Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59\(^{fyn}\) and to enhance neurite outgrowth

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In spite of advances in understanding the role of the cellular prion protein (PrP) in neural cell interactions, the mechanisms of PrP function remain poorly characterized. We show that PrP interacts directly with the neural cell adhesion molecule (NCAM) and associates with NCAM at the neuronal cell surface. Both cis and trans interactions between NCAM at the neuronal surface and PrP promote recruitment of NCAM to lipid rafts and thereby regulate activation of fyn kinase, an enzyme involved in NCAM-mediated signaling. Cis and trans interactions between NCAM and PrP promote neurite outgrowth. When these interactions are disrupted in NCAM-deficient and PrP-deficient neurons or by PrP antibodies, NCAM/PrP-dependent neurite outgrowth is arrested, indicating that PrP is involved in nervous system development cooperating with NCAM as a signaling receptor.

Introduction

The cellular prion protein (PrP) is a ubiquitous glycoprotein, prominently expressed in the brain and localized at the cell surface via a glycosylphosphatidylinositol (GPI) anchor. In prion diseases, PrP is converted to a conformationally altered form that accumulates in the brain (Prusiner, 1998; Weissmann and Flechsig, 2003). Mutations in the PrP gene have been linked to the Gerstmann-Sträussler-Scheinker syndrome, familial fatal insomnia, sporadic Creutzfeld-Jakob disease, and certain forms of dementia with cerebellar disorder and myopathy (Hsiao et al., 1989; Collinge, 1997).

Attempts to identify the functions of PrP are consistent with PrP functioning as a recognition molecule. PrP interacts or associates with the 67-kD laminin receptor, the 37-kD laminin receptor precursor protein, or the ECM glycoprotein laminin (Rieger et al., 1997; Graner et al., 2000; Gauczynski et al., 2001). PrP has been identified in a complex with the neural cell adhesion molecule (NCAM) by chemical cross-linking (Schmitt-Ulms et al., 2001). However, whether PrP binds directly to NCAM has remained unclear. In addition to its protective role in models of neurodegeneration due to oxidative stress, being probably linked to its metal ion binding ability (Milhavet and Lehmann, 2002), PrP has been implicated in neurite outgrowth and neuronal survival as a trans-interacting partner, that is, an interaction between the cell surface of one cell and a molecule from the ECM or from the cell surface of an adjacent cell. The binding partner for PrP at the neuronal cell surface has, however, remained elusive (Chen et al., 2003). Similar to the molecules associated with PrP, such as laminin and NCAM, PrP has been implicated in the physiology of neurons, affecting synaptic function (Collinge et al., 1994), neurite outgrowth, and neuronal survival (Chen et al., 2003). Because both PrP and NCAM have been implicated in signaling cascades involving the p59\(^{fyn}\) nonreceptor tyrosine kinase (fyn) (Beggs et al., 1997; Mouillet-Richard et al., 2000) and because fyn is involved in NCAM-induced neurite outgrowth (Beggs et al., 1994), we investigated whether the two molecules may functionally cooperate with each other by engaging in cis and/or trans interactions. Furthermore, it seemed important to characterize the involvement of lipid-enriched microdomains, the so-called lipid rafts, at the cell surface as a signaling platform for PrP, which localizes to lipid rafts because of its GPI anchor (Gorodinsky and Harris, 1995; Walmsley et al., 2003) and for NCAM, which can be sequestered to lipid rafts due to palmitoylation, which is essential for promotion of neurite outgrowth (Niethammer et al., 2002). Here, we show that PrP interacts directly with NCAM, and in a heterophilic cis and trans configuration recruits to and stabilizes NCAM in lipid rafts, thereby activating fyn to induce NCAM-dependent neuritogenesis.
Results

PrP directly interacts with NCAM

To obtain insights into the function of PrP in the developing brain, we first analyzed the association between PrP and NCAM in cultured hippocampal neurons. PrP partially colocalized with NCAM along neurites and in growth cones (Fig. 1 A). As a GPI-anchored protein, PrP mostly localizes to lipid rafts (Gorodinsky and Harris, 1995; Walmsley et al., 2003). We therefore analyzed whether NCAM colocalizes with PrP in lipid rafts by extracting neurons with cold 1% Triton X-100, a procedure used to isolate cytoskeleton-bound and raft-associated proteins (Ledesma et al., 1998; Niethammer et al., 2002; Leshchyns’ka et al., 2003). In extracted neurons, PrP showed a patchy distribution along neurites (Fig. 1 B) (Madore et al., 1999), showing that PrP accumulates in subdomains at neuronal plasma membranes. Similar to PrP, NCAM showed a patchy distribution in clusters along neurites (Fig. 1 B). Clusters of NCAM overlapped with PrP accumulations (mean correlation between distributions of two proteins, $r = 0.70 \pm 0.01$; Fig. 1 D). To verify whether this overlap was specific for NCAM, we analyzed the distribution of L1, another recognition molecule of the immunoglobulin superfamily and also present in lipid rafts of neurites (Nakai and Kamiguchi, 2002). In contrast to NCAM and PrP, L1 showed a more uniform distribution along extracted neurites (Fig. 1 C). The overall pattern of L1 and PrP localization was different from that between NCAM and PrP (mean correlation between distributions of L1 and PrP, $r = 0.27 \pm 0.02$; Fig. 1, E and F).

The similar localization of NCAM and PrP suggested that both proteins form a complex in lipid rafts. We thus cross-linked NCAM at the neuronal surface with NCAM antibodies applied to live neurons. NCAM clustering induced partial redistribution of PrP to NCAM-containing clusters (Fig. 2 A). We also noticed nonoverlapping clusters of NCAM and PrP (Fig. 2 A) in accordance with our observation that only a small portion of the neuronal NCAM140 and NCAM180 isoforms is in lipid rafts (Niethammer et al., 2002). To further investigate this phenomenon, we analyzed by an ELISA binding assay whether PrP and NCAM directly interact using recombinant PrP-Fc, which contains the extracellular domain of mouse PrP fused to the Fc portion of IgG (Chen et al., 2003), and NCAM purified from mouse brain. NCAM bound to PrP-Fc in a concentration-dependent manner, but not to BSA (Fig. 2 B). L1 also did not bind to PrP (Fig. 2 B), in accordance with results with extracted neurons (Fig. 1). NCAM is the carrier of polysialic acid (PSA), which may influence its binding to PrP. To verify whether PSA influences binding to PrP, we analyzed by ELISA the interaction between PrP and nonpolysialylated NCAM-Fc produced in CHO cells using recombinant PrP-AP, which contains the extracellular domain of mouse PrP fused to alkaline phosphatase (Chen et al., 2003). We found that nonpolysialylated NCAM-Fc also bound to PrP-AP in a concentration-dependent manner, but not to BSA (Fig. 2 C).

To investigate whether NCAM and PrP interact in brain tissue, we immunoprecipitated NCAM from brain homogenates and analyzed immunoprecipitates with antibodies against PrP. PrP coimmunoprecipitated with NCAM (Fig. 2 E), indicating that the two proteins are associated in brain. Another GPI-anchored immunoglobulin superfamily recognition molecule, F3/contactin, did not coimmunoprecipitate with NCAM, underscoring the specificity of NCAM interaction with PrP (Fig. 2 E).

To determine whether NCAM and PrP exist in a complex in the same plasma membrane microenvironment by inducing covalent binding between primary amino groups of adjacent proteins in the lipid raft fraction from total brain.

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**Figure 1.** NCAM colocalizes with PrP in lipid rafts. (A) Neurite with two growth cones (arrows) double-labeled with NCAM and PrP antibodies. PrP and NCAM partially colocalize along neurites and in growth cones. (B and C) Neurons were extracted in cold 1% Triton X-100 and labeled with PrP and NCAM antibodies (B) or PrP and L1 antibodies (C). Detergent-insoluble clusters of PrP overlap with accumulations of NCAM. L1 shows a distribution distinct from PrP. Bars, 10 \( \mu \)m. (D and E) Examples of linear regression graphs comparing distributions of PrP and NCAM (D) or PrP and L1 (E) are shown. Corresponding correlation coefficients ($r$) are presented. (F) The diagram shows mean correlation coefficients ($r$) comparing distributions of PrP and NCAM or PrP and L1. The correlation between localization of NCAM and PrP is significantly higher than between localization of L1 and PrP. Mean values $\pm$ SEM ($n > 30$) are shown. *, $P < 0.05$, t test.
PrP is involved in stabilization of NCAM in lipid rafts

NCAM140 and NCAM180 localize mostly to raft-free areas and redistribute to lipid rafts through palmitoylation of NCAM after NCAM activation (Niethammer et al., 2002; Leshchyns’ka et al., 2003). Because PrP localizes to lipid rafts, complex formation between NCAM and PrP should occur in lipid rafts. We thus hypothesized that interaction of NCAM with PrP may recruit NCAM to and stabilize it in lipid rafts. Indeed, absence of PrP in PrP<sup>−/−</sup> brains reduced the amount of NCAM140 and NCAM180 in lipid rafts (76.4 ± 6.7% in PrP<sup>−/−</sup> lipid rafts with PrP<sup>+/+</sup> values set to 100%; Fig. 3 A) and growth cones (66.13 ± 10% in PrP<sup>−/−</sup> lipid rafts with PrP<sup>+/+</sup> values set to 100%; Fig. 3 A). The GPI-anchored and lipid raft–localized NCAM120 was present in similar amounts in lipid rafts isolated from PrP<sup>+/+</sup> and PrP<sup>−/−</sup> brains, indicating that lipid rafts were isolated with the same efficacy. Levels of L1 were similar in lipid rafts isolated from brains of PrP<sup>−/−</sup> and PrP<sup>+/+</sup> mice (Fig. 3 B), confirming that PrP deficiency does not affect the overall raft composition. In contrast, overall levels of NCAM were increased in PrP<sup>−/−</sup> brains (Fig. 3 A), excluding the possi-

**Figure 2.** NCAM directly interacts with PrP. (A) Neurons incubated live with antibodies against NCAM to induce clustering of NCAM at the cell surface were fixed and labeled with antibodies against PrP. PrP partially redistributed to NCAM clusters (arrows). Bar, 10 μm. (B) Increasing concentrations of NCAM and L1, purified from mouse brain, were assayed by ELISA for their binding to plastic-bound PrP-Fc (5 μg/ml). BSA (3 mg/ml) served as a control. Mean values (OD<sub>405</sub>) ± SEM (n = 6) are shown. (C) Increasing concentrations of NCAM-Fc were assayed by ELISA for their binding to plastic-bound PrP-AP (5 μg/ml). BSA (3 mg/ml) served as a control. Mean values (OD<sub>405</sub>) ± SEM (n = 6) are shown. (D) NCAM from mouse brain and NCAM-Fc were immunoblotted (WB) with antibodies against NCAM and polysialic acid (PSA). Note that PSA immunoreactivity is found only on NCAM purified from mouse brain. The two bottom bands represent degradation products of NCAM-Fc (asterisk). (E) NCAM immunoprecipitates from the NCAM<sup>+/+</sup> mouse brain were immunoblotted (WB) with PrP or F3 antibodies. NCAM<sup>−/−</sup> brains served as a control for PrP immunoprecipitation. Brain homogenate (hom.) was also probed for F3. PrP, but not F3, coimmunoprecipitated with NCAM. (F) Lipid rafts (S) or lipid rafts treated with BS<sub>3</sub> (BS<sub>3</sub>) were immunoblotted with PrP antibodies (WB: PrP). Then, the membrane was stripped and labeled with NCAM antibodies (WB: NCAM). Note a PrP-immunoreactive band above 200 kD in the cross-linked material that overlaps with a shifted NCAM immunoreactive band (arrows). (G) Total membrane (S) or PrP immunoprecipitates from total membranes treated with BS<sub>3</sub> (IP: PrP) were immunoblotted (WB) with antibodies to NCAM, PrP, and L1. Immunoprecipitation performed with nonimmune IgG served as a control (IP, IgG). Note that NCAM, but not L1, coimmunoprecipitated with PrP.
bility that reduced levels of NCAM140 and NCAM180 in lipid rafts are due to reduced expression of NCAM in PrP−/− mice. We conclude that PrP is involved in stabilization of NCAM140 and NCAM180 in lipid rafts.

Cis and trans interactions between PrP and NCAM are important for NCAM stabilization in lipid rafts

Two distinct types of interaction between PrP and NCAM could account for the observed phenomena. PrP could stabilize NCAM in lipid rafts by cis interaction, i.e., both molecules associate in the plasma membrane of the same neuron. In accordance with this idea, NCAM and PrP were cross-linked with antibodies at live neuronal cell surfaces (Fig. 2 A). Chemical cross-linking of NCAM and PrP was also performed in low density cultures allowing only cis interactions between NCAM and PrP (unpublished data). To analyze the cis interaction in lipid rafts, we estimated the amount of detergent-insoluble NCAM in PrP+/+ and PrP−/− cultured hippocampal neurons. Only neurons without contacts were evaluated to assure that only cis interactions were analyzed. PrP−/− neurons extracted with cold Triton X-100 showed lower NCAM labeling intensity in NCAM clusters and along neurites when compared with PrP+/+ neurons (Fig. 4, C and D). This reduction was not due to a decrease in expression of NCAM in PrP−/− neurons because the mean labeling intensity of NCAM along neurites of nonextracted neurons was increased in PrP−/− neurons (Fig. 4, A and B), in accordance with our biochemical data (Fig. 3 A). We conclude that cis interactions between NCAM and PrP are important for stabilization of NCAM in lipid rafts.

PrP could also induce redistribution of NCAM to lipid rafts by trans interaction, i.e., NCAM binds to PrP on adjacent cells. To analyze the role of a trans interaction between NCAM and PrP, we applied soluble PrP-Fc to neurons from PrP−/− mice to evaluate the redistribution of NCAM to lipid rafts in
absence of NCAM-to-PrP cis interaction. Covalent chemical cross-linking of PrP-Fc with NCAM in brain homogenates indicated that PrP-Fc indeed bound to endogenous NCAM in a trans fashion (unpublished data). Application of PrP-Fc increased the detergent-insoluble NCAM fraction in NCAM clusters and along neurites of PrP<sup>−/−</sup> neurons (Fig. 4, E and F). Because increase of NCAM levels in lipid rafts after application of PrP-Fc suggested a PrP-Fc-induced redistribution of NCAM to lipid rafts, we measured the association of NCAM with lipid rafts in response to PrP-Fc application in PrP<sup>−/−</sup> neurons using the PI(4,5)P<sub>2</sub> raft marker (Laux et al., 2000; Niethammer et al., 2002). Amounts of PI(4,5)P<sub>2</sub> in detergent-insoluble NCAM clusters were increased after PrP-Fc application, indicating that PrP-Fc redistributed NCAM to lipid rafts (Fig. 4, G and H). The same result was obtained when endogenous PrP or ganglioside GM1 (Leshchyns’ka et al., 2003) were taken as raft markers (unpublished data). As for PrP<sup>−/−</sup> neurons, application of PrP-Fc to PrP<sup>+/+</sup> neurons increased levels of detergent insoluble NCAM in NCAM clusters and along neurites (Fig. 4, G and H). We conclude that trans interaction of PrP with NCAM induces redistribution of NCAM to lipid rafts.

NCAM activation with NCAM-Fc or NCAM antibodies induces redistribution of NCAM to lipid rafts being necessary for NCAM-mediated neurite outgrowth (Niethammer et al., 2002; Leshchyns’ka et al., 2003). Indeed, NCAM-Fc or NCAM antibodies increased PrP levels in NCAM clusters in PrP<sup>−/−</sup> neurons (Fig. 5, A and C), indicating that NCAM partially redistributes to lipid rafts as previously observed (Leshchyns’ka et al., 2003), and suggesting that association between NCAM and PrP is enhanced after NCAM activation. In spite of...
overall higher levels of NCAM expression in PrP<sup>−/−</sup> neurons (Fig. 4, A and B), NCAM levels in lipid rafts were reduced in stimulated PrP<sup>−/−</sup> neurons (Fig. 5, B and D), indicating that cis interactions between NCAM and PrP are important for redistribution of NCAM to lipid rafts in response to NCAM activation.

Activation of the fyn kinase is reduced in PrP<sup>−/−</sup> mice
Accumulation of NCAM in lipid rafts is necessary for NCAM-mediated neurite outgrowth and implies activation of the fyn kinase pathway (Niethammer et al., 2002). Total levels of fyn kinase immunoprecipitable from brain homogenates and lipid rafts of PrP<sup>−/−</sup> mice were increased when compared with PrP<sup>+/+</sup> mice (Fig. 6). However, levels of activated fyn were reduced in PrP<sup>−/−</sup> brain homogenates (ratio of activated fyn to the total fyn protein was 59.4 ± 15.3% for PrP<sup>−/−</sup> brains with PrP<sup>+/+</sup> set to 100%) and lipid rafts (ratio of activated fyn to the total fyn protein was 26.7 ± 9.8% for PrP<sup>−/−</sup> rafts with PrP<sup>+/+</sup> set to 100%). Because fyn forms a complex with NCAM (Beggs et al., 1997) and NCAM redistribution to lipid rafts activates NCAM-mediated fyn signaling (Niethammer et al., 2002; Bodrikov et al., 2005), reduction of activated fyn in PrP<sup>−/−</sup> brains could be due to reduction of activated fyn associated with NCAM.

To analyze this, we studied whether redistribution of NCAM to lipid rafts in response to stimulation with PrP-Fc would affect levels of activated fyn. Indeed, application of PrP-Fc to PrP<sup>−/−</sup> neurons increased levels of activated fyn along neurites and in NCAM clusters (Fig. 7, A and B). When PrP-Fc was applied to PrP<sup>+/+</sup> neurons, levels of activated fyn were also significantly increased along neurites and in NCAM clusters (Fig. 7, A and B), indicating that trans interactions between NCAM and PrP induce fyn activation. However, the efficacy of fyn activation was lower in PrP<sup>−/−</sup> cells (200 and 140% along neurites of stimulated PrP<sup>+/+</sup> and PrP<sup>−/−</sup> cells, respectively, with the level of activated fyn in control cells set to 100%), suggesting that cis interactions between NCAM and PrP are also important for fyn activation. To confirm this, we analyzed activation of fyn in response to NCAM-Fc in PrP<sup>−/−</sup> neurons, thereby excluding cis interactions between NCAM and PrP. Application of NCAM-Fc increased levels of activated fyn.
of GM1 containing lipid rafts with cholera toxin (Harder et al., 1998). Levels of GM1 were similar in neurites of NCAM and PrP are required for NCAM-mediated fyn activation, we analyzed activation of fyn in response to PrP-Fc application to NCAM−/− neurons: levels of activated fyn along neurites of NCAM−/− neurons were not changed (Fig. 7 E), indicating that NCAM is required for PrP-Fc induced fyn activation. In agreement, polyclonal PrP antibodies did not activate fyn, indicating that clustering of PrP alone is insufficient to activate fyn (Fig. 7 F). Interestingly, PrP antibodies completely inhibited NCAM-Fc–induced fyn activation (Fig. 7 F), probably by interfering with cis interaction between NCAM and PrP. Finally, levels of activated fyn coimmunoprecipitated with NCAM were also approximately two times lower in PrP−/− brains when compared with PrP+/+ brains (unpublished data), despite the overall increase of NCAM expression in PrP−/− brains. We conclude that NCAM is the receptor for PrP in trans and cooperates with PrP in cis to activate fyn.

Coexpression of NCAM140 with PrP enhances targeting of NCAM140 to lipid rafts and fyn activation in CHO cells

To exclude that enzymes responsible for NCAM palmitoylation or fyn activation were nonspecifically affected by PrP ablation, we investigated whether PrP expression in PrP-negative CHO cells would affect NCAM140 targeting to lipid rafts and fyn activation. CHO cells were stably transfected with NCAM140 or PrP alone or cotransfected with NCAM140 and PrP. In low density CHO cell cultures and thus absence of trans interactions between NCAM and PrP, levels of NCAM140 were higher in lipid rafts from cells cotransfected with NCAM140 and PrP when compared with NCAM140-only transfected cells (Fig. 8 A), further confirming that cis interactions between NCAM140 and PrP target NCAM140 to lipid rafts. Application of PrP-Fc to NCAM140-transfected cells increased levels of NCAM140 in lipid rafts (Fig. 8 C), indicating that trans interactions between NCAM and PrP also target NCAM140 to lipid rafts. Furthermore, both types of interactions increased levels of activated fyn in the cells (Fig. 8 B and D).

NCAM interaction with PrP enhances NCAM-mediated neurite outgrowth

Redistribution of NCAM to lipid rafts in response to NCAM homophilic binding is required for NCAM-mediated neurite outgrowth (Niethammer et al., 2002; Leshchyns’ka et al., 2003). In this paradigm, substrate-coated or soluble NCAM interacts with and signals through NCAM at the neuronal cell surface. Because the binding of PrP-Fc to NCAM also redistributes NCAM lipid rafts (Fig. 4 G), we investigated whether PrP-Fc promotes NCAM-mediated neurite outgrowth. PrP-Fc was thus applied to cultured hippocampal neurons and neurite length was measured after 24 h. PrP-Fc increased neurite lengths when compared with the control group (Fig. 9 A) as previously observed (Chen et al., 2003), suggesting that PrP-Fc–induced redistribution of NCAM to lipid rafts promotes neurite outgrowth. Alternatively, clustering of GPI-anchored raft-associated proteins may also activate intracellular signaling cascades, leading to enhanced neurite outgrowth (Doherty et al., 1993). To exclude the possibility...
that PrP-Fc acts via clustering of PrP at cell surfaces and not via NCAM, we incubated neurons with different concentrations of polyclonal PrP antibodies, thereby clustering PrP at the cell surface. Unexpectedly, we found that PrP antibodies inhibited neurite outgrowth (Fig. 9 B), indicating that clustering of PrP is not sufficient to induce neurite outgrowth. Furthermore, it suggested that PrP antibodies inhibit cis interactions between PrP and a binding partner at the cell surface that was required for neurite outgrowth. To directly assess the role of NCAM in PrP-Fc–induced neurite outgrowth, we treated NCAM-neurons with PrP-Fc and found that, in contrast to NCAM neurons, NCAM neurons did not respond to PrP-Fc (Fig. 9 C), confirming that NCAM is a major receptor for PrP in PrP-Fc–induced neurite outgrowth. To analyze the role of PrP-to-NCAM trans interaction in NCAM-mediated neurite outgrowth, we estimated neurite outgrowth in response to PrP-Fc in PrP neurons, thereby abolishing cis interactions between NCAM and PrP. PrP-Fc enhanced neurite lengths (Fig. 9 D), indicating that trans interactions between NCAM and PrP are involved in promoting neurite outgrowth. To analyze the role of cis interactions between NCAM and PrP in NCAM-mediated neurite outgrowth, we compared neurite outgrowth in response to NCAM activation in PrP-neurons transfected with GFP alone or GFP together with PrP. Transfected PrP was delivered to the cell surface and partially colocalized with NCAM (unpublished data). In GFP-transfected neurons, treatment with NCAM-Fc enhanced neurite outgrowth when compared with Fc-treated cells (controls), indicating PrP-independent response to NCAM. However, PrP-transfected neurons treated with NCAM-Fc produced even longer neurites (Fig. 9 E). This increase in the NCAM-Fc–elicited response thus evolves from an NCAM-to-PrP cis interaction. Furthermore, PrP antibodies completely abolished the NCAM-Fc–induced response in wild-type neurons (Fig. 9 F), probably by interfering with cis interactions.
between NCAM and PrP at the cell surface and with fyn activation (Fig. 7). The combined observations indicate that NCAM is a major neuronal receptor for PrP presented in a trans fashion. We also conclude that cis interactions between NCAM and PrP at the neuronal cell surface enhance NCAM-induced neurite outgrowth when NCAM is presented to neurons in a trans fashion.

Discussion

In this study we have shown that PrP and NCAM are not only associated with each other at the surface of hippocampal neurons, but also directly interact with each other—features that have not been described previously. We also showed that the GPI-linked PrP that cannot interact directly with intracellular signaling pathways recruits to and stabilizes the transmembrane NCAM isoforms in lipid-rich microdomains to activate fyn and promote NCAM-mediated neurite outgrowth by cis and trans interactions.

Transmembrane recognition molecules are often segregated from their signaling cascades by colocalizing receptors and their downstream effectors to distinct membrane subdomains, such as lipid rafts. The mechanisms that collect receptors to their signaling platforms have only started to emerge. We show that NCAM uses its PrP-guided enrichment in lipid rafts to activate fyn, which becomes associated with lipid rafts rapidly after synthesis (van’t Hof and Resh, 1997; Filipp et al., 2003). Interestingly, although the intracellular domain of NCAM does not contain sequences known to activate fyn, it directly associates with the receptor type protein phosphatase (RPTP)α, a fyn activator. At resting conditions, when not stimulated by cis or trans interactions, NCAM and RPTPα are segregated from fyn, which localizes to rafts. In response to NCAM activation NCAM binds RPTPα and both molecules are recruited to lipid rafts where RPTPα activates fyn (Bodrikov et al., 2005), a process that is regulated by PrP (Fig. 10). It is important in this respect that, in contrast to other GPI-anchored molecules such as Thy-1 (Doherty et al., 1993), clustering of PrP alone with PrP antibodies is not sufficient to induce neurite outgrowth but rather inhibits it by interfering with cis interactions between NCAM and PrP. Because PrP-Fc–induced fyn activation and neurite outgrowth are largely inhibited in NCAM−/− cells, the most plausible explanation is that NCAM is a major PrP receptor. These observations merge two previously separate venues of investigation, namely the requirement of NCAM to be localized to lipid rafts to induce neurite outgrowth via fyn activation together with FGF receptor activation (Niethammer et al., 2002) and the proposed activation of fyn kinase (Mouillet-Richard et al., 2000) by PrP, which binds to an unknown surface receptor (Chen et al., 2003). Whether NCAM cooperates with the laminin receptor, which is another surface receptor for PrP (Gauzynski et al., 2001), or whether PrP–laminin receptor interactions have distinct functions in neurite outgrowth remains to be investigated.

Remarkably, not only binding of NCAM to PrP in the neuronal plasma membrane (cis interaction), but also binding of NCAM to PrP in trans interaction, redistributes NCAM to lipid rafts. It is thus interesting that besides its lipid domain-
targeting GPI anchor, PrP may associate in trans interaction with lipid rafts via its ectodomain (Walmsley et al., 2003) and thereby may additionally recruit NCAM to lipid rafts. It is also conceivable that NCAM uses other signal transduction pathways independent of PrP and PrP's ability to recruit NCAM to lipid rafts for signal transduction. Because abnormalities in PrP\(^{-/-}\) mice have been described as undetectable or mild, although closer scrutiny has identified abnormalities, it is conceivable that triggered palmitoylation of NCAM and/or recruitment of NCAM to other signaling platforms may compensate for some PrP functions.

Functional interactions of PrP with its binding partner(s) have been suggested previously (Telling et al., 1995; Shmerling et al., 1998): a cis- and/or trans-interacting PrP activates an unknown binding partner competing with the dominant-negative mutant of PrP truncated at the amino terminus, leading to ataxia and cerebellar lesions (Shmerling et al., 1998). We have now identified NCAM as binding partner for PrP that can cooperate with PrP in neurite outgrowth. It is likely that cooperation between these molecules also occurs in the adult. It is thus noteworthy that both molecules modify synaptic activity (Collinge et al., 1994; Cremer et al., 1994; Luthi et al., 1994; Mallucci et al., 2002). Interestingly, defects in NCAM- and PrP-dependent regulation of synaptic activity may not only be due to developmental abnormalities, but are seen in mice conditionally ablated for NCAM and PrP expression at a juvenile state (Mallucci et al., 2002; Bukalo et al., 2004). Furthermore, mutations in PrP in both humans and mice lead to abnormal sleep patterns, resulting in fatal familial insomnia in humans (Gambetti et al., 2003). Likewise, NCAM is involved in regulation of circadian body functions by entraining glutamatergic activity of the suprachiasmatic nuclei to the solar day in photic and nonphotic settings (Kleene and Schachner, 2004). These findings are remarkable in view of PrP and NCAM signaling through fyn, which is also implicated in synaptic functions (Grant et al., 1992; Kojima et al., 1997).
Finally, consequences of the trans and cis interactions between NCAM and PrP for transmissible and nontransmissible prion diseases, leading to infectious propagation of the mutation with its loss-of-function or gain-of-function consequences, should be viewed in the context of PrP interacting heterophilically with other molecules, such as NCAM. Interestingly, the incubation period of the scrapie conformer of prion protein (PrPSc) is not altered in NCAM− /−/ − mice compared with NCAM+/+/+ mice, suggesting that NCAM does not affect PrPSc formation (Schmitt-Ulms et al., 2001). Also, it has been excluded that neurodegeneration occurs because of PrP deficiency (Mallucci et al., 2002). It is thus conceivable that interactions between PrP and NCAM are reduced by accumulation of PrPSc in the diseased nervous system. A reduced association between PrP and NCAM could also be caused by application of PrP antibodies that trigger rapid and extensive apoptosis in hippocampal and cerebellar neurons in vivo (Solforosi et al., 2004). Similarly, we found that application of PrP antibodies abrogates NCAM-induced neurite outgrowth. It is tempting to speculate that interference with NCAM-mediated signaling in the diseased brain may favor cell death and inhibit synaptic plasticity-related neuritogenesis. Because NCAM, on the one hand, and PrP, on the other, interact with different sets of cell surface and ECM molecules, the interplay of these interactions during development and in the adult brain will be the target of further investigations.

Materials and methods

Antibodies
Rabbit pAbs against the extracellular domain of NCAM (Niemann et al., 2002) were used in immunoprecipitation, immunoblotting, and immunocytochemical experiments and rat mAbs H28 against mouse NCAM (Gennarini et al., 1984) were used in immunocytochemical experiments. Both antibodies react with the three major isoforms of NCAM. Hybridoma clone H28 was provided by Dr. Christo Goridis (CNRS UMR 8542, Paris, France). Mouse mAbs 8H4 and rabbit pAbs 340 against PrP were ob-
tained from Dr. Man Sun Sy (Case Western Reserve University, Cleveland, OH); mouse mAbs against PI(4,5)P2 were from Dr. Kiyouki Fukami (University of Tokyo, Tokyo, Japan). Rat mAbs 555 against L1 were used in the ELISA binding assay and immunoblotting (Appel et al., 1993). The mAbs 735 against PSA have been described elsewhere (Frasch et al., 1985).

Goat pAbs m20 against PrP and rabbit pAbs and mouse mAbs against PrP-Fc containing the extracellular domain of the mouse PrP in fusion with AP was produced as described previously (Chen et al., 2003). NCAM and L1 were purified from adult mouse brain homogenate as described previously (Rathjen and Schachner, 1984; Frosch et al., 1985).

PrP expression vector was provided by Dr. Patricia Maness (University of North Carolina, Chapel Hill, NC). 24 h after transfection, 1 mg/ml G418 was added to the culture medium, which was replaced with fresh medium containing 1 mg/ml G418 every 3 d. After 3 wk in culture, single colonies were selected for subsequent screening. In experiments with stimulation, 2 µg/ml PrP-Fc or 8 µg/ml human Fc were applied to live CHO cells for 20 min in a CO2 incubator.

Subcellular fractionation and isolation of lipid-enriched microdomains

Rafts were prepared from crude membrane and growth cone fractions from 0–4-d-old mice as described previously (Leshchyns’ka et al., 2003). The same protocol was used to isolate lipid rafts from CHO cells.

Chemical cross-linking

Crude membrane fractions or lipid rafts from 0–4-d-old mice were incubated with 0.2 mM BS3 cross-linker (Pierce Chemical Co.) in 20 mM sodium phosphate buffer (pH 7.6) for 30 min at RT. The cross-linking reaction was stopped by the addition of 1 M Tris-HCl (pH 7.6) to a final concentration of 100 mM for 10 min. The cross-linked fractions were either separated by gel electrophoresis and then immunoblotted or were used for communoprecipitation. Chemical cross-linking in brain homogenates with PrP-Fc as bait was performed using Sulfo-SBED biotin label transfer reagent (Pierce Chemical Co.), following the manufacturer’s instructions.

Communoprecipitation

Homogenates were prepared from the brains of 0–4-d-old mice in 50 mM Tris-HCl buffer (pH 7.5), containing 0.32 M sucrose, 1 mM CaCl2, 1 mM MgCl2, and 1 mM NaHCO3. Samples containing 1 mg of protein were lysed for 30 min in 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl, 0.5% Triton X-100, 1% β-mercaptoethanol, 1 mM sodium fluoride, 2 mM NaVO4, 0.1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche). The lysate buffer containing this combination of detergents completely solubilizes lipid rafts and has been used in a number of studies involving communoprecipitation of lipid raft components (Tranter et al., 1999; Paratcha et al., 2001). Samples were then centrifuged for 15 min at 20,000 g and 4°C. Supernatants were cleared with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) for 3 h at 4°C and incubated with pAbs against NCAM, p59Fyn, or nonimmune rabbit Ig (overnight at 4°C), followed by precipitation with protein A/G-agarose beads (for 3 h at 4°C). The beads were washed 3× with RIPA buffer, once with PBS, and analyzed by immunoblotting (Leshchyns’ka et al., 2003). The approxi-
mation percentage of NCAM molecules bound to PrP in the total brain membranes (P) was quantified as: P = (NCAMPrP/NCAMtotal) × (PrPimmunoprecipitates/PrPtotal) × 100%, where NCAMtotal and NCAMPrP are labeling intensities of NCAM in the total brain membranes and PrP immunoprecipitates, respectively, and PrPtotal and PrPimmunoprecipitates are labeling intensities of PrP in the total brain membranes and PrP immunopectipitates, respectively.

**Gel electrophoresis and immunoblotting**

Proteins were separated by 8% SDS-PAGE and electroblotted onto nitrocellulose transfer membrane (PROTRAN; Schleicher & Schuell) overnight at 5 mA. Immunoblots were incubated with appropriate primary antibodies followed by incubation with peroxidase-labeled secondary antibodies and visualized using SuperSignal West Pico reagents (Pierce Chemical Co.) on BIOMAX film (Sigma-Aldrich). Molecular weight markers were prestained protein standards from Bio-Rad Laboratories. Chemiluminescence was quantified using TINA 2.09 software (University of Manchester, Manchester, UK). To allow quantitative comparisons of chemiluminescence between the lanes, the same amounts of total protein or equal amounts of immunoprecipitates were loaded in each lane and the intensity observed for the wild type was used for normalization. All preparations (brain homogenates, immunoprecipitations, or lipid rafts) were performed three times and at least two Western blots were performed with an individual sample (n = 6). In each experiment, when PrPimmunoprecipitates/PrPtotal and PrPimmunoprecipitates/PrPtotal × 100% were compared, PrPimmunoprecipitates/PrPtotal × 100% values were set to 1, and intensities for PrPimmunoprecipitates/PrPtotal × 100% values of all experiments were used to calculate mean values and SEM.

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**References**

Appel, F. J., Holm, J. F., Cosciencence, and M. Schachner. 1993. Several extracellular domains of the neural cell adhesion molecule L1 are involved in neurite outgrowth and cell body adhesion. *J. Neurosci.* 13:4764–4775.

Bhamidipati, A., S.A. Lewis, and N.J. Cowan. 2000. 149:1087–96. ADP ribosylation factors as trans-interacting partner for neurons is involved in neurite outgrowth and cell body adhesion. *J. Biol. Chem.* 275:3863–3875.

Collinge, J., M.A. Whittington, K.C. Sidle, C.J. Smith, M.S. Palmer, A.R. Clarke, and J.G. Jefferys. 1994. Prion protein is necessary for normal synaptic function. *Nature.* 370:295–297.

Cremer, H., R. Lange, A. Christoph, M. Plomann, G. Vopper, J. Roos, R. Brown, S. Baldwin, P. Kraemer, S. Scheff, et al. 1994. Inactivation of the N-CAM gene in mice results in the reduction of the olfactory bulb and deficits in spatial learning. *Nature.* 367:455–459.

Culligan, K., L. Glover, P. Dowling, and K. Ohlendieck. 2001. Brain dystrophic glycoprotein complex: persistent expression of β-dystroglycan, impaired oligomerization of Dp71 and up-regulation of utrophins in animal models of muscular dystrophy. *BMC Cell Biol.* 2:2.

Doherty, P., A. Singh, G. Rimon, S.R. Bolsover, and F.S. Walsh. 1993. Thy-1 antibody-triggered neurite outgrowth requires an influx of calcium into neurons via N- and L-type calcium channels. *J. Cell Biol.* 122:181–189.

Filipp, D., J. Zhang, B.L. Leung, A. Shaw, S.D. Levin, A. Veillette, and M. Julius. 2003. Regulation of Fyn through translocation of activated Lck into lipid rafts. *J. Exp. Med.* 197:1221–1227.

Frosch, M., I. Gorgen, G.J. Boulnois, K.N. Timmis, and D. Bitter-Suermann. 1985. NZB mouse system for production of monoclonal antibodies to weak bacterial antigens: isolation of an IgG antibody to the polysaccharide capsule of *Escherichia coli* K1 and group B meningococci. *Proc. Natl. Acad. Sci. USA.* 82:1194–1198.

Gambetti, P., Q. Kong, W. Zou, P. Parchi, and S.G. Chen. 2003. Sporadic and familial CD3 classification and characterization. *Br. Med. Bull.* 66:213–239.

Gauczynski, S., J.M. Peyrin, S. Haik, C. Leucht, C. Hundt, R. Rieger, S. Krase, and J.P. Laurent, M. Schachner. 2005. RPTPalpha knockout mice exhibit deficits in spatial learning. *Hum. Mol. Genet.* 82:1194–1198.

Gennarini, G., M. Him, H. Deagostini-Bazin, and C. Goridis. 1984. Studies on the transfembrane disposition of the neural cell adhesion molecule N-CAM. The use of liposome-inserted radioiodinated N-CAM to study its transbilayer orientation. *Eur. J. Biochem.* 142:65–73.

Gorodinsky, A., and D.A. Harris. 1995. Glycolipid-anchored proteins in neuroblastoma cells form detergent-resistant complexes without caveolin. *J. Cell Biol.* 129:619–627.

Graner, E., A.F. Mercadante, S.M. Zanata, O.V. Forlenza, A.L. Cabral, S.S. Veiga, M.A. Juliano, R. Roessler, R. Walz, A. Minetti, et al. 2000. Cellular prion protein binds laminin and mediates neurogenesis. *Brain Res. Mol. Brain Res.* 76:83–92.

Grant, S.G., T.J. O’Dell, K.A. Karl, P.L. Stein, P. Soriano, and E.R. Kandel. 1992. Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science.* 258:1903–1910.

Harder, T., P. Scheiffele, P. Verkade, and K. Simons. 1998. Lipid domain structure of the plasma membrane revealed by patching of membrane components. *J. Cell Biol.* 141:929–942.

Hsiao, K., H.F. Baker, T.J. Crow, M. Poulter, F. Owen, J.D. Terwilliger, D. Westaway, J. Ott, and S.B. Prusiner. 1989. Linkage of a prion protein missense variant to Gerstmann-Strassler syndrome. *Nature.* 338:342–345.

Kayaks, A., J. Yang-Snyder, M. Heroux, K.V. Shah, M. Bouvier, and R.T. Moon. 2004. Mutant Frazzled 4 associated with vitreoretinopathy traps wild-type Frazzled in the endoplasmic reticulum by oligomerization. *Nat. Cell Biol.* 6:52–58.

Kleene, R., and M. Schachner. 2004. Glycans and neural cell interactions. *Nat. Reviews.* 5:195–208.

Kojima, N., J. Wang, I.M. Mansuy, S.G. Grant, M. Mayford, and E.R. Kan. 1997. Rescuing impairment of long-term potentiation in fyn-deficient mice by introducing Fyn transgene. *Proc. Natl. Acad. Sci. USA.* 94:4761–4765.

Laux, T., K. Fukami, M. Thelen, T. Golub, D. Frey, and P. Caroni. 2000. GAP43, MARKCS, and CAP23 modulate PIP(4,5)P2 at plasmaemalff raft and regulate cell cortex actin dynamics through a common mechanism. *J. Cell Biol.* 149:1455–1472.

Ledesma, M.D., K. Simons, and C.G. Dotti. 1998. Neuronal polarity: essential role of protein-lipid complexes in axonal sorting. *Proc. Natl. Acad. Sci. USA.* 95:3966–3971.

Lemhann, S., and D.A. Harris. 1995. A mutant prion protein displays an aberrant membrane association when expressed in cultured cells. *J. Biol. Chem.* 270:24589–24597.

Leshchyns’ka, I., V. Sytnyk, J.S. Morrow, and M. Schachner. 2003. Neural cell adhesion molecules L1 and NCAM mediated neurite outgrowth and cell body adhesion. *J. Neurobiol.* 56:577–582.

Madore, N., K.L. Smith, C.H. Graham, A. Jen, K. Brady, S. Hall, and R. Morris. 1999. Functionally different GPI proteins are organized in different do-
mains on the neuronal surface. *EMBO J.* 18:6917–6926.

Mallucci, G.R., S. Ratte, E.A. Asante, J. Linehan, I. Gowland, J.G. Jefferys, and J. Collinge. 2002. Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *EMBO J.* 21:202–210.

Mihuvet, O., and S. Lehmann. 2002. Oxidative stress and the prion protein in transmissible spongiform encephalopathies. *Brain Res. Brain Res. Rev.* 38:328–339.

Mouillet-Richard, S., M. Ermonval, C. Chebassier, J.L. Laplanche, S. Lehmann, J.M. Launay, and O. Kellermann. 2000. Post-natal knockout of prion protein alters hippocampal CA1 properties, but does not result in neurodegeneration. *EMBO J.* 21:202–210.

Nakai, Y., and H. Kamiguchi. 2002. Migration of nerve growth cones requires detergent-resistant membranes in a spatially defined and substrate-dependent manner. *J. Cell Biol.* 159:1097–1108.

Niethammer, P., M. Delling, V. Sytnyk, A. Dityatev, K. Fukami, and M. Schachner. 2002. Cosignaling of NCAM via lipid rafts and the FGF receptor is required for neurotogenesis. *J. Cell Biol.* 157:521–532.

Paratcha, G., F. Ledda, L. Baars, M. Coulpier, V. Besset, J. Anders, R. Scott, and C.F. Ibanez. 2001. Released GFRα1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. *Neuron.* 29:171–184.

Pollerberg, E.G., R. Sadoul, C. Goridis, and M. Schachner. 1985. Selective expression of the 180-kD component of the neural cell adhesion molecule N-CAM during development. *J. Cell Biol.* 101:1921–1929.

Prusiner, S.B. 1998. Prions. *Proc. Natl. Acad. Sci. USA.* 95:13363–13383.

Rathjen, F.G., and M. Schachner. 1984. Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO J.* 3:1–10.

Rieger, R., F. Edenhofer, C.I. Lasmezas, and S. Weiss. 1997. The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat. Med.* 3:1383–1388.

Schmitt-Ulms, G., G. Legname, M.A. Baldwin, H.L. Ball, N. Bradon, P.J. Bosque, K.L. Crossin, G.M. Edelman, S.J. DeArmond, F.E. Cohen, and S.B. Prusiner. 2001. Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J. Mol. Biol.* 314:1209–1225.

Shmerling, D., I. Hegyi, M. Fischer, T. Blattler, S. Brandner, J. Gotz, T. Rulicke, E. Flechsig, A. Cozzio, C. von Mering, et al. 1998. Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. *Cell.* 93:203–214.

Solforosi, L., J.R. Criado, D.B. McGavern, S. Wirz, M. Sanchez-Alavez, S. Sugama, L.A. DeGiorgio, B.T. Volpe, E. Wiseman, G. Abalos, et al. 2004. Cross-linking cellular prion protein triggers neuronal apoptosis in vivo. *Science* 303:1514–1516.

Sytnyk, V., I. Leschyns'ka, M. Delling, G. Dityateva, A. Dityatev, and M. Schachner. 2002. Neural cell adhesion molecule promotes accumulation of TGN organelles at sites of neuron-to-neuron contacts. *J. Cell Biol.* 159:649–661.

Telling, G.C., M. Scott, J. Mastriani, R. Gabizon, M. Torchia, F.E. Cohen, S.J. DeArmond, and S.B. Prusiner. 1995. Prion propagation in mice expressing human and chimeric PrP transgenes implicates the interaction of cellular PrP with another protein. *Cell.* 83:79–90.

Trupp, M., R. Scott, S.R. Whitemore, and C.F. Ibanez. 1999. Ret-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells. *J. Biol. Chem.* 274:20885–20894.

van’t Hof, W., and M.D. Resh. 1997. Rapid plasma membrane anchoring of newly synthesized p59Fyn: selective requirement for NH2-terminal myristoylation and palmitoylation at cysteine-3. *J. Cell Biol.* 136:1023–1035.

Walmsley, A.R., F. Zeng, and N.M. Hooper. 2003. The N-terminal region of the prion protein ectodomain contains a lipid raft targeting determinant. *J. Biol. Chem.* 278:37241–37248.

Weissmann, C., and E. Flechsig. 2003. PrP knock-out and PrP transgenic mice in prion research. *Br. Med. Bull.* 66:43–60.

Weiss, S., D. Proske, M. Neumann, M.H. Groschup, H.A. Kretzschmar, M. Fammolok, and E.L. Winnacker. 1997. RNA aptamers specifically interact with the prion protein PrP. *J. Virol.* 71:8790–8797.