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System x_c^- as a potential target for novel neuroprotective strategies: focus on Parkinson's disease and its psychiatric comorbidities

Introduction on Parkinson's disease and its psychiatric comorbidities: In Parkinson's disease (PD), nigrostriatal degeneration causes a loss of dopamine in the striatum. As a result, the motor loop of the basal ganglia is dysregulated, resulting in hyperactivity of the subthalamic nucleus (STN) and subsequent overactivaton of the GABA-ergic output structures (i.e. substantia nigra pars reticulata (SNr), globus pallidus pars interna (GPi)) which in turn inhibits the thalamus. This results in motor impairment (bradykinesia, tremor and rigidity) due to reduced excitatory input from the thalamus to the motor cortex. The hyperactive glutamatergic STN neurons also project to the dopamine containing neurons located in the substantia nigra pars compacta (SNc) and may cause more neurodegeneration due to toxic glutamate levels (excitotoxicity). As such, SNc neuronal loss and STN overactivation sustain each other and may cause progression of the disease[1,2]. In this respect, the use of NMDA (ionotropic glutamate receptor) antagonists is clearly beneficial as neuroprotective effects can be observed. However, their use is hampered by neurological side effects that are the consequence of impairment of fast excitatory NMDA-mediated synaptic transmission[3,4]. Moreover, as recently described, the stimulation of extrasynaptic NDMA receptors triggers cell destruction pathways whereas the stimulation of synaptic NMDA receptors is involved in neuroprotection[5,6]. Antagonists of the postsynaptic group I metabotropic glutamate receptor (mGluR) mGluR5, have also been shown to be neuroprotective in the absence of overt side effects, as they negatively modulate NMDA responses without completely blocking synaptic transmission[7-9].

Besides the classical motor symptoms, 65% of PD patients suffer from neuropsychiatric symptoms such as depression and anxiety. The underlying mechanisms of depression and anxiety in PD are still unclear and might be attributed to a combination of medical, neurochemical and psychosocial factors. Interestingly, both depression and anxiety may precede PD onset, indicating they are not merely the result of the difficulties related to PD[10].

Despite therapeutic advances over the last decades, PD can still only be treated symptomatically. Moreover, medication that could treat the neuropsychiatric comorbidities, such as antidepressants or anxiolytic agents, has not been proven to be effective in PD and the risk of deterioration of PD as well as interactions with the PD medication are a major concern[10]. In conclusion, new insights into the molecular mechanisms leading to PD and its comorbidities are crucial as they might provide new targets for disease-modifying interventions.

Project background information: System x_c^- or the Na^+-independent cystine/glutamate antiporter, consisting of xCT as a specific subunit and 4F2hc, is located on glial cells and imports one cystine molecule in exchange for a glutamate molecule in an obligatory 1:1 exchange rate[11] (fig. 1). Increased activity can as such contribute to excitotoxic damage. In the context of our research focus, i.e. the role of glutamate transporters in neurological disorders[12-16], we were the first to propose a possible involvement of system x_c^- in the pathogenesis of PD. This hypothesis is based on the observation that xCT expression levels were increased in the ipsilateral striatum of the unilateral 6-hydroxydopamine (6-OHDA) hemi-Parkinson rat model[17]. To understand the functional meaning of this increased xCT expression levels, we used mice with a genetic deletion of xCT ($xCT^{-/-}$)[18] and tested their vulnerability for 6-OHDA-induced neurodegeneration. Although no effect was seen on striatal dopamine loss, the dopaminergic neurons in the SNc of these mice were significantly protected. Apart from the strong decrease in striatal extracellular glutamate levels, we could not observe neurochemical or anatomical changes in the $xCT^{-/-}$ brain under physiological conditions and therefore we concluded that the protective effects of the loss of system x_c^- should probably be linked to the decreased extracellular glutamate levels[19].

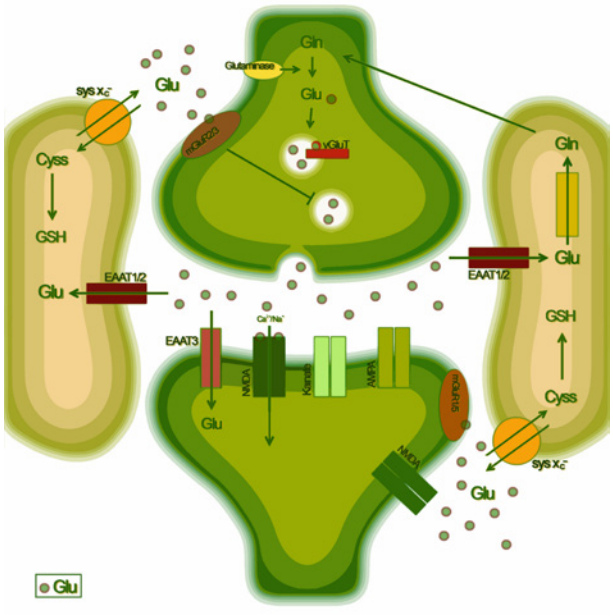

Figure 1: schematic representation of a glutamatergic synaps including glutamate reuptake transporters (EAAT), glutamate receptors (NMDA, AMPA, mGluR) and system x_c^- (sys x_c^-).

The **rationale for studying system x_c^- as a target for treating PD and its comorbidities** is based on the dual role this antiporter can have in neurological disorders that are characterized by increased levels of oxidative stress as well as excitotoxicity. Cystine is intracellularly reduced to cysteine, which is the rate limiting building block in the synthesis of glutathione (GSH), a major brain antioxidant. Consequently, the expression of system x_c^- can be induced by pathways that are activated by oxidative stress or other stress stimuli[20]. Indeed, we observed increased xCT expression in the ipsilateral striatum of the 6-OHDA hemi-Parkinson rat model[17]. However, each time a cystine molecule is imported, glutamate is released into the extracellular space and we demonstrated that under physiological conditions, in certain brain regions 60-70% of extracellular glutamate levels, as measured using *in vivo* microdialysis, originate from system x_c^- [16-19]. This **glutamate**, that is **released extrasynaptically**, can activate extrasynaptic NMDA receptors and mGluRs[21] and, as such, induce neurodegeneration when present in excess. **Our findings that mice lacking system x_c^- are protected against 6-OHDA-induced neurodegeneration[19] suggest 1/ that mice can perfectly deal with (oxidative) stress situations in the absence of system x_c^- and 2/ that avoiding the upregulation of system x_c^- in response to this cellular stress is protective, probably due to the fact that excessive glutamate release is prevented.** It thus seems that in certain pathological conditions, the brain induces excitotoxic damage in an attempt to protect itself against oxidative stress.

The goal of this project is to strengthen our hypothesis that inhibition of system x_c^- is a novel, neuroprotective strategy for the treatment of PD and its comorbidities. In order to do so, we proposed a validation plan consisting of **four specific aims**: **1/** investigate whether xCT expression levels are affected in PD-related structures of human PD patients, **2/** investigate the susceptibility of mice with a genetic deletion of xCT, and thus lacking functional system x_c^-, for lactacystin (LAC, inhibitor of the proteasome injected into the substantia nigra (SN)) and MPTP (1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine)-induced parkinsonism. Vice versa, investigate the effect of LAC- and MPTP-induced parkinsonism on the expression of xCT. **3/** unveil whether downregulation or inhibition of system x_c^- in the STN at a certain time in life and at a certain stage of the disease, instead of a genetic deletion, can still protect dopamine neurons from the SNc against toxin-induced degeneration. **4/** Ultimately, investigate the link between system x_c^- and anxiety/depression in healthy as well as parkinsonian mice. During the time-course of this project, **two additional studies**, related to this project, have been conducted in order to: **5**/ characterize antibodies directed against xCT and **6**/ investigate neuroprotective effects of zonisamide (ZNS).

Results obtained since the start of the project (2014-2015):
Results that were obtained in 2014 and already reported in our activity report of 2014 are in italic. For figures related to these results, we refer to our previous report.

1. Specific aim 1: Investigate xCT mRNA (real-time PCR) and protein (semi-quantitative Western blotting) levels in tissue samples of PD patients and healthy controls.

Up till now, we only collected some preliminary data on human tissue samples. With a limited number of samples, we could observe a trend towards increased xCT protein expression in cortex of PD patients compared to healthy controls and a decreased xCT expression in the SN of PD patients (fig. 2 in report 2014). In the near future, additional samples will be obtained from the Netherlands brain bank.

In this small number of samples we also investigated expression levels of proteins of the phosphoinositide 3-kinase (PI3K)/glycogen synthase kinase 3β (GSK-3β)/eukaryotic initiation factor 2α (eIF2α)/activating transcription factor (ATF4) pathway (collaboration with P. Maher, Salk Institute, San Diego, US and J. Lewerenz, University Hospital Ulm, Germany). We previously characterized this pathway as being responsible for the increased xCT expression levels in hippocampus of epileptic patients[22] as well as a chronic epilepsy mouse model (Leclercq, Van Liefferinge *et al.*, in preparation). Yet, in PD samples, there was no correlation between the changes in xCT expression and the proteins of this pathway. Further studies should identify the pathway that modulates xCT expression in PD.

2. For specific aim 2 we investigated whether our observations in the 6-OHDA model can be generalized to other PD models, by studying xCT expression in the LAC/SN model and the chronic, progressive MPTP model. At the same time, we compared the behavioral and neurochemical outcome of $xCT^{-/-}$ and $xCT^{+/+}$ mice in both models.

2.1. Characterization of the intranigral lactacystin mouse model

Since we are the first to use a mouse model for PD in which LAC is injected into the SN, we further characterized this model before investigating the involvement of system x_c^- in LAC-induced parkinsonism. A very thorough behavioral and neurochemical analysis was performed on three month old C57Bl/6 mice, one and three weeks after LAC injection.

In this **manuscript** *that was* **recently published**[23] *we report that unilateral administration of LAC to the SN of mice leads to acute and non-progressive dopaminergic neurodegeneration (fig. 3 in report 2014), in the presence of increased levels of Ser129-phosphorylated α-synuclein (fig. 4 in report 2014). These pathological changes induced the development of motor asymmetry and impairment, as assessed*

in various motor behavior paradigms (fig. 5 in report 2014). Furthermore, we detected signs of non-motor symptoms resembling early-stage Parkinson's disease, including somatosensory disturbances, akathisia (restlessness), perseverative behaviour (fig. 5 in report 2014), and anxiety-like behaviour (fig. 6 in report 2014). We conclude that the intranigral LAC mouse model can be a relevant model to study the involvement of proteasomal dysfunction and of authentically phosphorylated α-synuclein at Ser129 in the pathogenesis of sporadic Parkinson's disease.

We are currently studying STN hyperactivity in this LAC model. Ongoing experiments should reveal whether the increased VGLUT2 protein expression (vesicular glutamate transporter) that we observe in the Substantia nigra pars reticulata (SNr) originates in the STN (*in situ* hybridization for VGLUT2) and whether the latter region is hyperactive (CO staining/zif268 *in situ* hybridization), conform observations in PD patients.

Finally, using electron microscopy (collaboration with C. Meshul; VA Medical Center, Portland, Oregon, USA) we are studying ultrastructural changes in corticostriatal synapses in the nigral LAC mouse model (fig. 2). Previous findings indicate significant synaptic plasticity in the striatum of patients with PD, characterized by an increase in the size of the post-synaptic densities of dendritic spines in asymmetric contact with afferents most likely of cortical origin[24]. In line with the proposed hyperactivity of cortico-striatal terminals in PD, we provide evidence suggesting an increase in the activity of the cortico-striatal pathway following intranigral LAC administration. In particular, by performing VGLUT1-pre-embed diaminobenzidine immunolabeling followed by post-embed immunogold on slices covering the dorsolateral striatum, we observed a significant increase in the percentage of labeled terminals containing mitochondria. As mitochondria are recruited into the presynaptic terminal with increased activity[25], this might indicate an increase in the activity of the corresponding corticostriatal afferents. Consistent with this notion, the post-synaptic elements contacted by the labeled terminals exhibited plastic changes suggestive of increased synaptic activity following lesion, including an increase in the thickness and area of the post-synaptic density.

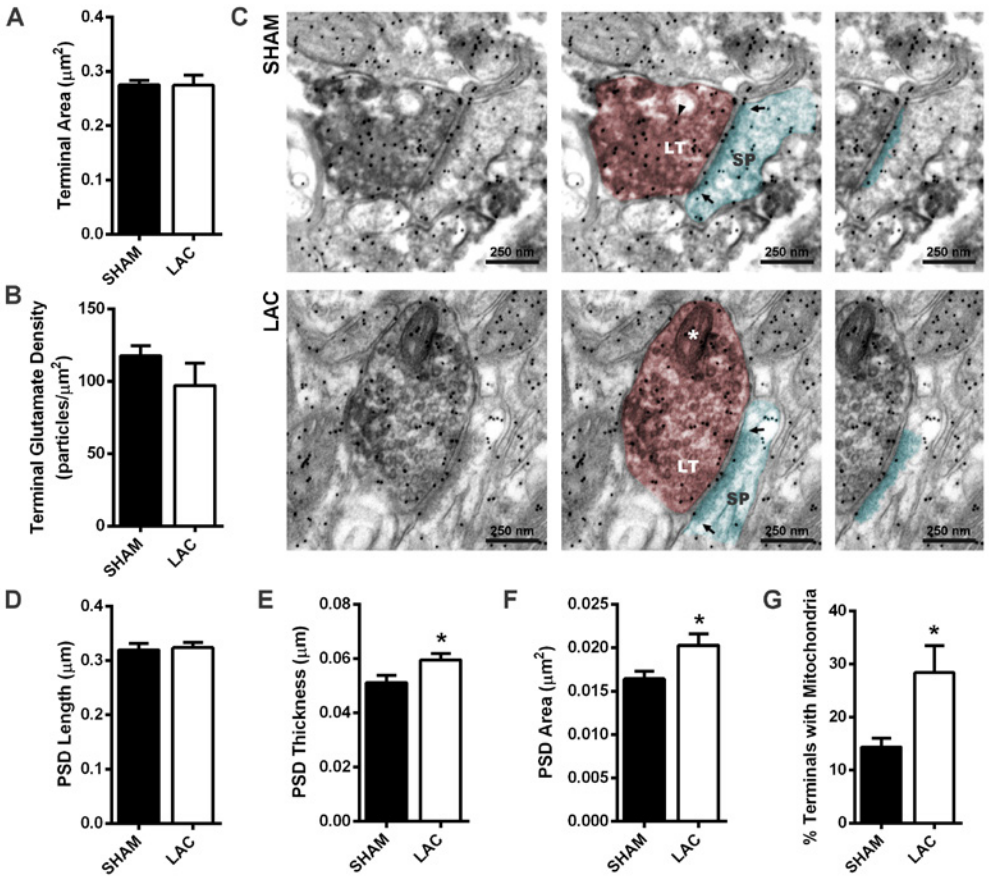

Figure 2: Plastic changes occurring at corticostriatal synapses in the lactacystin (LAC) mouse model of Parkinson's disease. Intranigral LAC injection led to significant modifications of corticostriatal synapses after 1 week, including increases in the thickness (**E**) and area (**F**) of the postsynaptic density (PSD) and percentage of corticostriatal terminals containing mitochondria (**G**). LAC administration did not influence the area (**A**) or density of glutamate (**B**) in corticostriatal terminals, nor did it change the length of the PSD (**D**). **C**: Representative electron micrographs of VGLUT1- pre-embed diaminobenzidine labeled terminals (LT, shaded red) contacting dendritic spines (SP, shaded teal) in the dorsolateral striatum of SHAM- (top; control mice) or LAC- (bottom) mice. The asymmetric axospinous contacts are defined with arrows. Glutamate molecules have been identified using post-embed glutamate immunogold (arrowhead). Note the increased presence of mitochondria in corticostriatal terminals of LAC-lesioned mice (asterisk). Right panel: The PSD has been highlighted (teal) in order to illustrate the increased thickness and total area following intranigral LAC injection. Data are presented as mean ± SEM and were generated from n=8 SHAM mice (22.5 ± 1.701 synapses/mouse) and n=7 LAC mice (24.86 ± 2.143 synapses/mouse). Statistical analysis was performed using an unpaired t test, *p<0.05.

2.2. Involvement of system x_c^- in lactacystin-induced parkinsonism

We next investigated the involvement of system x_c^- in LAC-induced parkinsonism. xCT expression levels were measured in striatum of LAC- and sham-injected mice using semi-quantitative Western blotting (fig. 7 in report 2014). Preliminary data on a limited number of mice show a strong trend towards increased xCT expression levels in parkinsonian striatum. However, groups need to be enlarged and additional brain regions will be investigated in the coming months.

Finally, the behavioral and neurochemical outcome of $xCT^{-/-}$ mice after LAC injection was compared to $xCT^{+/+}$ mice. In adult mice (three months old), we could not observe any differences in susceptibility for LAC-induced nigrostriatal degeneration (fig. 8 in report 2014) or behavioral impairment (fig. 9 in report 2014). On the other hand, in aged mice (i.e. 19-23 month old mice) we could clearly observe neuroprotection as the result of the loss of system x_c^- (fig. 10 in report 2014).

The reason for this age-dependent protection is currently being elucidated as part of a larger study in which we investigate the role of system x_c^- in (healthy) aging.

2.3. Involvement of system x_c^- in MPTP-induced parkinsonism

Next, we studied the involvement of system x_c^- in MPTP-induced parkinsonism, using the progressive, chronic MPTP model. Besides being progressive and chronic, this model has the advantage that no surgery is needed. MPTP is being delivered intraperitoneally instead of intracerebral. These experiments have been performed in collaboration with C. Meshul and are **published in Neuroscience Letters[26]***. xCT expression levels have been measured in midbrain and striatum of MPTP-treated C57Bl/6 mice and we compared MPTP-induced nigrostriatal degeneration between $xCT^{-/-}$ and $xCT^{+/+}$ mice.*

Our results indicate that the expression of xCT undergoes region-specific changes in MPTP-treated mice, with increased expression in the striatum (fig. 11A in report 2014), and decreased expression in the SN (fig. 11B in report 2014). Furthermore, mice lacking xCT were equally sensitive to the neurotoxic effects of MPTP, as they demonstrate similar decreases in striatal dopamine content, striatal tyrosine hydroxylase (TH) expression, nigral TH immunopositive neurons and forelimb grip strength, five weeks after commencing MPTP treatment (fig. 12 in report 2014). Altogether, our data indicate that progressive lesioning with MPTP induces striatal and nigral dysregulation of system x_c^-. However, loss of system x_c^- does not affect MPTP-induced nigral dopaminergic neurodegeneration and motor impairment in mice.

3. For specific aim 3, we will investigate whether a local and timed downregulation (or inhibition) of system x_c^- can have the same protective effects as observed in animals that are born with a total loss of system x_c^-.

These experiments will be performed in 2016.

4. For specific aim 4, we investigated in detail anxiety and depressive-like behavior in non-PD $xCT^{-/-}$ mice compared to $xCT^{+/+}$ littermates. Moreover, the effect of loss of system x_c^- on anxiety and depressive-like behavior in PD mice will be evaluated.

We have completed a very thorough phenotyping study of the $xCT^{-/-}$ vs. $xCT^{+/+}$ mice. These results are recently **published in Progress in Neuropsychopharmacology & Biological Psychiatry[27].**

We phenotyped adult and aged system x_c^- deficient mice in a battery of tests for anxiety- and depressive-like behavior (open field, light/dark test, elevated plus maze, novelty suppressed feeding, forced swim test, tail suspension test). Concomitantly, we evaluated the sensorimotor function of system x_c^- deficient mice, using motor and sensorimotor based tests (rotarod, adhesive removal test, nest building test). Our results indicate that loss of system x_c^- does not affect motor or sensorimotor function, in either adult or aged mice, in none of the paradigms investigated (fig. 13, 14 in report 2014). On the other hand, in the open field and light/dark tests, and forced swim and tail suspension tests respectively, we could observe significant anxiolytic (fig. 15 in report 2014) and antidepressive-like (fig. 16 in report 2014) effects in system x_c^- deficient mice that in certain cases (light/dark, forced swim) were age-dependent. These findings indicate that, under physiological conditions, nonvesicular glutamate release via system x_c^- mediates aspects of higher brain function related to anxiety and depression, but does not influence sensorimotor function. As such, modulation of system x_c^- might constitute the basis of innovative interventions in mood disorders.

5. Specificity of antibodies raised against xCT

Many studies aiming to unveil the distribution or the role of system x_c^-, rely on antibody recognition of its specific subunit, xCT. It is generally accepted however that antibody specificity is a major problem. Until 2004, the only possibility to detect system x_c^- was the use of an antibody against a selective substrate, aminoadipic acid[28]. After that, distinct antibodies raised against xCT appeared without consensus about the molecular weight of the antiporter. Based on literature, the xCT protein band would be expected around 55.5 kDa[29]. However, it has been reported that our own antibody[17] as well as the antibodies raised by Burdo *et al.* (2006)[30], Shih *et al.* (2006)[31] and La Bella *et al*. (2007)[32] recognize a fuzzy protein band at about 35 kDa. Antibody specificity of our own polyclonal antibody was confirmed on brain tissue of $xCT^{-/-}$ mice[18], using Western blotting[17]. The presence of nonspecific protein bands (present in both $xCT^{-/-}$ and $xCT^{+/+}$ tissue) shows that this antibody also recognizes unrelated proteins and as such cannot be used for immunohistochemistry[17]. Many researchers are still identifying the 55.5 kDa sharp immunoreactive band, observed when using most commercial antibodies in Western blot experiments and claimed to be xCT on the datasheets accompanying these antibodies, as xCT. Since often these antibodies are even used for immunohistochemistry, wrong conclusions can easily be drawn. Moreover, inappropriate negative controls are frequently being used to control for antibody specificity, such as pre-adsorption of the antiserum against the peptide or omission of the secondary antibody[33,34].

We therefore verified the specificity of the most commonly used commercial as well as some in-house-developed anti-xCT antibodies (table 1, 2), by comparing their immunoreactivity in mouse brain tissue of $xCT^{+/+}$ and $xCT^{-/-}$ littermates using Western blotting (fig. 3) and immunohistochemistry (fig. 4, 5). A specific signal should be observed exclusively in $xCT^{+/+}$ tissue and be absent in $xCT^{-/-}$ tissue. The Western blot screening results demonstrate that antibody specificity not only differs between batches produced by immunizing different rabbits with the same antigen, but also between bleedings of the same rabbit (table 1). A number of immunohistochemical protocols has been tested for all the anti-xCT antibodies that were specific on Western blots (*in table 1 and 2) in order to obtain a specific immunolabeling (fig. 4). Only one of our in-house-developed antibodies could reveal specific xCT labeling and exclusively on acetone-postfixated cryo-sections (fig. 5). Using this approach, we observed xCT protein expression throughout the mouse forebrain, including cortex, striatum, hippocampus, midbrain, thalamus and amygdala, with highest expression in regions facing the cerebrospinal fluid and meninges (fig. 5). **The results of this study were published in the Journal of Comparative Neurology[35].**

Table 1: In-house developed antibodies that were tested in this study (* specific protein band detected).

Antibody ID	Production Date	Rabbit ID no	Peptide	Peptide sequence	Concentration (µg/ml)
Antibody#496	2002-10-27	1B1685	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.079
Antibody#497	2002-10-27	1B1685	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.028
Antibody#510	2004-07-14	1B1685	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.183
Antibody#511	2004-07-14	1B1685	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.292
Antibody#512	2004-07-14	1B1685	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.108
Antibody#418	2003-01-13	2B0438	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.092
Antibody#422	2003-01-13	2B0438	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.067
Antibody#502	2004-07-14	2B0635	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.042
Antibody#501	2004-07-14	2B0635	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.158
*Antibody#500	2004-07-14	2B0635	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.225
Antibody#504	2004-07-14	2B0636	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.117
Antibody#505	2004-07-14	2B0636	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.058
Antibody#506	2004-07-14	2B0636	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.592
Antibody#509	2004-07-14	2B0664	MxCT(22-36)	GRLPSMGDQEPPGQE-(amide)	0.183
Antibody#508	2004-07-14	2B0664	MxCT(471-485)	DKKPKWFRRLSDRIT-(amide)	0.083
*Antibody#507	2004-07-14	2B0664	MxCT(492-502)	LEVVPEDSKEL-(COOH)	0.083
Antibody#453	2003-07-22	2B1085	MxCT(226-232)	SGRDTSL-(amide)	0.400
Antibody#456	2003-07-22	2B1085	MxCT(226-232)	SGRDTSL-(amide)	0.060
Antibody#465	2003-07-22	2B1085	MxCT(256-269)	TEEVDNPEKTIPLA-(amide)	0.033
*Antibody#470	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.140
Antibody#471	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.440
*Antibody#472	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.370
*Antibody#473	2003-07-22	2B1085	MxCT(345-356)	ASREGHLPEILS-(amide)	0.357
Antibody#466	2003-07-22	2B1086	MxCT(226-232)	SGRDTSL-(amide)	0.307
Antibody#467	2003-07-22	2B1086	MxCT(226-232)	SGRDTSL-(amide)	0.460
Antibody#469	2003-07-22	2B1086	MxCT(226-232)	SGRDTSL-(amide)	0.470
Antibody#461	2003-07-22	2B1086	MxCT(256-269)	TEEVDNPEKTIPLA-(amide)	0.300
*Antibody#474	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.240
Antibody#475	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.467
*Antibody#476	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.345
*Antibody#477	2003-07-22	2B1086	MxCT(345-356)	ASREGHLPEILS-(amide)	0.420
Antibody#517	2004-07-20	3B0042	MxCT(345-356)	ASREGHLPEILS-(amide)	0.087
Antibody#514	2004-07-20	3B0045	MxCT(226-232)	SGRDTSL-(amide)	0.062
Antibody#516	2004-07-20	3B0045	MxCT(256-269)	TEEVDNPEKTIPLA-(amide)	0.032
Antibody#518	2004-07-20	3B0045	MxCT(345-356)	ASREGHLPEILS-(amide)	0.050
*Antibody#622	2006-01-27	4B0425	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.092
*Antibody#618	2006-01-27	4B0447	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.045
*Antibody#619	2006-01-27	4B0447	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.035
Antibody#614	2006-01-27	4B0454	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.420
*Antibody#616	2006-01-27	4B0454	RxCT(471-502)	DKKPKWFRRLSDRITRTLQIILEVVP EDSKEL-(COOH)	0.337
Antibody#610	2006-01-27	4B0462	RxCT (1-37)	MVRKPVVATISKGGYLQGNVSGRLP SVGDQEPPGHEK-(amide)	0.027
Antibody#612	2006-01-27	4B0462	RxCT(471-502)	DKKPKWFRRLSDRITRTLQIILEVVP EDSKEL-(COOH)	0.307
*Massie et al., 2008	2006-02-23	SB1315	MxCT(216–229)	GQTHHFKDAFSGRD-(amide)	0.206

Table 2: Commercially available antibodies that were tested in this study (* specific protein band detected).

Peptide	Manufacturer	Catalog number	Lot number	RRID	concentration (µg/ml)
HxCT between (1-50)	*Abcam plc.	ab37185	GR115623	AB_778944	2.000
HxCT between (1-50)	*Abcam plc.	ab37185	GR120606	AB_778944	0.250
HxCT between (1-50)	Abcam plc.	ab37185	GR180673	AB_778944	2.000
HxCT between (1-50)	*Abcam plc.	ab93030	24670	AB_10563683	0.250
HxCT between (1-50)	*Thermo Fisher Scientific Inc.	PA1-46264	MD1415805	AB_2190850	0.500
HxCT between (1-50)	*Thermo Fisher Scientific Inc.	PA1-16893	OB1663384	AB_2286208	2.000
HxCT between (1-50)	Thermo Fisher Scientific Inc.	PA1-16893	PH1894974	AB_2286208	0.333
HxCT between (1-50)	*Novus Biologicals	NB 300-318	G3	AB_527560	0.200
HxCT between (1-50)	Novus Biologicals	NB 300-318	H1	AB_527560	0.500
MxCT N-terminal region	Transgenic Inc.	KE021	TG210612	AB_1627148	1.000

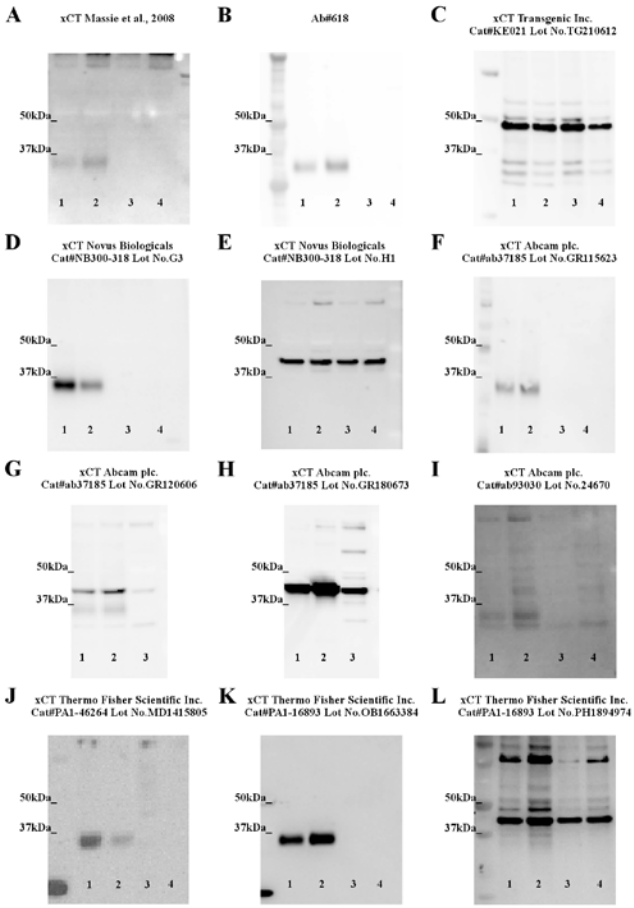

Figure 3: Specificity of anti-xCT antibodies: representative examples of the different Western blot experiments (2 concentrations of each sample were loaded; lanes 1-2: $xCT^{+/+}$ mouse brain tissue samples; lanes 3-4: $xCT^{-/-}$ mouse brain tissue samples).

After incubation with anti-xCT antibodies: Massie et al., 2008 **(A)** and Ab#618 **(B)** and commercial antibodies: Novus Biologicals Cat# NB 300-318 Lot No.G3 **(D)**, Abcam plc. Cat#ab37185 Lot No.GR115623 **(F)**, Abcam plc. Cat#ab37185 Lot No.GR120606 **(G)**, Abcam plc. Cat#ab93030 Lot No.24670 **(I)**, Thermo Fischer Scientific Inc. Cat#PA1-46264 Lot No.MD1415805 **(J)** and Thermo Fisher Scientific Inc. Cat#PA1-16893 Lot No.OB1663384 **(K)** specific xCT bands are observed of about 35 kDa in the $xCT^{+/+}$ samples which are absent in the $xCT^{-/-}$ samples.

No specific bands are discernable after incubation with the commercial anti-xCT antibodies: Transgenic Inc. Cat#KE021 Lot No.TG210612 **(C)**, Novus Biologicals Cat# NB 300-318 Lot No.H1 **(E)**, Abcam plc. Cat#ab37185 Lot No.GR180673 **(H)** and Thermo Fisher Scientific Inc. Cat#PA1-16893 Lot No.PH1894974 **(L)**. These antibodies give rise to a nonspecific protein band of about 40-50 kDa present in both $xCT^{+/+}$ and $xCT^{-/-}$ brain homogenates.

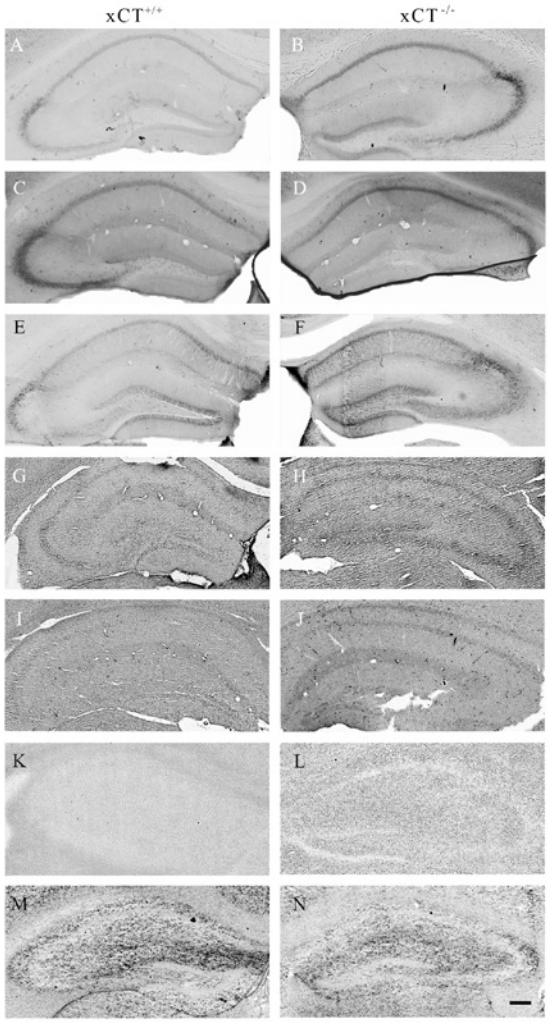

Figure 4: Specificity of anti-xCT antibodies: representative examples of distinct immunohistochemical experiments (left panels: xCT+/+ mouse brain samples; right panels: xCT-/- mouse brain samples).
(**A-B**) Cardiac perfusion with 4% formaldehyde (F) and incubation with our own xCT antibody Massie et al. 2008, trypsine digestion and Tris-buffered saline (TBS, 0,01M, 0,1% TritonX-100, pH 7,4) rinsing steps.
(**C-D**) Cardiac perfusion with 4% F and incubation with Thermo Fisher Scientific Inc. Cat#PA1-16893 Lot No.OB1663384, TBS (0,03% TritonX-100) rinsing steps.
(**E-F**) Cardiac perfusion with 4% F and incubation with Abcam plc. Cat#ab37185 Lot No.GR115623, phosphate-buffered saline (PBS) rinsing steps.
(**G-H**) Cardiac perfusion with 4% F, serial sucrose incubation (15-20-30%), snap-freezing and incubation with Abcam plc. Cat#ab37185 Lot No.GR115623.
(**I-J**) Cardiac perfusion with a mixture of 4% F and 0.35% glutaraldehyde, 48h incubation in 15% sucrose, snap freezing and incubation with Transgenic Inc. Cat#KE021 Lot No.TG210612.
(**K-L**) Snap-freezing, 10 min post-fixation in 4% F and incubation with Ab#618.
(**M-N**) Snap-freezing, 10 min post-fixation in ice-cold acetone and incubation with Novus Biologicals Cat# NB 300-318 Lot No.G3.
Scale bar in N = 250µm (applies to all panels)

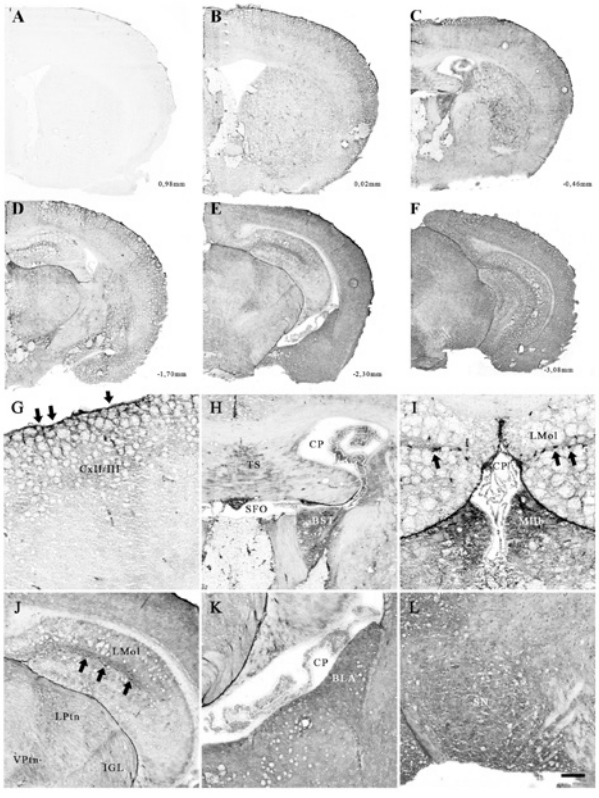

Figure 5: Regional distribution of xCT throughout the mouse forebrain, as determined using the Ab#618 antibodies to xCT. (**A-F**) Immunohistochemical labeling at distinct levels in the $xCT^{+/+}$ mouse brain (**B-F**) is absent in the $xCT^{-/-}$ mouse brain (**A**). Coordinates relative to bregma are depicted at right bottom. (**G-L**) Structures in $xCT^{+/+}$ mouse brain showing dense xCT immunoreactivity. **G**: leptomeninges (arrows), cortical layers II-III (CxII/III), **H**: subfornical organ (SFO), bed nucleus of the stria terminalis (BST), triangular septal nucleus (TS), choroid plexus (CP), **I**: choroid plexus (CP), medial habenular nucleus (MHb), lacunosum moleculare layer of the hippocampus (LMol), hippocampal vasculature (black arrows), **J**: lacunosum moleculare layer of the hippocampus (LMol), lateral posterior thalamic nucleus (LPtn), ventral posteromedial thalamic nucleus (VPtn), intergeniculate leaf (IGL), hippocampal vasculature (black arrows), **K**: choroid plexus (CP), basolateral amygdala (BLA), L: substantia nigra (SN). Scale bar in L = 175µm applies to G, I, L; 750µm for A-F; 375µm for H, J; 250µm for K.

6. Neuroprotective effects of zonisamide against lactacystin-induced neurodegeneration are independent of system x_c^-.

Zonisamide (ZNS), used in symptomatic treatment of PD[36-38], was recently reported to exert neuroprotection in rodent models[39-42]. One of the proposed neuroprotective mechanisms involves elevated xCT protein expression, inducing GSH synthesis[40]. This is however in contrast with our hypothesis that inhibition of system x_c^- might be a neuroprotective strategy for the treatment of PD[19]. Therefore, 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 LAC (or vehicle, Sham) was administered intranigrally to mice receiving repeated i.p. injections of either ZNS 30mg/kg or vehicle (placebo). 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. In a separate set of experiments, the impact of ZNS treatment on system x_c^- was investigated in naive, control conditions *in vivo* as well as *in vitro*. ZNS reduced the loss of nigral dopaminergic neurons following LAC infusion (fig. 6) and the degree of sensorimotor impairment (fig. 7). ZNS failed, however, to modulate xCT expression in basal ganglia of lesioned mice (fig. 8). Similarly, ZNS did not influence xCT or GSH levels in naive mice (fig. 9), nor did it alter system x_c^- activity or GSH content *in vitro* (fig. 10). 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. These results have been **submitted to Neuropharmacology** (Bentea *et al.*, under review).

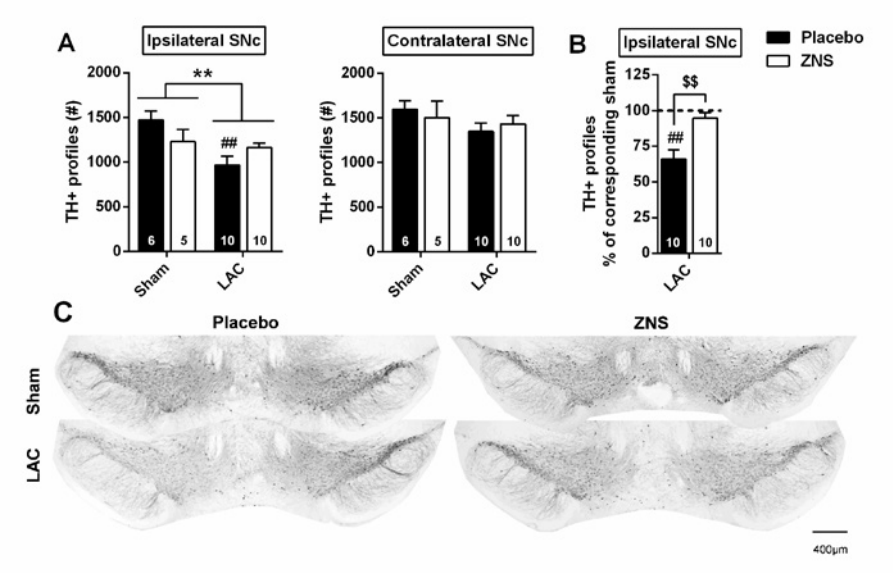

Figure 6: Repeated administration of zonisamide (ZNS) protects against lactacystin (LAC)-induced nigral neurodegeneration. (**A**) Tyrosine hydroxylase (TH) immunohistochemistry revealed a global loss of TH+ neurons after LAC lesion in the ipsilateral substantia nigra pars compacta (SNc). Significant loss of TH+ profiles was observed in the placebo-treated group, compared to their corresponding sham group. (**B**) ZNS treatment reduced the LAC-induced loss of SNc TH+ profiles. Bars represent mean ± SEM, **p<0.01 (two-way ANOVA), ##p<0.01 (Tukey's post-hoc comparison to corresponding sham), $$p<0.01 (Tukey's post-hoc comparison to placebo-treated). Sample sizes are mentioned in the bars. (**C**) Representative TH photomicrographs of the SNc of the four groups of mice. Scale bar 400 µm.

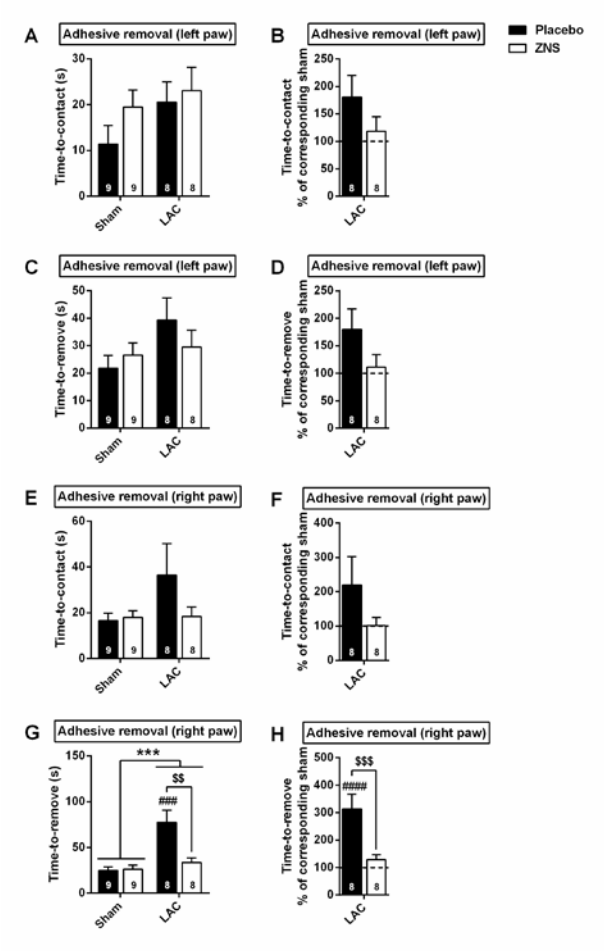

Figure 7: Repeated zonisamide (ZNS) treatment improves sensorimotor performance of lactacystin (LAC)-lesioned mice. (**A**-**D**) In the adhesive removal test, time-to-contact and time-to-remove the adhesive strips remained unaltered on the ipsilateral side. (**E**-**H**) On the contralateral side, LAC lesion induced a significant delay in the time-to-remove that was effectively reduced by ZNS treatment. Bars represent mean ± SEM, ***p<0.001 (two-way ANOVA), ###p<0.001, ####p<0.0001 (Tukey's post-hoc comparison to corresponding sham), $$p<0.01, $$$p<0.001 (Tukey's post-hoc comparison to placebo-treated). Sample sizes are mentioned in the bars.

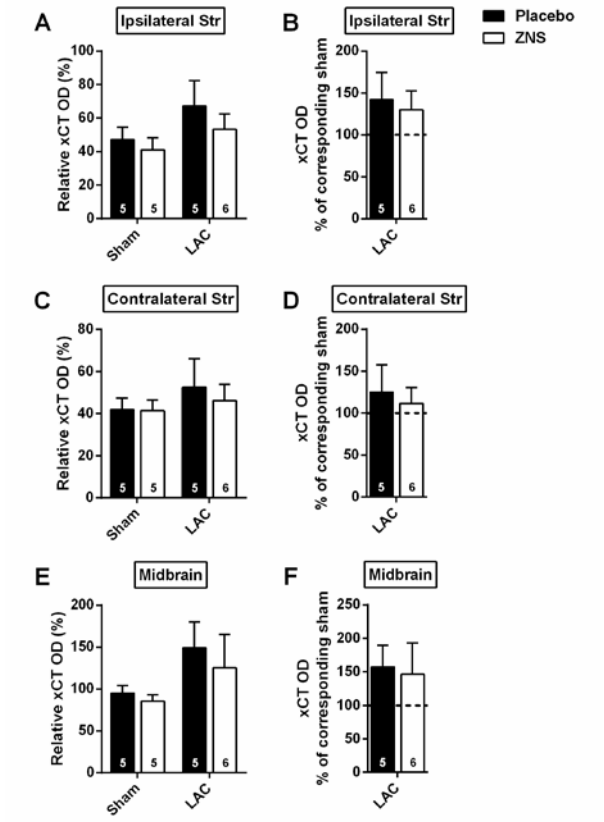

Figure 8: Repeated administration of zonisamide (ZNS) does not influence xCT protein expression one week after lactacystin (LAC) administration. xCT protein expression was not affected by LAC-lesion nor ZNS treatment, both in striatum (**A**-**D**) and midbrain (**E**, **F**). Bars represent mean ± SEM. Sample sizes are mentioned in the bars.

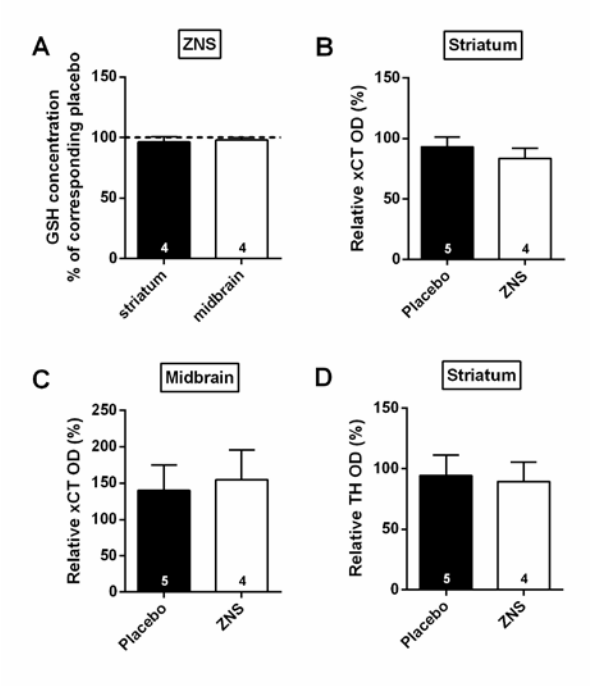

Figure 9: Repeated administration of zonisamide (ZNS) to naïve mice does not influence glutathione (GSH) or xCT expression levels in the basal ganglia. (**A**) No effect of a 14 day ZNS treatment on midbrain and striatal GSH levels in $xCT^{-/-}$ mice and their $xCT^{+/+}$ littermates. (**B**, **C**) Quantification of striatal (**B**) and midbrain (**C**) xCT expression levels by Western blotting in $xCT^{+/+}$ mice after ZNS treatment failed to reveal any significant difference compared to placebo controls. (**D**) Repeated administration of ZNS did not influence striatal protein expression of TH. Bars represent mean ± SEM. Sample sizes are mentioned in the bars.

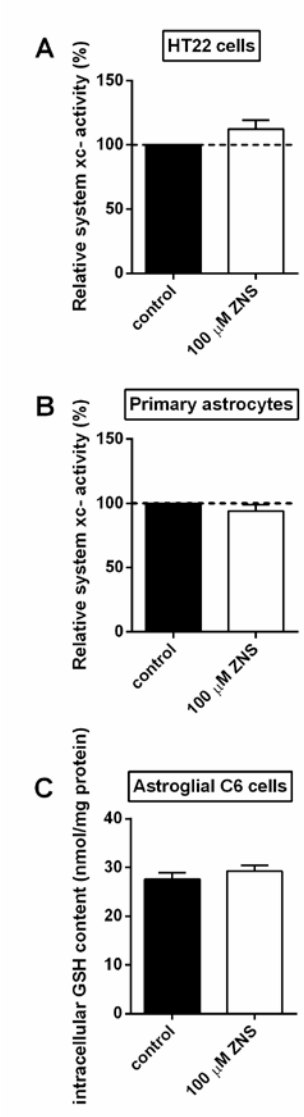

Figure 10: Zonisamide (ZNS) does not influence the activity of system x_c^- or intracellular glutathione (GSH) levels *in vitro*. (**A**-**C**) Lack of effect of 100 µM ZNS administration on relative system x_c^- activity in HT22 cells upon 24 h of ZNS (**A**) and microglia-free murine primary astrocytes upon 48 h of ZNS (**B**), and on intracellular GSH content in astroglial C6 cells upon 24 h of ZNS (**C**). Bars represent mean ± SEM of six (**A**) and five (**B**, **C**) independent experiments.

7. Summary of the main achievements/findings in the first two years (2014-2015) of this project:

1. In-depth analysis of the specificity problem of anti-xCT antibodies
2. Development and characterization of a new intranigral LAC mouse model for PD, showing α-synuclein accumulation as well as motor and non-motor impairment
3. Behavioral phenotyping of the xCT$^{-/-}$ mice, with clear anxiolytic and anti-depressive like effects
4. Further evidence for inhibition of system x_c^- as potential neuroprotective strategy for the treament of PD, without negative effects in the elder and possibly beneficial for neuro-psychiatric co-morbidities
5. Confirmation of system x_c^- - independent, neuroprotective effects of ZNS in the LAC model

8. Publications obtained (or under revision/submitted) in the frame (or with the support) of the current GSKE project (2014-2016)

- Bentea E, Van Liefferinge J, Martens K, Kobayashi S, Deneyer L, Verbruggen L, Demuyser T, Albertini G, Maes K, Sato H, Smolders I, Lewerenz J, Massie A. Zonisamide reduces lacatcystin-induced nigral dopaminergic neurodegeneration and sensorimotor impairment in mice via system x_c^- independent mechanisms. Neuropharmacology, submitted
- 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. Physiology & Behavior, in press.
- Massie A, Boillée S, Hewett S, Knackstedt L, Lewerenz J (2015) Main path and byways : non-vesicular glutamate release via system x_c^- as an important modifier of glutamatergic neurotransmission. Journal of Neurochemistry, in press.
- Van Liefferinge J, Bentea E, Demuyser T, Albertini G, Follin-Arbelet V, Holmseth S, Merckx E, Sato H, Aerts J, Smolders I, Arckens L, Danbolt NC, Massie A (2015) Comparative analysis of antibodies to xCT (Slc7a11) : forewarned is forearmed. Journal of Comparative Neurology, in press.
- El Arfani A, Albertini G, Bentea E, Demuyser T, Van Eeckhaut A*, Smolders I*, Massie A* (2015) Alterations in the motor cortical and striatal glutamatergic and D-serinergic system in the bilateral 6-hydroxydopamine rat model for Parkinson's disease. Neurochemistry International 88: 88-96. (*equally contributing authors)
- Bentea E*, Demuyser T*, Van Liefferinge J, Albertini G, Deneyer L, Nys J, Merckx E, Michotte Y, Sato H, Arckens L, Massie A*, Smolders I* (2015) Absence of system xc- in mice decreases anxiety and depressive-like behavior without affecting sensorimotor function or spatial vision. Progress in Neuro-Psychopharmacology & Biological Psychiatry 59: 49-58. (*equally contributing authors)
- Bentea E, Van Liefferinge J, Van der Perren A, 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 parkinsonism including non-motor impairment and alpha-synuclein phosphorylation at Ser129. Frontiers in Behavioral Neuroscience 9: 68.
- Bentea E*, Sconce MD*, Churchill MJ*, Van Liefferinge J, Sato H, Meshul CK, Massie A (2015) MPTP-induced parkinsonism in mice alters striatal and nigral xCT expression but is unaffected by the genetic loss of xCT. Neuroscience Letters, Neuroscience Letters 593: 1-6. (*equally contributing authors)
- El Arfani A, Bentea E, Aourz N, Ampe B, De Deurwaerdère P, Van Eeckhaut A, Massie A, Sarre S, Smolders I, Michotte Y (2014) NMDA receptor antagonism potentiates the L-DOPA-induced extracellular dopamine release in the subthalamic nucleus of hemi-parkinson rats. Neuropharmacology 85:198-205

9. Publications obtained in the frame (or with the support) of the previous GSKE project (2011-2013)

- De Bundel D*, Fafouri A*, Csaba Z*, Loyens E, Lebon S, El Ghouzzi, V, Peineau S, Vodjdani G, Kiagiadaki F, Aourz N, Coppens J, Walrave L, Portelli J, Vanderheyden P, Chai SY, Thermos K, Bernard V, Collingridge G, Auvin S, Gressens P, Smolders I*, Dournaud P* (2015) Trans-modulation of the somatostatine type 2 receptor trafficking by insulin-regulated aminopeptidase decreases limbic seizures. Journal of Neuroscience 35: 11960-75.
- Van Liefferinge J, Jensen C, Albertini G, Bentea E, Demuyser T, Merckx E, Aronica E, Smolders I, Massie A (2015) Altered vesicular glutamate transporter expression in human temporal lobe epilepsy with hippocampal sclerosis. Neuroscience Letters 590: 184-8.

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