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Age-dependent synuclein pathology following traumatic brain injury in mice

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Abstract

Synucleins (Syn), a family of synaptic proteins, includes α -Syn, which plays a pivotal role in Parkinson's disease and related neurodegenerative diseases (synucleinopathies) by forming distinct brain pathologies (Lewy bodies and neurites). Since traumatic brain injury (TBI) is a poorly understood risk factor for Parkinson's disease, we examined the effects of TBI in the young and aged mouse brain on α -, β -, and γ -Syn. Immunohistochemical analysis showed that brains from sham-injured young and aged mice had normal α - and β -Syn immunoreactivity (IR) in neuropil of cortex, striatum, and hippocampus with little or no γ -Syn IR. At 1 week post TBI, the aged mouse brain showed a transient increase of α - and β -Syn IR in the neuropil as well as an induction of γ -Syn IR in subcortical axons. This was associated with strong labeling of striatal axon bundles by antibodies to altered or nitrated epitopes in α -Syn as well as by antibodies to inducible nitric oxide synthase. However, these TBI-induced changes disappeared by 16 weeks post TBI, and altered Syn IR was not seen in young mice subjected to TBI nor in α -Syn knockout mice while Western blots confirmed that TBI induced transient alterations of α -Syn in the mouse brains. This model of age-dependent TBI-induced transient alterations in α -Syn provides an opportunity to examine possible links between TBI and mechanisms of disease in synucleinopathies.

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Keywords: Traumatic brain injury; Mouse brain; Neurodegenerative disease; Synuclein

Introduction

Synuclein (Syn) proteins are directly implicated in human diseases since α -Syn gene mutations have been identified in rare kindreds with familial Parkinson's disease (PD) (Polymeropoulos et al., 1997; Kruger et al., 1998), and α -Syn inclusions occur in hereditary and sporadic PD

(Arima et al., 1998; Tu et al., 1998; Arawaka et al., 1998; Spillantini et al., 1997, 1998; Irizarry et al., 1998; Baba et al., 1998; Duda et al., 2002). Previous studies have shown that α -Syn is the primary building block of filamentous Lewy bodies (LBs) and dystrophic Lewy neurites in PD and related disorders known as synucleinopathies (Baba et al., 1998; Giasson et al., 2000b). In addition, α -Syn forms filaments in vitro and α -Syn transgenic animals develop LB-like inclusions associated with neurodegeneration (Auluck et al., 2002; Conway et al., 1998; Feany and Bender 2000; Masliah et al., 2000; Giasson et al., 2000a; Paxinou et al., 2001). Moreover, α -Syn in human synucleinopathy brains is pathologically altered by ubiquitination, phosphor-

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Table 1
List of antibodies

Antibodies	Epitope/antigen	Dilution	Species	Reference
Syn202	α - and β -Syn	1:20K	Mouse	Giasson et al., 2000b
SNL-1	α -Syn (104–119)	1:5K	Rabbit	Giasson et al., 2000b
Syn207	β -Syn	1:20K	Rabbit	Giasson et al., 2000b
β -2	β -Syn	1:2K	Rabbit	This study
Syn102	α -, β -Syn, C-terminus (130–140)	1:1K	Mouse	Giasson et al., 2000b
Syn119	α -Syn, NAC	1:1K	Mouse	This study
Syn205	α -, β -Syn, C-terminus (130–140)	1:1K	Mouse	Giasson et al., 2000b
Syn214	α -, β -Syn, C-terminus (130–140)	1:1K	Mouse	Giasson et al., 2000b
Syn303	Conformational α - > β - > γ -Syn (α -Syn N-terminus)	1:500	Mouse	Duda et al., 2002
Syn505	Conformational α - > β - > γ -Syn (α -Syn N-terminus)	1:500	Mouse	Duda et al., 2002
Syn506	Conformational α - > β - > γ -Syn (α -Syn N-terminus)	1:500	Mouse	Duda et al., 2002
Syn514	Conformational α - > β - > γ -Syn (α -Syn N-terminus)	1:500	Mouse	Duda et al., 2002
γ -1	γ -Syn	1:1K	Mouse	Giasson et al., 2001
nSyn14	Nitrated α -Syn	Neat	Mouse	Giasson et al., 2000a
iNOS-2	iNOS	1:50	Rabbit	Santa Cruz Biotech
LN-39	APP	1:50	Mouse	Nakagawa et al., 2000
NFL	NFL	1:2k	Rabbit	Ishihara et al., 1999

ylation, nitration, and conformational modifications (Duda et al., 2002; Giasson et al., 2000a; Fujiwara et al., 2002).

Mechanistic insights into disease processes underlying PD and related disorders have evolved substantially since the link between these diseases and α -Syn pathology was discovered, but it is not yet known how normal α -Syn is transformed from a highly soluble synaptic protein into filamentous perikaryal aggregates in the disease state (Trojanowski and Lee., 2002). Although the genetic determinants of late-onset PD are uncertain (Tanner et al., 1999), traumatic brain injury (TBI) has been identified as an environmental risk factor for sporadic PD (Nayernouri, 1985; Factor and Weiner, 1991; Stern, 1991), in addition to exposure to neurotoxins (Langston et al., 1983; Hubble et al., 1993; Przedborski and Jackson-Lewis, 1998; Betarbet et al., 2000). Although the brains of patients with dementia pugilistica are characterized by plaques and tangles similar to those seen in Alzheimer's disease (AD), the abnormal accumulation of α -Syn in axonal swellings and dystrophic neurites in these brains suggests a possible link between TBI and the development of α -Syn pathology in PD (Newell et al., 1999), which could be mediated by TBI-induced oxidative and nitrative stress (Mesenge et al., 1988). Indeed, experimental TBI has been shown to cause oxidative stress in wild-type (WT) mice (Pratico et al., 2002) and in Tg mouse models (Tg2576) of AD-like amyloidosis (Uryu et al., 2002). Further, TBI-induced oxidative stress was associated with an augmentation of A β levels and deposits in the brains of Tg2576 mice (Uryu et al., 1993). Thus, emerging data indicate that TBI causes chronic as well as acute neurodegeneration (Maxwell et al., 1997; McIntosh et al., 1998; Uryu et al., 2002). Nonetheless, experimental evidence directly linking TBI to the development of α -Syn pathology or any other abnormalities in PD and related disorders has not been obtained.

Thus, we addressed this issue by examining the effects of

TBI on the expression of α -, β -, and γ -Syn in WT mice subjected to controlled cortical impact (CCI) injury, and our studies revealed that TBI induces transient nitrative alterations in α -Syn similar to those seen in PD and related synucleinopathies.

Materials and methods

Antibodies

The panel of antibodies to α -, β -, and γ -Syn as well as to other proteins studied here is summarized in Table 1 together with the provenance and citations on the characterization and properties of these antibodies.

Animals and injury

The CCI paradigm for TBI was applied to 4-month-old (young) ($n = 35$) and 24-month-old (aged) WT mice ($n = 25$) with (B6D2F1 background) and to 16-month-old α -Syn knockout (KO) mice ($n = 6$; kindly provided by Dr. Asa Abeliovich) (Abeliovich et al., 2000). The procedures for CCI have been described in detail elsewhere (Murai et al., 1998; Nakagawa et al., 1999, 2000). Briefly, an anesthetized mouse was mounted on the stereotactic frame, the scalp and temporal muscle were reflected, and 5-mm craniectomy was performed (centered between the lambda and bregma over the left parietal cortex) with the dura remaining intact. CCI brain injury was produced as modified from the original paradigm described by Dixon et al. (1991) by using a 3-mm metal impounder driven at a velocity of 5.0 m/s to a depth of 1.0 mm over a time frame of 100 ms as described (Murai et al., 1998; Nakagawa et al., 1999, 2000). A transducer attached to the device provided an analog signal recorded by a computer program (R.C. Electronics, Santa Barbara, CA)

for monitoring impact parameters. Uninjured control mice were surgically prepared in a similar manner, but they were not subjected to CCI injury. After CCI brain injury, the craniotomy was covered with a cranioplasty and the scalp was sutured. All mice were allowed to recover on a heating pad maintained at 37°C.

Sham and CCI mice were allowed to survive for various time periods, as follows: young mice for 1, 3, 5 days, and 1, 9, and 16 weeks post injury, aged mice for 1, 9, and 16 weeks post injury, and aged α -Syn KO mice for 1 week. All of these procedures were performed in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and they were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Immunohistochemistry

For immunohistochemical analyses, animals were deeply anesthetized and were perfused with saline followed by 10% neutral buffered formalin (NBF). The brain was removed and fixed overnight with fresh 10% NBF, embedded in paraffin, and a series of 6- μ m sections were cut for immunohistochemistry (IHC) with and without the use of antigen retrieval procedures as described (Uryu et al., 2002; Murai et al., 1998; Nakagawa et al., 1999, 2000). Sections from some mouse brains were pretreated prior to IHC with anti-mouse IgG Fab (30 μ l/ml) (Jackson ImmunoResearch Lab, Inc.) in donor horse serum (DHS) for 30 min to eliminate background due to residual endogenous mouse IgG. Sections were incubated with primary antibodies (see Table 1) overnight and with an appropriate biotinylated secondary antibody for 1 h. The avidin-biotin complex method was used according to instructions of the vendor (Vector Lab, Inc.) with 3,3'-diaminobenzidine as chromogen. The sections were counterstained with hematoxylin, dehydrated in series of ethanol, cleared in xylene, and coverslipped with paramount.

Colocalization of α -Syn with neurofilament (NF) subunits, or with A β precursor proteins (APP), was examined by applying double fluorescence IHC (FIHC). For Syn303 to α -Syn and the rabbit antiserum NFL to the low M_r NF subunit (NFL), double FIHC was performed by incubating sections with a mixture of the mouse monoclonal antibody (mAb) Syn303 and polyclonal NFL antiserum overnight and subsequently done with a mixture of Alexa 488-anti-mouse IgG and Alexa 594-anti-rabbit IgG (Molecular Probes, Inc.) for 2 h. For double FIHC using mAb Syn303 and mouse mAb LN-39 to APP, single FIHC was conducted with Syn303, which was visualized with Alexa 488-anti-mouse IgG followed by blocking sections with anti-mouse IgG Fab in DHS (30 min). Subsequently, another round of single FIHC was carried out by using the LN-39 mAb,

which was visualized by Alexa 594-anti-mouse IgG. Then, the sections were treated with 3% of Sudan Black for 10 min (Romijn et al., 1999) to suppress autofluorescence and mounted with Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Laboratories).

For all of IHC experiments, negative controls included the application of the same IHC protocols to sections using preimmune serum or DHS instead of primary antibody. Analysis of stained sections was done by bright-field and fluorescence microscopy by using an Olympus BX51 attached to a CCD camera (ProgResC14, Jenoptik Camera Europe Inc., Jena, Germany).

Western blot studies

For sequential biochemical fractionation and Western blot studies of α -Syn, selected unfixed brains were removed and frozen with dry ice powder. Cortical and subcortical regions subjacent to the impact site were excised from the left hemisphere of the CCI mice and equivalent areas from the right hemisphere also were excised for parallel studies as controls. Each sample was weighed and homogenized in 3 ml/g of high salt (HS) buffer (50 mM Tris, pH 7.5, 750 mM NaCl, 5 mM EDTA) and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl and 1 mg/ml each of pepstatin, leupeptin, *N*-tosyl-L-phenylalanyl chloromethyl ketone, *N*-tosyl-L-lysine chloromethyl ketone, and soybean trypsin inhibitor). The samples were sedimented at 100,000 $\times g$ for 20 min each. Pellets were reextracted with HS buffer followed by two sequential extractions with 3 ml/g of HS buffer containing 1% Triton X-100 (HS/T fraction). The pellets were homogenized in 500 ml of HS buffer/1 M sucrose and after centrifugation the floating myelin was discarded. The pellets were extracted with 2 ml/g of RIPA [50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] and sedimented at 100,000 $\times g$ for 20 min. Half of each pelleted sample was extracted with 1 ml/g SDS-sample buffer (SDS fraction) by sonication and heating to 100°C for 10 min in or 70% formic acids (FA fraction) by sonication. FA was removed by lyophilization and the dried material was resuspended in 1 ml/g of SDS-sample buffer by heating to 100°C for 10 min. Five microliters of each fraction was loaded on separate lanes of 13% polyacrylamide gels. Proteins were resolved on slab gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in buffer containing 48 mM Tris, 39 mM glycine, and 10% methanol. Membranes were blocked with a 5% solution of powdered milk dissolved in Tris-buffered saline-Tween (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20), incubated with primary antibodies followed with either a goat anti-mouse or rabbit IgG horseradish peroxidase (HRP)-conjugated an-

tibody (Jackson ImmunoResearch Lab), and visualized on films using enhanced chemiluminescence reagents (NEN, Boston, MA) or directly on the membrane with 3,3'-diaminobenzidine as the chromogen.

Results

Recognition of CCI-injured areas

APP immunoreactivity (IR) was used to detect axons damaged by CCI in brain subjacent to the impact site as described (Pierce et al., 1998). In the aged mouse at 1 week post TBI, APP IR was noted exclusively in areas ipsilateral to the site of CCI, including the somatosensory and cingulate cortex, the corpus callosum, dorsolateral thalamus striatum, globus pallidus and hippocampus, especially axons in CA3 and fimbria, but the most severely affected structures were striatal and pallidal axon bundles (Fig. 1A).

Transient effects of TBI on Syn IR in aged mouse brain

Staining patterns of α -, β -, and γ -Syn in normal mouse have been previously described (Giasson et al., 2001), and the brains of sham (uninjured) aged mice showed Syn IR consistent with this previous report, whereas the brains of mice subjected to CCI-induced TBI showed alterations in the distribution of α -, β -, and γ -Syn confined to those areas wherein APP IR was noted in damaged axons although these alterations were not as dramatic as those revealed by antibodies to APP (Fig. 1A).

In the sham-treated mouse brains, antibodies to α -Syn demonstrated light staining in the striatum (Fig. 1B) as well as in cortex, thalamus, and hippocampus (not shown). However, TBI induced by CCI caused a modest enhancement of α -Syn (SNL-1) IR between 1 and 9 weeks post injury (Fig. 1A and B), especially in the neuropil of cortex, striatum, and hippocampus (e.g., fimbria and CA3 region), but this was only evident on high magnification (Fig. 1A and B). The intensity of α -Syn IR was reduced by 16 weeks post injury to levels close to those seen in the sham-treated mice wherein no diffuse axonal injury profiles or spheroids were seen to exhibit APP IR or α -Syn IR (Fig. 1B).

As in normal mice, β -Syn (Syn207, β -2) exhibited modest IR in the neuropil of sham-treated mice in the same brain areas wherein α -Syn IR was located. However, the intensity of β -Syn IR was enhanced in the cortical neuropil near the area of impact, as well as in the subjacent striatum (Fig. 1A and B), the hilus of the hippocampus, and the dorsolateral thalamus (not shown) 1 week following TBI in aged mice, and this change also was evident only on high magnification (Fig. 1A and B). The enhanced intensity of β -Syn IR decreased at 9 weeks post injury, and by 16 weeks post injury, it was identical to the normal staining pattern (Fig. 1B); but infrequent β -Syn IR axonal spheroids were seen in axons of the corpus callosum and internal capsule (Fig. 1B, inset).

Scant γ -Syn (γ -1) IR was seen in the brains of sham mice, but it was induced by CCI in the corticofugal axons of the striatum at 1–9 weeks post injury (Fig. 1A and B). It then diminished to normal levels by 16 weeks post injury. Infrequent γ -Syn IR spheroids were seen in axons of the internal capsule and corpus callosum at 9 and 16 weeks post injury (Fig. 1B, inset).

Conformational/nitrative α -Syn alterations in aged mouse brains induced by TBI

Antibodies that recognize conformational (Syn303, Syn505, Syn506, and Syn514) and or nitrative (nSyn14) alterations of α -Syn showed no IR in the sham-treated mouse brain; but in the CCI-treated mice, these same antibodies produced increased IR at 1 week post injury restricted exclusively to the cortex near the injury site as well as axon bundles of the striatum, corpus callosum, globus pallidus, and the fimbria of brain ipsilateral to the site of CCI (Fig. 2A–C and Fig. 3). Double FIHC with Syn303 and NFL antibodies (Fig. 2C) showed that Syn303 IR colocalized with a subset of NFL-positive fibrous elements, while double FIHC using Syn303 and LN-39 antibodies revealed α -Syn IR colocalized with APP IR in damaged axons (Fig. 2D). The induction of conformational/nitrative alterations in α -Syn IR was noted only at 1 week post injury since this IR was markedly reduced or undetectable by 9 weeks post injury and beyond (Fig. 2A and B). Moreover, these transient alterations were most prominent in 24-month-old compared to 4-month-old CCI-treated mice (Fig. 3). IHC revealed that inducible nitric oxide synthase (iNOS) IR appeared in those areas of the old but not young CCI-treated mouse brains that contained conformational/nitratively altered α -Syn IR at 1 week post injury (Fig. 3), which suggests that iNOS induction may be linked to the abnormal transient nitration of α -Syn in damaged axons.

Despite that this α -Syn axonal pathology was most prominent in the 24-month-old CCI-treated mice, the brains of CCI-treated 4-month-old mice killed at 1 and 5 days post TBI showed IR for nitrated α -Syn in cortical neurons near the impact site (Fig. 4A), but antibodies to conformationally altered epitopes in α -Syn did not produce convincing IR (Fig. 4B). Similar staining patterns also were noted in aged mice (data not shown).

Finally, IHC with all of the antibodies against α -Syn used in the present study, including antibodies against conformationally and nitratively altered forms of α -Syn, failed to demonstrate any IR in aged α -Syn KO mice at 1 week post injury (Fig. 5A).

Western blot analyses of aged mouse brain following TBI

Brains from control or sham-treated WT mice and aged WT mice subjected to TBI that were examined 1 week post CCI showed comparable levels of monomeric α -Syn in Western blots of the high-salt and Triton X-100 fractions

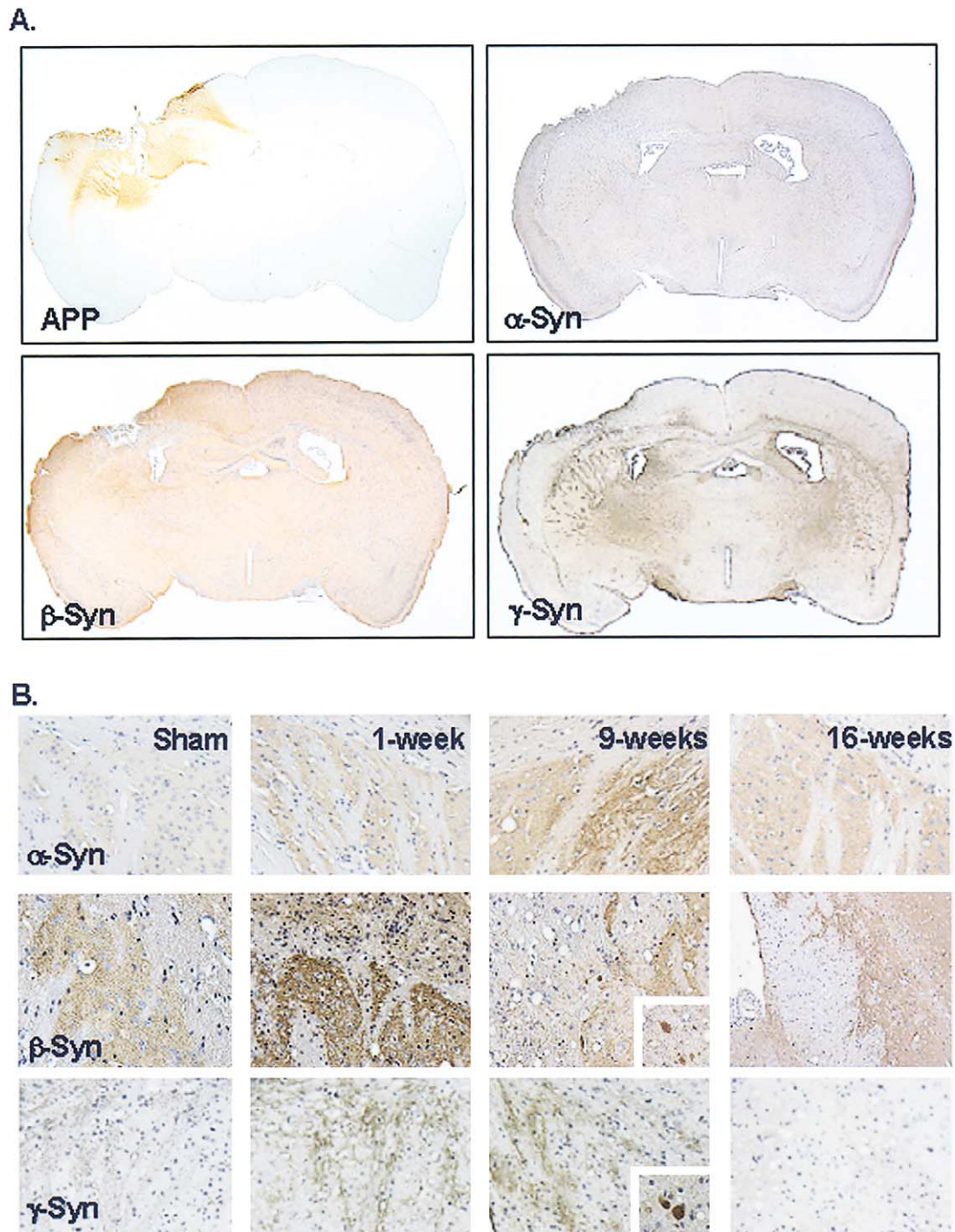


Fig. 1. In the aged wild-type mouse brain, A β precursor proteins (APP) immunoreactivity (IR) delineates the damaged area induced by controlled cortical impact (CCI) wherein altered synuclein (Syn) IR also occurs. (A) Low-power views of APP, α -, β -, and γ -Syn IR at 1 week post traumatic brain injury (TBI). APP IR identifies the area of CCI-induced damage and antibodies to α - (SNL-1), β - (Syn207), and γ -Syn (γ -1) also show variable changes in IR in the same region, but these are more evident when viewed at higher magnification as shown in B using a composite set of images. (B) High-power views of α -, β -, and γ -Syn IR to at 1, 9, and 16 weeks post TBI in the striatum. At 1 and 9 weeks post TBI, there is an increase axonal IR for γ -Syn, while only α - and β -Syn show increased neuropil IR. In addition, antibodies to β - and γ -Syn demonstrate scattered axonal white matter spheroids (inset). In contrast, at 16 weeks post TBI, the changes in Syn IR noted above have decreased to control levels.

(but not the SDS fraction) probed with Syn303 (Fig. 5B). In addition, Syn303 also recognized murine β -Syn, and it was evident that the brains from both the sham- and CCI-treated

mice also contained similar levels of β -Syn. However, using the same mAb and Western blot conditions, the brains from sham- and CCI-treated α -Syn KO mouse 1 week post injury

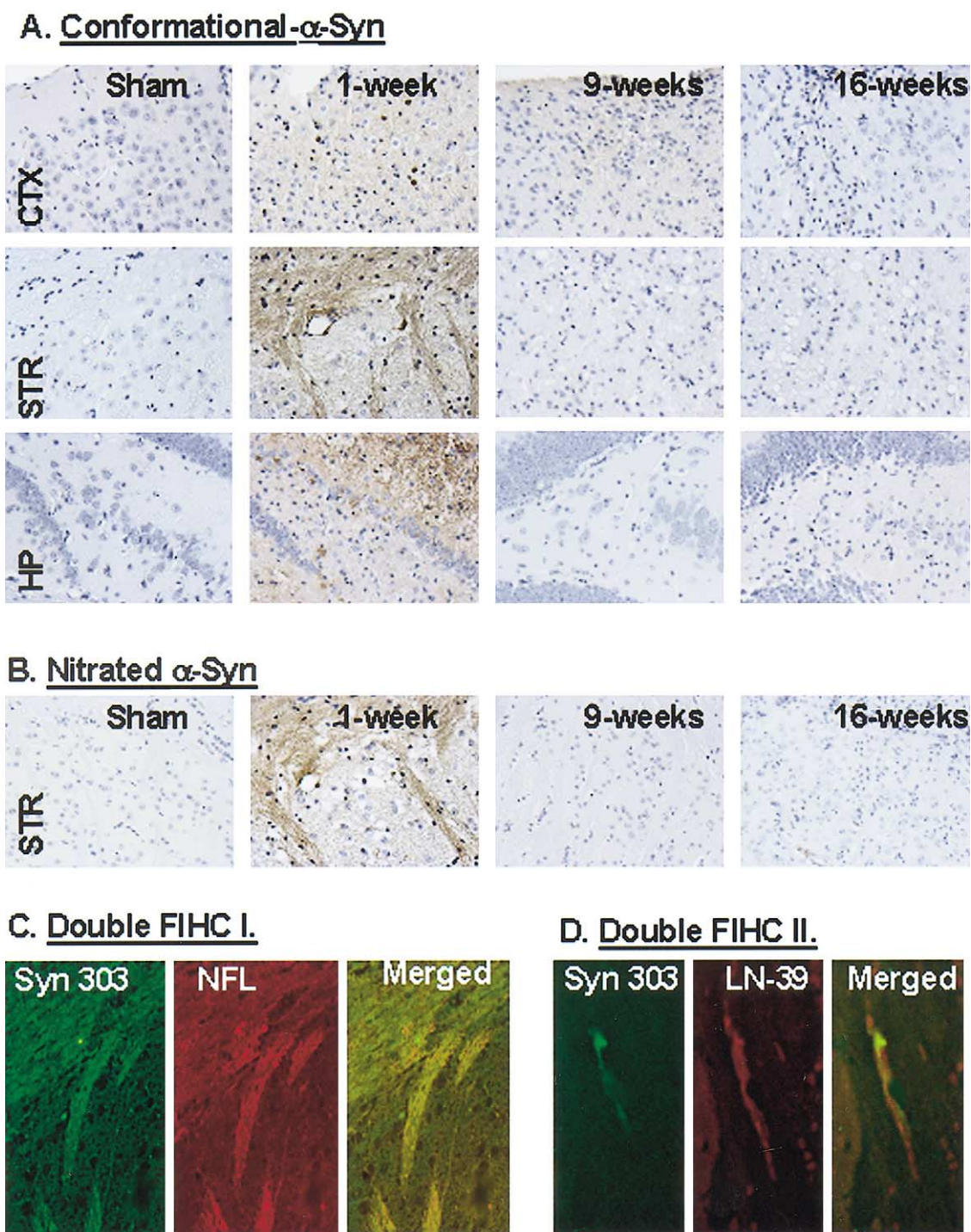


Fig. 2. In the aged mouse brain, α -Syn undergoes conformational and nitrative modifications associated with inducible nitric oxide synthase (iNOS) induction following traumatic brain injury (TBI). (A) High-power views of conformationally altered α -Syn (Syn303) immunoreactivity (IR) seen mainly at 1 week post TBI in the cortex, striatum, and hippocampus, but not in these same regions at 9 and 16 weeks post TBI or in sham-treated wild-type mice. (B) High-power views of nitrated α -Syn (nSyn14) IR at 1 week post TBI in the striatum, but not at 9 and 16 weeks post TBI. (C and D) High-power images of double fluorescence immunohistochemistry (FIHC) with anti-conformational α -Syn (Syn303) and anti-NFL antibodies (C) or α -Syn (Syn303) and APP (LN-39) antibodies at 1 week post TBI in the striatum (D). This double FIHC (see merged images) reveals that the abnormal α -Syn accumulates in axons in the controlled cortical impact-damaged area of the brain. NFL, low M_r NF subunit; APP, A β precursor protein.

showed no evidence of authentic monomeric α -Syn, although monomeric β -Syn was detected in the high-salt and Triton X-100 fractions but not in the SDS fraction (Fig. 5B).

Interestingly, 45- and 50-kDa M_r immunobands were noted in the SDS fraction of the brain from the TBI-treated WT mouse brain (Fig. 5B), but they were not seen in the SDS or

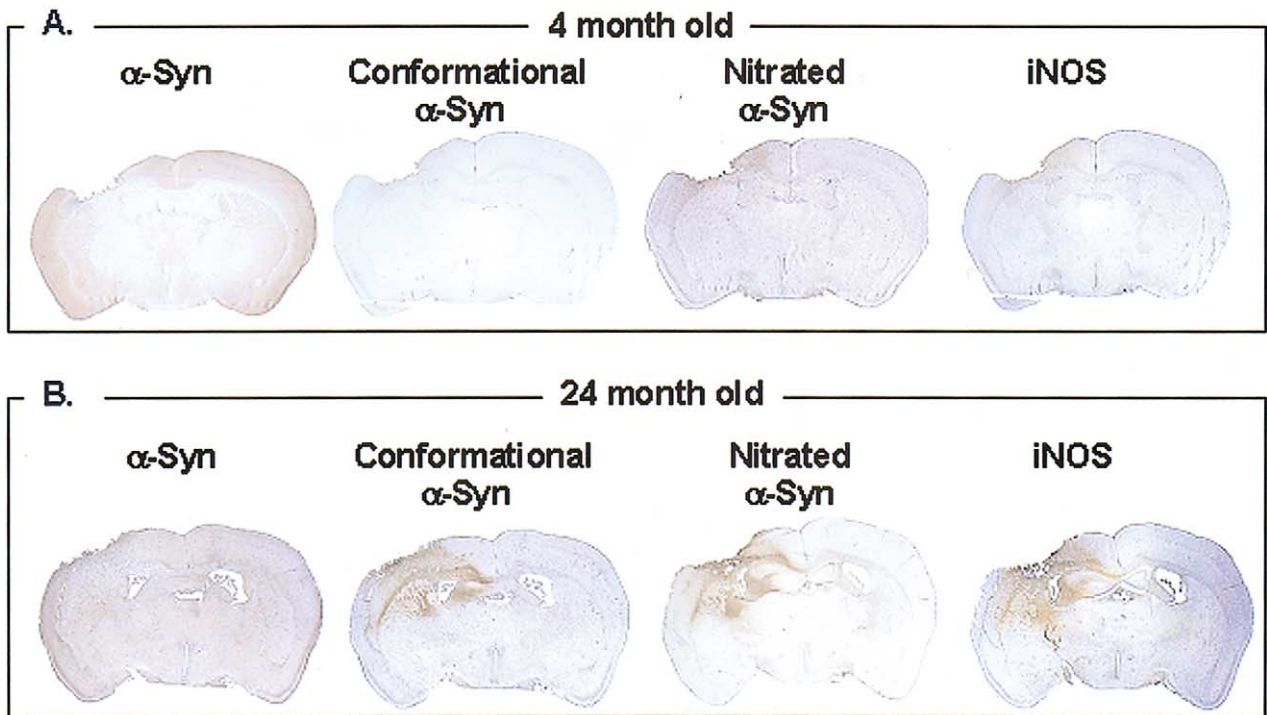


Fig. 3. Comparison of immunoreactivity (IR) for normal α -Syn, conformationally altered α -Syn, nitrated α -Syn, and inducible nitric oxide synthase (iNOS) IR between young and aged wild-type mouse brains at 1 week post-traumatic brain injury (TBI). The young (4 month old) mouse brain (A) shows little IR for conformationally altered (Syn303) or nitrated α -Syn (nSyn14) nor for iNOS (iNOS-2) at 1 week post TBI, compared to the localized enhanced IR for these proteins in the aged mouse brain (B) in the brain region subjacent to the impact site for controlled cortical impact.

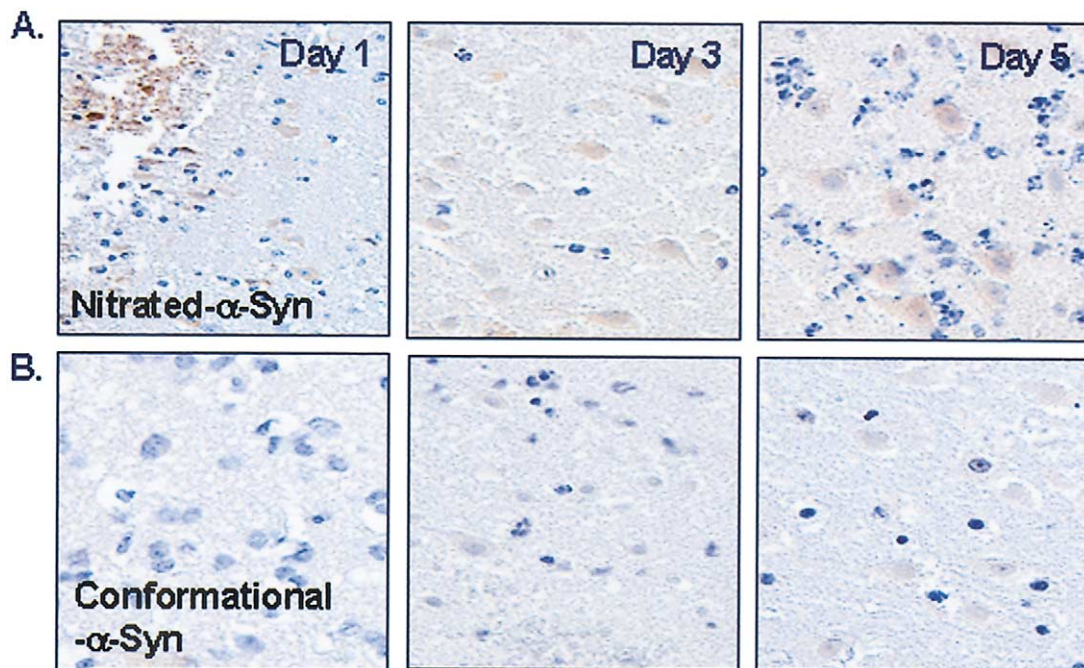


Fig. 4. Limited nitration of α -Syn detected in the brains of young wild-type mice following controlled cortical impact (CCI). (A) The young mouse brain shows nitrated α -Syn (nSyn14) immunoreactivity (IR) in cortical neurons subjacent to the site of CCI from 1 through 5 days post-traumatic brain injury (TBI), whereas this IR diminishes and is no longer detectable at 1 week post TBI. (B) Conformationally altered α -Syn (Syn303) IR is not seen in young mice at any time including the times shown in B.

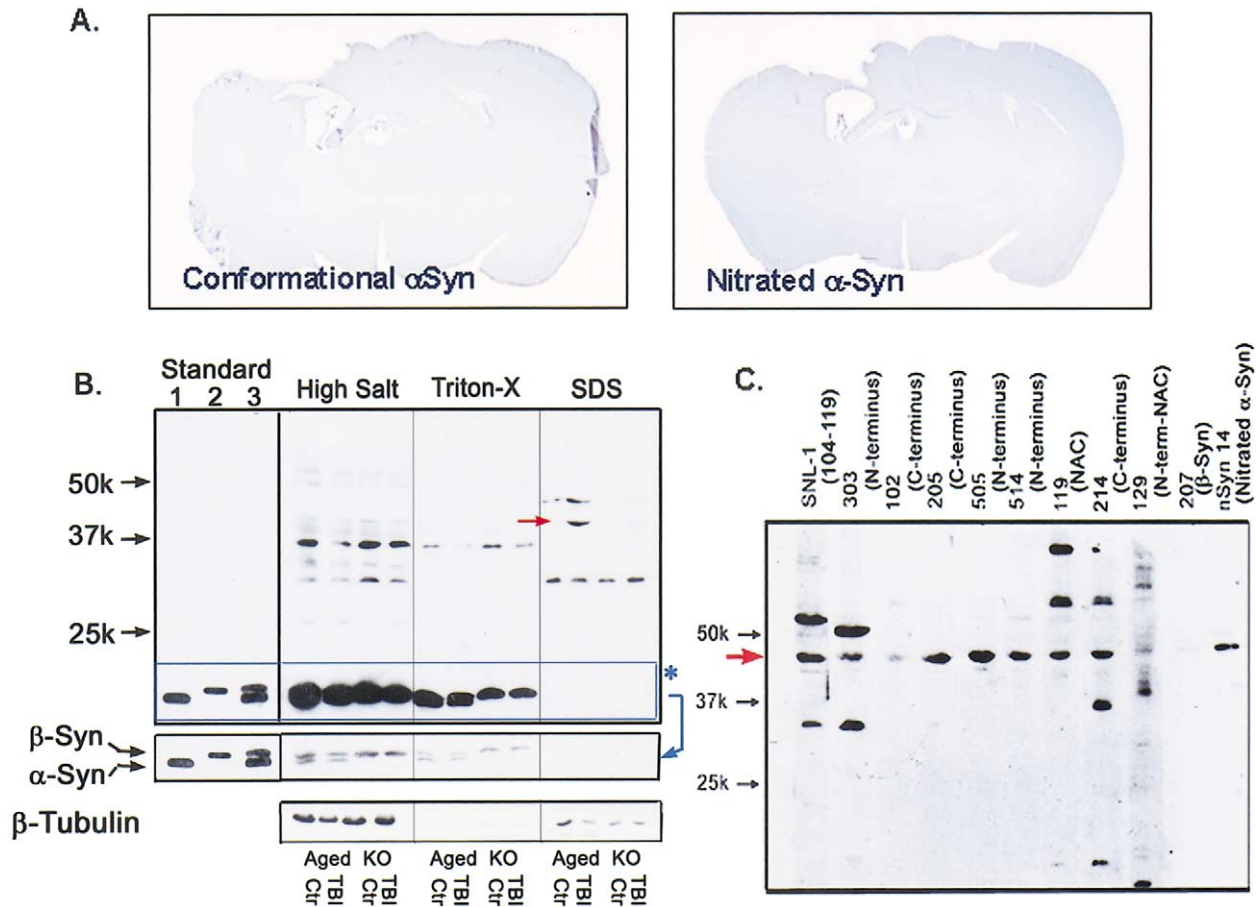


Fig. 5. (A). The aged α -Syn knockout (KO) mouse brain shows no α -Syn immunoreactivity (IR) following traumatic brain injury (TBI). Immunohistochemistry using antibodies to conformationally altered (Syn303) and nitrated (nSyn14) α -Syn shows no IR in the aged α -Syn KO mouse at 1 week post TBI, thereby confirming the specificity of the α -Syn IR seen in the aged wild-type (WT) mouse brain following TBI. (B and C) Western blot analysis shows a 45-kDa M_r protein in the insoluble fraction in the aged mouse brain following TBI that is recognized by multiple anti- α -Syn antibodies. (B) As molecular standards, 20 ng of recombinant human α -Syn (lane 1), β -Syn (lane 2), or α - and β -Syn (lane 3) were loaded in the far left panel. The high-salt and Triton-X fractions of aged control (Ctr) WT (Aged) and α -Syn KO (KO) mice subjected to controlled cortical impact (CCI) (TBI) show equivalent levels of monomeric α -Syn, but only the WT mice show monomeric α -Syn immunobands that migrate just below the β -Syn immunobands. This is seen more clearly in the less exposed portion of the same Western blot that is shown in the separate box below the illustration of the entire Western blot identified with an arrow and asterisk as well as arrows pointing to the α -Syn and β -Syn immunobands. Note that only the brain of the CCI-treated WT mouse shows a 45-kDa immunoband in the sodium dodecyl sulfate (SDS) fraction that is recognized by Syn303 (arrow in B). Samples were also analyzed by Western blotting for the presence of β -tubulin (bottom panels) as a control for the processing and gel loading of samples. (C) Numerous other anti- α -Syn antibodies, including those that recognize epitopes extending from the N-terminus to the C-terminus of α -Syn and nitrated α -Syn also consistently detect the same 45-kDa immunoband in the SDS fraction from the brain of the CCI-treated WT mouse, but this band is not detected by an anti- β -Syn (207) antibody.

any of the other fractions of the brains from the sham-treated WT mouse or the α -Syn KO mice. Since either or both of these bands could represent the transiently modified species of α -Syn induced by CCI that were detected by IHC, as described above, we examined these immunobands further.

Briefly, the SDS fractions of brain from the TBI-treated aged WT mice at 1 week post CCI were subjected to Western blot analysis with multiple additional antibodies to different α -Syn epitopes including those in the C-terminal (SNL-1, Syn102, Syn205, Syn214), central ("NAC") (Syn119, Syn129), and N-terminal (Syn303, Syn505, Syn506, Syn514) domains of α -Syn as well as to nitrated

epitopes (including Tyr39) in α -Syn (nSyn14) and with an antibody to β -Syn (Syn 207). Notably, all of the antibodies specific for epitopes in α -Syn consistently recognized a 45-kDa immunoband (Fig. 5C) that was not detected by the anti- β -Syn antibody and therefore probably corresponds to the transiently modified species of α -Syn induced by TBI in the aged WT mice. Although some of these anti- α -Syn antibodies inconsistently detected other higher and lower M_r protein bands (data not shown), they do not appear to be reproducible products of TBI-induced modifications in α -Syn because they were not recognized consistently by the entire panel of anti- α -Syn antibodies as was the case for the 45-kDa band described above.

Discussion

Here we show that TBI induced by the CCI paradigm transiently altered the distribution of α -Syn, and post-translationally modified this synaptic protein in a unique manner, but only in the aged (24 month old) WT mouse brain and not in young (4 month old) WT mice. Significantly, these changes in α -Syn IR in the brains of old WT mice subjected to CCI recapitulate some of the key features of α -Syn pathologies that are seen in the brains of patients with synucleinopathies such as accumulations of α -Syn in axons, and the generation of nitratively as well as conformationally modified forms of α -Syn. Further, similar to authentic human synucleinopathies, these transient modifications and alterations of α -Syn were specific largely for this member of the synuclein family of synaptic proteins since more modest transient increases in IR for β - and γ -Syn were detected by IHC in brain sections with antibodies to each of these other members of this family of proteins.

Since Braak and coworkers recently demonstrated that the presence of axonal α -Syn pathology in the brainstem of patients with PD is one of the initial morphological manifestations of disease (Braak et al., 1999, 2003) and Duda and his colleagues reported an unprecedented as well as extensive burden of α -Syn pathology in the striatum of the brains of patients with different LB disorders (i.e., PD, dementia with LBs or DLB, and AD plus DLB) (Duda et al., 2002), it is plausible to consider that the axonal deposits of α -Syn as filamentous inclusions or nonfilamentous aggregates are more significant mediators of neurological and behavioral impairments than LBs themselves in synucleinopathies, especially since the abundance of LBs does not always correlate robustly with such impairments (Hurtig et al., 2000). Thus, our studies here showing an intra-axonal localization of pathological α -Syn in the striatum of aged mouse brains following TBI using double FIHC with a combination of antibodies to α -Syn and either NFL or APP may facilitate efforts to dissect out the biological significance and underlying mechanisms of axonal α -Syn pathologies.

Comparison of the IHC and Western blot data here suggests that TBI induced a nitrative and conformational modification of α -Syn, especially in striatal axons, and the 45-kDa immunoband illustrated in Fig. 5C is likely to correspond to this transiently altered form of α -Syn microscopic pathology since it was recognized by multiple different antibodies to diverse α -Syn epitopes throughout the N-terminal to C-terminal extent of α -Syn as well as by antibodies that recognize nitrated and conformationally modified forms of this synaptic protein. In contrast, this 45-kDa protein was not recognized by antibodies specific for β -Syn. Although the relationship of this 45-kDa protein to different species α -Syn is not entirely clear, a recent report described the induction of a similar 45-kDa α -Syn--

like protein in PC12 cells treated with nerve growth factor (NGF) for 1 week (Stefanis et al., 2001), and since TBI induces neurotrophin upregulation (DeKosky et al., 1994), it is possible that generation of this 45-kDa protein from monomeric α -Syn is a consequence of TBI mediated by neurotrophins. Alternatively, this TBI-induced α -Syn-like protein may reflect the formation of a protein complex that results from the interaction of α -Syn with itself or with other protein(s) since there is evidence that α -Syn itself may function as chaperone that interacts with other proteins (Souza et al., 2000b).

Since it has been shown that nitration of α -Syn is a common feature of pathological forms of this protein in LBs of diverse synucleinopathies (Giasson et al., 2000a), it is significant that similar changes were transiently seen following CCI here in aged WT mice because it is known that TBI causes oxidative and nitrative stress (Clark et al., 2001; Liu et al., 2002), including in WT rodents (Semchuk et al., 1992) as well as in transgenic mouse models of AD-like amyloidosis (Uryu et al., 2002). Further, the induction of iNOS closely coincided with the temporal appearance of nitrated α -Syn in the brains of CCI-treated WT mice here, and this suggests that TBI-induced iNOS may play a role in transiently modifying the properties of α -Syn following TBI. Although it is unclear how nitrative stress contributes to the development of α -Syn pathology in human neurodegenerative diseases or TBI, previous studies suggest that oxidative/nitrative stress may play a role in the aggregation of α -Syn (Paxinou et al., 2001; Norris et al., 2003) or in stabilizing aggregated forms of α -Syn (Souza et al., 2000a). Thus, the data presented here suggest a possible link between TBI, oxidative/nitrative stress, and the generation of α -Syn pathology. Finally, the prominence of the enhanced α -Syn and β -Syn IR in axons and the neuropil at 1–9 weeks following TBI probably reflects a transient perturbation of axonal transport that leads to the accumulation of multiple organelles and proteins followed by the partial or complete reversal of impaired transport and diminution of the abnormal axonal α -Syn and β -Syn IR. Indeed, although the role that altered axonal transport plays in TBI and neurodegeneration is unclear, there is growing evidence to implicate perturbations of axonal transport in these disease processes. However, in selected at-risk individuals, these changes may not be completely reversed thereby predisposing these victims of head trauma to develop AD or PD many years after the initial episode of TBI. While the precise nature of the events that unfold over many years to link one or more episodes of TBI to a greater risk for developing neurological impairments is unclear, further investigations of experimental animal models of TBI such as those described here and transgenic α -Syn mice offer a tractable strategy for elucidating how TBI-induced pathological modifications of α -Syn contribute to chronic neurodegeneration and behavioral impairments.

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