Epileptic Disord 2003; 5 (Suppl 2): S 51–S 58

The genesis of epileptogenic cerebral heterotopia: clues from experimental models

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ABSTRACT – The pre-natal administration of methylazoxymethanol acetate (MAM) in rats is able to induce cerebral heterotopia that share striking similarities with those observed in human periventricular nodular heterotopia, a cerebral dysgenesis frequently associated with drug-resistant focal seizures. In the present study, we investigated the mode of neurogenesis in cerebral heterotopia of MAM-treated rats, by analyzing post-natal cytoarchitectural features and time of neurogenesis using bromodeoxyuridine immunocytochemistry. The cytoarchitectural analysis demonstrated the existence, in the early post-natal period, of white matter cellular bands in close anatomical relationship with the heterotopia, which most likely serve as a reservoir of young, migrating neurons for the newly forming heterotopia. The birth dating analysis demonstrated that the period of generation of neurons within the heterotopia and adjacent white matter bands, was extended in comparison to corticogenesis in normal rat brains. In addition, it demonstrated that the heterotopia were formed through a rather precise outside-in (for cortical and periventricular heterotopia) and dorso-ventral (for intra-hippocampal heterotopia) neurogenetic pattern. We hypothesize that the MAM-induced ablation of an early wave of cortical neurons is sufficient to alter *per se* the migration and differentiation of subsequently generated neurons, which in turn set the base for the formation of the different types of heterotopia. On this basis, we suggest a neurogenetic scheme for MAM-induced heterotopia that can also explain the origin and intrinsic epileptogenicity of periventricular nodular heterotopia in humans.

KEY WORDS: cortical development, neuronal migration, cerebral dysgenesis, methylazoxymethanol acetate, periventricular nodular heterotopia, epilepsy

Introduction

The final assembly of the mature central nervous system (CNS) is the result of an ordered sequence of temporally and spatially regulated events that are finely tuned by genetic and environmental factors. In the past decades, the cellular and molecular mechanisms involved in brain morphogenesis have been explored, and processes such as neurogenesis, radial and tangential migration, gliogenesis, and axonogenesis have been studied in detail in the normal developing brain [1-6]. In contrast to this, with the few exceptions of rare, genetically proven cases determined by impairment of genes involved in the processes of neuronal migration [7, 8], the etiology and pathogenic mechanisms responsible for the development of cerebral malformations in humans are largely unknown.

The importance of understanding how brain dysgeneses form during development is underscored by the fact that

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these malformations are, by far, more common than previously suspected. Indeed, human brain dysgeneses have been increasingly recognized in the last two decades as a relevant cause of mental retardation and neurological deficits including epilepsy [9, 10]. In particular, the widespread use of magnetic resonance imaging has revealed that patients with drug-resistant focal epilepsy are frequently (in about 20 to 40% of the cases) affected by developmental malformations of the cortex, and they frequently undergo epilepsy surgery for the relief of their intractable seizures [11, 12]. However, the mechanisms leading to epileptogenesis in human brain dysgenesis are still under investigation.

A possible approach to this specific issue is the use of appropriate animal models that could mimic patients with acquired or genetic structural brain abnormalities. Among the acquired models, the one based on the administration of the anti-mitotic agent methylazoxymethanol acetate (MAM) has long been used to induce developmental brain dysfunction in rodents (reviewed in [13]). We have already demonstrated that MAM-treated rats can be considered a useful model for human brain dysgeneses [14, 15]. Indeed, a double MAM administration in rats on embryonic day 15 induces cerebral heterotopia made up of hyperexcitable neurons that share striking similarities with those observed in human periventricular nodular heterotopia (PNH), a cerebral dysgenesis characterized by nodular masses of gray matter located in close apposition to the periventricular germinative neuroepithelium [16, 17].

The present study is part of a larger project investigating the mechanisms underlying the genesis and the hyperexcitability of heterotopic neurons in human PNH and in MAM-treated rats [14, 15, 18, 19]. The present study is based on the idea that the analysis of the development of cerebral heterotopia in MAM-treated rats may help us to understand not only how PNH develop in patients but also why they are so highly epileptogenic. Therefore, we have investigated the cytoarchitectural features of the heterotopia in the early post-natal period, and analysed the birthdating of the heterotopia by means of incorporation of the thymidine analogue bromodeoxyuridine (BrdU), in order to provide a scheme that may explain why heterotopia develop not only in this animal model but also in human PNH.

Materials and methods

Animal handling and cerebral tissue processing

All experimental procedures were carried out with care to minimize discomfort and pain to treated rats, in accordance with the guidelines of the European Communities Council (Directive of November 24, 1986, 86/609/EEC). Pregnant Sprague-Dawley rats received two intraperitoneal MAM acetate administrations (15 mg/kg maternal body weight, in sterile saline) on E15, the first injection at 12.00 a.m. and the second at 12.00 p.m., as previously reported [15]. On the same day, control pregnant rats were sham injected with the vehicle alone. The day after conception, determined by vaginal smear, was designated embryonic day 1 (E1). A group of MAM-treated and sham-operated pregnant rats were then subjected to intraperitoneal injections of 5-bromo, 2'-deoxy-uridine (BrdU, 50 mg/kg maternal body weight in sterile saline) at different embryonic ages (see below). The day after conception (as determined by vaginal smear) was designed embryonic day 1. Litters were born on day 22 or 23 of gestation, and the day of birth was designated post-natal day 1 (P1). The pups were housed under standard conditions, as previously reported [15].

At various post-natal ages (one day to four months of age), MAM/BrdU-treated and sham-operated control rats were deeply anesthetized with ice (from P1 to P5) or chloral hydrate (from P15 to adulthood; 1 mL/100 g body weight of a 4% solution) and perfused with 1% paraformaldehyde followed by 4% paraformaldehyde in 0.1 M phosphate buffered-saline (PBS) at pH 7.2. Brains were removed from the skull, post-fixed overnight in 4% paraformaldehyde, and cut with a vibratome into 40 to 50 µm thick coronal sections. Sections were collected in PBS and sodium azide (0.01%) in serial order. One out of three sections (for developing rats) or five sections (for adult rats) were counterstained with 0.1% thionine for the cytoarchitectural analysis, with particular attention to the developing heterotopia in the first two post-natal weeks. The adjacent sections were processed for BrdU immunocytochemistry as outlined below.

BrdU immunocytochemistry

To investigate the time of neurogenesis of the MAM-induced heterotopic neurons, seven different time points for the BrdU injections were chosen, at 24, 36, 48, 60, 84, 108, and 132 hours after the last MAM administration, referred to as E16/24, 17/12, 17/24, 18/12, 19/12, 20/12, and 21/12, respectively. Earlier BrdU administrations were not performed, given the already reported MAM-induced ablation of neuroblasts generated at the time of the MAM administration or shortly thereafter [20, 21].

To optimize BrdU immunocytochemistry, we performed several pilot experiments according to protocols reported in the literature [22, 23]. We obtained the best results with the following protocol. Free-floating sections were initially pre-treated with 2N HCl in PBS for 60 min to separate DNA strands, and then with sodium borate 0.1 M for 10 min to neutralize the acid. Sections were then treated with 1% H_2O_2 in PBS for 20 min to neutralize the endogenous peroxidase activity, rinsed in PBS, and incubated with 10% normal serum (NGS) and 0.2% Triton-X100 for 60 min, to mask non-specific adsorption sites and to increase the penetration of the reagents. Sections were then incubated overnight with anti-BrdU monoclonal antibodies (Becton-Dickinson, San José, CA, USA; or Boehringer-

Mannheim GmbH, Germany) diluted to 1:75. After rinsing in PBS, the sections were incubated with biotinylated goat anti-mouse IgG (GAM, Jackson, PA, USA; diluted to 1:200), rinsed in PBS, and then incubated with Extravidin (1:5000, Sigma-Aldrich, Milano, Italy). All immunoreagents were diluted in 1% NGS in PBS. Peroxidase staining was obtained by incubating the sections either in DAB 0.075% and H_2O_2 (0.002%) or in DAB (1.25 mg/mL), NAS (0.04%), NH_4Cl (0.004%), glucose (0.2%) and glucose oxidase (1.2 U/mL) in 0.05 M Tris-HCl at pH 7.6. The immunoreacted sections were mounted onto gelatin-coated glass slides, air-dried, dehydrated, and coverslipped with DPX. Slides were then analyzed and photographed with a Nikon Microphot FXA microscope.

Results

In the present study, we have specifically addressed the following issues: **1)** the development of MAM-induced heterotopia in the first two post-natal weeks; and **2)** the neurogenesis of the heterotopia, by means of BrdU immunocytochemistry.

Early post-natal development of the MAM-induced heterotopia

One of the striking cytoarchitectural features of the developing MAM-treated rat brains was the presence of elongated cellular bands of intensely thionine-stained cells within the subcortical white matter *(figure 1A)*, particularly evident during the first post-natal week. These cellular bands were always intermingled with many elongated cells, with morphological features typical of young, migrating neurons (*figure 1A*, arrowheads). In addition to that, they were always located in the white matter in close anatomical relationship to the cortical, periventricular, and intra-hippocampal heterotopia (*figure 1A-D*, arrows). They remained conspicuous during the first post-natal week, and they tended to disappear during the second post-natal week, being barely appreciable at P15.
The cortical heterotopia overlying the white matter bands were already present at birth, as round or elongated nod-

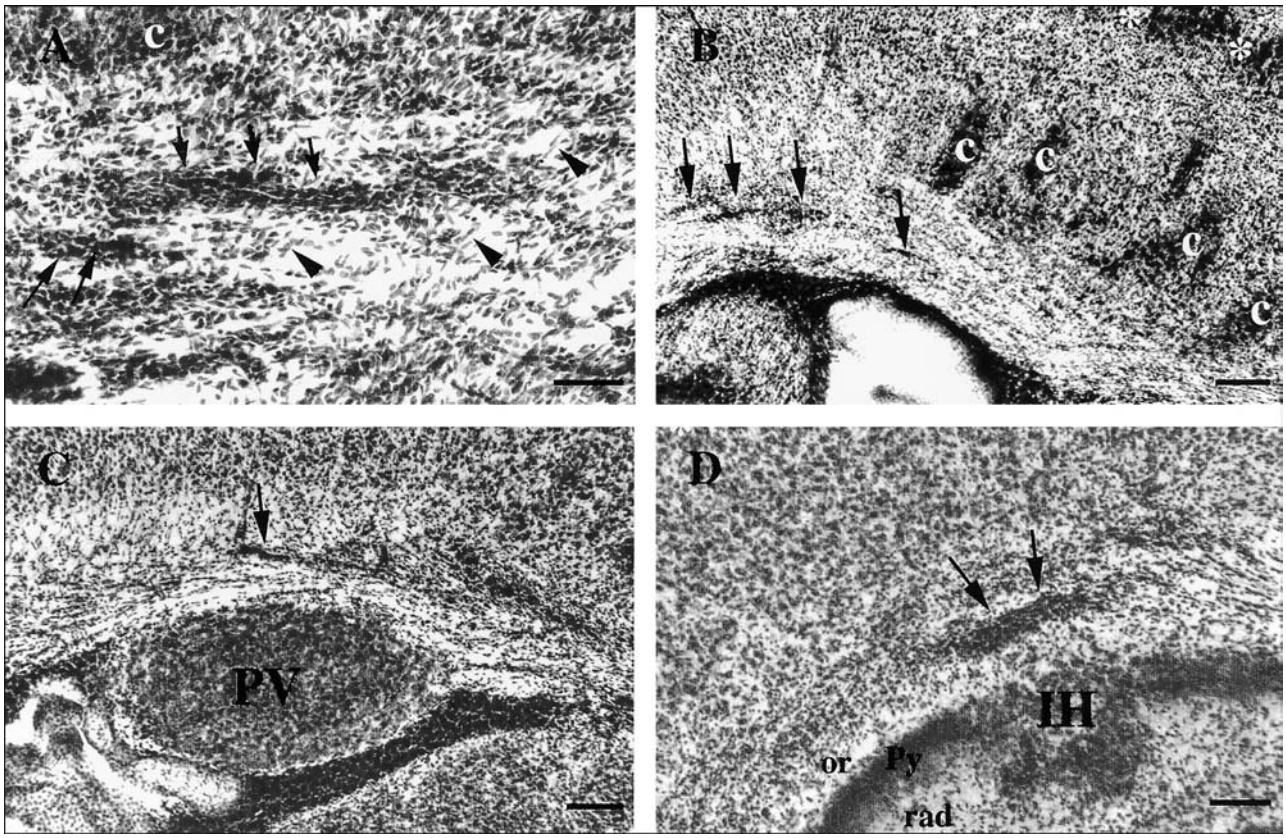

Figure 1. *Cytoarchitectural features of MAM-treated rat brains during early post-natal development. Thionine stain. (***A***) Cellular bands within the subcortical white matter (arrows) at* **P3**, *intermingled with migrating neurons (arrowheads). Note the close anatomical relationship with a cortical heterotopia* **(c)**. *(***B***) Cortical heterotopia* **(c)** *at* **P1**. *Note the reduction of cellular density in the cortical layers overlying the heterotopia (asterisks), and the cellular bands within the white matter (arrows). (***C-D***) Periventricular heterotopia* **(PV)** *at* **P3 (C)**, *and intra-hippocampal heterotopia at* **P5 (D)**. *Note the white matter cellular bands (arrows) close to the heterotopia.* **or***: stratum oriens;* **rad***: stratum radiatum;* **Py***: pyramidal cell layer. Calibration bars: 50 µm in* **A**, *150 µm in* **B**, *100 µm in* **C-D**.

ules located in the deep cortical layers, made up of densely packed neurons of small size and ill-defined morphology *(figure 1B)*. In the cortical areas surrounding the heterotopia, normal cortical layering was not recognizable, and the cortical areas above the heterotopia were consistently characterized by a clear reduction of cellular density (*figure 1B*, asterisks). During the first two post-natal weeks, the cortical heterotopia enlarged in size: the neurons located within the core of the heterotopia were still more darkly stained and densely packed than the surrounding neocortical neurons, whereas the neurons at the periphery of the heterotopia progressively displayed a less immature morphology. At P15, the cortical heterotopia displayed the anatomical features already described for the adult rats [15].

The periventricular heterotopia were also already present at birth, as round nodules located just dorsal to the germinative neuroepithelium and immediately beneath the white matter cellular bands *(figure 1C)*. As in cortical heterotopia, they also progressively increased in size during the first two post-natal weeks. The core of the nodules was made up of densely packed round cells, whereas darkly stained small cells, possibly representing young migrating neurons, were placed marginally at the border of the nodules. A thin space separating the cells at the border from those within the core of the nodules became evident from P5 onwards, most likely representing tangentially running fibers.

In contrast, intra-hippocampal heterotopia were never present at birth. At P1, sectors of the germinative neuroepithelium dorsal to the developing hippocampus were particularly thick, with thin columns of dark migrating neurons extending to the already formed pyramidal cell layer of CA1 and CA2. At P3, wedge-shaped masses of neurons filled the *stratum oriens* between the neuroepithelium and the pyramidal cell layer, whereas at P5 the intra-hippocampal heterotopia consistently disrupted the CA1 and CA2 pyramidal cell layer and invaded the *stratum radiatum (figure 1D)*. As for the other types of heterotopia, in the first post-natal week, thick cellular bands were consistently present within the subcortical white matter overlying the progressively forming intra-hippocampal heterotopia (*figure 1D*, arrows). At P15, the periventricular, as well as the intra-hippocampal nodules displayed the anatomical features of the adult rat [15].

BrdU labeling

Clear, inside-out, latero-medial, and rostro-caudal gradients of neurogenesis were observed in the MAM neocortex outside the heterotopia after BrdU injections from E16 to E21. In the hippocampus, CA3 neurons were first generated, followed by CA1 and CA2 neurons, and by granule cells in the dentate gyrus. In addition, a superficial to deep (or external to internal) gradient of neurogenesis was clearly present (not shown). All these results are consistent with already reported data on cortical and hippocampal neurogenesis [4, 24, 25].

In cortical *(figure 2A)* and periventricular (*figures 2B* and *3*) heterotopia, BrdU-labeled cells after the E16/E17 injections (i.e., from 24 to 48 hours after the last MAM administration), were mainly located along the borders of the heterotopia. In particular, the nuclei of the cells located at the periphery of the heterotopia were completely and intensely BrdU-immunoreactive, whereas the nuclear BrdU signal was progressively less intense moving toward the core of the heterotopia *(figure 3A)*. After E18/E19 BrdU administration (from 60 to 84 hours after the administration of MAM), labeled heterotopic neurons were located throughout the heterotopia, both at the periphery and within the core of the cortical *(figure 2A)* and periventricular (*figures 2B* and *3B*) heterotopic nodules. After E20/E21BrdU administration (from 108 to 132 hours after the administration of MAM), the number of BrdU immunoreactive neurons progressively decreased in both types of heterotopia. Labeled heterotopic neurons were confined inside the cortical *(figure 2A)* and periventricular (*figures 2B* and *3C*) heterotopia.

In the intra-hippocampal heterotopia *(figure 2C)*, after E16/E17 administration of BrdU, labeled cells were more evident in the dorsal part of the heterotopia, and the number of labeled cells and the intensity of labeling decreased moving ventrally *(figure 2C)*. After E18/E19 administration of BrdU, labeled cells were very numerous in both the dorsal and ventral aspects of the intra-hippocampal heterotopia, whereas after E20/E21 BrdU pulses, labeled neurons tended to decrease and to be confined in the more ventral aspects of the intra-hippocampal heterotopia *(figure 2C)*.

With regard to the cellular bands within the white matter, these were BrdU-negative after E16/E17 BrdU pulses, even if there were rare BrdU-positive cells after E17/24 injections. In contrast, after E18/E19 BrdU pulse numerous cells were BrdU-positive between P1 and P7 in the white matter bands close to the cortical and subcortical heterotopia and overlying the intra-hippocampal heterotopia. After E20/E21 administration, the white matter bands close to the heterotopia were still BrdU-positive in P1 to P7 MAM-rats, but less conspicuously so, if compared to those labeled after E18/19 injections. After E18/E21 injections, a few BrdU-positive neurons were still present in adult rats in the white matter, close to the heterotopic nodules.

Thus, taken together these experiments indicated that: i) the period of generation of neurons within the heterotopia and adjacent white matter bands was extended and it roughly overlapped that of superficial neocortical neurons; ii) the white matter cellular bands began to form after the heterotopia; and iii) the heterotopia were formed through an outside-in (for cortical and periventricular heterotopia) and dorso-ventral (for intra-hippocampal heterotopia) neurogenetic gradient.

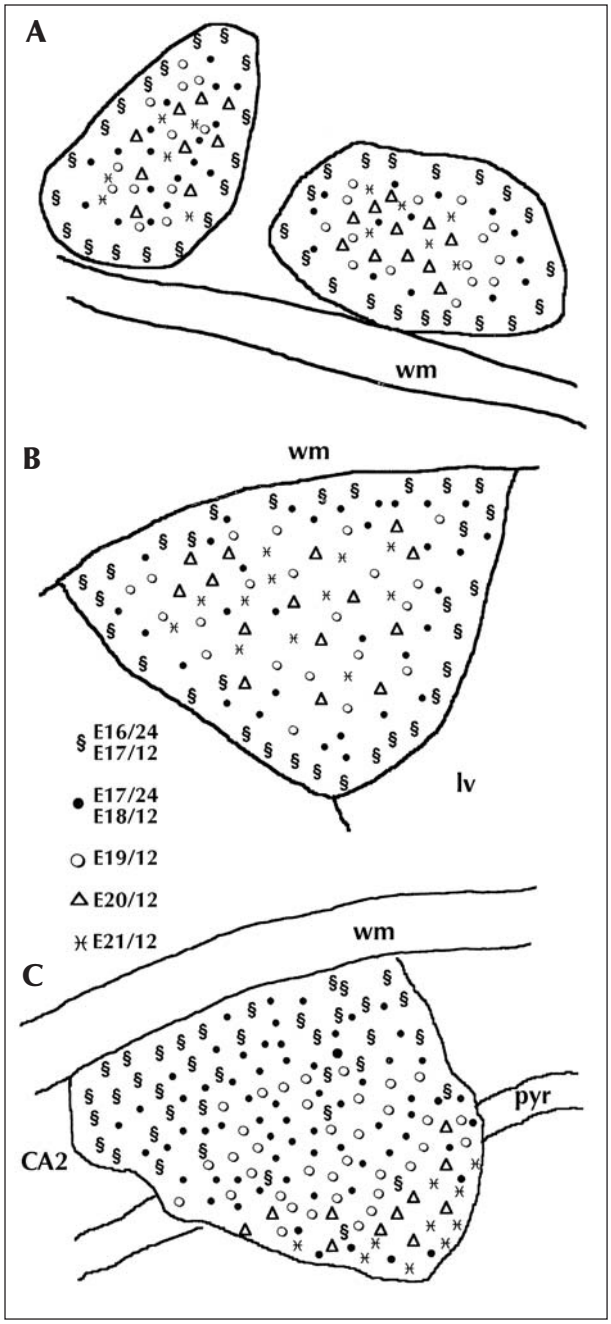

Figure 2. *Reconstructions of the distribution of BrdU-labeled cells within the cortical (***A***), periventricular (***B***), and intra-hippocampal (***C***) heterotopia after BrdU administration at different embryonic ages from E16/24 to E21/12. Note the outside-in gradient of neurogenesis in cortical and periventricular heterotopia, and the dorso-ventral neurogenetic gradient in intra-hippocampal heterotopia.*

Discussion

In the present study, we have investigated the ontogenesis of cerebral heterotopia in MAM-treated rats as a model for understanding the possible way by which periventricular nodular heterotopia develop in humans. These data should also help clarify why human PNH are so frequently associated with epileptic phenomena.

Numerous cellular bands of migrating neurons are present in the white matter of MAM rats in the early post-natal period, and this is particularly evident in the first post-natal week. In the normal embryonic rat brain, cells committed to the neocortex sojourn between E15 and E21 in cellular bands within the intermediate and subventricular zones before reaching their final cortical destination [4]. The white matter bands described here in the post-natal brain of MAM rats probably correspond to the superior bands of the pre-natal brain of normal rats [4], indicating that the MAM treatment determines a delayed, post-natal maturation of the heterotopia. These bands are made up mostly of immature neurons. Their close anatomical relationship with the different types of heterotopia suggest that these bands function, in the early post-natal period, as a reservoir of young, migrating neurons specifically committed to the newly forming heterotopia. They later disappear, but quite a few neurons remain within the white matter close to the heterotopia in adult MAM rats. The presence of many neurons within the white matter surrounding the heterotopia is also a feature of human PNH [19].

It has been already hypothesized that early generated and properly migrated neurons are of key importance for the migration and differentiation of subsequent waves of neurons [21]. Some features of the MAM rat brain in the early post-natal period seem to support this view. Firstly, a clear reduction of cellular density is consistently present in the superficial cortical layers overlying the cortical heterotopia. Secondly, the newly forming heterotopia are in close anatomical relationship with the cellular bands in the white matter. This suggests that the heterotopia themselves are able to influence and direct the migration of later-generated waves of neurons. Therefore, we hypothesized that the MAM-induced ablation of early-generated cortical neurons determines the presence of heterotopic cortical neurons in the deep cortical layers and periventricular white matter, and that, in turn, these heterotopic neurons influence the migration of later-generated neurons to determine the genesis of the heterotopia.

What is the progressive way by which heterotopia develop? Our birth-dating BrdU analysis has revealed a clear neurogenetic gradient in all MAM-induced heterotopia. Cortical and periventricular heterotopia are formed by progressive settling of neurons from the outside to the inside, whereas intra-hippocampal heterotopia are progressively formed through a dorsal to ventral migration of neurons *(figure 2)*. Similar findings have been obtained in the intra-hippocampal heterotopia induced by a single MAM administration [26]. Our data clearly demonstrate that in MAM rats the neurogenesis is not chaotic, but follows the general ontogenetic rules of cortical layering formation. In MAM rats however, as a consequence of the

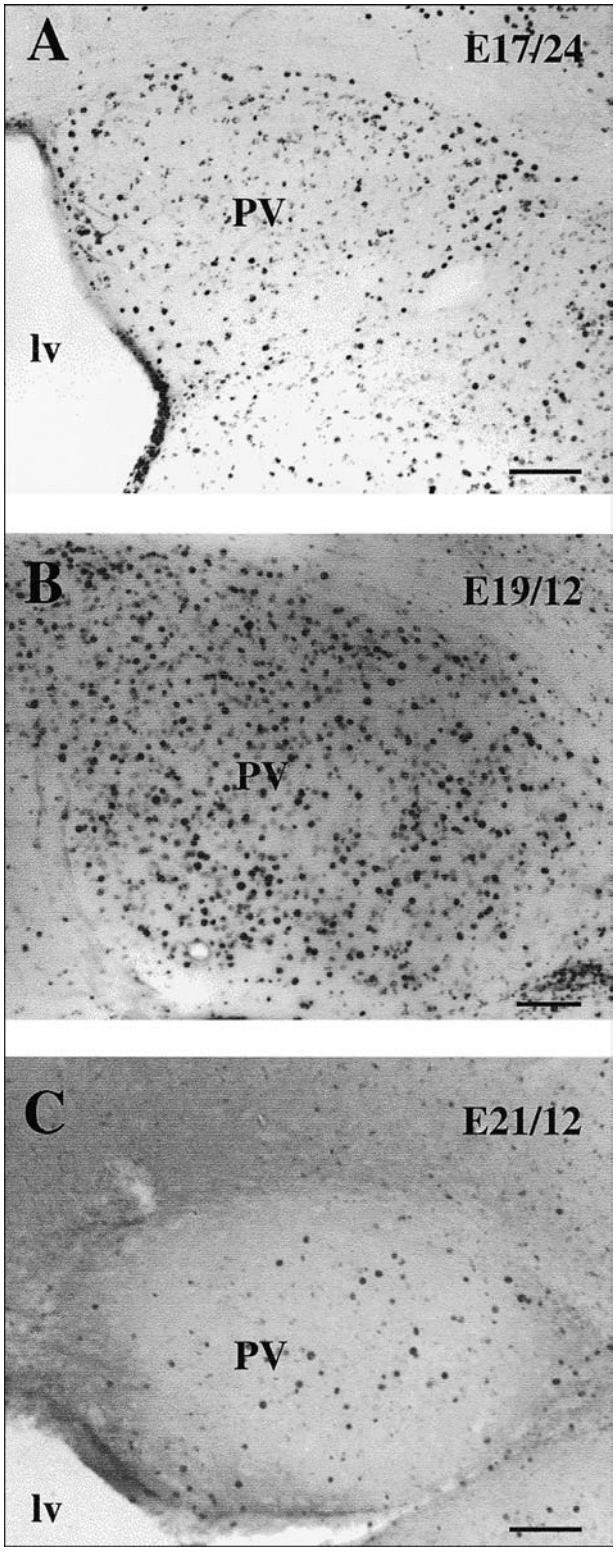

Figure 3. *BrdU labeling in periventricular heterotopia (PV). BrdU immunocytochemistry. Note that BrdU labeled cells progressively move from the periphery (E17/24 injections in* **A***) to the core of the heterotopia (E21/12 injections in* **C***).*
lv: lateral ventricle. Calibration bars: 100 µm.

MAM-induced ablation of early-generated neuroblasts, heterotopically-located neurons in the periphery of the cortical and periventricular heterotopia and in the dorsal part of intra-hippocampal heterotopia set the base for the subsequent migration of neurons into the heterotopia. To explain the existence of intra-hippocampal heterotopia and their delayed genesis [26-28], we hypothesize that hippocampal neurons may attract the young, migrating neurons sojourning in the white matter bands toward the pyramidal cell layer; the post-natal development of intra-hippocampal heterotopia could be related to the pre-natal physical separation between the hippocampus and the subcortical white matter, which would make the migration into the hippocampus impossible [26].

On the basis of this evidence we propose the following scheme for the genesis of all types of heterotopia *(figure 4)*. Firstly, the MAM administration at E15 kills a cellular subpopulation of neuroblasts committed to the neocortex *(figure 4A)*. Secondly, the following wave of neurons fails to migrate correctly into the developing cortical plate, possibly because of the absence of soluble factors and the alteration of the proper environment which would allow migration into the cortical plate. For this reason, heterotopic cortical neurons settle in the deep cortical layers, in the underlying white matter, and within the periventricular neuroepithelium *(figure 4A)*. Thirdly, these heterotopic neurons influence the migration of the subsequent waves of young neurons, which first sojourn in the developing white matter, and then migrate within the newly forming heterotopia *(figure 4B)*. Fourthly, this migration occurs in a rather precise neurogenetic order, outside-in for cortical and periventricular heterotopia and dorso-ventral for intra-hippocampal heterotopia *(figure 4C)*. Finally, heterotopic neurons send their axons to (and receive axons from) neocortical and archicortical areas *(figure 4C)*, thus establishing aberrant connections between the neocortex and the hippocampus [18, 26, 29].

If this series of neurogenetic events also takes place in the human brain, we can hypothesize that a cellular lesion, for instance ischemic, in a limited region of the germinative neuroepithelium, may eventually lead to the nodular heterotopia seen in human PNH patients. Moreover, if we take into account that a fraction of neurons within the heterotopia are characterized by hyperexcitable firing patterns [14, 28, 30], the progressive electrophysiological recruitment of cortical and subcortical neurons via the altered circuitry linking the heterotopic nodules to the adjacent neocortical and archicortical areas, may explain the genesis of sustained tonic discharges and therefore the clinical onset of epileptic discharges that are so frequently observed in PNH patients. □

Acknowledgements

This study was partially supported by grants and RF162/2001 from the Italian Ministry of Health and R/03/29 from the Mariani foundation for pediatric neurology.

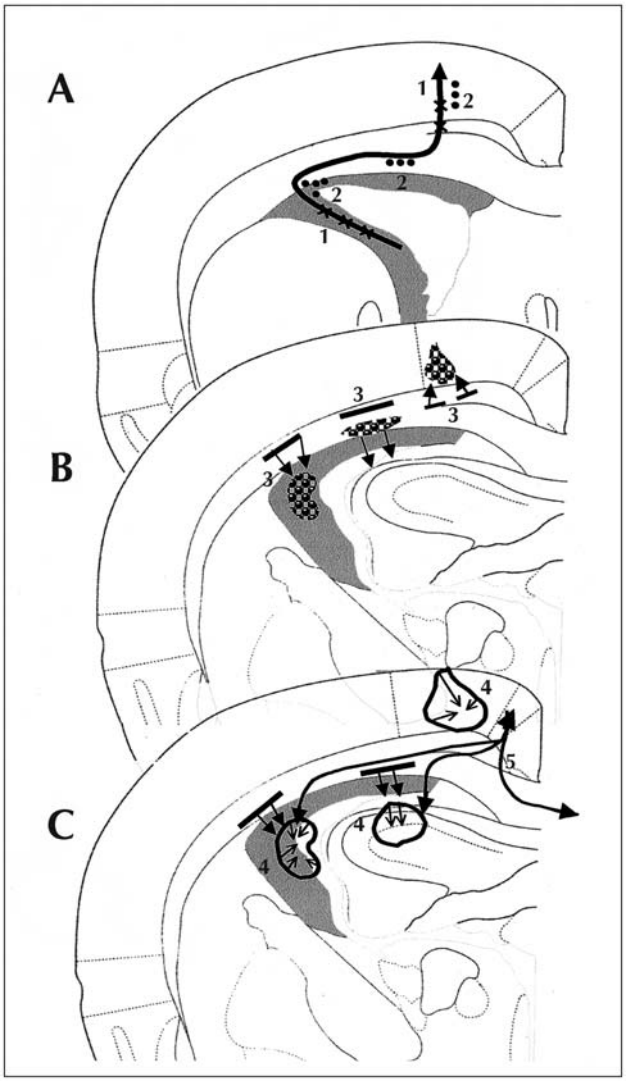

Figure 4. *Summary diagram illustrating the mode of neurogenesis of cerebral heterotopia suggested by the present experiments. See text for specifications.*

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