The reeler gene: a clue to brain development and evolution

ANDRÉ M. GOFFINET*

Department of Physiology, FUNDP Medical School, Namur, Belgium

ABSTRACT Reeler mutant mice are characterized by profuse anomalies of cell positioning in the telencephalic and cerebellar cortices as well as by distinct malformations in non-cortical structures such as the inferior olive, the facial nerve nucleus and other brainstem nuclei. Studies of the embryonic development of these structures reveal that the early cell patterns formed by reeler neurons is consistently affected, so that the reeler gene plays an important role in the development of nerve cell patterns. Comparative studies of cortical development in reptiles suggest further that the mammalian type of cortical architectonics has been acquired progressively during brain evolution, and reveal some similarities in early cortical organization between reeler and reptilian, particularly chelonian, embryos, most notably the presence of an inverted gradient of cortical histogenesis. These observations point to a possible role of the reeler gene in cortical evolution. Although the factors responsible for the formation of neural cell patterns are largely unknown, most data point to the importance of cell-cell interactions. Cell-interaction molecules have probably been acquired during brain evolution and the reeler gene could act by perturbing, directly or indirectly, such cell interactions. The characterization and thus the cloning of the reeler gene is therefore important for our understanding of brain development. Recent data on the fine chromosomal mapping of the mutation prior to its positional cloning are reported.

KEY WORDS: *reeler, brain development, evolution, gene mapping*

Introduction

The study of vertebrate brain development is challenging, especially due to the complexity and importance of the brain. So far most of the mechanisms which govern brain development remain mysterious. Yet, it is quite obvious that a better understanding of the basic principles involved in brain development is requisite to an effective management of medical problems such as brain malformations and mental retardation.

The purpose of this paper is to show that the reeler mutation in mice defines a key step in the genetic program of brain development and evolution, thus justifying attempts to characterize the reeler gene. The paper will be divided into three parts. First, the reeler mutation will be presented by focusing mostly on studies of cerebellar systems. Second, comparative embryological data on cortical development in reptiles and mice will serve to introduce the reeler gene in an evolutionary perspective. Finally, recent progress in mapping the reeler locus will be reported.

The reeler mouse: a model for developmental studies

The reeler mutation is an autosomal recessive trait, first isolated by Falconer (Falconer, 1951) and mapped to the fifth chromosome. A second allele of reeler appeared in 1969 in Orléans (Guenet, personal communication). The genetic symbols are rl and rl-Orl, for the classical and the Orléans alleles, respectively. Reeler mutants have various malformations of the central nervous system (Caviness and Rakic, 1978; Goffinet, 1984b; Rakic, 1985). We carried out studies on the embryonic development of the reeler nervous system, with the hope that the pathology during embryogenesis would reflect more closely the primary effect of the mutation and that comparisons of the brain malformation at various levels may help define a «common denominator» of the pleiotropic effects of the mutation. In this text, examples from studies of cerebellar systems are selected.

Cerebellar development in reeler mutant mice

The development of normal cerebellar systems is relatively well-understood. The cerebellum is thus a privileged locus for defining a possible mechanism of action of the mutation, the more so since several studies have focused on the analysis of the adult reeler cerebellum (Mariani *et al.*, 1977; Mikoshiba *et al.*, 1980; Mariani, 1982; Terashima *et al.*, 1986). The mouse cerebellar anlage appears at E12 as a thickening along the rostral lip of the fourth ventricle. Before E14, the cerebellum is poorly differentiated and appears similar in normal and reeler littermates. At early stages,

Abbreviations used in this paper: Mdr, multiple drug resistance; Sor, sorcin; En-2, engrailed-2; Pgy-1, P glycoprotein-1.

***Address for reprints**: Department of Physiology, FUNDP Medical School, B-5000 Namur, Belgium. FAX: 32-81-23.03.91

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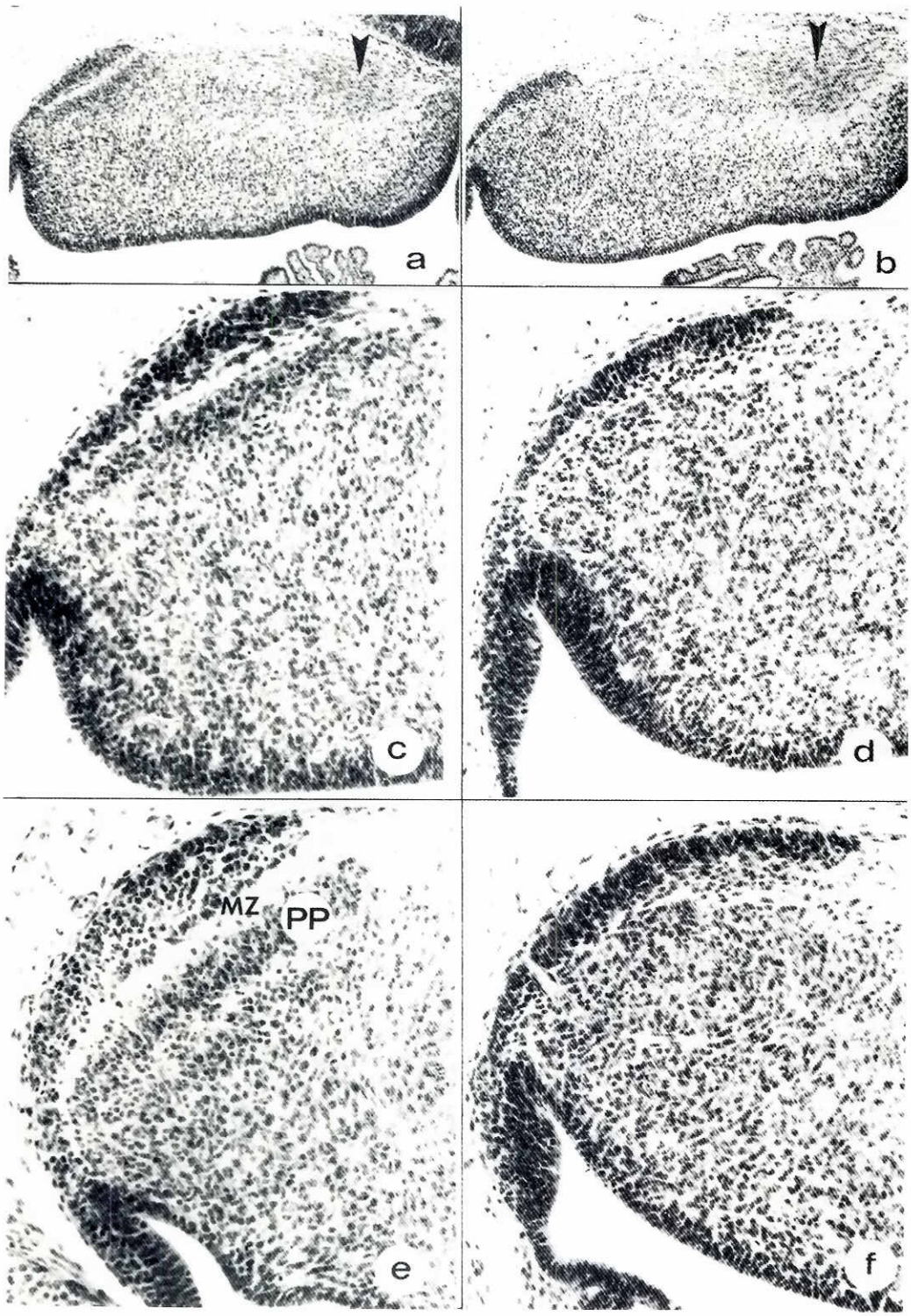

Fig. 1. Cerebellar development in normal and reeler mice. *Frontal sections in the embryonic cerebellum of normal* **(a, c, e)** *and reeler mutant* **(b, d, f)** *mice at E14* **(a-d)** *and E15* **(e,f).** *In the normal embryo, the Purkinje cell plate (PP) and incipient molecular layer (MZ) are evident at both stages, whereas Purkinje cells fail to organize into a plate and the MZ is poorly defined in reeler embryos. The arrowhead in a,b points to the neurons destined to the central cerebellar nuclei.*

E14 and E15 (Fig. 1), the cerebellar cortex is immature and the cerebellar primordium appears nearly similar in both genotypes. An intermediate zone of radially migrating cells is found external to the ventricular zone. The contingent of neurons destined to the future central cerebellar nuclei is found at the rostromedial level of the cerebellum (Korneliussen 1968; Altman and Bayer, 1978; Goffinet, 1983a, 1984b). The cerebellar cortex spreads around this central mass and is composed of four concentric zones. Externally, the layer of external granule cells extends from the rhombic lip to the rostral, well-defined border of the cortex. Beneath the external

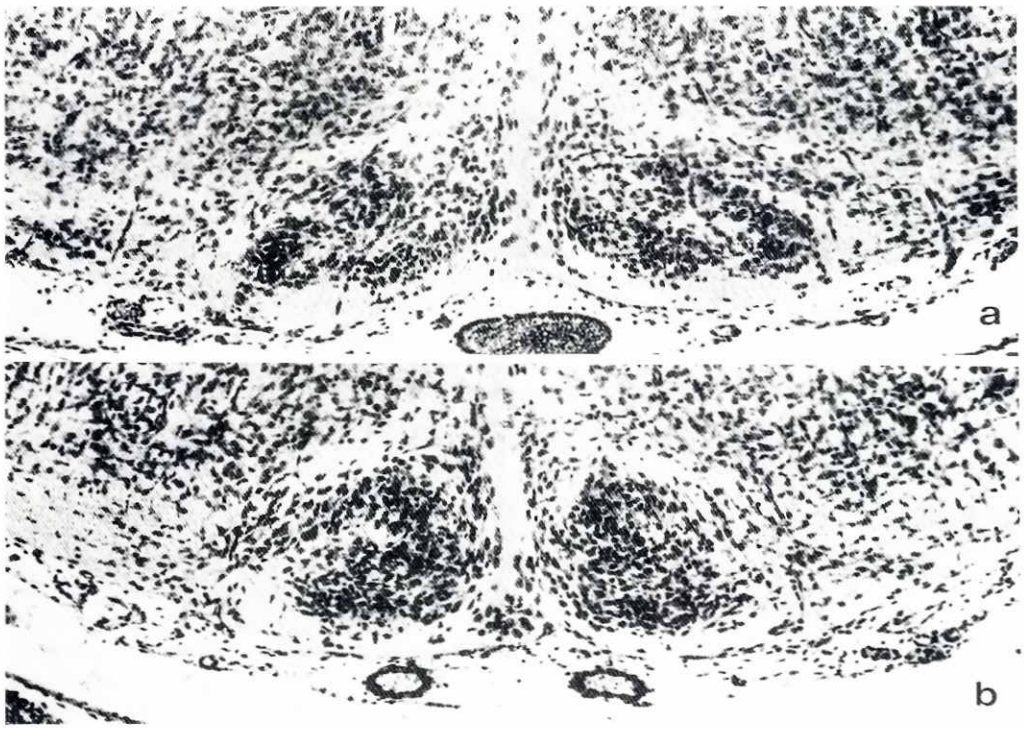

Fig. 2. Architectonics of the inferior olive in normal and reeler mice. *Photomicrographs of the normal* **(a)** *and reeler* **(b)** *inferior olivary complex at a late embryonic stage (E17). Despite its abnormal foliation, the reeler olive contains a normal contingent of apparently normal cells and the relative topography of its subcomponents is preserved.*

granular layer lies a cell-poor marginal zone covering the Purkinje cell layer. Purkinje cells are characteristically arranged into a multicellular array which we have named the «Purkinje cell plate» by analogy with the telencephalic cortical plate (Goffinet, 1983a). The majority of Purkinje cells are generated at E12, slightly later than nuclear neurons. The morphology of embryonic Purkinje cells in Golgi and EM preparations is typical of immature bipolar neurons with a dendritic bouquet and an immature axon. In reeler embryos, Purkinje cells are not arranged into a well-defined «Purkinje cell plate» but instead are scattered at the periphery of the cortex. Despite this defective cell pattern, the various types of neurons and glia are generated at normal stages and normally differentiated (Goffinet, 1983a). At late embryonic stages, from E16-E17, the cerebellar cortex grows in the tangential plane and begins to overlay the mass of the central nuclei. In parallel, the central nuclei begin to separate into their three components. This separation proceeds normally in reeler, although the architectonic differentiation of the dentate nucleus is not complete. From E17, the anomaly of the reeler cerebellar cortex begins to amplify. While the first evidence of cerebellar foliation is found in the normal cerebellum at this stage, no such event is present in reeler . Instead, Purkinje cells fail to become arranged into a tangential layer. Several of them settle in ectopic positions and aggregate to form various masses. The defect is purely one of cell patterns, and cell differentiation, by contrast, proceeds remarkably well. The hodology of the reeler cerebellum, particularly of olivocerebellar connections, is remarkably preserved (Steindler 1977; Goffinet *et al.*, 1984; Blatt and Eisenman, 1985, 1988; Shojaeian *et al.*, 1985). The situation is thus analogous to that in the forebrain in that the effect of the reeler mutation can be described as purely architectonic.

Development of the inferior olivary complex in normal and reeler mice

The embryonic development of the mouse inferior olivary complex can be schematically divided into three stages: neuron migration, early olivary nucleus and olivary foliation (Goffinet, 1983c). Neurons destined for the olive are generated in ventricular zones located around the fourth ventricle, the so-called « rhombic lip». They complete their last division at E11 and migrate tangentially along the external limiting membrane. Some cells leave the migratory pathway to join the olivary anlage by its lateral face; they form the deep branch of the migratory contingent. Other cells follow the superficial pathway and enter the olive radially from its ventral face. In the early olivary nucleus, no obvious divisions are seen and the differentiation of olivary cells, studied by the Golgi method and electron microscopy, proceeds similarly in normal and reeler fetuses. However, a subtle defect in cellular arrangements is seen in the reeler olive at E14 and E15. From E16, when neuron migration is complete, a progressive architectonic modeling leads to the fragmentation of the olivary mass and to the elaboration of its typical foliated shape. This process of «olivary foliation» is markedly defective in reeler mutants (Fig. 2). In the normal mouse, the olivary complex is divided into a dorsal accessory, a principal and a medial accessory olives. Some further divisions will not be considered here. In reeler mice, the dorsal accessory olive is shorter and blunted. The medial accessory olive is displaced medially and ventrally. The principal nucleus is not foliated but appears as a «whorl» of neurons in which it is difficult to recognize any consistent pattern. Despite the profuse architectonic anomalies, the differentiation of individual cells, of glial cells, as well as synaptogenesis are normal in reeler, and the topography of olivocerebellar connec-

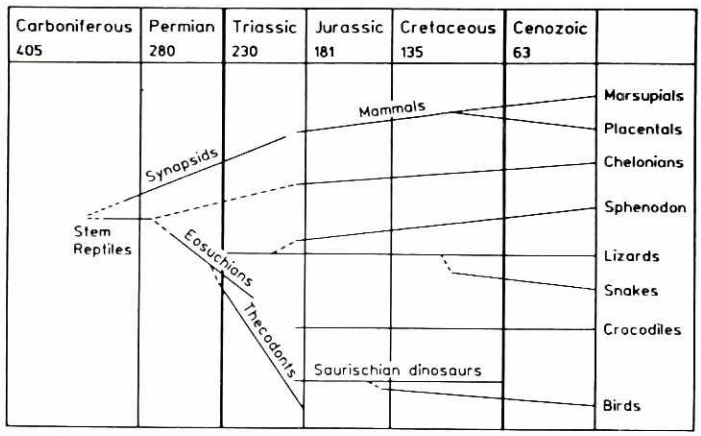

Fig. 3. Evolutionary filiations of upper vertebrates. *Time in Myrs since beginning of geological period. See text for discussion.*

tions is also grossly normal in the mutant. There are, however, subtle anomalies of olivocerebellar connectivity in reeler, such as an abnormal persistence of multiinnervation of Purkinje cells, a feature which is transient in normal animals but persists in the adult reeler mouse (Mariani, 1982; Shojaeian *et al.*, 1985; Blatt and Eisenman, 1988).

Action of the reeler gene: role of cell-cell interactions

In addition to the well-known defect in cortical lamination and anomalies of cerebellar systems, other targets of the reeler mutation include the cochlear nuclei (Martin, 1981), the lateral geniculate and tectum (Derer, Edwards, personal communications; Frost *et al.*, 1986), the lateral reticular nuclei, pontine nuclei, the habenula and the trigeminal complex (Goffinet, 1984a,b). It is thus reasonable to assume that the reeler mutation affects the whole CNS. All the effects of the reeler gene are consistent with the idea that the mutation acts at the end of migration by perturbing the early architectonic organization of postmigratory neurons.

In other words, we are led to postulate that a specific «event» is responsible for the early organization of nerve cell patterns in the embryonic brain of mammals, and that this «event» is dependent on the presence of a normal allele at the reeler locus. Several data suggest that early nerve cell patterns are not organized along morphogenetic gradients but result from an intrinsic, local property of the early neuroepithelium. Like others, we believe that the simplest hypothesis to consider is that early cell patterns result from local, intrinsic cell-cell interactions in embryonic brain tissue. The reeler gene would affect these cell-cell interactions, directly or indirectly.

At an early stage, brain tissue is mostly composed of two cell types, namely early neurons and radial neuroepithelial cells, and three types of interactions thus appear particularly worth considering: nèuronal-neuronal, neuronal-glial or both. Circumstantial evidence suggests that available observations are best explained by postulating homophylic interactions between immature neuronal cells at the end of migration (Goffinet, 1984b). This, however, remains to be demonstrated.

Comparative embryology of the cortical plate

Overview of the problem

The cerebral cortex is the product of a long evolutionary process, culminating in the development of the multilaminar mammalian pallium. In all mammals studied so far, the cerebral cortex develops according to a common sequence (reviewed by Caviness and Rakic, 1978; Goffinet, 1984b). As discussed above, an important event, somehow abnormal in reeler, must occur at the end of radial neuronal migration in order to «stabilize» early neural cell patterns. Although very little is known about this important morphogenetic step, the very fact that it is defective in reeler mutants suffices to show that it is submitted to a genetic control. As a corollary, the question of a possible phylogenetic control may be raised. In other terms: is the property of radial organization in the developing cortex present in every cortex or has it been acquired during cortical evolution, independently of the appearance of the cortex itself?

The most elegant way to examine this question would be to compare brain development in mammals and in their reptilian ancestors. However, since mammalian ancestors are extinct, the developmental question formulated above can only be approached indirectly by comparing cortical histogenesis in the main reptilian phyla. Therefore, the results of our studies need to be understood by reference to known filiations among living reptilian groups, of which only well-established features will be considered. As a basis for discussion, we shall use the «minimal consensus» cladogram shown in Fig. 3. A common reptilian ancestor (probably during the Pennsylvanian period) gave rise to several independent lines, four of which led to living reptiles, mammals and birds. A first branch, the synapsids, separated early and gave rise to mammals; a second branch led to chelonians, via a poorly understood lineage; a third branch led to Rhynchocephalia (of which *Sphenodon punctatus* is the only living remnant), as well as to lizards and their ophidian derivatives; the fourth branch gave rise to the crocodilians (via thecodonts) and to birds (via saurischian dinosaurs). The third and fourth branches form the diapsid lineage, the ancestors of which correspond to eosuchians. It is generally thought that chelonians (turtles) have evolved very little since the permian-triassic period and can be considered quite similar to stem reptiles, including mammalian ancestors. In order to cover the various branches of the evolutionary tree, cortical development has been examined in at least one species of turtles, squamates, crocodiles and in *Sphenodon*, and two representative species of the synapsid and squamate lineages have been analyzed in detail, using usual

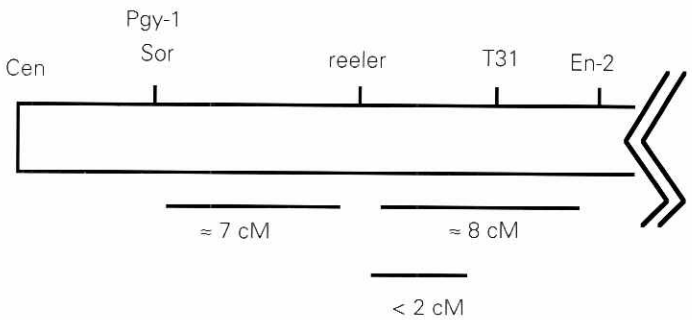

Fig. 4. Centromeric portion of mouse chromosome 5.

TABLE I

RECOMBINATION EVENTS OBSERVED BETWEEN LOCI IN THE BACKCROSS

Allelic combination	Locus						Number of informative meioses
	Pgy-1	*Sor*		*reeler*		*En-2*	
Parental	C	C		rl		C	53
	B6	B6		+		B6	55
Recombinant	C	C		rl	X	B6	6
	B6	B6		+	X	C	4
	C	C	X	+		B6	4
	B6	B6	X	rl		C	5

«C» and «B6» refer respectively to the *Pgy-1*, *Sor* and *En-2* alleles of the BALB/c and C57BL/6 strains, respectively. «X»: crossing-over.

histological techniques, the Golgi method, electron microscopy and tritiated thymidine to determine the timing of neuronal histogenesis (Goffinet, 1983b, 1984b; Goffinet *et al.*, 1986).

Cortical histogenesis in reptiles.

It is generally admitted that the cerebral cortex (or pallium) of reptiles in composed of four divisions: the dorsomedial cortex or hippocampus, the dorsal or general cortex, the lateral or pyriform cortex, and the dorsal ventricular ridge (DVR) which will not be considered here.

For reasons mentioned above, cortical development was studied comparatively in several reptilian species belonging to the various branches of the evolutionary tree. Morphological studies at critical stages of embryonic cortical development show that important species variations are present in the degree of radial organization of the cortical plate. The architectonics of the embryonic cortex is the most primitive in turtles. In these species, the radial cytological pattern is extremely poor, either at the level of neurons or of glial cells. By contrast, all lizards have a nicely ordered cortical plate at all developmental stages. This property is best appreciated in Golgi preparations which show the radial deployment of neuronal processes as well as of radial glial fibers. The level of cortical organization in other reptilian species can be described as intermediate. For example, snakes are in this respect quite similar to lizards, whereas the crocodilian cortex is characterized by a poor architectonic pattern. In *Sphenodon*, the medial cortex is extremely well developed and organized, while the dorsal cortex is rather reminiscent of the turtle, primitive, type.

The observations demonstrate that the radial organization of the cortical plate is not an «all or nothing» property. There are various degrees of organization in the different segments of the embryonic pallium. Among living species, turtles on the one hand, and lizards (and mammals) on the other, may be viewed as the extreme types of a spectrum of histological variations. According to the cladogram outlined above, radial cytoarchitectonics must then be regarded as a property which has been acquired gradually, independently and to variable extents after phyletic divergence. It was probably not present in the ancestral cortex of stem reptiles and has not been inherited by the different phyla from their common root. It is thus a case of homoplasy (as opposed to homology), due to evolutionary convergence. As in other cases of homoplasy, it reveals that «similar solutions to biological problems have occurred independently» (Northcutt, 1981) and presumably correspond to especially efficient solutions.

The embryological studies on the reptilian cortex have been complemented by autoradiographic analyses of neurogenesis in the cortex of a turtle *(Emys orbicularis)* and a lizard *(Lacerta trilineata)*, two species selected because of their widely different types of cortical organization. The data confirm the general pattern of neurogenesis suggested by embryological studies and allow interesting comparisons between neurogenesis in mammals and reptiles, particularly regarding histogenetic gradients. In embryos injected with thymidine before the stage of neuronal generation, all cortical neurons are labeled, indicating that only precursors are present in the ventricular zones at this time. Thymidine administration later than the stages of neurogenesis results only in the labeling of glial and mesodermal cells, so that ependymal, astrocytic and satellite cells differentiate or at least continue to proliferate after the majority of the neurons are formed. In the three cortical fields (medial, dorsal and lateral cortices), neurogenesis follows a lateral to medial as well as an anterior to posterior gradient, and thus correlates with the pattern of development seen in embryological preparations of reptiles and mammals. In addition to the tangential gradients, radial histogenetic gradients are also found. In the lateral and dorsal cortices, radial histogenesis follows an outside to inside gradient. That is to say, late-generated cells settle at deeper levels in the cortex than older cells. At the level of the medial cortex, however, a difference is apparent between the primitive cortical type (turtles) and the more elaborate pattern (lizards). In turtle cortex, an outside to inside gradient is evident. By contrast, it is impossible to define any gradient in the lacertilian medial cortex.

It is worth recalling that the mammalian cortex is formed by an «inverted» histogenetic gradient (see Caviness and Rakic, 1978 for discussion). That is, younger cells traverse layers of previously established neurons to settle at progressively more superficial levels. This important property of the mammalian cortex is thus absent in reptile cortex. If the phylogenetic assumptions outlined above are acceptable, it follows that the presence of an «inverted» histogenetic gradient must be considered an acquisition, a step in the evolution of the cerebral cortex from stem reptiles to mammals (synapsid radiation). Most interestingly, in the reeler mutant mouse, histogenesis no longer follows the normal inverted gradient, but proceeds from outside to inside as it does in the primitive reptilian cortex. The data thus shows that the evolutionary acquisition of an inside to outside pattern of cortical development is contingent upon the presence of a normal gene at the reeler locus (see Goffinet, 1984b for discussion).

Towards the characterization of the reeler gene

The studies summarized above point to the importance of the reeler locus during neurogenesis and brain evolution. The product of the normal allele of the *reeler* locus is unknown, so that cloning of the locus is a prerequisite to any understanding of the mode of action of the mutation. Although the location of *reeler* on the proximal part of chromosome 5 has been known for many years, the map of this chromosomal region remains quite imprecise. There-

TABLE II

RECOMBINATION BETWEEN REELER AND T31H IN N2 ANIMALS

brain phenotype	karyotype	
	normal	T31H/+
normal (rl/+), n= 24	0	24
reeler (rl/rl), n= 23	23	0

fore, as a first step towards establishing a genetic map of the centromeric end of chromosome 5, we assessed the localization of *reeler* relative to three markers of this region, namely the breakpoint of the translocation *T(5;12)31H*, the P-glycoprotein-1 locus *(Pgy-1)* – also called multiple drug resistance-1 *(Mdr-1)* – and the engrailed-2 gene *(En-2)*. The location of *reeler* relative to nearby markers was more precisely defined by measuring recombination frequencies between *reeler*, *Pgy-1*, the sorcin locus *Sor* and *En-2*, as well as between *reeler* and *T31H*. (Dernoncourt *et al.*, 1991; Goffinet and Dernoncourt, 1991).

Animal strains and crosses

The *Orléans* allele of the *reeler* mutation is on BALB/c (C) background and noted C-rl^{Orl}. The original allele of *reeler* (Falconer, 1951) on C57Bl/6 (B6) background was transferred to BALB/c background by more than 10 backcrosses and the resulting congenic strain is noted C.B6-*rl*. The *T(5;12)31H* translocation, is a reciprocal translocation between chromosomes 5 and 12, and results in the formation of balanced carrier animals with a very long 12^5 chromosome and a very short 5^{12} chromosome. Carrier males are sterile and the translocation is maintained by mating female heterozygous carriers with normal males (Beechey *et al.*, 1980). Carrier females also give birth to viable, sterile animals who are tertiary monosomic (≈4%) or tertiary trisomic (≈10%) for the small 5^{12} chromosome.

For studies of recombination between *reeler*, *Pgy-1*, *Sor* and *En-2*, backcross mice were generated as follows. Fertile homozygous rl^{Orl}/rl^{Orl} males were mated with normal C57Bl/6J females, yielding F1 $rl^{Orl}/+$ offspring. 127 N2 mice were obtained by backcrossing $rl^{Orl}/+$ F1 females with homozygous rl^{Orl}/rl^{Orl} males. Recombination between *reeler* and the *T(5;12)31H* breakpoint was studied in another set of backcross animals obtained as follows. rl^{Orl}/rl^{Orl} males were mated with females heterozygous for the *T31H* translocation. *T31H* heterozygous F1 females, selected by karyotype analysis, were backcrossed with rl^{Orl}/rl^{Orl} males to yield N2 animals, of which 31 were available for analysis. Backcross animals were typed for the *reeler* trait by their behavior and macroscopic examination of the cerebellum. The *En-2* locus was studied with the mp2 probe (Joyner and Martin 1987), the *Pgy-1* locus with probe pCHP-1 (Riordan *et al.*, 1985) and the sorcin *(Sor)* locus with probe pCP7 (Van der Bliek *et al.*, 1986).

Mapping results

The reeler *locus is proximal to* T31H

When crossed to mice with a normal karyotype, *T(5;12)31H/+* females produce about 4% of tertiary monosomic offspring with a chromosomal constitution of the type: $5 + 12 + 12^5$ (Beechey *et al.*, 1980). These animals have lost the 5^{12} chromosomal segment, composed of the centromeric part of chromosome 5 and the telomeric segment of chromosome 12 distal to the breakpoint. Since the *reeler* mutation is recessive, advantage was taken of this cytogenetic peculiarity to assess whether, when mated to a *T(5;12)31H/+* female, a *rl/rl* male could produce tertiary monosomic offspring with a reeler cerebellar phenotype (*rl/0* constitution). Two animals were found with the *reeler* cerebellar phenotype and were confirmed as being tertiary monosomic by karyotype of spleen cells.

Pgy-1 (Mdr-1) *and* En-2 *are respectively proximal and distal to the* T31H *breakpoint*

In order to estimate the respective location of the *Pgy-1* and *En-2* loci relative to *T31H*, an equal amount of DNA isolated from mice who were disomic, tertiary monosomic (one dose of chromosome 5^{12}) and tertiary trisomic (three doses of 5^{12}) was dot-hybridized to the pCHP-1 and mp2 probes. A ß-actin probe was used as a control for a gene with normal dosage in all three genotypes. ß-actin and mp2 did not show any evidence of gene dosage, whereas the pCHP-1 probe for the *Pgy-1* locus clearly gave different hybridization signals with a gene-dose effect related to the diploid, tertiary haploid or tertiary triploid status of the DNA. This demonstrated that the *Pgy-1* locus is proximal, whereas the *En-2* gene is distal relative to the *T31H* translocation breakpoint.

Backcross analyses and genetic distances

The results of the backcross analyses are shown in Tables I and II and summarized in Fig. 4. Of the 127 N2 backcross mice tested, a total of 9 recombination events were observed between reeler and *Pgy-1* and *Sor*, while 10 recombinations occurred between *reeler* and *En-2*. No recombination between *Pgy-1* and Sor was observed, and the recombination between *Pgy-1* and *Sor* on the one hand, and *En-2* on the other were 19, the sum of recombinants for each group separately. No double recombinant was found. These frequencies yield distance estimates of 7.9±2.4 cM between *En-2* and *reeler*, and of 7.1±2.3 cM between *Pgy-1* and *Sor*, and reeler. The distance separating *Pgy-1* and *Sor* from *En-2* is estimated to be 15.0±3.2 cM. Those recombination frequencies strongly show that *reeler* is located in between the *Pgy-1/Sor* and the *En-2* genes, separated from these two groups by a distance of 7 to 8 cM. Inasmuch as *reeler* and *Pgy-1* are known to map centromeric to *En-2* (Lyon and Kirby 1991), our results indicate the following order: centromere, *Pgy-1/Sor - reeler - En-2*. The strong linkage between *Pgy-1* and *Sor* indicates that the sorcin gene lies in the vicinity of the P-glycoprotein gene in mice as well as in hamster; this suggests that the several genes of the hamster Multiple Drug Resistance *(Mdr)* amplicon may be similarly linked in the Mdr gene family of the mouse (Raymond *et al.*, 1990). The distance between *reeler* and *En-2* was previously estimated indirectly to be 6 to 9 centimorgans (Martin *et al.*, 1990; Hillyard *et al.*, 1991; Lyon and Kirby 1991), in close agreement with the present, direct estimate of ≈8 cM. On the other hand, the present measurement of a distance of ≈7 cM between *reeler* and the *Mdr* complex suggests that the reeler locus is farther from the centromere than the current estimate of 4 cM.

As shown in Table II, no recombination was found in the 31 N2 backcross animals of the *T31H-reeler* panel. Due to difficulties in breeding mice carrying both *reeler* and the *T(5;12)H* breakpoint, the number of backcross animals is low. It is sufficient, however, to locate the *T31H* breakpoint within 3±3 cM from *reeler*, thus confirming that the *T31H* breakpoint maps between *reeler* and *En-2* and showing that it is the closest marker of *reeler* to date. So far as we

know, however, there is no straightforward technique to clone sequences corresponding to a translocation breakpoint so that this marker cannot be used to approach the *reeler* locus further.

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