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THRESHOLD FOR EPILEPTIFORM ACTIVITY IS ELEVATED IN PRION KNOCKOUT MICE

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Abstract—Prion protein (PrP) is abundant in the nervous system, but its role remains uncertain. Prion diseases depend on an aggregation of the protein that is likely to interfere with its normal function. Loss of function does not in itself cause neurodegeneration, but whether it contributes to the clinical features of the disease remains an open question. Patients with classical Creutzfeldt-Jakob disease (CJD) have a higher than expected incidence of epilepsy. To study the mechanisms by which loss of PrP function may underline changes in vulnerability to epilepsy in disease, we used several acute epilepsy models: we applied a variety of convulsant treatments (zero-magnesium, bicuculline, and pentylenetetrazol) to slices in vitro from PrP knockout (Prnp0/0) and control mice. In all three epilepsy models, we found that longer delays and/or higher concentrations of convulsants were necessary to generate spontaneous epileptiform activity in Prnp0/0 mice. These results together indicate an increased seizure threshold in Prnp0/0 mice, suggesting that loss of PrP function cannot explain a predisposition to seizures initiation in CJD. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: prion, epilepsy, hippocampus, pentylenetetrazol, bicuculline, zero-magnesium.

In prion diseases, the prion protein (PrP) aggregates, which is likely to interfere with its normal function. While knockout of PrP in adult neurons excludes loss of PrP function as the cause of neurodegeneration (Mallucci et al., 2002; Mallucci and Collinge, 2004), it is unclear to what extent this functional loss contributes to the development of symptoms in the established disease. PrP knockout (Prnp0/0) mice do not develop prion disease, but previous reports have found reduced afterhyperpolarizing potentials (Collinge et al., 1996; Herms et al., 2001; Mallucci et al., 2002), enhanced calcium ion buffering (Powell et al., 2008), and decreased synaptic inhibition (Collinge et al., 1994) in hippocampus and cerebellar neurons. These changes are expected to increase excitability and may reduce seizure threshold in these animals. In fact, one study reported increased severity of seizures and mortality caused by seizures in Prnp0/0 mice (Walz et al., 1999). Consistent with this, Creutzfeldt-Jakob disease (CJD) patients present with various forms of epilepsy (Rees et al., 1999; Parry et al., 2001; Neufeld et al., 2003).

The aim of the present study was to investigate the role of normal prion protein in the generation of epileptiform activity in the hippocampus using Prnp0/0 mice. We used three acute epilepsy models in vitro, because these recording conditions allow the analysis of detailed basic mechanisms of any change in epileptic threshold in Prnp0/0 mice. Differences in sensitivity to these models would provide insights into the cellular mechanisms that depend on normal prion function.

Zero-magnesium (0-Mg2+). Magnesium was omitted from the extracellular solution. This releases the Mg2+-dependent block of N-methyl-D-aspartic acid (NMDA) receptors and leads to a hyperexcitable network, able to generate prolonged seizure-like events in vitro (Mody et al., 1987; Schneiderman and MacDonald, 1987; Stanton et al., 1987). This mechanism differs substantially from that of the other agents, allowing the assessment of epileptic threshold independently of a direct cellular effect of convulsants.

Pentylenetetrazol (PTZ) produces paroxysmal depolarizations and hyperpolarizations in CA3 in vitro (Piredda et al., 1985; Bingmann and Speckmann, 1986) and is widely used in vivo to produce epileptic seizures. PTZ suppresses inhibition by gamma-aminobutyric acid (GABA) (MacDonald and Barker, 1978; Leweke et al., 1990) by competing with GABA for the GABAA receptor (Huang et al., 2001) and also modulates resting membrane potential, action potential generation, voltage-dependent calcium channels, and ionotropic glutamate receptors (Piredda et al., 1985; Omrani et al., 2003).

Bicuculline is commonly used to induce epileptiform bursts in vitro (MacDonald and Barker, 1978) and epileptic seizures in vivo (Millan et al., 1991). Bicuculline is a GABAA receptor antagonist (Curtis et al., 1970) which also can depress the slow afterhyperpolarization (Johnson and Seutin, 1997).

EXPERIMENTAL PROCEDURES

Data were obtained from control mice (FVB), and Prnp0/0 mice of the original “Zurich I” background (Bueeler et al., 1992) fully back-
crossed onto an FVB background. Animals were adults (>12 weeks) weighing 30–40 g. Genotype was verified by PCR on the DNA extracted from tail tips (Bueler et al., 1992). All experiments were carried out under the regulation of, and in compliance with, the United Kingdom Animals (Scientific Procedures) Act of 1986 and associated guidelines.

In vitro epileptic activity

Animals were anesthetized with an intraperitoneal injection of a ketamine (75 mg kg⁻¹)/medetomidine (1 mg kg⁻¹) mixture and then killed by cervical dislocation. Transverse slices 400 μm-thick were cut from the ventral pole of the hippocampus. Slices were allowed to recover submerged in a storage chamber at room temperature, before being placed at 32 °C in an interface chamber, with a regular flow (2 ml/min) of artificial cerebrospinal fluid (aCSF). Composition of the aCSF in mM was: NaCl, 125; KCl, 3–5 (varied as indicated for each model); NaHCO₃, 26; NaH₂PO₄, 1.25; CaCl₂, 2; MgCl₂, 1; glucose, 10; pH 7.4, equilibrated with 95%O₂/5%CO₂.

Field potentials were recorded using 2–5 MΩ glass pipettes filled with aCSF, and amplified using Neurolog AC-coupled NL 104 preamplifiers (Digitimer Ltd, Welwyn, UK) or Axoclamp-2A (Axon Instruments, Burlington, CA, USA) DC amplifier. Signals were low-pass filtered at 2 kHz and digitized at 5 kHz using a CED 1401 (CED, Cambridge, UK). Signals recorded with the Axoclamp-2A were high-pass filtered offline with settings resembling those of the NL 104 preamplifiers (0.2 Hz and 0.1 Hz, respectively). Recordings were analysed using Signal and Spike2 software (CED, Cambridge, UK). Stimuli were delivered with a twisted 50 μm Ni/Cr wire bipolar electrode placed on the Schaffer collaterals in stratum radiatum, and recordings were made from hippocampal areas CA3 and CA1, and from entorhinal cortex (EC), as indicated in the Results.

Epileptiform activity in vitro was induced in one of the following ways:

1) Omitting MgCl₂ (Mody et al., 1987; Schneiderman and MacDonald, 1987; Stanton et al., 1987) in otherwise normal aCSF (3 mM KCl).

2) Adding increasing concentrations of PTZ (1.4 mM, 2.2 mM, 2.9 mM, and 3.6 mM at intervals of 30 min) to aCSF containing 5 mM KCl (Piredda et al., 1985). Because few bursts develop spontaneously with application of PTZ only (Bingmann and Speckmann, 1986), we also stimulated the Schaffer collaterals every 30 min, starting from 25 min after the first application of PTZ, using a stimulus intensity that produced a half-maximal response in CA3 at the start of the experiment.

3) Adding bicuculline methiodide (BMI) at increasing concentrations (2 μM, 3 μM, and 4 μM at intervals of 30 min) to aCSF containing 4 mM KCl.

All drugs were obtained from Sigma (Poole, UK).

Statistics

Statistical analysis included Student’s t-test, ANOVA, Fisher exact test and Chi-square test and was performed using SYSTAT 10.0. In figures and text, values are expressed as mean ± SEM.

RESULTS

0-Mg²⁺-induced epileptic activity

All slices eventually developed spontaneous bursts in 0-Mg²⁺ aCSF. We measured bursts in the EC and hippocampal regions CA1 and CA3. Typically, short (duration <1 s), small amplitude interictal bursts developed first (Fig. 1A, B). These bursts generally appeared in EC first, and tens of minutes later in CA areas (Fig. 1B, right panel). Even when developed in CA areas, they initially occurred independently of EC, and only later synchronized between the two regions. The delay to interictal bursts in EC was significantly longer in slices from Prnp0/0 mice (Fig. 1B; t-test: P<0.05; n=18 slices from 10 Prnp0/0 mice and 18 slices from nine control mice). Events in CA3, which occurred later than in EC, did not differ between genotypes.

Seizure-like events (SLE; duration >8 s) appeared some time after the interictal bursts (Fig. 1A, C). SLE differed from interictal bursts in their duration, and in their morphology (early high frequency activity followed by series of bursts). They resembled interictal bursts in first appearing in EC, and only later in CA areas; initially SLE occurred independently in the two regions. The delay to SLE in EC (but not in CA3) was also significantly longer in slices from Prnp0/0 mice (Fig. 1C; t-test: P<0.05).

With time, SLE interval and duration gradually shortened to convert into continuous large amplitude bursts recurring every 1–2 s (Fig. 1D), referred to as recurrent activity (Dreier and Heinemann, 1990). The delay to recurrent activity did not differ between Prnp0/0 and control mice in EC, but was significantly longer in CA3 of slices taken from Prnp0/0 mice than from controls (t-test; P<0.05). In both groups of mice, events in CA1 (not shown) were the same as those described for CA3.

PTZ-induced epileptic activity

Applying PTZ led to a sequence of progressively more intense epileptic activity, presumably associated with the build up of PTZ concentration in the slice. After the start of PTZ application, a variety of small spontaneous bursts (less than 0.2 mV) typically developed in CA3 (Fig. 2A, lower left panel). Subsequently larger bursts appeared (Fig. 2A, lower right panel). Large bursts did not develop gradually from small bursts, but appeared abruptly and unambiguously (Fig. 2A). They could easily be identified by their larger amplitude and complexity: a combination of population spikes (reflecting highly synchronized activity), superimposed on slow shifts in potential. This was in contrast to the small bursts, which did not have highly synchronized activity.

A significantly smaller proportion of slices from Prnp0/0 mice showed spontaneous epileptiform activity (Fig. 2B; Chi-square, P<0.01). Small bursts developed in 4 out of 10 slices from five Prnp0/0 mice and 11 out of 12 slices from six control mice (Fig. 2B, right panels). Large bursts developed in only one of the Prnp0/0 slices, in contrast to 8 out of 12 control slices, a significantly smaller proportion (Fisher exact test, P<0.01).

When bursts were present, their duration was significantly shorter in Prnp0/0 slices (Fig. 2C; t-test, P<0.05; small bursts: n=4 Prnp0/0 mice, n=11 control mice; large bursts: n=1 Prnp0/0 mice, n=8 control mice). Statistical tests were made on small bursts only because large bursts occurred in only one Prnp0/0 slice. The average burst frequency was slightly lower in Prnp0/0 slices, but this difference was not significant (Fig. 2D). There was no
difference in burst amplitude (Fig. 2E), or in the delay to the first occurrence of bursts from the initial drug application (Fig. 2F).

**Bicuculline-induced epileptic activity**

Distinct populations of small (<0.3 mV) bursts appeared at low doses of bicuculline and large bursts appeared abruptly in response to increasing concentrations (Fig. 3A), resembling the results in PTZ. Large bursts had not only larger amplitudes, but also comprised highly synchronized activity (population spikes) superimposed on slow shifts in potential, again resembling PTZ. There was no difference between the Prnp0/0 and control groups in the number of slices that developed bursts: small bursts developed in all slices, and large bursts occurred in the same proportion of slices in each group (6 of 13 slices in both groups). However, the delay from first application of bicuculline to both of the types of epileptiform events was significantly longer in Prnp0/0 mice (ANOVA, P<0.05; Fig. 3B), suggesting an increased threshold for epileptiform activity in Prnp0/0 mice.

**DISCUSSION**

**Increased threshold for epileptiform activity in Prnp0/0 mice**

Using three different convulsants, we observed higher threshold for epileptiform activity and reduced bursting in
slices taken from Prnp0/0 mice. The consistent result from these experiments is that hippocampal slices from Prnp0/0 mice are more resistant than controls to the induction of epileptic activity.

This was not predicted by our previous findings of impairments of both fast inhibitory mechanisms (Collinge et al., 1994) and pyramidal cell afterhyperpolarizations (Colling et al., 1996; Mallucci et al., 2002). Each of these physiological phenotypes should, in theory, increase excitability in the hippocampus and lead to a higher propensity for epileptiform activity when slices and/or animals are challenged with epileptogenic treatments. In fact, increased excitability was observed by Khosravani et al. (2008), who found shorter latency to 0-Mg2+ epileptiform discharges and larger and longer responses in CA1 following stimulation of Schaffer collaterals; these results, however, failed to be reproduced in our lab and we were unable to show any difference in CA1 field responses to normal single and paired stimulations of afferents without convulsants (unpublished observation). This may be due to the difference in the ages of the animals used in the different studies (>84 days in our study vs. 30–45 days in the study by Khosravani et al.): Khosravani et al. (2008) attributed the excitability of their hippocampal slices in Prnp0/0 mice to the unsilencing of NMDA receptors, and NMDA currents decrease with age (Magnusson, 1998; Zhao et al., 2009).

Lack of enhanced seizure activity may be due to developmental adaptations that compensate for deficits in inhibition and afterhyperpolarizations in knockout mice. The use of the ventral end of the hippocampus in the present study and of the dorsal end in the earlier work could also be a factor: the ventral end of the hippocampus is more sensitive to convulsant treatments than the dorsal (Gilbert et al., 1985; Bragdon et al., 1986). While it would be interesting to determine whether position within the hippocampus affects the results, the more sensitive end is perhaps more relevant to the intact animal. Perhaps more important is that earlier studies (Collinge et al., 1994; Colling et al., 1996; Mallucci et al., 2002; Khosravani et al., 2008) used the hippocampal CA1 area but epileptiform bursts typically start in entorhinal cortex and/or CA3 and then propagate to CA1. In our present study 0-Mg2+ interconcentration (see Experimental procedures), the likelihood of spontaneous epileptiform discharges increased. Small bursts (amplitude <0.2 mV) occurred first, and were followed by large bursts in some slices. Large bursts developed suddenly, and were easily distinguished from small bursts by their combination of population spikes superposed on a slower response (right panel). In this slice, small bursts started in 2.9 mM PTZ perfusion; by the time concentration was changed to 3.6 mM PTZ, large bursts had also already started. (B) A significantly smaller proportion of slices from Prnp0/0 mice showed spontaneous epileptiform activity (Chi-square, P<0.01). At the concentrations used, most bursts in Prnp0/0 mice were small, only one of 10 slices developed large bursts. (C) Bursts also had a significantly shorter duration in Prnp0/0 slices (Heat, P<0.05). The spontaneous frequency (D), amplitude of bursts (calculated relative to maximal population spike before application of convulsant) (E), and delay to first bursts (measured from the first application of PTZ) (F) were similar in Prnp0/0 and control slices. * indicates statistical significance (P<0.05).

Fig. 2. Seizure threshold in response to pentylenetetrazol (PTZ) treatment was increased in slices from Prnp0/0 mice. (A) Trace that provides an example of PTZ-induced epileptiform activity in area CA3. Beginning of trace is at time of first PTZ application. Except for their duration (see below), bursts in Prnp0/0 mice were indistinguishable from those in control mice (shown). With time and increasing PTZ
Mechanisms underlying the reduced sensitivity to convulsants

The observation that slices from Prnp0/0 mice showed consistently reduced sensitivity to several epileptogenic treatments with different cellular actions suggests that the difference is most likely to be in the neuronal networks generating the epileptic activity. Both PTZ and, more selectively, bicuculline impair fast inhibitory transmission which suggests that the Prnp0/0 phenotype is not due to differences in functional inhibition (Collinge et al., 1994). PrP may play a role in controlling oxidative stress (Vassallo and Herms, 2003). Oxidation can affect many neuronal properties, including the depression of NMDA receptor-mediated conductances (Tang and Aizenman, 1993), which may be directly relevant to our results on the O-Mg2+ model here, and indirectly to the bicuculline model (Herron et al., 1995). Impaired oxidative stress management (Brown et al., 1997) may also explain higher mortality rates of Prnp0/0 mice during seizures (Walz et al., 1999); despite elevated thresholds for seizure-like activity, neuronal tissue in Prnp0/0 mice may be more vulnerable to the intensity of epileptic activity. Finally, reduced calcium elevations caused by increased extrusion properties have been reported in neurons of Prnp0/0 mice (Herms et al., 2000; Fuhrmann et al., 2006; Powell et al., 2008). This, combined with the reduced synaptic transmission in oxidative stress conditions also observed in Prnp0/0 mice (Herms et al., 1999), could contribute to the general decrease in network excitability that we observe here. Walz et al. (2003) have found an association between the outcome of epilepsy surgery and a polymorphism of PRNP, with their allele having a five-fold worse risk of continuing seizures after temporal lobectomy, suggesting that the relationship between PrP and epilepsy has a clinical as well as a scientific significance. There are also scattered case studies of apparent associations between the onset of CJD and sustained forms of epilepsy (Rees et al., 1999; Parry et al., 2001; Neufeld et al., 2003). However, our results argue that if the development of seizures can be related to prion disease, then it would have to be attributed to a new function taken by either PrPSc or the hypothetical toxic intermediate PrPSc (Hill et al., 1999; Mallucci and Collinge, 2005) during diseases rather than to the loss of function of the normal PrP.

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