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Effect of 3-nitropropionic acid on sirtuin gene expression in Sirt3 deficient mice

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ABSTRACT

Huntington's disease (HD) is an autosomal inherited progressive neurodegenerative disorder which is caused by the CAG trinucleotide repeat in the huntingtin gene. The mutation induces mitochondrial dysfunction in neurons, which leads to striatal neuronal loss. The efficacy of the available therapies is limited, thus acquisition of more data about the pathomechanism of HD and development of new strategies is urgent.

Sirtuins (Sirt1-7) belong to the histone deacetylase family, and interestingly they have been associated with HD, however, their role in HD is still not fully understood. To clarify the role of sirtuins in HD, we utilized a 3-nitropropionic acid (3-NP) induced HD model and assessed alterations in gene expression using RT-PCR. Moreover, we studied the extension of neurodegeneration in the striatum, and behavioural changes. Furthermore, we involved Sirt3 knockout (Sirt3KO) mice to investigate the impact of Sirt3 deficiency in the expression of the other sirtuins.

Our results showed that with 3-NP treatment, the mRNA level of Sirt2,5,7 changed significantly in wild-type (WT) mice, whereas in Sirt3KO animals there was no change. Interestingly, Sirt3 deficiency did not exacerbate 3-NP-mediated striatal neuronal loss, while Sirt3KO animals showed higher mortality than WT littermates. However, the absence of Sirt3 did not affect the behaviour of animals. Finally, we demonstrated that the changes in the expression of sirtuins are age- and sex- dependent.

According to our findings, there is evidence that Sirt3 has a major impact on the regulation of other sirtuin isoforms, survival and neuroprotection. However, this neuroprotective effect does not manifest in the behaviour.

Keywords: Sirtuins, Huntington's disease, 3-nitropropionic acid, Neurodegeneration Sirtuin3 knockout

1. Introduction

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disease caused by the expansion of CAG trinucleotide repeats in the huntingtin gene (HTT), resulting in a mutant gene product and mutant huntingtin protein (mHTT) [1]. Under physiological conditions HTT is crucial for energy metabolism and mitochondrial functions. However, in HD the mHTT, containing an expanded polyglutamine repeat in the N-terminal region, provokes neuronal dysfunction, mitochondrial damage, disruption of transcription, and, thereby, cell death [2]. Several signalling pathways are involved in the pathomechanism of HD, including the Sirtuin family, which has come to prominence in the last couple of years as attempts are made to clarify their role in HD. mHTT can directly interact with the signalling pathway of Sirtuin-PGC-1α axis by affecting mitochondrial biogenesis, and normal mitochondrial function [3].

Sirtuins are nicotinamide adenine dinucleotide (NAD^+) dependent histone deacetylases, which are involved in several biological functions, such as energy metabolism, mitochondrial function, stress response, cell survival and aging, but most importantly, their role is emerging in different neurological diseases [4]. Currently, seven mammalian sirtuin homologs are known (Sirt1-7), which have different enzymatic functions, and are localized in distinct cellular compartments. Sirt1, Sirt6, Sirt7 are located in the nucleus, whereas Sirt3, Sirt4, Sirt5 are mitochondrial, and Sirt2 is mainly cytoplasmic proteins [5]. In terms of HD, the most investigated sirtuins are Sirt1, Sirt2 and Sirt3, however as the roles of other sirtuins (Sirt4-7) are less known, it is important to discern them.

There are some preliminary reports showing protective effect of Sirt1 in HD models. Where Sirt1 transgenic (Sirt1 overexpression) and N171-82Q HD mice were crossed, the impaired motor functions, brain atrophy and metabolic abnormalities caused by mHTT significantly improved [6]. mHTT can interact with Sirt1 and inhibit its deacetylating activity, thereby decreasing the level of important neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and metabolic regulators [7]. Moreover, it is widely known that BDNF is indispensable to normal brain function [8], and its expression is promoted by Sirt1 in HD. Despite of its increased activity, Sirt1 is incapable of protecting the neurons from mHTT toxicity [7].

Elevated levels of Sirt2 have been shown in both mouse model and human post-mortem brains in relation with HD, thus the inhibition of Sirt2 has proven to be neuroprotective. However its reduction has no positive effect on HTT protein levels or on the progression of the disease in the HD R6/2 mouse model [9].

Sirt3 is located in the mitochondrial matrix and is associated with the activation of lifespan mechanisms, moreover it is involved in fatty acid oxidation, adenosine triphosphate (ATP) production and antioxidant protection [10]. Sirt3 has important roles in the regulation of mitochondrial function, maintenance of mitochondrial integrity and regulation of succinate dehydrogenase activity [11]. Several data proved the neuroprotective effect of Sirt3, which reacts to mitochondrial stress by initiating deacetylation of other substrates and prohibits the formation of the mitochondrial permeability transition pore [12], [13], [14]. Sirt3 has direct effect on Peroxisome proliferator–activated receptor gamma coactivator-1 alpha (PGC-1α), a master regulator of mitochondrial biogenesis, which is connected to ROS reduction [15]. However, the role of Sirt3 is highly contested, thus it needs to be further investigated. Salvatori et al. found that Sirt3 deficient mice do not display reduced survival [15], while Naia et al. showed that Sirt3 knockout mice showed decreased survival [12]. Interestingly, Sirt3 knockout animals have increased acetylation of mitochondrial proteins, thus altered mitochondrial metabolism, which leads to declined ATP production and increased oxidative damage [14].

In our study, we utilized the 3-NP induced HD model, where the 3-NP irreversibly inhibits the mitochondrial complex II, resulting in decreased energy metabolism and excitotoxicity through increased Ca^{2+} influx [16]. *In vivo* experiments demonstrated that 3-NP treatment resulted in reduction of both striatal neuronal markers like gamma-aminobutyric acid (GABA) and dopamine [17], therefore this toxin model is convenient for the study striatal degenerations [18]. Besides, the behavioural changes can possibly show hyperactivity, general uncoordination, bilateral dystonia and gait abnormalities [18]. In 3-NP model, knocking out of Sirt3 increased the toxicity, striatal neuronal cell loss, and decreased the survival. It has been showed previously that in cell cultures derived from Sirt3KO mice the neurons are more sensitive to 3-NP treatment [13].

In this study, we examined the effects of 3-NP on sirtuin (Sirt1-7) gene expression using RT-PCR and also the neurodegeneration of striatal neurons using NeuN staining. In addition, Open-field and Rota-Rod were carried out to assess alterations in motor functions. Previously, the effect of Sirt3 deficiency on the expression of other sirtuin isoforms had not been investigated, thus we performed mRNA expression analysis on Sirt1,2,4,5,6,7. Moreover, we also studied the regional specificity and age-dependent expression patterns of sirtuins in male and female WT and Sirt3KO mice.

2. Material and methods

2.1. Ethical statement

The studies were in accordance with the Ethical Codex of Animal Experiments and were approved by the Committee of the Animal Research of University of Szeged (XI./1944/2022). The protocol for animal care was approved both by the Hungarian Health Committee (40/2013 (II.14.)) and by the European Communities Council Directive (2010/63/EU).

2.2. Animals

Wild-type C57BI/6J (WT) and Sirtuin3 knock out (Sirt3KO) mice (B6.129S6(Cg)-Sirt3$^{tm1.1Fwa}$/J) (12, 16 week old, male and female) were involved in this study. The animal strain was originally obtained from Jackson Labs (Jackson Laboratories, USA). The animals were housed in cages under standard conditions under a 12–12 h light–dark cycle at 24 ± 1 ºC and 45-55% relative humidity, with free access to food and water in the Animal House of the Department of Neurology, University of Szeged.

2.3. Treatment

3-Nitropropionic acid (3-NP; Sigma Chemical, USA) was dissolved in phosphate-buffered saline (PBS; pH adjust 7.4) and was administered intraperitoneally (i.p.) to 12 and 16 week old male and female WT and Sirt3KO mice to induce toxicity. The 12 week old animals received 50 mg/kg body weight injections twice a day for 5 days, while the 16 week old animals were treated only once a day for 5 days (as older animals are more sensitive to the toxin, thus avoiding higher mortality). The control groups were injected with PBS. Male and female animals of 12 and 16 week of age were randomly divided into four groups: (female 12 week old: WT control n=6, WT 3-NP treated n=7, Sirt3KO control n=6, Sirt3KO 3-NP treated n=7; male 12 week old: WT control n=9, WT 3-NP treated n=10, Sirt3KO control n=10, Sirt3KO 3-NP treated n=11; female 16 week old: WT control n=5, WT 3-NP treated n=7, Sirt3KO control n=5, Sirt3KO 3-NP treated n=5; male 16 week old: WT control n=4, WT 3-NP treated n=8, Sirt3KO control n=5, Sirt3KO 3-NP treated n=6). During treatment behavioural tests were performed on 16 week old animals **(Figure 1.)**.

2.4. Behavioral tests

2.4.1. RotaRod test

The motor ability and sense of balance were tested by using the rotarod apparatus (TSE RotaRod V4.2.6 system (TSE Laboratory). The animals were given a prior training session before the test. On the first day of training the mice were placed on the rod apparatus, they performed three separate trials with 30 min resting periods between the trials. In the first trial the rod was immobile, but in second and third trials the speed of rotation was 5 rpm, for 300 s. On the second day the conditions were the same as the day before, but the speed was 10 rpm for 300 s. The test was on the third day of 3-NP treatment, where the rotation was gradually accelerate from 5 to 40 rpm for 300s [19].

2.4.2. Open-field test

To test the exploratory ability and spontaneous locomotor behaviour of animals we applied Open-field test under dim light. The Open-field apparatus consists of a 48x48x40 cm box, which has special sensors inside to detect the movements and locations of animals. Each animal was placed individually into the centre of the open field apparatus for 15 minutes (analysed in three 5 min periods). During this period the ambulation distance, ambulation time, immobility time and rearing count were measured with the Conducta v3.1 system (Experimetria Ltd.) [19].

2.5. Sample Handling

On the 5th day of 3-NP treatment, ninety minutes after the last injection, the animals were deeply anesthetized with isoflurane (Forane; Abott Laboratories Hungary Ltd., Budapest, Hungary) and immediately perfused transcardially with artificial cerebrospinal fluid (composition in mM: 122 NaCl, 3 KCl, 1 Na_2SO_4, 1.25 KH_2PO_4, 10 D-glucose, 1 $MgCl*6H_2O$, 2 $CaCl_2*2H_2O$, 6 $NaHCO_3$) for 2 min with a flow rate of 10 ml/min by an automated peristaltic perfusor. The brains were rapidly removed from the skull on ice and immediately halved at the midline. The right hemisphere was dissected into the following brain areas: striatum, cortex, cerebellum, and were stored at -80°C until the Real-time Polymerase chain reaction (RT-PCR) analysis. The left hemisphere was postfixed in 4 w/v% paraformaldehyde for 24 hours, and then transferred into 10 v/v%, 48 hours later into 20 v/v%, and then 72 hours later into 30 v/v% sucrose until immunohistochemical sample processing.

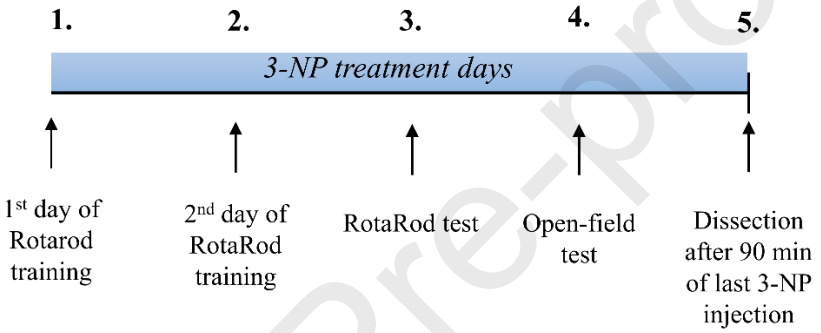

Figure 1 *Experimental timeline*

2.6. Real-Time PCR Analysis

For RT-PCR analysis, total RNA was isolated from the dissected brain areas of 12 and 16 week old animals, with Trizol reagent according to the manufacturer's protocol (Molecular Research Center, USA). RNA concentrations were determined using a MaestroNano spectrophotometer. cDNA was synthesized from 1 µg of total RNA with random hexamer primers (RevertAid First Strand cDNA Synthesis Kit Thermo Scientific, USA). The synthesized cDNA was stored at -20°C. RT-PCR analysis was performed with a CFX 96 Real Time System (Bio-Rad, USA) to detect changes in mRNA expression, using various Sirtuin primer pairs at a final volume of 20 µl, applying syber green labels (PCR Biosystems, USA). The primer sequences are presented in **Table 1**. Thermal cycling conditions for Sirtuin 1,2,5,6,7 were: 95°C for 3 min, followed by 39 cycles of 95°C for 10 s, and 66°C for 30 s; and for Sirtuin 3,4 they were: 95°C for 3 min, followed by 39 cycles of 95°C for 10 s, and 57°C for 30 s. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method [20]. We applied 18S rRNA as an endogenous control (Applied Biosystems, USA). The expression of Sirt3 is not detectable in Sirt3KO animals, thus it is not presented on diagrams.

Gene	Forward	Reverse
Sirt1	5’-GTTTCTGTCTCCTGTGGGATTC-3’	5’-AAACATGGCTTGAGGGTCTG-3’
Sirt2	5’-TGGCCTGGCTGGGTGACTGT-3’	5’-GGCAGGCGGTGGGGACTTTC-3’
Sirt3	5’-GCATCAGCACACCCAGT-3’	5’-CAACATGAAAAAGGGC-3’
Sirt4	5’-GAAAGAGGCGGACTCCCTAC-3’	5’-CGGGTCTATTAAAGGCAGCA-3’
Sirt5	5’-GCCACCGACAGATTCAGGTT-3’	5’-CCACAGGGCGGTTAAGAAGT-3’
Sirt6	5’-CCTGGTCAGCCAGAACGTAG-3’	5’-GTGTCTCTCAGCTCCCCTCT-3’
Sirt7	5’-AGGAGGAGGTGTGTGATGA-3’	5’-AGAAGCTGCTGTGCTGATT-3’

Table 1.: *Primer sequences used for RT-PCR analysis*

2.7. Immunohistochemistry and intensity measurement

For immunostaining, 30-µm-thick serial sections were cut from the entire striatum. The brains of 12 week old animals were used for immunohistochemistry. The sections were consecutively collected into 12 wells, the distance between the sections was 360 µm. The sections were washed in PBS for 2x10 min, and then were transferred into methanol, 0.3 w/v% H_2O_2 for 30 min. After that the sections were washed in PBS-T (1 v/v% Triton-X 100) for 2x10 min, and then were blocked for 1 h with PBS-T containing 10 v/v% normal horse serum (NHS). The sections were incubated in PBS-T, 2 v/v% NHS, containing anti-NeuN primary antibody at 1:10,000 dilution (Millipore, USA) for one night at room temperature. After washing in PBS-T for 2x10 min, the sections were incubated in biotinylated anti-mouse IgG secondary antibody at 1:600 dilution containing PBS-T and 2 v/v% NHS for 2h. After incubation, the sections were washed in PBS-T for 2x10 min, followed by the application of avidin-biotin-peroxidase complex at 1:600 dilution in PBS-T for 2h. The sections were rinsed in PBS for 2x10 min, and visualized with nickel ammonium sulfate-intensified 3,3’diaminobenzidine (Sigma-Aldrich, USA). The sections were dried overnight on glass slides, and covered using DPX as mounting medium [1]. The sections were analyzed using a Zeiss Axio Imager M2 Upright Microscope (Carl Zeiss MicroImaging, Germany) supplied with an AxioCam MRC camera. Photographs were taken at 10x magnification with ZEN 2.6 pro (blue edition) program and intensity measurements were also carried out with ZEN 2.6 pro [19]. We analysed the sections with the support of The Mouse Brain in Stereotaxic Coordinates (written by George Paxinos and Keith B.J. Franklin 2001). For analysis, we used six sections from every animal, the range was from Bregma 1.10

mm to Bregma -0.22 mm. Briefly, in every consecutive section we measured the intensity of whole striatum and the corresponding corpus callosum, and substracted the values from each other to normalize for variations in background stain. After adjusting the differences for the parameters of the corresponding striatum, we calculated the average intensity values. Thereafter, we compared these derived values in control and 3-NP treated animals.

2.8. Statistics

The statistical analyses were carried out with the IBM SPSS Statistics 28.0 software (SPSS Inc., USA). We used the Shapiro-Wilk test to check the normality of data. For intensity measurements and the comparison of relative sirtuin gene expression due to toxic treatments, most of our data showed Gaussian distributions and displayed non-significant differences in the homogeneity of variances (analysed with the Levene's test), thus we used parametric statistics One-Way Anova and for multiple comparisons LSD post hoc test. Most of the behavioural tests' data showed non-Gaussian distributions and non-significant differences in the homogeneity of variances, therefore we used non-parametric statistics (Kruskal-Wallis). A $p < 0.05$ value was regarded as significant. Data are presented as mean ± SEM.

3. Results

3.1. 3-NP toxin treatment alters the expression of sirtuins in WT more than in Sirt3KO mice in striatum

We showed that in female WT mice, the 3-NP toxin treatment increased the expression level of Sirt2, Sirt3 and Sirt5 in striatum (Sirt2: p=<0.001; Sirt3: p=0.045; Sirt5: p=0.032), while Sirt1 and Sirt7 levels were significantly reduced (Sirt1: p=0.029; Sirt7: p= 0.015). By contrast, in the Sirt3KO toxin treated group we demonstrated no alterations in Sirt2, Sirt5 or Sirt7 expression, however Sirt1 reduction remained significant (p=0.006) **(Figure 1. A)**. In male 3-NP treated WT mice, the expression levels of sirtuins in striatum showed similar patterns to female mice, we detected a significant increase in the case of Sirt2 (p=0.042), but significant decreases in Sirt1 and Sirt7 (Sirt1: p=0.032; Sirt7: p=0.023). In Sirt3KO 3-NP groups Sirt1 expression remained low, as in female Sirt3KO 3-NP groups (p=0.001) **(Figure. 1. B)**.

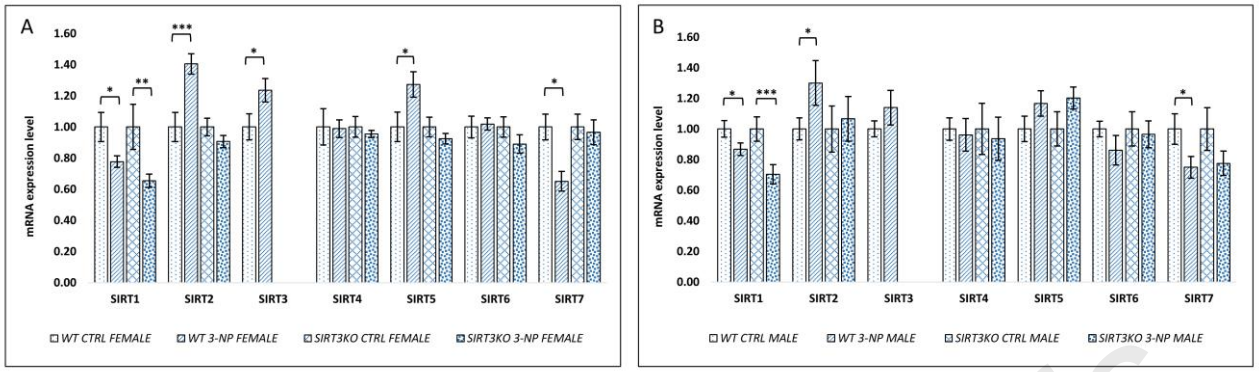

Figure 1. A: *Relative mRNA expression of SIRT1-7 in the striatum of 12 week old female WT/Sirt3KO control and 3-NP treated groups* (WT ctrl n=6; WT 3-NP n=7; Sirt3KO ctrl n=6; Sirt3KO 3-NP n=7). **B:** *Relative expression of SIRT1-7 in the striatum of 12 week old male WT/Sirt3KO control and 3-NP treated groups (*WT ctrl n=9; WT 3-NP n=10; Sirt3KO ctrl n=10; Sirt3KO 3-NP n=11). *One-Way Anova, LSD post hoc test; mean ± SEM;* p<0.05; **p<0.01; ***p<0.001.*

We also examined the expression of sirtuins in the cortex and cerebellum. In the cortex of female mice, we detected a significant increase only in Sirt5 expression in the WT 3-NP toxin treated group (p=0.012), while in the Sirt3KO 3-NP treated group Sirt2 and Sirt5 levels were raised (Sirt2: p=0.048; Sirt5: p=0.030) **(Figure 2. A)**. In the male WT 3-NP treated group Sirt3 and Sirt5 were significantly increased (Sirt3: p=0.009; Sirt5: p= 0.014). On the other hand, in the Sirt3KO group Sirt1 expression declined **(Figure 2. B)**. In the cerebellum most of the expression of sirtuins did not change significantly, in neither male nor in female groups **(Supplementary Figure 1.)**.

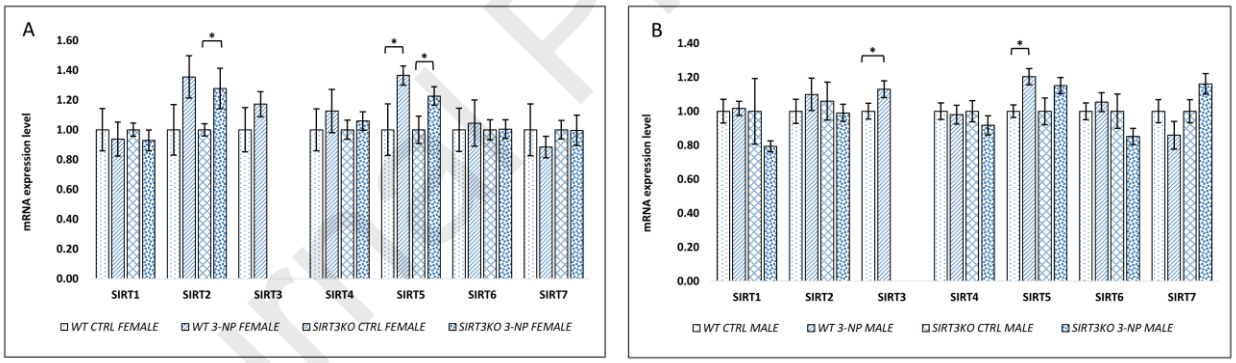

Figure 2. A: *Relative expression of SIRT1-7 in the cortex of 12 week old female WT/Sirt3KO control and 3-NP treated groups (*WT ctrl n=6; WT 3-NP n=7; Sirt3KO ctrl n=6; Sirt3KO 3-NP n=7). **B:** *Relative expression of SIRT1-7 in the cortex of 12 week old male WT/Sirt3KO control and 3-NP treated groups (*WT ctrl n=7; WT 3-NP n=7; Sirt3KO ctrl n=6; Sirt3KO 3-NP n=7). *One-Way Anova, LSD post hoc test; mean ± SEM; *p<0.05.*

As we could only detect notable changes in the striatum of 12 week old mice, we examined the expression of sirtuins only in that brain area of 16 week old animals. In the female WT 3-NP group only Sirt5 increased (p=0.002), whereas in the Sirt3KO 3-NP group we detected signifcant decreases in Sirt5 and Sirt7 (Sirt5: p=0.002; Sirt7: p=0.028) **(Figure 3. A)**. In the 16 week old WT male group 3-NP treatment there was enhanced Sirt2 expression (p=0.015), however in the Sirt3KO 3-NP group the expression of Sirt6 decreased significantly (Sirt6: p=0.025) **(Figure 3. B)**.

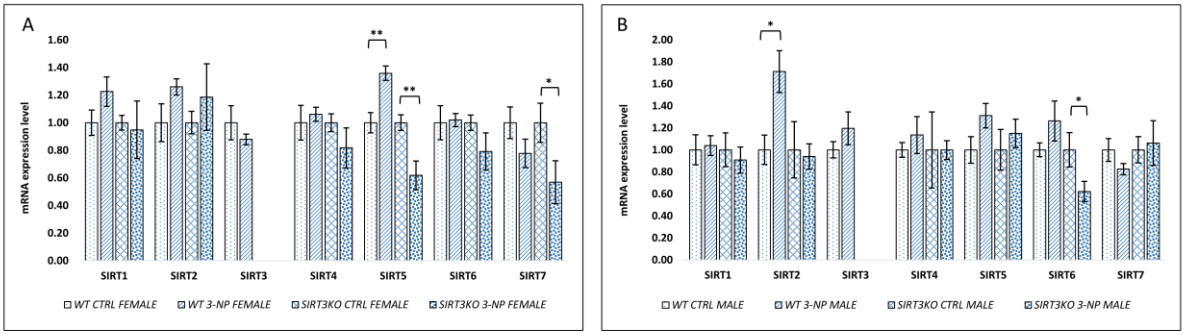

Figure 3. A: *Relative expression of SIRT1-7 in the striatum of 16 week old female WT/Sirt3KO control and 3-NP treated groups (*WT ctrl n=5; WT 3-NP n=7; Sirt3KO ctrl n=5; Sirt3KO 3-NP n=5). **B:** *Relative expression of SIRT1-7 in the striatum of 16 week old male WT/Sirt3KO control and 3-NP treated groups (*WT ctrl n=4; WT 3-NP n=8; Sirt3KO ctrl n=5; Sirt3KO 3-NP n=6). *One-Way Anova, LSD post hoc test; mean ± SEM; *p<0.05; **p<0.01.*

3.2. Expression of sirtuins changes depending on age and sex

We examined the age-dependent expression of sirtuins, comparing 12 to 16 week old animals. In WT female animals Sirt5 and Sirt7 expression decreased significantly (Sirt5: p=0.012; Sirt7: p=0.009), while in case of Sirt3KO mice we detected significant reductions of Sirt1 and Sirt2 expression by the age of 16 week (Sirt1: p= 0.034; Sirt2: p=0.004) **(Figure 4. A)**. In 16 week old WT male animals Sirt4 and Sirt6 expression was raised, while Sirt7 expression was significantly lowered (Sirt4: p=0.033; Sirt6: p=0.031; Sirt7: p=0.016). On the other hand, in 16 week old Sirt3KO animals, we detected a significant decrease in Sirt7 expression (p=0.003) **(Figure 4. B)**.

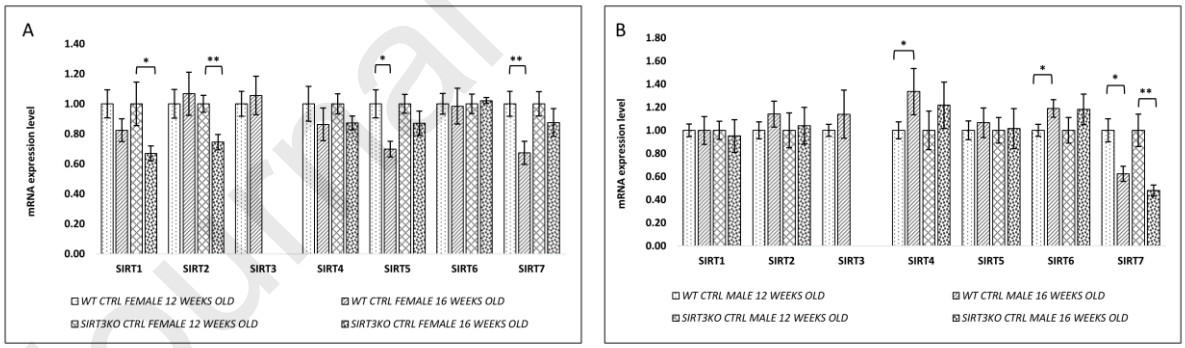

Figure 4. A*: Relative expression of SIRT1-7 in the striatum of 12 and 16 week old female WT/Sirt3KO control groups (WT ctrl female 12 week old n=6; WT ctrl female 16 week old n=5; Sirt3KO ctrl female 12 week old n=6; Sirt3KO ctrl female 16 week old n=5).* **B:** *Relative expression of SIRT1-7 in the striatum of 12 and 16 week old male WT/Sirt3KO ctrl groups (WT ctrl male 12 week old n=9; WT ctrl male 16 week old n=4; Sirt3KO ctrl male 12 week old n=6; Sirt3KO ctrl male 16 week old n=5). Independ Samples T-test; mean ± SEM; *p<0.05; **p<0.01.*

In addition, we analysed the expression of sirtuins depending on sex. In the female WT group we observed significant differences in Sirt2 and Sirt3 expression compared to male animals (Sirt2: p=0.037; Sirt3: p=0.001). Moreover, in Sirt3KO animals only Sirt5 increased (p=0.026) **(Figure 5. A).** We also examined the expression of sirtuin in 16 week old female and male animals in WT and Sirt3KO groups. In the case of WT female animals Sirt1, Sirt5 and Sirt6

expression declined significantly (Sirt1: p=0.050; Sirt5: p=0.031; Sirt6: p=0.047). In Sirt3KO groups, Sirt2 decreased, while Sirt5 increased (Sirt2: p= 0.030; Sirt5: p= 0.042) **(Figure 5. B)**.

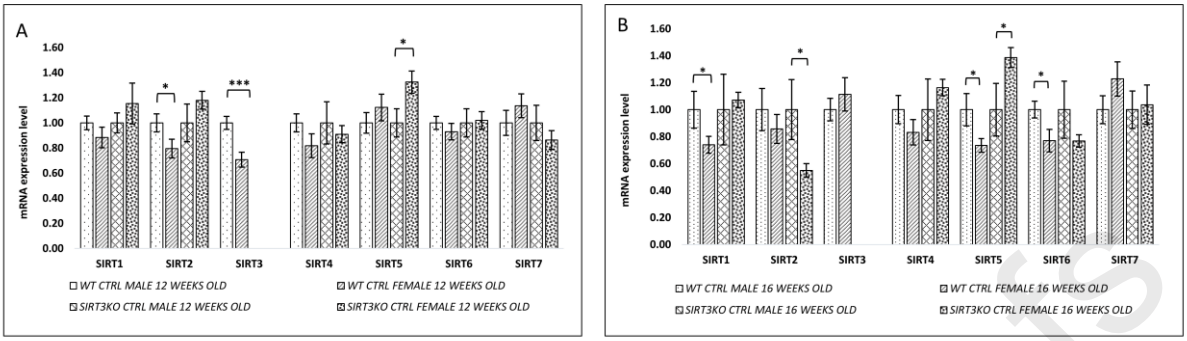

Figure 5. A: *Relative expression of SIRT1-7 in the striatum of 12 week old male-female WT/Sirt3KO ctrl groups (WT ctrl male 12 week old n=9; WT ctrl female 12 week old n=6; Sirt3KO ctrl male 12 week old n=10; Sirt3KO ctrl female 12 week old n=6)* **B:** *Relative expression of SIRT1-7 in the striatum of 16 week old male-female WT/Sirt3KO groups groups (WT ctrl male 16 week old n=4; WT ctrl female 16 week old n=5; Sirt3KO ctrl male 16 week old n=5; Sirt3KO ctrl female 16 week old n=5). Independ Samples T-test; mean ± SEM; *p<0.05; ***p<0.001.*

In next step, we studied the expression of WT/Sirt3KO male and WT/Sirt3KO female groups both in 12 week and 16 week old animals. In female WT/Sirt3KO 12 week old animals we detected significant changes in Sirt1, Sirt2, Sirt4, Sirt5 expression (Sirt1: p=0.041; Sirt2: p=<0.001; Sirt4 p=0.037; Sirt5: p=0.024) **(Figure 6. A)**. In both WT and Sirt3KO males we did not observe any significant changes **(Figure 6. B)**. In the case of 16 week old animals, in female WT/Sirt3KO animals only Sirt5 showed a significant increase (p=0.001) **(Figure 6. A)**. In male groups only Sirt6 expression changed significantly (p=0.029) **(Figure 6. B)**.

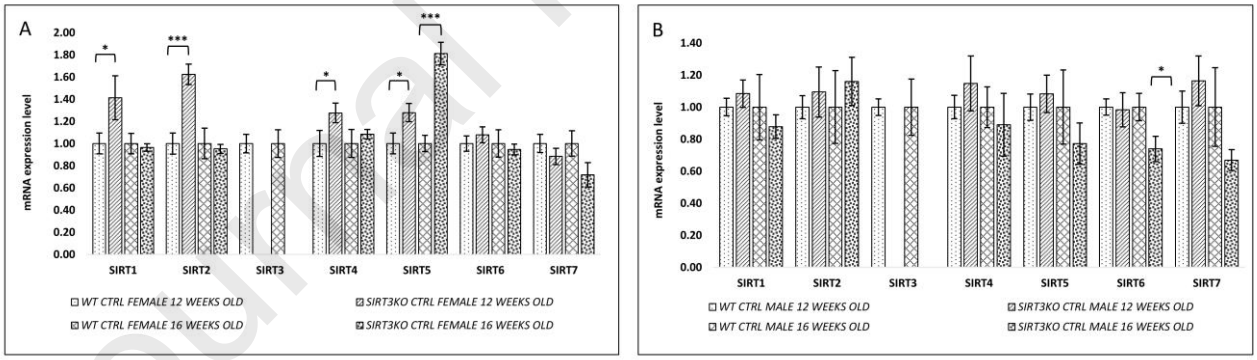

Figure 6. A: *Relative expression of SIRT1-7 in the striatum of 12/16 week old female WT/Sirt3KO control groups (WT ctrl female 12 week old n=6; Sirt3KO ctrl female 12 week old n=6; WT ctrl female 16 week old n=5; Sirt3KO ctrl female 16 week old n=5).* **B:** *Relative expression of SIRT1-7 in the striatum of 12/16 week old male WT/Sirt3KO control groups (WT ctrl male 12 week old n=9; Sirt3KO ctrl mae 12 week old n=10; WT ctrl male 16 week old n=4; Sirt3KO ctrl male 16 week old n=5). Independ Samples T-test; mean ± SEM; *p<0.05; **p<0.01; p<0.001**.*

3.3. Sirt3KO alleviates 3-NP-mediated striatal neuronal loss

To evaluate neuronal loss in striatum due to 3-NP treatment, we performed NeuN nuclei immunostaining and implanted intensity measurement. During intensity measurement the brightness of the image was calculated. Toxin-induced neuronal loss led to fewer NeuN stained neurons, thus we could detect higher intensity, which refers to higher neuronal loss.

There was significantly higher neuronal loss in the female 12 week old WT 3-NP treated group (p=0.012) compared to WT control group, whereas in case of Sirt3KO 3-NP treated group we did not detect any alteration compared to Sirt3KO control **(Figure 9. A)**. For male mice, we could not detect significant differences in Sirt3KO 3-NP treated group compared to Sirt3KO control group **(Figure 9. B)**. NeuN nuclei immunostaining is represented in **Figures 7-8.**

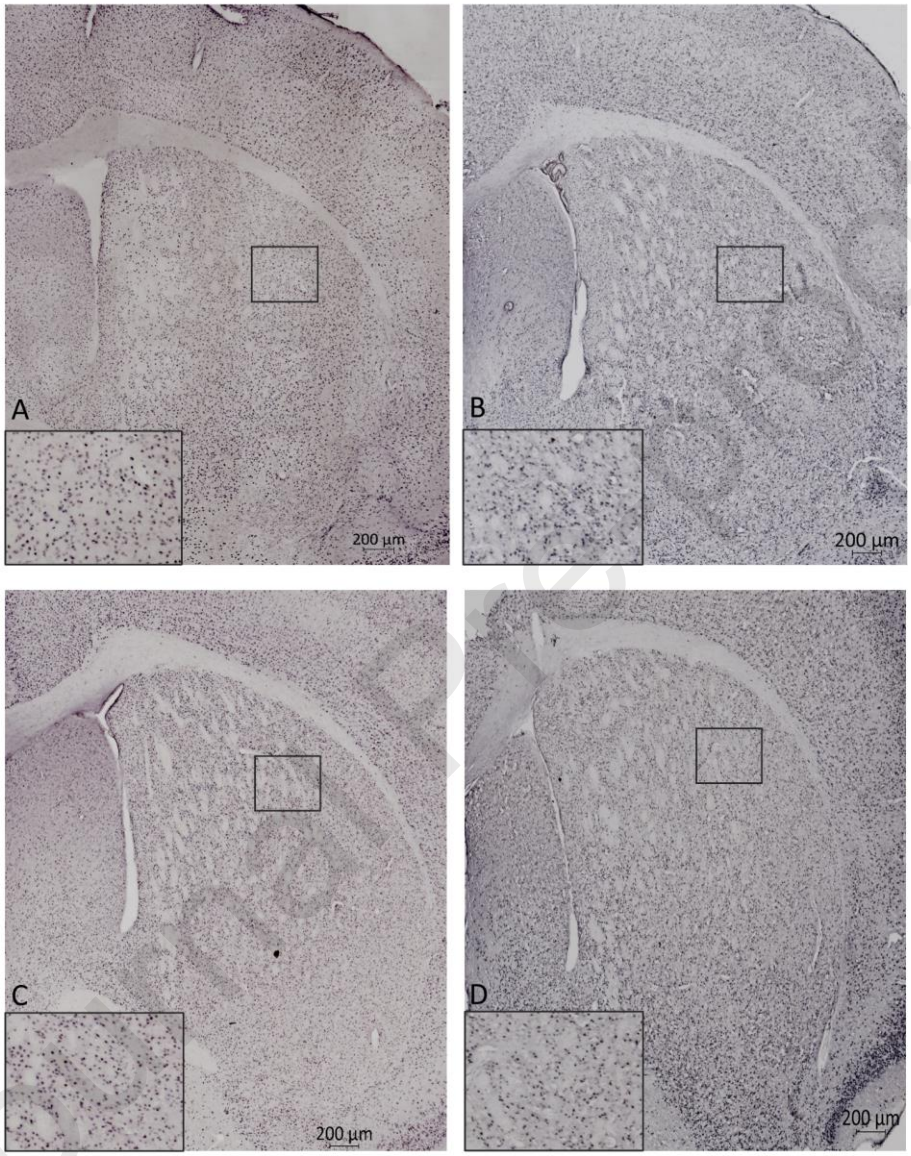

Figure 7. NeuN staining *in striatum of female 12 week old animals.* **A:** *WT ctrl*; **B:** *WT 3-NP*; **C:** *Sirt3KO ctrl*; **D:** *Sirt3KO 3-NP.*

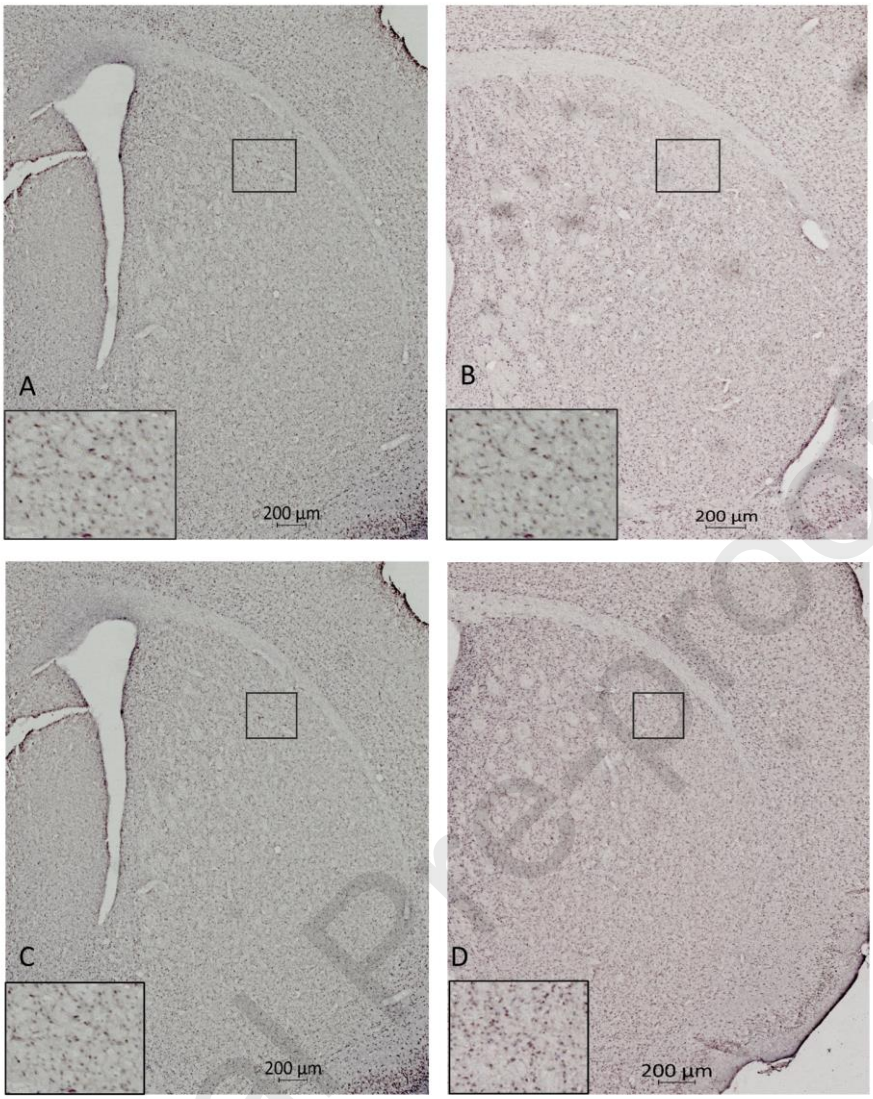

Figure 8. NeuN staining of striatum of *male 12 week old animals.* **A:** *WT ctrl*; **B:** *WT 3-NP*; **C:** *Sirt3KO ctrl***; D:** *Sirt3KO 3-NP.*

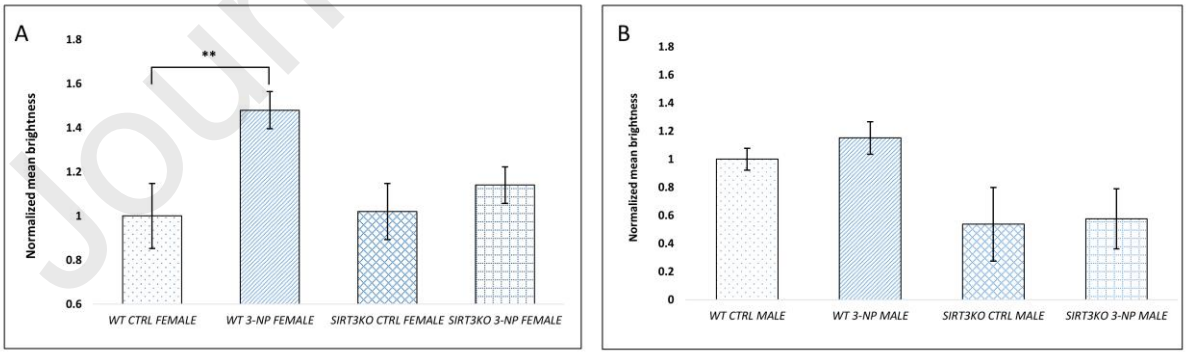

Figure 9. *Effect of 3-NP treatment on striatal cells in 12 week old female WT/Sirt3KO animals (WT ctrl n=6, WT 3-NP n=7, Sirt3KO ctrl n=6, Sirt3KO 3-NP n=7)* ***(A)*** *and in 12 week old male WT/Sirt3KO animals (WT ctrl n=9, WT 3-NP n=10, Sirt3KO ctrl n=10, Sirt3KO 3-NP n=11)* ***(B)****. We implanted instensity measurement to indentify the neuronal loss. One-Way Anova, LSD post hoc test; mean ± SEM; **p<0.01.*

3.4. Sirt3KO 3-NP treated male mice show higher mortality than WT littermates

The calculation of 3-NP induced mortality showed severe mortality in the case of 12 week old male animals, however female animals displayed only mild sensitivity to the toxin **(Figure 10. A-B)**. Based on this observation we assumed that 16 week old animals will be more sensitive to 3-NP treatment, thus we reduced it to one treatment per day to avoid high mortality. As a result, none of them died during the treatment (not presented).

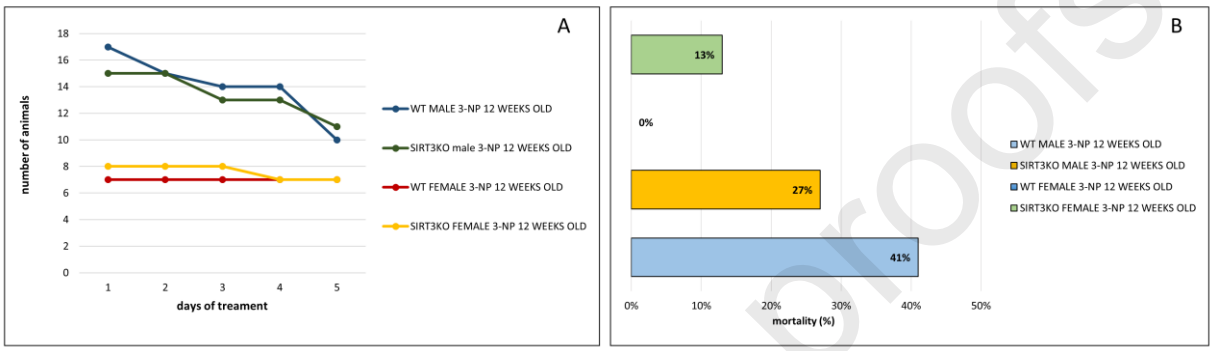

Figure 10: A: *Number of animals that have died during the experiment by time presented in timepoint.* **B:** *Mortality of the 12 weeks old animals at the end of the treament presented in percentage (%).*

3.5. Male and female mice performed differently during Rota-Rod test

In Rota-Rod test, there was no significant difference between groups of male mice **(Figure 11. A)**. However, due to 3-NP treatment the sense of balance of Sirt3KO female animals was significantly worse than in the Sirt3KO control group (p=<0.001) **(Figure 11. B)**.

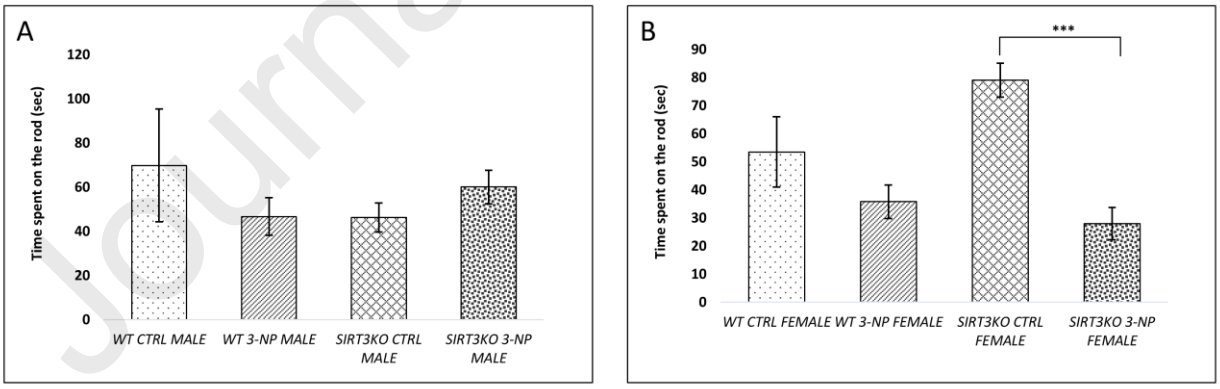

Figure 11. A: *Rotarod results for 16 week old male WT/Sirt3KO control and 3-NP treated groups (WT ctrl n=4, WT 3-NP n=8, Sirt3KO ctrl n=5, Sirt3KO 3-NP n=6)* **B:** *Rotarod results for 16 week old female WT/Sirt3KO control and 3-NP treated groups (WT ctrl n=5, WT 3-NP n=7, Sirt3KO ctrl n=5, Sirt3KO 3-NP n=5). Kruskal-Wallis test; mean ± SEM; p<0.001***.*

3.6. The absence of Sirt3 does not affect the exploratory ability and the spontaneous locomotion in the Open-field test

In the Open-field test, 16 week old WT 3-NP treated male mice performed worse than WT control groups for ambulation distance (p=0.017), ambulation time (p=0.017), immobility time (p=0.027) and rearing count (p=0.017). The results for the Sirt3KO control and Sirt3KO 3-NP groups were almost the same as the WT groups (ambulation distance (p=0.004), (ambulation time (p=0.006) and immobility time (p=0.006) **(Figure 12. A-D)**. 16 week old WT and Sirt3KO 3-NP treated female mice performed worse than control littermates for ambulation distance (WT ctrl and WT 3-NP: p=0.012; Sirt3KO ctrl and Sirt3KO 3-NP: p=0.009), ambulation time (WT ctrl and WT 3-NP: p=0.003; Sirt3KO ctrl and Sirt3KO 3-NP: p=0.003) and rearing count (WT ctrl and WT 3-NP groups: p=0.012; Sirt3KO ctrl and Sirt3KO 3-NP: p=0.009). In regard to immobility time, we detected significant changes only in Sirt3KO groups (Sirt3KO ctrl and Sirt3KO 3-NP: p=0.002) **(Figure 12. A-D)**.

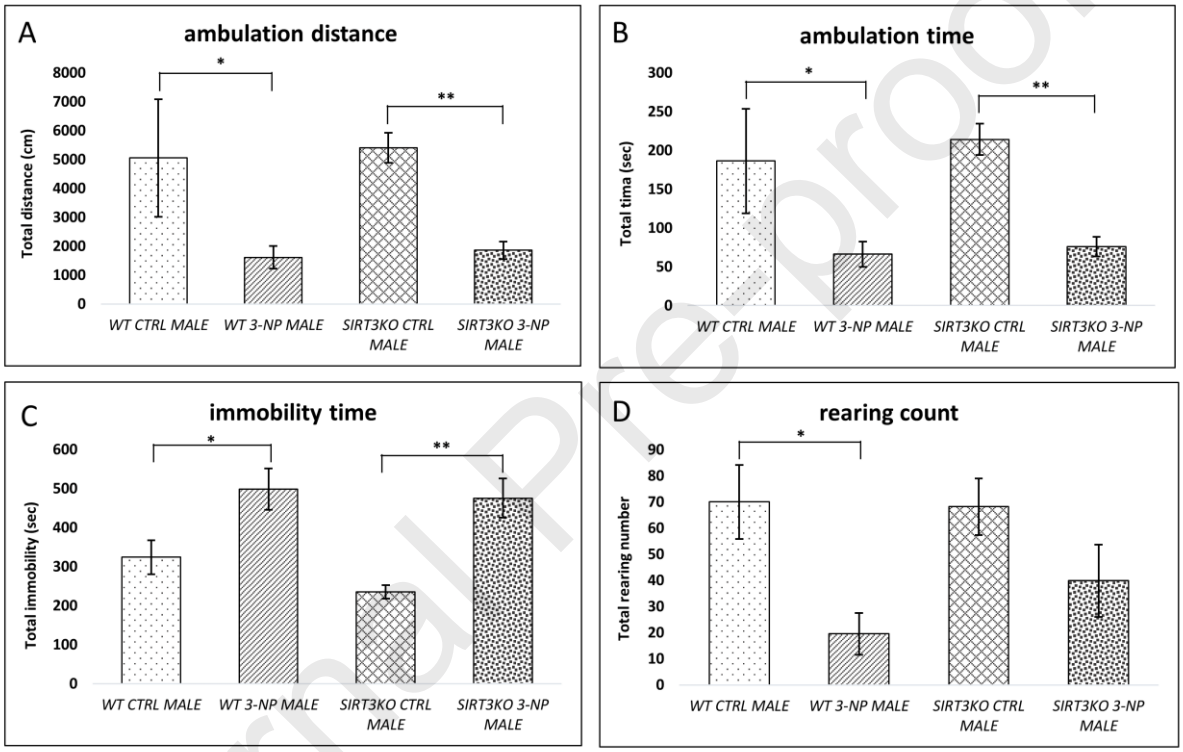

Figure 12. A-D: *Open-field test of 16 week old male WT/Sirt3KO ctrl and 3-NP treated groups (WT ctrl n=4, WT 3-NP n=8, Sirt3KO ctrl n=5, Sirt3KO 3-NP n=6). : Kruskal-Wallis test; mean ± SEM; *p<0.05; **p<0.01.*

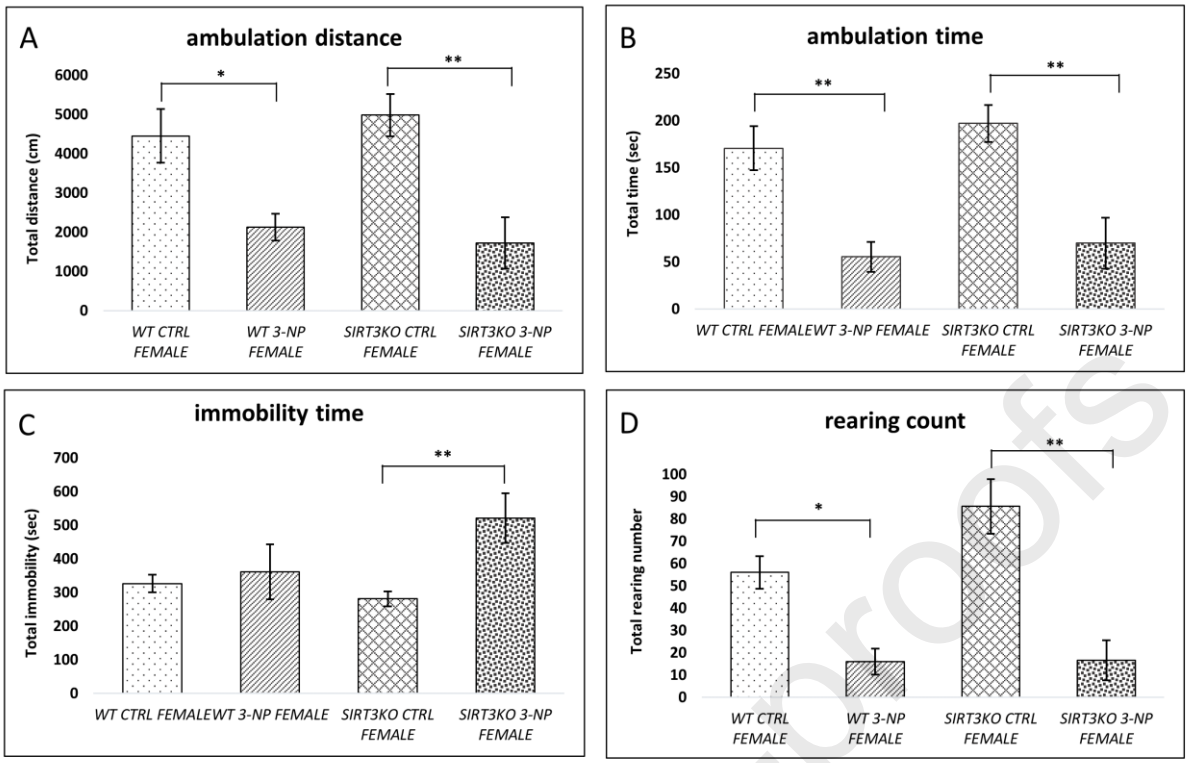

Figure 12. A-D: *Open-field test of 16 week old female WT/Sirt3KO ctrl and 3-NP treated groups (WT ctrl n=5, WT 3-NP n=7, Sirt3KO ctrl n=5, Sirt3KO 3-NP n=5). Kruskal-Wallis test; mean ± SEM; *p<0.05; **p<0.01.*

4. Discussion

In the present work, we investigated the effect of 3-NP toxin treatment on the expression levels of all seven sirtuin homologs in both wild-type and Sirt3KO mice in different brain areas. It has been previously stated that HD histopathological alterations include striatal atrophy, firstly affecting the basal ganglia, and predominantly the GABAergic medium spiny neurons of the striatum [2], [22]. Our findings provide additional evidence that sirtuin expression changes occurred mainly in the striatum, thus further confirming that this brain region is primarily affected in HD. Ibrahim et al. showed that as a consequence of 3-NP treatment Sirt1 protein level declines [23]. Moreover, another study suggested that Sirt1 is downregulated in HD models, whereas Sirt2 mRNA level is increased in human post-mortem striatum [24], [25]. Our results demonstrated that 3-NP treatment decreases Sirt1 levels. Intriguingly, in absence of Sirt3, Sirt1 mRNA expression remains reduced, which indicates that the 3-NP treatment had the effect of reducing Sirt1, and not the absence of Sirt3. We found that Sirt2 expression increased in response to 3-NP treatment in the presence of Sirt3, however in the absence of Sirt3, its level did not change, indicating a connection between these two genes. Interestingly, in our study 3-NP treatment did not affect Sirt4 or Sirt6 gene expression. Naia et al. hypothesized that the activity of Sirt3 in HD cells is caused by defence mechanisms, not by a toxic response [12]. In the absence of Sirt3 due to toxic treatment, we did not detect any sirtuin baseline level changes, thus we consider that compensatory or defence mechanisms could not start without Sirt3. Moreover, Sirt3KO mice showed mild sensitivity to the toxin, because the expression of other sirtuins remained unchanged. Our

results showed that mitochondrial Sirt3 and Sirt5 levels increased in response to toxic treatment, therefore we suspect that this increase is a protective mechanism against the neurotoxicity of 3-NP on mitochondria. Currently, we have limited knowledge about the role of Sirt7 in HD. We found that Sirt7 level reduction is mainly notable in toxin-treated WT animals, which again indicates an interaction in gene expression and may represent either protective or toxic effects of this disease. However, for better understanding we need to further investigate Sirt7. Interestingly, in the case of elder female animals Sirt5 and Sirt7 level decreased, while in male animals just Sirt6 decreased. We consider that, along with 3-NP, age can also affect these changes.

Braidy et al. found that the mRNA expression levels of sirtuins fluctuate with age. Sirt1 levels increase in the frontal, temporal, occipital lobes and hippocampus by the age of 24 months in female rat brain, but Sirt2 levels increase only in the occipital lobe. Sirt3,4,5,6 levels decrease in the hippocampus and frontal lobe. Sirt7 levels increase only in the frontal lobe [26]. However, another study presented that during ageing Sirt1 levels decreased, while Sirt2 levels increased in mouse brain and spinal cord [27]. We found that in the striatum by the age of 16 weeks, a consequent decrease could be seen in case of Sirt7 levels. Moreover, in the presence of Sirt3, in female animals only Sirt5 decreased, unlike in male animals, where Sirt4 and Sirt6 increased. In Sirt3KO, female animals showed lower Sirt1 and Sirt2 expression. Previously, it has been published that Sirt3 level declines significantly in the cortex and hippocampus of rat brain [14]. On the contrary, we detected no alterations in Sirt3 expression in the striatum by the age of 16 weeks.

According to our knowledge, there is a lack of data on the sex-dependent expression of sirtuins in mice, thus we investigated the expression changes between sexes. We found that in most cases Sirt5 changed, which indicates that Sirt5 fluctuates the most depending on sex.

Substantial amounts of data indicate that 3-NP treatments affect motor performance in mice. Naia et al. found that Sirt3 knockout animals perform worse on RotaRod compared to wild-type littermates [12]. Moreover, Aiwu et al. showed that 3-NP treatment decreases the motor performance of Sirt3KO mice [28]. We found that 3-NP treated groups performed worse than control groups during behavioural tests. Most of our data indicate that there is no difference between WT and Sirt3KO 3-NP treated groups, which suggests that Sirt3 does not affect the motor skills. Interestingly, we observed that the mortality of younger WT male animals was higher than Sirt3KO groups, thus we assume they are more sensitive to the toxin. In elder mice, probably as a result of the reduced dose of 3-NP, we could not detect significant changes according to mortality. The decreased sensitivity of Sirt3KO animals to the 3-NP toxin could derive from different activity in mitochondrial complexes. Ahn et al. found that under physiological conditions, Sirt3 can interact with Complex I, but mitochondria from knockout animals showed selective inhibition of Complex I activity [29]. Cimen et al. found that the activity of Complex II in Sirt3 knockout mice showed a 30% reduction compared to wild-type [11]. Nevertheless, we cannot rule out that in Sirt3 deficient animals the decreased mitochondrial activity provides a lower level of free radical production, thus a decreased sensitivity to 3-NP. Aiwu et al. showed that with a Sirt3 deficiency the 3-NP treatment resulted in increased striatal neurotoxicity in mice and, based on NeuN staining, the neuronal damage was larger in these animals [28]. Moreover, Sirt3 can protect neurons from toxicity [28], therefore it can protect neurons against excitotoxic injury by supressing oxidative stress [30]. Contradictorily, our data indicated that in young female animals cell death was higher in WT

3-NP groups than in Sirt3KO 3-NP animals, thereby striatal neuronal loss was more significant in the presence of Sirt3, which raises a question about the protective effect of Sirt3.

5. Conclusion

In this study, we provided evidence that without Sirt3 the 3-NP toxic treatment has different effects on sirtuin gene expression when compared to physiological conditions. However, our approach has limitations, in particular, this is toxin-induced and not a transgenic model of HD. This model displays just a portion of the symptoms of Huntington's disease, but demonstrates striatal damage through 3-NP toxicity, which is involved in the disorder. The most prominent alteration we found is the expression of Sirt7, which declined in almost every WT animal. Moreover, it is also interesting that Sirt2 and Sirt5 levels did not change in Sirt3KO animals in contrast with WT littermates. These findings ensure that Sirt3 has an important role in the regulation of sirtuin expression, and raises questions about the role of Sirt7 in the disease. Furthermore, the behavioural and immunohistochemistry investigations did not demonstrate neuroprotective effects of Sirt3, therefore it is questionable whether Sirt3 has any negative effects on the disease. We demonstrated changes in the expression of sirtuins depending on age and sex. However, our study did not expand to the protein level, which would be interesting to investigate in the future.

All together, these results confirm that the sirtuin system is very complex and has an important role in this disorder, although further study is required for better understanding of the molecular pathomechanism of Huntington's disease.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon request.

Conflict of interest

The authors declare that they have no competing interests.

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Authors' contributions

O.H. performed the experiments; analysed and interpreted the data; wrote the paper. P.K. conceived and designed the experiments; contributed reagents, materials, analysis tools or data; wrote the paper. All authors reviewed the manuscript.

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Additional information

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In this study we clearly demonstrate that 3–nitropropionic acid neurotoxicity affects the sirtuin genes expression and these gene expression changes differs in case of SIRT3 deficiency as demonstrated by RT-PCR, immunohistochemistry and behavioural tests.