Lack of riluzole efficacy in the progression of the neurodegenerative phenotype in a new conditional mouse model of striatal degeneration

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Background. Huntington's disease (HD) is a rare familial autosomal dominant neurodegenerative disorder characterized by progressive degeneration of medium spiny neurons (MSNs) located in the striatum. Currently available treatments of HD are only limited to alleviating symptoms; therefore, high expectations for an effective therapy are associated with potential replacement of lost neurons through stimulation of postnatal neurogenesis. One of the drugs of potential interest for the treatment of HD is riluzole, which may act as a positive modulator of adult neurogenesis, promoting replacement of damaged MSNs. The aim of this study was to evaluate the effects of chronic riluzole treatment on a novel transgenic mouse model of HD based on the genetic ablation of the transcription factor TIF-IA. This model is characterized by selective and progressive degeneration of MSNs.

Methods. Selective ablation of TIF-IA in MSNs (TIF-IA^{D1RCre} mice) was achieved by Cre-based recombination driven by the dopamine 1 receptor (D1R) promoter in the C57Bl/6N mouse strain. Riluzole was administered for 14 consecutive days (5 mg/kg, i.p.; 1x daily). Behavioral analysis included a motor coordination test performed on 13-week-old animals on an accelerated rotarod (4 to 40 r.p.m.; 5 min). To visualize the potential effects of riluzole treatment, the striata of the animals were stained by immunohistochemistry (IHC) and/or immunofluorescence (IF) with neuronal markers (NeuN) and specific markers associated with neurodegeneration (GFAP, 8OHdG). Additionally, the morphology of dendritic spines of neurons was assessed by a commercially available FD Rapid Golgi Stain™ Kit.

Results. A comparative analysis of IHC staining patterns with chosen markers for the neurodegeneration process in MSNs did not show an effect of riluzole on delaying the progression of MSN cell death despite an observed enhancement of neurogenesis as visualized by the Ki67 marker. A lack of a riluzole effect was also reflected by the behavioral phenotype associated with MSN degeneration. Moreover, the analysis of dendritic spine morphology did not show differences between mutant and control animals.

Discussion. Despite the observed increase in newborn cells in the subventricular zone (SVZ) after riluzole administration, our study did not show any differences between riluzole-treated and non-treated mutants, revealing a similar extent of the neurodegenerative phenotype evaluated in 13-week-old TIF-IA^{D1RCre} animals. This could be due to either the treatment paradigm (relatively low dose of riluzole used for this study) or the possibility that the effects were simply too weak to have any functional meaning. Nevertheless, this study is in line with others that question the effectiveness of riluzole in animal models and raise concerns about the utility of this drug due to its rather modest clinical efficacy.

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Author contributions:

GK designed the study, analyzed data and wrote the paper; GK and KRZ performed the riluzole injections and the behavioral study; GK and MB performed immunohistochemistry; MB bred and genotyped the mice; PC performed the analysis of dendritic spine morphology; IN supervised the study, critically revised and made final approval of the manuscript.

Abstract

Background. Huntington's disease (HD) is a rare familial autosomal dominant neurodegenerative disorder characterized by progressive degeneration of medium spiny neurons (MSNs) located in the striatum. Currently available treatments of HD are only limited to alleviating symptoms; therefore, high expectations for an effective therapy are associated with potential replacement of lost neurons through stimulation of postnatal neurogenesis. One of the drugs of potential interest for the treatment of HD is riluzole, which may act as a positive modulator of adult neurogenesis, promoting replacement of damaged MSNs. The aim of this study was to evaluate the effects of chronic riluzole treatment on a novel transgenic mouse model of HD based on the genetic ablation of the transcription factor TIF-IA. This model is characterized by selective and progressive degeneration of MSNs.

Methods. Selective ablation of TIF-IA in MSNs (TIF-IA^{D1RCre} mice) was achieved by Cre-based recombination driven by the dopamine 1 receptor (D1R) promoter in the C57Bl/6N mouse strain. Riluzole was administered for 14 consecutive days (5 mg/kg, i.p.; 1x daily). Behavioral analysis included a motor coordination test performed on 13-week-old animals on an accelerated rotarod (4 to 40 r.p.m.; 5 min). To visualize the potential effects of riluzole treatment, the striata of the animals were stained by immunohistochemistry (IHC) and/or immunofluorescence (IF) with neuronal markers (NeuN) and specific markers associated with neurodegeneration (GFAP, 8OHdG). Additionally, the morphology of dendritic spines of neurons was assessed by a commercially available FD Rapid Golgi Stain™ Kit.

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Discussion. Despite the observed increase in newborn cells in the subventricular zone (SVZ) after riluzole administration, our study did not show any differences between riluzole-treated and non-treated mutants, revealing a similar extent of the neurodegenerative phenotype evaluated in

13-week-old TIF-IA^{D1RCre} animals. This could be due to either the treatment paradigm (relatively low dose of riluzole used for this study) or the possibility that the effects were simply too weak to have any functional meaning. Nevertheless, this study is in line with others that question the effectiveness of riluzole in animal models and raise concerns about the utility of this drug due to its rather modest clinical efficacy.

Introduction

Huntington's disease (HD) is a rare (1:10000) familial autosomal dominant neurodegenerative disorder caused by an expanded stretch of polyglutamine (polyQ) repeats in the protein huntingtin (Hannan 2005) and characterized by progressive degeneration of medium spiny neurons (MSNs) located in the striatum. The disease inevitably culminates with death and cures to at least retard its progression are unavailable so far. Currently available treatments are limited to alleviating some of the symptoms, mainly involuntary movements, associated with the disease. Despite the known origin, there is a lack of understanding of the complex pathogenesis of HD, which affects multiple functions and regulatory pathways, making the development of efficient therapeutics challenging (Kazantsev & Hersch 2007). Classic pharmacological models of HD are based on applying a neurotoxin, 3-nitropropionic acid (3-NP) (Tunez et al. 2010), however this approach leads to immediate neuronal death, which substantially narrows the opportunity to observe the pathological changes associated with the slow neurodegenerative process. On the other hand, many transgenic animal models of HD, even though created by replicating the same genetic malfunction directly responsible for HD in humans, do not fully recapitulate the HD-like phenotype, including profound neuronal loss (or at least not to the expected extent) (Kreiner 2015).

Designed cell therapies for neurodegenerative diseases are mostly based on the replacement of lost neurons through transplantation or activation of neuronal progenitor cells (Emsley et al. 2005). In rodent models of HD, induced neurogenesis in MSNs is thought to be evoked primarily due to neuronal precursors derived from the subventricular zone (SVZ) of the lateral ventricles. The SVZ represents the largest reservoir of adult stem-like progenitors and in normal conditions gives rise to new olfactory bulb interneurons (Bonfanti & Peretto 2007). Stimulation of postnatal neurogenesis is being considered as a potential therapeutic target in several neurodegenerative diseases including HD (Abdipranoto et al. 2008; Lindvall & Kokaia 2010; Ransome et al. 2012). One of the drugs of potential interest for the treatment of HD is riluzole, already approved for the treatment of amyotrophic lateral sclerosis (ALS) (Miller et al. 2012). Riluzole, by interfering with glutamatergic neurotransmission, reduces excitotoxicity and acts as a positive modulator of adult neurogenesis, promoting replacement of damaged MSNs (Katoh-Semba et al. 2002; Squitieri et al. 2008; Veyrac et al. 2009). Moreover, this compound was shown to be effective in attenuating several clinically relevant symptoms in a variation of an

animal MPTP model representing the early phase of Parkinson's disease (PD) (Verhave et al. 2012). Nevertheless, there are still concerns about its utility due to its rather modest clinical efficacy.

To address this question, we applied a novel approach using a mouse model of HD based on the activation of an endogenous suicide mechanism achieved by genetic ablation of the transcription factor TIF-IA, an essential regulator of polymerase I activity (Kreiner et al. 2013). Inactivation of TIF-IA blocks the synthesis of ribosomal RNA, leading to nucleolar disruption and p53-mediated apoptosis (Yuan et al. 2005). Loss of TIF-IA in neuronal progenitor cells results in mice born without a brain (Parlato et al. 2008), but when it is lost in mature neurons, the major features of the neurodegenerative process are recapitulated. Namely, inactivation of the TIF-IA gene in striatal MSNs (TIF-IA^{D1RCre} mice) recapitulates the phenotypic alterations associated with selective striatal neurodegeneration, including increased oxidative damage and inflammatory response, finally leading to MSN cell death and resulting in an HD-like phenotype (i.e., impairment of motion control and clasping behavior) (Kreiner et al. 2013). In contrast to the majority of other models of neurodegeneration, TIF-IA^{D1Cre}-mutant mice are characterized by the progressive degeneration of targeted neurons over a long period of time (several weeks), mimicking the typical hallmark of the disease (Kreiner et al. 2013). Preliminary analysis of adult TIF-IA^{D1RCre}-mutant mice indicated the occurrence of neurogenesis in the striatum. Thus, the progressive TIF-IA-driven neurodegeneration in these mice offers the unique advantage to study if, in such conditions, the endogenous progenitors potentially involved in putative neuroprotective mechanisms can be modulated by experimental treatments.

Materials & Methods

Mice

Selective ablation of TIF-IA in the MSNs (TIF-IA^{D1RCre} mice) was achieved by *Cre/loxP* recombination in the C57Bl/6N mouse strain. Transgenic mice hosting Cre recombinase under the dopamine 1 receptor (D1R) promoter were crossed with animals harboring the floxed TIF-IA gene as described previously (Kreiner et al. 2013). Mutant TIF-IA^{D1RCre} mice were kept together with their control (Cre-negative) littermates in self-ventilated cages (Allentown, USA) under standard laboratory conditions (12 h light/dark cycle, food and water ad libitum). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of

Laboratory Animals of the National Institutes of Health. The protocol for the behavioral study was approved by the Animal Ethical Committee at the Institute of Pharmacology, Polish Academy of Sciences (Permit Number: 951, issued: June 28, 2012).

Drug treatment

The 9-week-old mice (approx. 2 months old) were divided into 4 experimental groups (control+VEH, control+RIL, mutant+VEH, mutant+RIL), receiving either riluzole (RIL; 5 mg/kg, i.p.; Sigma-Aldrich Chemical Co., St. Louis, USA) or vehicle (VEH; 10% DMSO) for 14 consecutive days (1x daily).

Behavioral analysis

A coordination test was performed on 13-week-old animals on an accelerated rotarod (Ugo Basile, Italy), and the time spent on the accelerating rod (4 to 40 r.p.m. within 5 min) was measured. Additionally, the weight of the animals was consistently monitored during the time of drug application and on the day before the behavioral test.

Immunohistochemistry

To visualize the potential effects of drug treatment, the striata of animals were subject to *post-mortem* staining using immunohistochemistry (IHC) and/or immunofluorescence (IF) with specific markers as described previously (Chmielarz et al. 2013; Kreiner et al. 2013). Briefly, the mice were sacrificed by cervical dislocation, and their brains were excised, fixed overnight in 4% paraformaldehyde (PFA), embedded in paraffin and sectioned on a rotary microtome. Chosen sections from corresponding regions of the striatum in mutant and control animals were incubated overnight at 4 °C with primary anti-NeuN (1:500, Millipore), anti-GFAP (1:500, Millipore), and anti-8OHdG (1:200, Millipore) antibodies. Visualization of antigen-bound primary antibodies was carried out using a proper biotinylated secondary antibody together with the Avidin–Biotin Complex (ABC; Vector Laboratories, USA) followed by diaminobenzidine treatment (DAB; Sigma-Aldrich, USA) or an anti-rabbit Alexa-488-coupled secondary antibody (Invitrogen, USA).

Dendritic spine morphology

Morphological analysis of dendritic spines was assessed as described previously (Chmielarz et al. 2015). Briefly, following extraction, the brains were rinsed in distilled water, impregnated with the use of the FD Rapid Golgi Stain™ Kit (FD NeuroTechnologies, USA), and incubated in 30% sucrose for 3-7 days. Vibratome (Leica, Germany) sections were cut to 100 µm thick and

mounted on Super Frost Plus slides (Thermo Scientific, USA) and stained using solutions provided in the kit. The dendritic spines were counted on the dorsal striatum between Bregma 1.1 and 0.0. Dendritic spines were counted on at least 10 μM long fragments of 3rd and 4th row dendrites. There were 3 pieces counted from each neuron and 5 neurons counted for each animal. Only completely stained neurons not obscured by neighboring neurons within the striatum were considered. Spine counting and optical imaging were performed by an experimenter blind to the genotype of the animal on a Nikon Eclipse 50i (Nikon, Japan) equipped with a CCD camera connected to a computer equipped with NIS Elements BR 30 software.

Statistical analysis

Statistical analysis was performed with Graph Pad Prism 5.01. Data were evaluated by 2-way analysis of variance (2-way ANOVA) followed by Bonferroni test for comparison of biologically relevant groups.

Results

Lack of riluzole efficacy on progression of MSN cell death despite enhancement of neurogenesis.

A comparative analysis of immunohistochemical staining patterns with chosen markers characteristic for neurodegenerative process in MSNs (marker for labeling immature neurons, NeuN; an oxidative stress indicator marker, 8-hydroxydeoxyguanosine, 8OHdG; astrocyte marker, GFAP) did not show any visual differences between mutant TIF-IA^{D1RCre} mice with or without riluzole treatment (**Fig. 1A-C**). The expression of all of the above-mentioned markers seems to be similar in the mutant TIF-IA^{D1RCre} mice, showing similar enhancement of inflammatory processes, oxidative stress and neuronal loss. Only the expression of Ki67 (marker of neurogenesis) showed substantial enhancement in the region of the SVZ in both control and mutant riluzole-treated animals (**Fig. 1D**).

Lack of riluzole efficacy on the behavioral phenotype associated with MSN degeneration.

Chronic riluzole administration did not prevent the drop in weight and impaired motor coordination of 13-week-old mutant TIF-IA^{D1RCre} mice as demonstrated by the rotarod test (**Fig. 2**). The riluzole administration had no effect on control animals, while the different effect of the

introduced mutation is reflected in a 2-way ANOVA, which reveals a treatment (riluzole) x genotype interaction for genotype [$F(131.38) = 80.39$ ($p < 0.0001$)] but not for riluzole itself.

Riluzole does not affect dendritic spine morphology in TIF-IA^{D1RCre} mice.

Our previous research done on TIF-IA^{D1RCre} mice clearly showed that although the neurodegeneration (cell loss) is not observed earlier than in 13-week-old animals, some symptoms of cellular impairment can be seen 2-4 weeks in advance (Kreiner et al. 2013). Taking this into account, we checked whether chronic riluzole treatment could have any positive effects on neural cell morphology. Nevertheless, the performed analysis of the morphology of dendritic spines did not show any differences between mutants and controls (**Fig. 3**). There was also no effect of riluzole application on dendritic spine morphology in control animals.

Discussion

To elucidate whether the enhancement of adult neurogenesis induced by chronic riluzole administration can have any positive influence on the progression of the neurodegenerative phenotype observed in TIF-IA^{D1RCre} mice, we evaluated several markers of neurodegeneration known to be differentially expressed in these mice at 13 weeks old as described previously (Kreiner et al. 2013). Additionally, we also screened their behavioral phenotype by assessing motor coordination. Despite an observed increase in newborn cells in the SVZ after riluzole administration as visualized by Ki-67 staining (an effective marker of proliferating cells (Kee et al. 2002)), neither experimental approach showed any differences between riluzole-treated and non-treated mutants, revealing a similar extent of the neurodegenerative phenotype evaluated in 13-week-old animals (**Fig. 1-2**). The mice were characterized by the same expression of induced-GFAP and 8OHdG and profoundly reduced staining intensity for NeuN in the striatum (**Fig. 1A-C**). This was reflected by the impairment in motor coordination on the rotarod test, and again, no differences were observed between riluzole- and vehicle-treated mutants (**Fig. 2**). Overall, these experiments did not show any beneficial effects of riluzole administration on the progress of the mutation.

The rationale for this research was supported by preliminary studies, in which we observed an increase in neurogenesis within the SVZ in 9-week-old TIF-IA^{D1RCre} mice. It seemed

that further enhancement of this process by riluzole administration can bring considerable benefits in the form of slowing down the progression of the mutation. Our transgenic models based on the conditional ablation of transcription factor TIF-IA have already been positively verified as a possible tool to study the mechanisms of action of other pharmacotherapies. In particular, we showed that the progression of neurodegenerative phenotype in the TIF-IA^{DATCre} mice (PD model) can be postponed by L-DOPA (Rieker et al. 2011) or reboxetine treatment (Rafa-Zabłocka et al. 2014).

It can be argued that either the treatment paradigm was not appropriate to achieve the expected drug efficacy or the effects were simply too weak to have any functional meaning. Regarding the first issue, the dose of riluzole in chronic experiments performed on rodents does indeed range from 1 to 40 mg/kg (Besheer et al. 2009; Carbone et al. 2012; Fumagalli et al. 2006; Sepulveda et al. 1999), and is predominantly 20 mg/kg when used to evoke a neurogenesis response. Therefore, the dose used in our experiment was in the lower range of the therapeutic window. The reason for choosing this particular dose was determined by the lethargy and spastic gait followed by a high mortality rate of the mice treated with 20 and 10 mg/kg. This problem has also been reported by other researchers when rats were treated with similar doses and exhibited locomotor ataxia and lethargy (Kitzman 2009; Simard et al. 2012). We presume that this phenomenon is associated with the specific mouse strain (C57Bl/6N) rather than with the introduced mutation since the problem affected both control and TIF-IA^{D1RCre} mice. Nevertheless, it has to be emphasized that even the dose of 5 mg/kg was able to induce cell proliferation within the SVZ region as visualized by Ki67 staining (**Fig. 1D**) and there are existing reports that prove a similar dose to be effective (Kitzman 2009).

In addition, in order to evaluate whether riluzole can exert any influence on affected MSNs, we performed a quantitative analysis of dendritic spine morphology at the stage when the neurons were still present in the striata of TIF-IA^{D1RCre} mice. We analyzed 11-week-old mice, as this is the stage where no cell loss has been observed but the cascade of molecular events leading to degeneration has already been prompted (Kreiner et al. 2013). Nevertheless, this analysis did not show any changes in dendritic spine morphology, supporting the observation that riluzole seems to not be effective in the investigated model.

Surprisingly, we were not able to find any abnormalities in the morphology of dendritic spines in the non-treated TIF-IA^{D1RCre}-mutant mice despite the clear neurodegenerative

phenotype that has already been documented. This issue has not been addressed in our previous work. However, the occurrence of such changes is not always correlated with neurodegeneration (Dickstein et al. 2010) or may be a subsequent event. On the other hand, lack of spine pathology might also be attributed to the relatively early stage of pathology observed in 13-week-old TIF-IA^{D1RCre} mutants, as other authors have shown that spine pathology was present in late (36-weeks old) (Spires et al. 2004), but not early (20-weeks old) (Nithianantharajah et al. 2009), symptomatic stages of the R6/1 Huntington disease model.

The lack of analysis of other time points (i.e., 16-week-old animals or older) can be regarded as a drawback of the experimental design. This is mainly due to the relatively low number of animals in the cohort, which is restricted by current strict animal welfare regulations. Nevertheless, since the phenotype of riluzole-treated TIF-IA^{D1RCre} mice is non-distinguishable from untreated mutants (regarding both the behavioral and histological levels) at the age of 13 weeks (where the cells are already starting to degenerate), it would be hard to imagine that any differences would be observed at a later period. Moreover, lack of differences at this pivotal stage does not provide any support for the investigation of earlier time points, which could have been interesting if we had observed findings differentiating the animals at 13 weeks.

In spite of expectations based on previously gathered evidence in preclinical studies and the use of riluzole in clinics for the treatment of ALS, a recent study also yielded disappointing results concerning this drug. Despite being an expensive drug, it does not stop the progression of ALS and is not always well tolerated, making the efficacy of riluzole in the treatment of ALS inconclusive (Ginsberg & Lowe 2002). Moreover, experiments performed on animal models assessing riluzole as a potential treatment for HD and spinocerebellar ataxia (SCA) had no beneficial effects (Hockly et al. 2006; Schmidt et al. 2016). In clinical trials of anti-HD treatment, there was also no clear neuroprotective effect of riluzole administration, and its effects were narrowed only to reduced chorea (Frank 2014). Thus, our study seems to be in line with others that question the effectiveness of riluzole in animal models and raise concerns about the utility of this drug due to its rather modest clinical efficacy (Limpert et al. 2013).

Conclusions

Despite an observed increase in newborn cells in the SVZ after riluzole administration, our study did not show any differences between riluzole-treated and non-treated mutants,

revealing a similar extent of the neurodegenerative phenotype evaluated in 13-week-old TIF-IA^{D1RCre} animals, a new transgenic model resembling HD-like neurodegeneration. This lack of an observed effect could be due to either the treatment paradigm or the possibility that the effects were simply too weak to have any functional meaning. Nevertheless, this study is in line with others that question the effectiveness of riluzole in animal models and raise concerns about the utility of this drug due to its rather modest clinical efficacy.

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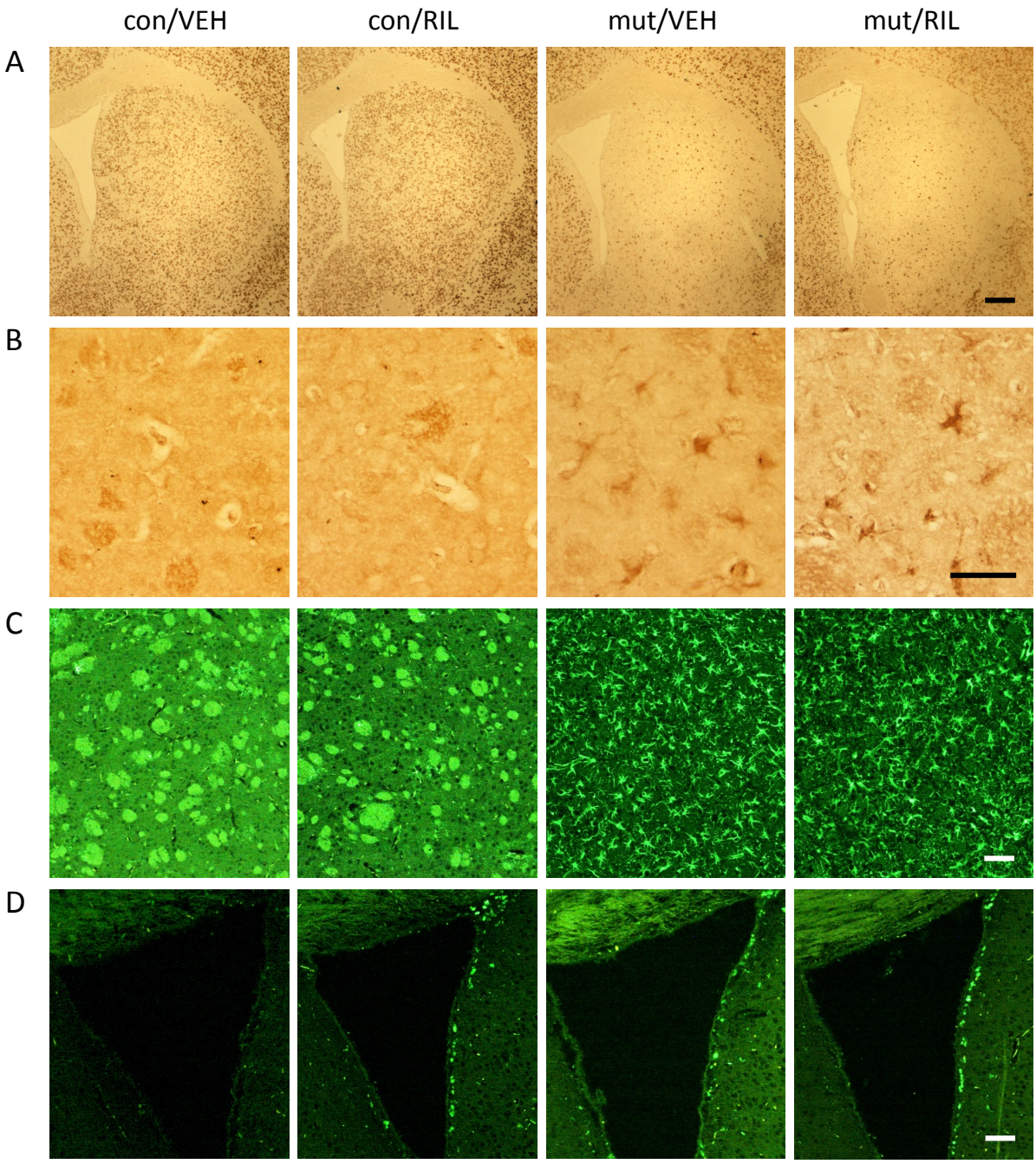
Fig. 1. Representative images of immunohistochemical analysis showing staining of striata with the NeuN (**A**), induction of oxidative stress detected by the anti-8OHdG antibody **(**B**)**, astrogliosis visualized by the GFAP-specific antibody **(C)**, and proliferating cells in the region of the SVZ shown by the Ki67 marker (**D**) in control (con) and TIF-IA^{D1RCre}-mutant (mut) mice. RIL – riluzole, VEH – vehicle. Scale bars: 5 µm (**A**), 25 µm (**B**), 50 µm (**C, D**).



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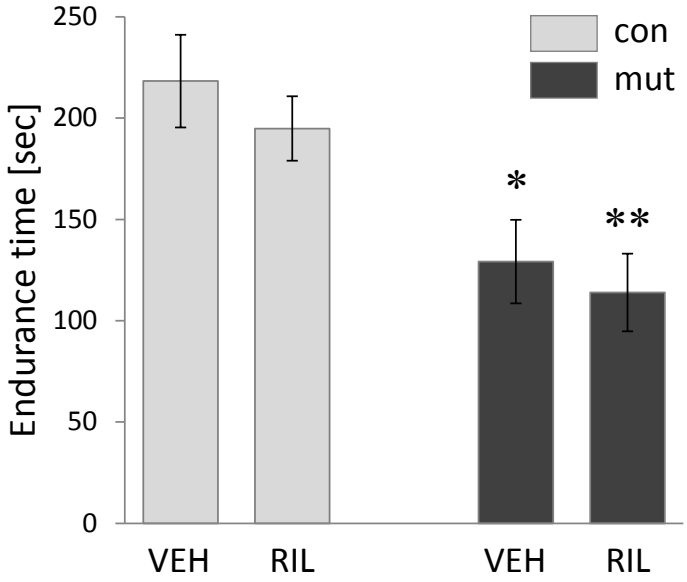
298 **Fig. 2.** Assessment of motor coordination of control (con) and TIF-IA^{D1RCre}-mutant (mut) mice
299 demonstrated by endurance in the rotarod test. Values for endurance time are represented by
300 means±S.E.M. (n = 7–8; * $p < 0.05$; ** $p < 0.01$). RIL – riluzole, VEH – vehicle.



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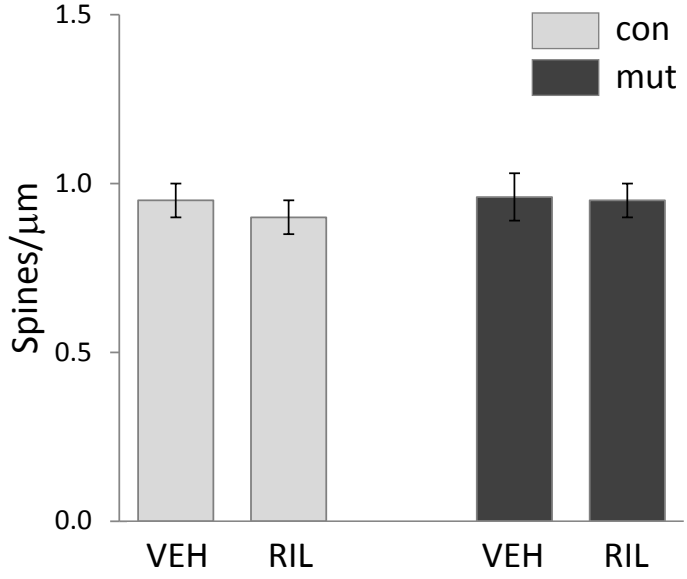
303 **Fig. 3.** Number of dendritic spines in the control (con) and mutant (mut) mice. The spines were
304 counted in the dorsal striatum (Bregma 1.1 – 0.0). Data are represented by the means±SEM (n =
305 3–4). RIL – riluzole, VEH – vehicle.



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