Published in final edited form as:

Cerebellum. 2015 October ; 14(5): 516–527. doi:10.1007/s12311-015-0673-9.

α -synuclein expression in the mouse cerebellum is restricted to VGluT1 excitatory terminals and is enriched in unipolar brush cells

Sun Kyong Lee¹, Roy V. Sillitoe², Coralie Silva¹, Marco Martina¹, and Gabriella Sekerkova¹

¹Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, IL, 60611

²Department of Pathology and Immunology, Department of Neuroscience, Baylor College of Medicine, Jan and Dan Duncan Neurological Research Institute of Texas Children's Hospital, Houston, TX, 77030

Abstract

α -synuclein has a crucial role in synaptic vesicle release and synaptic membrane recycling. Although its general expression pattern has been described in the cerebellum, the precise cerebellar structures where α -synuclein is localized are poorly understood. To address this question, we used α -synuclein immunohistochemistry in adult mice cerebellar sections. We found that α -synuclein labels glutamatergic but not glycinergic and GABAergic synaptic terminals in the molecular and granule cell layers. α -synuclein was preferentially expressed in parallel and mossy fiber synaptic terminals that also express vesicular glutamate transporter 1 (VGluT1) while it was not detected in VGluT2-positive climbing fibers. α -synuclein was particularly enriched in lobules IX and X, a region known to contain high density of unipolar brush cells (UBCs). To elucidate whether the α -synuclein-positive mossy fibers belong to UBCs, we double labeled cerebellar sections with antibodies to α -synuclein and UBCs type specific markers (calretinin for type I and metabotropic glutamate receptor 1 α (mGluR1 α) for type II UBCs), and took advantage of organotypic cerebellar cultures (in which all mossy fibers are UBC axons) and moonwalker mice (in which almost all UBCs are ablated) and found that both type I and type II UBCs express α -synuclein. In moonwalker mutant cerebella, the α -synuclein/VGluT1 immunolabeling showed dramatic decrease in the vestibulocerebellum that correlated with the absence of UBC. α -synuclein appears to be an excellent marker for intrinsic mossy fibers of the VGluT1 subset in conjunction with UBCs of both subtypes.

Keywords

VGluT1; VGluT2; granule cells; moonwalker mice; mossy fiber terminals; vestibulo-cerebellum

Introduction

α -synuclein function may be central to the pathogenesis of several neurodegenerative diseases, including Parkinson's disease, dementia with Lewy bodies, multiple system atrophy and Alzheimer's disease [1–6]. The hallmark of these neurodegenerative diseases, collectively termed synucleopathies, is the aggregation of α -synuclein in cytoplasmic deposits such as the Lewy bodies and senile plaques [1, 2]. α -synuclein is a small, highly conserved presynaptic protein that is enriched in the vertebrate central nervous system (CNS). It plays a role in synaptic transmission and synaptic plasticity under physiological conditions [1, 7–11]. During normal synaptic function α -synuclein behaves as a non-classical chaperone for soluble N-ethylmaleimide-sensitive (NSF) protein receptor (SNARE) complex assembly and regulates vesicle trafficking and recycling [9, 12, 13].

α -synuclein protein [14] and mRNA (*in situ* hybridization (ISH) [15]) are both abundantly and widely expressed in different neuronal populations throughout the CNS. Despite its abundance, the brain expression of the α -synuclein is not ubiquitous, but instead it's rather selective to specific neuronal populations [16]. This is especially true for the cerebellum, where expression of α -synuclein transcripts restricted to the granule cell layer, and no mRNA signal is detectable in Purkinje cells and stellate/basket cells in molecular layer [15]. Remarkably, α -synuclein expression is particularly intense in the vestibulo- cerebellum, encompassing the nodulus (lobule X), uvula (lobule IX), and flocculus/paraflocculus complex. These cerebellar regions are known to contain a high density of unipolar brush cells (UBCs), a special class of excitatory interneurons [17, 18]. In this study we investigated the presence of α -synuclein in different cerebellar circuitries, with special emphasis on the UBCs. We used immunohistochemical labeling of cerebellar cryosections as well as *in vitro* slice cultures of wild type mouse. Because α -synuclein staining appears in register with regions that contain UBCs, we took advantage of moonwalker (*Mwk*) mice, in which type II UBCs are completely absent [18] to verify that α -synuclein expression is indeed enriched in these neurons.

We found that α -synuclein is associated with excitatory presynaptic terminals, and in particular with VGluT1-expressing terminals of granule cells and UBCs in the cerebellar cortex and that its expression is dramatically reduced in the *Mwk* mouse. Interestingly, intrinsic mossy fiber terminals (MFTs, the UBC axons) that also express VGluT1 contain much higher levels of α -synuclein than the extrinsic MFTs originating from pre-cerebellar nuclei.

Materials and Methods

Animals and tissue preparation

This study was carried out on mice in accordance with the protocols approved by the Northwestern University and Baylor College of Medicine Animal Care and Use Committee. The following mouse strains were used: CD1 as wild-type; α -synuclein knockout (α -synuclein KO) (129X1-Sncatm1Rosl/J; Jackson Laboratories); heterozygous moonwalker (*Mwk*⁺) (ENU-mutagenized mice [19, 20]) and their control littermates.

Adult mice of both sexes (32 to 60 day old, N=12) were anesthetized with sodium pentobarbital (60 mg/kg body weight) and then perfused through the ascending aorta with saline followed by freshly depolymerized 4% paraformaldehyde in 0.12 M phosphate buffer (PB), pH 7.4. One hour after the perfusion, the brains were dissected out and cryoprotected in a series of sucrose solutions in phosphate buffered saline (PBS), 10%, 20%, and then 30%. Cryoprotected cerebella were sectioned in the sagittal or coronal planes at 25 μ m on a freezing-stage microtome and the sections were collected serially in multiwell plates. No differences were observed in the α -synuclein distribution between the 32 and the 60 day old mice.

Organotypic cultures

Organotypic cerebellar slice-cultures were prepared from postnatal day (P) 8–10 mice as described previously [21]. The mice were euthanized by CO₂ inhalation and then immediately perfused with saline (0.9% NaCl). Next the cerebellum was dissected out and sagittally sliced at 250 μ m using a McIlwain tissue chopper. Lobule X was isolated from residual meninges in cold 1X Hank's balanced salt solution (HBSS, Life Technologies) and then placed on the membrane surface of Millicell-CM culture plate inserts (Millipore) equilibrated in plating medium (Basal medium eagle containing 25% horse serum, 1/4X HBSS, 1mM L-glutamine, 0.1% D-glucose, 1% penicillin/streptomycin, and 0.05% fungizone). At 7 days in vitro (DIV), the medium was replaced with maintaining medium (Basal medium eagle containing 15% horse serum, 1/4X HBSS, 1mM L-glutamine, 0.1% D-glucose, 1% Penicillin/streptomycin, and 0.05% fungizone). The medium was changed every 2–3 days and cultures were grown for 22–24 DIV at 37°C in a humidified atmosphere with 5%CO₂/95%O₂. The slices were then fixed for 30 min at room temperature with 4% paraformaldehyde in 0.12 M PB after which they were kept in PBS at 4°C.

Immunohistochemistry

Bright-field microscopy—Cryosections were processed for immunohistochemistry according to an avidin/biotin amplification protocol. Briefly, the endogenous peroxidase activity was blocked in 0.3% H₂O₂ and 10% methanol in Tris buffered saline (TBS; 100mMTris and 150mM NaCl; pH7.4). Non-specific binding was suppressed in a blocking solution containing 5% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in TBS with 0.2% Triton X-100. Sections were then incubated overnight, or up to 2 days at 4°C with a primary antibody. Bound antibodies were detected using biotinylated anti-mouse, -guinea pig or -rabbit IgG (GE Healthcare), ABC Elite kit (Vector Laboratories) and diaminobenzidine (DAB; Sigma). Control sections incubated without primary antibodies were free of immunoreaction products.

Immunofluorescence—To study colocalization of different proteins, cryosections and organotypic slice cultures were immunolabeled with a combination of antibodies. Briefly, after 1 hour pretreatment with a blocking solution containing 5% NGS and 1% BSA in TBS with 0.2% Triton X-100 the sections were incubated for 2–3 days at 4°C in a cocktail of primary antibodies raised in different species. Bound primary antibodies were visualized by secondary antibodies coupled to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen).

Primary antibodies—The following primary antibodies were used: rabbit, mouse, sheep, and guinea pig anti- α -synuclein, mouse and rabbit anti-calretinin, mouse anti-glutamate acid decarboxylase 67 (GAD67), guinea pig anti-glycine transporter 2 (GlyT2), rabbit anti- 25-kDa heat shock protein (Hsp25), mouse and rabbit anti-mGluR1 α , guinea pig anti-vesicular glutamate transporter 1 (VGluT1), and guinea pig anti- vesicular glutamate transporter 2 (VGluT2). Detailed specifications of these antibodies are listed in Table 1.

Specificity of α -synuclein antibodies—The specificity of these antibodies was tested on α -synuclein KO mice brain sections (Fig. 1). 2 of the 3 antibodies tested proved to be specific; the monoclonal antibody from Encor Biotechnology(MCA-3H9) and the sheep polyclonal antibody from Santa Cruz Biotechnology (AB5336) (Fig. 1A,B,E,F). Both these antibodies showed immunostaining (see also Fig. 2A) in brain regions that were previously reported to express α -synuclein either by in ISH (see Allen Brain Atlas)[15] or by immunohistochemistry using a proprietary antibody[14], and no staining in brain sections from α -synuclein KO mice (Fig. 1E,F). Surprisingly, the antibody raised in guinea pig (PC325; Calbiochem) proved to be non-specific as it produced the same staining pattern in the wild-type and α -synuclein KO mice (Fig. 1C,G) and thus was not used in the study. Finally we tested one pan synuclein antibody raised in rabbit (C0336; Assay Biotech). In the wild type cerebellum, this antibody showed the same staining pattern as the monoclonal α -synuclein antibody (Fig. 1D); in double immunolabeled sections the two antibodies co-localized in the MFTs (Fig. 1I–J). No immunostaining was detected in the cerebellum of the KO mouse (Fig. 1H).

Image acquisition

Images of cerebellar sections were acquired with a Spot RT CCD video-camera (Diagnostic Instruments) mounted on a Nikon Eclipse E800 microscope. Laser scanning confocal images were obtained with a Nikon PCM 2000 Confocal Microscope System using Simple PCI Program. Images were analyzed individually or in z-stacks of different depths. For co-localization experiments, type FF immersion oil was used with either a 40x plan-fluor lens (numerical aperture 1.3) or a 60x plan-apochromatic lens (numerical aperture 1.4). To minimize channel spillover the images were sequentially acquired. All images were processed with Adobe Photoshop CS5. Brightness and contrast were adjusted.

Results

α -synuclein in the cerebellar cortex

While α -synuclein inclusions are the hallmark of diffuse Lewy body disease, including Parkinson's, they are also the hallmark of multiple system atrophy-cerebellar (MSA-C), a cause of adult onset cerebellar ataxia [3, 22] in which the patients CSF appears particularly favorable to α -synuclein aggregation [23]. Despite the evidence pointing to the cerebellum as an important target of synucleinopathies, the normal distribution of α -synuclein in the cerebellum has not been thoroughly investigated. We studied wild-type mouse brain in sections immunolabeled with commercially available α -synuclein antibodies (Table 1, Fig. 1)

In wild-type animals intense staining was observed in forebrain regions, including olfactory bulb, cortex, hippocampus, septum, striatum and thalamus. The mesencephalon and the hindbrain showed very little immunostaining with the exception of the substantia nigra (Fig. 2A). The cerebellum showed a distinct immunostaining pattern that is described in detail below (Fig. 2A,B). α -synuclein immunostaining was strictly associated with neurons, most specifically with axonal terminals (Fig. 2C–E); glial cells were not labeled.

α -synuclein antibodies revealed a distinct staining pattern in the cerebellar cortex (Fig. 2A,B). The posterior cerebellum, especially the vestibulo-cerebellum (nodulus and uvula) showed intense immunolabeling, while the immunolabeling of the anterior cerebellum was moderate-to-faint. In both areas the immunostaining was localized to fibers and presynaptic terminals in the molecular and granule cell layers (Fig. 2B–E). We did not detect staining in the somata of any cerebellar neurons. To further identify the terminals displaying α -synuclein labeling we employed double immunolabeling using cell-type specific antibodies recognizing different cerebellar markers. VGluT1 and VGluT2 were used to identify excitatory glutamatergic terminals of the granule cells and climbing fibers, respectively, in the molecular layer, as well as MFTs in the granule cell layer [24–26]. GAD67 immunolabeling was used to identify inhibitory terminals of GABAergic neurons including Purkinje cells, Golgi cells, stellate cells, and basket cells [27]. Finally, Glyt2 immunolabeling was used to identify inhibitory glycinergic terminals of Golgi cells [27]. Double immunolabeling experiments revealed that α -synuclein is exclusively associated with VGluT1-positive (VGluT1⁺) excitatory terminals (Fig. 3).

α -synuclein in granule cell synaptic terminals of the molecular layer

At higher magnification, immunostaining with α -synuclein antibody revealed a homogenous punctate-like labeling of synaptic terminals (Fig. 3A–D). When sections were co-labeled with antibodies recognizing VGluTs we observed extensive co-localization of α -synuclein with VGluT1 in the granule cell terminals (Fig. 3A) but not with VGluT2, which labeled the synaptic terminals of climbing fibers (Fig. 3B). GAD67 antibody labeled the terminals of inhibitory neurons, but none of these terminals contained α -synuclein (Fig. 3C). Similarly, GlyT2⁺ terminals were free of α -synuclein labeling (Fig. 3D). Based on these results we conclude that α -synuclein is expressed by granule cells and that the protein is preferentially accumulated in the synaptic terminals.

α -synuclein in MFTs of the granule cell layer

In the granule cell layer α -synuclein was distinctly associated with the MFTs (Figs. 2C–E and 3E–H). All α -synuclein-positive MFTs contained VGluT1 but not all VGluT1⁺ MFTs contained α -synuclein. Figure 4 illustrates the differential distribution of the α -synuclein-positive and VGluT1⁺ MFTs. In the nodulus and ventral uvula (folium IXc) most of the MFTs displayed both proteins (Fig. 4A), while in other lobules the frequency of double labeling varied greatly (Fig. 4B–D). High densities of VGluT1⁺/ α -synuclein⁺ MFTs were observed in lobules I, (Fig. 4D) II, VIb and VII, while in other lobules only about 1/4 of VGluT1⁺ MFTs displayed α -synuclein (Fig. 4B). We have previously described a narrow area between folium IXa and IXb, which is defined by the absence of UBCs and their

terminals [18]. This “no UBC zone” was also easily recognized in α -synuclein immunolabeled sections by the obvious lack of immunolabeling (Figs. 2A,B, 4B).

Distribution of VGluT1 and VGluT2 is complementary in most brain structures with the exception of a few brain areas in which neurons co-expressing both proteins were reported [28–31]. Among the neuronal structures in which these two transporters colocalize are the MFTs in the cerebellum [24, 25, 29]. Notably, only a subset of MFTs contains both VGluT1 and VGluT2 and the frequency of these double labeled MFTs varies in different cerebellar lobules [25]. In line with these observations, we found that most MFTs displayed either α -synuclein or VGluT2 staining (Figs. 3F, 5). Only a small subset of α -synuclein⁺ MFTs displayed a faint VGluT2 immunostaining (Fig. 5). In lobule X we observed very few double immunostained α -synuclein⁺/VGluT2⁺ MFTs (Fig. 5A), while the double labeled terminals were more frequent in lobules I, VIb and VIII (Fig. 5B,C). Because all α -synuclein-positive MFTs contained VGluT1, it can be inferred that the observed α -synuclein⁺/VGluT2⁺ MFTs probably also contain VGluT1. GABAergic and glycinergic terminals in the granule cell layer did not contain α -synuclein (Fig. 3G and 3H).

α -synuclein in cerebellar UBCs

Our data show that α -synuclein is associated with excitatory terminals including a subset of MFTs in the granule cell layer. The cerebellar granule cell layer contains two distinct mossy fiber systems; the canonical mossy fiber system of afferent fibers originating from the precerebellar nuclei, and the intrinsic mossy fiber system formed by the axons of UBCs [17]. UBC axons often terminate in the same lobule where their parent soma are located [17, 32] and therefore the vestibulo-cerebellum contains the highest concentration of intrinsic MFTs. Nunzi and Mugnaini [21] estimated that about 50% of MFTs in the vestibulo-cerebellum are provided by UBCs.

The distribution of α -synuclein labeling in the granule cell layer of the vestibulo-cerebellum was reminiscent of the typical UBC distribution pattern (compare Figs. 2A,B and 6A; see also [18]) e.g. intense immunolabeling in nodulus and folium IXc, moderate labeling in folium IXb and faint-to-moderate immunolabeling in the rest of cerebellum with the exception of the transition zone between IXa and IXb, where the immunolabeling was absent (Figs. 2A,B and 6C,D). MFTs of both UBC types contain VGluT1 [26]. In the nodulus, α -synuclein and VGluT1 co-localized in MFTs (Fig. 4A) indicating the presence of α -synuclein in UBC terminals. UBCs are classified in two subclasses on the basis of size, distribution, electrophysiological properties and expression of selective markers [18, 33, 34]. To identify the UBC population expressing α -synuclein we employed double immunostaining with UBC type specific markers. Type I UBCs were visualized by calretinin antibody (Figs. 6C, 7A,B). This immunostaining resulted in labeling of all cytoplasmic compartments including the soma, dendritic brush, axon and MFTs (Fig. 7B₂). Notably α -synuclein showed a striped pattern in folium IXc that was in register with UBC rich stripes visualized by calretinin immunolabeling (Fig. 6B,C; [18, 35]) as well as with HSP25⁺ Purkinje cell bands (Fig. 6D; [36, 37]). Double labeling experiments revealed that all calretinin⁺ MFTs contained α -synuclein. These double labeled MFTs represented only about 1/3 of all α -synuclein⁺ MFTs. Type II UBCs were labeled with mGluR1 α antibody

(Fig. 7C) and although this antibody is a reliable marker for type II UBCs, it only labeled their dendritic brushes and somata (Fig. 7C₂); thus in double immunostaining experiments α -synuclein-labeled terminals could not be unequivocally assigned to type II UBCs (Fig. 7C). However, the presence of numerous α -synuclein⁺/calretinin⁻/VGluT1⁺ MFTs indicates that not only type I but also type II UBCs contain α -synuclein.

α -synuclein in MFTs of type I and type II UBC types *in vitro*.

Our data suggest that both UBC types express α -synuclein. Yet we cannot exclude that the α -synuclein⁺/calretinin⁻ (also VGluT1⁺) MFTs represent extrinsic mossy fibers and thus do not belong type II UBCs.

To address this problem we utilized organotypic slice cultures prepared from the P8-P10 cerebellar nodulus. In these cultures, the extrinsic mossy fibers degenerate while both types of UBCs survive long-term – up to 30 DIV – and form an intricate mossy fiber network [21, 26]. In the current study nodular slices were cultured for 22–24 DIV. Immunohistochemical analysis confirmed that both UBC subtypes were present in these cultures. Type I and type II UBCs were visualized by calretinin (Fig. 8A) and mGluR1 α (Fig. 8B) immunostaining, respectively. Consistent with our tissue staining experiments, we found that calretinin labeled the MFTs of type I UBCs. These terminals also contained α -synuclein (Fig. 8A). However, the majority of α -synuclein⁺ MFTs lacked calretinin immunolabeling. Because in these cultures the only source of MFTs are the UBCs, we can conclude that both UBC types contain α -synuclein.

α -synuclein in UBC deficient moonwalker mouse

Our experiments show expression of α -synuclein in type II UBCs *in vitro*, however neurons under artificial conditions may express proteins that they do not produce *in vivo*. For example, type I UBCs were shown to contain VGluT2 in organotypic cultures [26], yet we were unable to confirm this finding in cerebellar cryosections. In lobule X only a few calretinin⁺ MFTs contained faint VGluT2 immunolabeling (not shown). This result is consistent with the ISH data published on the Allen brain atlas [15] showing no VGluT2 ISH signal in the cerebellum. This observation prompted us to further investigate the presence of α -synuclein in type II UBCs *in vivo*.

To this end, we took advantage of *Mwk* mutant mice, in which type II UBCs are completely ablated by 1 month of age (in Fig. 9 compare panel A with panel B; [20]). Type I UBCs are still found in these mice, but their number is reduced dramatically (in Fig. 9 compare panel C with panel D; [20]). When cerebellar sections from *Mwk* mice were labeled with α -synuclein we found a dramatic decrease in α -synuclein⁺ MFTs (Fig. 9E–H) in lobule X and ventral IX. The distribution of the α -synuclein MFTs in these lobules was in register with the distribution of calretinin⁺ UBCs and their terminals (compare panels F and D in Fig 9). Double immunostaining with calretinin and α -synuclein revealed an almost complete co-localization of these two proteins in the remaining MFTs (Fig.10). These results further support the conclusion that both cell types – type I and type II UBCs – express α -synuclein.

Discussion

This study shows that α -synuclein is present in cerebellar glutamatergic terminals. In the molecular layer α -synuclein is present in the granule cell terminals while in the granule cell layer α -synuclein is associated with MFTs. Moreover we show that α -synuclein is especially enriched in the intrinsic MFTs (the terminals of UBC axons). Consistent with this observation, α -synuclein is dramatically decreased in the vestibulo-cerebellum of the *Mwk* mouse, a model of cerebellar ataxia in which UBCs are almost completely ablated.

Previously it has been shown that α -synuclein containing terminals are localized in both the molecular and the granule cell layers [24, 25, 31], yet the origin of these terminals had not been determined. Here we took advantage of cell type specific markers recognizing glutamatergic (VGluT1 and VGluT2; VGluT3 is not present in the cerebellum [38]), GABAergic and glycinergic terminals to identify the nature of the synuclein-positive terminals in the cerebellum. We found that only the glutamatergic terminals contain α -synuclein. Similar findings were reported in the hippocampus where the excitatory, but not the inhibitory, neurons were found to express α -synuclein [16]. Our data showing that synuclein is selectively present in glutamatergic terminals supports previous findings showing that α -synuclein promotes SNARE complex formation at the presynaptic membranes [10, 12]. Importantly, the SNARE proteins are not expressed in GABAergic terminals (with the exception of the terminals of basket cells [39]) and the soluble NSF attachment protein-25 (SNAP-25, a component of the SNARE complex) has been also associated with glutamatergic but not GABAergic synapses [40, 41].

Intriguingly we found that VGluT1 is coexpressed with α -synuclein in cerebellar synaptic terminals. On the other hand, VGluT2 expressing climbing fibers and a majority of MFTs do not contain α -synuclein. Our data indicate that the α -synuclein⁺/VGluT2⁺ MFTs represent a subset of the VGluT1⁺ MFTs based on pieces of evidence: 1) all α -synuclein⁺ MFTs express VGluT1, and 2) VGluT1 and VGluT2 are coexpressed in a small subset of MFTs [24, 25, 29]. VGluT1 and VGluT2 exhibit a complementary expression pattern in the CNS with few exceptions [24, 31]. However the physiological significance of the differential expression of these transporters is still unknown. Freneau and colleagues [28] suggested that the glutamate transporters differentially affect glutamate packaging and release. They proposed that VGluT1 expression is dominant in synapses with low probability release, like in the parallel fibers, while VGluT2 expression may correlate with high probability release, for example in climbing fibers [42]. Our data however show that MFTs are heterogeneous with regard to VGluT expression, as they can express VGluT1, VGluT2 or, in a small subset, both these transporters. Thus, it may be suggested that release probability varies between different MFTs; this latter hypothesis is in line with the observation of large fiber-to-fiber variation in MF synaptic strength [43]. Interestingly, in the vestibulocerebellum of *Mwk* mice, in which UBCs are absent, VGLUT1 expression is decreased, thus suggesting that intrinsic MFTs preferentially express this transporter, which may suggest that intrinsic MFs form synapses with lower release probability than extrinsic MFTs. α -synuclein, however, is also enriched in UBC axons. It was suggested that loss of α -synuclein reduces synaptic efficacy at the Schaeffer collateral-CA1 synapse [44], implying that its presence in presynaptic sites correlates with high release probability. However, further targeted

electrophysiological recordings will be required to ascertain whether intrinsic UBC MFs are high or low probability synapses.

Our data, on the other hand, may indirectly provide some hints as to the functional roles of UBCs. MSA-C is characterized by synuclein inclusions in glia as well as by neuronal cell death and ubiquitin-positive neuritic degeneration, which is particularly pronounced in the vermis [45] a UBC-rich area [18]. Because UBC terminals are enriched in α -synuclein, it may be suggested that, similar to the moonwalker ataxia [20], MSA-C may also be characterized by severe UBC loss. It would be interesting to test this hypothesis on human samples. A positive finding may further support the idea that UBC loss is important for the development of ataxia.

Acknowledgments

This study was funded by NIH grant NS09904 (MM) and NS089664 (RVS).

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Figure 1.

Specificity of α -synuclein antibodies. Cerebellar cryosections prepared from wild-type (A–D;I) and α -synuclein KO (E–H) mice immunostained with α -synuclein antibodies raised in mouse (Ms; Encor Biotechnology), sheep (Sh; Santa Cruz Biotechnology), and guinea pig (GP; Calbiochem), and pan-synuclein antibody raised in rabbit (Rb). A–D, In wild-type mice all the tested antibodies showed a similar staining pattern. A punctate like immunostaining in the molecular layer (ml), MFT staining in the granule cell layer (gcl) and no staining in the Purkinje cell layer (Pcl). E–H, No immunolabeling is detected in the α -synuclein KO sections using antibodies raised in mouse (E), sheep (F) and rabbit (H). Antibody raised in guinea pig showed the same staining pattern in the α -synuclein KO and the wild-type mouse (G). I₁₋₃, Colocalization of mouse anti- α -synuclein and rabbit anti-pan-synuclein antibodies in the same MFTs. Scale bars: A–H 50 μ m; I₁₋₃ 20 μ m

Figure 2.

α -synuclein immunolabeling in wild-type mouse brain. A–C, Parasagittal cryosection labeled with an immunoperoxidase protocol using mouse anti- α -synuclein antibody (Encor Biotechnology). A, Intense immunolabeling is present in the forebrain structures: olfactory bulb (OB), cortex (Cx), septum (Sep), striatum (Str), hippocampus (Hip), and thalamus (Th). The midbrain and the hindbrain contain very little immunostaining with exception of substantia nigra (SN) and cerebellum (Cer). In the cerebellum, α -synuclein show a distinct pattern, with more intense immunolabeling in the posterior cerebellum (lobule VIIb–X) than in the anterior cerebellum (lobule I–VIa). The UBC free zone (asterisk) is not labeled with α -synuclein antibody. SC, superior colliculus; IC, inferior colliculus. Anterior (A) and posterior (P) directions are indicated by the double-headed arrow. B, α -synuclein immunostaining in the nodulus (lobule X) and uvula (lobule IX) show intense immunostaining in the granule cell layer (gcl) of nodulus and folium IXc. In folium IXb the immunolabeling is less intense; folium IXa and lobule VIII show only moderate immunostaining. The transition zone between IXa and IXb contains no α -synuclein immunolabeling (asterisks; area delineated with dash lines). ml, molecular layer. C, α -synuclein⁺ MFTs in gcl of nodulus (arrowheads). D,E, Comparison of α -synuclein immunolabeling between lobule X and VIII; immunofluorescence staining using mouse anti- α -synuclein antibody. The MFTs (arrowheads) in gcl of lobule X are clearly recognizable by intense α -synuclein immunolabeling. In lobule VIII the α -synuclein⁺ MFTs (arrows) are smaller and with moderate immunolabeling. Scale bars: A 1mm; B 200 μ m; C 10 μ m; D,E 20 μ m

Figure 3.

α -synuclein colocalizes with VGluT1 in cerebellar synaptic terminals. Double immunolabeling with α -synuclein (Encor Biotechnology) and cell type specific marker antibodies. Images illustrate the immunostaining pattern in the molecular (A–D) and granule cell layer (E–H) of the nodulus. A–D, In the molecular layer the α -synuclein immunolabeling shows a distinct punctate immunolabeling of synaptic terminals (A₁, B₁, C₁, D₁). VGluT1 (A₂) and VGluT2 (B₂) immunostaining were used to visualize the excitatory synaptic terminals of granule cells and climbing fibers, respectively. GAD67 (C₂) and GlyT2 (D₂) label the inhibitory GABAergic and glycinergic terminals. Merged images in panels A₃, B₃, C₃ and D₃ show that only the VGluT1⁺ granule cell terminals contain α -synuclein. E–H, In the granule cell layer the α -synuclein immunolabeling is exclusively localized to MFTs (E₁, F₁, G₁, H₁). VGluT1 (E₂) and VGluT2 (F₂) antibodies also label MFTs, while GAD67 (G₂) and GlyT2 (H₂) antibodies label the GABAergic and glycinergic terminals. Merged images in panels E₃, F₃, G₃ and H₃ show that only the VGluT1⁺ MFTs contain α -synuclein. *Scale bars*: A–H 5 μ m

Figure 4. Distribution of α -synuclein⁺ and VGluT1⁺ MFTs in different cerebellar regions. A–D, Single color images illustrate the distribution of the α -synuclein⁺ (A₁–D₁) and VGluT1⁺ (A₂–D₂) MFTs. Merged images (A₃–D₃) show that all α -synuclein-labeled MFTs contain VGluT1 (arrowheads) but not all VGluT1⁺ MFTs contain α -synuclein (arrows). In the nodulus (lobule X) almost all MFTs display both proteins (A). In contrast the transition area between folium IXa and IXb (no UBC zone) lacks α -synuclein labeling (B). Scale bars: A–H 20 μ m

Figure 5.

Distribution of α -synuclein⁺ and VGluT2⁺ MFTs in different cerebellar regions. A–C, Single color images illustrate the distribution of the α -synuclein⁺ (A₁–C₁) and VGluT2⁺ (A₂–C₂) MFTs. Merged images (A₃–C₃) show that most MFTs display either α -synuclein (crossed arrow) or VGluT2 (arrows). Lobule X (A₃) contains only a few α -synuclein⁺ and VGluT2⁺ double labeled MFTs (arrowhead). About half of the α -synuclein⁺ MFTs are VGluT2⁺ in lobule VIII (B₃) while in lobule I (C₃) most MFTs contain both proteins. *Scale bars: A–H 20 μ m*

Figure 6. α -synuclein is expressed in zonal compartments in the adult mouse cerebellum. A, distribution map of UBC in mouse cerebellum. The highest densities of both UBC types are found in lobule X and folium IXc (red area). Folium IXb contains mostly type II UBCs (green area). Few UBCs are found in the rest of the cerebellar lobules (blue colored areas) with exception of transition zone between folium IXa and IXb where no UBCs are found (black area; arrow). Anterior (A) and posterior (P) directions are indicated by double headed arrow. B, Schematic diagram of the cerebellum shown in wholemount. The red box denotes the region shown in panels C and D. C,D, α -synuclein-labeled zonal “clusters” in the granular layer of lobule IX (C_1 , D_1) align with either calretinin immunoreactive UBC clusters (C_2 , C_3) or HSP25⁺ Purkinje cell zones (D_2 , D_3). Notably, the boundaries of the α -synuclein⁺ clusters are not as sharply defined as the Purkinje cell zones (D_3). The midline and lateral zones are labeled as 1 and 2, respectively (arrowheads). Scale bar, 125 μ m.

Figure 7.

α -synuclein in the axonal terminals of UBCs. A,B, Type I UBCs (arrows) are visualized with calretinin immunolabeling. This staining afforded labeling of all UBC compartments (B₂); soma (arrows), dendritic brush (br) and axon including the synaptic terminals (MFTs; blue arrowheads). All calretinin⁺ MFTs contain α -synuclein (blue arrowheads), however many α -synuclein⁺ MFTs (white arrowheads) do not express calretinin. C, Type II UBCs are visualized by mGluR1 α immunolabeling. This staining labels only the soma (arrows) and the dendritic brush (br). Double immunolabeling with α -synuclein shows the presence of α -synuclein⁺ MFTs (arrowheads) in areas that are rich with type II UBCs. *Scale bar*, A₁–A₃ 20 μ m; B₁–B₃ 10 μ m; C₁–C₃ 10 μ m

Figure 8.

α -synuclein is expressed in intrinsic MFTs *in vitro*. Organotypic slice-cultures of the mouse nodulus grown for 24 DIV, and double immunolabeled with α -synuclein (Encor Biotechnology) and UBC type specific markers. A,B, Type I UBCs (arrows) are visualized with calretinin immunolabeling. This staining visualizes all UBC compartments (B₂); soma (arrows), dendritic brush (br) and axon including the synaptic terminals (MFTs; blue arrowheads). All calretinin⁺ MFTs contain α -synuclein (blue arrowheads), however many α -synuclein⁺ MFTs (white arrowheads) do not express calretinin. C, Type II UBC are visualized by mGluR1 α immunolabeling. This staining labels only the soma (arrows) and dendritic brush (br) of UBCs. Double immunolabeling with α -synuclein shows MFTs (arrowheads) in areas that are rich with type II UBCs. *Scale bar*, A₁-A₃ 20 μ m; B₁-B₃ 10 μ m; C₁-C₃ 10 μ m

Figure 9.

Reduced α -synuclein immunolabeling in 1 month-old *Mwk* mutant mouse correlates with the loss of UBCs. A-D, mGluR1 α (A,B) and CR (C,D) immunoperoxidase labeling of type II UBCs and type I UBCs, respectively. Comparison of wild-type (A,C) and *Mwk* (B,D) mice coronal sections taken from the same brain areas reveals the absence of GluR1 α ⁺ type II UBCs (B) and severe depletion of calretinin⁺ type I UBCs (D) from the granule cell layer (gcl) of the nodulus (lobule X) and folium IXc. The Purkinje cells arbor in the molecular layer (ml) show distinct mGluR1 α labeling (A,B). E-H, α -synuclein immunofluorescence labeling of wild-type (E,G) and *Mwk* (F,H) mice. The *Mwk* nodulus (F,H) contains significantly less α -synuclein⁺ MFTs than the wild-type nodulus (E,G). G and H are enlarged magnifications of the boxed images in E and F, respectively. Scale bar, A-F 500 μ m; G,H 100 μ m

Figure 10.

α -synuclein and calretinin are colocalized in MFTs in the nodulus of 1month- old *Mwk* mutant mouse. At this age, only a few calretinin⁺ UBCs (arrows) are present. Arrowheads point to α -synuclein⁺/calretinin⁺ profiles in MFTs. Panel B₃ is an enlarged image of the boxed area in panel A. Scale bar, A 20 μ m; B₁–B₃ 10 μ m

Table 1

List of primary antibodies used in this study

Antibody	Species	Dilution	Immunogen	Source	Characterization References
α-synuclein	mouse	1:3,000	full length human protein epitope from amino acids 61–95	EnCor Biotechnology Cat.N.: MCA-3H9	α-synuclein KO mice*
α-synuclein	sheep	1:1,000	synthetic peptide corresponding to amino acids 108–120 human α-synuclein	Santa Cruz Biotechnology Cat.N.: AB5336	α-synuclein KO mice*
α-synuclein	guinea pig	1:5,000	synthetic peptide corresponding to of the C-terminus rat α-synuclein	Calbiochem Cat.N.: PC325	α-synuclein KO mice**
pan-synuclein	rabbit	1:1,000	synthetic peptide derived from human α-synuclein	Assay Biotech Cat.N.:C0336	co-labeling with mouse anti-α-synuclein***
Calretinin	mouse	1:2,000	full-length recombinant human CR	Swant, Switzerland Cat.N.:6B3	Sekerkova et al. 2014
Calretinin	mouse	1:1,000	full-length recombinant human CR	Millipore Cat.N.: MAB1568	Kim et al. 2012
Calretinin	rabbit	1:5,000	full-length recombinant human CR	Swant, Switzerland Cat.N.:7696/3H	Sekerkova et al. 2014
GAD67	mouse	1:1,000	recombinant GAD67 protein	Millipore Cat.N.:MAB5406	Nakayama et al. 2012
GLYT 2	guinea pig	1:5,000	Synthetic peptide from the C-terminus as predicted from the cloned rat GLYT2	Millipore Cat.N.:AB1173	Milanease et al. 2014
Hsp25	rabbit	1:500	Recombinant mouse Hsp25 protein	Assay Designs Enzo Life Sciences Cat.N.:SPA-801	Armstrong et al., 2000, 2001
mGluR1α	mouse	1:800	fusion protein containing sequences from C-terminus of rat mGluR1α	Pharmingen Cat.N.: 556389	Nunzi and Mugnaini 2009
mGluR1α	rabbit	1:500	peptide representing amino acid residues 945-1127 of mouse mGluR1α	Frontier Institute Co, Japan Cat.N.:Rb-A1811-1	Sekerkova et al., 2014
VGLUT1	guinea pig	1:5,000	synthetic linear peptide from rat VGLUT1 protein	Millipore Cat.N.:AB2251	Nunzi et al.2003
VGLUT2	guinea pig	1:5,000	synthetic linear peptide from rat VGLUT2 protein	Millipore Cat.N.:AB5905	Nunzi et al.2003

* Sections from SNCA KO mouse labeled with these antibodies showed no immunolabeling.

** Sections from SNCA KO mouse labeled with these antibodies showed the same immunolabeling as the wild-type animals (fig x).

*** In cerebellar sections the rabbit anti-pan synuclein showed complete colocalization with mouse anti-α-synuclein (suppl. FigX).