Aβ neurotoxicity depends on interactions between copper ions, prion protein, and *N*-methyl-D-aspartate receptors

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N-methyl-D-aspartate receptors (NMDARs) mediate critical CNS functions, whereas excessive activity contributes to neuronal damage. At physiological glycine concentrations, NMDAR currents recorded from cultured rodent hippocampal neurons exhibited strong desensitization in the continued presence of NMDA, thus protecting neurons from calcium overload. Reducing copper availability by specific chelators (bathocuproine disulfonate, cuprizone) induced nondesensitizing NMDAR currents even at physiologically low glycine concentrations. This effect was mimicked by, and was not additive with, genetic ablation of cellular prion protein (PrP^C), a key copper-binding protein in the CNS. Acute ablation of PrP^C by enzymatically cleaving its cell-surface GPI anchor yielded similar effects. Biochemical studies and electrophysiological measurements revealed that PrP^C interacts with the NMDAR complex in a copper-dependent manner to allosterically reduce glycine affinity for the receptor. Synthetic human Aβ$_{1-42}$ (10 nM–5 μM) produced an identical effect that could be mitigated by addition of excess copper ions or NMDAR blockers. Taken together, Aβ$_{1-42}$, copper chelators, or PrP^C inactivation all enhance the activity of glycine at the NMDAR, giving rise to pathologically large nondesensitizing steady-state NMDAR currents and neurotoxicity. We propose a physiological role for PrP^C, one that limits excessive NMDAR activity that might otherwise promote neuronal damage. In addition, we provide a unifying molecular mechanism whereby toxic species of Aβ$_{1-42}$ might mediate neuronal and synaptic injury, at least in part, by disrupting the normal copper-mediated, PrP^C-dependent inhibition of excessive activity of this highly calcium-permeable glutamate receptor.

NMDA receptor | Alzheimer's disease | Aβ oligomer | 5XFAD mouse | Cu

The *N*-methyl-D-aspartate receptor (NMDAR) is a key ionotropic glutamate receptor in the mammalian CNS, playing a critical role in a range of functions including development, memory, and learning (1, 2). Although the primary activator of these receptors is glutamate, they typically also require the binding of the coagonist glycine (or D-serine) to the NR1 subunit of the receptor complex. Glycine binding results in enhanced peak current amplitude and slowing of receptor desensitization (3), an essential intrinsic mechanism that terminates receptor activity during prolonged agonist exposure to protect neurons from toxic calcium entry (3, 4). Indeed, excessive NMDAR activity has been implicated in the pathophysiology of several neurodegenerative disorders such as Alzheimer's disease (AD), but the underlying molecular mechanisms are poorly understood (5–7). One fundamental feature of AD pathogenesis is an excessive production and accumulation of Aβ peptides in the brain. Although the most characteristic histopathologic feature includes neurofibrillary tangles within neurons and deposition of congophilic β-amyloid plaques in the cortex (8, 9), recent work suggests that soluble oligomeric Aβ peptides are the neurotoxic species, rather than the large amyloid deposits (10). However, the precise mechanisms of Aβ neurotoxicity remain unclear (11), nor is the relationship between Aβ and NMDARs clearly

elucidated. Lauren and colleagues (12) recently reported that Aβ$_{1-42}$ oligomers are high-affinity ligands of cellular prion protein (PrP^C) and that PrPc is required to mediate the effect of these toxic species. This observation was recently confirmed by Barry and coworkers (13) who showed that PrP^C was required for Aβ oligomer-mediated suppression of in vivo long-term potentiation (LTP). Along these lines, Gimbel and colleagues (14) reported that memory impairment observed in a transgenic mouse model of Alzheimer's disease was abolished upon deletion of PrP^C. Finally, Collinge and coworkers (15) showed that low-molecular-weight, Aβ-derived diffusible ligand prepared from human Alzheimer's brain disrupts hippocampal synaptic plasticity in a PrP^C-dependent manner. Interestingly, their observed disruption of LTP induction is reminiscent of the effect of tonic activation of NMDARs (16). Data from our laboratory have revealed that PrP^C forms a signaling complex with NMDARs (17). Furthermore, our work showed that knockout of PrP^C in mouse hippocampal neurons enhances NMDAR currents by slowing their deactivation kinetics, perhaps due to an increase in receptors containing the NR2D subunit. Given that Aβ can regulate NMDA receptors (18), we studied the mechanistic relationship between Aβ, NMDA receptors, and PrP^C.

Results

Aβ$_{1-42}$ Induces Steady-State NMDAR Current. To determine whether Aβ can directly regulate NMDAR function, we acutely applied Aβ$_{1-42}$ to cultured hippocampal pyramidal neurons and examined its effect on NMDAR receptor function using whole-cell voltage clamp. Synapses are among the very first structures to be compromised during the early stages of AD (10, 19). Thus, NMDAR-dependent currents were elicited in the presence of 300 nM glycine, a concentration chosen to mirror the submicromolar levels of this coagonist estimated to be present under physiological conditions in the synapse (20, 21). Under our experimental conditions, application of 500 μM NMDA evoked robust inward currents that exhibited near-complete current decay after several seconds in both rat (Fig. 1*A*) and mouse (see below) hippocampal neurons. In contrast, although Aβ$_{1-42}$ itself exhibited no effect, NMDA currents elicited in the presence of 1 μM unfractionated Aβ$_{1-42}$ (which, like the intact brain, contains a mixture of monomers and oligomers) showed a substantial steady-state component that persisted for many seconds. The effects of Aβ$_{1-42}$ on NMDAR current kinetics developed
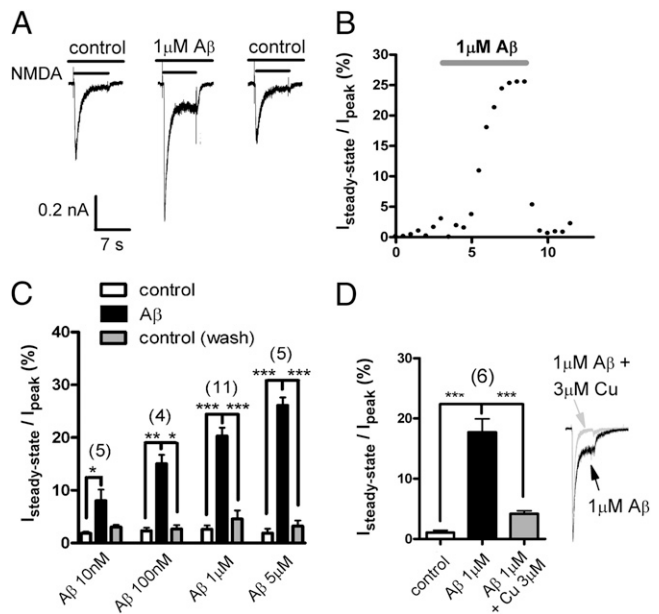
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Fig. 1. Soluble $A\beta_{1-42}$ increased steady-state NMDAR current. (*A*) Representative currents from a rat hippocampal neuron in response to 500 μM NMDA exhibited virtually complete desensitization in 300 nM glycine. $A\beta_{1-42}$ peptide (1 μM) induced a pronounced steady-state current, which was reversible. (*B*) Representative time course of the effects of $A\beta_{1-42}$ on the steady-state NMDA current. (*C*) Bar graph showing mean ± SEM steady-state current as a percentage of peak in control neurons (white bars) and after exposure to unfractionated $A\beta_{1-42}$ (10 nM–5 μM; black bars). The induction of steady-state current by $A\beta_{1-42}$ was completely reversible (gray bars). (*D*) Addition of excess copper ions almost completely reversed the effect of $A\beta_{1-42}$, suggesting that the effect is mediated by alteration of copper ion availability at the receptor. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Fig. 2. Copper chelators induced steady-state NMDAR current. (*A*) Chelating copper with bathocuproine disulfonate (BCS) reversibly induced steady-state NMDAR current in rat hippocampal neurons. (*B*) Representative time course of development of the steady-state current after addition of BCS. (*C*) Mean ± SEM steady-state current as a percentage of peak. There was minimal steady-state current in the absence of copper chelators (white bars), whereas copper chelators induced a substantial steady-state component. (*D*) Specificity of BCS was determined by replacing metal ions. Addition of 2 μM copper to 10 μM BCS had no effect as the chelator remained substantially in excess. In contrast, addition of 2 μM zinc completely abolished the steady-state current, indicating that BCS does not appreciably bind zinc at the concentrations used. Adding excess copper to BCS predictably abolished the steady-state current. (*E*) Effect of BCS was not additive with that of $A\beta_{1-42}$. (*Inset*) Representative traces scaled to normalized peak currents. (*F*) Disrupting copper homeostasis by exposure of neurons to BCS (10 μM) induced significant neuronal death as measured by TUNEL labeling. $A\beta_{1-42}$ was equally toxic, but scrambled $A\beta_{1-42}$ (subjected to the same oligomerization procedure as normal sequence peptide) was not. NMDAR block with 5,7-dichlorokynurenic acid (DCKA, 100 μM) or replenishment with $CuSO_4$ (4 μM) protected neurons from injury. Neurons exposed to NMDA and D-serine (both 500 μM) served as positive controls. Values are mean ± SEM. $*P < 10^{-5}$; $^{\#}P > 0.95$ vs. control.

slowly over several minutes, were rapidly reversed upon washout, and could be observed at concentrations as low as 10 nM (Fig. 1 *B* and *C*). Application of 100 nM scrambled $A\beta_{1-42}$ did not significantly alter NMDA currents ($I_{steady state} = 5.6 \pm 2.9\%$ of I_{peak}, $P = 0.29$ vs. control, $n = 7$). These robust effects of $A\beta_{1-42}$ appear to contrast with recent findings from cultured cortical neurons (22) where no alteration of NMDA currents was observed as a result of chronic treatment with Aβ. However, as is often the case with NMDAR activity measurements, this prior study used high concentrations of the coagonist glycine (20 μM). Glycine and the other classical NMDAR coagonist D-serine are effective chelators of copper ions, and exogenous copper is known to decrease NMDAR-mediated currents (23–25). Given that these coagonists slow NMDAR desensitization (3) and that $A\beta_{1-42}$ also binds copper ions with picomolar or higher affinity (26, 27), we hypothesized that $A\beta_{1-42}$ might modulate NMDAR kinetics by altering copper regulation. Consistent with such a mechanism, addition of 3 μM copper to 1 μM $A\beta_{1-42}$ to yield an excess of copper restored the normal decay of NMDAR currents (Fig. 1*D*). Our findings thus suggest that $A\beta_{1-42}$ might produce a pathological enhancement of NMDAR-mediated current by altering copper availability at the receptor complex.

Copper Ions Modulate NMDAR Kinetics. To separate copper-dependent effects from other possible actions of $A\beta_{1-42}$, we performed analogous experiments using the selective copper ion chelator bathocuproine disulfonate (BCS) (*Materials and Methods*) (28). Application of BCS (1–10 μM) closely mimicked the effects of $A\beta_{1-42}$ on NMDAR kinetics, as did application of another copper chelator, cuprizone (Fig. 2 *A–C*). Although both BCS and cuprizone are considered selective copper chelators, it was important to rule out the possibility that the observed effects were due to chelation of other metals such as zinc, a known
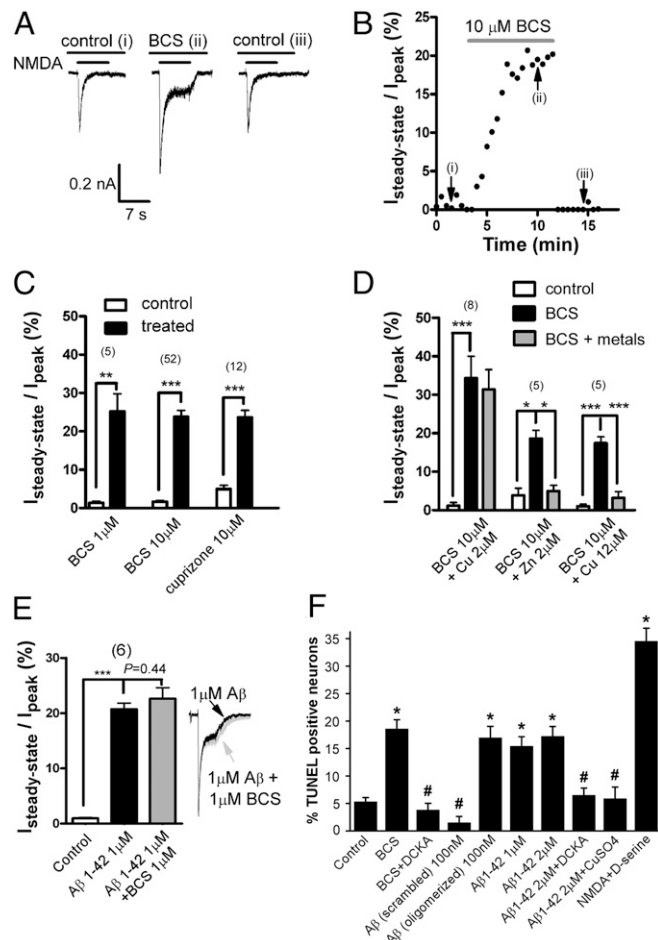
regulator of NMDARs (29). We therefore took advantage of the known NMDAR-blocking effects of micromolar zinc (30). Application of 2 μM zinc to cells bathed in 10 μM BCS markedly reduced the steady-state current (Fig. 2*D*), indicating that BCS did not appreciably chelate zinc ions. In contrast, application of 2 μM copper in the presence of excess BCS had no effect under these conditions, confirming the selectivity of BCS for copper. Raising copper levels to 12 μM, to yield an excess of this metal ion, resulted in near-complete elimination of the steady-state current. Together, these data confirm that the observed effects of BCS are due to selective chelation of copper rather than zinc and
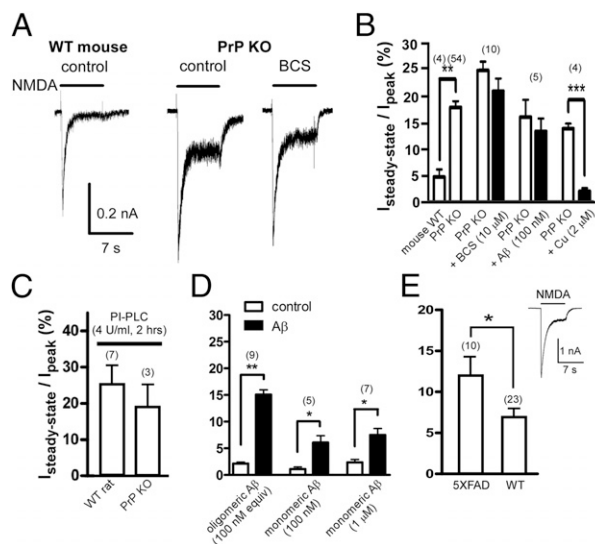
Fig. 3. Prion protein influenced NMDAR kinetics and partially mediated the effect of Aβ$_{1-42}$. (*A*) Representative traces from hippocampal neurons showing large steady-state currents only in the PrP^C-nulls; copper chelation with BCS had no additional effect. (*B*) Mean ± SEM steady-state current as a percentage of peak recorded from PrP knockout neurons. Mere absence of PrP^C resulted in an ~20% steady-state current, similar in magnitude to BCS-treated neurons (cf. Fig. 2). Neither BCS nor Aβ$_{1-42}$ was additive. **$P < 0.01$; ***$P < 0.001$. (*C*) Acute cleavage of the PrP^C GPI anchor by PI-PLC induced substantial steady-state current in WT rat neurons, which was not additive in PrP-null neurons. (*D*) Mean ± SEM steady-state current as a percentage of peak from rat neurons showing that oligomeric Aβ$_{1-42}$ was particularly effective at inducing steady-state NMDAR current. *$P < 0.05$; **$P < 0.01$. (*E*) Quantification of the steady-state current recorded in 300 nM glycine from cultured 5XFAD mouse neurons vs. WT littermates (*$P < 0.05$, t test). (*Inset*) Representative current trace from a 5XFAD neuron.

Fig. 4. PrP^C reduced the affinity of the NMDAR for glycine. (*A*) Glycine dose dependence of the percentage of steady-state current in wild-type and PrP-null mouse neurons. The data were fitted with a modified Hill equation. Note the leftward shift in the glycine dose dependence and the greater maximal effect in PrP-null mouse neurons. *$P < 0.05$ wild type vs. PrP-null. (*B*) Representative traces from rat hippocampal neurons obtained in the absence or presence of 10 μM BCS and corresponding bar chart depicting the size of steady-state current. Consistent with the data in *A*, BCS had little effect at low glycine (0.1 μM) compared with control, but, in contrast, induced a significant steady-state current at 0.3 and 1 μM glycine, indicating a shift to a higher apparent glycine affinity in the absence of copper. *$P < 0.05$; ***$P < 0.001$. (*C*) Coimmunoprecipitation of NR1 subunits and PrP^C from rat brain homogenate under control conditions after addition of 10 μM CuSO$_4$ or after chelation of copper with BCS. NR1 and PrP^C coimmunoprecipitated, with the strength of this association being dependent on ambient copper levels. The blot was probed with an NR1 antibody (expected molecular weight for NR1 is ~120 kDa) and is a representative example of four separate experiments; equal amounts of lysate were used for each condition. The empty lanes reflect a bead-only control ("Beads") and a coimmunoprecipitation control experiment from PrP-null mouse brain. The bar graph is a quantification of band intensities normalized to that observed under control conditions.

establish ambient copper ions as a potent regulator of NMDAR function. The effects of BCS were not additive with those of 1 μM Aβ$_{1-42}$, further supporting the notion that this peptide's effects on NMDAR kinetics were also due to alterations in copper ion availability (Fig. 2*E*). Finally, internal dialysis of neurons with either 10 μM BCS ($I_{steady\ state}$ = 6.3 ± 0.8% of I_{peak}, $n = 3$) or 1 μM Aβ$_{1-42}$ ($I_{steady\ state}$ = 4.1 ± 0.8% of I_{peak}, $n = 3$) had no significant effect on NMDAR kinetics, indicating that copper-dependent regulation of the receptor involves a location accessible from the extracellular space.

Akin to inactivation of voltage-gated calcium channels, decay of NMDAR-mediated current prevents toxic calcium overload of neurons. Hence, one might expect that an increase in steady-state NMDAR-mediated current due to copper chelation by BCS or Aβ$_{1-42}$ would be neurotoxic. As shown in Fig. 2*F*, this is indeed the case. Exposure of hippocampal cultures to BCS or Aβ$_{1-42}$ resulted in a similar degree of neuronal cell death, which was significantly abrogated by NMDAR block with the selective antagonist 5,7-dichlorokynurenic acid (100 μM) or by addition of excess copper ions.

Cellular Prion Protein Modulates NMDAR Kinetics. We next explored the physiological mechanism by which ambient copper might act on the NMDAR. The CNS expresses a broad spectrum of copper-binding proteins (31). One attractive candidate is PrP^C, a widely expressed cuproprotein (32) that is anchored to the extracellular surface of cell membranes and that is especially abundant at synapses (33). Moreover, the expression of PrP^C has been shown to influence synaptic transmission, although the mechanism remains unclear (34, 35). PrP^C contains a number of octarepeat regions (36) that can bind several copper ions with affinities spanning many orders of magnitude (37). Given that PrP^C can directly interact with Aβ$_{1-42}$ (12) and also regulate NMDAR activity (17), our current findings raised the possibility
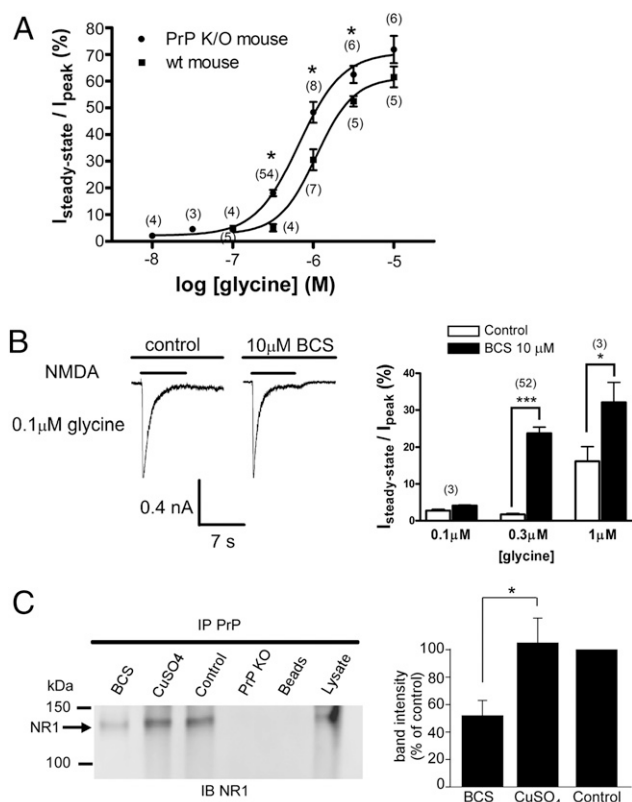
that PrP^C may function to modulate NMDARs in a copper-dependent manner. To test this hypothesis, we performed whole-cell recordings from hippocampal pyramidal cells cultured from PrP^C knockout mice. When activated by NMDA and 300 nM glycine, these neurons exhibited a steady-state current (Fig. 3*A*) that could not be further augmented by addition of either 10 μM BCS or 100 nM Aβ$_{1-42}$ (Fig. 3*B*). Experiments involving recombinant NMDARs composed of NR1/NR2B subunits expressed in *Xenopus* oocytes in the absence and the presence of recombinant PrP^C yielded results similar to those obtained with hippocampal neurons (Fig. S1). Finally, we acutely interfered with PrP^C function in rat hippocampal neurons by enzymatically cleaving the GPI anchor on PrP^C using phosphatidylinositol-specific phospholipase C (PI-PLC), which would cause the protein to be removed from its extracellular location. Under these conditions, we observed a steady-state current that was similar in magnitude to that observed in PrP^C-null mouse neurons or to

wild-type neurons treated with copper chelators (Fig. 3C). These observations indicate that the steady-state currents seen in PrP^C-null mice were indeed due to the absence of PrP^C rather than a compensatory mechanism. In contrast, application of PI-PLC did not produce additional effects in PrP^C-null mouse neurons (Fig. 3C), indicating that if other proteins were removed from the cell surface by cleavage of their GPI anchors, they did not affect the NMDAR-mediated currents that we recorded. Moreover, the steady-state current observed in PrP^C-null neurons could be eliminated upon application of 2 μM exogenous copper (Fig. 3B), indicating that copper has the ability to regulate NMDAR current kinetics even in the absence of PrP^C, perhaps by acting directly at the receptor complex. This result, together with the fact that tissue culture media contain ~100 nM to low-micromolar copper, might explain why neuronal cultures from PrP^C-null mice remain healthy (17), whereas cell death is observed under conditions where copper ions are deliberately chelated to near-zero concentrations (Fig. 2F). Similarly, resting copper levels might be sufficiently elevated in brains of PrP-null mice to explain why these animals do not exhibit significant early neurodegeneration.

In light of our data implicating PrP^C in copper regulation of NMDARs, it is important to note that, although both monomeric and oligomeric species of Aβ$_{1-42}$ interact with copper (26, 38), PrP^C selectively interacts with Aβ$_{1-42}$ oligomers (12). Soluble Aβ$_{1-42}$ normally used in our experiments contained a mixture of monomers and oligomers, and this is further complicated by the fact that copper ions are known to promote oligomerization (39). To determine which of the Aβ species mediated the observed modulation of NMDAR kinetics, we varied the relative proportion of monomeric and oligomeric Aβ$_{1-42}$ species using established protocols (40) (*SI Materials and Methods* and Fig. S2). We found that 100 nM of oligomer-enriched Aβ$_{1-42}$ triggered a steady-state current that was notably more pronounced than the steady-state current evoked by either 100 nM or 1 μM of the mainly monomeric form (Fig. 3D).

To determine the effect of natural Aβ species on NMDAR current activity, we cultured hippocampal neurons from hemizygous 5XFAD mice and their control littermates. 5XFAD mice generate large quantities of natural Aβ$_{1-42}$ and are considered a suitable animal model of AD (41). As shown in Fig. 3E,

neurons cultured from these mice exhibited a significantly greater tonic steady-state current that is consistent with an increase in naturally produced ambient Aβ$_{1-42}$ levels.

Taken together, our data indicate that PrP^C plays an essential role in copper-dependent effects on NMDAR kinetics in neurons. Moreover, the potent effects of Aβ on NMDAR currents might be mediated either by direct chelation of copper by the peptide or by interference with the normal PrP^C-dependent regulation of the receptor; either mechanism results in excessive NMDAR-mediated currents and neuronal damage.

PrP^C and Copper Regulate Glycine Affinity. Next, we sought mechanistic insight into the molecular mode of action of copper and PrP^C on NMDAR kinetics. Our observations in the presence of Aβ oligomers, copper chelators, or upon inactivation of PrP^C, all strongly parallel the effects of increasing concentrations of the coagonist glycine (4). Conversely, at a fixed concentration of glycine, an increase in glycine affinity for the receptor would be expected to produce a similar enhancement of the steady-state current. This led us to hypothesize that PrP^C, in its copper-bound form, reduces glycine affinity for the receptor complex, enhancing desensitization and reducing steady-state current. We therefore compared the glycine dose dependence of NMDAR current decay in WT and PrP^C-null mouse neurons. As shown in Fig. 4A, in neurons lacking PrP^C, glycine was more potent at inducing steady-state NMDAR currents over a concentration range considered physiological (20, 21), consistent with the notion that PrP^C normally reduces the apparent affinity of glycine for the receptor. Chelation of ambient copper with BCS (Fig. 4B) produced a similar shift in glycine dose dependence as that observed with PrP^C knockout, suggesting that the effects of PrP^C on reducing glycine affinity require copper binding. A direct modulatory effect of PrP^C on NMDARs was further suggested by our observation that PrP^C coimmunoprecipitated in a copper-dependent manner with NR1—the obligatory NMDAR subunit that contains the glycine-binding site (Fig. 4C). In contrast, interactions between mGluR1 receptors and PrP^C (42) were not affected by chelating copper (Fig. S3). Together, these data represent a unique mechanism whereby PrP^C suppresses NMDAR activity by regulating glycine interactions with the receptor complex in a manner strongly dependent on copper ions.
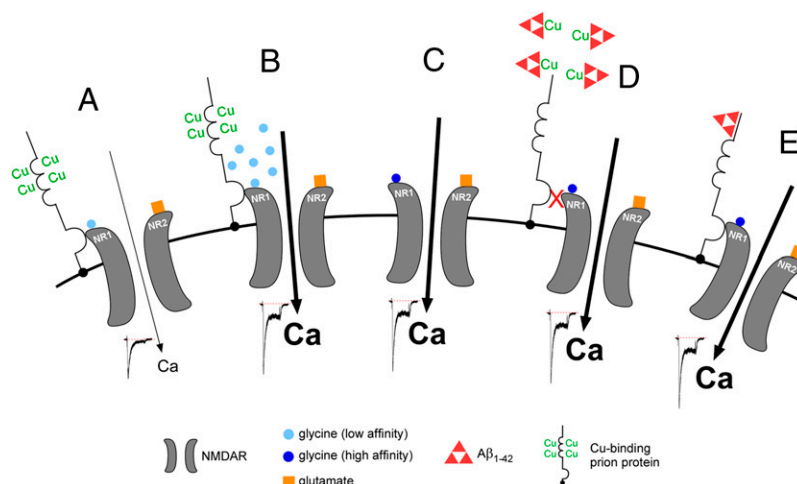

Fig. 5. Proposed model linking copper-dependent effects of PrP^C and Aβ$_{1-42}$ to NMDAR activity. (*A*) NMDARs are closely associated with PrP^C protein (as shown in Fig. 4C) (17). Under physiological conditions, copper-bound PrP^C reduces glycine affinity for the receptor complex, thus enhancing NMDAR desensitization and limiting calcium flux through the receptor. (*B*) At high-glycine concentrations, receptor occupancy increases even though affinity remains low, augmenting the proportion of steady-state current. (*C*) In the absence of endogenous PrP^C, the glycine affinity is enhanced, leading to steady-state currents. (*D*) Under pathological conditions of excessive Aβ production, the peptide acts as a high-affinity copper chelator, preventing copper ions from normally binding to PrP^C; this effect might alter the ability of PrP^C to normally regulate NMDAR desensitization and/or cause dissociation of the two proteins (Fig. 4C), mimicking the genetic or biochemical ablation of PrP^C. The result is an increased glycine affinity, leading to prolonged steady-state current and pathological calcium influx. (*E*) Aβ oligomers act as high-affinity ligands for PrP^C protein (12), and this interaction might also disturb PrP^C-dependent modulation of NMDAR kinetics.

Discussion

Copper is a critically important transition metal involved in numerous metabolic pathways (31, 43). Under our conditions, we identify copper as an endogenous, PrP^C-dependent regulator of NMDARs that prevents excessive activity of the receptor by enhancing desensitization, thus limiting potentially toxic steady-state NMDAR-mediated currents. At physiological levels of synaptic glycine (20, 21), ambient levels of copper greatly limited steady-state NMDAR currents, thus preventing calcium overload and neurotoxicity. In contrast, when even nanomolar amounts of oligomerized Aβ$_{1-42}$ peptide were added, pathological non-desensitizing currents were observed in the presence of physiologically relevant concentrations of glycine due to interference with the normal copper-dependent regulation of NMDARs. Such dysregulated NMDAR currents might contribute to neurotoxicity in AD. Copper-dependent regulation of the NMDAR is also known to affect LTP (44), a correlate of learning and memory, and is thus additionally relevant to AD and related disorders. The pathophysiological importance of these copper-dependent phenomena may have been overlooked previously because most investigations of NMDAR activity are conducted in relatively high (10–50 μM) concentrations of glycine or D-serine where NMDAR desensitization is already dramatically reduced.

In the present study, both copper chelators and Aβ$_{1-42}$ produced similar effects on NMDAR current decay, and their effects were not additive. This, together with the reversal of the steady-state current upon readdition of copper, and the very high affinity of Aβ peptide for copper ions (26, 27), suggests that Aβ$_{1-42}$ might mediate its functional effects on NMDAR activity by disturbing copper homeostasis near the receptor. The corollary would be that under conditions of excess production/liberation of Aβ as in AD, the homeostasis of ambient copper ions is altered, in turn causing an increase in NMDAR current producing potentially toxic calcium influx. With time, synaptic elements would be damaged by chronic calcium overload, leading to synaptic loss, which is one of the earliest manifestations of AD (9, 19). This might also offer a mechanistic explanation for a recent report in a mouse model of AD showing increased spontaneous calcium transients in cortical neurons that could be blocked by NMDAR antagonists (45).

It is thought that free copper is virtually absent in biological fluids, instead existing bound to various amino acids and proteins (34). Our data show that one such copper binding protein, PrP^C, is a key regulator of NMDAR activity. We propose that PrP^C, in its copper-loaded state, binds to the NMDAR complex (Fig. 4C) to allosterically reduce its glycine affinity, thereby increasing desensitization. When copper is chelated (i.e., by BCS or monomeric Aβ$_{1-42}$) or when PrP^C is absent or functionally compromised (by GPI anchor cleavage or binding to Aβ oligomers, for example), glycine affinity is enhanced, reducing receptor desensitization and producing pathologically large, steady-state currents that contribute to neuronal damage (Fig. 5).

We have previously reported that PrP^C can be coimmunoprecipitated with NR2D subunits (17). Our finding that PrP^C and NR1 subunits (which are common to all subtypes of NMDARs) can be coimmunoprecipitated is consistent with the existence of an NMDAR–PrP^C-signaling complex. This assay does not allow us to discern whether PrP^C interacts with the NMDAR complex via NR1 or NR2 subunits. However, given that the NR1 subunit contains the glycine-binding site and that PrP^C regulates glycine affinity, a direct interaction with NR1 is plausible.

It has been shown that PrP^C is a conduit for mediating neurotoxic effects of various β-sheet rich aggregates including Aβ, and that toxicity is prevented by NMDA receptor antagonists (46). As noted earlier, Strittmatter and colleagues (12) reported that Aβ$_{1-42}$ oligomers are high-affinity ligands of PrP^C, which in turn is required for Aβ-mediated neurotoxicity and suppression of LTP (13–15). Together, these findings are consistent with the mechanism of Aβ-mediated suppression of NMDAR desensitization that we report here. Other groups, however, have disputed the above findings, reporting that PrP^C is not required for Aβ-induced interference with synaptic activity (47–49). It is possible that the discordant results might arise from different amounts of copper and/or glycine present in the various preparations used in these studies. Indeed, our data show that copper, at sufficiently high concentrations, is able to regulate NMDAR function in the absence of PrP^C (Fig. 3B), perhaps as a result of direct interactions with the receptor or due to formation of copper–glycine complexes that might render this coagonist ineffective. Depending on the combination of copper and glycine concentrations in any given preparation, Aβ may thus affect NMDA receptor currents independently of PrP^C through direct copper chelation, i.e., by virtue of the extremely high affinity of Aβ for this metal (26, 27). Altogether, the discordant findings with regard to the necessity for PrP^C in Aβ-mediated neurotoxicity can be reconciled by our model that indicates that Aβ can induce pathologically large, steady-state NMDAR currents under conditions of *physiological* glycine and copper concentrations via interactions with PrP^C (Fig. 5). Notably, the electrophysiological effects of excess Aβ$_{1-42}$ produced by cultured 5XFAD neurons were indistinguishable from those of copper chelation, synthetic Aβ$_{1-42}$ in its various forms, or PrP^C ablation (Figs. 1–3). Brains of these mice contain various Aβ$_{1-42}$ complexes ranging from low-*n* oligomers to higher-molecular-weight Aβ-derived diffusible ligands (50, 51). On the basis of our own data and the reported interactions of larger Aβ aggregates with PrP^C (12, 15), it is likely that various species of Aβ contributed to inducing the significant steady-state NMDAR currents. Importantly, however, our results show that naturally overproduced Aβ$_{1-42}$ closely recapitulated what we observed with synthetic peptide.

In summary, the copper-dependent mechanism that we describe here might explain, at least in part, the neurodegeneration observed in AD. This, in turn, could pave the way for the design of effective therapeutics aimed at targeting such a mechanism; state-dependent inhibition or suppression of steady-state NMDAR current might therefore be a promising approach. In contrast and by extension, copper chelator therapy might have unexpected deleterious effects.

Materials and Methods

Experimental procedures pertaining to biochemistry, *Xenopus* oocyte recordings, TUNEL assays, and data analysis are presented in *SI Materials and Methods*.

Neuronal Primary Culture. Pregnant Sprague–Dawley rats were purchased from Charles River and maintained in compliance with the University of Calgary and Sanford-Burnham Medical Research Institute Animal Care and Use Policies. Wild-type and PrP knockout mice (Zuerich 1 strain outbred to a pure C57 genetic background by Frank Jirik's laboratory, University of Calgary, Calgary, AB, Canada) were prepared as described (17). 5XFAD mice were purchased from The Jackson Laboratory. Rat and mouse hippocampal neurons were prepared from P0-2 pups as described by us previously in detail (17). Total copper in culture media was measured at ~100 nM.

Electrophysiology of Hippocampal Neurons. Unless stated otherwise, chemicals were obtained from Sigma-Aldrich. Whole-cell voltage-clamp recordings were performed on hippocampal pyramidal neurons after 10–15 d in culture at room temperature using an Axopatch 200B amplifier (Axon Instruments). The holding potential was −60 mV throughout. The external solution contained 140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 25 mM Hepes, and 33 mM D-glucose, pH adjusted to 7.4 with NaOH. Total measured copper was ~50 nM before addition of any exogenous copper. To obtain NMDA currents, the external solution was supplemented with 0.5 μM TTX (Tocris Bioscience), 100 μM picrotoxin, 15 μM 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt, and different concentrations of glycine as indicated. The internal pipette solution was composed of 140 mM CsCl, 11 mM EGTA, 1 mM CaCl$_2$, 2 mM MgCl$_2$ and 10 mM Hepes, pH adjusted to 7.3 with CsOH. The internal solution was supplemented with 4 mM K$_2$ATP and 0.6 mM GTP, which were added directly to the internal solution immediately before use. Aβ$_{1-42}$ sodium salt was purchased from Anaspec (catalog no. 60883) or rPeptide. Drug delivery was controlled by a rapid microperfusion system (AutoMate Scientific), which was composed of a ValveLink 8.2 controller, an eight-channel mini Lee valve, an eight-channel perfusion pencil, a 250-μm diameter removable tip, and a pressurized superfusion device to achieve fast switching of solution. The perfusion tip was positioned a few hundred micrometers from the cell and kept as constant as possible throughout the experiments.

The solution was perfused with a pressure of 1.6–1.8 psi. The solution exchange was computer controlled for timing and initiation using a Digidata 1320A interface (Molecular Devices). NMDAR-mediated currents were evoked by application of NMDA (500 μM, Tocris Bioscience) for 7 s at 30-s intervals. In a typical experiment, a stable baseline current was obtained by applying external recording solution from a pair of channels. After reaching a stable state, the solution was switched to one that contained NMDA with or without other compounds. The steady-state current ($I_{steady-state}$) was determined as the nondesensitizing current amplitude at the end of a 7-s NMDA application. Solution could be rapidly washed out by switching back to a channel that contained the external recording solution. Trace copper was chelated using the canonical Cu^{2+}-selective chelator cuprizone (52) or BCS (28). Although the latter has been reported to preferentially bind Cu^+ over Cu^{2+} (53), BCS also interacts with Cu^{2+} with high affinity to form bis $[Cu(BCS)_2]^{2-}$ complexes (54, 55), or the protonated form of BCS [H·BCS]$^-$ associates with Cu^{2+} to form complex species such as $[Cu^{2+} (BCS) (H_2O)_x]$ and a proton (56).

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