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Synaptic zinc contributes to motor and cognitive deficits in 6-hydroxydopamine mouse models of Parkinson's disease

Joanna Sikora[a,b], Brigitte L. Kieffer[c], Pierre Paoletti[d], Abdel-Mouttalib Ouagazzal[a,*]

[a] Laboratoire de Neurosciences Cognitives, Aix-Marseille Univ, CNRS, LNC, UMR 7291, 13331 Marseille, France
[b] Aix-marseille Université, Marseille, France
[c] Douglas Research Center, Department of Psychiatry, McGill University, Montréal, Canada
[d] Institut de Biologie de l'Ecole Normale Supérieure (IBENS), Ecole Normale Supérieure, Université PSL, CNRS, INSERM, Paris, France

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ABSTRACT

Hyperactivity of glutamatergic corticostrial pathways is recognized as a key pathophysiological mechanism contributing to development of PD symptoms and dopaminergic neurotoxicity. Subset of corticostriatal projection neurons uses Zn^{2+} as a co-transmitter alongside glutamate, but the role of synaptically released Zn^{2+} in PD remains unexplored. We used genetically modified mice and pharmacological tools in combination with 6-hydroxydopamine (6-OHDA) lesion models of PD to investigate the contribution of synaptic zinc to disease associated behavioral deficits and neurodegeneration. Vesicular zinc transporter-3 (ZnT3) knockout mice lacking releasable Zn^{2+} were more resistant to locomotor deficit and memory impairment of nigrostriatal dopamine (DA) denervation compared to wildtype littermates. The loss of striatal dopaminergic fibers was comparable between genotypes, indicating that synaptically released Zn^{2+} contributes to behavioral deficits but not neurotoxic effects of 6-OHDA. To gain further insight into the mechanisms of Zn^{2+} actions, we used the extracellular Zn^{2+} chelator CaEDTA and knock-in mice lacking the high affinity Zn^{2+} inhibition of GluN2A-containing NMDA receptors (GluN2A-NMDARs). Acute chelation of extracellular Zn^{2+} in the striatum restored locomotor deficit of 6-OHDA lesion, confirming that synaptic Zn^{2+} suppresses locomotor behavior. Disruption of the Zn^{2+}-GluN2A interaction had, on the other hand, no impact on locomotor deficit or neurotoxic effect of 6-OHDA. Collectively, these findings provide clear evidence for the implication of striatal synaptic Zn^{2+} in the pathophysiology of PD. They unveil that synaptic Zn^{2+} plays predominantly a detrimental role by promoting motor and cognitive deficits caused by nigrostriatal DA denervation, pointing towards new therapeutic interventions.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder in the elderly characterized by resting tremor, rigidity, bradykinesia and gait disturbance. PD patients also experience a range of non-motor symptoms, including autonomic dysfunction, sleep disturbances, depression, anxiety, and cognitive impairment, which can often appear in the early stages of the disease (Papagno and Trojano, 2018; Whittington et al., 2006; Zis et al., 2015). The pathological hallmarks of PD include the loss of dopaminergic neurons in the nigrostriatal system coupled with the presence of Lewy bodies composed predominantly of misfolded α-synuclein protein (α-syn). The clinical symptoms of PD have been largely attributed to depletion of dopamine (DA) at striatal level and the subsequent dysfunction of cortico-basal ganglia-thalamo-cortical circuits (Obeso et al., 2000). The pathogenic pathways leading to neurodegeneration remain poorly understood and several factors have been implicated, including mitochondrial dysfunction, aberrant protein folding (*e.g.*, α-synuclein misfolding into toxic conformation), oxidative stress and neuroinflammation (Fujita et al., 2014; Lotharius and Brundin, 2002; Russo et al., 2014).

Compelling evidence support a role of zinc in PD pathogenesis. In PD patients, excessive zinc deposits are found in the substantia nigra region and the striatum (Dexter et al., 1991). Accordingly, studies using experimental models of PD showed that cytosolic accumulation of labile Zn^{2+} is a component of the pathogenic events leading to DA neuron death (Lee et al., 2009; Sheline et al., 2012; Tamano et al., 2018a, 2018b). For instance, intracellular zinc chelation protects dopaminergic neurons against neurotoxic effects of MPTP, 6-OHDA and paraquat (Sheline et al., 2012; Tamano et al., 2018a, 2018b). Conversely, systemic and intracerebral injections of zinc induce dopaminergic neurodegeneration and exacerbate nigrostriatal DA neuron loss induced by different neurotoxins (Hussain and Ali, 2002; Kumar et al., 2012, 2010;

* Corresponding author at: Laboratoire de Neurosciences Cognitives, UMR 7291 (AMU-CNRS), 3 Place V. Hugo, 13331 Marseille, France.
E-mail address: Abdel-mouttalib.ouagazzal@univ-amu.fr (A.-M. Ouagazzal).

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Lo et al., 2004; Yang et al., 2016). Further evidence for detrimental role of zinc in PD comes from genetic and molecular studies showing that the PD-associated human PARK9 (ATP13A2) gene encode for a cation pump (lysosomal type 5 P-type ATPase) that acts as a transporter for lysosomal sequestration of cytoplasmic Zn^{2+} (Kong et al., 2014; Tsunemi and Krainc, 2014). *In vitro*, PARK9 deficiency causes an alteration of intracellular Zn^{2+} homeostasis that in turn initiates a cascade of deleterious events (mitochondrial dysfunction, oxidative stress and α-syn aggregation) that are all involved in PD pathogenesis (Kong et al., 2014; Tsunemi and Krainc, 2014). Contrasting with the above findings, several studies reported a neuroprotective action of zinc. Zinc has been shown to reduce oxidative stress induced by 6-OHDA (Méndez-Álvarez et al., 2002) and prevents methamphetamine-induced dopaminergic neurotoxicity *in vitro* (Ajjimaporn et al., 2008, 2005). Zinc supplementation was also demonstrated to confer neuroprotection against rotenone-induced Parkinsonism in rats (Mbiydzenyuy et al., 2018) and extend lifespan in drosophila model of PD (Saini and Schaffner, 2010). In view of these findings, it emerges that zinc may play a contrasting role in PD depending of the etiological factors underlying development of the disease.

To date, most published studies relates to the pathogenic role of intracellular Zn^{2+} dyshomeostasis in PD. In the brain, however, a substantial amount (~10%) of total Zn^{2+} is packed within synaptic vesicles of a subset of glutamatergic terminals (Frederickson et al., 2000; Danscher and Stoltenberg, 2005; Paoletti et al., 2009; Sensi et al., 2011). This pool of vesicular (and chelatable) Zn^{2+} is particularly abundant in the forebrain (neocortex, hippocampus, striatum, amygdala) where zinc positive neurons form a dense associational network (Frederickson et al., 2000; Danscher and Stoltenberg, 2005). During synaptic activity, vesicular zinc is released alongside glutamate and acts as a potent extracellular modulator of neurocircuits by interacting with many synaptic receptors, such as NMDA and $GABA_A$ receptors (Anderson et al., 2017; Kodirov et al., 2006; Sensi et al., 2011, 2009; Vergnano et al., 2014; Vogt et al., 2000). Under pathological conditions (excitotoxicity), excess synaptic zinc can however enter postsynaptic neurons through ion channels and participates to neuronal injury (Sensi et al., 2011). Accordingly, synaptic zinc was implicated in neuronal damage and death after seizures, traumatic brain injury and ischemia, pathological events that all involve excessive glutamate release (Frederickson et al., 2005a, 2005b, 1987; Morris and Levenson, 2012; Sensi et al., 2011). Recently, extracellular Zn^{2+} influx into nigral dopaminergic neurons was also implicated in neurodegeneration induced by dopaminergic neurotoxins (Tamano et al., 2018a, 2018b). However, the sources of extracellular Zn^{2+} remain undetermined because glutamatergic neuron terminals in the substantia nigra are devoid of vesicular Zn^{2+} (Frederickson, 1989; Frederickson et al., 2000).

The striatum is heavily innervated by zinc-containing glutamatergic terminals arising from the cerebral cortex and the amygdala (Frederickson et al., 2000). In striatal slices, exogenous Zn^{2+} directly alters the activity of cholinergic interneurons and GABAergic spiny projection neurons (Berg and Bayliss, 2007; Blomeley and Bracci, 2008; Jiang et al., 2009; Yan and Surmeier, 1997). Exogenous Zn^{2+} also inhibits the excitatory responses of striatal neurons to cortical stimulations (Eseames et al., 1998). *In vivo*, local infusions of Zn^{2+} into the striatum impair locomotor activity in rats (Yakimovskii, 2012) and induce degeneration of dopaminergic terminals (Lo et al., 2004). Overactivity of glutamatergic corticostriatal transmission has been implicated in development of PD symptoms and the progression of the disease, but the role of synaptically released zinc in these processes remains elusive. In the present study, we used vesicular zinc transporter-3 (ZnT3) knockout mice that lack vesicular Zn^{2+} (Cole et al., 1999) to investigate the contribution of synaptically released Zn^{2+} to motor and cognitive deficits caused by nigrostriatal dopamine pathway deafferentation. The extracellular Zn^{2+} chelator CaEDTA, and knock-in mice lacking high affinity Zn^{2+} inhibition of NMDARs (GluN2A-H128S KI mice; Nozaki et al., 2011), were also used to gain further insight into the mechanisms of synaptic zinc actions.

2. Materials and methods

2.1. Animals

Eight weeks old C57/BL6J male mice were purchased from Charles River Laboratories (France). ZnT3 knockout (KO) mice were generously provided by Professor Richard Palmiter (University of Washington, USA) and maintained in pure BL6J genetic background. GluN2A-H128S knock-in (KI) mice were generated as described before (Nozaki et al., 2011) and maintained in 70% BL6JX129/SvPass genetic background. Mice were housed in groups of 3–4 in individually ventilated cages (Techniplast) and kept in 12 h light/dark cycle (light on at 7:00, off at 19:00) with water and food *ad lib*. Behavioral testing was performed during the light cycle between 09 h00 and 17 h00. All experimental procedures were conducted with the approval of the French national ethics committee (CE071) and in accordance with the EEC (2010/63/UE) guidelines for care and use of laboratory animals.

2.2. Drugs

6-Hydroxydopamine hydrochloride (6-OHDA, Tocris Bioscience), was dissolved in a vehicle solution (0.9% sterile NaCl containing 0.02% ascorbic acid). The extracellular zinc chelator, EDTA calcium disodium salt (CaEDTA, Sigma-Aldrich), was dissolved in 0.9% sterile NaCl.

2.3. Stereotaxic surgery and intrastriatal microinjections

2.3.1. Partial and full unilateral intrastriatal 6-OHDA lesions

Mice were anesthetized with a mixture of ketamine and xylazine (100 and 10 mg/kg, respectively) and mounted in a stereotaxic frame (Kopf Instruments). For partial unilateral dopamine lesion, 1 µl of 6-OHDA (4 µg/µl, free base) was infused into the left or right striatum (counterbalanced among mice) over 3 min at the following coordinates: AP + 1.0 mm, L ± 1.5 mm and DV −3.2 mm from the skull surface, according to the atlas of Paxinos and Franklin (2001). For full unilateral lesion, 2 µl of 6-OHDA (5 µg/µl, free base) was infused in the striatum over 6 min at the same coordinates. Control mice (Sham) receive an injection of the corresponding volume of vehicle in the same conditions.

2.3.2. Partial bilateral intrastriatal 6-OHDA lesion

Mice received bilateral injections of 1 µl of 6-OHDA (4 µg/µl, free base) into the striatum over 3 min at the same coordinates as before. Sham mice were injected bilaterally with the same volume of vehicle.

2.3.3. Cannula implantation

This procedure was performed immediately after full unilateral intrastriatal 6-OHDA lesion. 23-gauge-7 mm long stainless-steel guide cannula was positioned in the striatum at the following coordinates: AP + 1.0 mm, L ± 1.5 mm and DV −2.2 mm from the skull surface, according to the atlas of Paxinos and Franklin (2001). The cannula was fixed to the skull with anchoring screws and dental cement. Wire stylets (7 mm, gauge) were inserted into the cannula to prevent occlusion.

2.3.4. Intrastriatal chelation of extracellular Zn^{2+}

We used the cell-impermeable Zn^{2+} chelator, CaEDTA. This compound displays a high specificity and affinity for extracellular Zn^{2+}, and is therefore widely used for studying the functional role of synaptic Zn^{2+} *in vitro* and *in vivo* (Frederickson et al., 1990; Radford and Lippard, 2013; Takeda et al., 2006). The dose of CaEDTA and pretreatment time were chosen based on previous behavioral studies (Daumas et al., 2004; Lassalle et al., 2000; Suzuki et al., 2015). Drug infusions were made with stainless-steel injector needle (30-gauge) that protruded the guide cannula by 1 mm below into the striatum. Mice received unilateral injection of 0.5 µl of CaEDTA (300 mM) at rate of

0.1 µl/min *via* Hamilton syringe mounted on microdrive pump (Harvard apparatus, France). Behavioral testing was carried 15 min after local injection.

2.4. Apparatus and behavioral testing

Behavioral testing was carried a minimum 2 weeks after the post-operative period. All experiments were performed on 3–4 months old mice. Experimental cohorts of ZnT3 KO, GluN2A-H128S KI mice and their wildtype (WT) counterparts were littermates. Cohorts of female ZnT3 KO, GluN2A-H128S KI mice and their WT counterparts were used for studying the effects of partial unilateral 6-OHDA lesion on motor behavior. One cohort of male ZnT3 KO and WT mice was used for studying the effect of partial bilateral 6-OHDA lesion on recognition memory performance. Finally, one cohort of male BL6 mice was used for studying the effect of intrastriatal CaEDTA infusion on locomotor deficits induced by full 6-OHDA lesion.

2.4.1. Locomotor activity

Testing was carried in actimetry cages (20x11x17cm, Imetronic, France) fitted with infra-red beams frames that provides automated measures of locomotor activity and rears. The effect of synaptic Zn^{2+} elimination or GluN2A-H128S mutation on locomotor deficits induced by partial unilateral intrastriatal 6-OHDA lesion was assessed during 60 min testing period. The effect of intrastriatal chelation of extracellular Zn^{2+} on locomotor deficits induced by full unilateral intrastriatal 6-OHDA lesion was assessed during 10 min testing period.

2.4.2. Recognition memory

Testing was performed in five-choice operant chambers (Coulbourn Instruments, Allentown, USA) as previously described (Reiss et al., 2014). The procedure comprised an acquisition session followed 24 h by a retention session. In the acquisition session, mice were exposed for 30 min to the testing chamber in the presence of two adjacent nose-poke modules (NPM, spaced 4 cm apart and turned on with a blinking cue light) presented either in right or left corner of the front wall. The spatial location of NPM was counterbalanced between mice for each treatment. In the retention session, mice were reexposed 15 min to the chamber in the presence of the familiar NPM presented in the same corner, and two novel adjacent NPM (turned on with constant cue light) placed 8 cm apart in the opposite corner. Number of nose poking was monitored during the acquisition and retention sessions to assess baseline exploratory activity and recognition memory, respectively. Discrimination index (DI) was expressed by the ratio (100× total number of exploration of novel NPM) / (total number of exploration of all NPM)). A RI of 50% corresponds to chance level whereas a higher DI reflects a good recognition memory.

2.5. Western blotting

Animals were sacrificed by decapitation, striatal tissues were collected and homogenized in modified RIPA buffer (50 mM Tris, 1% SDS, 0.5 mM EDTA, 1% NP-40, 1% DOC) containing protease inhibitor cocktail (cOmplete™, Sigma Aldrich). 15 µg of total proteins were separated using 12% SDS-polyacrylamide gel and transferred to a 0.45 µm EMD Millipore Immobilon PVDF membrane. Membranes were blocked with blocking buffer (Invitrogen™ Molecular Probes™ WesternDot™ 625 Kit) for 1 h at RT and incubated overnight at 4 °C with rabbit anti-TH (1/6000, Synaptic Systems, 213,102), rabbit anti-ZnT3 (1/4000, Alamone, AZT-013), mouse anti-VGLuT1 (1/5000, Synaptic Systems, 135,011). Next day, membranes were incubated with mouse anti- β-actin (1/10000 or 1/20000, Sigma Aldrich, A5316) for 1 h at RT and procced further with WesternDot™ Kit. Densitometric analysis was performed using *E*-BOX CX5 imager and Vision Capt software (Vilber).

2.6. Histology

Mice were perfused transcardially with 4% paraformaldehyde and the brains were removed, postfixed, and cryoprotected. Free floating coronal sections (40 µm thick) containing substantia nigra pars compacta (SNc) were incubated with rabbit anti-TH antibody (1/1000, Synaptic Systems, 213102) overnight at 4 °C then 2 h with Alexa Fluor 594 goat anti-rabbit antibody (1/500, Jackson Laboratory, 111-585-003) at RT. Sections were mounted onto SuperFrost Plus glass slides (VWR) and coverslipped with Roti®-Mount FluorCare mounting medium (Carl Roth). Immunofluorescence was analyzed by a laser confocal microscopy (Zeiss LM710, Germany) at 20 X magnification. TH positive cells were counted through SNc region based on the stereotaxic mouse atlas (Paxinos and Franklin, 2001) delineating the SNc from the ventral tegmental area by the medial optic tract. Counting was done manually in four regions of interest (ROIs) per hemisphere, covering the whole medio-lateral extension of the structure (−2.80 to −3.28 mm relative to bregma, 3 sections per mouse), using cell counter plugin of FIJI software (ImageJ, National Institutes of Health).

Placements of the injection needle tips were examined in coronal striatal slices stained with DAB immunochemistry for TH-positive fibers and cresyl violet. Images were acquired using Leica DM LB Microscope at 2.5 X magnification equipped with Nikon digital camera DXM1200. Mice with injection needle placements outside of the striatum were excluded from analysis.

2.7. Statistical analysis

All data are expressed as mean group value ± standard error of the mean (SEM). Data were analyzed by one-way or two-way ANOVA, with 6-OHDA lesion and genotype or pharmacological treatment as the independent factors (GraphPad Prism 6.0). When relevant, Dunnett post-hoc test was conducted for individual comparisons. Komolgorov-Smirnov test was performed prior to ANOVA analysis to ensure that the assumption of normality was not violated. One sample Student *t*-test was used to compare discrimination index values to the chance level (50%). For partial bilateral DA depletion, mice with striatal TH loss ≤20% or ≥ 80% were excluded from the study. For all comparisons the significance criterion was $p < .05$.

3. Results

3.1. Nigrostriatal dopaminergic lesion upregulates striatal VGluT1 but not ZnT3 expression

The degeneration of the nigrostriatal dopaminergic pathway in PD patients and animal models triggers a complex compensatory remodeling of glutamatergic systems within the striatum, namely changes in the expression of the vesicular glutamate transporter 1 (VGluT1) protein, the presynaptic marker of corticostriatal glutamate afferents (Liguz-Lecznar and Skangiel-Kramska, 2007; Villalba et al., 2015). Yet, the impact of dopamine (DA) lesion on vesicular zinc transporter level has not been studied. We preformed partial and full unilateral intrastriatal 6-OHDA lesions in BL6 mice to examine whether striatal ZnT3 level varies depending on the severity of the denervation of nigrostriatal dopaminergic pathway. The vesicular zinc transporter ZnT3 is uniquely responsible for Zn^{2+} loading into synaptic vesicles and thereby serves as a selective molecular marker of zinc-positive fibers (Cole et al., 1999).

We assessed the magnitude of nigrostriatal dopaminergic lesions by analyzing tyrosine hydroxylase (TH) protein level in the striatum (Fig. 1A). As expected, a significant decrease of TH level was observed in the striatum ($F_{2,18} = 61.4, p < .05$). Partial 6-OHDA lesion reduced by 60% TH level ($p < .05$, Dunnett post-hoc test), while full 6-OHDA lesion produced a nearly complete TH loss (95% loss, $p < .05$, Dunnett post-hoc test). No change in striatal TH level was observed in the non-

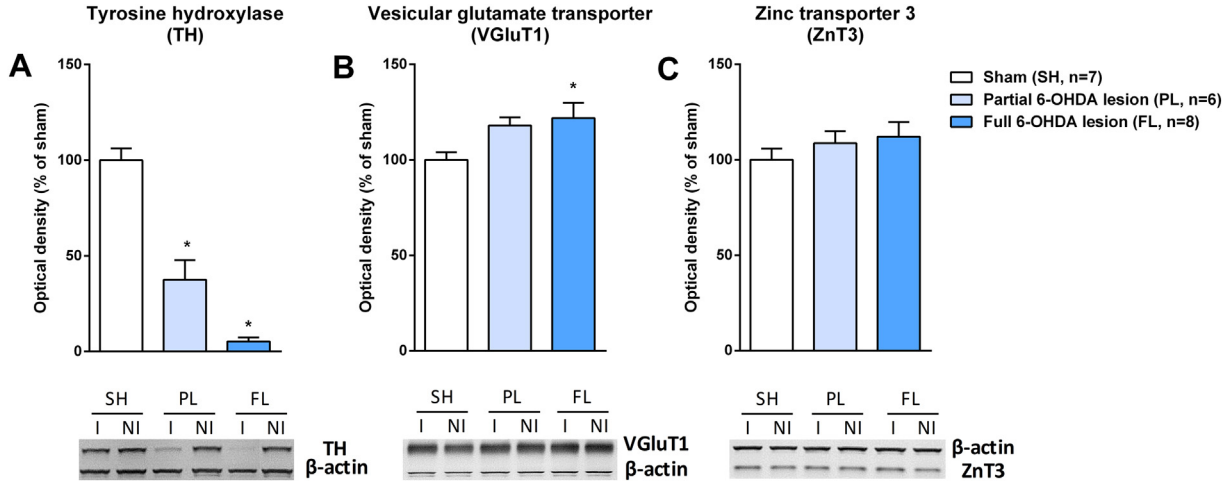


Fig. 1. Effects of nigrostriatal dopaminergic lesions on striatal VGluT1 and ZnT3 protein levels. (A) Upper panel depicts densitometric analysis of tyrosine hydroxylase (TH) protein expression in the striatum after partial and full unilateral intrastriatal 6-OHDA lesions. Lower panel depicts a representative blot of TH protein of injected (I) and non-injected (NI) striatal sides with β-actin as the reference. (B) Upper panel shows densitometric analysis of vesicular glutamate transporter 1 (VGlut1) protein expression upon partial and full 6-OHDA lesions. Lower panel shows a representative blot of VGlut1 protein of injected (I) and non-injected (NI) striatal sides with β-actin as the reference. (C) Upper panel depicts densitometric analysis of vesicular zinc transporter-3 (ZnT3) protein expression upon partial and full 6-OHDA lesions. Lower panel shows a representative blot of ZnT3 protein of injected (I) and non-injected (NI) striatal side with β-actin as the reference. Data expressed as mean % of change from corresponding Sham group ± SEM. *$p < .05$ *vs* Sham group, Dunnett's post-hoc test following a significant one-way ANOVA.

injected side after partial or full 6-OHDA lesion (data not shown).

In accordance with previous studies (Kashani et al., 2007; Massie et al., 2010; Raju et al., 2008), nigrostriatal DA denervation increased the total amount of VGluT1 protein in the striatum ($F_{2,18} = 3.68$, $p < .05$, Fig. 1B) and a significant effect was obtained with full 6-OHDA lesion ($p < .05$, Dunnett post-hoc). By contrast, no significant changes in total amount of striatal ZnT3 protein were observed following partial or full 6-OHDA lesion ($F_{2,18} = 0.86$, $p > .05$, Fig. 1C).

3.2. Vesicular zinc mediates locomotor deficits but not neurotoxic effects of 6-OHDA

The precise pathophysiological role of synaptic Zn^{2+} in PD is difficult to predict because extracellular Zn^{2+} interacts with numerous synaptic targets (Vogt et al., 2000; Kodirov et al., 2006; Paoletti et al., 2009; Sensi et al., 2011; Vergnano et al., 2014; Anderson et al., 2017) and can also translocate into injured dopaminergic axon terminals and contribute to neurotoxicity (Sensi et al., 2011). To address these issues, ZnT3 KO mice and their wildtype (WT) littermates were subjected to a partial unilateral intrastriatal 6-OHDA lesion. Partial 6-OHDA lesion model produces a moderate striatal DA depletion and mild motor impairments in mice, which makes it is particularly suitable for revealing potential beneficial or deleterious effects of vesicular Zn^{2+} elimination. As expected, lesioned WT mice displayed motor deficits ($F_{1,26} \geq 10.52$, $p < .05$ for Fig. 2A and B) that were reflected by a significant decrease in spontaneous locomotor activity ($p < .05$, Dunnett post-hoc test, Fig. 2A) and rearing behavior ($p < .05$, Dunnett post-hoc test, Fig. 2B) compared to sham counterparts. Sham ZnT3 KO mice had a normal locomotor phenotype (Fig. 2A and B) indicating that vesicular Zn^{2+} elimination did not alter motor behavior, in agreement with previous studies (Cole et al., 1999). Interestingly, a striking motor improvement was observed in lesioned ZnT3 KO mice. Two-way ANOVA revealed a significant effect of genotype ($F_{1,26} \geq 4.63$, $p < .05$, Fig. 2A and B) and post-hoc analysis confirmed the lack of effect of 6-OHDA lesion on ZnT3 KO mice ($p > .05$, Dunnett post-hoc test, Fig. 2A and B). We then examined the susceptibility of ZnT3 KO mice to neurotoxic effects of 6-OHDA by analyzing changes in total amount of TH in the striatum. Sham ZnT3 KO mice had a normal baseline TH level in the striatum ($F_{1,26} = 0.10$, $p > .05$, Fig. 2C). Lesioned WT and ZnT3 KO mice showed a significant loss of striatal TH ($F_{1,26} = 64.86$, $p < .05$), but no difference was observed between the two genotypes ($p < .05$, Dunnett post-hoc test). No changes in striatal TH level were detected in non-injected side after 6-OHDA lesion and/or vesicular Zn^{2+} depletion ($p > .05$, Two-way ANOVA, data not shown). These results show that ZnT3 KO mice are more resistant to motor deficits than neurotoxic effects of 6-OHDA.

The above findings suggest that synaptically released Zn^{2+} facilitates expression of locomotor deficits by altering synaptic transmission rather than by promoting 6-OHDA neurotoxicity. To test this hypothesis, we examined whether acute chelation of extracellular Zn^{2+} directly in the striatum could improve locomotor deficits of DA lesion. To do so, we used the classical 6-OHDA lesion model of PD, which consists of full unilateral denervation of the nigrostriatal DA pathway. This intrastriatal 6-OHDA lesion model produces severe motor impairments and is therefore complementary to the partial unilateral 6-OHDA lesion model. As can be seen in Fig. 3A, intrastriatal infusion of the extracellular Zn^{2+} chelator CaEDTA had no effect by itself on baseline locomotor activity, but improved locomotor deficit induced by 6-OHDA lesion. Two-way ANOVA showed a significant lesion x treatment interaction ($F_{1,23} = 4.74$, $p < .05$) and post-hoc analysis indicated that only 6-OHDA/NaCl group had a significantly lower locomotor activity level compared to Sham/NaCl group ($p < .05$, Dunnett post-hoc test, Fig. 3A). Full 6-OHDA lesion also severely impaired rearing behavior ($F_{1,23} = 12.84$, $p < .05$), but CaEDTA treatment had no beneficial effect on this behavioral deficit ($p < .05$ *vs* Sham/NaCl group, Dunnett post-hoc test, Fig. 3B). Immunohistochemical staining of TH in the striatum confirmed complete loss of striatal dopaminergic fibers (Fig. 3C) and quantitative analysis of TH immunofluorescence in the SNc (Fig. 3D) revealed 85% and 90% loss of TH positive cells in 6-OHDA/NaCl and 6-OHDA/CaEDTA groups, respectively ($F_{1,23} = 236.60$, $p < .05$, Fig. 3E). Collectively, these findings show that blockade of synaptic Zn^{2+} action, using genetic and pharmacological means, improves locomotor deficits in 6-OHDA lesioned mice.

3.3. Vesicular zinc mediates memory deficits but not neurotoxic effects of 6-OHDA

PD patients also suffer from a range of non-motor symptoms, such as cognitive impairments, that often appear in the early stages of the disease (Das et al., 2019; Davidson et al., 2006; Whittington et al.,

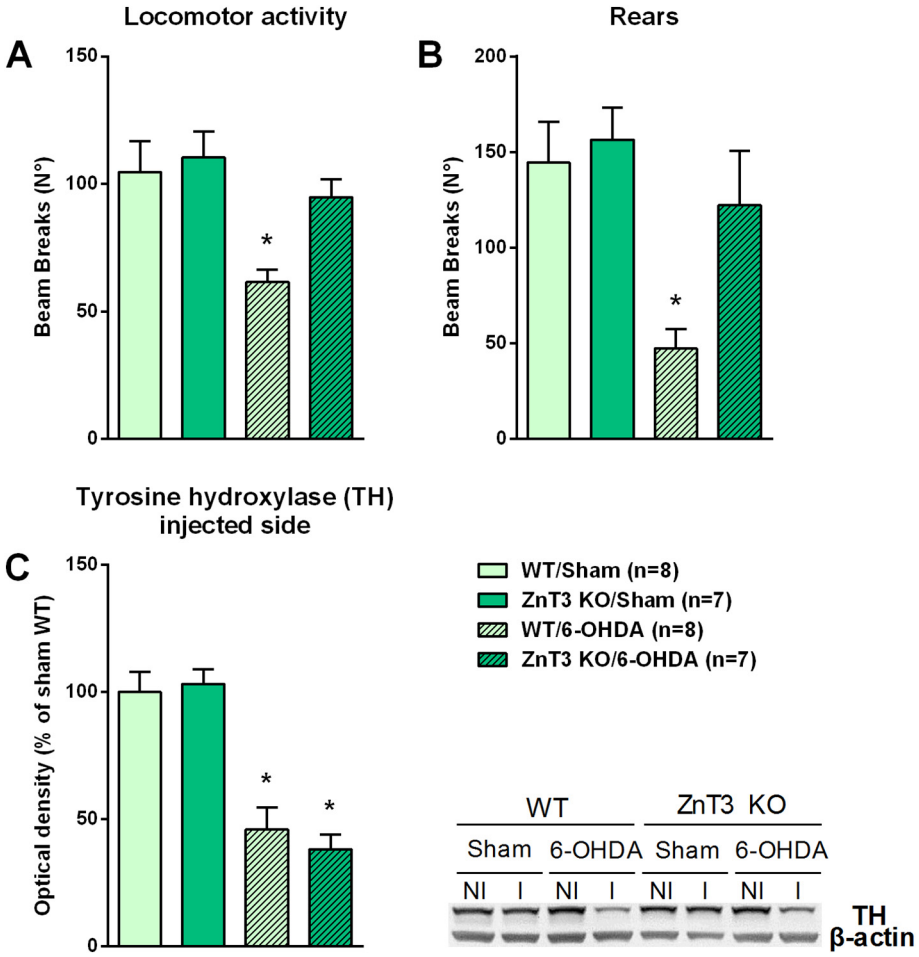


Fig. 2. Effects of vesicular zinc elimination on 6-OHDA-induced locomotor deficits and neurotoxicity. (A) Locomotor activity and (B) rearing behavior of ZnT3 KO and WT mice subjected to partial unilateral intrastriatal 6-OHDA lesion. Corresponding control groups, Sham/ZnT3 KO and Sham/WT mice, received intrastriatal injection of vehicle. Mice were tested for 1 h in actimetry cages. (C) Left panel depicts densitometric analysis of striatal TH level of injected side. Right panel shows representative blots of TH protein of injected (I) and non-injected (NI) striatum from lesioned WT and ZnT3 KO mice and their corresponding control groups. Data expressed as mean ± SEM, *$p < .05$ *vs* Sham/WT group, Dunnett's post-hoc test following a significant two-way ANOVA.

2006). Cognitive symptoms of PD have been largely attributed to dysfunction of the corticostriatal glutamatergic circuits. Yet, the contribution of synaptic Zn^{2+} to memory decline in the context of PD has not been investigated. To tackle this issue, we used a partial bilateral intrastriatal 6-OHDA lesion model and a recently developed automated recognition memory task for mice (Reiss et al., 2014), as deficits in different aspects of recognition memory are common in newly diagnosed as well as advanced-stage PD patients (Whittington et al., 2006; Papagno and Trojano, 2018). Because ZnT3 KO mice exhibit memory impairments with aging (Adlard et al., 2010; Sindreu and Storm, 2011), we first verified whether recognition memory was intact in young adult ZnT3 KO mice (4 months old). During the acquisition session, ZnT3 KO mice displayed a normal exploratory behavior, as reflected by number of nose pokes (WT mice: 26.9 ± 1.9 and ZnT3 KO mice: 26.8 ± 4.0, $p > .05$, Student's *t*-test). When submitted 24 h later to the retention session, ZnT3 KO and WT mice displayed good discrimination performances (WT mice: 69.9 ± 5.4 and ZnT3 KO mice: 70.6 ± 7.1 $p < .05$ *vs* chance level, One-sample Student t-test) and no difference was detected between the two genotypes ($p > .05$, Student's t-test).

Having confirmed that ZnT3 KO mice have intact recognition memory, we then performed partial bilateral 6-OHDA lesion on naive batch of mice of the same age to study the effect of DA lesion on learning/memory performance. As illustrated in Fig. 4A, Sham ZnT3 KO mice showed good discrimination scores comparable to Sham WT mice ($p < .05$ *vs* chance level, One-sample Student's t-test, Fig. 4A), consistent with previous findings. Lesioned WT mice had a poor discrimination performance ($p > .05$, *vs* chance level, One-sample Student's t-test), while lesioned ZnT3 KO mice performed significantly better, scoring above chance level ($p < .05$, One-sample Student's t-test, Fig. 4A), as Sham mice. Two-way ANOVA showed a significant lesion x genotype interaction ($F_{1,36} = 6.56$, $p < .05$) and post-hoc analysis confirmed that only lesioned WT mice displayed a significant memory impairment compared to Sham WT mice ($p < .05$, Dunnett post-hoc test). During the acquisition session, partial bilateral 6-OHDA lesion had no effect on exploratory behavior ($F_{1,36} = 1.47$, $p > .05$, Fig. 4B). Sham ZnT3 KO mice displayed a lower exploration level compared to Sham WT mice ($p < .05$, Dunnett post-hoc test), but this locomotor phenotype was not observed in lesioned ZnT3 KO mice ($p < .05$, Dunnett post-hoc test, Fig. 4B) or in the previous experiment (data above). Finally, we studied the susceptibility of ZnT3 KO and WT mice to neurotoxic effects of 6-OHDA by analyzing changes of striatal TH level (Fig. 4C). Bilateral partial 6-OHDA lesion produced a significant TH loss in the striatum ($F_{1,36} = 61.79$, $p < .05$) that was comparable between ZnT3 KO and WT mice ($p < .05$, Dunnett post-hoc). Overall, the above findings show that ZnT3 KO mice display strong resistance to memory deficit but not to neurotoxic effects induced by 6-OHDA.

3.4. Disruption of the zinc-GluN2A interaction does not impact locomotor deficits and neurotoxic effects of 6-OHDA

The above findings indicate that synaptically released Zn^{2+} contributes to behavioral deficits of 6-OHDA lesion by altering synaptic transmission rather than promoting neurotoxicity. To determine whether detrimental action of Zn^{2+} involves NMDAR-dependent mechanisms we used knock-in (KI) mice carrying a point mutation (histidine to serine) at position 128 in the high-affinity (nanomolar) Zn^{2+} binding site of the GluN2A subunit (GluN2A-H128S mutation). We previously showed that this mutation selectively eliminates zinc inhibition of GluN2A-NMDARs and completely abolishes analgesia induced by

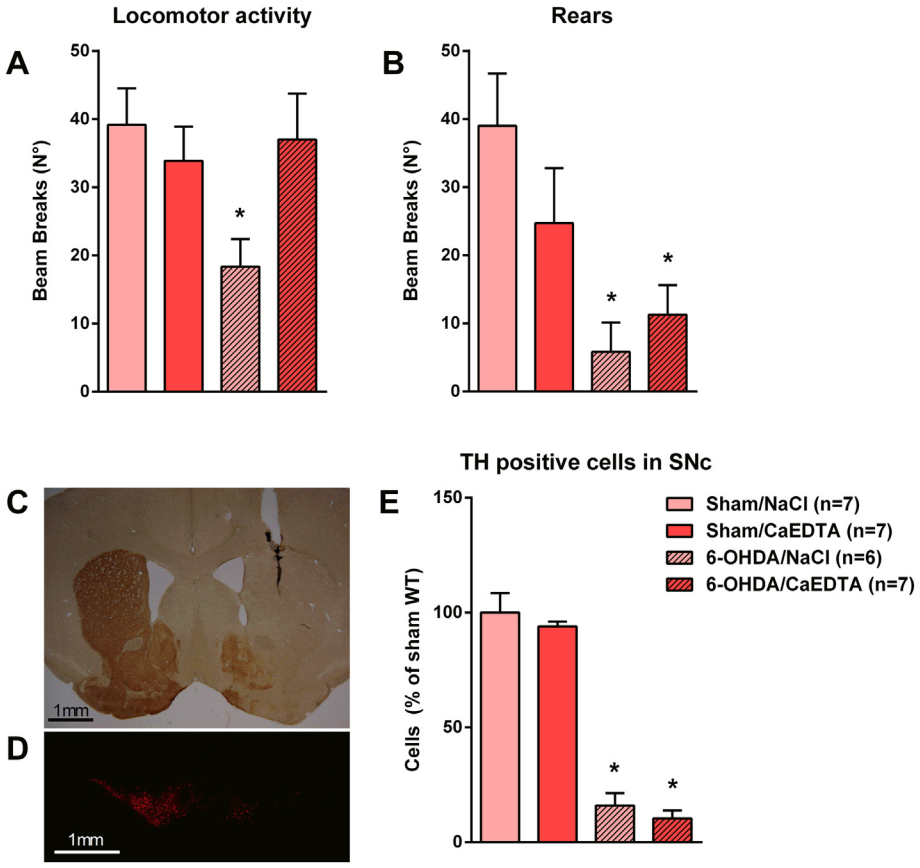


Fig. 3. Effect of intrastriatal chelation of extracellular zinc on 6-OHDA-induced locomotor deficits. (A) Locomotor activity and (B) rearing behavior of BL6J mice subjected to full unilateral intrastriatal 6-OHDA lesion. Mice received CaEDTA (Sham/CaEDTA and 6-OHDA/CAEDTA) or vehicle (Sham/NaCl and 6-OHDA/NaCl) injection into dorsal striatum and tested 15 min later in the actimetry cages for 10 min. (C) Representative coronal section showing loss of TH-positive fibers and injector needle placement in the dorsal striatum. Scale bar: 1 mm. (D) Representative coronal immunofluorescence image showing TH-positive cells in the non-injected side and severe degeneration in lesioned side of the substantia nigra pars compacta (SNc). Scale: 1 mm. (E) Quantification of TH-positive cells in lesioned side of the SNc. Data expressed as mean ± SEM, *$p < .05$ *vs* Sham/NaCl group, Dunnett's post-hoc test following a significant two-way ANOVA.

exogenous Zn^{2+} under acute and chronic pain conditions (Nozaki et al., 2011; Vergnano et al., 2014). We first studied the susceptibility of GluN2A-H128S KI and WT mice to motor deficits induced by partial unilateral intrastriatal 6-OHDA lesion, as performed with ZnT3 KO mice. Sham GluN2A-H128S KI mice displayed a normal locomotor phenotype compared to Sham WT mice ($F_{1,22} = 0.15$, $p > .05$, Fig. 5A and B). Partial unilateral 6-OHDA lesion significantly reduced locomotor activity (Fig. 5A) and rearing behavior (Fig. 5B) in WT mice ($p < .05$, Dunnett post-hoc test, $F_{1,22} \geq = 8.47$, $p < .05$ for both measures), and similar deficits were obtained in GluN2A-H128S KI mice ($p < .05$, Dunnett post-hoc test). We next examined whether GluN2A-H128S mutation changes the susceptibility to 6-OHDA neurotoxicity. Immunoblot analysis of TH in the striatum showed a normal baseline level in Sham GluN2A-H128S KI mice ($F_{1,22} = 0.68$, $p > .05$, Fig. 5C). Partial 6-OHDA lesion significantly reduced striatal TH level in WT and GluN2A-H128S KI mice ($F_{1,22} = 37.11$, $p < .05$), and no difference was detected between genotypes ($p < .05$ *vs* Sham WT, Dunnett post-hoc, Fig. 5C). No change in striatal TH level was detected in non-injected side (data not shown).

4. Discussion

Histochemical studies revealed that the striatum contains large amounts of chelatable Zn^{2+} packed into synaptic vesicles of glutamatergic axon terminals (Danscher and Stoltenberg, 2005; Frederickson et al., 2000). Zinc-positive glutamatergic afferents onto striatum originate largely from different cortical areas. In contrast, thalamo-striatal glutamatergic inputs are devoid of synaptic Zn^{2+} (Frederickson et al., 2000; Sorensen et al., 1995). Likewise, no vesicular Zn^{2+} staining is visible in other basal ganglia nuclei outside the striatum (Danscher and Stoltenberg, 2005; Frederickson et al., 2000). The high vesicular Zn^{2+} content in the striatum, the main relay structure between neocortex and basal ganglia, raises intriguing questions about the functional role of synaptically released Zn^{2+} in normal and pathological conditions. The current study shows that Zn^{2+} elimination from synaptic vesicles or extracellular Zn^{2+} chelation in the striatum prevents locomotor deficits but not neurotoxic effects of 6-OHDA. Elimination of synaptic Zn^{2+} was also effective in preventing memory impairment induced by DA lesion. Collectively, these findings demonstrate that synaptically released Zn^{2+} acts as an endogenous neuromodulator critically implicated into motor and non-motor deficits induced by nigrostriatal DA denervation.

Corticostriatal glutamatergic systems undergo complex structural and functional changes upon degeneration of midbrain DA neurons (Liguz-Lecznar and Skangiel-Kramska, 2007; Villalba et al., 2015). Accordingly, we found that unilateral lesion of nigrostriatal dopaminergic pathway increases the expression of striatal VGluT1 protein, the presynaptic marker of corticostriatal glutamate afferents (Liguz-Lecznar and Skangiel-Kramska, 2007). Significant VGluT1 upregulation was detected upon full but not partial 6-OHDA lesion indicating that striatal VGlut1 level varies as function of the severity of the nigrostriatal DA pathway denervation. These findings corroborate those reported in PD patients and animal models showing high VGluT1 levels in the striatum (Kashani et al., 2007; Massie et al., 2010; Raju et al., 2008). They further suggest that vesicular glutamate storage may undergo dynamic regulation during the progression of PD depending on endogenous DA levels. Unlike VGluT1, we show that the expression of striatal ZnT3, the marker of zinc-containing glutamatergic terminals (Cole et al., 1999), remains unchanged upon 6-OHDA lesions. Consistent with this observation, Rojas et al., (2005) showed that DA lesion has a limited impact on striatal synaptic Zn^{2+} content in rats. They found that synaptic vesicular Zn^{2+} staining in the striatum was reduced immediately (1 and 24 h) but not 7 days after intracerebroventricular administration of the Parkinsonian mimetic MPP^+. The dissociation between the effect of DA lesion on striatal VGluT1 and ZnT3 levels is intriguing and suggests that regulation of vesicular glutamate and Zn^{2+} storage may be uncoupled in the context of PD. The upregulation of VGluT1 may lead

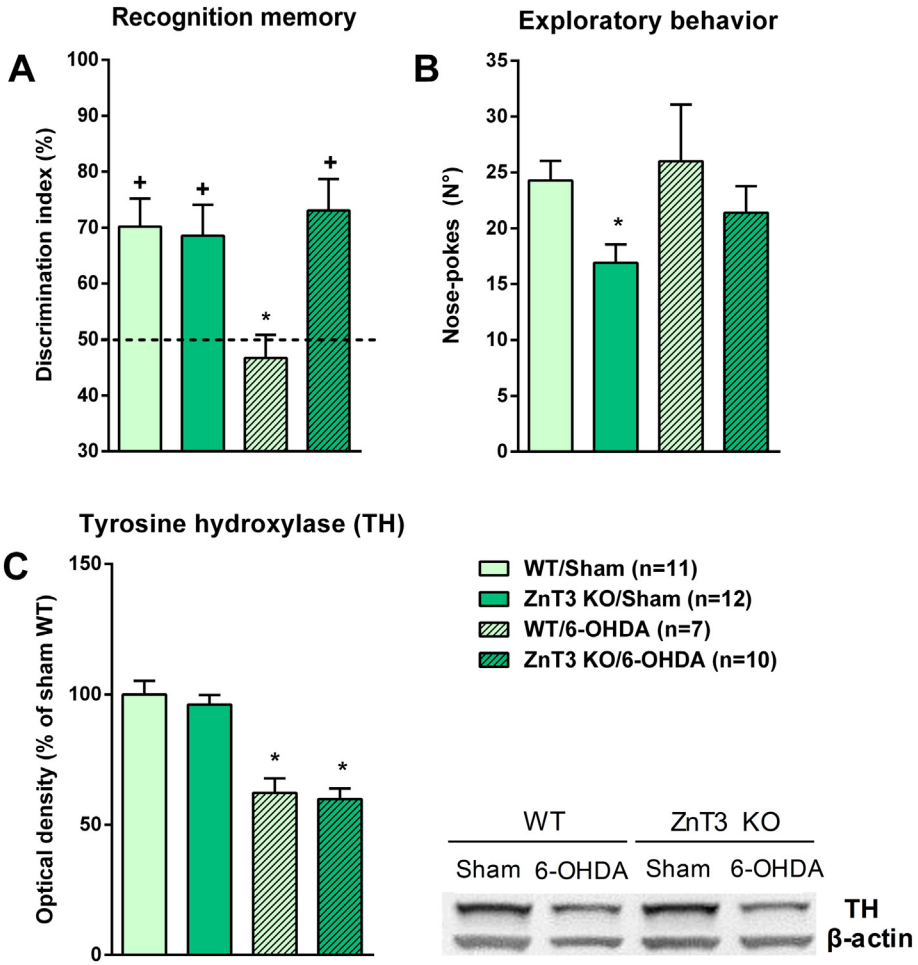


Fig. 4. Effects of vesicular zinc elimination on 6-OHDA–induced memory deficit and neurotoxicity. (A) Recognition memory performances of ZnT3 KO and WT mice subjected to partial bilateral intrastriatal 6-OHDA lesion. Corresponding control groups, Sham/ZnT3 KO and Sham/WT mice, received intrastriatal injection of vehicle. Mice were submitted to 30 min acquisition session in the presence of blinking nose-poke modules (NPKMs) and preference for novel (non-blinking) over familiar NPKMs was assessed the following day during 15 min retention session. The dashed line materializes the chance level of 50% indicating equal exploration of both NPKMs. (B) Baseline exploratory activity assessed by monitoring the number of spontaneous nose-pokes in the acquisition session. (C) Left panel depicts densitometric analysis of striatal TH level from both striata pooled together. Right panel shows representative blots of TH protein from lesioned WT and ZnT3 KO mice and their corresponding control groups. Data expressed as mean ± SEM, $*p < .05$ *vs* chance level, One-sample *t*-test. $*p < .05$ *vs* Sham/WT group, Dunnett's post-hoc test following a significant two-way ANOVA.

to increased glutamate loading in synaptic vesicles and excess glutamate release, thereby contributing to the deleterious overactivity of corticostriatal glutamatergic systems (Calabresi et al., 2000; Dzahini et al., 2010; Robinson et al., 2003). However, it cannot be excluded that VGluT1 overexpression may also reflect increased synaptogenesis in the striatum. Further studies are needed to clarify this issue and determine whether VGluT1 upregulation occurs in zinc-positive terminals or is restricted to zinc-negative ones.

Our results reveal that elimination of synaptic Zn^{2+} strongly diminish motor and cognitive deficits triggered by DA lesion. On the other hand, it did not afford neuroprotection against 6-OHDA neurotoxicity. Analysis of TH protein expression in the striatum revealed a comparable dopaminergic axonal degeneration in ZnT3 KO and WT mice subjected either to partial unilateral or bilateral 6-OHDA lesion. The later finding was somewhat unexpected given the well-known neuronal cytotoxicity of labile Zn^{2+} and the bulk of studies implicating zinc dysfunctions in cell death of dopaminergic neurons in PD (see Introduction). For instance, Zn^{2+} injected into the striatum alone or in combination with dopamine causes degeneration of nigrostriatal DA pathway (Lo et al., 2004). Zn^{2+} infused into the striatum was also demonstrated to undergo retrograde transport to the substantia nigra (Takeda et al., 1998), raising the possibility that synaptically released Zn^{2+} from corticostriatal synapses might translocate into injured dopaminergic terminals and participate to the ongoing axonal degeneration. The absence of neuroprotective phenotype in lesioned ZnT3 mice rules out this possibility and indicates that extracellular concentrations of synaptically released Zn^{2+} do not reach neurotoxic levels within the striatum to promote further 6-OHDA neurotoxicity. Studies on animal models of PD showed that cytosolic accumulation of neurotoxic Zn^{2+} in DA neurons is a key component of the pathogenic events leading to neuronal degeneration (Lee et al., 2009; Sheline et al., 2012; Tamano et al., 2018a, 2018b). Our findings suggest that Zn^{2+} released from intracellular stores (lysosomes, mitochondria and metal-binding proteins) rather than influx of synaptically released zinc may be the main source of neurotoxic Zn^{2+} underlying dopamine cell death.

The fact that ZnT3 KO mice displayed resistance to motor and cognitive deficits but not to neurotoxic effects of 6-OHDA suggests that synaptically released Zn^{2+} in the striatum mediates behavioral deficits caused by DA depletion. Accordingly, acute infusion of the extracellular Zn^{2+} chelator CaEDTA into the striatum restored locomotor deficit induced by full unilateral 6-OHDA lesion. At the dose tested, CaEDTA was less effective on rearing deficit, likely because of the drastic effect of the complete DA depletion. On the whole, these findings extend those reported with exogenous zinc ($ZnCl_2$) showing that elevated extracellular Zn^{2+} level in the striatum impairs locomotor behavior in rats (Yakimovskii, 2012; Yakimovskii and Stepanov, 2011). They also provide the first direct evidence that endogenous striatal Zn^{2+} released at synaptic sites negatively modulates motor function under PD conditions. The lack of effect of CaEDTA by itself on baseline locomotor activity and the normal locomotor phenotype of ZnT3 KO mice indicate that the detrimental modulatory action of synaptic Zn^{2+} on motor behavior requires DA lesion. Such conditional involvement of endogenous synaptic Zn^{2+} signaling may likely occur through multiple mechanisms. Enhanced vesicular Zn^{2+} content/release seems however less likely because no upregulation of striatal ZnT3 level or neuroprotection could be detected in lesioned Bl6 mice and ZnT3 KO mice, respectively. The negative findings obtained upon elimination of Zn^{2+} action on GluN2A-NMDARs, the prime target of synaptic Zn^{2+} (Chen

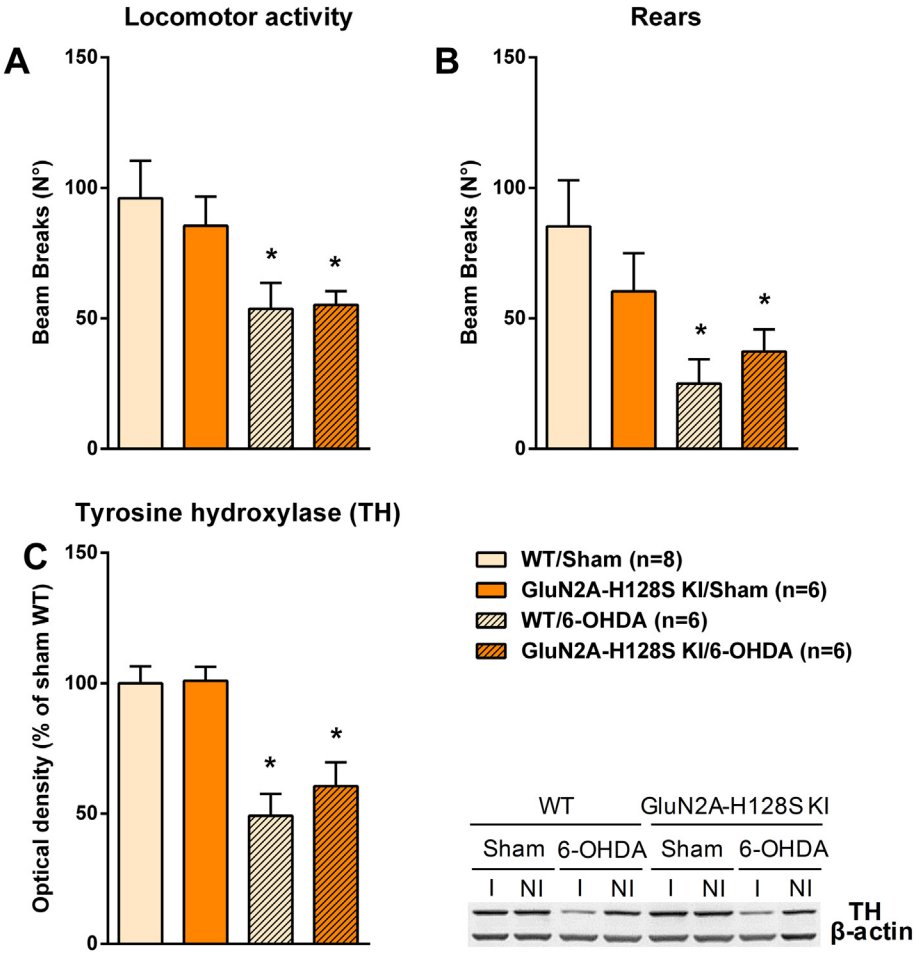


Fig. 5. Effects of disruption of Zinc-GluN2A interaction on 6-OHDA-induced locomotor deficits and neurotoxicity. (A) Locomotor activity and (B) rearing behavior of GluN2A-H128S KI and WT mice subjected to partial unilateral intrastriatal 6-OHDA lesion. Corresponding control groups, GluN2A-H128S KI and Sham/WT mice, received intrastriatal injection of vehicle. Mice were tested for 1 h in actimetry cages. (C) Left panel depicts densitometric analysis of striatal TH level of injected side. Right panel shows representative blots of TH protein of injected (I) and non-injected (NI) striatum from lesioned WT and GluN2A-H128S KI mice and their corresponding control groups. Data expressed as mean ± SEM, $^{*}p < .05$ *vs* Sham/WT group, Dunnett's post-hoc test following a significant two-way ANOVA.

et al., 1997; Low et al., 2000; Paoletti et al., 2000, 1997; Vergnano et al., 2014) also argue in the same direction. As demonstrated by numerous studies, DA lesion produces widespread compensatory changes within the striatum, namely an increase in the expression of synaptic receptors and their sensitivity for neurotransmitters (Villalba et al., 2015; Zhai et al., 2018). Thus, one conceivable mechanism could be an increased sensitivity of post-synaptic proteins to Zn^{2+} action, since zinc can directly interact with a myriad of synaptic targets, including voltage-gated ion channels, neurotransmitter receptors and transporters (Vogt et al., 2000; Kodirov et al., 2006; Paoletti et al., 2009; Sensi et al., 2011; Vergnano et al., 2014; Anderson et al., 2017).

The partial bilateral 6-OHDA lesion that we used is a well-established model in rodents for studying cognitive and emotional deficits associated to early stages of PD (Eagle et al., 2015; Leonibus et al., 2009, 2007; Masini et al., 2017; Rane et al., 2012; Ztaou et al., 2018). Unlike unilateral DA lesion, it does not produce locomotor asymmetry (spontaneous circling behavior), and thereby it has a minor impact on locomotor and exploratory behavior. Accordingly, the recognition memory impairment exhibited by lesioned mice was independent of any decrease in exploratory activity. Likewise, the rescue of recognition memory deficits observed in lesioned ZnT3 KO mice was not accompanied by changes in exploratory behavior, thus reflecting a genuine beneficial effect of synaptic Zn^{2+} elimination on learning/memory performance. Early studies using ZnT3 KO mice and chelating agents demonstrated that vesicular Zn^{2+} is quite essential for normal cognitive behavior (Adlard et al., 2010; Daumas et al., 2004; Lassalle et al., 2000; Martel et al., 2010; Sindreu et al., 2011; Sindreu and Storm, 2011; Takeda et al., 2010). Such important physiological role was consistently shown to be subserved by synaptically released Zn^{2+} in limbic system, especially in the hippocampus and the amygdala where vesicular Zn^{2+} is particularly abundant. Here, we reveal a detrimental role of striatal synaptic Zn^{2+} within the basal ganglia system in mediating memory deficit associated to DA deficiency. As for locomotor behavior, the inhibitory influence of Zn^{2+} on learning/memory performance could only be revealed upon DA lesion, likely due to the compensatory synaptic changes that take place within the striatum. In the present study we did not assessed the impact of 6-OHDA lesion on the content of noradrenaline and serotonin, which have been also implicated in non-motor symptoms associated to PD. However, previous studies using similar partial bilateral 6-OHDA lesion procedure in mice showed either no or a marginal loss in these two neurotransmitters (Bonito-Oliva et al., 2014b, 2014a; Branchi et al., 2010; Delaville et al., 2011). Furthermore, the recognition memory impairments and emotional deficits induced by the partial bilateral 6-OHDA lesion has been shown to be caused by dopamine loss (Bonito-Oliva et al. 2014a and 2014b). However, it cannot be excluded that deleterious action of synaptic Zn^{2+} may involve noradrenergic and/or serotoninergic mechanisms.

Deciphering how synaptic Zn^{2+} contributes to behavioral deficits induced by 6-OHDA lesion is a challenging task owing to the lack of pharmacological tools able to selectively interfere with its synaptic targets. Synaptically released Zn^{2+} within the striatum may exert both inhibitory and disinhibitory modulatory actions on motor and cognitive functions depending on the synaptic targets and their localizations, even though the net behavioral effect is mainly detrimental. NMDA receptors (NMDARs) are one of the best characterized targets of synaptic zinc (Paoletti et al., 2009; Vergnano et al., 2014) and have been incriminated in development of behavioral deficits and neurotoxicity in PD. To examine how disruption of Zn^{2+} action on NMDARs impacts locomotor deficits and neurotoxic effects of 6-OHDA we used GluN2A-H128S KI mice that specifically lack the high-affinity (nanomolar) zinc

inhibition of GluN2A-NMDARs (Nozaki et al., 2011). Unlike complete elimination of synaptic Zn^{2+} that prevented locomotor deficits of partial unilateral DA lesion, disruption of Zn^{2+} action on GluN2A-NMDARs had no notable consequence in lesioned mice. The loss of striatal dopaminergic fibers was also similar between GluN2A-H128S KI and WT mice indicating that the mutation did not change the susceptibility of mice to 6-OHDA neurotoxicity. The lack of phenotype in lesioned GluN2A-H128S KI may be due to compensatory mechanisms that may operate upon the loss of Zn^{2+} inhibition to maintain normal GluN2A-NMDAR function. However, behavioral phenotyping of GluN2A-H128S KI mice revealed improved learning/memory abilities in a range of cognitive tasks (contextual fear conditioning, spatial memory and recognition memory paradigms; data not shown) confirming that synaptic Zn^{2+} inhibits mnemonic function by dampening NMDAR activity. Among glutamatergic receptors, GluN2A-NMDARs stand out for their exquisite zinc sensitivity (Chen et al., 1997; Low et al., 2000; Paoletti et al., 2000, 1997). The fact that abrogation of Zn^{2+} action on these receptors had no impact on expression of locomotor deficits and neurotoxic effects of 6-OHDA is very intriguing. It implies that under pathological conditions, such as those associated to PD, vesicular Zn^{2+} released alongside glutamate may act through glutamatergic-independent mechanisms to promote motor deficits. Such unexpected detrimental action of synaptic Zn^{2+} may emerge as consequence of the structural and functional compensatory changes that take place upon DA lesion. Other potential neurotransmitter systems that could likely relay the detrimental influences of synaptic Zn^{2+} in the context of DA lesion could be GABAergic and cholinergic systems. In striatal slices, Zn^{2+} has been shown to disinhibit cholinergic interneurons (CINs) by blocking postsynaptic $GABA_A$ currents (Yan and Surmeier, 1997). Zn^{2+} also enhances the excitability of CINs by blocking leak K^+ channels (TASK-3, Berg and Bayliss, 2007). Such combined Zn^{2+} actions on CINs might result in enhanced striatal cholinergic transmission, which ultimately could promote both motor and memory deficits of DA lesion. In support of this idea, studies from our laboratory showed that optogenetic inhibition of CINs could restore motor deficits induced by full unilateral 6-OHDA lesion (Maurice et al., 2015) as well as recognition memory impairment caused by partial bilateral 6-OHDA lesion in mice (Ztaou et al., 2018). Series of studies are therefore needed to identify which synaptic targets precisely mediate the actions of vesicular Zn^{2+} in the context of nigrostriatal DA lesion.

In conclusion, the present study provides the first empirical evidence implicating endogenous pools of striatal synaptic Zn^{2+} in the pathophysiology of PD. Excessive glutamatergic corticostriatal transmission has long been recognized for its contribution to development of PD symptoms and neurotoxicity leading to neuronal degeneration. Our findings suggest that Zn^{2+} released from corticostriatal terminals may play predominantly a deleterious role alongside glutamate by promoting motor and cognitive symptoms associated to the disease. Zinc chelation is currently considered as a potential therapeutic approach to neuroprotection in neurodegenerative disease such as Alzheimer disease and PD (Ayton et al., 2015; Stelmashook et al., 2014). Our findings suggest that such therapeutic strategy might prove also beneficial for symptomatic relief in patients with PD.

Declaration of Competing Interest

All authors report no biomedical financial interest or potential conflicts of interest.

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