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Embryonic and Early Postnatal Abnormalities Contributing to the Development of Hippocampal Malformations in a Rodent Model of Dysplasia

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Abstract

While there are many recent examples of single gene deletions that lead to defects in cortical development, most human cases of cortical disorganization can be attributed to a combination of environmental and genetic factors. Elucidating the cellular or developmental basis of teratogenic exposures in experimental animals is an important approach to understanding how environmental insults at particular developmental junctures can lead to complex brain malformations. Rats with prenatal exposure to methylazoxymethanol (MAM) reproduces many anatomical features seen in epilepsy patients. Previous studies have shown that heterotopic clusters of neocortically-derived neurons exhibit hyperexcitable firing activity and may be a source of heightened seizure susceptibility, however the events that lead to the formation of these abnormal cell clusters is unclear. Here, we used a panel of molecular markers and birthdating studies to show that in MAM-exposed rats, the abnormal cell clusters (heterotopia) first appear postnatally in the hippocampus (P1–P2) and that their appearance is preceded by a distinct sequence of perturbations in neocortical development: (i) disruption of the radial glial scaffolding with premature astroglial differentiation and (ii) thickening of the marginal zone with redistribution of Cajal-Retzius neurons to deeper layers. These initial events are followed by disruption of the cortical plate and appearance of subventricular zone nodules. Finally, we observed the erosion of neocortical subventricular zone nodules into the hippocampus around parturition followed by migration of nodules to hippocampus. We conclude that prenatal MAM exposure disrupts critical developmental processes and prenatal neocortical structures ultimately resulting in neocortical disorganization and hippocampal malformations.

Keywords

Cajal-Retzius; Cortical Dysplasia; Epilepsy; Hippocampus; Reelin

Normal brain development involves a series of complex and highly interrelated events. Genetic and/or environmental defects in this process, result in a variety of brain malformations. With recent advances in neuro-imaging, malformations of cortical development (MCDs) are now recognized as a significant clinical problem. Children with MCD often exhibit medically intractable forms of epilepsy and in some cases autism, schizophrenia, or mental retardation (Sisodiya et al., 2004). Although our general knowledge

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of how dysplastic neurons function is rapidly emerging, our understanding of how a malformed brain develops remains quite limited and would greatly benefit from systematic analysis of appropriate animal models. It has been especially challenging to model diffuse malformations of cortical development where the etiology is complex. While there are certain MCD disorders with a known causative genetic alteration such as Tuberous Sclerosis complex (Dabora, et al., 2001) or Lissencephaly-1 (Kato and Dobyns, 2003), many syndromes have not been linked to a specific gene mutation and instead appear to be associated with environmental factors (Palmini, et al. 1994).

In recent years, several rodent models mimicking specific aspects of brain malformations seen in humans have been described. Broadly, these can be classified as genetic- or injury-based models. For example, knockout mice were generated based on human genes associated with subcortical lband heterotopia (*doublecortin*) and lissencephaly (*Lis1*) (Hirotsume et al., 1998; Corbo et al., 2002). Other MCD models, such as gamma-irradiation and freeze-lesion (Roper, 1998; Jacobs et al., 1999), use pre- or perinatal injury to disrupt early stages of neurodevelopment; these animals are characterized by reproducible regions of dysplasia and have proven particularly useful for studying functional alterations that occur in a malformed brain. Our laboratory has focused on an injury-based model, prenatal exposure to a DNA methylating agent methylazoxymethanol (MAM)(Nagata and Matsumoto, 1969; Cattaneo et al., 1995). Rats exposed to MAM *in utero* develop cortical and hippocampal malformations (microdysgenesis, disorganized cortical lamination, hippocampal heterotopia) that resemble abnormalities reported in humans (Chevassus-au-Louis et al., 1998b; Colacitti et al., 1999). These animals exhibit a heightened susceptibility to seizures (Baraban and Schwartzkroin, 1996; Germano et al., 1996; Chevassus-au-Louis et al., 1998a), pharmaco-resistance to available antiepileptic drugs (Smyth et al., 2001) and impaired synaptic plasticity (Ramakers et al., 1993). MAM-exposed rats also share several anatomical and molecular features of pediatric MCD-associated epilepsy syndromes e.g., developmental dysgenesis observed in human patients with FCD and PVH (Emery et al., 1997; Colacitti et al., 1999; Crino, 2003). At a potentially functional level, alterations in glutamate receptor subunit expression were detected in tissue from patients with FCD, as well as in the MAM brain (Rafiki et al., 1998; Andre et al., 2004; Calcagnotto and Baraban, 2005). Finally, the location of abnormal clusters of neurons in the ventricular region along the temporal horns (Raymond et al., 1994) or in the hippocampus proper (Sloviter et al., 2004) is similar under both conditions. Given that the hippocampus of MAM-exposed rats are populated by distinct clusters of identifiable heterotopic neurons with anatomical similarities to humans, and that these heterotopic neurons functionally contribute to a hyperexcitable state (reminiscent of the human condition), we set out to understand the process by which these heterotopic clusters arose.

How a malformed brain develops after a temporally discrete environmental insult, either clinically or in an experimental model, remains a relatively unexplored area of research. While a better understanding of the electrophysiological function of a malformed brain could lead to clear implications for how seizures or cognitive deficit occur, understanding how a malformed brain develops could lead to early intervention. Here we begin to address this issue using the MAM-exposed rat model of MCD. Our studies are based on recent data suggesting a developmental link between hippocampal heterotopic and neocortical neurons. First, molecular and electrophysiological analysis of the MAM model revealed a striking similarity between heterotopic cells in the hippocampus and supragranular cells in layers II–III of neocortex (Castro et al., 2002). Second, tracing studies demonstrated reciprocal connections between cells within the hippocampal heterotopia and in the neocortex (Colacitti et al., 1998). Third, BrdU birthdating studies indicated that neocortical cells are born and settle into the early postnatal hippocampus in an organized gradient within heterotopia (Battaglia et al., 2003). Taken together, these findings suggest that heterotopia

originate within the neocortical neuroepithelium and subsequently erode through the hippocampal neuroepithelium to reach their final location. Examination of this hypothesis will shed light, not only on how malformations are formed in the MAM model, but will also provide insights as to general mechanisms that may occur in patients with MCD, particularly those of unknown etiology.

Materials and Methods

MAM Exposure

Pregnant Sprague-Dawley rats were injected with 25 mg/kg methylazoxymethanol (MAM; Midwest Research Institute). Intraperitoneal injections (0.3 ml in DMSO) were made on day 15 of gestation (E15). All procedures using animals were in accordance with ethical guidelines set forth in the *NIH Guide for Care and Use of Laboratory Animals* and approved by the University of California, San Francisco Committee on Animal Research. Studies were designed to minimize animal suffering and the overall number of animals used for experimentation. Animals were perfused at ages of interest with 4% paraformaldehyde in PBS. Brains were subsequently removed, fixed overnight in 4% paraformaldehyde, and cryoprotected in 30% sucrose. Litter size for MAM treatments varied between 8 and 12 pups. Although litter-to-litter variability in MAM exposure can occur, we only performed studies in litters where clear evidence of an abnormal heterotopic cell cluster in the CA1-CA2 region of hippocampus could be verified beyond postnatal day 5 (P5) in at least two offspring. Severe disorganization was also seen in the rat cortices of MAM-exposed rats used for these studies. MAM-exposed animals had much smaller cortices but care was taken to compare anatomic levels that were approximately equivalent based on subcortical structures.

Cresyl violet staining and immunohistochemistry

Cryosectioned brain slices from animals of various ages were stained with a 1% cresyl violet solution and dehydrated for cover slipping. Immunostaining using the ABC Vectastain kit involved pretreatment of sections with 1.5% hydrogen peroxide for 20 minutes. After overnight incubation with the primary antibodies, the secondary detection was done for two hours with either biotinylated anti-mouse or anti-rabbit IgG antibodies. Colorimetric detection was done with exposure to Diaminobenzidine (DAB, Sigma). For double-labeling experiments, antibody detection was done with AlexaFluor secondary antibody conjugates for mouse, rabbit, or rat IgG antibodies (Molecular Probes). For all immunohistochemistry studies, a minimum of four MAM-exposed and non-exposed brains were used and the experiments repeated at least once to confirm the results.

We generally used antibodies that have been well characterized in the literature, and to ensure the absence of background from the secondary antibodies we included negative controls in our immunostain runs where we incubated the tissue in secondary antibody without prior exposure to a primary antibody. The β -catenin antibody is a mouse monoclonal generated using full length mouse β -catenin as the immunogen (BD Transduction Laboratories; mouse, 610153; 1:1000), its specificity was characterized by western blot (see technical formation from technical documents posted on the BD Transduction Laboratories website (<http://www.bdbiosciences.com/ptDatabaseList.jsp>) and the pattern of epithelial staining we observed is similar to that described in the literature (Tateishi, et al. 2001; Persad, et al., 2001). The nestin antibody is a mouse monoclonal (Developmental hybridoma Bank, mouse, rat401; 1:100) raised using whole homogenized rat brain as an immunogen (Hockfield, et al., 1985); this antibody was subsequently used to clone nestin (Lendahl et al., 1990) and the staining pattern that we have observed is similar to that seen in both of these references. The GFAP antiserum was generated in rabbit using

purified bovine GFAP as the immunogen (Chemicon; rabbit, AB5804; 1:1000), is specific for GFAP on western blots (Darman et al., 2004) and stains adult brain sections from mouse or rat in the characteristic pattern for GFAP in the dentate gyrus (data not shown). TuJ1 is a mouse monoclonal antibody generated using purified rat microtubules as the immunogen (Covance; mouse, MMS-435P; 1:1000); this clone was subsequently found to be specific for β III-tubulin by western blot (manufacturer's technical information <http://store.crpinc.com/datasheet.aspx?catalogno=MMS-435P>) and is a selective marker for neuronal microtubules (Menezes and Luskin, 1994). The CSPG antibody is a mouse monoclonal raised using ventral membranes of chicken gizzard fibroblasts as the immunogen (Sigma, mouse monoclonal CS-56; 1:1000) and the pattern of staining in control rats was similar to that published by others (Bicknese et al., 1994). The Reelin monoclonal was raised in mouse using the N-terminal of Reelin as an immunogen (Chemicon; mouse, MAB5364, Clone G10; 1:1000) and this monoclonal was shown to be specific for an epitope between aa 164–405 and is selective for Reelin by Western blotting (de Bergeyck, et al., 1998); the staining pattern in control rats was typical as that seen in the literature and we further confirmed the specificity of this antibody for Reelin in rats by staining tissue from rats with Reelin mutations (KZC rats; data not shown). The Calretinin antiserum was raised in rabbits using recombinant rat calretinin as the immunogen (Chemicon; rabbit, AB5054; 1:1000) and is specific for Calretinin on western blots (according to the manufacturer's data sheet) and the staining pattern that we observed in control rats was identical to that shown in the literature (Fonseca et al., 1995; Meyer et al., 2002). The BrdU antibody is a rat monoclonal and doesn't react with control tissue from rats not given BrdU (AbCam; rat, ab6326; 1:200). The GABA antiserum was raised in rabbit using GABA coupled to BSA with paraformaldehyde (Sigma; rabbit, A2052; 1:2500). The Calbindin antibody was raised in rabbit using recombinant Calbindin as the immunogen (Chemicon; rabbit, AB1778; 1:1000) and is specific for calbindin on western blots according to the manufacturer's website (<http://www.chemicon.com/Product/ProductDataSheet.asp?ProductItem=AB1778>). The specificity of the Calbindin and GABA antisera were further confirmed by staining tissue from the brains of $DI \times 1/2$ mutant mice, which have dramatic reductions in the number of GABAergic, Calbindin-positive interneurons (Pleasure et al., 2000).

In situ hybridization

Fluorescent *in situ* hybridization—Tissue was fixed in 4% PFA for 30 minutes, treated with proteinase K (50mg/ml) for 1.5 minutes, and fixed again with 4% PFA for 30 minutes. Acetylation was performed with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 minutes, followed with three 1× PBS washes. Slides were incubated with hybridization buffer (50% formamide, 5× SSC, 0.3mg/ml yeast tRNA, 100ml/ml Heparin, 1× Denhart's, 0.1% Tween 20, 0.1% CHAPS, 5mM EDTA) for 30 minutes at 65°C, followed by overnight incubation with digoxigenin-labeled Reelin probe (IMAGE clone 734262) and fluorescein-labeled p73 probe. (IMAGE clone 6812399) Three high stringency washes were performed with 0.2× SSC at 65 °C. Slides were then washed in 0.1M TRIS-HCl, 0.15M NaCl, pH 7.5 (TN), treated with 1.5% peroxide in TN for 20 min., washed with TN, and blocked for 30 min. with 0.5% Blocking Reagent (Perkin Elmer) in TN. Slides were then incubated with peroxidase (HRP)-conjugated anti-fluorescein antibody (Roche; 1:1500) diluted in blocking buffer for 2 hours, followed by signal amplification using TSA Plus Fluorescence System (Perkin Elmer). TSA amplification was followed by HRP inactivation using 0.01N HCl for 15 min. at room temperature, and then an HRP-conjugated anti-digoxigenin antibody was applied for 2 hours, followed by signal amplification using a different fluorescent color tyramide reagent.

BrdU labeling

MAM-treated and untreated pregnant rats were i.p.-injected with 100 mg BrdU per kilogram body weight at gestational days 12, 14, 16, 17, or 18. Embryos were collected at gestational day 19 and processed as described before. To stain for BrdU-positive cells, tissue was treated with 2N HCl for 30 minutes in a 37° C incubator. After several quick PBS washes, the tissue was neutralized with 0.1 M NaBorate pH 8.5 for 15 minutes. The sections were incubated overnight with anti-BrdU antibody (AbCam) and detected as described above.

Statistics

All values are expressed as means $\pm$ standard errors of the mean. For statistical analysis, the Student's t-test was used and results designated significant at a level of $P < 0.05$.

Results

Timeline of cortical and hippocampal malformations

A striking anatomical observation and area of intense study in the MAM model has been the abnormal cell cluster (i.e., heterotopia) found in the CA1-CA2 regions of hippocampus (Baraban et al., 1995; Chevassus-au-Louis et al., 1998; Battaglia et al., 2003; arrow in Fig. 1E). Hippocampal heterotopic cells exhibit hyperexcitable firing activity, lack Kv4.2 potassium channels and may be a source of epileptic activity in these animals (Baraban et al., 2000; Castro et al., 2001). To examine the early postnatal development of heterotopia, we sacrificed rats at various ages after exposure to MAM at E15 and histologically examined hippocampal anatomy. Distinct CA1 heterotopias were first observed at P2 (Fig. 1D); hippocampal malformations were never observed at P0 (0/10 animals) but occasionally seen at P1 (2/10 animals). We examined serial sections throughout the hippocampal formation at these three ages to form a complete representation of the anterior-posterior extent of hippocampus. This excluded the possibility that heterotopic cells were missed in P0 and P1 animals. At P0-P2, cells were observed as accumulations in the neocortical subventricular zone and ventricular zone, taking the form of nodules protruding into the lateral ventricle and abutting the apposed hippocampal neuroepithelium (arrow in Figs. 1B and C, C'). We also noted severe disruption in cortical layering (arrowheads in Fig. 1B) at the earliest postnatal age examined; the hippocampus from MAM-exposed rats, aside from being slightly smaller than that of controls, appeared otherwise normal. Hippocampal malformations were never observed in age-matched control rats not exposed to MAM *in utero*.

The formation of heterotopic neocortical cell clusters in hippocampus could be related to earlier changes in neocortical organization, therefore we histologically examined prenatal neurodevelopment. Indications that neocortical organization was disrupted were seen as early as E17, two days after MAM exposure (Fig. 2B). At this embryonic age, the cortical plate (CP) was thinner than in age-matched control brains and had several apparent gaps (arrow in Fig. 2B'). The ventricular zone (VZ) in MAM-exposed animals was significantly thinner than that of a normal brain, an expected finding because MAM acts as an anti-proliferative agent and should initially reduce the number of progenitor cells (Cattaneo et al., 1995). Four days after MAM exposure (E19), the cortical plate and intermediate zone had lost their distinct boundaries, with the CP lacking a tightly packed laminar organization (compare Figs. 2C and 2D). The subventricular zone was severely affected as well (arrowheads in Fig. 2D'), with a patchy distribution of cellular nodules mixed with areas of reduced cellular density. In addition, cortical thickness and hippocampal size were already reduced by E19 and this continued into adulthood. In a subset of animals, we quantified differences in neocortical thickness (from the VZ to pial surface) at E19 in comparable areas of dorsal neocortex. We found that control neocortex was 1.8 times wider than age-matched

MAM neocortex; control cortical width averaged 596 μ m compared to an average MAM cortical width of 332 μ m (Fig. 2E; $n = 5$ per group; $p = 4e-7$).

Breakdown in cell-cell adhesion provides an avenue for invasion of hippocampus

Adhesive junctions represent key modulators of cell-cell contact and β -catenin, an intracellular component of these junctions (Chenn et al., 1998), serves as a marker of the integrity of these structures. At E19, we detected hints of cellular disorganization in the neocortical and hippocampal ventricular zones indicated by altered cellular orientation and the appearance of cells with less clearly organized β -catenin staining (data not shown). However, by P2, when heterotopic cells were noted in the hippocampus proper (see Fig. 1), we observed significant disruptions in cell-cell junctions at areas around the heterotopia (Fig. 3D). In age-matched control brains, the region where the neocortical and hippocampal ventricular zones abutted are characterized by tight cell associations on both the neocortical and hippocampal side of this border (white dashed line, Fig 3B). In the MAM-exposed animal, the area around a heterotopia in CA1 had a more dispersed pattern of β -catenin staining (black dashed line surrounds heterotopia Fig 3D). In an example where the nodule had not yet reached the hippocampus (Fig. 3E–F), β -catenin staining in the nodule is chaotically organized (black dashed line) and bridges the normally well organized boundaries between neocortex and hippocampus; meanwhile, the adjacent neocortico-hippocampal border retained its integrity and well organized β -catenin staining pattern (as marked by the white dashed line).

Early disruption in cortical development in the MAM-exposed neocortex

Studying the evolution of the appearance of the hippocampal heterotopia showed that there were initial changes already taking place in the embryonic neocortex. These alterations contributed to the formation of VZ/SVZ nodules that then eroded into the hippocampus to subsequently become the heterotopia found in the postnatal hippocampal formation. One of the first and most dramatic changes we noted in MAM-exposed brains was disruption of the radial glial scaffold. To examine this we used antibodies to nestin and vimentin, two intermediate filament proteins expressed in radial glial processes, that both gave similar results. For 24 hours after MAM exposure the radial glial morphology appeared normal with no apparent difference in the scaffolding between E16 MAM-treated and control brains (Fig 4A–D). However, by E19, many radial glial fibers in MAM-exposed brains were thicker (see arrow in Fig 4H) and had lost their typical orientation, with fibers oriented horizontally to the VZ (see arrowhead in Fig 4H). We also noted a concomitant increase in GFAP immunoreactivity, suggesting a premature differentiation of radial glia into GFAP-positive astrocytes (Fig 4J). A similar phenomenon was identified in ferrets exposed to MAM at an equivalent gestational stage (Hasling, et al., 2003).

Other aspects of neocortical organization were also defective in the embryonic MAM-treated brain. The CP, intermediate zone, and subplate, usually well-defined structures, were poorly delineated (Fig. 5D and H). TuJ1 staining, which identifies newly differentiated neurons (Menezes and Luskin, 1994), showed well-defined organization of the neocortex in E19 control animals (Fig. 5A and C). Note the high density of fibrillary TuJ1 staining in the intermediate zone, presumably arising from neurites, beneath the heavily labeled cells in the CP. In the MAM-treated brain, however, the orderliness of the neocortical structure was absent (Fig. 5B and D). There is no evidence of an organized strip of cells that normally labeled the cortical plate, and the fibers crossing through the intermediate zones were no longer in tight bundles nor were they restricted to the intermediate zone. In addition, where normally TuJ1 staining is minimal in the proliferative regions of the VZ/SVZ at this age, in the MAM-treated brains, there was more labeling in this region. The increase in TuJ1 staining in the VZ suggests that cells that have already differentiated into neurons are

remaining in these proliferative zones and failing to migrate to the CP. Staining for Doublecortin and Lis1, additional markers of postmitotic neurons, confirmed the disruption in cortical plate formation (data not shown).

The subplate has been reported to be rich in chondroitin sulfate proteoglycans (CSPGs), a component of the pericellular and extracellular matrices and antibodies to CSPG stain the SP strongly (Bicknese et al., 1994), but is also quite abundant in the pial basement membrane and more weakly seen in the CP and the upper intermediate zone as the neocortex matures (Miller, et al., 1995). CSPG staining in MAM-exposed tissue showed disruption of the SP staining and thinning of the pial basement membrane CSPG (Fig. 5E–H). These defects were equally apparent at all anatomic levels and the entire dorsal-ventral extent of the cortex.

We also compared the organization of interneurons in the E19 neocortex using GABA and calbindin staining (calbindin is expressed in approximately 80% of subcortically originating tangentially migrating GABAergic interneurons – Pleasure et al., 2000). In control brains (Fig 6A, C), there appeared to be three separate layers highly populated with labeled cells – the MZ, the lower IZ and the SVZ – reflecting the presumed migratory streams, in addition, individual cells were seen distributed in the CP, presumably reflecting cells adopting laminar positions. In comparison, labeled cells in MAM-treated brains (Fig 6B, D) were scattered throughout the neocortex with no obvious organization.

Abnormalities in the marginal zone in brains exposed to MAM

The previously described effects of MAM took place on steps in neocortical development that were in progress during, and after, the time of administration. However, we noted additional changes involving cells in a structure known to be well established before administration of MAM. The marginal zone (MZ), the future layer I, is the most superficial neocortical layer and is rich in a unique cell-type, the Cajal-Retzius (C-R) cells. These horizontally-oriented cells are born before the peak of neurogenesis, between E12–E14 in rat, and settle against the pial basement membrane (Meyer et al., 1998; Morante-Oria et al., 2003). Normally, this layer is quite thin, only one or two cells in width. Reelin, a glycoprotein secreted by C-R cells that plays a key role organizing proper neuronal migration, can be used as an antibody marker for C-R cells (Zecevic and Rakic, 2001). Reelin staining in MAM-exposed animals showed an apparent increase in the number of reelin-positive cells as early as E18, increasing marginal zone width to several cells in thickness (Fig. 7B and D). By E19, strongly reelin-positive cells from the widened MZ were dispersed into deeper neocortical layers (Fig. 7F and arrow in H). Quantification of these changes in reelin-positive cells showed a dramatic alteration both in the overall distribution of these cells as well as the number of cells present in the MZ. We compared the number of reelin-positive cells (counted in a box extending 400µm along the surface of the MZ and including all the strongly reelin-positive cells underlying) in E19 control and MAM brains. The number of reelin-positive cells in control brains (black bar) and MAM-exposed brains (white bar) averaged 32.6 ± 2.7 cells for control brains ($n=4$) and 61.1 ± 5.8 cells for MAM-exposed brains ($n=5$) (Figure 6I). We also analyzed the proportion of reelin-positive cells that had redistributed to deeper cortical layers; cells a minimum of three-cell widths in distance from the marginal surface were considered to be “deep”. In the E19 control brain, a negligible number of reelin-positive cells had left the surface, while many reelin-positive cells in the E19 MAM-exposed brain were more than three cell diameters away from the MZ. Control brains (black bar) had an average of $0.02\% \pm 0.01$ deep cells ($n=4$) while MAM-exposed brain had an average of $42.4\% \pm 0.06$ deep cells ($n=5$) (Fig 7J). Both the changes in the density of C-R cells and the number in deeper cortical layers were highly statistically significant ($p < 0.05$, Student’s t-test).

In addition to C-R cells, the MZ contains a population of GABAergic interneurons. Since some GABAergic interneurons have been found to be reelin-positive in mice, we wanted to confirm that the reelin-positive cells apparently redistributed to deeper cortical layers were C-R cells and not GABAergic interneurons. To address this, we examined the distribution of calretinin, a Ca^{2+} binding protein expressed in C-R cells (Meyer et al., 1998) and p73, a p53 related protein that is a highly selective marker for C-R cells (Meyer et al., 2002). Since GABA is exclusively found in interneurons (Stuhmer et al., 2002) we used this as a marker for this population of cells. In E19 control and MAM-exposed brains, calretinin and reelin were co-localized in cells throughout the entire MZ (Fig. 8C and arrowheads in C'). In the normal rat brain, calretinin also stained many subplate neurons (arrowhead in Fig. 8A; Fonseca et al., 1995), and again, we observed apparent disruption of subplate organization in embryonic MAM-exposed animals (compare Figs. 8A and 8C). In addition, all reelin-positive cells seen in deeper brain layers were also p73-positive (Figs. 9D' and E') while none of the deeper strongly reelin-positive cells were GABA-positive (arrows in Fig. 8E'). Therefore, the population of cells that make up the enlarged MZ and the redistributed reelin-positive cells in deeper layers are C-R cells (Fig. 8F, F').

It seemed likely that the apparent increase in C-R cell density in the marginal zone is partly due to the relative thinness of the cortical wall and smaller brain size leading to smaller brain surface area (and ultimately a smaller MZ volume). However, to exclude the possibility that the increase reflected production of newly born C-R cells after MAM exposure outside of the normal C-R cell genesis period, we performed birthdating with BrdU administered on E12, E14, E16, E17 and E18 and double-labeled with anti-reelin and anti-BrdU antibodies. In this analysis we found double-labeled cells only in brains from both control and MAM animals (analyzed at E19) that were given BrdU at the earlier dates (E12 and E14), reflecting the normal period of neurogenesis for C-R cells (Fig. 9); surface reelin-positive cells in control and MAM-exposed brains were BrdU-labeled (arrows in Fig. 9A'–D'). In the teratogen-exposed brains, some of the ectopic reelin-positive cells were BrdU-positive, consistent with their identity as C-R cells (arrowheads in Figs. 9B' and D'); because BrdU was given in a single pulse, not all cells that were born during the E12–14 window were labeled. We observed no co-labeling in control brains given BrdU at E16–18 (Figs. 10A and C), as expected, or in MAM animals (Figs. 10B and D), indicating that there was no reemergence of C-R cell neurogenesis following MAM exposure. BrdU-labeling was also interesting because it substantiated observed defects in neocortical organization induced by MAM exposure.

Interestingly, these birthdating experiments also yielded further evidence of the disruption of neocortical lamination induced by MAM exposure. Control brains from rats given BrdU at E12 or E14 showed BrdU-labeled cells in deeper layers of the cortical plate (in addition to the C-R cells in Layer 1) with a superficial clear zone, reflecting the production and migration of later born neurons superficially to form Layers 2–4. In E19 MAM brains this normally well ordered process was disrupted and BrdU-positive cells generated at E12 and E14 were chaotically distributed throughout all cortical layers (Figs. 9A' vs. B' and C' vs. D').

Discussion

The earliest work on the MAM-treated rat focused on characterizing the overall reduction in brain size and dramatic histological changes induced by use of a cytotoxic agent. (Haddad et al., 1969; Johnston and Coyle, 1979). Neocortical organization was disarrayed with regions of severe dysplasia, abnormal orientation of cortical pyramidal neurons, a reduced corpus callosum and heterotopias in the CA field of the hippocampus (Singh, 1977; Dambaska et al., 1982). This hippocampal heterotopia became a region of intense focus in the MAM rat

model. Anatomical analysis showed the absence of the hippocampal heterotopia at P0 but its presence at P5, the earliest date investigated (Singh, 1977; Chevassus-Au-Louis et al., 1998a). An earlier hypothesis regarding the origin of the CA heterotopias held that hippocampal pyramidal cells dyslaminated leading to the formation of the heterotopia (Singh, 1977; Zhang et al., 1995)

However, subsequent studies indicated that heterotopic cells were more related to neocortical cells than to neighboring CA₁ pyramidal cells. The progression of the expression pattern of calbindin and parvalbumin in heterotopic cells more closely resembled that of supragranular (layers II/III) cells in the neocortex (Chevassus-Au-Louis et al., 1998a). BrdU birthdating showed that the heterotopic cells were born between E16 to E20, with the majority of them born at E18 (Chevassus-Au-Louis et al., 1998a) Battaglia et al., 2003) coinciding with the birthdate of neurons that make up the superficial neocortical layers (Bayer et al., 1991), but not with neurogenesis in rat CA1 (mainly between E14 and E17) (Super et al., 1998). Furthermore, tracing studies showed that there were reciprocal connections between heterotopic cells and neocortical motor areas (Colacitti et al., 1998, Chevassus-au-Louis et al., 1998a). These connections were shown to be functionally relevant as stimulation of afferent/efferent projections to neocortex evoked responses within heterotopia; conversely, stimulating within heterotopia elicited responses in cortical cells (Chevassus-au-Louis et al., 1998b; Baraban et al., 2000). Further electrophysiological studies showed that this abnormal cluster is a focus of hyperexcitability (Baraban et al., 1995, 2000; Castro et al., 2001) and may be neocortical in origin (Castro et al., 2002). Heterotopic cells lacked hippocampal markers, such as SCIP, Math-2 and Neuropilin-2, but exhibited strong expression of a layer II/III marker (Id-2). In addition, the intrinsic firing properties of these two populations of cells were remarkably equivalent. All of these lines of evidence suggested that the heterotopic cells were likely destined for superficial layers of neocortex and not the CA1–CA2 pyramidal cell region of hippocampus.

How these hyperexcitable, potentially epileptogenic neurons migrate from their presumed neocortical location to a hippocampal location remained unknown. Previous work suggested that there was a bridge from the VZ and SVZ to hippocampus in early postnatal pups exposed to MAM (Colacitti et al., 1998; Battaglia et al., 2003). We now show that heterotopic cells begin as cell clusters in the ventricular zone of the embryonic and early postnatal neocortex. They continue to grow and protrude into the ventricle by P0/P1 and eventually break through the neocortico-hippocampal junction to reach the hippocampus at P2. We believe that the formation of these cell clusters requires that cells dissociate from the neuroepithelium and subventricular zone and then erode into hippocampus; disruption of cell-cell contacts at these areas would permit such a change by weakening cell associations that maintain tissue integrity. Because the radial glial network is preserved within the hippocampus, the nodular cell cluster can use them as guides to travel and incorporate themselves within the nearest anatomical region, which typically is the CA1–CA2 sub-field.

Because hippocampal heterotopias appear to be a consequence of earlier neocortical events, we chose to step backwards and characterize, for the first time, the precise neocortical defects present in the embryonic brain of rats exposed to MAM. From these studies, it seems likely that the root cause of heterotopia formation lies in the mechanism(s) of embryonic cortical disorganization. We noted significant aberrations in cortical organization several days after drug exposure that worsened with age. Within two days, the MAM brain demonstrated signs of architectural breakdown in the cortical plate and subplate. Concurrent with these changes were other global alterations that subsequently typified this malformed brain: (i) disruption of the radial glial scaffold, (ii) loss of the intermediate zone, and (iii) an overall decrease in cortical size. These defects, while interesting, are not surprising given that the teratogen (MAM) is administered at a time when these aspects of normal

development are in progress. Moreover, like human embryos exposed to a teratogen or other deleterious environmental insult, multiple problems can occur when prenatal development is disrupted. Because rat corticogenesis takes place between E14–E17 (Super et al., 1998) and the lamination of the neocortex continues several days postnatally, it was foreseeable that these events are vulnerable to an early insult, such as exposure to MAM. When structures as fundamental as the radial glia scaffold break down, the consequences are far-reaching. However, we were most intrigued by the change we saw in the pre-existing marginal zone of the MAM brain. This region, the antecedent to Layer I, has already been established as of E14 in the rat, and reelin-positive C-R cells that constitute the MZ have settled by this age. Interestingly, the cells that we observe thickening the MZ and entering deeper areas are not mis-located interneurons but likely C-R cells that are displaced. Therefore C-R cell localization is more plastic than originally thought and their positioning may require maintenance throughout embryonic development.

Prenatal MAM exposure in the ferret leads to similar changes in radial glia and the marginal zone (Hasling et al., 2003; Gierdalski and Juliano, 2002). In the ferret, investigators were able to rescue these alterations by exposing MAM slice cultures to media from control slices; they conclude that a secreted factor can repair the glial scaffold and return displaced reelin-positive cells to the cortical surface. However, it is unclear how effective the rescue was and whether the abnormal reelin-positive cells are indeed C-R cells or interneurons. Ferret slices for the rescue studies were made at a postnatal age; by this time in the rat, most reelin-positive cells are now interneurons, and there has also been a dramatic loss of C-R cells via developmentally regulated cell death. While very suggestive, further studies are required to clarify the changes and dynamic nature of the MZ in MAM-exposed animals. Another interesting question would be to understand how disruption of the MZ contributes to the disruption of neocortical lamination. For example, does the disorganization of C-R cells at the MZ lead to both a breakdown of radial glial fibers and a loss of the reelin gradient required for proper neuronal migration? Furthermore, do the disruptions of two of the twin pillars of the organization of radial migration – the radial glia and the C-R cells – directly contribute to the formation of nodular heterotopia in the SVZ/VZ and thereby lead indirectly to the availability of these nodules to generate hyperexcitable hippocampal heterotopia? There is suggestive evidence for a role of C-R cells and reelin in preserving the integrity of the radial glial scaffold (Super, et al., 2000; Luque, et al., 2003). Since C-R cells are displaced to deep layers and misoriented it is possible that the abnormal distribution of these “master” organizers of radial migration may substantially contribute to the dyslamination phenotype. The alterations of the MAM-treated MZ also highlights how regions not thought to be vulnerable after a certain time point can still be disrupted by an insult. The changes are especially intriguing in light of some preliminary studies in patients with varying types of MCDs. In a study on patients with polymicrogyria, investigators found an increased number of C-R cells, as identified by reelin expression and morphological features that persist into later ages, particularly around areas of architectural disruption (Eriksson et al. 2001). Increased calretinin-positive cells in Layer I were seen in temporal cortical tissue from patients with focal cortical dysplasia (Garbelli et al., 2001), and greater numbers of reelin-positive cells in Layer I have been detected in patients with microdysgenesis (Thom et al., 2003). Reelin-positive cells are seen in the adult brain at the surface though their role later in life is unclear (Deguchi et al., 2003). These clinical characterizations examined tissue from older patients with different types of MCDs but they suggest histological alterations occurring in Layer I similar to those seen in the MAM-exposed rat. No direct interpretation can be made with regards to the relationship between the Layer I changes and the dysplasia in the rest of the cortex; however the effect in this early layer supports the idea that the marginal zone, though already established, can still be disturbed.

The MAM model is useful in understanding the developmental mechanisms underlying human MCDs because many of these disorders are associated with a pre- or perinatal insult. Ingestion of a toxin (Choi, et al., 1978) or a traumatic injury (Lombroso 2000) have been linked to disordered brain architecture; these cases also have an associated epileptic syndrome. One attempt to compare the contribution of prenatal events and genetic background to the occurrence of disorders of cortical malformation suggests a propensity to arise from environmental factors, such as maternal drug exposure, head trauma, and maternal health status (Palmini, et al. 1994). The accuracy of these numbers might be affected by unrecognized pre- or perinatal events or by reported events that did not truly play a role in the malformation. Nonetheless, these reports demonstrate the important role that environmental factors play in the frequency of MCDs. As such, injury-induced models, such as the MAM-exposed rat, may better reflect the etiology of the majority of cases of cortical malformation and provide important insight into the pathogenesis underlying these cases. Such information can offer new therapeutic targets for the treatment or prevention of malformations of cortical development.

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Figure 1. Time course of the emergence of hippocampal heterotopia in the MAM-exposed brain
A–B: Comparison of normal (**A**) and MAM-exposed (**B**) rat brains at P0. At this age, nodules in the cortical neuroepithelium (arrow in **B**) protruded into the ventricle. The cortical plate was also obviously disrupted (arrowhead in **B**); these abnormalities were not present in control brain sections. **C and C':** MAM-exposed rat hippocampal section at P1. The arrow indicates a nodule bridging the neocortical-hippocampal junction. **D:** At P2, heterotopia (arrow) reached the hippocampus proper, physically integrating into the CA subfields. **E:** A section at P22 showing the disruption of the CA1 field by heterotopic neocortical neurons (arrow). Scale bars: **A,C,E** = 200mm; **B,D** = 500mm; **C'** = 100mm

Figure 2. Histologic analysis of neocortical changes in MAM-exposed rats

A and B: Coronal brain sections of normal (**A**) and MAM-exposed (**B**) animals at gestational day 17 (E17). **A' and B':** Higher magnification pictures of normal (**A'**) and MAM (**B'**) neocortex demonstrated early evidence of disruption of the cortical plate with the appearance of gaps (arrow in **B'**). **C and D:** At E19, the differences between normal (**C**) and MAM (**D**) brains were more apparent. The entire neocortex and hippocampus were smaller in the MAM brain. **C' and D':** Higher magnification images of the neocortex of normal (**C'**) and MAM (**D'**) brains showed that the cortical plate was dramatically thinner and indistinct. In general the laminar architecture of the normal neocortex (labels in **C'**) was obliterated, also the VZ and SVZ showed collections of cells (arrowhead in **D'**) alternating with cell poor areas indicated severe disorganization of the structural integrity of these layers. **E:** Quantification of cortical thickness shows that the normal neocortex was almost twice as wide as that of an MAM-exposed brain; normal thickness averaged $596 \pm 17.2\text{mm}$ while in the MAM brain, the average was $332 \pm 8.6\text{mm}$; $n=5$ per group. CP = cortical plate; IZ = intermediate zone; SVZ = subventricular zone; VZ = ventricular zone; Scale bars: **A,B** = 500mm; **C,D** = 200mm; **A'–D'** = 50mm.

Figure 3. Heterotopia formation is associated with disorganization of cell-cell contacts
A–B: In P2 control brains there were tightly organized cell-cell contacts at the point of apposition of the neocortical and hippocampal ventricular zones (white dashed line in **B**). **C and D:** In a brain from a MAM-exposed rat at P2, a heterotopic cluster of neurons with chaotic β -catenin staining (outlined by the black dashed line in **D**) had disrupted the formation of the CA1 subfields. **E and F:** In a case where the heterotopia was still bridging the neocortical and hippocampal ventricular zones (**E**), the cells away from the nodule maintained their well ordered β -catenin staining (dashed white line in **F**) but in the region of the nodule it was clear that the normal architecture of this junction was compromised and β -catenin staining was chaotic (dashed black line in **F**). Scale bars: low magnification = 250mm; high magnification = 130mm.

Figure 4. Disintegration of the radial glial scaffold

A–D: Comparison of nestin expression, a marker for radial glia, at E16, one day after MAM exposure. The control brain (**A** and **C**) and the MAM brain (**B** and **D**) had similar nestin staining patterns. **E–H:** By E19, radial glial fibers were severely disrupted in the MAM neocortex (**F** and **H**). Instead of the normal radial orientation (**G**), the radial glial scaffold was tangled (arrowhead in **H**) with many abnormally thick processes (arrow). **I and J:** GFAP expression at E19 demonstrated early astrocytic differentiation in the MAM brain (**J**) when compared to control (**I**). Scale bars: **A,B,E,F,I,J** = 200mm; **C,D** = 100mm; **G,H** = 50mm.

Figure 5. Disruption of the laminar architecture in the MAM-treated rat brain

A–D: Tuj1 expression, a marker for differentiated neurons, normally shows a tightly organized pattern in the E19 control brain. In brains from MAM-treated animals Tuj1 staining is spread throughout the entire neocortex and there was no obvious intermediate zone or organized cortical plate structure. **E–H:** Chondroitin Sulfate Proteoglycan (CSPG) staining was present in the pial basement membrane and the subplate region of the E19 control rat (**E** and arrow in **G**). In the brains from MAM-treated animals the subplate was disrupted and not visible as an organized structure (**H**). Scale bars: **A**, **B**, and **E** = 200mm; **C**, **D** = 50mm.

Figure 6. Disorganization of interneuronal localization in MAM brains

A: and **C:** GABA-positive (**A**) and Calbindin-positive (**C**) interneurons were well-organized in the E19 control neocortex, localized especially within the MZ and the intermediate zone. A few labeled cells were also seen in the CP. **B** and **D:** In the MAM-treated brain, both GABA-positive (**B**) and Calbindin-positive (**D**) cells were scattered throughout the neocortex and there was no distinct localization as was seen in the control brain. Scale bar: **A,B** = 80mm; **C,D** = 50mm

Figure 7. Marginal Zone abnormalities in MAM brains

A–D: Changes in the superficial layer of the MAM brain were first evident at E18 by reelin expression. In the normal rat brain (**A**), the MZ was a thin layer containing with reelin-positive cells at the surface (**C**). The MZ in the MAM brain (**B**) was thicker and had more reelin-positive cells. Higher magnification showed that the MZ was considerably thicker in MAM-exposed animals compared to controls (**D**). **E–H:** By E19, reelin-positive cells were still confined to the MZ of control brains (**E** and **G**), but in the MAM neocortex the MZ was thickened (**F**) and many reelin-positive cells were in deeper cortical layers (**G**). **I:** Quantification of the number of reelin-positive cells along the surface of the MZ compared the E19 control brain (black bar; 32.6 cells; SEM ± 2.1) to the E19 MAM-treated brain (white bar; 61.1; SEM ± 5.8; $p = 1.3E-05$). **J:** Quantification of the percentage of deep reelin-positive cells that escaped from the MZ (ie were located > 2 cell diameters away from the MZ) in the E19 control and MAM brains. The percent of deep reelin-positive cells was 0.02% in control brains and 42.4% in MAM-treated brains ($p = 5E-08$). ; Scale bars: low magnification = 200mm; high magnification = 100mm

Figure 8. Reelin-positive cells are Cajal-Retzius cells

Double immuno-labeling for reelin and calretinin (**A, A', D, D'**) showed that many of the reelin-positive cells in the MZ of E19 control brains (**A, A'**) also expressed calretinin (CR). In the MAM brain (**C, C'**) both the reelin-positive cells in MZ and those in deeper layers co-labeled with calretinin (arrowheads in **D'**). Calretinin expression also delineated the subplate in the rat (arrowhead in **A**) and showed that the integrity of this structure was lost in the MAM-treated brain (agreeing with the CSPG data shown above). (**B, B', D, D'**) Double *in situ* hybridization for reelin and p73 expression showed co-labeling of reelin with p73, a very specific marker of C-R cells. Even the chaotically organized reelin-positive cells in deeper layers were double labeled with p73 (arrowheads in **D'**). Double immunostaining for reelin and GABA expression (**E, E'**) in the E19 MAM brain showed that the reelin-positive cells did not co-label with GABA (**E, E'**, arrowheads in **E'**). Scale bars: low magnification = 130mm; high magnification = 40mm.

Figure 9. Displaced reelin-positive cells are born early in corticogenesis

When BrdU was given at E12 and E14, there was labeling for BrdU in some reelin-positive cells in both E19 control and MAM brains, including some of those reelin-positive cells in deeper cortical layers (arrows in **A'–D'**). Scale bars: low magnification = 130mm; high magnification = 40mm.

Figure 10. Increased proliferation does not account for the increase of reelin-positive cells at the MZ in the MAM brain

When rats were given BrdU at E16 and E18, neither the control E19 (A and C) or the E19 MAM brains (B and D) demonstrated double labeling with BrdU and reelin. Thus, reelin-positive cells were not born at this later time period. In addition, note the dramatic decreases in numbers of BrdU labeled cells and disorganization of the subventricular zone in the MAM treated animals given BrdU after MAM treatment (E16 and E18 injections). Scale bars: low magnification = 130mm; high magnification = 40mm.