Experimental Neurology 290 (2017) 15–28

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Research paper

Zonisamide attenuates lactacystin-induced parkinsonism in mice without affecting system x_c^-

Eduard Bentea [a], Joeri Van Liefferinge [b], Lise Verbruggen [a], Katleen Martens [a], Sho Kobayashi [c,1], Lauren Deneyer [a], Thomas Demuyser [b], Giulia Albertini [b], Katrien Maes [b], Hideyo Sato [d], Ilse Smolders [b], Jan Lewerenz [e], Ann Massie [a,*]

[a] Department of Pharmaceutical Biotechnology and Molecular Biology, Center for Neurosciences, Vrije Universiteit Brussel, Brussels, Belgium
[b] Department of Pharmaceutical Chemistry and Drug Analysis, Center for Neurosciences, Vrije Universiteit Brussel, Brussels, Belgium
[c] Department of Food and Applied Life Sciences, Yamagata University, Yamagata, Japan
[d] Laboratory of Biochemistry and Molecular Biology, Department of Medical Technology, Niigata University, Niigata, Japan
[e] Department of Neurology, Ulm University, Ulm, Germany

ARTICLE INFO

Article history:
Received 8 July 2016
Received in revised form 15 December 2016
Accepted 21 December 2016
Available online 24 December 2016

Keywords:
Zonisamide
Neuroprotection
Parkinson's disease
Lactacystin
xCT
Glutathione

ABSTRACT

Zonisamide (ZNS), an anticonvulsant drug exhibiting symptomatic effects in Parkinson's disease (PD), was recently reported to exert neuroprotection in rodent models. One of the proposed neuroprotective mechanisms involves increased protein expression of xCT, the specific subunit of the cystine/glutamate antiporter system x_c^-, inducing glutathione (GSH) synthesis. Here, we investigated the outcome of ZNS treatment in a mouse model of PD based on intranigral proteasome inhibition, and whether the observed effects would be mediated by system x_c^-. The proteasome inhibitor lactacystin (LAC) was administered intranigrally to male C57BL/6J mice receiving repeated intraperitoneal injections of either ZNS 30 mg kg^{-1} or vehicle. Drug administration was initiated three days prior to stereotaxic LAC injection and was maintained until six days post-surgery. One week after lesion, mice were behaviorally assessed and investigated in terms of nigrostriatal neurodegeneration and molecular changes at the level of the basal ganglia, including expression levels of xCT. ZNS reduced the loss of nigral dopaminergic neurons following LAC injection and the degree of sensorimotor impairment. ZNS failed, however, to modulate xCT expression in basal ganglia of lesioned mice. In a separate set of experiments, the impact of ZNS treatment on system x_c^- was investigated in control conditions *in vivo* as well as *in vitro*. Similarly, ZNS did not influence xCT or glutathione levels in naive male C57BL/6J mice, nor did it alter system x_c^- activity or glutathione content *in vitro*. Taken together, these results demonstrate that ZNS treatment provides neuroprotection and behavioral improvement in a PD mouse model based on proteasome inhibition *via* system x_c^- independent mechanisms.

1. Introduction

The serendipitous discovery of the antiparkinsonian effects of zonisamide (ZNS), an anticonvulsant drug, in a Japanese Parkinson's disease (PD) patient with epilepsy (Murata et al., 2001) stimulated interest in this drug for the symptomatic management of motor symptoms in PD. Subsequent double-blind, placebo-controlled clinical trials provided support for this hypothesis, as the combinatorial treatment of ZNS and the dopamine (DA) precursor L-3,4-dihydroxyphenylalanine (L-dopa) was found to improve all cardinal symptoms of PD with a low incidence of adverse effects (Murata et al., 2004, 2007). These results prompted the marketing of the drug in Japan (under the commercial name Trerief®) as adjunctive therapy to L-dopa in previously treated patients with PD (January 2009; Dainippon Sumitomo Pharma Co.) (Yang and Perry, 2009). More recently, ZNS has been found to reduce the "off" time in L-dopa treated PD patients experiencing wearing-off phenomena (Murata et al., 2015) and to improve motor function of patients with early stage PD (Maeda et al., 2015), supporting the clinical implementation of this drug in PD.

Several pharmacological mechanisms contributing to DA release and synthesis might explain the antiparkinsonian action of ZNS (Miwa,

Abbreviations: 5HT, serotonin; 5HIAA, 5-hydroxyindoleacetic acid; 6-OHDA, 6-hydroxydopamine; DA, dopamine; DAT, dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; ECL, enhanced chemiluminescence; GSH, glutathione; HVA, homovanillic acid; i.p., intraperitoneal; L-Dopa, L-3,4-dihydroxyphenylalanine; LAC, lactacystin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; SNc, substantia nigra pars compacta; STN, subthalamic nucleus; TH, tyrosine hydroxylase; ZNS, zonisamide.

* Corresponding author: Department of Pharmaceutical Biotechnology and Molecular Biology, Center for Neurosciences (C4N), Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium.
E-mail address: Ann.Massie@vub.ac.be (A. Massie).
[1] Current affiliation: Department of Biochemistry and Molecular Biology, Graduate School of Medical Science, Yamagata University, Yamagata, Japan.

http://dx.doi.org/10.1016/j.expneurol.2016.12.009
0014-4886/

2007). Chronic administration of ZNS has been shown to increase protein levels and activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis (Murata, 2004), to inhibit the DA metabolizing enzyme MAO-B (Sonsalla et al., 2010) and to increase intracellular DA levels in striatum of rats (Okada et al., 1995). Furthermore, acute administration of therapeutic doses of ZNS has been found to increase extracellular DA levels in striatal microdialysate samples of intact freely moving rats (Okada et al., 1995). Concomitantly, non-DA-ergic pathways might also be involved in the observed antiparkinsonian effects. ZNS has been shown to suppress tacrine-induced tremulous jaw movements, a proposed model of pharmacologically induced parkinsonian tremor *via* non-DA-ergic mechanisms (Miwa et al., 2008). In addition, administration of ZNS inhibits the striato-pallidal GABA-ergic neurotransmission *via* activation of striatal δ1 receptors (Yamamura et al., 2009). Recently, it was proposed that decreases of both striato-pallidal GABA-ergic and subthalamo-nigral glutamatergic transmission following ZNS might be mediated by stimulating astroglial release of endogenous group II and III metabotropic glutamate receptor ligands resulting from the kynurenine pathway (Fukuyama et al., 2014).

In addition to its symptomatic effects, several research groups recently reported neuroprotective effects of ZNS in toxin- and genetic-based PD rodent models (Arawaka et al., 2014; Asanuma et al., 2010; Sano et al., 2015; Sonsalla et al., 2010). One of the proposed neuroprotective mechanisms involves increased xCT protein expression, the functional subunit of the cystine/glutamate antiporter system x_c^-, demonstrated by an increase in xCT-like immunoreactivity upon immunofluorescence (Asanuma et al., 2010). System x_c^- is a membrane-bound Na^+-independent transporter that imports one molecule of cystine in exchange for one glutamate molecule (Lewerenz et al., 2013; Massie et al., 2015). *In vitro*, imported cystine, after intracellular reduction to cysteine, is an essential building block of glutathione (GSH), the major brain antioxidant. In cell cultures, system x_c^- seems to be essential for maintenance of intracellular GSH levels and antioxidant defenses, given that genetic deletion (Sato et al., 2005) or inhibition (Albrecht et al., 2010) of system x_c^- leads to GSH depletion and cell death by oxidative stress. On the other hand, mice deficient in xCT ($xCT^{-/-}$ mice) do not show decreased striatal (Massie et al., 2011) or hippocampal (De Bundel et al., 2011) GSH levels or signs of increased oxidative stress, indicating that system x_c^- might play a smaller role in regulating GSH levels *in vivo*, or alternatively that other pathways compensate for the loss of system x_c^- and support normal GSH synthesis. Whereas Asanuma and colleagues attributed ZNS-induced neuroprotection in the unilateral 6-hydroxydopamine (6-OHDA) mouse model to the enhancement of system x_c^- and subsequent increase in GSH levels (Asanuma et al., 2010), we observed protective effects of a genetic loss of system x_c^- against 6-OHDA-induced neurodegeneration and ascribed this effect to reduced levels of extracellular glutamate (Massie et al., 2011). In accordance, other studies suggest that in diverse neurological disorders enhancement of system x_c^- might trigger excitotoxicity due to excessive non-vesicular glutamate release (Evonuk et al., 2015; Mesci et al., 2015; Qin et al., 2006; Robert et al., 2015).

The proteasome inhibition model of PD was recently described as an alternative toxin-based model (Bentea et al., 2015; Mackey et al., 2013; McNaught et al., 2002; Xie et al., 2010), originating from the discovery of decreased catalytic activities of the 20S proteasome in the Substantia Nigra pars compacta (SNc) of PD patients (McNaught et al., 2003). Impairment in the function of the ubiquitin-proteasome system leads to build-up of damaged or misfolded proteins, and is believed to contribute to the formation of intracellular protein aggregates and neurodegeneration in PD (Ebrahimi-Fakhari et al., 2012; Olanow and McNaught, 2006). Similar to the proposed pathophysiological role of proteasome inhibition in the human disorder, stereotaxic delivery of proteasome inhibitors such as lactacystin (LAC) in rodents has been found to trigger DA-ergic neurodegeneration and accumulation of disease-linked proteins such as α-synuclein (Fornai et al., 2003; Mackey et al., 2013; Vernon et al., 2010; Xie et al., 2010) and Ser129-phosphorylated α-synuclein (Bentea et al., 2015), a post-translationally modified form enriched in Lewy bodies (Fujiwara et al., 2002).

Although ZNS has been previously shown to provide neuroprotection against classical PD neurotoxins such as 6-OHDA (Asanuma et al., 2010) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sonsalla et al., 2010), it is currently unknown whether it would also protect nigral DA-ergic neurons against proteasome-inhibition mediated toxicity. In addition, the precise molecular mechanisms underlying the protective effects of ZNS in PD animal models are incompletely understood, while the effects of the drug on PD-related non-motor symptoms remain so far insufficiently unexplored. In order to address these issues, we designed a pharmacological approach in which mice received ZNS prior to, as well as following, intranigral LAC administration, and were investigated in terms of damage to the nigrostriatal pathway and both motor and non-motor function, with particular emphasis on anxiety-like behavior. In addition, we tested the proposed function of ZNS to induce system x_c^- and GSH synthesis in different *in vitro* and *in vivo* experimental set-ups. Finally, given that ZNS has been proposed to influence the activity of the indirect pathway of the basal ganglia (Fukuyama et al., 2014; Yamamura et al., 2009), we also investigated whether repeated ZNS administration modulates subthalamic nucleus (STN) hyperactivity following LAC lesion using c-fos immunohisto chemistry.

2. Materials and methods

2.1. Animals and treatment

Animal experiments were conducted in accordance to the national guidelines on animal experimentation and were approved by the Ethical Committee for Animal Experimentation of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel. All animals were housed under standardized conditions, with free access to food and water. All efforts were made to minimize animal suffering. The results are presented in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010).

A first set of experiments was designed to investigate the effect of chronic ZNS treatment on nigrostriatal DA-ergic degeneration, behavioral impairment, STN hyperactivity and xCT expression changes in the LAC hemi-parkinson model (see Fig. 1A for experimental design). A total of 57 male C57BL/6J mice (Charles River Laboratories, France), 12–14 weeks of age, were used for these experiments. Half of the mice ($n = 28$) were once daily injected intraperitoneally (i.p.) with 30 mg kg^{-1} ZNS sodium salt (Tocris Bioscience) dissolved in NaCl 0.9% for 10 days, starting three days before LAC or sham lesioning, while the other half ($n = 29$) received a corresponding placebo treatment and were once daily injected i.p. with NaCl 0.9% for the same amount of time. The protocol of ZNS administration was based on a previous study indicating that repeated i.p. injections of 30 mg kg^{-1} ZNS for 7 days is able to protect nigral DA neurons against intrastriatal 6-OHDA administration in mice (Asanuma et al., 2010). From the 28 mice receiving ZNS, 16 were stereotaxically injected with LAC and 12 with vehicle. From the 29 mice receiving placebo, 17 were stereotaxically injected with LAC and 12 with vehicle. Following stereotaxic surgery, 1 sham-lesioned placebo-treated, 2 sham-lesioned ZNS-treated and 2 LAC-lesioned placebo-treated mice died. Accounting for post-operative mortality, the final group sizes of the study included $n = 11$ sham-lesioned placebo-treated, $n = 10$ sham-lesioned ZNS-treated, $n = 15$ LAC-lesioned placebo-treated and $n = 16$ LAC-lesioned ZNS-treated mice. All mice were investigated in motor and non-motor based paradigms at 7 days post-stereotaxic surgery (*i.e.* one day after the last ZNS/placebo injection) (Fig. 1A). After neurobehavioral analyses, mice were sacrificed by cervical dislocation and brains were quickly removed. A first group of mice (Group A: $n = 6$ sham-lesioned placebo-treated mice, $n = 5$ sham-lesioned ZNS-treated mice, $n = 10$ LAC-lesioned placebo-treated mice, $n = 10$ LAC-lesioned ZNS-treated mice)

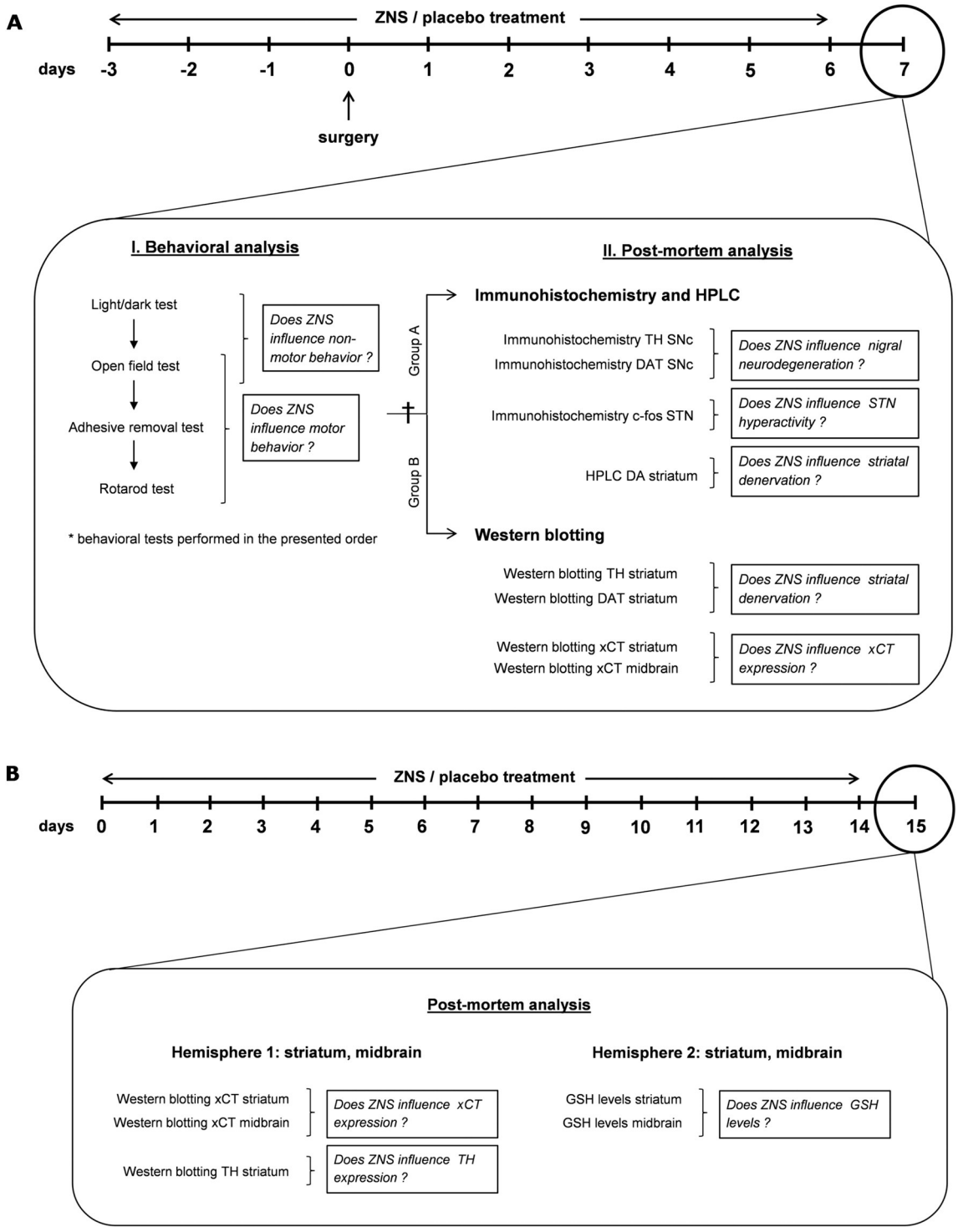

Fig. 1. Experimental design for investigating the effect of repeated ZNS treatment in mice stereotaxically injected with LAC or vehicle (A) or in naive mice (B). ZNS was injected daily, i.p., at a dose of 30 mg kg^{-1}, dissolved in NaCl 0.9%. Control mice were subjected to the same treatment protocol, but received only vehicle (NaCl 0.9%).

was used to investigate changes in DA neurons at the level of the SNc and expression of c-fos at the level of the STN by immunohistochemistry, while from the rostral part of the brain striata were dissected for neurochemical analysis of total monoamine contents. From the second group of mice (Group B: $n = 5$ sham-lesioned placebo-treated mice, $n = 5$ sham-lesioned ZNS-treated mice, $n = 5$ LAC-lesioned placebo-treated mice, $n = 6$ LAC-lesioned ZNS-treated mice), midbrain and striata were quickly dissected and homogenized for protein extraction and Western blotting experiments. Mice were randomly allocated to either Group A or Group B until both groups were fully allocated to the designed sample size. Due to the aggressive nature of mice in the current batch, all animals were single housed during the entire study period to avoid severe injuries and the requirement of end-point euthanasia.

In a second set of experiments, naive male C57BL/6J mice were once daily injected i.p. with 30 mg kg^{-1} ZNS sodium salt (Tocris Bioscience) dissolved in NaCl 0.9% for 14 days ($n = 4$) or received a corresponding placebo treatment ($n = 5$) (see Fig. 1B for experimental design). This experimental protocol was based on a previous study indicating that

repeated i.p. injections of 30 mg kg^{-1} ZNS for 14 days increases GSH levels in the striatum and ventral midbrain of naive mice (Asanuma et al., 2010). One day after the last ZNS administration, mice were sacrificed by cervical dislocation and striatum and midbrain dissected out immediately. One striatum and one half of the midbrain were homogenized for protein extraction and the other striatum and second half of the midbrain were homogenized for total GSH content measurement.

2.2. Stereotaxic surgery

As previously described (Bentea et al., 2015), mice were anesthetized with a mixture of ketamine (100 mg kg^{-1} i.p.; ketamine 1000 Ceva, Ceva Sante Animale, Brussels, Belgium) and xylazine (10 mg kg^{-1} i.p.; rompun 2%, Bayer N.V., Brussels, Belgium) and positioned on a stereotaxic frame (Kopf Model 963 Ultra Precise Small Animal Stereotaxic Frame, David Kopf Instruments, California, USA). A microinjection of 1.5 μL freshly prepared LAC solution (2 μg/μL in NaCl 0.9%; Cayman Chemicals, Michigan, USA) was performed at a flow rate of 0.5 μL/min into the left SNc, using the following coordinates: AP-3.0, LM-1.0, DV-4.5 from bregma (Paxinos and Franklin, 2007). Sham-operated mice received the same volume of vehicle (NaCl 0.9%) at the same coordinates. Mice received 4 mg kg^{-1} ketoprofen i.p. (Ketofen, Merial, Brussels, Belgium) for post-operative analgesia.

2.3. Neurobehavioral assessment

Mice were subjected to distinct motor and non-motor based paradigms, one week after LAC administration. Behavioral tests were conducted during the light phase (8:00 a.m.–5:00 p.m.). Mice were acclimatized to the testing room at least 1 h before the start of each behavioral test. Assessment was performed by scientists blinded for lesion and treatment condition.

2.3.1. Rotarod test

Motor coordination and balance were assessed using an accelerating rotarod system (TSE RotaRod Advanced, TSE Systems). Prior to surgery and the initial ZNS administration, mice were trained for 5 min at a constant speed of 5 rpm. The second phase of training consisted of 3 repeated trials of 1 min at a fixed speed of 5 rpm, with 3 min of rest in-between. To assess rotarod performance at baseline and after lesion, mice underwent five repeated trials starting at a constant speed of 5 rpm for 30 s and continued with a 5–25 rpm accelerating protocol during 200 s (maximum total rod time of 230 s). The mean of the five test trials was used for statistical analysis.

2.3.2. Adhesive removal test

Sensorimotor performance was investigated using the adhesive removal test. All mice were first habituated to a transparent test box for 60 s. Small adhesive strips (0.3 × 0.4 cm) were taped on the plantar surface of both forelimbs with equal pressure. Mice were immediately placed back in the test box for assessment, until both strips were removed. Prior to surgery, the mice were trained for 5 days. The adhesive placement order (left forepaw or right forepaw first) was alternated for each day of training during the first 4 days and randomized for the last day of training and for the test session. During the test session, two parameters were measured: time-to-contact and time-to-remove. Time-to-contact, or the time needed to sense the adhesive, visually observed as mouth to paw contact, reflects correct paw and mouth sensitivity. Time-to-remove, or the time required to completely remove the adhesive, is representative for sensorimotor performance. If a mouse did not feel or remove an adhesive during the trial, a maximum time of 120 s was given. Mice demonstrating freezing behavior during assessment of baseline values were excluded from subsequent analyses.

2.3.3. Open field test

All mice were monitored for 60 min in a Plexiglas box (60 × 60 cm; height 60 cm; center of the arena defined as the central 40 × 40 cm zone), using an over-head video tracking system connected to Ethovision 3.0 (Noldus). The light levels in the room created a luminance of 150 lx at the center of the open field. Different behavioral parameters were integrated by the software, including motor features (distance traveled, immobility, rearing), measures of perseverative behavior (meander) and anxiety-like behavior (time in center). In addition, all parameters were also analyzed for the first 5 min of the trial.

2.3.4. Light/dark test

In the light/dark test, each mouse was placed in a dark shelter (30 × 30 cm; height 8.5 cm) positioned in a corner of a brightly lighted open field arena (luminance outside the shelter 700 lx, inside the shelter 0.5 lx). Anxious behavior was assessed by comparing the innate exploratory activity of the mice with the preference for an enclosed, safe shelter during a 5 min trial. Time spent outside the shelter and the latency to exit the shelter were measured.

2.4. Neurochemical analysis

Dissected striata were weighed and homogenized in 400 μL antioxidant solution (0.05 M HCl, 0.5% $Na_2S_2O_5$, and 0.05% Na_2EDTA) containing 10 ng /100 μL 3,4-dihydroxybenzylamine as internal standard. Homogenates were centrifuged for 20 min at 10000 × *g* at 4 °C. Supernatants were diluted 1:5 in 0.5 M acetic acid and 20 μL of this sample dilution were analyzed for DA, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5HT) and 5-hydroxyindoleacetic acid (5HIAA) on a narrow-bore (C18 column, 5 μm, 150 × 2.1 mm; Altima Grace, Lokeren, Belgium) liquid chromatography system with an electrochemical detector (Antec, Leiden, The Netherlands).

2.5. Immunohistochemistry

40 μm vibratome sections were cut from the post-fixed caudal part of the brain (Leica Microsystems, Germany) and stored in serial order in 10 mM PBS supplemented with 1.5 mM sodium azide at 4 °C. Sections of the SNc were selected to investigate the presence of TH and dopamine transporter (DAT) expressing neurons, while on sections of the STN c-fos positive neurons were stained. All incubations were performed at room temperature. Primary antibodies consisted of rabbit anti-TH antibody (AB152, Millipore, Temecula, CA, USA; 1:2000 in Tris-buffered saline), rabbit anti-DAT antibody (sc-14002, Santa Cruz Biotechnology, Inc., CA, USA; preference for recognizing non-glycosylated DAT enriched in DA-ergic cell bodies (Afonso-Oramas et al., 2009); 1:400 in 2% pre-immune goat serum) and rabbit anti-c-fos antibody (sc-52, Santa Cruz Biotechnology, Inc., CA, USA; 1:2000 in 20% pre-immune goat serum). The staining protocol for all three antibodies was identical and described before (Bentea et al., 2015). Immunoreactivity was visualized using 3,3′-diaminobenzidine as chromogen. Photomicrographs were taken of the stained sections, and cell counts were performed using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). The total numbers of TH and DAT positive profiles in the SNc were counted by an investigator blinded to treatment in six serial sections throughout the entire rostro-caudal extent of this brain region (AP −2.92 to −3.64 from bregma). Total numbers of c-fos positive profiles in the STN were counted blindly in 4 serial sections throughout the entire rostro-caudal extent of the STN (AP −1.70 to −2.30 from bregma), and averaged for each mouse. C-fos labeling was categorized either as absent (<0.5 average cell counts), sparse (between 0.5 and 1.5 average cell counts), moderate (between 1.5 and 5 average cell counts) or extensive (>5 average cell counts). The method of cell-surface counting applied in the present study leads to a strong underestimation of the total number of labeled neurons within the region analyzed when compared to more elaborate, gold-standard 3D

methods of analysis, for instance using stereology. However, linear regression analyses performed on nigral sections immunolabeled for DAT from a subset of 8 mice (5 sham-lesioned placebo-treated and 3 LAC-lesioned placebo-treated) revealed a significant correlation between cell counts obtained using the currently described methodology and cell counts obtained by stereology (data not shown), indicating that our cell-surface counts change in proportion to the total number of labeled cells.

2.6. Western blotting

After dissection, brain tissue was homogenized in 300 μL extraction buffer consisting of 2% sodium dodecyl sulfate (SDS), 60 mM Tris, 100 mM dithiothreitol, with phosphatase and protease inhibitor cocktails (Sigma-Aldrich), pH 7.5. After homogenization, samples were incubated for 30 min at 37 °C and centrifuged for 10 min at 10000 rpm at 4 °C. Supernatants were stored at −20 °C. Total protein concentrations were measured using a Qubit fluorometer (Invitrogen, Groningen, the Netherlands). Equal concentrations of protein were loaded and separated by SDS-polyacrylamide gel electrophoresis (4–12% gel; Bio-Rad Laboratories, Belgium), under reducing conditions. For TH immunoblotting, samples were transferred to a polyvinylidene fluoride membrane using a semi-dry blotting system (Trans-Blot Turbo Transfer System; Bio-Rad Laboratories). For DAT and xCT immunoblotting, samples were transferred to a polyvinylidene fluoride membrane using a wet blotting system (Criterion Blotter; Bio-Rad Laboratories). All incubations were performed at room temperature unless stated otherwise. Nonspecific binding was blocked by incubating the membranes for 1 h in 5% enhanced chemiluminescence (ECL) Advance Membrane Blocking Agent (GE Healthcare, The Netherlands). Next, membranes were incubated overnight with primary antibody: rabbit polyclonal anti-TH antibody (AB152, Millipore, Temecula, CA, USA; 1:2000 diluted in Tris-saline buffer), rabbit polyclonal anti-DAT antibody (AB1591P, Millipore, Temecula, CA, USA; preference for recognizing glycosylated DAT enriched in DA-ergic terminals (Afonso-Oramas et al., 2009); 1:750 diluted in blocking agent, incubation at 4 °C) and rabbit polyclonal anti-xCT antibody (Massie et al., 2008) (1:5000 diluted in blocking agent, incubation at 4 °C). The following day, membranes were incubated for 30 min with horse-radish-peroxidase conjugated anti-rabbit Immunoglobulin G antiserum (1:4000 for TH; 1:10000 for DAT; 1/15000 for xCT; DakoCytomation, Belgium) and immunoreactive proteins were visualized using ECL (ECL Prime for TH and DAT detection; ECL Select for xCT detection; GE Healthcare, The Netherlands). After immunodetection, membranes were washed overnight and incubated in stripping buffer (containing 7.8 g β-mercaptoethanol, 20 g SDS, 7.57 g Tris, in 1000 mL MilliQ water; pH 6.7), after which ServaPurple total protein stain (SERVA Electrophoresis GmbH, Germany) was performed following the manufacturer's instructions. Densitometric analysis of the immunoreactive bands was performed using the ImageJ software (National Institute of Health, Bethesda, Maryland, USA) and normalized to the densities of the total protein stain detected on the same membrane. Western blotting experiments were done in triplicate. The anti-TH antibody selectively labeled a single band at ~62 kDa. Specificity of the xCT antibody was confirmed during our experiments, as the immunoreactive band observed at ~35 kDa corresponding to the xCT protein was not observed in $xCT^{-/-}$ brain protein samples (data not shown) (Van Liefferinge et al., 2016). Specificity of the band corresponding to glycosylated DAT (~70 kDa) was confirmed by including a sample of cerebellum as negative control (data not shown) (Freed et al., 1995).

2.7. Glutathione measurement

Total GSH content was measured in striatal and midbrain tissue using the QuantiChrom GSH Assay kit according to the instructions of the manufacturer (BioAssay Systems).

2.8. Cell culture, measurement of system x_c^- activity and intracellular glutathione levels

Primary astrocytes from C57BL/6 newborn mice were prepared as described previously (Wiesner et al., 2013) with the difference that confluent cultures typically three to four weeks after plating were sequentially treated with cytosine β-D-arabinofuranoside and L-leucine methyl ester (Sigma-Aldrich) to remove microglia (Hamby et al., 2006). Typically, three to four days after microglia removal, astrocytes were detached from the cultures flasks by using 0.05% trypsin/EDTA (Invitrogen) and plated onto 24-well plates. After reaching confluence, pure astrocytes were treated with ZNS at a final concentration of 100 μM for 48 h. HT22 cells were grown as described previously (Lewerenz et al., 2012). For uptake assay, 2.5×10^4 cells were seeded onto 24-well plates in the presence or absence of 100 μM ZNS and cells were grown for 24 h. The system x_c^- activity was measured as homocysteate-inhibitable uptake of ^{3}H-L-glutamate (Lewerenz et al., 2012). The culturing of astroglial C6 cells and the measurement of total intracellular GSH content after 100 μM ZNS treatment for 24 h were conducted as described previously (Massie et al., 2011).

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.1 software. For analyzing the proportion of LAC-lesioned mice in the placebo-treated and ZNS-treated groups showing different stages of c-fos labeling, we applied the Chi-square test. For all other analyses, we employed the two-way ANOVA followed by Bonferroni post-hoc tests. As our experiments were designed to inquire whether ZNS treatment is able to interfere with LAC-induced neurotoxicity, post-hoc tests compared sham-lesioned placebo-treated mice to LAC-lesioned placebo-treated mice and sham-lesioned ZNS-treated mice to LAC-lesioned ZNS-treated mice. The α-value was set at 0.05.

3. Results

3.1. ZNS reduces the loss of nigral DA-ergic neurons but does not influence the loss of striatal DA-ergic fibers following LAC lesion

To evaluate whether repeated ZNS administration affects LAC-induced nigral neurodegeneration, various markers of the nigrostriatal pathway were comparatively assessed. Two-way ANOVA (with lesion and treatment as factors) revealed a global effect of LAC lesion on the number of TH+ profiles at the ipsilateral SNc [lesion factor: $F(1,27) = 8.800$, $p < 0.01$]. A significant interaction between lesion and treatment on the loss of ipsilateral nigral TH+ neurons was observed [lesion × treatment factor: $F(1,27) = 5.147$, $p < 0.05$], with a significant decrease of ~35% TH+ profiles in placebo-treated ($p < 0.01$) but no decrease in ZNS-treated mice when compared relative to their corresponding sham mice (Fig. 2A). Comparable results were obtained using DAT as marker of DA-ergic neurons. Administration of LAC led to a global loss of nigral DAT+ profiles [lesion factor: $F(1,27) = 4.617$, $p < 0.05$] in the presence of a strong trend for an interaction between lesion and treatment [lesion × treatment factor: $F(1,27) = 3.906$ $p = 0.058$]. Post-hoc testing revealed a significant loss of ~30% DAT+ profiles in placebo-treated ($p < 0.01$) but not ZNS-treated mice when compared to their corresponding sham mice (Fig. 2B).

At the level of the striatum, LAC induced a global loss of ipsilateral DA content [lesion factor: $F(1,26) = 7.103$, $p < 0.05$], in the absence of significant interaction effects [lesion × treatment factor: $F(1,26) = 0.4656$, $p > 0.05$]. However, using post-hoc tests, this decrease in DA content could be specifically attributed to a decrease of ~30% DA content in placebo-treated mice ($p < 0.05$), with no change in ZNS-treated mice relative to their corresponding sham (Fig. 2C).

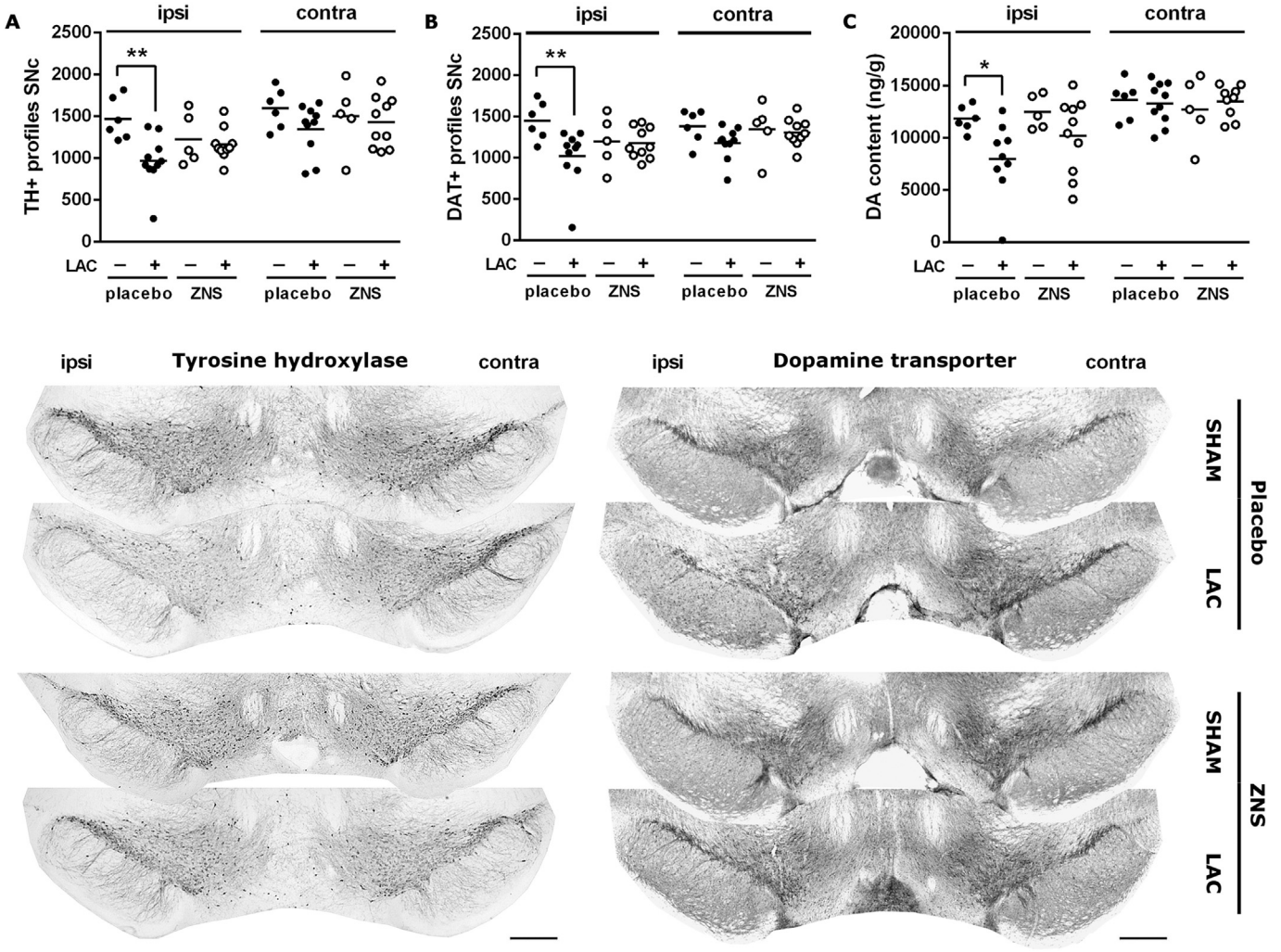

Fig. 2. Repeated administration of ZNS protects against LAC-induced nigral neurodegeneration. (A, B) TH (A) and DAT (B) immunohistochemistry revealed a loss of DA-ergic neurons after LAC injection in the ipsilateral SNc, which was significantly prevented by ZNS treatment. (C) LAC induced a global loss of ipsilateral striatal DA levels, which was specifically attributed to a decrease in DA levels in placebo-treated mice. Lower panels: Representative TH (left) and DAT (right) photomicrographs of the SNc (scale bar 400 μm). Data are presented as scatter dot plot (horizontal line indicates mean). $^{*}p < 0.05$, $^{**}p < 0.01$ (two-way ANOVA followed by Bonferroni post-hoc test). DA dopamine, DAT dopamine transporter, LAC lactacystin, SNc substantia nigra pars compacta, TH tyrosine hydroxylase, ZNS zonisamide.

In contrast, the detrimental effect of LAC on striatal DA-ergic fibers was observed to a similar extent in both treatment groups. Intranigral LAC injection led to an overall reduction of TH expression in the ipsilateral striatum of both treatment groups [lesion factor: $F(1,17) = 27.58$, $p < 0.0001$] with a significant decrease of ~65% and ~55% TH expression in placebo-treated ($p < 0.001$) and ZNS-treated mice ($p < 0.05$) respectively, when compared relative to their corresponding sham mice (Fig. 3A). Equivalent effects were found when probing the expression of DAT, as intranigral LAC induced a general decrease in DAT expression in the ipsilateral striatum [lesion factor: $F(1,17) = 122.7$, $p < 0.0001$] with significant losses of ~80% and ~90% DAT expression in placebo-treated ($p < 0.001$) and ZNS-treated mice ($p < 0.001$)

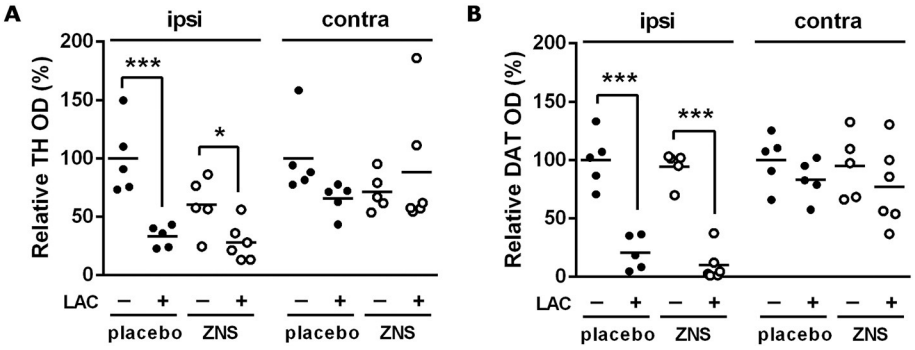

Fig. 3. Repeated administration of ZNS does not influence LAC-induced striatal DA-ergic denervation. (A, B) Treatment with ZNS did not rescue the decrease in TH (A) or DAT (B) protein expression levels in the ipsilateral striatum after LAC lesion. Data are presented as scatter dot plot (horizontal line indicates mean). $^{*}p < 0.05$, $^{***}p < 0.001$ (two-way ANOVA followed by Bonferroni post-hoc test). DA dopamine, DAT dopamine transporter, LAC lactacystin, OD optical density, TH tyrosine hydroxylase, ZNS zonisamide.

respectively, when compared relative to their corresponding sham mice (Fig. 3B).

ZNS has been found to increase striatal DA turnover in mice and common marmosets treated with MPTP (Yabe et al., 2009), which could mediate partial functional recovery of striatal DA-ergic neurotransmission. In order to assess DA metabolism, we quantified the main DA metabolites, DOPAC and HVA, in striatal homogenate samples. Our results failed to reveal any significant changes in the levels of DOPAC, either following LAC lesion or ZNS treatment (Fig. 4A). Unilateral injection of LAC led to a bilateral increase of striatal HVA [ipsilateral striatum, lesion factor: $F(1,22) = 5.391$, $p < 0.05$; contralateral striatum, lesion factor: $F(1,21) = 6.313$, $p < 0.05$], which occurred in the absence of significant lesion × treatment interaction effects for either the ipsilateral striatum [lesion × treatment factor: $F(1,22) = 0.113$, $p > 0.05$] or the contralateral striatum [lesion × treatment factor: $F(1,21) = 0.003$, $p > 0.05$] (Fig. 4B). When computing striatal DA turnover [(DOPAC + HVA) / DA], LAC was found to lead to a bilateral increase in DA turnover [ipsilateral striatum, lesion factor: $F(1,22) = 5.473$, $p < 0.05$; contralateral striatum, lesion factor: $F(1,21) = 5.460$, $p < 0.05$] to similar extents in both treatment groups [ipsilateral striatum, lesion × treatment factor: $F(1,22) = 0.542$, $p > 0.05$; contralateral striatum, lesion × treatment factor: $F(1,21) = 0.005$, $p > 0.05$] (Fig. 4C). Our results, therefore, indicate a compensatory increase in striatal DA turnover following LAC lesion, which was unaffected by ZNS treatment.

In addition to its documented effects on the DA system, chronic administration of ZNS has been found to increase striatal levels of 5HT and of its main metabolite 5HIAA (Okada et al., 1999). Therefore, we evaluated 5HT as well as 5HT turnover (5HIAA/5HT) in our striatal homogenate samples. We failed however to reveal any significant effect of LAC lesion or ZNS treatment on striatal content of 5HT (Fig. 4D) or 5HIAA (Fig. 4E). We noted a bilateral increase in 5HT turnover (5HIAA/5HT) following LAC lesion [ipsilateral striatum, lesion factor: $F(1,22) = 6.405$, $p < 0.05$; contralateral striatum, lesion factor: $F(1,22) = 10.09$, $p < 0.01$], which occurred in the absence of significant interaction effects for either the ipsilateral striatum [lesion × treatment factor: $F(1,22) = 1.169$, $p > 0.05$] or the contralateral striatum [lesion × treatment factor: $F(1,22) = 0.356$, $p > 0.05$] (Fig. 4F). Collectively, repeated ZNS administration did not significantly influence striatal monoaminergic neurotransmission in sham- or LAC-lesioned mice.

3.2. ZNS does not modulate the activity of the STN following LAC lesion

Diminished lesion-induced STN hyperactivity might mediate some of the symptomatic or neuroprotective effects of ZNS (Fukuyama et al., 2014; Yamamura et al., 2009). In order to investigate this hypothesis, we performed c-fos immunohistochemistry of the STN in the different experimental groups and categorized the staining based on the extent of c-fos labeling as either absent, sparse, moderate or extensive. C-fos, the protein product of the immediate-early gene *c-fos*, is a marker of activated neurons (Chaudhuri, 1997) and was previously found to accumulate in the STN following acute or chronic MPTP administration (Shaw et al., 2012). In the ipsilateral STN, sham lesion led to a pattern of absent-to-sparse labeling in both the placebo- and ZNS-treated groups, reflecting low expression of c-fos under these conditions (Fig. 5). LAC-lesion led to development of ipsilateral STN hyperactivity to a moderate-to-extensive degree in 40% of mice, while concomitant ZNS treatment did not significantly change the proportion of lesioned mice showing STN hyperactivity (Chi-square test, $p = 0.156$) (Fig. 5). The pattern of c-fos labeling on the contralateral STN was absent-to-sparse in all experimental groups.

3.3. ZNS improves sensorimotor function following LAC lesion

Next, we evaluated the effects of ZNS treatment on behavioral outcome after LAC-induced parkinsonism, using the rotarod, open field and adhesive removal tests. No significant differences were recorded during the rotarod baseline experiments indicating equal acquisition of rotarod motor skills (data not shown). One week following surgery, LAC-injected mice displayed a global impairment in motor coordination and balance compared to the sham-treated mice [lesion factor: $F(1,48) = 4.924$, $p < 0.05$], irrespective of treatment [lesion × treatment factor: $F(1,48) = 0.301$, $p > 0.05$] (Fig. 6), indicating that ZNS was unable to rescue the decrease in rotarod performance following lesion.

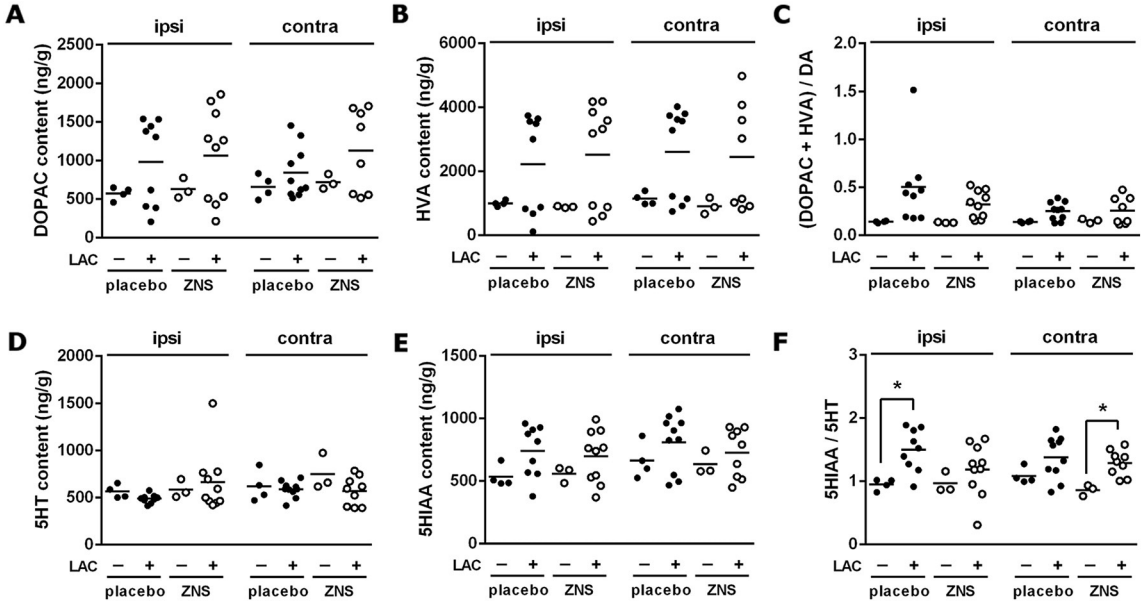

Fig. 4. Repeated ZNS administration does not influence striatal DA or 5HT metabolism in the LAC model. (A) The levels of DOPAC were unaffected by LAC lesion or ZNS treatment in the ipsilateral or contralateral striatum. (B) LAC administration led to a bilateral increase in the striatal levels of HVA independent of treatment. (C) Intranigral LAC caused a bilateral increase in the rate of DA turnover [(DOPAC + HVA) / DA] in both placebo- and ZNS-treated mice. (D, E) Neither LAC lesion nor ZNS treatment influenced the total content of 5HT, or its metabolite 5HIAA, in the ipsilateral or contralateral striatum. (F) LAC administration led to a bilateral increase in striatal 5HT turnover (5HIAA/5HT) that was independent of treatment. Data are presented as scatter dot plot (horizontal line indicates mean). $^*p < 0.05$ (two-way ANOVA followed by Bonferroni post-hoc test). 5HT 5-hydroxytryptamine, 5HIAA 5-hydroxyindoleacetic acid, DA dopamine, DOPAC 3,4-dihydroxyphenylacetic acid, HVA homovanillic acid, LAC lactacystin, ZNS zonisamide.

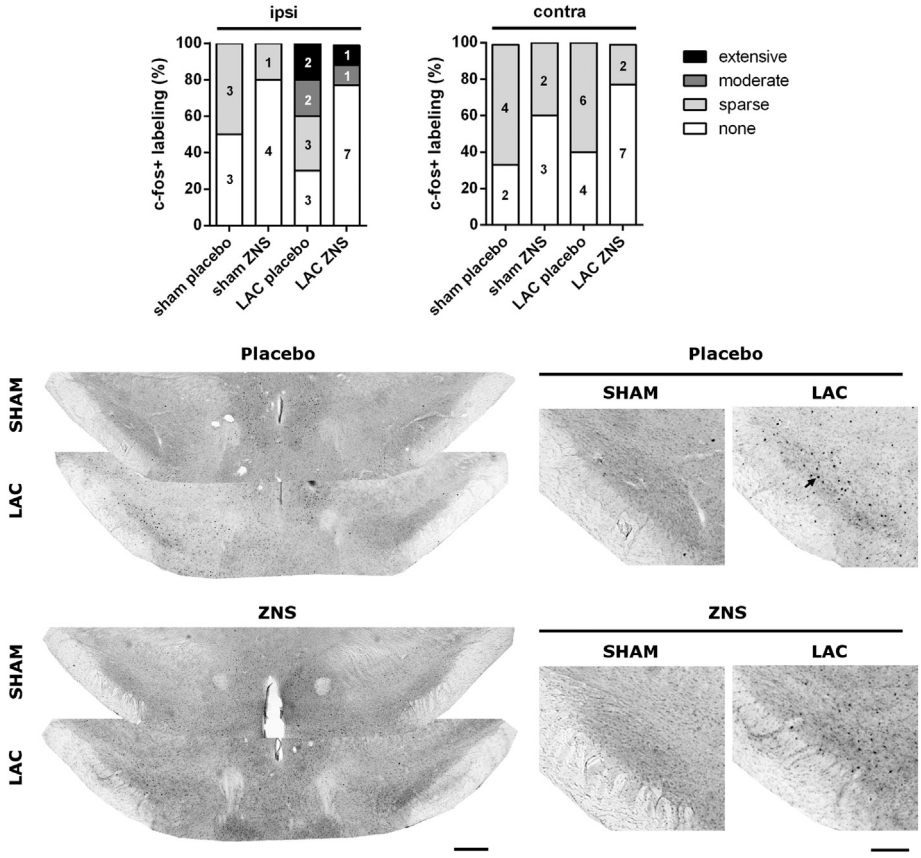

Fig. 5. Repeated administration of ZNS does not influence the activity of the STN at one week after LAC administration. The pattern of c-fos immunoreactivity in the ipsilateral STN of the LAC model was not significantly influenced by ZNS treatment, with moderate-to-extensive c-fos labeling observed in 40% of LAC-lesioned placebo-treated mice *vs.* 22% of LAC-lesioned ZNS-treated mice ($p = 0.156$). The pattern of c-fos labeling in the contralateral STN was absent-to-sparse in all groups. Sample size indicated in the figure. Lower panels, left: Representative photomicrographs of c-fos labeling in the STN, indicating the absence of staining in the ipsilateral STN of two sham-lesioned mice, and extensive staining in the ipsilateral STN of two LAC-lesioned mice (scale bar 400 μm). Right: High magnification photomicrographs of the ipsilateral STN demonstrate the presence of c-fos immunoreactive cells at one week after intranigral LAC injection (arrow) (scale bar 200 μm). LAC lactacystin, STN subthalamic nucleus, ZNS zonisamide.

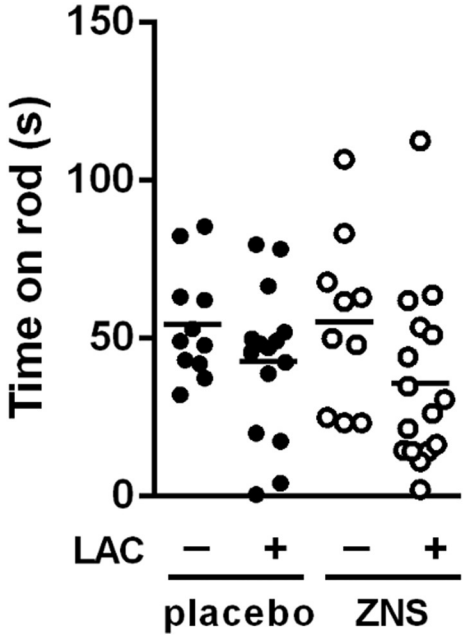

Fig. 6. Repeated ZNS treatment does not influence rotarod performance at one week after LAC lesion. Data are presented as scatter dot plot (horizontal line indicates mean). LAC lactacystin, ZNS zonisamide.

In the open-field test, we analyzed spontaneous motor activity by measuring distance traveled, immobility, rearing and meandering over the entire 60 min trial, as well as during the first 5 min of the test. None of the parameters were affected by either LAC lesion or ZNS treatment during the first 5 min of the test (Fig. 7A–D). Similarly, distance traveled and immobility were unaffected by either lesion or treatment when evaluated over the entire 60 min trial (Fig. 7E, F). We could detect a global decrease in rearing over 60 min that occurred to a similar extent in placebo- and ZNS-treated mice [lesion factor: $F(1,47) = 5.503$, $p < 0.05$] (Fig. 7G). In addition, we identified a decrease in meander over the 60 min trial [lesion factor: $F(1,47) = 6.430$, $p < 0.05$], which was attributed by post-hoc testing to a decrease in LAC-lesioned mice receiving placebo treatment ($p < 0.05$), but not in lesioned mice receiving ZNS treatment (Fig. 7H). Overall, the open-field test revealed only modest changes in spontaneous activity in LAC-injected mice, which were largely unaffected by ZNS treatment.

Finally, we employed the adhesive removal test to investigate sensorimotor function. No significant differences were observed during the adhesive removal baseline experiments indicating equal acquisition of motor skills (data not shown). The time to contact and to completely remove the adhesive strips was unaltered by LAC lesioning and ZNS treatment on the ipsilateral side [time-to-contact, lesion factor: $F(1,30) = 2.174$, $p = 0.1508$; time-to-remove, lesion factor: $F(1,30) = 3.040$, $p = 0.0915$] (Fig. 8A, B). On the contralateral side, similarly, no significant effect could be detected for time-to-contact between the different conditions [lesion factor: $F(1,30) = 1.983$, $p = 0.1693$] (Fig. 8C). On the other hand, LAC lesion was found to lead to a general increase in the time-to-remove the adhesive from the contralateral paw [lesion

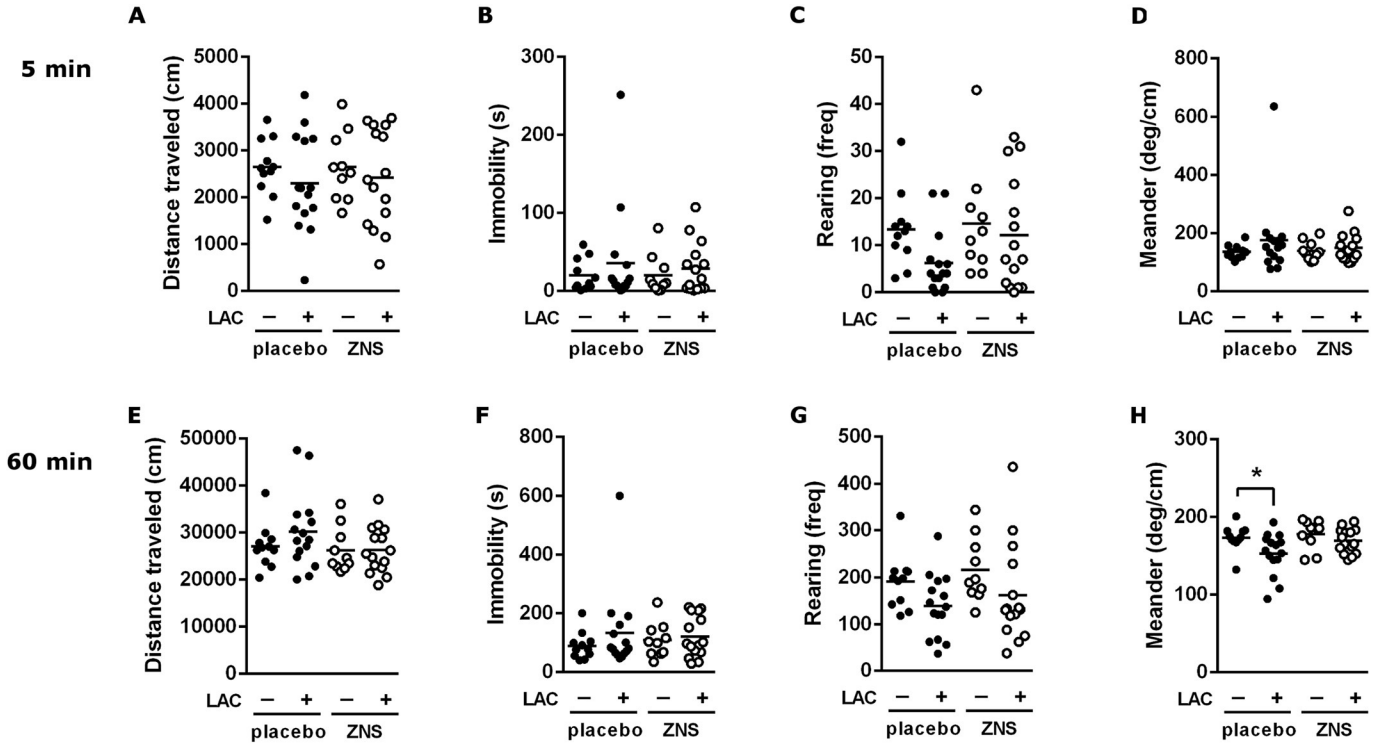

Fig. 7. Repeated ZNS treatment does not influence spontaneous motor activity of LAC-lesioned mice. Administration of ZNS did not influence distance traveled, immobility time, or rearing in mice receiving LAC, either when these parameters were integrated over the first 5 min of the trial (A–C), or over the entire 60 min trial (E–G). Similarly, ZNS did not influence the meander of LAC-lesioned mice during the first 5 min of the test (D). However, chronic ZNS treatment prevented the decrease in meander observed after LAC lesion when evaluated over the entire 60 min trial (H). Data are presented as scatter dot plot (horizontal line indicates mean). $^{*}p < 0.05$ (two-way ANOVA followed by Bonferroni post-hoc test). LAC lactacystin, ZNS zonisamide.

factor: $F(1,30) = 16.81$, $p < 0.001$] indicating unilateral sensorimotor dysfunction (Fig. 8D). Interestingly, an interaction effect between lesion and treatment was observed for time-to-remove the adhesive from the contralateral paw [lesion × treatment factor: $F(1,30) = 9.540$, $p < 0.01$]. Time-to-remove was significantly increased in the LAC-lesioned placebo-treated group ($p < 0.001$), whereas this parameter did not change after LAC lesioning combined with ZNS treatment when compared relative to their corresponding sham groups (Fig. 8D), suggesting that ZNS reduces the degree of sensorimotor dysfunction following lesion.

3.4. ZNS influences LAC-induced anxiety-like behavior

During the first 5 min of the open-field test, LAC injection significantly reduced the time in center with ~55% in placebo-treated mice ($p < 0.05$) but had no apparent effect in ZNS-treated mice when compared relative to their corresponding sham mice (Fig. 9A). No effect of LAC lesion or ZNS treatment could be observed on the time spent in the center, when this parameter was averaged over the entire 60 min trial (Fig. 9B). However, when evaluating the 60 min trial over consecutive 5 min time bins, in order to reveal habituation to the novel environment of the open-field arena, ZNS demonstrated an anxiolytic effect, as ZNS-treated LAC-lesioned mice habituated quicker than placebo-treated LAC-lesioned mice [treatment factor: $F(1,348) = 7.832$, $p < 0.01$] (Fig. 9E). Surprisingly, ZNS had an opposite effect on habituation in the sham operated groups, where it led to impaired habituation to the arena [treatment factor: $F(1,228) = 25.39$, $p < 0.001$] (Fig. 9E).

In the light/dark test, a global decrease in the time outside the shelter [lesion factor: $F(1,48) = 5.725$, $p < 0.05$] (Fig. 9C) and increase in the latency to exit the shelter [lesion factor: $F(1,48) = 9.497$, $p < 0.01$] (Fig. 9D) were detected at one week after LAC injection,

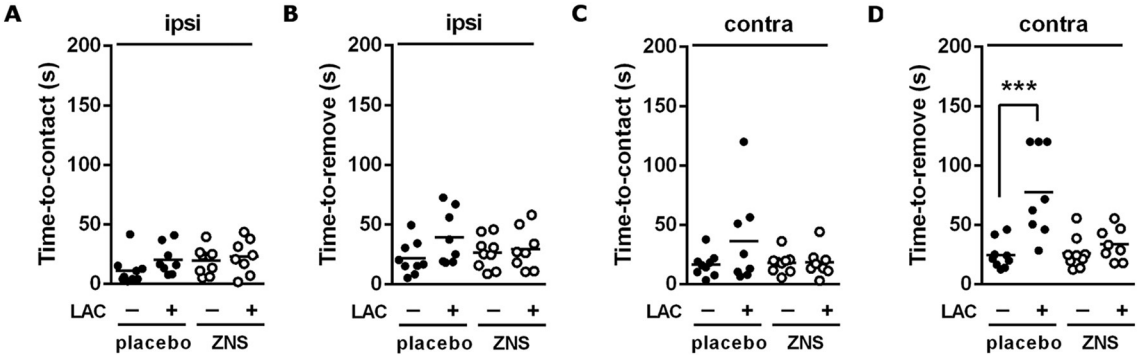

Fig. 8. Repeated ZNS treatment improves sensorimotor performance of LAC-lesioned mice. In the adhesive removal test, time-to-contact (A) and time-to-remove (B) the adhesive strips remained unaltered on the ipsilateral side. On the contralateral side, intranigral LAC injection did not significantly change the time-to-contact (C), but induced a delay in the time-to-remove that was effectively reduced by ZNS treatment (D). Data are presented as scatter dot plot (horizontal line indicates mean). $^{***}p < 0.001$ (two-way ANOVA followed by Bonferroni post-hoc test). LAC lactacystin, ZNS zonisamide.

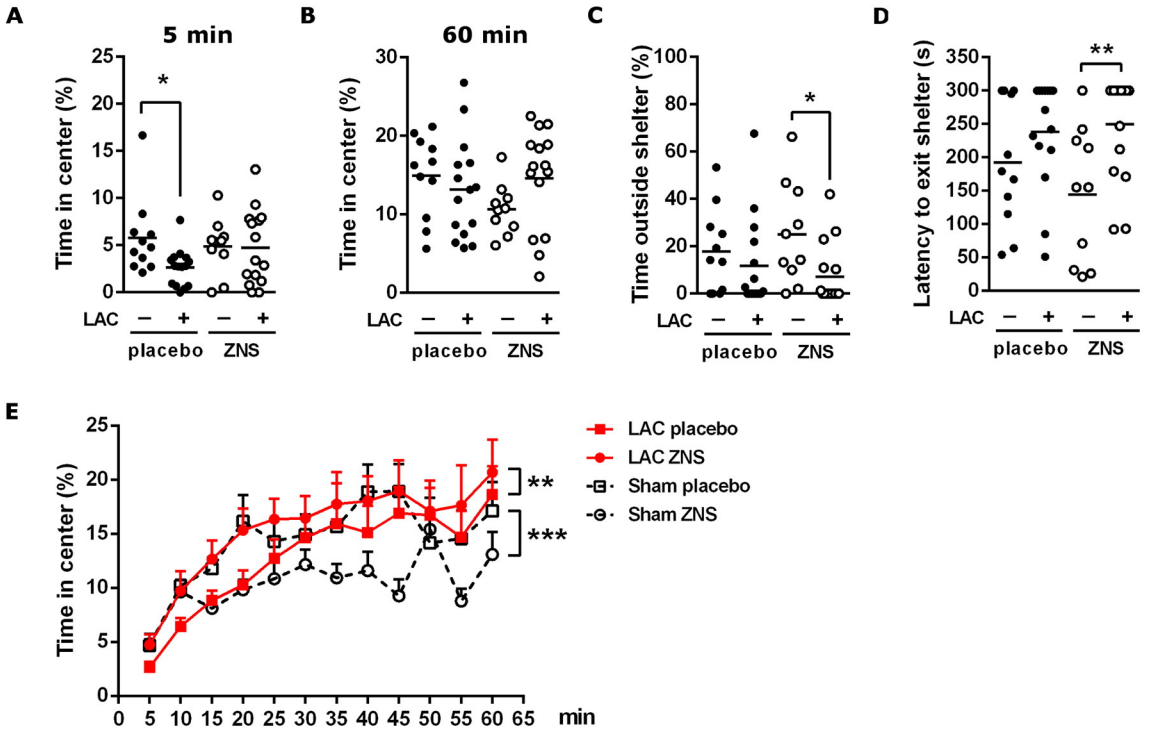

Fig. 9. Repeated administration of ZNS has contrasting influences on anxiety-like behavior in the open-field test and the light/dark test at one week after LAC lesion. (A) During the first 5 min of the open-field test, LAC-lesioned placebo-treated mice spent less time in the center of the arena, and this avoidance behavior was not observed in LAC-lesioned ZNS-treated mice. (B) No effect of either LAC lesion or ZNS treatment was observed when the time in center was averaged over the entire 60 min trial. However, LAC-lesioned ZNS-treated mice habituated quicker to the novel environment of the open-field arena compared to LAC-lesioned placebo-treated mice, as evaluated by plotting the time spent in the center over 60 min (E). (C, D) In the light/dark test, LAC lesion led to a decrease in time spent outside the shelter (C) and an increase in latency to exit shelter (D), only when combined with ZNS treatment. In A–D, data are presented as scatter dot plot (horizontal line indicates mean). $^{*}p < 0.05$, $^{**}p < 0.01$ (two-way ANOVA followed by Bonferroni post-hoc test). In E, data are presented as mean + standard error of the mean. $^{**}p < 0.01$, $^{***}p < 0.001$ (two-way ANOVA, global treatment effect). LAC lactacystin, ZNS zonisamide.

reflecting an increase in anxiety-like behavior. Post-hoc analyses revealed that these anxiogenic effects were specifically attributed to changes observed in LAC-lesioned ZNS-treated mice. When combined with ZNS treatment, LAC lesion led to a decrease with ~70% time spent outside the shelter ($p < 0.05$) and a concomitant increase with ~75% in the latency to exit the shelter ($p < 0.01$), whereas these

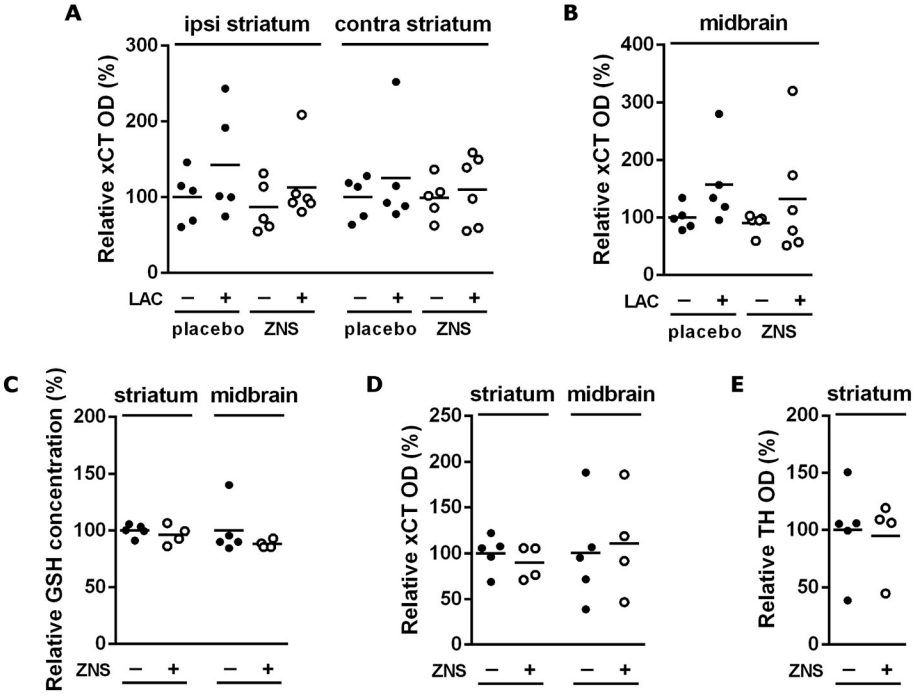

Fig. 10. Repeated administration of ZNS does not influence xCT protein expression or GSH levels in the basal ganglia. (A, B) In the LAC model, xCT protein levels remained unchanged in placebo- and ZNS-treated mice in both the striatum (A) and the midbrain (B). (C, D) Similarly, in naive mice, repeated ZNS treatment did not influence GSH levels (C) or xCT protein expression (D) in either the striatum or the midbrain. (E) Repeated administration of ZNS to naive mice did not influence striatal protein expression of TH. Data are presented as scatter dot plot (horizontal line indicates mean). GSH glutathione, LAC lactacystin, OD optical density, ZNS zonisamide.

parameters remained unaffected in placebo-treated LAC-lesioned mice. As such, although ZNS treatment demonstrated an anxiolytic profile in the LAC group in the open field test, it potentiated the anxiogenic effects of LAC in the light/dark test.

3.5. ZNS does not modulate the expression of xCT in the basal ganglia following LAC lesion

Neither LAC lesion nor ZNS treatment had an effect on xCT expression, both in the ipsilateral and contralateral striatum (Fig. 10A). When we evaluated xCT expression in the midbrain, we similarly saw no effect after LAC injection or ZNS treatment (Fig. 10B).

3.6. Prolonged ZNS does not alter total GSH content and xCT protein expression in mouse basal ganglia under physiological conditions

Repeated ZNS administration for 14 days to naive C57BL/6J mice did not influence midbrain or striatal GSH levels (Fig. 10C). Moreover, no effect on midbrain or striatal xCT expression (Fig. 10D) or striatal TH expression (Fig. 10E) could be observed in naive mice after two weeks of ZNS treatment compared to placebo-treated controls.

3.7. ZNS does not alter intracellular GSH content and system x_c^- activity in cultured cells

Previous findings suggest that exposing cultured astroglial C6 cells to 100 μM ZNS for 24 h leads to an increase in xCT expression and stimulation of GSH production (Asanuma et al., 2010). In our experiments, however, incubation of astroglial C6 cells for 24 h with 100 μM ZNS did not affect total intracellular GSH content (Fig. 11A). Similarly, we did not observe any significant effect of 100 μM ZNS treatment on system x_c^- activity in the hippocampal HT22 cells (24 h incubation) (Fig. 11B) or in microglia-free murine primary astrocytes (48 h incubation) (Fig. 11C).

4. Discussion

ZNS, a drug clinically used to provide symptomatic benefits in PD patients in Japan (Yang and Perry, 2009), has been shown to exert neuroprotective properties in toxin- and genetic-based models of PD such as the 6-OHDA model (Asanuma et al., 2010), the MPTP model (Sonsalla et al., 2010), the α-synuclein viral vector model (Arawaka et al., 2014) and the *Engrailed* genetic mouse model (Sano et al., 2015). Various mechanisms have been proposed to contribute to the observed neuroprotective effects including enhancement of antioxidant systems (Choudhury et al., 2012), anti-inflammatory properties (Yokoyama et al., 2010), stimulation of neurotrophic factor synthesis (Choudhury et al., 2012; Sano et al., 2015), suppression of endoplasmic reticulum stress (Tsujii et al., 2015) and protection against mitochondrial complex I dysfunction (Costa et al., 2010). Whether ZNS interferes with proteasome inhibition-induced neurodegeneration, an important pathogenic pathway leading to protein accumulation in PD (Olanow and McNaught, 2006), remains so far unexplored. In the present study, we report the therapeutic effect of repeated ZNS treatment in a mouse model of PD based on intranigral administration of proteasome inhibitor LAC.

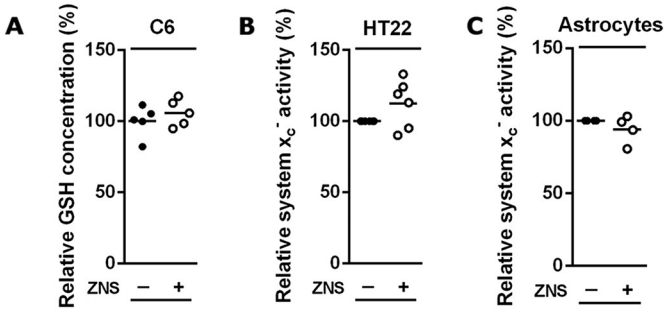

Fig. 11. ZNS does not influence the activity of system x_c^- or intracellular GSH levels *in vitro*. (A–C) Lack of effect of 100 μM ZNS administration on intracellular GSH content in astroglial C6 cells upon 24 h of ZNS (A), and on relative system x_c^- activity in HT22 cells upon 24 h of ZNS (B) and microglia-free murine primary astrocytes upon 48 h of ZNS (C). Data are presented as scatter dot plot (horizontal line indicates mean), and were obtained from five (A), six (B), or four (C) independent experiments each normalized to the corresponding control cells treated with vehicle. GSH glutathione, ZNS zonisamide.

Our present findings indicate that treatment with ZNS (30 mg kg^{-1} per day) starting three days prior to lesion and continued for a succession of six days post-lesioning effectively protects against LAC-induced loss of nigral DA-ergic neurons. Although not counted stereologically, and as such representing an underestimation of the total number of neurons, immunohistochemical experiments identifying two independent markers of DA-ergic neurons, TH and DAT, revealed that the number of nigral DA-ergic neurons remained largely unchanged following lesion in mice treated with ZNS, while being decreased with 30–35% in control (placebo-treated) mice. Surprisingly, we failed to detect a concomitant protection of striatal DA-ergic fibers, as striatal expression of TH and DAT decreased post-lesion to similar extents in both treatment groups. Previously, administration of ZNS to MPTP-treated mice was reported to reduce the loss in the number of nigral TH + neurons at one week post-lesioning compared to placebo-treated MPTP mice. Particularly, this neuroprotection was not accompanied by a corresponding decrease in striatal DA loss (Choudhury et al., 2011). However, the levels of striatal DA progressively increased in ZNS-treated MPTP-lesioned mice over time with a significant recovery only at 9 weeks post-lesion (Choudhury et al., 2011), indicating that ZNS treatment can initiate a gradual recovery of damaged DA-ergic neurons following lesion. Interestingly, post-treatment with ZNS (drug initiated one day post-lesion) was found to significantly attenuate 6-OHDA-induced nigral DA neuronal loss and to increase mRNA expression of neurotrophic factors such as mesencephalic astrocyte-derived neurotrophic factor and vascular endothelial growth factor (Choudhury et al., 2012). Furthermore, ZNS has been found to induce the expression of brain-derived neurotrophic factor (Sano et al., 2015) and increase the production of S100β, an astrocytic factor with neuroprotective and neurotrophic functions (Asanuma et al., 2010; Choudhury et al., 2011). Based on these findings, continuous treatment with ZNS prior to and following the LAC lesion might have stimulated recovery of nigral DA-ergic neurons damaged by the injection of LAC. While we observed a complete protection of nigral neurons at one week post-lesion, we failed to see a similar preservation of striatal DA-ergic innervation. Possibly, more time would be required for post-lesion axonal sprouting and re-population of striatal DA-ergic terminals.

Concomitantly, we evaluated the effect of ZNS on motor function in a series of behavioral tests. Whereas in the open-field test we could not detect any differences in spontaneous locomotor activity between the four groups, in the rotarod test we detected a global impairment in motor performance due to LAC lesion indicating deterioration in motor coordination and balance, irrespective of placebo- or ZNS-treatment. Our observations are in contrast with two previous studies reporting improved rotarod performance after ZNS (Sano et al., 2015; Yokoyama et al., 2010). A first plausible explanation for these contradictory findings might be the fact that the neuroprotective effects of ZNS in our current model did not translate into a significant preservation of striatal DA-ergic fibers. Moreover, we administered the last dose of ZNS one day before testing, to exclude direct symptomatic effects of ZNS, while in both other studies ZNS was also given on the day of testing. Interestingly, in the *Engrailed* genetic model of PD, Sano and coworkers observed a gradual improvement of rotarod performance with ZNS on repeated rotarod trials (Sano et al., 2015). As the performance of ZNS-treated *Engrailed* mice was not significantly different to that of placebo-treated control mice during the first trial, ZNS might have provided a positive effect on motor learning as opposed (or in addition) to the effect on motor performance in the rotarod test. At the

same time, given that the rotarod performance can be influenced in conditions of stress (Mizoguchi et al., 2002), the effects of ZNS on anxiety-like behavior in our study (further described below) might have biased the evaluation of the drug in this behavioral paradigm.

To the best of our knowledge, we are the first to investigate the effect of chronic ZNS treatment on sensorimotor function in a model of PD. In the adhesive removal test, we observed contralateral sensorimotor impairment and impaired skilled and coordinated movement at one week after LAC lesion. This motor impairment was effectively reduced by repeated ZNS administration with treated mice reaching the same level of motor performance as sham-lesioned mice. The behavioral improvement following ZNS might be linked to both nigral, as well as extra-nigral, effects resulting from chronic treatment. Interestingly, reduced somatodendritic DA release in the substantia nigra can impair motor function in the absence of significant changes in striatal DA levels, while an increase in nigral DA release may partially counteract the loss of striatal DA and improve motor function (Andersson et al., 2006). As such, it can be speculated that the normalized sensorimotor performance of LAC-lesioned ZNS-treated mice might result, at least partly, from the neuroprotective effect of ZNS leading to increased somatodendritic DA release. Alternatively, other mechanisms acting downstream of the striatum in the basal ganglia might regulate the effects of ZNS on behavior. Of note, ZNS has been shown to inhibit the indirect pathway of the basal ganglia (Fukuyama et al., 2014; Yamamura et al., 2009), an effect that might contribute to its symptomatic effects in PD. To investigate possible non-DA-ergic pathways mediating the improvement in sensorimotor function, we used c-fos, an inducible transcription factor and a protein product of an immediate-early gene (Chaudhuri, 1997), to identify activated neurons in the STN. Our findings indicate that LAC lesion leads to moderate-to-extensive c-fos labeling in the STN in 40% of the lesioned mice, a proportion that was, however, not significantly changed following ZNS administration. These findings indicate that the improvement of sensorimotor function following ZNS treatment was not attributed to a decrease in the activity of the STN following lesion.

Beside motor symptoms, non-motor symptoms such as anxiety, depression or cognitive decline, have an important impact on PD patients' quality of life (Chaudhuri and Schapira, 2009). Up to date, the effect of ZNS on anxiety has only been investigated in an open label study that suggested that adjunctive ZNS treatment reduces anxiety in patients with refractory anxiety (Kinrys et al., 2007). In a recent open-label trial in early stage PD, ZNS treatment improved the Unified Parkinson's Disease Rating Scale part III score, however did not influence non-motor symptoms, as assessed using the Non-Motor Symptom scale that includes, besides others, a domain of mood and cognition (Maeda et al., 2015). In contrast, a case report describing treatment with ZNS of a patient with PD dementia suffering from concurrent seizures demonstrated an improvement in mood as evaluated using the Unified Parkinson's Disease Rating Scale part I (Tombini et al., 2013). Still, the effects of ZNS on co-morbid anxiety in PD remain largely unknown and are so far unexplored in animal models of PD. Our findings highlight conflicting effects of ZNS treatment in two behavioral paradigms for anxiety-like behavior, the open-field test and the light/dark test. In the open-field test, LAC lesion decreased the time spent in the center of the arena in placebo-treated mice, an effect which was not observed in lesioned mice treated with ZNS. Similarly, ZNS facilitated the habituation of the LAC-lesioned mice to the novel environment of the open-field test, as evaluated by the time spent in the center of the arena during 60 min. In contrast to the anxiolytic profile of ZNS in the open field test, administration of the drug potentiated the LAC-induced anxiety-like behavior in the light/dark test. It is conceivable that the difference of effects of ZNS in the two behavioral paradigms might be due to the fact that they evaluate different dimensions of anxiety-like behavior (Ramos, 2008). Despite the construct similarity between the two approach-avoidance paradigms, the light/dark test introduces an important variable determining avoidance behavior, namely an increase in light contrast in the open, exposed area of the open field arena. Notably, previous findings indicate that changes in the light intensity during approach-avoidance paradigms, such as the elevated plus maze, can switch the anxiogenic profile of 5HT1A receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin to an anxiolytic profile (Handley et al., 1993). It is tempting to hypothesize that changes in the light intensity in the light/dark test compared to the open field test might have influenced the effects of ZNS on anxiety-like behavior. Although we did not detect any significant changes of ZNS treatment on striatal 5HT-ergic neurotransmission, ZNS has been shown to influence hippocampal 5HT-ergic transmission (Okada et al., 1999), which might be linked to its intricate effect on anxiety-dependent behavior. This is however speculative and further experiments are necessary in order to better define the anxiety-mediating effects of ZNS in PD, which are of particular interest given the contemporary use of the drug for the management of motor symptoms.

System x_c^- is a plasma membrane antiporter that couples cystine uptake to non-vesicular glutamate release to the extracellular environment (Massie et al., 2015). The imported cystine can serve as a building block for the production of GSH and stimulate antioxidant defenses. At the same time, an increase in extracellular glutamate release by system x_c^- might be detrimental, given that this neurotransmitter acts as an excitotoxin at high concentrations. In a previous study, Asanuma and co-workers reported neuroprotective effects of ZNS in the 6-OHDA PD model *via* enhancement of system x_c^- and subsequent increase in GSH synthesis (Asanuma et al., 2010). Concomitantly, genetic loss of system x_c^- has been shown to be neuroprotective in the 6-OHDA mouse model for PD (Massie et al., 2011), while several other *in vivo* studies postulate that upregulation of system x_c^- contributes to excitotoxicity through release of toxic amounts of non-vesicular glutamate (Evonuk et al., 2015; Mesci et al., 2015; Qin et al., 2006; Robert et al., 2015). In order to further investigate the possible involvement of system x_c^- in the effects mediated by ZNS, we evaluated the expression of the specific xCT subunit of the antiporter, following LAC lesion. We could not detect any changes in xCT protein expression in mouse basal ganglia after lesion, irrespective of treatment. As such, system x_c^- does not seem to be a mediator of the neuroprotective action of ZNS.

Consequently, we repeated the chronic, daily administration of ZNS 30 mg kg^{-1} for 14 days described previously (Asanuma et al., 2010) to naive C57BL/6J mice. In contrast to the observations of Asanuma and collaborators, we could not observe any change in GSH levels in striatum or midbrain after a 14 days treatment period with ZNS. In addition, we could not detect any difference in xCT expression level in striatum or midbrain after ZNS, indicating that ZNS does not induce system x_c^- expression *in vivo*. Although we were not able to confirm an effect of ZNS on striatal or midbrain GSH levels after repeated administration during 14 days to naive mice, we do not exclude the possibility that GSH levels might have increased after repeated administration of ZNS during 10 days to LAC-lesioned mice. Our data suggest, however, that if such changes did occur, they would not have been mediated by changes in xCT expression.

Finally, ZNS treatment had no effect on system x_c^- activity in HT22 cells and microglia-free murine primary astrocytes. The lack of effect of ZNS on system x_c^- in HT22 cells is in line with the observations of Yurekli and collaborators in PC12 neuronal cells (Yurekli et al., 2013). Although they reported that ZNS can increase GSH levels in PC12 cells, they only observed this effect when comparing the ZNS-treated cells to MPP^+ treated cells and not to control cells (Yurekli et al., 2013). Moreover, ZNS treatment did not affect intracellular GSH levels of C6 glioma cells, which is once more in contrast to the observation of Asanuma et al. who showed that ZNS increases GSH levels in this astrocytic cell line (Asanuma et al., 2010).

The reason for the discrepancies of the current data and the results published by Asanuma and collaborators remains to be resolved. Besides the genetic background of the mice, environmental factors might affect the data, as well as the nature of the primary xCT antibody used

Table 1
Overview of the effects of repeated administration of ZNS in mice unilaterally lesioned with LAC.

Outcome	Effect
Nigral DA-ergic neurodegeneration (TH and DAT IHC)	+
Striatal DA loss (HPLC)	+
Striatal DA turnover (HPLC)	=
Striatal 5HT-ergic neurotransmission (HPLC)	=
Striatal DA-ergic denervation (TH and DAT WB)	=
STN hyperactivity (c-fos IHC)	=
Motor coordination and balance (rotarod)	=
Spontaneous motor activity (open field)	=
Sensorimotor function (adhesive removal)	+
Anxiety-like behavior (open field)	+
Anxiety-like behavior (light/dark test)	−
Striatal and midbrain xCT expression (WB)	=

+ positive effect, = no effect, − negative effect, DA dopamine, DAT dopamine transporter, HPLC high-performance liquid chromatography, IHC immunohistochemistry, TH tyrosine hydroxylase, WB western blotting.

in the previous study, which, at least in our hands, does not seem to specifically label xCT (Van Liefferinge et al., 2016). We failed to find, however, conclusive evidence from both *in vitro* or *in vivo* settings indicating that ZNS is an effective inducer of system x_c^-.

In conclusion, ZNS has been found to provide neuroprotection against proteasome inhibition-induced nigral DA-ergic neurodegeneration *via* system x_c^- -independent mechanisms. Treatment with ZNS significantly improved sensorimotor function of LAC-lesioned mice and had a complex influence on anxiety-like behavior (Table 1).

Several limitations of the present study should be, at the same time, acknowledged. First, we have initiated treatment with ZNS prior to LAC lesion. While such a neuroprotective design can provide evidence that ZNS interferes with the subsequent toxicity of proteasome inhibitors, future studies whereby the drug is administered post lesion are of interest and can inform whether this intervention would be successful in a clinical setting, where neurodegeneration is an ongoing chronic process. Second, given their aggressive nature, the mice included in the present study were single housed during the duration of the experiment, in order to avoid severe injuries that could have disabled subsequent behavioral analyses and cause premature end-point euthanasia. Single housing can have significant impacts on behavioral phenotype, including an increase in anxiety-like behavior (Demuyser et al., 2016). Presumably, single housing in combination with the additional stressors present in our experimental design (such as stereotaxic surgery and chronic i.p. injections), led to an increase in the basal levels of anxiety-like behavior. However, as all mice in our study were single housed (including sham-operated and placebo-treated), we believe that the effect of single housing affected all experimental groups, allowing us to draw dependable conclusions on the effects of LAC lesion or ZNS treatment. Finally, in our study we have restricted to the inclusion of only male mice, and extrapolation of our findings to the population of PD patients requires confirmation of our findings in female animals. Such studies should be, however, carefully controlled for hormonal cycle, given that hormones such as estrogen can have modulatory effects on neurodegeneration or behavior (Liu and Dluzen, 2007).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by grants of the Fund for Scientific Research Flanders (FWO Belgium, grant G.0384.12N), the Queen Elisabeth Medical Foundation (G.S.K.E.) and the Vrije Universiteit Brussel (grant SRP40). Joeri Van Liefferinge, Thomas Demuyser and Katrien Maes were funded by the Agency for Innovation by Science and Technology (IWT, Belgium). Eduard Bentea was funded by the FWO. The authors also acknowledge Ms. R. Berckmans and Mr. Frank Van Der Kelen for their excellent technical assistance.

References

Afonso-Oramas, D., Cruz-Muros, I., Alvarez de la Rosa, D., Abreu, P., Giraldez, T., Castro-Hernandez, J., Salas-Hernandez, J., Lanciego, J.L., Rodriguez, M., Gonzalez-Hernandez, T., 2009. Dopamine transporter glycosylation correlates with the vulnerability of midbrain dopaminergic cells in Parkinson's disease. Neurobiol. Dis. 36, 494–508.

Albrecht, P., Lewerenz, J., Dittmer, S., Noack, R., Maher, P., Methner, A., 2010. Mechanisms of oxidative glutamate toxicity: the glutamate/cystine antiporter system x_c^- as a neuroprotective drug target. CNS Neurol. Disord. Drug Targets 9, 373–382.

Andersson, D.R., Nissbrandt, H., Bergquist, F., 2006. Partial depletion of dopamine in substantia nigra impairs motor performance without altering striatal dopamine neurotransmission. Eur. J. Neurosci. 24, 617–624.

Arawaka, S., Fukushima, S., Sato, H., Sasaki, A., Koga, K., Koyama, S., Kato, T., 2014. Zonisamide attenuates alpha-synuclein neurotoxicity by an aggregation-independent mechanism in a rat model of familial Parkinson's disease. PLoS One 9, e89076.

Asanuma, M., Miyazaki, I., Diaz-Corrales, F.J., Kimoto, N., Kikkawa, Y., Takeshima, M., Miyoshi, K., Murata, M., 2010. Neuroprotective effects of zonisamide target astrocyte. Ann. Neurol. 67, 239–249.

Bentea, E., Van der Perren, A., Van Liefferinge, J., El Arfani, A., Albertini, G., Demuyser, T., Merckx, E., Michotte, Y., Smolders, I., Baekelandt, V., Massie, A., 2015. Nigral proteasome inhibition in mice leads to motor and non-motor deficits and increased expression of Ser129 phosphorylated alpha-synuclein. Front. Behav. Neurosci. 9, 68.

Chaudhuri, A., 1997. Neural activity mapping with inducible transcription factors. Neuroreport 8, iii–vii.

Chaudhuri, K.R., Schapira, A.H., 2009. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. Lancet Neurol. 8, 464–474.

Choudhury, M.E., Moritoyo, T., Kubo, M., Kyaw, W.T., Yabe, H., Nishikawa, N., Nagai, M., Matsuda, S., Nomoto, M., 2011. Zonisamide-induced long-lasting recovery of dopaminergic neurons from MPTP-toxicity. Brain Res. 1384, 170–178.

Choudhury, M.E., Sugimoto, K., Kubo, M., Iwaki, H., Tsujii, T., Kyaw, W.T., Nishikawa, N., Nagai, M., Tanaka, J., Nomoto, M., 2012. Zonisamide up-regulated the mRNAs encoding astrocytic anti-oxidative and neurotrophic factors. Eur. J. Pharmacol. 689, 72–80.

Costa, C., Tozzi, A., Luchetti, E., Siliquini, S., Belcastro, V., Tantucci, M., Picconi, B., Ientile, R., Calabresi, P., Pisani, F., 2010. Electrophysiological actions of zonisamide on striatal neurons: selective neuroprotection against complex I mitochondrial dysfunction. Exp. Neurol. 221, 217–224.

De Bundel, D., Schallier, A., Loyens, E., Fernando, R., Miyashita, H., Van Liefferinge, J., Vermoesen, K., Bannai, S., Sato, H., Michotte, Y., Smolders, I., Massie, A., 2011. Loss of system $x(c)^-$ does not induce oxidative stress but decreases extracellular glutamate in hippocampus and influences spatial working memory and limbic seizure susceptibility. J. Neurosci. 31, 5792–5803.

Demuyser, T., Deneyer, L., Bentea, E., Albertini, G., Van Liefferinge, J., Merckx, E., De Prins, A., De Bundel, D., Massie, A., Smolders, I., 2016. In-depth behavioral characterization of the corticosterone mouse model and the critical involvement of housing conditions. Physiol. Behav. 156, 199–207.

Ebrahimi-Fakhari, D., Wahlster, L., McLean, P.J., 2012. Protein degradation pathways in Parkinson's disease: curse or blessing. Acta Neuropathol. 124, 153–172.

Evonuk, K.S., Baker, B.J., Doyle, R.E., Moseley, C.E., Sestero, C.M., Johnston, B.P., De Sarno, P., Tang, A., Gembitsky, I., Hewett, S.J., Weaver, C.T., Raman, C., DeSilva, T.M., 2015. Inhibition of system x_c^- transporter attenuates autoimmune inflammatory demyelination. J. Immunol. 195, 450–463.

Fornai, F., Lenzi, P., Gesi, M., Ferrucci, M., Lazzeri, G., Busceti, C.L., Ruffoli, R., Soldani, P., Ruggieri, S., Alessandri, M.G., Paparelli, A., 2003. Fine structure and biochemical mechanisms underlying nigrostriatal inclusions and cell death after proteasome inhibition. J. Neurosci. 23, 8955–8966.

Freed, C., Revay, R., Vaughan, R.A., Kriek, E., Grant, S., Uhl, G.R., Kuhar, M.J., 1995. Dopamine transporter immunoreactivity in rat brain. J. Comp. Neurol. 359, 340–349.

Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M.S., Shen, J., Takio, K., Iwatsubo, T., 2002. Alpha-synuclein is phosphorylated in synucleinopathy lesions. Nat. Cell Biol. 4, 160–164.

Fukuyama, K., Tanahashi, S., Hoshikawa, M., Shinagawa, R., Okada, M., 2014. Zonisamide regulates basal ganglia transmission via astroglial kynurenine pathway. Neuropharmacology 76 Pt A, 137–145.

Hamby, M.E., Uliasz, T.F., Hewett, S.J., Hewett, J.A., 2006. Characterization of an improved procedure for the removal of microglia from confluent monolayers of primary astrocytes. J. Neurosci. Methods 150, 128–137.

Handley, S.L., McBlane, J.W., Critchley, M.A., Njung'e, K., 1993. Multiple serotonin mechanisms in animal models of anxiety: environmental, emotional and cognitive factors. Behav. Brain Res. 58, 203–210.

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8, e1000412.

Kinrys, G., Vasconcelos e Sa, D., Nery, F., 2007. Adjunctive zonisamide for treatment refractory anxiety. Int. J. Clin. Pract. 61, 1050–1053.

Lewerenz, J., Sato, H., Albrecht, P., Henke, N., Noack, R., Methner, A., Maher, P., 2012. Mutation of ATF4 mediates resistance of neuronal cell lines against oxidative stress by inducing xCT expression. Cell Death Differ. 19, 847–858.

Lewerenz, J., Hewett, S.J., Huang, Y., Lambros, M., Gout, P.W., Kalivas, P.W., Massie, A., Smolders, I., Methner, A., Pergande, M., Smith, S.B., Ganapathy, V., Maher, P., 2013. The cystine/glutamate antiporter system x(c)(−) in health and disease: from molecular mechanisms to novel therapeutic opportunities. Antioxid. Redox Signal. 18, 522–555.

Liu, B., Dluzen, D.E., 2007. Oestrogen and nigrostriatal dopaminergic neurodegeneration: animal models and clinical reports of Parkinson's disease. Clin. Exp. Pharmacol. Physiol. 34, 555–565.

Mackey, S., Jing, Y., Flores, J., Dinelle, K., Doudet, D.J., 2013. Direct intranigral administration of an ubiquitin proteasome system inhibitor in rat: behavior, positron emission tomography, immunohistochemistry. Exp. Neurol. 247, 19–24.

Maeda, T., Takano, D., Yamazaki, T., Satoh, Y., Nagata, K., 2015. Zonisamide in the early stage of Parkinson's disease. Neurol. Clin. Neurosci. 3, 127–130.

Massie, A., Schallier, A., Mertens, B., Vermoesen, K., Bannai, S., Sato, H., Smolders, I., Michotte, Y., 2008. Time-dependent changes in striatal xCT protein expression in hemi-Parkinson rats. Neuroreport 19, 1589–1592.

Massie, A., Schallier, A., Kim, S.W., Fernando, R., Kobayashi, S., Beck, H., De Bundel, D., Vermoesen, K., Bannai, S., Smolders, I., Conrad, M., Plesnila, N., Sato, H., Michotte, Y., 2011. Dopaminergic neurons of system x(c)(−)-deficient mice are highly protected against 6-hydroxydopamine-induced toxicity. FASEB J. 25, 1359–1369.

Massie, A., Boillee, S., Hewett, S., Knackstedt, L., Lewerenz, J., 2015. Main path and byways: non-vesicular glutamate release by system x_c^- as an important modifier of glutamatergic neurotransmission. J. Neurochem. 135, 1062–1079.

McNaught, K.S., Bjorklund, L.M., Belizaire, R., Isacson, O., Jenner, P., Olanow, C.W., 2002. Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. Neuroreport 13, 1437–1441.

McNaught, K.S., Belizaire, R., Isacson, O., Jenner, P., Olanow, C.W., 2003. Altered proteasomal function in sporadic Parkinson's disease. Exp. Neurol. 179, 38–46.

Mesci, P., Zaidi, S., Lobsiger, C.S., Millecamps, S., Escartin, C., Seilhean, D., Sato, H., Mallat, M., Boillee, S., 2015. System x_c^- is a mediator of microglial function and its deletion slows symptoms in amyotrophic lateral sclerosis mice. Brain 138, 53–68.

Miwa, H., 2007. Zonisamide for the treatment of Parkinson's disease. Expert. Rev. Neurother. 7, 1077–1083.

Miwa, H., Hama, K., Kajimoto, Y., Kondo, T., 2008. Effects of zonisamide on experimental tremors in rats. Parkinsonism Relat. Disord. 14, 33–36.

Mizoguchi, K., Yuzurihara, M., Ishige, A., Sasaki, H., Tabira, T., 2002. Chronic stress impairs rotarod performance in rats: implications for depressive state. Pharmacol. Biochem. Behav. 71, 79–84.

Murata, M., 2004. Novel therapeutic effects of the anti-convulsant, zonisamide, on Parkinson's disease. Curr. Pharm. Des. 10, 687–693.

Murata, M., Horiuchi, E., Kanazawa, I., 2001. Zonisamide has beneficial effects on Parkinson's disease patients. Neurosci. Res. 41, 397–399.

Murata, M., Hasegawa, K., Kanazawa, I., 2004. Randomized, double-blind study of zonisamide with placebo in advanced Parkinson's disease. Mov. Disord. 19 (Suppl. 9), S198.

Murata, M., Hasegawa, K., Kanazawa, I., 2007. Zonisamide improves motor function in Parkinson disease: a randomized, double-blind study. Neurology 68, 45–50.

Murata, M., Hasegawa, K., Kanazawa, I., Fukasaka, J., Kochi, K., Shimazu, R., 2015. Zonisamide improves wearing-off in Parkinson's disease: a randomized, double-blind study. Mov. Disord. 30, 1343–1350.

Okada, M., Kaneko, S., Hirano, T., Mizuno, K., Kondo, T., Otani, K., Fukushima, Y., 1995. Effects of zonisamide on dopaminergic system. Epilepsy Res. 22, 193–205.

Okada, M., Hirano, T., Kawata, Y., Murakami, T., Wada, K., Mizuno, K., Kondo, T., Kaneko, S., 1999. Biphasic effects of zonisamide on serotonergic system in rat hippocampus. Epilepsy Res. 34, 187–197.

Olanow, C.W., McNaught, K.S., 2006. Ubiquitin-proteasome system and Parkinson's disease. Mov. Disord. 21, 1806–1823.

Paxinos, G., Franklin, K.B.J., 2007. The Mouse Brain in Stereotaxic Coordinates. Academic Press, San Diego, CA.

Qin, S., Colin, C., Hinners, I., Gervais, A., Cheret, C., Mallat, M., 2006. System x_c^- and apolipoprotein E expressed by microglia have opposite effects on the neurotoxicity of amyloid-beta peptide 1-40. J. Neurosci. 26, 3345–3356.

Ramos, A., 2008. Animal models of anxiety: do I need multiple tests? Trends Pharmacol. Sci. 29, 493–498.

Robert, S.M., Buckingham, S.C., Campbell, S.L., Robel, S., Holt, K.T., Ogunrinu-Babarinde, T., Warren, P.P., White, D.M., Reid, M.A., Eschbacher, J.M., Berens, M.E., Lahti, A.C., Nabors, L.B., Sontheimer, H., 2015. SLC7A11 expression is associated with seizures and predicts poor survival in patients with malignant glioma. Sci. Transl. Med. 7, 289ra86.

Sano, H., Murata, M., Nambu, A., 2015. Zonisamide reduces nigrostriatal dopaminergic neurodegeneration in a mouse genetic model of Parkinson's disease. J. Neurochem.

Sato, H., Shiiya, A., Kimata, M., Maebara, K., Tamba, M., Sakakura, Y., Makino, N., Sugiyama, F., Yagami, K., Moriguchi, T., Takahashi, S., Bannai, S., 2005. Redox imbalance in cystine/glutamate transporter-deficient mice. J. Biol. Chem. 280, 37423–37429.

Shaw, V.E., Peoples, C., Spana, S., Ashkan, K., Benabid, A.L., Stone, J., Baker, G.E., Mitrofanis, J., 2012. Patterns of cell activity in the subthalamic region associated with the neuroprotective action of near-infrared light treatment in MPTP-treated mice. Parkinsons Dis. 2012, 296875.

Sonsalla, P.K., Wong, L.Y., Winnik, B., Buckley, B., 2010. The antiepileptic drug zonisamide inhibits MAO-B and attenuates MPTP toxicity in mice: clinical relevance. Exp. Neurol. 221, 329–334.

Tombini, M., Pellegrino, G., Di Pino, G., Assenza, G., 2013. Zonisamide for seizures in Parkinson's disease with dementia. Seizure 22, 324–325.

Tsujii, S., Ishisaka, M., Shimazawa, M., Hashizume, T., Hara, H., 2015. Zonisamide suppresses endoplasmic reticulum stress-induced neuronal cell damage in vitro and in vivo. Eur. J. Pharmacol. 746, 301–307.

Van Liefferinge, J., Bentea, E., Demuyser, T., Albertini, G., Follin-Arbelet, V., Holmseth, S., Merckx, E., Sato, H., Aerts, J.L., Smolders, I., Arckens, L., Danbolt, N.C., Massie, A., 2016. Comparative analysis of antibodies to xCT (Slc7a11): forewarned is forearmed. J. Comp. Neurol. 524, 1015–1032.

Vernon, A.C., Johansson, S.M., Modo, M.M., 2010. Non-invasive evaluation of nigrostriatal neuropathology in a proteasome inhibitor rodent model of Parkinson's disease. BMC Neurosci. 11, 1.

Wiesner, D., Merdian, I., Lewerenz, J., Ludolph, A.C., Dupuis, L., Witting, A., 2013. Fumaric acid esters stimulate astrocytic VEGF expression through HIF-1alpha and Nrf2. PLoS One 8, e76670.

Xie, W., Li, X., Li, C., Zhu, W., Jankovic, J., Le, W., 2010. Proteasome inhibition modeling nigral neuron degeneration in Parkinson's disease. J. Neurochem. 115, 188–199.

Yabe, H., Choudhury, M.E., Kubo, M., Nishikawa, N., Nagai, M., Nomoto, M., 2009. Zonisamide increases dopamine turnover in the striatum of mice and common marmosets treated with MPTP. J. Pharmacol. Sci. 110, 64–68.

Yamamura, S., Ohoyama, K., Nagase, H., Okada, M., 2009. Zonisamide enhances delta receptor-associated neurotransmitter release in striato-pallidal pathway. Neuropharmacology 57, 322–331.

Yang, L.P., Perry, C.M., 2009. Zonisamide: in Parkinson's disease. CNS Drugs 23, 703–711.

Yokoyama, H., Yano, R., Kuroiwa, H., Tsukada, T., Uchida, H., Kato, H., Kasahara, J., Araki, T., 2010. Therapeutic effect of a novel anti-parkinsonian agent zonisamide against MPTP (1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine) neurotoxicity in mice. Metab. Brain Dis. 25, 305–313.

Yurekli, V.A., Gurler, S., Naziroglu, M., Uguz, A.C., Koyuncuoglu, H.R., 2013. Zonisamide attenuates MPP+-induced oxidative toxicity through modulation of Ca2+ signaling and caspase-3 activity in neuronal PC12 cells. Cell. Mol. Neurobiol. 33, 205–212.