Epileptogenesis is associated with enhanced glutamatergic transmission in the perforant path

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Abstract

The perforant path provides the main excitatory input into the hippocampus, and has been proposed to play a critical role in the generation of temporal lobe seizures. It has been hypothesized that changes in glutamatergic transmission in this pathway promote the epileptogenic process and seizure generation. We therefore asked whether epileptogenesis is associated with enhanced glutamatergic transmission from the perforant path to dentate granule cells. We used a rat model of temporal lobe epilepsy in which spontaneous seizures occur following an episode of pilocarpine-induced status epilepticus. Whole-cell patch clamp recordings were obtained from dentate granule cells in hippocampal slices from control and epileptic animals, three weeks after pilocarpine-induced status epilepticus. The paired pulse ratio of perforant path-evoked AMPA receptor-mediated EPSCs was reduced in tissue obtained from epileptic rats. This is consistent with an increase in release probability. NMDA receptor-mediated EPSCs were also prolonged. This prolongation could not be accounted for by decreased activity of glutamate transporters or by a change in NMDA receptor subunit composition in dentate granule cells, implying a change in NMDA receptor kinetics. This change in NMDA receptor kinetics was associated with the emergence of significant synaptic cross-talk, detected as a use-dependent block of receptors activated by medial perforant path synapses following lateral perforant path stimulation in MK-801. Enhanced glutamatergic transmission and the emergence of cross-talk among perforant path-dentate granule cell synapses may contribute to lowering seizure threshold.
INTRODUCTION

The hippocampus and parahippocampal gyri play an integral part in the generation of seizures in mesial temporal lobe epilepsy, and it is thus crucial to understand the propagation of excitation through these structures. The entorhinal cortex provides the major excitatory input to the hippocampus via the perforant path, which targets neurons in the fascia dentata and in the CA1-3 regions. The axonal tracts that form the perforant path split into two anatomically and functionally distinct pathways, the medial (MPP) and the lateral (LPP) perforant path, which travel along the middle and the outer third of stratum lacunosum-moleculare, respectively, and target different sections of the granule cell dendritic tree (Hjorth-Simonsen and Jeune 1972; Steward 1976; Witter 1993). Furthermore, the MPP and LPP have different targets within the hippocampus proper (Sewards and Sewards 2003). In addition to these distinctive anatomical properties, there are marked neurophysiological and pharmacological differences between the MPP and LPP (Bough et al. 2004; Colino and Malenka 1993; Dahl et al. 1990; Do et al. 2002; McNaughton 1980; Min et al. 1998; Pelletier et al. 1994; Rush et al. 2001). These two pathways may have distinct functional roles, relaying different information; the MPP provides feedback information into the hippocampus, whilst the LPP provides the main external input (Sewards and Sewards 2003).

Dentate granule cells have been proposed to act as a brake against seizure propagation through the limbic circuitry via the perforant path, and changes in dentate granule cell excitability have been implicated in epileptogenesis (Behr et al. 1998; Collins et al. 1983; Heinemann et al. 1992; Lothman and Bertram 1993). During the kindling process, there is a transient enhancement of NMDA receptor mediated transmission at
the LPP-dentate granule cell synapse (Behr et al. 2001; Sayin et al. 1999). In addition, more permanent changes in NMDA receptor opening times have been described in granule cells both from kindled animals and from tissue resected from patients with temporal lobe epilepsy (Kohr et al. 1993; Lieberman and Mody 1999).

Here we apply several complementary methods to investigate glutamatergic transmission at the perforant path-granule cell synapse in a post-status epilepticus model of temporal lobe epilepsy. The goals of this study are to determine whether transmission is indeed enhanced at the perforant path to dentate granule cell synapse, and whether this is accompanied by increased cross-talk within the perforant path that may disrupt normal physiological function. We find that epilepsy is associated with a decrease in the paired pulse ratio (PPR) of AMPA and NMDA receptor-mediated EPSCs in the LPP, consistent with an increase in release probability (Pr). In addition, stimulating the LPP elicits NMDA receptor-mediated EPSCs with a more prolonged time course in granule cells from epileptic than from control tissue; this cannot be explained by decreased activity of glutamate transporters or by a change in NMDA receptor subunit composition. Furthermore repetitive stimulation of the LPP results in detectable cross-talk from the LPP to the MPP in epileptic animals. These results reveal several mechanisms that point to an enhancement of glutamatergic transmission that may promote seizure generation and spread.
MATERIALS AND METHODS

Epilepsy Model

Limbic status epilepticus (SE) was induced in adult male Sprague Dawley rats (8 weeks old, ~250 g) by intraperitoneal (i/p) pilocarpine injection (320 mg/Kg)(Turski et al. 1989). To lessen peripheral cholinergic effects, scopolamine methyl nitrate (1 mg/Kg, i/p) was administered 30 min before and 30 min after pilocarpine (Chandler et al. 2003). The onset of SE was defined as the appearance of Stage 3 seizures(Racine 1972), followed by continuous clinically detectable seizure activity. Clinically overt SE was terminated 90 min after its onset by injection of diazepam (10 mg/Kg, i/p). The animals were monitored daily for the appearance of spontaneous recurrent seizures. All rats with SE were observed to have spontaneous seizures by two weeks (maximal severity 3-4 of the Racine scale). Rats were sacrificed 3 weeks after the pilocarpine injection.

All animal procedures followed the Animal (Scientific Procedures) Act, 1986.

Tissue preparation and reverse transcription-PCR

Control (n = 5) and post-SE rats (n = 5) were killed with an overdose of pentobarbital (500 mg/Kg, i/p). We dissected out both hippocampi, and kept one of them for the electrophysiological recordings, and the other for the RNA processing. The dentate gyrus was separated from the hippocampus proper under a light microscope, and the samples were rapidly frozen and stored at –80°C until the RNA extraction. We homogenized each tissue sample, and extracted the total RNA with Trizol according to the directions of the manufacturer (Invitrogen, Paisley, UK). 1-3 µg of each RNA
sample was reverse-transcribed using random hexamers and Superscript II (Invitrogen, Paisley, UK). Each RT reaction was amplified using degenerate primers designed to amplify all four rat NR2 subunits, essentially as described by Hynd et al. (2003). The degenerate primer sequences were: NR2up TRGCNGCCTTCATGATCCA; NR2down CAGCTKGCTRCTCATCAC. The resulting PCR product was digested with MboII, phenol: chloroform extracted, and separated on a 3% agarose gel stained with ethidium bromide. The bands were quantified using non-saturating exposures on GeneGenius (Syngene, Cambridge, UK).

*Electrophysiology and data analysis*

Adult control rats (8-11 weeks old, ~250 g) and rats 3 weeks following pilocarpine-induced SE were deeply anesthetized with pentobarbital (500 mg/Kg, i/p) and decapitated, and their brains were submerged in ice-cold carbogenated (95% O₂-5% CO₂) sucrose solution containing (in mM): 70 sucrose, 80 NaCl, 2.5 KCl, 7 MgCl₂, 0.5 CaCl₂, 25 NaHCO₃, 1.25 NaH₂PO₄, and 22 glucose. The whole hippocampi were dissected from surrounding brain tissue and placed in agar blocks prior to slicing. Transverse hippocampal slices (300 µm thick) from all hippocampal levels were obtained using a Leica VT1000S vibrating blade microtome. After cutting slices were transferred to an interface chamber containing EBSS medium (Invitrogen, Paisley, UK) supplemented with 1 mM CaCl₂ and 2 mM MgCl₂. Slices were stored in this solution for more than 1 h, and then transferred to a submersion-type recording chamber superfused with carbogenated artificial CSF containing (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO₄, 2.5 CaCl₂, 26.2 NaHCO₃, 1 NaHPO₄ and 22 glucose (21-23 ºC).
Stimuli (100 µs square pulses; 0.1 Hz frequency) were delivered via bipolar stainless steel electrodes positioned in *stratum moleculare*, ~100 µm away from the recorded cell. Field potential recordings were made with glass electrodes filled with artificial CSF (tip resistance ~1 MOhm). Whole-cell recordings were made from dentate gyrus granule cells using patch pipettes filled with an intracellular solution containing (in mM): 97.5 Cs gluconate, 17.5 CsCl, 10 HEPES, 10 1,2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid (BAPTA), 8 NaCl, 2 MgATP, 0.3 GTP, 5 QX314 Br (pH 7.2, osmolarity 290 mOsm). The series resistance was monitored throughout the experiments using a −5 mV voltage step command, was in the range 15-30 MΩ, and was not compensated. Cells in which the series resistance varied > 20% were discarded from the analysis. All the experiments were performed in the presence of the GABA_A receptor antagonist picrotoxin (PTX, 100 µM). In some experiments the following cocktail of drugs was added to avoid interference from heterosynaptic signaling by GABA_B, group II and III metabotropic glutamate, opioid, A_1 and muscarinic receptors (in µM): 5 CGP52432, 100 (RS)-α-Methylserine-O-phosphate (MSOP), 1 LY341495, 10 naloxone, 0.2 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 1 atropine. Evoked AMPA EPSCs were recorded by holding cells at $V_H = -70$ mV, whereas NMDA EPSCs were recorded at $V_H = +40$ mV, in the presence of 25 µM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt (NBQX). Currents were acquired with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) using a 2 kHz low-pass filter, digitized at 5 kHz, and stored on a personal computer for off-line analysis (LabView, National Instruments Corporation, Newbury, UK). PPR analysis was performed on the average of 20 single or double EPSCs. The two averaged traces were then corrected for their offset, normalized by the peak of the first EPSC, and then subtracted one from the other.
PPR was calculated as the amplitude of EPSC$_2$ in the subtracted trace (and corresponded to EPSC$_2$/EPSC$_1$).

In the MK-801 experiments, NMDA EPSC amplitudes were measured as the average current over a 100 ms time interval after onset, in order to reduce the noise-related error of individual EPSC measurements. Five minutes were allowed for wash-in of MK-801, and 15-20 min for washout. To reduce possible bias caused by incomplete washout of MK-801, we analyzed only the first 5 trials after restarting stimulation (Scimemi et al. 2004). Wherever two inputs were compared, the stimulus intensity was adjusted to obtain EPSCs of similar amplitudes in both pathways.

Data are expressed as means ± S.E.M., and were considered significant at P < 0.05, as determined by using Student’s paired or unpaired $t$ test (when measures were normally distributed) or Wilcoxon matched pair or Mann-Whitney U test (when distributions were not normal).

Drugs were purchased from Tocris Cookson (Bristol, UK) and Sigma (St. Louis, MO), except for QX314 Br (Alomone Laboratories, Jerusalem).

**RESULTS**

*Paired-pulse responses in the MPP and LPP*

MPP and LPP EPSCs were evoked in granule cells through stimulating electrodes positioned in the middle and outer thirds of the dentate molecular layer, respectively (Abraham and McNaughton 1984)(Fig. 1A). In control animals, two consecutive stimuli delivered with a 50 ms inter-pulse interval with GABA$_A$ receptors blocked yielded paired pulse depression (PPD) of AMPA EPSCs in the MPP and paired pulse
facilitation (PPF) in the LPP (Fig1. B,C). This is consistent with the different PPRs in these two pathways observed by others (Colino and Malenka 1993; McNaughton 1980; Min et al. 1998). In contrast, when the same protocol was applied to the epileptic hippocampus, although we still detected a clear PPD in the MPP, PPF in the LPP was no longer present (Fig. 1B,C). The difference in the PPR in the LPP of control and epileptic dentate granule cells was significant at P = 0.02. Using field potential recordings in the outer third of the molecular layer of the dentate gyrus at 34°C, we confirmed that this change in PPR was not temperature dependent (PPR of LPP in control 1.30 ± 0.10, n = 4 and in epileptic 1.08 ± 0.02, n = 6, P = 0.02 for difference).

Such a change in PPR could be due to a change in the modulation of the LPP synapses by endogenous factors; indeed epileptogenesis may affect the modulation of perforant transmission by metabotropic glutamate receptors (Bough et al. 2004; Klapstein et al. 1999). We therefore repeated the measurement of the PPR in the presence of a cocktail of drugs at concentrations that we and others have previously shown to block GABA_B receptors, group II and III mGluRs, muscarinic ACh receptors, A_1 adenosine receptors and opioid receptors in acute hippocampal slices (see Methods for details) (Bramham and Sarvey 1996; Doherty et al. 2004; Foster and Deadwyler 1992; Manzoni et al. 1994; Wagner et al. 1992). In the presence of these drugs, the PPRs in the MPP and LPP from control tissue were not significantly different from those observed in the presence of PTX alone (P = 0.96 for MPP; P = 0.51 for LPP). This implies that these G protein-coupled receptors (GPCRs) do not contribute to PPR in either pathway. We repeated the measurement of PPR in epileptic tissue in the presence of the cocktail of antagonists (Fig. 1D). The reduction
in PPR in LPP in epileptic tissue persisted when the GPCRs were blocked, further arguing against a role for these receptors in PPR. This reduction of PPR in the LPP with epilepsy is consistent with a higher baseline Pr in the epileptic hippocampus.

An alternative explanation for the reduced PPR in LPP in epileptic tissue is that the stimulating electrode positioned in the outer third of the molecular layer activated some MPP axons, possibly as a result of axonal re-arrangements. We tested for such contamination by stimulating the MPP and then LPP with a 50 ms interval and looking for evidence of cross-inhibition. The LPP was not significantly changed by prior stimulation of the MPP in either control or epileptic tissue (Fig. 1D), arguing against a major contamination from MPP fibers when stimulating the LPP.

The change in PPR with epilepsy is present at high and low Pr synapses

Since there is a non-uniform distribution of Pr at cortical synapses (Hessler et al. 1993; Rosenmund et al. 1993), changes in PPR at LPP synapses could be due to a change in Pr across all synapses or a selective loss of low Pr synapses. In order to distinguish between these two possibilities, we took advantage of the use-dependent block of NMDA receptors by MK-801. MK-801 initially preferentially blocks NMDA EPSCs at high Pr synapses (Manabe and Nicoll 1994), and this is reflected in a gradual increase in PPR during MK-801 application (Manabe and Nicoll 1994). We therefore recorded paired NMDA EPSCs at \( V_H = +40 \text{ mV} \) in the cocktail of metabotropic receptor blockers (as used in Fig. 1D), supplemented with NBQX (25 \( \mu \text{M} \)) to block AMPA/kainate receptors. After establishing a stable baseline, we tested the effect of MK-801 (40 \( \mu \text{M} \)). Consistent with an increased Pr, there was a trend for the reduction in the amplitude of NMDA EPSCs in the presence of MK-801 to be
faster in epileptic granule cells (data not shown). Similar to the PPR for AMPA EPSCs and in agreement with a higher baseline Pr at synapses in the epileptic tissue, the PPR for NMDA EPSCs calculated across a set of 20 traces before applying MK-801 was significantly greater in control than in epileptic granule cells (P < 0.01, Fig. 2 left panel). The PPR progressively increased throughout the MK-801 application, reaching a steady state ~20 min after the beginning of the application (Fig. 2). The increase in the PPR normalized by the baseline PPR followed a similar time course and reached the same steady state in control and epileptic tissue (Fig. 2 right panel). This result suggests that the Pr at the LPP dentate granule cell synapses is increased across all synapses (high and low Pr) during epileptogenesis. It also argues further against the hypothesis that the LPP in epileptic tissue was contaminated by MPP fibers to a greater extent than in control tissue.

The time course of NMDA EPSCs is prolonged in epileptic tissue

The results presented so far are consistent with an increase in Pr at LPP terminals during epileptogenesis. A transient enhancement of NMDA receptor mediated transmission at the LPP-dentate granule cell synapse has been previously reported during kindling (Behr et al. 2001; Sayin et al. 1999), and has been suggested to be involved in the epileptogenic process. We therefore asked whether a similar phenomenon occurs in the post-status epilepticus model of epileptogenesis. We evoked NMDA EPSCs of similar amplitudes in the epileptic and control slices, and measured their time course by dividing the charge transfer over a 975 ms time window by their peak amplitude. The time course of NMDA EPSCs evoked by LPP stimulation was significantly (P < 0.01) greater in epileptic than control tissue (Fig. 3A). A similar, albeit smaller, increase in NMDA EPSC time course was seen in MPP.
This finding is consistent with an increased open probability of NMDA receptors in epileptic animals (Kohr et al. 1993; Lieberman and Mody 1999), but could additionally be explained by a change in glutamate uptake (which can shape the decaying phase of NMDA EPSCs; Arnth-Jensen et al. 2002; Diamond 2001) or in NMDA receptor subunit composition (Chen et al. 1999; Lozovaya et al. 2004; Prybylowski et al. 2002). The following experiments were designed in order to distinguish between these possible mechanisms.

**Effects of glutamate uptake on NMDA receptor responses**

We first tested whether a reduction of glutamate uptake could explain the slower time course of NMDA EPSCs in epileptic tissue. In order to block both neuronal and glial transporters, we perfused slices with the glutamate transporter inhibitor DL-threo-beta-benzyloxyaspartate (TBOA 50 µM) (Fig. 3B). This evoked a large outward current that did not differ significantly between epileptic and control tissue (Fig. 3B, left panel). It also produced a significant increase in the time course of EPSCs (control: 204 ± 14% of the baseline t-course, n = 10, P < 0.01; epileptic: 165 ± 20% of the baseline t-course, n = 10, P < 0.01; P = 0.12 for comparison between control and epileptic) (Fig. 3B, right panel). Although these effects of TBOA on the holding current and on the time course of NMDA EPSCs tended to be smaller in epileptic tissue, this trend did not reach significance, arguing against a major difference in the activity of glutamate transporters.

**Role and expression of different NMDA receptor subunits**
NMDA receptors containing NR2B subunits have slower kinetics than those that contain NR2A subunits (Erreger et al. 2005). We asked whether a different ratio of NR2B/NR2A receptors underlies the different time course of EPSCs following LPP stimulation in the epileptic hippocampus. We delivered single pulses to the LPP to evoke similar amplitude NMDA EPSCs in control and epileptic granule cells. We then measured the effect of the NR2B antagonist ifenprodil (5 μM) on the amplitude and on the decay of the EPSCs (Fig. 4A). Ifenprodil reduced the peak amplitude of NMDA EPSCs by $49 \pm 3\%$ in control ($n = 10$) and by $41 \pm 3\%$, in epileptic tissue ($n = 12$); there was no significant difference between the reduction in control and epileptic tissue ($P = 0.06$), arguing against a major change in the contribution of the NR2B subunit. The effect of ifenprodil on the EPSC time course was similar in control and epileptic cells (Fig. 4A, right panel), reducing it to $90 \pm 4\%$ in control ($n = 10$) and to $83 \pm 3\%$ in epileptic tissue ($n = 12$, $P = 0.17$ for comparison).

As a further test of the possibility that the change in decaying phase of EPSCs resulted from a shift in NMDA receptor subunit composition we estimated the relative abundance of NR2A and NR2B transcripts in control and epileptic tissues using RT-PCR (Fig. 4B). There was no significant change in the ratio of NR2B/NR2A in RNA from control and epileptic dentate gyrus (control: $1.28 \pm 0.01$, $n = 7$; epileptic: $1.21 \pm 0.05$, $n = 9$, $P = 0.33$) or hippocampus proper (control: $1.15 \pm 0.02$, $n = 8$; epileptic: $1.20 \pm 0.03$, $n = 9$, $P = 0.13$). As a control of the sensitivity of the method, we repeated the test on RNA purified from the cerebellum of control rats. The NR2B/NR2A ratio was $0.17 \pm 0.03$ ($n = 3$, $P < 0.001$ for comparison with control dentate gyrus and hippocampus), consistent with previous reports of a lower NR2B/NR2A ratio in this area (Petralia et al. 1994; Zhong et al. 1995). Also
consistent with previous reports (Farrant et al. 1994), we found a higher NR2C/NR2A subunit ratio in the cerebellum (1.25 ± 0.06, n = 3) than in the control dentate gyrus (0.12 ± 0.01, n = 4, P < 0.001) and hippocampus (0.13 ± 0.01, n = 5, P < 0.001). (All ratios were adjusted to correct for the different lengths of the DNAs, data not shown). These results indicated that our assay was sensitive to changes in levels of NMDA receptor NR2 subunits.

Thus, neither the electrophysiological data, nor the RT-PCR experiments, lend any support to the hypothesis that a significant change in the NR2B/NR2A distribution occurs in the dentate gyrus during epilepsy.

Cross-talk from the LPP to the MPP can be detected in epileptic tissue

The results thus far suggest that there are no major changes in local glutamate uptake or NMDA receptor subunit composition. However, the change in decay time is consistent with an increased affinity of the NMDA receptor for glutamate, resulting in a prolonged opening time. An increase in receptor affinity should enhance the ability of the receptors to detect spill-over of glutamate, and this alone or in combination with increased Pr at the LPP could increase synaptic cross-talk. The MPP and LPP are distinct, spatially separated, pathways. We therefore asked if epileptogenesis could enhance cross-talk from LPP to MPP. In order to assay long-range cross-talk, we applied a highly sensitive approach that relies on the use-dependent blocker MK-801 (Carter and Regehr 2000; Scimemi et al. 2004). We added the cocktail of drugs used for the experiments described in Fig. 1D to the perfusing solution, and verified that stimulating the MPP and LPP evoked AMPA receptor-mediated responses that were independent as confirmed by testing cross-PPR as previously described. We then
recorded baseline NMDA EPSCs at $V_{H} = +40$ mV, with AMPA-kainate receptors blocked, elicited by stimulating the MPP and LPP alternately every 10 s. MK-801 (4 µM) was subsequently washed in for 5 min and the LPP was stimulated alone (5 pulses at 20 Hz, repeated every 10 s), until the LPP EPSC amplitude decreased to ~15% of baseline. Stimulation of the MPP was resumed 15-20 min after washout of MK-801.

When the first 5 MPP responses after MK-801 washout were compared to the baseline EPSCs, they showed a significant reduction in amplitude in epileptic tissue (Fig. 5A). In order to control for the effect of background activation of NMDA receptors by spontaneous glutamate release, we repeated the experiment without stimulating the LPP during the MK-801 application. The reduction of MPP EPSCs was significantly ($P = 0.01$) greater after LPP stimulation than in the no-stimuli control experiments (Fig. 5A,B). This argues for substantial cross-talk between LPP and MPP synapses in epileptic tissue. Specifically, it implies that the same NMDA receptors can be activated by glutamate released by either pathway.

When the same protocol was applied to the tissue obtained from control rats, the reduction in the EPSC amplitude in the MPP was not significantly different from that observed in the corresponding no-stimuli control experiments (NS-Ctrl $0.59 \pm 0.03$ of baseline, $n = 8$; trains of stimuli $0.50 \pm 0.06$ of baseline, $n = 7$, $P = 0.07$). Thus, significant cross-talk between LPP and MPP was only revealed in the epileptic tissue.

DISCUSSION
We have shown that an experimental model of temporal lobe epilepsy is associated with enhanced glutamatergic transmission at the perforant path to dentate granule cell synapse. This enhancement in glutamatergic transmission is manifest as an increased Pr, and prolonged NMDA receptor mediated EPSCs; these result in the emergence of detectable cross-talk from the lateral to the medial perforant path.

Change in Pr in the lateral perforant path

One of the features that distinguishes the LPP from the MPP is a difference in PPR of the EPSC; this has been proposed to be due to a difference in release probabilities at these two pathways (Colino and Malenka 1993; McNaughton 1980; Min et al. 1998). We found that the PPR was decreased at the LPP to dentate granule cell synapse during epileptogenesis. This decrease persisted despite blocking pre-synaptic adenosine, metabotropic glutamate, GABA<sub>B</sub> and opiate receptors, arguing against a change in tonic presynaptic modulation of transmission, but consistent with an increase in baseline Pr. A potential pitfall is that the increase in Pr might be explained by contamination of the LPP by MPP axons, because this pathway exhibits PPD in control animals. This is, however, unlikely for the following reasons. First, we standardized the stimulating electrode positions to recruit the two pathways selectively (Abraham and McNaughton 1984). Second, we confirmed that there was no significant cross-interaction of the pathways by stimulating each pathway in turn. Finally, if the change in PPR was due to a greater contamination of the LPP by MPP axons, this would lead to the prediction that progressively blocking NMDA receptors with MK-801 would lead to a change in PPR with a different evolution in control and epileptic tissue. Instead, the normalized PPR increased in a similar way, arguing for a uniform increase in Pr.
There have been variable observations of changes in PPF at the LPP to dentate granule cell synapse during epileptogenesis (Bough et al. 2004; Klapstein et al. 1999). To some extent, this may be due to selection bias; LPP synapses are identified by the presence of PPF (Bough et al. 2004). Our result is, however, consistent with a study of kindled animals in which a long lasting decrease in PPF was observed at the LPP (Klapstein et al. 1999), although the authors could not exclude contamination by MPP.

Changes in NMDA receptor kinetics

As a further evidence of an enhancement in glutamatergic transmission from entorhinal cortex to dentate granule cells, we observed a prolongation of the NMDA receptor EPSC. This could be explained by a change in glutamate uptake (Arnth-Jensen et al. 2002; Diamond 2001), a change in glutamate diffusion (Savtchenko and Rusakov 2005), a change in NMDA receptor subtype (Chen et al. 1999; Lozovaya et al. 2004; Prybylowski et al. 2002) or a change in NMDA receptor kinetics (Jahr 1992; Rosenmund et al. 1993).

Blocking glutamate uptake had a similar effect on the NMDA EPSC decay in both epileptic and control granule cells, suggesting that glutamate transporter function in the granule cell molecular layer does not change significantly with epileptogenesis. This is in keeping with the finding that during epileptogenesis changes in glutamate transporter expression in the dentate molecular layer seem to be restricted to the inner molecular layer (Gorter et al. 2002). Thus a change in glutamate uptake cannot explain the prolongation of the NMDA EPSC in epilepsy.
Could the prolonged response be due to changes in NMDA receptor subtype expression? Changes in NMDA receptor subtype have been found in experimentally induced cortical malformations and human cortical dysplasia (Andre et al. 2004; Calcagnotto and Baraban 2005; Hagemann et al. 2003; Ying et al. 2004) and would be expected to change NMDA receptor current kinetics. However, we observed no change in either the proportion of the NMDA EPSC mediated by NR2B or the mRNA ratios for NMDA receptor subtypes in the dentate gyrus (although this result does not specifically address the NMDA receptors expressed at the lateral perforant path synapse).

The most likely explanation of the prolonged response is thus a change in NMDA receptor open probability. Such a phenomenon is supported by evidence from dissociated granule cells from kindled rats and human tissue from patients with epilepsy (Kohr et al. 1993; Lieberman and Mody 1999). In these studies, the increase in opening time was explained not by a change in receptor subtype, but by a change in the phosphorylation state of the NMDA receptor.

Changes in synaptic cross-talk in epileptogenesis
Enhanced glutamate release in the LPP and an increase in NMDA receptor open probability should increase synaptic cross-talk in epileptic animals. There may also be additional untested factors such as synaptic rearrangements and changes in diffusivity (Rusakov and Kullmann 1998; Savtchenko and Rusakov 2005). We therefore asked if we could detect glutamate spill-over from the LPP to the MPP in epileptic tissue. In vivo, the pyramidal cells in the entorhinal cortex fire at approximately 10 Hz (Frank
et al. 2001). However, the enthorhinal cortex from epileptic rats and humans exhibits high frequency oscillations that may contribute to the excitatory input to dentate granule cells (Bragin et al. 2004; Bragin et al. 2002). We therefore asked whether there was increased cross-talk between the pathways when there was a burst of action potentials in the lateral perforant path. In this circumstance, we detected a significant interaction between the pathways in the epileptic, but not in the control animals. Although it is not possible to predict the consequences of this for entorhinal-hippocampal function in vivo because of differences in diffusion, temperature, glutamate release and pattern of firing, this result does indicate that there is a greater propensity for cross-talk between the lateral and medial perforant path in epilepsy.

**Implications of these changes**

The dentate granule cells have been proposed to act as a brake on activity transmitted from the entorhinal cortex to the hippocampus that dysfunctions during epileptogenesis (Behr et al. 1998; Collins et al. 1983; Heinemann et al. 1992; Lothman and Bertram 1993). Indeed, there is increasing evidence that the entorhinal cortex plays a critical role in mesial temporal lobe epilepsy (Jones et al. 1992; Spencer and Spencer 1994). An increase in Pr will lead to a greater activation of glutamatergic synapses, and thus enhanced neurotransmission. The prolonged decay of NMDA receptor responses will also result in enhanced transmission, but importantly has further implications for dentate function. During kindling, a transient increase in NMDA receptor mediated transmission has been found that may promote the kindling process (Behr et al. 2001; Sayin et al. 1999). Inhibition of NMDA receptors retards the kindling process (McNamara et al. 1988), and also can inhibit epileptogenesis in post-status epilepticus models (Prasad et al. 2002; Rice and
DeLorenzo 1998). The finding of increased NMDA receptor mediated transmission at the perforant path to dentate granule cell may thus represent a common mechanism that enhances the epileptogenic process. We also found enhanced cross-talk between the pathways with high frequency stimulation. This increased spill-over may promote hyperexcitability. In addition, a decrease in perforant pathway specificity with epileptogenesis may adversely affect hippocampal function. Thus enhanced glutamatergic transmission at the perforant path to granule cell synapse may promote seizure generation and epileptogenesis whilst disrupting hippocampal function.

Acknowledgements

This work was supported by the European Commission (contract no. QLG3-CT-200102004), and the Medical Research Council (UK). We would like to thank Dmitri Rusakov for his helpful comments.
REFERENCES


FIGURE LEGENDS

Figure 1. Loss of PPF in the LPP during epilepsy

A: schematic illustration of the experimental design. Bipolar stimulating electrodes were placed in the middle and outer third of stratum lacunosum-moleculare to activate MPP and LPP fibres respectively. B: delivering two pulses to the MPP at 20 Hz revealed PPD, whereas LPP stimulation resulted in PPF. Applying the same stimulation protocol to the epileptic hippocampus resulted in PPD in the MPP, but PPF in the LPP was no longer observed. All traces represent the average of 20 consecutive trials normalized by the peak amplitude of the first EPSC, recorded in the presence of PTX (100 µM) to block GABA_A receptors. C: summary of the PPR measured in control and epileptic granule cells following MPP (MM) and LPP (LL) stimulation. The PPR is significantly decreased in epileptic granule cells when delivering pulses to the LPP. D: summary histogram of the PPR measured following stimulation of MPP and LPP in the presence of a cocktail of PTX and drugs to block metabotropic receptors (see Text). Data were collected from control (left panel) and epileptic granule cells (right panel). Even in the presence of the cocktail, PPR in LPP was still significantly decreased during epilepsy. Using the same stimulation parameters, single pulses were applied to the MPP and then LPP at 50 ms interval (ML); this demonstrated no significant pathway interaction, thus indicating the independency of the two pathways.

*: P < 0.05, **: P < 0.01, ***: P < 0.001

Figure 2. Comparison of the effect of MK-801 block on paired NMDA EPSCs.

left panel: A baseline of 20 min was recorded before adding MK-801 (40 µM) to the perfusing solution. Note that for NMDA EPSCs, as well as for AMPA EPSCs, the
PPR was smaller in the epileptic granule cells. Right panel: Time course of the effect of MK-801 on the PPR normalized by the PPR measured during baseline. MK-801 induced a gradual increase in PPR, that reached a steady state after ~20 min of application. These results support the hypothesis that different populations of synapses with different Pr might exist in the LPP, the high Pr ones being the first to be blocked by MK-801. The similar effect of MK-801 on the PPR in epileptic granule cells suggests that the higher Pr occurs during epilepsy homogeneously across high and low Pr synapses.

**Figure 3.** The slower kinetics of NMDA EPSCs during epilepsy cannot be explained by a decrease activity of glutamate transporters. *A*: traces in the left and middle panel represent the average of 10 consecutive trials recorded in control (n = 7, black traces) and epileptic granule cells (n = 6, grey traces) following MPP (left panel) and LPP stimulation (right panel). The histogram in the right panel summarizes the analysis of the time course of EPSCs evoked by stimulating the two pathways (M = MPP, L = LPP) in tissue from control and epileptic (E) animals. EPSCs last for longer in epileptic granule cells after either MPP or LPP stimulation (*: P < 0.05, **: P < 0.01). In epileptic granule cells, the time course of EPSCs in the LPP is larger than in the MPP (P < 0.05). *B*: effect of TBOA (50 µM) on the holding current (I_{hold}, left panel) and on the time course of EPSCs (right panel). TBOA pronouncedly increased both I_{hold} and the time course to the same extent in control and epileptic granule cells, suggesting that the role of glutamate transporters in the dentate gyrus does not change during epilepsy.
**Figure 4.** The role and the expression of the NR2B subunit do not change during epilepsy. 

*A (left panel):* average of 10 consecutive traces of NMDA EPSCs evoked by LPP stimulation in control and epileptic granule cells before (black traces) and after (grey traces) blocking NR2B receptors with ifenprodil (5 µM). The traces on the right have been normalized by the peak amplitude of EPSCs in order to show the effect of ifenprodil on the time course. 

*A (middle and right panel):* summary histograms of the effect of ifenprodil on the peak amplitude and on the decay of NMDA EPSCs. Ifenprodil significantly decreased the amplitude and the time course of EPSCs to the same extent in control and epileptic granule cells (*: P < 0.05, ***: P< 0.001), suggesting that the contribution of the NR2B subunit to synaptic transmission does not undergo detectable changes in the epileptic dentate gyrus. 

*B (left panel):* results of degenerate RT-PCR analysis of NR2 subunit expression in tissue samples from the dentate gyrus (DG), *hippocampus proper* (HP) and cerebellum (CB) of control and epileptic rats. Left panels show representative degenerate NR2 subunit PCR products. The full-length PCR product generated from the NR2 degenerate primers is at ~500 nt for all four NR2 subunits (uncut PCR both control and Ep). The lanes labelled DG, and HP show the degenerate PCR product after 1 h digestion with MboII (New England Biolabs, USA) to distinguish between the four NR2 subunits. In these lanes, the top band, at 430 nt, is NR2B. The second band, at 370 nt, corresponds to NR2A. NR2D which would generate a band at 310 nt was not detectible in these samples. NR2C generated two faint bands at 230 and 250 nt. Note that these bands were much more readily detectable in the CB. The complementary bands to NR2A and NR2B were < 100 nt and are not visible on the gel. MW is the 2-Log DNA Ladder from New England Biolabs (USA). These gels are over-exposed to show NR2C. 

*B (right panel):* ratio of NR2B/NR2A intensity in control and Ep tissues. Comparison of the
intensity of the NR2B fragment (430 nt) relative to the NR2A fragment (370 nt), adjusted for the difference in length. For these measurements the exposure was adjusted so that no bands were saturated, to prevent distortion of the ratios. For all samples the mean ratio is presented ± S.E.M.

**Figure 5.** Inter-synaptic cross-talk from the LPP to the MPP occurs during epilepsy when repetitive stimuli are applied to the LPP. *A (left panel):* normalized NMDA EPSCs amplitude averaged across control (n = 8) and epileptic granule cells (6) during a control experiment in which the MPP was stimulated before and after application of MK-801 (4 µM). No stimulus was delivered to any pathway in the presence of the NMDA receptor blocker. There is however a decrease in the EPSC amplitude that may be the result of spontaneous activation of NMDA receptors. *A (middle panel):* one-cell example of test experiments. The open and closed circles represent the normalized amplitude of NMDA EPSCs in the MPP and in the LPP, respectively. After establishing a stable baseline of EPSCs, MK-801 (4 µM) was washed in for 5 min. Stimulation was then resumed only in the LPP with a train of 5 pulses at 20 Hz applied every 10 s, until the EPSCs amplitude was ~15% of baseline. MPP stimulation was resumed 15-20 min after MK-801 washout. *A (right panel):* traces represent the average of 5 normalized EPSCs recorded from the MPP (grey traces) and LPP (black traces) before and after MK-801 application in control and epileptic granule cells. *B: summary histogram of the NMDA EPSC amplitude following MPP stimulation normalized to baseline before MK-801. The results were then normalized to those obtained in the no-stimuli control. Each bar represents the results obtained in a series of no-stimuli control (NS-Ctrl) and test experiments (Stim), in which 5 pulses were delivered to the LPP in the continuous presence of*
MK-801. In epileptic granule cells, a significantly greater reduction of the EPSC amplitude was seen when the LPP was stimulated than in control experiments. ** $P = 0.01$
**Figure 1** (Scimemi et al.)
Figure 2 (Scimemi et al.)
Figure 3 (Scimemi et al.)
**Figure 4** (Scimemi et al.)
Figure 5 (Scimemi et al.)