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REORGANIZATION OF BARREL CIRCUITS LEADS TO THALAMICALLY-EVOKED CORTICAL EPILEPTIFORM ACTIVITY

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Abstract

We studied circuit activities in layer IV of rat somatosensory barrel cortex containing microgyri induced by neonatal freeze lesions. Structural abnormalities in GABAergic interneurons are present in the epileptogenic paramicrogyral area (PMG) and we therefore tested the hypothesis that decreased postsynaptic inhibition within barrel microcircuits occurs in the PMG and contributes to epileptogenesis when thalamocortical afferents are activated. In thalamocortical (TC) slices from naïve animals, single electrical stimuli within the thalamic ventrobasal (VB) nucleus evoked transient cortical multi-unit activity lasting 65 ± 42 ms. Similar stimuli in TC slices from lesioned barrel cortex elicited prolonged 850 ± 100 ms paroxysmal discharges that originated in the PMG and propagated laterally over several mm. Paroxysmal discharges were shortened in duration by ~70 % when APV was applied, and were totally abolished by CNQX. The cortical paroxysmal discharges did not evoke thalamic oscillations. Whole cell patch clamp recordings showed that there was a shift in the balance of TC evoked responses in the PMG that favored excitation over inhibition. Dual whole-cell recordings in layer IV of the PMG indicated that there was selective loss of inhibition from fast-spiking interneurons to spiny neurons in the barrel circuits that likely contributed to unconstrained cortical recurrent excitation with generation and spread of paroxysmal discharges.

Keywords

thalamocortical slices; epileptogenesis; inhibition; interneurons; microgyri

Introduction

Integration and processing of sensory information carried by thalamocortical (TC) axons to the neocortex requires precisely timed activation of excitatory and inhibitory neurons (Sillito, 1977; Simons, 1978; Agmon and Connors 1991, 1992; Kim et al., 1995; Porter et al, 2001). Inhibitory interneurons play an important role in gating sensory input and shaping receptive fields (Sillito, 1977), as well as limiting recurrent excitation present among spiny neurons (Feldmeyer et al 1999; Sun et al, 2006). A cohort of morphologically distinct excitatory cells and inhibitory interneurons has been described in layer IV barrels (Keller and White, 1987; Woolsey, 1967). Different subtypes of inhibitory neurons located in the barrel cortex are also known to form inhibitory networks coupled by electrical and chemical synapses that have important functional roles (Beierlein et al., 2000; Gibson et al., 1999). Using paired intracellular recordings we previously studied the properties of synaptic connections between spiny neurons (i.e. spiny stellate and pyramidal cells) and interneurons, as well as integration of TC input, in layer IV barrels of rat TC slices (Sun et al. 2006). We found that, while inhibition from regular-spiking non-pyramidal (RSNP) interneurons to spiny neurons is comparable in strength to excitatory connections, inhibition mediated by fast-spiking (FS) interneurons is ten times more powerful. TC excitatory postsynaptic potentials (EPSPs) elicit reliable and precisely timed action potentials in FS neurons, and a small number of FS neurons mediate TC

feed-forward inhibition onto each spiny neuron, inhibition that can powerfully shunt TC-mediated excitation. The ready activation of FS cells by TC input coupled with powerful feed-forward inhibition would profoundly influence sensory processing in vivo and protect against runaway synaptic excitation as occurs during epileptiform discharges (Sun et al., 2006).

In the present experiments, we examined the properties of neuronal responses to TC inputs in layer IV of rat barrel cortex in areas known to become epileptogenic after a developmental cortical injury. A focal transcortical freeze lesion at P1 in rat (Dvorak and Feit, 1977; Dvorak et al, 1978; Jacobs et al, 1996) results in development of a cortical microgyrus that is structurally very similar to that seen in human epileptogenic polymicrogyria. Two anatomical alterations, present in the hyperexcitable cortex adjacent to the malformation, namely abnormal hyperinnervation by thalamocortical projections (Jacobs et al, 1999c) and loss of parvalbumin-containing interneurons (Rosen et al, 1998), would be predicted to disrupt normal responses to TC inputs in layer IV of barrel cortex and contribute to epileptogenesis (see Jacobs et al, 1999b for review). We tested this hypothesis in thalamocortical slices cut through microgyral sensory cortex.

At least 30 human syndromes result from disruption of the normal cortical developmental pattern (Schwartzkroin and Walsh, 2000). Human neocortical malformations are induced by both genetic and “epigenetic” influences, i.e., trauma and other insults to the immature brain that occur during pregnancy, or during the birth process (Evrard, 1988; Evrard, 1997; Raymond et al., 1995; Weinberger and Lipska, 1995). Malformations resulting from aberrant patterns of brain development are highly correlated with childhood seizure syndromes, as well as cognitive disabilities and other neurological disorders. A better understanding of the mechanisms underlying hyperexcitability in malformed cortex is potentially important for development of novel therapeutic strategies. A number of animal models have been studied that mimic various aspects of the histopathology and cortical hyperexcitability found in human epileptogenic developmental malformations (Lee et al, 1997; Roper, 1998; Chevassus-Au-Louis et al., 1999a; Chevassus-Au-Louis et al., 1999b; Chevassus-Au-Louis and Represa, 1999; Jacobs et al., 1996; 1999a,c). In the freeze lesion model of cortical microgyria (Dvorák and Feit, 1977; Dvorák et al 1978), cellular recordings and intracellular staining have shown that the region generating the epileptiform activity (the paramicrogyral zone or PMG) is not congruent with the microgyrus itself, but rather adjacent to it (Jacobs et al., 1999a). This adjacent region shows normal gross cytoarchitecture, however changes in synaptic strength and postsynaptic receptors are present (DeFazio and Hablitz, 1999; DeFazio and Hablitz, 2000; Jacobs and Prince, 2005; Hagemann et al, 2003). Further, there is evidence for structural reorganization including increased density of thalamocortical fibers and gross reorganization of cortical barrel structures in the PMG (Jacobs et al., 1999c) and other abnormalities of afferent and efferent connectivity (Rosen et al, 2000). There is also evidence for loss of parvalbumin-containing GABAergic interneurons within the PMG (Rosen et al, 1998) as in irradiated rat models of cortical dysplasia and human dysplastic cortex (Roper et al., 1997; Roper, 1998; Spreafico et al, 1998; Alonso-Nanclares et al, 2005; but see Schwarz et al, 2000).

While enhanced afferent and recurrent glutamatergic connections and/or loss of GABAergic inhibition might provide a sufficient substrate for generation of epileptiform activities, more detailed information is required to understand the role of maladaptive reorganization of local neocortical microcircuitry in epileptogenesis. One approach to this problem is to assess alterations in normal intracortical circuits activated by specific sensory inputs in areas of cortical malformations. In the present experiments, we took advantage of the preservation of thalamic projections to somatosensory cortex in rat thalamocortical slices (Agmon and Connors, 1991; Sasaki et al, 2006; Inoue and Imoto, 2006) to study maladaptive synaptic connectivity and evoked paroxysmal activities in neocortical circuits of epileptogenic barrel cortex adjacent to freeze lesion-induced microgyri. Dual whole-cell patch-clamp recordings

in layer IV of the paramicrogyral cortex indicated that there was selective loss of inhibition from fast-spiking interneurons to spiny neurons in the barrel circuits that likely contributed to un-constrained cortical recurrent excitation and paroxysmal discharges.

Methods and Materials

All experiments were performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Stanford University Institutional Animal Care and Use Committee. Freeze lesions were made as previously described (Jacobs et al., 1996). Sprague-Dawley rat pups <48 hrs old were immersed in ice for ~4 minutes until movements and responses to tail pinch were absent. The skull was exposed through a scalp incision and a freezing probe with a 1 mm diameter circular tip, cooled to -50 - -60 °C, was placed on the skull over somatosensory cortex ~3 mm from the midline and 0.5 mm rostral to the bregma for 5-6 sec. The scalp was then sutured, the pup warmed and returned to the dam. This procedure routinely resulted in the development within barrel cortex of a microsulcus, consisting of an invagination of the cortical surface, and an associated 4 layered microgyrus measuring ~500 μ m across. The microgyrus was clearly evident in fixed and live TC slices after P12 (e.g. Fig 1 and Jacobs et al, 1999c).

Brain slice preparation: Thirteen to 16 days later, rats were anesthetized with pentobarbital (55 mg/kg, i.p.), decapitated, and brains removed and placed into cold (~ 4 °C) oxygenated slicing medium containing (in mM): 2.5 KCl, 1.25 NaH₂PO₄, 10.0 MgCl₂, 0.5 CaCl₂, 26.0 NaHCO₃, 11.0 glucose, and 234.0 sucrose. The site of the previous freeze lesion could be seen as a small depression in the pial surface that corresponded to the site of the microsulcus. The brains were quickly removed and thalamocortical (TC) slices (300-400 μ m) cut with a vibratome (TPI, St. Louis, MO) in the above solution, according to methods of Agmon and colleagues (Agmon and Connors, 1991). Slices were transferred to a holding chamber, where they were incubated (35°C) for at least 1 hr in artificial cerebrospinal fluid (ACSF) (below). Individual slices were then transferred to a recording chamber fixed to a modified microscope stage, and allowed to equilibrate for at least 30 min before recording. Slices were minimally submerged and continuously superfused with oxygenated ACSF at the rate of 4.0 ml/min. The perfusion solution contained (in mM): 126.0 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1.0 MgCl₂, 2.0 CaCl₂, 26.0 NaHCO₃, and 10.0 glucose. Solutions were gassed with 95% O₂/5% CO₂ to a final pH of 7.4 at a temperature of 35 ± 1 °C.

Silver staining was used to illustrate patterns of degenerating axonal fibers in the area in and around the microgyrus in some sections. Slices were fixed in 4% paraformaldehyde, and 40 μ m sections cut and mounted on gelatin coated slides. Sections were postfixated for 3 min in acid formalin, rinsed in glass distilled water and put in a solution of 15% silver nitrate, 10% potassium nitrate and 5 % glycine for 15 minutes. Sections were then placed in a reducing solution of 1% pyrogallol, 1% nitric acid and 50% alcohol for 1 minute, rinsed with glass distilled water, fixed for 5 minutes in 5% sodium thiosulfate, dehydrated, cleared and mounted.

The barrel subfield and thalamus in TC slices were identified using a low-power objective (2.5 $\times$) as previously described (Agmon and Connors, 1991). A water immersion objective (40 $\times$) with Nomarski optics and infrared video was used to visualize individual neurons in the barrel and single or dual whole-cell patch-clamp recordings were obtained. Recording pipettes were fabricated from capillary glass (World Precision Instruments, M1B150F-4), using a Sutter Instrument P80 puller, and had tip resistances of 2-5 M Ω when filled with the intracellular solutions below. A Multiclamp 700A amplifier (Axon Instruments, Foster City, CA) was used for voltage and current clamp recordings from pairs of neurons. Biocytin (0.5%; Vector Labs, Burlingame, CA) was regularly added to the patch pipette solution for subsequent anatomical analysis. Patch pipette saline was modified according to Brecht and Sakmann (2002) and was

composed of (in mM): 100 K-gluconate, 10.0 phosphocreatine-Tris, 3.0 MgCl₂, 0.07 CaCl₂, 4 EGTA, 10.0 HEPES, 4.0 Na₂-ATP, and 1.0 Na-GTP, pH adjusted to 7.4 and osmolarity adjusted to 280 mOsm·l⁻¹; E_{Cl}⁻ = -77 mV. In several experiments, an intracellular solution containing 14 mM Cl⁻ was used to give an estimated E_{Cl} of ~-55 mV (e.g. Figs 4 & 7). Data were accepted for analysis when access resistance in whole-cell recordings ranged from 4 to 12 MΩ, and was stable (<25% change) during the recording. Extracellular multiple-unit activities were recorded using monopolar tungsten electrodes (0.2–2 MΩ; Frederick Haer, Brunswick, ME) and a Grass amplifier (bandwidth, 0.03–3 kHz). Current and voltage protocols were generated using PCLAMP8 software (Axon Instruments).

A sharpened bipolar tungsten electrode, placed in the internal capsule or VPM, was used to activate TC afferents. The approximate position of the stimulating electrode was within the TC projections, as estimated from the results of DiI applications reported by others (Agmon and Connors, 1991; Agmon et al., 1995). In some slices, the stimulating electrode was repositioned closer to the internal capsule or striatum (CPu) to obtain an optimal TC response. The stimulus intensity was initially set at a subthreshold level and then gradually increased to evoke all-or-none EPSCs and increased further to obtain maximal amplitude responses. Monosynaptic excitatory postsynaptic potentials or currents (EPSPs or EPSCs) were evoked in fast spiking (FS) interneurons or spiny neurons by TC stimuli. The term “spiny neurons” is used throughout to refer to both spiny stellate and pyramidal cells (Sun, et al. 2006). Pyramidal cells and interneurons were visually identified during recordings (Sun, et al. 2006) and cell type confirmed with biocytin labeling (below). In some experiments, TC slices were maintained in an interface chamber and recordings of cortical and thalamic evoked multiunit activity were obtained with tungsten microelectrodes (Figs. 2 & 3).

Unitary PSP latency in paired recordings was defined as the time from the peak of the presynaptic AP to the peak of the post-synaptic PSP, while AP latency was the time from either the onset of an intracellular current pulse or an extracellular fiber stimulation to the peak of the evoked AP. Spontaneous IPSCs were recorded in spiny neurons as outward currents under voltage clamp at holding potentials more positive than -45 mV (Fig 4), whereas sEPSCs were recorded as inward currents at E_{Cl}⁻: ~ -77 mV. Cross correlations were calculated with Clampfit 8.0.

After recording, brain slices were fixed in 100 mM phosphate-buffered (PB) solution, pH 7.4, containing 1% paraformaldehyde and 2.5% glutaraldehyde at 4°C for at least 24 hr. Endogenous peroxidase was blocked by incubation in 1% H₂O₂ for 15-20 min. After several rinses in PB solution, slices were transferred to 1% avidin-biotinylated horseradish peroxidase complex containing 0.1% Triton X-100 in PB (0.1 M, pH 7.4; ABC-Elite Camon, Wiesbaden, Germany) and left overnight at 4°C while being shaken lightly. The next day, slices were reacted using 3, 3'-diaminobenzidine (DAB; Sigma) and 0.01% H₂O₂ until dendrites and axonal arbors were clearly visible (~2-5 min). Slices were mounted on glass slides, embedded in DPX-mounting media (Aldrich, Milwaukee), and coverslipped.

Cytochrome oxidase staining (Wong-Riley, 1979) was used to identify the barrel structures in layer IV. Brain slices were stored overnight in 4% paraformaldehyde at 4°C. After several rinses in PB solution, they were re-sectioned at 100 μm and sections incubated in a PB solution containing 50 mg DAB, 15-30 mg cytochrome c, and 20 mg catalase/100 ml at 37°C for 2 hr in the dark. The reaction was stopped when individual barrels were clearly distinguishable from the background. After several rinses in PB solution, sections were mounted on glass slides, air-dried, defatted in absolute alcohol and xylene, embedded in DPX-mounting media (Aldrich, Milwaukee), and coverslipped. Photomicrographs were converted into grayscale images and imported into IgorPro (Wavemetrics, Lake Oswego, OR) for quantitative analysis.

Neuronal three-dimensional reconstruction and morphometric measurements of dendritic and axonal arbors were made with NeuroLucida software (MicroBrightField, Colchester, VT). Cells and their processes were drawn with a camera lucida using 40× or 100× objectives. Biocytin histochemistry often resulted in background staining that allowed delineation of cortical laminae and barrel borders. In some slices, cytochrome oxidase histochemistry and labeling of single biocytin-filled neurons were combined to reveal the dendritic and axonal organization with respect to the barrel structure.

Results

Histological sections showed that freeze lesion-induced microgyri, consisting of areas of abnormal lamination, were present within the barrel field (Fig. A1, M.G.; Jacobs et al., 1999c). The location of the microgyrus with respect to the barrel field could be seen in live images of TC slices (not shown), allowing electrophysiological recordings to be made from identified sites. After recording, slices were recovered and cytochrome C (CO) and Nissl stains used to establish the precise location of microgyri within the barrel cortex. The widths of microgyri varied from 0.1-0.6 mm (e.g. Fig 1A1). In 6/9 TC slices, enhanced CO staining was detected at the border between the “normal” 6 layered cortex and the 3 layered microgyrus (e.g. Fig 1A), suggesting that increased activity might occur in this region (see also Jacobs et al, 1999a,c). In addition, consistent with previous results showing increased density of afferents and abnormal barrel structures (Jacobs et al, 1999c), TC sections cut through the region of malformed neocortex showed enhanced silver stain in layer IV adjacent to the microgyrus (PMG) region in 6/9 slices from 3 animals (e.g. Fig 1B).

Single thalamic stimuli elicited robust paroxysmal discharges in the FL cortex

We next examined the electrophysiological responses evoked by thalamic stimuli in vitro. Multiunit neocortical and thalamic activities were recorded in an interface chamber with tungsten microelectrodes from various locations in the neocortex (S1 and adjacent region) and thalamic relay nuclei. In naïve untreated TC slices, single electrical stimuli applied to VB evoked transient cortical multi-unit activity lasting 65 ± 42 ms (not shown; $n = 6$). In the FL cortex, single stimuli elicited robust, prolonged paroxysmal discharges with a duration of 850 ± 100 ms (Fig 2, $n = 6$). The duration of these epileptiform responses was shorter in minimally submerged slices used for voltage clamp recordings (cf Figs. 2 and 7). Dual extracellular recordings showed that the epileptiform discharges were highly synchronous when electrodes were separated by <0.5 mm. The most intense paroxysmal activities were always recorded from the PMG, close to the border of the microgyrus ($n = 6$ slices from 3 rats; Figs. 2 & 3B). Within the microgyri, however, evoked activities were brief and less intense (mean durations 45 ± 15 ms, $n = 6$ slices from 3 rats, Fig 3B1). When one recording electrode was positioned laterally at various distances from the center of the lesion (i.e., the microsulcus), abnormal TC evoked activities could be recorded for up to ~ 5 mm. The epileptiform discharges propagated laterally with a latency of ~ 100 ms at a distance of ~ 5 mm (mean propagation speed, as measured in layer IV, was 50 ± 9 mm/sec, Fig 2B, $n = 6$). Paired simultaneous recordings in different laminae (excluding layer I) at the same distance from the microsulcus showed that the abnormal evoked activities were present and had a similar duration in layers II-VI (cf. traces 1 and 2 in Fig 2A). The paroxysmal discharges were reduced in duration by about 70% ($\pm 10\%$) in APV ($50 \mu\text{M}$) and abolished by CNQX ($10 \mu\text{M}$), suggesting that the abnormal activities were mediated via activation of NMDA and AMPA receptors. Simultaneous thalamic and cortical recordings demonstrated that neocortical paroxysmal activity evoked by thalamic stimuli did not recruit thalamic oscillations (5 of 5 slices, e.g. Fig. 3A), suggesting that the aberrant and prolonged neocortical discharge arose through recruitment of intracortical, but not thalamocortical circuits.

A shift in excitatory/inhibitory balance in the PMG

Layer IV neurons in the barrel cortex are the major targets for thalamic afferents (Agmon and Connors, 1991; Simons, 1978; Simons and Woolsey, 1979; Woolsey and Van der Loos, 1970). Anatomical results show that barrel structures in this lamina are abnormally formed (Jacobs et al, 1999c), and extracellular recordings indicate that epileptiform activities are generated in layer IV (Figs. 2 and 3). We therefore examined the mechanisms underlying the TC evoked paroxysmal activities by obtaining whole-cell patch clamp recordings from neurons located in layer IV of barrel cortex. In spiny cells of control slices ($n = 30$ cells), supramaximal extracellular TC stimuli evoked monosynaptic EPSCs followed by multi-peaked polysynaptic responses composed of mixed excitatory and inhibitory components (Fig. 4B). We initially used conditions that maximized detection of both excitatory and inhibitory (GABA_A receptor-mediated) signals. Thus intracellular pipette solution contained slightly elevated $[\text{Cl}^-]$ (14 mM, estimated $E_{\text{Cl}} \approx E_{\text{GABA-A}}: -55$ mV), and the voltage clamp holding potential was set at a value positive to this (-40 mV trace of Fig 4B1). Under these conditions, long-latency (150 ± 45 ms, $n = 10$ cells), presumed polysynaptic IPSCs were recorded as outward currents interspersed with polyphasic excitatory inward responses. At a V_{hold} of either -60 or -80 mV, the inhibitory responses became inward, and thalamically-evoked polysynaptic responses consisted of polyphasic inward currents likely composed of mixed EPSCs and IPSCs (Fig 4B1). In current clamp recordings, using patch pipettes containing physiological $[\text{Cl}^-]$ (7 mM, estimated $E_{\text{Cl}} \approx E_{\text{GABA-A}}: -77$ mV), the TC stimulus evoked a single spike followed by a long-lasting (100s of ms) hyperpolarizing inhibitory response (8 ± 3 mV, $n = 10$ cells; Fig 4B2, control trace). To determine the role of inhibitory signaling in regulating the TC evoked response, we applied the GABA_A receptor antagonist bicuculline (10 μM), which resulted in the equivalent TC stimulus evoking an EPSP that triggered a spike followed by barrage of depolarizing PSPs and spikes resembling a paroxysmal depolarization shift (Matsumoto and Ajmone-Marsan, 1964; Prince, 1968; Fig 4B2, BMI trace). These results suggest that, in the normal barrel cortex in vitro, synchronous TC inputs evoke complex local cortical network activities that are predominantly composed of inhibitory responses that limit concurrent excitation (Sun et al., 2006).

In FL-containing slices, supramaximal TC stimuli evoked multi-peaked polysynaptic responses. In voltage clamp recordings from neurons located far from the PMG region, the TC-evoked responses contained both inhibitory and excitatory synaptic events (Fig 5A3 & B). In cells located in the PMG region, the same stimuli evoked predominantly inward currents (Fig 5A2 & B). Dual intracellular recordings of nearby cells in either region showed that the evoked activities were very similar in duration and amplitude, indicating that the polysynaptic currents are likely generated by local network activities. To access the contributions of inward and outward currents to the overall polysynaptic local network activities, we compared the total charge of the PSCs at different distances from the edge of the microgyrus. In neurons located close to the border of the microgyrus, the overall charge of evoked polysynaptic events was positive (Fig. 5B, 0.3 mm), whereas there was a progressive shift in the negative direction for neurons closer to the PMG (Fig 5B, 0.6 and 0.9 mm). These results suggest that in the epileptogenic PMG zone, the balance between excitation and inhibition in the local network is shifted toward enhanced excitatory activities. The generation of paroxysmal network activities may result from either enhanced recurrent excitatory or reduced inhibitory synaptic connections.

To determine the strength of excitatory or inhibitory synaptic events in the epileptogenic PMG region, we obtained simultaneous whole-cell recordings from 30 pairs of cells in layer IV barrels near the PMG, of which 9 pairs were synaptically connected. Interneurons and excitatory neurons were distinguished according to (1) their dendritic structures (spiny or aspiny), (2) firing properties during current injections (McCormick et al, 1985; Wang et al.,

2002; Gupta et al., 2000) and (3) the GABAergic or glutamatergic nature of the unitary synaptic connection made by the neuron, which was routinely verified by estimating the reversal potential of the synaptic response and/or demonstrating sensitivity to CNQX (10 μ M) or gabazine (10 μ M). Unitary synaptic events were initially examined under current clamp by repeatedly eliciting short trains of action potentials (APs) alternately in presumed presynaptic and postsynaptic neurons (e.g. Fig 6B1). Synaptic connections were then confirmed by evoking > 100 single action potentials at 1 Hz with brief depolarizing current pulses in presynaptic neurons. Both unitary excitatory postsynaptic potentials (uEPSPs; Fig 6) and uIPSPs (Fig 7) had short, fixed latencies (e.g. Fig 6B2; 7B). In 4 of the 9 synaptically connected pairs within PMG sites, presynaptic cells were spiny neurons and unitary EPSCs were recorded from the postsynaptic cell (Fig 6B). In 5 pairs, presynaptic cells were FS interneurons and postsynaptic uIPSCs were evoked (Fig 7B). Additional data from excitatory (11) and inhibitory (6) pairs recorded in layer IV of naïve untreated brains were previously published (Sun et al., 2006) and have been included as a control group in this study for comparison (Table 1). The recording conditions and ages of naïve animals and those with microgyri induced by freeze lesions were very similar, and data were obtained by the same investigator (Q-Q Sun).

Functional excitatory connections between adjacent layer IV neurons were not up-regulated in the epileptogenic PMG region

Each pair of synaptically coupled neurons in the PMG. was within the same barrel. There were no significant differences in amplitude, τ_{rise} or τ_{decay} for uEPSPs between excitatory neuronal pairs in naïve TC slices versus those in the PMG region of FL slices (Fig. 8). Analysis of recovered biocytin-filled pairs of neurons with a 100x objective (Fig 6A) demonstrated that the number of putative connections ($n = 4 \pm 2$ for 4 pairs of spiny cells in PMG; vs. $n = 4 \pm 1$ for 11 spiny cell pairs in naïve brain slices) and the location of the connections (i.e. distance from the cell body) were similar in PMG and naïve slices.

Intracortical inhibition from basket cells is selectively down-regulated in the epileptogenic PMG region

Unitary IPSPs between layer IV inhibitory and excitatory neurons were examined in 5 FS-excitatory pairs from naïve slices and 5 pairs located in the PMG region of microgyrus-containing slices. In 4 of 5 pairs in the PMG, the postsynaptic excitatory neuron was a spiny stellate cell and in the remaining pair it was a star pyramidal neuron. All postsynaptic neurons in the naïve cortex were star pyramidal cells. The properties of uIPSPs in the PMG cells were quite different from those in spiny cells of naïve slices in that the mean conductance was less than half of control values (Fig 8B1, $p < 0.05$). However, no significant differences were found in rise time (τ_{rise}) or decay time (τ_{decay}) for uIPSPs in control cortex versus PMG (Fig 8B2-4).

The balance between inhibitory and excitatory synaptic strength is shifted towards excitation in the epileptogenic PMG region

Reduced inhibition from FS to stellate cells would alone be expected to increase network excitability, but the ratio between unitary EPSC vs IPSC strengths would also be an important factor in determining whether afferent inputs recruit polysynaptic circuits. In naïve untreated slices, the amplitude of uIPSPs received by layer IV stellate cells was ~4-5 times larger than that of uEPSPs (5.0 ± 0.7 mV for uIPSPs vs. 1.1 ± 0.4 mV for uEPSPs, $p < 0.05$) and the decay time constant (τ_{decay}) was about 4 times longer for uIPSPs than for uEPSPs ($p < 0.05$). Thus, within this layer inhibition from FS to stellate cells is relatively stronger than the recurrent excitation between stellate cells. In the PMG region of FL treated slices, peak conductance of uIPSCs was dramatically reduced (Figure 8B) and approached that for uEPSCs, which were not affected (Figure 8A).

These results confirm our previous observations (Sun, et al. 2006) that in naïve slices, inhibition from single FS interneurons onto excitatory cells is far stronger than the unitary excitation that FS interneurons receive from single local excitatory neurons. The strength of this inhibition from FS cells onto spiny neurons is significantly decreased in the PMG. This down-regulation may be due in part to a reduction in the number of putative inhibitory synaptic contacts per pair, as the number of close appositions between FS cell axons and spiny cells in pairs of labeled neurons appeared to be decreased from controls. In our earlier study (Sun et al, 2006), basket cells in layer 4 formed $\sim 30 \pm 5$ putative contacts per spiny cell. In the 4 labeled pairs from the PMG, the average number of close appositions formed by the axon of a single FS cell onto a spiny neuron was $\sim 10 \pm 3$ (e.g. Fig. 7A2). However there were insufficient numbers of labeled pairs in the PMG to establish that this trend was significant.

Spontaneous excitatory and inhibitory synaptic events

We measured the properties of spontaneous (s) IPSCs and sEPSCs to determine whether the specific deficits in FS to stellate cell inhibition in the PMG would be correlated with overall changes in synaptic input. Spontaneous synaptic events were recorded under voltage-clamp with pipette solution containing physiologically relevant $[Cl^-]$ (7 mM, estimated E_{Cl^-} : -77 mV) at different holding potentials (Methods). In spiny neurons located in the PMG, where we observed a reduction in uIPSC strength (Figure 8), the amplitude of sIPSCs was unexpectedly increased both at distal and proximal PMG zones (Table 1, area 1 and 2). In addition, sIPSC frequency was significantly increased, especially in the proximal PMG (FL area 1, Table 1). By contrast, in cells located within the microgyrus, the amplitude of sIPSCs was smaller than controls and the frequency was unaffected (Table 1, FL area 0). Spontaneous EPSCs were largely unaffected, although there was a significant increase in their frequency in the proximal PMG (Table 2, FL area 1), the same location in which sIPSC frequency was elevated, indicating a general increase in connectivity within this zone.

Discussion

We examined some possible mechanisms underlying epileptogenesis in rat barrel cortex subjected to a neonatal freeze lesion that produced a microgyral developmental malformation. The cell type, neuronal intrinsic properties and synaptic connections in barrel cortex are well characterized (Feldmeyer et al., 1999; Feldmeyer et al., 2002), making this a useful model in which to examine the relationship between epileptogenesis and the structural reorganization (e.g. Jacobs et al, 1999c; Rosen et al 2000), and alterations in GABAergic inhibitory circuitry (Rosen et al, 1998; Jacobs and Prince, 2005) that are known to be present. Our results identify reductions in inhibitory synaptic strength as one major factor underlying hyperexcitability in barrel cortex adjacent to experimental microgyri.

The principal finding in these experiments is a reduction in inhibition from FS interneurons onto spiny cells in the area adjacent to the microgyrus. There are known alterations in microgyral cortex that could affect the strength of postsynaptic inhibition, including a transient reduction in the number of interneurons expressing parvalbumin (PV; presumed FS cells) that is present within the microgyrus and adjacent cortex until $\sim P21$, as well as a decrease in the density of PV-containing cells in the PMG that persists into adulthood (Rosen et al, 1998). Changes in GABA_A receptors are also present (Zilles et al, 1998) and are inferred from altered responses to GABAergic agonists (DeFazio and Hablitz, 1999). Depletion of FS interneurons should not affect the amplitude of uIPSCs in the current experiments if the remaining FS cells show similar connectivity. Notably, our recordings were obtained at P13-16 at a time when presumed FS cells still show reduced PV expression (Rosen et al, 1998). This may indicate that structural and functional maturation of these cells is delayed, affecting their ability to release GABA or the numbers of efferent connections to spiny neurons (e.g. see results and

Fig. 7). In future experiments, it would be of interest to assess the immunoreactivity to PV and the axonal arbors of these presumably immature interneurons. Of relevance to this possibility are our previous findings that anatomical abnormalities suggesting delayed maturation occur in calbindin-containing interneurons within the microgyrus proper (Kharazia et al, 2003). Alterations in postsynaptic GABA_A receptors such as decreased receptor density could also reduce postsynaptic conductance (Fig. 8B1) without a significant change in the kinetics of the evoked uIPSCs (Fig.8B 3, 4).

Our results in spiny cells in layer IV of the PMG zone agree in some respects with those previously obtained from pyramidal cells in layer V of the epileptogenic area in the microgyrus model (Jacobs and Prince, 2005). In both studies, the conductance of sIPSCs was increased while that of sEPSPs was not. However in layer V, the conductance of monosynaptic IPSCs was unchanged from control, in contrast to the significant reduction in uIPSCs in spiny cells of layer IV reported here. There is precedent for a differential reduction in IPSCs in layer IV versus layer V pyramidal cells in epileptic *tottering* mice, presumably due to differences in presynaptic Ca⁺⁺ channels in terminals of FS cells (Sasaki et al, 2006). It is also likely that other subtypes of interneurons contribute to sIPSCs and polysynaptic evoked IPSCs in both layer IV and V, perhaps accounting for the discrepancy between reduced conductance of uIPSCs from FS cells and the increase in sIPSC conductance in the current results. Other possible explanations include an increase in the contacts provided by one interneuron subtype and a decrease in those from another (Buckmaster and Dudek, 1997); and a reduction in overall interneuron number with compensatory sprouting and formation of inhibitory synapses by surviving interneurons (Nieoullon and Dusticier 1981; Katsumaru et al. 1986; Davenport et al. 1990; Wittner et al, 2001).

Another potential mechanism underlying epileptogenesis associated with microgyri is enhanced excitatory connectivity, as evidenced by the increased frequency of sEPSCs and mEPSCs in subgroups of layer V PMG cells and the marked reductions in sIPSC frequency induced by perfusion with glutamate receptor blockers (Jacobs and Prince, 2005), as well as by increases in sEPSC frequency in the present experiments (Table 2). The increased density of axons in the immediate paramicrogyral area (Fig.1B), enhanced glutamate immunoreactivity of axonal projections in this area (Humphreys et al, 1991) and increased thalamocortical projections to areas of aberrantly structured barrels (Jacobs et al,1999c) all suggest restructuring of excitatory inputs. Whether this restructuring also affects recurrent connections of spiny cells in the PMG is not known. In both hippocampal slice cultures (McKinney et al, 1997), and epileptogenic partially isolated cortex (Jin et al, 2006), it appears that aberrant excitatory connectivity involves individual pyramidal cells making contacts on a wider field of targets, rather than more synapses on single postsynaptic cells. There was no significant increase in the amplitude of uEPSCs in the PMG, suggesting that enhanced connectivity between spiny cells, if present, involves more widespread projections, rather than increased connectivity between cell pairs. Of course, the lateral spread of epileptiform activity (Fig.2B) might result from decreased GABAergic inhibition alone (Prince and Wilder, 1967; Miles and Wong, 1987; Chagnac-Amitai and Connors, 1989).

GABAergic cells in the neocortex and hippocampus provide stability to the principal neuronal population by feed-back and feed-forward inhibition that prevents development and spread of epileptogenic discharges (Prince and Wilder, 1967; Wong and Prince, 1979; Chagnac-Amitai and Connors, 1989). A decrease in normally powerful thalamocortically evoked GABAergic inhibition from FS interneurons in barrel cortex should have important functional consequences due to a resulting prolonged excitation of spiny (excitatory) cells (Sun et al, 2006). In addition, GABAergic neurons actively participate in cortical maturation in rodents, as well as in primates and humans (Owens et al, 1999; Levitt et al, 2004) Much of cortical development occurs late in gestation and postnatally, making these high risk periods when pathological processes can

lead to developmental abnormalities and associated neuropsychiatric disorders (Marin and Rubenstein, 2001; Marin and Rubenstein, 2003). Defects in either the production or migration of cortical GABAergic neurons or GABA receptor abnormalities can result in a hyper-excitabile cortex, epilepsy and cognitive dysfunction (Powell et al., 2003; Marini et al., 2003; Harkin et al., 2002; Hensch et al., 1998). Apparent *increases* in cortical inhibition, described in other epilepsy models (Buhl et al., 1996; Klaassen et al., 2006) are likely due to GABAergic synchronization of excitatory cortical activity necessary to initiate the spread of epileptiform activity (Engel, 1995; Kohling et al., 2000; Troyer et al. 1992; Michelson and Wong 1994; Avoli et al. 2002; Khazipov and Holmes 2003).

In the neonatal freeze lesion model, the most epileptogenic zone is the cortical region immediately adjacent to the microgyrus (Jacobs et al., 1996; 1999a,c). This paramicrogyral zone shows grossly normal cytoarchitectonic organization, however alterations in postsynaptic receptors and synaptic strength are present (DeFazio and Hablitz, 1999; DeFazio and Hablitz, 2000; Jacobs and Prince, 2005) as well as abnormal connectivity (Jacobs et al., 1999c; Rosen et al., 2000) and widespread reductions in the density of PV-containing GABAergic interneurons (Rosen et al., 1998; but see Schwarz et al. 2000). Apparent loss of inhibitory neurons or decreases in functional inhibition has also been reported in cortical dysplasia produced by fetal irradiation (Chen and Roper, 2003; Roper et al., 1997; Roper, 1998; Zhu and Roper, 2000) and in human dysplastic cortex (Calcagnoto et al., 2005), and is a common finding in other models of chronic epileptogenesis (Ribak et al., 1986; Lowenstein et al., 1992; Buckmaster and Dudek, 1997; Cossart et al., 2001; reviewed in Prince and Jacobs, 1998)..

Our results show that the strength of unitary inhibitory responses from FS cells onto spiny cells is severely affected in barrels within the PMG region, while excitatory connections between two spiny cells within a barrel circuit are not changed. The selective down-regulation of basket cell output may be sufficient to induce epileptiform activities in neocortex. Results suggest that thalamically evoked cortical responses are predominantly regulated by basket cells whose output is a key factor in controlling excitability in the thalamocortical circuit. Disorders in these neurons or their functional connections can lead to the generation and propagation of epileptiform discharges.

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Figure 1.

Cytochrome C staining of layer IV barrels in rat somatosensory cortex containing a freeze lesion-induced microgyrus (M.G.). **A1)** Photomicrograph of a fixed thalamocortical (TC) slice stained with cytochrome C. White arrows point to barrels that appear as darker areas in layer IV separated by lighter septa. Scale bar: 0.5 mm. **A2)** Pixel intensities were measured by a single pixel width line scan as a function of distance from the microgyrus within the center of layer IV. Peaks in this intensity function identify barrel regions (arrows 1-5, as in A1) separated by the low-intensity septal regions. Note that the first barrel (No. 1) in the PMG region has the most intense cytochrome C immunoreactivity. X axis in A2, B2: distance (μm) from edge of microgyrus. **B1)** Darkfield micrograph of silver staining in TC section cut through layers I-VI of S-1 region of malformed neocortex (P40). Large white arrows: area of enhanced silver staining in layer IV adjacent to the microgyrus (M.G.). Roman numerals: cortical lamina. Scale bar: 0.5 mm. **B2)** Photographs were converted into grayscale images and analyzed as in A2. The brightness of pixels is quantified across the barrels in layer IV. The area near the MG region had intense silver staining, while cortex within the MG itself showed little staining.

Figure 2.

Single electrical stimuli applied to the thalamic VB complex evoke epileptiform activities in the somatosensory cortical slices of FL treated rats. **A)** Top: Camera lucida reconstruction of TC slice from a FL rat. A microgyrus (MG, black arrow) was present in the face representation of barrel cortex. Barrels (black regions in A and B) were visualized with bright-field microscopy in live slices, and also in fixed slices stained with cytochrome C. Scale bars in A,B, top: 0.5 mm. A1,A2: Dual simultaneous recordings of multiunit extracellular epileptiform activities in paramicrogyral layer IV (1) and layer V (2), evoked by single electrical stimuli (10V, 5 μ S, black arrow) applied to VB. In this Fig. and Fig. 3, TC projection is shown in gray. nRT: thalamic reticular nucleus; VB: thalamic ventrobasal complex; Str: striatum. IV: layer IV. The evoked epileptiform activities in layers IV (A1) and V (A2) were similar in latency and duration at the 2 recording sites shown in the diagram above. **B)** Top: Electrodes were repositioned in the same slice, as shown in upper panel. B1,2: Multi-unit extracellular activities, evoked with same stimuli as in A1-2, recorded in layer IV at sites close to (B1) and at a distance (B2) from the border of the microgyrus. The epileptiform multi-unit activities (black) and field potentials (gray) had a shorter latency and longer duration at site 1 than site 2. Calibrations in A2 for traces of A and B.

Figure 3.

Lack of thalamic or microgyral involvement in VB-evoked epileptiform responses in TC slices of FL treated rats. A,B, top: diagrams of slices as in Fig.2 showing locations of the microgyrus, barrels, and stimulating and recording electrodes. Scale bars: 0.5 mm. **A)** The thalamic stimulus evoked an epileptiform event at long latency in layer IV of the paramicrogyral zone (site 1) but not in simultaneous recording from the relay nucleus (site 2). **B)** Thalamic stimuli evoked long duration multiunit epileptiform activities in layer IV of paramicrogyral cortex (site 2) but not within the microgyrus itself (site 1).

Figure 4.

Characterization of thalamically evoked synaptic activities in control layer IV neurons from unlesioned rat. **A1**) Diagram as in Figs. 2,3. **A2**) Whole-cell current clamp recording from a spiny neuron located in a layer IV barrel, showing typical responses to 100 pA depolarizing and hyperpolarizing current pulses. **B1**) Voltage-clamp recording ($V_{\text{hold}} = -40, -60$ and -80 mV) from cell of A. Single thalamic stimuli (TC, black arrow) evoke a short latency, presumed monosynaptic EPSC followed by di- and polysynaptic excitatory (inward, below dashed line) and inhibitory synaptic currents (outward, above dashed line.). $E_{\text{Cl}} = -55$ mV. **B2**) Current clamp recording from the neuron in (A), showing suprathreshold thalamically evoked depolarizing response followed by polysynaptic hyperpolarizing IPSPs (black trace). Dashed line: resting V_{m} . Bath application of bicuculline methiodide (BMI, $10 \mu\text{M}$), eliminated the hyperpolarizing responses and led to development of a prolonged large amplitude depolarizing response (gray trace).

Figure 5.

Thalamically evoked synaptic activities in layer IV neurons located at different distances from the microgyrus. **A1)** Diagram of slice and sites of recording. Simultaneous intracellular recordings were made from pairs of layer IV spiny neurons, each pair in a different barrel, as shown. **A2)** Single thalamic stimuli (arrows) evoked monosynaptic EPSCs followed predominantly by polysynaptic excitatory (inward) currents in cells a & b. **A3)** The same thalamic stimulus evoked monosynaptic EPSCs followed by inward and outward polysynaptic events in cells c & d. **B)** Box/whisker and scatter plots (open circles) of total synaptic charge of the thalamically evoked polysynaptic events in all recorded layer IV neurons located in areas 1 (within MG, 120 ± 540 pA*ms); 2 (0.2-0.4 mm from margin of MG, $13,500 \pm 7570$ pA*ms); 3 (0.6 mm from MG, 490 ± 6890 pA*ms), and 4 (0.9 mm from MG, 520 ± 5740 pA*ms) in TC slices from FL rats (see Fig. 1A). Normal: Responses in layer IV neurons of naive rats (4700 ± 3650 pA*ms). ***: $p < 0.001$.

Figure 6.

Unitary excitatory responses in PMG cortex. **A1)** Camera lucida reconstruction of a synaptically connected pair in layer IV, consisting of a star pyramidal neuron (cell body shown in blue; cell **a** in A2) and a spiny stellate cell (cell body shown in cyan; cell **b** in A2). Blue: dendrites; red: axons. Scale bar: 100 μm . Yellow dots indicate sites of putative contacts between axons of presynaptic cell (blue soma) and dendrites of postsynaptic cell (cyan soma) as identified from 100x microscopic images. **A2)** Photomicrograph of the same cell pair (rotated slightly CW compared to A1). Note that the main axon (large arrows), originating from the axon hillock of cell **a**, forms several collaterals along its path into deeper cortical layers. One of the branches (dashed small arrow) forms putative contacts on the dendrites of cell **b**. Another recurrent branch projects into layer IV, and also forms putative contacts with cell **b** (close appositions are not visible in this focal plane). Calibration: 50 μm . **B1)** Current clamp recording in both cells. Presynaptic APs in cell **a** (bottom trace, 20 trials averaged) evoke uEPSPs in cell **b**. **B2)** APs in cell **a** evoke fixed latency variable amplitude uEPSCs in voltage clamped cell **b** (6 traces overlaid). Vertical line: peak of APs in cell **a**.

Figure 7.

Unitary inhibitory responses mediated by a fast-spiking basket cell in PMG cortex. **A1)** Camera lucida reconstruction of a synaptically connected fast-spiking basket cell (green cell body) - spiny stellate neuron (red cell body) pair. Blue: dendrites; Red: axons. Gray shading: barrel structure. Scale bar: 20 μm . Yellow dots indicate putative contacts between axons of presynaptic fast-spiking cell and dendrites of postsynaptic spiny stellate cell as identified from 100x microscopic images. **A2)** Enlarged view of A1 (rotated CW approximately 160 degrees) showing putative synaptic contacts from cell **a** onto the soma and dendrites of cell **b**. Close appositions were not seen between fast-spiking cell **a** and another adjacent spiny neuron (cell body in purple). **B)** Unitary synaptic currents (B1) and potentials (B2) elicited in cell **b** by single APs in the presynaptic FS cell (**a**, black trace). AP in FS cell **a** (bottom trace) evokes a depolarizing uIPSC in spiny stellate cell (top trace). $E_{\text{Cl}} = -55 \text{ mV}$.

Figure 8.

Comparison of properties of uIPSCs and uEPSCs in normal and FL cortices **A)** Mean value and standard error for uEPSPs recorded in layer IV of naïve brain (white bars throughout) and PMG of FL cortices (black bars). **A1)** Amplitudes of uEPSPs recorded under current clamp mode (V_m : -55 mV). **A2)** Coefficient of variation (C.V.) for the amplitudes of uEPSPs. **A3)** Decay time constant (τ) as measured from single exponential decay for the uEPSPs. **A4)** Rise time constant (τ) as measured from single exponential equation for the uEPSPs. N.S.: no statistical significance. $N = 11$ pairs for controls, and 4 pairs for PMG. **B)** Mean value and standard error for uIPSCs recorded in layer IV of naïve brain and PMG of FL cortices. V_m : -40 mV; E_{Cl} : -55 mV. **B1)** Conductance for uIPSCs recorded in voltage clamp mode. **B2)** Coefficient of variation (C.V.) for the amplitudes of uIPSCs. **B3)** Decay time constant (τ) as measured from single exponential decay for the uEPSCs. **B4)** Rise time constant (τ) as measured from single exponential equation for the uEPSCs. N.S.: no statistical significance. $N = 11$ pairs for controls, and $N = 5$ pairs for PMG.

Table 1

Properties of sIPSCs in spiny neurons in control and FL cortices

	Peak Amp (pA)	Half-width (ms)	Rise Time (ms)	Decay Time (ms)	Area (pA · ms)	Freq. (Hz)	N
Control	10.2±2.0	14.2±2.1	3.2±0.4	19.6±3.6	238±40	5.6±1.2	12
FL area 0	6.9±1.8*	13.0±1.6	2.9±0.3	15.4±1.7	144±17*	4.7±1.3	8
FL area 1	14.7±3.3*	12.8±3.2	3.3±0.6	21.7±4.4	214±105	7.2±1.1*	12
FL area 2	18.3±5.1*	16.6±3.1	3.3±0.4	21.5±1.3	258±88	4.7±0.9	6

p<0.05 vs. controls, FL area 0: within the microgyrus; FL area 1: area adjacent to border of the microgyrus (on average 0.3 mm from MG); FL area 2: area far away (~1 mm) from microgyrus.

Table 2

Properties of sEPSCs in spiny neurons of control and FL treated cortices

	Peak Amp (pA)	Half-width (ms)	Rise Time (ms)	Decay Time (ms)	Area (pA · ms)	Inst. Freq. (Hz)	N
Control	-11.8±1.9	8.8±1.0	3.3±0.5	10.8±1.1	-125±17	9.4±1.1	12
FL area 0	-8.1±0.8	8.6±0.9	2.7±0.3	9.7±1.0	-88±12	8.1±1.2	8
FL area 1	-12.8±1.6	6.2±1.3	4.3±0.6	7.5±1.3	-139±26	15.6±1.9*	12
FL area 2	-8.3±1.1	9.2±1.2	2.7±0.3	8.5±1.3	-96±25	8.3±1.4	6

* : P<0.05 vs. controls. FL area 0: within the microgyrus; FL area 1: area adjacent to border of the microgyrus (on average 0.3 mm from MG); FL area 2: area farther away (~1 mm) from microgyrus.