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Combined effects of prenatal inhibition of vasculogenesis and neurogenesis on rat brain development

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Abstract

Malformations of cortical development (MCD) are one of the most common causes of neurological disabilities including autism and epilepsy. To disrupt cortical formation, methylazoxymethanol (MAM) or thalidomide (THAL) has been used to affect neurogenesis or vasculogenesis. Although previous models of MCD have been useful, these models primarily attack a single aspect of cortical development. We hypothesized that simultaneous prenatal exposure to MAM or THAL will lead to the development of a novel and specific type of brain maldevelopment. Rats were prenatally exposed to MAM and THAL. At early postnatal days, brains displayed abnormal ventricular size and hemispheric asymmetry due to altered brain water homeostasis. The postnatal brain was also characterized by gliosis in regions of focal leakage of the blood brain barrier. These morphological abnormalities gradually disappeared at adult stages. Although the adult MAM-THAL rats showed normal cortical morphology, abnormal hippocampal connectivity and mossy fiber sprouting persisted well into adulthood.

Keywords

Blood–brain barrier; Cerebrovascular function; Angiogenesis; In utero; Epilepsy; Hydrocephalus

Introduction

Dysplastic lesions are a common cause of epilepsy and developmental delay, and the associated clinical spectrum of epilepsy is broad (Schwartzkroin and Walsh, 2000; Sisodiya, 2004; Battaglia and Bassanini, 2006). Other consequences of malformations of cortical development (MCD) are cognitive impairment, spasticity, congenital hemiparesis and visual field defects. The overall progression to MCD occurs during cortical formation and results from disrupted neuronal and glial migration. While these neuroglial mechanisms have been well characterized, vasculogenesis also plays an important role in brain development (Hallene et al., 2006; Bassanini et al., 2007). For example, while the effect of prenatal exposure to MAM is one affecting neurogenesis (Bardosi et al., 1985a; Battaglia et al., 2003a,b; Fonnum and Lock, 2004), MAM exposure produces a similar result as prenatal exposure to thalidomide, which causes MCD by primarily affecting vasculogenesis (Franks et al., 2004; Hallene et al., 2006).

Abnormalities of cortical structure, generated as a product of aberrant pattern of brain development, are often associated with but not limited to seizures and chronic epileptic condition (Barkovich et al., 1992; Sancini et al., 1998). Both motor and cognitive delays are

frequently impaired as a consequence of MCD. Teratogen exposure (drugs and/or environmental poisons), maternal trauma, infection and stroke are all factors that might interfere with the normal progression of brain development and give rise to aberrant patterns of cortical structure. Acquired cortical dysplasia appears to result from a progressive process (i.e., that may continue beyond the time of insult), affecting not only the primary region of lesion but also surrounding 'normal' tissue (Marin-Padilla, 2000).

Injection of MAM acetate into pregnant rats at day 14/15 of gestation exposes the fetuses to an agent that disrupts cell proliferation at a time when neocortical and hippocampal neurons and glia are being formed (Bassanini et al., 2007; Battaglia et al., 2003b; Chevassus-Au-Louis et al., 1999). The most salient result of this manipulation is some cortical thinning and the generation of cortical heterotopia— abnormally placed clusters of cortical neurons with abnormal intrinsic properties, altered receptor characteristics, and aberrant connectivity (Chevassus-Au-Louis et al., 1999; Colacitti et al., 1998, 1999). According to a recent study, the affected animals have spontaneous seizures (Harrington et al., 2007). A number of laboratories have shown that these animals have lower seizure thresholds than normal controls in response to a variety of epileptogenic agents (flurothyl, hyperthermia, kindling, etc) (Baraban and Schwartzkroin, 1996).

In addition to neurogenesis, vasculogenesis also plays an important role in MCD and epilepsy or autism. Some reports have emphasized that MAM-treated animals display an array of pathological features including vascular dysgenesis (Bardosi et al., 1985a,b; Bardosi et al., 1987) and prenatal exposure to thalidomide is a recognized cause of autism (Trottier et al., 1999). E15 exposure in rats to the angiogenesis inhibitor thalidomide inhibits vasculogenesis in vitro at concentrations that result in significant morphological alterations in cortical and hippocampal regions of rats prenatally exposed to this vasculotoxin. Abnormal neuronal development was associated with vascular malformation and a leaky blood–brain barrier (BBB). Altering this process leads to failure of normal cerebrovascular development and has a profound implication for CNS maturation. Based on these results, we hypothesize that simultaneous prenatal exposure to MAM and thalidomide will generate a combined toxic effect on neurogenesis and vasculogenesis (Hallene et al., 2006) leading to the development of a novel and specific type of brain maldevelopment relevant to the study of a broad spectrum of neurological diseases including epilepsy, mental retardation and autism.

Materials and methods

Animal handling

E12 pregnant Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN, USA). Animals were housed at the Cleveland Clinic Foundation animal facility where food, water, and enrichment materials were provided in accordance with CCF and the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national (D.L.n.116, G.U., suppl.40, Feb. 18, 1992; UK legislation under the 1986 Animals [Scientific Procedures] Act) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Chemicals and reagents

Methylazoxymethanol acetate was purchased from The National Cancer Institute (Bethesda, MD). Thalidomide was purchased from Sigma Aldrich (St. Louis, MO). Isoflurane was from Hospira Inc (Lake Forest, IL). Sodium Pentobarbital was from Abbott Laboratories (Abbott

Park, IL). FITC-albumin and mouse anti-glial fibrillary acidic protein (GFAP) and β -actin monoclonal antibodies were from Sigma Aldrich. Mouse anti-neuronal nuclei (NeuN) monoclonal antibody was from Chemicon International (Temecula, CA). VEGF monoclonal antibodies were from Abcam (Cambridge, MA). Texas-Red dye conjugated Affinipure donkey anti-mouse IgG was from Jackson ImmunoResearch (West Grove, PA). Rabbit anti-mouse IgG HRP-conjugated secondary antibody was from Dako Corporation (Carpinteria, CA). 12% pre-cast Tris–Glycine gels were from Biorad Labs (Hercules, CA). Western lighting chemiluminescence reagent plus was from PerkinElmer Las Inc (Boston, MA). Restore plus western blot stripping buffer was from Pierce (Rockford, IL).

Prenatal exposure to MAM and THAL

For prenatal exposure to MAM and THAL alone, pregnant Sprague–Dawley rats received MAM (15 mg/kg of maternal body weight, i.p. injection, in sterile saline, 12 h apart) or THAL (30 mg/kg maternal body weight, i.p. injection in sterile saline, 12 h apart) on day E15. For prenatal exposure to combined MAM and THAL, the rats received MAM (15 mg/kg of maternal body weight, i.p. injection, in sterile saline) and THAL (30 mg/kg maternal body weight, i.p. injection in sterile saline), 12 h apart, or MAM (7.5 mg/kg of maternal body weight, i.p. injection, in sterile saline) and THAL (15 mg/kg maternal body weight, i.p. injection in sterile saline), 12 h apart, or vehicle alone (sterile saline) on day E15.

Postnatal brain samples preparation

At various postnatal (PN) stages, the offspring of control or MAM-THAL rats were deeply anesthetized with isoflurane (PN1–PN9) or by an i.p. injection of 40 mg/kg sodium pentobarbital (for adult animals). Animals were moved to a designated surgery area and positioned supine on a surgical towel to expose the ventral surface, and the thoracic skin was removed. After surgical exposure of the ribcage, the midline thoracic cutis was incised and the sternum opened. The apex of the left ventricle was cannulated with a 24-gauge polyethylene catheter (BDInsyte, BDVialon, Becton Dickinson, Mountain View, CA, USA), and perfused (1 ml/min) with a FITC-albumin (1% in 0.1 M phosphate-buffered saline). The right atrium was incised to prevent elevation in systemic blood pressure. Immediately following perfusion, animals were decapitated and the brains removed. The lag time between decapitation and immersion fixation never exceeded 2 min; brains were fixed for 24 h in 10% formalin solution, then cryoprotected in 30% sucrose solution for 2 days. Brains were then frozen for later processing.

Immunohistochemistry analysis

Frozen brain samples were mounted on a cryostat and cut in the coronal plane at 35 μ m thickness. Sections were collected and floated in 0.1 M phosphate buffer solution (PBS) and mounted on positive-charged, glass slides (Fisher Scientific, Pittsburgh, PA). Sections were dried and were processed with Cresyl Violet and/or fluorescence to visualize blood vessels and cells. Immunostaining was performed as previously described (Bassanini et al., 2007). Primary antibodies used were: mouse anti-NeuN monoclonal antibody (1:500); mouse anti-GFAP monoclonal antibody (1:200). Secondary antibody was as follows: Texas-Red dye conjugated Affinipure donkey anti-mouse IgG (1:100). Briefly, the sections were incubated with blocking solution containing 3% goat serum (DAKO) and 1% Bovine serum albumin (Roche Diagnostics, Indianapolis, IN) at room temperature (RT) for 1 h. Washed 3 times in PBS, the sections were incubated with primary antibodies in blocking solution at RT for 2 h. Washed 3 times in PBS, the sections were incubated with secondary antibody in blocking solution at RT for 1 h. After immunostaining, the slides were coverslipped using Vectashield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA), then viewed under immunofluorescent microscopy. The neuron

densities in CA3 of hippocampus were analyzed by using Phoretix 1D Advanced Software (Newcastle upon Tyne, UK).

Densitometric analysis of brain sections of MAM-THAL rats

Cresyl violet stained sections were chosen at random from MAMTHAL rats at postnatal day 1, 3, 4, 6, and 39 for analysis using QCapturePro software (QImaging). Each section was chosen from a rostral or caudal portion of the brain to measure densities throughout the brain. Sections were chosen from the dorsal part of cortex to take images under light microscope. The images were captured and analyzed by QCapturePro software to measure the density of each region of interest. QCapturePro software quantified the number of pixels in each region of the section, to determine intensity of staining. Cortical staining intensity was correlated to cortical tissue through an analysis of the number of pixels present within the chosen section. Locations of decreased pixel density were determined to be areas of edema. Edema was quantified by the absence of cortical tissue in brain sections. All calculations were normalized to eliminate background, using CorelDRAW software prior to QCapturePro analysis.

Brain water content analysis

Fresh brain samples were collected from MAM-THAL and control rats at postnatal day 2, 9 and 29 to analyze their densities by using the gradient column method (Marmarou et al., 1978). Briefly, kerosene and bromobenzene were mixed to generate solutions of specific gravity (SG) of 0.9500 and 1.0700 separately. The denser mixture (1.0700) was placed in a 250-ml graduated cylinder first and the lighter liquid (0.9500) carefully layered above it using a sterilized glass pipette. The gradient was prepared by mixing the solutions with a soft copper wire coiled at one end until the mixing is even. Standards were prepared with K₂SO₄ and dried overnight at 100 °C. The SG standards used are as follows: 1.0200 (24.7), 1.0300 (37.0), 1.0400 (49.2), 1.0500 (61.3), and 1.0600 (73.3). A standard or sample is added to the column and its position was determined 1–2 min later using the graduated scale on the cylinder. The percentage of water calculation was made based on a formula as simply (100-% Wet weight). This yielded to a linear correlation when plotted against percent water. This relationship was used to measure specific gravity in subsequent experiments. Data are expressed as mean ± S.E.M of value from three experiments.

Western blot analysis

Brains from control and MAM-THAL rats at postnatal day 1, 9 and 29 were removed, snap frozen in liquid nitrogen, and stored at –80 °C until use. Protein extracts from frozen brain samples were dissolved in a radial immunoprecipitation assay (RIPA) buffer (0.5% deoxycholic acid, 1% NP-40, 0.1% SDS) containing protease inhibitors (0.17 mg/ml PMSF, 2 µg/ml leupeptin, and 0.7 µg/ml aprotinin; Sigma). Prior to electrophoresis, protein extracts were denatured by heating at 100 °C for 5 min in a running buffer solution containing RIPA, β-mercaptoethanol, and Bromophenol Blue tracking dye. The acrylamide gels were run for 2.5–3 h at constant voltage (80 V) until the Bromophenol Blue tracking dye migrated to the bottom edge of the gel. Proteins from each gel were transferred onto a blot of PVDF membrane using a 192 mM glycine, 25 mM Tris-base, 20% methanol buffer system at constant current (40 mA) overnight at 4 °C. Primary antibodies used were: Mouse anti-VEGF monoclonal antibody (1:1000); mouse anti-GFAP monoclonal antibody (1:1000) and mouse anti-β-actin monoclonal antibody (1:5000). All primary antibodies were probed overnight at 4 °C. Blots were washed and treated with the following secondary antibody: rabbit anti-mouse IgG HRP-conjugated secondary antibody (1:2000). Protein concentration was estimated according to the Bradford assay method. Relative expressions of protein were determined by densitometric analysis using Phoretix 1D Software.

TIMM staining

Adult control, MAM-THAL and MAM rats ($n = 3$ for each group) were anesthetized and perfused through the ascending aorta with 10 ml of heparinized saline, followed by 400 ml of 0.37% sodium sulfide (N_2S) and 500 ml of 4% paraformaldehyde (PFA) in 0.1 M PBS. The brains were then collected and placed in 4% PFA in 0.1 M PBS overnight, then in 30% sucrose in 0.1 M PBS. Brains were frozen and mounted on the freezing stage of a sliding microtome, where they were sectioned at 30 μm . One out of 5 continuous sections was chosen to mount on the slide. Slide-mounted sections were placed in a solution consisting of 180 ml gum Arabic (50% w/v), 30 ml 2.25 M citrate buffer, 45 ml 0.5 M hydroquinone and 50 ml 0.04 M silver lactate (all chemicals were from Sigma Chemical Co except for the citrate buffer ingredients, which were from Fisher Scientific, Denver). Sections that were developed for approximately 70 min in darkness at room temperature were rinsed in water and lightly counterstained with Cresyl violet. Images were taken under a bright field microscope. TIMM staining was quantified for terminal sprouting in CA3, supragranular region and inner molecular layers according to (Holmes et al., 1999). Also, the intensities of TIMM positive deposit on the inner molecular layer were analyzed using QCapturePro software. Data are expressed as means $\pm$ S.E.M of value from three experiments.

Statistical analysis

Data were analyzed with Origin 7.1 software (Microcal) and Jump 7.0. Statistical significance was determined by ANOVA and data are shown as mean $\pm$ SEM. The analysis of variance technique was achieved by computing the mean within each group and the sum of squared distances from each point to the group mean. Sums of squares were pooled and the total response variation less this residual determined the amount of variation explained by the analysis of variance model.

Results

For the experiments described herein, we used a total of 192 rat pups from 5 control, 5 MAM-THAL, 3 MAM and 3 THAL treated dams (Table 1).

Dose dependent effects and gross anatomical alteration in MAM-THAL rats

To study the combined effects of MAM and THAL on cortical development, we initially chose a dosage based on previous protocols used for exposure to MAM and THAL alone (Bassanini et al., 2007; Hallene et al., 2006). E15 fetuses were exposed to MAM and THAL (12 h apart) at half dosage of each drug given individually (Fig. 1A). The combined drugs were toxic to the fetus, resulting in pre-delivery death. As can be seen in Fig. 1A, the brain of aborted fetus exposed to these concentrations had significantly smaller brain compared to a normal brain at the same developmental stage. Due to the lethality of this dosage, we attempted to reduce toxicity by halving the dosage of each drug while leaving the schedule of treatment unvaried (Fig. 1B). At this regimen, no prenatal fetal absorption was observed. Examination of gross brain anatomy unveiled that MAM-THAL rats were microcephalic and that this mild form of microcephaly persisted until adulthood and did not depend on gender (Fig. 1B).

Morphological changes in postnatal development after exposure to MAM-THAL

The overall morphology of postnatal brain of 16 control and 16 MAM-THAL rats was studied. As depicted in Fig. 2A, At early postnatal stages PN1–PN4 Cresyl violet staining showed that rat brains treated with MAM-THAL presented ventricular and hemispheric asymmetry that was never observed in untreated or in animals exposed to either toxin alone (Bassanini et al., 2007; Hallene et al., 2006). Those abnormalities gradually disappeared in

adult stages. The percentages of hemispherical asymmetry were qualified to show significant differences at early postnatal stages (Fig. 2B).

Brain edema of MAM-THAL rats at the early postnatal stages

At early postnatal stages (PN1–PN4), MAM-THAL rats exhibited edema in Cresyl violet stained sections. Hydrocephaly, here defined as ventricular enlargement, and clusters of neuronal cells were observed (Fig. 3). In order to quantify levels of edema within the cortex, two independent densitometric analysis methods were applied. QCapturePro software was used to measure cortical staining intensity in Cresyl-violet stained sections of MAM-THAL rats at early postnatal day 1, 3, 6 and at adulthood stage (Fig. 3). At postnatal days PN1 and PN3, cortical staining was indicative of edema. From PN6 onward, cortical tissue density became more uniform, suggesting edema reabsorption. Once rats reached adulthood, cortical staining intensity reverted to what seen in control rat brain, implicating that brain edema gradually disappeared over time.

The gradient column method was used to quantify brain edema by analyzing water content in whole brain. Fig. 4A shows that MAM-THAL brain displayed a significant increase in water content during early postnatal stages (PN2 and PN9) when compared to control. Surprisingly, at adult stage, MAM-THAL rat brain displayed a significant water content decrease in comparison to control rat brains. Also, brain water content of both MAM-THAL and control rats were significantly lower in adult stages compared to that of early postnatal stages PN2 and PN9. Perivascular edema was never observed in postnatal brain of rats exposed to MAM or THAL alone (Bassanini et al., 2007; Hallene et al., 2006). One of the main characteristics of MAM-THAL cortex was the “Swiss cheese” appearance due to frequent loss of parenchymal content in segregated regions.

Neuronal disorganization, focal leakage of blood brain barrier (BBB) associated with reactive gliogenesis at early postnatal stages of MAM-THAL rats

In MAM-THAL rats at early postnatal stages (PN1–PN4), immunofluorescent staining with anti-NeuN antibody and intravascular FITC-albumin showed neuronal clustering and disorganization of pial and parenchymal blood vessels. On the contrary, control rats showed organized, uniform neuronal staining and blood vessel formation (Fig. 5A). FITC leakage was seen around blood vessels, suggesting focal BBB leakage (Cavaglia et al., 2001). Immunofluorescent staining with mouse anti-GFAP monoclonal antibody showed GFAP+ cells superimposed to the BBB leakage area in MAM-THAL rats, suggesting that reactive gliosis occurred in areas of BBB leakage (Fig. 5B). The phenomenon was only seen in MAM-THAL rats at early postnatal (PN1–PN4), but not at adult stages.

Abnormal vasculogenesis, gliogenesis in MAM-THAL rats

In order to study the pathophysiological mechanisms of MAM-THAL exposure, western blot experiments were performed on whole brain samples to quantify the expression of the vasculogenesis marker VEGF and the glial marker GFAP throughout the early postnatal and adult stages for MAM-THAL and control rats. As shown in Fig. 6, VEGF and GFAP were downregulated at early postnatal stages of MAM-THAL rats compared to that of control group, but gradually restored to levels comparable to control.

Abnormal morphology and aberrant mossy fiber sprouting in the DG and CA3 of hippocampus of MAM-THAL adult rats

The overall changes seen in MAM-THAL rats are summarized in Fig. 7. In addition, hippocampal abnormalities were observed in the dentate gyrus and CA3 field, as shown in Fig. 8A. Compared to control rats, the head of the dentate gyrus was longer in MAM-THAL

rats and shorter in MAM rats. In addition, the granule cell layer displayed frequent indentations (indicated by asterisks). Two distinct TIMM positive layers were seen in CA3 region of MAM-THAL rats. Analysis of images of the stratum pyramidal of CA3 region showed uniform TIMM staining in control rats and a continuous or near-continuous dense laminar band of TIMM positive granules in MAM-THAL rats (Fig. 8B). Analysis of mossy fiber scores in CA3 showed that most images were ranked 1–2 in control rats (i.e., minimal or no sprouting), and 5 in MAM-THAL rats (intense staining indicating robust sprouting). There was a statistically significant difference between MAM-THAL vs. control for CA3 scoring ($p < 0.001$) (Fig. 8C).

In control rats, analysis of images of the dentate gyrus revealed the presence of few mossy fiber sprouts in the granule cell layer and no TIMM positive granules in the super granular region and inner molecular layers. In contrast, both signals were robust in MAM and MAM-THAL rats (Fig. 9A, B, C). Analysis of the scores of mossy fiber sprouting in DG showed that most sections were ranked 0–1 in control rats, score 2 in MAM-THAL and 1–2 in MAM rats (Fig. 9D). There was a statistically significant difference between MAM-THAL vs. MAM ($p < 0.05$) and MAM-THAL vs. control ($p < 0.001$) in terms of intensity of inner molecular layer TIMM staining (Fig. 9E).

Immunofluorescent staining with NeuN antibodies on neighboring brain sections showed increased thickness and dispersion of the upper granule layers in MAM-THAL rats, while both granule cell layers were thick and dispersed in MAM rats (Fig. 10A). There was a statistically significant difference between the ratio of upper granule layer/lower granule layer staining between MAM-THAL vs. control and MAM rats ($p < 0.001$) (Fig. 10B). In addition, CA3 neurons were fewer and more dispersed in MAM and MAM-THAL rats compared to control rats (Fig. 10C). There were statistically significant differences for the density of neurons between MAM, MAM-THAL vs. control ($p < 0.001$) and MAM vs. MAM-THAL rats ($p < 0.05$) (Fig. 10D).

Discussion

Rodent models of MCDs can be produced by prenatal exposure to neurogenesis inhibitors (e.g., MAM) or to inhibitors of vasculogenesis (THAL) (Bassanini et al., 2007; Hallene et al., 2006; Battaglia and Bassanini, 2006). Since both neurogenesis and vasculogenesis are parallel features of neurodevelopment (Carmeliet and Tessier-Lavigne, 2005), we tested the possibility of generating a new type of MCDs by prenatal exposure to combined MAM and THAL. The main new findings of our work are: 1) early postnatal rat brains treated with MAM and THAL display morphological and pathophysiological abnormalities distinct from those seen in MAM or THAL alone models; 2) these abnormalities are largely reversible with time, which is in sharp contrast to what is seen after single drug exposure; and 3) the hippocampus of MAM-THAL rats is selectively affected by this combined prenatal treatment: loss of CA3 neurons, and mossy fiber sprouting were in fact seen only in adult animals after double, but not single, exposure.

Morphological abnormalities and time dependence

MAM is a DNA-methylating toxin which inhibits cell growth and blocks the development of proliferating cells including brain endothelium (Battaglia and Bassanini, 2006). Based on the time-window of the neuronal development and the choice of the exact time of drug administration, it is possible to disrupt the development of specific brain areas (Battaglia and Bassanini, 2006; Colacitti et al., 1999). THAL was originally developed as a hypnotic, sedative and anxiolytic drug that was first used in 1955 (Franks et al., 2004). The mechanism of action of THAL is complex and not yet fully understood, but there is good evidence to suggest anti-angiogenic properties (D'Amato et al., 1994). Our initial dosage of

combined MAM and THAL showed unexpected toxic effects to fetus not observed after MAM or THAL alone, which suggested a yet unreported synergism. The prenatal toxicity of the combined injection was effectively aborting gross brain development; as shown in Fig.1; the combined injection of high concentrations of MAM-THAL completely arrested brain development in utero.

At lower concentrations, the effects of MAM-THAL were characterized by an unexpected neonatal pathology, consisting of widespread cerebral edema. The nature of these edematous changes was short-lived and characterized by vasogenic (BBB leakage, Fig. 5) and perhaps cytotoxic (abnormal cell clustering, Fig. 4) features. In addition, a significant number of animals presented with hydrocephalic features (Fig. 2) with pronounced ventricular enlargement and hemispheric mass asymmetry. To our knowledge, this is the first report of congenital hydrocephalus in an animal model based on prenatal exposure to neurotoxins.

Loss of brain water homeostasis in MAM-THAL animals was revealed by a variety of experimental approaches, including measurements of extracellular space and direct assessment of water in fresh brain samples. Both revealed a significant increase in water which preferentially localized to the rostral portion of the developing cortex. A consequence of large water shifts was the displacement of developing neurons in bundle-like formations (Fig. 4) and the appearance of reactive astrocytes in regions of blood-brain barrier leakage. Thus, it appears that parenchymal water mismanagement (presumably by BBB endothelial cells) leads to both hydrostatic (displacement) and neurobiological (gliosis) effects.

Long term connectivity abnormalities in MAM-THAL rats

The developing brain is particularly susceptible to seizures (Stafstrom et al., 2006). In the study of epilepsy, animal models serve numerous functions. They provide opportunities to investigate and elucidate basic mechanisms, to study the neurologic consequences of seizures at various stages of brain development, to devise new diagnostic approaches, and to test and/or develop new antiepileptic medications (AEDs) and other therapeutic modalities. So far, most of the studies aimed at development of models of adult disease, and most experimental manipulations were performed on adult animals. However, epilepsy is most common in children, and in particular seizures in the newborn are lacking appropriate neonatal animal models.

One concern is the fact that our data provide no direct evidence that the MAM-THAL is a model of epilepsy or seizures. Similarly, the evidence that MAM-treated rats are “epileptic” is based on only one report (Harrington et al., 2007). Seizures have never been recorded in THAL animals albeit hyperexcitability in vitro was reported (Hallene et al., 2006). Evaluating seizures in newborn rats is extremely difficult since EEG recordings are not possible in the first post-natal hours/days. We thus used TIMM staining to assess whether temporal lobe reorganization occurred during brain development in MAM-THAL rats.

From recent studies on the kindling and status epilepticus models, which have been used most extensively to investigate temporal lobe epilepsy, it is now clear that the brain reorganizes itself in response to seizure activity. Hippocampal Timm's staining was analyzed to quantify the extent of hippocampal rearrangement in the post-natal consequences of post-natal hypoxia and encephalopathy (Williams et al., 2004). Our results are essentially similar, at least at the qualitative level, inasmuch in adult MAM-THAL animals the most significant defect was hippocampal mossy fiber sprouting in otherwise unremarkable brain histology. The important feature of these models is, in our opinion, the fact that they achieve a similar morphological outcome by entirely different means. Whether or not MAM-THAL experience seizures is being studied by continuous EEG monitoring.

Relevance to human pathology

There are several well established models of epilepsy or seizures based on exposure to chemical agents such as cholinergic or glutamatergic agonists (pilocarpine, (Cavalheiro et al., 2006) or kainic acid (Fisher and Alger, 1984). In addition, experimental seizures are commonly induced by electrical stimulation or “kindling” (Racine, 1972). Other models of seizure disorders are based on the induction of abnormal brain connectivity by prenatal exposure to toxins, such as those described here. A combination of these two approaches has also been used to demonstrate that prenatal exposure lowers seizure threshold to neurochemical stimulation (Chevassus-Au-Louis et al., 1998). It is clear that epilepsy is only one of the several consequences of MCD, and recent evidence points to a single molecular mechanism leading to different phenotypes (Sherr, 2003). This may suggest that the current models of post-natal seizures may in fact also be useful to study other neurodevelopmental disorders.

Whichever the model, most studies dealing with epilepsy have so far focused on adult animals. In contrast, to mimic perinatal insults, researchers have relied on hypoxia/ischemia (Vannucci and Vannucci, 2005). This latter approach is based on the clinical reality of profound pro-epileptogenic changes induced by perinatal metabolic distress or disturbances of cerebral blood flow (Vannucci, 2000). Since for our studies we used an entirely different model to induce brain damage (a chemical insult), the question remains on how this relates to the human condition. In other words, why do MAM-THAL cause changes that are similar to what expected with a hypoxic–ischemic insult?

This question can be addressed by two considerations. First, given the powerful anti-angiogenic effect of THAL-induced VEGF inhibition it is reasonable to expect that in treated animals the vascular supply may have been reduced to levels insufficient to support brain development, thus imitating the effects of ischemia or hypoxia. On the other hand, the effects of ischemia itself (whether induced chemically or otherwise) cause loss of blood–brain barrier function (Huang et al., 1999; Krizanac-Bengez et al., 2006), which was observed in our model. Thus, it is possible the MAM-THAL actually recapitulate some of the features of perinatal hypoxia–ischemia models due to a direct action on blood vessel development.

The spectrum of conditions that lead to unilateral hydrocephalus or brain edema is consistent with abnormal production or release of VEGF or other signaling molecules that contribute to water homeostasis. Thus, the effects of combined MAM and THAL appear to be due to downstream actions, rather than a direct effect of these drugs on vasculogenesis and BBB permeability. It is thus possible that this model may bear significance to study several known causes of loss of brain homeostasis perhaps opening new tools for drug development.

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Fig. 1.

Schematic representation of the time course of MAM and THAL exposure paradigms and analysis of gross anatomical alterations in control, MAM, THAL and MAM-THAL rat brain. (A) Protocol of the prenatal lethal treatment at day E15 with MAM in sterile saline (15 mg/kg maternal body weight) and THAL in DMSO (30 mg/kg maternal body weight), administered 12 h apart. (A1) Morphology of E21 brain sagittal sections of control and MAM-THAL rats. *CxP*: cortical plate; *Str*: striatum; *Ventr*: ventricle; *p-m*: pons–medulla. Note the gross morphological alterations in treated animals. See text for details. (B) Protocol of treatment with MAM (7.5 mg/kg maternal body weight) and THAL in DMSO (15 mg/kg maternal body weight), 12 h apart. (B1) Gross anatomical alterations of brain in control, MAM, THAL and MAM-THAL rats at postnatal day 1, 3 and 4. Note the significant microcephaly in MAM brain, and the less pronounced delay in growth in MAM-THAL animals.

Fig. 2.

Hemispheric asymmetry and altered ventricular size in MAM-THAL progeny. (A) Cresyl violet stained brain sections of control and MAM-THAL rats at early postnatal day 1, 3, 4 and adult stage reveal reversible changes occurring postnatally. The schematic of the rat brain shows the approximate level at which the image was taken. The quantification in (B) shows the presence of hemispheric asymmetry at birth and the subsequent return to normal size in adulthood. We analyzed 10 contiguous sections from 3 animals per group. ** indicates $p < 0.001$.

Fig. 3.

Densitometric analysis of Cresyl violet stained slides of MAM-THAL rats reveals postnatal brain edema. Cresyl violet stained sections were used to quantify brain edema in MAM-THAL rats at early postnatal and adult stage. The images in the center panel show the position and region being analyzed. QCapturePro software was used to perform a densitometric analysis of sections by measuring the density of stained cortical tissue from the rostral to caudal brain. Densities were normalized to section staining levels to eliminate background. Locations of edema are reflected by decreased 1/optical density value. *Call.*, corpus callosum; *ento.*, entorhinal cortex, *hippo.*, hippocampus.

Fig. 4.

Gravimetric analysis of brain edema in MAM-THAL rats confirms morphological quantification of brain edema. (A) Densitometric analysis of brain samples of control and MAM-THAL rats by gradient column method. Note the significant increase in brain water content in post-natal rat brain exposed to MAM-THAL. Also note the transient nature of this phenomenon and the full reversal to control value in adulthood. ** indicates $p < 0.05$; * indicates $p < 0.001$. (B) Cresyl violet staining of brain sections of MAM-THAL rats at PN3 reveals the “Swiss cheese” appearance of MAM-THAL neonatal brain. Also note the bundled appearance of neurons (white arrow).

Fig. 5. Abnormal vasculature and BBB leakage are topographically related to reactive gliosis in MAM-THAL rat brain. (A) Immunofluorescent staining of brain sections of control and MAM-THAL rats at postnatal day 1, 3. We used anti-NeuN antibodies and merged the images with green fluorescent signals from blood vessels perfused with FITC-albumin. White arrows indicate abnormal blood vessels in MAM-THAL rats. (B) GFAP immunofluorescence (red) staining of brain sections of control and MAM-THAL rats perfused with FITC-albumin (green) at postnatal day 3. The white boxes highlight the area of FITC-albumin leakage.

Fig. 6. MAM-THAL effects encompass expression of markers of vasculogenesis and gliogenesis. The effects of treatment were time dependent and largely reversible. GFAP and VEGF expression was significantly decreased after birth, i.e., at the time of most significant differences in terms of water content and BBB leakage.

Fig. 7.

Summary of time dependent anomalies of prenatal MAM-THAL exposure on postnatal brain development. Continuous 35 μ m brain sections of MAM-THAL rats were analyzed at the postnatal days indicated. The color-coded key represents different abnormal findings and points to their preferred location. The data show the findings from all the animals used for the study and have topographic but not quantitative value.

Fig. 8. Hippocampal morphology, cytology and connectivity are altered in adult animals after prenatal exposure to MAM-THAL. (A) Cresyl violet staining reveals significant differences between normal, MAM-THAL and MAM treated animals. In particular, while MAM alone caused a pronounced shrinkage of the hippocampus, the effects of MAM-THAL were more subtle and primarily limited to the dentate gyrus. The asterisks indicate indentation in the granule cell layers which were commonly seen in MAM-THAL, but not MAM animals. (B) TIMM stained brain sections further reveal abnormal circuitry development in MAM and MAM-THAL animals. Note the double layered sprouting in the CA3 region of treated animals. Due to shrinkage of the whole hippocampal structure, the TIMM stain in MAM

animals appears to end abruptly. (C) Quantitative analysis and scoring of mossy fiber sprouting in CA3 region of hippocampus reveals statistically significant differences between control and MAM-THAL rats. ** $p < 0.001$. The bar indicates 500 μm .

Fig. 9. Bright-field photomicrographs from TIMM-stained sections demonstrate the extent of mossy fiber sprouting in the dentate gyrus of adult rats after prenatal exposure. (A–C). Morphology of TIMM stained brain sections from rostral to caudal in control, MAM and MAM-THAL rats. Sprouting was analyzed in the “tip”, “mid” and “crest” portions of the supragranular region and in the inner molecular layer. The arrows indicate the TIMM positive deposit in the inner molecular layer. (D) Statistical analysis of the scoring of mossy fiber sprouting in the dentate gyrus region of continuous brain sections. (E) Statistical analysis of the intensity of mossy fiber sprouting in the inner molecular layer of continuous

brain sections. iml: inner molecular layer; g: granule cell layers; H: *hilus*. ** $p < 0.05$, ** $p < 0.001$.

Fig. 10.

Decreased neuronal density in the CA3 region of MAM-THAL and MAM animals and abnormal layering of dentate gyrus in MAM-THAL dentate gyrus. (A–B) Apparent proliferation of the upper molecular layer in MAM-THAL animals correlates with reduced CA3 neuronal density in the same animals (C–D). The data in A and B were obtained by dividing the thickness of the upper and lower cell layers at the levels indicated. A value of 50% represents equal thickness. Note the apparent proliferation in MAM-THAL upper blade granule cell layers compared to either MAM or naïve animals. However, MAM-treated animals displayed a significant reduction of neuronal cell numbers in the CA3 region. This

suggest that DG thickness and CA3 neuronal density are not necessarily related. ** $p < 0.05$, ** $p < 0.001$. Bar indicates 100 μm .

Table 1

Summary of the animals and drugs used for the experiments (5 control dams were also used)

Drug used	Dosage	Interval	Number of dams	Number of pups
MAM	15 mg/kg of maternal body weight	12 h	3	39
THAL	30 mg/kg maternal body Weight	12 h	3	32
MAM + THAL (High dose)	MAM: 15 mg/kg of maternal body weight THAL: 30 mg/kg maternal body weight	12 h	3	0
MAM + THAL (Low dose)	MAM: 7.5 mg/kg of maternal body weight THAL: 15 mg/kg maternal body weight	12 h	5	63