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Abnormal Connections in the Malformed Cortex of Rats with Prenatal Treatment with Methylazoxymethanol May Support Hyperexcitability

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Key Words

Development · Hippocampus · Mossy fibres · Epilepsy · Cortical dysplasia · Heterotopia · Neuronal migration disorders

Abstract

Prenatal treatment with methylazoxymethanol (MAM) in rats generates animals with a diffuse cortical malformation associated with hyperexcitability. These alterations are reminiscent of the cortical malformations associated with epilepsy in children. We hypothesised that one of the mechanisms supporting hyperexcitability in MAM rats could be the presence of abnormal cortical connections in the malformed cortex. Using a variety of anatomical techniques, we provide evidences for three types of such abnormal connections: (i) tangential bundles of corticocortical fibres in and below the neocortical molecular layer; (ii) partial deafferentation of neocortical heterotopias by afferent cortical fibres whatever their location; (iii) exuberant innervation of hippocampal CA3 pyramidal cells by mossy fibres that form ectopic mossy boutons on their basal dendrites. We conclude that these abnormal intrinsic cortical connections may support the propagation of paroxymal activity in the neocortex of MAM-treated rats.

Introduction

In humans, cortical malformations are an important cause of childhood epilepsy [Taylor et al., 1971; Palmini et al., 1991, Aicardi, 1994; Raymond et al., 1995; Robain, 1996]. These symptomatic epilepsies are frequently pharmacoresistant. When left uncontrolled, seizures have deleterious consequences on psychomotor development, often leading to mental retardation [Dulac, 1997]. The pathophysiological mechanisms of epileptogenicity in malformed cortex remain poorly understood.

One reliable model to investigate this issue experimentally consists in prenatal treatment with the antimitotic agent methylazoxymethanol (MAM) [Johnston and Coyle, 1982; Cattabeni and Di Luca, 1997]. When administered at embryonic day 14 (E14) in rats, MAM causes cell death in the proliferative neuro-epithelium and secondary disorganisation of radial glial cells [Ashwell, 1992; Zhang et al., 1995] that guide neuroblast migration to the cortical plate. Consequently, the so-called MAM rats are microcephalic, with a particular reduction of the external cortical layers [Dambska et al., 1982; Yurkewicz et al., 1984], and exhibit a variety of migration disorders such as subcortical, subpial and deep cortical layers and intrahippocampal neocortical heterotopias [Singh, 1977; Collier and Ashwell, 1993].

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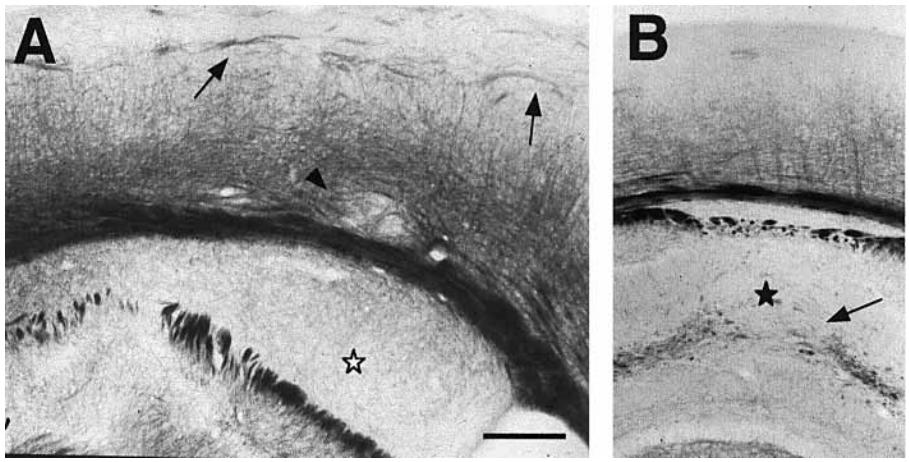


Fig. 1. Gold axonal impregnations in MAM-treated rats. **A** coronal section demonstrating the presence of abnormal tangential fibres (arrows) below the molecular layer, and the partial differentiation of deep layers (arrowhead) and subcortical (star) heterotopias. **A** Intrahippocampal heterotopias (star) are also apparently deafferentated and appear to perturbate the pathway of hippocampal fibres (arrow). **A** Scale bar = 250 μm; the same length corresponds to 100 μ in **B**.

MAM rats grow and develop normally but show poor learning abilities [Balduini et al., 1986] as well as hyperactivity [Vorhees et al., 1984]. More recently, it has been shown that adult MAM rats have an increased sensitivity to a number of convulsing agents including kainic acid [Germano and Sperber, 1997, 1998], flurothyl [Baraban and Schwartzkroin, 1996] and kindling [Chevassus-au-Louis et al., 1998a]. This hyperexcitability is already present at postnatal day 14 [De Feo et al., 1995; Germano et al., 1996] which is equivalent to early childhood in humans. These data show that MAM-treated rats can be used as experimental models of epilepsy associated with cortical malformation [for a recent review, see Chevassus-au-Louis et al., 1999], although spontaneous seizures have not been reported to date in these animals.

Several investigators have searched for altered physiological properties of cortical neurons in MAM-treated rats that may support hyperexcitability. Indeed, abnormal firing patterns have been described in a subset of pyramidal neurons encountered in the hippocampal CA1 region [Baraban and Schwartzkroin, 1995], in the external neocortical layers and in subcortical heterotopic nodules [Sancini et al., 1998]. These widespread impairments provide a physiological substrate for the initiation of paroxysmal activity.

Our group has focused its interest on the propagation – not initiation – of epileptic activity in the malformed cortex of MAM-treated rats. We thus reported that the integration of intrahippocampal heterotopias in both the neocortical and the hippocampal network allowed the spread of hippocampal paroxysmal activity to the neocortical mantle [Chevassus-au-Louis et al., 1998b]. Here, we extend these findings and provide anatomical evidence for other pathological corticocortical connections that may support the generalisation of paroxysmal activity in the malformed cortex of MAM rats.

Material and Methods

Wistar rats were used throughout the experiments. Pregnant rats were injected intraperitoneally with 25 mg/kg MAM (Sigma) in saline at E14 (first gestation day as E0) between 1 and 3 p.m. After normal delivery, litter size was reduced to 8 and the pups grew with free access to food and water under diurnal conditions with lights on from 8.00 to 20.00 h. The animals used in this study were sacrificed at the age of 2–3 months.

As a first step in the search for abnormal connections, we performed axonal staining using the highly sensitive gold method initially described by Schmued [1990]. In our hands, this method turned out to stain almost all axonal processes and not only myelinated axons as initially described, as evidenced by the comparison with ajdacent sections stained for myelin using the conventional fast blue method. After these preliminary explorations, several complementary experiments were conducted, according to protocols previously published by our group, namely carbocyanine tracing [Chevassus-au-Louis et al., 1988b], immunohistochemistry [Rafiki et al., 1998] using a polyclonal antibody against dynorphin (Pensinsula, 1:10,000), Timm staining [Represa et al., 1991] and rapid Golgi staining [Represa et al., 1993].

Results

Neocortex

In control rats, gold impregnations demonstrated a dense network of radially organised axons, spanning from the white matter. In MAM rats, these radial fibres could also be observed. Nonetheless, their fibres were sparse in subcortical heterotopias and did not penetrate the deep layer heterotopias (fig. 1). In addition, abnormal tangen-

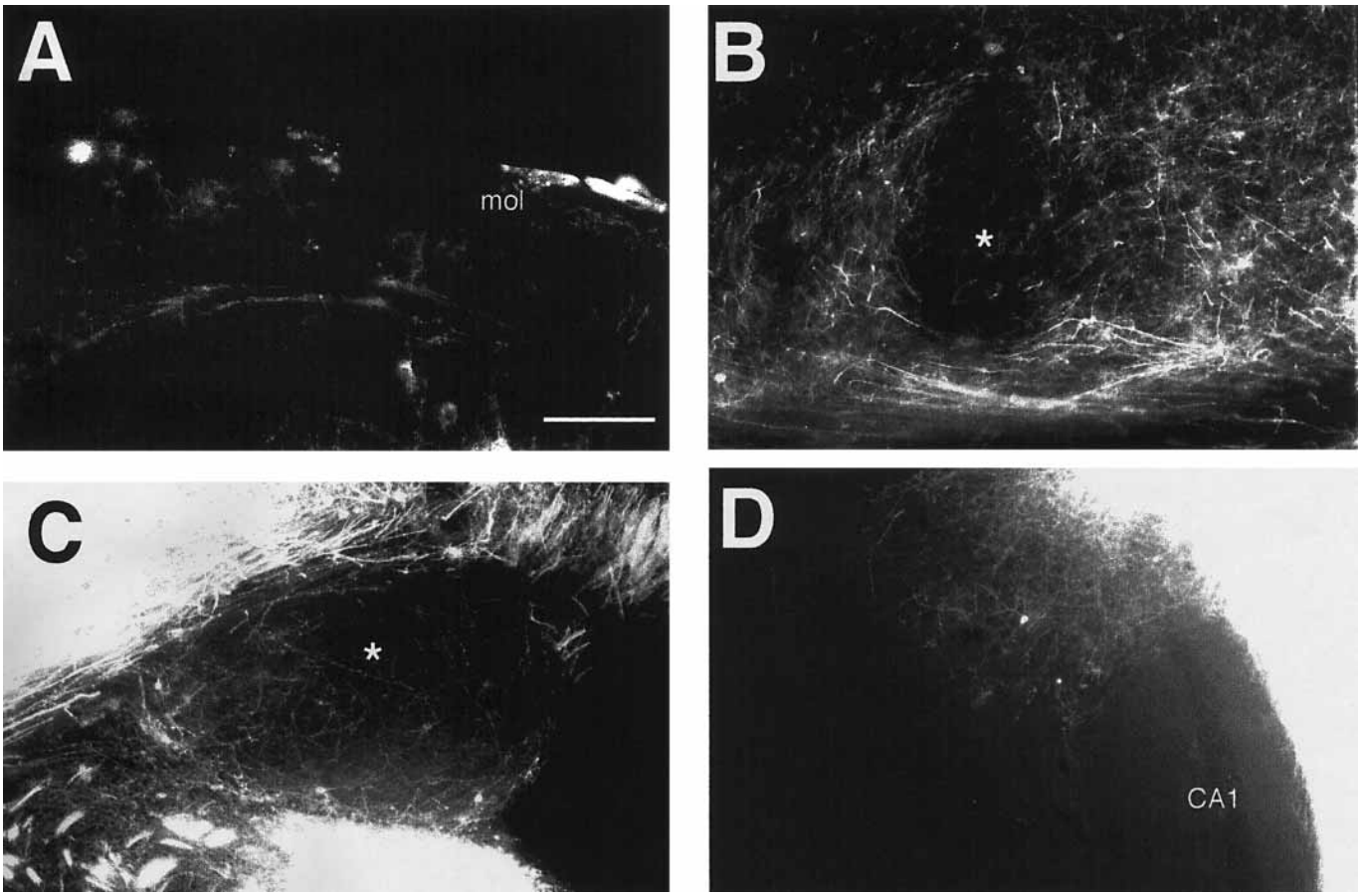


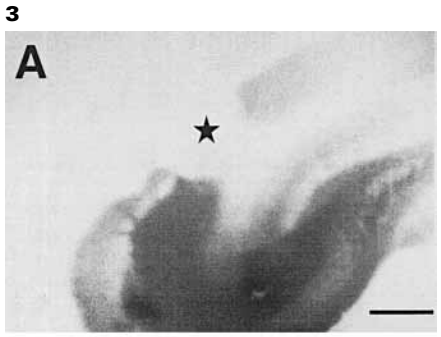
Fig. 2. Carbocyanine staining of neocortical fibres. **A** Tangential fibres (mol = molecular layer) were observed below the molecular layer after a local application of diI in the dorsal neocortex. This indicates that these fibres are intrinsic cortical fibres. Deep layers (**B**), subcortical (**C**) and intrahippocampal (**D**) heterotopias (stars) are contacted by relatively few neocortical fibres (low density), as compared to adjacent cortical regions. **A** Scale bar = 50 μm; the same length corresponds to 100 μm in **B**–**D**.

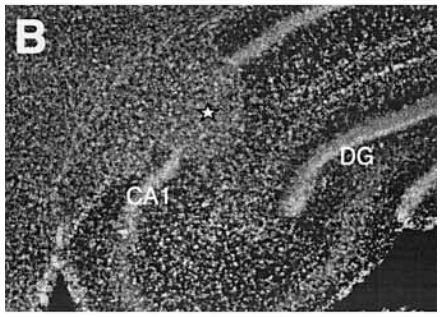
tial fibres, sometimes organised in bundles, were observed to course in and immediately below the molecular layer for distances up to 1 mm. These fibres were oriented in both the mediolateral and caudorostral directions as evidenced by their presence in both coronal and parasagittal sections. Such fibres were never observed in control brains, nor in the almost normal ventrolateral cortex of MAM-treated rats, which clearly indicates their pathological nature.

In order to know if these fibres were corticocortical or thalamocortical, we performed an extensive labelling of the neocortical network using large injections of diI in various regions of the neocortex. Tangential fibres coursing in the molecular layer, similar to those described in gold impregnations, were observed (fig. 2A). This indicates that at least some of these fibres are intrinsic cortical fibres. Carbocyanine-labelled fibres and some retrogradely labelled neurons were also observed in intracortical (fig. 2B), subcortical (fig. 2C) and intrahippocampal (fig. 2D) heterotopias. Again, the density of these fibres was clearly lower than that in adjacent normotopic neocortical regions, therefore suggesting a partial deafferentation of heterotopias by cortical fibres.

Hippocampus

Gold impregnations in the hippocampus of control rats allowed the visualisation of three important hippocampal fibre tracts: (i) the mossy fibres from the dentate gyrus to





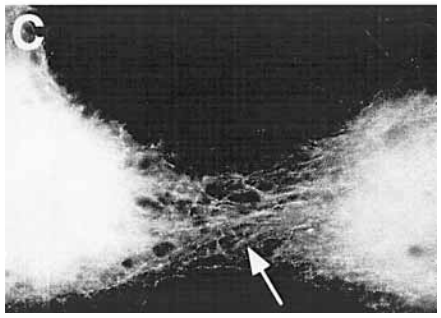
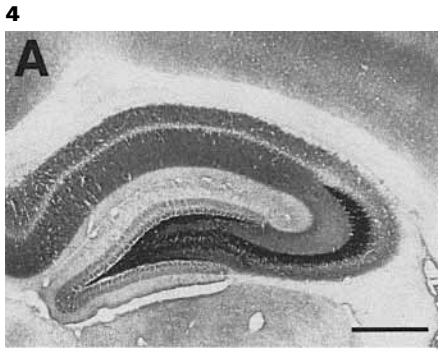
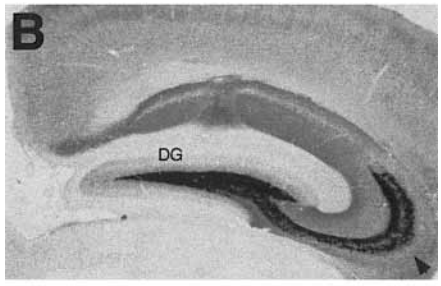


Fig. 3. Carbocyanine tracing of Schaffer/commissural fibres. A cristal of carbocyanine was inserted in the CA3 area and labelled fibres are depicted (**A**, negative picture for the purpose of contrast effects, with cyto-architectonics provided by bisbenzidine staining in **B**). Stars = Heterotopia; DG = dentate gyrus. **C** Schaffer/commissural fibres appear to avoid the heterotopic core (arrow) although some fibres can be seen in its ventral part at the higher magnification. **A**, **B** Scale bar = 125 μm; the same length corresponds to 50 μm in **C**.





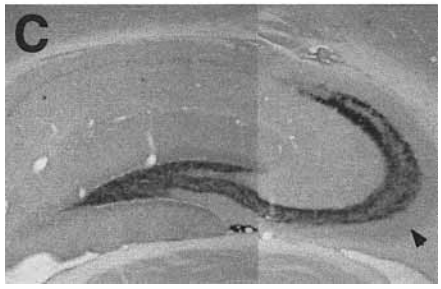


Fig. 4. Mossy fibres in the CA3 stratum oriens of MAM rats. **A** Timm staining in a control rat showing mossy fibres confined to the stratum lucidum of CA3. By contrast in MAM rats, both Timm staining (**B**) and dynorphin immunostaining (**C**) reveal a double band of mossy fibres in the stratum lucidum and in the stratum oriens (arrowheads) of the CA3. No signs of mossy fibres were found in the molecular layer of the dentate gyrus (DG). Scale bar = 150 μm.

the CA3 region that terminated exclusively in the stratum lucidum; (ii) the Schaffer/commissural pathway from CA3 to the stratum radiatum of CA-1; (iii) the temporoammonic pathway from the entorhinal cortex to the stratum lacunosum of CA1 and the dentate gyrus. This pattern was also observed in MAM rats with the following specificities: (i) intrahippocampal neocortical heterotopias in the CA1 region appeared to be avoided by the Schaffer/commissural network (fig. 1B); (ii) a dense axonal plexus was present in both the stratum lucidum and stratum oriens of the CA3 region.

In order to confirm the absence of CA3 projections in CA1 heterotopias, Schaffer/commissural fibres were labelled by a carbocyanine injection in the CA3 region in MAM rats. This led to the labelling of a very dense meshwork of fibres in the fimbria and in the stratum radiatum of CA1 (fig. 3A, B) and of retrogradely labelled granule cells in the dentate gyrus. In agreement with gold impregnations, CA1 heterotopias were largely avoided by Schaffer/commissural fibres, although a few labelled fibres could be observed in the most ventral part of the heterotopias (fig. 3C).

The projections from the dentate gyrus to CA3 were specifically labelled using Timm staining. This demonstrated the presence of an aberrant band of Timm staining in the stratum oriens of CA3 (fig. 4B) that was never observed in control Wistar rats (fig. 4A). The same held true when different mossy fibre markers were used such as dynorphin immunostaining (fig. 4C). No signs of Timm deposits or dynorphin immunostaining were found in the molecular layer of the dentate gyrus.

Mossy fibres and target cells were then analysed on Golgi preparations. This technique allows the identification of individual mossy fibres by the presence of their characteristic mousses (synaptic boutons). In addition, cells innervated by these fibres develop giant complex

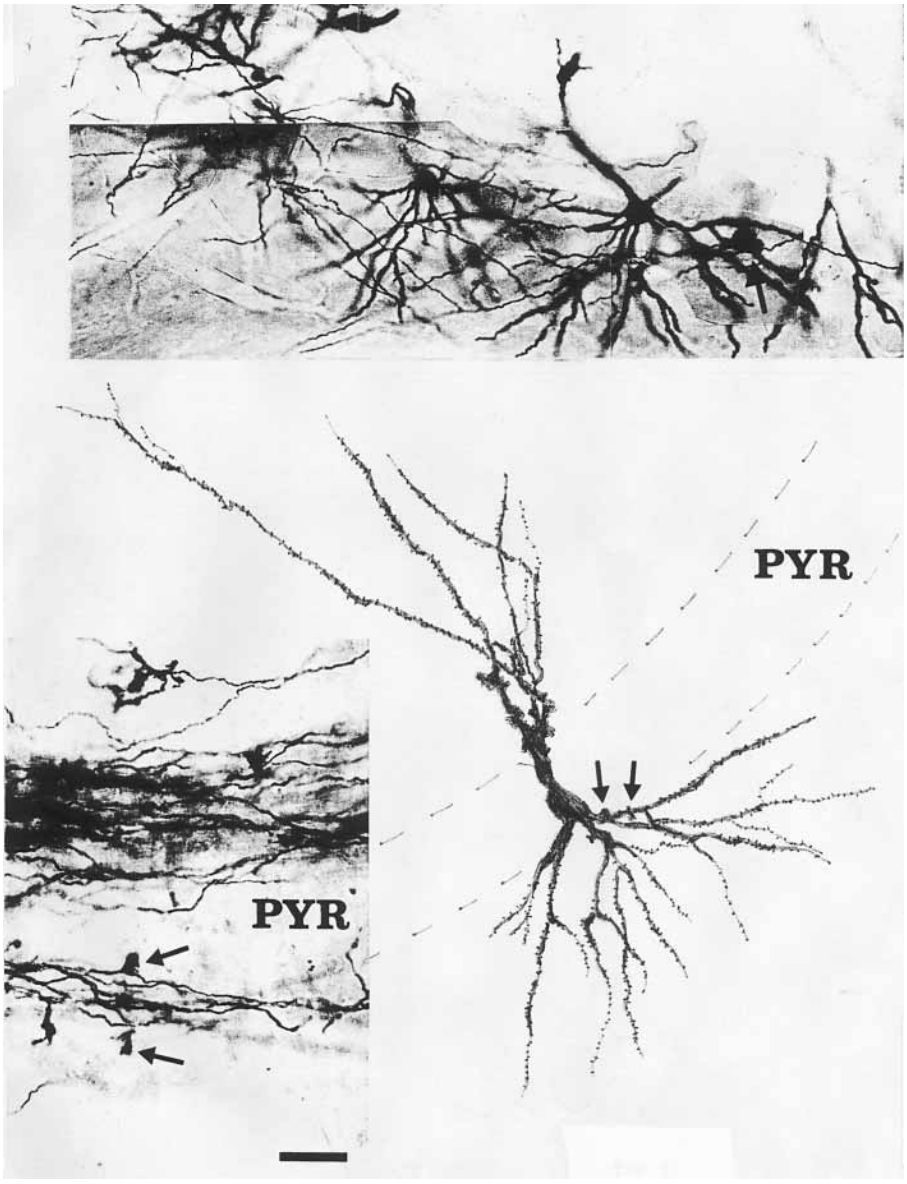


Fig. 5. Golgi staining demonstrates the presence of ectopic mossy boutons on basal dendrites of CA3 pyramidal cells. Micrographs showing mossy fibres in both stratum luidum and stratum oriens of the CA3 region of MAM rats. A camera lucida drawing displays a CA3 pyramidal cell in a MAM rat with typical but ectopic thorny excrescences on its basal dendrites (arrows). PYR = Stratum pyramidale. Scale bar = 50 μm.

spines, the so-called thorny excrescences. As shown in figure 5, mossy fibres were again observed in the stratum lucidum of the CA3 and in the stratum oriens of MAM rats, reinforcing the previous observations in Timm-stained preparations. Pyramidal cells clearly showed thorny excrescences at the normal location, i.e. on their apical dendrites, and in ectopic position in basilar dendrites (fig. 5). These observations suggest that mossy fibres do innervate basal dendrites of CA3 pyramidal cells.

Discussion

We have provided evidence for the existence of three types of abnormal projections in the cortex of MAM rats: (i) the presence of tangential intrinsic cortical fibres in the neocortical molecular layer; (ii) an abnormal innervation of the basal dendrites of CA3 pyramidal cells by mossy fibres; (iii) a partial deafferentation of neocortical heterotopias. These three projections will now be discussed from a developmental – what do these projections indicate regarding the mechanisms involved in axonal growth and

synaptogenesis? – and functional – may these projections contribute to hyperexcitability? – point of view.

Tangential Fibres in the Molecular Layer

Using gold axonal impregnations, we have observed abnormal tangential fibres in the neocortex of MAM rats that were intrinsic cortical fibres as demonstrated using carbocyanine tracing. In rats, the supragranular layers are an important source of corticocortical fibres [Nensen and Altman, 1982; Miller and Vogt, 1984]. We suggest that the dramatic reduction of these layers in MAM rats may cause cortical fibres originating from less affected neocortical regions (for instance frontal or ventral regions which are almost normal) to travel a long tangential distance in the search for a postsynaptic target.

Functionally, these fibres may form connections between normally unconnected cortical areas, but formally, this is hard to prove because the identification of cortical areas is almost impossible in MAM rats due to cyto-architectonic alterations. Nonetheless, it is possible to speculate on some pathophysiological consequences of these pathological intrinsic neocortical connections. In a different model of cortical malformations yet with similar histological alterations [Roper, 1998], Roper et al. [1997] have demonstrated that induced seizure activity in neocortical slices did propagate further in malformed neocortex than in control. We propose that this excessive propagation is supported by the presently described abnormal tangential connections. Moreover, we have observed similar subpial fibres in several cases of human malformations [Chevassus-au-Louis and Robain, unpubl. results], which suggests that the occurrence of abnormal corticocortical connections could be a general pathophysiological mechanism in cortical malformation.

Partial Deafferentation of Neocortical Heterotopias

We have observed that neocortical heterotopias are largely avoided by surrounding fibres, whatever their location. For instance, intrahippocampal neocortical heterotopias were shown, in agreement with a previous report [Singh, 1978], to be avoided by the environmental fibres of the Schaffer/commissural pathway. This suggests that some of the factors that promote the growth of these fibres are absent in heterotopic masses. Two recent in vitro studies have provided relevant insights on the molecular cues involved in this process. Chédotal et al., [1998] have demonstrated that hippocampal axons were repelled by neocortical explants that lacked the semaphorins necessary for their proper growth. Mann et al. [1998] have shown that the growth and branching properties of limbic axons were slowed down in tissue lacking the limbic associated membrane protein, a limbic system marker that we showed to be absent in CA1 heterotopias [Chevassus-au-Louis et al., 1998c]. These data suggest that some membrane- or substrate-bound factors that differ between heterotopic tissue and its environment may contribute to the partial deafferentation in the heterotopic neocortex.

On the other hand, the description of some afferent fibres in heterotopic regions [Colacitti et al., 1998; Jones et al., 1982; present report], although in lower density than in normotopic areas, suggests that some diffusible factors released by heterotopias are able to attract growing afferent fibres up to heterotopia. Similar arguments were advanced in two other works. O'Leary et al. [1990] observed that heterotopic basilar pons neurons induced by prenatal irradiation were normally contacted by reoriented axons from deep cortical layers. Frappé et al. [1999] have recently shown that frontal cortex grafted in the occipital cortex during the neonatal period in rats is contacted by the same afferent thalamic fibres than frontal normotopic cortex. The molecular characteristics of such diffusible guidance factors remain unknown.

From a physiological point of view, it is tempting to speculate that this partial deafferentation may be compensated by a development of local connections, in a manner similar to what has been described in the experimentally deafferentated adult neocortex [Salin et al., 1995]. In agreement with this view, exuberance of local axons in pyramidal neurons of subcortical heterotopic nodules has been described [Colacitti et al., 1999]. Excessive development of the excitatory local network would provide a potent system of synchronisation of firing among the population of heterotopic neurons and therefore could contribute to the formation of true epileptogenic foci in heterotopias.

Double Innervation of CA3 Pyramidal Cells by Mossy Fibres

We have observed an aberrant band of mossy fibres in the stratum oriens of CA3. There are two possible non-exclusive hypotheses to account for the formation of this projection. On the one hand, this may be a consequence of a modification of the ratio between the number of granule cells and the number of CA3 pyramidal cells. In this interpretation, the antimitotic effect of MAM would have decreased the number of CA3 pyramidal neurons that are generated in a time window (roughly E16–E18) affected by the antimitotic effect of MAM without affecting the number of granule cells that are generated later in development [Altman and Bayer, 1990; Bayer, 1980]. This

would cause a constant number of granule cell axons to innervate a smaller territory and thus the colonisation of normally non-innervated territories such as the stratum oriens. On the other hand, recent in vitro (Ikegaya, 1999] and in vivo [Holmes et al., 1999] experiments have shown that epileptiform activity during the period of mossy fibre growth may cause an aberrant pattern of Timm staining very similar to what we observed in MAM-treated rats. Since it has been shown that some hippocampal neurons have abnormal excessive firing properties [Baraban and Schwartzkroin, 1995], it is conceivable that a latent hyperexcitability of the developing hippocampal network in MAM rats may contribute to the formation of an abnormal pattern of mossy fibres.

We have shown that Timm staining in the CA3 stratum oriens is associated with the formation of ectopic mossy boutons on the basal dendrites of CA3. This is important in the light of the provocative demonstration that interneurons are more frequent postsynaptic partners of mossy fibres than CA3 pyramidal cells [Acsady et al., 1998]. Our Golgi study allowed us to conclude that the abnormal pattern of mossy fibres clearly causes at least some CA3 pyramidal neurons to receive a double excitatory drive on both their apical and basal dendrites. This may contribute to trigger an abnormal firing pattern of CA3 neurons.

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