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To cite this version:
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Received June 23, 2009; Accepted July 23, 2009

ABSTRACT – Epilepsy-associated glioneuronal malformations (malformations of cortical development [MCD]) include focal cortical dysplasias (FCD) and highly differentiated glioneuronal tumors, most frequently gangliogliomas. The neuropathological findings are variable but suggest aberrant proliferation, migration, and differentiation of neural precursor cells as essential pathogenetic elements. Recent advances in animal models for MCDs allow new insights in the molecular pathogenesis of these epilepsy-associated lesions. Novel approaches, presented here, comprise RNA interference strategies to generate and study experimental models of subcortical band heterotopia and study functional aspects of aberrantly shaped and positioned neurons. Exciting analyses address impaired NMDA receptor expression in FCD animal models compared to human FCDs and excitatory imbalances in MCD animal models such as lissencephaly gene ablated mice as well as in utero irradiated rats. An improved understanding of relevant pathomechanisms will advance the development of targeted treatment strategies for epilepsy-associated malformations.

Key words: epileptogenesis, glutamate receptors, methylazoxymethanol, pilocarpine, in utero irradiation, MAEUK proteins, MCD, animal model

Circumscribed malformative lesions of the CNS comprise a wide spectrum of neuroradiological and histomorphological alterations that are an important cause of developmental disabilities and focal epilepsy in humans. They are clinically as well as histologically diverse, ranging from subtle architectural aberrations to highly dysplastic lesions with respect to cytological and structural characteristics. Present classifications of malformations of cortical development (MCDs) are based on histological features such as loss of cortical lamination, glio-neuronal and/or neuronal heterotopias, the occurrence of

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Presented at the “Malformations of Cortical Development and Epilepsy” Symposium, organized in Istanbul, Turkey (October 30-31, 2008) by the ILAE Commissions of Neurobiology and Therapeutic strategies together with the Cerrahpasa Medical Faculty of Istanbul University.
The use of RNA interference to generate and study experimental models of subcortical band heterotopia

The first two presentations by Lo Turco and Represa describe experimental models of subcortical band heterotopia (SBH). In human patients, SBH is a cortical malformation characterized by the presence of large clusters of neurons within the white matter underlying a relatively normal cortex, determining the so-called “double cortex”. This condition has been reported in a woman with mutation of the DCX gene, and in patients with mutations of TUBA1A and LIS1 genes (Guerrini et al., 2008) but other genes are most likely involved. SBH is associated with mental retardation and intractable infantile epilepsies. The available animal models of band heterotopia are impinged by intrinsic limitations. DCX<sup>-/-</sup> and LIS1<sup>-/-</sup> mice have been generated but they display no neocortical abnormality, therefore lessening their impact on the field (Cahana et al., 2001; Corbo et al., 2002; Hirotsune et al., 1998). An interesting model of band heterotopia is the TISH mutant rat (Schottler et al., 1998); however, the gene responsible for this phenotype remains unknown and the phenotype involves quite different molecular and cellular mechanisms. More recently a similar “orphan” model of band heterotopia, HeCo mutant mice, has been reported (Croquelois et al., 2008), but the model has not been yet fully characterized. Consequently, we have still little information on the neurobiology of SBH and the cellular and network properties of neurons within the “double cortex”.

In contrast, the in utero knock-down of DCX RNA produces a morphologically relevant band heterotopia in rodents (Bai et al., 2003) providing an excellent tool to investigate this issue.

The group of LoTurco addresses the issue of whether neocortical malformations and associated functional impairments can be reversed by reactivating developmental programs in the developing brain once malformations are detected. They rely on an animal model of SBH in which RNA interference (RNAi) is first used to induce the brain malformation, then conditional re-expression of the targeted gene is used to restart migration after the malformation has already formed (Manent et al., 2009; figure 1). The authors found that it is possible to re-start migration in the early postnatal rat brain, and this reactivation of migration reduces the size of SBH and restores neuronal patterning. They observed further that the capacity to regress heterotopia and restore normal migration patterns shows a critical period in early postnatal development. Moreover, reactivation of migration reduces seizure threshold to injection of PTZ to levels similar to that of malformation-free control animals. These results suggest that future approaches directed at restarting migration during a critical period of development may reduce the risk of seizure development in malformed cerebral neocortex.

The group of Represa relies on the same RNAi preparation to compare the neuronal and network properties of heterotopic neurons, cortical neurons overlying the heterotopia and control neurons to identify how heterotopic neurons generate adverse patterns that will impact cortical activity (Ackman et al., 2009; figure 2). By combining dynamic calcium imaging and anatomo-electrophysiological techniques, they demonstrate that DCX<sup>+</sup>EGFP<sup>+</sup> labelled heterotopic neurons that fail to migrate, develop extensive axonal subcortical projections, retain immature properties and display a dramatic absence of GABA-mediated signalling (Ackman et al., 2009). Cortical neurons overlying the heterotopia exhibit, in contrast, a massive increase of ongoing glutamatergic synaptic currents reflecting a strong reactive plasticity. Neurons in both experimental fields are more frequently coactive in coherent synchronized oscillations than control cortical neurons. In addition, both fields display network-driven oscillations during evoked epileptiform burst. Represa reported preliminary data supporting the notion that epileptiform activities initiate from neurons on the cortex overlying the heterotopia and involve the heterotopic neurons in a relatively synchronized way (Ackman et al., 2009). These results show that DCX cortex involves major alterations not only in neurons that fail to migrate but also in their programmed target areas. Based on these observations, the author suggests that this duality plays a major role in cortical dysfunction of DCX brains.
These observations are in agreement with previous reports from human patients depicting BOLD changes on EEG-fMRI analysis during ictal and interictal events in both fields (Kobayashi et al., 2006). The analysis of other models of brain malformation, such as MAM- or γ-ray-treated rats (Colacitti et al., 1999; Roper, 1998), suggests similarly that ectopic neurons are not epileptogenic per se but they participate in the epileptiform activities generated by convulsant agents or protocols, through their strategic position at the frontier of two different fields, the hippocampus and the somatosensory cortex. Indeed, ectopic neurons in MAM rats are neurons of cortical origin that maintain their cortical and molecular approach to investigate possible molecular mechanisms of epileptogenesis shared by both human patients and experimental models. They have analyzed epileptogenic cortical samples from human patients affected by type IA and IIB FCD obtained during epilepsy surgery. The experimental counterpart is provided by a “double-hit” rat model, generated by inducing brain malformations with prenatal exposure to methylazoxymethanol (MAM) and spontaneous recurrent seizures induced by post-natal pilocarpine treatment in MAM-treated adult rats. Their results clearly indicate the presence of NMDA receptor complex abnormalities in both type IA and IIB focal cortical dysplasia. In type IA FCDs, a selective increase in the regulatory subunit NR2B was present in all considered cases. NR2B up-regulation was greater in the total homogenate than the post-synaptic membrane fraction, suggesting

Figure 1. Temporal control of Dcx expression in misplaced neurons by conditional re-expression of the Dcx gene (from Manent et al., 2009). A) A schematic diagram of the experimental approach to verify the hypothesis that re-expression of Dcx can re-start migration in the early postnatal rat brain, reduce the size of SBH and restore neuronal patterning. B) A 4-hydroxytamoxifen (4-OHT)–activatable Cre recombinase composed of two estrogen receptor (ER) binding domains is expressed under the control of the CAG promoter. In the presence of 4-OHT, recombination occurs and DCX-eGFP is expressed. C) Confocal images showing transfected neurons in neocortex of P15 rats that have received 4-OHT or vehicle injection at birth. Rats were electroporated at E14 with four plasmids. In 4-OHT–treated rats (left), DCX-GFP is expressed and Dcx is detectable with antibodies in transfected misplaced neurons. In vehicle-treated rats (right), no signal is detected in the green channel or with Dcx antibodies. D) Summary of the transfection conditions. E) Schematic diagram of experiments. Scale bar, 50 µm.

Morphological and molecular comparison between human patients and experimental models of focal cortical dysplasia

Battaglia et al. are currently investigating the NMDA receptor complex and related protein composition in focal cortical dysplasia (FCD), which is the more common type of MCD in human patients, characterized by a severe epilepsy course, often requiring epilepsy surgery for seizure control. They have used the same combined morphological and molecular approach to investigate possible molecular mechanisms of epileptogenesis shared by both human patients and experimental models. They have analyzed epileptogenic cortical samples from human patients affected by type IA and IIB FCD obtained during epilepsy surgery. The experimental counterpart is provided by a “double-hit” rat model, generated by inducing brain malformations with prenatal exposure to methylazoxymethanol (MAM) and spontaneous recurrent seizures induced by post-natal pilocarpine treatment in MAM-treated adult rats. Their results clearly indicate the presence of NMDA receptor complex abnormalities in both type IA and IIB focal cortical dysplasia. In type IA FCDs, a selective increase in the regulatory subunit NR2B was present in all considered cases. NR2B up-regulation was greater in the total homogenate than the post-synaptic membrane fraction, suggesting
that mechanisms other than increased ionic influx through the post-synaptic membrane may sustain hyperexcitability in dysplastic neurons (Finardi et al., 2006; figure 3). By contrast, in type IIB FCDs, a more intense NR2B staining was particularly evident in the apical dendrites and enlarged dendritic spines of giant pyramidal neurons. Accordingly, the molecular analysis showed a significant and selective increase of NR2A/B subunits and their scaffolding proteins, SAP97 and PSD95, in the post-synaptic membrane compartments. Since this up-regulation was not accompanied by an increased activation state of CREB and ERK1/2, it may reflect the presence of excessive extra-synaptic NMDA receptors, which may trigger intracellular pathways in the giant dysplastic cortical neurons underlying their pathological features.

In the analysis of the double-hit MAM/pilocarpine model, the authors observed that the onset of spontaneous seizures was anticipated and that seizure number and frequency was increased when compared to control rats treated with pilocarpine. These data confirm that in the MAM model the presence of brain malformations reduces the threshold to pro-convulsant stimuli. In addition, the
histological and molecular analysis of fractions of cortical malformed tissue enriched in post-synaptic densities, revealed a clear-cut increase in the expression level of the NMDA regulatory subunits in the chronic phase of epilepsy, with a consistent recruitment of the same subunits in the apical dendrites of large and dysplastic pyramidal neurons. The NR2A/B up-regulation was associated with increased cell size and expression of cytoskeletal elements, similarly to what was observed in giant dysplastic pyramidal neurons of human FCD IIB patients.

Taken together, data collected both from human MCDs tissues and MAM-pilocarpine treated rats support the existence of a relevant plasticity of the NMDA receptor complex, which could play a role in the basic mechanisms...
of hyperexcitability associated with MCDs. Moreover, they indicate that MAMPicroinjection rats represent a suitable double-hit model to study the pathogenic mechanisms of epilepsy and possibly the pathological neuronal plasticity associated with repeated seizures in human MCDs.

Altered balance between excitation and inhibition in different animal models of MCDs

The last two speakers of the basic science session, Baraban and Roper utilize similar neurophysiological approaches to investigate the balance between excitatory and inhibitory inputs in two different animal models, the Lis1+/– mice and the in utero irradiated rats. It is well known from human studies that a heterozygous mutation or deletion of the lissencephaly gene Lis1 is associated with severe disruption of cortical and hippocampal lamination, cognitive deficit and severe seizures. Mice with one null allele of Lis1 (Lis1+/– mice) exhibit significant hippocampal malformations and slowed migration of interneuron precursors. In addition, preliminary video-EEG data demonstrated spontaneous electrographic seizures in some of these mice. Baraban et al. first analyzed glutamate-mediated synaptic excitation of this model. Isolated whole-cell excitatory postsynaptic current (EPSC) on visually identified pyramidal neurons in disorganized CA1 regions of hippocampal slices prepared from Lis1+/– mice were recorded. They observed a two-fold increase in spontaneous and miniature EPSC frequency with no change in amplitude or decay kinetics, and using paired-pulse and 10 Hz stimulation protocols, a dramatic increase in presynaptic excitation. Deficits in paired-pulse facilitation could be restored to wild-type levels with the addition of cadmium chloride, a voltage-activated calcium channel blocker. Electron microscopy analysis revealed a significant increase in synaptic vesicle density with no change in the size of the active zone or terminal area. Taken together, this first set of experiments suggests the existence of an excitable and pro-epileptic circuit in the malformed hippocampus of Lis1 mutant mice. Second, they analyzed GABA-mediated synaptic inhibition by recording isolated whole cell inhibitory postsynaptic currents (IPSCs) on visually identified CA1 pyramidal neurons in slices prepared from Lis1+/– mice. They observed a 32% increase in spontaneous IPSC frequency in Lis1+/– mice compared with normotopic CA1 pyramidal neurons in age-matched controls. This increase was not associated with a change in spontaneous IPSC decay or miniature IPSC frequency. Mean IPSC amplitude was increased, and event histograms indicated a greater number of large (> 125 pA) events. Tonic inhibition, response to paired-pulse stimulation and evoked IPSC decay kinetics were not altered. Consistent with increased synaptic inhibition, Lis1+/– interneurons also exhibited more spontaneous firing in cell-attached recordings and increased excitation as measured by voltage-clamp recording of spontaneous excitatory postsynaptic currents (EPSCs) onto interneurons (Jones and Baraban, 2007; figure 4). This up-regulation of inhibition may be a compensatory response to the increased excitation and hyperexcitability observed in these animals. In addition to neurophysiological abnormalities, the authors also observed a significant disruption of the subgranular zone and glial fibrillary acidic protein-immunoreactive radial astrocytes in the dentate gyrus of adult LIS1 mice. Using pulse-chase bromo-deoxyuridine (BrdU) labeling combined with neuronal and glial antibody staining, they provide evidence for ectopic adult neurogenesis in LIS1 mice. A gradually decreased survival rate for these newborn granule cells was also demonstrated in LIS1 mice seven days after BrdU injection. This reduced survival rate was associated with impaired neuronal differentiation 28 days after BrdU administration. Thus, LIS1 haploinsufficiency can lead to abnormal cell proliferation, migration and differentiation in the adult dentate gyrus. A similar approach was undertaken by Roper and colleagues using a different model, the in utero irradiated rats (Chen et al., 2007; figure 5). This model was first described to study abnormal brain development (Cowan and Geller, 1960) but epilepsy was not a focus of these early investigations. The protocol involves exposure of a pregnant dam to external radiation (225 cGy of radiation from a linear accelerator source) on the 17th day of gestation (E17). The offspring are born on E21 and weaned on P21. Cortical malformation in this model is the result of two related processes: cellular damage from the initial radiation injury and continued addition of new cells to the neocortex after injury. The initial damage is extensive and necrosis can be seen throughout the neocortex, particularly in the intermediate layer, with relative sparing of progenitor cells in the ventricular zone and neurons already migrated into the cortical plate. There is some evidence that migrating, immature neurons are the most susceptible to localise at the radiation injury (Altman et al., 1968). Histologically, treated animals are microcephalic, with loss of lamination, abnormal spatial orientation of neurons, periventricular and subcortical heterotopia, focal areas of heterotopic neurons in the hippocampus and hypoplasia or agenesis of the corpus callosum. Neither giant dysmorphic neurons nor balloon cells were ever described. Therefore, in utero irradiation is an injury-based model of diffuse cortical malformation similar to type I FCD, according to the Palmini classification system (Palmini et al., 2004). Spontaneous seizures have been previously described in these animals (Kellinghaus et al., 2004). In this model, a striking feature reported by the authors is the impaired development of the inhibitory system in the dysplastic cortex, demonstrated by using both histological...
and physiological methods. In early experiments performed in adult irradiated and control animals, two-dimensional cell counting data showed a 50% reduction in the density of parvalbumin (PA) and calbindin D28k (CB) immunoreactive interneurons with no reduction in total neuronal density (Roper et al., 1999). This evidence suggests a relative reduction in the number of inhibitory interneurons in the dysplastic cortex. It should be underlined that a relative loss of cortical interneurons was also reported in studies from human FCD patients (Calcagnotto et al., 2005).

They have also evaluated functional connectivity in dysplastic cortex using spontaneously occurring post-synaptic currents (PSCs) (Zhu and Roper, 2000). Using whole cell patch clamp methods, they recorded excitatory and inhibitory PSCs (EPSCs and IPSCs) from large pyramidal cells in irradiated and control neocortex. They found a reduction in the frequency of spontaneous and miniature (post-tetrodotoxin) IPSCs. Conversely, spontaneous EPSCs had an increased frequency in dysplastic cortex, and miniature EPSCs were no different from controls. Consistent with the immunohistochemical findings, this evidence suggests a reduction in inhibitory connections as compared to excitatory contacts in the irradiated cortex. Identical findings were obtained in the subcortical heterotopic grey matter of irradiated rats (Chen and Roper, 2003).

The authors also recorded EPSCs from surviving interneurons in the irradiated rats identifying two types of interneurons on the basis of their physiological properties (Xiang et al., 2006): fast-spiking with short-term depression (type I) and regular-spiking with short-term facilitation (type II) interneurons. Both types of interneurons showed a reduced frequency of EPSCs in dysplastic cortex, therefore suggesting the existence of a reduced excitatory drive on the surviving interneurons in this model. The authors also addressed the issue of short-term plasticity (STP), which reflects dynamic function of cortical synapses and exerts relevant effects on the neuronal circuitry. In normal layer V pyramidal cells, repetitive activation of pre-synaptic axons results in short-term depression of EPSCs at age two weeks, then switching to short-term facilitation at age four weeks (Reyes and Sakmann, 1999). This developmental switch involves increased activation of pre-synaptic type 2/3 metabotropic glutamate receptors (Chen and Roper, 2004), most likely resulting from increased extracellular glutamate concentrations during this age period. In irradiated rats, the normal switch to short-term facilitation did not occur in cortical pyramidal neurons (Chen and Roper, 2007). Indeed, these animals demonstrated the persistence of short-term depression at two and four weeks, and in

Figure 4. An increase in excitatory drive onto GABAergic interneurons underlies enhanced activity of inhibitory systems in the hippocampus of Lis1 mice (from Jones and Baraban, 2007). A) Top: whole cell voltage-clamp recording of sEPSCs on a WT interneuron. Bottom: same cell recorded in current-clamp mode. B) Top: sEPSC recording from a Lis1 interneuron. Bottom: same cell recorded in current-clamp mode. C) Mean sEPSC frequency is increased in Lis1 interneurons (p < 0.05, Student’s t-test). D) Mean sEPSC amplitude is similar between WT and mutant mice. E) Mean sEPSC decay time is similar between WT and mutant mice.
adulthood. This suggests an abnormal persistence of increased release probability in the excitatory pre-synaptic terminals synapsing on large pyramidal cells, therefore potentially leading to increased excitability of the dysplastic cortex.

In summary, both groups have demonstrated a number of abnormalities in the balance between inhibition and excitation in the malformed cortical circuitry in the two models of genetic and acquired cortical malformations which may be relevant to increase the propensity for epileptiform activity.

Figure 5. In utero irradiated rat model of cortical dysplasia. Pyramidal cells from the dysplastic cortex (CD) are spatially disorganized and display a regular spiking pattern to suprathreshold current pulses. A) Low power photomicrographs of coronal sections of control (left) and dysplastic (right) cortex with cresyl violet staining. B) Photomicrographs (upper) show two biocytin-stained pyramidal cells from dysplastic cortex. Arrows point to pia. Traces (lower) show spike patterns of these two cells to depolarizing current injection (300 ms, 300 pA).
Conclusion

The development and characterization of clinically relevant models of cortical malformations is a major challenge for basic science to study the mechanisms underlying aberrant neuronal migration and the associated epileptic phenotype. The basic science session held at “Malformations of Cortical Development and Epilepsy” Symposium has provided a multidisciplinary approach to this issue: the use of modern molecular biology techniques associated with the close comparison at morphological, molecular and neurophysiological levels, between experimental and human malformed cortical samples. A major task remains to carefully characterize the epileptic phenotype, or decreased seizure threshold, in the genetically determined acquired models of cortical malformations. These research efforts are instrumental to increase our understanding of the pathogenetic mechanisms and to envisage novel targets and effective therapeutic approaches for treating epilepsy in human MCD patients. □

Disclosures.
None.

References


