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ORIGINAL ARTICLE

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Corticothalamic and thalamocortical pathfinding in the mouse: dependence on intermediate targets and guidance axis

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Abstract Recently, increasing attention has been paid to the study of intermediate targets and their relay guidance role in long-range pathfinding. In the present study, mechanisms of corticothalamic and thalamocortical pathfinding were investigated in C57BL/6 mice using in vitro DiI labeling and in vivo cholera toxin labeling. Specifically, three important intermediate targets, the subplate, ganglionic eminence, and reticular thalamic nucleus, were studied for their role in corticothalamic and thalamocortical pathfinding. The results show that the neuroepithelium of the ganglionic eminence is a source of pioneer neurons and pioneer fibers. Through radial and tangential migration, these pioneer neurons and fibers can approach the differentiating field of the ganglionic eminence, the subplate and thalamic reticular nucleus to participate in the formation of these three intermediate targets. Furthermore, the subplate, ganglionic eminence and thalamic reticular nucleus are linked by pioneer neurons and fibers to form a guidance axis. The guidance axis and the three important intermediate targets provide an ideal environment of contact guidance and chemical guidance for the corticothalamic and thalamocortical pathfinding. The concept of a "waiting time" in the subplate and the thalamic reticular nucleus is likely due to the expression of a guidance effect, so that the thalamocortical and corticothalamic projections can be deployed spatially and temporally to the subplate and thalamic reticular nucleus before these projections enter their final destinations, the neocortex and thalamus.

Keywords Ganglionic eminence · Subplate · Reticular thalamic nucleus · Pioneer neuron · Pioneer fiber

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Abbreviations *CLSM:* confocal laser scanning microscope · *CP:* cortical plate · *DF:* differentiating field · *E:* embryonic day · *GE:* ganglionic eminence · *IC:* internal capsule · *IZ:* intermediate zone · *MZ:* marginal zone · *NP:* neuroepithelium · *P:* postnatal day · *PB:* phosphate buffer · *PBS:* phosphate-buffered saline · *Po:* posterior group nucleus · *Pr5:* principle sensory trigeminal nucleus · *SI:* somatosensory cortex · *SP:* subplate · *SZ:* subventricular zone · *RT:* reticular thalamic nucleus · *V:* ventricle · *VPM:* ventral posterior medial nucleus · *VZ:* ventricular zone · *WGA:* wheat germ agglutinin · *WM:* white matter

Introduction

Studies of axonal pathfinding in diverse species show that growing axons use several types of guidance cues, successively or in combination, to find the path toward their final target. On a cellular level, changes in the course of growing axons, or sorting out among several types of fibers, are apparent in spatially restricted regions, termed "decision regions" or "intermediate targets" such as the motoneuronal plexus (Tosney and Landmesser 1985; Tosney 1991), and the optic chiasm (Godement et al. 1987; Wizenmann et al. 1993; Marcus et al. 1995). Such regions may display guidance for the ingrowing fibers (Felsenfeld et al. 1994; Molnár 2000; Marin et al. 2002). The ganglionic eminence (GE) is another intermediate target that operates in this way (Métin and Godement 1996; Métin et al. 1997). In addition, several studies implicate the role of early neurons in guiding the growth of later-growing fibers, or fibers originating from other areas (Ho and Goodman 1982; Sretavan et al. 1994; Frotscher 1998). The earliest-projecting neurons might rely solely on cues within the neuroepithelium for their guidance, whereas later-projecting neurons could use a greater variety of cellular and molecular cues. Selective axonal guidance by pioneer neurons is also implicated in the development of the long-distance pathways that reciprocally connect thalamic nuclei and cortical areas.

A set of pioneer and transitory neurons in the cortex, the "subplate cells" (Bayer and Altman 1990), could be involved in guiding thalamic fibers toward and into their cortical target areas (De Carlos and O'Leary 1992; O'Leary and Koester 1993; Molnár and Blakemore 1995). In carnivores, the axons of the subplate (SP) cells project toward the thalamus and could constitute a pioneer pathway that growing thalamic axons follow to reach the cortex (McConnell et al. 1989). Ablation of SP neurons leads to a failure of thalamic fibers to recognize their normal cortical target areas (Ghosh et al. 1990). Recently, the reticular thalamic nucleus (RT) also was found to exert the function of an intermediate target that participates in pathway guidance (Ulfig et al. 1998). Therefore, the RT, GE and SP are regarded as "transient structures" and cooperate to guide corticothalamic and thalamocortical pathfinding (Ulfig et al. 2000).

The most useful models for long-range pathfinding are the corticothalamic and thalamocortical pathways. The primary somatosensory cortex (SI) of rodents is characterized by the clustering of layer 4 neurons into aggregates known as "barrels" (Woolsey and Van der Loos 1970; Killackey 1973). In the posteromedial portion of the rodent primary somatosensory cortex, the arrangement of barrels corresponds to the discrete matrix of whiskers on the snout of the animal. Each single barrel represents a structural unit that corresponds to a single, isomorphic whisker. Mapping studies have demonstrated that neurons within a cortical column, both above and below a barrel, respond preferentially to deflections of one "principal" whisker and respond more weakly to adjacent whiskers (Armstrong-James and Fox 1987). Thus, each barrel cortical module seems to form the center of a cortical column that extends from layer 1 to the white matter (Durham and Woolsey 1985; McCasland and Woolsey 1988; Chmielowska et al. 1989). As such, the rodent somatosensory pathway provides an excellent model system to study the spatial and temporal properties of cortical neurons following the activation of a discrete peripheral unit, the single whisker.

Anatomical studies provide clear evidence for a one-to-one relationship between single whiskers and corresponding modules in the principal trigeminal nucleus, in the ventral posterior medial nucleus (VPM), and in the barrel cortex (Chmielowska et al. 1989; Lu and Lin 1993; Veinante and Deschenes 1999). The main sensory input to the neocortex is derived from neurons in the thalamus, which in turn makes a reciprocal connection. In the radial domain, thalamic axons project mainly to cortical neurons in layer 4 and, to a lesser extent, to neurons in layers 5 and 6. Neurons in layer 6 project back to the thalamus. Well-defined thalamic nuclei innervate specific cortical areas tangentially (Coleman and Mitrofanis 1996). Ontogenetic studies have revealed substantial species interspecific differences in the development of thalamocortical and corticothalamic projections. Thus, in cats and monkeys, thalamocortical axons reach the appropriate cortical regions and then wait in the subplate for several weeks before invading the cortical plate (Rakic 1977; Ghosh and Shatz 1992). In rodents, the existence of a waiting period has been questioned. Some studies of outgrowing thalamic axons in fetal rats determined that they accumulated in the intermediate and subplate zones for a few days before their invasion of the cortex (Lund and Mustari 1977; Molnár et al. 1998b). Other studies of fetal and postnatal development in rats demonstrated that thalamic axons penetrate the deeper layers of the cortex immediately after their arrival (Catalano et al. 1991, 1996). The latter results are in accordance with studies of thalamocortical projections of several rodents and other species (Miller et al. 1993).

Although much is known about the development of corticofugal and thalamocortical fibers as they grow in the immediate vicinity of the cortex, several characteristics of the pathfinding between the thalamus and the cortex are poorly understood. In particular, how are the earliest corticofugal fibers guided toward the thalamus, and how are the earliest thalamic fibers guided toward the cortex? In fact, three intermediate targets have recently been identified along the path between the neocortex and the dorsal thalamus in the rat. The SP, GE and RT seem to be relays to guide corticothalamic axons toward the thalamus, and thalamocortical axons toward the neocortex (Mitrofanis and Guillery, 1993). The present study uses neuroanatomical tracing techniques to investigate the genesis of the GE, SP and RT, as well as thalamocortical and corticothalamic pathfinding, in prenatal and postnatal C57BL/6 mice. Our observations suggest that the intermediate targets (SP, GE and RT) are linked by pioneer neurons and pioneer fibers to form an SP-GE-RT guidance axis. This SP-GE-RT guidance axis and the intermediate targets along the axis guide both thalamocortical and corticothalamic pathfinding.

Materials and methods

Animals

All experiments were carried out in accordance with institutional guidelines for animal welfare; the facility is AAALAC accredited. The Principles of Laboratory Animal Care (NIH Publication No. 85-23 1985 version) were followed. Male and female C57BL/6 mice (Jackson Labs) were placed in breeding cages in a standard laboratory animal housing environment with the light cycle of 12 h on, 12 h off. Embryonic (*E* days postconception, *E0* the day a vaginal plug is found in mated females) or postnatal (*P* days postnatal, *P0* the first 24 h after birth) offspring were produced from timed pregnancies. Pups were born on E19, prenatal mice were obtained by Caesarian section. A total of 124 pups at E14–18 and P0–17 were used for the study.

To obtain embryonic mice at appropriate ages, the pregnant dams were anesthetized (sodium pentobarbital, 40 mg/kg, i.p.) and fetuses were excised. The fetal skulls were opened carefully. Whole brains were taken out of skulls using a fine spatula, and immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.2) for 1–2 days at 4°C. To obtain postnatal mice at designated ages, the postnatal pups were removed from the mother, anesthetized (sodium pentobarbital, 40 mg/kg, i.p.), and perfused transcardially with 4% paraformaldehyde in PB (pH 7.2). The brains were exposed in the skulls and immersion-fixed in 4%

paraformaldehyde in PB (pH 7.2) for 1–2 days at 4°C. After this, the brains were taken out of the skulls using a fine spatula.

DiI labeling in vitro

The lipophilic dye, DiI (1, 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, D-282, Molecular Probes, Oregon), was chosen for its anterograde and retrograde labeling properties. Following crystal placement, DiI produces labeling by diffusing within the lipid bilayer of cell membranes that physically contact the crystal. The placement sites were in SI, GE or VPM, and a dissection microscope was used to facilitate crystal placement. For thalamus and somatosensory cortex placement, the two hemispheres were separated mid-sagittally along the longitudinal fissure by a sharp razor blade to expose the thalamus. In some cases, one or two small DiI crystals (150 μm size) were implanted in the VPM in the thalamus to examine the development of thalamocortical projections and corticothalamic neurons in the somatosensory cortex. In the youngest ages sampled, the position of the VPM was approximated at one-third the depth of the ventricle and just lateral enough for the DiI crystal to be positioned deep to the neuroepithelium. In the mid-sample ages, the VPM was approximated by its position below the posteroinferior termination of the hippocampus. In the oldest ages sampled, the VPM was approximated by a position half-way between dorsal/ventral and medial/lateral limits of the cortex. In other cases, one or two crystals of DiI were directly placed into SI, so that neurons and fibers in the thalamus could be labeled. In all ages sampled, the position of SI was approximated at the mid-point of the anterior/posterior dimension of either the cortical plate or the cortex, depending on the age. For the GE placement, a coronal cut removed the anterior one-third of the brain in order to expose the GE to place DiI inside it. The neocortex, thalamus and GE, itself, were examined to determine the GE's development and the migration of pioneer neurons and fibers toward the neocortex and thalamus. After crystal placement, the brains were incubated in phosphate-buffered saline (PBS) with 0.2% sodium azide and 0.1% paraformaldehyde in the dark at 37°C for 1–2 weeks. The brains were coronally sectioned with a vibratome at 100 μm thickness. Serial sections were mounted on glass slides with a coverslip using 65% glycerin in PBS (Elberger and Honig 1990). Sections were observed and images captured using the 568 nm laser line on a confocal laser scanning microscope (CLSM, Bio-Rad MRC1024).

Cholera toxin subunit B and wheat germ agglutinin labeling in vivo

Alexa Fluor 594-conjugated cholera toxin subunit B or Alexa Fluor 594-conjugated wheat germ agglutinin (WGA) was injected in vivo into SI or VPM. Mice at P0-P15 were anesthetized (sodium pentobarbital, 40 mg/kg, i.p.). Pups were fixed in a perinatal headholder modified for use with a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA.). The head skin was disinfected and incised. After the skull was exposed, bregma was used as the main bony landmark. The relevant positions of SI and VPM were determined on the skull surface. In P0 pups, the injection sites for SI and VPM were 2 mm and 4 mm below the skull, respectively. These depths were adjusted according to the age of the pup. Over these areas, a small hole was made with fine scissors or a 30 gauge needle. Then, 0.1 μl cholera toxin subunit B or 0.1 μl WGA (2.0 mg/ml cholera toxin subunit B or 2.0 mg/ml WGA in 0.1 M PB, Molecular Probes, Oregon) was injected slowly into the SI in cortex or VPM in thalamus with a 0.5 μl Hamilton syringe. After suturing the skin, the pups survived in the mothers' care for 2 days. Transcardial perfusion and postfixation were carried out according to the DiI labeling protocol given above. Coronal vibratome sections 100 μm thick were prepared to examine the retrogradely labeled neurons in somatosensory cortex and thalamus using a CLSM with a 568 nm laser line.

Nissl counterstaining

After checking for the presence of labeling using an epifluorescence microscope, some sections with DiI or cholera toxin labeling were selected for Nissl counterstaining. The sections were incubated in 0.5% fluorescent Nissl stain solution (NeuroTrace 500/525 green fluorescent Nissl stain solution, Molecular Probes, Oregon) in 0.1 M PB for 20 min at room temperature. Then the counterstained sections were covered with 65% glycerin in PBS. Finally, the red/green double labeling images were captured with 568/488 nm laser light on the CLSM.

Results

GE and the formation of SP-GE-RT guidance axis

Neuroepithelium of GE, the source of pioneer neurons and pioneer fibers

The GE in the embryo is the anlagen of the basal ganglia and is located between the telencephalon and diencephalon. Although the structures of GE and neocortex are different, their developmental processes are similar. Like the anlagen of the 6-layered neocortex, the ventricular zone (VZ), subventricular zone (SZ), intermediate zone (IZ), cortical plate (CP) and marginal zone (MZ), could be identified in the early anlagen of the GE. The CP is also called a "differentiating field" in GE, because it contains many undifferentiated pioneer neurons, and it is the rudiment of the parenchyma of GE. The striatum and pallidum are derived from the GE (Altman and Bayer 1995; Jain et al. 2001). In the present study, 6-layered lamination could be seen in GE as early as E14. At that stage, the GE was characterized by an extremely thick neuroepithelium. Within the neuroepithelium there were many undifferentiated cells which had two long processes extending through the whole epithelium (Fig. 1). Moreover, the round or ovoid-like pioneer neurons in the SZ and IZ were believed to migrate from the neuroepithelium (Fig. 1, also see Altman and Bayer 1995; Jain et al. 2001). As early as E14, the differentiating field was thin, and it was in the proximity of the VZ and IZ (Fig. 1). At E18-P0, with the internal capsule (IC) penetrating the GE and the pioneer neurons and fibers entering the area, the differentiating field increased in thickness and became more expansive (Figs. 2 and 3). In the marginal zone, the homologue of layer 1 in the neocortex, there were many irregular or astrocyte-like cells with multiple processes (Fig. 3). The morphological characteristics and developmental alterations seen in Figs. 1, 2 and 3 imply that GE has a conspicuously proliferative ability to produce pioneer neurons and fibers.

Pioneer neurons, pioneer fibers and the formation of the SP-GE-RT guidance axis

Evidence suggests that the GE is the source of the pioneer neurons and pioneer fibers in the early embryo (Tamamaki et al. 1999). Through radial and tangential migra-

tion, the neuroepithelium of GE exports many pioneer neurons inside and outside of GE. At E14, the pioneer neurons left the neuroepithelium for the VZ and IZ (Fig. 1), where they continued their journey both tangentially and radially. Tangentially, the pioneer neurons from the GE migrated in opposite directions to the neocortex and thalamus (Figs. 1, 4, 5 and 6). Generally, these pioneer neurons had long projections directed toward the GE (see also Deng and Elberger 2001), with the migration paths probably through the IZ to the neocortex or the thalamus. Upon reaching the neocortex, some pioneer neurons in the IZ would merge into the SP. These pioneer neurons would remain in the SP and differentiate into SP neurons there (Figs. 1, 4; see also Deng and Elberger 2001). The SP neurons are crucial to the formation of the CP and to thalamocortical pathfinding. The present study also shows that the pioneer neurons and fibers migrate and grow into the RT in a similar pattern. These neurons and fibers mainly remained in the RT (Figs. 5, 6). In comparison, the formation of the GE parenchyma depended on radial migration. These radial pioneer neurons left the IZ and migrated into the CP (differentiating field) with an "inside-out" sequence (Fig. 1). There, they would participate in the formation of the GE parenchyma. Thus, the pioneer neurons and fibers participate in the formation and connection of the three important intermediate targets, the SP, GE and RT. Due to their functional significance in corticothalamic and thalamocortical pathfinding, this structural connection is called the SP-GE-RT guidance axis (Ulfig et al. 1998, 2000).

The roles of intermediate targets and the SP-GE-RT guidance axis

The SP-GE-RT guidance axis with the three different intermediate targets inside has been described. The abundant pioneer neurons and fibers enabled the SP-GE-RT guidance axis to provide guidance for the corticothalamic and thalamocortical pathfinding. A contact guidance is suggested as the main mechanism for pioneer neurons and fibers (Frotscher 1998; Ceranik et al. 1999). The present study provides morphological and developmental evidence for the guidance function of the SP-GE-RT guidance axis; this guidance effect was much more obvious within the intermediate targets of the GE, SP and RT.

As the mid-point of the SP-GE-RT guidance axis, the GE received fibers from the subcortical and corticofugal pathways as early as E15 (Fig. 7). With increasing age, more and more subcortical and corticofugal fibers joined the IC, giving it a substantial size by P0 (Figs. 2, 3). The GE acted as a relay station in the pathfinding process. The fibers from neocortex to thalamus (the corticofugal fibers) or from thalamus to neocortex (thalamocortical fibers) entered and left GE in an orderly way. The subcortical and corticofugal fibers were diverged and converged inside the IC (Figs. 2, 3, 7). Further detail, in which the corticofugal projections were converged into the IC, and the subcortical projections from the IC were diverged into the neocortex, is shown in Fig. 8. This shows a clear guidance effect in GE, so that the corticothalamic and thalamocortical pathfinding were "relay-guided" in their particular direction. In comparison, the SP and RT were the entrance and exit positions located at the distal positions of the SP-GE-RT axis. When the axons passed out from the ends of the guidance axis, they were diverged out to their destinations of the neocortex or thalamus. Meanwhile, when axons entered into the guidance axis, they got converged inside the distal positions. For instance, the RT converged thalamocortical fibers into the guidance axis and diverged into corticothalamic fibers leaving the axis (Fig. 7). The SP functioned in a similar way. Thus, the SP-GE-RT guidance axis offered a path for the corticothalamic and thalamocortical pathfinding, and it collected the corticothalamic and thalamocortical fibers inside the guidance axis. Moreover, the three intermediate targets, SP, GE and RT, acted as the three main points of for providing guidance.

Thalamocortical neurons and thalamocortical pathfiding

The Pr5-VPM-barrel cortex axis is described as the transmission mechanism for the trigeminal somatosensory pathway (Williams et al. 1994; Pierret et al. 2000). As the last relay chain of the axis, the neurons in the VPM project their axons to the somatosensory cortex as a direct thalamocortical path. Therefore, the VPM is often used as an injection site to label the thalamocortical pathway that extends to the barrel cortex (Fig. 9) (Marotte et al. 1997; Molnár and Cordery 1999; Pierret et al. 2000). In the present study, both the SI and VPM were used as tracing sites, so that the thalamocortical neurons in the thalamus and the thalamocortical projections in the SI could be labeled in vivo and in vitro.

The genesis of thalamocortical neurons

As early as E15, labeled fibers and neurons could be observed in the thalamus when DiI was placed in SI cortex. The first labeled neurons were in the RT at E15 (Fig. 7), and postnatally, thalamocortical neurons were found in the VPM (Fig. 10). The thalamocortical neurons extended their axons from RT into the SP-GE-RT guidance axis (Figs. 10, 11). Like DiI,the cholera toxin labeled the thalamocortical neurons in the VPM and the neurons in RT as well (Fig. 12). Due to its retrograde labeling, no fibers were visible in this image. The thalamocortical neurons in the VPM were large and astrocyte-like with multiple dendrites and an axon directed toward the neocortex (Figs. 11, 13).

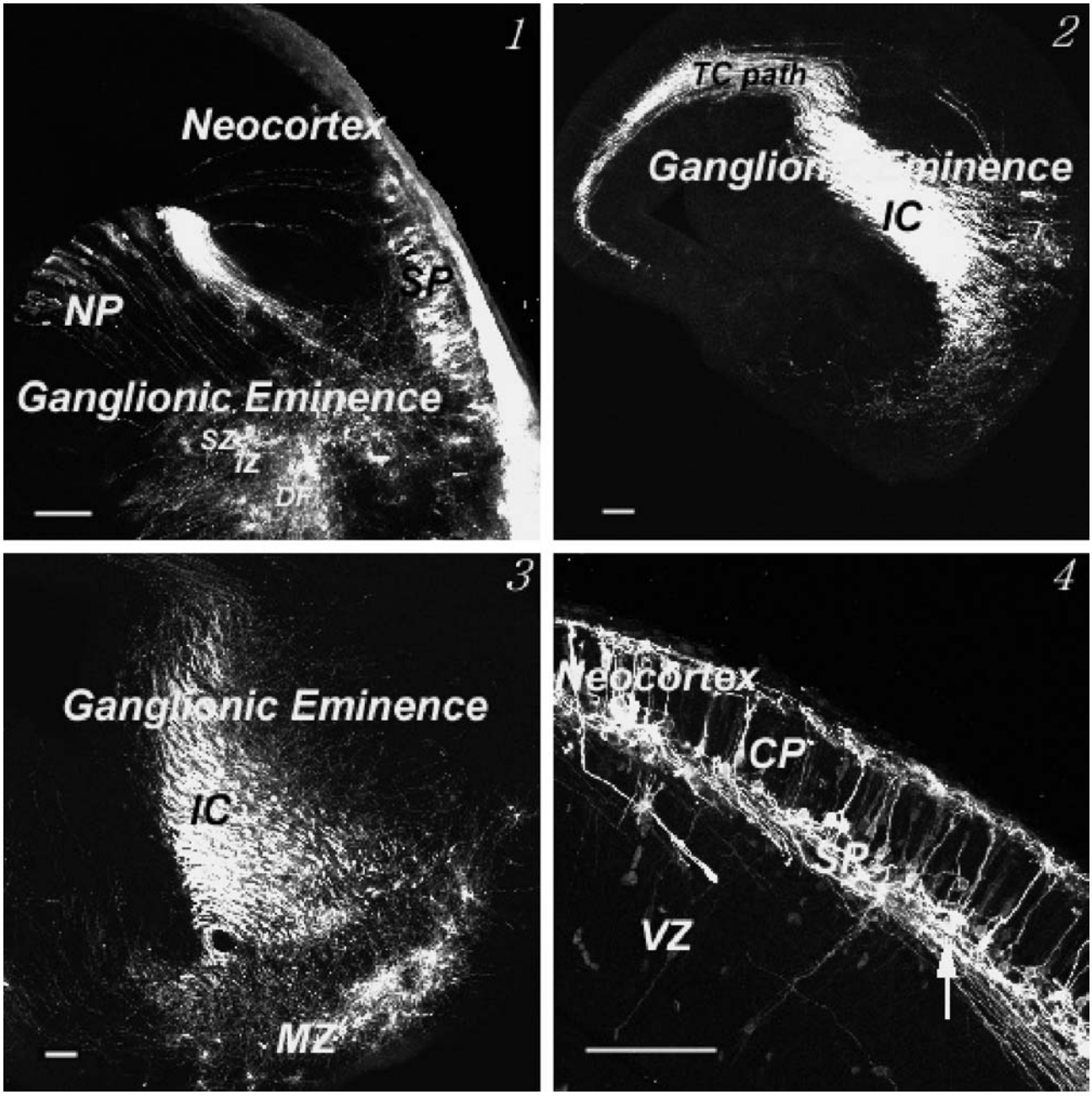


Fig. 1 E14 embryo. A crystal of DiI was placed into the GE. The photo shows the GE and neocortex. In the GE, the thick neuroepithelium (*NP*) was very distinct. Undifferentiated cells were arrayed in the neuroepithelium. The undifferentiated cell usually had two long processes spanning the whole ventricular zone. Many pioneer neurons migrated from the neuroepithelium into the subventricular zone (*SZ*), intermediate zone (*IZ*) and differentiating field (*DF*). The neocortex was adjacent to the GE. Tangentially, many pioneer neurons and fibers have migrated and grown from the GE into the subplate (*SP*) of neocortex. The ventricle (*V)* is indicated. *Bar* 50 μm

Fig. 2 E18 embryo. A crystal of DiI was placed in the VPM of the thalamus. A substantial internal capsule (*IC*) penetrated through the GE. After a small turn, the thalamocortical path entered into the neocortex. Instead of invading into the cortical plate, they "waited" in the IZ and SP until P0. With the IC expanding, the differentiating field occupied a large part of the GE. The ventricle (*V*) is indicated in the photo. *Bar* 50 μm

Fig. 3 P0 pup. DiI was placed in the VPM. The thalamocortical projections were located in the internal capsule (*IC*). The marginal zone (*MZ*) was outside of the differentiating field. There were some astrocyte-like cells in the MZ. *Bar* 50 μm

Fig. 4 E15 embryo. DiI was placed in the GE. Pioneer neurons and fibers had migrated and grown from the GE tangentially to form the subplate (*SP*) in the neocortex. The SP would become one of the important intermediate guidance targets for corticothalamic and thalamocortical pathfinding. The ventricular zone (*VZ*) and cortical plate (*CP*) are indicated. *Bar* 50 μm

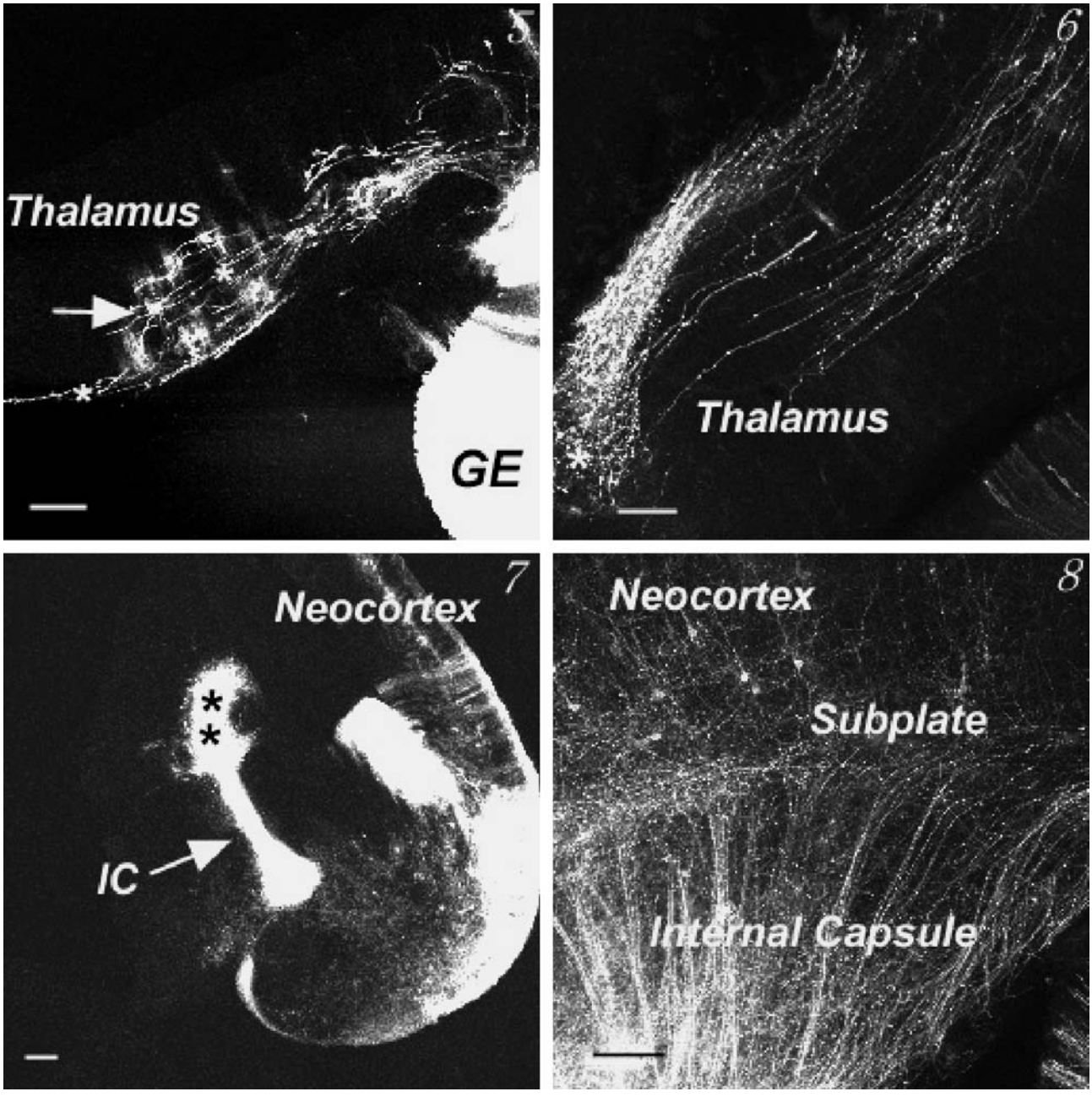


Fig. 5 E14 embryo. DiI was placed in the GE. The pioneer neurons (*arrow*) migrated, and the pioneer fibers (*asterisks*) grew tangentially into the thalamus from the ganglionic eminence (*GE*). This suggests that pioneer neurons and pioneer fibers participate to form the reticular thalamic nucleus (RT). *Bar* 50 μm

Fig. 6 E14 embryo. DiI was placed in the GE. In the thalamus the pioneer neurons and fibers were migrating from the GE tangentially. The precursor of the RT is located at the termination (*asterisk*) of the migrating fibers and neurons. *Bar* 50 μm

Fig. 7 E15 embryo. DiI was placed in the SI of the neocortex. The subcortical and corticofugal fibers were converged in the internal capsule (*IC*, *arrow*). The end of the IC was the reticular thalamic nucleus (*asterisks*), one of the intermediate targets for the corticothalamic and thalamocortical pathfinding. *Bar* 50 μm

Fig. 8 P9 pup. DiI was placed in the VPM. Through the subplate (*SP*), the corticofugal fibers left the neocortex for the internal capsule. Also through the SP the thalamocortical projection entered the neocortex from the internal capsule. The SP was the link between the neocortex and the internal capsule and guided thalamocortical and corticothalamic pathfinding through the divergence and convergence effect. It was also the first intermediate guidance target for corticothalamic pathfinding and the last intermediate guidance target for thalamocortical pathfinding. *Bar* 50 μm

SP-GE-RT guidance axis and the pathfinding of thalamocortical projections

The thalamocortical pathfinding had closely intrinsic connections with the intermediate targets and the SP-GE-RT axis as well. In the early stages (E14–17), the intermediate targets and the SP-GE-RT guidance axis were formed. The pioneer neurons and pioneer fibers in the SP-GE-RT axis provided an anatomic path for the thalamocortical pathfinding.

The information that the thalamocortical pathfinding came into and went out of the SP-GE-RT guidance axis was pieced together using the data from SI and VPM tracing. With the SI tracing, the thalamocortical projections came from thalamocortical neurons in the VPM. Then they entered the SP-GE-RT guidance axis to accomplish their pathfinding from the thalamus to the neocortex. The RT's guidance attracted the axons into the entrance of the SP-GE-RT guidance axis. Inside the SP-GE-RT guidance axis, the axons grew into the IC in the GE (Fig. 10, 11). In comparison, with VPM tracing, the thalamocortical projections appeared in the SP-GE-RT guidance axis as early as E14. At that time, a few fibers were recognized as forming the IC. At E15, the IC was tight and compact, and later it became more expansive. Inside the IC, the pathway was organized in bundles that traveled in parallel (Figs. 2, 3). The expansive IC changed its direction shortly before entering the neocortex, and the IC fiber bundle became thinner after it entered the neocortex (Figs. 2, 3). Once reaching the neocortex, the IC ran tangentially through the IZ. The thalamocortical projections could be seen in the IZ and SP as early as E14, and as the axons grew into the neocortex through the upper IZ they were tipped with growth cones. At this age, the overlying subplate had not yet been invaded. The thalamocortical projections occupied the SP by E15. After E15, the IZ and SP were well filled by ingrowing axons (Fig. 2). As many authors have described, these thalamocortical projections in the neocortex often stayed in the IZ and SP until P0 (Figs. 2 and 14). After P0 the fibers in the SP and IZ started to invade the CP. The invasion was so rapid that the axons extended through the CP in only 1–2 days (Fig. 14). The delay in the SP has been called the "waiting time". At the same time, the thalamocortical projections arborized into many collaterals in the CP. These fibers mainly distributed in layers 5 and 6, and few of them could penetrate from layer 4 into layer 1 (Figs. 15 and 16). After P3 the collaterals of the thalamocortical projections spread in layers 5 and 6 very densely.

The corticothalamic neurons and their pathfinding

The somatosensory cortex produces a corticofugal pathway into the thalamus. The neurons that send projections to the thalamus, the "corticothalamic neurons", are mainly located in layers 5 and 6. Veinante et al. (2000) believe that corticofugal axons from layers 5 and 6 have different distribution patterns in the thalamus. The axons of layer 6 cells head directly toward the dorsal thalamus and distribute arrays of small terminations in the RT, VPM and Po (posterior group nucleus). The axons of layer 5 cells terminate mainly in the Po and RT. In contrast, Marotte et al. (1997) observed that the neurons in both layers 5 and 6 terminated their projections in the VPM and Po.

The genesis of corticothalamic neuron and the formation of "barrel fields"

With DiI labeling in the VPM, corticothalamic neurons could not be found in the neocortex until P2 when some scattered neurons were located in layers 5 and 6 (Fig. 15). With increasing age, more and more corticothalamic neurons were labeled in layers 5 and 6 and at P5, the corticothalamic neurons were predominant in these two layers (Figs. 17, 18). In order to label the corticothalamic neurons retrogradely, cholera toxin or WGA was injected in vivo into the VPM (Fig. 9). The results from in vivo tracing were compatible with in vitro DiI labeling. The animals were sacrificed 2 days after injection. As early as P2, the corticothalamic neurons could be seen in the CP, but layers 5 and 6 could not be distinguished from each other and at P5, the neurons in layers 5 and 6 started to separate from each other (Fig. 19). At P16, layer 5 and layer 6 were well defined (Fig. 20) and the corticothalamic neurons showed the typical shape of pyramidal cells. For instance, the soma appeared large and triangular, and a long axon was extended from its basal region, while an apical dendrite and several lateral basilar dendrites also originated from the soma. Although their cell bodies were located in layer 5 or 6, the apical dendrite usually extended to the pia while it also formed several branches in layer 1 (Figs. 17 and 18). In comparison, the lateral basilar dendrites were usually arborized several times in layers 5 and 6. Along with the collaterals of thalamocortical projections, the lateral basilar dendrites formed a dense fiber plexus in layers 5 and 6 (Figs. 18, 21, 22).

From age P7, the corticothalamic neurons and the fiber plexus in layers 5 and 6 tended to aggregate together to form barrel-like structures in the SI (Fig. 23). These fiber plexuses were composed of the collaterals of thalamocortical projections and the basilar dendrites from corticothalamic neurons in layers 5 and 6, as described above. However, the septae between the barrels were free of cell bodies and fibers. After P7, the aggregation of cell bodies and fiber plexuses were more and more obvious (Fig. 24). In the present study, the barrel-like structure was easily reproduced by DiI labeling; these results were compatible with observations using cytochrome oxidase histochemistry, even though the aggregated neurons were in layers 5 and 6.

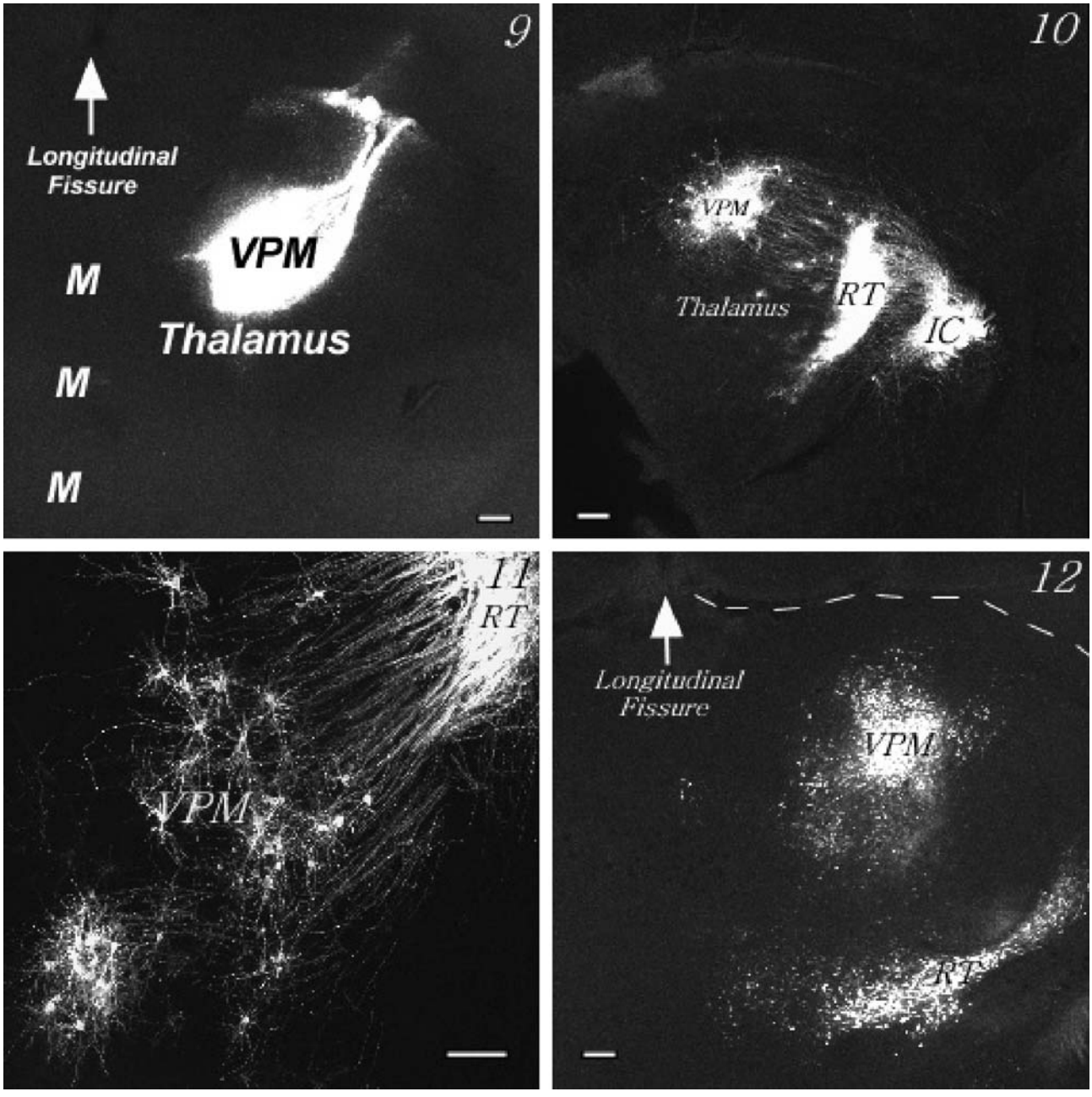
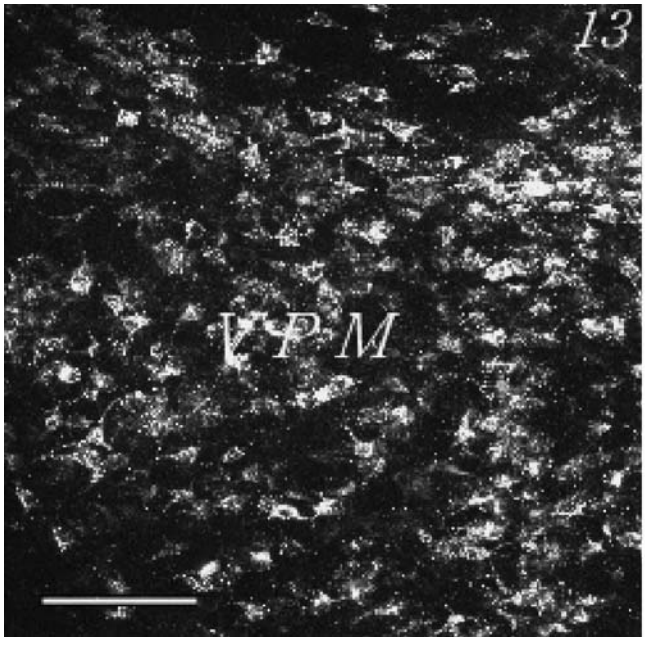


Fig. 9 P10 pup. Cholera toxin was injected into the VPM. The injection site in the VPM is indicated. *M* shows the midline of the thalamus. The longitudinal fissure (*arrow*) is at the upper left of the photo. *Bar* 50 μm

Fig. 10 P6 pup. DiI was placed in SI cortex. The IC (the segment in the cerebral peduncle), RT and VPM are indicated. The thalamocortical fibers came from the thalamocortical neurons in the VPM. The fibers gathered in the RT before entering the GE. Meanwhile, the corticofugal fibers terminated in the thalamus through the GE and RT. The RT was the first intermediate target for the thalamocortical pathfinding and the last intermediate target for the corticothalamic pathfinding. *Bar* 50 μm

Fig. 11 P0 pup. DiI was placed in SI. The thalamocortical neurons in the VPM were large and astrocyte-like with multiple dendrites and a long axon extended toward the RT. *Bar* 50 μm

Fig. 12 P2 pup. Cholera toxin was injected in the SI. The thalamocortical neurons in the VPM were labeled retrogradely, and some neurons also were labeled in the RT. The *arrow* points to the longitudinal fissure, and the *dashed line* marks the border between the neocortex and thalamus. *Bar* 50 μm



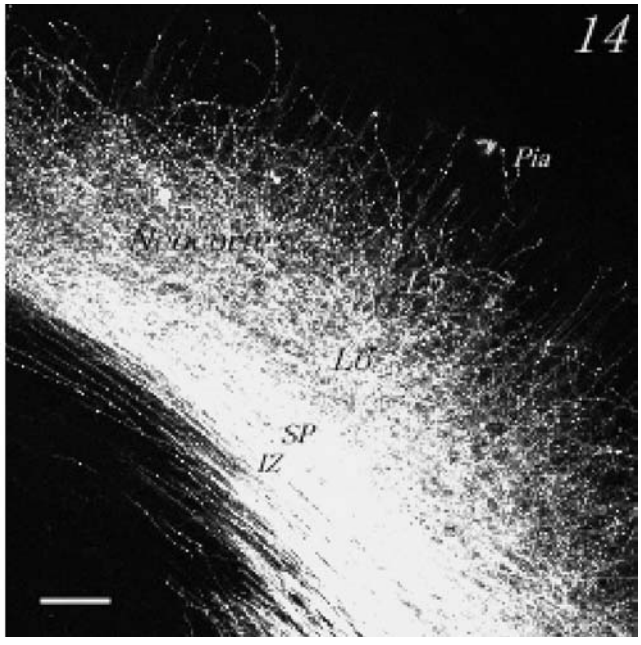
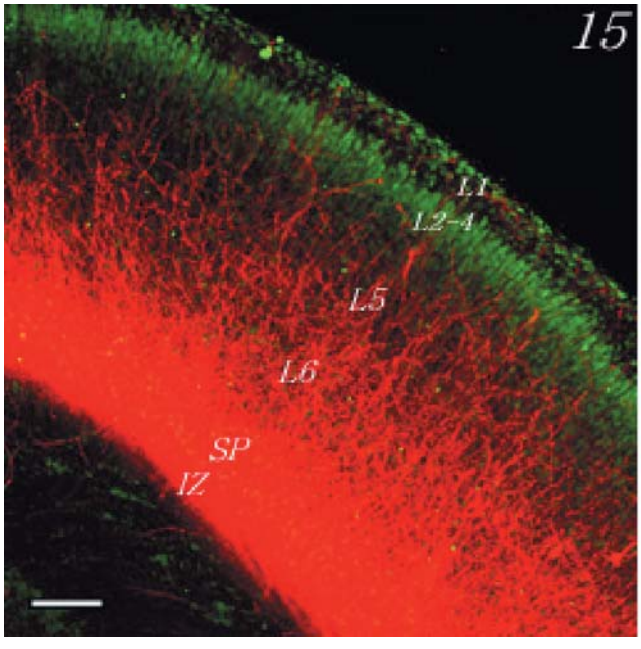


Fig. 13 P16 pup. Cholera toxin was injected into the SI. The magnified thalamocortical neurons were located in the VPM. *Bar* 50 μm

Fig. 14 P0 pup. DiI was placed in the VPM. At this stage, the thalamocortical projections that had "waited" in the SP started to invade into the cortical plate. Collaterals of the thalamocortical projections were mainly distributed in layer 5 and 6. The pia, layer 5 (*L5*), layer 6 (*L6*), subplate (*SP*) and intermediate zone (*IZ*) are indicated. *Bar* 50 μm



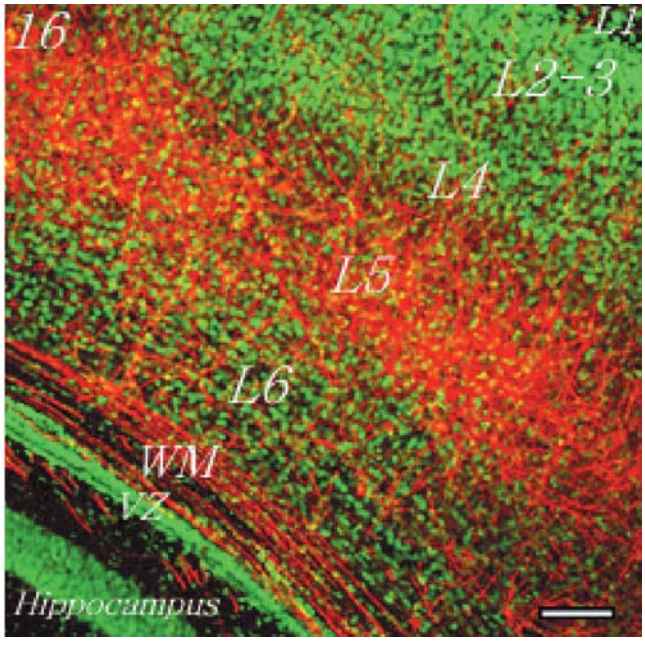


Fig. 15 P2 pup. DiI was placed in the VPM to label thalamocortical projections (*red*). The projections extend into the neocortex, seen in *green* due to Nissl counterstaining. Robust thalamocortical projections in the SP and IZ had invaded into layers 5 and 6. Some scattered corticothalamic neurons were also labeled retrogradely. The lamination of the neocortex was marked with L1 (*layer 1*), L2–4 (layers *2–4*), L5 (*layer 5*), L6 (*layer 6*), SP (*subplate*) and IZ (*intermediate zone*). *Bar* 50 μm

Fig. 16 P7 pup. DiI was placed in the VPM. The image was captured in the neocortex with green Nissl counterstaining. Collaterals of the thalamocortical projections (*red*) were located in layers 5 and 6. Few of the collaterals invaded layers 1–4. The white matter (*WM*) and ventral zone are indicated. *Bar* 50 μm

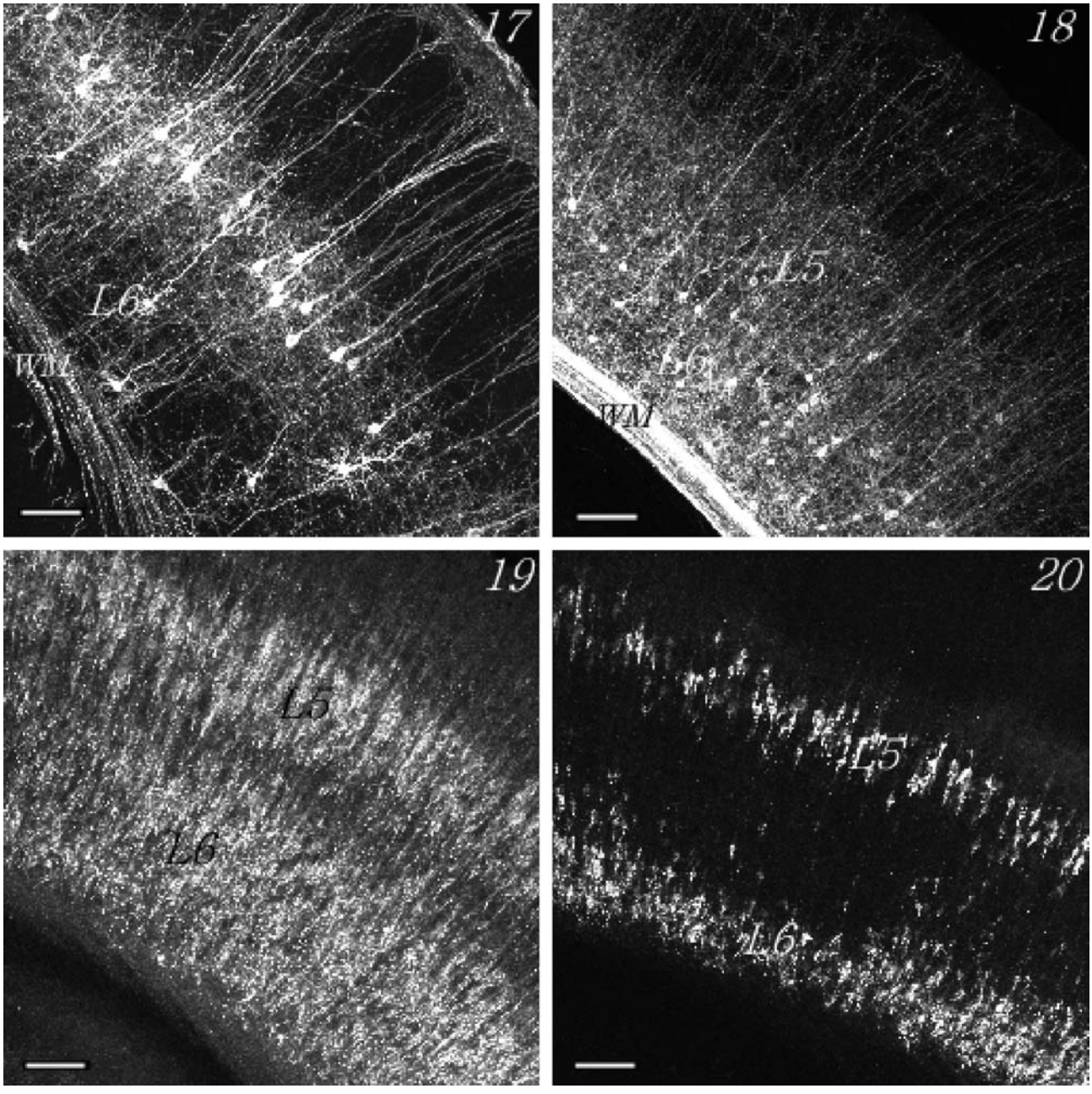


Fig. 17 P7 pup. DiI was placed in the VPM. Many corticothalamic neurons were arrayed in layer 5. They appeared as typical pyramidal cells with multiple lateral basilar dendrites and a single apical dendrite extended toward the pia. The white matter (*WM*) is indicated. *Bar* 50 μm

Fig. 18 P10 pup. DiI was placed in the VPM. The corticothalamic neurons were arrayed in layer 6. The lamination of the neocortex and white matter are marked. *Bar* 50 μm

Fig. 19 P5 pup. Cholera toxin was injected in the VPM on P3. The corticothalamic neurons were labeled in neocortex. The neurons in layers 5 and 6 started to separate from each other. *Bar* 50 μm

Fig. 20 P16 pup. Cholera toxin was injected in the VPM on P14. The corticothalamic neurons were labeled in separated layers 5 and 6. *Bar* 50 μm

SP-GE-RT guidance axis and the corticothalamic pathfinding

The corticothalamic projection could be labeled as early as E15 with DiI. The corticothalamic pathfinding left layers 5 and 6 in the SI and then entered the SP-GE-RT guidance axis. At the entrance, the SP converged these fibers (Figs. 8, 17, 21, 22). In the IC, the fibers oriented themselves toward the cerebral peduncle, which is located in the edge of the ventral diencephalon (Fig. 10). Then the fibers exited the SP-GE-RT guidance axis at the RT (Fig. 7, 10). Like the SP in thalamocortical pathfinding, the RT delayed the corticothalamic projections into the VPM. After 2 days delay or waiting at the RT, the

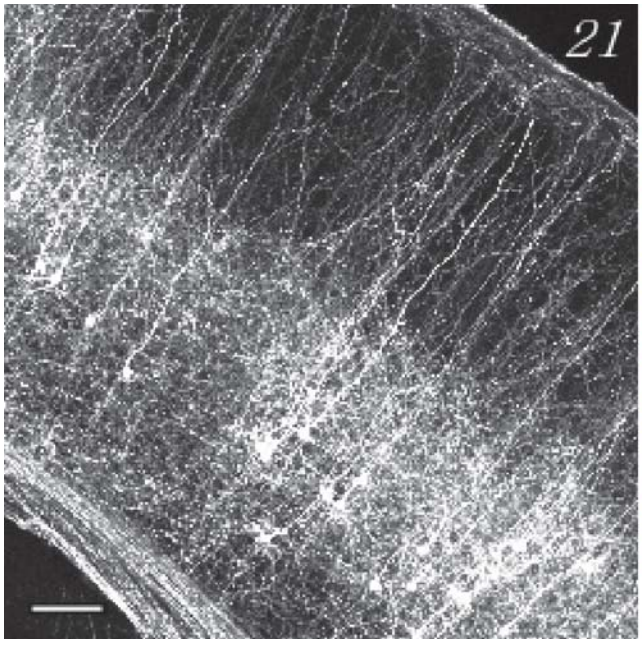


Fig. 21 P7 pup. DiI was placed in the VPM. The corticothalamic neurons and the thalamocortical projections were mainly located in layers 5 and 6. *Bar* 50 μm

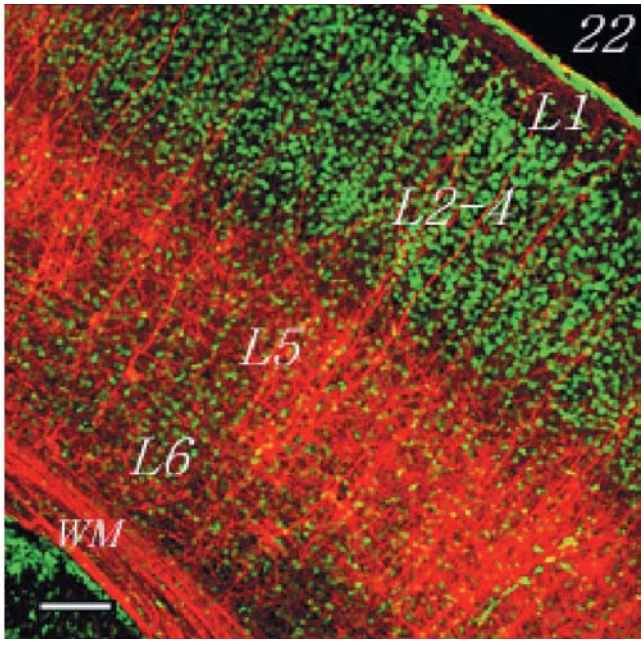


Fig. 22 The same section seen in Fig. 21 shown here in color along with fluorescent Nissl counterstaining. The cortical lamination was distinct. Layers 1–6 and white matter (WM) are indicated. *Bar* 50 μm

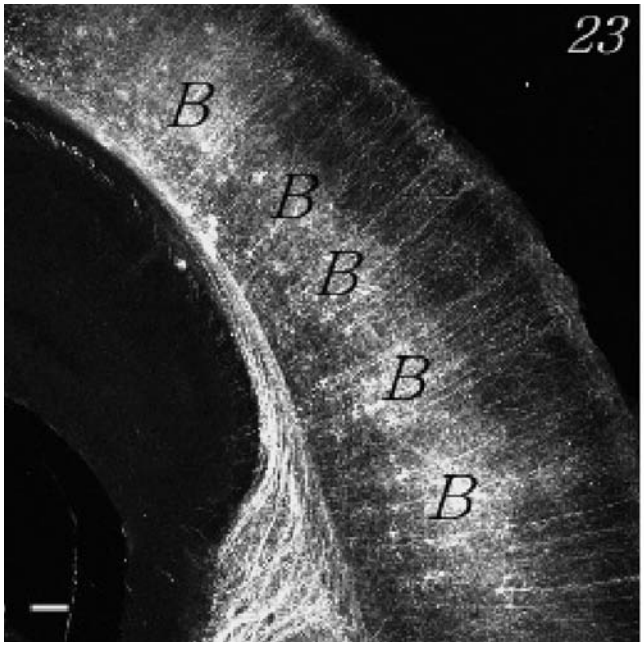


Fig. 23 P7 pup. DiI was placed in the VPM. The corticothalamic neurons and fiber plexuses gathered together in the SI to form the barrels (*B*). *Bar* 50 μm

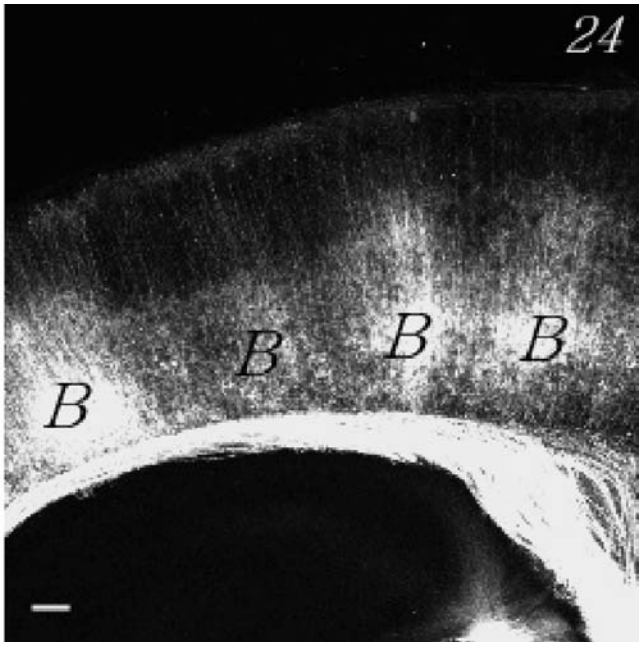


Fig. 24 P10 pup. DiI was placed in the VPM. The barrel (*B*) columns were more obvious. *Bar* 50 μm

corticothalamic projections finally reached their destination. They occupied a large part of the ventral posterior thalamus, including the VPM (Fig. 10).

Discussion

Recently, the GE is thought to be able to produce numerous pioneer neurons and pioneer fibers. These pioneer neurons and fibers, which can migrate and grow

tangentially or radially to various places within the brain, are crucial to brain development, especially to the formation of the neocortex (O'Rourke et al. 1995; Tamamaki et al. 1997, 1999; Parnavelas 2000; Soria and Fairen 2000; Deng and Elberger 2001). Moreover, studies show that the GE also is an intermediate target for corticofugal and thalamocortical axons (Métin and Godement 1996). Intermediate targets provide the relay guidance for long-range pathfinding. Structurally, the intermediate target is a cellular group located along the pathfinding route that is usually associated transiently with growing axons. Studies of the early growth of both corticofugal and thalamocortical fibers in hamster embryos reported that corticofugal and thalamocortical fibers invade the GE from different directions by E12 (Métin and Godement 1996). In the GE at E12, corticofugal fibers are close to, and associate with, cells that are likely to be neuronal. Co-culture explants of cortex and GE from either hamster or mouse embryos showed that the GE, but not other tested brain regions, was able to specifically orient the growth of cortical axons. Therefore, it appeared that the GE may be an intermediate target in the pathfinding of axons between the cortex and the thalamus. Furthermore, Richards et al. (1997) demonstrated that early cortical axon growth was directed toward the nascent IC, but outgrowth was non-directed and suppressed when co-cultured with the dorsal telencephalon, suggesting that the IC releases a chemoattractant for cortical axons. Netrin-1 was suggested as the main chemoattractant in the IC (Métin and Godement 1996; Métinet al. 1997). Nkx2-1 was proposed as another chemoattractant candidate for intermediate guidance (Métin and Godement 1996). Not only the GE, but the SP and RT were also found to be involved in the intermediate guidance (Ulfig et al. 1998, 2000); their corresponding chemoattractants were found. The transcription factor, Tbr1, was reported to participate in guiding neuron migration and axon projections in the SP and layer 6 (Hevner et al. 2001). The SP, GE and RT were suggested to cooperatively guide the thalamocortical and corticothalamic pathfinding (Mitrofanis and Guillery 1993; Métin et al. 1997; Adams et al. 1997; Ulfig et al. 1998, 2000). It was concluded that the "RT with another group of cells, the perireticular nucleus (in internal capsule) and cells of the cortical subplate, are prominent along the course of axons linking the cortex and thalamus early in development" (Adams et al. 1997). Furthermore, Adams et al. conjectured that the fiber reorganization produced in the RT was a fundamental requirement for linking orderly maps between the thalamus and corresponding cortex.

The formation of intermediate targets and the SP-GE-RT guidance axis

As a source of pioneer neurons, the GE's conspicuous proliferative characteristic persists throughout nearly the entire fetal period (Tamamaki et al. 1999; Ulfig et al. 2000; Yelnik 2002). Recently, data also showed that the GE is a crucial structure for thalamocortical and corticofugal pathfinding in the embryonic stage (Molnár 2000). In the present study, the GE played this important role for the genesis of the SP and RT and the formation of the SP-GE-RT guidance axis. Its effects were found in the following observations:

1. The neuroepithelium of GE produced numerous pioneer neurons. These pioneer neurons and fibers could migrate and grow into SP, RT and the parenchyma of GE, and they were the formative basis of the SP-GB-RT guidance axis. In the present study, GE's extraordinarily thick neuroepithelum determined that it could produce much more pioneer neurons into the VZ and IZ. As early as E14, with the tracing in GE, the some neurons and fibers could be labeled in SP of the neocortex and RT of the thalamus. These small, oval-like and long projection neurons were believed to be the pioneer neurons (see also Deng and Elberger 2001). Tangentially the pioneer neurons migrated along the VZ and IZ channel (Tamamaki et al. 1997; Lavdas et al. 1999; Deng and Elberger 2001). Here, they participated in the formation of the other two intermediate targets, the SP and RT. Meanwhile, some pioneer neurons migrated radially. These cells would remain in the differentiating field, the future parenchyma of GE, with an "inside-out" pattern (de Lambert and Goffinet 1998). Thus, under the participation of the pioneer neurons and fibers, the anlagen of three intermediate targets, the SP, GE and RT, were finally formed.
2. The SP-GE-RT guidance axis provided a path for the thalamocortical and corticofugal pathfinding. The present study showed that thalamocortical and corticofugal fibers grew along the SP-GE-RT guidance axis. Because the SP-GE-RT guidance axis was filled with numerous pioneer neurons and pioneer fibers, contact guidance mechanisms were utilized, as the pioneer neurons used elsewhere (Métin et al. 1997; Frotscher 1998; Ceranik et al. 1999).
3. The intermediate targets were the three main points of guidance. The locations of the SP, GE and RT were critical. The GE was located between the telencephalon and diencephalon. It was the mid-point of the SP-GE-RT guidance axis and the pivot point of the thalamocortical and corticofugal pathways, whereas the SP and RT were at the ends of the SP-GE-RT guidance axis. Their locations made the SP, GE and RT easy to relay-guide and sort out the incoming and outgoing fibers in the SP-GE-RT axis.

In contrast to contact guidance, chemical guidance was proposed as the guidance mechanism in the intermediate targets as well. Several chemoattractants that exist in the IC, SP and RT were cloned (Métin and Godement 1996; Métin et al. 1997; Richards et al. 1997; Braisted et al. 1999), for example, Netrin-1, NKx2–1, Tbr1 etc., which are produced in the GE, SP and RT (Métin et al. 1997;

Hevner et al. 2001). The present study has provided morphologic evidence that the three intermediate targets inside the SP-GE-RT guidance axis provide relay guidance for thalamocortical and corticofugal pathfinding. The fibers were sorted out in the three intermediate targets. The "waiting time" was probably the result of a guidance effect in the SP and RT. Through the analysis above, we hypothesize that the pioneer neurons and fibers contact-guided the thalamocortical and corticofugal fibers along the SP-GE-RT axis, while the three intermediate targets relay-guided and sorted out the fibers through chemical guidance as well.

The waiting period in SP and RT

The accumulation of thalamocortical projections below the cortical plate, which is such a striking feature of development in carnivores (Shatz and Luskin 1986; Ghosh and Shatz 1992) and primates (Rakic 1977; Kostovic and Rakic 1990), was first described by Lund and Mustari (1977) in the rat, however the existence of a "waiting period" in rodents has recently been questioned (Marotte et al. 1997). Catalano et al. (1991, 1996) and Kageyama and Robertson (1993) observed thalamic fibers simply advancing steadily into the cortex as it matured. Nevertheless, in the present study, a "waiting period" was a necessary process in the thalamocortical and corticothalamic pathfinding.

We found that thalamocortical projections reached the neocortex as early as E14, but they waited in the SP and did not invade the CP of the somatosensory cortex until P0. Similarly, the corticothalamic path had reached the RT at E15, but it did not terminate in the VPM until E17. The delay or "waiting time" in the SP and RT appeared distinct. What is the significance of the waiting period? The guidance function of the intermediate targets is often used to explain the cause of waiting periods in the SP and RT (Molnár and Cordery 1999; Ulfig et al. 2000). As a "waiting compartment", the SP is required for the formation of mature cortical connections, because within the SP various cortical afferents establish synaptic contacts for a prolonged period before entering the cortical plate (Ulfig et al. 2000). The same occurs within the RT (Molnár and Cordery 1999; Ulfig et al. 2000). The corticothalamic projections shared nearly the same growth path with the thalamocortical projections, but the directions were opposite to each other. The waiting period in the corticothalamic pathfinding occurred in the RT and was the fundamental requirement for corticothalamic pathfinding (Molnár and Cordery 1999; Ulfig et al. 1998, 2000). Molnár and Ulfig believed that through the diverging, and then converging of the projections inside the intermediate targets, the two-way connection become linked in an orderly mirror-like map between the thalamus and corresponding cortex (Adams et al. 1997).

Recently, the mechanism for the neocortex to regulate thalamocortical pathfinding through a waiting period was considered. One explanation is that the SP regulates thalamocortical pathfinding through expression of membrane-bound growth-permissive properties in the perinatal period (Götz et al. 1992; Molnár and Blakemore 1995). This conclusion is supported elsewhere (Henke-Fahle et al. 1996); they believed that some glycoprotein and carbohydrate epitopes contributed to the segregation of afferent and efferent axons and timing of thalamocortical innervation at later developmental stages. The other explanation for a waiting time came from the evidence in the spatial and temporal developmental of the corticothalamic neurons. As the target of the thalamocortical projection, the layer 4 neurons appeared postnatally in the mouse (Bayer and Altman 1991). Therefore, the thalamocortical projections should "wait" a while until the appearance of their targets, the dendrites of layer 4 neurons.

Corticothalamic neurons and thalamocortical projections in the barrel column

Cortical columns are the structural and functional units of the neocortex that are particularly prominent in the "barrel" field of the somatosensory cortex. Each barrel is relatively independent with few connections between the adjacent barrels (Petersen and Sakmann 2000; Miller B et al. 2001). A barrel is a group of neurons in layer 4 of the somatosensory cortex that is part of a cortical column (Miller KD et al. 2001). These columns include neurons above and below layer 4. The spatial patterning of intrinsic projections in layer 4 follows the architecture of the barrels and the septae that separate them, whereas projections in the other layers do not (Hoeflinger et al. 1995; Rhoades et al. 1996). Connections in the supragranular and infragranular layers (layers 2/3 and 5/6, respectively) are more widespread than connections in layer 4. The overall pattern resembles a distorted "hourglass" that is narrowest in layer 4 (Aroniadou-Anderjaska and Keller 1996). Layer 5 and 6 neurons are the main source of corticostriatal and corticothalamic projections (Clasca et al. 1995; Levesque et al. 1996; Kakei et al. 2001). The functions of neurons in layer 4 are to amplify and relay the incoming excitation from the periphery, while the signals from layer 4 neurons can be received by the corticothalamic neurons. There, layer 5 and 6 pyramidal cells integrate neuronal activity both within and across cortical columns and subsequently distribute it to both cortical and subcortical brain regions (Feldmeyer and Sakmann 2000). To explain the function of integration of layer 5 and 6 neurons, the hypothesis of corticothalamic feedback is widely accepted (Destexhe 2000; Ghazanfar et al. 2001, Hillenbrand and van Hemmen 2001). As a link of a circuit, corticothalamic neurons in layers 5 and 6 can feedback the signals to the thalamus, in order to adjust the input from the thalamus.

The thalamocortical projections mainly terminated in layers 5 and 6. It is known that the thalamocortical projections transfer their sensory signals to granular cells in layer 4 (Miller KD et al. 2001). The principle neurons

in layer 4 are the cortical input neurons that amplify and relay incoming excitation from the periphery (Feldmeyer and Sakmann 2000; Lübke et al. 2000). They are intrinsic projection neurons whose axons terminate inside the cortex, including layers 5 and 6. Due to the main recipients of the impulse from thalamocortical projections, their basal dendrites are spiny and mainly located in layers 5 and 6 (Feldmeyer and Sakmann 2000). In the present study, the barrel-like structures were found in SI at age P7 with DiI labeling, as Marotte et al. reported (1997). Because of the DiI application site in the VPM, both corticothalamic neurons and thalamocortical projections in the neocortex were labeled. Although layer 4 neurons are believed to be the main composition of the barrel, the corticothalamic neurons and the fiber plexus in layers 5 and 6 still showed the barrel-like formation in the present study. In other words, the barrels in the SI were composed of only corticothalamic neurons and fiber plexuses, which were the basilar dendrites of the corticothalamic neurons and the collaterals of thalamocortical projections in layers 5 and 6; there was no participation of layer 4 neurons. Neither DiI labeling in vitro nor cholera toxin labeling in vivo could visualize the neurons in layer 4. This was because the neurons in layer 4 had only short-ranged projections in the intrinsic cortex rather than long-ranged projections toward the thalamus. This phenomenon also implied that layer 4 neurons were the recipients of signals from thalamocortical projections. Moreover, the location of the basilar dendrites of layer 4 neurons also could explain why the collaterals of thalamocortical projections mainly terminate in layers 5 and 6.

Numerous studies have referred to the structure and function of the barrel field in SI (Chmielowska et al. 1989; Lu and Lin 1993; Veinante and Deschenes 1999). As a relatively independent unit of structure and function with a one-to-one relationship between single whiskers and corresponding modules in the principal trigeminal nucleus, in VPM, and in the barrel cortex, the barrel's formative mechanism has attracted wide interest (Chmielowska et al. 1989; Lu and Lin 1993; Veinante and Deschenes 1999; Pierret et al. 2000). In the present study, we tried to answer the question from the angle of thalamocortical and corticothalamic pathfinding. As a last relay chain, the neurons in the VPM project their axons to the somatosensory cortex directly. Our results showed that the thalamocortical and corticothalamic fibers grew along the SP-GE-RT guidance axis, and moreover, their direction and growth speed were deployed and readjusted by the three intermediate targets. Therefore, we hypothesize that the process of the thalamocortical and corticothalamic pathfinding under guidance of the SP-GE-RT guidance axis probably provided the developmental basis of barrel formation and the one-to-one mirror-like connection between SI cortex and thalamus.

In conclusion, the present study provided a number of key observations. The neuroepithelium in GE could produce numerous pioneer neurons. Through radial and tangential migration, these pioneer neurons entered the GE parenchyma, the SP and the RT to participate in the formation of three intermediate targets, the GE, SP and RT. The SP-GE-RT guidance axis is a path for the thalamocortical and corticothalamic pathfinding. Inside the SP-GE-RT guidance axis, there were many pioneer neurons and fibers. Those pioneer neurons and fibers provided contact guidance for the thalamocortical and corticothalamic pathfinding along the SP-GE-RT guidance axis. The intermediate targets were believed to be the three main guidance points for the thalamocortical and corticothalamic pathfinding. Substantial support for a waiting period in rodents was provided. Here, under the participation of chemical guidance, through diverging and converging, the fibers' growth directions and speed were determined inside the intermediate targets, so that the one-to-one mirror-like relationship between SI cortex and thalamus was established. The barrel field of the somatosensory cortex could be labeled with DiI tracing in VPM. The barrel was composed of corticothalamic neurons and the fiber plexus in layers 5 and 6 without the participation of layer 4 neurons.

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