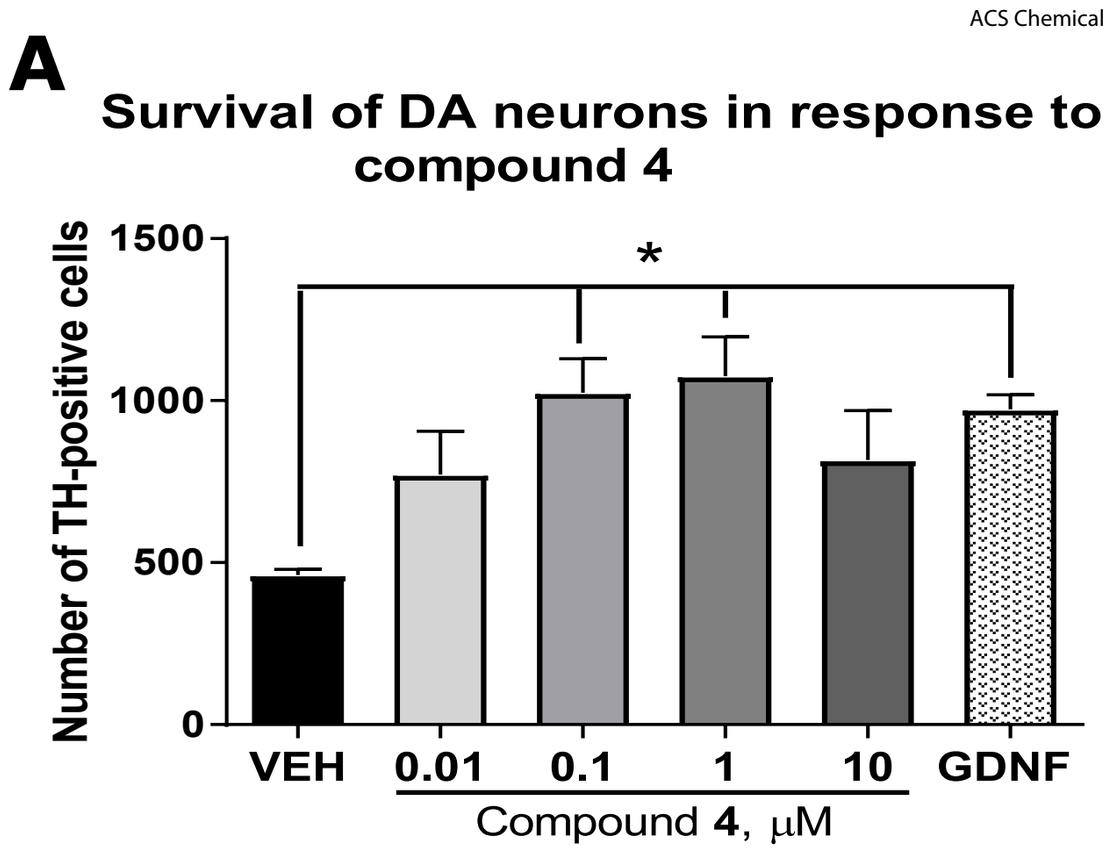


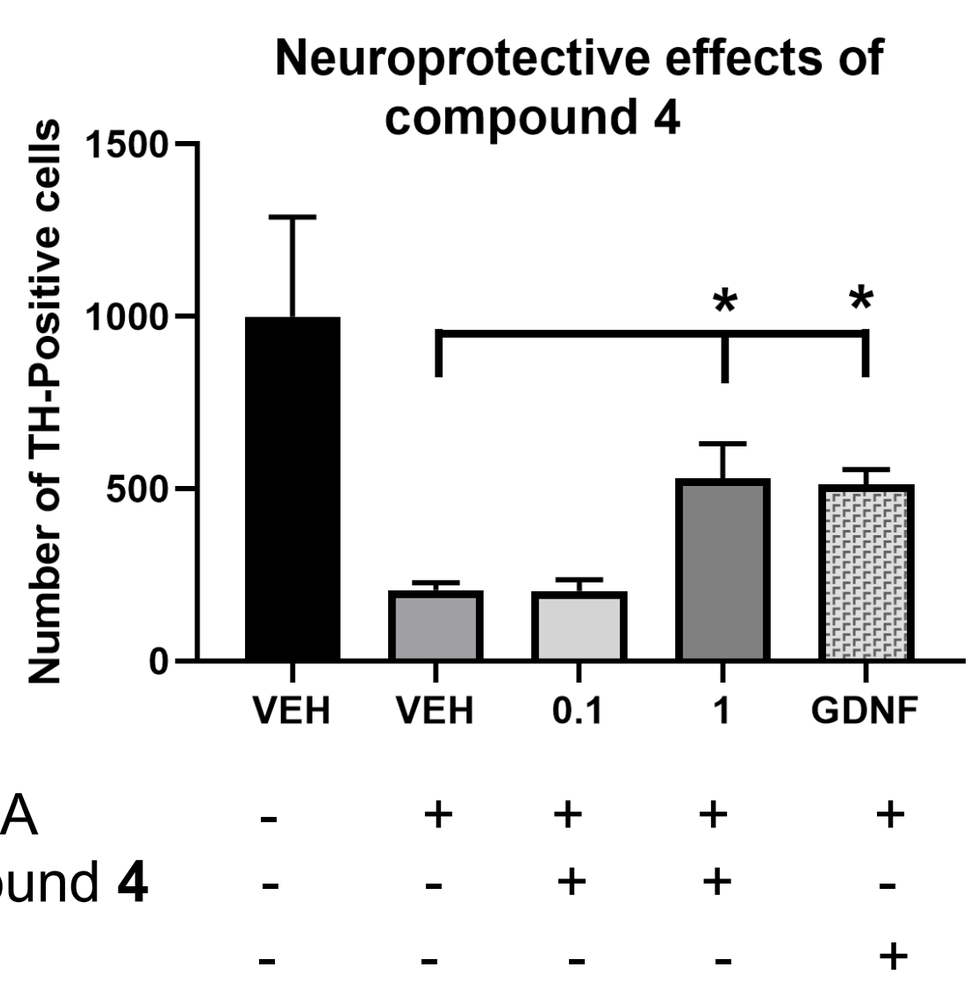


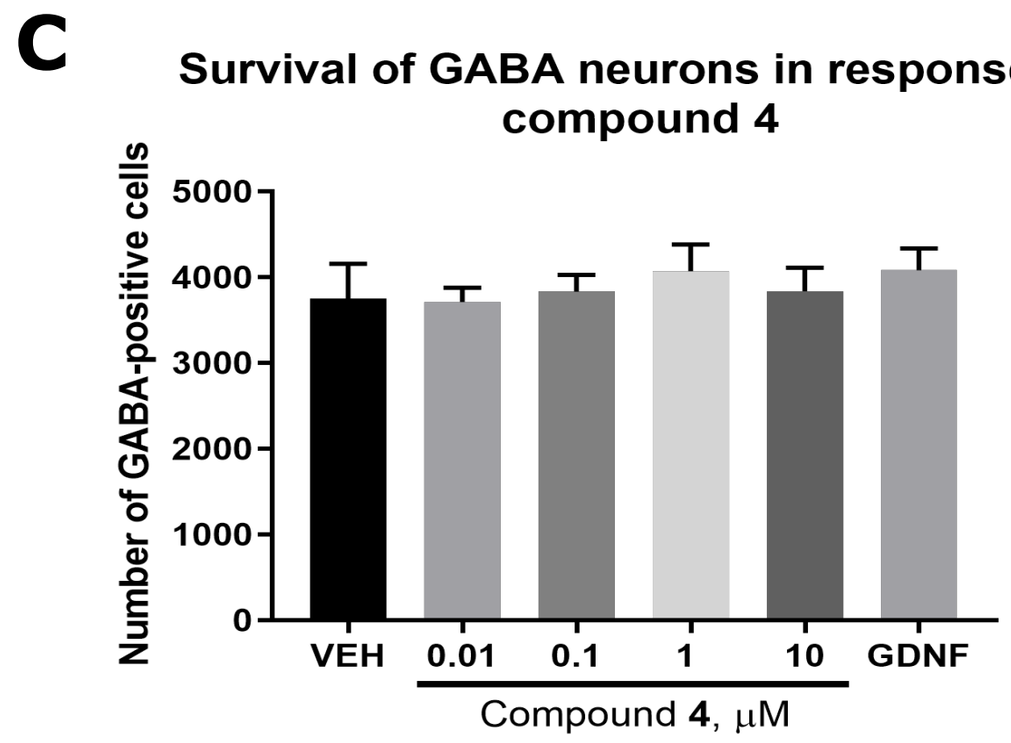


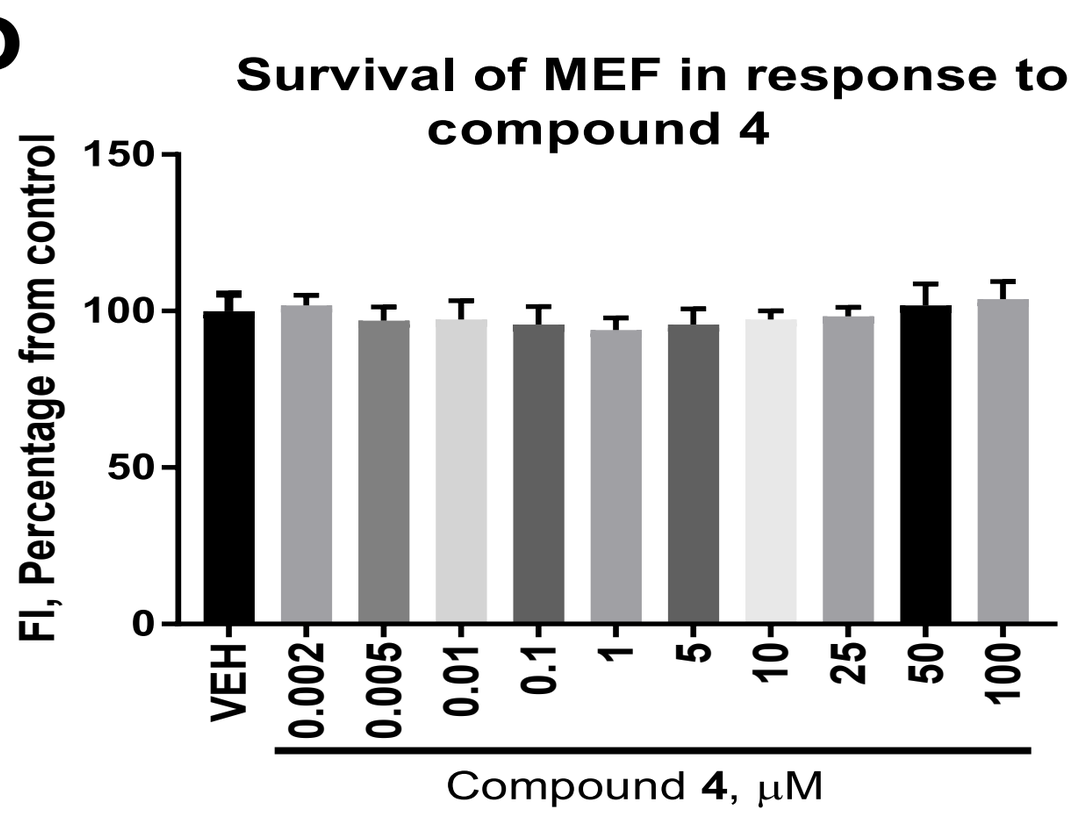


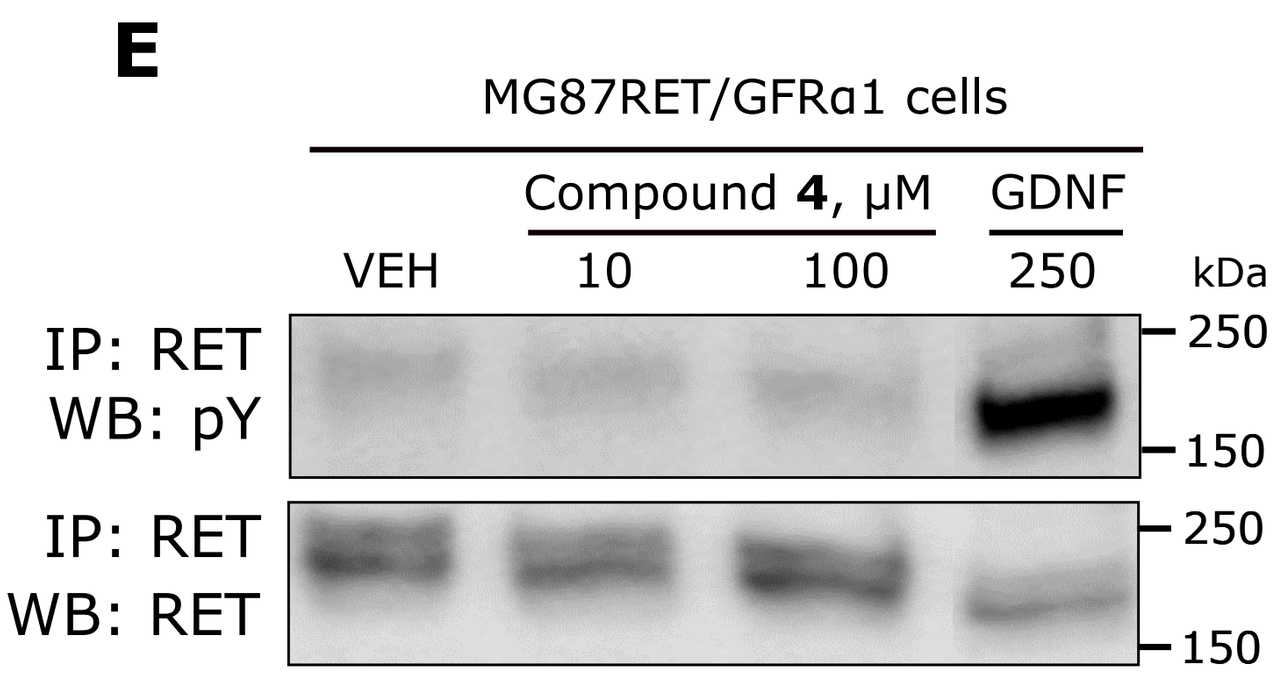


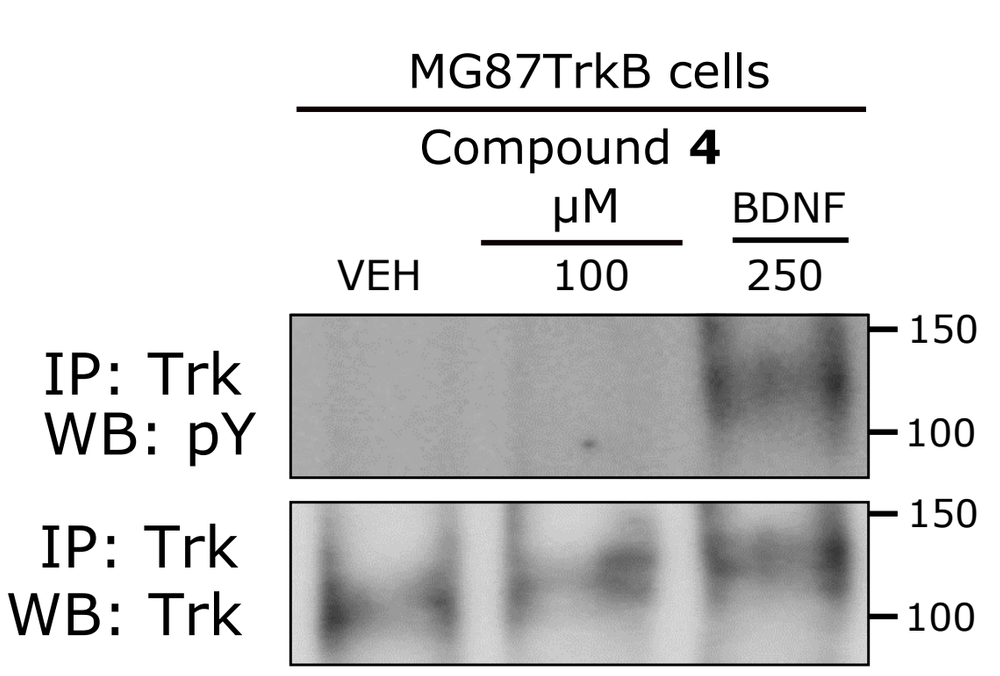

A
Survival of DA neurons in response to compound 4
Number of TH-positive cells
1500
1000
500
0
*
VEH
0.01
0.1
1
10
GDNF
Compound 4, μM

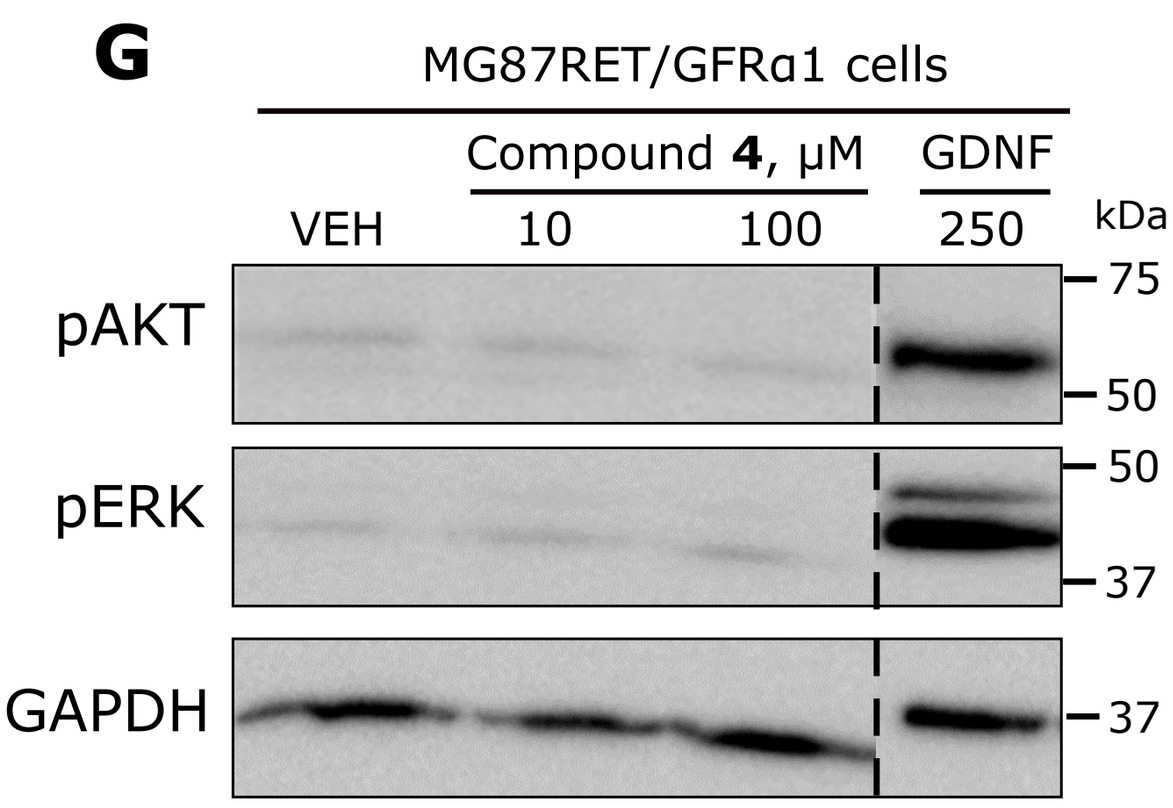

B
Neuroprotective effects of compound 4
Number of TH-Positive cells
1500
1000
500
0
*
*
VEH
VEH
0.1
1
GDNF
6-OHDA - + + + +
Compound 4 - - + + -
GDNF - - - - +

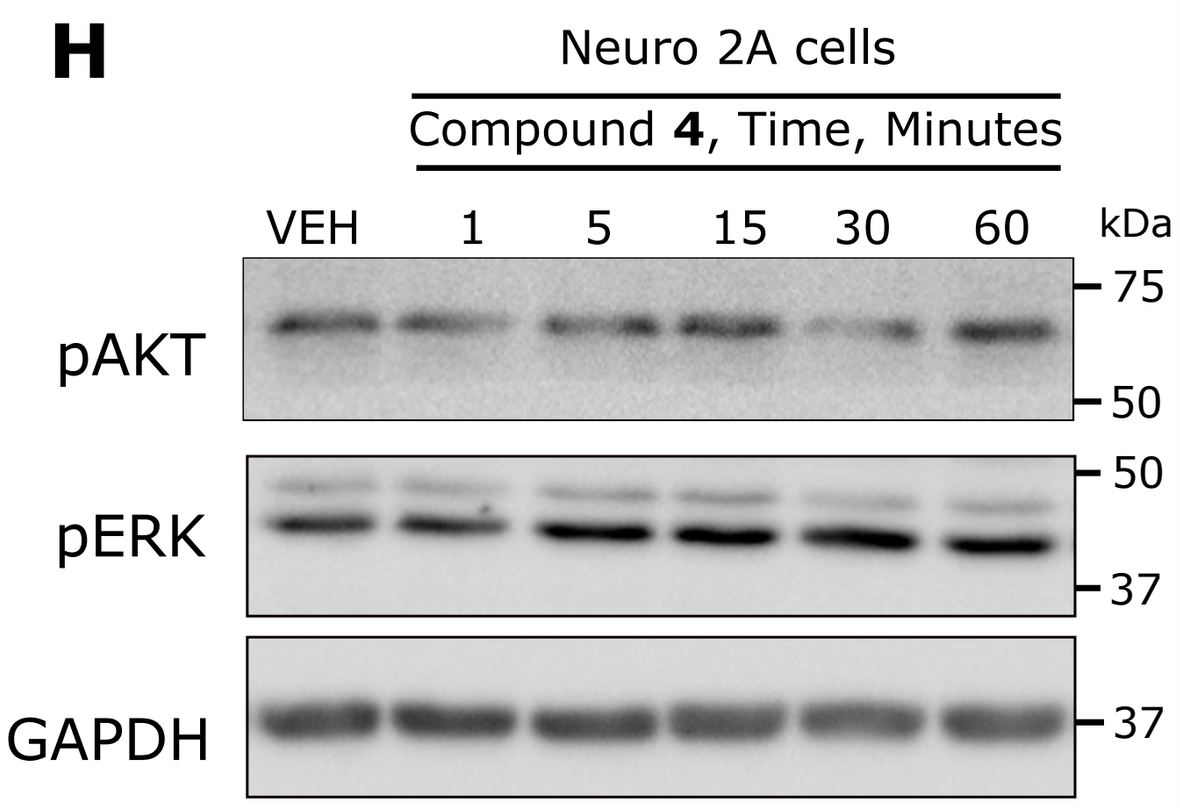

C
Survival of GABA neurons in response to compound 4
Number of GABA-positive cells
5000
4000
3000
2000
1000
0
VEH
0.01
0.1
1
10
GDNF
Compound 4, μM

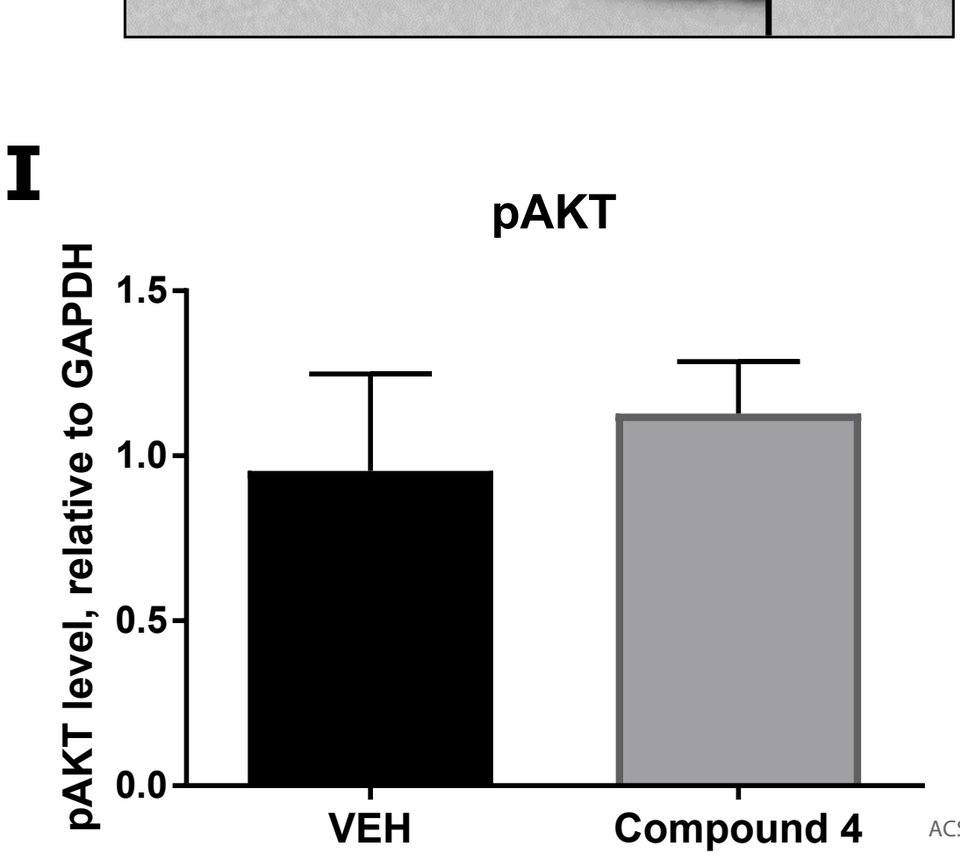

D
Survival of MEF in response to compound 4
FI, Percentage from control
150
100
50
0
VEH
0.002
0.005
0.01
0.1
1
5
10
25
50
100
Compound 4, μM

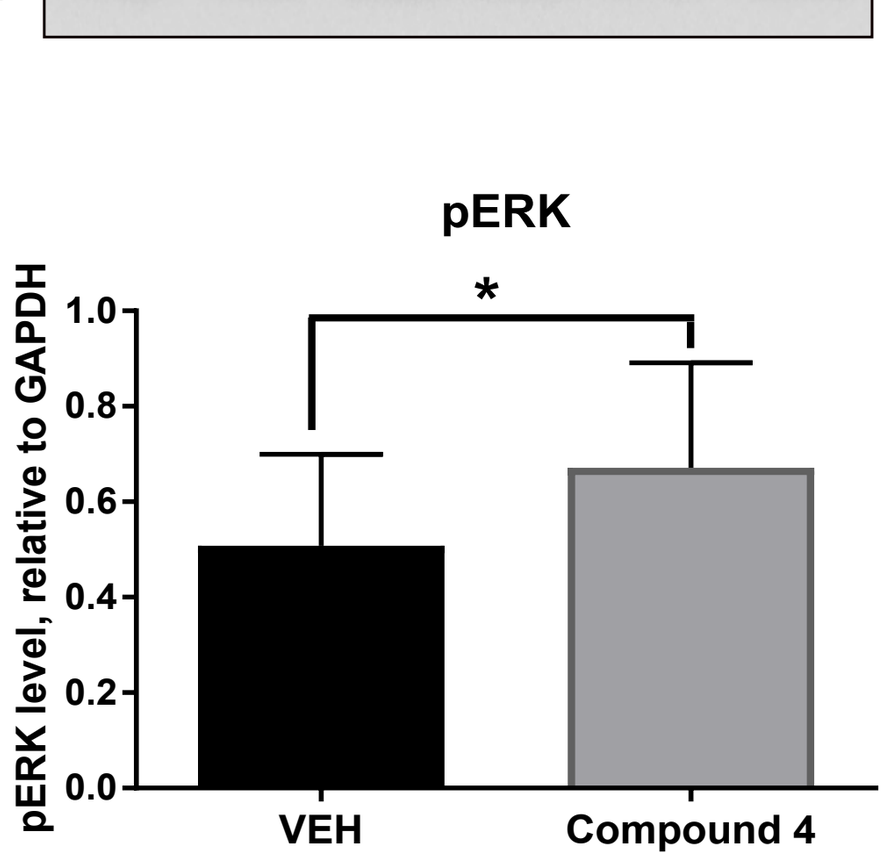

E
MG87RET/GFRα1 cells
Compound 4, μM
GDNF
VEH
10
100
250
kDa
IP: RET
WB: pY
250
150
IP: RET
WB: RET
250
150


F
MG87TrkB cells
Compound 4
μM
BDNF
VEH
100
250
IP: Trk
WB: pY
150
100
IP: Trk
WB: Trk
150
100


G
MG87RET/GFRα1 cells
Compound 4, μM
GDNF
VEH
10
100
250
kDa
pAKT
75
50
pERK
50
37
GAPDH
37


H
Neuro 2A cells
Compound 4, Time, Minutes
VEH
1
5
15
30
60
kDa
pAKT
75
50
pERK
50
37
GAPDH
37


I
pAKT
pAKT level, relative to GAPDH
1.5
1.0
0.5
0.0
VEH
Compound 4


J
pERK
pERK level, relative to GAPDH
*
1.0
0.8
0.6
0.4
0.2
0.0
VEH
Compound 4