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RESEARCH ARTICLE

Prokaryotic SPHINX replication sequences are conserved in mammalian brain and participate in neurodegeneration

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Abstract

A new class of viral mammalian Slow Progressive Hidden INfections of variable (X) latency ("SPHINX") DNAs, represented by the 1.8 and 2.4 kb nuclease-protected circular elements, were discovered in highly infectious cytoplasmic particles isolated from Creutzfeldt-Jakob Disease (CJD) and scrapie samples. These DNAs contained replication initiation sequences (REPs) with approximately 70% homology to those of environmental *Acinetobacter* phage. Antibodies against REP peptides from the 1.8 kb DNA highlighted a 41 kDa protein (spx) on Western blots, and in situ studies previously revealed its peripheral tissue expression, for example, in pancreatic islet cells, keratinocytes, kidney tubules, and oocytes but not pancreatic exocrine cells, alveoli, and striated muscle. To determine if spx concentrated in specific neurons and synapses, and also maintained a conserved pattern of architectural organization in mammalian brains, we evaluated mouse, rat, hamster, guinea pig (GP), and human samples. Most outstanding was the cross-species concentration of spx in huge excitatory synapses of mossy fibers and small internal granule neuron synapses, the only excitatory neuron within the cerebellum. Spx also localized to excitatory glutamate type synapses in the hippocampus, and both cerebellar and hippocampal synaptic spx was demonstrable ultrastructurally. Studies of two well-characterized models of sporadic CJD (sCJD) revealed novel spx pathology. Vacuolar loss of cerebellar synaptic complexes, thinning of the internal granule cell layer, and fibrillar spx accumulations within Purkinje neurons were prominent in sCJD GP brains. In rats, comparable spx fibrillar changes appeared in hippocampal pyramidal neurons, and they preceded prion protein misfolding. Hence, spx is an integral player in progressive neurodegeneration. The evolutionary origin, spread, and neuropathology of SPHINX 1.8 REP sequences opens another unanticipated chapter for mammalian symbiotic interactions with environmental microbes.

KEYWORDS

amyloids, CISS circular DNAs, endosymbiosis, excitatory synapses, latent viruses, phage virome, sporadic Creutzfeldt-Jakob Disease

NOTE: Unipolar brush cells in the vermis and floccullor lobe of the cerebellum are also excitatory and Fig. 3A (arrows) show label in their dendrites or cell bodies (communicated by Constantino Sotelo, see: Mugnaini, Serkova & Martina, Brain Res. Reviews 66: 220-245, 2011

1 | INTRODUCTION

The causes of late-onset neurodegeneration can be complex and heterogeneous. Unrecognized environmental, toxic, and microbial agents can initiate and contribute to the development of major common diseases such as Alzheimer's Disease (AD). The current strong focus on end-stage amyloid and a few other fibril-forming proteins have diverted investigation of unsuspected microbial causes of late-onset neurodegeneration[1,2] and these have some precedent. Influenza viruses have caused late-onset neurofibrillary tangles (NFTs) in Parkinson's neurodegeneration in a "hit and run" pattern, as in the 1918 epidemic, and activated subacute myxo-paramyxoviral infections can induce NFTs.[3] NFTs are also prominent in AD and in posttraumatic dementia. Thus, this NFT phenotype fails to discriminate viral, traumatic, and other initiating causes of AD. Targeting environmental infectious pathogens can have major beneficial health effects for neurodegenerative diseases. Indeed, epidemic "mad cow" disease, an infectious transmissible encephalopathy (TSE) that has transmitted a variant form of Creutzfeldt-Jakob Disease (vCJD) to people, was dramatically reduced simply by removing infectious animals and their products from the food chain. We have worked extensively with TSEs because these agents cause stealth infections that can remain quiescent in lymphoreticular cells for many years without inciting neurodegenerative sequelae. The essential molecular components of these infectious agents remain undefined,[4,5] and they present an intriguing evolutionary mystery. As other latent viruses, TSE agents do not provoke an acute lymphocytic reaction but they can induce early innate immune responses as they begin to exponentially replicate in the brain.[1] When released from complex animal surveillance systems by transfer to cells in culture, TSE agents replicate at a 10 to 30 fold faster rate.[6] Animal host responses include dendritic cell and microglial changes that occur well before any pathological forms of host-encoded prion protein (PrP) appear in brain.[7-9] Elucidating intrinsic TSE agent molecules can help prevent TSEs and offer new insights in other neurodegenerative diseases of unknown cause. The spx replication initiation sequence (REP) protein here appears as a novel player in this context and can discriminate between the responses of two different species to the same sporadic CJD (sCJD) agent.

Although normal host PrP is required for productive TSE infections,[10] PrP is not recognized as foreign. Indeed, PrP amyloid can trap and remove the infectious agent from cells.[11] It is widely believed that host PrP, without nucleic acid, spontaneously misfolds to become the causal infectious TSE agent.[12,13] PrP misfolding has also been claimed to encode all the different TSE agent strains.[14] Less well known are the many observations that show PrP is insufficient to replicate infection or produce stable individual strains. TSE infectious agent strains show vastly different clinical behaviors, incubation times, species transmissibility, and regional pathology. In normal mice with the same PrP gene, distinct agents also elicit identical PrP misfolded band patterns in the brain, a finding that does not fit the protein strain-encoding hypothesis.[15] Moreover, PrP band patterns change when TSE agents are propagated in different cell types of a single animal, yet the agent strain remains unchanged. Long-term cell type-specific PrP misfolding and glycosylation patterns in culture also do not alter the strain properties.[16] Additionally, some TSE agents can also interfere with the infection by a second TSE strain whereas others can add their infectivity, and this viral behavior is not predicted by any specific PrP misfolding.[17-19] Two reports claim recombinant PrP itself, without mammalian nucleic acids, can become infectious when misfolded into fibrils in vitro,[20,21] but thousands of experiments elsewhere have failed to reproduce this result.[22-24] Moreover, highly infectious brain particles of viral size approximately 20-nm diameter, comparable to those seen only in infected brain and cell sections,[25] have lacked detectable PrP by deep proteomic sequencing.[5] In contrast, highly infectious cytoplasmic particle preparations always contain nucleic acids of >500 nucleotides, and particle infectivity and nucleic acids are both destroyed simultaneously by nucleases whereas PrP remains unaffected.[4] The discovery of a new class of circular Slow Progressive Hidden INfections of variable (X) latency ("SPHINX") DNAs derived from a logical interrogation for a protected TSE agent genome.

Because an approximately 20 nm, infectious particle can accommodate a circular DNA genome of 1 to 5 kb,[26] nucleic acids protected in cytoplasmic particle fractions were analyzed using rolling circle amplification.[26] At least four related circular DNA elements were identified in brain and cultures. Two of these circular elements with REP motifs (of 1.758 and 2.36 kb) were completely sequenced, along with copurifying circular mitochondrial DNA of 16 kb.[26] Whereas protected mitochondrial sequences had a 100% homology with those in the database, both REPs had only an approximately 70% significant homology (e^{-105}) to small REP segments of an *Acinetobacter* phage virus, for example, 676/976 bases. Extensive tests failed to show these new circular DNAs were in any chemicals or reagents used. Both of these "SPHINX" DNAs were highly enriched in infected preparations and were subsequently identified in non-infectious material by both polymerase chain reaction and antibody studies. Their origin and representation were unclear, as was their potential contribution to

neurodegeneration. The 1.8 kb DNA was preferentially targeted for further study because it contained a classical iteron initiation repeat. It was also independently isolated and verified in Europe in three bovine sera clones that were 100% homologous to our 1.758 nucleotide sequence. An association with neurodegeneration was also substantiated because two multiple sclerosis brain samples contained 1.766 nucleotide sequences with a 98% nucleotide identity to SPHINX 1.8.[27]

We generated REP-specific antibodies to first delineate the expression of the 1.8 kb sequence in different tissues and cells. Two rabbits both produced antisera against a REP peptide not present in the mammalian database. Both sera-labeled a nonglycosylated 41 kDa protein band, close to its predicted 38 kDa size, and labeling was completely blocked by the REP peptide.[28] The highest specific titer serum was affinity purified. On Western blots, this immunoglobulin G showed a strong 41 kDa signal at 1:5000 dilution that was completely blocked by the REP peptide in Western blots, cell culture, and peripheral tissue sections as reported previously.[2,28] Because large anterior motor horn neurons appeared to receive synaptic boutons with high concentrations of spx by light microscopy, it was important to extend and substantiate this at the ultrastructural level. We here report that cerebellum shows a striking concentration of synaptic spx in a highly organized architectural pattern. Dendritic claws of excitatory internal granule cell (GC) neurons[29] receiving large pontine excitatory synapses showed strong spx accumulations, and these GC cells synchronize temporospatial coordination[30] that is critical for survival. Each GC cell axon then ascends through the molecular layer (as climbing parallel fibers) and branches to synapse with as many as 100 Purkinje cell dendrites. As demonstrated here, their spx positive axonal boutons surrounded Purkinje cell dendrites in the molecular layer, and this feature was also conserved in different species. Additionally, although previous Western blots failed to show major spx differences in PrP knockout vs normal mice, nor in CJD and scrapie-infected brains vs controls,[28] in situ evaluation of brain tissue, as detailed here, demonstrated novel spx neurodegenerative changes, as did the hippocampus, another region rich in excitatory glutamate synapses. These changes included abnormal collections of spx in the Purkinje perikaryon and obvious loss of internal granule neurons with their dendritic synapses. Tracer studies in AD have shown degeneration of GC neurons that is unrelated to β-amyloid neuritic plaques[31] and spx analysis may also be informative in different AD subsets. On an evolutionary scale, spx elements represent the tip of the iceberg of new and unsuspected environmental symbiotes that can cause or propel neurodegeneration.

Finally, from a broader evolutionary perspective, SPHINX DNAs appear to be the only viral phage REP reported to reside in the cytoplasm of mammalian cells. The only other protected prokaryotic genome maintained in the cytoplasm of mammals is the 16 kb mitochondrial DNA that was acquired early in evolution by endosymbiosis.[32] REP circular DNAs fall within the broad class of circular single-stranded "circular single stranded viruses (CISS)" viruses that are found within many plant and invertebrate cells.[33] It is surprising that comparable prokaryotic elements have not been detected previously in mammals, possibly because of their relatively low cytoplasmic copy number or protection. In contrast, human endogenous retroviruses (LINES), first isolated and sequenced here in 1982,[34] derive from full length retroviruses and constitute 2% to 5% of nuclear chromosomal DNA, a huge amount. Along with other endogenous retroviral sequences (ERVs), these elements were retrotranscribed and inserted as DNA early in evolution. During their long infectious and symbiotic interactions with mammals, LINES and other ERVs have developed unique sequence signatures that allow them to define functionally cohesive megabase gene regions in chromosomes,[35,36] promote tissue-specific patterns of gene expression,[37] modulate innate immunity,[38] and attack competitor species.[36] Interestingly, LINES are highly expressed in the brain and their gag proteins can also copurify with infectious particles.[39] However, unlike retroviral integrants, no SPHINX REP sequences have been identified in the genome. Although the function of SPHINX 1.8 or other members of this family is unknown, this cytoplasmic DNA may modify its host, and also be sculpted or altered by its host over time. The synaptic concentration of spx, as well as its other tissue-specific features, give credence to an evolving nonrandom symbiotic modulation, possibly one that interacts with endogenous elements such as retroviruses.

The present studies define potential new functions for spx in neuronal subsets and also demonstrate the participation of spx in neurodegenerative processes that precede abnormal PrP accumulation. The ways phage segments can invade and spread to other organs including the brain are briefly discussed, realizing that underlying reasons for their endogenous survival remain unknown.

2 | METHODS

2.1 | Light microscopy preparations

Archival human and TSE experimental animal tissue were sectioned and dewaxed as previously for detection of spx using a previously characterized affinity purified rabbit antiserum.[2,28] Briefly, sections were blocked in

10% normal goat serum and 3% fish gelatin in Tris-buffered saline (TBS) at 22°C for 1 hour and then exposed to Rabbit anti-spx at 1:5000 in TBS-0.1% Tween 20 (TBS-T) at 4°C overnight. As previously, this represents a 1:10,000 dilution of the purified antibody since it was diluted 1: 1 with glycerol for cryoprotection. TBS-T washes were followed by Goat anti-rabbit biotin in TBS-T at 1:5000 for 1.5 hours at 37°C, TBS-T washes and then exposed for 30 minutes to strepavidin-alkaline phosphatase and developed with Vector Red Laboratories per kit instructions. For enhanced penetration of very old formalin fixed brain, heat-citrate autoclaving as described[8] or 2 M GdnHCl exposure for 30 minutes was done after dewaxing and these treatments did not affect spx localization or peptide blocking of the anti-spx antibody as previously shown.[2,28] A Bio-rad confocal microscope was used to collect fluorescent optical slices, which were then deconvolved using the Bio-rad software, and some stacks were then rotated using commercial Zen software with the help of Dr. Larry Rizzolo.

2.2 | Electron microscopy

This followed a similar sequence of antibody exposure except 4% paraformaldehyde-0.1%glutaraldehyde perfused mouse brains were sliced approximately 50-μm thick with a vibratome and slices blocked in 3% normal goat serum, 1% bovine serum albumin, 0.1% lysine, 0.1% glycine for 24 hours before exposure for 2 days to anti-spx antibody followed by biotinylated anti-rabbit antibody (Vector 1:250) and Avidin-Biotin peroxidase (Vector 1:200) that was visualized with diaminobenzidine. Sections were exposed to 1% osmium tetroxide and dehydrated in increasing ethanol concentrations. 1% uranyl acetate was added to the 70% ethanol to enhance ultrastructural membrane contrast. Flat embedding was done in Durcupan with ultrathin sections collected on formvar-coated single-slot grids and analyzed with Tecnai 12 Biotwin Electron Microscope. For enhanced penetration, slices were equilibrated in 20% glycerol and freeze-thawed ×3 or exposed to 2M GdnHCl for 8 to 10 minutes before blocking. Both treatments increased the number of synapses labeled but did not alter the pattern of labeling as shown representatively in Figure 3A,B; these were the same as with TBS-T. Sections were osmified, embedded in plastic, and thin sectioned for electron microscopy

3 | RESULTS

3.1 | Light microscopy of spx

Cerebellar folia have a well defined and conserved architecture in mammals with three distinct macroscopic layers. This layered architecture facilitates unambiguous identification of specific neuronal cell types with known physiological functions. Different patterns of spx protein are detected in each layer. Alkaline phosphatase labeling was used to detect a red spx signal by transmitted light microscopy and also showed a corresponding intense red fluorescence by epifluorescence as depicted respectively in Figures 1A-D. At low magnification A, a section of the human cerebellum, the outer molecular layer (m) displays intense red signal throughout. This layer is separated from the internal granule cell layer (g) by a row of Purkinje neuronal cell bodies (P) as shown in C and D. Each Purkinje cell body receives inputs via its complex dendritic arborizations within the molecular layer, and each Purkinje dendritic tree receives excitatory synapses from thousands of GC axons. The GC cell bodies and their stubby branched dendrites reside in layer g where their small neuronal nuclei are stained dark blue in C. Note the many separate and intense discrete red bodies of 2 to 6 μm in this granule cell layer. These are more dramatically accentuated by fluorescence microscopy in D where the perikaryon and nuclei show minimal spx fluorescence. The large congregation of spx signals (rosettes) in this layer correspond to mossy fiber synapses that decorate the GC dendritic claws (at arrows). Mossy neuron synapses, like those of GC neurons, are excitatory and their perikarya reside far away in pontine nuclei of the spinal cord. In Figure 1E, a guinea pig (GP) cerebellum shows the same large spx positive GC clusters as seen in the human section. It also highlights many discrete synapse-sized red boutons around minimally labeled processes cut longitudinally in the molecular layer. A plethora of these synapses are excitatory GC axon synapses from their climbing and branched parallel axon fibers. Each GC axon can synapse on as many as 100 Purkinje cell dendrites. This section also cuts through a normal Purkinje cell body (P) that shows a much weaker cytoplasmic spx signal. Figure 1E also shows a stellate interneuron in the molecular layer with its dendrite surrounded by intense spx signals (arrows). These stellate neurons receive excitatory GC inputs as well as inhibitory inputs from other neurons.

The overall architectural pattern of spx, with high concentrations in rosette synapses on GC dendrites and in numerous GC synapses surrounding Purkinje dendrites in the molecular layer was consistently seen in all the mammals examined (human, GP, hamster, rat, and mouse). As in previous studies using procedures to enhance tissue penetration,[2] neither 2 M GdnHCl that can loosen protein binding to nucleic acids nor other unmasking procedures, altered the pattern, specificity of spx labeling or its peptide blocking. The strongly positive mossy fiber synapses in the GC layer contrasts with minimally labeled cytoplasm of GC cell bodies and other

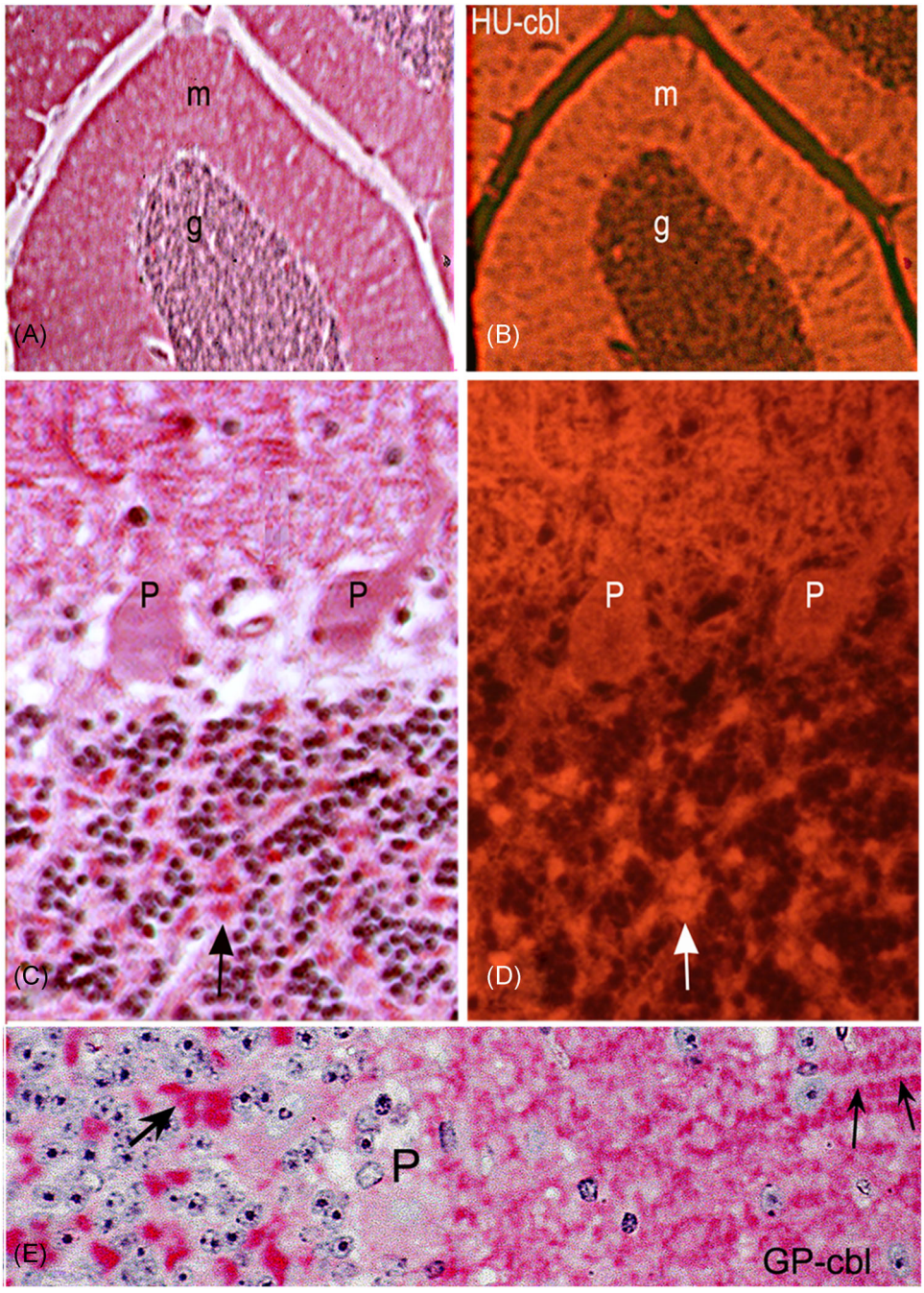

FIGURE 1 Transmitted light with counterpart fluorescent epi-illumination of red spx signals at low (A, B) in low and higher magnification (C, D) of the human cerebellum. Outer molecular layer (m) with blue stained nuclei in the internal granule cell layer (g). Purkinje cells (P) with dendrites in the outer molecular layer with abundant synapses, many of which come from small granule neuron mossy fiber axons. Arrow in C/D shows complex spx+large excitatory synapses from distant neurons in the pons. (E) Shows guinea pig (GP) cerebellum with same spx architectural and synaptic pattern. Large arrow again shows mossy neuron synaptic complex and small arrows point to spx+boutons on a stellate neuron dendrite in the molecular layer with low spx signal. The Purkinje neuron perikaryon P also has low spx

adjacent fibers (see C and E). The weak labeling of the GC perikaryon also contrasts with their synapses in distant regions of the molecular layer. Similarly, the strong concentration of spx in mossy fiber synapses, but not their axons traveling through the cerebellar white matter, implicates inapparent transport of spx to synapses and/or synthesis of spx within these synaptic regions. It remains unknown if SPHINX 1.8 DNA circles are clustered, transcribed, and translated within synaptic regions. In any case, synaptic spx is in a perfect position

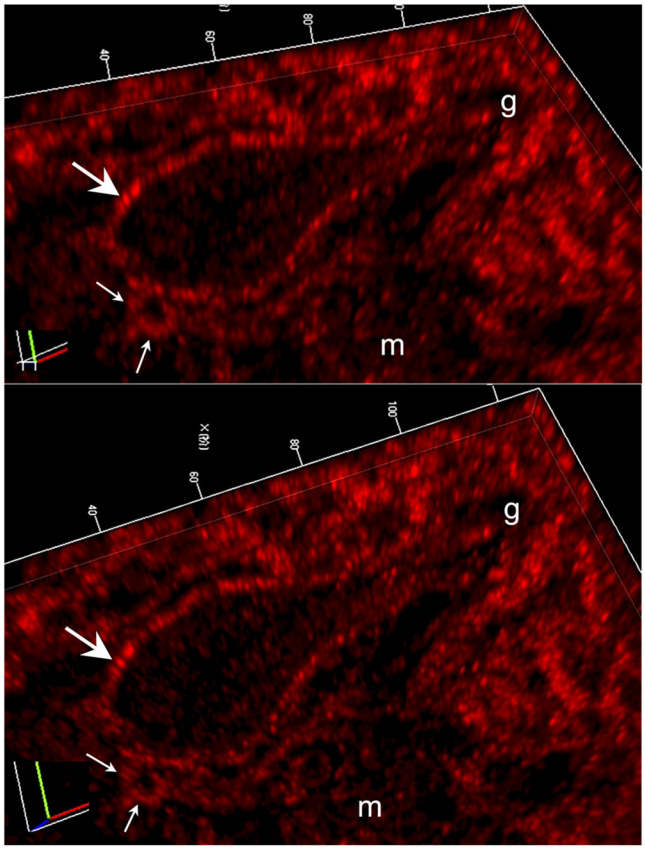

FIGURE 2 Shows a deconvolved fluorescent stack in two close three-dimensional rotation views of human cerebellum show cross-section of a large Purkinje perikaryon, with low spx signal studded with spx+round boutons consistent with synapses as at large arrow. Its dendrite is also surrounded by spx+boutons in the molecular layer and prominent larger spx+synapses in the internal granule layer (g)

to enforce both rapid and long term plasticity that is critical for the many complex integrative functions of the cerebellum.

To better resolve the structure of spx positive clusters we first used confocal microscopy deconvolution and reconstruction on approximately 6 μm sections that allow excellent penetration of the vast majority of cells and their organelles. This analysis delineated the discrete spx positive boutons on Purkinje cell dendrites within the molecular layer (m). Figure 2 shows a sample of a human cerebellum section in two three-dimensional rotations. One large diameter Purkinje cell apical dendrite (between small arrows) is surrounded by discrete round spx bodies consistent with the small axonal synapses from many GC neurons. There is also strong spx labeling of small round boutons surrounding the Purkinje cell body (large arrow) whereas spx labeling in the cytoplasm of the Purkinje perikaryon is relatively weak. The granule cell layer (g) contains much larger spx synaptic signals, and again, surrounding neuronal and glial processes as well as GC cell bodies display minimal signal.

3.2 | Ultrastructural concentrations of spx in specific synapses

An even better resolution was obtained in ultrastructural studies. The specific labeling of mossy fiber excitatory synapses (also known as rosettes) around GC dendritic claws was confirmed unequivocally by immuno-electron microscopy. As shown in Figure 3 of mouse cerebellum, peroxidase precipitates delineate prominent spx within the huge mossy fiber rosettes around the GC claws. In A, two large positive processes (large arrows) are strongly labeled with a smaller positive process between them (smaller arrow near vessel V) in this preparation

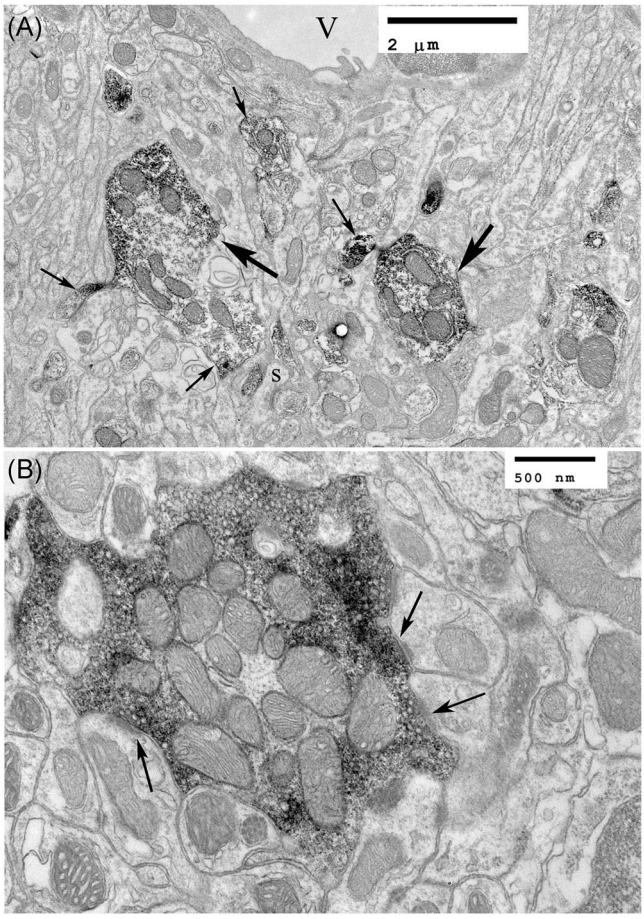

FIGURE 3 Mouse cerebellum. A, Ultrastructural localization of spx in mossy neuron excitatory synapses (dense precipitates in complex large processes at arrows). V is. vessel. Permeabilized with 2 M GdnHCl. B, Shows another labeled process in a higher power with characteristic numerous small vesicles that are also packed with mitochondria. Arrows point to postsynaptic densities of the delicate granule cell dendrites innervated by this mossy fiber's synapses. This preparation was permeabilized only by freeze-thawing (see methods)

pretreated with 2 M GdnHCl for enhanced permeabilization. These three positive processes are consistent with a mossy fiber rosette where only a few small diameter irregular connecting processes are apparent (small arrows) in thin section. Notably, the leftmost process is 4 μm in length, and the span of this synapse is 8.6 μm wide if the large rightmost process is included. This size corresponds to the large synapses seen by light microscopy above, as well as by other classical studies demonstrating a fundamentally conserved fine structure and organization of mossy fiber synapses in species from amphibian through mammals.[40] Note the many mitochondria stuffed into these broad terminals, another characteristic of mossy neuron synapses. In Figure 3B, where only freeze-thawing was used for permeabilization, and no heat or GdnHCl, the spx positive processes again demonstrate all the characteristics of mossy fiber inputs[40]; multiple synaptic vesicles are seen in the cytoplasm, this process makes many synapses on unlabeled small granule cell dendrites, as at arrows, and the GC dendrites also display postsynaptic densities at these synapses (arrows). Again, mitochondria are even more abundant in this terminal, a feature that may relate to a replicative and synthetic ability of mitochondria (and possibly SPHINX 1.8 DNA) at sites distant from their perikarya in chicks and mammals.[41,42]

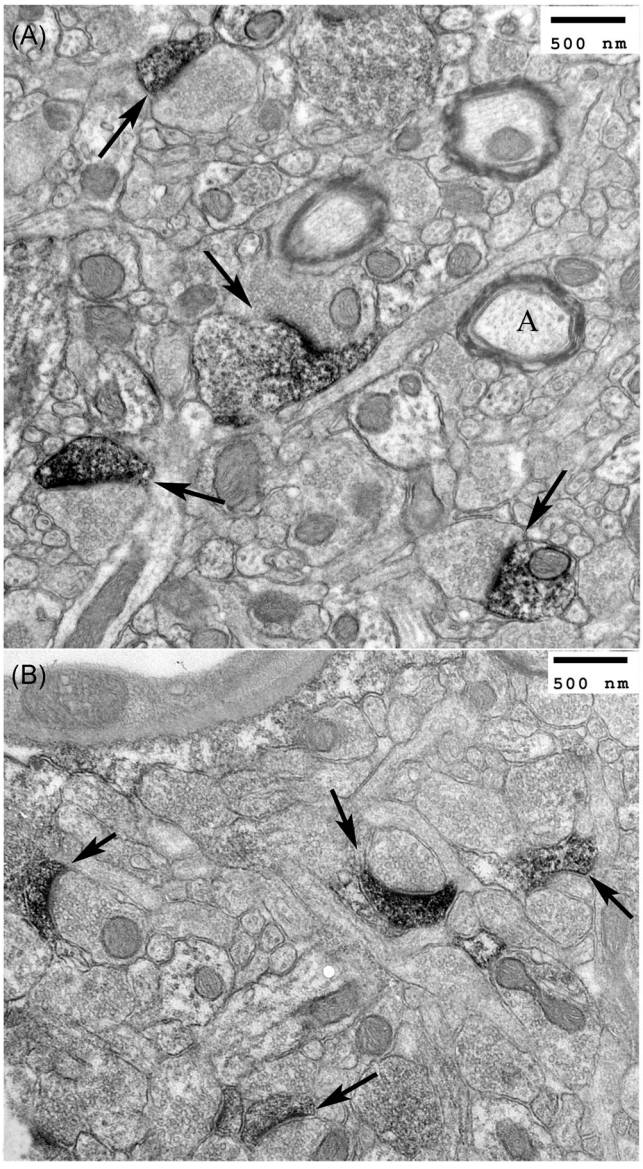

FIGURE 4 A, Mouse hippocampus CA3 shows labeling of ovoid synapses (arrows) consistent with glutamate excitatory function. Myelinated axon at A. In B, mouse cortex again shows highly selective labeling of synapses

3.3 | Spx in other excitatory synapses and cell bodies

The high concentration of spx at identified excitatory mossy neuron glutamate synapses does not exclude its localization at other nonexcitatory synapses in the cerebellum and elsewhere. Nor does it exclude spx in the cell body of selected other neurons. Although characterizing all spx positive synapses in the cerebellum with double labeling for functional discrimination is not in the realm of this paper, we evaluated spx in CA3 of hippocampus ultrastructurally for more information. This region also has distinct anatomical layers that contain other types of mossy fiber neurons and small dentate neurons. It is responsible for complex integrative physiology in memory and can also be a target of AD early neurodegeneration. CA3 again revealed labeling of specific synapses, many of which are known to have excitatory properties by light microscopy (data not shown) and this was confirmed ultrastructurally. Figure 4A shows a section through the CA3 with four spx positive postsynaptic termini that are consistent with those containing excitatory glutamate receptors. Three of these synapses have an ovoid shape and are approximately 0.4 μm in diameter, characteristics of recurrent synapses that transmit excitatory glutamate from CA3 pyramidal cells to other pyramidal neurons and to interneurons.[43] These synapses, as glutamate excitatory cerebellar synapses, have a key role in the synchronous physiology of the hippocampus and integrative memory. For reference, this section also contains three myelinated axons, for example, at A, that show no spx signal, and this corresponds to the lack of spx in myelinated axons elsewhere. Figure 4B from the mouse cerebral cortex permeabilized by freeze-thawing also shows selective spx labeling of small ovoid synapses, comparable to those in CA3.

We also examined large ganglia of the sympathetic-parasympathetic system in the peritoneum, a completely different monotypic group of large neurons. These large

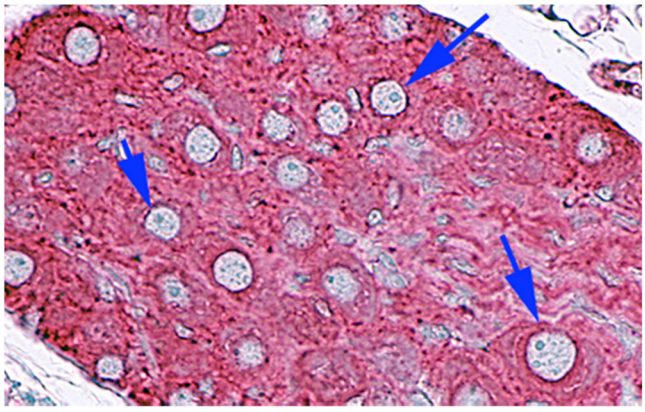

FIGURE 5 Unlike cerebellar Purkinje neurons and pyramidal neurons, large sympathetic ganglia neurons in the peritoneum show strongly labeled spx+perikarya with a strong perinuclear label (arrows). Tubular labeled structures in the cytoplasm are probably on the rough endoplasmic reticulum

neurons, unlike cerebellar Purkinje neurons and hippocampal pyramidal neurons, clearly display intense spx signal around their nuclei (Figure 5, arrows), within tubal structures likely to be endoplasmic reticulum, and at the membrane. This demonstrates that the intracellular localization of spx is highly specific for different neuronal cell types where it may subserve different functions. In peripheral tissues, the intracellular localization of spx is also cell type specific.[2]

3.4 | Novel neurodegenerative spx pathology

In infectious TSEs, and in many other dementing neurodegenerative diseases of unknown cause, the cerebellum is often ignored as a focus of pathology. By Western blot, regions of thalamus and cortex with CJD and scrapie terminal spongiform changes and PrP pathology failed to show any obvious quantitative or qualitative spx changes as compared with controls.[28] On the other hand, normal looking brain with exponentially replicating CJD and scrapie infectious agents, but no detectable PrP pathology, have been shown to express significant molecular markers of innate immunity during the initial phases of infection. Large increases in astrocytic messenger RNA and myeloid/microglial activation also precede behavioral and PrP pathology.[1,7,8] To see if we had overlooked potential changes in spx that might be regional and/or neuron-type specific, we evaluated the cerebellum of mice, hamsters, rats, and GP with proven TSE infections from a wide variety of distinct agent strains, for example, sCJD, RML scrapie, 263k scrapie, Asiatic FU-CJD.[15] Figure 6 shows there are unique as well as shared pathological manifestations of spx in different agent disease models.

Figure 6A is from a GP-infected with the human sCJD agent. This clinically ill animal does not display classical TSE spongiform change in the cerebellum, yet its Purkinje cells (P) show abnormal collections of spx in the perikaryon, and these are present in all except the one remaining more normal neuron (at arrow). Spx is concentrated more at the base of the Purkinje cell as compared with the emerging apical dendrite. Moreover, these intracellular intense spx signals are reminiscent of AD NFTs, especially as they extend in more distal Purkinje dendrites in the molecular layer, as shown in Figure 6A (at both sides of m). This layer also contains a few strongly positive round spx bodies representing cross-sections of degenerating dendrites. Purkinje cell neurodegeneration is ongoing, and astrocytes have collected (at As) in regions where Purkinje cells have died and are missing. The intense bouton-like staining in the molecular layer is markedly diminished here (compare Figure 1E). Layer g also shows fewer large spx synaptic rosettes, indicating distal pontine excitatory neurons are degenerating. In sum, there is a remarkable amount of unsuspected spx pathology in specific cells of the cerebellum that is novel and not evident by Western blotting of heterogeneous cellular lysates.

Rats have a very different immune system than GP (a non-rodent species) and the sCJD agent wreaks morphologic havoc in different regions of rat brain. In Figure 6B, an sCJD infected rat with progressive clinical signs over 40 days still maintained normal appearing Purkinje perikarya with low spx signal, unlike the GP. This difference represents a species-specific response to a single TSE agent, one that can also profoundly alter incubation time.[15] This terminally ill rat at 294 days postinoculation also displays some vacuolar degeneration in cerebellar dendrites (small arrows), but unlike the GP, multiple small synaptic boutons are still strongly positive and abundant in the molecular layer where they surround neuronal dendrites in a typical normal arrangement (as at large arrow). The large spx synapses in the GC layer are also preserved. We, therefore, assumed that sCJD in rats provoked little cerebellar pathology. However, in evaluating inoculated rats killed well before clinical signs in this time course series[8] we were surprised to find major early pathological changes in the rat cerebellum. Figure 6C shows a rat with no clinical signs killed at 150 days postinoculation, ie, approximately 150 days before the terminal disease of cagemates.[8] There are profound vacuolar changes within the GC layer. Only very few positive spx synaptic rosettes (arrows) are seen, and this indicates early selective vacuolar disintegration of pontine mossy fibers and their synapses. Additionally, the molecular layer is only weakly positive for spx at this early time point. Notably, myelinated tracts (mye) in the

cerebellum that carry the mossy fiber axons (as virtually all myelinated tracts in the normal brain) also show no obvious spx signal, indicating spx may be translated and possibly transcribed around the synaptic rosette.

The early vacuolization in this cerebellum mimics the previous demonstration of TSE vacuoles elsewhere in the rat cerebrum in healthy appearing rats at 150 days, a time well before abnormal PrP responses are detected; significant PrP accumulates between 250 to 300 days in this rat model.[8] It also demonstrates that spx can be useful as an early marker of neurodegeneration. This concept is furthered by the remarkable concentration and abnormal fibrillar aggregates of spx in hippocampal pyramidal perikarya and their dendrites (double-headed arrows) as early as 150 days postinoculation (Figure 6D). Only a few of these pyramidal neurons may be normal (arrows). The massive abnormal spx fibrillary-like degenerative changes are not unique to GP Purkinje cells but are also a dramatic feature of hippocampal pyramidal neurons in a region known to have predominantly excitatory glutamate synapses. This conservation of pathology can also be useful in evaluating other diseases such as Parkinson's Disease.

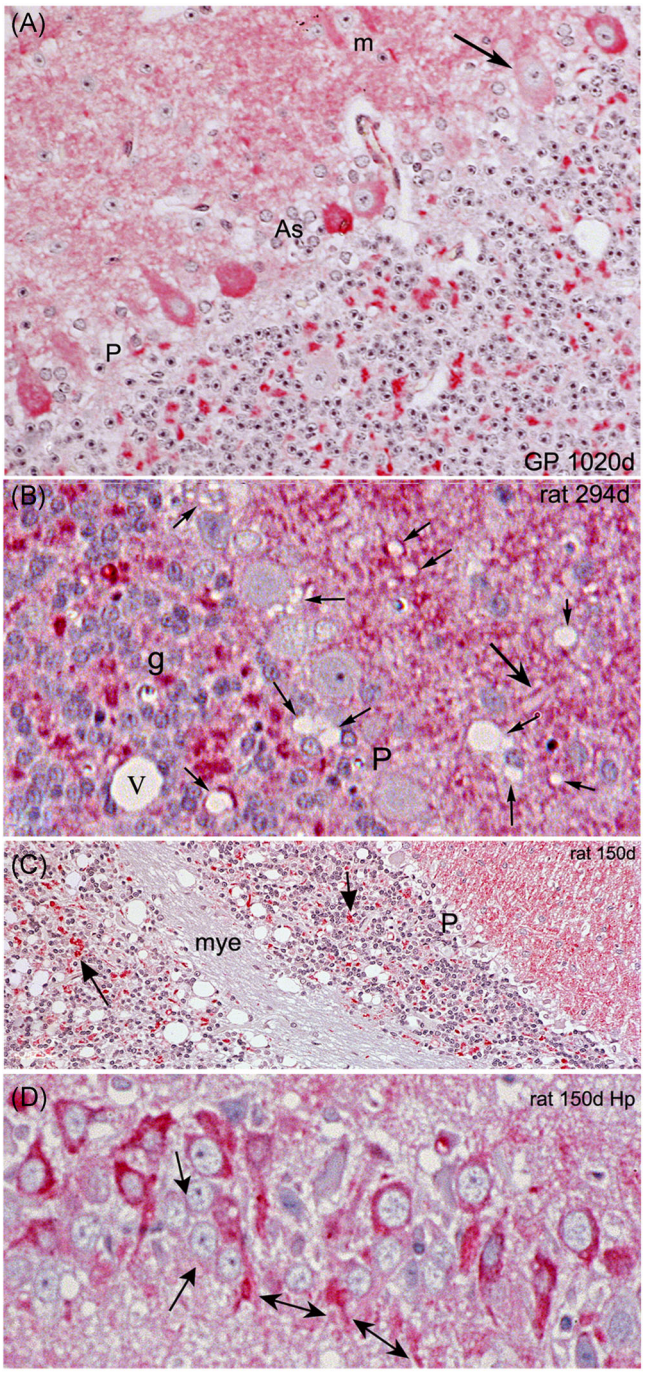

FIGURE 6 A, Guinea pig with sCJD shows pathological aggregates of spx in Purkinje perikarya; some of these have a fibrillar appearance and are also seen in the dendrites around m. Only one semi-normal cerebellar Purkinje cell with low spx is seen (arrow) and others have been lost (as at P). Astrocytes are also seen where Purkinje neurons have disintegrated (As). Note the reduced number of large mossy neuron synapses in the granule cell layer and the molecular layer synapses also show less label than normal. Spongiform change (vacuoles) are rare in this ill guinea pig at 1020 days (<3 years old); guinea pigs have lived for 12 years in our lab, ie, this is not an old animal. B, Clinically ill rat with sCJD at 294 days shows some vacuoles (small arrows) and a vessel (v) but does not show Purkinje cell spx accumulation in the perikarya. A large arrow shows normal spx+boutons and synapses in the GC layer are numerous. C, In contrast to the ill rat in B, this normal appearing rat killed at 150 days in the same series shows numerous vacuoles in the GC layer with dramatic loss of most mossy synapses. The myelin (mye) with axons shows negligible spx. Purkinje cells (P layer) do not show fibrillar accumulations of spx although many are missing. D, Instead, rat hippocampus at 150 days shows abnormal collections of spx in pyramidal neuron perikarya extending into their dendrites. The labeled dendrites (double-headed arrows) appear as fibrillary tangles and only a few more normal appearing pyramidal neurons remain (single-headed arrows). Early vacuolization also present. GC, granule cell; sCJD, sporadic Creutzfeldt-Jakob Disease

4 | DISCUSSION

Study of spx in the cerebellum here simplified the identification of well defined neuronal subsets with known functional attributes. The cerebellum participates in coordinating motor learning and balance, as well as other important behaviors such as cognition, emotion, and spatial navigation that are often damaged in neurodegenerative diseases. Spx exhibited a conserved architectural pattern in mammals. The localization of spx in synapses, with particularly high concentrations in excitatory glutamate synapses, was striking and was maintained in evolution, as demonstrated in the

representative rodent, GP, and human cerebellar samples shown here. Previous studies have produced good evidence that this spx localization reflects a microbial phage REP segment. Binding of this spx antibody to Western blot lysates, and to various mammalian tissue cell types in major organs, was completely inhibited by inclusion of its cognate peptide.[2,28] Both rabbit sera also decorated the same 41 kDa band of REP, in good accord with its predicted 38 kDa size, and a transiently transfected REP construct studied elsewhere also appeared to be 41 kDa.[2,28] Neither the REP antigenic peptide nor its corresponding complete REP DNA has been detected in any mammalian database up to the present time, and even chaotropic 2M GdnHCl treatment that enhanced penetration of antibody did not alter the fundamental pattern or conservation of spx labeling ultrastructurally. One of the most interesting facets of spx was its preferential localization in synapses as compared with much weaker signals in the perikaryon of cerebellar neurons. This was evident in GC cell bodies vs their synaptic boutons on Purkinje cell dendrites. The consistent lack of spx signal in white matter tracts that carry mossy fiber axons from the pons over long distances vs their terminal synapses on GC neurons in the cerebellum also suggests that spx may be translated near the synaptic terminus. Indeed, cytoplasmic SPHINX1.8 DNA, like mitochondrial DNA, may also reside, replicate and actively transcribe in synaptic regions.

Is there precedence for this unusual concept in mammalian cells? It is often implied that mitochondrial DNA replication, essential for its survival in the cytoplasm, is completely dependent on nuclear DNA functions. Nuclear-encoded DNA polymerase γ, mitochondrial DNA helicase, and mitochondrial single-stranded DNA-binding protein together facilitate mitochondrial ss DNA replication.[44] However, mitochondria have been shown to replicate in severed chick axons,[41] and increases in replication in distal axons has been demonstrated in a Parkinson's Disease stress model.[42] SPHINX DNAs may also replicate, be transcribed, and translated in distal mammalian axons. Interestingly, the tight packing of mitochondria at mossy fiber synapses on cerebellar GC dendrites suggests it may collaborate in the processing and functions of SPHINX DNA at this site. It will be of great interest to find if other spx REPs, such as the one encoded by SPHINX 2.36 DNA also concentrate in synapses where they may enhance each others' replication and functions as originally suggested.[26] In any case, the persistence of the SPHINX1.8 REP at specific synapses implicates both evolutionary divergence and positive selection. This REP sequence is only 70% homologous to its best phage counterpart in the environment, implicating a relatively high mutation rate, as found for mitochondria. At the same time, the REP antigenic peptide is conserved in diverse mammals, a feature that underscores its selective maintenance.

The most, fundamental and fascinating question is how and where any phage REP sequence may have entered mammals to eventually insert into the oocyte. One source is the gut. Abundant Gram-negative bacteria, including *Acinetobacter*, carry as many as 10^{31} phage viruses, the most numerous biomass of the gut.[45] Most of these virome sequences have not been sequenced and/or do not have sequence matches in the current database. Phages possess a unique capability of bypassing anatomical and physiological barriers, quickly permeate across the endothelium barrier, and can appear in blood (reviewed in[46]), a classic viral route of dissemination. Phages also readily penetrate the brain, especially via a nasal route. The sturdy filamentous portion of the phage, or segments of "circular nanoparticles" show even greater and prolonged penetration.[46] We presume that some of these segments, such those containing special REPs, confer a selective advantage that favors them in evolution, and REPs might possibly participate in cytoplasmic DNA repair. Clearly, not all of the many circular DNAs in the SPHINX family are maternally inherited or endogenous, and a number of other family members with strong homology to the SPHINX 2.36kb element from cow sera and dairy products have been proposed as environmental risk factors for breast and colon cancer.[47] On the other hand, the findings here, along with at least two additional SPHINX elements isolated from brain, encourage further investigations of their role(s) in synaptic plasticity, neurodegeneration, and neoplasia. Complementary elements may work together combinatorically to enhance specific functions and pathology.[26]

In terms of pathology, the discovery of specific spx fibril like accumulations in particular large neurons undergoing neurodegeneration are remarkable. Such changes were not entirely surprising because previous experiments here showed that approximately 10% of spx protein was resistant to limited proteinase K digestion,[28] a feature of abnormal PrP and other fibrillar accumulations seen in AD and Parkinson's disease. In TSEs, pathology in specific neuronal cell types, such as cerebellar Purkinje neurons of GP vs hippocampal pyramidal neurons of rat was species dependent because both were infected with the same sCJD agent. Nevertheless, the same spx fibrillary pathology was observed within both types of neurons. Moreover, pyramidal spx accumulations in the perikaryon and dendrites occurred >100 days before clinical signs in rat sCJD, which suggests spx could be of use in detecting early disease, possibly via altered spx protein in cerebropinal fluid (CSF) or serum. The simultaneous loss of mossy fiber synapses, with profound vacuolization in the GC layer of the cerebellum, also shows spx might be an early diagnostic tool because such vacuoles are concentrated

ultrastructurally in dendrites and synaptic regions.[48] Spx pathology discriminated different models of CJD, and the spx fibrillar and synaptic changes may be a common neurodegenerative phenotype; it might define new subsets of neurodegenerative disease caused by traumatic, vascular, and environmental toxic and microbial agents. This presents another opportunity to understand distinctive types of progressive late-onset neurodegeneration.

In conclusion, the fastidious and conserved residence of spx in specific synapses, its ability to highlight previously inapparent TSE agent-induced changes, and its appearance early in disease, all demonstrate that spx participates in neurodegenerative processes. Future molecular studies can use spx to target new and unsuspected pathways for progressive molecular changes. On a broader evolutionary scale, the findings here emphasize the importance of deeper analyses of unknown phage and microbial elements that can reside covertly in mammalian cells. Some of these sequences may initiate or cause progressive late-onset disease, especially in concert with other environmental or endogenous viral sequences or physiological stresses.[1]

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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