Choreography of Early Thalamocortical Development

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Thalamic axons, which carry most of the information from the sensory environment, are amongst the first projections to reach the cerebral cortex during embryonic development. It has been proposed that the scaffold of early generated cells in the ventral thalamus, internal capsule and preplate play a pivotal role in their deployment through sharp gene expression boundaries. These ideas were recently evaluated in various strains of mutant mice. In *Tbr1*, *Gbx2*, *Pax6* KO both thalamic and corticofugal projections fail to traverse the striatocortical junction. In both *Emx2* and *Pax6* KO brains, the misrouted thalamic afferents are accompanied by displacements of the pioneering projections from the internal capsule. Regardless of their altered route, thalamic afferents in the *reeler* and *L1* KO mice seem to be able to redistribute themselves on the cortical sheet and establish normal periphery-related representation in the somatosensory cortex. Early neural activity delivered through the thalamic projections is thought to be involved in the realignment process of thalamic axons at the time of their accumulation in the subplate layer. However, axonal growth and the early topographic arrangement of thalamocortical fiber pathways appear normal in the *Snap25* KO, where action potential mediated synaptic vesicle release is disrupted. We therefore suggest that intercellular communication mediated by constitutive secretion of transmitters or growth factors might play a dominant role during early thalamocortical development.

Introduction

Cerebral cortex receives most of its input from the environment through thalamic connectivity (Jones, 1985). Although all cortical regions receive some form of thalamic input, there are marked differences in the orientation, density and modality of the thalamic innervation of different cortical areas (Caviness and Frost, 1980). The early steps of thalamocortical fiber deployment depend on various factors. Selective fasciculation and contact guidance, the release of positive and negative neurotropic factors, gradients of cortical gene expression and early neuronal activity are all believed to play important roles (DeCarlos and O'Leary, 1992; Bolz *et al*., 1993; Allendoerfer and Shatz, 1994; Métin *et al*., 1997; Catalano and Shatz, 1998; Braisted *et al*., 2000; Vanderhaeghen *et al*., 2000; Fukuchi-Shimogori and Grove, 2001). Tracing experiments revealed that the thalamic projections from different thalamic nuclei traverse the subdivisions of the forebrain in an organized fashion (Molnár and Blakemore, 1995). In the internal capsule their relative position is maintained although they undergo some transformation, including an ~90° anticlockwise rotation. Early tracing experiments revealed that even at this immature stage, before the formation and differentiation of most thalamic nuclei, predictions can be made from the particular position of a cell group in the dorsal thalamus as to the target site in the cortex (Blakemore and Molnár, 1990; Catalano *et al*., 1996). Thalamic projections are established in this basic topography [Embryonic day (E)15/16 in mouse] at a time when thalamic nuclei and the different forebrain compartments have not even fully differentiated.

How Do Thalamic Projections Cross Subdivisions Marked by Gene Expression Boundaries to Reach the Cortex?

In rodent, during the second and third week of gestation the forebrain is undergoing spectacular changes. During this period the embryonic forebrain will differentiate into distinct domains, termed prosomeres, each with specific morphological features and gene expression patterns (Puelles and Rubenstein, 1993). This is the period, in mice E13–16, when thalamocortical and corticofugal axons have to travel through numerous rapidly forming subdivisions and boundaries of the embryonic brain. These critical boundaries outlined by distinct molecular properties (Puelles *et al*., 2000), include the diencephalic–telencephalic and the pallial–subpallial (PSPB) or striatocortical boundaries.

Both thalamocortical and corticofugal projections show puzzling behavior at these boundaries during their growth. The developing thalamocortical axons first proceed ventrally from the dorsal thalamus and then turn dorsolaterally at the diencephalic–telencephalic junction, where they enter the internal capsule. They rapidly advance amongst a largely transient population of cells in this region (Métin and Godement, 1996), but then pause before traversing the corticostriatal junction. The earliest corticofugal projections, most of which originate from preplate neurons also pause at this boundary around E13/14 in mouse. Although projections from different cortical regions arrive at this zone at slightly different times, the front of the early corticofugal projection lines up along the striatocortical junction. Subsequent to their interaction with the striatocortical junction, the thalamocortical and corticothalamic fibers resume their advance, intimately associated with each other, and proceed towards their targets (Fig. 1*A*).

Numerous transcription factors are expressed (*Pax6*, *Tbr1*, *Ngn2*, *Emx2*, *Otx2*) or absent (*Emx1*) in the region of the ventral pallium (Puelles *et al*., 2000), thus having a modulatory potential for molecular patterning at the PSPB and axonal pathfinding. While the PSPB represents a perpendicular barrier zone that thalamic afferents and corticofugal projections have to cross, it also guides the laterally migrating neurons towards various structures in the lateral pallial region (Bayer and Altman, 1991).

Early-generated, largely transient neuronal populations are known to extend pioneering axonal projections through these critical boundaries. It was proposed that these cells provide scaffolds or temporary targets, 'guide-post cells', for the developing axonal projections (McConnell *et al*., 1989; Mitrofanis and Guillery, 1993). It has been suggested that the early outgrowth of thalamocortical axons from the diencephalon might be governed by pioneering projections from internal capsule cells (Métin and

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Godement, 1996), while the crossing of the PSPB might be dependent on selective fasciculation with corticofugal projections (Molnár and Blakemore, 1995). It has been postulated in the 'handshake hypothesis' that axons from the thalamus and from the early-born cortical preplate cells meet and intermingle in the basal telencephalon, so that thalamic axons grow over the scaffold of preplate axons and become 'captured' for the waiting period in the subplate (Blakemore and Molnár, 1990). However, it still has not been unequivocally demonstrated whether selective fasciculation of the two fiber sets is essential for their crossing of this boundary. It is even less clear whether the order in which the thalamocortical and corticofugal projections interact holds any importance. We believe that the detailed examination of thalamocortical development and these critical forebrain regions in normal and various mutant mice shall help to resolve these questions.

Possible Role of Early Functional Interactions in Thalamocortical Self-organization

The first post-mitotic neurons of the cortex form the preplate. The preplate is subsequently split into a superficial marginal zone and a deep layer called subplate. The cortical plate then forms between these two layers, as neurons migrate from the ventricular and subventricular zones, passing through the intermediate zone and the subplate *en route*. Migration into the cortical plate occurs in an 'inside-out' order (Rakic, 1977). The thalamic projections arrive to the cortex through the intermediate zone, which lies between the first-generated cells of the subplate layer and the subventricular zone. Upon thalamic fiber arrival, only an immature cortical plate, non-permissive for thalamic ingrowth is present, and therefore the thalamic fibers accumulate in the subplate before the cortex gradually becomes mature enough (Lund and Mustari, 1977; Rakic, 1977; Shatz and Luskin, 1986).

It has been proposed that while the thalamic axons accumulate in the subplate they engage in activity-dependent interaction with these cells and this might lead to their realignment before they enter the cortex (Catalano and Shatz, 1998; Krug *et al.*, 1998). Thalamic axons are known to develop numerous transient side branches (Naegele *et al.*, 1988; Ghosh and Shatz, 1992; Catalano *et al.*, 1996) as they accumulate in the subplate. The side branch formation might be regulated by electric fields of the activity patterns along the axons. Catalano and Shatz (Catalano and Shatz, 1998) delivered TTX (sodium channel blocker) into the putative visual cortex of cat fetuses, at the time thalamic projections were arriving to the subplate. Lateral Geniculate Nucleus developed projections to cortical areas that they normally bypass and failed to enter the visual cortex. The few fibers that did enter had an aberrant topography within the cortical plate. Although this experiment suggests that even the initial phases of thalamocortical targeting might depend on early activation patterns, the exact nature of the required neural activity is not known. We set out to investigate the pattern of cortical activation after direct thalamic stimulation during the period of thalamic fiber accumulation using an optical recording technique. There are various forms of neurotransmitter release, which could activate recipient cells (Rizo and Südhof, 2002). The *Snap25* KO mouse (Washbourne *et al.*, 2002), where the action potential-regulated synaptic vesicle release is disrupted, provides an excellent model system to evaluate the contribution of regulated and spontaneous neurotransmitter release in embryonic cortical development.

Overview of Methods

We used carbocyanine dye tracing to visualize thalamocortical and corticofugal projections as well as immunohistochemistry for L1 and TAG-1, respective markers of the two axonal systems, in wild-type, heterozygote and null mutants for *Emx2*, *Pax6*, *Snap25*, *L1* and *reeler* mouse at ages ranging from E13.5 to birth (E19.5–20). Detailed description of the methods can be found in previous works (Molnár *et al.*, 1998a,b; López-Bendito *et al.*, 2002; Jones *et al.*, 2002). In those mutants which survive into the postnatal periods (*L1* KO and *reeler*) we also examined the cytoarchitecture of the barrel field with Nissl staining and the periphery-related thalamocortical fiber patterning with cytochrome oxidase (CO) (Molnár and Hannan, 2000; Erzurumlu and Kind, 2001). In *L1* KO and *reeler* mice, we used [^{14}C]deoxyglucose (DG) mapping in combination with Nissl and CO staining to examine whether or not a functional cortical representation of the mystacial whiskers can be related to a cytoarchitectonic organization in the mutant. For the DG experiments, all whiskers were clipped except the three caudal-most whiskers of rows B and D, with which mice actively explored a stimulus-rich cage. We performed DG autoradiography on the coronal plane to reveal a columnar activation pattern and on tangential plane, to investigate the cortical whisker representation (Bronchti *et al.*, 1999a).

For the optical recording, we used 400 μm thick cut slices from embryonic (E17–E21) and postnatal (P0–P10) rat brains (Agmon *et al.*, 1993). These were stained with a voltage-sensitive dye (RH482) and images were captured in a Fuji Deltaron 1700 differential image acquisition system after selective thalamic stimulation (Higashi *et al.*, 2002).

Results

We will discuss our recent results in four groups. (i) First, we shall analyze mutants where the thalamic projections have altered or defected growth through the forebrain subdivisions (including *Emx2*, *Pax6/LacZ*, Table 1). Ideas of early axon guidance mechanisms will be tested, with special attention to the role of pioneer projections. (ii) We shall then examine the initial thalamocortical topography in mutants (including *reeler*, *L1*) where thalamic axons eventually arrive to the cortex via an altered route. *Emx2* shall also be mentioned in this group. The question here is to examine whether thalamic afferents, capable of correcting the defects in their trajectory closer to their targets, are able to form normal cortical representations. (iii) Since one of the candidates for the realignment of these projections is the neural activity transmitted via thalamic projections to the immature cortex, we shall review evidence for functional interactions between thalamic fibers and cortex at the time of their arrival. (iv) We shall finally examine the development of thalamocortical outgrowth and entry to the cortex in the *Snap25* KO mice (Table 1). In this mutant, all neurons, including thalamic cells, fail to release neurotransmitters in a manner regulated by action potentials.

Altered Growth of Thalamic Axons Through the Forebrain Subdivisions: Lessons from Emx2 KO, Pax6/LacZ, L1 KO

Emx2 KO

Emx2 is a member of the empty spiracles family of genes, and its expression in the anterior CNS of the developing mouse embryo follows a rostro-caudal gradient (Simeone *et al.*, 1992; Gulisano *et al.*, 1996; Bishop *et al.*, 2000; Mallamaci *et al.*, 2000) (Fig. 1*A*). *Emx2* homozygous mutant mice die perinatally. Previous studies have shown that the lack of *Emx2* results in some forebrain structural abnormalities. The most striking abnormalities are the severe reduction of cortical hemisphere size and the disruption in cortical lamination (Yoshida *et al.*, 1997; Mallamaci *et al.*, 2000). Lamination defects have been reported to occur in the

neocortex of *Emx2* KO mice, but the diencephalon develops relatively normally (Suda *et al*., 2001). A disproportional, but orderly, arealization of the *Emx2* mutant neocortex reflected by an expansion of rostral areas and a contraction of caudal areas has been described (Bishop *et al*., 2000; Mallamaci *et al*., 2000). This shift in areal identity in the cerebral cortex of *Emx2* KO is matched by the altered distribution of thalamocortical projections. In order to understand how the altered thalamocortical axons occur in the *Emx2* KO mice, we specifically tested the idea of whether thalamocortical axons become misrouted close to the start of their path towards the cortex, where cues other than the ones intrinsic to the cortex could influence their behavior. The tracing studies demonstrated that in *Emx2* KO mice a large proportion of early thalamocortical projections were misrouted at the ventral border between the diencephalon and telencephalon. This abnormality occurred in conjunction with displaced connectivity of the internal capsule cells at the diencephalic–telencephalic junction. Interestingly, most of the aberrant thalamic projections compensated for the ventral entry to the telencephalon and still ascended to the cortex, but their arrival was slightly delayed (López-Bendito *et al*., 2002) (Fig. 1*B*).

Pax6/LacZ KO

Pax6 has been found to have an essential role in brain morphogenesis (Walter and Gruss, 1991; Caric *et al*., 1997; Stoykova *et al*., 1997; Warren *et al*., 1999), and has been implicated in early axonal pathfinding (Mastick *et al*., 1997; Kawano *et al*., 1999; Pratt *et al*., 2000). A *Pax6* positive group of cells was observed to extend along the PSPB, from the ventricular/subventricular zones towards the mantle of the basal telencephalon (Stoykova *et al*., 1997; Smith-Fernandez *et al*., 1998; Puelles *et al*., 2000) (Fig. 1*A*). Loss of *Pax6* function in the $Pax6^{Sey/Sey}$ mutant brains, results in a prominent ventralization of the molecular characteristics of the region of the ventral pallium that leads to severe morphological malformation in the basolateral cortex (Stoykova *et al*., 2000; Toresson *et al*., 2000; Yun *et al*., 2001) (Fig. 1*C*). We used a *Pax6/LacZ* KO mouse, in which the endogenous *Pax6* expression is reflected by β-galactosidase activity (St Onge *et al*., 1997), to study the consequences of the loss of *Pax6* function on thalamocortical and corticofugal axon pathfinding. In *Pax6* heterozygote brains, we correlated the *Pax6* expression pattern to the different steps of thalamocortical development during the period E14.5–18.5. Carbocyanine dye tracing in *Pax6* HT and *Pax6* wild-type brains revealed that, corticofugal and thalamocortical axons temporarily arrest their growth at E14.5 at the border of the β-galactosidase-positive region at the PSPB before they continue towards their targets. However, in *Pax6* KO embryos, corticofugal and thalamocortical were unable to encounter each other at the PSPB and reach their final targets. Instead of crossing this boundary, they tended to descend into the ventral pallium in large aberrant fascicles (Fig. 1*C*). In addition, cells normally situated in the ventral thalamus and internal capsule, were displaced into the hypothalamus and ventral pallium. These pathfinding defects were confirmed by immunohistochemistry for L1 and TAG-1, markers of the early axonal connections. The aberrant development of axonal connections in absence of *Pax6* function appear to be related with ultrastructural defects of cells along the PSPB, as well as to a failure of axonal guidance molecule expression, including Sema3C, Sema5A and possibly Netrin-1 (Jones *et al*., 2002).

Initial Thalamocortical Topography in Mutants (Including Reeler, L1 and Emx2) where Thalamic Axons Reach the Cortex Via an Altered Route

Reeler

The selective fasciculation of thalamic and preplate projections was proposed as a mechanism for thalamic afferents to traverse the internal capsule and advance to the cortex (Molnár and Blakemore, 1995). The unique pattern of thalamic fiber ordering in *reeler* mice supports this notion. In this mutant, the cortical plate develops below the early-generated preplate cells and therefore separates the incoming thalamic fibers from their target cells now located in a 'superplate' (Caviness and Rakic, 1978). The embryonic cortical plate is thought to be a non-permissive environment for thalamic fiber ingrowth (Götz *et al*., 1992). The existence of privileged pathways for axon growth could explain how thalamic axons in *reeler* are able to penetrate the cortical plate and steer up to reach the equivalent cells in the superplate, whilst ignoring the hostile territory of cortical plate cells around them (Molnár *et al*., 1998b). Thalamic projections follow the same pattern of development observed in wild-type rodents, but in relation to the displaced superplate cells. Thalamic projections loop up to the superplate before they descend and branch and arborize in the cortical plate. We investigated how this altered thalamic input established periphery-related pattern and cytoarchitecture in the primary somatosensory cortex (S1).

Nissl sections of the mutant mice did not show clearly defined barrel boundaries, but CO staining revealed normal periphery-related pattern in a region corresponding to S1 (Polleux *et al*., 1998; Bronchti *et al*., 1999b). This suggests that in *reeler* the majority of the thalamic fibers assume normal periphery-related pattern in the barrel cortex, but the cell patterning in the barrel field might be impaired. We examined the DG uptake after clipping all the mystacial whiskers with the exception of the three caudal-most of rows B and D. DG uptake examined on the coronal plane, revealed a columnar activation pattern with a highest DG uptake in the intermediate layers. This however was not confined to the upper part of the column, rather, showing a more diffuse activation pattern. In the tangential plane, DG uptake showed that the cortical activation pattern, and thus the areal distribution of whisker representation in *reeler*, is organized in an identical manner to that in normal mice (Bronchti *et al*., 1999b). The abnormal trajectory to the cortex in the *reeler* does not seem to alter the ordered functional whisker representation.

L1 KO

The molecular mechanisms of contact guidance between thalamocortical and corticothalamic projections throughout the internal capsule are not known. Subplate neurons express immunoreactivity to the surface molecules L1 and TAG1 (Godfraind *et al*., 1988; Denaxa *et al*., 2001) and fibronectin (Stewart and Pearlman, 1987), which could provide highly attractive substrates for the growth of thalamic axons in an otherwise relatively non-permissive environment. A mouse deficient for L1 was produced (Cohen *et al*., 1997), hence we were interested in examining the thalamocortical pathfinding and topography in this mutant. Our carbocyanine dye tracing experiments in embryonic and early postnatal L1 KO mouse brains revealed that fasciculation problems occur at the PSPB (Molnár *et al*., 1999). Thalamic fibers gather in larger fiber bundles in the striatum and their path is different from the ones observed in normal mice. In spite of this abnormality thalamic

fibers reach the cortex, and DiI crystal placement in embryonic and early postnatal animals reveals backlabeled thalamic cells and thalamic fibers with normal gross topography. Nissl sections of the mutant mice show clearly defined barrel boundaries and CO staining reveals normal periphery-related pattern in a region corresponding to SI primary somatosensory cortex. This suggests that the majority of the thalamic fibers branch and arborize normally and assume periphery-related pattern in the barrel cortex (Molnár *et al*., 1999). We used DG mapping in combination with Nissl and CO staining to examine whether or not a functional cortical representation of the mystacial whiskers can be related to a cytoarchitectonic organization in the mutant.

Table 1
Abnormalities in thalamocortical development in mutant mice

Gene mutated	Type of mutation	Phenotype	Mechanism	Reference
Altered early thalamocortical development through forebrain subdivisions in various mutant mice				
Mash1	Targeted KO	No thalamic fiber outgrowth beyond internal capsule	Not known: internal capsule cells with thalamic projections are missing.	Tuttle *et al*., 1999
Tbr1	Targeted KO	Thalamocortical and corticothalamic axon elongation defect	Not known	Hevner *et al*., 2001
Pax6$^{Sey/Sey}$	Spontaneous mutation	Thalamocortical and corticothalamic axon elongation disrupted at the internal capsule	Not known	Kawano *et al*., 1999; Hevner *et al*., 2002
Pax6/LacZ	Targeted KO	Disturbed thalamocortical and corticothalamic axon elongation at the CS	Altered projections from internal capsule. Compact cell mass at the PSPB. Altered expression of cell adhesion molecules.	St Onge *et al*., 1997; Jones *et al*., 2002
Gbx2	Targeted KO	Thalamocortical and corticothalamic axon elongation disrupted at the internal capsule	Not known	Miyashita-Lin *et al*., 1999; Hevner *et al*., 2002
Cortical topography of thalamic projections after altered thalamocortical deployment				
Reeler	Spontaneous mutation	Preplate does not split and has altered position (superplate). Cortical plate develops inverted.	Thalamic axons cross cortical plate to reach superplate	Caviness and Rakic, 1978; Yuasa *et al*., 1994; Molnár *et al*., 1998b
L1	Targeted KO	Thalamocortical axon fasciculation defects in internal capsule and at the SC junction	Selective fasciculation defect mediated by L1	Cohen *et al*., 1997; Molnár *et al*., 1999
Sema6A	Targeted KO	Disturbed thalamocortical development at diencephalic–telencephalic border	Altered Sema6A expression	Leighton *et al*., 2001
Mutants with altered neurotransmitter release				
Snap25	Targeted KO	Normal forebrain development in the absence of regulated synaptic vesicle release	Normal prenatal thalamocortical development	Washbourne *et al*., 2002; Molnár *et al*., 2002b
Munc13	Targeted KO	Normal forebrain development in the absence of regulated synaptic vesicle release	Thalamocortical development not yet examined	Varoqueaux *et al*., 2002
Munc18	Targeted KO	Normal forebrain development in the absence of regulated synaptic vesicle release	Thalamocortical development not yet examined	Verhage *et al*., 2000

Figure 1. Schematic diagram representing sections through the forebrain of a wild type, *Emx2* KO and *Pax6* KO brain. The diagrams illustrate the relationship between the developing reciprocal thalamocortical (green) and corticofugal (blue) projections and the early subdivisions of the forebrain around embryonic day 18.5. *Pax6* expression is marked with shaded blue, *Emx2* expression is labeled with brown in the wild-type (WT). Pax6 promoter activity is marked by light blue as was revealed with β-galactosidase staining in the *Pax6/LacZ* null mutant. Both *Pax6* and *Emx2* are expressed outside the dorsal cortex. Pink dots indicate cells in the ventral thalamus and internal capsule with projections to the dorsal thalamus (DT). By E18.5 thalamocortical projections reached the appropriate cortical areas while associating with an early corticofugal fiber scaffold. In the *Emx2* KO animals, fasciculation abnormalities occur, and some thalamic projections are misrouted ventrally at the diencephalic–telencephalic border. The aberrant thalamocortical and corticofugal fiber trajectories follow the displaced thalamic projections established earlier by the internal capsule cells. Thalamic projections arrive to the *Emx2* KO cortex slightly delayed compared to WT. In the *Pax6/LacZ* null mutant, cells with dorsal thalamic projections are located in the ventral internal capsule and in the hypothalamus. Some thalamocortical projections follow this aberrant route and descend into the hypothalamus and a subset of fibers cross the diencephalic–telencephalic boundary at altered sites. Although some thalamic projections reach the pallial–subpallial boundary, they fail to interact with the corticofugal projections, which instead of turning into the internal capsule, aim to the ventral pallium. The reporter gene expression pattern is displaced ventrolaterally at the pallial–subpallial boundary and a non-labeled cellular mass (CM) protrudes into the lateral ventricle. Figure based on Jones *et al*. and López-Bendito *et al*. (Jones *et al*., 2002; López-Bendito *et al*., 2002).

Figure 2. The figure summarizes the embryonic (*A–D*) and early postnatal (*E–G*) cortical activation patterns in rat revealed with optical recording with voltage sensitive dyes after dorsal thalamic stimulation in thalamocortical slice preparations. Each age is represented by a column; comprised of reference image (top row) and a selected frame of the recording at the peak of the response (time is indicated in milliseconds) viewed with ×4 objectives on the Fuji Deltaron 1700 differential image acquisition system. The lower row indicates the image obtained with the same conditions, but after the bath application of DNQX and APV. Short depolarization, which probably represent spikes, reach the cortex by E17 (*A*). Sustained depolarization, corresponding to postsynaptic responses appear in the subplate at E19 (*C*). These are blocked after the application of DNQX and APV (C2). The responses extend through the cortical plate by E21 (*D*). During postnatal ages the optical response develops much stronger than seen prenatal stages and then periphery-related columnar patterning was apparent from P2 (*F*), which is refined to layers 4 and 6 by P10 (G). Figure based on Higashi *et al*. (Higashi *et al*., 2002).

Figure 3. Comparison of thalamocortical fiber outgrowth and topography of the early innervation of the cortex revealed with carbocyanine dye tracing from the dorsal thalamus (*A* and *B*) or from cortex (*C* and *D*) in *Snap25* KO brain at E18.5. Corresponding coronal sections are photographed with appropriate filters to reveal the bisbenzimide counterstain (blue) and the DiI labeling (orange/red). Thalamic axons traverse the internal capsule as an organized array of fiber bundles, then defasciculated and turn dorsally to extend through the intermediate zone and subplate below the cortical plate. Although there are slight individual variations in maturity within individual litter, there is no consistent difference between *Snap25*$^{+/+}$ (*A*) and *Snap25*$^{-/-}$ (*B*) brains in the state of advancement (arrows), branch formation of thalamocortical fibers. (*C*, *D*) Normal targeting of thalamic axons revealed from multiple carbocyanine dye placements to the cortex at E18.5. Posterior cortical crystal placement (a) revealed a group of cells in the lateral thalamus (a′), whereas progressively more anterior crystal placements (b, c) labeled more medial thalamic regions (b′, c′). The pattern was identical in wild-type and null mutants. Scale bar: 200 μm for *A–D*. Figure is based on Molnár *et al*. (Molnár *et al*., 2002).

The stimulation of the three caudal-most whiskers of rows B and D during active exploration of a stimulus-rich cage results in a normal pattern of cortical distribution both on coronal and tangential planes. Thus, a modified organization of fiber fascicles in the internal capsule of the L1 KO mice does not seem to alter the ordered, functional whisker representation.

Evidence for Early Thalamocortical Transmission in the Cerebral Cortex

It was demonstrated that the peripheral sensory organs already generate spontaneous activity patterns at ages (Galli and Maffei, 1988; Meister *et al.*, 1991) when the sensory afferents begin to reach the thalamus. These activity patterns could elicit EPSPs on thalamic projecting neurons (Mooney *et al.*, 1996) which are capable of relaying them to cortex (Friauf and Shatz, 1991), and thus these activity patterns may alter the forming terminals within the subplate and cortical plate by controlling side branch formation.

To gain an insight into the formation of early thalamocortical synapses, we recorded optical images, using voltage sensitive dyes, in the cerebral cortex of prenatal rats by selective thalamic stimulation (Higashi *et al.*, 2002) of thalamocortical slice prep-

Figure 1

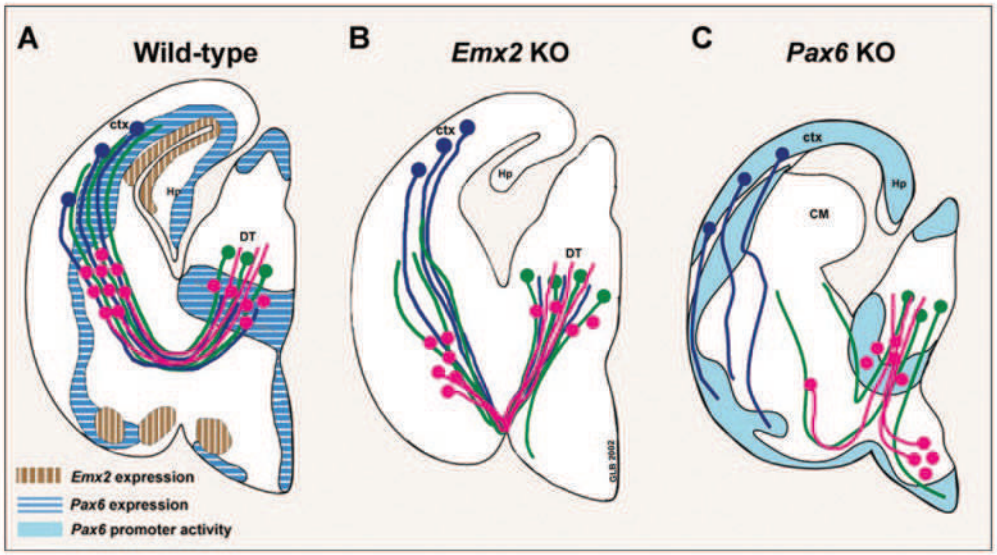


Figure 2

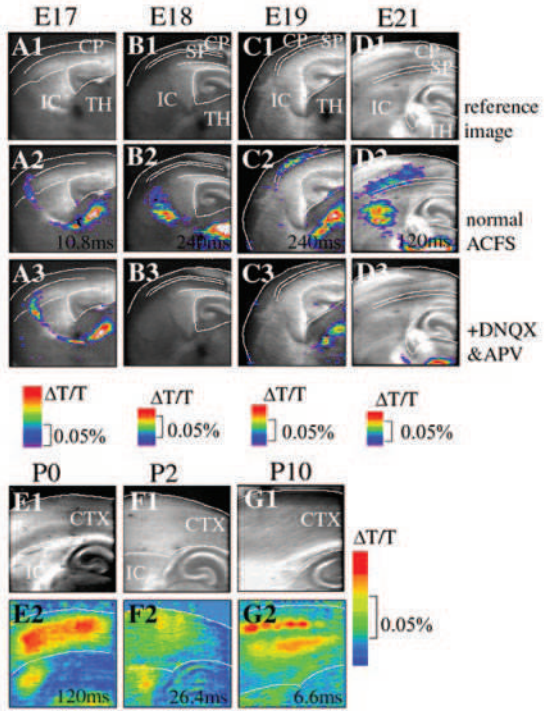
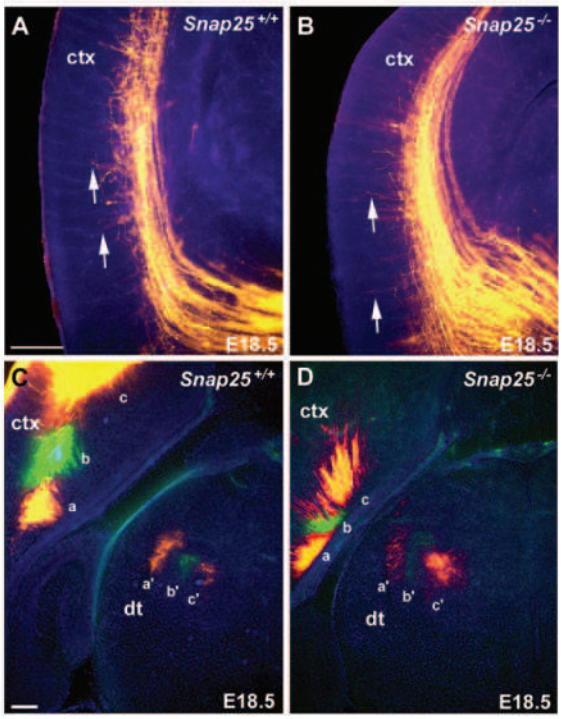


Figure 3



arations (Agmon *et al*., 1993). The embryonic developmental pattern in rat is similar to that in mice, but in rat the developmental stage is ~1 day less advanced. At E17, thalamic stimulation elicits excitation that rapidly propagates through the internal capsule to the cortex. These responses last <<10–15 ms, and are not affected by the application of glutamate receptor antagonists, suggesting they might reflect presynaptic fiber responses (Fig. 2*A*1–3). At E18, long-lasting (>>300 ms) responses appear in the internal capsule. These responses are abolished by perfusion of glutamate receptor antagonists, which indicates synapse-mediated activation of internal capsule cells (Fig. 2*B*1–3). At E19, distinct long-lasting responses appears mainly in the cortical subplate (Fig. 2*C*). By E21, shortly before birth, the deep cortical layers are also activated in addition to the subplate. The laminar location of the responses was determined in the same slices by Nissl-staining or birthdating with bromodeoxyuridine (BrdU) at E13 (Higashi *et al*., 2002). Our results demonstrate that there is a delay of several days between the arrival of thalamocortical axons at the subplate at E16 and the appearance of functional thalamocortical synaptic transmission at E19. Since thalamocortical connections are already functional within subplate and in deep cortical plate at embryonic ages, prenatal thalamocortical synaptic connections could influence cortical circuit formation before birth.

Development of Thalamocortical Projections in the Absence of Regulated Neurotransmitter Release

There are various forms of neurotransmitter release, which could activate recipient cells. Most synaptic vesicle release occurs from nerve terminals after they have been triggered by action potentials. The spontaneous vesicle release is not regulated and the paracrine type, non-vesicular release, does not require synaptic vesicle fusion mechanisms (Rizo and Südhof, 2002).

Snap25 KO

Disruption of the gene encoding SNAP25, a component of the SNARE complex required for regulated neuroexocytosis, eliminates evoked but not spontaneous neurotransmitter release (Washbourne *et al*., 2002). The *Snap25* null mutant mouse provides an opportunity to test whether synaptic activity is required for prenatal neural development. We find that evoked release is not needed for at least the gross formation of the embryonic forebrain, since the major features of the diencephalon and telencephalon are normal in the null mutant mouse. Tracing of the thalamocortical fiber pathway reveals normal growth kinetics and fasciculation patterns between E17.5 and E19. As in normal mice, thalamocortical axons of the mutant reach the cortex, accumulate below the cortical plate, and then start to extend side-branches in the subplate and deep cortical plate (Fig. 3*A*,*B*). Multiple carbocyanine dye placements in the cortical convexity reveals a normal overall topography of both early thalamocortical and corticofugal projections (Fig. 3*C*,*D*) (Molnár *et al*., 2002). Unfortunately the mutants die at birth and thus, the period of thalamic axon branching and termination in layer 4 and the barrel formation remain out of our reach.

Discussion

During their growth, thalamic fibers change their fasciculation pattern and growth kinetics when they cross gene expression boundaries along their trajectory towards the cortex. There seem to be at least two especially critical zones for axon outgrowth in the embryonic forebrain. One critical zone is at the diencephalic–telencephalic border, and the other one at the striatocortical junction, the PSPB. Thalamic axons prove to be very sensitive indicators of regionalization defects in the developing forebrain. Altered gene expression patterns along the thalamocortical path or defects of the thalamic cells themselves (Pratt *et al*., 2000) can arrest or modify their development at specific sites. Having examined the aberrant development of thalamocortical projections in various mutants some basic principles are beginning to emerge.

Aberrant Early Pioneer Projections from Internal Capsule Cells Are Associated with Axon Growth Defects at the Diencephalic–Telencephalic Boundary

In *Mash1* homozygous mice, the internal capsule cells with thalamic projections are missing and thalamocortical axons fail to enter the internal capsule (Tuttle *et al*., 1999). Impairment in the early growth of thalamic axons has also been reported in *Sema6A* KO brains (Leighton *et al*., 2001). In *Emx2* homozygous mice, the early internal capsule projections take an aberrant ventral route at the diencephalic–telencephalic boundary, and some of the thalamocortical axons follow them as they traverse that region (López-Bendito *et al*., 2002). The interaction between thalamic and corticofugal projections is more of a 'low five' rather than handshake in the *Emx2* KO. Both sets of projections get derailed ventrally, both cross the diencephalon in an aberrant pattern.

In *Pax6* KO mice, it was observed that if the internal capsule cell projections to thalamus are aberrant, then the thalamic projections also take this aberrant path. It is known that the diencephalic–telencephalic junction zone is compromised in *Mash1*, *Emx2* and *Sema6A* mutants, but the causal relationship between these events is not understood. The pattern in *Mash1*, *Emx2* and *Pax6* KO mice is compatible with the suggestion that ventral thalamic, and internal capsule cells and their projections, guide the outgrowth of thalamic projections. This hypothesis will have to be abandoned if normal thalamic projections are described in the absence of these pioneering pathways, or, if aberrant thalamic development not accompanied by the aberrant development of the thalamic projections occurs at this segment.

Disrupted Thalamocortical Development at the Striatocortical Junction Is Accompanied by Anomalous Corticofugal Projections

The second common theme emerging from recent studies on mutants is that both thalamocortical and corticothalamic projections are disrupted at the striatocortical junction (Hevner *et al*., 2001, 2002; Jones *et al*., 2002). It is surprising how many homeobox related genes can alter thalamic development at the PSPB, which appears as a rather vulnerable region following modifications in gene expression. Synchronized choreography of thalamocortical development and forebrain regionalization is needed for the successful completion of the hurdle to cross this region. Any spatial/temporal misalignment can have dramatic effects. Errors occurring in corticothalamic and thalamocortical pathfinding within the region of the internal capsule were described in mice with mutations of transcription factor genes expressed in cortex (*Tbr1*), thalamus (*Gbx2*), or in both (*Pax6*) (Kawano *et al*., 1999; Miyashita-Lin *et al*., 1999; Hevner *et al*., 2001, 2002). In the *Pax6/LacZ* mutant, for example, the majority of corticofugal fibers do not turn from cortex to the internal capsule, instead they continue to descend to ventral pallium. The thalamocortical fibers fail to enter the cortex, and the lateral pallial sector is morphologically compromised (Stoykova *et al*., 2000; Jones *et al*., 2002). We consider the

aberrant fiber growth pattern observed in *Pax6* KO only as limited support for the handshake hypothesis, since the phenotype is rather complicated (Fig. 1*C*). There are numerous mutants [*Tbr1*, *Gbx2*, see Hevner *et al.* (Hevner *et al.*, 2002)] with much less severe abnormalities at this region; nevertheless thalamic fibers fail to cross. It is puzzling that the gene expression is localized proximal or distal to the site of the actual guidance defect at the striatocortical junction. It is conceivable that an intact expression of certain receptors or surface molecules is required by both sets of projections to react to guidance signals in the region. A better understanding of the interaction between gene expression and environmental factors is needed at different sectors of the fiber trajectories.

Map Formation in the Cortex after Altered Thalamocortical Deployment

A significant fraction of thalamic projections in *Emx2* KO mice is misrouted at the diencephalic–telencephalic boundary and this abnormality is associated with displaced projections of internal capsule cells and disrupted entorhinal and perirhinal cortical projections. As we discuss above, it appears that most of these misrouted thalamocortical projections recovered and ascended from the ventral telencephalon towards different cortical regions. The misrouting caused a considerable delay in thalamic fibers arriving to the cortex, which might contribute to the altered cortical topography. The abnormalities likely to be related to earlier guidance defects, some of which are located at the diencephalic–telencephalic boundary where *Emx2* has strong expression during development. Although this thalamic axon guidance defect is linked with the altered *Emx2* expression pattern in the dorsal cortex and with aberrant growth of some of the early corticofugal projections at the striatocortical junction, it most probably occurs independently from the cortical *Emx2* expression. Similar abnormalities were found in the *Sema6A* mutant mouse (Leighton *et al.*, 2001), and *Sema6A* might be one of the candidate genes responsible for some of the abnormalities observed in *Emx2* mutants.

It seems that a precise trajectory might not be crucial for the final pattern of cortical targeting. *L1*, *reeler* mice provide examples for cases where thalamic projections arrive to the cortex through aberrant routes, yet they form a normal periphery-related pattern in the primary somatosensory cortex. It will be interesting to further study these interesting paradigms in mutants during their postnatal development. The challenge is to dissect the role of the gene expression patterns *en route* to the cortex and within the cortex itself.

Axonal Pathfinding and Cortical Regionalization Defects Might Be Independent

There is a continuing debate as to how the developing pallium influences thalamocortical development, and in turn whether thalamocortical afferents affect the development of the cortex. Early gene expression patterns are normal in the absence of thalamic input during embryonic life (Nakagawa *et al.*, 1999; Miyashita-Lin *et al.*, 1999). It has been demonstrated that there is a shift in the areal identity in the cerebral cortex of *Emx2* and $Pax6^{Sey/Sey}$ mutant mice, which is matched with altered distribution of thalamocortical projections in the *Emx2* KO brains (Bishop *et al.*, 2000; Mallamaci *et al.*, 2000). In the *Pax6* KO brains, no thalamic projections reach the cortex, suggesting that the shift in cortical representation observed in the *Pax6* KO cortex might, in fact, be independent from thalamic axon targeting and does not rely on the presence or absence of thalamic projections. Our tracing studies demonstrated that in both *Emx2* KO (López-Bendito *et al.*, 2002) and *Pax6* KO mice (Jones *et al.*, 2002), a large proportion or the entire thalamocortical axon population was misrouted at the border between the diencephalon and telencephalon. In addition, in the *Pax6* KO an additional defect prevented the thalamic afferents from invading the cortex through the striatocortical boundary. It will be exciting to investigate the formation of cortical areas in mutants with axonal targeting abnormalities, but without primary cortical alterations. Will the thalamic projections compensate for their targeting error, or they shift the cortical representation?

What Form of Intercellular Communication Is Needed for the Remodeling of the Initial Thalamocortical Map?

In contrast with the initial gross deployment of thalamocortical and corticothalamic connections, the remodeling of cortical circuitry during thalamic fiber invasion is probably a more complex process in which patterns of afferent and local activity, expression of surface molecules and growth factors, and cell death all play crucial roles (Katz and Shatz, 1996). In turn, the remodeling may lead to the formation of new synapses and therefore to new distributions of activity. Early thalamic projections are capable of eliciting sustained depolarization patterns in the subplate at the time of side-branch formation (Friauf and Shatz, 1991; Higashi *et al.*, 2002; Molnár *et al.*, 2003). This early interaction is different from the mature postnatal form, being relatively smaller, but much longer. Our data in *Snap25* KO mice suggest that axonal growth and early topographic arrangement of these fiber pathways do not rely on activity-dependent mechanisms requiring evoked neurotransmitter release. We propose that other forms of intercellular communication might still play a part. There are three forms of neurotransmitter release, which could activate recipient cells: (i) regulated synaptic vesicle release, which is absent in *Snap25* KO; (ii) spontaneous vesicle release, which is absent in *Munc13* and *Munc18* KO mice; and (iii) paracrine type, non-vesicular release, which is still present in *Munc18* and *13* KO. With these currently available mutants it should be possible to create various *in vivo* and *in vitro* paradigms to determine which mechanisms are required for different periods of cortical and thalamocortical development. In these experiments, neuronal interactions will have to be better defined, since it is no longer sufficient to determine whether developing neurons play a 'melody' or not; it will be necessary to define what melody and whether they sing, hum or just whisper it.

Conclusion

Transgenic mouse models continue to provide a very powerful tool to evaluate the sequence of various axon guidance mechanisms in the developing brain. Detailed analysis can reveal causal relationships between forebrain patterning, thalamic growth and cortical regionalization. Although the adult organization of thalamocortical projections is intriguingly complex, we are beginning to understand how the initial layout of this complicated pathway is constructed with a cascade of simple rules.

Notes

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