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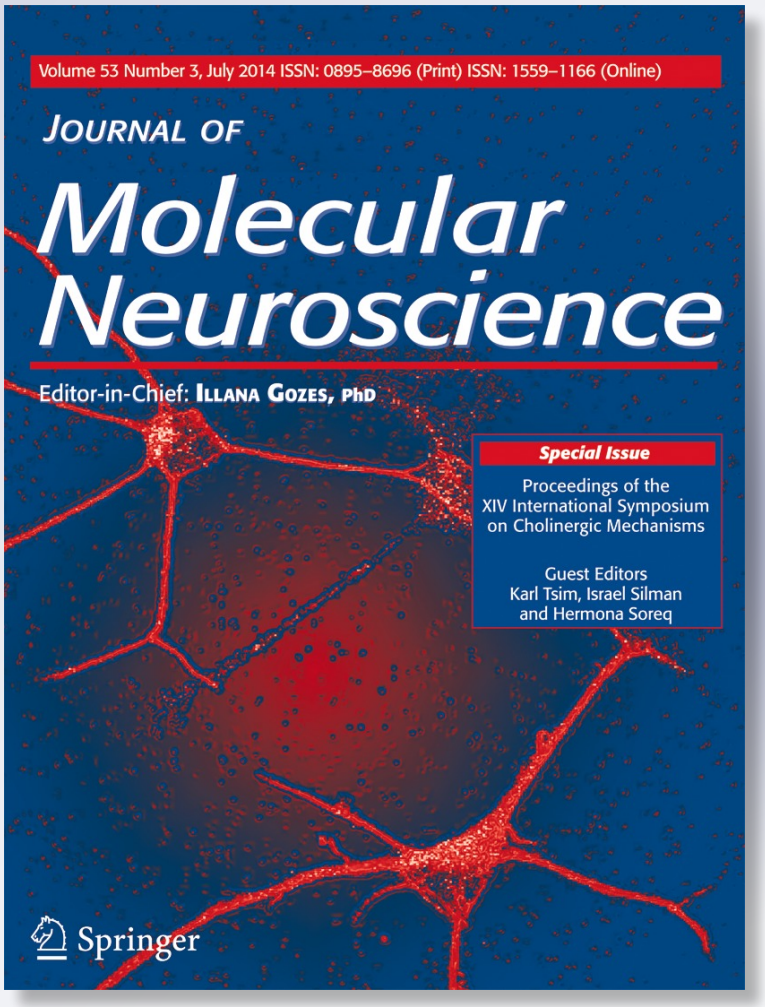

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lynx1 Supports Neuronal Health in the Mouse Dorsal Striatum During Aging: an Ultrastructural Investigation

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Abstract Nicotinic acetylcholine receptors have been shown to participate in neuroprotection in the aging brain. Lynx protein modulators dampen the activity of the cholinergic system through direct interaction with nicotinic receptors. Although lynx1 null mutant mice exhibit augmented learning and plasticity, they also exhibit macroscopic vacuolation in the dorsal striatum as they age, detectable at the optical microscope level. Despite the relevance of the lynx1 gene to brain function, little is known about the cellular ultrastructure of these age-related changes. In this study, we assessed degeneration in the dorsal striatum in 1-, 3-, 7-, and 13-month-old mice, using optical and transmission electron microscopy. We observed a loss of nerve fibers, a breakdown in nerve fiber bundles, and a loss of neuronal nuclei in the 13-month-old lynx1 null striatum. At higher magnification, these nerve fibers displayed intracellular vacuoles and disordered myelin sheaths. Few or none of these morphological alterations were present in younger lynx1 null mutant mice or in heterozygous lynx1 null mutant mice at any age. These data indicate that neuronal health can be maintained by titrating lynx1 dosage and that the lynx1 gene may participate in a trade-off between neuroprotection and augmented learning.

Keywords Nicotinic acetylcholine receptors · Cholinergic system · Prototoxins · Neurotoxins · Neurodegeneration · Plasticity · Learning and memory

Introduction

Throughout life the cholinergic system provides important control over a number of neurotransmitter systems that participate in a variety of brain functions (Miwa et al. 2011; Picciotto 2003). Cholinergic activation can have beneficial effects, promoting plasticity, learning (Levin et al. 2005; Picciotto et al. 1995), and neuroprotective mechanisms (Bordia et al. 2008; Quik et al. 2007; Ryan et al. 2001; Stevens et al. 2003). Overactivation of the cholinergic system has been linked to cell death (Orb et al. 2004; Orr-Urtreger et al. 2000), whereas, hypoactivity of the cholinergic system has been linked with memory impairments in conditions such as Alzheimer's disease (Albuquerque et al. 2009; Decker and McGaugh 1991; Terry and Buccafusco 2003). Epidemiological evidence supports an inverse correlation between smoking and neurodegenerative disorders such as Parkinson's disease, implying a further connection between nicotinic acetylcholine receptors and neurodegeneration; smoking seems to provide some neuroprotection against Parkinson's disease (Huang et al. 2007; Picciotto and Zoli 2008). Titrating the amount of cholinergic activation is therefore critical for normal brain function and for neuronal health.

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During normal aging, cholinergic neurons and specific nicotinic acetylcholine receptor subtypes are eliminated selectively or reduced (Albuquerque et al. 2009; Terry and Buccafusco 2003). This loss is thought to underlie cognitive decline, which is not a pathology, but part of the aging process (Picciotto 2003). In certain pathologies, such as Alzheimer's disease and other dementias, there is a selective loss of various nicotinic acetylcholine receptor subtypes (Albuquerque et al. 2009), cholinergic neurons (Decker and McGaugh 1991; Schliebs and Arendt 2011), or axons of cholinergic neurons (Geula et al. 2008), which all play a role in the clinical manifestation of these diseases. In neurodegenerative disorders, therapies using acetylcholinesterase inhibitors can augment the contribution of the cholinergic system in improving cognition (Terry and Buccafusco 2003). These therapies inhibit the enzyme that breaks down the neurotransmitter acetylcholine, acetylcholinesterase, and thus raise overall acetylcholine levels in the brain. Balancing this activity is critical because moderate activation of the cholinergic system can enhance cognitive functions, whereas overactivation can be detrimental (Picciotto 2003). Thus, this inverted U-shaped relationship between activity and function requires tight regulation (Miwa et al. 2011).

Nicotinic acetylcholine receptors are ligand-gated ion channels of the cholinergic system that have been implicated in neuronal degeneration (Terry and Buccafusco 2003) and survival (Dajas-Bailador et al. 2000; Kaneko et al. 1997; Stevens et al. 2003). Activation of these receptors leads to a rise in intracellular Ca^{2+} levels (Berger et al. 1998), partially because some subtypes allow Ca^{2+} to flux directly into the cell (Yamauchi et al. 2011). Animal studies indicate a neuroprotective effect of nicotine in mouse models of neurodegeneration (Ryan et al. 2001), suggesting a role of nicotinic acetylcholine receptors in neuroprotective mechanisms (Picciotto and Zoli 2008; Stevens et al. 2003). It is well known that regulation of the activity of the cholinergic system is important in protecting neurons as they age (Albuquerque et al. 2009; Terry and Buccafusco 2003).

Toxin-like modulators of the lynx family provide promising avenues for manipulation of cholinergic system activation levels. Lynx molecules are endogenous proteins that bind tightly to nicotinic acetylcholine receptors and modulate their function (Ibanez-Tallon et al. 2002; Miwa et al. 1999; Tekinay et al. 2009). Lynx1KO mice exhibit elevated nicotinic receptor responses, greater plasticity (Morishita et al. 2010), and increased learning and memory (Miwa et al. 2006). Our previous studies also showed that neurons from lynx1KO mice exhibit elevated intracellular Ca^{2+} levels and enhanced nicotine-evoked rises in Ca^{2+} (Miwa et al. 2006). Lynx1KO neurons, however, are also more susceptible to glutamate-mediated neurotoxicity, and nicotine neuroprotection in vitro is abolished. Optical microscopic examination revealed that homozygous lynx1KO mice at the age of 12 months and older display vacuoles in the striatum and brain stem. This vacuolation can be ameliorated by crossing lynx1KO mice to $\alpha 7$ or $\beta 2$ null mutant mice, indicating that nicotinic acetylcholine receptor subunits are downstream effectors of lynx1 signaling.

Despite the link between levels of nicotinic receptor activity and neuron vulnerability, the role of lynx1 in this vacuolating degenerative process is unknown. To date, no detailed examination of the pathological features in the lynx1 mouse model has been undertaken. We therefore investigated the degeneration in the dorsal striatum as a function of age using optical and transmission electron microscope (TEM) ultrastructural analyses. Using TEM, we found two types of degeneration occurring in the dorsal striatum. One occurs at the level of fascicle disorganization, with a loss of nerve fibers and a loss of neuronal cell bodies. The other is an internal degeneration, which appears to be a degenerative change in the axoplasm of axon fibers. The fascicles were compact with distinct boundaries in wild-type brains but were indistinct with fuzzy margins in lynx1KO brains. When we examined axon and nerve fibers in cross section within the fascicles using TEM, we discovered unusual morphological alterations, such as disordered myelin sheaths, axoplasmic anomalies, and intracellular vacuoles.

We have reported that lynx mRNA levels can be regulated by environmental and other manipulations (Miwa et al. 2011). This opens up the possibility that the brain may be subjected to altered amounts of susceptibility to neuronal damage. To understand the role of lynx1 dosage in cholinergic activity, we compared the effect of partial removal of lynx1, exhibited in heterozygous mice, to complete removal of lynx1 as exists in homozygous knockout mice.

The lynx1KO animals were largely normal during development and into early adulthood. We found that lynx1KO animals over 1 year of age have clear ultrastructural differences in the striatum, but this is largely spared in the heterozygous mice. This age-related degenerative phenotype in lynx1KO mice implicates lynx1 in neuronal health during aging.

Materials and Methods

Animals lynx1KO mice were maintained as a backcross on a C57BL/6 (Charles River) background for >8 generations. Heterozygous lynx1KO mice were intercrossed to derive a mixture of wild-type, heterozygous, and homozygous lynx1KO mice. Lynx1KO mice used in this study were all homozygous for lynx1KO. Animals were treated according to

the guidelines detailed in the *Guide for the Care and Use of Laboratory Animals*, at the OLAR facility, California Institute of Technology, IACUC protocol number 1386-10G. All samples (1, 3, 7, and 13 months old of wild type and lynx1KO) were prepared and analyzed with the investigator blinded to genotype.

Electron Microscopic Analyses Adult mice were used to examine the ultrastructure of nerve fibers in the dorsal striatum. Animals were perfused transcardially with saline/heparin followed by 1.25 % glutaraldehyde and 1.5 % sucrose in 0.1 M phosphate buffer (PB). Brains were post-fixed and sectioned coronally at 60-μm thickness on a vibrating microtome with 5 % sucrose in 0.1 M PB. Sections were then post-fixed with 2.0 % glutaraldehyde and 1.5 % sucrose in 0.1 M cacodylate buffer (pH 7.4). Osmolarity was maintained at ~400 mOs throughout the fixation process (Cragg 1980). Sections were then incubated in 2 % osmium tetroxide with 0.8 % K ferricyanide and 5 % sucrose in 0.1 M Na cacodylate buffer for 1 h followed by 2 % aqueous uranyl acetate pre-staining for 30 min in darkness. Sections were dehydrated in an acetone series and then embedded in EMBed 812 (Electron Microscopy Sciences). The samples were then cut into 60-nm thin sections with a diamond knife (DiATOME) on a Leica Ultracut UCT ultramicrotome. The thin sections were collected on a TEM grid then post-stained with a 2 % uranyl acetate and Reynolds' lead citrate. For TEM imaging, we used a Tecnai T12 operating at 120 kV, equipped with a Gatan Ultrascan 2 k×2 k CCD camera. For analysis in the dorsal striatum, we acquired many images to create montages of fascicles. Micrographs were quantified using Illustrator and ImageJ software. The number of fascicles was determined using Cell Counter and area measurements (number of fibers/μm^2).

Quantitative PCR Tissue was quickly dissected on a cooled dissecting surface, and placed into RNAlater stabilization reagent (Qiagen). Tissue was homogenized using a Bullet Blender homogenizer (Next Advance) with RNAse-free metal beads to disrupt tissue. Homogenized tissue was quickly centrifuged, and RNA was purified from supernatant using RNeasy columns (Qiagen). RNA was analyzed on a Bioanalyzer and subjected to reverse transcription (RT) reactions using 500-ng RNA (Quanta BioSciences). Quantitative PCR (qPCR) was performed in triplicate using a lynx1 TaqMan assay with GAPDH and HPRT as reference genes (Life Technologies) at a cDNA concentration of 1 ng per well. Experimental design and analysis was performed as per MIQE guidelines.

Western Blot Protein extracts were prepared using a buffer solution of 2 % sodium dodecyl sulfate (SDS) and 2 % BME in phosphate buffer saline (PBS). Tissue was homogenized using Bullet Blender homogenizer. Extracts were centrifuged and supernatants run out on a 7 % SDS PAGE gel. Gels were blotted onto nitrocellulose membranes and blocked with 5 % milk in TBS-T. Primary antibodies were applied at 1:500 dilution and secondary antibodies incubated at 1:1,000. Quantitation was performed using Li-COR system to measure anti-lynx1 bands, normalized to the actin bands for each sample. Triplicate samples were analyzed for each mouse brain.

Immunohistochemistry We conducted immuno-labeling using the avidin/biotin complex (Spencer-Segal et al. 2011). Thirteen-month-old mice were perfused transcardially with saline/heparin followed by 4 % paraformaldehyde/0.1 % glutaraldehyde in 0.1 M PB and post-fixed for 2 h and then stored in 25 % sucrose/0.1 M PB overnight. Brains were sectioned coronally at 30-μm thickness on a vibrating microtome with 5 % sucrose in 0.1 M PB. Sections were treated with 1 % sodium borohydride in 0.1 M PB for 2×20 min followed by avidin/biotin blocking agent for 15 min each, and by blocking buffer (2 % normal goat serum, 1 % bovine serum albumin, 0.1 % CWFS in phosphate buffer saline PBS), and then incubated in mouse anti-Neuronal NeuN (Chemicon) for 3 days at 4 °C. The control sections were treated with non-specific IgG (Sigma-Aldrich). Sections were washed with incubation buffer (0.1 % BSA-*c* in PBS) for 30 min. The sections were then incubated in biotinylated goat anti-mouse IgG (H + L) antibody (Kirkegaard & Perry Laboratories, Inc.) for 2 days at 4 °C in darkness. After washing with incubation buffer for 3×5 min, sections were post-fixed with 2 % glutaraldehyde in 0.1 M PB for 15 min. Sections were then labeled with peroxidase-conjugated streptavidin (DAKO). Labeling was visualized with stable diaminobenzidine (Invitrogen) and hydrogen peroxide. Labeled sections mounted on slides were examined by a Leica DM2500P microscope with 10× and 63× objectives.

Images were imported into Photoshop and divided into quadrants. Quadrant files were imported into ImageJ, and the number of NeuN positive cells were quantified using the Cell Counter plug-in. Numbers for each quadrant were averaged and analyzed using ANOVA with post hoc Tukey honest significant difference (HSD) test.

Results

We performed a TEM evaluation of lynx1KO mouse striatum, an area of substantial disruption at the light microscopic level. Within the striatum, nerve fibers (marked as N in Figs. 1 and 2) run in compact bundles, which we refer

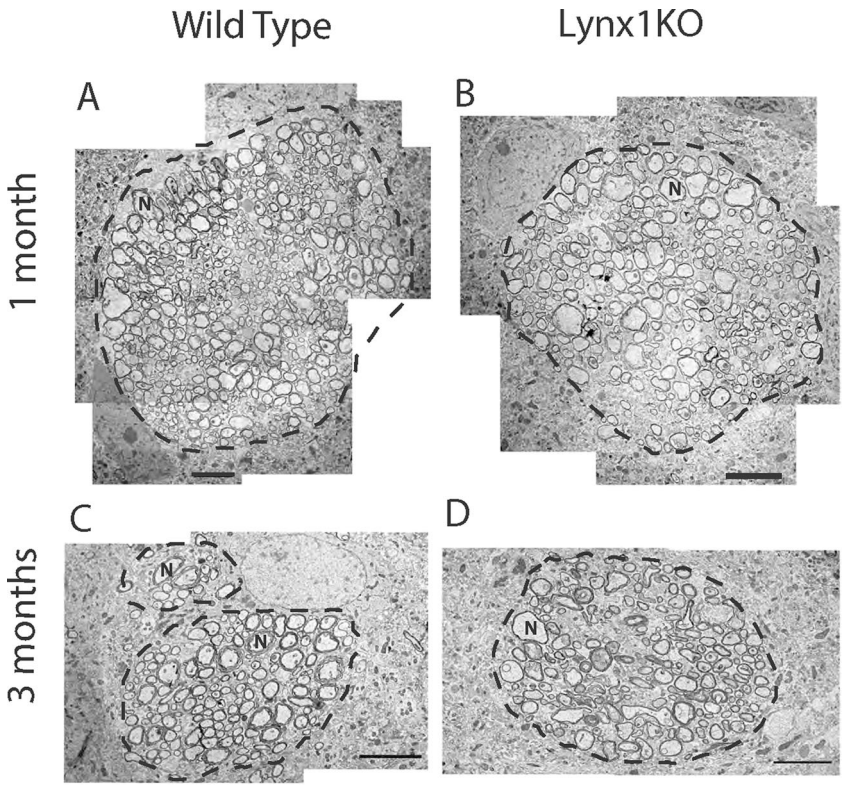

Fig. 1 Electron micrographs of fascicles in the dorsal striatum of 1- and 3-month-old mice in cross section. In young striatal bundles, the nerve fibers (marked as *N*) are closely packed for both wild-type and lynx1KO mouse, so that the boundary of an individual bundle is clearly demarcated (indicated with a *dashed line*). These schematic images are shown in Fig. 9a. **a** Ultrastructural montage of a nerve fiber bundle of a 1-month-old wild-type mouse. **b** Ultrastructural montage of a nerve fiber bundle of a 1-month-old lynx1KO mouse. **c** Ultrastructural montage of a nerve fiber bundle of a 3-month-old wild-type mouse. **d** Ultrastructural montage of a nerve fiber bundle of a 3-month-old lynx1KO. *All scale bars* 5 μm

to as fascicles (drawn in dotted lines). In coronal cross sections, the majority of these fascicles have distinct boundaries demarcating them from the striatal neuropil, which is the rest of the striatal tissue external to these fascicles. Our past histological studies of 6-, 9-, 12-, 15-, and 18-month-old mice showed that lynx1KO animals start to exhibit macroscopic vacuolar degeneration in the tissues of the dorsal striatum at 12 months of age. We therefore employed TEM to investigate the degeneration of striatal tissues in more detail in animals aged 1, 3, 7, and 13 months.

Loss of lynx1 in Aging Alters the Shape of Axon Bundles, and Their Borders Are No Longer Defined

We generated cross section montages of the nerve fiber bundles in the dorsal striatum. In 1-month-old wild-type (Fig. 1a) and lynx1KO (Fig. 1b) mice, fiber bundles appeared largely similar. In 3-month-old mice, both the wild-type (Fig. 1c) and lynx1KO (Fig. 1d) mouse striata were unchanged from 1-month-old striata; the packing density of bundles within lynx1KO mice resembled wild type. In 7-month-old mice, fascicles appeared largely similar in the wild type (Fig. 2a) and lynx1KO (Fig. 2b), and the density of nerve fibers (marked N in figures) within the fascicles continued to look largely similar between the two genotypes, indicating that there were only subtle differences between wild-type and lynx1KO mice in the dorsal striatum up to that point.

In 13-month-old mouse brains, however, there was a striking difference in the structures in the dorsal striatum (Fig. 2c, d). In the montages of the lynx1KO mouse brains, there were no defined borders to the nerve fascicles (Fig. 2d). The borders of the fascicles were indistinct (marked "?" in Fig. 2d), discontinuous in places, and the nerve fibers within the fascicle were dispersed with gaps between them (Fig. 2d). In the wild-type mouse brains of the same age, the border of the fascicles continued to appear smooth and regular, and the overall geometries of these fascicles appeared contiguous and intact (Fig. 2c). We quantified the nerve fiber density in striatal montages to compare young (1- and 3- month-old) vs. aged (13-month-old) brains (Fig. 2e). We found a significant effect by ANOVA [$F(3,11)=6.11$, $p=0.01$] when comparing the 13-month-old lynx1KO fiber density ($M = 56.4\%$, SEM = 9.3) to that of 13 month old wild type ($M = 100.0\%$, SEM = 5.8), young wild type ($M = 88.2\%$, SEM = 11.0), and young lynx1KO samples ($M = 77.1\%$, SEM = 4.2). Data were normalized and presented as a percentage of the density in the 13-month-old wild-type condition. Post hoc comparisons using the Tukey HSD test indicated that the mean score for the 13-month-old homozygous KO condition was significantly different from the 13-month-old wild-type condition ($p<0.01$). Because we did not detect significant differences in nerve fiber density between wild type and lynx1KO brains in young animals, we conclude that the reduction in nerve fiber density in the 13-month-old lynx1KO is a progressive effect that occurs during aging

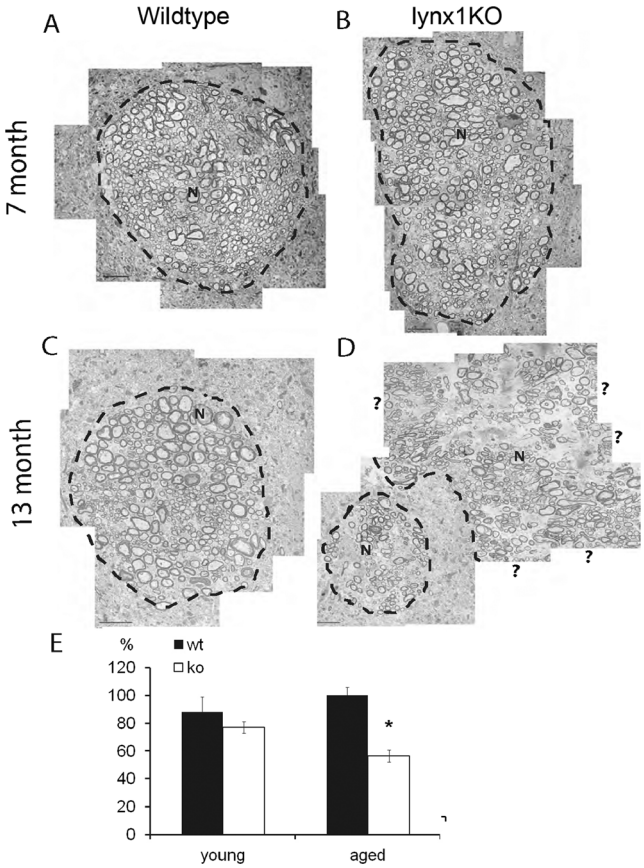

Fig. 2 Electron micrograph of a nerve fiber bundle in dorsal striatum of 7- and 13-month-old mice in cross section, for both wild type and lynx1KO. In 13-month-old striatal bundles from wild-type animals, the axon fibers (*N*) are still tightly packed, as drawn with *dashed lines*. However, the axon fibers from the 7-month-old lynx1KO appear slightly dispersed and the 13-month-old lynx1KO fibers appear to be highly dispersed. **a** Ultrastructural montage of a nerve fiber bundle of a 7-month-old wild type. **b** Ultrastructural montage of a nerve fiber bundle from a 7-month-old lynx1KO mouse. **c** Ultrastructural montage of a nerve fiber bundle from a 13-month-old wild-type animal. **d** Ultrastructural montage of a nerve fiber bundle from a 13-month-old lynx1KO. Although some of the bundles (*lower left fascicle*) appear to be packed, many (*upper right*) have lost their shape so that the boundary cannot be easily demarcated (shown by the "*?*" marks). *All scale bars* 5 μm. **e** Bar graph of nerve fiber density in a fascicle (*y*-axis is # of fibers/μm^2). Data normalized and presented as percentage of the 13-month-old wild-type density. $p < 0.01$ [$F(3,11) = 6.11$] by ANOVA. Post hoc Tukey HSD test indicates a significant difference in the mean density when comparing the 13-month-old lynx1KO fiber density (56.4 ± 9.3 %, $n = 3$) to that of the 13-month-old wild-type density (100.0 ± 5.8 %, $n = 5$). Density in young wild-type (88.2 ± 11.0 %, $n = 4$) and young lynx1KO samples (77.1 ± 4.2 %, $n = 3$) was not significantly different. *y*-axis is in percentage

Aging lynx1KO Mice Exhibit Internal Disruptions Within Axon Fiber Bundles

At higher TEM magnification, we examined the nerve fibers within the fascicles. The nerve fibers were markedly different between the 13-month-old lynx1KO (Fig. 3c, d) and age-matched wild-type sections (Fig. 3a, b). Whereas the wild-type axons had ordered myelin sheaths, the lynx1KO axons were highly disordered (marked SD in Fig. 3d). In addition, the extent of myelination appears to be reduced in the lynx1KO samples as compared to the wild-type samples. In the lynx1KO mice (Fig. 3c, d), some of the nerve fibers have vacuoles (marked NV in Fig. 3), which were not observed in the wild-type sections.

Aging lynx1KO Mice Display Degeneration in Striatal Neurons

We wanted to ascertain whether the morphological alterations of aging lynx1KO brains were confined to nerve fibers traversing the striatum or if other sub-regions, such as the regions between the fascicles—the striatal neuropil—were also affected. We therefore performed TEM on the striatal neuropil, which contain relatively more cell bodies and fewer nerve fibers of passage than in the fascicular region. These regions contain dendritic (marked as DR in Fig. 4) and somatic (Fig. 5) structures. In the 13-month-old sections of wild type (Fig. 4a, b), it was evident that nerve fibers in the region were surrounded by a dense network of tissue containing many dendrites. The lynx1KO, however, exhibits a striking disruption in the striatal neuropil (Fig. 4c, d), with areas between dendrites and other structures having a very low density (marked E in Fig. 4c, d). Furthermore, we saw signs of degeneration in the nucleus in the lynx1KO samples, with cases of both condensed nuclei and cleared nuclei (Fig. 5b). In the wild-type samples, however, the nuclei appeared to be intact with no condensation (Fig. 5a).

Age-Related Degenerative Features Are Spared in Heterozygous lynx1KO Mice

Because of the severity of the phenotype in aged mice without lynx1, we wanted to determine if partial dosage of lynx1 would result in a less severe phenotype. We confirmed by qPCR analysis that heterozygous lynx1KO mice had a 50 % reduction in lynx1 transcript levels in total brain extracts as compared to those of wild-type mice (Fig. 6c). We noted a corresponding reduction of lynx1 protein by Western blot analysis (Fig. 6a) from protein extracts of heterozygous brains compared to wild-type levels. Quantitative assessment indicates that lynx1 protein is expressed at about 50 % of wild-type levels of protein (Fig. 6b).

We generated cross section montages of the fascicles in the dorsal striatum. Interestingly, in the 13-month-old heterozygous mice (Fig. 7a), there was little evidence of such structural alteration as can be observed in homozygous lynx1KO aging mice. At higher TEM magnification, we examined the nerve fibers (marked as N in Fig. 7b) within the fascicles in the heterozygous lynx1KO mice. The heterozygous mice fibers had ordered myelin sheaths, same as the wild-type mice. In the striatal neuropil, the regions between the fascicles contain relatively few nerve fibers of passage (Fig. 7c). This tissue

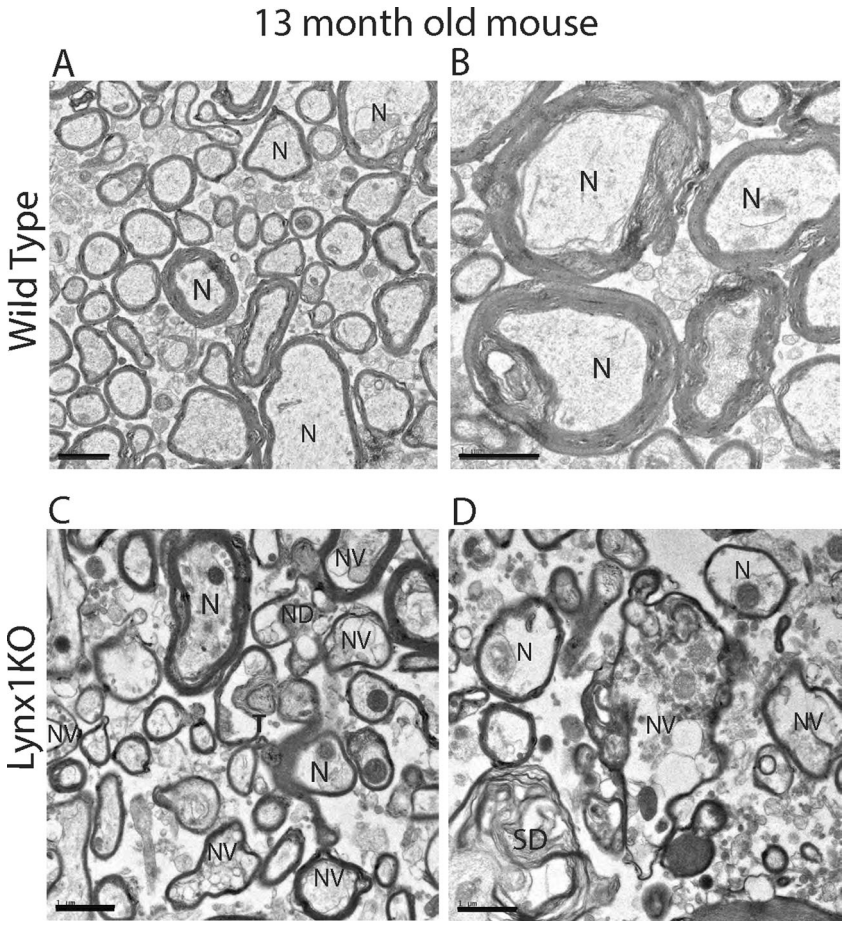

Fig. 3 Nerve fibers in the fascicles are markedly disrupted in lynx1KO mice. High-power micrographs of the 13-month-old lynx1KO striatal fascicles reveal disruption in the region between individual nerve fibers. In some cases, the diameters of the axons themselves, or the myelin sheaths surrounding them, appeared shrunken. In some cases, the myelin appear to be degenerated (marked as *SD*), and the interior of the axon appears vacuolated (marked as *NV*). **a** 13-month-old wild-type mouse brain. **b** 13-month-old wild-type mouse brain, higher power. **c** 13-month-old lynx1KO mouse brain. **d** 13-month-old lynx1KO mouse brain, higher power. All scale bars 1 μm

contained many dendrites (marked as DR in Fig. 7c) and was densely filled, as seen in the wild-type case. In the heterozygous lynx1KO mice, the nuclei appeared to be intact with no condensation (marked as Nucleus in Fig. 7a). Nerve fiber density measurements of fascicle montages did not show a significant difference between aged wild-type and heterozygous animals, while aged homozygous lynx1KO animals showed a significant reduction in nerve fiber density. There was a significant effect of genotype on fiber density at 13 months, at the $p<0.01$ level for the three conditions [$F(2,10)=10.02$, $p=0.004$] (Fig. 7d). Post hoc comparisons using the Tukey HSD test indicated that the mean score for the homozygous KO condition ($M=0.34$, SEM=0.02) was significantly different from the wild-type ($M=0.61$, SEM=0.04) condition ($p<0.01$). The heterozygous ($M=0.44$, SEM=0.05) condition, however, showed no significant differences with either the wild-type or homozygous KO groups. Taken together, these results suggest that the complete removal of lynx1 is detrimental to axon fiber retention during aging. Partial loss of lynx1, however, is apparently sufficient to support neuronal health, even after prolonged periods of reduction (Fig. 7d).

In order to compare the loss of neuronal cell bodies within the striatum between the 13-month-old wild-type, heterozygous lynx1KO, and lynx1KO mice, we performed immunocytochemical analysis of the dorsal striatum using anti-NeuN to label neuronal cell bodies (Fig. 8). We quantified the NeuN positive cells in wild-type (Fig. 8a) vs. heterozygous lynx1KO (Fig. 8b) and lynx1KO (Fig. 8c) brain sections of the striatal neuropil. There was a significant effect of genotype on neuronal cell body number at 13 months, at the $p<0.05$ level for the three conditions [$F(2,21)=19.8$, $p<0.0001$] (Fig. 8d). Post hoc comparisons using the Tukey HSD test indicated that the mean score for the homozygous KO condition ($M=960$, SD 85.8) was significantly different from the wild-type ($M=1{,}194$, SD 93.9) and the heterozygous ($M=1{,}171$, SD 63.2) conditions. There were no significant differences, however, between the wild-type vs. heterozygous groups. Taken together, these results suggest that the complete removal of lynx1 is detrimental to striatal neuronal health, but titrating lynx1 dosage could spare neurons from degeneration. Significant loss in the neuropil, also demonstrates that the aging phenotype is not confined to fibers of passage through the striatum, but involves striatal neurons themselves This observation could explain why, in the dorsal striatum of wild-type mice and heterozygous mice, axon bundles run in compact fascicles which show distinct boundaries, but in aged lynx1KO mice, the boundaries of fascicles become indistinct.

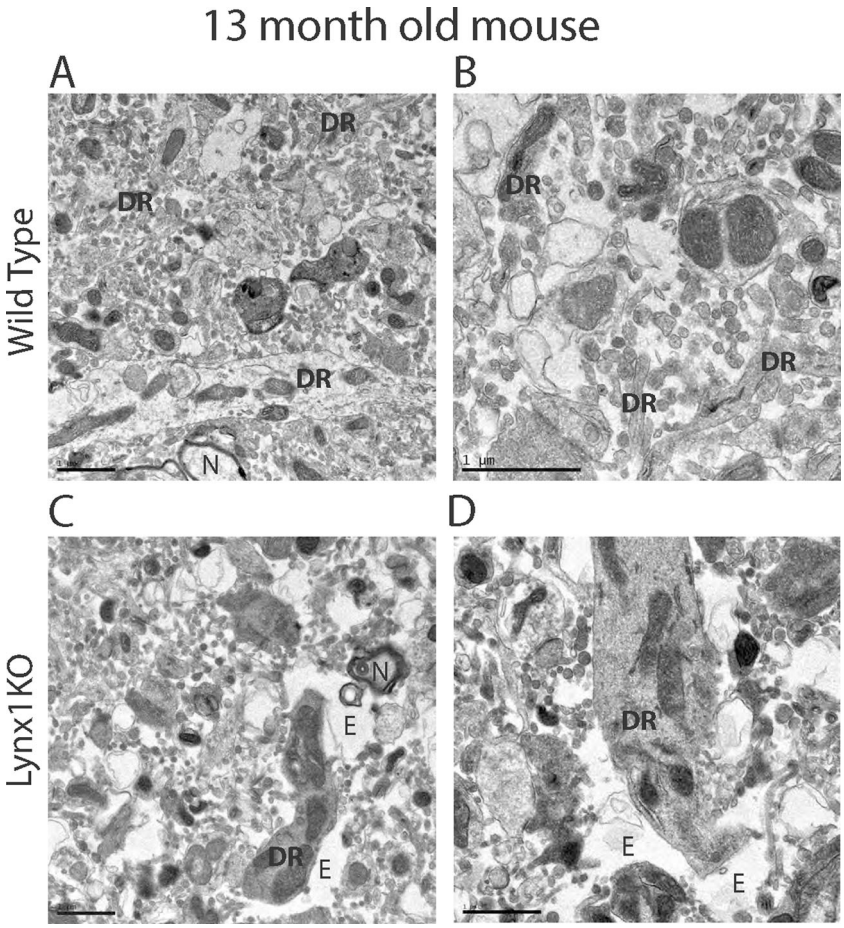

Fig. 4 Microscopic examination of 13-month-old striatal neuropil. **a** Electron micrograph of wild-type mouse. Part of an individual nerve fiber (marked as *N*) is shown at bottom. **b** Electron micrograph of wild-type mouse at higher magnification. For wild-type animals, the nerve fiber bundles are still packed by parts of dendritic (marked as *DR*) and neuronal cell bodies. **c** Electron micrograph of lynx1KO mouse. **d** Electron micrograph of lynx1KO mouse at higher magnification. The tissues in the lynx1KO mouse contain many areas that lack intracellular structures as compared to the wild-type mice. Areas between the dendrites appear empty (marked as *E*). *All scale bars* 1 µm

Striatal Degeneration as a Trigger for Vacuole Formation

The external degeneration observed in 13-month-old animals might have triggered the loss of axon bundles, producing the vacuoles observed in the previous study (Miwa et al. 2006). We suggest here that fascicles may have been lost when the samples in the previous study were switched suddenly from high to low osmolarity in the solution. This change in osmolarity could be associated with a transient phase of "curling" in the previous study during the staining procedure. In summary, we observed two major differences in the aged lynx1KO brain. One is the degeneration of the striatal neuropil, while the other is the degeneration within axons of fascicular fibers coursing through the striatum. The most extreme ultrastructural change

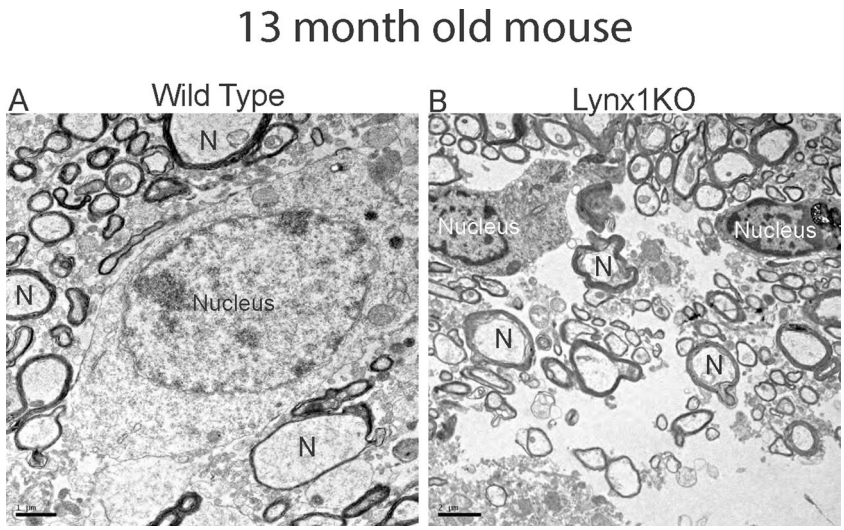

Fig. 5 Electron micrograph of the somatic structures in the striatal neuropil between fascicles shows disruption of nuclei evident in aging lynx1KO brains. Lynx1KO samples appear to contain condensed and clear nuclei, and empty spaces between nerve structures in the striatum. **a** 13-month-old wild-type striatum. **b** 13-month-old lynx1KO striatum. Cell nuclei appear condensed or cleared. All scale bars 1 µm

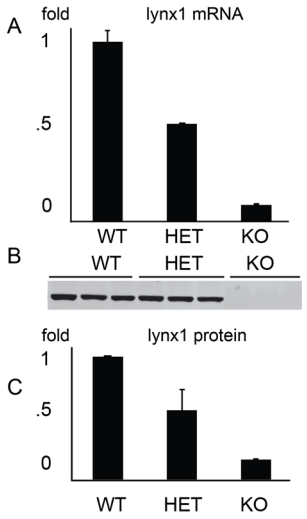

Fig. 6 Measurements of lynx1 levels using qPCR and Western analyses. **a** qPCR analysis indicates 50 % of lynx1 transcript levels in heterozygous lynx1KO mice as compared to those of wild-type mice. *y*-axis is fold level of expression of lynx1 relative to wild-type levels. **b** Western blot of total brain protein extracts from 13-month-old mice run out in triplicate and detected with anti-lynx1 antibodies. **c** Quantitation of lynx1 bands indicates protein levels in the heterozygous mice are ~50 % of wild-type levels and absent in homozygous lynx1KO extracts. *y*-axis is fold level of lynx protein relative to wild-type levels

manifested itself at 13 months of age, while 1- and 3-month-old lynx1KO mice exhibited little to no difference in neuronal structures. The similarity between wild-type and heterozygous samples suggests that lynx1 is protective to neurons, even at half dosage of the protein.

Discussion

We characterized the dorsal striatum morphologically and found changes in the cellular ultrastructure of the lynx1KO mouse brain compared to wild-type mouse brains during aging. Before 1 year of age, we saw no substantial difference between wild-type and lynx1KO mouse brains. In 13-month-old lynx1KO animals, however, there was evidence of altered morphology of the fascicles and their integrity appeared weakened. In montages of the fascicles the density of fiber packing was reduced in lynx1KO mice, and there was evidence of clearing of both nerve fibers and neuronal cell bodies. In this study, we therefore asked what would be the consequence of restricting the degree of lynx1 inhibition over the cholinergic system and capitalized on the partial lynx1 dosage within heterozygous lynx1KO mice to carry out our investigations. We found that with partial lynx1 reduction, it is possible to ameliorate the neuronal loss associated with the aging lynx1KO brains.

Types of Altered Morphology Between and Within Axons

The external degeneration observed in 13-month-old animals might have triggered the loss of axon bundles, producing the vacuoles observed in the previous study. The fact that this was observed only in the lynx1KO mice suggests that the tissue was weakened or cleared out by the degeneration. To summarize the idea that the observed morphology of lynx1KO degeneration within the striatum could lead to weakened fascicle structure, we represented each individual axon by a cotton-tipped swab and each fascicle by bundles of swabs (Fig. 9a, b). The wild-type and heterozygous axons are regular and parallel, represented by parallel swabs (Fig. 9a). In contrast, the lynx1KO axons are skewed (Fig. 9b), perhaps because the loss of neuronal cell bodies and clearing of dendritic tissues decreases the external mechanical constraints that force the packing of the fascicles. Furthermore, we saw the altered ultrastructure of axoplasm in the dorsal striatum. This alteration may signify that pathology is transported via axoplasmic flow from defects or signals that originate in cell bodies located outside the striatum.

Age-Related Changes in Cholinergic Signaling

Aging is associated with changes in cholinergic signaling (Terry and Buccafusco 2003; Albuquerque et al. 2009) and loss of cholinergic neurons (Decker and McGaugh 1991; Schliebs and Arendt 2011), as well as loss of axons of cholinergic neurons (Geula et al. 2008). The aging brain may also be harmed by heightened nicotinic acetylcholine receptor function, which could generate excessive calcium signaling. As nicotinic receptor modulators, lynx1 molecules may be required to balance nicotinic receptor activity in the aging brain. We have reported elevated baseline Ca^{2+} levels and elevated nicotine-evoked Ca^{2+} transients in primary neuronal-cultured neurons from lynx1KO mice (Miwa et al. 2006). This is consistent with the demonstrated ability of nicotinic receptors to flux Ca^{2+} directly (Colquhoun and Patrick 1997; Yamauchi et al. 2011) or to induce the release of Ca^{2+} from intracellular calcium stores via downstream effectors (Stevens et al. 2003; Vijayaraghavan et al. 1992). In addition to displaying elevations in Ca^{2+} levels, lynx1KO neurons are more susceptible to toxic levels of glutamate. Pre-incubating neurons with low doses of nicotine fails to protect lynx1KO mouse neurons, while this neuroprotective process is robust in wild-type neurons (Miwa et al. 2006). This suggests that lynx1 is playing a critical role in protecting neurons via nicotinic receptor-mediated Ca^{2+} dynamics. Elevated intracellular Ca^{2+} levels and exaggerated responses of nicotinic acetylcholine receptors in the lynx1KO mouse would make neurons more susceptible to insults over time. A definitive mechanism of action for the degenerative pathway awaits further investigation.

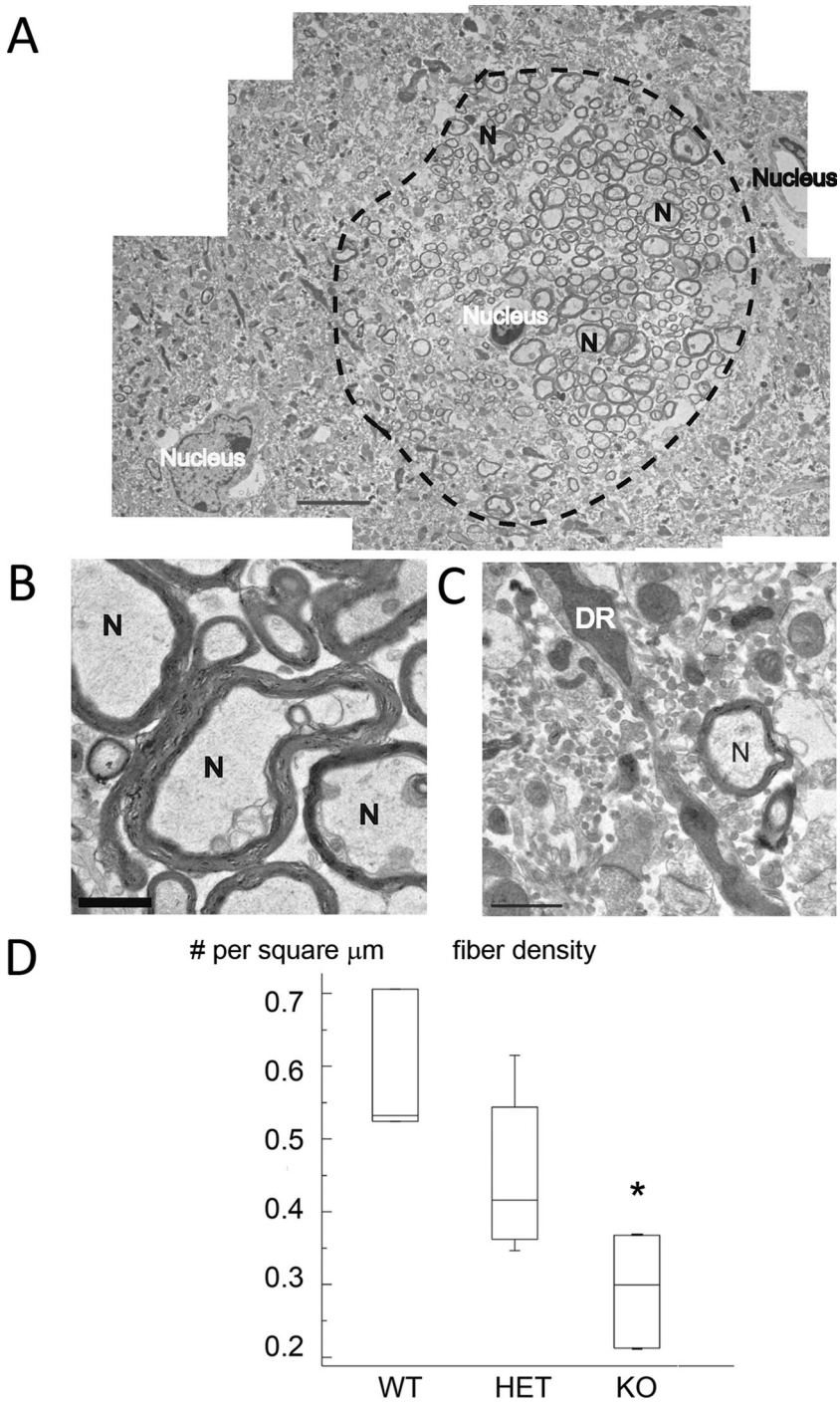

Fig. 7 Microscopic examination of 13-month-old heterozygous lynx1KO mice reveals that titrating lynx1 dosage protects neurons. **a** The nerve fibers (marked as *N*) are closely packed in heterozygous lynx1KO mice, so that the boundary of an individual bundle is clearly demarcated (indicated with a *dashed line*). These schematic images are shown in Fig. 9a. Scale bar 5 μm. **b** Part of an individual nerve fiber (marked as *N*) is shown. Scale bar 1 μm. **c** Electron micrograph of the somatic structures in the striatal neuropil between fascicles shows that the nerve fibers (marked as *N*) are still packed by the parts of dendritic structures (marked as *DR*). Scale bar 1 μm. **d** Bar graph of axon fiber density. *y*-axis is number of axons in a fascicle/area in square micrometer. Wild type is 0.61±0.04, n=4, heterozygous lynx1KO of 13 months old is 0.44±0.05, n=5, and lynx1KO is 0.34±0.02, n=3 $F(2,10)=10.02$; $p=<0.01$. Tukey HSD test indicates a significant difference in the mean scores between the KO and the wild-type condition ($p<0.01$), whereas there were no sigificant differences with the heterozygous condition. *y*-axis is # of fibers/μm^2

Genetic, biochemical, and pharmacological studies have demonstrated that nicotinic receptors are downstream effectors of lynx1. The striatal degeneration observed at the light microscopic level was ameliorated by crossing the lynx1KO mice to the α7 and β2 KO mouse lines (Miwa et al. 2006). This indicates that lynx operates through both subtypes, in line with evidence that lynx1 can form stable interactions with both subtypes in binding studies (Ibanez-Tallon et al. 2002). As further support for pan-specific interactions with nicotinic receptors, pharmacological agents blocking both subtypes, when given as a cocktail, can block plasticity effects in lynx1KO mice (Morishita et al. 2010). Multiple nicotinic receptor subtypes in the striatum have been reported in rodents (Hill et al. 1993; Perez et al. 2008; Zhang et al. 1998), primates (McCallum et al. 2006), and humans (Court and Clementi 1995). α7 (O'Neill et al. 2002; Toulorge et al. 2011), β2 (Stevens et al. 2003; Zoli et al. 1999), and other nAChRs (Khwaja et al. 2007) have been implicated in neuroprotection

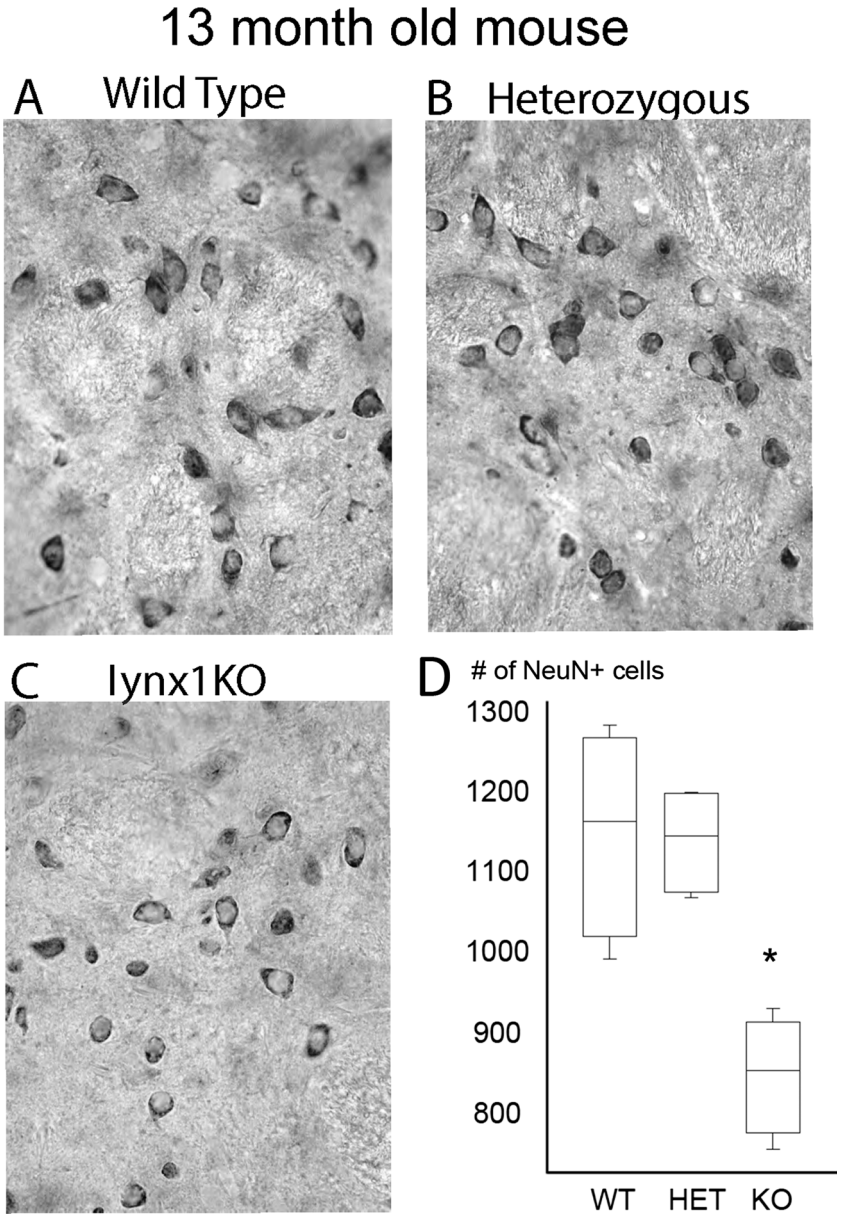

Fig. 8 Neurons are lost in aging dorsal striatum of lynx1KO mice, but titrating lynx dosage protects neurons. All images taken at 63× magnification. **a** 13-month-old wild-type mouse brain stained with neuronal marker NeuN. **b** 13-month-old heterozygous lynx1KO mouse brains stained with NeuN. **c** 13-month-old lynx1KO mouse brains stained with NeuN. **d** Box plot of number of neurons as assessed by NeuN positive staining. *y*-axis plots the number of NeuN positive neurons per region

(Quik and Kulak 2002). The requirements for neuroprotection are influenced by many factors, including developmental stage, receptor subtype, and dosage (Picciotto and Zoli 2008). Lynx1 appears to be critical for nicotine-mediated

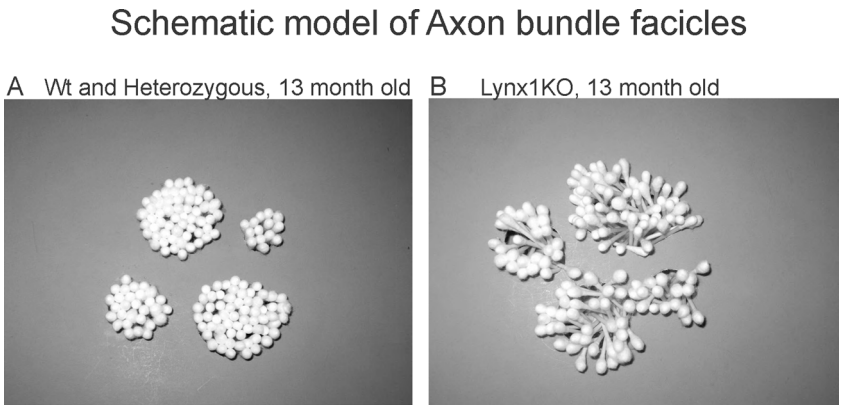

Fig. 9 **a** and **b** are schematic models of axon bundle fascicles of 13-month-old wild-type and heterozygous lynx1KO mice, and the dispersed bundles described as of 13-month-old lynx1KO, respectively, illustrated by the clumping of cotton-tipped swabs which represent myelinated axon fibers

neuroprotection (Miwa et al. 2006), and thus, lynx1 adds another layer of complexity to our understanding of nicotinic receptors and neuroprotection.

The lynx1KO degeneration occurs at therapeutically relevant sites in the brain. The dorsal striatum and the neurons which course through it are important targets for the neuroprotective effects of nicotine. Striatal dopamine has been shown to be sensitive to nicotine neuroprotection (Costa et al. 2001; Dajas et al. 2001). In vitro studies invoke changes in intracellular Ca^{2+} levels (Toulorge et al. 2011). Whereas nerve fibers traversing the striatum were significantly affected, hippocampal sections appeared to be normal at all time-points (data not shown), suggesting this susceptibility was specific to cell type. Even though we have observed marked regional differences in the degeneration in aged lynx1KO mice, these differences do not correlate with areas with the highest levels of lynx1 expression. Lynx1 mRNA levels are highest in select brain stem nuclei, CA3 of the hippocampus, and deep cerebellar nuclear neurons, with moderate levels of expression throughout the entire brain (Miwa et al. 1999). The greatest levels of degeneration seen at the light microscopic level are observed in the dorsal striatum, cerebellar peduncles, brain stem, and, to a lesser extent, the corpus callosum (Miwa et al. 2006). The selective degeneration in the striatum may reflect a selective vulnerability of these neurons to non-cell-autonomous stressors caused by the absence of lynx1.

Evidence is emerging that lynx1 levels can change due to physiologically relevant events and different environmental and network factors (Miwa et al. 2011; Miwa et al. 2012; Pfeffer et al. 2009; Sekhon et al. 2005). The sparing of neurons in heterozygous mice reveals that partial reductions in lynx levels may be well tolerated in the brain. This may provide a demonstration of haplosufficiency of the lynx1 gene with respect to neuronal protection. If this extends to learning and cognitive functions, then the lynx1 gene in the brain may be involved in a trade-off between neuroprotection and augmented learning. If expression levels are altered in specific environmental conditions to optimize this trade-off, the degree of cholinergic tone would be able to change in response to situational or environmental demands.

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