

A soluble derivative of PrP^C activates cell-signaling and regulates cell physiology through LRP1 and the NMDA receptor

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Cellular prion protein (PrP^C) is a widely expressed glycosylphosphatidylinositol-anchored membrane protein. Scrapie prion protein is a misfolded and aggregated form of PrP^C responsible for prion-induced neurodegenerative diseases. Understanding the function of the nonpathogenic PrP^C monomer is an important objective. PrP^C may be shed from the cell surface to generate soluble derivatives. Herein, we studied a recombinant derivative of PrP^C (soluble cellular prion protein, S-PrP) that corresponds closely in sequence to a soluble form of PrP^C shed from the cell surface by proteases in the A Disintegrin And Metalloprotease (ADAM) family. S-PrP activated cell-signaling in PC12 and N2a cells. TrkA was transactivated by Src family kinases and extracellular signal-regulated kinase 1/2 was activated downstream of Trk receptors. These cell-signaling events were dependent on the *N*-methyl-D-aspartate receptor (NMDA-R) and low-density lipoprotein receptor-related protein-1 (LRP1), which functioned as a cell-signaling receptor system in lipid rafts. Membrane-anchored PrP^C and neural cell adhesion molecule were not required for S-PrP-initiated cell-signaling. S-PrP promoted PC12 cell neurite outgrowth. This response required the NMDA-R, LRP1, Src family kinases, and Trk receptors. In Schwann cells, S-PrP interacted with the LRP1/NMDA-R system to activate extracellular signal-regulated kinase 1/2 and promote cell migration. The effects of S-PrP on PC12 cell neurite outgrowth and Schwann cell migration were similar to those caused by other proteins that engage the LRP1/NMDA-R system, including activated α_2 -macroglobulin and tissue-type plasminogen activator. Collectively, these results demonstrate that shed forms of PrP^C may exhibit important biological activities in the central nervous system and the peripheral nervous system by serving as ligands for the LRP1/NMDA-R system.

Host-encoded cellular prion protein (PrP^C) is a widely expressed glycosylphosphatidylinositol (GPI)-anchored membrane protein (1, 2). In prion diseases, scrapie prion protein (PrP^{Sc}), a misfolded conformer of PrP^C, functions as an infectious agent, recruiting PrP^C monomers into multimolecular assemblies, which accumulate to high levels in the central nervous system (CNS). Whereas PrP^C is monomeric and predominantly α -helical in structure, PrP^{Sc} is rich in β -sheets and

insoluble in nondenaturing detergents (3, 4). PrP^{Sc} aggregates include oligomers and insoluble fibrils, which continuously accumulate to cause the onset and progression of neurodegeneration and gliosis (2, 5).

Understanding the activity of naturally occurring PrP^C is an important objective. Because PrP^C is GPI-anchored (6), it localizes largely to lipid rafts, which are known to be important for assembly of molecular scaffolds involved in signal transduction (7). PrP^C also may be shed from cell surfaces to generate soluble derivatives. Proteases in the A Disintegrin And Metalloprotease (ADAM) family have been implicated in PrP^C shedding, including ADAM8, ADAM9, and ADAM10 (8–10).

There is considerable evidence that membrane-anchored and soluble PrP^C derivatives participate in signal transduction to regulate processes such as neuronal differentiation and synapse formation (11–14). Neural cell adhesion molecule (NCAM) has been implicated in PrP^C-activated cell-signaling (11, 13, 15). Metabotropic glutamate receptor type 5 (mGluR5) and the *N*-methyl-D-aspartate receptor (NMDA-R) function with membrane-anchored PrP^C to activate signal transduction in response to α -synuclein oligomers (14). Irrespective of the receptors involved, activation of Src family kinases (SFKs) appears to be a key step in the pathway by which PrP^C regulates cell physiology (11, 13, 14).

Low-density lipoprotein receptor-related protein (LRP1) is an endocytic and cell-signaling receptor expressed by diverse cell types (16). In the CNS, LRP1 is expressed by neurons and concentrated in postsynaptic densities, where it regulates synaptic plasticity (17–19). In response to ligands, including activated α_2 -macroglobulin and tissue-type plasminogen activator (tPA), LRP1 activates SFKs in neurons and neuron-like cell lines (20). This pathway requires the NMDA-R in addition to LRP1; together these receptors form a cell-signaling receptor complex that functions in lipid rafts (21–23). Other receptors that may be recruited into the LRP1/NMDA-R complex to activate signal transduction include Trk receptors and p75^{NTR} (22, 24). Important consequences of SFK activation, downstream of LRP1 and the NMDA-R, include Trk receptor transactivation and extracellular signal-regulated kinase 1/2 (ERK1/2) activation (20). Activation of the LRP1/NMDA-R signaling receptor complex induces neurite outgrowth in neurons and migration of Schwann cells (SCs) (20, 25).

In addition to LRP1 and the NMDA-R, activation of cell-signaling by tPA is reported to require membrane-anchored PrP^C

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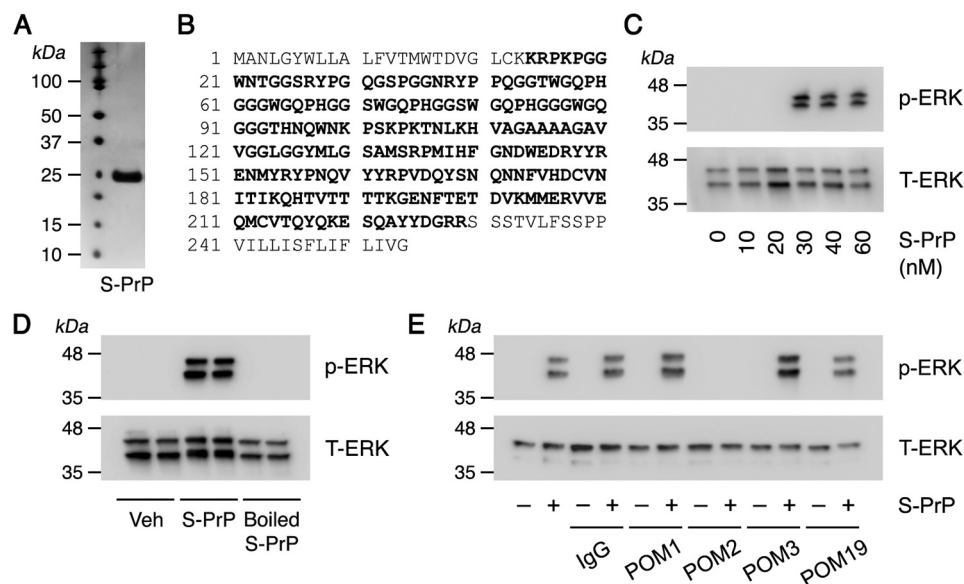


Figure 1. S-PrP initiates cell-signaling in PC12 cells. A, S-PrP (1.8 μ g) was subjected to SDS-PAGE and silver staining. B, tryptic peptides identified in S-PrP by LC-MS/MS are shown in bold in relation to the sequence of PrP^C. C, PC12 cells were treated with increasing concentrations of S-PrP for 10 min. D, PC12 cells were treated with S-PrP (40 nM), boiled S-PrP (40 nM), or vehicle (Veh; 20 mM sodium phosphate and 150 mM NaCl, pH 7.4) for 10 min. E, PC12 cells were treated with S-PrP (40 nM) or vehicle in the presence of nonspecific IgG, POM1, POM2, POM3, or POM19 (10 μ g/ml) for 10 min. In panels C–E, phosphorylated ERK1/2 (p-ERK) and total ERK1/2 (T-ERK) were determined by immunoblot analysis. The blots shown are representative of at least three independent studies.

(22, 26, 27). Herein, we studied the cell-signaling activity of a recombinant product, soluble cellular prion protein (S-PrP), corresponding closely in structure to the soluble form of PrP^C released by ADAM10 (8). We demonstrate that S-PrP activates ERK1/2 by a pathway that requires LRP1, the NMDA-R, SFKs, and Trk receptors. Membrane-anchored PrP^C and NCAM were not required. S-PrP induced neurite outgrowth in PC12 cells and SC migration. Our results support a model in which the LRP1/NMDA-R signaling receptor complex is capable of mediating biological activities of soluble PrP^C derivatives and possibly membrane-anchored PrP^C expressed by adjacent cells.

Results

S-PrP initiates cell-signaling in neuron-like cells

A recombinant derivative of PrP^C (S-PrP), corresponding roughly in sequence (residues 23–231) to the PrP^C ectodomain fragment shed by ADAM10 (8), was expressed in *Escherichia coli*. SDS-PAGE and silver staining confirmed that a single major product with the anticipated molecular mass was purified (Fig. 1A). To authenticate this recombinant protein, the only detected band was excised from the gel and, following reduction and alkylation, proteolyzed with trypsin. Tryptic peptides were identified by LC-MS/MS. Over 98% of the identified peptides corresponded in sequence to mouse PrP^C. Fig. 1B shows that the identified peptides covered the PrP^C sequence from amino acids 24–228, closely matching the anticipated structure of S-PrP and excluding the known N-terminal PrP^C signal peptide and C-terminal GPI-anchor signal sequence (28).

Neuron-like PC12 pheochromocytoma cells were treated with S-PrP at increasing concentrations for 10 min. ERK1/2 phosphorylation was determined by immunoblot analysis and evident in cells treated with PrP^C at concentrations of 30 nM or

higher (Fig. 1C). To confirm that the effects of S-PrP on ERK1/2 phosphorylation were not due to endotoxin contamination, S-PrP was boiled at 100 °C for 5 min before addition to PC12 cell cultures. This procedure inactivates most proteins but fails to affect the bioactivity of endotoxin (29). Fig. 1D shows that boiling completely eliminated the ability of S-PrP to activate ERK1/2.

Next we tested the ability of four PrP^C-specific monoclonal antibodies to inhibit ERK1/2 activation in response to S-PrP (30). POM2 recognizes the N-terminal octarepeat region and thus has the greatest potential to sterically interfere with interactions involving the absolute N terminus, the octarepeat region, or the central unstructured region. POM3 recognizes an epitope C-terminal to the POM2 epitope and near the center of PrP^C. POM1 and POM19 recognize epitopes in the C-terminal globular region of PrP^C (30). PC12 cells were treated with 40 nM S-PrP in the presence of each antibody (10 μ g/ml) for 10 min. Only POM2 blocked ERK1/2 phosphorylation in response to S-PrP (Fig. 1E). The activity of POM2 confirms that the effects of S-PrP are not due to a contaminant such as endotoxin. Furthermore, the selective activity of POM2 suggests that the N-terminal “nonglobular” region of S-PrP is most likely involved in triggering signal transduction.

Activation of cell-signaling by S-PrP requires LRP1 and lipid rafts

We showed previously that LRP1 localizes transiently to lipid rafts (31) and that this subpopulation of LRP1 is essential for LRP1-initiated cell-signaling (23). Because LRP1 and membrane-anchored PrP^C apparently function as co-receptors to trigger cell-signaling events initiated by the LRP1 ligand, tPA (27), we tested whether LRP1 is necessary for S-PrP-initiated

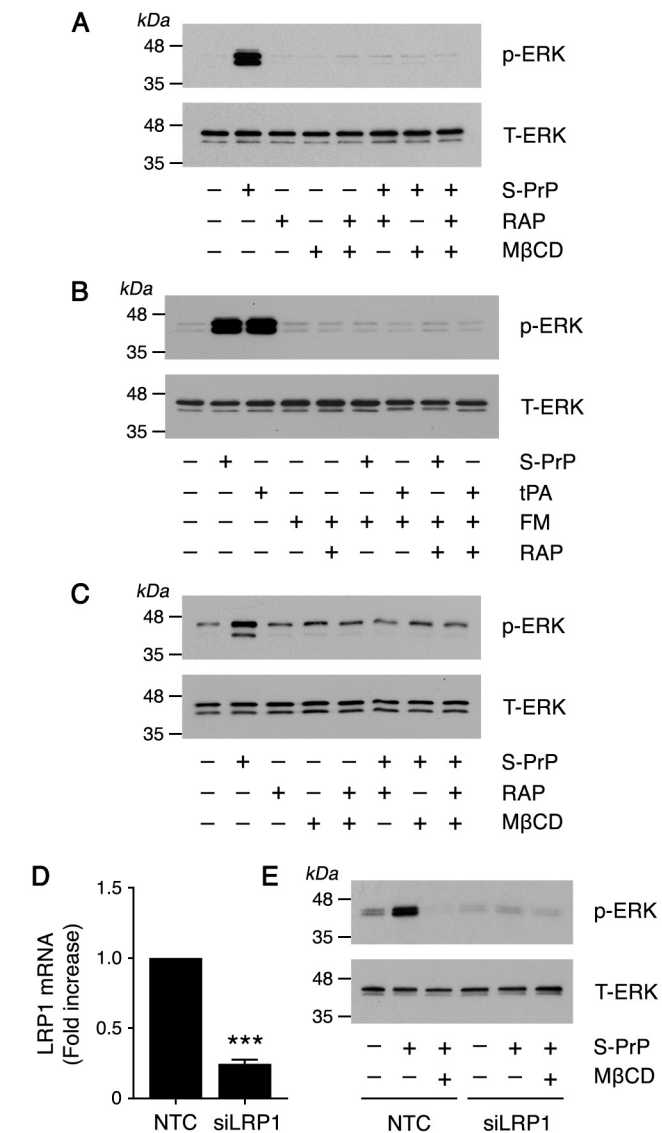


Figure 2. LRP1 and lipid rafts are required for S-PrP-initiated cell-signaling. A, PC12 cells were pretreated with RAP (150 nM) and/or with M β CD (1 mM), as indicated, and then with S-PrP (40 nM) or vehicle (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) for 10 min. B, PC12 cells were pretreated with FM (25 μ M) and/or with RAP (150 nM) and then with S-PrP (40 nM), EI-tPA (12 nM), or vehicle, as indicated. C, N2a cells were pretreated with RAP (150 nM) or M β CD (1 mM) and then with S-PrP (40 nM) or vehicle for 10 min. D, PC12 cells were transfected with LRP1-specific or NTC siRNA. Relative LRP1 mRNA expression was determined by RT-qPCR, ($n = 4$, *** $p < 0.001$). E, PC12 cells were transfected with LRP1-specific or NTC siRNA. The cells were then treated with M β CD (+) or vehicle (–) for 30 min, followed by S-PrP (40 nM) or vehicle. In panels A–C and E, equal amounts of cellular protein (20 μ g) were subjected to SDS-PAGE. Immunoblot analysis was performed to detect phosphorylated ERK1/2 (p-ERK) and total ERK (T-ERK). The blots shown are representative of at least three independent studies.

cell-signaling. To begin, we treated PC12 cells with S-PrP together with receptor-associated protein (RAP), which binds to LRP1 and inhibits binding of all other known ligands that activate signal transduction (16, 22, 32). In the absence of RAP, 40 nM S-PrP induced ERK1/2 phosphorylation in PC12 cells; however, this response was entirely blocked by 150 nM RAP (Fig. 2A). ERK1/2 phosphorylation in response to S-PrP also was blocked by methyl- β -cyclodextrin (M β CD), a cholesterol sequestration reagent known to disrupt lipid rafts (33). These

results suggest that lipid raft-associated LRP1 is involved in S-PrP-initiated cell-signaling.

To further test whether intact lipid rafts are required for S-PrP-initiated cell-signaling, we pretreated PC12 cells with fumonisin B1 (FM, 25 μ M) for 24 h. FM blocks synthesis of sphingolipids that are key components of lipid rafts (34). Fig. 2B shows that FM blocked ERK1/2 phosphorylation in response to enzymatically inactive (EI) tPA, as previously demonstrated (23), and in response to S-PrP.

Similar results were obtained when we studied mouse N2a neuroblastoma cells (Fig. 2C). ERK1/2 was phosphorylated in N2a cells treated with 40 nM S-PrP. The response to S-PrP was blocked by 150 nM RAP and by pre-treating the cells with M β CD. Thus, S-PrP activates cell-signaling in two distinct neuron-like cell lines via a pathway that is inhibited by RAP and by disrupting lipid rafts.

In addition to LRP1, RAP blocks binding of ligands to other receptors in the LDL receptor gene family (35). To confirm the importance of LRP1 in S-PrP-activated signaling, we silenced *LRP1* gene expression in PC12 cells with siRNA. Fig. 2D shows that LRP1 mRNA expression was significantly decreased in cells transfected with LRP1-specific siRNA compared with cells transfected with nontargeting control (NTC) siRNA. Fig. 2E shows that LRP1 gene-silencing blocked activation of ERK1/2 in response to S-PrP. M β CD inhibited ERK1/2 phosphorylation in cells transfected with LRP1-specific or NTC siRNA.

Activation of cell-signaling by S-PrP requires the NMDA-R

LRP1 activates cell-signaling in response to diverse ligands by functioning as part of a system of receptors, including the NMDA-R, which plays an essential role (21, 22, 36). To test whether S-PrP-activated cell-signaling requires the NMDA-R, we treated PC12 cells with dizocilpine (MK801), a noncompetitive antagonist of the NMDA-R (37). MK801 blocked ERK1/2 phosphorylation in response to the known LRP1 ligand, tPA, which was introduced as an EI derivative (Fig. 3A), as anticipated (22). MK801 also blocked ERK1/2 phosphorylation in response to S-PrP. To further test the activity of the NMDA-R in S-PrP-activated signaling, we treated PC12 cells with dextromethorphan hydrobromide (DXM), a noncompetitive NMDA-R antagonist (38). DXM blocked ERK1/2 phosphorylation in response to S-PrP (Fig. 3B).

Finally, to confirm the importance of NMDA-R in S-PrP-activated signaling, we applied a gene-silencing approach, targeting the GluN1 NMDA-R subunit with siRNA in PC12 cells, as previously described (22). The GluN1 subunit is essential for assembly of intact NMDA-R heterotetramers (39). Our gene-silencing approach decreased GluN1 mRNA expression by $80 \pm 3\%$, as determined by RT-quantitative PCR (qPCR) ($n = 3$). Fig. 3C shows that GluN1 gene-silencing blocked ERK1/2 activation in response to S-PrP.

S-PrP induces neurite outgrowth in PC12 cells

ERK1/2 activation has been implicated in neuronal differentiation and in neurite outgrowth in PC12 cells (40). We examined the effects of S-PrP on PC12 cell neurite outgrowth. Fig. 4 shows that S-PrP promoted neurite outgrowth in PC12 cells

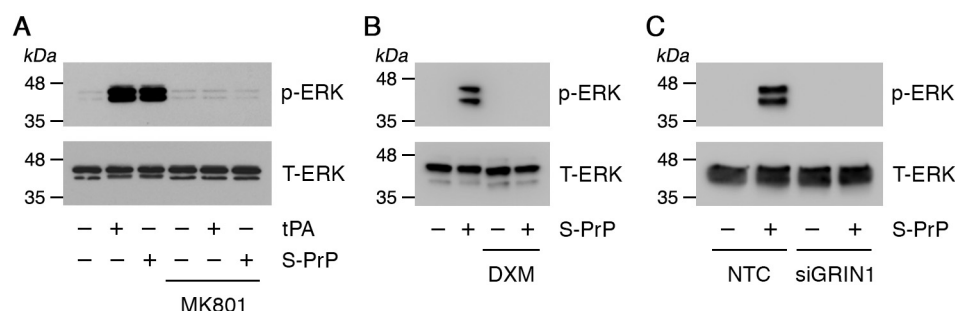


Figure 3. NMDA-R is required for ERK1/2 activation by S-PrP. A, PC12 cells were pretreated with 1 μ M MK801 or vehicle (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) for 30 min, and then with S-PrP (40 nM) or Et-TPA (12 nM), as indicated, for 10 min. B, PC12 cells were pretreated with 10 μ M DXM or vehicle for 30 min, and then with S-PrP (40 nM) or vehicle for 10 min. C, PC12 cells were transfected with GluN1-specific (*siGRIN1*) or NTC siRNA. The cells were then treated with S-PrP (40 nM) or vehicle for 10 min. Equal amounts of cellular protein (20 μ g) were loaded into each lane and subjected to SDS-PAGE. Immunoblot analysis was performed to detect phosphorylated ERK1/2 (p-ERK) and total ERK1/2 (T-ERK). The blots shown are representative of at least three independent studies.

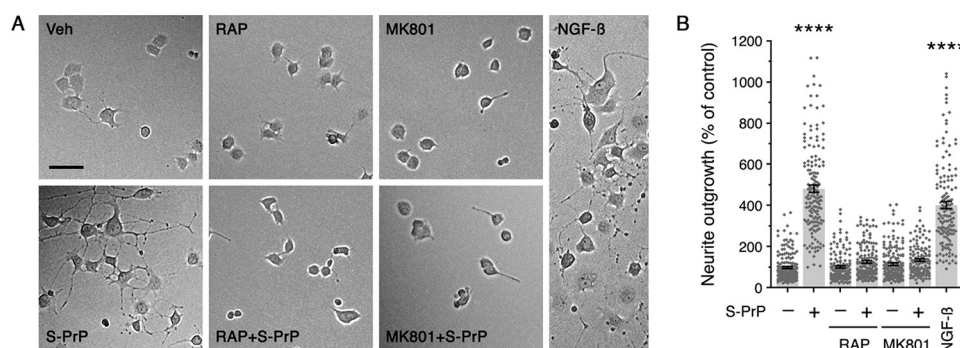


Figure 4. S-PrP promotes neurite outgrowth in PC12 cells. A, PC12 cells were treated with 40 nM S-PrP (bottom row) or vehicle (20 mM sodium phosphate and 150 mM NaCl, pH 7.4; top row) for 48 h in the presence or absence of 150 nM RAP or 1 μ M MK801. NGF- β (50 ng/ml) was added to separate cultures as a positive control. Neurite outgrowth was detected by phase contrast microscopy. Representative images are shown (scale bar, 20 μ m). B, neurite length was determined for 50 cells, chosen at random, in three different experiments (**** p < 0.0001).

maintained in serum-free media (SFM) for 48 h. The extent of neurite outgrowth was similar to that detected in cells treated with nerve growth factor (NGF)- β . RAP and MK801 did not independently regulate neurite outgrowth; however, both reagents entirely blocked the response to S-PrP, suggesting that LRP1 and the NMDA-R are necessary for S-PrP-induced PC12 cell neurite outgrowth.

Trk is transactivated by SFKs upstream of ERK1/2 in S-PrP-treated PC12 cells

SFKs have been implicated in cell-signaling events initiated by PrP^C in association with NCAM and mGluR5 (11, 13, 14). SFKs also are activated in neurons and neuron-like cells when specific ligands such as tPA or activated α_2 -macroglobulin bind to LRP1 (20). Activated SFKs transactivate Trk receptors, which leads to ERK1/2 activation (20).

Fig. 5A shows that TrkA was phosphorylated in PC12 cells treated with 40 nM S-PrP for 10 min. *LRP1* gene-silencing blocked Trk phosphorylation in response to S-PrP, as did MK801. These results suggest that TrkA is activated in S-PrP-treated cells downstream of LRP1 and the NMDA-R. To test whether SFK activity is necessary for TrkA phosphorylation and ERK1/2 activation in response to S-PrP, PC12 cells were pretreated for 2 h with the SFK-selective inhibitor, PP2 (1 μ M). PP2 blocked TrkA phosphorylation in response to S-PrP (Fig. 5B). Similarly, PP2 blocked ERK1/2 phosphorylation in response to S-PrP (Fig. 5C). These results support a model in

which S-PrP transactivates TrkA through a pathway that requires SFKs. ERK1/2 is activated downstream of TrkA.

To further test this model, PC12 cells were treated for 2 h with the Trk tyrosine kinase inhibitor, K252a (10 nM), and then with S-PrP. K252a blocked ERK1/2 phosphorylation in response to S-PrP (Fig. 5D).

To test whether S-PrP-activated cell-signaling in PC12 cells requires membrane-associated PrP^C, we silenced *PRNP* gene expression in PC12 cells with siRNA. Fig. 5E shows that gene-silencing was nearly complete at the protein level, as determined by immunoblot analysis. When PrP^C-deficient PC12 cells were treated with S-PrP (40 nM), ERK1/2 was activated, suggesting that membrane-associated PrP^C may not be necessary for this response.

Next, we studied whether NCAM is necessary for activation of cell-signaling by S-PrP in PC12 cells. *NCAM1* gene expression was silenced in PC12 cells with siRNA. Fig. 5F shows that NCAM protein expression was nearly but not entirely eliminated by gene-silencing. In PC12 cells in which *NCAM1* was silenced, S-PrP (40 nM) still activated ERK1/2, suggesting that the LRP1/NMDA-R-dependent pathway by which S-PrP activates cell-signaling is probably distinct from the previously described NCAM pathway (11, 13, 15).

The results of our cell-signaling experiments suggested a model in which SFKs and Trk receptors function downstream of LRP1 and the NMDA-R in the pathway by which S-PrP activates ERK1/2. To test whether SFKs and Trk receptors are

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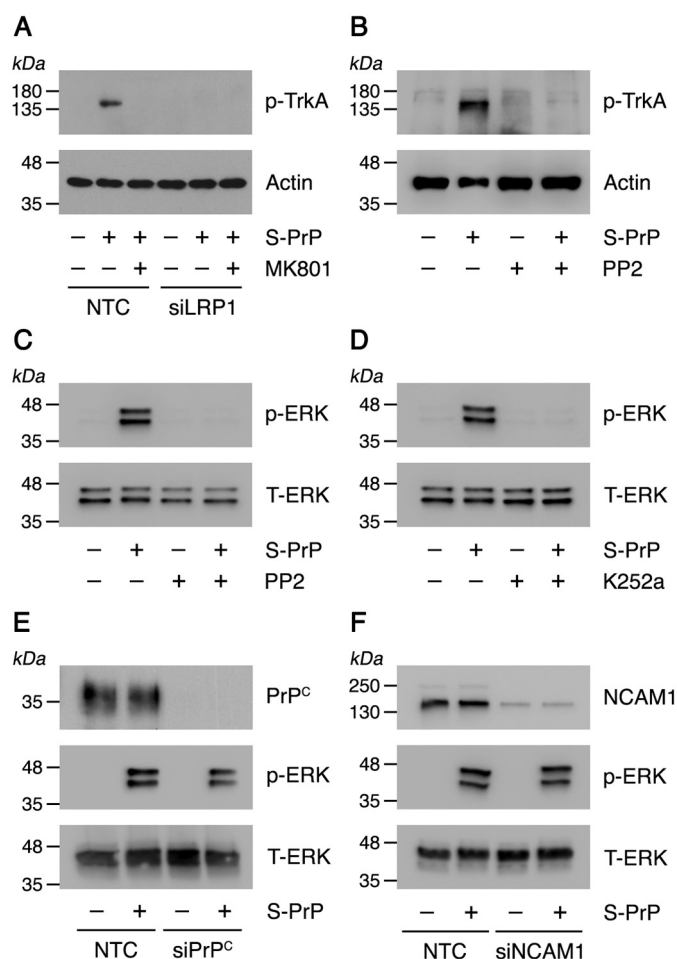


Figure 5. TrkA is phosphorylated and required for ERK1/2 activation in PC12 cells treated with S-PrP. A, PC12 cells were transfected with LRP1-specific or NTC siRNA. The cells were treated with 1 μ M MK801 or vehicle (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) for 30 min, and then with S-PrP (40 nM) or vehicle. B–C, PC12 cells were treated with 1 μ M PP2 or vehicle for 2 h, and then with S-PrP (40 nM) or vehicle. D, PC12 cells were treated with 10 nM K252a or vehicle for 2 h, and then with S-PrP (40 nM) or vehicle. E, PC12 cells were transfected with PrP^C-specific or NTC siRNA. The cells were then treated with S-PrP (40 nM) or vehicle. F, PC12 cells were transfected with NCAM1-specific or NTC siRNA. The cells were then treated with S-PrP (40 nM) or vehicle. Equal amounts of cellular protein (20–40 μ g) were subjected to SDS-PAGE. Immunoblot analysis was performed to detect phosphorylated TrkA (p-TrkA), actin, phosphorylated ERK1/2 (p-ERK), total ERK1/2 (T-ERK), PrP^C, and NCAM1. The blots shown are representative of at least three independent studies.

necessary for S-PrP-induced PC12 cell neurite outgrowth, we treated PC12 cells with 40 nM S-PrP in the presence and absence of PP2 and K252a for 48 h. Both reagents blocked neurite outgrowth in response to S-PrP (Fig. 6).

S-PrP activates cell-signaling in SCs by a pathway that requires the NMDA-R and LRP1

SCs express LRP1 and the NMDA-R (41, 42). In response to specific ligands that bind to the LRP1/NMDA-R complex, ERK1/2 is activated and increased SC migration is observed (25, 41–43). As a second model system to test whether the LRP1/NMDA-R system activates cell-signaling in response to S-PrP, we treated primary cultures of rat SCs with S-PrP (40 nM) for 10 min. Fig. 7A shows that ERK1/2 was activated and

that the response was blocked by RAP (150 nM) and MK801 (1 μ M).

Next, we studied the effects of S-PrP on SC migration using Transwell cell migration devices. SCs were treated with S-PrP in the presence or absence of RAP or MK801 in the upper chamber and then allowed to migrate for 48 h. Representative images of cells that migrated to the underside surfaces of Transwell membranes are shown in Fig. 7B. The results of three separate experiments with different SC preparations are summarized in Fig. 7C. S-PrP significantly increased SC migration. RAP and MK801 blocked SC migration in response to S-PrP. Thus, S-PrP promotes SC migration by a pathway that is inhibited by antagonists of LRP1 or the NMDA-R.

Discussion

PrP^C is expressed at high levels by neurons and astrocytes in the CNS, by neurons in the peripheral nervous system (PNS) and by diverse cell types outside the nervous system (44, 45). Nevertheless, mice in which the gene encoding PrP^C, *PRNP*, is globally deleted develop normally (46). In neurons and non-neuronal cells, PrP^C has been reported to inhibit apoptosis, although this activity may be context-specific (47–50). In the PNS, *PRNP* gene deletion causes a chronic form of demyelinating polyneuropathy (51). Because conditional deletion of *PRNP* in SCs did not replicate the demyelinating disorder, it is assumed that SCs respond to membrane-anchored PrP^C expressed by adjacent neurons or to soluble PrP^C derivatives, similar to S-PrP, released from neurons.

Membrane-anchored PrP^C has been shown to function in concert with LRP1 to initiate cell-signaling in response to tPA (26). This is intriguing because there are similarities in the reported activities of tPA and PrP^C relative to neuronal cell physiology. For example, tPA is reported to be neuroprotective and inhibit neuron apoptosis (52–54). The effects of tPA on cell survival in the CNS are independent of its activity as a protease and instead dependent on interaction with the LRP1/NMDA-R system (52–54). The LRP1/NMDA-R system also promotes survival of SCs in the PNS (42).

We studied a soluble recombinant derivative of PrP^C that is almost full-length. Due largely to the activity of ADAM gene family members, as much as 10–15% of membrane-anchored PrP^C may be released as soluble derivatives (7–9, 55, 56). By studying recombinant S-PrP, we hoped to uncover activities attributable to soluble derivatives of PrP^C. Our studies also may model interactions of membrane-anchored PrP^C with receptors expressed by closely associated cells in trans configuration.

The results presented here show that S-PrP activates ERK1/2 in PC12 cells, N2A cells, and SCs. The response requires the NMDA-R and LRP1 and causes changes in cell physiology that are anticipated for this receptor system, including neurite outgrowth in PC12 cells (20, 57) and increased SC migration (25, 42). SFKs played a critical role in S-PrP-initiated cell-signaling by transactivating Trk receptors, which are apparently upstream of ERK1/2, as previously reported (20). Inhibiting SFKs with PP2 blocked Trk receptor transactivation, ERK1/2 activation, and the effects of S-PrP on PC12 neurite outgrowth. Interestingly, membrane-anchored PrP^C did not appear to be

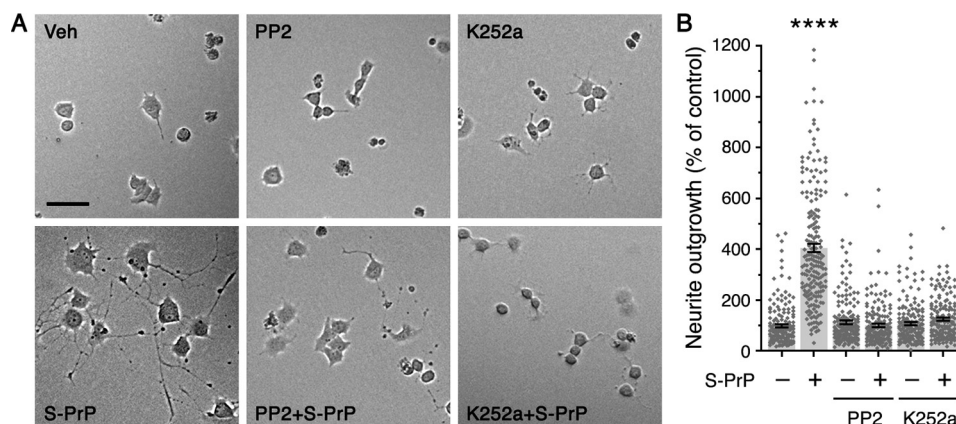


Figure 6. SFKs and Trk receptors are necessary for S-PrP to promote neurite outgrowth in PC12 cells. A, PC12 cells were treated with S-PrP (40 nM) or vehicle (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) for 48 h in the presence or absence of 1 μ M PP2 or 10 nM K252a, as indicated. Neurite outgrowth was detected by phase contrast microscopy. Representative images are shown (scale bar, 20 μ m). B, neurite length was determined for 50 cells, chosen at random, in three different experiments (**** p < 0.0001).

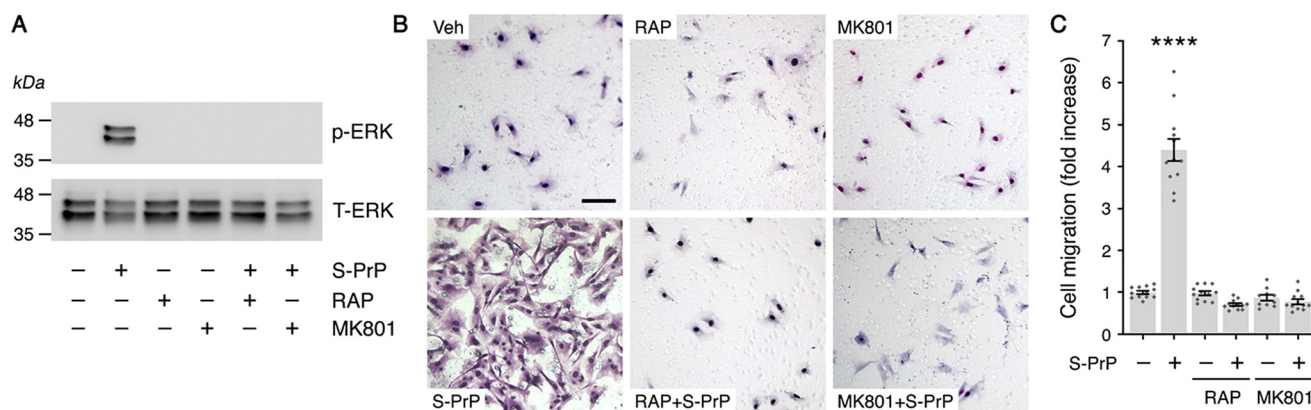


Figure 7. S-PrP promotes SC migration. A, SCs cells were pretreated with 150 nM RAP, 1 μ M MK801, or vehicle (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) for 30 min, as indicated, and then with S-PrP (40 nM) for 10 min. Equal amounts of cellular protein (20 μ g) were subjected to SDS-PAGE. Immunoblot analysis was performed to detect phosphorylated ERK1/2 (*p-ERK*) and total ERK1/2 (*T-ERK*). The blots shown are representative of at least three independent studies. B, representative images of Schwann cells that migrated through Transwell membranes to the lower surfaces. The cells were treated with S-PrP (40 nM), RAP (150 nM), MK801 (1 μ M), or vehicle, as indicated. All reagents were added to both Transwell chambers. Migration was allowed to proceed for 4 h at 37 $^{\circ}$ C (scale bar, 10 μ m). C, quantification of cell migration results. Data are expressed as the mean \pm S.E. (n = 3, one-way analysis of variance with Tukey's post hoc; **** p < 0.0001 compared with the vehicle control).

necessary for activation of cell-signaling by S-PrP, in contrast with the results obtained in a previous study of tPA-initiated cell-signaling through LRP1 (26).

We confirmed the purity of S-PrP by SDS-PAGE, silver staining, and LC-MS/MS. Because cell-signaling activity was entirely eliminated when S-PrP was boiled, it is highly unlikely that endotoxin contributed to the activity of S-PrP (29). Furthermore, a single mAb that recognizes PrP^C, POM2, entirely neutralized S-PrP activity, again indicating that S-PrP is the active agent. Nevertheless, we cannot be certain that our entire preparation of recombinant S-PrP, expressed in bacteria, refolded equivalently to PrP^C that is shed from eukaryotic cells. S-PrP lacks glycosylation, which is present in PrP^C expressed by mammalian cells (58). However, we are encouraged that S-PrP initiated cell-signaling at concentrations as low as 30 nM, which is similar to the concentration of tPA required to trigger cell-signaling via the LRP1/NMDA-R system (22, 59). If refolding of recombinant S-PrP was imperfect and a fraction of the preparation was incapable of binding to the LRP1/NMDA-R system, then the re-

mainder of the S-PrP preparation probably functions at concentrations lower than 30 nM.

Although LRP1 ligands are diverse in structure and function (16, 35), the structural elements required for LRP1-binding appear to be conserved. Lys residues, frequently positioned in tandem, are major elements of the LRP1-binding sites in activated α_2 -macroglobulin, RAP, and plasminogen activator inhibitor-1 (60–62). Adjacent Lys residues are present in the unstructured N-terminal region of PrP^C, close to the absolute N terminus and between the octarepeat and hydrophobic regions (see Fig. 1B). The Lys residues that are more distant from the N terminus are adjacent to His residues implicated in copper-binding, which regulates the activity of PrP^C in neuritogenesis (63). The POM2 epitope is positioned in the primary structure of PrP^C so that POM2 binding may sterically inhibit interaction of LRP1 with Lys residues either at the absolute N terminus or between the octarepeat and hydrophobic regions (30).

Our studies demonstrating an essential role of the NMDA-R in mediating cell-signaling initiated by S-PrP are intriguing

A soluble PrP^C derivative activates cell-signaling

given prior studies demonstrating possible interactions between PrP^C and the NMDA-R. PrP^C has been reported to attenuate neuronal excitotoxicity by regulating GluN2D-containing NMDA-Rs and to co-immunoprecipitate with the NMDA-R (64). PrP^C and the NMDA-R are both key components of the receptor system activated by α -synuclein oligomers (14). In this pathway, the G protein-coupled receptor, mGluR5, plays an essential role by activating Fyn, which targets the NMDA-R as a substrate.

Like the NMDA-R, Trk receptors are targeted as substrates by SFKs. This interaction results in Trk receptor transactivation (65). Because inhibiting the NMDA-R with MK801 blocked Trk receptor transactivation in response to tPA (22) and S-PrP (Fig. 5A), we hypothesize that SFKs are activated downstream of the NMDA-R in response to LRP1 ligands, an apparent difference compared with the pathway reported for α -synuclein oligomers (14). Nevertheless, assessing the role of mGluR5 as a component of the NMDA-R/LRP1 cell-signaling receptor system appears justified.

LRP1 localizes primarily in clathrin-coated pits and undergoes rapid endocytosis and recycling, apparently in the presence or absence of ligands (66, 67). A small fraction of LRP1 transiently localizes to lipid rafts before transferring to clathrin-coated pits; however, this subpopulation of LRP1 may be very important because lipid raft-associated LRP1 is apparently responsible for initiating cell-signaling in response to LRP1 ligands (22, 30). The ability of S-PrP to stimulate cell-signaling was blocked when lipid rafts were disrupted, consistent with this model. It thus appears that lipid raft microdomains play an instrumental role in the cell-signaling activity of membrane-anchored PrP^C and soluble derivatives of this protein.

Not all LRP1 ligands trigger cell-signaling equivalently. The pathway described herein, in which S-PrP activates ERK1/2 downstream of the NMDA-R and Trk, is observed with quite a few LRP1 ligands (20, 22). However, other LRP1 ligands, such as myelin-associated glycoprotein, force assembly of a cell-signaling receptor complex that includes p75^{NTR} instead of Trk receptors and activate RhoA instead of ERK1/2 (24). RAP activates neither of these pathways but instead serves as an antagonist of cell-signaling responses mediated by LRP1 in conjunction with all other co-receptors, including the NMDA-R, Trk receptors, and p75^{NTR} (22). The ability of diverse LRP1 ligands to force assembly of different cell-signaling receptor complexes and thereby activate different cell-signaling pathways provides a novel form of specificity to a receptor system with over 100 structurally diverse ligands (35). Engaging LRP1 and associated co-receptors represents a novel mechanism by which S-PrP may regulate cell physiology in diverse cell types including neurons and glia in the CNS and PNS.

Experimental procedures

Proteins and reagents

S-PrP (residues 23–231) was expressed in *E. coli* BL21 cells using the T7 promoter. His-tagged S-PrP was isolated from inclusion bodies, denatured in guanidinium hydrochloride, purified by Ni²⁺-nitrilotriacetic acid agarose affinity chromatography, oxidized, and refolded out of denaturant. The N-ter-

минаl histidine tail was proteolytically dissociated with thrombin. The thrombin was then removed by ion exchange chromatography. S-PrP preparations were examined by non-reducing SDS-PAGE and visualized by silver staining (Pierce Silver Stain Kit).

The monoclonal antibodies POM1, POM2, POM3, and POM19, directed against different epitopes of mouse PrP^C, were purified as previously described (30). Human enzymatically inactive tissue-type plasminogen activator (EI-tPA), which carries the S478A mutation and is thus inactive as a protease, was from Molecular Innovations. EI-tPA carries a second mutation (R275E) that disallows conversion of the single-chain form into two-chain tPA. We previously demonstrated that human EI-tPA and mouse EI-tPA signal equivalently in rodent cells (59). Endotoxin-free, monomeric RAP was provided by Dr. Travis Stiles (Novoron Bioscience). NGF- β was from Invitrogen. MK801 was from Cayman Chemical. M β CD and FM were from Sigma-Aldrich. PP2 and DXM were from Abcam. K252a was from Cell Signaling Technology.

Cell culture

Rat PC12 cells were from the ATCC (CRL-1721) and subjected to quality control tests by the ATCC. PC12 cells were cultured in DMEM (high glucose; Gibco) containing 10% heat-inactivated FBS (Gibco), 5% heat-inactivated horse serum (HyClone), penicillin (100 units/ml), and streptomycin (1 mg/ml), in plates that were pre-coated with 0.01 mg/ml type IV collagen (Sigma-Aldrich). Cells were passaged no more than eight times. Mouse N2a neuroblastoma cells were a generous gift from Dr. Katerina Akassoglou (Gladstone Institute of Neurological Disease, University of California, San Francisco, CA). N2a cells were cultured in DMEM containing 10% FBS, penicillin, and streptomycin.

SCs were isolated from sciatic nerves of 1-day-old Sprague-Dawley rats. The SCs were enriched and separated from fibroblasts using fibronectin-specific antibody and rabbit complement cytotoxicity. Final preparations consisted of 98% SCs, as determined by immunofluorescence microscopy for S100 β , which is a specific SC marker. Primary SC cultures were maintained in plates coated with 1 μ g/ml poly-D-lysine in low-glucose DMEM containing 10% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, 21 μ g/ml bovine pituitary extract (Sigma-Aldrich), and 4 μ M forskolin (Cell Signaling Technology) and were passaged no more than six times before conducting experiments. Our protocol for isolating SCs from animals was approved by the University of California San Diego Institutional Animal Care and Use Committee.

Analysis of S-PrP by LC-MS/MS

S-PrP (10 μ g) was reduced, denatured, and subjected to SDS-PAGE. A single band was observed. The band was excised, reduced, alkylated, and trypsin-digested using the In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific). Digested peptides were passed through C18 spin tips (Thermo Fisher Scientific) and eluted in 80% acetonitrile and 0.1% formic acid. Samples were then vacuum-dried, equilibrated in 1% acetonitrile and 0.1% formic acid, bomb-packed into 70 μ m C18 infused

capillaries, and eluted in a positive ion nanospray with a 1–90% acetonitrile gradient using an Agilent 1200 series liquid chromatography injection system. Peptides were detected using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) using Xcalibur 2.1 (Thermo Fisher Scientific). For assignment, raw files were searched against the *Mus musculus* proteome (UniProt taxonomy ID 10090), containing 21,986 entries, using Proteome Discoverer Software 2.0 with SEQUEST HT and MS Amanda search engines (Thermo Fisher Scientific). Our search parameters identified fixed modifications, including cysteine carbamidomethylation, variable methionine oxidation, lysine carbamylation, and N-terminal acetylation and oxidation. The maximum number of missed cleavages permitted was two. Mass tolerance for precursor ions was set to 50 ppm and for fragment ions, 0.6 Da. Peptides were not considered if they had an Xcorr threshold $\leq 1\%$. A strict peptide false positive rate of 5% was used to accept proteins based on spectral match. Protein samples were analyzed in technical duplicates.

Gene-silencing

Rat-specific ON-TARGETplus SMARTpool siRNA, which targets *LRP1*, the GluN1 subunit of the NMDA-R (*GRIN1*), *NCAM1*, and membrane-anchored PrP^C (*PRNP*) were from Horizon Discovery, as was NTC siRNA. PC12 cells (2×10^6) were transfected with siRNA by electroporation using the Cell Line Nucleofector Kit V (Lonza). Gene-silencing was determined at the mRNA level by RT-qPCR or at the protein level by immunoblot analysis. Experiments were performed 36–48 h after transfection.

RT-qPCR

RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel) and reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using TaqMan gene expression products (Thermo Fisher Scientific). The relative change in mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH mRNA as an internal normalizer.

Analysis of cell-signaling

PC12 cells, N2a cells, and SCs were cultured until ~70% confluent. The cells were then transferred into SFM for 2 h before adding S-PrP, EI-tPA, or vehicle. Some cultures were pretreated with 1 mM M β CD for 30 min or with 25 μ M FM for 24 h. RAP (150 nM), MK801 (1 μ M), and DXM (10 μ M) were added 30 min before S-PrP or EI-tPA, as indicated. PP2 (1 μ M) and K252a (10 nM) were added 2 h before S-PrP. Incubations with S-PrP or EI-tPA were conducted for 10 min. The cells were then rinsed twice with ice-cold PBS. Extracts of PC12 cells, N2a cells, and SCs were prepared in RIPA buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor mixture, and phosphatase inhibitor mixture). The protein concentration in cell extracts was determined by bicinchoninic acid assay. An equivalent amount of cellular protein (20–40 μ g) was subjected to 10% SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes. The membranes

were blocked with 5% nonfat dried milk and incubated with primary antibodies from Cell Signaling Technology that target phospho-ERK1/2 (1:1000, 4370), total-ERK1/2 (1:1000, 4695), phospho-TrkA (1:1000, 9141), NCAM1 (1:1000, 99746T), and β -actin (1:5000, 3700). The membranes were washed and treated with horseradish peroxidase-conjugated secondary antibody (The Jackson Laboratory). Immunoblots were developed using SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) and imaged using Blue Devil autoradiography film (Genesee Scientific) or the Azure Biosystems c300 system.

Neurite outgrowth

PC12 cells were plated at 1×10^5 cells/well and maintained in serum-containing medium for 24 h. The medium was then replaced with SFM supplemented with S-PrP (40 nM), RAP (150 nM), MK801 (1 μ M), PP2 (1 μ M), K252a (10 nM), NGF- β (50 ng/ml), or vehicle, as indicated, for 48 h. The cells were imaged by phase contrast microscopy, using a Leica DMi8 microscope (Leica Microsystems) equipped with a Leica DFC3000 G digital camera and Leica Application Suite X software. Neurite length was determined in 50 cells/replicate using ImageJ software (National Institutes of Health). Results were subjected to statistical analysis using GraphPad Prism.

SC migration

SC migration was studied using 6.5-mm Transwell chambers with 8 μ m pores (Corning), as previously described (25, 42). The bottom surface of each membrane was coated with 10 μ g/ml fibronectin. SCs in Sato medium (68) supplemented with 1 mg/ml BSA were pretreated with S-PrP (40 nM), in the presence or absence of RAP (150 nM) or MK801 (1 μ M), for 10 min at 37 °C. The cells (10^4) were then transferred to upper Transwell chambers. The bottom chamber contained Sato Medium with 1 mg/ml BSA, 10% FBS, and the same proteins that were added to the top chamber. Cell migration was allowed to occur for 4 h at 37 °C in 5% CO₂. The upper surface of each membrane was cleaned with a cotton swab. The membranes were then stained with PROTOCOL Hema 3. The number of cells on the bottom surface of each membrane was quantified using ImageJ. Four fields were examined per filter. Each condition was studied in triplicate.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.2 (GraphPad Software). All results are expressed as the mean \pm S.E. Neurite outgrowth and cell migration data were analyzed by one-way analysis of variance followed by a Tukey's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Data availability

The complete list of peptides identified in our LC-MS/MS analysis of S-PrP, with corresponding charge, modifications, and identification scores are provided in Supplemental File 1.

The raw MS files are available in the public repository Figshare (10.6084/m9.figshare.12762176).

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Abbreviations—The abbreviations used are: CNS, central nervous system; DXM, dextromethorphan hydrobromide; tPA, tissue-type plasminogen activator; EI, enzymatically inactive; FM, fumonisin B1; GPI, glycosylphosphatidylinositol; LDL, low-density lipoprotein; LRP1, LDL receptor-related protein-1; M β CD, methyl- β -cyclodextrin; NCAM, neural cell adhesion molecule; NMDA-R, N-methyl-D-aspartate receptor; NTC, nontargeting control; PNS, peripheral nervous system; PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; RAP, receptor-associated protein; SC, Schwann cell; SFK, Src family kinase; SFM, serum-free media; S-PrP, soluble cellular prion protein; ADAM, a disintegrin and metalloprotease; ERK, extracellular signal-regulated kinase; mGluR, metabotropic glutamate receptor; MK801, dizocilpine; qPCR, quantitative PCR; NGF, nerve growth factor.

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