Neuron Dysfunction Is Induced by Prion Protein with an Insertional Mutation via a Fyn Kinase and Reversed by Sirtuin Activation in *Caenorhabditis elegans*

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Although prion propagation is well understood, the signaling pathways activated by neurotoxic forms of prion protein (PrP) and those able to mitigate pathological phenotypes remain largely unknown. Here, we identify *src-2*, a Fyn-related kinase, as a gene required for human PrP with an insertional mutation to be neurotoxic in *Caenorhabditis elegans*, and the longevity modulator *sir-2.1*/SIRT1, a sirtuin deacetylase, as a modifier of prion neurotoxicity. The expression of octarepeat-expanded PrP in *C. elegans* mechanosensory neurons led to a progressive loss of response to touch without causing cell death, whereas wild-type PrP expression did not alter behavior. Transgenic PrP molecules showed expression at the plasma membrane, with protein clusters, partial resistance to proteinase K (PK), and protein insolubility detected for mutant PrP. Loss of function (LOF) of *src-2* greatly reduced mutant PrP neurotoxicity without reducing PK-resistant PrP levels. Increased *sir-2.1* dosage reversed mutant PrP neurotoxicity, whereas *sir-2.1* LOF showed aggravation, and these effects did not alter PK-resistant PrP. Resveratrol, a polyphenol known to act through sirtuins for neuroprotection, reversed mutant PrP neurotoxicity in a *sir-2.1*-dependent manner. Additionally, resveratrol reversed cell death caused by mutant PrP in cerebellar granule neurons from *prnp*-null mice. These results suggest that Fyn mediates mutant PrP neurotoxicity in addition to its role in cellular PrP signaling and reveal that sirtuin activation mitigates these neurotoxic effects. Sirtuin activators may thus have therapeutic potential to protect from prion neurotoxicity and its effects on intracellular signaling.

Introduction

Misfolded prion proteins are involved in several neurodegenerative disorders including Creutzfeldt–Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, and scrapie in goat and sheep. Human prion disease may be sporadic, acquired through exposure to prion-infected tissues, or inherited through mutations of the prion protein gene *PRNP* (Prusiner and Hsiao, 1994). There is a wide variety of phenotypic variation in CJD that is not encountered in other neurodegenerative disorders

(Aguzzi et al., 2008a). Additionally, the genetic modifiers of prion diseases remain unknown, notably those that may be associated with inherited CJD. The genetic modification of prion disease may involve the genes that modulate pathogenic PrP toxicity. Interestingly, the activity of cellular prion protein (PrP^C), a glycoprotein anchored at the membrane, involves numerous ligands and signaling pathways (Westergaard et al., 2007; Aguzzi et al., 2008b; Linden et al., 2008). Signaling by PrP^C may notably involve caveolin-1, FYN kinases, and ERK1/2 kinases (Mouillet-Richard et al., 2007) as well as the cAMP/protein kinase A, MAP kinase, and PI3-kinase/Akt pathways (Linden et al., 2008), suggesting that the modulators of prion neurotoxicity may belong to the “core signaling machinery” essential for neuron activity and, more largely, for cell survival.

The conversion of PrP^C or mutant PrP into the misfolded isoform (PrP^{Sc}) confers partial resistance to proteolytic digestion and detergent insolubility (McKinley et al., 1983; Bolton et al., 1984) and may be central to prion disease pathogenesis (Aguzzi et al., 2008b). However, PrP molecules carrying pathogenic mutations can be neurotoxic in the absence of PrP^C (Lehmann and Harris, 1996; Telling et al., 1996; Chiesa et al., 1998), and their

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study may provide insight into the cellular pathways for prion neurotoxicity (Solomon et al., 2009). Among these mutations, insertional mutations consisting of 1–9 additional copies of an octarepeat are associated with dominantly inherited forms of CJD (Prusiner and Hsiao, 1994). These mutations may direct the folding of the protein toward the PrP^{Sc} conformation, and could initiate a self-conversion process (Leliveld et al., 2006). We previously identified a peculiar French family with early age at onset, prominence of psychiatric symptoms, lengthy clinical course, and a neuropathological phenotype of Gerstmann–Sträussler–Scheinker syndrome associated with eight extra octarepeats in PrP (PG13-PrP) (Laplanche et al., 1999). Here, we expressed PG13-PrP in nematode touch receptor neurons to establish a model of prion neurotoxicity. These neurons control the response to gentle touch, a well defined phenotype suitable in quantitative studies of neurotoxicity. While *Caenorhabditis elegans* contains proteins with putative prion-like properties (Iwasaki et al., 1992), *C. elegans* does not have a PrP homolog and is thus suitable to manipulate the PrP^C-independent effects of prion neurotoxicity. Additionally, *C. elegans* was instructive to uncover the neuroprotective role of longevity modulators such as the sirtuin *sir-2.1/SIRT1* in animals expressing a N-terminal portion of the Huntington's disease (HD) protein (Parker et al., 2005), a protein not conserved in nematodes. Here, the biochemical characterization and the manipulation of mutant PrP in *C. elegans* together indicated that sirtuin activation protects from a component of mutant PrP neurotoxicity that involves a Fyn-related kinase activity. Our data also indicate that sirtuin activators have therapeutic potential to protect from the effects of prion neurotoxicity on intracellular signaling.

Materials and Methods

C. elegans

Reporter constructs. The various constructs, human wild-type PrP, mutated PrP with 8 additional octarepeat (PHGGGWGQ) expansion, and GFP, were cloned into the EcoRV–EcoRI site of the *P_{mec-7}* plasmid, pPD118.44 (gifts from Andy Fire, Washington University, St. Louis, MO), to create *P_{mec-7}*-GFP and *P_{mec-7}*-PrP constructs. The normal Wt-PrP and the mutated PG13-PrP constructs were derived from human DNA (Laplanche et al., 1999). Primers used to amplify of human PrP were as follows: PrP-f, 5'-CCGGATATCCGGATGGCGAACCTTGCTGCTGGA-3'; PrP-r, 5'-CGGGAATTCGGTCATCATATCCCACTATCAGGAAGATG-3'. All constructs were sequenced to verify integrity.

Genetic manipulations. Nematodes were maintained following standard methods (Brenner, 1974). Some nematode strains used in this work were provided by the *Caenorhabditis elegans* Genetics Center CGC, which is funded by the National Institutes of Health National Center for Research Resources. Transgenic animals were generated by standard transformation techniques (Mello and Fire, 1995). The various PrP-encoding plasmids were coinjected with a wild-type *lin-15* and *P_{Sir-1}*-GFP marker plasmid into the gonads of young adult *lin-15(n765)* hermaphrodites. The concentrations used were 20 ng/μl for PrP-encoding plasmids, 20 ng/μl for GFP marker-encoding plasmids (*P_{Sir-1}*-GFP and *P_{mec-7}*-GFP), and 50 ng/μl for the wild-type *lin-15* marker. Extrachromosomal arrays showing a similar level of transgene expression as shown by RT-PCR and Western blotting were retained for further studies. Mutant strains were first crossed with *lin-15(n765)* males. These strains were then crossed with our PrP strains (two strains/PrP allele), rescuing the *lin-15(n765)* phenotype. The mutants used in this study included *sir-2.1(ok434)*, *gel-3(sir-2.1(O/E))* (Tissenbaum and Guarente, 2001), and *src-2(ok819)* (Hirose et al., 2003).

Mechanosensory assays. Touch response assays and visualization of *C. elegans* transgenics were performed as described previously (Parker et al., 2001). Touch tests involved scoring for the response to light touch at the tail by using a fine hair. Touch test were performed by scoring 10 touches

at the tail of the animal for a minimum of 200 animals per genotype and with three independent assays performed. Ordinarily, wild-type animals will respond by backing away from touch. The responses were recorded for every animal such that, for example, 3 responses out of 10 at the tail is given as 30% responsiveness, and the mean values for responsiveness were retained for comparison of nematode groups. Wt-PrP animals and PG13-PrP animals were used as internal controls for all of the independent assays performed, and percentage rescue was calculated as (test% – control%)/(Wt-PrP% – PG13-PrP%), where “test” and “control” refer to the genetic background tested. With this formula, a negative value means aggravation, and the greatest amount of rescue as expected by comparing the baseline for Wt-PrP and that for PG13-PrP (maximal achievable rescue) is 100%.

Biochemical analysis and Western blotting. For protein extraction, nematodes were collected and incubated in M9 buffer with 4% glucose during 15 min and were concentrated after centrifugation at 1000 × *g* for 30 s. They were then sonicated during 30 s in lysis buffer containing PBS, 0.1% Triton X-100, and a protease inhibitor cocktail (Roche). Protein concentrations were determined using the BCA method (Pierce) according to the manufacturer's instructions. To test for PrP expression, protein samples were directly subjected to Western blotting analysis. To test for protease resistance, the homogenates were digested with proteinase K (PK, Roche) at final concentration ratio total protein/PK: 1/10,000⁶ for 1 h 45 min at 37°C. To test for PrP solubility, worm homogenates were incubated with 2× detergent buffer (PBS, 2% *N*-laurylsarcosyl, and 2% SDS) for 30 min under agitation and at room temperature (v/v). One fraction of the samples was saved for Western blotting (total fraction) and the remaining was centrifuged 30 min at 15,000 × *g* and at room temperature. The supernatants were removed to fresh tubes and the pellets resuspended in 1× detergent buffer (PBS, 1% *N*-laurylsarcosyl, and 1% SDS). Protein concentrations were determined for each fraction. For Western blotting analysis, proteins (40 μg/line) were separated by SDS-PAGE 12%. After blot transfer, nitrocellulose membranes were incubated overnight at 4°C with the PrP antibody 3F4 (Covance; 1:2000). The blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Biorad; 1:5000), and peroxidase activity was detected using ECL Plus reagent (GE Healthcare). Signal quantification was performed using standard scanning and image analysis tools.

PrP analysis based on the scrapie-associated fibril protocol. This analysis was performed as previously described (Levavasseur et al., 2008; Brandel et al., 2009). Brain samples were homogenized at 20% (w/v) using a Ribolyser (Bio-Rad) in 5% glucose. *C. elegans* proteins were prepared as indicated above. One volume of a 20% NaCl solution and one volume of a detergent solution (20% *N*-laurylsarcosyl, 2% SB3–14, and Tris 20 mM, pH 7.4) were added to one volume of 20% brain homogenate. This mixture had a final pH of 7.6 and was digested with proteinase K at 10 μg/ml for 1 h at 37°C. PrP resistant to PK was then purified according to the scrapie-associated fibril protocol including a centrifugation at 27,500 × *g* for 2 h at 20°C on 20% sucrose cushion. Samples were subjected to SDS-PAGE 12%. Proteins were electroblotted onto nitrocellulose membrane. Immunoblotting was performed using the mouse monoclonal antibodies 3F4 at 0.2 μg of IgG/ml and detected with an ECL kit (GE Healthcare).

RNA isolation and RT-PCR. The expression level of PrP mRNAs was determined using quantitative RT-PCR. RNA was isolated from young adult animals upon synchronization using sonication followed by extraction with a Qiagen RNeasy kit and DNase I (Sigma) treatment (as per the manufacturer's protocol). Single-strand cDNA synthesis was performed using oligoDT priming and 200 ng of total RNA with Taqman Reverse Transcriptase Multiscribe (Applied Biosystems). Quantitative PCR was performed using SYBR Green with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and the following oligonucleotides: 5'–3', PrP-f (TGCTGGATGCTGGTTCTCTT), PrP-r (GGCTGCCCCAGTGTTCCTCA), col-1f (CCAACATCTCCAAGTTATGAAA), and col-1r (GATTGTATGTCGCGAGGAT). Assays and data analysis were performed according to the manufacturer's protocol (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, PerkinElmer). All samples were run at least in triplicate using *Col-1* as the calibrator gene with a dilution of 1:100 of cDNA. The amount of target, normalized to an endogenous reference (PrP) and relative to the calibrator (*Col-1*), was

calculated using the $2^{-\Delta\Delta CT}$ method and statistical significance determined using paired *t* tests (Livak and Schmittgen, 2001).

Immunohistochemistry and fluorescence microscopy. Nematodes were permeabilized and fixed as previously described (Finney and Ruvkun, 1990). The PrP antibody SAF-32 (CEA; 1:400) was incubated with the specimens overnight at 4°C. The fluorescently conjugated Cy-3 secondary antibody (Alexa; 1:800) was applied for 2 h at room temperature. Specimens were washed and stained with DAPI before mounting. Animals were examined with a Zeiss Axioscope epifluorescence microscope (Zeiss) equipped with a Coolsnap camera and MetaView software (Universal Imaging). Transgenic animals were examined for aggregation and axonal morphology of PLM cells using a $\times 100$ objective. A minimum of 100 animals/strain was scored. Examination of PLM cell morphology was performed using a $\times 63$ objective, and 100 animals/strain were scored.

Confocal microscopy. Confocal sections of 0.4 μm were taken, and GFP expression and PrP immunostaining were detected by using FITC and Cy-3 filters, respectively. This was conducted on a confocal microscope (TCS, Leica) and image analysis performed using the ImageJ software. A minimum of 10 animals/strain was scored.

Drug assays in *C. elegans*. Quinacrine and resveratrol were obtained from Sigma. Synchronized L1 larvae were obtained by hypochlorite extraction and incubated with drugs in 96-well plates in 50 μl of S-Media with bacteria (OP50), 30 $\mu\text{g}/\text{ml}$ streptomycin, and 1% DMSO, at 20°C until they reached young adulthood. Animals were then transferred to agar plates, allowed to recover, and assayed for light touch response. A minimum of 200–250 worms/test were scored per dose, and at least four independent assays performed. Wt-PrP animals and PG13-PrP animals were used as internal controls for all of the independent assays performed, and percentage rescue was calculated as $(\text{test}\% - \text{control}\%)/(\text{Wt-PrP}\% - \text{PG13-PrP}\%)$, where “test” and “control” refer to the genetic background tested. Scoring for abnormalities in PLM cells of treated animals was performed as described above.

Mouse cerebellar granule neurons

Cell culture and transfection. Primary cultures of cerebellar granule neurons (CGN) were established as previously described (Cronier et al., 2007). Briefly, CGN cells were extracted from 5- or 6-d-old PrP knock-out mice Zurich I (Büeler et al., 1992) by enzymatic and mechanical dissociation. Batches (5 millions cells) of freshly dissociated cerebellar granule neurons were transfected using Amaxa technology with 10 μg of endofree purified plasmids (Qiagen) encoding either Wt-PrP or PG14-PrP, and PrP constructs inserted in a pcDNA3 vector were a kind gift from Dr. S. Lehmann (CNRS, Montpellier, France) (Lehmann and Harris, 1995). Typical transfection yields were $\sim 50\%$. Cells were then plated at a density of 1900 cells/ mm^2 on plastic culture wells coated with 10 $\mu\text{g}/\text{ml}$ poly-D-lysine and incubated at 37°C with 6% CO_2 . Cells were cultured for 3 d in an antioxidant-containing medium (DMEM-GlutaMAX I (Invitrogen) supplemented with 10% FCS (BioWhittaker), 20 mM KCl, penicillin, and streptomycin (Invitrogen) and completed with N2 and B27 (Invitrogen). Seventy-two hours after transfection (day 3), the cell culture medium was changed for an antioxidant-free medium containing vehicle (DMSO) or 1 μM resveratrol (DMEM-GlutaMAX I, supplemented with 10% FCS, 20 mM KCl, penicillin and streptomycin, N2, and antioxidant-free B27 supplement). Seventy-two hours after treatment (day 6), cells were fixed and processed for immunocytochemistry.

Immunofluorescence. Cells were fixed in PBS containing 4% paraformaldehyde and 4% sucrose for 10 min at room temperature and permeabilized (5 min with PBS and 0.1% Triton X-100). PrP was detected using mAb 4F2 and neuronal population was specifically labeled by using polyclonal Ab raised against MAP2 (Sigma). Cells were then incubated with appropriate Alexa-conjugated secondary antibodies and nuclear marker 4',6-diamidino-2-phenylindole (Sigma) and finally mounted in Fluoromount (Sigma).

Neuronal survival assessment. Neurons from the same transfection batches were processed for PrP immunostaining 72 h after transfection (day 3) and 72 h after treatment with vehicle or resveratrol (day 6). The number of PrP-expressing neurons for each condition was assessed by counting 10 different microscopic fields per well (three wells per condition) and expressed as a ratio of PrP-expressing neurons versus the total

number of cells. This provided a relative PrP expression factor (PF). Neuronal survival of PrP-expressing neurons was then assessed by comparing the PrP expression factors at day 6 versus day 3. Neuronal survival was expressed as the ratio $(\text{PF at day 6})/(\text{PF at day 3})$. Results represent the means of three independent experiments.

Statistical analysis

Statistics of nematode data were performed using one-way ANOVA followed by a Fisher's *post hoc* PLSD test. Statistics of mouse neuron data were performed using the student paired *t* test. All experiments were repeated at least three times. $p < 0.05$ was considered significant.

Results

PG13-PrP expression causes neuronal dysfunction in *C. elegans*

To test whether mutant PrP may be neurotoxic in *C. elegans*, we used the promoter of the *mec-7* (β -tubulin) gene (Finney and Ruvkun, 1990) to express PG13-PrP in touch receptor neurons (supplemental Fig. S1A, available at www.jneurosci.org as supplemental material) in the form of extrachromosomal arrays. We generated four wild-type PrP (Wt-PrP) and four PG13-PrP lines, all of them carrying transgenes at the expected size (supplemental Fig. S1B, available at www.jneurosci.org as supplemental material) and expressing transgenes at similar levels as tested by quantitative RT-PCR (supplemental Fig. S1C, available at www.jneurosci.org as supplemental material). These animals were tested for response to light touch at the tail, a phenotype under the control of two neurons, namely the PVM-L and PVM-R cells. The expression of Wt-PrP did not modify touch response at the tail compared to wild-type animals N2 (Fig. 1A). Compared to Wt-PrP expression, PG13-PrP expression produced a progressive neuronal dysfunction phenotype as tested in L3 and L4 larvae and young adult animals (Fig. 1A). The distribution of individual responses to light touch at the tail of adult PG13-PrP animals was typical of defective mechanosensation (Fig. 1B). To test for the pattern of prion protein expression, we crossed two extrachromosomal lines of each genotype (Wt-PrP or PG13-PrP) to animals stably expressing (integrated array) GFP in touch receptor neurons. The analysis of double transgenic animals confirmed that prion proteins were expressed in PVM cells (Fig. 1C) and that they were addressed at the cell surface membrane (Fig. 1D), as expected. This analysis also revealed that PG13-PrP formed clusters in cell bodies and axons similarly to what has been described for mouse PG14-PrP in mouse brain neurons (Medrano et al., 2008), a phenotype not detected for Wt-PrP (Fig. 1D,E). The analysis of double transgenic animals at the old adult stage further indicated that mutant PrP expression does not cause cell death (Fig. 1F–H).

Mutant PrP in nematode neurons is partially resistant to proteinase K and shows insoluble properties

Previous studies have indicated that mutant PrP may acquire PK resistance, a property characteristic of the pathogenic isoform. To test whether PG13-PrP may be PK resistant in *C. elegans* neurons, we analyzed protein extracts from transgenic worms by Western blot. First, we observed that Wt-PrP and the PG13-PrP proteins had an expected apparent molecular weight of 28–34 kDa and 34–40 kDa, respectively (Fig. 2A), consistent with the pattern of migration of the human prion protein (Aguzzi et al., 2008a). These experiments also confirmed that transgenic lines expressed similar amount of PrP, either wild-type or mutant. As expected, control animals (N2) were deprived of prion protein immunoreactivity (Fig. 2A). To determine whether PG13-PrP may be resistant to proteolysis in *C. elegans* neurons, we incubated transgenic

Figure 1. Human mutant PrP produces a progressive neuronal dysfunction in *C. elegans* transgenics. **A**, Progressive loss of response to touch at the tail caused by mutant (PG13-PrP) PrP expression. Four lines of each genotype (Wt-PrP or PG13-PrP), all of them expressing similar levels of transgene (see supplemental Fig. 1, available at www.jneurosci.org as supplemental material, and Fig. 4A), were tested at different developmental stages (L3 larvae, L4 larvae, and young adults). Each bar corresponds to the mean $\pm$ SEM for touch response at the tail. *** $p < 0.0001$ versus control (mean value for the four Wt-PrP lines). **B**, Compared to a population of Wt-PrP animals, PG13-PrP animals show a shift toward insensitivity in the distribution of responsiveness/animal. The responses to light touch at the tail were recorded for every animal and expressed as mean percentage of responsiveness/animal $\pm$ SEM as shown on the x-axis. **C**, PrP is expressed in *P_{mec-7}* target cells. Two lines expressing either Wt-PrP or PG13-PrP were crossed with animals stably expressing GFP under the control of *P_{mec-7}*. Shown here is a representative immunohistochemical analysis of PrP localization in PLM cells from young adults, with Nomarski analysis shown in the left panel. Magnification is $\times 63$ and scale bar is 5 μ m. Posterior is to the bottom in all panels. **D**, PrP is expressed at the cell body membrane, with accumulation detected in the cell body and axons for PG13-PrP. PrP expression was assessed using confocal analysis of PLM neurons in animals coexpressing GFP and PrP (2 lines/genotype) upon PrP immunostaining. Shown here are representative images of the most frequent phenotypes observed in PLM cells. Wt-PrP primarily shows diffuse expression at the cell body membrane, with few signals detected in the axon in some occasions. PG13-PrP expression shows clustering in the cell body and along the axonal process (white arrows). Magnification is $\times 100$ and scale bar is 2 μ m. **E**, Quantification of PrP clusters in PLM cells. Neurons expressing PG13-PrP exhibit a higher level of PrP clustering (mean $\pm$ SEM) in the cell body and axon. *** $p < 0.001$ compared to Wt-PrP. **F**, Representative confocal images of the most frequent morphology of PLM neurons in day-4 adults coexpressing GFP and PrP. R and L refer to the right and left PLM cells. Magnification is $\times 63$ and scale bar is 5 μ m. Posterior is to the left in all panels. **G**, Quantification of PLM neurons showing a normal morphology in young (day 1) and old (day 4) adults. Two lines/genotype were scored, and 80–90% of

nematode homogenates with a range of PK concentrations. A weaker resistance was observed for PG13-PrP compared to human sporadic CJD brain samples (see PK at 10 μ g/ml), whereas nematode homogenates containing Wt-PrP and human control brain samples were fully digested at these concentrations (Fig. 2B,C). The sensitivity to PK was different between the Wt-PrP lines and PG13-PrP lines (Fig. 2D), and the pattern of digestion for PG13-PrP showed a 19 kDa PK-resistant band similarly to that classically observed in human CJD brain extract (Cali et al., 2006). However the 19 kDa band was difficult to detect in *C. elegans* samples under the protocol used herein. To reach a better comparison of the nematode and human samples, we purified PK-resistant PrP by using the scrapie-associated fibril protocol including a centrifugation step on a 20% sucrose cushion (Levavasseur et al., 2008; Brandel et al., 2009). Additionally, we used brain samples from a patient with Gerstmann-Sträussler-Scheinker syndrome and the PG13 mutation as a control. Samples from this patient and from PG13-PrP-expressing animals showed a comparable pattern of PK-resistant PrP, with additional bands at 17–18 kDa and 7–8 kDa compared to PK-resistant PrP from sporadic CJD (Fig. 2E). Finally, we tested for PG13-PrP solubility into detergents. To this end, we separated nematode homogenates into soluble and sedimented protein fractions after incubation with detergents and centrifugation. Consistent with previous studies (Yuan et al., 2006), $\sim 70\%$ of PG13-PrP was detected in the sedimented fraction, whereas $\sim 96\%$ of Wt-PrP was detected in the soluble fraction (Fig. 2F), indicating that PG13-PrP has insoluble properties.

Quinacrine reduces PG13-PrP neurotoxicity and proteinase-resistant PrP levels in nematodes

To test whether the phenotypes produced by PG13-PrP in nematodes may be manipulated by pharmacological means, we used the tricyclic derivative of acridine quinacrine, a known inhibitor of PrP^{Sc} production (Korth et al., 2001). Micro-

normal PLM cells were detected in mutant PrP and control animals at the old adult stage. **H**, Image of PLM cell degeneration in old adults. A weak level of cell body loss or axonal degeneration (white arrows) was observed in all the genetic background tested, thus corresponding to normal cellular senescence. Magnification is $\times 63$ and scale bar is 5 μ m. Posterior is to the left in all panels.

molar concentrations of quinacrine reversed the abnormal behavior of PG13-PrP animals, with no effect detected in Wt-PrP animals (Fig. 3A; for raw touch responsiveness data, see supplemental Fig. S2, available at www.jneurosci.org as supplemental material). This therapeutic activity was unrelated to a change in transgene expression and prion protein expression (Fig. 3B,C, top). Consistent with its mode of action as an inhibitor of PrP conversion (Korth et al., 2001), quinacrine reduced the level of PK-resistant forms of PG13-PrP (Fig. 3C, bottom).

Mutant PrP is neurotoxic via the FYN-related kinase *src-2*

Having shown that PG13-PrP is able to produce a neurological phenotype in nematode neurons that involves the production of PK-resistant molecules, we sought to examine whether mutant PrP neurocytotoxicity may also involve signaling pathways associated with cellular PrP signaling. To this end, we tested for the role of Fyn kinase since this kinase and member of the large Src oncogene related family tyrosine kinases (SFK family) was previously reported to have a role in cellular PrP signaling (Mouillet-Richard et al., 2000). Kinases of the SFK family are associated with plasma membranes and they are involved in initiating a variety of cellular events such as cell growth and division, cell attachment and movement, cellular differentiation, cell survival, and apoptosis (Thomas and Brugge, 1997). The *C. elegans* genome contains two fyn-related kinases, namely *src-1* and *src-2/kin-22* (Hirose et al., 2003), and we elected to use *src-2* since it is the most characterized gene (Hirose et al., 2003). To test for the role of *src-2* in PG13-PrP neurotoxicity, PrP nematodes were crossed with the loss-of-function (LOF) mutant *src-2(ok819)* (Hirose et al., 2003) and assessed for response to light touch. The LOF mutation *src-2(ok819)* strongly reduced the neuronal dysfunction induced by PG13-PrP expression, whereas it had no effect in Wt-PrP animals (Fig. 4A; for raw touch responsiveness data, see supplemental Fig. S3A, available at www.jneurosci.org as supplemental material). This effect was unrelated to a change in PG13-PrP expression (supplemental Fig. S4A, available at www.jneurosci.org as supplemental material). Importantly, this effect was also unrelated to a change in PK-resistant PrP levels (supplemental Fig. S4B, available at www.jneurosci.org as supplemental material). These results indicated that PG13-PrP is neurotoxic in *C. elegans* via the *src-2*/FYN-related kinase and that *src-2* modifies mutant PrP neurotoxicity regardless of the conversion of mutant PrP into a PK-resistant prion molecule.

Figure 2. Biochemical properties of human prion protein expressed in *C. elegans* neurons. **A**, Similar expression levels for transgenic PrP proteins as shown by Western blotting in the four lines expressing Wt-PrP and four lines expressing PG13-PrP. A molecular weight shift observed for PG13-PrP as expected. Human brain homogenate and protein extracts from the N2 nematode strain were used as controls. **B**, Calibration of resistance to PK digestion using PK at 1000 to 0.1 $\mu\text{g/ml}$. PG13-PrP is partially resistant to PK digestion in a PK-concentration-dependent manner, whereas Wt-PrP is not resistant to PK at all the enzyme concentrations tested. **C**, The resistance of PG13-PrP to PK digestion is weaker than that of sporadic CJD PrP^{Sc}. PG13-PrP is resistant to PK in a dose-dependent manner (10 $\mu\text{g/ml}$ and 0.1 $\mu\text{g/ml}$) at concentrations that fully degrade PrP from healthy human brain (human CTL). At these concentrations, PrP from human brain CJD (Hum CJD) is PK resistant, indicating that PG13-PrP show partial and weaker PK resistance compared to PrP from human CJD. **D**, Resistance to PK digestion in lines expressing PG13-PrP. Prion protein in the PG13-PrP lines are partially protected from PK digestion, whereas Wt-PrP lines do not show PK resistance. Healthy (CTL) and sporadic disease (CJD) human brain homogenates were used as digestion controls. **E**, Western blot signature of PK-resistant PrP associated with the PG13 mutation. T1, Control sample with PK-resistant PrP type 1 from patient with sporadic CJD. T2A, Control sample with PK-resistant PrP type 2A from a patient with sporadic CJD. FC, Frontal cortex from a patient with the PG13 mutation. Tha, Thalamus from a patient with the PG13 mutation. WT, *C. elegans* line expressing wild-type PrP. PG13, *C. elegans* line expressing PG13-PrP. Samples from a patient with the PG13 mutation and from PG13-PrP-expressing animals showed a comparable pattern of PK-resistant PrP^{res} with, compared to PrP^{res} type from sporadic CJD, additional bands at 17–18 and 7–8 kDa. **F**, PG13-PrP is insoluble. Upon fractionation, Wt-PrP is primarily found in the soluble fraction, whereas mutant PrP is primarily found in the sedimented fraction.

Sir-2.1/SIRT1 rescues neuronal dysfunction in mutant PrP nematodes without reducing proteinase-resistant PrP levels

Next, we examined whether the sirtuin and key longevity modulator *sir-2.1*/SIRT1 (Tissenbaum and Guarente, 2001), a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase, may be able to protect nematode neurons from mutant PrP. We elected to study the effects of *sir-2.1*/SIRT1 on mutant PrP neurotoxicity because sirtuin manipulation has therapeutic potential in several models of degenerative diseases,

Figure 3. Quinacrine protects *C. elegans* neurons from the effects of PG13-PrP expression. **A**, Quinacrine reversed neuronal dysfunction (mean $\pm$ SEM) in PG13-PrP animals with no effect detected in Wt-PrP animals. Three independent lines were tested per genotype. $^{***}p < 0.05$ and $^{***}p < 0.0001$ versus untreated PG13-PrP animals. **B**, Quinacrine does not alter PG13-PrP transgene expression as detected by RT-PCR. **C**, Representative Western blot showing that quinacrine does not alter the expression level of the mutated prion protein and restores sensitivity to PK digestion.

including Huntington's disease (Parker et al., 2005), microglia-dependent amyloid β toxicity (Chen et al., 2005), inherited Parkinson's disease (Outeiro et al., 2007), and oculopharyngeal muscular dystrophy (OPMD) (Catoire et al., 2008). The LOF mutant *sir-2.1(ok434)* enhanced neuronal dysfunction induced by PG13-PrP expression with no effect detected in animals expressing Wt-PrP (Fig. 4A; for raw touch responsiveness data, see supplemental Fig. S3A, available at www.jneurosci.org as supplemental material). This effect was unrelated to a change in PG13-PrP expression (supplemental Fig. S4C, available at www.jneurosci.org as supplemental material) and to a change in proteinase-resistant PrP levels (supplemental Fig. S4D, available at www.jneurosci.org as supplemental material). Consistently, increased *sir-2.1* dosage (*sir-2.1(O/E)*) reversed the neuronal dysfunction induced by PG13-PrP expression with no effect detected in animals expressing Wt-PrP (Fig. 4A; for raw touch responsiveness data, see supplemental Fig. S2A, available at www.jneurosci.org as supplemental material). This effect was unrelated to a change in PG13-PrP expression (supplemental Fig. S4E, available at www.jneurosci.org as supplemental material) and to a change in PK-resistant PrP levels (supplemental Fig. S4F, available at www.jneurosci.org as supplemental material). Thus, SIR-2.1 activation protects neurons from mutant PrP neurotoxicity, and this effect is not due to inhibition of mutant PrP conversion into a PK-resistant PrP molecule.

Resveratrol rescues mutant PrP neurotoxicity in nematode and mouse neurons

Having shown that Sir2 protects from PG13-PrP neurotoxicity in *C. elegans*, we sought to examine whether a similar effect may be achieved by pharmacological means. To this end, we used resveratrol, a polyphenolic compound known to act via Sir2 for protection of diseased neurons in several models of neurodegenerative diseases (Chen et al., 2005; Parker et al., 2005; Kim et al., 2007). Resveratrol reversed neuronal dysfunction in PG13-PrP animals with no effect detected in Wt-PrP animals (Fig. 4B; for raw touch responsiveness data, see supplemental Fig. S3B, available at www.jneurosci.org as supplemental material). This effect was not due to a change in PrP transgene expression (supplemental Fig. S4E, available at www.jneurosci.org as supplemental material). Additionally, the neuroprotective effect of this compound

Figure 4. Modulation of PG13-PrP cytotoxicity in *C. elegans* neurons by *src-2/FRK*, *sir-2.1/SIRT1*, and resveratrol. **A**, The LOF *src-2(ok819)* greatly reduced neuronal dysfunction in PG13-PrP animals with no effect seen in Wt-PrP animals. This effect was not due to a change in the resistance to PK digestion (see supplemental Fig. S4A, available at www.jneurosci.org as supplemental material), nor to a change in PG-13-PrP expression levels (supplemental Fig. S4B, available at www.jneurosci.org as supplemental material). Increased *sir-2.1* dosage specifically rescues neuronal dysfunction in PG13-PrP animals, whereas a *sir-2.1* deletion allele (*ok434*) enhanced neuronal dysfunction. These effects were not due to a change in the resistance to PK digestion (see supplemental Fig. S4C,E, available at www.jneurosci.org as supplemental material), nor to a change in PG-13-PrP expression levels (supplemental Fig. S4D,F, available at www.jneurosci.org as supplemental material). Results are given as the percentage of rescue (mean $\pm$ SEM), a negative value means aggravation, and the maximal achievable rescue is 100% (see Materials and Methods). Three independent lines were tested per genotype. $^{***}p < 0.0001$; $^{**}p < 0.001$ compared to PrP transgenics. **B**, Resveratrol rescues neuronal dysfunction in PG13-PrP animals with no effect seen in Wt-PrP animals. This effect was not due to a change in PG13-PrP expression levels (supplemental Fig. S2G, available at www.jneurosci.org as supplemental material). Rescue by resveratrol is lost in PG13-PrP transgenics harboring *sir-2.1* LOF. Three independent lines were tested per genotype. $^{**}p < 0.001$; $^{*}p < 0.05$ compared to animals treated with vehicle.

was lost in the *sir-2.1* LOF (*ok434*) background (Fig. 4B; for raw touch responsiveness data, see supplemental Fig. S3B, available at www.jneurosci.org as supplemental material), indicating that the rescue of mutant PG13-PrP toxicity by resveratrol is mediated by Sir2.

We also tested whether resveratrol may protect mouse neurons against mutant PrP. To this end, primary cultures of cerebellar granule neurons from mice null for PrP (*Prnp*^{-/-}) were transfected with mouse Wt-PrP and PG14-PrP. Costaining of

Figure 5. Resveratrol protects mouse neurons from cell death induced by human mutant (PG14-PrP) prion protein expression. **A**, Representative images of cerebellar granule neurons transfected with Wt-PrP or PG14-PrP constructs. To detect intracellular aggregated form of PG14, cells were permeabilized and immunostained after 4 d of culture (green, dendritic MAP2; red, PrP; blue, DAPI). Wt-PrP and PG14-PrP are expressed in cell bodies and neuronal processes (note that intracellular Wt-PrP staining is fainter than membrane-bound PrP signal in **C**). Accumulation and aggregation in neuronal processes (white arrows) is observed for PG14-PrP. Magnification is $\times 400$ and scale bar is $10\ \mu\text{m}$. **B**, Quantification of PG14-PrP-induced neuronal death and neuroprotection by resveratrol. Data are expressed as the mean ratio $\pm$ SEM of surviving cells at day 6 compared to day 3 after transfection. A ratio equal to 1 means no cell death. PG14-PrP expression strongly alters neuronal survival ($***p < 0.0001$), whereas Wt-PrP expression has no effect. Resveratrol (duration of treatment: 72 h starting at day 3 after transfection) prevents neuronal death induced by PG14-PrP expression. $***p < 0.0001$. **C**, Representative fields of cerebellar granule neurons transfected with Wt-PrP or PG14-PrP and treated for 72 h with either vehicle or resveratrol. Membrane-bound (no permeabilization) forms of PrP are immunostained after 6 d in culture (green, membrane-bound PrP; blue, cell nuclei, DAPI). Note the drastic reduction in PrP-expressing cell number caused by PG14-PrP expression in untreated neurons. Magnification is $\times 200$ and scale bar is $20\ \mu\text{m}$. Inset [red, membrane-bound PrP; yellow, nuclear DAPI (pseudocolor)] shows a healthy Wt-PrP-expressing neuron, whereas remaining PG14-PrP-expressing neurons shows condensed chromatin (white arrow) with dystrophic neurites.

mouse neurons with MAP2 antibodies (staining of dendrites only in cerebellar granule neurons) and PrP antibodies indicated that PrP was primarily expressed at the periphery of cell bodies and along neuritic processes, with a significant level of intracellular protein accumulation and presence of dystrophic dendrites observed for mutant PrP (Fig. 5A), which is consistent with previous

observations on PG14-PrP pattern of expression (Medrano et al., 2008). Mutant PrP caused death of mouse neurons at day 6 after transfection, while Wt-PrP showed no effect, and resveratrol strongly reversed the toxic effect of PG14-PrP with no effect detected in Wt-PrP neurons (Fig. 5B). Rescue by resveratrol was unrelated to a change in mutant PrP expression levels as inferred from analysis of dendrites (Fig. 5C). It is interesting to note that resveratrol treatment did not modify the clustered pattern of PG14-PrP expression along neuritic processes (Fig. 5C). Thus, resveratrol protects PrP-null mouse neurons from cell death induced by mutant PrP.

Discussion

Our data indicate that human mutant PrP expression is able to alter neuronal cell activity in *C. elegans* transgenics, providing a unique system to uncover the modifiers of the early phases (cell dysfunction without cell death) of protein toxicity *in vivo*. The *C. elegans* amenability to genetic and pharmacological analyses may indeed uncover aspects of PrP cytotoxicity difficult to manipulate *in vivo* by other means. Furthermore, the availability of a highly penetrant neurotoxic phenotype in *C. elegans* provides a model suitable in suppressor screens. Interestingly, these animals recapitulate an important component of mutant PrP toxicity, namely, the production and toxicity of PK-resistant and insoluble PrP (Telling et al., 1996; Chiesa et al., 1998). Additionally, these animals reveal that the alteration of proteins known to be involved in cellular PrP signaling such as Fyn-related kinases is involved in mutant PrP neurotoxicity (Mouillet-Richard et al., 2007). Human PrP is usually N-glycosylated in two sites and produces a typical triple-band pattern in Western blot experiments (Lawson et al., 2005). In nematodes, Wt-PrP and PG13-PrP also showed the triple-band pattern reminiscent of the protein glycosylation of PrP.

Although PK resistance of mutant PrP in nematodes was weaker than that of PrP^{Sc} from human CJD brains, mutant PrP produced a strong neurological phenotype in nematodes. This is consistent with the notion that PK resistance and toxicity may be not strictly correlated as observed in Tg mice (Fischer et al., 1996; Manson et al., 1999). The production of PK-resistant PrP and the alteration of intracellular signaling are two pathological components likely to underlie neurotoxicity in mutant PrP nematodes. Regarding the first component, the rescue of neuronal dysfunction by quinacrine, an inhibitor of pathogenic PrP forms *in vitro* and in cell cultures (Korth et al., 2001), indicates that the self-conversion of mutant PrP into PK-resistant prion molecules can be pharmacologically manipulated in these nematodes. This provides a basis and method to search for new inhibitors of pathogenic forms of PrP *in vivo*. This may enhance antiprion drug discovery since the ability of putative antiprion drugs such as quinacrine to protect from pathogenic PrP in mice may be questionable (Collins et al., 2002; Gayraud et al., 2005). Regarding the second component, the strong reduction of neuronal dysfunction in the *src-2* LOF background with no effect on PK-resistant PrP levels indicates that mutant PrP may directly alter intracellular signaling. This observation reveals that a particular Fyn homolog in *C. elegans* is involved in PG-13 PrP neurotoxicity, suggesting that Fyn kinases may be involved in prion neurotoxicity in addition to their role in cellular PrP signaling (Mouillet-Richard et al., 2007). Future studies will determine whether FYN kinases may play a role in mutant PrP neurotoxicity and whether the effectors involved in cellular PrP signaling downstream to FYN such as ERK1/2 (Schneider et al., 2003) may also participate to mutant PrP signaling.

Together with the fact that human prionopathies do not affect a specific neuronal cell type or brain area (DeArmond and Prusiner, 1995; DeArmond and Bouzamondo, 2002), the biochemical and biological features of mutant PrP expression in the touch receptor neurons of *C. elegans* transgenics identify these animals as a relevant model to study the pathogenesis and modification of inherited CJD. Other simple models of prionopathies have been described. A truncated and cytosolic mouse prion protein (MoPrP(23–231)) fused to CFP was previously expressed in nematode muscle cells and reported to alter several processes including development and mobility (Park and Li, 2008). This study concluded that the prion folding mechanism may be similar in nematodes and mammals, which is also supported by our comparison of the PK resistance of pathogenic PrP in nematode neurons and human CJD brain. However, the type of cell targets (muscle cells) and PrP species (cytoplasmic protein) used in this study (Park and Li, 2008) may limit the relevance of this model to study the neurotoxic effects of the full-length prion protein. An octarepeat-expanded form of PrP was previously expressed in the *Drosophila* eye, however with no phenotype produced, a situation likely to result from the existence of a mechanism that suppresses the production of misfolded PrP (Deleault et al., 2003). Here, our data support the notion that octarepeat expansion may confer to PrP the ability to misfold into pathogenic conformers and produce neurotoxicity as previously documented by the expression of mouse PG14-PrP in BHK cells (Ivanova et al., 2001) and transgenic mice (Chiesa et al., 1998). Our data are also consistent with the ability of mutant PrP expression to produce a neurological phenotype in invertebrates as previously reported for the P101L mouse PrP in *Drosophila* (Gavin et al., 2006). Among the mechanisms that may contribute to mutant PrP neurotoxicity is the delayed exit from the endoplasmic reticulum as documented for PG14-PrP in BHK cells (Ivanova et al., 2001). Although we cannot exclude this possibility, our genetic data in nematodes (modulation by *src-2/FRK* and *sir-2.1/SIRT1*) suggest that mutant PrP neurotoxicity may primarily arise from the cell surface, affecting downstream signaling.

Sirtuins are key longevity/cell survival modulators, and their manipulation is neuroprotective in several models of degenerative disease pathogenesis including Wallerian degeneration (Araki et al., 2004), HD (Parker et al., 2005), microglia-mediated amyloid- β toxicity (Chen et al., 2005), Alzheimer's disease/tauopathies and amyotrophic lateral sclerosis (Kim et al., 2007), α -synuclein toxicity (Outeiro et al., 2007), and OPMD (Catoire et al., 2008). It is intriguing that in models of expanded polyglutamine toxicity (HD), increased *sir-2.1/SIRT1* dosage protects diseased cells (Parker et al., 2005), whereas in models of expanded polyalanine toxicity (OPMD), it is detrimental to cell survival, with *sir-2.1/SIRT1* inhibition being beneficial (Catoire et al., 2008). It is also intriguing that both SIRT1 activation (Chen et al., 2005; Parker et al., 2005) and SIRT2 inhibition (Outeiro et al., 2007) may be neuroprotective. Whether sirtuins may be activated or inhibited to achieve neuroprotection is still debated (Dillin and Kelly, 2007). Since sirtuins belong to a network of genes that is finely tuned in response to stress (Brunet et al., 2004), the net outcome of manipulating sirtuins in diseased cell protection may be greatly influenced by (1) the sirtuin being manipulated (SIRT1, SIRT2, and SIRT3 have different subcellular localizations and activities), (2) the level of acetylation of the sirtuin targets that are affected by the pathology (Catoire et al., 2008), and (3) the strength of cellular stress (cell death vs cell dysfunction) induced in the model system (Parker et al., 2005; Pallos et al., 2008). Regarding sirtuins and prions, it was recently reported

by Chen et al. (2008) that the onset of disease induced by infection of mice with the RML prion strain is delayed in SIRT1 KO mice. This appears to contrast with protection of nematode neurons by increased *sir-2.1/SIRT1* dosage. However, infectivity and neurotoxicity may be distinct properties of PrP (Solomon et al., 2009) and the two studies address different prion pathologies. The study by Chen et al. (2008) primarily addressed infectivity, showing that SIRT1 may influence PrP expression and that delayed onset of prion disease (severe neuronal loss and gliosis) in SIRT1 KO mice may involve a reduction of PrP expression, which is a well known determinant of prion infectivity. This protective mechanism is not addressed in PG13-PrP nematodes since *C. elegans* does not have a prion protein. Nonetheless, the data from Chen et al. (2008) together with those reported herein suggest that sirtuins may protect against certain pathological components of prion diseases while exacerbating others, highlighting the complexity of manipulating prion diseases.

Resveratrol is neuroprotective against expanded huntingtin (Parker et al., 2005) and microglia-dependent amyloid- β toxicity (Chen et al., 2005) and active in models of Alzheimer's disease/tauopathies and amyotrophic lateral sclerosis (Kim et al., 2007). While resveratrol may be an indirect sirtuin activator and may activate other cellular targets such as AMP kinase (Dasgupta and Milbrandt, 2007), many of resveratrol's effects are consistent with activation of SIRT1 and modulation of its targets (Baur, 2009). In support of this notion, neuroprotection from expanded polyglutamines by resveratrol was lost in nematodes with *sir-2.1* LOF and in huntingtin knock-in mouse striatal cells treated with the sirtuin inhibitor sirtinol (Parker et al., 2005). Consistently, the rescue of mutant PrP neurotoxicity by resveratrol was lost in *sir-2.1* mutants. The protection from mutant PrP by resveratrol in nematode neurons and *prnp*-null mouse neurons thus calls for sirtuin activators to be evaluated in animal models of inherited prion diseases. Future studies will address the activity of SIRT1-selective activators (Milne et al., 2007; Feige et al., 2008) in this respect.

In conclusion, we generated *C. elegans* transgenics that allow the neurotoxicity of mutant PrP to be manipulated *in vivo*. Mutant PrP forms clusters in neurons, is partially resistant to proteinase K, shows insoluble properties, and induces neuronal dysfunction. The *src-2/Fyn*-related kinase mediates a neurotoxic component that is unrelated to the conversion of mutant PrP into a proteinase-resistant prion. Increased *sir-2.1/SIRT1* dosage protects from this neurotoxic phenotype without affecting proteinase-resistant PrP, and the indirect sirtuin activator resveratrol protects nematode and mouse neurons from mutant PrP toxicity. Collectively, these data suggest that SIRT1 activators have therapeutic potential in prion diseases to protect neurons from the detrimental effects of misfolded PrP conformers on intracellular signaling and homeostasis.

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