Title
Mechanisms of abnormal development in an injury-induced model of cortical malformations

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Mechanisms of abnormal development in an injury-induced model of cortical malformations

by
Mercedes Paredes

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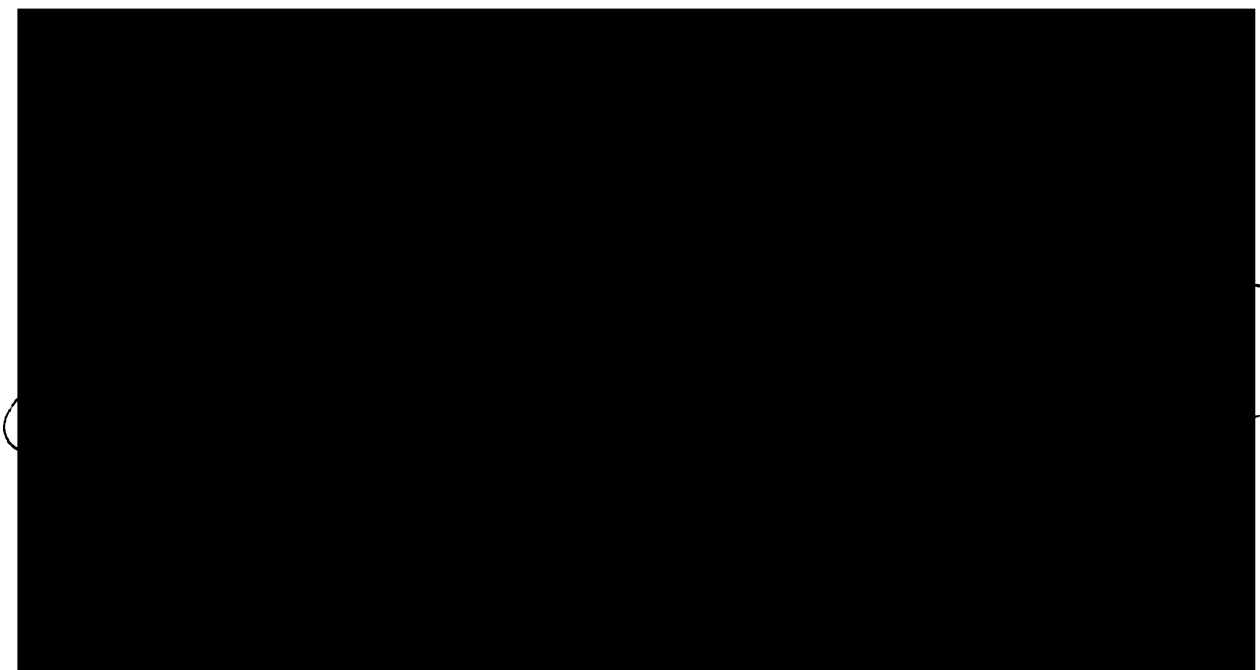

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Dedication

To my mother, Castula, and my sister, Alice: my greatest supporters who always believed in me and encouraged me to do the same. They taught me to follow my dreams.

To my granddad, Howard Salisbury, who always reminds me to do what is right.

To my best buddy Erin, who always reminds me of the beauty in the world and of the good that science can do.

And to my best friend, Peter, who's friendship and patience are priceless in my life.

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I would like to thank Scott Baraban for 4 great years of mentorship and guidance. He cared not just about my scientific development but also about my career and personal growth. I appreciate the vast freedom he allowed me with my project and the way he supported me in all aspects of my training as a physician-scientist. I also learned more about ERAs and WHIPs than I thought possible!

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My family and friends who from afar cheered me on also deserve tremendous thanks. Erin DePietro, my dearest friend, always reminded me why I started on this long path.

My mother, Tula, and sister, Alice, were my cheerleaders and taught me what determination means. Coshitas rule!

Lastly, and importantly, I want to thank Peter Castro, whose devotion and belief in me make me look to a brighter future.

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Abtract

Malformations of cortical development are frequently associated with severe conditions such as autism, mental retardation, and pediatric cases of epilepsy. Current studies have focused on understanding the mechanisms underlying the epileptogenesis. However the more general and far-reaching question of how the malformed brain develops remains unanswered. The aim of this dissertation was to characterize the defects in cortical development that occur in the teratogen-induced model of cortical dysplasia, the methylazoxymethanol (MAM)-treated rat. The adult MAM-treated brain is microcephalic with regions of cortical dysplasia, loss of lamination, and nodular heterotopias in the hippocampus. Loss of cell adhesion and increased cell death at the cortico-hippocampal junction during the first two days after birth allow for the erosion of ventricular nodules into CA1 field of the hippocampus. Thus, the hippocampal heterotopia has neocortical origins. Prenatal injury also causes earlier damage to the embryonic rat brain. Structures of ongoing developmental processes were severely affected. The radial glial scaffold had broken down and prenatal architecture (including elements such as the cortical plate and ventricular zone) was completely disordered. In addition, the marginal zone, an already established region, was disorganized by teratogen exposure. The unexpected disorganization of the marginal zone was a result of loss of stromal-derived factor-1 (SDF-1). SDF-1 was sufficient and necessary for reelin-(+) cells to localize to the meningeal surface in the MAM brain *in vitro*. This finding revealed that a global insult (teratogen exposure) can result in a specific alteration (decrease in SDF-1 in the meninges). The normal marginal zone also required SDF-1 for structural

maintenance since blocking SDF-1 activity *in vitro* and *in vivo* resulted in marginal zone disorganization. Significantly, the changes discovered in the MAM-treated rat brain are similar to the defects seen in cortical tissue from patients with FCD. There is disruption of the superficial layer (MZ in the MAM-exposed brain and layer I tissue from FCD patients) and a dramatic decrease in the expression of cortical GABA transporters. Thus the findings on how the MAM-treated rat brain develops should provide insight into the processes taking place in patients with malformations of cortical development.

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Chapter 1:

Introduction

Malformations of Cortical Development (MCD) are a group of clinical disorders that arise from defects occurring during normal brain development, particularly during the steps of migration, differentiation, or organization. MCD include clinical conditions such as lissencephaly, polymicrogyria, focal cortical dysplasia, and periventricular nodular heterotopias. This term encompasses disorders that are often associated with sequelae such as epilepsy, autism, developmental retardation, and congenital motor problems (Palmini, 2000; Sisodiya, 2004). It is the second most common cause of refractory epilepsy (Koepp and Duncan, 2004). The number of recognized cases of cortical malformations has increased with the continuous improvement of neuroimaging techniques that allow for occult structural pathologies to be identified (Sisodiya, 2004). With this improved awareness, there is now more effort directed toward understanding the developmental processes that, when disturbed, result in a malformed brain.

Normal cortical development

Neocortical development involves a series of complex and interrelated processes (Figs.1 and 2). After neural tissue has been specified, the cortex arises from the rostral part of a structure called the neural tube. This region quickly divides itself in half into the left and the right telencephalic vesicles, the two cortical hemispheres (Monuki and Walsh, 2001). Early on, these vesicles consist mainly of neuroepithelial cells that are undergoing cell division as the emergent cortex expands. The first differentiated neurons to emerge form the preplate, consisting mainly of two unique cell populations: Cajal-Retzius cells and subplate cells (Marin-Padilla, 1998). The subsequent postmitotic neurons leave the

proliferative zone of the neuroepithelium lining the ventricles (the ventricular zone). They split the preplate into a superficial marginal zone and a deeper subplate as they migrate to form the cortical plate, which becomes future layers II-VI. Signals from Cajal-Retzius cells in the marginal zone guide the neurons as they radially migrate towards the pial surface to position themselves in a laminar fashion. The sequential waves of neurons that leave the ventricular zone must move past the previous set already in place; thus the deeper cortical layers consist of cells born earlier than those in the superficial layers. The final phase in this process is the maturation of the neurons and the establishment of synaptic contacts (Bielas et al., 2004). The intricacy of these steps presents many opportunities where errors resulting in a malformed brain.

Animal models

Animal models for MCD have offered insights into both the genetic basis of these disorders as well as the functional abnormalities present in a malformed brain. There are two general types of models for cortical malformations: genetic-based and injury-induced. Genetic-based models have been useful in deciphering the role of specific genes in the formation of a disorganized neocortex. Injury-induced models have provided a better understanding of the functional abnormalities that are associated with a malformed brain.

Genetic models

Genetic mutations seen in patients with MCD provided a starting point for generating rodent models. Two genes, *doublecortin (DCX)* and *lissencephaly-1 (LIS1)*, have been implicated as the major cause of classical type I lissencephaly, manifested by a

smooth brain surface and abnormal cortical layering. The DCX gene on the X-chromosome has been implicated in sporadic lissencephaly in men and subcortical band heterotopia in women (Gleeson et al., 1998; Sisodiya, 2004). It encodes a microtubule-associated protein highly expressed in migrating neuroblasts (Gleeson et al., 1999) and, together with LIS1, is important for proper neuronal radial migration (Tanaka et al., 2004). A DCX knockout in the mouse surprisingly had no obvious defects in neurogenesis or neocortical lamination. It did have abnormal hippocampal architecture, particularly in the CA_3 field, with associated behavioral deficits (Corbo et al., 2002). A rat model for the double cortex syndrome was achieved by the application of RNA interference (RNAi) technology. Use of *in utero* RNAi to knock down DCX in rats resulted in disrupted radial migration and a neocortex with subcortical band heterotopias and abnormal cortical layering, similar to what is seen in the human condition (Bai et al., 2003). Another model with a spontaneous mutation and similarities to patients with subcortical band heterotopia is the *tish* (telencephalic internal structural heterotopia) rat (Lee et al., 1997). They have bilateral cortical heterotopia that are separated from the normal cortical layers by white matter and likely originated from a secondary proliferative zone (Lee et al., 1998). The cells in the heterotopia have typical projections to subcortical areas (such as the thalamus and spinal cord) but internally have abnormal lamination and neuronal polarity. It is not known what the mutation in the *tish* rat is but the mode of inheritance (autosomal recessive) indicates that it is not the DCX gene.

Mutations in a related protein, LIS1, lead to isolated lissencephaly sequence (ILS) and Miller-Dieker syndrome (MDS). Patients with MDS also have craniofacial defects along with severe lissencephaly. This gene encodes a subunit of platelet-activating factor

acetlyhydrolase (PAFAH). In aspergillus, proteins related to LIS1 participate in nuclear migration (Xiang et al., 1995), and it seems that the mammalian homologue also plays a role in neuronal migration (Hirotsune et al., 1998; Shu et al., 2004). Mice lacking LIS1 have brain defects that are gene-dose dependent. Mice with only one copy of the gene have defects in the olfactory bulb, cortex, and hippocampus. Reducing LIS1 gene activity even further caused more severe problems, and also involved the cerebellum. The defects in the hippocampus of LIS1 heterozygotes have associated physiological abnormalities such as hyperexcitable connectivity between two major hippocampal structures, the dentate gyrus and CA_3, and increased propensity for interictal bursting (Fleck et al., 2000).

Mutants for genes involved in regulating neuronal migration have also served as useful models. These mice do not necessarily have a mutation known to be related with a human disorder. Yet they still shed light on the molecular players required for particular steps during normal cortical development. Mutants for both cyclin-dependent kinase 5 (cdk5), and its activator, p35, have abnormal cortical layering (Gilmore et al., 1998; Wenzel et al., 2001) and the two genes have been shown to be involved in neuronal migration (Xie et al., 2003; Hammond et al., 2004). p35 mutants have additional dysplasia in the hippocampal formation and abnormal hippocampal circuitry that likely contributes to the epileptiform activity originating in the dentate gyrus (Patel et al., 2004). Mutants for components of the meningeal basement membrane, such as integrins and laminin (Graus-Porta et al., 2001; Halfter et al., 2002), also have severe defects in cortical organization resulting in dysplasia. It is becoming clearer what key pathways are required for proper basement membrane assembly and structure, which subsequently

influences neocortical development. One recent example is the targeted knockout of focal adhesion kinase (FAK), a tyrosine receptor kinase that is highly expressed in the developing brain and glia and is activated by binding to components of the extracellular matrix. Loss of this kinase in neuronal and glial precursors led to disrupted cortical lamination and neuronal ectopias that invaded the marginal zone (Beggs et al., 2003). Specific removal of FAK in meningeal fibroblasts, but not in neurons, showed that FAK is required by meningeal cells to maintain the integrity of the basement membrane. Cortical disorganization in the FAK mutants is not a result of a neuronal deficiency of FAK but is a direct effect of the breakdown of the basement membrane at the meninges.

Injury-induced models

Injury-based models have been useful in providing insight into the basis of the functional abnormalities observed in the malformed brain. While there are many human disorders of MCD that have an associated genetic mutation, a substantial proportion of cases have no clear genetic predisposition (e.g. family history of congenital malformation or mental retardation), but instead are related to an environmental, potentially non-specific, *in utero* insult (e.g. ischemia, toxin exposure, or infection). One retrospective study by Palmini, et al. examined the frequency of malformations associated with either genetic factors or environmental harm and found that a majority of cases of MCD had an associated insult without any genetic predilection, suggesting that injurious external events can play a significant role in the pathogenesis of MCD (Palmini et al., 1994). Thus, studies designed to understand teratogenic models of MCD are an important accompaniment to the study of genetic causes.

Rats exposed to γ-irradiation *in utero* exhibit many anatomical and histological features of cortical dysplasia such as microcephaly, heterotopic neurons in the hippocampal formation, and diffuse cortical disorganization (Roper, 1998). These animals have spontaneous seizures, and histological analysis of the irradiated brains showed a decrease in interneurons. Electrophysiological studies have substantiated the decrease in inhibition, as indicated by reduced inhibitory currents (Zhu and Roper, 2000). An injury-induced model that specifically mimics polymicrogyria, the freeze-lesion model, has also added to our knowledge of the origins of epileptogenesis. The brains of early postnatal pups are exposed to a freezing probe, which results in brains that have small involutions (microgyri) with only 4 layers at the site of the lesion. Extracellular recordings show that the area around the microgyria is hyperexcitable (Jacobs et al., 1996). More detailed examination using multi-electrode recordings at the site of injury revealed that the epileptogenic zone is separate from the microgyrus proper. In fact, separating the microgyrus from this zone did not eliminate epileptiform activity (Jacobs et al., 1999). This information is significant in our understanding of what regions are crucial to generating epileptiform activity in the malformed brain. For many cases of intractable epilepsy, the best treatment option is surgical resection of the epileptogenic tissue (Hamiwka et al., 2005), and more than 50% of the lesion had to be removed to maximize the efficacy of surgery (Palmini et al., 1991). The results from the freeze-lesion model highlight the need to differentiate lesion areas with histological abnormalities from the actual zone of epileptogenesis.

The MAM-treated rat model

Among the aquired models of MCD, the teratogen-induced model, the MAM-treated rat, is one that has been used by many groups to understand the physiological and morphological defects in the abnormal brain. Pregnant rats receive an i.p. injection of methylazoxymethanol (MAM) on gestational day 15 (E15) and the resulting pups have microcephalic brains with cortical dyslamination and abnormal cell clusters (heterotopias) along the lateral ventricle and within the CA fields of the hippocampus (Baraban et al., 2000).

Anatomical alterations in the MAM-treated rat

Early studies on the MAM rat described the overall reduction in brain size and dramatic histological changes (Haddad et al., 1969; Johnston and Coyle, 1979). The architecture of the neocortex was disorganized with regions of severe dysplasia, the presence of cortical pyramidal neurons with abnormal orientation and dendritic morphology, a reduced corpus callosum, and ectopic cell clusters, or heterotopias, in the CA field of the hippocampus (Singh, 1977; Dambska et al., 1982). Anatomical analysis showed the absence of the hippocampal heterotopia at P0 but its presence as of P5, the earliest date investigated (Singh, 1977; Chevassus-Au-Louis et al., 1998a). Furthermore, there were prenatal indications that radial glial elements had been disrupted. The early hypothesis about the CA ectopia was that hippocampal pyramidal cells rearrangement, resulting from MAM exposure, lead 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 CA_1 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 and E20, with the majority of them born at E18 (Battaglia et al., 2003). The BrdU labeling of the hippocampal heterotopia occurred in a dorsal-ventral gradient. The birthdate of the cells that formed the heterotopia coincided with the birthdate of neurons that make up the superficial neocortical layers (Bayer et al., 1991). Further characterization of these heterotopic cells confirmed that they are more similar to supragranular cells from cortical layers II/III than to hippocampal pyramidal cells (Castro et al., 2002). Molecular markers of CA field pyramidal cells, such as Math-2 and SCIP, were absent in the heterotopia. However, Id-2, normally present in layer II/III cells, was expressed in heterotopic cells. Thus the evidence pointed to the hippocampal heterotopia having origins in the neocortex.

Physiological defects in the MAM-treated rat

The MAM-treated brain is hyperexcitable: it has a lower threshold and latency to epileptiform activity when exposed to proconvulsants such as kainic acid and flurothyl *in vivo* (Baraban and Schwartzkroin, 1996) and elevated extracellular potassium *in vitro* (Baraban et al., 1997). Early physiological studies on the MAM-treated brain also focused on the hippocampal heterotopia. It was a striking anatomical aberration and was identified as the origin of epileptogenesis. Isolated heterotopia within the CA_1-CA_2

regions of hippocampus can generate epileptiform burst activity independent of CA_3 synaptic input (Baraban et al., 2000). Neuronal tracing experiments on the heterotopic cells showed reciprocal connectivity between those cells and cortical cells as well as neighboring pyramidal cells in the CA_1 field (Chevassus-Au-Louis et al., 1998a; Colacitti et al., 1998). Some of these anomalous connections were physiologically active. Stimulation of white matter evoked responses in both hippocampal heterotopic cells and neocortical supragranular cells as well as stimulation of afferent fibers from the CA_3 field (Schaffer collaterals) and entorhinal cortex (Chevassus-Au-Louis et al., 1998b). Therefore the heterotopic cells were incorporated into both the neocortical and hippocampal circuits. It also allowed propagation of epileptiform activity to the neocortex: discharges in the hippocampal heterotopia that were evoked by stimulation of either the dentate gyrus or Schaffer collaterals also spread to the neocortex.

Further characterization of the heterotopic cells gave clues into the mechanisms underlying their functional significance. These cells have fast spiking properties that are not normally seen in pyramidal cells (Williams et al., 1994). Histological studies revealed that heterotopic cells lacked the A-type Kv4.2 potassium channel and its associated current, which is present in the neighboring CA1 pyramidal (normotopic) cells (Castro et al., 2001). Kv4.2 potassium channels are normally expressed in hippocampal pyramidal cells and mediate the fast, transient potassium current, I_A. This current regulates firing frequency, action potential (AP) properties, and synaptic integration at dendrites (Cai et al., 2004). Loss of this current likely contributes to the intrinsic hyperexcitibility of these neurons. Firing characteristics and AP properties of heterotopic

cells more closely resembled that of supragranular cells than hippocampal pyramidal cells.

In addition to the intrinsic changes in the heterotopic cells, the inhibitory system was altered in the region of the heterotopia. Extracellular field recordings provided the first clue that the inhibitory system had changed. Paired-pulse facilitation (PPF), one measure of use-dependent synaptic plasticity, compares the size of two stimuli that have been given in sequence and can be calculated over several time intervals. It is measured as the difference between amplitude of the response to the second and first stimuli divided by the amplitude of the response to the first amplitude. PPF was missing in recordings within the heterotopia of MAM slices (Fig. 3) suggestive that the inhibitory input that usually limits the initial response was altered. Voltage-clamp studies confirmed a change in the inhibitory system and showed that inhibitory post synaptic currents (IPSCs) onto heterotopic cells were prolonged (Calcagnotto et al., 2002). One explanation for the change in PPF and the longer IPSCs would be the presence of a modification in the GABA receptor in the MAM-treated brain function that would affect its function. Application of exogenous GABA onto acute slices and measuring the percent inhibition of the recorded population spike did not show any difference in GABA inhibition between recordings from normal CA_1 or the heterotopic region (Fig. 4). Voltage–clamp studies also did not report changes in inhibitory currents that would indicate a change in GABA receptors. However, the addition of agents that impede GABA reuptake did not affect the duration of these inhibitory currents (Calcagnotto et al., 2002). This indication of a change in GABA transporters was confirmed by immunohistochemistry. The expression of GABA transporters, GAT-1 and GAT-3, was

selectively reduced in heterotopic cells compared to the normotopic region in CA_1 (Fig. 5). The inhibitory system in the MAM-treated brain was enhanced in a region that is hyperexcitable, a potential compensation for the presence of heterotopic cells in the hippocampus. In tissue from patients with Focal Cortical Dysplasia (FCD) and refractory epilepsy, expression of GAT-1 and GAT-3 was also reduced in dysplastic regions (Fig. 6), suggesting a similar mechanism of compensation taking place in the human brain.

Histological evidence pointed to the emergence of the hippocampal heterotopia being an early postnatal event (Chevassus-Au-Louis et al., 1998a; Battaglia et al., 2003). Physiological evidence also corroborated this view. Elevation of external potassium, $[K+]_o$, can induce bursting in the CA fields, as early as P5 (Dzhala and Staley, 2003). Differences in epileptiform activity could be detected with extracellular recordings in the CA1 field of control slices and in the heterotopia of MAM slices (Fig. 7A). In recordings from early acute postnatal slices, 30% (3/10) of the control slices (P7-P11) had epileptiform activity at 6mM $[K+]_o$ while 80% of slices from MAM-treated brains burst at this same concentration (Fig. 7A vs. B). Thus, hyperexcitability is present early in the MAM-treated brain.

One hypothesis is that the incidence of bursting was associated with the concurrent loss of I_A current in heterotopic cells in the hippocampus. Whole-cell patch clamp recordings from animals, as early as P0 when only ventricular nodules are visible, suggested otherwise. Interestingly, at the early ages, the I_A current was still detectable in the majority of heterotopic cells (Fig. 7B). However, the percent of cells that had this current decreased with increasing age: by P11, only 50% of cells had the normal I_A. By P15, none of the recorded cells in the heterotopia had the current. The loss of the fast,

transient I_A is a gradual process (Fig. 7C) and likely the hyperexcitability arising from the hippocampal heterotopia in the MAM-treated brain is the summation of multiple changes that contribute to the physiological defect.

In light of how early the functional changes were taking place and the paucity of detailed information on the early anatomical deficits, we chose to look at prenatal development after MAM administration. Such an investigation would help identify the developmental processes that when disrupted lead to both a structurally and physiologically abnormal brain.

Figure Legends

Figure 1.1. General architecture of the prenatal rat brain. The marginal zone is the most superficial layer in the embryonic brain. It is populated mainly by Cajal-Retzius cells. Underneath is the cortical plate (CP), future layers II-VI, which has postmitotic and newly differentiated neurons. Below the cortical plate is the intermediate zone (IZ), a cell sparse region through which postmitotic neurons migrate. The most basal layers are the ventricular zone and subventricular zones (VZ/SVZ). These are the proliferative layers where neuronal production takes place. The VZ contains radial glia that serve as neuronal precursors and the scaffolding that neurons use to migrate to the cortical plate.

Figure 1.2. Schematic of early stages of neocortical development. The structure begins as a pseudostratified cerebral wall which then forms a preplate (PP). This is a period of intense cell division at the ventricular zone. In the subsequent step, postmitotic neurons migrate along radial glia to form a cortical plate (CP) that separates the preplate into the deeper subplate and superficial marginal zone. This migration occurs in an orderly fashion with newly born cells moving past the older ones to begin pattering the embryonic brain in an inside-out fashion. During the last stages of development, the cortical plate has expanded and now has more complex layering and axonal growth. These steps ultimately lead to the adult six-layered neocortex.

Figure 1.3. Paired Pulse Facilitation (PPF) was lost in the heterotopia of the MAM-treated rat brain. (A) Schematic of the set for extracellular field recordings. In acute slices from control rats, the recording electrode was placed within the CA_1 field of the hippocampus. Recordings in slices from the MAM-treated rat brain were taken from within the heterotopic cluser in CA_1. (B) PPF between 2 stimuli at a 60 msec interval was seen only in control slices. The second stimulus (P2) was larger than the first one giving a PPF of greater than 100%. (B1) PPF was absent in MAM slices. P2 was the same size as P1, giving a P2/P1 value of 100%. (C) Quantification of P2/P1 values taken over 10-250msec intervals shows that in MAM slices, the ratio never rises above 100%. Normally, as seen in control slices, PPF rises to a maximum at the intermediate time intervals and then stabilizes with the longer intervals, in this case to 120% facilitation.

Figure 1.4. The GABAergic system is altered in the MAM-treated rat brain. (A) Application of 5mM exogenous GABA normally decreases the amplitude of the population spike; n=5 slices per condition. (B) Quantification of this effect was graphed as percent inhibition. This percent was the difference of the amplitude of the population spike before and after GABA administration over the pre-treatment amplitude. Whether recordings were taken in the CA_1 field of control and MAM slices or within the heterotopia of the MAM slice, the effect of GABA was equivalent.

Figure 1.5. The MAM-treated rat brain has decreased cortical GABA transporters. (A, A1) Coronal hippocampal section of a control brain shows GAT-1 labeling around cell bodies in CA_1-CA_3 stratum pyramidal and granule cells of the dentate gyrus. (B, B1)

GAT-1 labeling for a coronal hippocampal section from a MAM-treated rat at low (B)- and high (BD1)-power magnification shows diffuse GAT-1 labeling around cell bodies in the nodular heterotopia. GAT-3 labeling was also diffuse within the heterotopia of sections from the MAM brain (D) compared to the staining in control sections (C). Magnification: A1, B1, C, D, 1.6×; A2, B2, 132×. Scale bars: A1, B1, 600 μm; A2, B2, 80 μm; C, D, 500 μm.

Figure 1.6. Dysplastic tissue from patients with FCD has decreased of cortical GABA transporters. Immunostaining for GAT-1 in (A) control tissue and (B) tissue from a patient with focal cortical dysplasia shows a loss of the transporter protein in the abnormal tissue. The expression of GAT-3 in (C) control tissue and (D) FCD tissue had a similar finding (magnification: X200; inset magnification: X400).

Figure 1.7. A developmental timeline of the physiological alterations in the MAM-treated brain. (A) The extracellular potassium concentration $[K+]_o$ was raised to induce bursting in acute hippocampal slices. Traces are of recordings from slices at P7. Normal $[K+]_o$ is 3mM, which did not cause epileptiform activity in either control or MAM slices. Raising $[K+]_o$ to 6mM induces bursting in the majority of MAM slices but only occasionally in control slices. Using the highest $[K+]_o$ of 8.5mM results in epileptiform activity in both control and MAM slices. (B) Whole recordings of normal CA_1 pyramidal (normotopic) cells and hippocampal heterotopic cells before P11 show the presence of the fast, transient I_A current (arrow) in both cell types. After this age, the I_A current is still present in pyramidal cells and is sensitive to 4-AP, a I_A current blocker. However,

the heterotopic cells no longer have this current. (C) A breakdown by age of the loss of the I_A current. The current is present in the majority of cells before P11. At this time, there is a shift where most cells now have lost this current.

References

Bai J, Ramos RL, Ackman JB, Thomas AM, Lee RV, LoTurco JJ. 2003. RNAi reveals doublecortin is required for radial migration in rat neocortex. Nat Neurosci 6(12):1277-1283.

Baraban SC, Bellingham MC, Berger AJ, Schwartzkroin PA. 1997. Osmolarity modulates K+ channel function on rat hippocampal interneurons but not CA1 pyramidal neurons. J Physiol 498 (Pt 3):679-689.

Baraban SC, Schwartzkroin PA. 1996. Flurothyl seizure susceptibility in rats following prenatal methylazoxymethanol treatment. Epilepsy Res 23(3):189-194.

Baraban SC, Wenzel HJ, Hochman DW, Schwartzkroin PA. 2000. Characterization of heterotopic cell clusters in the hippocampus of rats exposed to methylazoxymethanol in utero. Epilepsy Res 39(2):87-102.

Battaglia G, Pagliardini S, Saglietti L, Cattabeni F, Di Luca M, Bassanini S, Setola V. 2003. Neurogenesis in cerebral heterotopia induced in rats by prenatal methylazoxymethanol treatment. Cereb Cortex 13(7):736-748.

Bayer SA, Altman J, Russo RJ, Dai XF, Simmons JA. 1991. Cell migration in the rat embryonic neocortex. J Comp Neurol 307(3):499-516.

Beggs HE, Schahin-Reed D, Zang K, Goebbels S, Nave KA, Gorski J, Jones KR, Sretavan D, Reichardt LF. 2003. FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. Neuron 40(3):501-514.

Bielas S, Higginbotham H, Koizumi H, Tanaka T, Gleeson JG. 2004. Cortical neuronal migration mutants suggest separate but intersecting pathways. Annu Rev Cell Dev Biol 20:593-618.

Cai X, Liang CW, Muralidharan S, Kao JP, Tang CM, Thompson SM. 2004. Unique roles of SK and Kv4.2 potassium channels in dendritic integration. Neuron 44(2):351-364.

Calcagnotto ME, Paredes MF, Baraban SC. 2002. Heterotopic neurons with altered inhibitory synaptic function in an animal model of malformation-associated epilepsy. J Neurosci 22(17):7596-7605.

Castro PA, Cooper EC, Lowenstein DH, Baraban SC. 2001. Hippocampal heterotopia lack functional Kv4.2 potassium channels in the methylazoxymethanol model of cortical malformations and epilepsy. J Neurosci 21(17):6626-6634.

Castro PA, Pleasure SJ, Baraban SC. 2002. Hippocampal heterotopia with molecular and electrophysiological properties of neocortical neurons. Neuroscience 114(4):961-972.

Chevassus-Au-Louis N, Congar P, Represa A, Ben-Ari Y, Gaiarsa JL. 1998. Neuronal migration disorders: heterotopic neocortical neurons in CA1 provide a bridge between the hippocampus and the neocortex. Proc Natl Acad Sci U S A 95(17):10263-10268.

Chevassus-Au-Louis N, Rafiki A, Jorquera I, Ben-Ari Y, Represa A. 1998. Neocortex in the hippocampus: an anatomical and functional study of CA1 heterotopias after prenatal treatment with methylazoxymethanol in rats. J Comp Neurol 394(4):520-536.

Colacitti C, Sancini G, Franceschetti S, Cattabeni F, Avanzini G, Spreafico R, Di Luca M, Battaglia G. 1998. Altered connections between neocortical and heterotopic areas in methylazoxymethanol-treated rat. Epilepsy Res 32(1-2):49-62.

Corbo JC, Deuel TA, Long JM, LaPorte P, Tsai E, Wynshaw-Boris A, Walsh CA. 2002. Doublecortin is required in mice for lamination of the hippocampus but not the neocortex. J Neurosci 22(17):7548-7557.

Dambska M, Haddad R, Kozlowski PB, Lee MH, Shek J. 1982. Telencephalic cytoarchitectonics in the brains of rats with graded degrees of micrencephaly. Acta Neuropathol (Berl) 58(3):203-209.

Dzhala VI, Staley KJ. 2003. Excitatory actions of endogenously released GABA contribute to initiation of ictal epileptiform activity in the developing hippocampus. J Neurosci 23(5):1840-1846.

Fleck MW, Hirotsune S, Gambello MJ, Phillips-Tansey E, Suares G, Mervis RF, Wynshaw-Boris A, McBain CJ. 2000. Hippocampal abnormalities and enhanced excitability in a murine model of human lissencephaly. J Neurosci 20(7):2439-2450.

Gilmore EC, Ohshima T, Goffinet AM, Kulkarni AB, Herrup K. 1998. Cyclin-dependent kinase 5-deficient mice demonstrate novel developmental arrest in cerebral cortex. J Neurosci 18(16):6370-6377.

Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, Walsh CA. 1998. Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. Cell 92(1):63-72.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA. 1999. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23(2):257-271.

Graus-Porta D, Blaess S, Senften M, Littlewood-Evans A, Damsky C, Huang Z, Orban P, Klein R, Schittny JC, Muller U. 2001. Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron 31(3):367-379.

Haddad RK, Rabe A, Laqueur GL, Spatz M, Valsamis MP. 1969. Intellectual deficit associated with transplacentally induced microcephaly in the rat. Science 163(862):88-90.

Halfter W, Dong S, Yip YP, Willem M, Mayer U. 2002. A critical function of the pial basement membrane in cortical histogenesis. J Neurosci 22(14):6029-6040.

Hamiwka L, Jayakar P, Resnick T, Morrison G, Ragheb J, Dean P, Dunoyer C, Duchowny M. 2005. Surgery for epilepsy due to cortical malformations: ten-year follow-up. Epilepsia 46(4):556-560.

Hammond V, Tsai LH, Tan SS. 2004. Control of cortical neuron migration and layering: cell and non cell-autonomous effects of p35. J Neurosci 24(2):576-587.

Hirotsune S, Fleck MW, Gambello MJ, Bix GJ, Chen A, Clark GD, Ledbetter DH, McBain CJ, Wynshaw-Boris A. 1998. Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality. Nat Genet 19(4):333-339.

Jacobs KM, Gutnick MJ, Prince DA. 1996. Hyperexcitability in a model of cortical maldevelopment. Cereb Cortex 6(3):514-523.

Jacobs KM, Hwang BJ, Prince DA. 1999. Focal epileptogenesis in a rat model of polymicrogyria. J Neurophysiol 81(1):159-173.

Johnston MV, Coyle JT. 1979. Histological and neurochemical effects of fetal treatment with methylazoxymethanol on rat neocortex in adulthood. Brain Res 170(1):135-155.

Koepp MJ, Duncan JS. 2004. Epilepsy. Curr Opin Neurol 17(4):467-474.

Lee KS, Collins JL, Anzivino MJ, Frankel EA, Schottler F. 1998. Heterotopic neurogenesis in a rat with cortical heterotopia. J Neurosci 18(22):9365-9375.

Lee KS, Schottler F, Collins JL, Lanzino G, Couture D, Rao A, Hiramatsu K, Goto Y, Hong SC, Caner H, Yamamoto H, Chen ZF, Bertram E, Berr S, Omary R, Scrable

H, Jackson T, Goble J, Eisenman L. 1997. A genetic animal model of human neocortical heterotopia associated with seizures. J Neurosci 17(16):6236-6242.

Marin-Padilla M. 1998. Cajal-Retzius cells and the development of the neocortex. Trends Neurosci 21(2):64-71.

Monuki ES, Walsh CA. 2001. Mechanisms of cerebral cortical patterning in mice and humans. Nat Neurosci 4 Suppl:1199-1206.

Palmini A. 2000. Disorders of cortical development. Curr Opin Neurol 13(2):183-192.

Palmini A, Andermann E, Andermann F. 1994. Prenatal events and genetic factors in epileptic patients with neuronal migration disorders. Epilepsia 35(5):965-973.

Palmini A, Andermann F, Olivier A, Tampieri D, Robitaille Y. 1991. Focal neuronal migration disorders and intractable partial epilepsy: results of surgical treatment. Ann Neurol 30(6):750-757.

Patel LS, Wenzel HJ, Schwartzkroin PA. 2004. Physiological and morphological characterization of dentate granule cells in the p35 knock-out mouse hippocampus: evidence for an epileptic circuit. J Neurosci 24(41):9005-9014.

Roper SN. 1998. In utero irradiation of rats as a model of human cerebrocortical dysgenesis: a review. Epilepsy Res 32(1-2):63-74.

Shu T, Ayala R, Nguyen MD, Xie Z, Gleeson JG, Tsai LH. 2004. Ndel1 operates in a common pathway with LIS1 and cytoplasmic dynein to regulate cortical neuronal positioning. Neuron 44(2):263-277.

Singh SC. 1977. Ectopic neurones in the hippocampus of the postnatal rat exposed to methylazoxymethanol during foetal development. Acta Neuropathol (Berl) 40(2):111-116.

Sisodiya SM. 2004. Malformations of cortical development: burdens and insights from important causes of human epilepsy. Lancet Neurol 3(1):29-38.

Tanaka T, Serneo FF, Higgins C, Gambello MJ, Wynshaw-Boris A, Gleeson JG. 2004. Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration. J Cell Biol 165(5):709-721.

Wenzel HJ, Robbins CA, Tsai LH, Schwartzkroin PA. 2001. Abnormal morphological and functional organization of the hippocampus in a p35 mutant model of cortical dysplasia associated with spontaneous seizures. J Neurosci 21(3):983-998.

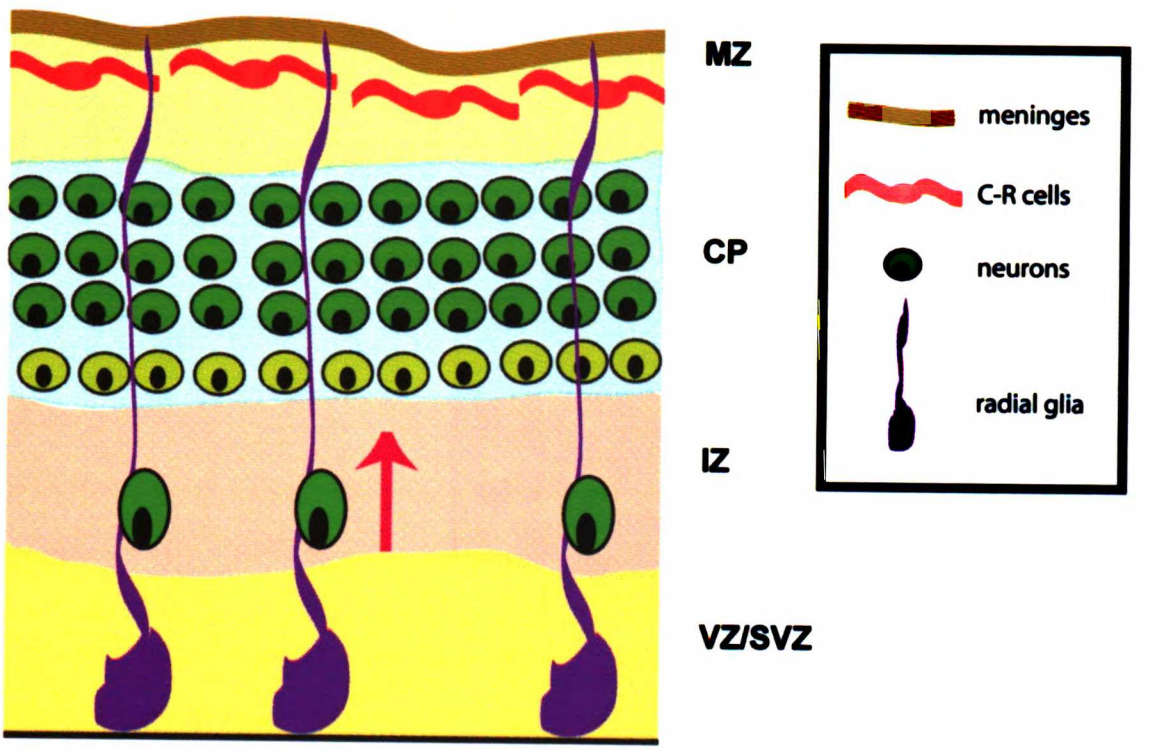
Williams S, Samulack DD, Beaulieu C, LaCaille JC. 1994. Membrane properties and synaptic responses of interneurons located near the stratum lacunosum-moleculare/radiatum border of area CA1 in whole-cell recordings from rat hippocampal slices. J Neurophysiol 71(6):2217-2235.

Xiang X, Osmani AH, Osmani SA, Xin M, Morris NR. 1995. NudF, a nuclear migration gene in Aspergillus nidulans, is similar to the human LIS-1 gene required for neuronal migration. Mol Biol Cell 6(3):297-310.

Xie Z, Sanada K, Samuels BA, Shih H, Tsai LH. 2003. Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. Cell 114(4):469-482.

Zhang LL, Collier PA, Ashwell KW. 1995. Mechanisms in the induction of neuronal heterotopiae following prenatal cytotoxic brain damage. Neurotoxicol Teratol 17(3):297-311.

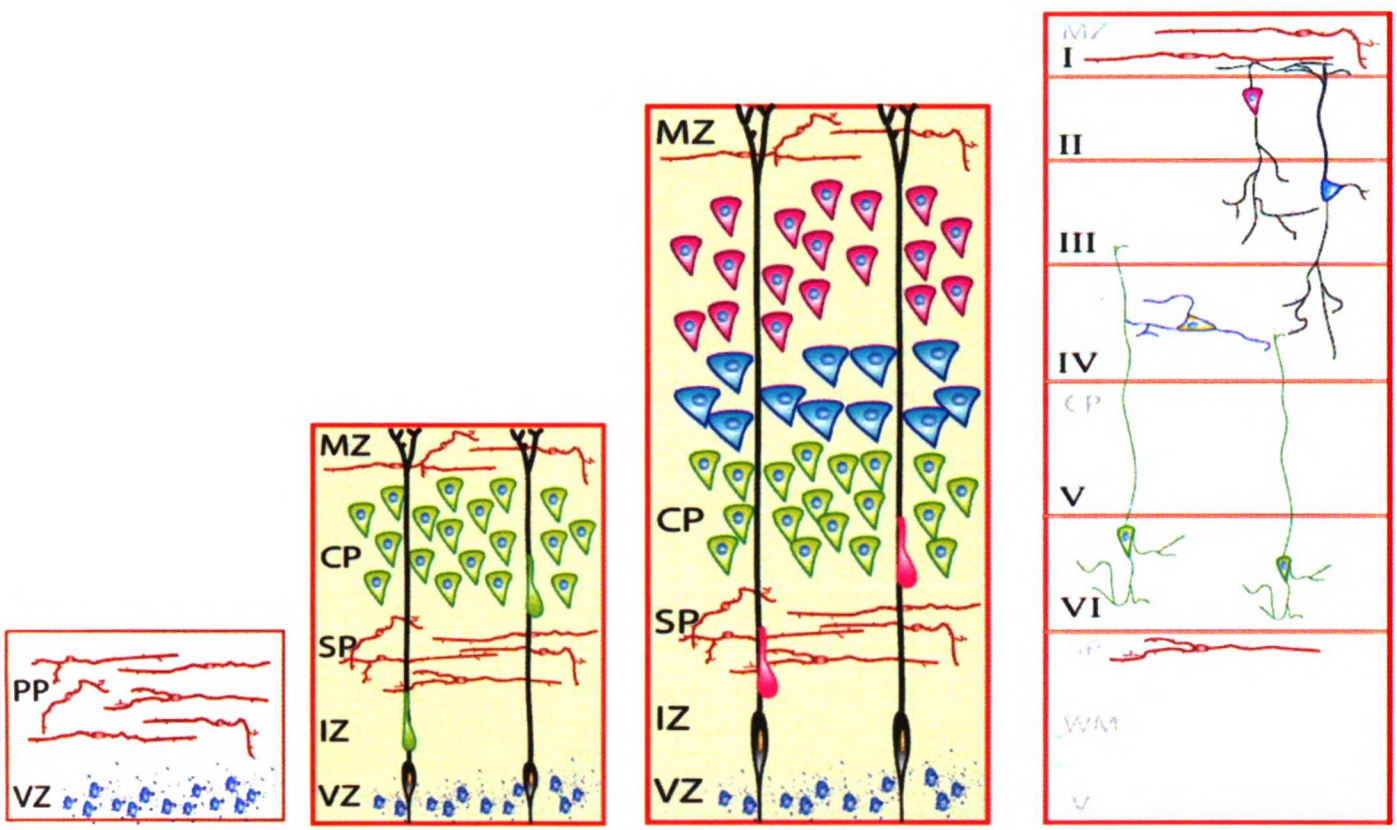
Zhu WJ, Roper SN. 2000. Reduced inhibition in an animal model of cortical dysplasia. J Neurosci 20(23):8925-8931.



normal late embryonic rat neocortex

Figure 1.1

Stages of Cortical Development



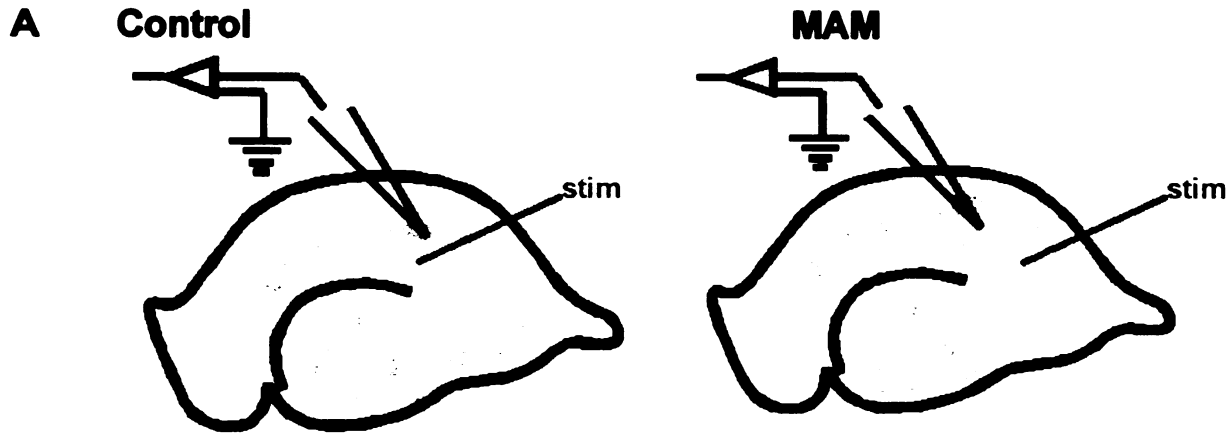
Preplate (proliferation)

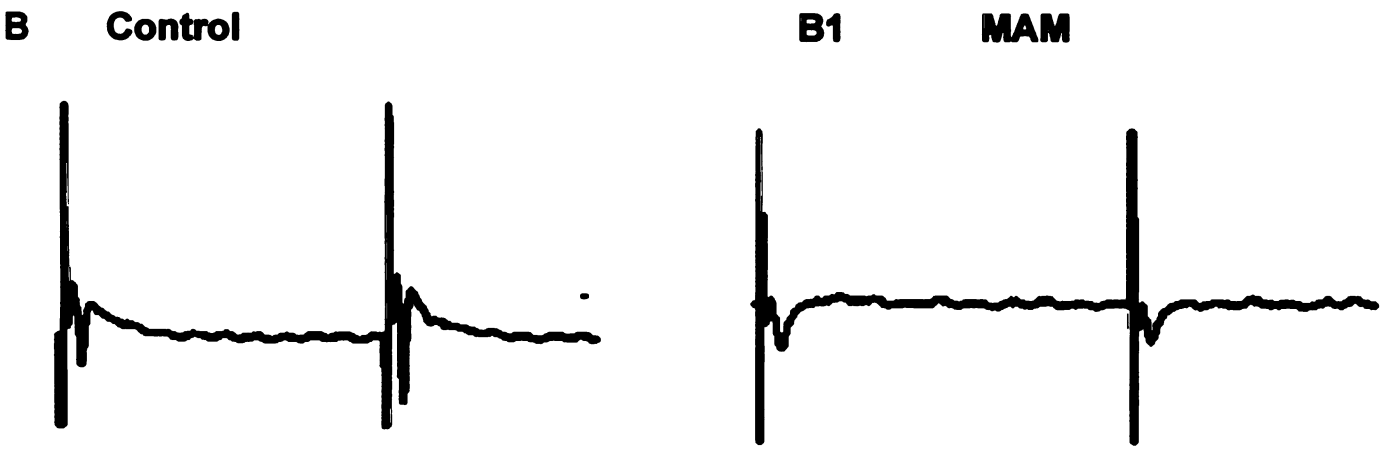
Cortical Plate (migration, patterning)

Late Embryonic (layering, axon growth)

Adult

Figure 1.2


A
Control
MAM
stim
stim


B
Control
B1
MAM

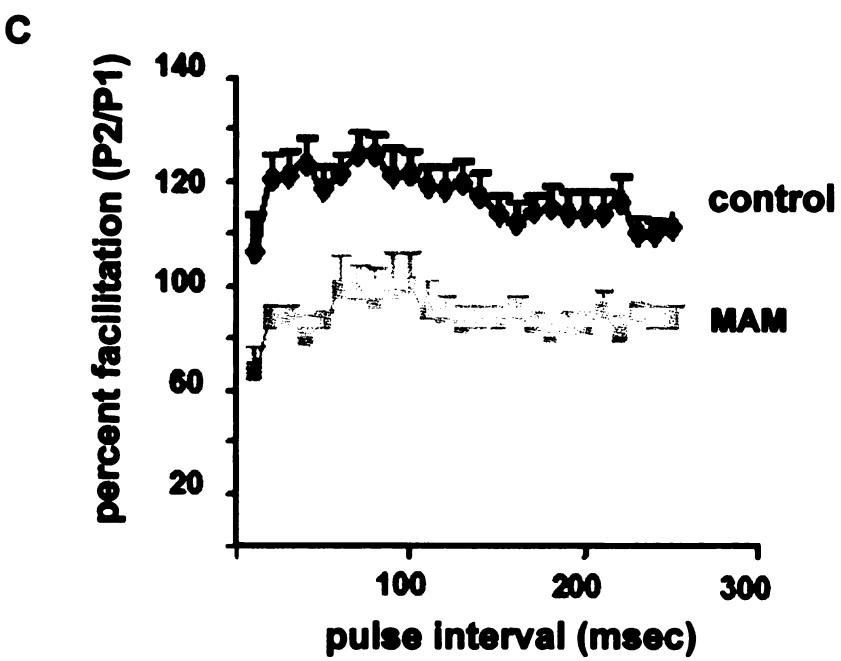

C
140
120
100
60
20
percent facilitation (P2/P1)
control
MAM
100
200
300
pulse interval (msec)

Figure 1.3

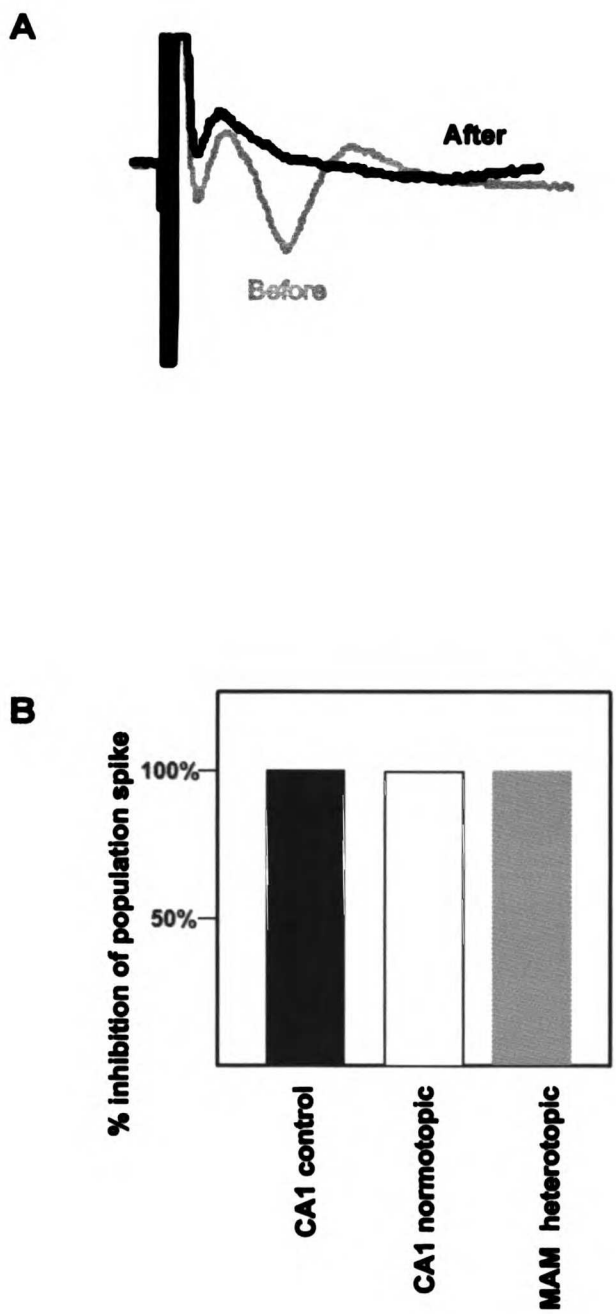

A
After
Before
B
% inhibition of population spike
100%
50%
CA1 control
CA1 normotopic
MAM heterotopic

Figure 1.4

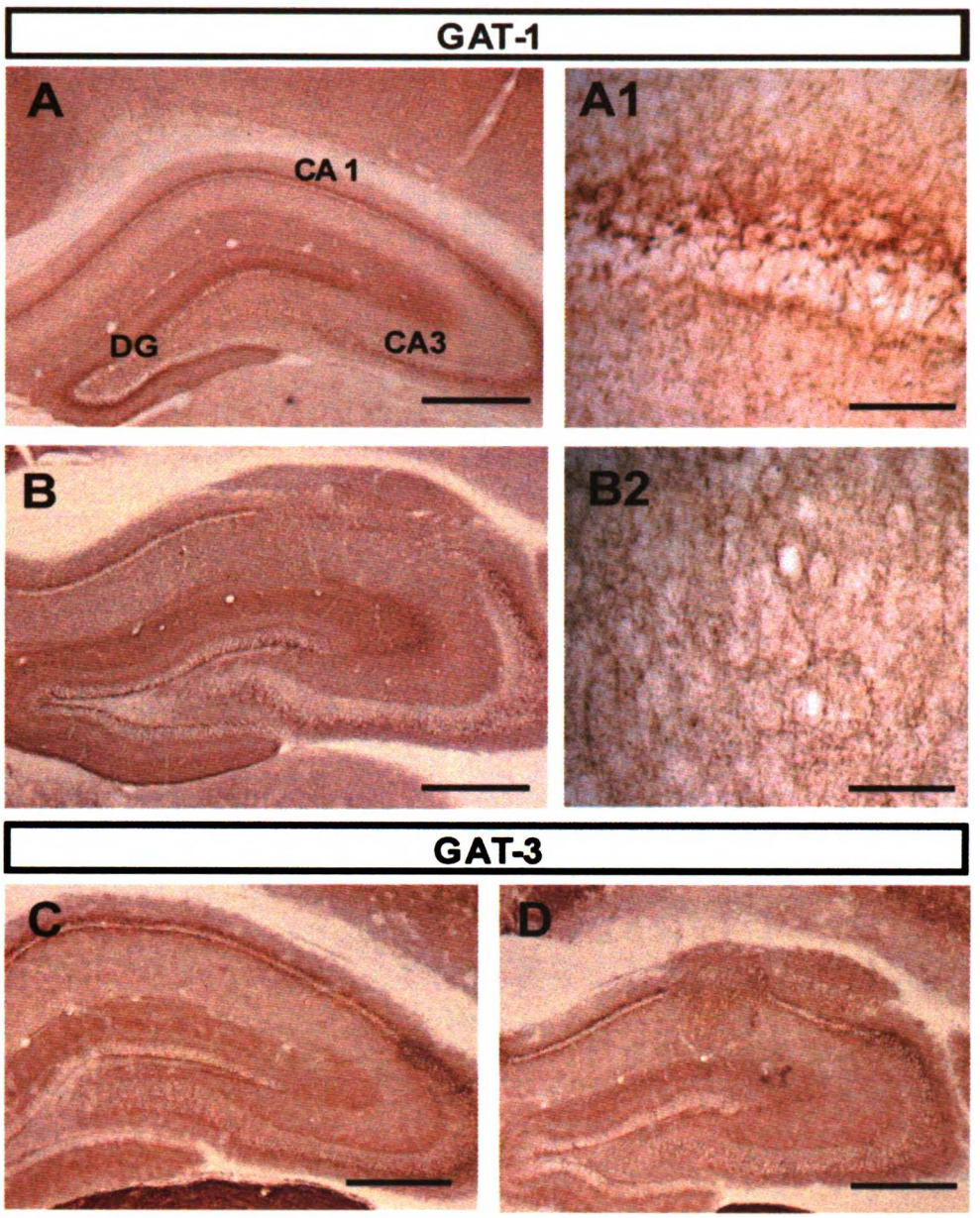


Figure 1.5

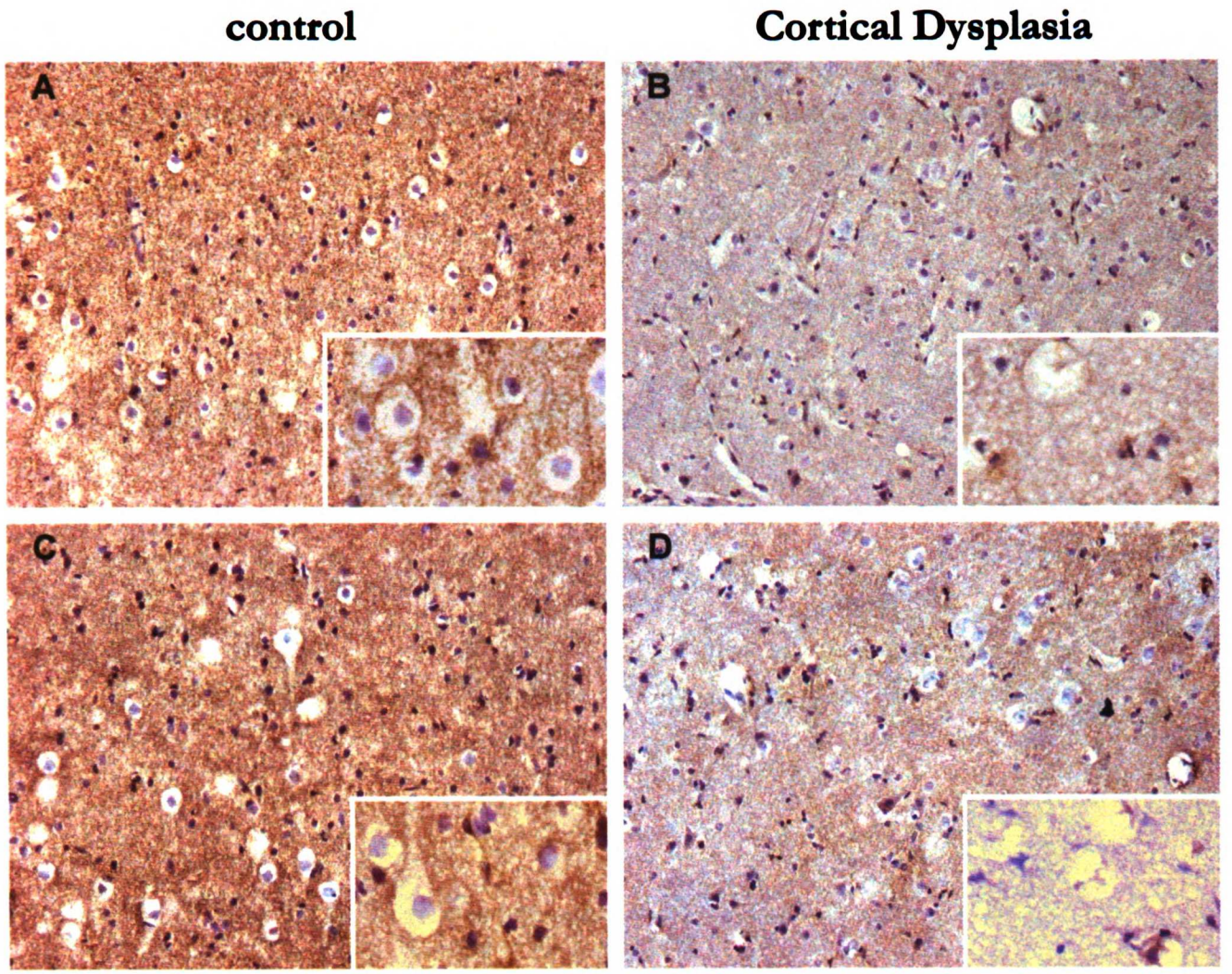
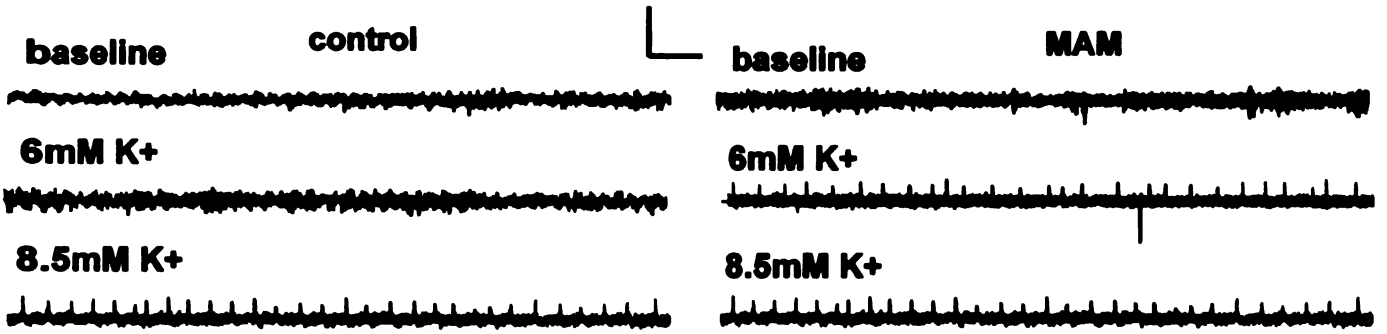
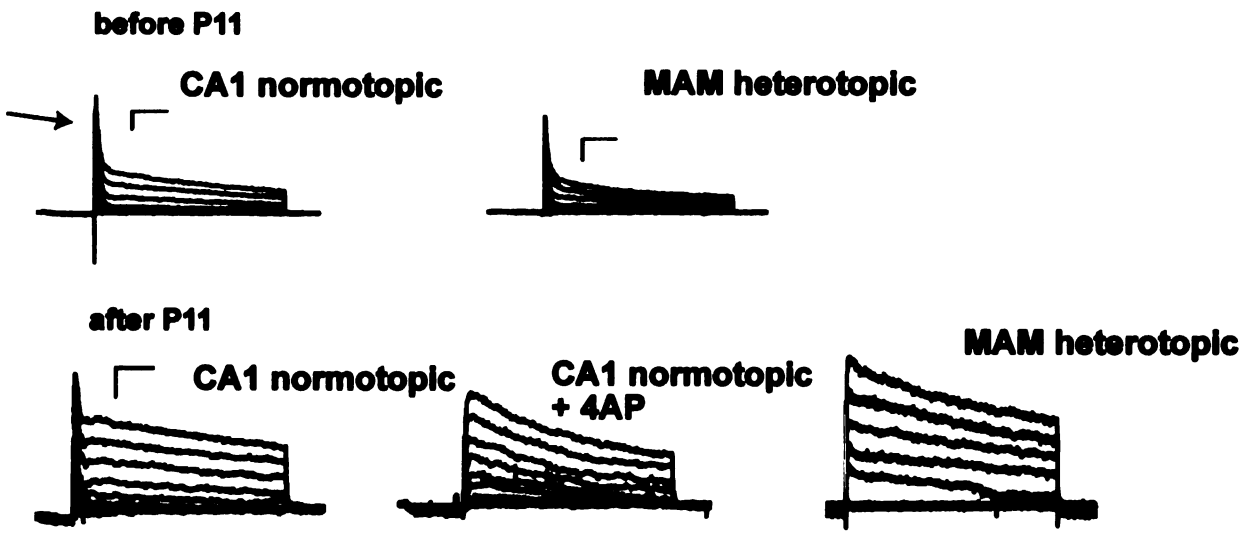


Figure 1.6



B



C

Age	% cells lacking I_A
P0	0 (0/3)
P5	20 (1/5)
P7	13 (1/8)
P11	50 (4/8)
P15	100 (4/4)

Figure 1.7

Chapter 2:

Embryonic and Early Postnatal Abnormalities Contributing to the Development of hippocampal malformations in a Rodent Model of Dysplasia

EMBRYONIC AND EARLY POSTNATAL ABNORMALITIES CONTRIBUTING TO THE DEVELOPMENT OF HIPPOCAMPAL MALFORMATIONS IN A RODENT MODEL OF DYSPLASIA

Abbreviated Title: Development in the MAM Model

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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.

Normal brain development involves a series of complex and highly interrelated events. Genetic and/or environmental defects during 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 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 band heterotopia (*doublecortin*) and lissencephaly (*Lis1*) (Hirotsune 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. Similar sections were stained with antibodies against the following: β-catenin (BD Transduction Laboratories; mouse, 610153; Tateisshi, et al. 2001; Persad, et al., 2001), nestin (Developmental hybridoma Bank, mouse, rat401; Hockfield, et al., 1985), GFAP (Chemicon; rabbit, AB5804; Jungling, et al. 2003), TuJ1 (Covance; mouse, MMS-435P; Jepsen et al., 2000; Byu land, et al., 2003), CSPG (Sigma, mouse monoclonal CS-56; Avnur and Geiger, 1984), reelin

(Chemicon; mouse, MAB5364; de Bergeyck, et al., 1998), Calretinin (Chemicon; rabbit, AB5054 Liu, et al., 2003), and BrdU (AbCam; rat, ab6326; Chauvet, et al., 2004), and GABA. 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 used antibodies that have been well characterized in literature. However, to ensure the reliability of the staining, we included negative controls in our immunostain runs where we incubated the tissue in secondary antibody without prior exposure to a primary antibody. Reelin antibody specificity was further confirmed by staining tissue from rats with mutations in the reelin gene (KZC rats), which showed no detectable staining (data not shown). GABA and calbindin antibody staining were confirmed by staining the tissue from the brains of Dlx1/2 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 (50μg/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 1x PBS washes. Slides were incubated with hybridization buffer (50% formamide, 5x SSC, 0.3mg/ml yeast tRNA, 100µl/ml Heparin, 1x 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 0.2x 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 ± 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 μm compared to an average MAM cortical width of 332 μm (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 postions. 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 administraion. 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 & 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) (Fig. 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%±0.01 deep cells (n=4) while MAM-exposed brain had an average of 42.4%±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; Dambska 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 it's 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 CA_1 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. None the less, 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.

Figure Legends

Figure 2.1

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** = 200μm; **B,D** = 500μm; **C'** = 100μm

Figure 2.2

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 ± 17.2μm while in the MAM brain, the average was 332 ± 8.6μm; n=5 per group. CP = cortical plate; IZ = intermediate zone; SVZ = subventricular zone; VZ = ventricular zone; Scale bars: **A,B** = 500μm; **C,D** = 200μm; **A'-D'** = 50μm.

Figure 2.3

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 = 250μm; high magnification = 130μm.

Figure 2.4

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** = 200μm; **C,D** = 100μm; **G,H** = 50μm.

Figure 2.5

Disruption of the laminar architecture in the MAM-treated rat brain. **A-D:** Tuj1 expression, a marker for the cortical plate and subplate, 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:** Chondroitan 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** = 200μm; **C, D** = 50μm.

Figure 2.6

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** = 80μm; **C,D** = 50μm.

Figure 2.7

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 = 200μm; high magnification = 100μm

Figure 2.8

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**, arrowheads in **E'**). Scale bars: low magnification = 130μm; high magnification = 40μm.

Figure 2.9

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

Figure 2.10

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 and18, 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 = 130μm; high magnification = 40μm.

References

Andre VM, Flores-Hernandez J, Cepeda C, Starling AJ, Nguyen S, Lobo MK, Vinters HV, Levine MS, Mathern GW. 2004. NMDA receptor alterations in neurons from pediatric cortical dysplasia tissue. Cereb Cortex 14:634-46.

Avnur Z, Geiger B. 1984. Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. Cell. 38(3):811-22.

Baraban SC, Schwartzkroin PA. 1995. Electrophysiology of CA1 pyramidal neurons in an animal model of neuronal migration disorders: prenatal methylazoxymethanol treatment.
Epilepsy Res 22:145-56.

Baraban SC, Wenzel HJ, Hochman DW, Schwartzkroin PA. 2000. Characterization of heterotopic cell clusters in the hippocampus of rats exposed to methylazoxymethanol in utero. Epilepsy Res 39:87-102

Battaglia G, Pagliardini S, Saglietti L, Cattabeni F, Di Luca M, Bassanini S, Setola V. 2003. Neurogenesis in cerebral heterotopia induced in rats by prenatal methylazoxymethanol treatment. Cereb Cortex 13:736-48.

Bayer SA, Altman J, Russo RJ, Dai XF, Simmons JA. 1991. Cell migration in the rat embryonic neocortex. J Comp Neurol. 307(3): 499-516.

Benardete EA, Kriegstein AR. 2002. Increased excitability and decreased sensitivity to GABA in an animal model of dysplastic cortex. Epilepsia 43:970-82.

Bicknese AR, Sheppard AM, O'Leary DD, Pearlman AL. 1994. Thalamocortical axons extend along a chondroitin sulfate proteoglycan-enriched pathway coincident with the neocortical subplate and distinct from the efferent path. J Neurosci 14:3500-10.

Bylund M, Andersson E, Novitch B, Muhr J. 2003. Vertebrate neurogenesis is counteracted by Sox1–3 activity Nature Neuroscience **6**, 1162 – 1168.

Castro PA, Cooper EC, Lowenstein DH, Baraban SC. 2001. Hippocampal heterotopia lack functional Kv4.2 potassium channels in the methylazoxymethanol model of cortical malformations and epilepsy. J Neurosci 21:6626-34.

Castro PA, Pleasure SJ, Baraban SC. 2002. Hippocampal heterotopia with molecular and electrophysiological properties of neocortical neurons. Neuroscience 114:961-72

Calcagnotto ME, Baraban SC. 2005 Prolonged NMDA-mediated response, altered ifenprodil sensitivity, and generation of epileptiform-like events in the malformed hippocampus of methylazoxymethanol exposed rats. J. Neurophysiol. 94:153-162

Cattaneo E, Reinach B, Caputi A, Cattabeni F, Di Luca M. 1995. Selective in vitro blockade of neuroepithelial cells proliferation by methylazoxymethanol, a molecule capable of inducing long lasting functional impairments. J Neurosci Res 41:640-7.

Chauvet N, Privat A, Prieto M. 2004. Differential expression of p120 catenin in glial cells of the adult rat brain. J Comp Neurol.1;479(1):15-29.

Chenn A, Zhang YA, Chang BT, McConnell SK. 1998. Intrinsic polarity of mammalian neuroepithelial cells. Mol Cell Neurosci 11:183-93.

Chevassus-au-Louis N, Ben-Ari Y, Vergnes M. 1998a. Decreased seizure threshold and more rapid rate of kindling in rats with cortical malformation induced by prenatal treatment with methylazoxymethanol. Brain Res 812:252-5.

Chevassus-Au-Louis N, Congar P, Represa A, Ben-Ari Y, Gaiarsa JL. 1998b. Neuronal migration disorders: heterotopic neocortical neurons in CA1 provide a bridge between the hippocampus and the neocortex. Proc Natl Acad Sci USA 95:10263-8.

Choi BH, Lapham LW, Amin-Zaki L, Saleem T. 1978. Abnormal neuronal migration, deranged cerebral cortical organization, and diffuse white matter astrocytosis of human fetal brain: a major effect of methylmercury poisoning in utero. J Neuropathol Exp Neurol. 37(6):719-33.

Colacitti C, Sancini G, DeBiasi S, Franceschetti S, Caputi A, Frassoni C, Cattabeni F, Avanzini G, Spreafico R, Di Luca M, Battaglia G. 1999. Prenatal methylazoxymethanol treatment in rats produces brain abnormalities with morphological similarities to human developmental brain dysgeneses. Neuropathol Exp Neurol 58:92-106.

Corbo JC, Deuel TA, Long JM, LaPorte P, Tsai E, Wynshaw-Boris A, Walsh CA. 2002. Doublecortin is required in mice for lamination of the hippocampus but not the neocortex. J Neurosci 22:7548-57.

Cowen D, Geller LM, Wolf A. 1970. Healing in the cerebral cortex of the infant rat after closed-head focal injury J Neuropathol Exp Neurol 29:21-42.

Dabora SL, Jozwiak S, Franz DN, Roberts PS, Nieto A, Chung J, Choy YS, Reeve MP, Thiele E, Egelhoff JC, Kasprzyk-Obara J, Domanska-Pakiela D, Kwiatkowski DJ. 2001. Mutational analysis in a cohort of 224 tuberous sclerosis patients indicates

increased severity of TSC2, compared with TSC1, disease in multiple organs. Am J Hum Genet. 68(1):64-80

Dambska M, Haddad R, Kozlowski PB, Lee MH, Shek J. 1982. Telencephalic cytoarchitectonics in the brains of rats with graded degrees of micrencephaly. Acta Neuropathol.58(3):203-9.

Deguchi K, Inoue K, Avila WE, Lopez-Terrada D, Antalffy BA, Quattrocchi CC, Sheldon M, Mikoshiba K, D'Arcangelo G, Armstrong DL. 2003. Reelin and disabled-1 expression in developing and mature human cortical neurons. J Neuropathol Exp Neurol 62:676-84.

de Bergeyck V, Naerhuyzen B, Goffinet AM, Lambert de Rouvroit C. 1998. A panel of monoclonal antibodies against reelin, the extracellular matrix protein defective in reeler mutant mice. J Neurosci Methods. 1;82(1):17-24.

Dvorak K, Feit J. 1977. Migration of neuroblasts through partial necrosis of the cerebral cortex in newborn rats-contribution to the problems of morphological development and developmental period of cerebral microgyria. Histological and autoradiographical study. Acta Neuropathol (Berl) 38:203-12.

Emery JA, Roper SN, Rojiani AM. 1997. White matter neuronal heterotopia in temporal lobe epilepsy: a morphometric and immunohistochemical study. J Neuropathol

Exp Neurol 56:1276-82.

Eriksson SH, Thom M, Heffernan J, Lin WR, Harding BN, Squier MV, Sisodiya SM. 2001. Persistent reelin-expressing Cajal-Retzius cells in polymicrogyria. Brain 124:1350-61.

Fonseca M, del Rio JA, Martinez A, Gomez S, Soriano E. 1995. Development of calretinin immunoreactivity in the neocortex of the rat. J Comp Neurol 361:177-92.

Garbelli R, Frassoni C, Ferrario A, Tassi L, Bramerio M, Spreafico R. 2001. Cajal-Retzius cell density as marker of type of focal cortical dysplasia. Neuroreport 12:2767-71.

Gierdalski M, Juliano SL. 2003. Factors affecting the morphology of radial glia. Cereb Cortex 13:572-9.

Haddad RK, Rabe A, Laqueur GL, Spatz M, Valsamis MP. 1969. Intellectual deficit associated with transplacentally induced microcephaly in the rat. Science. 163(862):88-90.

Hasling TA, Gierdalski M, Jablonska B, Juliano SL. 2003. A radialization factor in normal cortical plate restores disorganized radial glia and disrupted migration in a

model of cortical dysplasia. Eur J Neurosci. Feb;17(3):467-80.

Hockfield S, McKay RD. 1985. Identification of major cell classes in the developing mammalian nervous system. J Neurosci. 5(12):3310-28.

Hirotsune S, Fleck MW, Gambello MJ, Bix GJ, Chen A, Clark GD, Ledbetter DH, McBain CJ, Wynshaw-Boris A. 1998. Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality.Nat Genet 19:333-9.

Jacobs KM, Hwang BJ, Prince DA. 1999. Focal epileptogenesis in a rat model of polymicrogyria. J Neurophysiol 81:159-73.

Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, Seto E, Hedrick SM, Mandel G, Glass CK, Rose DW, Rosenfeld MG. . 2000. Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell. 15;102(6):753-63.

Johnston MV, Coyle JT. 1979. Histological and neurochemical effects of fetal treatment with methylazoxymethanol on rat neocortex in adulthood. Brain Res. 170(1):135-55.

Jungling K, Nagler K, Pfrieger FW, Gottmann K. 2003. Purification of embryonic stem cell-derived neurons by immunoisolation. FASEB J. 17(14):2100-2.

Kato M, Dobyns WB. 2003. Lissencephaly and the molecular basis of neuronal migration. Hum Mol Genet. 2003 12 Spec 1: R89-R96.

Li MO, Sarkisian MR, Mehal WZ, Rakic P, Flavell RA. 2003. Phosphatidylserine receptor is required for clearance of apoptotic cells. Science 302:1560-3.

Liu S, Wang J, Zhu D, Fu Y, Lukowiak K, Lu YM. 2003. Generation of Functional Inhibitory Neurons in the Adult Rat Hippocampus. J of Neuroscience 23:732-736.
Lombroso CT. 2000. Can early postnatal closed head injury induce cortical dysplasia. Epilepsia. 41(2):245-53.

Luque JM, Morante-Oria J, Fairen A. 2003. Localization of ApoER2, VLDLR and Dab1 in radial glia: groundwork for a new model of reelin action during cortical development. Brain Res Dev Brain Res. Feb 16;140(2):195-203.

Maldonado M, Baybis M, Newman D, Kolson DL, Chen W, McKhann G 2nd, Gutmann DH, Crino PB. 2003. Expression of ICAM-1, TNF-alpha, NF kappa B, and MAP kinase in tubers of the tuberous sclerosis complex. Neurobiol Dis 14:279-90.

Menezes J and Luskin M. 1994 Expression of Neuron-Specific Tubulin Defines a Novel Population in the Proliferative Layers in the Developing Telencephalon. J Neurosci. 14(9): 5399-5416.

Meyer G, Perez-Garcia CG, Abraham H, Caput D. 2002. Expression of p73 and Reelin in the developing human cortex. J Neurosci. 22(12):4973-86.

Meyer G, Soria JM, Martinez-Galan JR, Martin-Clemente B, Fairen A. 1998. Different origins and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. J Comp Neurol 397:493-518.

Miller B, Sheppard A, Bicknese A, Pearlman A. 1995. Chondroitin Sulfate Proteoglycans in the Developing Cerebral Cortex: The Distribution of Neurocan Distinguishes Forming Afferent and Efferent Axonal Pathways. J Comp Neurol. 355: 615-628.

Morante-Oria J, Carleton A, Ortino B, Kremer EJ, Fairen A, Lledo PM. 2003. Subpallial origin of a population of projecting pioneer neurons during corticogenesis. Proc Natl Acad Sci USA. 100:12468-73.

Nagata Y, Matsumoto H. 1969. Studies on methylazoxymethanol: methylation of nucleic acids in the fetal rat brain. Proc Soc Exp Biol Med 132:383-5.

Ohtsuka Y, Yoshinaga H, Kobayashi K. 2000. Refractory childhood epilepsy and factors related to refractoriness. Epilepsia 41[Suppl. 9]:14-7.

Palmini A, Andermann E, Andermann F. 1994. Prenatal events and genetic factors in epileptic patients with neuronal migration disorders. Epilepsia. 35(5):965-73.

Palmini A, Gambardella A, Andermann F, Dubeau F, da Costa JC, Olivier A, Tampieri D, Gloor P, Quesney F, Andermann E,Paglioli E, Paglioli-Neto E, Andermann LC, Leblanc R, Kim, H. 1995. Intrinsic epileptogenicity of human dysplastic cortex as suggested by corticography and surgical results. Ann Neurol 37:476-87.

Persad S, Troussard AA, McPhee TR, Mulholland DJ, Dedhar S. 2001. Tumor suppressor PTEN inhibits nuclear accumulation of β-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. J Cell Biol. 11;153(6):1161-74.

Pleasure SJ, Anderson SA, Hevner RF, Bagri A, Marin O, Lowenstein DH, Rubenstrain JL. 2000 Cell Migration for the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. Neuron. 28(3):727-740.

Rafiki A, Chevassus-au-Louis N, Ben-Ari Y, Khrestchatisky M, Represa A. 1998. Glutamate receptors in dysplasic cortex: an in situ hybridization and immunohistochemistry study in rats with prenatal treatment with

methylazoxymethanol. Brain Res 782:142-52.

Ramakers GM, Urban IJ, De Graan PN, Di Luca M, Cattabeni F, Gispen WH. 1993. The impaired long-term potentiation in the CA1 field of the hippocampus of cognitive deficient microencephalic rats is restored by D-serine. Neuroscience 54:49-60.

Raymond AA, Fish DR, Stevens JM, Sisodiya SM, Alsanjari N, Shorvon SD. 1994. Subependymal heterotopia: a distinct neuronal migration disorder associated with epilepsy. J Neurol Neurosurg Psychiatry 57:1195-202.

Roper SN. 1998. In utero irradiation of rats as a model of human cerebrocortical dysgenesis: a review. Epilepsy Res 32:63-74.

Singh SC. 1977. Ectopic neurones in the hippocampus of the postnatal rat exposed to methylazoxymethanol during foetal development. Acta Neuropathol. 40(2):111-6.

Sisodiya SM. 2004. Malformations of cortical development: burdens and insights from important causes of human epilepsy. Lancet Neurol 3:29-38.

Sloviter RS, Kudrimoti HS, Laxer KD, Barbaro NM, Chan S, Hirsch LJ, Goodman RR, Pedley TA. 2004. "Tectonic" hippocampal malformations in patients with temporal lobe epilepsy. Epilepsy Res 59:123-53.

Smyth MD, Barbaro NM, Baraban SC. 2002. Effects of antiepileptic drugs on induced epileptiform activity in a rat model of dysplasia. Epilepsy Res 50:251-64.

Stuhmer T, Puelles L, Ekker M, Rubenstein JL. 2002. Expression from a Dlx gene enhancer marks adult mouse cortical GABAergic interneurons. Cereb Cortex 12:75-85.

Super H, Del Rio JA, Martinez A, Perez-Sust P, Soriano E. 2000. Disruption of neuronal migration and radial glia in the developing cerebral cortex following ablation of Cajal-Retzius cells. Cereb Cortex. Jun;10(6):602-13.

Super H, Soriano E, Uylings HB. 1998. The functions of the preplate in development and evolution of the neocortex and hippocampus. Brain Res Rev 27:40-64.

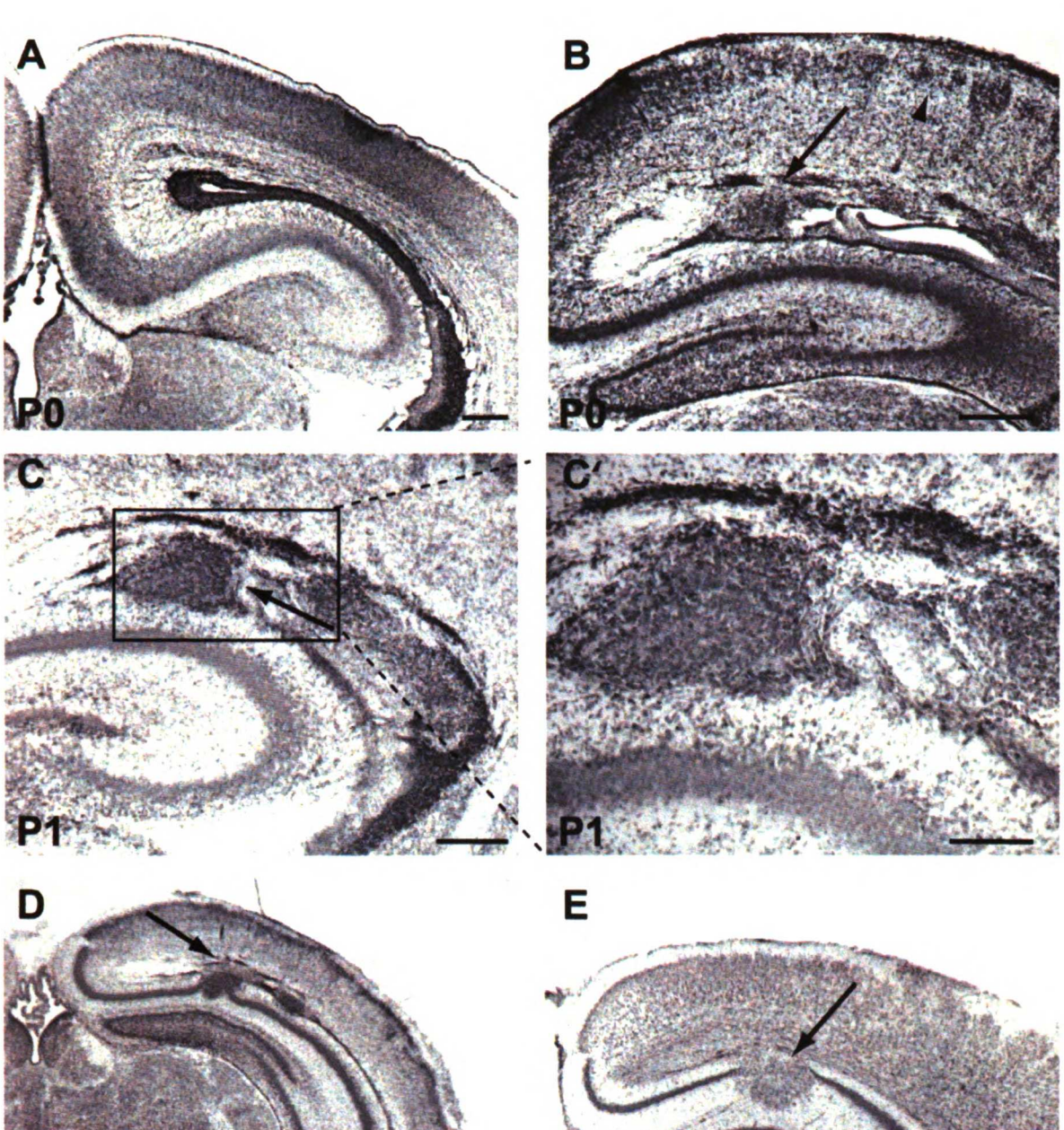
Tateishi K, Omata M, Tanaka K, Chiba T. 2001. The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. J Cell Biol. 12;155(4):571-9.

Thom M, Harding BN, Lin WR, Martinian L, Cross H, Sisodiya SM. 2003. Cajal-Retzius cells, inhibitory interneuronal populations and neuropeptide Y expression in focal cortical dysplasia and microdysgenesis. Acta Neuropathol (Berl) 105:561-9.

White LD, Barone S Jr. 2001. Qualitative and quantitative estimates of apoptosis from birth to senescence in the rat brain. Cell Death Differ 8:345-56.

Zecevic N, Rakic P. 2001. Development of layer I neurons in the primate cerebral cortex. J Neurosci. 21(15):5607-19.

Zhang LL, Collier PA, Ashwell KW. 1995. Mechanisms in the induction of neuronal heterotopiae following prenatal cytotoxic brain damage. Neurotoxicol Teratol. 17(3):297-311.



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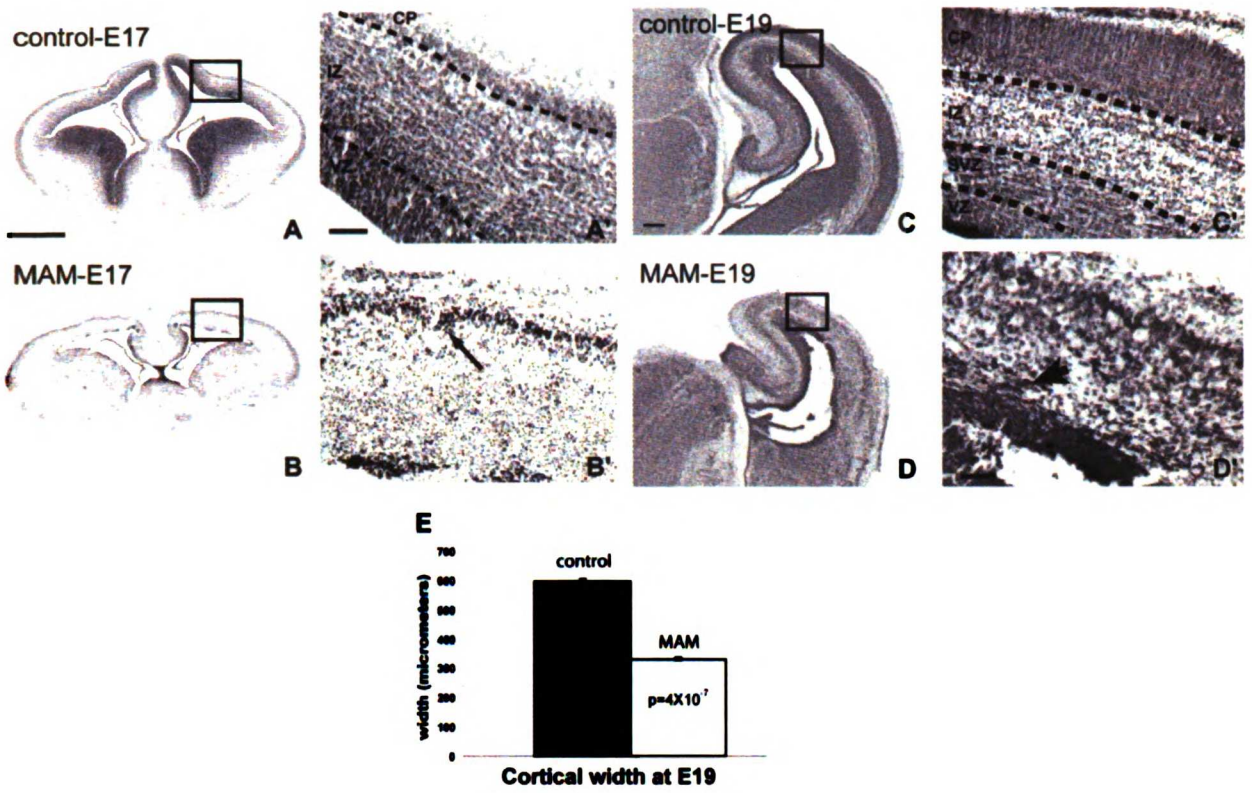
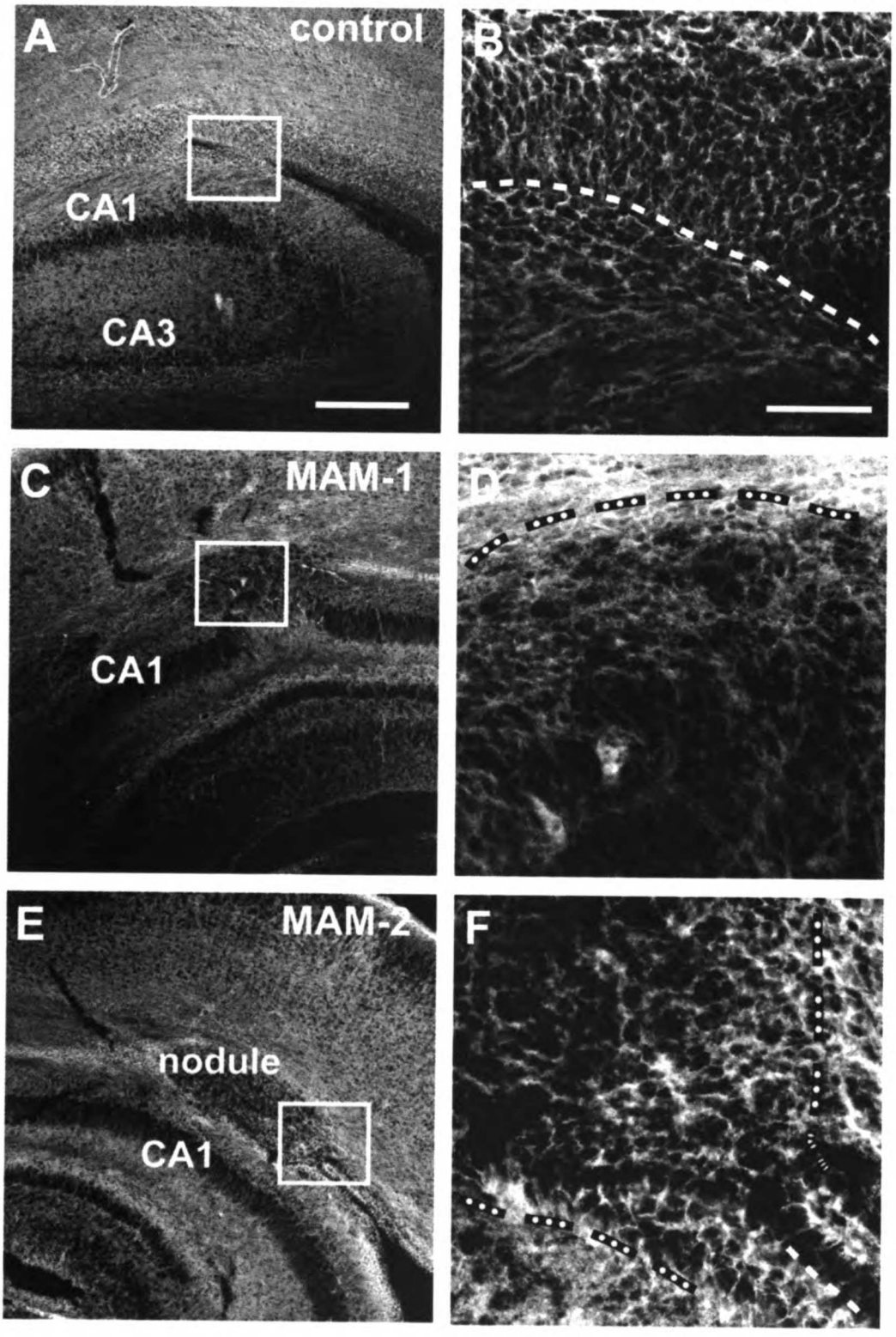

control-E17
A
CP
IZ
VZ
A'
control-E19
C
CP
IZ
SVZ
VZ
C'
MAM-E17
B
B'
MAM-E19
D
D'
E
control
MAM
p=4X10⁻⁷
width (micrometers)
Cortical width at E19

Figure 2.2



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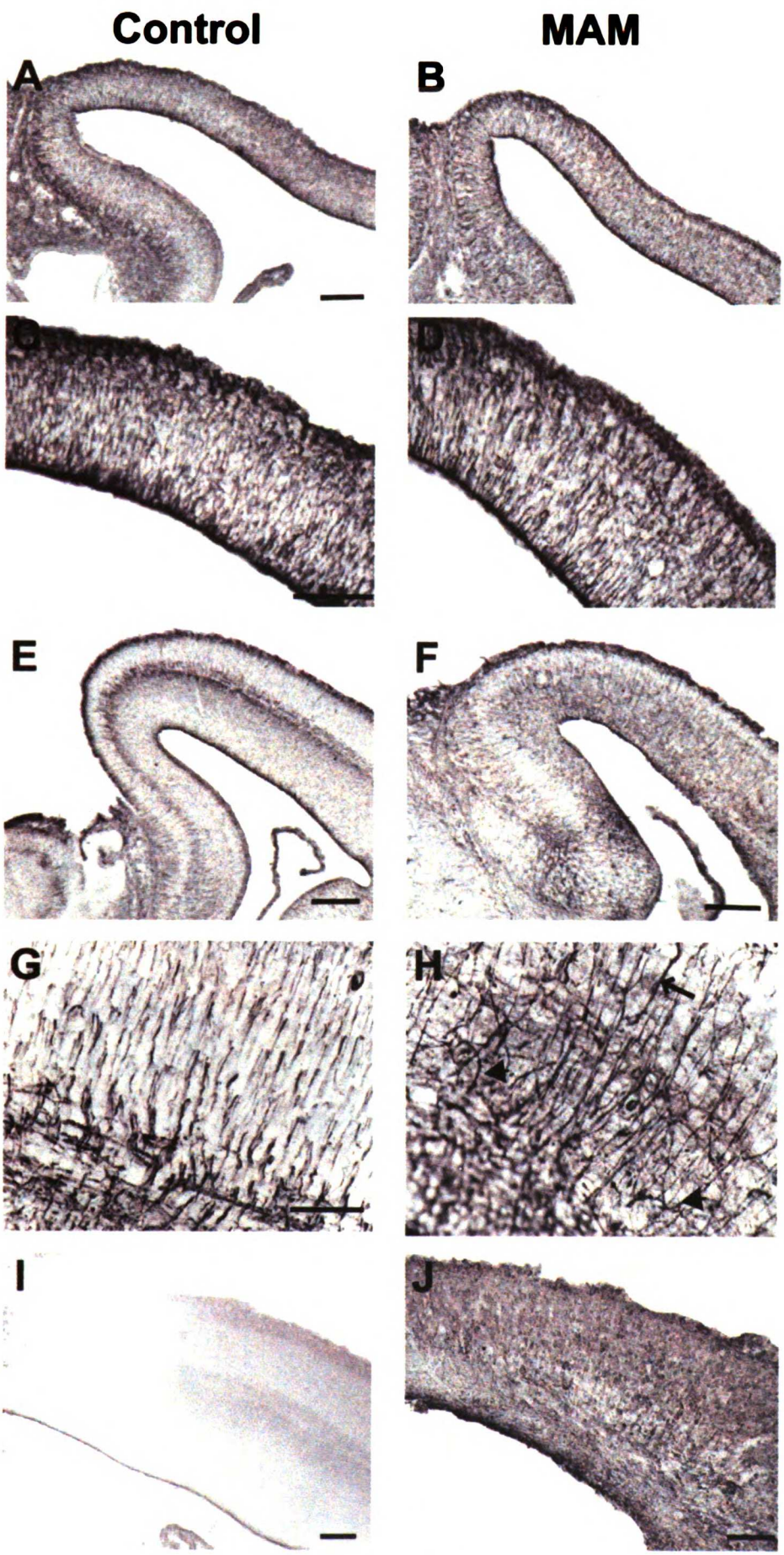

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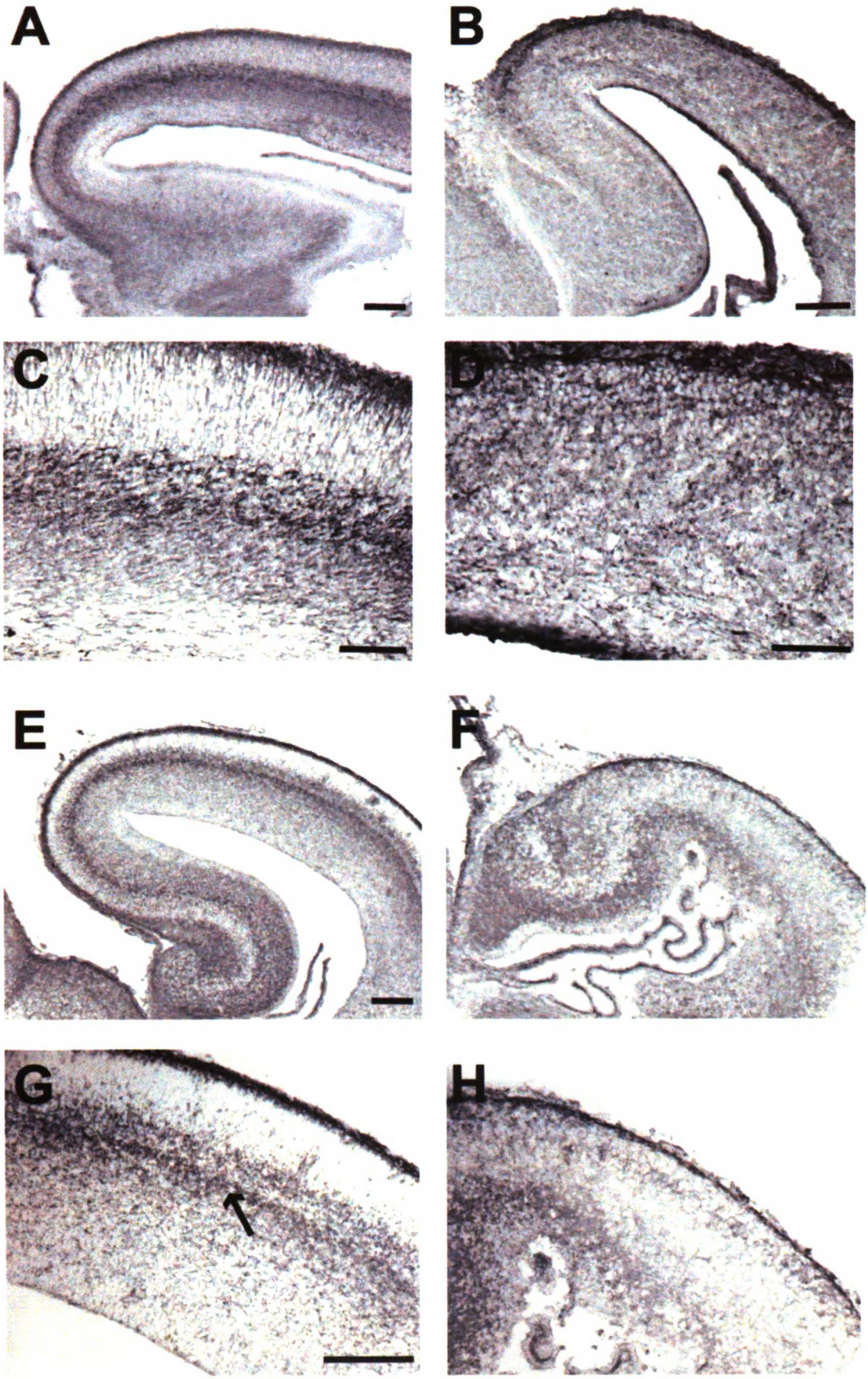

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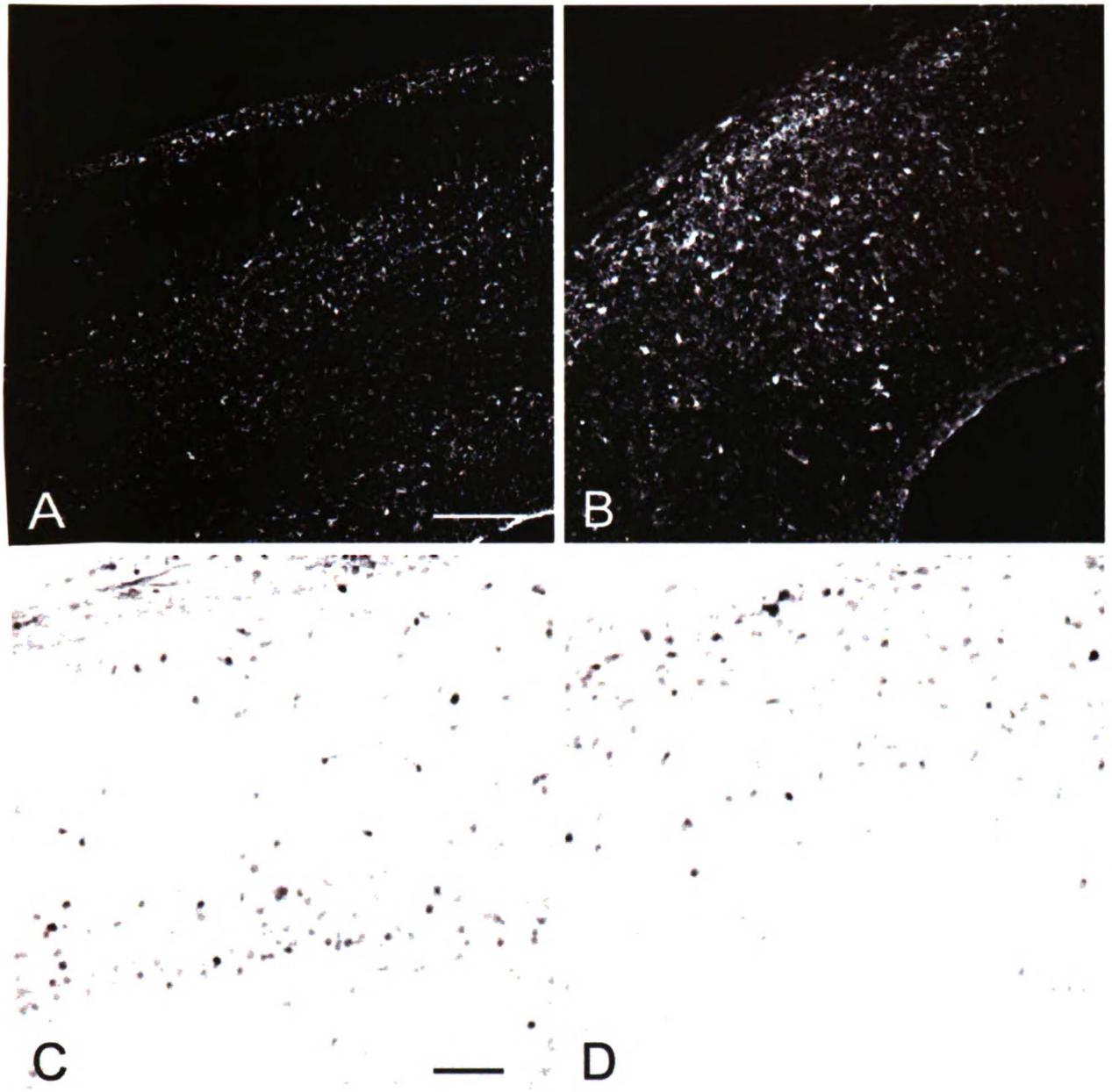

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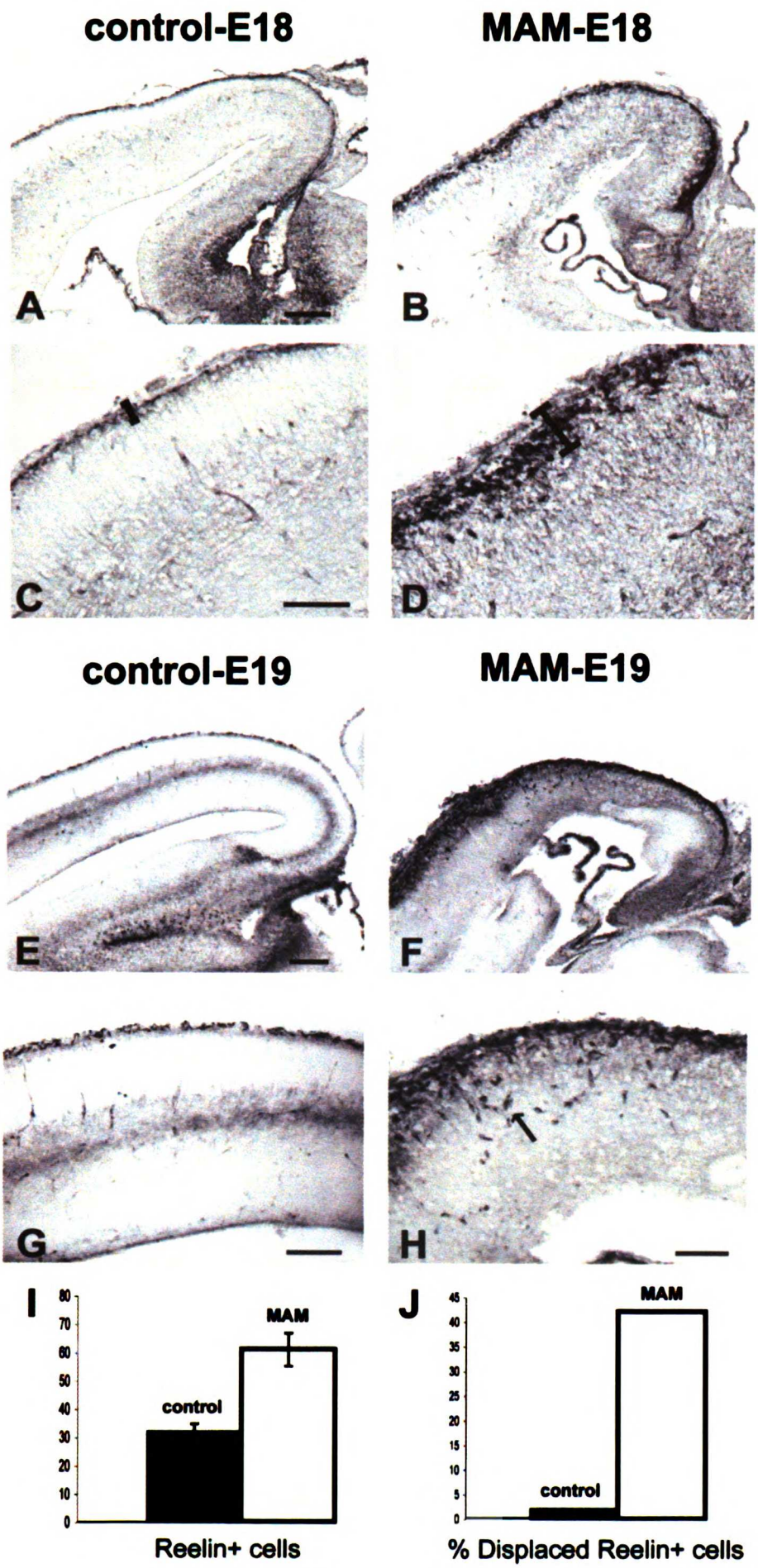

control-E18
MAM-E18
A
B
C
D
control-E19
MAM-E19
E
F
G
H
I
MAM
control
Reelin+ cells
J
MAM
control
% Displaced Reelin+ cells

Figure 2.7

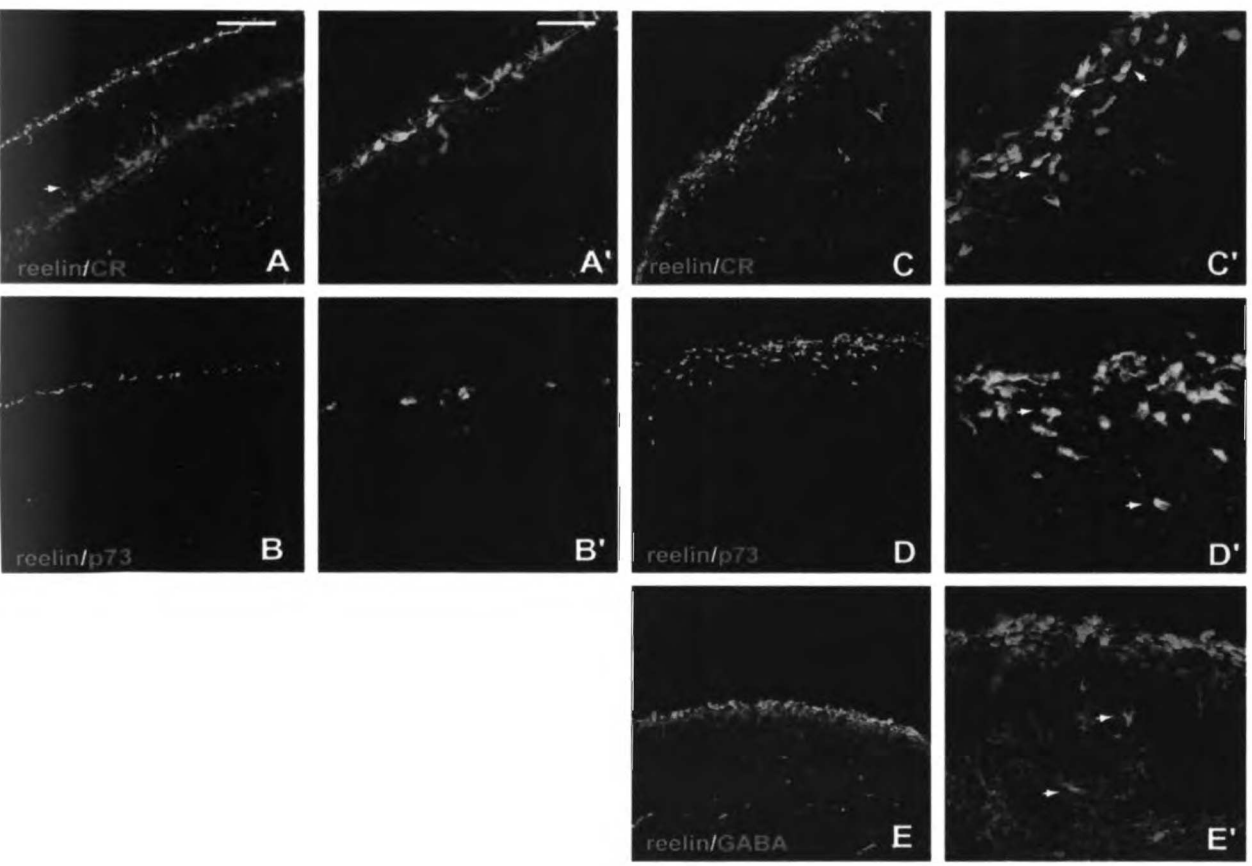
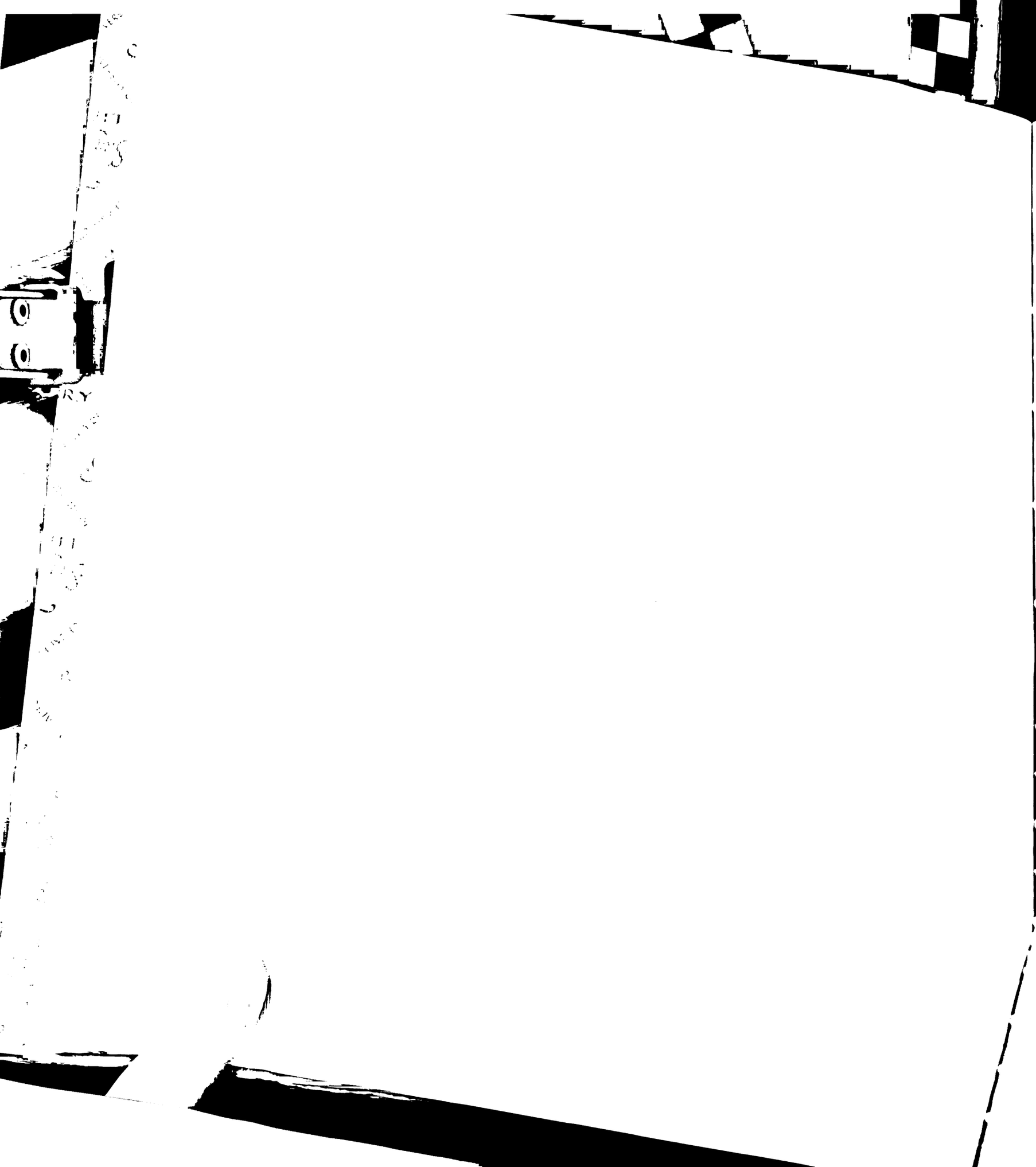
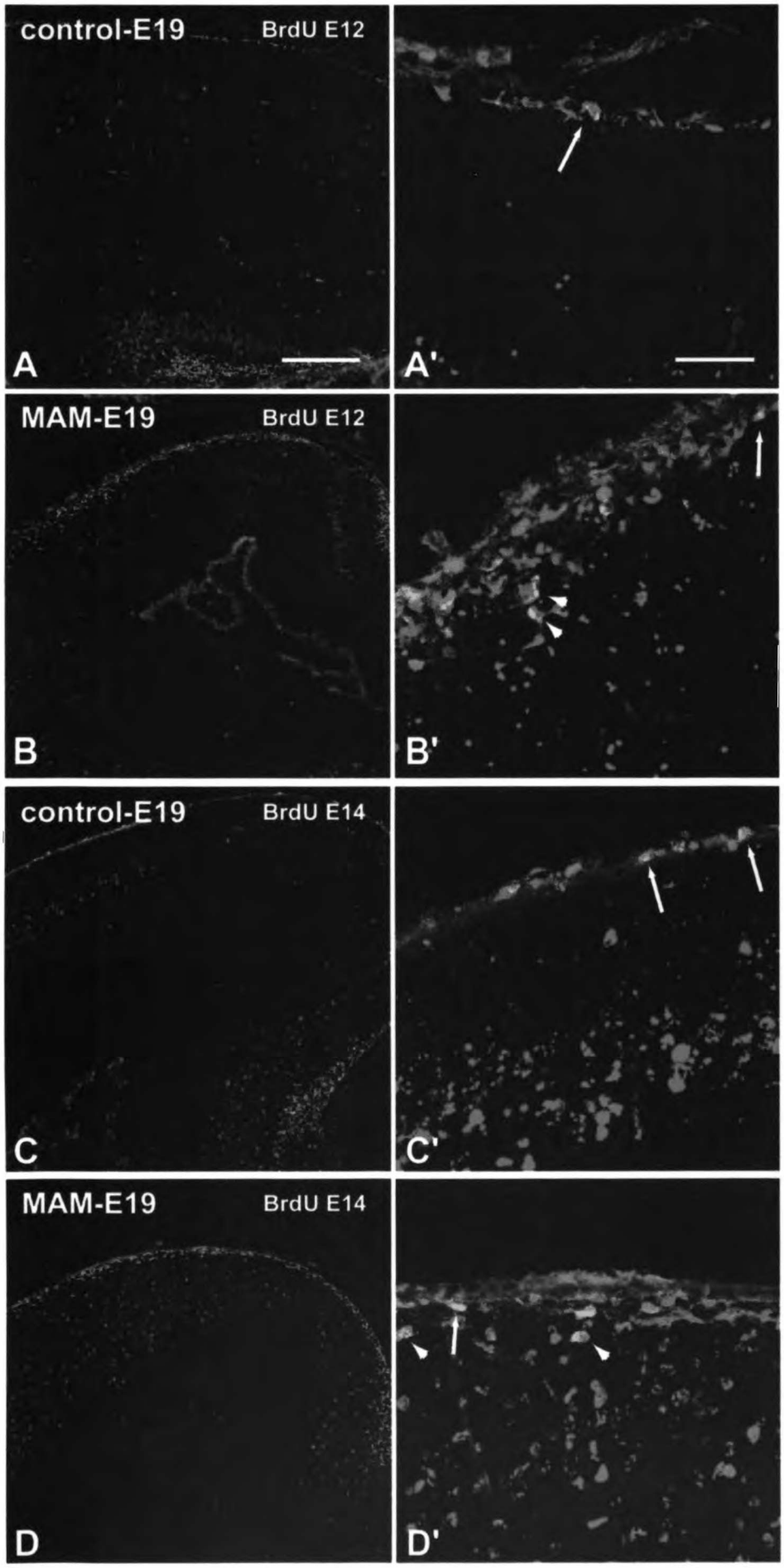

reelin/CR
A
A'
reelin/CR
C
C'
reelin/p73
B
B'
reelin/p73
D
D'
reelin/GABA
E
E'

Figure 2.8




control-E19
BrdU E12
A
A'
MAM-E19
BrdU E12
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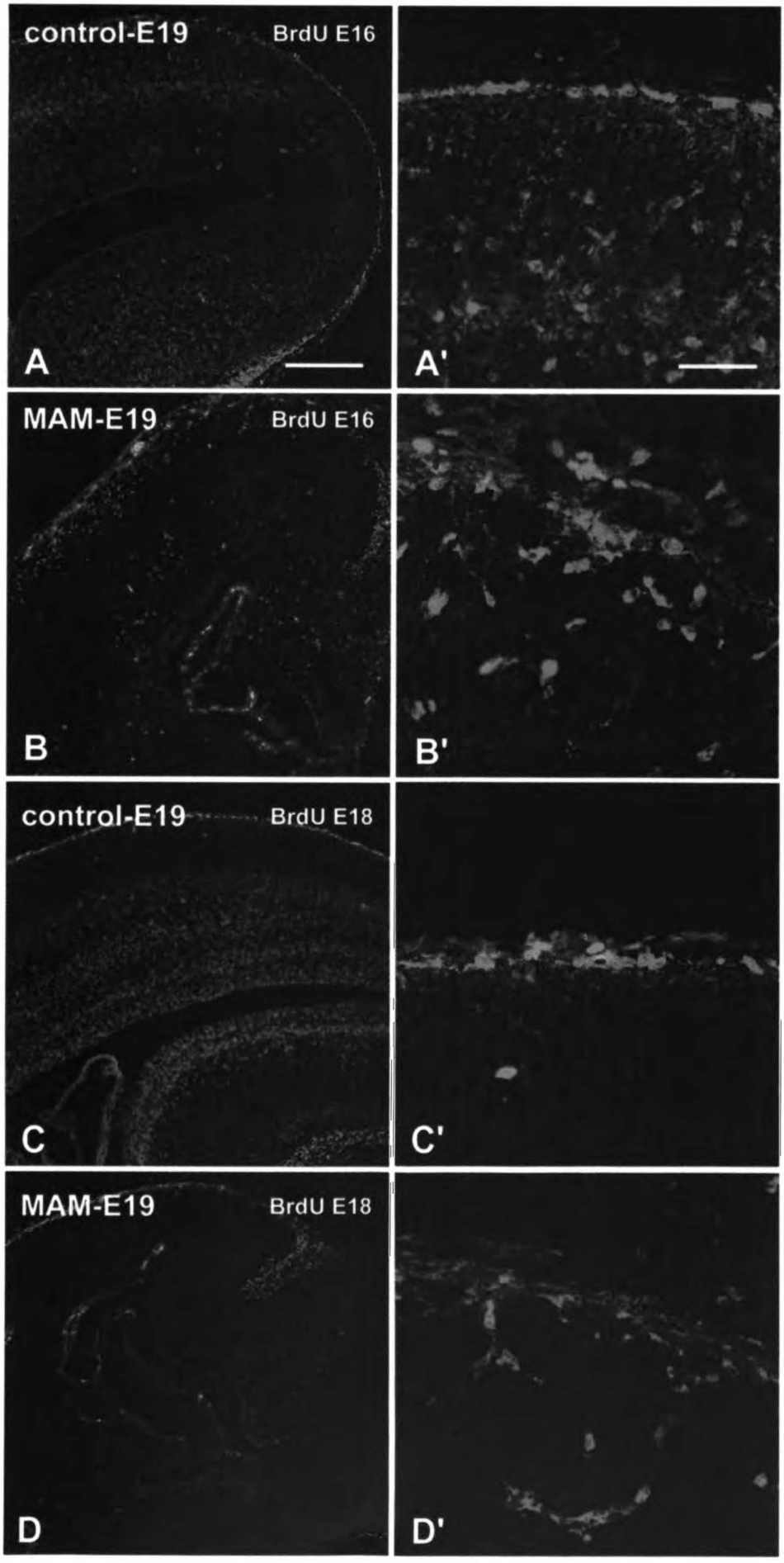
control-E19
BrdU E14
C
C'
MAM-E19
BrdU E14
D
D'

Figure 2.9


control-E19
BrdU E16
A
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MAM-E19
BrdU E16
B
B'
control-E19
BrdU E18
C
C'
MAM-E19
BrdU E18
D
D'

Figure 2.10

Chapter 3:

SDF1 repairs marginal zone dysplasia in a teratogenic model of cortical malformation

SDF1 repairs marginal zone dysplasia in a teratogenic model of cortical malformation

By

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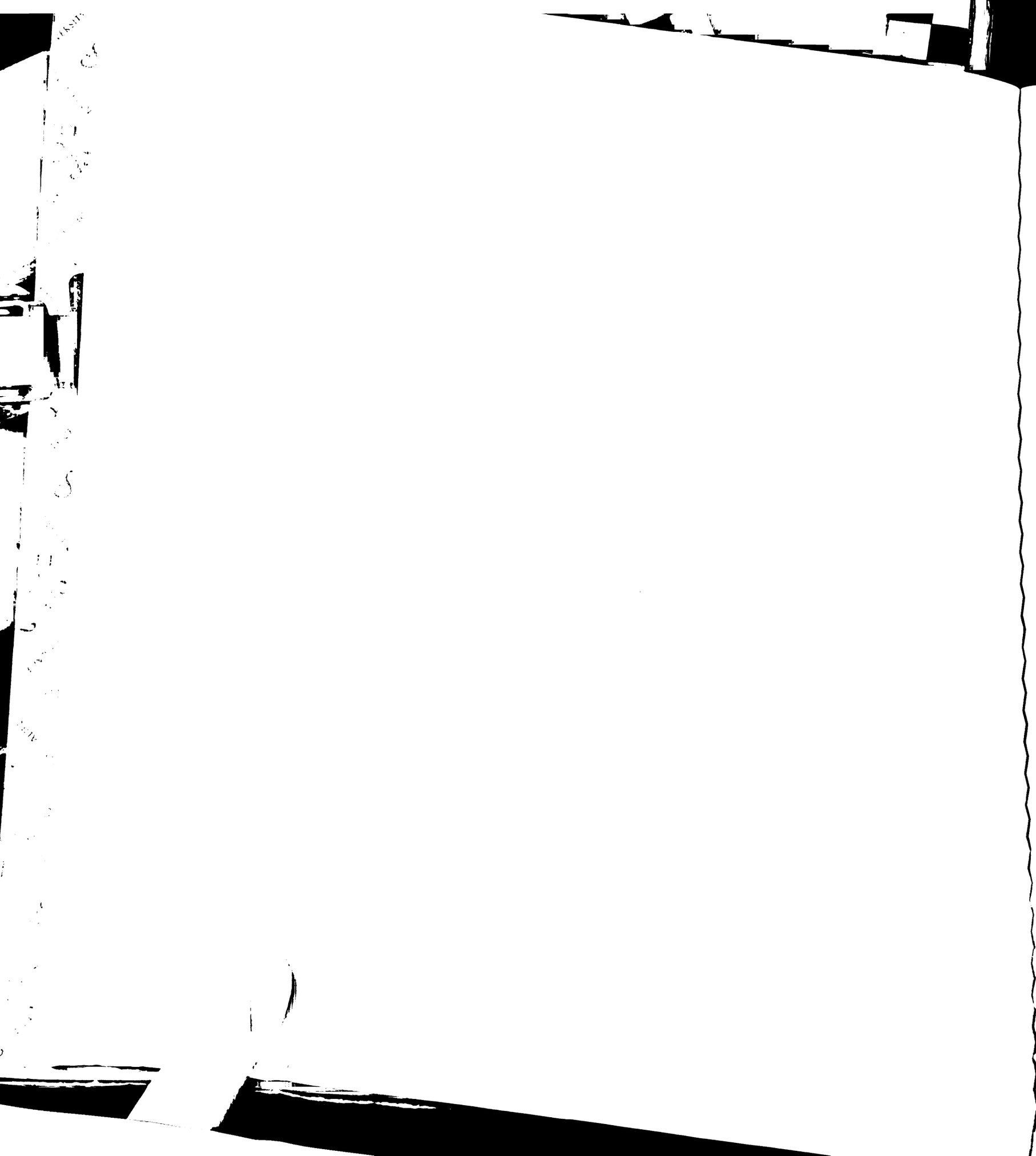
Normal brain development involves a series of highly complex and interrelated steps. The intricate nature of these steps presents opportunities for errors to occur, resulting in defects in the developing brain, clinically referred to as malformations of cortical development. The marginal zone and Cajal-Retzius cells are key players in cortical development and are established early. There is little understanding of why it becomes so disorganized in a variety of cortical malformations. Here we establish that marginal zone disorganization in a widely used teratogenic animal model of malformations of cortical development is caused a decrease of chemokine SDF1 (CXCL12) at the leptomeninges. Furthermore, we establish that SDF1 signaling is required for the maintenance of marginal zone structure during normal cortical development, revealing that cortical layering need not be a static process but likely requires input from locally produced molecular cues.



The six-layered mammalian neocortex is formed by glutamatergic projection neurons, generated in the proliferative zone lining the ventricles. These neurons migrate radially to split the previously formed preplate into a superficially placed marginal zone, containing neurons of cortical Layer I and the subplate, the boundary with the future white matter. Cajal-Retzius cells are a unique cell type populating the marginal zone, which is directly below the meninges. Cajal-Retzius cells regulate neuronal migration into the cortical plate by secreting the glycoprotein reelin (Ogawa, et al. 1995), as well as producing other factors that help support radial glial fibers, the primary conduits for neuronal migration (Super, et al., 1997; Anton, et al., 1997). Very recent studies suggest that Cajal-Retzius cells are generated in up to three discrete germinative zones and then migrate long distances tangentially to establish themselves in the marginal zone early during gestation (Takiguchi-Hayashi, et al., 2004; Muzio, et al., 2005; Bielle, et al., 2005). Loss of Cajal-Retzius cells (or reelin expression) results in loss of the inside-out pattern of radial migration required to generate the normal laminar organization of neocortex (Super, et al., 2000; Ogawa, et al., 1995). In humans, reelin mutations lead to a subtype of Lissencephaly, a malformation of cortical development (MCD), with associated cerebellar hypoplasia (Kato and Dobyns, 2003). Other types of MCD such as polymicrogyria (excessive folding and disorganization of the gyral pattern of the cortex) and focal cortical dysplasia (chaotic disorganization of the normal laminar pattern of organization) also have associated defects in layer I, suggestive of problems in the marginal zone during brain development (Garbelli, et al., 2001; Eriksson, et al., 2001). There have been tremendous advances in understanding the basis of normal and abnormal

cortical development by studying genetic causes of MCD (Olson and Walsh, 2002). However, the most common cases of MCD presenting in humans, especially focal cortical dysplasia (usually in young adults presenting to epilepsy centers) are sporadic and thought to be due to an interplay of environmental and genetic factors (Montenegro, et al., 2002; Sisodiya, 2004). While it is now clear that the marginal zone and Cajal-Retzius cells are key players in cortical development, there is little understanding of why it becomes so disorganized in a variety of MCD. Here we establish that marginal zone disorganization in a widely used teratogenic animal model of MCD is caused by defective chemokine SDF1 (CXCL12) signaling. Furthermore, we establish that SDF1 signaling is required for the maintenance of marginal zone structure during cortical development, revealing that cortical layering need not be a static process but likely requires input from locally produced molecular cues.

We previously observed severe alterations in marginal zone architecture of rats exposed *in utero* to a teratogen, methylazoxymethanol (MAM), at gestational day 15 (E15). MAM is a DNA-alkylating agent that presumably promotes toxicity predominantly by causing the accumulation of defects during DNA replication of dividing cells (Kisby, et al., 1999). However, in the case of marginal zone disruption following MAM exposure, it appears that the effect on Cajal-Retzius cells was via a different, non-cell autonomous mechanism because, at the time of drug exposure, Cajal-Retzius cells had long finished being produced. In fact, since MAM was administered after Cajal-Retzius cells had already occupied the marginal zone (Meyer, et al., 1998; Marin-Padilla, 1998), it was apparent to us that events started by MAM exposure led to

active destruction of marginal zone organization rather than simply a failure to properly form (in submission). The MAM-exposed marginal zone appeared thickened and many reelin-positive Cajal-Retzius cells redistributed to deeper cortical layers (Fig 1A-B). Dispersed cells reached far into the cortical plate and were randomly oriented within the neocortex. Our previous studies established that these cells were Cajal-Retzius cells by expression of multiple markers (in submission). There were compelling similarities between this staining pattern and reelin expression in brain tissue from focal cortical dysplasia patients who underwent surgical resection for epilepsy treatment (Sisodiya, 2004). Tissue from control patients (controls were patients with temporal lobe epilepsy with hippocampal resections but with normal non-spiking temporal neocortex) showed a rather cell sparse layer I and only small numbers of strongly reelin-positive cells in Layer I (Fig. 1C). In contrast, tissue from a patient with focal cortical dysplasia exhibited many more reelin-positive cells and severe disruption of the laminar architecture of the superficial cortical layers, with deep displacement and chaotic polarity of reelin-positive cells (Fig. 1D).

Similar disorganization following MAM exposure was previously observed in cortical slice cultures from MAM-exposed ferret kits and the authors showed that a secreted factor released from control slices was able to rescue the positioning defect (Hasling, et al., 2003). These data suggest that marginal zone defects following MAM exposure are reversible and we reasoned that a teratogenic insult may cause cortical dysplasia by affecting only a small number of molecular pathways. We found that in E20 cortical slice cultures from unexposed rat brains grown for 2 days *in vitro* (2DIV), the

majority of reelin-positive cells were located in the marginal zone adjacent to the leptomeninges (Fig 2A). Brain slices from age-matched MAM-exposed rats demonstrated the dispersal of reelin-positive cells away from the neocortical surface and a loss of longitudinal orientation (Fig 2B). We quantified this abnormality by examining the percentage of strongly reelin-positive cells confined to the marginal zone versus those dispersed into deeper layers (greater than 2 cell widths from the pial surface of the cortical slice). Control slices had significantly fewer reelin-positive cells in deeper layers than MAM-exposed (Control: 24.5% ± 1.1%; MAM: 43.7% ± 1.7; $p < 0.001$, $n = 5$ slices per condition). To determine if secreted factors from normal slices could rescue this defect we cultured age-matched slices from control and MAM-exposed animals together in shared media. The marginal zone of co-cultured control slices was similar to that of slices grown on their own. However, the marginal zone of co-cultured MAM slices was different from those of MAM slices grown alone (Fig 2C). Quantification of these changes (Fig 2G) showed that the percentage of dispersed or "deep" reelin-positive cells in the marginal zone from MAM slices grown alone was statistically greater than that in the marginal zone of MAM slices co-cultured with control slices (43.7% in slices grown alone vs. 25.8% in co-cultured MAM-exposed; $p < 0.001$; $n = 5$ slices per condition). Thus co-culture with control slices essentially rescues the marginal zone disruption caused by MAM exposure. We also observed the "rescue" of the marginal zone when we used conditioned media (media incubated with control slices) to culture MAM-exposed (MAM: 46.2% ± 5.8%; MAM-exposed in conditioned media: 19% ± 3.1%; $p < 0.05$; $n =$ 4 slices per group). It is important to note that this is a true rescue rather than a failure of the marginal zone defect to occur since our previous *in vivo* analysis showed that by

gestational age of slice preparation the marginal zone disruption was already quite advanced (in submission) and the co-culture apparently reversed this process.

We next hypothesized that the MAM-exposed rat brain was lacking a factor that was normally present in the leptomeninges. This factor would maintain Cajal-Retzius cell localization in the marginal zone adjacent to the neocortical surface by a chemotactic mechanism. Previous studies suggested several candidate factors able to affect Cajal-Retzius cell positioning under some conditions (Ringstedt, et al., 1998; Brunstrom, et al., 1997; Stumm, et al., 2003; Alcantara, et al., 2005). We exposed slices from MAM-exposed brains separately to Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-4 (NT-4), and Stromal-Derived Factor-1 (SDF1) for 2DIV and observed the subsequent positioning of reelin-positive cells in the marginal zone. Slices with 50ng/ml BDNF or NT-4 added to the media showed no rescue of the MAM phenotype (BDNF: 45.5% ± 8.8% displaced cells; NT-4: 40% ± 4.7% displaced cells; no factor: 39% ± 2.2%) (Fig 2H). However, MAM-exposed slices incubated with 100ng/ml SDF1 appeared quite similar to MAM-exposed co-cultured with control slices when stained for reelin (Fig 2E). Quantification showed that SDF1 rescued the MAM marginal zone phenotype (39% ± 2.2% for MAM slices alone vs. 20.1% ± 1.1% for SDF1 treated slices; $p < 0.001$; $n = 6$ slices per condition) (Fig 2H). Thus, SDF1 was sufficient to rescue the localization of Cajal-Retzius cells with levels comparable to control slices or slices treated with conditioned medium.

To determine if SDF1 signaling was also required to rescue Cajal-Retzius cell positioning we examined whether interrupting SDF1 signaling would prevent rescue. SDF1 is the only known ligand for the chemokine receptor, CXCR4, and several CXCR4 antagonist compounds have been devised (Donzella, et al., 1998; Tamamura, et al., 1998). We added a CXCR4 antagonist, AMD3100, to co-cultured MAM slices. The addition of AMD3100 to co-cultured MAM slices blocked rescue (25.9% in co-cultured MAM vs. 44.6% in co-cultured MAM slices with AMD3100; $p = 0.006$; $n=5$ per condition). Interestingly, there was no significant difference between the percent of displaced cells in MAM-exposed slices grown alone and in co-cultured MAM-exposed slices incubated with AMD3100 (Fig 2G). Therefore, SDF1 activity is necessary and sufficient for the rescue of Cajal-Retzius cell displacement.

Our data indicated that SDF1 rescued the marginal zone phenotype in MAM-exposed rats, but was SDF1 signaling likely to be involved in the causation of this defect? We reasoned that MAM exposure could lead to alterations of either SDF1 expression or its cognate receptor, CXCR4 (previously shown to be expressed in Cajal-Retzius cells (Stumm, et al., 2003), in the developing brain and that this might be sufficient to induce Cajal-Retzius cell displacement from the meninges. Double *in situ* hybridization showed that in both control and MAM-exposed brains, reelin-positive cells at the marginal zone expressed CXCR4 (Fig 3B and E). In addition, the displaced reelin-positive cells of the MAM-exposed brain were also CXCR4-positive (Fig 3E and F). Thus, MAM does not appear to cause Cajal-Retzius cell displacement by creating a subset of cells unable to respond to SDF1. However, we did find dramatic decreases in SDF1 expression in the

meninges of the MAM-exposed brain (Fig. 3H). In controls, reelin-expressing cells at the marginal zone were situated compactly against the SDF1-rich meninges (Fig. 3G). SDF1 expression in the meninges of the MAM-exposed brain was remarkably lower. Associated with this decrease was a thickening of the marginal zone and scattering of reelin-positive Cajal-Retzius cells (Fig 3H). Taken together, these data indicated that while there was a reduction in SDF1 expression in the meninges of MAM-exposed animals, Cajal-Retzius cells in the marginal zone retained CXCR4 expression. Displaced Cajal-Retzius cells, though disorganized, would likely respond to SDF1. This explains how the localization of Cajal-Retzius cells in the MAM-exposed brain could be affected in culture by manipulating the SDF1/CXCR4 pathway.

It seemed from our expression studies that MAM-exposure caused decreased SDF1 expression in the meninges. Consequently, we wondered if MAM induced direct injury to embryonic meninges. MAM is an alkylating agent with anti-proliferative effects (Kisby, et al., 1999), and the meninges undergo dramatic expansion at the same time as cortical expansion. We therefore examined whether MAM induced apoptotic cell death in the meninges 24 hours after injury. The E16 MAM-exposed brain had a remarkable and widespread increase in TUNEL labeling compared to control. TUNEL labeling was high throughout most of the E16 neocortex: in the cortical plate, intermediate zone, ventricular zone and meninges. Most of this is consistent with the widespread effects of MAM on the cortical ventricular and subventricular zones observed previously (in submission), but the presence of apoptotic cells in the meninges but not the marginal zone indicated that MAM exposure may cause direct toxicity to the meninges. We used laminin (Fig. 4E), an

extracellular matrix component of the pial basement membrane produced by meningeal cells (Halfter, et al., 2002; Beggs, et al., 2003) to delineate the meninges. Double labeling for laminin and TUNEL-positive cells confirmed that apoptotic cells were likely to be meningeal cells (Fig. 4F, G). Despite the evident increase in meningeal cell death at E16, laminin staining showed only very mild alterations in the basement membrane by E19 (Fig. 4H). 24 hours after teratogen insult, examination of another major constituent of the meningeal basement membrane, chondroiton sulfate proteoglycan (CSPG) (McCarthy et al., 1989), showed that it was acutely unaffected (Fig. 5A-B). However, 4 days after exposure, the meningeal basement membrane of the MAM-exposed brain was markedly thinner (Fig. 5C-D). Quantification of the basement membrane thickness, as defined by CSPG staining, showed a significant difference between control brains and MAM-treated brains (control: 19μm±0.58 μm; MAM: 10μm±1.1 μm; n=3 per set; $p<0.05$) .

Our data indicated that SDF1 was a key player in the mechanism of marginal zone disruption following teratogenic insult by MAM. We wanted to determine if Cajal-Retzius cells depended on SDF1 for their position in the normal marginal zone, since our co-culture studies indicated that this was the case. When we used AMD3100, not only was rescue of the MAM slice prevented, but in addition, there were more deep reelin-positive cells in the control slices. To further study the effect of blocking endogenous SDF1/CXCR4 signaling, we incubated control slices separately with two different CXCR4 antagonists, AMD3100 and TN14003, and quantified the displacement of Cajal-Retzius cells from the marginal zone. Slices with both CXCR4 blockers showed a similar magnitude of marginal zone disorganization (Fig 6A). The proportion of deep reelin-positive cells in the blocker-treated slices was comparable to that in MAM slices

(Control: 22.1% ± 1.7% deep cells; AMD3100-exposed: 49.0 % ± 4.8% deep cells; TN14003-exposed 49.1% ± 3.7% deep cells; $p < 0.001$; n=7 slices per condition) (Fig 6B). Therefore, we hypothesized that SDF1 is necessary for maintaining the normal organization of the marginal zone. We then confirmed an *in vivo* role for SDF1 in marginal zone organization in the rat brain by *in utero* intraventricular injections of the CXCR4 blocker, TN14300. We administered either saline solution (control) or TN14003 into E20 or E21 rat embryos, which we then collected at E22, 24 hours or 48 hours post-surgery. The marginal zone of saline-injected embryos was normal up to 48 hours after surgery. Reelin-positive Cajal-Retzius cells were found in a single-cell layer adjacent to the meninges (6C-D; n=3 per each age of injection). However, the TN14003-treated E22 embryos showed early signs of marginal zone disruption with Cajal-Retzius cells displaced from the meningeal surface as early as 24 hours after drug injection (Fig. 6E-F; n=4 embryos). The disruption was even more severe 48 hours after exposure to the drug (Fig. 6G-H; n=4 embryos).

Our data revealed similarities in the developmental organization of the cortex and cerebellum. SDF1 was first implicated as a chemoattractant in leukocyte chemotaxis, but was soon found to play several roles in brain development (Nagasawa, et al., 1996; Zou, et al., 1998; Ma, et al., 1998; Tran and Miller, 2003). In the nervous system, SDF1 regulates the migration of sensory neuron precursor cells in the dorsal root ganglion (Belmadani, et al., 2005), and acts as a chemoattractant for embryonic cerebellar neurons (Zhu, et al., 2002) and dentate granule neurons (Bagri, et al., 2002). In the cerebellum, SDF1 is secreted by the meninges. This ligand transiently keeps progenitor cells in the developing cerebellar cortex in the external granular layer (EGL), in proximity to the

overlying meningeal cells (Reiss, et al., 2002). Sulfated proteoglycans within the leptomeningeal basement membrane concentrate factors produced by meningeal cells, such as SDF1 (Reiss, et al., 2002). In fact, there has been evidence for direct localization and presentation of SDF1 by basement membrane proteoglycans in other systems as well (Netelenbos, et al., 2003). Our data suggest that a similar mechanism is acting on Cajal-Retzius cells to retain them adjacent to the meninges. The ability of SDF1 to bind to proteoglycans is the likely reason that conditioned media and SDF1 added to media are capable of rescuing Cajal-Retzius cell positioning in a localized manner. A variety of mice with mutations in components of the meningeal basement membrane (such as laminin γ1 and beta-1 class integrins) have been described and many of these lead to alterations in the organization of Cajal-Retzius cells in the marginal zone (Halfter, et al., 2002; Graus-Porta, et al., 2001) similar to those seen in the marginal zone of the MAM-exposed brain. Our data suggest that when the basement membrane of the leptomeninges is disrupted, SDF1 cannot be properly concentrated at the meningeal surface, where it functions to restrict Cajal-Retzius cells to the superficial layer.

Our current study has two major findings with significant implications. First, we show that a form of marginal zone dysplasia seen in the MAM teratogenic model of MCD, which mimics key aspects of human MCD (Colacitti, et al., 1999), is caused by interruption of SDF1 signaling. Even after established, this phenotype can be rescued, a finding that could have therapeutic potential. Second, we show that SDF1 is required during normal corticogenesis to maintain Cajal-Retzius cell position in the marginal zone. The plastic nature of cellular position in the marginal zone further indicates potentially

important lessons for understanding cortical development in general. It is generally assumed that neuronal laminar positioning is a stable event once established, but this may not necessarily be true. Since, Cajal-Retzius cells are central regulators of radial migration in the cortex it is possible that their displacement may have secondary effects on neocortical neuronal positioning in other layers. Also, it is quite possible that other, currently unknown, signals may regulate the stability of laminar positioning of other types of cortical neurons. Thus, we conclude that marginal zone architecture is dependent on continuous signaling by SDF1 and the integrity of the meningeal basement membrane. Therefore the marginal zone is a dynamic structure requiring maintenance throughout neocortical development. The conservation of marginal zone structure is critical as the cortex expands over a long time frame and is dependent on the appropriate signals from this region. Since Cajal-Retzius cells in the marginal zone play a crucial role in proper neocortical organization, damage to this area would have severe consequences in later neocortical development.

Figure 3.1. Brains from MAM-treated rats have marginal zone dysplasia similar to that in patients with focal cortical dysplasia. Reelin expression visualized by *in situ* hybridization revealed the difference between Cajal-Retzius cells at the marginal zone in E19 control (C) and MAM-exposed (D) rat brains. The marginal zone in MAM-treated brains is several cell-layers thick, and many reelin-positive cells have no contact with either the meninges or neighboring Cajal-Retzius cells. The Cajal-Retzius cells in the marginal zone of the MAM-treated brain are also randomly oriented, unlike those in the control marginal zone, which have horizontal polarity. Hematoxylin and reelin immunostaining on surgical tissue from a non-MCD patient (A; patient had temporal lobe sclerosis) and a patient with Focal Cortical Dysplasia (B). Immunostaining shows absence of labeled cells in layer I of non-MCD tissue but their presence scattered throughout the superficial region of the MCD tissue. This pattern was replicated in 4 other patients with focal cortical dysplasia. Scale bar: A,B- 130μm; C,D-100μm

Figure 3.2. Slice culture studies show that SDF1 is is necessary and sufficient to maintain normal positioning of Cajal-Retzius cells in the marginal zone of the MAM-treated brain. (A) Example of a 2DIV cortical slice used. Boxed area shows the region on slices used to quantify the number of reelin-positive cells. (B) 2DIV cortical slices from E20 control rats show a normal marginal zone, with reelin-positive cells positioned individually along the meninges. (C) 2DIV slices from E20 MAM-treated brains show marginal zone disorganization similar to that seen *in vivo*, with a thicker marginal zone and many scattered reelin-positive cells. (D) Co-cultured MAM slices showed fewer dispersed reelin-positive cells in the marginal zone compared to MAM slices cultured alone. (E)

Addition of a CXCR4 antagonist, AMD3100, to 2DIV co-cultured slices increased the scattering of reelin-positive cells in the MAM slices. Many labeled cells in the slices lost contact with the meninges and no longer shared a common orientation. (F) 2DIV slices from E20 MAM brains were grown with SDF1. The localization of reelin-positive cells in these slices was similar to that seen in control slices and co-cultured MAM slices. (G) Quantification of the proportion of deep reelin-positive cells demonstrates that co-cultured cortical slices from E20 MAM-treated brains had fewer cells separated from the marginal zone. When AMD3100 was added to the co-cultures, we observed a subsequent increase in the percent of deep reelin-positive cells in the co-cultured MAM slice. (H) Quantification of the effect that exposure to BDNF, NT-4 or SDF1 and NT-4 had on the percent of deep reelin-positive cells in MAM slices. MAM slices cultured with SDF1 had significantly fewer deep reelin-positive cells than MAM slices grown without any factor. Scale bar: 100μm

Figure 3.3. Displaced Cajal-Retzius cells in the MAM-exposed brain expressed CXCR4 but the overlaying leptomeninges showed a dramatic decrease in SDF1 expression. Fluorescent double *in situ* hybridization performed on E19 SD rat brain sections illustrated that Cajal-Retzius cells, as labeled by reelin (A), also expressed the SDF1 receptor, CXCR4 (B). Cajal-Retzius cells in the MAM-treated brain were also double-labeled with reelin (D) and CXCR4 (E), including the scattered Cajal-Retzius cells that had left the neocortical surface (arrow in F). SDF1 was normally expressed in the meninges (G) of E19 rat brains, and reelin-positive cells were located directly below, in the marginal zone (A). There was a tight apposition of Cajal-Retzius cells against the

SDF1-rich meninges. (H) SDF1 expression in the meninges was lower in equivalent MAM-exposed brain sections, and Cajal-Retzius cells were scattered below the meninges that lacked SDF1. Scale bar: A-40μm ;G-130μm

Figure 3.4. There was an acute increase in cell death and damage in the meninges of the MAM-treated rat brain. (A) E16 control rat brains had a basal level of TUNEL labeling throughout the cortical plate, which is normally observed in the developing neocortex. The basement membrane of the leptomeninges, as marked by laminin immunostaining (B) had been laid down. (C) The merged picture of the E16 brain showed minor TUNEL labeling in the meninges. (D) There was a dramatic increase in TUNEL labeling 24 hours after MAM administration, indicative of higher levels of cell death and damage. This increase in the E16 MAM-treated bain took place throughout the neocortex, especially in the cortical plate, the intermediate zone, and the meninges. (E) The meningeal basement membrane, as demarcated by laminin staining, was still intact at E16. (F) The overlap of TUNEL labeling and laminin expression illustrates an increased amount of cell death in the meninges. (G) Higher magnification of the meninges of the MAM-treated brain shows the numerous TUNEL labeled cells within the laminin staining. (H) At E19, the control rat neocortex had very little TUNEL labeling and kept a thick ECM at the meninges. (I) The E19 MAM rat brain has very few TUNEL-positive cells, though still more than in control brains, and a mildly thinner meningeal basement membrane. Scale bar: A,H-130μm ;G-40μm

Figure 3.5. The meninges of the MAM-treated rat brain had a thinner basement membrane compared to the normal brain. Staining for CSPG labels proteoglycans highly present in the neocortical meningeal basement membrane that can concentrate factors such as SDF1. CSPG expression was similar between the E16 control (A) and MAM-treated (B) rat brains. However, 4 days after teratogen exposure, the meningeal basement membrane in the E19 MAM neocortex (D) was noticeably thinner than that in the control E19 neocortex (C). Scale bar: 40μm

Figure 3.6. Administration of CXCR4 blocker disorganized the marginal zone of the embryonic SD rat brain, both *in vitro* and *in vivo*. (A) E20 control slices were cultured for 2DIV with TN14003. The marginal zone in these sections was less compact, and reelin-labeled cells were separated from each other and from the meninges. The percentages of deeper cells in the pharmacologically-treated control slices were comparable to those in MAM slices. A similar result was observed in control slices treated with 2.5mg/ml AMD3100. (B) Quantification of the effect of application of two different CXCR4 antagonists showed that interrupting SDF1 binding in control slices induced a MAM-like phenotype. (C and D) In saline-injected rat embryos, Cajal-Retzius cells, as marked by reelin immunostaining, were situated against the meninges and formed a single-cell-layer thick marginal zone. (E and F) In E22 TN14003-injected rat embryos, several Cajal-Retzius cells at the marginal zone had distanced themselves from the meninges after 24 hours. (G and H) The disorganization remained in the E22 brain when the CXCR4 antagonist was given at E20 (48 hours before). Each picture represents a single

embryonic brain. Dashed line demarcates upper border of the marginal zone. Scale bar: 40μm

Methods

MAM Exposure

Pregnant Sprague-Dawley (SD) rats were injected with 25 mg/kg methylazoxymethanol (MAM; Midwest Research Institute, Kansas City, MO). Intraperitoneal injections (0.3 ml in dimethyl sulfoxide) 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 were 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 E16 and E19 with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brains were subsequently removed, fixed overnight in 4% paraformaldehyde, cryoprotected in 30% sucrose, and frozen in Optimal Cutting Temperature Compound (Tissue-Tek; Sakura, Torrance, CA). Litter size for rats treated with MAM varied between 8 and 12 pups.

Immunohistochemistry

Cryosectioned brain slices (14μm) were stained with antibodies against the following: laminin g1 (rabbit; Sigma, St. Louis, MO), TuJ1 (mouse; Covance, Princeton, NJ), CSPG (mouse; Sigma), reelin (mouse; Chemicon, Temecula, CA), and BrdU (rat; AbCam, Cambridge, MA). Immunostaining was performed using the ABC Vectastain kit (Burlingame, CA). Sections were pretreated with 1.5% hydrogen peroxide for 20 minutes. After overnight incubation with the primary antibodies, the secondary detection was performed by 2 hour incubation with either biotinylated anti-mouse or anti-rabbit IgG antibodies. Colorimetric detection was achieved after exposure of the sections to Diaminobenzidine (Sigma). We used AlexaFluor secondary antibody conjugates for mouse, rabbit, or rat IgG antibodies (Molecular Probes, Carlsbad, CA) for fluorescent immunohistochemistry.

In all the immunohistochemistry studies, we used a minimum of four MAM-exposed and non-exposed brains, and all experiments were repeated at least once to confirm the results. We used antibodies that have been well characterized in the literature. However, to ensure the reliability of the staining, we included negative controls in our immunostaining experiments in which we incubated the tissue in secondary antibody without prior exposure to a primary antibody.

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 using 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 minutes, followed by 3 PBS washes. Slides were incubated with hybridization buffer (50% formamide, 5x SSC,

0.3mg/ml yeast tRNA, 100ml/ml Heparin, 1x Denhart's, 0.1% Tween 20, 0.1% CHAPS, 5mM EDTA) for 30 minutes at 65°C, followed by overnight incubation with a digoxigenin-labeled reelin probe and a fluorescein-labeled CXCR4 or SDF1 probe. Three high-stringency washes were performed with 0.2x SSC at 65°C. Slides were then washed in TN buffer (0.1M TRIS-HCl, 0.15M NaCl, pH 7.5), treated with 1.5% peroxide in TN buffer for 20 minutes, washed again with TN buffer, and blocked for 30 minutes with 0.5% Blocking Reagent (Perkin Elmer, Wellesley, MA) in TN buffer. 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 the TSA Plus Fluorescence System (Perkin Elmer). TSA (tyramide signal amplification) was followed by HRP inactivation using 0.01N HCl for 15 minutes at room temperature. An HRP-conjugated anti-digoxigenin antibody was then applied for 2 hours, followed by signal amplification using a different fluorescent color tyramide reagent from the first amplification.

TUNEL labeling

Cell death detection was performed on fixed, frozen sections according to the Promega DeadEnd Colormetric TUNEL System. Briefly, mounted tissue was refixed in 4% PFA for 20 minutes. After several PBS washes, the samples were treated with Proteinase K (20 mg/ml) for 40 seconds (exposure time was empirically determined. Following several PBS washes, tissue was immersed in equilibration buffer before the end-labeling reaction. Tissue was incubated in the TdT reaction mix for 60 minutes at 37° C. The

reaction was stopped by immersion in 2X SSC. After several washes the tissue was exposed to FITC-conjugated streptavidin for signal detection.

BrdU labeling

MAM-treated and untreated pregnant rats were injected intraperitoneally with 100 mg BrdU per kilogram body weight at gestational day 15.5, and embryos were collected at E16 and processed as described for MAM exposure. 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 sodium borate pH 8.5 for 15 minutes. The sections were incubated overnight with anti-BrdU antibody (AbCam) and detected as described for fluorescent immunostaining.

Cortical Slice Cultures

We dissected brains from E20 untreated and MAM-treated SD rats in cold 1X Kreb's solution (126mM NaCl, 2.5mM KCl, 1.2mM NaH2P04, 1.2mMMgCl2, 2.5mMCaCl2) and prepared 250mm coronal cortical sections on a Leica vibratome. Slices were grown on Nucleopore Track-Etch membrane filters (Whatman, Florham Park, NJ) in serum free media (Neurobasal media 1X, B-27 supplement, 0.5% glucose, Penicillin/Streptomycin, 2mM GlutaMAX-1). Slices were allowed to recover for 2-3 hours before pharmacological treatments were begun. For control slices (where there was no treatment), the media was changed to new media. After 2DIV, slices were fixed in 4% PFA, cryoprotected in 30% sucrose, and frozen. For analysis, slices were recut on a cryostat into 14mm-thin sections and immunostained for reelin. For pharmacological

treatments, we used 50ng/ml of BDNF and 100ng/ml SDF-1α (Peprotech). AMD3100 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: bicyclam JM-2987 (hydrobromide salt of AMD3100) and was used at a final concentration of 2.5μg/ml. TN14003 was a generous gift from Dr, Nobutaka Fujii (Kyoto University) and was used at a final concentration of 1□M

In utero TN14003 administration

SD pregnant rats at gestational day E20 and E21 were anesthetized with 100mg/kg ketamine, 6mg/kg Xylazine, and 2mg/kg Acepromazine and their uterus was exposed through the abdominal wall. The control solution was 0.9% saline solution with 0.04% trypan blue. The TN14003 was diluted in water and 0.04% trypan blue to a final concentration of 1mM. For each litter, the embryos from the left horn were injected with 1-2μl saline solution and the embryos from the right horn were injected with 1-2μl TN14003 .

Statistics

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

References

Alcantara S, Pozas E, Ibanez CF, Soriano E. 2005. BDNF-modulated Spatial Organization of Cajal-Retzius and GABAergic Neurons in the Marginal Zone Plays a Role in the Development of Cortical Organization. Cereb Cortex.

Anton ES, Marchionni MA, Lee KF, Rakic P. 1997. Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. Development 124(18):3501-3510.

Bagri A, Gurney T, He X, Zou YR, Littman DR, Tessier-Lavigne M, Pleasure SJ. 2002. The chemokine SDF1 regulates migration of dentate granule cells. Development 129(18):4249-4260.

Beggs HE, Schahin-Reed D, Zang K, Goebbels S, Nave KA, Gorski J, Jones KR, Sretavan D, Reichardt LF. 2003. FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. Neuron 40(3):501-514.

Belmadani A, Tran PB, Ren D, Assimacopoulos S, Grove EA, Miller RJ. 2005. The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J Neurosci 25(16):3995-4003.

Bielle F, Griveau A, Narboux-Neme N, Vigneau S, Sigrist M, Arber S, Wassef M, Pierani A. 2005. Multiple origins of Cajal-Retzius cells at the borders of the developing pallium. Nat Neurosci 8(8):1002-1012.

Brunstrom JE, Gray-Swain MR, Osborne PA, Pearlman AL. 1997. Neuronal heterotopias in the developing cerebral cortex produced by neurotrophin-4. Neuron 18(3):505-517.

Colacitti C, Sancini G, DeBiasi S, Franceschetti S, Caputi A, Frassoni C, Cattabeni F, Avanzini G, Spreafico R, Di Luca M, Battaglia G. 1999. Prenatal methylazoxymethanol treatment in rats produces brain abnormalities with morphological similarities to human developmental brain dysgeneses. J Neuropathol Exp Neurol 58(1):92-106.

Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, Maddon PJ, Allaway GP, Sakmar TP, Henson G, De Clercq E, Moore JP. 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat Med 4(1):72-77.

Eriksson SH, Thom M, Heffernan J, Lin WR, Harding BN, Squier MV, Sisodiya SM. 2001. Persistent reelin-expressing Cajal-Retzius cells in polymicrogyria. Brain 124(Pt 7):1350-1361.

Garbelli R, Frassoni C, Ferrario A, Tassi L, Bramerio M, Spreafico R. 2001. Cajal-Retzius cell density as marker of type of focal cortical dysplasia. Neuroreport 12(12):2767-2771.

Graus-Porta D, Blaess S, Senften M, Littlewood-Evans A, Damsky C, Huang Z, Orban P, Klein R, Schittny JC, Muller U. 2001. Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. Neuron 31(3):367-379.

Halfter W, Dong S, Yip YP, Willem M, Mayer U. 2002. A critical function of the pial basement membrane in cortical histogenesis. J Neurosci 22(14):6029-6040.

Hasling TA, Gierdalski M, Jablonska B, Juliano SL. 2003. A radialization factor in normal cortical plate restores disorganized radial glia and disrupted migration in a model of cortical dysplasia. Eur J Neurosci 17(3):467-480.

Kato M, Dobyns WB. 2003. Lissencephaly and the molecular basis of neuronal migration. Hum Mol Genet 12 Spec No 1:R89-96.

Kisby GE, Kabel H, Hugon J, Spencer P. 1999. Damage and repair of nerve cell DNA in toxic stress. Drug Metab Rev 31(3):589-618.

Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA. 1998. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc Natl Acad Sci U S A 95(16):9448-9453.

Marin-Padilla M. 1998. Cajal-Retzius cells and the development of the neocortex. Trends Neurosci 21(2):64-71.

McCarthy KJ, Accavitti MA, Couchman JR. 1989. Immunological characterization of a basement membrane-specific chondroitin sulfate proteoglycan. J Cell Biol 109(6 Pt 1):3187-3198.

Meyer G, Soria JM, Martinez-Galan JR, Martin-Clemente B, Fairen A. 1998. Different origins and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. J Comp Neurol 397(4):493-518.

Montenegro MA, Guerreiro MM, Lopes-Cendes I, Guerreiro CA, Cendes F. 2002. Interrelationship of genetics and prenatal injury in the genesis of malformations of cortical development. Arch Neurol 59(7):1147-1153.

Muzio L, Mallamaci A. 2005. Foxg1 confines Cajal-Retzius neuronogenesis and hippocampal morphogenesis to the dorsomedial pallium. J Neurosci 25(17):4435-4441.

Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382(6592):635-638.

Netelenbos T, van den Born J, Kessler FL, Zweegman S, Merle PA, van Oostveen JW, Zwaginga JJ, Huijgens PC, Drager AM. 2003. Proteoglycans on bone marrow endothelial cells bind and present SDF-1 towards hematopoietic progenitor cells. Leukemia 17(1):175-184.

Ogawa M, Miyata T, Nakajima K, Yagyu K, Seike M, Ikenaka K, Yamamoto H, Mikoshiba K. 1995. The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. Neuron 14(5):899-912.

Olson EC, Walsh CA. 2002. Smooth, rough and upside-down neocortical development. Curr Opin Genet Dev 12(3):320-327.

Reiss K, Mentlein R, Sievers J, Hartmann D. 2002. Stromal cell-derived factor 1 is secreted by meningeal cells and acts as chemotactic factor on neuronal stem cells of the cerebellar external granular layer. Neuroscience 115(1):295-305.

Ringstedt T, Linnarsson S, Wagner J, Lendahl U, Kokaia Z, Arenas E, Ernfors P, Ibanez CF. 1998. BDNF regulates reelin expression and Cajal-Retzius cell development in the cerebral cortex. Neuron 21(2):305-315.

Sisodiya SM. 2004. Malformations of cortical development: burdens and insights from important causes of human epilepsy. Lancet Neurol 3(1):29-38.

Stumm RK, Zhou C, Ara T, Lazarini F, Dubois-Dalcq M, Nagasawa T, Hollt V, Schulz S. 2003. CXCR4 regulates interneuron migration in the developing neocortex. J Neurosci 23(12):5123-5130.

Super H, Del Rio JA, Martinez A, Perez-Sust P, Soriano E. 2000. Disruption of neuronal migration and radial glia in the developing cerebral cortex following ablation of Cajal-Retzius cells. Cereb Cortex 10(6):602-613.

Super H, Martinez A, Soriano E. 1997. Degeneration of Cajal-Retzius cells in the developing cerebral cortex of the mouse after ablation of meningeal cells by 6-hydroxydopamine. Brain Res Dev Brain Res 98(1):15-20.

Takiguchi-Hayashi K, Sekiguchi M, Ashigaki S, Takamatsu M, Hasegawa H, Suzuki-Migishima R, Yokoyama M, Nakanishi S, Tanabe Y. 2004. Generation of reelin-positive marginal zone cells from the caudomedial wall of telencephalic vesicles. J Neurosci 24(9):2286-2295.

Tamamura H, Xu Y, Hattori T, Zhang X, Arakaki R, Kanbara K, Omagari A, Otaka A, Ibuka T, Yamamoto N, Nakashima H, Fujii N. 1998. A low-molecular-weight inhibitor against the chemokine receptor CXCR4: a strong anti-HIV peptide T140. Biochem Biophys Res Commun 253(3):877-882.

Tran PB, Miller RJ. 2003. Chemokine receptors: signposts to brain development and disease. Nat Rev Neurosci 4(6):444-455.

Zhu Y, Yu T, Zhang XC, Nagasawa T, Wu JY, Rao Y. 2002. Role of the chemokine SDF-1 as the meningeal attractant for embryonic cerebellar neurons. Nat Neurosci 5(8):719-720.

Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393(6685):595-599.

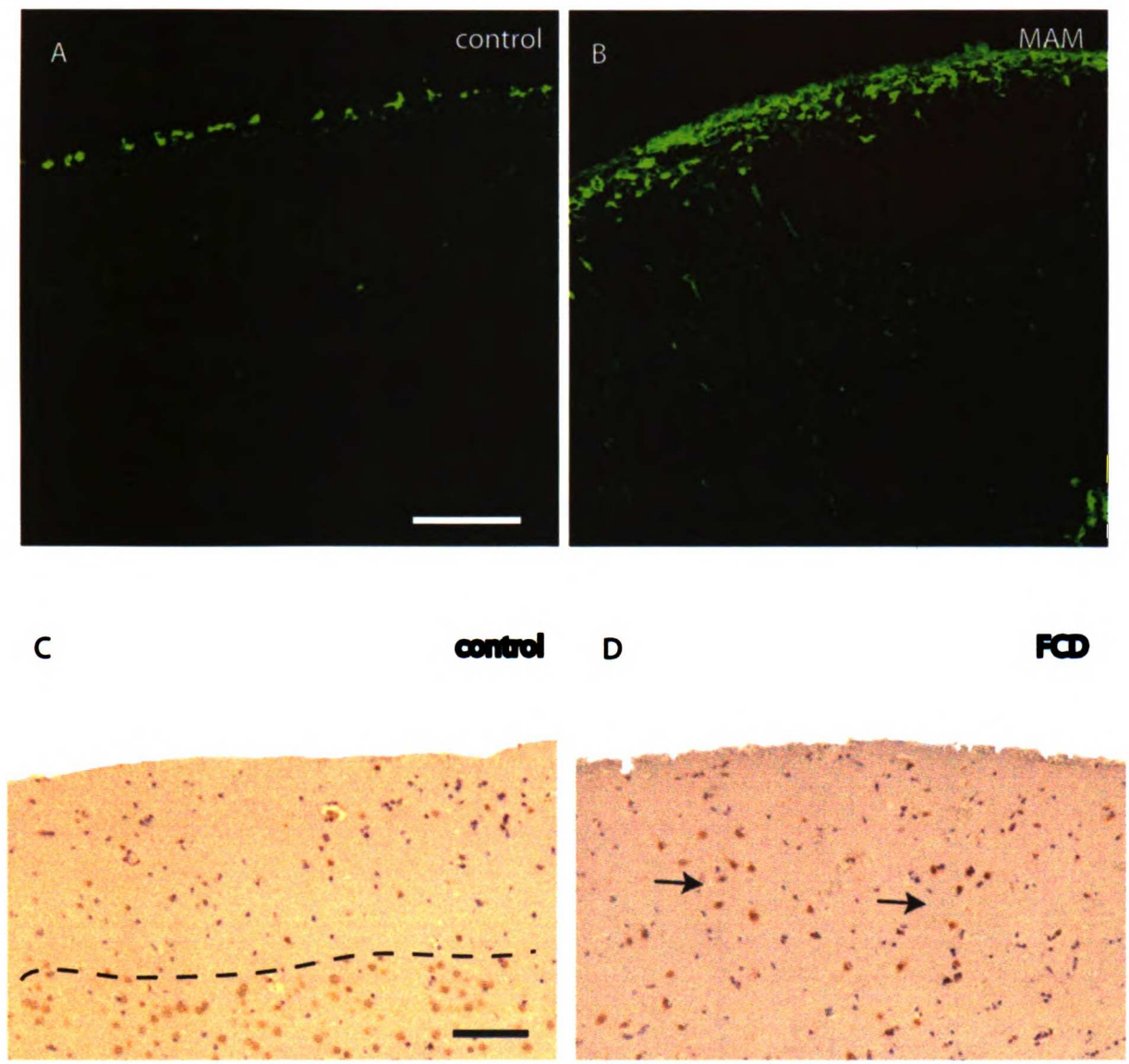
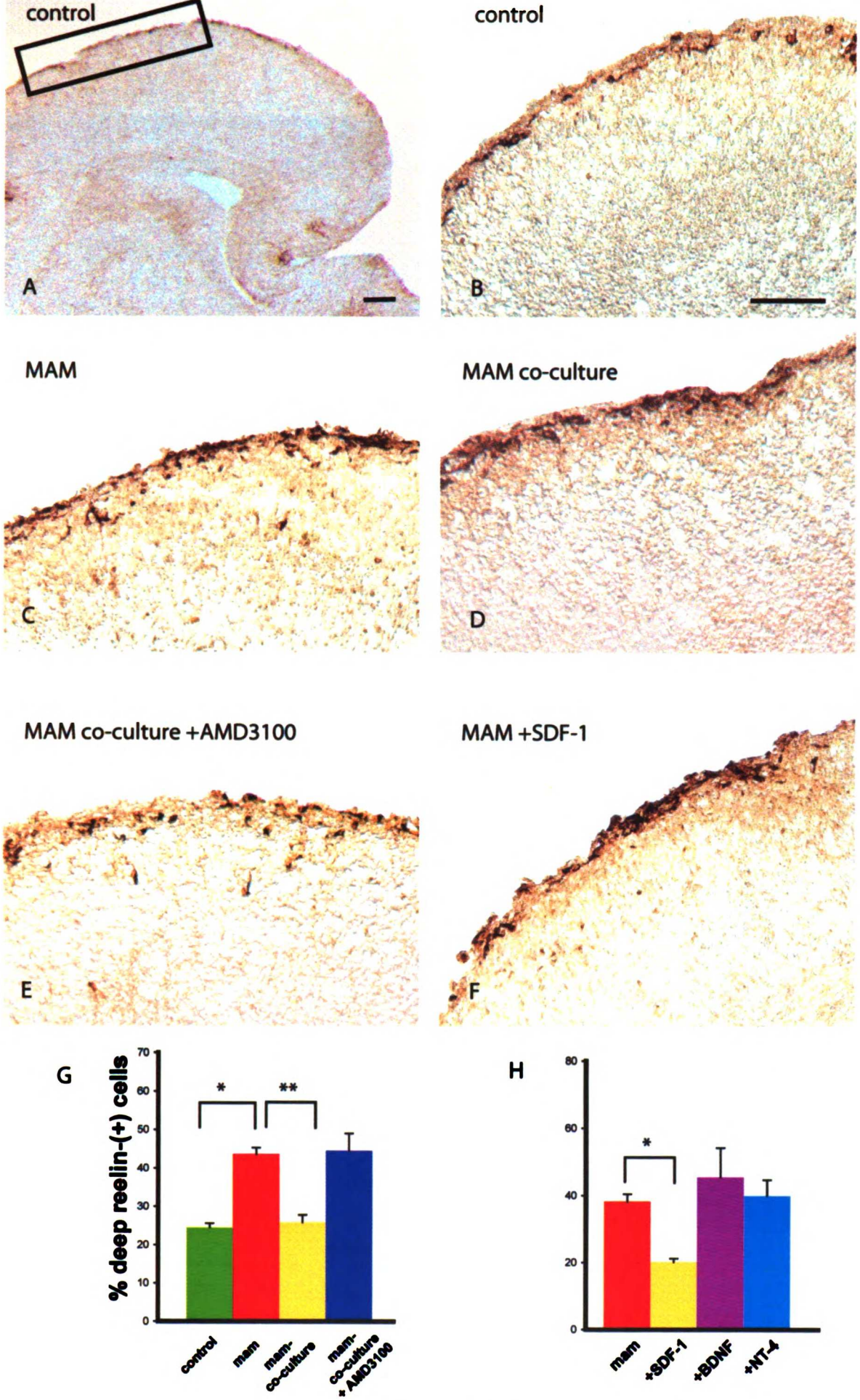


Figure 3.1


control
A
control
B
MAM
C
MAM co-culture
D
MAM co-culture +AMD3100
E
MAM +SDF-1
F
G
% deep reelin-(+) cells
0
10
20
30
40
50
60
70
*
**
control
mam
mam-co-culture
mam-co-culture +AMD3100
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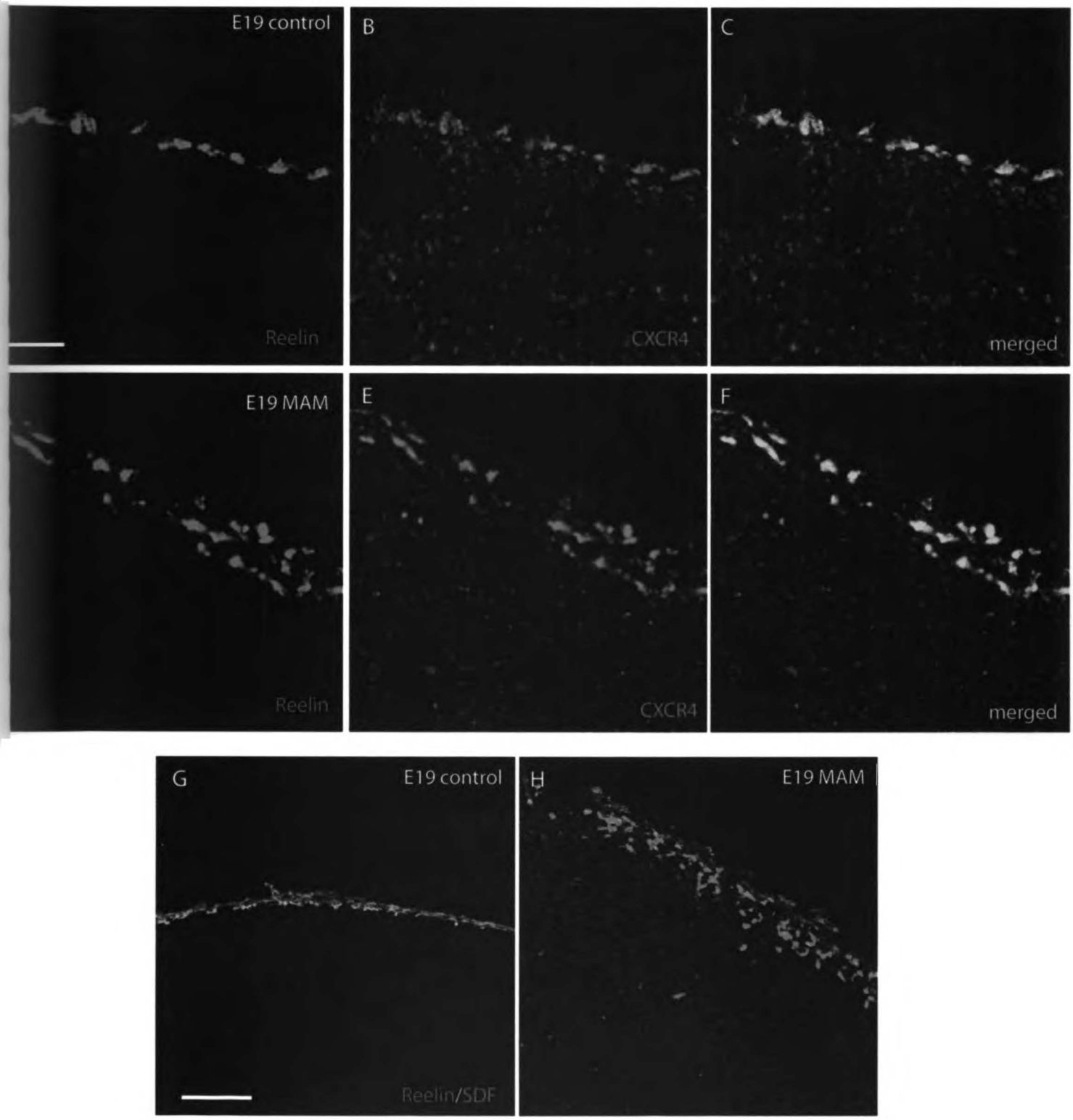
mam
+SDF-1
+BDNF
+NT-4

Figure 3.2


E19 control
B
C
Reelin
CXCR4
merged
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H
E19 MAM
Reelin/SDF

Figure 3.3

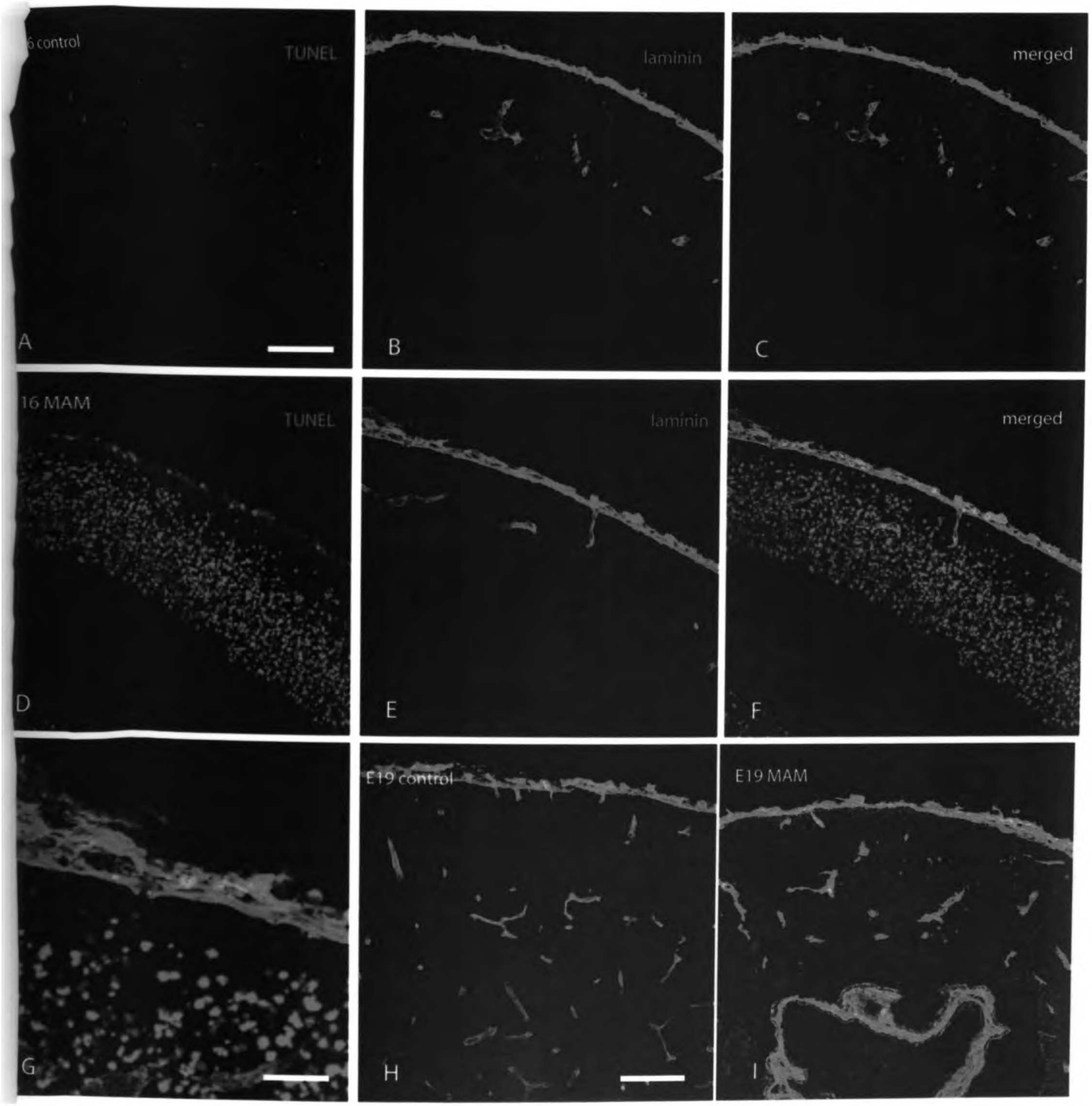

6 control
TUNEL
A
laminin
B
merged
C
16 MAM
TUNEL
D
laminin
E
merged
F
G
E19 control
H
E19 MAM
I

Figure 3.4

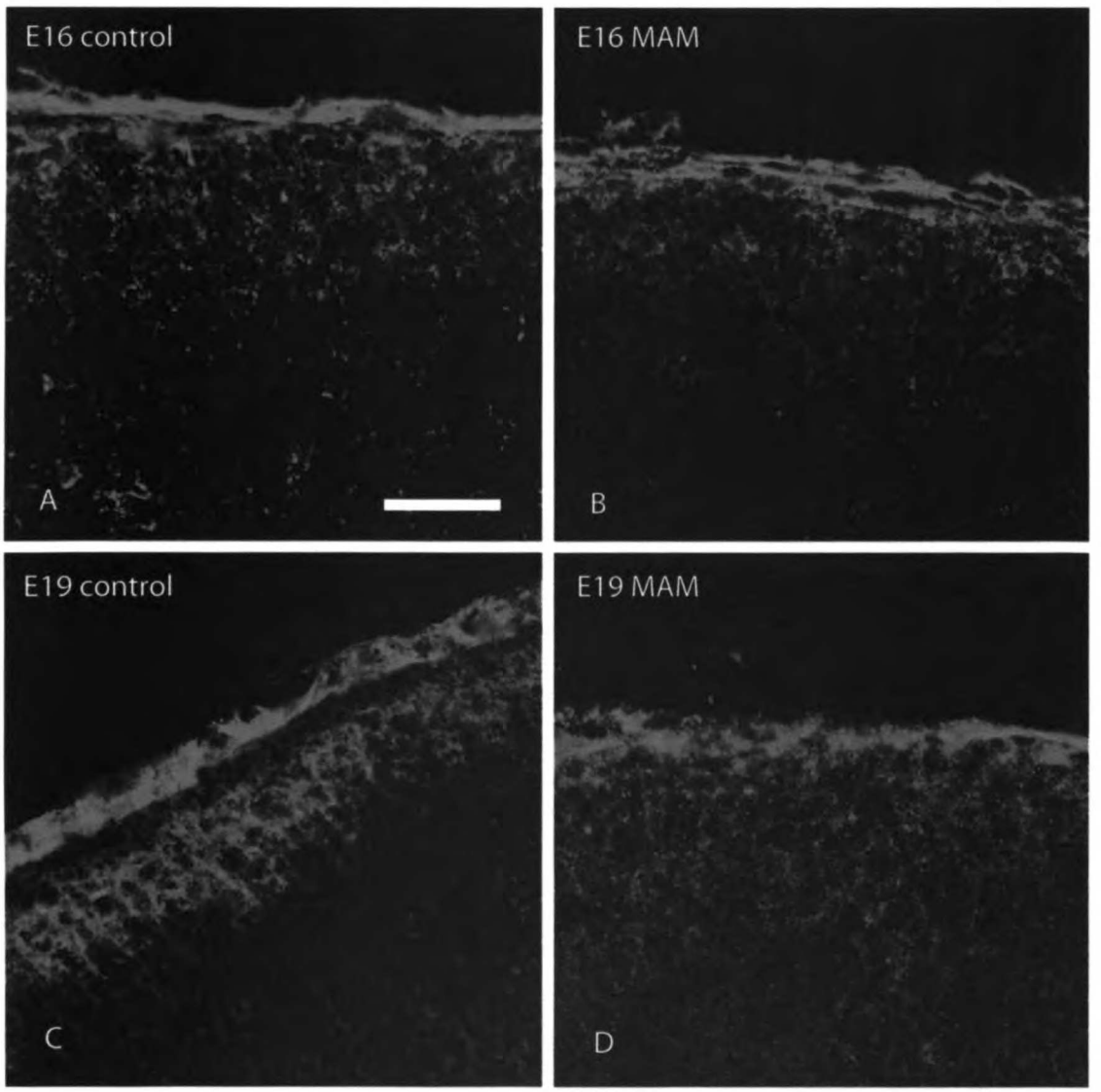
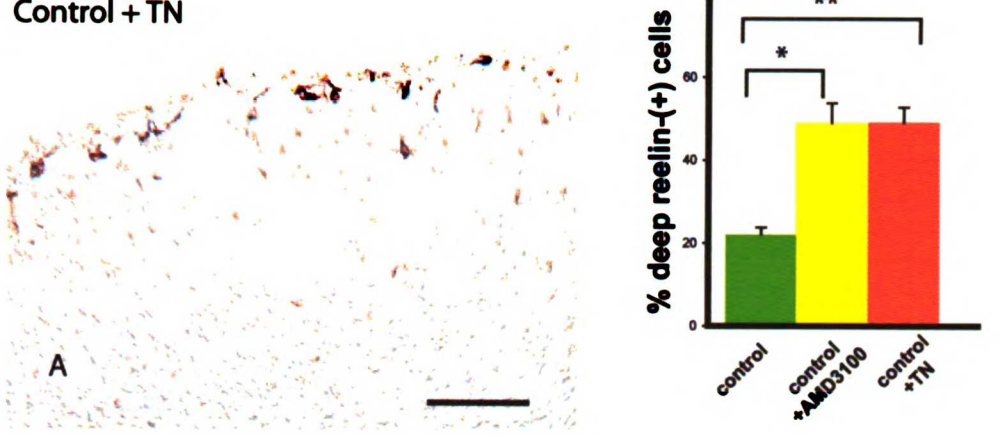


Figure 3.5


Control + TN
A
% deep reelin-(+) cells
0
20
40
60
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**
control
control +AMD3100
control +TN

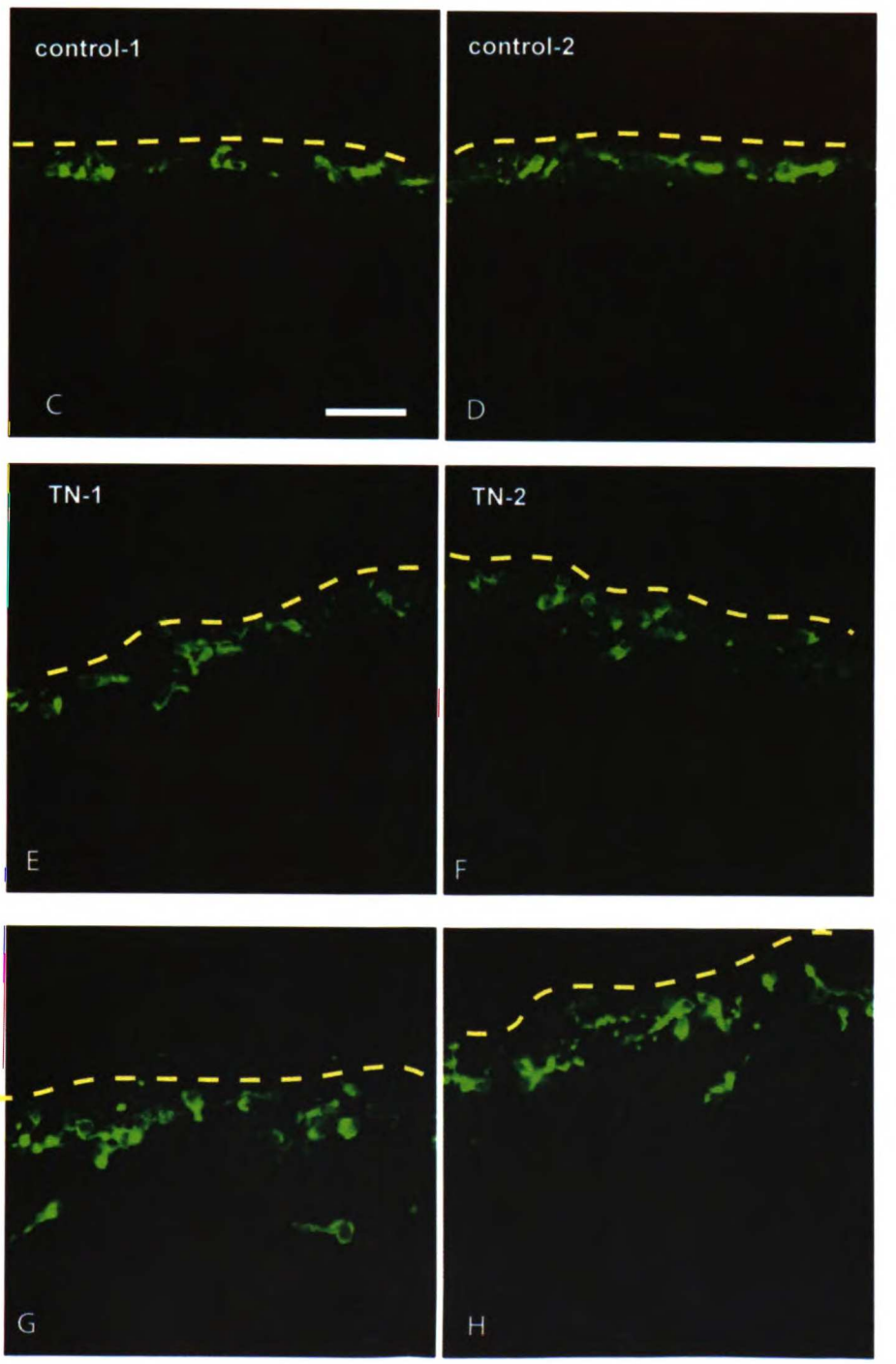

control-1
control-2
C
D
TN-1
TN-2
E
F
G
H

Figure 3.6

Chapter 4:

Discussion

The MAM-treated Rat as a model for Focal Cortical Dysplasia

The MAM-treated rat was a useful model for Malformations of Cortical Development (MCD) because it closely mimics the architectural and electrophysiological changes that take place in patients with MCD. Recordings from invasive electroencephalograms using depth electrodes demonstrated the presence of epileptiform activity arising from the hippocampal malformation (unpublished data). These animals do not have spontaneous seizures. This resembles the clinical situation where patients with cortical malformations are often undetected earlier in life or show only mild symptoms later in life (Schwartzkroin and Walsh, 2000). The disorganization in cortical layering in the MAM rodent model is similar to what is evidenced in many patients with cortical dysplasia. The presence of heterotopic cell clusters in the ventricle is reminiscent of the pathology that characterizes patients with periventricular heterotopias (PVH). Hippocampal heterotopias rarely, if ever, occur in humans. This may be due to the geometrical constraints imposed by the brain structure: the hippocampal formation is situated away from the ventricles such that anomalies within the ventricular lining cannot reach the hippocampus as they do in the rodent brain. As mentioned previously, tissue from young patients with focal cortical dysplasia shows an increase in scattered reelin-positive cells in layer I as is seen in the marginal zone of young MAM-treated brains. This pattern raises the possibility of changes in the marginal zone of the developing human neocortex similar to that found in the prenatal MAM-treated rat.

Not only does the MAM-treated brain replicate most of the anatomical defects seen in patients with focal cortical dysplasia, but there is also evidence that it is

representative of the functional aberrations as well. Whole-cell recordings of neurons in the dysplastic regions show that these cells have alterations in the inhibitory system. The frequency of IPSCs is decreased in tissue from patients with focal cortical dysplasia compared to non-focal cortical dysplasia control tissue from patients with mesial temporal lobe sclerosis. The distribution of interneurons was also shown to be abnormal throughout dysplastic neocortex and was associated with the inhibitory changes. In addition, spontaneous and evoked IPSCs from cells in the dysplastic areas are prolonged compared to IPSCs in control tissue (in press). This was similar to what was seen in equivalent recordings in the MAM-treated rat neocortex (Calcagnotto et al., 2002). The mechanism underlying the alterations in focal cortical dysplasia tissue was similar to that in the MAM-treated rat model: a decrease in GABA transporters, GAT-1 and GAT-3, in the dysplastic area likely caused the increase in the duration of inhibitory currents.

The MAM-treated rat, and other injury-induced models for MCD, is an important component in our efforts to understand the pathogenesis of a malformed brain. The majority of cases of MCD are a result from either an environmental insult or an environmental event overlying a genetic predisposition (Palmini et al., 1994). Therefore these models best exemplify the human condition. This is particularly important in disorders, such as focal cortical dysplasia, where no genetic link has been made.

MAM-treated rat model provided an opportunity to study abnormal brain developmental processes with no previous genetic disturbance or subsequent genetic compensation

Another unique aspect of the MAM model is the temporal control that it allows over the injury (i.e., until the point that the drug is given, it is assumed that cortical development is normal in the MAM brain). Any developmental steps that are affected would be ones that are either still in progress or require upkeep. The idea that embryonic structures need maintenance even after they have been established is important. This opens up new questions about what cortical structures are vulnerable to a prenatal insult. One cannot assume that because a structure, such as the marginal zone, is completed that it can no longer be damaged. Instead factors from neighboring regions, such as the meninges, are needed to keep the integrity of that structure. This interpretation could be made because we know that all the components that made the marginal zone were normal and that any changes caused by the teratogen happened after the marginal zone was established. Another important issue remains: how is a central neocortical structure such as the marginal zone maintained? In the case of the marginal zone disruption in the MAM-treated rat, a specific molecule and signaling pathway was found to be the key player in the phenotype. Once initial findings in these animals are uncovered, more precise clues to the mechanisms underlying both the developmental and functional pathology of MCD would require the use of genetic models.

A new role for SDF-1 in neocortical development

SDF-1 has been implicated in the proliferation and localization of cerebellar granule cells, dentate gyrus development, interneuronal migration, and the migration of sensory neuron precursors (Bagri et al., 2002; Lazarini et al., 2003; Stumm et al., 2003; Belmadani et al., 2005). The MAM rat revealed a new role: localization of Cajal-Retzius cells to the marginal zone (Fig. 1). Further characterization of this role would be important.

One fundamental question would be how the signaling transduction pathway acting in Cajal-Retzius cells regulates their organization. CXCR4 is a member of the heterotrimeric G-protein linked seven-transmembrane receptor family (Lazarini et al., 2003; Tran and Miller, 2003). G-proteins coupled to CXCR4 on astrocytes, neurons, and microglia link it to two signaling pathways: the extracellular signaling-regulated kinase (ERK) pathway and the phospholipase C pathway. Which specific players are present and how these pathways are modulated in Cajal-Retzius cells would be of significant interest. Also, SDF-1 can have roles in chemotaxis, proliferation, and cell survival depending on the cell type expressing CXCR4. One can imagine that SDF-1 might have other roles in Cajal-Retzius cells, depending on the time window. These multiple roles would have to be distinguished by the different components of the signaling pathways triggered by SDF-1 binding to CXCR4.

Another intriguing question with regard to the role of SDF-1 in marginal zone organization is how its effect on Cajal-Retzius cells is differentiated from that on interneurons. GABAergic interneurons also express CXCR4 as they migrate from the

ganglionic eminence into the cortical plate (Stumm et al., 2003). How does response to SDF-1 in these cells differ from that in Cajal-Retzius cells, both in terms of the signaling components used and the purpose it serves?

One can also move beyond the MAM rat model and ask what other components of the basement membrane are needed to preserve marginal zone architecture. As noted in the previous chapter, there are mutants of basement membrane proteins that have severe cortical dysplasia and ectopias in the marginal zone. An overarching issue is identifying the signaling pathway or elements of the meningeal basement membrane that are needed to preserve the developmental framework (such as the marginal zone and radial glial association to the meninges) required to keep proper neocortical development.

Conclusion

The goal of this dissertation was to characterize the steps in neocortical development that have been disrupted in the MAM-treated rat, an injury-induced model for cortical dysplasia. This model replicated much of the anatomical alterations (loss of cortical lamination and heterotopic cells) seen in patients with MCD and provided insight into the functional abnormalities (loss of specific currents and alteration in the inhibitory system) that can arise in such a malformed brain. We began by asking how heterotopic cells that are of cortical origin reached the hippocampus. These studies revealed how related the hippocampal heterotopia and the cortical malformation were and the necessity to investigate the defects in embryonic development in the MAM-treated brain. Our prenatal investigations showed that specific developmental steps were damaged by teratogen exposure. The embryonic regions at risk included not only structures that were "in progress" during the time of injury, but also an area that had been completed beforehand. Thus regions that were originally thought not to be susceptible to late prenatal injury should still be regarded with care. One such structure was the marginal zone. Further investigation in the MAM-treated brain and in the normal rat brain showed that SDF-1 was required for maintenance of this area. Thus, even though the marginal zone is made before MAM exposure, the damage to cells and areas that are involved in marginal zone upkeep has extensive consequences. These findings offer two major implications. They propose that early embryonic structures are dynamic: even after they have been fully established areas, such as the marginal zone, are dependent on neighboring regions to support their upkeep. In addition, it also offered new areas of

intervention against pre- and peri-natal injury. Global insult, such as teratogen exposure, can have widespread results. Nonetheless, specific molecules can be identified that are directly affected by such an injury. They provide potential targets for the development of new therapies for injuries that can lead to malformations of cortical development.

Fig. 4.1 A novel role for SDF-1 at the marginal zone of the CNS. In the normal brain (control) SDF-1 is expressed by the meninges. This acts as a chemoattractant for Cajal-Retzius cells in the MZ and keeps them localized to the superficial layer. In this situation, proper neuronal migration takes place (in the direction of the arrow). In the MAM-treated rat brain, however, the meninges is damages by teratogen exposure. This leads to a reduction of SDF-1 at the cortical surface. Cajal-Retzius cells no longer maintain their position and are dispersed throughout the neocortex. This leads to a disruption in the radial glia scaffold and abnormal neuronal migration (broken arrow). MZ=marginal zone, CP=cortical plate, IZ=intermediate zone, VZ/SVZ=ventricular zones.

References

Bagri A, Gurney T, He X, Zou YR, Littman DR, Tessier-Lavigne M, Pleasure SJ (2002) The chemokine SDF1 regulates migration of dentate granule cells. Development 129:4249-4260.

Belmadani A, Tran PB, Ren D, Assimacopoulos S, Grove EA, Miller RJ (2005) The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J Neurosci 25:3995-4003.

Calcagnotto ME, Paredes MF, Baraban SC (2002) Heterotopic neurons with altered inhibitory synaptic function in an animal model of malformation-associated epilepsy. J Neurosci 22:7596-7605.

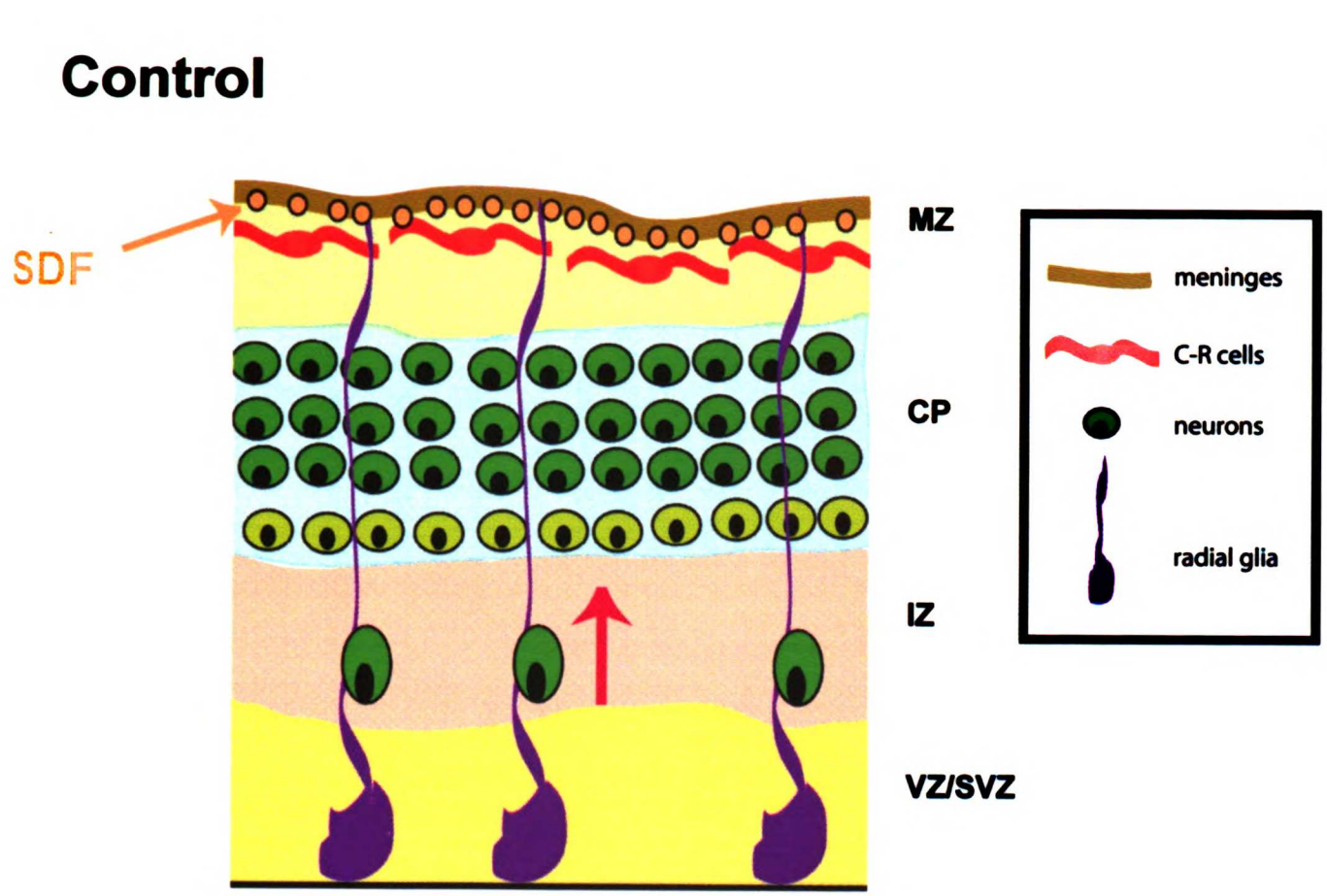
Lazarini F, Tham TN, Casanova P, Arenzana-Seisdedos F, Dubois-Dalcq M (2003) Role of the alpha-chemokine stromal cell-derived factor (SDF-1) in the developing and mature central nervous system. Glia 42:139-148.

Palmini A, Andermann E, Andermann F (1994) Prenatal events and genetic factors in epileptic patients with neuronal migration disorders. Epilepsia 35:965-973.

Schwartzkroin PA, Walsh CA (2000) Cortical malformations and epilepsy. Ment Retard Dev Disabil Res Rev 6:268-280.

Stumm RK, Zhou C, Ara T, Lazarini F, Dubois-Dalcq M, Nagasawa T, Hollt V, Schulz S (2003) CXCR4 regulates interneuron migration in the developing neocortex. J Neurosci 23:5123-5130.

Tran PB, Miller RJ (2003) Chemokine receptors: signposts to brain development and disease. Nat Rev Neurosci 4:444-455.


Control
SDF
MZ
CP
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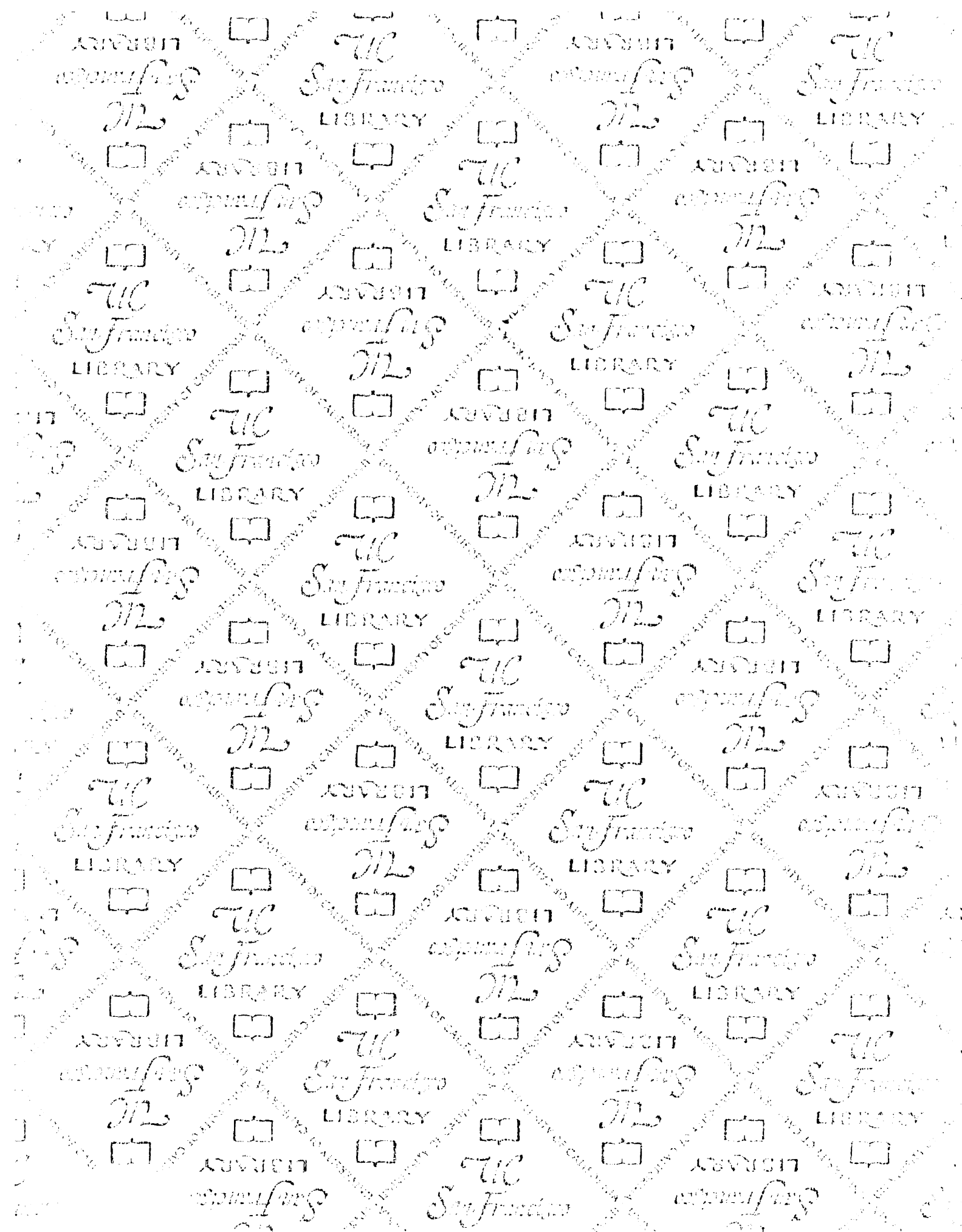
VZ/SVZ
meninges
C-R cells
neurons
radial glia
MAM

Figure 4.1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