Neuroprotective role of PrP<sup>C</sup> against kainate-induced epileptic seizures and cell death depends on the modulation of JNK3 activation by GluR6/7–PSD-95 binding

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ABSTRACT Cellular prion protein (PrP<sup>C</sup>) is a glycosyl-phosphatidylinositol–anchored glycoprotein. When mutated or misfolded, the pathogenic form (PrP<sup>Sc</sup>) induces transmissible spongiform encephalopathies. In contrast, PrP<sup>C</sup> has a number of physiological functions in several neural processes. Several lines of evidence implicate PrP<sup>C</sup> in synaptic transmission and neuroprotection since its absence results in an increase in neuronal excitability and enhanced excitotoxicity in vitro and in vivo. Furthermore, PrP<sup>C</sup> has been implicated in the inhibition of N-methyl-D-aspartic acid (NMDA)–mediated neurotransmission, and prion protein gene (Prnp) knockout mice show enhanced neuronal death in response to NMDA and kainate (KA). In this study, we demonstrate that neurotoxicity induced by KA in Prnp knockout mice depends on the c-Jun N-terminal kinase 3 (JNK3) pathway since Prnp<sup>−/−</sup> Jnk3<sup>−/−</sup> mice were not affected by KA. Pharmacological blockage of JNK3 activity impaired PrP<sup>C</sup>-dependent neurotoxicity. Furthermore, our results indicate that JNK3 activation depends on the interaction of PrP<sup>C</sup> with postsynaptic density 95 protein (PSD-95) and glutamate receptor 6/7 (GluR6/7). Indeed, GluR6–PSD-95 interaction after KA injections was favored by the absence of PrP<sup>C</sup>. Finally, neurotoxicity in Prnp knockout mice was reversed by an AMPA/KA inhibitor (6,7-dinitroquinoxaline-2,3-dione) and the GluR6 antagonist NS-102. We conclude that the protection afforded by PrP<sup>C</sup> against KA is due to its ability to modulate GluR6/7-mediated neurotransmission and hence JNK3 activation.

INTRODUCTION

The abnormal processing of cellular prion protein (PrP<sup>C</sup>) gives rise to PrP<sup>Sc</sup>, or pathogenic prion, which is the etiologic agent of several transmissible spongiform encephalopathies (reviewed in Aguzzi et al., 2007; Aguzzi and Calella, 2009; Linden et al., 2008). These devastating encephalopathies are characterized by severe histological changes, including extensive neuronal death, reactive gliosis, and neuroinflammation, concomitant to the accumulation of the aggregated misfolded form of PrP<sup>Sc</sup> in affected brains. Although necessary for prion propagation and infectivity (Weissmann et al., 1994), the repertoire of physiological roles of PrP<sup>C</sup> remains to be fully determined. Indeed, PrP<sup>C</sup> has been implicated in a wide range of neuronal functions, such as copper homeostasis (Brown et al., 1997, 1998; Vassallo and Hermès, 2003), neurotransmission (Maglio et al., 2004;
Prestori et al., 2008; Rangel et al., 2009), stem-cell proliferation and differentiation (Steele et al., 2006), malignant glioma proliferation (Erlich et al., 2007), neurite outgrowth (Chen et al., 2003; Sattuccone et al., 2005), and, more recently, putative cross-talk between neurodegenerative diseases (Lauren et al., 2009; Gavin et al., 2010; Gunther and Strittmatter, 2010).

Early in vitro studies with prion protein gene Prnp<sup>−/−</sup> mice (Zürich I) (Bueler et al., 1992) showed that cultured Prnp<sup>−/−</sup> neurons were more prone to die than Prnp<sup>+</sup> neurons after serum withdrawal or other treatments (Kuwahara et al., 1999). Subsequent studies (Brown et al., 2002; Rambold et al., 2008; Aude-Garcia et al., 2011) indicated that PrP<sub>C</sub> might play a neuroprotective function in the context of excitotoxic lesions. Indeed, it was demonstrated in rats that PrP is overexpressed in ischemic brains and protects cortical neurons after ischemia (McLennan et al., 2004, 2006; Shyu et al., 2005; Spudich et al., 2005; Weise et al., 2006; Mitteregger et al., 2007). In fact, cells overexpressing PrP<sub>C</sub> show increased toxicity after endoplasmic reticulum stress (Anantharam et al., 2008). Neuroprotective effects of PrP<sub>C</sub> after central nervous system insults have been associated with both extracellular and intracellular effectors (e.g., the stress-induced phosphoprotein 1) (Zanata et al., 2002) and are mediated by PI3K-mTOR signaling (Martins et al., 2002) and are mediated by PI3K-PI53-activating kinase 3 (Akt3) (Zürich I) (Bueler et al., 2010; Gunther and Strittmatter, 2010).

In neurons, PrP<sub>C</sub> does not act as a major modulator in a panel of neurodegenerative diseases (McLennan et al., 2005; Weise et al., 2011) indicated that PrP-mediated JNK3 activity to external stimuli (e.g., box handling) (Table 1).

Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mice were highly susceptible to KA, showing a greater number (from five to eight) of severe seizures (grade VI). In addition, they maintained grade IV–VI seizures for 2–3 h after the first episode, whereas and Prnp<sup>+/−</sup>Jnk3<sup>−/−</sup> and Prnp<sup>+/−</sup>Jnk3<sup>o/o</sup> mice displayed only grade III seizure. Furthermore, three Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> animals died during the experiments. These data corroborate previous results (Walz et al., 1999; Rangel et al., 2007, 2009) indicating increased sensitivity to KA in Prnp knockout mice. We established that the minimal concentration of KA required to induce seizures in the Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> animals was 35–40 mg/kg body weight, which is similar to that required for Prnp<sup>−/−</sup>Jnk3<sup>o/o</sup> mice. At this concentration, all Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> animals died shortly after a second injection. These results suggested an active role of JNK3 in Prnp<sup>−/−</sup> susceptibility to KA. Indeed, Prnp<sup>+/−</sup>Jnk3<sup>−/−</sup> and Prnp<sup>−</sup>/Jnk3<sup>−/−</sup> mice were not affected by KA treatment as described (Yang et al., 1997).

Decreased seizures in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mice correlated with lower number of dying cells in the hippocampus

Protein expression was analyzed by Western blot (Figure 1A), which showed that PrP expression was similar in Prnp<sup>+/−</sup>Jnk3<sup>−/−</sup> and Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mice. In addition, JNK3 expression was similar in Prnp<sup>+/−</sup>Jnk3<sup>−/−</sup> and Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mice, and neither of these proteins was detected in the double-knockout mice, as expected (Figure 1A). Next we analyzed in more detail the time course of the seizure score after KA injection (Figure 1B). Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mice showed maximum scores (V–VI) between 90 and 180 min after the first KA injection. To determine whether the severity of seizure observed in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> correlates with neuronal loss and reactive glial changes in the hippocampus after KA injection, we carried out several histochemical and immunohistochemical analyses (Figure 1, C and D).

First, we analyzed the pattern of neurodegeneration in the Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup>, Prnp<sup>+/−</sup>Jnk3<sup>−/−</sup>, and Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mice 24 h after KA treatment by using Fluoro-Jade B staining (Schmued and Hopkins, 2000; Figure 1C). Numerous Fluoro-Jade B–positive pyramidal neurons were observed in the CA1 and CA3 regions of Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> hippocampus. In the other genotypes Fluoro-Jade B labeling was rare and generally pale (Figure 1C). To further corroborate the death of these neurons, we applied in parallel
sections the in situ nick-translation technique with biotinylated-UTP to determine the DNA fragmentation level (TUNEL technique, by using ApopTag kit; Heatwole, 1999; Figure 1D). Distribution of TUNEL-positive nuclei largely correlated with the pattern of Fluoro-Jade B labeling observed in the CA1 and CA3 of Prnp<sup>o/o</sup> Jnk3<sup>+/+</sup> mice (Figure 1, D and E). Cell counts showed statistical significance (see Materials and Methods for details) when comparing data of Prnp<sup>o/o</sup> Jnk3<sup>+/+</sup> mice with any of the other two genotypes.

| Genotype. | Animal | Number of seizures | Number of blinkings | Behavioral stages | Prioritary stage |
|-----------|--------|--------------------|---------------------|------------------|-----------------|
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 1      | 1                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 2      | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 3      | 1                  | 1                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 4      | 1                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 5      | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 6      | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 7      | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 8      | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 9      | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 10     | 1                  | 0                   | I–III            | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 11     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 12     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 13     | 8                  | 4                   | I–VI             | †               |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 14     | 5                  | 3                   | I–VI             | IV–VI           |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 15     | 6                  | 4                   | I–VI             | V–VI            |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 16     | 6                  | 3                   | I–VI             | †               |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 17     | 5                  | 2                   | I–VI             | V–VI            |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 18     | 6                  | 3                   | I–VI             | †               |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 19     | 6                  | 4                   | I–VI             | V–VI            |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 20     | 5                  | 2                   | I–VI             | IV–VI           |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 21     | 4                  | 2                   | I–VI             | V–VI            |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 22     | 8                  | 5                   | I–VI             | V–VI            |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 23     | 6                  | 3                   | I–VI             | IV–VI           |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 24     | 7                  | 5                   | I–VI             | V–VI            |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 25     | 1                  | 1                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 26     | 2                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 27     | 1                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 28     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 29     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 30     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 31     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 32     | 2                  | 2                   | I–III            | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 33     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 34     | 1                  | 1                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 35     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 36     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 37     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 38     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 39     | 0                  | 0                   | I–II             | II              |
| Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> | 40     | 0                  | 0                   | I–II             | II              |

See Results for stage classification.

**TABLE 1:** Effect of KA-induced status epilepticus and death in Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup>, Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup>, Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup>, and Prnp<sup>+/+</sup> Jnk3<sup>+/+</sup> mice.
Acute down-regulation of PrP<sup>C</sup> expression in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> primary neuronal cultures increased susceptibility to KA

We examined whether the neurotoxic effects of KA are modified when PrP<sup>C</sup> expression levels are acutely altered in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> neurons. Thus, Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> primary cortical neurons were infected with a lentivirus carrying a small interfering RNA (siRNA) interference sequence for Prnp. Cells infected with lentivirus showed an 81% decrease in PrP<sup>C</sup> expression (Figure 2A). After exposure to KA, cells infected with lentivirus showed a statistically significant 29% decrease in survival (Figure 2B) compared with parallel enhanced green fluorescent protein (eGFP)–infected cultures (Figure 2B). Thus, although similar data were previously described in neuroblastoma cells (N2A) using interference RNA against Prnp (Rangel et al., 2007), this is the first observation of KA-mediated cell death after acute PrP<sup>C</sup> down-regulation in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> neurons. This finding reinforces the notion that decreased expression of PrP<sup>C</sup> compromises neuronal viability after acute KA treatment.

Differential activation of JNK3 and extracellular regulated kinases 1/2 in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mouse hippocampus treated with KA

Because genetic deletion of Jnk3 on a Prnp<sup>−/−</sup> background overcomes the intrinsic susceptibility to KA of PrP<sup>C</sup>–deficient mice, we next developed several experiments to identify changes in JNK3 activation in the hippocampus after KA treatment (Figure 3). Mice were injected with KA as described and killed 6 h later (see Materials and Methods for details). Activation of extracellular regulated kinase (ERK) 1/2, JNK3, c-Jun, and p53 was analyzed in protein samples from the hippocampus of treated mice by Western blot and compared with vehicle-injected mice. In the absence of KA, ERK1/2 was activated in vehicle-injected mice. In the absence of PrP<sup>C</sup>, ERK1/2 was activated in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mouse hippocampus (Figure 3A). These data corroborate the reported data of Rangel et al. (2007), who showed that down-regulation of PrP<sup>C</sup> compromises neuronal viability after acute KA treatment.

**FIGURE 1:** KA-dependent sensitivity, seizure behavior, neurotoxicity and apoptosis in the different genotypes studied. (A) Western blot of JNK3, PrP<sup>C</sup>, and tubulin in protein extract from the hippocampi of the different mouse strains used in this study (Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup>, Prnp<sup>−/−</sup>Jnk3<sup>+/−</sup>, Prnp<sup>−/−</sup>Jnk3<sup>+/+</sup>, Prnp<sup>+/−</sup>Jnk3<sup>−/−</sup>, and Prnp<sup>+/−</sup>Jnk3<sup>+/−</sup>); tubulin was used as a loading control. (B) Comparison of seizure responses in littermates of Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup>, Prnp<sup>−/−</sup>Jnk3<sup>+/−</sup>, Prnp<sup>−/−</sup>Jnk3<sup>+/+</sup>, and Prnp<sup>+/−</sup>Jnk3<sup>−/−</sup> mice to intraperitoneal injection of KA (6 mg/kg body weight) or 0.1 M PBS. KA-injection timing is indicated below the graph. Seizures were scored as indicated in Materials and Methods. Eight mice in each group were observed and scored to determine the time-dependent seizure score. (C) Photomicrographs showing examples of the pattern of Fluoro-Jade B and DAPI staining of hippocampal region of Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup>, Prnp<sup>−/−</sup>Jnk3<sup>+/−</sup>, and Prnp<sup>−/−</sup>Jnk3<sup>+/+</sup> mice after 24 h of KA treatment. Dying cells positive for Fluoro-Jade B are located in the pyramidal cell layer of Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> (arrows). (D) Examples of TUNEL-positive cells in CA1–CA3 hippocampal regions of Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup>, Prnp<sup>−/−</sup>Jnk3<sup>+/−</sup>, and Prnp<sup>−/−</sup>Jnk3<sup>+/+</sup> mice after 24 h of KA treatment. (E) Quantification of Fluoro-Jade B and TUNEL-positive cells in the CA1 and CA3 regions of the hippocampus in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup>, Prnp<sup>−/−</sup>Jnk3<sup>+/−</sup>, and Prnp<sup>−/−</sup>Jnk3<sup>+/+</sup> mice after 24 h of KA treatment. Results are obtained from nine mice per genotype. DG, dentate gyrus; CA1–3, hippocampal regions 1 and 3; gl, granule cell layer; h, hilus; ml, molecular layer; so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Scale bar, C, top, 200 μm; C, bottom, 100 μm; D, 100 μm. Asterisks in E indicate statistical significance (***p < 0.001, ANOVA test).
ERK1/2 activation in Prnp knockout mice (Brown et al., 2002). In parallel to the phosphorylation of ERK1/2 in all genotypes after KA treatment, an increase in p-JNK3 and p-c-Jun Ser
\[\text{ERK1/2 activation in Prnp knockout mice (Brown et al., 2002). In parallel to the phosphorylation of ERK1/2 in all genotypes after KA treatment, an increase in p-JNK3 and p-c-Jun Ser}\]

parallel to the phosphorylation of ERK1/2 in all genotypes after KA treatment. Double-labeling techniques with glial fibrillary acidic protein (GFAP) and p-ERK1/2 revealed a second group on p-ERK1/2-stained cells at CA1 and CA3 regions (Figure 3C). However, detailed observation of astroglial cells in these mutants revealed an increased reactivity of astrocytes in Prnp\(^{+/+}\)Jnk3\(^{+/+}\)hippocampus (relevant GFAP content and thick glial expansions), in contrast to moderate reactivity in Prnp\(^{+/+}\)Jnk3\(^{+/+}\)and absence in Prnp\(^{+/-}\)Jnk3\(^{+/-}\)mice (Figure 3D). Thus these data indicate that most p-ERK1/2 staining observed in Prnp\(^{+/-}\)Jnk3\(^{+/-}\)and Prnp\(^{+/-}\)Jnk3\(^{+/-}\)after KA treatment is mainly associated with mossy fiber staining with the participation of GFAP-reactive cells.

The JNK3 pathway is implicated in c-fos and c-jun activation after glutamate treatment (Brami-Cherrier et al., 2007). To discriminate the particular contribution of JNK3 to c-jun and c-fos expression in our experiments, we performed quantitative real-time PCR (RT-qPCR; Figure 4). Data obtained demonstrate that both c-jun and c-fos were overexpressed in the hippocampus of Prnp\(^{+/-}\)Jnk3\(^{+/-}\)mice 6 h after KA administration (4.10- and 49.2-fold increase, respectively; Figure 4A). Deletion of Jnk3 in Prnp\(^{+/-}\)mice offset the c-jun and c-fos increase by 34 and 25% respectively, compared with Prnp\(^{+/-}\)Jnk3\(^{+/-}\)levels. These results support the idea that other KA-activated pathways are the main contributors to the overexpression of both early genes. To examine this hypothesis, we analyzed the differential expression of cyclooxygenase-2 (cox-2), a molecular target of JNK activation in neurodegeneration and inflammation activated by activator protein-1 (AP-1; Hunot et al., 2004). RT-qPCR of cox-2 revealed statistically significant overexpression in the Prnp\(^{+/-}\)Jnk3\(^{+/-}\)hippocampus after KA treatment. Furthermore, stronger decrease of cox-2 was observed in Prnp\(^{+/-}\)Jnk3\(^{+/-}\) (60% compared with those reported by c-jun and c-fos) (Figure 4B). The results indicate that the absence of Jnk3 is not the only factor mediating the early changes in gene expression mediated by KA.

**Pharmacological inhibition of JNK in Prnp\(^{+/-}\)Jnk3\(^{+/-}\) hippocampal slices overcame the neurotoxic effect of KA**

To ascertain whether neurotoxic differences are cell specific or associated with an increased glutamatergic input from the entorhinal cortex, we performed a parallel study in isolated hippocampal slices (Figure 5). Prnp\(^{+/-}\)Jnk3\(^{+/-}\) and Prnp\(^{+/-}\)Jnk3\(^{+/-}\) hippocampal slices were treated with KA or phosphate-buffered saline (PBS), following the addition (when indicated) of two different JNK inhibitors: the pharmacological inhibitor SP600125 and the peptide TAT-JIP, a specific inhibitor derived from the minimal JNK-binding region of the scaffold protein JNK-interacting protein 1 (JIP-1) coupled to the short cell-permeable TAT sequence (Bonny et al., 2001; Figure 5A). After propidium iodide (PI) incubation, we observed few randomly distributed, labeled cells in the CA1–CA3 of Prnp\(^{+/-}\)Jnk3\(^{+/-}\) (Figure 5B). The inhibition of JNK activity with SP600125 and TAT-JIP in Prnp\(^{+/-}\)Jnk3\(^{+/-}\)-derived slices decreased the fluorescence levels by 54 and 42%, respectively, compared with parallel KA-treated slices without the inhibitors (Figure 5B and C). These data indicate that increased cell death of hippocampal Prnp\(^{+/-}\)Jnk3\(^{+/-}\) neurons is associated with intrinsic synaptic properties.

**Communoprecipitation of PrPC with PSD-95 and GluR6/7 in the PSD fractions**

To investigate the participation of PrPC in the neurotoxic effects of KA, we examined whether PrPC exerts its neuroprotective function by modulating the intracellular signaling triggered by KA receptor after ligand binding. Because neuronal depolarization mediated by KA requires the interaction of the GluR6/7 subunit of KA receptor with PSD-95 and MLK3 to form a trimeric complex (Pei et al., 2006; Li et al., 2010), we analyzed biochemically the distribution of GluR6/7 and PrPC in the Prnp\(^{+/-}\)Jnk3\(^{+/-}\), Prnp\(^{+/-}\)Jnk3\(^{+/-}\), and Prnp\(^{+/-}\)Jnk3\(^{+/-}\) hippocampus and whether changes in JNK3 alter the normal distribution of...
Figure 6B). Reactive glia are not completely abolished by the genetic deletion of PrP. Carulla et al. (2005), and NS-102 is a good functional antagonist of PrP.

Figure 1. Scale bars, B, 150 μm; C, 100 μm; and D, 100 μm.

FIGURE 3: KA-induced neurotoxicity in Prnp knockout hippocampal mouse correlates with JNK3 activation. (A) Western blot for p-JNK, JNK3, p-ERK1/2, ERK1/2, p-c-Jun, c-Jun, p-p53, and tubulin from hippocampus extracts of Prnp+/+Jnk3+/+, Prnp+/+Jnk3−/−, and Prnp−/−Jnk3−/− mice after 6 h of KA treatment (6 mg/kg body weight). KA-induced neurotoxicity in the Prnp−/−Jnk3−/− hippocampus is cell-specific and dependent on JNK3 activity. (B) Photomicrograph illustrating the pattern of p-ERK1/2 in the different phenotypes after 24 h of KA treatment. Notice the relevant increase of p-ERK1/2 in Prnp−/−Jnk3−/− and Prnp+/−Jnk3−/− hippocampus. (C) High magnification of the CA1 region of hippocampus from Prnp−/−Jnk3−/−, after 24 h of KA treatment, immunostained with p-ERK1/2 and GFAP antibodies. Note that numerous cells are double labeled (arrows). (D) Photomicrograph illustrating examples of the CA1 region of the three genotypes analyzed after 24 h of KA treatment and immunostained for GFAP. Levels of reactive glia are not completely abolished by the genetic deletion of Jnk3. Abbreviations as in Figure 1. Scale bars, B, 150 μm; C, 100 μm; and D, 100 μm.

PrP−/−. Enriched PSD fractions were isolated from the hippocampus of Prnp+/+Jnk3+/+, Prnp+/+Jnk3−/−, and Prnp−/−Jnk3−/− mice 6 h after administration of KA and processed for GluR6/7, PSD-95, and PrP by Western blot (Figure 6A). Correct purification of the PSD fractions was corroborated since the percentage of PSD-95 in this fraction was 91.6% of total PSD-95. Results revealed no changes in subcellular distribution of these proteins among genotypes. As expected, PrP−/− was detected in all cell compartments but enriched in PSD fractions, where GluR6/7 and PSD-95 were also found.

These results suggested a putative interaction between GluR6/7 and PrP. To explore this possibility, we performed coimmunoprecipitation experiments from whole Prnp+/+Jnk3−/− hippocampus protein extracts and PSD fractions using two different PrP antibodies (6H4 and SAF61; see Materials and Methods; Figure 6B). Results indicated that PrP interacts with PSD-95 and GluR6/7 both in whole-hippocampus extracts and in purified PSD. As expected, both interactions were stronger in the PSD fraction (Figure 6B). Taken together, these results implicate PrP in the binding of PSD-95 with the KA receptor containing GluR6/7. This would allow the formation of the ternary complex (GluR6/7–PSD-95–MLK3), which in turn activates the JNK3 pathway. Thus, to determine this, we immunoprecipitated PSD-95 in hippocampal protein extracts from Prnp+/+Jnk3−/− and Prnp−/−Jnk3−/− mice to measure the amount of GluR6/7 coimmunoprecipitated in the presence or absence of KA (Figure 6C). Immunoblots showed that the interaction between GluR6/7 and PSD-95 was higher in the hippocampus of Prnp−/−Jnk3−/− compared with Prnp+/+Jnk3−/− mice in the presence of KA (1.01 vs. 2.13, respectively; Figure 6C). Taken together, these data indicate that, in the absence of PrP−/−, KA treatment increased the binding of GluR6/7-containing KA receptors to PSD-95, which correlates with increased activation of JNK3.

Increased KA-mediated neurotoxicity in Prnp−/− mice was reversed by 6,7-dinitroquinoxaline-2,3-dione and NS-102

To examine the role of GluR6/7 in KA-induced neurotoxicity in Prnp+/+Jnk3−/− and Prnp−/−Jnk3−/− mice, we injected the AMPA/KA inhibitor 6,7-dinitroquinoxaline-2,3-dione (DNQX) and the GluR6 antagonist NS-102 (Verdoorn et al., 1994) prior to the intraperitoneal injection with KA (Figure 7). DNQX inhibits AMPA/KA receptors in vivo (Alford and Grillner, 1990) and inhibits JNK3 activation after excitotoxic insults (Tian et al., 2005), and NS-102 is a good functional antagonist of GluR6 in vivo in response to excitotoxic insults (Tian et al., 2005;
DISCUSSION
Differential participation of ERK1/2, JNK3 kinases, and early gene expression in KA-mediated neuronal death in Prnp\(^{−/−}\) mice

In this study we demonstrate that abolition of JNK3 activity (either genetically or pharmacologically) blocks the neurotoxic activity induced by KA in Prnp\(^{−/−}\) mice. These data indicate that the signal for neuronal cell death in Prnp knockout mice is mainly associated with JNK3 activity. In addition, to avoid the effects of the increased oxidative phenotype in absence of Prnp (Wong et al., 2001; Brown et al., 2002), we show that an acute decrease in PrP\(^{C}\) expression in cultured neurons from Prnp\(^{+/+}\)/Jnk3\(^{−/−}\) mice also increased their susceptibility to KA. In fact, absence of Jnk3 completely blocked the epileptogenic effects of KA in Prnp\(^{−/−}\) mice, and the original Jnk3\(^{−/−}\) mice were characterized as being resistant to KA, with similar scores to those reported here (Yang et al., 1997; de Lemos et al., 2010).

Although basal p-ERK1/2 is higher in Prnp\(^{−/−}\) mice (Brown et al., 2002; Chiarini et al., 2002; Nicolas et al., 2007; Rangel et al., 2007; and present results), treatment with KA induces overphosphorylation of ERK1/2, which is not affected by the absence of Jnk3. In contrast, ERK1/2 activation is abolished in Jnk3\(^{−/−}\) mice 3 h after KA treatment (de Lemos et al., 2010). Thus it is reasonable to consider that changes in p-ERK1/2 in Prnp\(^{−/−}\)/Jnk3\(^{−/−}\) mice after KA treatment are mainly associated with the Prnp\(^{−/−}\) background. p-ERK1/2 is expressed by mossy fibers of granule cells, as well as by reactive, GFAP-positive astrocytes, after KA treatment; in contrast, JNK activation mainly takes place in dying neurons (CA1–CA3 pyramidal neurons; Rangel et al., 2007).

Our histological and biochemical analyses demonstrate that an increased number of GFAP-positive cells can be seen in the double-knockout hippocampus without relevant cell death. These data also contrast with those obtained with Jnk3\(^{−/−}\) mice (Yang et al., 1997; de Lemos et al., 2010). Although we cannot rule out an effect of activated p-ERK1/2/GFAP-positive astrocytes on JNK-activated dying neurons, it seems that intrinsic ERK1/2 phosphorylation (which is not blocked by deletion of Jnk3) in granule cells could participate in the sustained c-jun overexpression in response to KA in a Prnp\(^{−/−}\) background. On the other hand, c-jun was upregulated in Prnp\(^{−/−}\)/Jnk3\(^{−/−}\) mice after KA treatment, but no neurodegeneration was observed when compared with both Prnp\(^{−/−}\)/Jnk3\(^{−/−}\) and Prnp\(^{+/+}\)/Jnk3\(^{−/−}\) mice in brain sections when examined by different methods (Fluoro-Jade B or TUNEL). Thus c-Jun overexpression cannot be associated with cell death and needs to be phosphorylated by JNK3 to induce cell death. Indeed, JNK3 phosphorilates c-Jun at Ser-63 and Ser-73, and abrogation of c-Jun phosphorylation using alanine mutants for c-Jun at Ser-63 and Ser-73 confers resistance to epileptic seizures and neuronal apoptosis induced by KA (Behrens et al., 1999). On the other hand, c-fos was upregulated only in dying Prnp\(^{−/−}\)/Jnk3\(^{−/−}\)

 Hu et al., 2008; Chen et al., 2009). First, we counted the seizures and the blinking episodes in treated mice (Figure 7A). Pharmacological treatments indicate that preinjection with DNQX and, more effectively, with NS-102 reduced the number of all of the episodes counted in Prnp\(^{−/−}\)/Jnk3\(^{−/−}\) mice. Prnp\(^{+/+}\)/Jnk3\(^{−/−}\) mice were almost insensitive to KA, as described earlier (Figure 7A). Again, both treatments reduced the number of Fluoro-Jade B-positive cells in the hippocampus (Figure 7B) and the presence of p-c-Jun in Prnp\(^{−/−}\)/Jnk3\(^{−/−}\) hippocampus in Western blots (Figure 7C). In addition, we examined the effect of DNQX and NS-102 on c-Fos expression, which was overexpressed in the hippocampus of Prnp\(^{−/−}\)/Jnk3\(^{−/−}\) mice after KA injections (see earlier discussion). Blockage of both AMPA/KA receptors and GluR6 prevented c-Fos overexpression in CA1 (Figure 7D) and increased immunostaining of p-ERK1/2 in mossy fibers of Prnp\(^{−/−}\)/Jnk3\(^{−/−}\) (Figure 7E). Measure of the fluorescence in the mossy fibers with ImageJ software (square regions of interest of 75 × 75 μm, six measures per section) indicates a statistically significant reduction of 80 and 75% of p-ERK1/2 staining after DNQX and NS-102 treatments respective (Figure 7F).
cells, and Jnk3 deletion did not totally decrease c-fos levels, suggesting its implication in different KA-signaling mechanisms. In addition, our data also implicate p53 in these results since phosphorylation of p53 follows the activity pattern of JNK3 (only activated in Prnp<sup>−/−</sup>Jnk3<sup>+/+</sup> after KA treatment). This is compatible with the recent proposal that p53 activation after glutamatergic stimulus depends on JNK activity (Choi et al., 2011).

JNK3-mediated signaling activates the AP-1 complex, which triggers the expression of early response genes such as c-fos or c-jun, as well as the up-regulation of cox-2, which is involved in the inflammatory response after KA treatment (Tu and Bazan, 2003). In fact, pharmacological inhibition or genetic deletion of cox-2, but not Cox-1, increases susceptibility to KA-induced hippocampal excitotoxicity (Toscano et al., 2008). In our study, cox-2 was up-regulated after KA treatment in Prnp<sup>−/−</sup>Jnk3<sup>+/+</sup> mice, whereas in Prnp<sup>−/−</sup>Jnk3<sup>−/−</sup> mice, cox-2 expression was largely reduced (up to 60%), indicating a reduction in inflammatory responses in the double-knockout mice. Indeed, a direct relation between JNK activity, c-Jun phosphorylation, and cox-2 up-regulation has been demonstrated in several models (Waetzig et al., 2005), and the up-regulation of Cox-2 mediated by JNK is necessary and required for neurodegeneration in a mouse model of Parkinson disease (Hunot et al., 2004).

A putative role of PrP<sup>C</sup> at the neuronal membrane as modulator of GluR6/7–mediated neurotransmission and JNK3 activity

GluR6 knockout and Jnk3<sup>−/−</sup> mice are resistant to KA treatment. In fact, it has been reported that GluR6 binds to PSD-95, and this binding is necessary to trigger GluR6-dependent neurotransmission (Savinainen et al., 2001) due to the formation of a stable trimeric complex GluR6–PSD-95–MLK3 (Li et al., 2010). In this study we found large colocalization of PrP<sup>C</sup> with GluR6/7 in the PSD. Moreover, Prnp deletion favors the interaction between PSD-95 and GluR6/7 in the presence of KA. Taken together, our results suggest that absence of PrP<sup>C</sup> enhances the interaction of the ternary complex PSD-95 and GluR6 and most probably MLK3 at the synapse, thus enabling GluR6-mediated intracellular signaling. This in turn leads to JNK3 activation among that of other kinases.

As mentioned, PrP<sup>C</sup> has been detected in axon and synaptic contacts, although whether its location is presynaptic or postsynaptic is not fully determined (Fournier et al., 2000; Fournier, 2008). Here we report that a fraction of PrP<sup>C</sup> localized to the PSD, where neurotransmitter receptors are located. Recent studies indicate that PrP<sup>C</sup> is a constitutive basal protector against NMDA-mediated toxicity. Deletion of the Prnp gene induces enhanced...
prolonged NMDA-evoked currents in hippocampal neurons, which increases neuronal excitability and glutamate excitotoxicity (Khosravani et al., 2008). The same authors described changes in some parameters associated with AMPA/KA-mediated miniature inhibitory postsynaptic currents and γ-aminobutyric acid A-mediated miniature inhibitory postsynaptic currents (Khosravani et al., 2008). These findings are in concordance with our data (Rangel et al., 2007, 2009), which demonstrated that enhanced neurotoxicity to KA in hippocampal slices of Prnp−/− mice has both a KA receptor and a NMDA receptor component. However, the molecular mechanism by which PrPc inhibits NMDA and KA receptors is unclear. PrPc may either influence agonist binding, favor the closed state of the channel, or interfere with the signal transmission, thus forming a negative modulator complex (Steele, 2008). In any case PrPc expression does not appear to be critical for neuronal glutamate transport (Thais et al., 2006). Irrespective of the molecular mechanisms responsible for these effects, our data indicate that PrPc (both for KA and NMDA receptors) inhibits glutamatergic neurotransmission and that the absence of PrPc enhances neurotoxic signaling. Furthermore, it is tempting to speculate on a similar protective effect in other brain injuries that share a similar activation mechanism through the receptor complex GluR6/7–PSD-95–MLK3, where signaling is transmitted downstream into the JNK3 pathway. Further studies would be needed to confirm this hypothesis. In this regard, increased levels of PrPc in plasma have been found after acute ischemia in rats and in human stroke patients (Mitsios et al., 2007). Indeed, Weise et al. (2004) suggested that the extent of the up-regulation of PrPc in ischemic brains depends on the severity of ischemia and may therefore reflect the extent of ischemia-induced neuronal damage. We believe that discerning the functional roles of PrPc at the synapse, as well as its specific interacting partners in normal and pathological conditions at the synapse, would help us to understand neurotoxicity in synapse-associated neurodegenerative diseases and ischemic processes. Figure 8 shows a general scheme depicting the role of the GluR6/7–JNK3 signaling pathway in mediating the neurotoxic effects of KA in mouse hippocampus in the absence of PrPc.

MATERIALS AND METHODS

Animals

Prnp knockout mice Zürich I (Prnp−/−) were purchased from the European Mouse Mutant Archive (EMMA, Monterotondo, Italy). Prnp+/− mice were backcrossed with C57BL/6J mice from 10 generations up to obtain 92–95% of C57BL/6J microsatellite markers (Charles River background analysis) from the previous 46–48% of the Zürich I mice. Jnk3 knockout mice were generated as described elsewhere (Yang et al., 1997). Double-knockout animals (Prnp+/−Jnk3+/−) were generated by crossing backcrossed Prnp+/− mice with Jnk3−/− mice (70–75% of C57BL/6J microsatellite markers). To avoid putative background-specific differences between mice, all of the experiments were conducted using littermates.
Reagents and antibodies
KA, PI, SP600125, TAT-JIP peptide, DNQX, NS-102, and antitubulin antibody were from Sigma (Poole, United Kingdom). Anti-p-ERK1/2, total anti-ERK1/2, p-c-Jun (Ser-63), anti-p-JNK, anti-p-p53 (Ser-15), and total anti-JNK3 antibodies were from Cell Signaling (Beverly, MA). Anti-c-Jun antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-Fos was from Calbiochem (Darmstadt, Germany). Postsynaptic density 95 (anti-PSD-95) and glutamate receptor 6/7 (anti–GluR6/7) antibodies and Fluoro-Jade B were from Millipore (Billerica, MA). SAF61 antibody was from Spi-Bio (Cayman Chemical, Massy, France), and 6H4 antibody was from Prionics (Schlieren, Switzerland). SYBR green was from Roche (Basel, Switzerland).

KA injections
To induce convulsive nonlethal seizures in mice, we developed a progressive KA treatment by administering several consecutive intraperitoneal (i.p.) injections of the glutamate agonist KA dissolved in 0.1 M PBS, pH 7.2 (Rangel et al., 2007). PBS 0.1 M, pH 7.2 (vehicle), was injected as control. Adult animals (Prnp<sup>o/o</sup>Jnk3<sup>+/+</sup>; 2–3 mo old) were weighed and i.p. injected with two injections of KA (6 mg/kg body weight) at time 0 and 30 min. Seizure intensity after KA injections was evaluated as described previously during the 4 h after the first KA injection (Peng et al., 1997; Lee et al., 2000). Tissue samples were analyzed at the indicated times.

FIGURE 7: AMPA/kainate receptor inhibition decreased the KA-enhanced neurotoxicity in Prnp<sup>o/o</sup>Jnk3<sup>+/+</sup> mice. (A) Seizures and blinking events for KA-injected mice. Injections were carried out with KA (6 mg/kg body weight), PBS, and the inhibitors DNQX and NS-102 as indicated on the timeline. (B) Examples of Fluoro-Jade B and DAPI staining in hippocampal CA1 region of Prnp<sup>o/o</sup>Jnk3<sup>+/+</sup> mice 24 h after injection of KA in the presence or absence of DNQX and NS-102. (C) Western blots of p-c-Jun, c-Jun, and tubulin from the hippocampus of the animals 4 h after injection of KA in presence or absence of DNQX and NS-102. (D) Photomicrographs of c-Fos immunoreactivity in the hippocampal CA1 region of Prnp<sup>o/o</sup>Jnk3<sup>+/+</sup> mice 24 h after injection of KA in the presence or absence of DNQX and NS-102. Representative images from three different experiments are shown. (E) Photomicrographs of p-ERK1/2 immunoreactivity in the hippocampus of Prnp<sup>o/o</sup>Jnk3<sup>+/+</sup> mice 24 h after injection of KA in the presence or absence of DNQX and NS-102. Representative images from three different experiments are shown. (F) Histogram illustrating quantitative results of the fluorescence levels analyzed in E. Data are represented as mean ± SEM. Asterisks indicate statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001; ANOVA test). Abbreviations as in Figure 1. Scale bars, B, 100 μm; E, 200 μm.
Fluoro-Jade B staining of dying neurons in brain sections
Coronal brain sections were obtained as described and rinsed for 2 h in 0.1 M Tris, pH 7.4, mounted, and air dried at room temperature overnight. The next day, sections were pretreated for 3 min in absolute ethanol, followed by 1 min in 70% ethanol and 1 min in distilled water. They were then oxidized in a solution of 0.06% KMnO4 for 15 min. After three rinses of 1 min each in distilled water, the sections were incubated for 30 min in a solution of 0.001% Fluoro-Jade B (Chemicon) containing 0.01% of DAPI (Sigma) in 0.1% acetic acid. The slides were rinsed in deionized water for 3 min each, dried overnight, cleared in xylene, and cover-slipped with Eukitt (Merck, Darmstadt, Germany) and examined using an Olympus (Hamburg, Germany) BX61 epifluorescence microscope.

Neuronal primary cultures and treatments
Primary hippocampal neurons were prepared from the hippocampus of perinatal (embryological day 18 to day of birth) animals. Briefly, hippocampal neurons were grown on dishes coated with poly-d-lysine (30 μg/ml) in Neurobasal medium supplemented with B27, 0.5 mM glutamine, 12.5 mM glutamate, and antibiotics (all purchased from Invitrogen, Carlsbad, CA). At 9 d in vitro (DIV), hippocampal neurons were infected with lentivirus carrying the eGFP construct (Morales et al., 2008) or the siRNA for Prnp. Lentiviral particles were produced by transient transfection of 293FT cells with Lipofectamine 2000 (Invitrogen), using Mission short hairpin RNA prion protein NM_011170 (Sigma), the second-generation packaging construct psPAX2 (Tronolab, Lausanne, Switzerland), and the envelope plasmid pMD2G (Tronolab). 293FT cells were cultured in DMEM supplemented with 10% fetal calf serum and without antibiotics before transfection. Medium was changed and supplemented with antibiotics after 6 h. Vector supernatants containing viral particles were harvested (~24 and 48 h later and concentrated by ultracentrifugation (2 h at 26,000 × g at 4°C). After 5 d of infection, cultured neurons were treated with KA (150 μM dissolved in 0.1 M PBS; for 8 h). After treatment, cultures were rinsed twice in KA-free culture medium. The next day, cultures were incubated for 2 h with WST-1 reagent (Roche), and the absorbance (450 nm) was measured in a multiwell plate reader (Merck ELISA System MIOS).

Organotypic slice cultures of hippocampus
Hippocampal slice cultures were prepared from day of birth as described elsewhere (Stoppini et al., 1991, 1993; del Rio and Soriano, 2010). Animals were anaesthetized, and brains were removed aseptically from the skull. Horizontal slices (325–350 μm thick) of the hippocampus were obtained using a McIlwain tissue chopper (Mickle Laboratory, Guildford, United Kingdom). Sections were maintained and selected in cold MEM supplemented with glutamine (2 mM; MEM dissecting salt solution) for 45 min. Selected slices were then cultured using the membrane interface method (Stoppini et al., 1991). Slices were placed on 30-mm-diameter sterile membranes (Millicell-CM, Millipore) and transferred to six-well tissue culture plates (Nunc, Roskilde, Denmark). Cultures were incubated with 1.2 ml of culture medium (50% MEM, 25% horse serum, 25% Hank's buffered salt solution, containing 2 mM glutamine and 0.04% NaHCO3, adjusted to pH 7.3). The membrane cultures were maintained in a humidified incubator at 37°C in a 5% CO2 atmosphere. The medium was changed every 2 d. All culture reagents were purchased from Invitrogen-Life Technologies (Meriebeke, Belgium).

Neuronal death after treatments in organotypic slice cultures was monitored by PI uptake (Rangel et al., 2007). After 9 d of culture, hippocampal slice cultures were serum deprived for 3 h, and 150 μM of KA was added. Cultured were exposed to protein JNK

Immunohistochemistry
After KA treatment, mice were perfused with phosphate buffered 4% paraformaldehyde, pH 7.3, postfixed overnight in the same fixative, and cryoprotected in 30% sucrose. Coronal sections (30 μm thick) were obtained in a freezing microtome (Leica, Wetzlar, Germany). Free-floating sections were rinsed in 0.1 M PBS, and endogenous peroxidases were blocked with 3% H2O2 and 10% methanol dissolved in 0.1 M PBS. After extensive rinsing, sections were incubated in 0.1 M PBS containing 0.2% gelatin, 10% normal serum, 0.2% glycine, and 0.2% Triton X-100 for 1 h at room temperature. Sections were then incubated overnight at 4°C with the distinct primary p-ERK1/2 and GFAP antibodies. After washing in 0.1 M PBS containing 0.2% Triton X-100, sections were incubated with Alexa Fluor 488– and/or Alexa Fluor 568–tagged secondary antibodies (1:200 diluted; Molecular Probes, Eugene, OR), washed in 0.1 M PBS counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1 μM in 0.1 M PBS; 10 min), and mounted in Fluoromount (Vector Labs, Burlingame, CA). Dying cells in histological sections were measured using the S7100 ApopTag peroxidase in situ apoptosis (TUNEL) detection kit, following the manufacturer’s instructions (Chemicon, Temecula, CA).

FIGURE 8: Proposed scheme illustrating the neurotoxic effects of KA in mouse hippocampus in the absence of PrpC. In the absence of Prnp (knockout, siRNA), KA activates neurotoxic signaling in the hippocampal cells, favoring the interaction of PSD-95 with GluR6/7, inducing c-Jun and c-Fos overexpression and JNK3 activation, which, in turn, provokes the phosphorylation of c-Jun, leading to neurotoxicity and cell death.
Inhibitors (SP600125 20 μM or TAT-JIP 10 μM) for 45 min before KA treatment. After treatment, slices were rinsed twice in KA-free culture medium. The next day, slices were incubated for 2 h with 1 μg/ml of PI dissolved in culture medium. PI-treated slices were fixed for 1 h with 4% buffered paraformaldehyde, pH 7.2. After rinsing, cultures were mounted in Fluoromount and photodocumented in an Olympus confocal microscope. To determine the maximum PI fluorescence for quantification, total fluorescence in the pyramidal layer of the hippocampus was measured in a 500-μm segment of the layer in the CA1–CA3 regions after incubation with 10 mM glutamate for 10 min, using Quantity One Imaging and Analysis software (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom).

PSD purification from mice hippocampus

The PSD fraction was purified from adult mice (10–12 mo old) as indicated (Coba et al., 2009). Briefly, tissue was weighed, and 1 ml of homogenization buffer (0.32 M sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid [HEPES], pH 7.4, 2 mM EDTA, 2.5 mM N-ethylmaleimide, containing protease and phosphatase inhibitors) was added for every 125 mg of tissue. Samples were homogenized in a glass–Teflon Dounce homogenizer (40 strokes) and centrifuged at 800 × g for 10 min at 4°C. The supernatant was centrifuged at 15,000 × g for 15 min at 4°C, and the pellet was resuspended in 0.5 ml of Triton buffer: 50 mM HEPES, pH 7.4, 2 mM EDTA, 5 mM EGTA, 1% Triton X-100, 2.5 mM N-ethylmaleimide, containing protease and phosphatase inhibitors) for every 125 mg of tissue. The pellet was resuspended in 125 μl of resuspension buffer—50 mM Tris, pH 7.4, 1% SDS (when used for Western blotting) or 50 mM Tris, pH 8.5, 1% deoxycholate (when used for immunoprecipitation experiments)—for every 125 μg of tissue. Samples were incubated for 10 min at room temperature and centrifuged at 50,000 × g for 15 min at 4°C.

Western blotting

Tissue samples from the hippocampus of KA-treated or vehicle-treated mice were homogenized (10% wt/vol) in ice-cold lysis buffer—50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% (wt/vol) Triton X-100, 0.5% (wt/vol) Nonidet P-40 (IGEPAL; Sigma), glycerol 10%, 1 mM EDTA, 1 mM EGTA, and protease and phosphatase inhibitors—using a motor-driven, glass–Teflon homogenizer in ice and centrifuged at 15,000 × g for 20 min. After protein quantification, tissue extracts were boiled in Laemmli sample buffer at 100ºC for 5 min, followed by 6–10% SDS–PAGE electrophoresis, electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore), and processed for immunoblotting and the ECL Plus kit (Amersham Pharmacia Biotech, GE Healthcare Bio-Sciences, Piscataway, NJ).

Immunoprecipitation

Cell extracts from total hippocampus or hippocampal PSD fractions were processed for immunoprecipitation experiments. For total hippocampus, cell extracts were processed as indicated for Western blot. After protein quantification, 1 mg of hippocampal protein extract was mixed with 10 μl of protein A/G–Sepharose (Sigma) previously equilibrated with lysis buffer—50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and protease and phosphatase inhibitors—for 2 h (preclearing). Samples were centrifuged, and the supernatant was mixed with the indicated immunoprecipitation antibodies plus 10 μl of protein A/G–Sepharose overnight at 4°C. The immunocomplexes were washed in lysis buffer and once in lysis buffer plus 0.25 M NaCl. For immunoprecipitation from PSD hippocampal fractions, PSD fractions were diluted with RIPA buffer: 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0, up to 500 μl. Samples were processed as described earlier but using RIPA buffer instead of lysis buffer for immunoprecipitation washes. Proteins attached to Sepharose beads were eluted with SDS–PAGE sample buffer, subjected to SDS–PAGE, transferred to PVDF membranes, and probed with the GluR6/7 and PSD-95 antibodies.

RT-qPCR

RT-qPCR was performed on total RNA extracted with the mirVana isolation kit (Ambion, Austin, TX) from the hippocampus of analyzed mice. Purified RNAs were used to generate the corresponding cDNAs, which served as PCR templates for mRNA quantification. The primers used in this study for RT-qPCR validation are given in Supplemental Figure S1. PCR amplification and detection were performed with the Roche LightCycler 480 detector, using 2x SYBR Green Master Mix (Roche) as reagent, following the manufacturer’s instructions. The reaction profile was as follows: denaturation–activation cycle (95°C for 10 min) followed by 40 cycles of denaturation–annealing–extension (95°C, 10 min; 72°C, 1 min; 98°C, continuous).

mRNA levels were calculated using the LightCycler 480 software. The data were analyzed using the ΔΔCt method, which provides the target gene expression values as fold changes in the problem sample compared with a calibrator sample. Both problem and calibrator samples were normalized by the relative expression of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

Statistical analysis

All data are presented as mean ± SEM. Pairwise one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparisons test was used for comparison. The operations were performed using Systat Software (Chicago, IL) or Statgraphics (StatPoint Technologies, Warrenton, VA). Statistical significance was set at *p < 0.05, **p < 0.01, and ***p < 0.001.

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