Prion protein is essential for the RE1 silencing transcription factor (REST)-dependent developmental switch in synaptic NMDA receptors

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Abstract

It is important that the correct amounts of GluN2 subunits are maintained, as they determine NMDAR functional properties, which are crucial to neuronal communication, synaptogenesis and cognitive function. The transcriptional repressor RE1 silencing transcription factor (REST) is critical for the postnatal developmental switch in NMDARs. However, the mechanisms triggering REST and the link between NMDARs and REST are unclear. Here we show a new physiological essential role for cellular prion protein (PrPC) in REST-dependent homeostasis and the developmental switch of NMDARs. REST and REST-associated proteins were overactivated in the hippocampi of Prnp knockout mice (Prnp0/0) compared with wild-type Prnp (+/+) mice. This coincided with the disruption of the normal developmental switch from GluN2B-to-GluN2A in vivo. PrPC co-located with REST under physiological environments and mediated the translocation of REST in conditioners of NMDARs in vitro in Prnp+/- hippocampal neurons. Regardless of whether REST was knocked down or overexpressed, deletion of PrPC not only disrupted REST-mediated distribution of mitochondria, but also prevented REST-regulated expression of GluN2B and GluN2A in Prnp0/0. Importantly, these effects were rescued after overexpression of full-length PrPC through restoration of NMDAR2 subunits and their distributions in dendritic processes in Prnp0/0. Consistently, knockdown of PrPC in Prnp+/- had a similar effect on Prnp0/0. Furthermore, PrPC colocalized with both GluN2B and GluN2A in Prnp+/-.

Introduction

N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels critical for synaptogenesis and neuronal communication1. These heterotetrameric channels are formed by the assembly of two obligatory GluN1 and two GluN2/GluN3 subunits2,3. NMDARs subunit composition is plastic and diverse, leading to abundant receptor subtypes, each with its own biophysical and pharmacological properties4. The subunit isoform of NR2 (GluN2) is a key determinant of the functional capabilities of NMDARs, including activation, deactivation and desensitization kinetics4. A developmental switch from containing primarily GluN2B-to-GluN2A occurs during postnatal development in NMDARs5. This switch, as well as the correct GluN2A/GluN2B ratio, is critical for
neural circuitry, hippocampus-dependent learning, plasticity-induced alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and spine growth. A previous report has shown that the transcriptional repressor element 1-silencing transcription factor (REST), also known as neuron-restrictive silencer factor, participates in the postnatal switch in synaptic NMDAR subunit by decreasing GluN2B expression through epigenetic remodeling of Grin2b at rat hippocampal synapses. During postnatal development, REST shows both target and temporal characteristics in differentiated neurons for GluN2B. REST acts as a multiple hub in coordination with other factors regulating the multiple aspects of neurogenesis and preserving the distinct neural phenotype. However, as a switch from primarily GluN2B-to-GluN2A, the mechanisms that trigger REST expression in differentiated neurons, and the following long-term increase in GluN2A expression during postnatal development, are still unclear.

Although under normal conditions NMDA receptors mediate important physiological functions such as learning and memory, they also take part in glutamate excitotoxicity, which can occur in response to ischemia and is related to many neurodegenerative diseases, including Alzheimer’s Disease (AD). Hence, strict regulation of NMDAR activity plays a key role in homeostasis by preventing excitotoxicity. It is well established that misfolded forms of cellular prion protein (PrPC) transform into the β-sheet-rich, aggregate-prone scrapie conformation (PrPSc), resulting in several progressive fatal diseases, known as prion diseases. These transmissible spongiform encephalopathies include bovine spongiform encephalopathy, scrapie, Creutzfeld–Jakob disease, Gerstmann–Straussler–Scheinker syndrome in humans, and Jakob disease, Gerstmann–Scheinker syndrome in humans. Although research has been conducted exploring the harmful effects of misfolded or aggregated prion proteins, the physiological roles of PrPC remain only partly understood.

Recent studies have demonstrated that PrPC also communicates with NMDARs; PrPC-deficient mice exhibit enhanced NMDAR-dependent neuronal excitability and are more susceptible to NMDA-induced excitotoxicity. Research has cumulatively demonstrated that the absence of PrPC increased NMDAR glycine affinity, resulting in persistent NMDAR activity after prolonged agonist treatment. PrPC and GluN2D are found in the same protein complex and colocalize in hippocampal neurons. However, in the absence of PrPC, it is still not clear whether other subunits of NMDARs are altered. Therefore, more details of PrPC-mediated regulation of NMDARs need to be revealed.

Here, we explore the relationship between PrPC and REST-dependent developmental switch from GluN2B-to-GluN2A. Our data show that a lack of PrPC gives rise to REST overexpression and disorder of REST-associated proteins in the hippocampus of neonatal mice. REST-dependent epigenetic remodeling of the developmental switch of NMDARs is also suppressed in vivo. Comparing wild-type (WT; Prnp+/+) and Prnp knockout (Prnp0/0) mice primary cultured hippocampal neurons, we found that PrPC not only affects the translocation of REST, but also partially mediates REST-regulated mitochondria distribution and the developmental switch in synaptic NMDARs. The adverse effects induced by overactivated REST in Prnp0/0 neurons are recapitulated by overexpression of exogenous PrPC. Thus, we demonstrate a novel functional role for native PrPC as an essential modulator of REST-dependent NMDARs activity, and provide more evidence to support the hypothesis that PrPC is a regulator of NMDARs.

Results

Overexpression of REST and alterations of REST-associated proteins in the hippocampi of postnatal Prnp0/0 mice

In almost all tissues, including the brain, epigenetic modification of chromatin is an important modulator of gene expression. First, we confirmed the developmental expression of PrPC in the hippocampus of Prnp+/+ neonatal mice. Consistent with previous reports, PrPC levels increased during the first two postnatal weeks, reached a peak at P13 and were slightly diminished in the adult (Fig. 1a, b). Second, we tested and compared postnatal expression of REST, postsynaptic density 95 kDa protein (PSD-95), synaptophysin (SYP), GluN2B, GluN2A and NMDAR1 in the hippocampal homogenates of Prnp+/+ and Prnp0/0 neonatal mice.

In the WT group, contrary to the tendency of PrPC, a transient, but marked increase in REST abundance occurred at postnatal day 3 (P3; Fig. 1a, b), with a subsequent, and constant, abundance of GluN2B protein from P7 until P13. REST abundance then declined ~2.25-fold (relative to P3) to a level that persisted until adulthood (Fig. 1e, f). GluN2A protein was barely detectable at P3, and its levels progressively increased ~6.43-fold by P30 relative to that at P3, and continued to increase until P60 (nearly ninefold) (Fig. 1e, g). NMDAR1 levels gradually increased in a similar way to GluN2A (Fig. 1e, h).

In the Prnp0/0 group, REST expression significantly increased after P5 (~9.32-fold) and the highest levels were seen at P13 (~28.32-fold) relative to P3 (Fig. 1c, d). Although GluN2B levels progressively declined after P3 (Fig. 1i, j), GluN2B showed higher expression from P3 to P30 compared with the WT group (Fig. 2a, d). GluN2A was barely detectable before P5, but its expression markedly increased from P7 to P13 (Fig. 1k). NMDAR1 was strongly expressed from P3 to P7 and decreased to undetectable levels at P60 (Fig. 1i, l).

Compared with WT controls, Prnp0/0 mice showed significantly higher expression of REST (Fig. 2c), GluN2B (Fig. 2d) and GluN2A (Fig. 2e) during postnatal...
development (Fig. 2a). NMDAR1 expression was higher from P3 to P9 but decreased remarkably after P30 (Fig. 2f). Levels of SYP and PSD-95, the pre- and postsynaptic markers, showed a consistent upward trend and were not significantly different between the two groups.

We recently demonstrated that β-catenin was expressed in parallel with REST under physiological22 and pathological conditions in LRP6-Wnt-β-catenin signaling pathways23,24. Conversely, total GSK3β protein has an inverse relationship with REST24. Our results reveal that: (1) total β-catenin and p-β-catenin (Ser552)/total β-catenin protein had different expression patterns between WT and PrP-null mice: the former remained relatively high after P7 (Fig. 1m, Fig. S1E, and Fig. S1F), whereas the latter was more highly expressed at the beginning of P3 (Fig. 1n, Fig. S1I, and Fig. S1J). (2) Comparing WT and Prnp0/0 mice, total β-catenin was still more highly expressed in the Prnp0/0 group, and p-β-catenin (Ser552)/total β-catenin protein was generally more highly expressed in the latter, except at P9, but significantly decreased at P60 (Fig. 2b, g, h). (3) Although total GSK3β protein and p-GSK3β (Ser9)/total GSK3β protein had similar expression patterns in both groups (Fig. 1m, Fig. S1G, and Fig. S1H; Fig. 1n, Fig. S1K, and Fig. S1L), GSK3β protein levels were lower in Prnp0/0 mice except at P11, and p-GSK3β (Ser9)/total GSK3β protein was higher in the latter until P60. These findings are consistent with the state of REST in different groups.
Fig. 2 (See legend on next page.)
LiCl, respectively (Fig. S3). Nuclear REST was markedly increased in synapses, and a larger increase of PrPC in the soma. Lithium chloride (LiCl), a selective REST agonist (10 mM, 48 h), remarkably induced the nuclear localization of REST in primary cultured WT and Prnp0/0 neurons by immunofluorescence (IF) and western blot (WB) analysis. In the WT group, PrPC colocalized with REST in the cytoplasm under normal conditions, but also for neuroprotection in neurodegenerative diseases, such as prion diseases \(^{23,24}\) and AD\(^{22}\). Thus, to further study the potential effect of PrPC on REST, we compared the expression and distribution of REST in primary cultured WT and Prnp0/0 hippocampal neurons by immunofluorescence (IF) and western blot (WB) analysis. In the WT group, PrPC colocalized with REST in the cytoplasm under normal conditions. N-methyl-D-aspartic acid (NMDA), a selective NMDAR agonist (10 μM, 24 h), stimulated the accumulation of REST partly in synapses, and a larger increase of PrPC in the cytoplasm under normal conditions. Overall, Prnp0/0 mice exhibited REST overexpression and disordered postnatal developmental switching from GluN2B-to-GluN2A, together with overactivation of β-catenin and suppression of GSK3β.

**PrPC is essential for REST functional translocation**

Previous reports have shown that activation and translocation of REST is a universal feature in response to stressors\(^{25}\). Nuclear REST is a key factor, not only for developmental switch of NMDARs under physiological conditions, but also for neuroprotection in neurodegenerative diseases, such as prion diseases \(^{23,24}\) and AD\(^{22}\). Thus, to further study the potential effect of PrPC on REST, we compared the expression and distribution of REST in primary cultured WT and Prnp0/0 hippocampal neurons by immunofluorescence (IF) and western blot (WB) analysis. In the WT group, PrPC colocalized with REST in the cytoplasm under normal conditions. N-methyl-D-aspartic acid (NMDA), a selective NMDAR agonist (10 μM, 24 h), stimulated the accumulation of REST partly in synapses, and a larger increase of PrPC in the soma. Lithium chloride (LiCl), a selective REST agonist (10 mM, 48 h), remarkably induced the nuclear translocation of REST (Fig. 3a, b) (Fig. S2), a result consistent with our previous report. However, in the absence of PrPC, REST had very little response to the agonist and most of it located in the cytoplasm (Fig. 3a, c). Quantitative WB analysis revealed results consistent with IF (Fig. 3d–i). Although total REST in the Prp-null group was 1.31-fold higher than in the WT group under normal conditions, total REST significantly decreased by 40.41% and 32.22% after exposure to NMDA and LiCl, respectively (Fig. 3d, g). What’s more, in the Prnp0/0 groups, compared with the control, total REST decreased significantly more, by 45.44% and 51.62% in the NMDA and LiCl, respectively (Fig. S3). Nuclear REST was markedly lost in each group when PrPC was absent (Fig. 3e, h). This demonstrates that the functional translocation of REST in response to NMDA and LiCl treatment depends on the presence of PrPC.

**PrPC is essential for REST-dependent NMDAR expression**

REST provides a regulatory hub that coordinately regulates multiple physiological and pathological pathways of neuronal development and neurological diseases in vitro and in vivo\(^{10}\). Although REST-dependent epigenetic remodeling is critical to ischemia-induced neuronal death\(^{26}\), other reports reveal that overexpression of REST plays a critical neuroprotective role\(^{22,24}\). Therefore, we examined whether overexpression or knockdown of REST could functionally recover NMDARs expression in the absence of PrPC. Thus, we examined the expression of GluN2B, GluN2A and NMDAR1 when REST was blocked or overexpressed in WT or PrP-null neurons. Importantly, overexpression of REST significantly increased the level of GluN2B by 7.47-fold compared with the HA vector control in the WT group. Conversely, knockdown of REST markedly suppressed the level of GluN2B by 32.16% but increased the expression of Glu2A 1.57-fold compared with WT control (Fig. 4a, b). By contrast, neither knockdown nor overexpression of REST had any significant effect on NMDAR expression (Fig. 4a, b). This strongly suggests that PrPC is essential for REST-dependent NMDAR expression, especially for GluN2B. Additionally, the number of normal mitochondria, mediated by the neuroprotective function of REST\(^{24}\), decreased (Fig. 4e, f). Even though REST was overexpressed, compared with the WT group, the mitochondrial number decreased by 7.92% (Fig. 4e, f) in the Prnp0/0 group, implying that the presence of PrPC might have an effect on the REST-mediated density of mitochondria.

**REST effects on mitochondria partially depend on PrPC**

It has been shown that Aβ induces an extrasynaptic NMDAR-dependent increase in nitric oxide, leading to mitochondrial dysfunction and synapse deficiency\(^{27,28}\). Overexpression of REST in primary cortical neurons alleviates neurotoxicity peptide (PrP106–126)-induced neuronal oxidative stress and mitochondrial damage\(^ {24}\). Importantly, loss of PrPC results in decreased mitochondrial numbers and abnormal mitochondrial morphology\(^ {29}\). In light of these previous studies, we further explored the potential relationship of PrPC with REST-regulated mitochondrial numbers and distribution.

The NMDAR antagonist dizocilpine maleate (MK-801) improves mitochondrial function and energy status\(^ {30}\), linking NMDAR activation and mitochondrial function\(^ {31}\). WT and Prnp0/0 hippocampal neurons were treated with either NMDA (10 μM, 24 h) or MK-801 (Dizocilpine) (10 μM, 24 h) to observe the density and distribution of mitochondrial numbers.
In both the WT and Prnp0/0 groups, NMDA significantly decreased mitochondrial numbers by 60.49% and 63.01%, respectively, compared with controls. MK-801 markedly increased mitochondrial numbers in the WT and Prnp0/0 groups by 1.61-fold and 1.53-fold, respectively, relative to corresponding control.

Exposure of hippocampal neurons to NMDA significantly increased the number of cells harboring clustered perinuclear mitochondria in both WT and Prnp0/0 groups, whereas the number in the latter group increased slightly. As overexpression of REST had a diminished effect on mitochondrial numbers in the Prnp0/0 group compared with the WT group, (mitochondrial number decreased by 7.92%), through a PrPC rescue experiment we directly explored the role of PrPC in REST-regulated mitochondria. A REST-HA vector was co-transfected with a HA vector into WT or Prnp0/0 hippocampal neurons, or co-transfected with a PrP-HA vector into Prnp0/0 hippocampal neurons. As expected, co-overexpression of PrPC and REST in Prnp0/0 cultures partially restored mitochondrial numbers compared with the Prnp0/0 group transfected with REST alone (Fig. 5d, e). Moreover, the number of cells harboring clustered perinuclear mitochondria also significantly decreased when PrP C and REST were co-transfected into Prnp0/0 mice (Fig. 5d, f).

PrPC plays a critical role in REST-regulated GluN2A and GluN2B expression

Previous reports have demonstrated that native PrPC mediates an important neuroprotective role through its...
Fig. 4 (See legend on next page.)
ability to inhibit NR2D subunits. Although NR2B subunits did not immunoprecipitate with PrP<sup>C</sup> in that study, PrP<sup>P</sup> may interact with NMDARs subunits through other and/or indirect ways, an interaction that needs further exploration. So, to examine whether there is an association between NR2 subunits and PrP<sup>C</sup>, we analyzed the surface expression of Glu2A, Glu2B, and in WT hippocampal neurons using immunolabel reactivity (Fig. 6a). Although Glu2A expression was slight, Glu2B and PrP<sup>C</sup> could well localize in the place where Glu2A existed (Fig. 6b). We then stained total Glu2A and total Glu2B along dendritic processes in WT and Prnp<sup>0/0</sup> neurons. The specific dendritic marker, microtubule-associated protein (MAP2) was used to reveal dendrites by IF. Glu2A-positive and Glu2B-positive puncta along processes were quantitatively analyzed. PrP<sup>C</sup> rescue experiments were also used to explore the function of PrP<sup>C</sup> by IF and WB. First, WT cultures were transfected with three different short hairpin RNA (shRNA)-mediated PrP knockdown vectors (Prnp<sup>−/−</sup> + Sh-PrP) and compared with the PrP-null cultures (Prnp<sup>0/0</sup>) to exclude the interfering factor of genetic background in the knockout mice. In a second experiment, PrP-null cultures were transfected with HA-PrP vector (Prnp<sup>0/0</sup> + HA-PrP) to observe the function of full-length PrP<sup>C</sup>. Third, PrP-null cultures were transfected with a WT PrP<sup>C</sup> vector (Prnp<sup>0/0</sup> + WT) and compared with the HA-PrP vector group to remove the potential influence of the HA tag to the location of PrP<sup>C</sup> and to further confirm the function of full-length PrP<sup>C</sup>. In a final experiment, PrP-null cultures were transfected with a PrP<sup>C</sup> mutant (D177N point mutation) [Prnp<sup>0/0</sup> + Prp(D177N)] that is less efficiently trafficked to the surface than the WT PrP and accumulates in the cytoplasm even without proteasome inhibition. These cultures were compared with the WT PrP<sup>P</sup> to further confirm the role of full-length PrP<sup>C</sup>. Remarkably, both in the Prnp<sup>−/−</sup> + Sh-PrP group and in the Prnp<sup>0/0</sup> group, Glu2A puncta significantly decreased by 37.70% and 52.17% relative to the WT group (23.07 ± 0.05 per 100 μm of dendrites), respectively. Conversely, Glu2B was present in more continuously abundant puncta, increased 1.25-fold (Prnp<sup>−/−</sup> + Sh-PrP) and 1.30-fold (Prnp<sup>0/0</sup>) over the WT group (33.67 ± 0.06 per 100 μm of dendrites) (Fig. 6c, d). On the other hand, overexpression of HA-PrP vector or WT PrP vector had similar effects. They both partly restored the densities of GluN2A puncta (Prnp<sup>0/0</sup> versus Prnp<sup>0/0</sup> + HA-PrP: 11.01 ± 0.07 versus 20.33 ± 0.09 per 100 μm of dendrites; Prnp<sup>0/0</sup> versus Prnp<sup>0/0</sup> + WT PrP: 11.01 ± 0.07 versus 23.03 ± 0.05 per 100 μm of dendrites) and reduced the densities of GluN2B puncta (Prnp<sup>0/0</sup> versus Prnp<sup>0/0</sup> + HA-PrP: 43.67 ± 0.04 versus 35.07 ± 0.07 per 100 μm of dendrites; Prnp<sup>0/0</sup> versus Prnp<sup>0/0</sup> + WT PrP: 43.67 ± 0.04 versus 34.33 ± 0.05 per 100 μm of dendrites). However, the D177N mutant had no significant effect on the expression of GluN2A (12.33 ± 0.07 per 100 μm of dendrites) or GluN2B (42.83 ± 0.03 per 100 μm of dendrites) (Fig. 6f, g). Total neurite length (the cumulative length of all neurites of a single neuron) was not significantly different in the groups tested (n = 30 randomly selected neurons per coverslip within each experiment. Each experiment was repeated with, at least, three independent cultures). The average length of neurites in each group is shown in Figure 6e, h.

Consistently, quantitative WB analysis revealed the following. (1) Knockdown of PrP<sup>C</sup> in WT culture remarkably induced the expression of REST (increased to 17.14-fold of the Prnp<sup>0/0</sup> group), stimulated the level of GluN2B (increased to 20.68-fold of the Prnp<sup>0/0</sup> group) and suppressed the level of GluN2A (decreased to 63.54% of the Prnp<sup>0/0</sup> group). However, NMDAR1 expression was not significantly different, and negative control vector (NC) showed similar results to control group (Fig. 6i, l). (2) PrP<sup>C</sup> overexpression significantly inhibited the expression of REST (decreased to 21.94% of the Prnp<sup>0/0</sup> group), contributing to the suppression of GluN2B (decreased to 39.50% of the Prnp<sup>0/0</sup> group) and the promotion of GluN2A (increased to 2.33-fold of the PrP-null group). NMDAR1 expression was not significantly different in the two groups (Fig. 6j, m). (3) Both HA-PrP vector and WT PrP vector had similar degrees of functional action to REST, GluN2B and GluN2A. However, PrP (D177N) mutant failed to recover the expression of these proteins in Prnp<sup>0/0</sup> culture (Fig. 6k, n). Overall, our data indicate a critical and novel relationship between full-length PrP<sup>C</sup>, GluN2A and GluN2B. This is mediated by REST to maintain the correct proportions of NMDAR subunits.
Fig. 5 (See legend on next page.)
Fig. 5 PrPC effects on the density and distribution of mitochondria. a Following treatment with NMDA or MK-801 (or no treatment), Prnp+/− and Prnp−/− mice primary hippocampal neurons were stained with MitoTracker Red to visualize mitochondria and analyzed by fluorescence microscopy. Nuclei (blue) are stained with DAPI. Scale bars = 10 μm. b Quantification of mitochondrial number (a) in a segment of neuronal process 200 μm in length beginning from the cell body of neurons (n = 30). **p < 0.01 vs corresponding control. c Quantification analysis of (a) to confirm the number of cells harboring perinuclearly clustered mitochondria. For this quantification, mitochondria of at least 500 cells per experiment were determined in a blinded manner. Quantifications were based on triplicates of at least three independent experiments. *p < 0.05 vs corresponding control. d Co-transfection of PrPC and REST rescued the density and distribution of mitochondria. Scale bars = 10 μm. e Quantification of mitochondrial number (d) in a segment of neuronal process 200 μm in length beginning from the cell body of neurons (n = 30). **p < 0.01 vs Prnp+/−. *p < 0.05 is Prnp−/− + HA-PrP vs Prnp−/−. f Quantification analysis of (d) to confirm the number of cells harboring perinuclearly clustered mitochondria. For this quantification, mitochondria of at least 500 cells per experiment were determined in a blinded manner. Quantifications were based on triplicates of at least three independent experiments. *p < 0.05 vs Prnp+/−. *p < 0.05 is Prnp−/− + HA-PrP vs Prnp−/−

Discussion

The importance of a developmental switch maintaining the correct ratio of GluN2B-to-GluN2A

NMDARs are necessary regulators of brain plasticity. During the development of synapse structure and function, they transform precise patterns of neuronal activity into long-term changes that are thought to underlie higher cognitive functions. Moreover, NMDARs switch their subunits from predominantly GluN2B to primarily GluN2A during early postnatal development. This subunit switch is evolutionarily conserved from amphibians to mammals and occurs all over the CNS during a time window coinciding with synapse growth and neuronal circuitry refinement. However, the mechanisms responsible for the GluN2B-to-GluN2A subunit exchange have yet to be fully defined. In neurodegenerative diseases, recent studies also indicate that GluN2B-containing NMDARs have an essential role in mediating the adverse effects of Aβ. GluN2B antagonists can rescue Aβ-induced damage of long-term potentiation and synaptic impairment. However, all of the clinical trials of first-generation NMDAR antagonists were disappointing as a result of unendurable side-effects and short therapeutic windows. Another potential limitation is the lack of subunit selectivity of the drugs. GluN2B-specific antagonists offer significant neuroprotection with a better side-effect profile. Conversely, activation of GluN2A may exhibit pro-survival effects via CREB signaling, although the neuroprotective role of GluN2A is still controversial. Additionally, in patients with Parkinson’s disease, the degeneration of nigral dopaminergic neurons gives rise to overactivation of glutamatergic projections. In the striatal membrane of 1-3,4-dihydroxyphenylalanine (L-DOPA)-treated dyskinesia animals, the increased synaptic abundance of GluN2A and redistribution of GluN2B from synaptic to extrasynaptic regions demonstrate that selectively targeting specific NMDARs might be more hopeful. Understanding the precise molecular mechanisms responsible for the GluN2B-to-GluN2A exchange could provide new perspectives for the development of therapeutic strategies.

PrPC plays a novel and critical role in the REST-dependent developmental switch in synaptic NMDA receptors

During embryogenesis in pluripotent stem cells and neural progenitors, REST is a widely expressed gene-silencing factor. REST plays an important role for synaptic function via epigenetic remodeling by silencing coding and noncoding neuronal genes. At the gene level, REST takes part in the postnatal switch in synaptic NMDARs by reducing GluN2B expression through epigenetic remodeling of Grin2b. Some questions remain unanswered, such as what mechanism turns on REST expression in differentiated neurons, and which factor regulates the long-term increase in GluN2A expression during postnatal development.

Mature PrPC is a glycoprotein attached by a carboxyl (C)-terminal glycosylphosphatidylinositol (GPI) anchor to the extracellular leaflet of the plasma membrane. PrPC can undergo different types of physiological cleavage, producing N2 and C2 fragments. PrPC contacts with, and signals through, multiple cell surface proteins and signaling pathways. This highlights the need for a better understanding of the mechanisms of PrPC interaction with its binding factors, both physiologically and pathologically. Comparing WT and Prnp−/− revealed that PrPC expression at synapses contributes to hippocampal synaptic function and exhibits neuroprotection by regulating neuronal excitability. In particular, PrPC requires copper to facilitate S-nitrosylation-mediated NMDAR suppression. However, GluN2B subunits did not immunoprecipitate with PrPC in a previous study. and no studies have yet explored the relationship between PrPC and GluN2A. PrPC might interact with GluN2B through indirect ways, an aspect needing further research. Nucleocyttoplasmic transport is thought to be important for REST as a transcriptional repressor regulating neuronal gene expression. In this study, nuclear REST was markedly lost in the absence of PrPC, demonstrating that the functional translocation of REST in response to NMDA and LiCl treatment depends on the presence of PrPC. Overexpression of exogenous PrPC rescued excessive REST-induced GluN2A and GluN2B imbalance by...
Fig. 6 (See legend on next page.)
increasing the expression of GluN2A and inhibiting the level of GluN2B in PrP-null neurons. To our knowledge, our study is the first to demonstrate that PrP<sup>+</sup>-mediated REST-dependent expression of GluN2A and GluN2B contributes to maintain the correct subunit proportions of NMDARs and cellular homeostasis, deserving to be further explored as a novel and viable therapeutic target against dysfunctions of glutamatergic transmission.

**Materials and methods**

### Animals

Prnp<sup>+/0</sup> mice on a C57BL/6J × 129Sv genetic background were kindly supplied by Dr. Charles Weissmann<sup>40</sup>. Control C57BL/6J mice with no genomic modifications (WT), 6–8 weeks of age and weighing 18–25 g, were obtained from Beijing Experimental Animal Center. Mice were housed 3–4 per cage and under controlled environmental conditions (21–23 °C; 40–60 % humidity; 12-h light/dark cycle) with free access to water and standard pelleted food. All animal experiments were conducted in accordance with the guidelines of Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals and approved by the Beijing Municipality Administration Office of Laboratory Animals (BAOLA).

### Plasmids and transfection

The pCMV-HA-REST vector of full-length REST (cat no. PPL50007-2A<sup>24,25</sup>) was obtained from Public Protein/Plasmid Library (Nanjing, China, GeneShare Technology, Co, Ltd). The pGPH1/GFP/Neo-REST-Mus shRNA vectors<sup>24,25</sup>, the pGPH1/GFP/Neo-PRNP-Mus shRNA vectors (Table 1) and the negative control vector (NC)—which has the same number of corresponding bases as shRNA-REST or shRNA-PRNP but does not target any known gene—were all obtained from GenePharma (Suzhou, China). The full-length mouse prion protein (PRNP) complementary DNA (cDNA) was originally cloned from total brain of C57BL/6J mice by PCR. The primers were designed according to the gene sequence of Mus musculus prion protein (PRNP) mRNA in GenBank (Gene ID: 19122). Oligonucleotides 5′-CGGAATTCGGATACAGCGCTAAGAAGTCGCGAACCTTGGCTACTG-3′ (forward) and 5′-CCACGATCAGGAAGATGA-3′ (reverse) were used to introduce EcoRI and XhoI sites flanking the cDNA (The underlined alphabets denote the "Restriction enzyme cutting sites" of EcoRI and XhoI, respectively.). The PCR product was cloned into vector pCMV-HA (Clontech, Kyoto, Japan) to generate plasmid pCMV-HA-PRNP (HA-PrP) by standard molecular biology techniques and confirmed by sequencing. All the primers were synthesized by Sangon Company (Shanghai, China). Full-length mouse PrP with 3F4 epitope (WT PrP<sup>+</sup>) (Addgene plasmid # 13917) and moPrP<sup>3F4</sup> (D177N) (Addgene plasmid # 1319) were gifts from Susan Lindquist<sup>40</sup>. For

| Table 1 Sequences of REST-targeting shRNA(Sh-REST), PRNP-targeting shRNA(Sh-PrP) |
|---------------------------------|---------------------------------|
| Name                           | Sequence (5′→3′)                 |
| **REST-mediated shRNA:**       |                                 |
| Target sequence 1              | GGCTGTGGCCTACAATACCAACC         |
| Target sequence 2              | GTGCAATTATGTTGGCCTCTAA          |
| Target sequence 3              | GGATTCCACAGCGGAGAAGT            |
| **PRNP-mediated shRNA:**       |                                 |
| Target sequence 1              | GCACCGTTACCCCACCTCATCCTTATGTTTTTC |
| Target sequence 2              | GCCACGGAGAATTGCTTTTTT           |
| Target sequence 3              | GTGACTATGTTGGACTGATG            |

(see figure on previous page)
transfection\textsuperscript{23,24,47,48}, cultured primary hippocampal neurons were washed with Opti-MEM (Gibco) and then transfected with the appropriate plasmids using the Lipofectamine\textsuperscript{TM} Ltx & Plus\textsuperscript{TM} Reagent (Thermo Fisher, Waltham, MA, USA) in Opti-MEM according to the manufacturer’s instructions. The amounts of plasmids and reagents were 2 μg and 3 μl per 12-well, respectively. Forty-eight hours after transfection, cells were observed using an upright fluorescence confocal microscopy (Olympus, Tokyo, Japan) or subjected to immunoblot analyses.

**Statistical analysis**

All assays were performed at least three times. Data are expressed as means ± S.D. For in vivo experiments, six hippocampal homogenates were separately collected and tested in each group. One-way ANOVA, followed by Dunnett’s test was performed for whole hippocampal lysate experiments in Figure 111. Two-way ANOVA was tested in each group. One-way ANOVA, followed by Student’s t-test or two-way ANOVA was performed for whole hippocampal lysate experiments in Figure 111. Two-way ANOVA was performed for Figure 2. Other comparisons for parametric data were made using Student’s t-test or one-way ANOVA followed by post hoc Turkey’s test or two-way ANOVA test using the SPSS software (version 13.0: SPSS Inc., Chicago, IL, USA), GraphPad Prism 5 software (La Jolla, CA, USA) and Image J (National Institutes of Health, USA). P < 0.05 was considered statistically significant\textsuperscript{24,25}.

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**Authors’ contributions**

Conceived and designed the experiments: Z.Q.S. Performed the experiments: Z.Q.S. and W.Y. Analyzed the data: Z.Q.S. Wrote the paper: Z.Q.S. Edited the paper: Z.Q.S., W.Y., G.Y.C., X.M.Z., L.F.Y and D.M.Z. Contributed reagents/materials/analysis tools: D.M.Z. All authors read and approved the final manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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