Status epilepticus-induced pathologic plasticity in a rat model of focal cortical dysplasia

Francesca Colciaghi,1,* Adele Finardi,1,* Angelisa Frasca,2 Silvia Balosso,2 Paola Nobili,1 Giovanni Carriero,3 Denise Locatelli,1 Annamaria Vezzani2 and Giorgio Battaglia1

1 Molecular Neuroanatomy and Pathogenesis Unit, Neurological Institute ‘C. Besta’, via Temolo 4, 20126 Milano, Italy
2 Laboratory of Experimental Neurology, Department of Neuroscience, Mario Negri Institute for Pharmacological Research, Via G. La Masa 19, 20156 Milano, Italy
3 Experimental Neurophysiology and Epileptology Unit, Neurological Institute ‘C. Besta’, via Celoria 11, 20133 Milano, Italy

*These authors contributed equally to this work.

Correspondence to: Giorgio Battaglia,
Molecular Neuroanatomy and Pathogenesis Unit,
Neurological Institute ‘C. Besta’,
via Temolo 4,
20126 Milano, Italy
E-mail: giorgio.battaglia@istituto-besta.it

We have generated an experimental ‘double-hit’ model of chronic epilepsy to recapitulate the co-existence of abnormal cortical structure and frequently recurrent seizures as observed in human focal cortical dysplasia. We induced cortical malformations by exposing rats prenatally to methylazoxymethanol acetate and triggered status epilepticus and recurrent seizures in adult methylazoxymethanol acetate rats with pilocarpine. We studied the course of epilepsy and the long-term morphologic and molecular changes induced by the occurrence of status epilepticus and subsequent chronic epilepsy in the malformed methylazoxymethanol acetate exposed brain. Behavioural and electroencephalographic analyses showed that methylazoxymethanol acetate pilocarpine rats develop more severe epilepsy than naïve rats. Morphologic and molecular analyses demonstrated that status epilepticus and subsequent seizures, but not pilocarpine treatment per se, was capable of affecting both cortical architectural and N-methyl-D-aspartate receptor abnormalities induced by methylazoxymethanol acetate. In particular, cortical thickness was further decreased and N-methyl-D-aspartate regulatory subunits were recruited at the postsynaptic membrane. In addition, methylazoxymethanol acetate pilocarpine rats showed abnormally large cortical pyramidal neurons with neurofilament over-expression. These neurons bear similarities to the hypertrophic/dysmorphic pyramidal neurons observed in acquired human focal cortical dysplasia. These data show that status epilepticus sets in motion a pathological process capable of significantly changing the cellular and molecular features of pre-existing experimental cortical malformations. They suggest that seizure recurrence in human focal cortical dysplasia might be an additional factor in establishing a pathological circuitry that favours chronic neuronal hyperexcitability.

Keywords: malformation of cortical development; double-hit model; epilepsy; seizures; NMDA receptor

Abbreviations: MAM = methylazoxymethanol acetate; NMDA = N-methyl-D-aspartate; PILO = pilocarpine
Introduction

Malformations of cortical development are brain abnormalities resulting from alterations of the normal process of cortical ontogenesis (Barkovich et al., 2001). In both genetic and acquired types of malformation of cortical development, epilepsy is the most common clinical manifestation (Battaglia et al., 2006; Saillour et al., 2009; Leventer et al., 2010), and seizures are frequently drug resistant. This is particularly relevant in focal cortical dysplasia (Blümcke et al., 2011), which is the consequence of altered late corticogenesis (Andres et al., 2005) or perinatal brain damage (Marin-Padilla, 1999) and the more common type of malformation of cortical development in patients undergoing epilepsy surgery for the relief of intractable seizures (Palmini et al., 1991a, b; Fauser et al., 2004; Lerner et al., 2009). The molecular basis of epileptogenicity in focal cortical dysplasias is not properly understood. We still need to clarify why dysplastic neurons are hypereexcitable; why epilepsy occurs in some dysplastic lesions only; and why epilepsy develops at different postnatal ages despite the presence of cortical malformation from birth.

Recent data have suggested the involvement of N-methyl-D-aspartate (NMDA) receptors in hypereexcitability leading to epilepsy in patients with focal cortical dysplasia (Mikuni et al., 1999; Ying et al., 1999; Najm et al., 2000; Crino et al., 2001; Moddel et al., 2005; Finardi et al., 2006; Takase et al., 2008). NMDA receptors are glutamate-gated ion channels composed of heteromeric assemblies of different subunits clustered in the postsynaptic densities and they are key players in neuronal excitability and excitotoxicity. The role of NMDA receptor complex in focal cortical dysplasia-related epilepsy is under debate: NR2B subunit up-regulation (Ying et al., 1998; Najm et al., 2000; Moddel et al., 2005; Finardi et al., 2006) versus decreased receptor activity and NR2B expression (André et al., 2004) were reported by different groups in dysplastic neurons of human focal cortical dysplasias. These discrepancies underscore the necessity of validating human data in animal models that closely mimic human pathology (Najm et al., 2007).

A widely used animal model of human malformations of cortical development is the rat prenatally exposed to methyloxyxymethanol acetate (MAM), characterized by cortical malformations and heterotopia (Baraban and Schwartzkroin, 1995; Chevassus-au-Louis et al., 1998a; Colacitti et al., 1999; Battaglia et al., 2003a). Heterotopic cortical neurons of MAM rats show abnormal firing properties and NMDA receptor abnormalities (Sancini et al., 1998, Baraban et al., 2000; Gardoni et al., 2003), but MAM rats rarely develop spontaneous seizures (Harrington et al., 2007) indicating that malformed cortical areas are not sufficient to trigger severe epilepsy. We have therefore administered pilocarpine in adult MAM rats to: (i) generate a ‘double-hit’ model recapitulating the typical features of human malformations of cortical development, i.e. abnormal cortical structure and recurrent seizures; and (ii) investigate whether status epilepticus and subsequent seizures contribute to modify the MAM induced brain abnormalities.

Our data emphasize a crucial role of status epilepticus in triggering a pathological process that affects the cortical, cellular and molecular abnormalities induced by MAM, in particular by inducing changes in pyramidal neurons that become reminiscent of hypertrophic/dysmorphic cortical neurons observed in acquired human focal cortical dysplasia. We hypothesize that these changes may further affect the propensity of the malformed cortex to generate recurrent seizures.

Materials and methods

MAM and pilocarpine administration

Procedures were carried out with care to minimize discomfort and pain to treated rats, in accordance with the European Community Council guidelines (1986/609/EEC). Procedures of MAM administration have previously been reported (Colacitti et al., 1999). Pregnant Sprague–Dawley rats received two intraperitoneal doses of MAM (15 mg/kg maternal body weight, in sterile saline) at the same embryonic day (E15) 12 h apart. Control pregnant rats were sham injected with vehicle alone.

Young adult male naïve (from saline-treated dams) and MAM-rats (from MAM-treated dams) (280–350 g, 2–3 months old) were used for inducing status epilepticus. We used male rats only to avoid the confounding factor related to the oestrous cycle in females, which has been reported to affect seizures (Scharffman and MacLusky, 2006; Scharfman et al., 2009). Rats were pretreated intraperitoneally with N-methylscopolamine (0.5–1 mg/kg) to minimize peripheral cholinergic activation (Clifford et al., 1987). Thirty minutes later, naïve and MAM rats were intraperitoneally injected with pilocarpine (PILO and MAM–PILO rats, respectively) or with vehicle alone (control and MAM rats). In pilot experiments, different pilocarpine doses were injected (320–360 mg/kg in 29 naïve and 270–320 mg/kg in 36 MAM rats) to choose the dose inducing highest status epilepticus incidence with lowest mortality in each group.

After establishing the optimal dose, 30 naïve and 54 adult MAM rats were treated with pilocarpine (360 mg/kg for PILO rats and 270 mg/kg for MAM–PILO) and visually observed for 90 min to detect status epilepticus onset by two investigators blind to the identity of the experimental groups. The onset of status epilepticus was defined by the time from pilocarpine injection to the occurrence of continuous seizure activity (Stages 4 or 5 in the Racine scale, see below). Twenty-five PILO and 39 MAM–PILO rats developed status epilepticus (Fig. 1). Five PILO and 15 MAM–PILO rats not experiencing status epilepticus were not further analysed. All rats experiencing status epilepticus received phenobarbital (intraperitoneal, 20 mg/kg) 90 min after onset to reduce mortality. Vehicle-treated control and MAM rats were similarly treated. Pilocarpine treated rats were hydrated subcutaneously with Lactate Ringer’s solution and fed by the operators to improve survival. Eighteen hours after status epilepticus onset, subgroups of PILO, MAM–PILO rats and relative controls (n = 4 in each group) were sacrificed by perfusion and their brains morphologically analysed (thionine staining, fluoro-jade and neuronal nuclei labelling) to evaluate the extent of status epilepticus-induced acute brain damage.

Video monitoring and quantification of spontaneous behavioural seizures

PILO (n = 16) and MAM–PILO (n = 24) rats surviving status epilepticus were video-recorded (from 6 p.m. to 10 a.m. every day, starting 48 h after status epilepticus) to detect clinical seizure onset. All rats surviving status epilepticus developed spontaneous recurrent seizures, as assessed by subsequent video monitoring; therefore, they were...
defined chronic epileptic rats. A subgroup of randomly chosen PILO \((n = 7)\) and MAM–PILO \((n = 9)\) rats were video monitored after epilepsy onset every 5 days (from 6 p.m. to 10 a.m.) for 2 months to quantify seizure activity. In the remaining rats (including the EEG recorded rats, see below), the occurrence of spontaneous recurrent seizures was verified by random video monitoring. The epileptic rats were used for EEG, immunocytochemical or molecular analysis (Fig. 1).

Seizures were graded using the classification by Pinel and Rovner [Stages 0–5: stages outlined by Racine (1972); Stage 6: cluster of multiple Stage 5 seizures; Stage 7: jumping and running seizures; Stage 8: Stage 7 plus tonic hindlimb extension and tail rigidity, sometimes culminating in death; (Pinel and Rovner, 1978a, b)]. Stages 4–7 were considered in seizure assessment.

**Rats not experiencing status epilepticus**

To verify direct effects of pilocarpine on brain architecture, we prevented status epilepticus by administrating intraperitoneal diazepam \((10 \text{mg/kg 5 min before and 5 mg/kg 1 h after pilocarpine; Fritsch et al., 2010})\). Rats were implanted with cortical screw electrodes 1 week before experiment (see below). Video–EEG monitoring was performed for 24 h after pilocarpine injections in six naïve and eight MAM rats treated with diazepam and in two naïve and four MAM rats injected with pilocarpine alone. One month later, rats treated with diazepam were video–EEG recorded for 8 days \((24 \text{ h/day})\) to exclude the occurrence of spontaneous seizures (Fig. 1; six PILO/no status epilepticus, eight MAM–PILO/no status epilepticus rats).

**EEG recordings during chronic epilepsy**

One month after epilepsy onset, randomly chosen MAM–PILO \((n = 4)\) and PILO \((n = 4)\) rats were stereotaxically implanted with electrodes under Equithesin anaesthesia \((1\% \text{ pentobarbital/4}\% \text{ v/v chloral hydrate; } 3.5 \text{mg/kg intraperitoneally})\). Two recording cortical screw electrodes were implanted upon the dura mater in the right side of the frontoparietal cortex (from bregma, mm: AP, \(-0.3\); \(-2.3\); L, \(-2\); Paxinos and Watson, 1986). A ground lead was positioned near the lambda and a reference lead in the nasal sinus. The electrodes were connected to a multiple socket and secured to the skull with acrylic dental cement. After 1 week of recovery, EEG monitoring was continuously performed \((24 \text{ h/day})\) for eight consecutive days to evaluate frequency and duration of spontaneous recurrent seizures through a Grass Telefactor Comet EEG System \((0.1–100 \text{Hz}, \text{ data stored at 400 samples/s, Astro-Med SRL})\). All EEG tracings (including those during acute status epilepticus) were independently analysed by two investigators blind to the treatment.

**Cerebral tissue preparation**

Ten PILO and 14 MAM–PILO epileptic rats (including EEG recorded rats, Fig. 1), six PILO and eight MAM–PILO rats treated with diazepam (no status epilepticus rats), and an additional five control and five MAM rats not exposed to pilocarpine were anaesthetized with chloral hydrate \((1 \text{ml/100 g body weight of a 4\% solution})\) and perfused with 4\% paraformaldehyde in 0.1 M phosphate buffered saline at pH 7.2. Brains were removed from the skull, post-fixed overnight, and cut with a vibratome into 40–50-\(\mu\)m thick coronal sections, collected in serial order. One series of sections \((1 \text{out of 7 sections})\) was counterstained with 0.1\% thionin and the adjacent sections were processed for immunocytochemistry and immunofluorescence.

For western blot analysis, four PILO and five MAM–PILO epileptic rats (randomly chosen from those rats video monitored, Fig. 1) and the relative controls \((four \text{ control and five MAM})\) were sacrificed by decapitation and their brains immediately removed. Cortical heterotopic areas of MAM and MAM–PILO rats and corresponding areas from control and PILO rats were dissected out from the surrounding normal cerebral areas under microscopic guidance (Gardoni et al., 2003). At the time of sacrifice, epileptic rats were seizure-free for at least 4 h.

**Fluoro-jade staining and immunocytochemistry**

For fluoro-jade staining, sections were mounted in distilled H\(_2\)O on glass slides, air-dried, immersed in a series of graded ethanol
(50, 75, 100, 75, 50%, 3 min each step) and washed in distilled H$_2$O for 3 min. Sections were then treated with 0.06% potassium permanaganate for 15 min, washed three times, immersed in fluoro-jade (0.001% fluoro-jade in 0.1% acetic acid; Histo-Chem, Inc.) for 30 min, and rinsed in distilled H$_2$O. After drying, slides were clarified in xylene and coverslipped with distyrene plasticizer xylene (DPC; BDH Lab Supplies).

The immunocytochemistry and immunofluorescence procedures have previously been described (Finardi et al., 2006). The following primary antibodies were used: monoclonal anti-SMI 311 non-phosphorylated neurofilaments, 1:500 (Sternerberg Monoclonals Inc.); monoclonal anti-neuronal nuclei specific DNA binding protein, 1:3000 (Chemicon International Inc.); monoclonal anti-NR1, 1:100 (Pharmingen); polyclonal anti-NR2AB, 1:1000 (Affinity BioReagents, Golden); polyclonal anti-parvalbumin 1:10 000 (Swant), as marker for GABAergic inhibitory interneurons. Selected sections were processed for double-labelling sequential immunofluorescence. Fluorescent sections were examined on a Radiance 2100 confocal microscope (Bio-Rad).

**Sub-cellular fractioning and western blot analysis**

About 100 mg of fresh cortical tissues was used to obtain purification of postsynaptic enriched fractions. We used a modified Triton insoluble fraction (Gardoni et al., 2009) preparation with sequential protein extraction in different pH-controlled buffers to separate pre- and post-synaptic membranes (Phillips et al., 2001). The P2 synaptosome-enriched pellet was resuspended in hypotonic buffer and centrifuged at 10 000g for 1 h. The pellet was resuspended in glass–glass potter in 1% Triton–x 100, 20 mM Tris HCl (pH 6) buffer containing ethylenediaminetetraacetic acid-free protease inhibitors (complete-ethylenediaminetetraacetic acid free, Boehringer Mannheim GmbH). After 15 min extraction (4°C), samples were centrifuged at 100 000g for 1 h to obtain a membrane pellet (Triton insoluble fraction/pH6) containing both pre- and postsynaptic membrane proteins (Feligioni et al., 2006). To purify postsynaptic membranes from presynaptic proteins, the Triton insoluble fraction/pH6 pellet was further resuspended in a glass–glass potter with a pH 8 Triton 1% buffer. After 15 min extraction (4°C), samples were centrifuged at 100 000g for 1 h to obtain post synaptic pel lent (Triton insoluble fraction/pH 8) and presynaptic supernatant (Triton soluble fraction/pH 8). The Triton insoluble fraction/pH 8 pellet was homogenized in complete-ethylenediaminetetraacetic acid free/20 mM HEPES. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (7% acrylamide), electroblotted onto nitrocellulose and probed with antibodies in 3% non-fat milk or albumin: monoclonal anti-NR1 (1:1000; Pharmingen), anti-NR2A (1:500; Zymed), anti-PDS95 (1:1000; Affinity BioReagents, Golden), anti-synaptophysin (1:3000; Chemicon); polyclonal anti-NR2B (1:500; Zymed), anti-NR2AB (1:1000; Chemicon). A monoclonal antibody against F-actin was used as loading control (1:3000; Chemicon). The optical density of immunoreactive bands was analysed with Quantity–One software (BioRad), normalized versus F-actin signals and compared among groups.

**Data analysis and statistical evaluation**

To evaluate cortical thickness, three thionine-stained coronal sections from the frontoparietal cortex (anterior commissure –0.2 mm, mid-thalamic, –2.3 mm and posterior thalamic –4.8 mm from bregma) were taken 18 h after acute status epilepticus (n = 4 for each group), from epileptic rats 2 months after seizure onset (n = 5 for each group) and from rats experiencing neither status epilepticus nor seizures (n = 4 for each group). Sections were photographed and cortical thickness measured with a Nikon Digital Sight Camera System. Three measures of sensorimotor (Fig. 5B1) or corresponding heterotopic cortical areas were taken per section and averaged to a single value. Values of the three sections per rat were averaged and used to calculate the mean cortical thickness in each rat.

To evaluate the extent of pilocarpine-induced neuronal injury, cell counting was performed in three fluoro-jade stained and three adjacent neuronal nuclei-immunoreacted sections from the frontoparietal cortex (–0.2, –2.3, –4.8 mm from bregma) of four PILO and four MAM–PILO rats sacrificed 18 h after onset of status epilepticus. Sections were independently analysed with the Axioplan 2 Imaging laser scanning system (Zeiss) by two investigators. Neurons stained with fluoro-jade or neuronal nuclei were counted in 0.1 mm$^2$ cortical subfields in layers III and V of PILO or in supra- and infra-granular layers of MAM–PILO rats. We counted neuronal nuclei neurons displaying a nucleus on the plane of the sections and all fluoro-jade positive cells. The fluoro-jade/neuronal nuclei density ratio was quantified in the different layers considered for each rat, and the value averaged for each group. To quantify the density of neurons with NR2AB dendritic staining at the postsynaptic membrane, two coronal sections through the rostral cortex (1.7/0.2 mm from bregma) and two sections through the heterotopic frontoparietal cortex and hippocampus (–1.8/–3.8 mm) were chosen from five MAM–PILO rats, and neurons counted in three adjacent non-overlapping 0.2 mm$^2$ subfields per section. Density values (number of cells/area) from hippocampal CA1 and CA2, rostral and heterotopic cortex were averaged per individual rat, and data from different cortical areas compared and statistically analysed. Although our method bears some limitations if compared with stereological analysis, any counting bias should equally affect the diverse samples considered.

To evaluate somatic area, apical dendrite thickness, cell and nuclear diameter of pyramidal neurons, SMI 311 neurons were identified using a Nikon Microphot FXA microscope with Nomarski differential interference contrast at ×400 magnification, photographed with a Nikon Coolpix camera and analysed with the Image-Pro Plus software. Three sections through the sensorimotor cortex were analysed per rat at the same levels considered for evaluating cortical thickness. At least 100 neurons displaying a nucleus on the plane of the section were analysed from each group (n = 5 rats each group). Cell areas and diameters and apical dendritic thickness (measured at 5 and 15 μm from the upper edge of the nucleus) from the different groups were pooled together and compared. Statistical analyses were performed using one- or two-way analysis of variance followed by Bonferroni as post hoc comparison test.

**Results**

**Pilocarpine dosage setting**

Our pilot dose–response experiments revealed that doses of pilocarpine able to induce status epilepticus in naïve rats induced a very high mortality rate in MAM rats. This evidence is in agreement with the intrinsic neuronal hyperexcitability and higher susceptibility to pro-convulsive agents of MAM rats (Sancini et al., 1998; Chevassus-Au-Louis et al., 1999; Battaglia and Bassanini, 2006). We therefore reduced pilocarpine doses in MAM rats to obtain incidence of status epilepticus and mortality rate similar to naïve rats. We found that 350–360 mg/kg pilocarpine...
Figure 2 Short-term neuropathological effects of status epilepticus. Photomicrographs of representative thionine staining (A–D) and fluoro-jade labelling (E–G) of coronal cortical sections obtained from PILO (A, C and E) and MAM–PILO (B, D, F and G) rats 18 h after onset of status epilepticus. Note in (A) and (C) the presence of two continuous bands of oedema (asterisks) in the neocortex of PILO rats. Inset in (C) magnifies oedematous layer V of PILO rats. Arrows in (B) and (D) mark subcortical heterotopia of MAM–PILO rats. The fluoro-jade-positive neurons were clearly concentrated in layers II and III of PILO (E) and in infragranular layers (F) and subcortical heterotopia (arrow in G) of MAM–PILO cerebral cortex. (H) Quantification of degenerating neurons (percentage ratio of fluoro-jade/neuronal nuclei positive neurons). Significantly higher density of degenerating cells in layer III versus V of PILO (34.9 ± 3.81% versus 9.6 ± 1.82%; ****p < 0.0001) and in infragranular versus supragranular layers of MAM–PILO rats (22.96 ± 4.84 versus 6.1 ± 1.24; **p < 0.01) were found. The overall density of degenerating cortical neurons was higher in PILO than MAM–PILO rats (22.25 ± 2.5% versus 14.5 ± 3%; *p < 0.05). Data are presented as mean percentage ± SD (n = 4 rats from each group). Scale bars: 1 mm in (A–D) (inset, 15 µm); 100 µm in (E–G). SE = status epilepticus.
induced status epilepticus in naïve rats (PILO) more frequently than 320–340 mg/kg (11 out of 13 versus 9 out of 16 rats) with slightly higher mortality (18% versus 11%). In MAM–PILO rats, 270–290 mg/kg pilocarpine induced status epilepticus in the same percentage of rats as those treated with 300–320 mg/kg (11 out of 16 rats versus 14 out of 20 rats), but the lower dosage induced lower mortality (27% versus 50%). We therefore selected for subsequent experiments 360 mg/kg in naïve rats (PILO) and 270 mg/kg in MAM rats (MAM–PILO) as optimal doses to induce status epilepticus in at least 70% of rats with low mortality and similar time of onset and severity of status epilepticus.

**Short-term neuropathological effects of status epilepticus**

We verified the effects of acute status epilepticus in four PILO and four MAM–PILO rats 18 h after onset. As illustrated in Fig. 2, the thionine stained cortical sections from PILO rats (treated with 360 mg/kg pilocarpine) were consistently characterized by two bands of oedema, as revealed by the clearly reduced intensity of staining involving layers II, III and V (Fig. 2A and C, asterisks). In contrast, no oedema was observed in corresponding sections from MAM–PILO rats treated with 270 mg/kg pilocarpine (Fig. 2B and D). In keeping with the presence of oedema, cortical thickness was significantly increased in PILO rats as compared with untreated controls (control: 1576.18 ± 63.38 μm; PILO 18 h: 1809.88 ± 101.77 μm; mean ± SD *P < 0.05), whereas this parameter was not changed in MAM–PILO versus untreated MAM rats (MAM: 866 ± 90.2 μm; MAM–PILO 18 h: 855.50 ± 87.5 μm; not significant). The staining pattern of fluoro-jade, a marker for degenerating neurons, was also different in PILO versus MAM–PILO rat brains (Fig. 2E–G). In PILO rats, fluoro-jade-positive pyramidal neurons were particularly concentrated in superficial cortical layers II–III and, to a lesser extent, in layer V (Fig. 2E). In MAM–PILO rats, they were mainly present in the infragranular layers and subcortical heterotopia (Fig. 2F and G). Quantification of degenerating neurons revealed that fluoro-jade neurons were significantly more abundant in layer III than layer V of PILO rats (35% versus 9%; *P < 0.001, Fig. 2H). In contrast, fluoro-jade neurons were significantly more numerous in infra- than supra-granular layers of MAM–PILO rats.
layers of MAM–PILO rats (22% versus 6%; \( P < 0.01 \), Fig. 2H).
The overall density of degenerating cortical neurons (fluoro-jade labelled over total neurons) was higher in PILO than MAM–PILO cortex (22% versus 14%, \( P < 0.05 \), Fig. 2H).

### Status epilepticus and spontaneous seizures in MAM and control rats

Thirty naïve rats and 54 MAM rats were treated with optimal pilocarpine doses (as described above). Incidence of status epilepticus induction (25/30 in naïve and 39/54 in MAM–rats) and mortality were comparable in both groups and not different from those observed in pilot experiments. Time of status epilepticus onset after pilocarpine was similar in both groups (Fig. 3A). Clinical and EEG features of status epilepticus in naïve and MAM rats were also comparable, with continuous repetitive discharges of fast spikes intermingled with high amplitude sharp wave/slow wave complexes (Fig. 4Aa1 and Bb1). In contrast, the estimated epilepsy onset (the occurrence of the first clinical seizure recorded in our video monitoring protocol) was significantly earlier in MAM–PILO (\( n = 24 \)) than PILO (\( n = 16 \)) rats (\( P < 0.05 \), Fig. 3B).
Figure 5  Morphological effects of chronic epilepsy in cortical tissue. (A1–A4 and B1–B4) Low resolution photographs of representative brains (A) and coronal cortical sections (B, thionine stain) obtained from PILO (A2, B2) and MAM–PILO (A4, B4) rats 2 months after epilepsy onset and from age-matched vehicle injected controls (CTR A1, B1 and MAM A3, B3). Note the reduction in brain size and cortical thickness in MAM–PILO versus MAM rats. Arrow in A4 marks an evident sink on the external surface of the posterior cortex.
Seizure frequency and severity

To evaluate epilepsy severity (frequency and semiology of seizures), we quantified seizure activity in a subset of seven PILO and nine MAM–PILO rats for 2 months after seizure onset. The mean number of Stages 4–7 seizures over a 16 h recording period (12 total overnight recording sessions, one every 5 days) was consistently higher in MAM–PILO than in PILO rats (P < 0.05; Fig. 3C). The average cumulative seizures number per rat observed during the 2-month period was 11.6 ± 3.3 in PILO versus 30.0 ± 6.4 in MAM–PILO rats (P < 0.05; Fig. 3D).

EEG recordings in epileptic MAM versus control rats

To further characterize epilepsy features, we continuously EEG recorded a subgroup of four PILO and four MAM–PILO rats for 8 days, beginning at ~40 days after spontaneous seizure onset. Seizures recorded from the cortical surface had a shorter average duration in PILO rats (21.7 ± 8.1 s) than in MAM–PILO rats (46.3 ± 12.7 s; P < 0.01, Fig. 4C versus E and panel F). EEG seizures in PILO rats were consistently characterized by abrupt onset of spiking activity at ~15 Hz (Fig. 4C, box c2), followed by post-ictal delta slow activity at about 3 Hz (box c3). In contrast, EEG seizures in MAM–PILO rats were characterized by a recruiting rhythmic activity at 7–8 Hz (Fig. 4E, box e1) followed by spiking activity of progressively increased frequency and voltage (box e2), and severe post-ictal depression (box e3). In addition, continuous interictal spiking activity was observed in MAM–PILO (Fig. 4D, box d1) but not in PILO rats. Taken together, these EEG findings confirmed what was shown by video recordings in a different cohort of rats, i.e., the greater severity of seizures in the MAM–PILO group.

Rats not experiencing status epilepticus

Rats treated with pilocarpine and diazepam did not experience status epilepticus (six naïve and eight MAM rats; Fig. 1) as demonstrated by the absence of epileptiform activity during 24 h EEG analysis (Fig. 4Aa2 and Bb2). Thirty days after administration of diazepam and pilocarpine, these rats were continuously video–EEG recorded for 8 days. In contrast to what was observed in rats experiencing status epilepticus, neither electroclinical seizures nor interictal EEG activities were ever observed (Fig. 4G and H).

Morphological abnormalities in the epileptic brain

We evaluated the effect of status epilepticus and subsequent seizures on brain morphology by comparing brain cytoarchitecture in epileptic MAM–PILO and PILO rats versus vehicle-treated MAM and control rats (Fig. 5). As previously reported (Colacitti et al., 1999), the thickness of motor and somatosensory cortical areas was significantly reduced in MAM versus control rats (Fig. 5E; P < 0.001). Seizures in naïve rats (PILO group) reduced hippocampal size (Fig. 5B2), but they did not significantly modify neocortical thickness (Fig. 5E). In contrast, sensorimotor cortical thickness was additionally reduced in MAM–PILO versus MAM rats (P < 0.05, Figs 5B3–4 and E). The cortical thinning of posterior temporal, entorhinal and underlying hippocampal areas was so pronounced in MAM–PILO brains to determine cortical sinks visible on the external brain surface (Fig. 5A2).

At the microscopic level, the more striking feature in the MAM–PILO cortex was the presence of abnormally large pyramidal neurons, frequently grouped in cell clusters, with increased expression of SMI311 positive neurofilaments, particularly in the apical dendrites (Figs 5C3 and 8A–D). These abnormal neurons were detected mainly in the heterotopic cortical areas, corresponding to the entire rostrocaudal extent of sensorimotor cortex (Colacitti et al., 1999; Gardoni et al., 2003). Neuronal cell diameters were significantly enlarged, ranging from 16.02–24.80 μm (mean ± SD: 19.8 ± 2.05 μm) as compared with 9.27–15.28 μm in normal layer III pyramidal neurons (mean ± SD: 12.51 ± 1.2 μm; P < 0.01). Cell nuclear diameters were also significantly enlarged ranging from 12.08–17.78 μm compared with 5.76–10.45 μm in normal layer III pyramidal neurons (MAM–PILO: 13.97 ± 1.55 μm; control: 7.83 ± 1.1 μm; P < 0.01). We quantified soma size and apical dendrite thickness of these SMI311 pyramidal neurons in the four groups of rats. Both cell parameters were significantly increased in MAM–PILO rats when compared with altered pyramidal neurons of MAM rats (P < 0.05; Fig. 5F and G). Histograms of size frequencies from the four groups confirmed the overall greater soma size of SMI311 neurons in MAM–PILO versus MAM rats. The diffuse increase in cell size and the presence of about 15% of SMI311 neurons with size > 600 μm², never observed in pyramidal neurons from control brains (Fig. 5D), likely indicates a diffuse acquired process. SMI311 pyramidal neurons with soma size...
Figure 6  NMDA over-expression in cortical pyramidal neurons of chronic epileptic MAM–PILO rats. (A–D) A clear increase of the NR2AB signal is present in the postsynaptic membranes of cell bodies and apical dendrites (arrows in D) of pyramidal neurons of MAM–PILO as compared with MAM rats (C), with evidence of enlarged spines (arrowhead in D). No increased NR2AB signal was observed either in control (A) or PILO (B) rats. (E–G) Confocal immunofluorescence staining of NR2AB subunits in rostral (E) and heterotopic (F) neocortex.
>600 µm² were <1% in the cortex of PILO rats (Fig. 5D2). Further, double-labelling experiments revealed that the cell bodies and dendrites of enlarged pyramidal neurons over-expressing SMI311 positive neurofilaments were surrounded by parvalbumin-positive boutons, likely representing GABAergic terminals (Fig. 8E, F and G). This morphological feature was not present in MAM–rats or in PILO rats (data not shown).

To exclude that the observed morphological changes were determined by pilocarpine itself rather than being the result of status epilepticus and seizures, we prevented status epilepticus occurrence in PILO and MAM–PILO rats and evaluated the long-term morphological outcome. Neither changes in cortical thickness (Supplementary Fig. 1A–C) nor abnormalities in soma size of SMI311 pyramidal neurons (Supplementary Fig. 1D–F) were found in PILO and MAM–PILO rats in the absence of status epilepticus and seizures.

**NMDA receptor complex in epileptic malformed brain**

Since NMDA receptor complex alterations were previously described in human epileptogenic dysplastic brain (Moddel et al., 2005; Finardi et al., 2006), we analysed the expression and subcellular localization of NMDA receptor subunits in cortical pyramidal neurons of epileptic MAM–PILO and PILO rats. Immunocytochemistry and immunofluorescence analyses did not reveal consistent differences in the intensity and localization of NR1 staining in PILO and MAM–PILO rats when compared with corresponding control groups (data not shown). In contrast, a remarkable increase of somatic and dendritic membrane staining for NMDA regulatory subunits (NR2A/B) was observed in pyramidal neurons from MAM–PILO rats in heterotopic cortical areas (Fig. 6D) as compared with pyramidal neurons from corresponding areas of MAM rats (Fig. 6C). No increase in NR2AB immunostaining was observed in pyramidal neurons from corresponding sensorimotor areas of control and PILO rats (Fig. 6A and B).

As illustrated in Fig. 6E, F and G, confocal immunofluorescence data demonstrated that NR2A/B up-regulation in MAM–PILO brains was evident in pyramidal neurons of heterotopic cortex (Fig. 6F), less pronounced in the rostral cortex (Fig. 6E), which is less affected by MAM treatment, and not present in the hippocampus (Fig. 6G). Quantification of pyramidal neurons with NR2A/B dendritic staining at the postsynaptic membrane revealed that the density of these neurons was significantly higher in heterotopic versus rostral cortical areas of MAM–PILO rats (Fig. 6J). Similar neurons were never found in the hippocampus (Fig. 6J).

Double-labelling experiments demonstrated that in the heterotopic cortex of MAM–PILO rats the NR2AB up-regulation in pyramidal neurons was consistently associated with increased expression of SMI311 neurofilaments (Fig. 6H and I). In addition, we found a remarkable degree of overlapping between soma size frequencies of pyramidal neurons either SMI311 immunopositive or with NR2AB up-regulation at the postsynaptic membrane (Fig. 6K), strongly suggesting that the two phenomena occurred in the very same cells. We also evaluated NR2AB staining in the somatosensory or heterotopic cortex from PILO or MAM–PILO rats experiencing neither status epilepticus nor seizures. No pyramidal neurons with NR2AB over-expression were found in these groups of rats (data not shown).

Morphology data were corroborated by western blot analysis of heterotopic or sensorimotor cortices from five MAM–PILO, five MAM, four PILO and four control rats. We obtained a progressive purification of postsynaptic density proteins, as demonstrated by the selective enrichment of PSD95 and the almost complete absence of synaptophysin in the final Triton insoluble fraction/pH 8 pellet (Fig. 7A). In cortical homogenates, we found a significant decrease of NR2A/NR2B (but not NR1) subunits in both epileptic MAM–PILO versus MAM rats (P < 0.01, Fig. 7B–C) and epileptic PILO versus control rats (P < 0.05, data not shown). In contrast, a significant increase of NR2A/NR2B (but not NR1) subunits was present only in postsynaptic density-enriched fraction of MAM–PILO versus MAM rats (P < 0.01/0.05, Fig. 7B and C), but not in epileptic PILO versus control rats (data not shown). The NR2A/NR2B subunit up-regulation in postsynaptic fraction is consistent with what observed in apical dendrites and spines of enlarged pyramidal neurons (Fig. 6D, F, H and I).

**Discussion**

Using a double-hit model (MAM–PILO rats), we recapitulated the two main features of human malformations of cortical development, namely abnormal cortical structure and recurrent spontaneous seizures. MAM rats have long been used to model human brain malformations (Chevassus-au-Louis et al., 1998a; Colacitti et al., 1999; Gardoni et al., 2003; Calcagnotto and Baraban, 2005), but they suffer from the virtual lack of seizure occurrence (Harrington et al., 2007). Therefore, no information is available

---

**Figure 6 Continued**

and in the hippocampus (G) of chronic epileptic MAM–PILO rats. Note that the NR2AB up-regulation is more evident in the enlarged neurons of the heterotopic (F) than the rostral (E) cortex, and not evident in the hippocampal CA2 region (G). (H and I) Confocal immunofluorescence of NR2AB (green)/SMI311 (red) double-labelling in enlarged pyramidal neurons of MAM–PILO rats. Increased cell size and neurofilament expression are associated with NR2AB recruitment at the postsynaptic membrane (arrows). Arrowhead in (H) marks an enlarged dendritic spine. (J) Histogram reporting the density of enlarged pyramidal neurons with NR2AB dendritic staining at the postsynaptic membrane in different cerebral areas of MAM–PILO rats. Higher density was found in heterotopic versus rostral cortical areas (11.15 ± 3.0 versus 2.19 ± 2.1 cells/0.2 mm²; ***P < 0.001, data presented as mean density ± SD in n = 5 MAM–PILO rats). No neurons were detected in the hippocampus. (K) 3D histogram comparing size frequency distributions of single-labelled SMI311 and NR2AB positive neurons in heterotopic MAM–PILO neocortex (n = 5 rats). Scale bars: 25 µm. Het ctx = heterotopic cortex; Hipp = hippocampus; Ros ctx = rostral cortex.
Status epilepticus-induced changes in MAM rats

Membranes were obtained for NR2A and NR2B of epileptic cortical homogenate and parallel increases in postsynaptic percentage of values in MAM rats. Significant reductions in the months after epilepsy onset. Optical density ratio is expressed as

\[
\frac{\text{optical density ratio of NMDA subunits/actin in control MAM}}{\text{clonal antibody (data not shown). (right). Similar results were obtained using anti-NR2AB polyclonal antibody. The double blots were revealed with anti-PSD-95 (postsynaptic marker) and synaptophysin (presynaptic marker) antibodies. The double pH-controlled Triton extractions yielded highly purified postsynaptic (Triton insoluble fraction/acid 8, lane 7) proteins. (B) Western blot (WB) of the heterotopic cortex from two representative MAM and two representative epileptic MAM–PILO rats sacrificed 2 months after epilepsy onset. NR1 protein levels did not change in both the homogenate (left) and postsynaptic membrane enriched fractions (Triton insoluble fraction/acid 8, right). In contrast, NR2A and NR2B levels were decreased in total homogenate (left) while they were increased in postsynaptic membranes (right). Similar results were obtained using anti-NR2AB polyclonal antibody (data not shown). (C) Histogram showing the optical density ratio of NMDA subunits/actin in control MAM \( n = 5 \) and chronic epileptic MAM–PILO rats \( n = 5 \) sacrificed 2 months after epilepsy onset. Optical density ratio is expressed as percentage of values in MAM rats. Significant reductions in the cortical homogenate and parallel increases in postsynaptic membranes were obtained for NR2A and NR2B of epileptic.

Figure 7 Altered subcellular distribution of NMDA regulatory subunits in chronic epileptic MAM–PILO rats. (A) Subcellular fractionation used in the present study. Lane 1: total heterotopic cortical homogenate; lanes 2 and 3: Triton insoluble and soluble fractions after single Triton extraction; lanes 4–7: Triton insoluble and soluble fractions after double pH-controlled Triton extractions. Equal amount of proteins (15 μg) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and blots were revealed with anti-PSD-95 (postsynaptic marker) and anti-synaptophysin (presynaptic marker) antibodies. The double Triton extraction yielded highly purified postsynaptic (Triton insoluble fraction/acid 8, lane 6) and presynaptic (Triton soluble fraction/acid 8, lane 7) proteins. (B) Western blot (WB) of the heterotopic cortex from two representative MAM and two representative epileptic MAM–PILO rats sacrificed 2 months after epilepsy onset. NR1 protein levels did not change in both the homogenate (left) and postsynaptic membrane enriched fractions (Triton insoluble fraction/acid 8, right). In contrast, NR2A and NR2B levels were decreased in total homogenate (left) while they were increased in postsynaptic membranes (right). Similar results were obtained using anti-NR2AB polyclonal antibody (data not shown). (C) Histogram showing the optical density ratio of NMDA subunits/actin in control MAM \( n = 5 \) and chronic epileptic MAM–PILO rats \( n = 5 \) sacrificed 2 months after epilepsy onset. Optical density ratio is expressed as percentage of values in MAM rats. Significant reductions in the cortical homogenate and parallel increases in postsynaptic membranes were obtained for NR2A and NR2B of epileptic.

regarding the impact of seizures on the malformed brain (Choi et al., 2005).

Here, combined behavioural, EEG, morphological and molecular analyses demonstrate that the occurrence of status epilepticus eventually exacerbates the cyto-architectural and NMDA cortical abnormalities induced by MAM administration. Prenatal MAM is capable of inducing microcephaly and alterations of apical and basal dendritic morphology (Garbossa and Vercelli, 2003), but the increased cell, nuclear and dendritic size, expression of neurofilaments and postsynaptic localization of NR2A/NR2B subunits are specific for cortical pyramidal neurons in MAM rats only after experiencing status epilepticus. We have focused our analysis mainly on the neocortex rather than the hippocampus, since MAM-induced abnormalities are specific for the neocortex, and even hippocampal heterotopia are of neocortical origin (Chevassus-Au-Louis et al., 1998b, c; Battaglia et al., 2003a, b).

Epileptic MAM–PILO rats show neocortical abnormalities that bear similarities with those observed in human focal cortical dysplasias (Blümcke et al., 2011). Some pathological features we have described differ from those observed in type IIB focal cortical dysplasia. This condition is not associated with cortical atrophy, and our model does not reproduce grey–white matter blurring and balloon cells (Taylor et al., 1971; Andres et al., 2005; Blümcke et al., 2011). MAM–PILO rats could mimic acquired forms of cortical dysplasia (type III focal cortical dysplasias, Blümcke et al., 2011) because of the cortical disorganization and atrophy and the presence of hypertrophic neurons. However, some differences exist: in MAM–PILO rats hypertrophic pyramidal neurons are more frequent; brain damage occurs during early corticogenesis and not perinatally; and brain damage is less severe than that reported in acquired human focal cortical dysplasias (Marin-Padilla, 1999; Marin-Padilla et al., 2002). Whatever the precise relationship between our double-hit model and a specific type of human focal cortical dysplasia could be, the altered cortical laminarization with no distinction between layers (Fig. 8A), the significantly enlarged cell body (Fig. 8B), neurofilament accumulation (Fig. 8C and D) and increased parvalbumin-positive puncta surrounding soma and proximal dendrites of abnormal pyramidal neurons of MAM–PILO rats (Fig. 8E, F and G) are all features described in patients with focal cortical dysplasia (Sprefico et al., 1998; Marin-Padilla, 1999; Alonso-Nanclares et al., 2005; Blümcke et al., 2009, 2011). Therefore, some of the biological phenomena responsible for these changes in MAM–PILO epileptic brains might also occur in human focal cortical dysplasia, underscoring the instrumental value of our model for investigating human focal cortical dysplasia pathogenesis (Marin-Padilla, 1999).

Seizure analysis confirmed the previously reported greater susceptibility of MAM rats to pro-convulsant agents (de Feo et al., 1995; Germano et al., 1998; Chevassus-Au-Louis et al., 1998b;
Figure 8  Morphological features of enlarged pyramidal neurons of chronic epileptic MAM–PILO rats. (A) Low-power microphotograph of a thionine-stained coronal section from heterotopic cortex. Note the absence of recognizable layering and the presence of heterotopic neurons in layer I (arrows) and white/grey matter interface (arrowheads). (B and D) High-power photomicrographs of thionine stained (B) or SMI311 positive enlarged pyramidal neurons (C and D). Note the clustering of abnormal neurons [in (B) and (C)], and the increased
Battaglia and Bassanini, 2006). Indeed, MAM rats developed status epilepticus with a dose of pilocarpine ineffectve in normal rats, the onset of spontaneous seizures was accelerated, and epilepsy was more severe. In contrast, cortical damage observed 18 h after status epilepticus induction was greater in PILO (i.e. in naïve rats treated with higher pilocarpine doses) than MAM–PILO rats. This finding suggests that the more severe epilepsy in MAM–PILO rats is not related to a direct pilocarpine effect, rather to the intrinsic hyperexcitability of the MAM model. The causal relationship between status epilepticus (and not pilocarpine), seizures, and morphologic changes is demonstrated by MAM–PILO/no status epilepticus rats, which are not characterized by chronic epilepsy or by any morphologic change.

The EEG data demonstrated that spontaneous seizures, as recorded from the cortical surface, were consistently longer in MAM–PILO than PILO rats. The continuous interictal spiking (Fig. 4D) observed in MAM–PILO rats was similar to what is commonly observed in scalp and stereo-EEG recordings from patients with focal cortical dysplasia (Palmini et al., 1995; Gambardella et al., 1996; Tassi et al., 2002).

Morphological and molecular data demonstrated that the occurrence of status epilepticus eventually modified the structure of heterotopic cortical pyramidal neurons. At the morphological level, the reduction of cortical thickness, the increased size, apical dendrite thickness and neurofilament expression of malformed cortical pyramidal neurons were all features significantly affected by status epilepticus and subsequent seizures. Thus, our data extend to the malformed brain previous findings of seizure-related structural changes in experimental and human epilepsy (Dudek and Sutula, 2007). The enlarged pyramidal neurons, already reported in dysplastic and non-dysplastic epileptic patients (Andres et al., 2005), suggest the existence of increased excitatory activity in MAM–PILO cortex (Cepeda et al., 2005). The GABAergic terminals enveloping the dysplastic neurons, also reported in human focal cortical dysplasias (Spreafico et al., 1998; Alonso-Nanclares et al., 2005) and in experimental models of acquired focal cortical dysplasia (Marín-Padilla et al., 2003), might be a compensatory mechanism of GABAergic interneurons mediating inhibition onto abnormal excitatory neurons. The western blot data demonstrated subcellular redistribution of NMDA receptors and the specific recruitment of NR2A/B subunits to the postsynaptic membrane in the enlarged abnormal pyramidal neurons.

As glutamatergic excitation requires postsynaptic NMDA anchoring, the NR2A/NR2B over-expression in enlarged pyramidal neurons of MAM–PILO rats may enhance NMDA-mediated cortical excitability. The postsynaptic recruitment of both NR2A and NR2B subunits, which are differentially regulated during normal brain development (Monyer et al., 1994), may reflect abnormalities in NMDA receptor composition, potentially exerting relevant downstream effects on enlarged pyramidal neurons. The decrease of NR2A/B subunits observed in the homogenate fraction can be accounted for by post-translational modifications, such as phosphorylation (Huo et al., 2006) or proteolytic C-terminal cleavage (Simpkins et al., 2003; Yuen et al., 2008), which may alter the antigenicity of full-length subunits. Although these post-translational changes need to be further investigated, our results clearly highlight the NMDA receptor complex as a key component of the synaptic remodelling in MAM–PILO rat brains. NMDA up-regulation was previously described in the dysplastic neurons of epileptic patients with focal cortical dysplasia (Mikuni et al., 1999; Najmi et al., 2000; Moddel et al., 2005; Finardi et al., 2006), thus indicating that the association of malformed neurons and NMDA abnormalities is a biological marker of the epileptic malformed brain in both experimental settings and humans (Qu et al., 2009).

The morphological/biochemical features of MAM–PILO rats are not present in MAM rats without seizures (Gardoni et al., 2003), or in epileptic PILO rats or in MAM–PILO rats just after status epilepticus, thus indicating that neither cortical malformations nor status epilepticus alone are sufficient to determine the reported abnormalities. It is likely that the combination of cortical malformation and status epileptics sets in motion the pathological plasticity leading to cellular and molecular alterations. Further experiments are needed to reveal whether this process is progressive and further affected by spontaneous seizures. In addition, longer follow-up in epileptic PILO rats will be necessary to verify whether the observed changes might also eventually be detectable in chronic non-malformed epileptic rat brain.

In conclusion, our data demonstrate that status epilepticus in MAM–PILO rats triggers a pathological process capable to affect the pre-existing cytologic and NMDA abnormalities. The present data have relevant translational implications since the comparison with human focal cortical dysplasia patients suggests the existence of similar maladaptive neuronal plasticity, which may affect the propensity of generating seizures.

Acknowledgements

We thank Dr Stefania Bassanini for providing relevant contributions in the early phases of this work and Professor Giuliano Avanzini for critically reading the article.

Funding

Italian Ministry of Health (to G.B., in part); the ‘Sovvenzione Globale Ingenio – Regione Lombardia’ from the Social European Fund (Grant n. A0000844 to A.F., in part); Fondazione Monzino (to A.V., in part).

Figure 8 Continued

expression of neurofilaments [in (C) and (D)] in clearly misoriented dendrites. Cell/nuclear diameters in (D) (bar): 20.26 μm/12.16 μm. (E and F) High-power photomicrographs of parvalbumin-immunoreactive synaptic boutons surrounding enlarged pyramidal neurons (arrows). (G) Confocal immunofluorescence image of parvalbumin (green)/SMI311 (red) double-labelling showing increased parvalbumin synaptic boutons (arrows) contacting an enlarged pyramidal neurons. Scale bars: 250 μm in (A); 20 μm in (B–F); 25 μm in (G). PV = parvalbumin.
Supplementary material

Supplementary material is available at Brain online.

References


Huo JZ, Dykstra CM, Gurd JW. Increase in tyrosine phosphorylation of the NMDA receptor following the induction of status epilepticus. Neurosci Lett 2006; 401: 266–70.


Yuen EY, Ren Y, Yan Z. Postsynaptic density-95 (PSD-95) and calcineurin cleavage in cortical neurons. Mol Pharmacol 2008; 74: 360–70.