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Minireview

Genetic Mechanisms Specifying Cortical Connectivity: Let's Make Some Projections Together

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Great neuroanatomists of the twentieth century recognized that the cerebral cortex of mammals is the single most complex structure of the central nervous system both in terms of neuronal diversity and connectivity. Understanding the cellular and molecular mechanisms specifying the afferent and efferent connectivity in the neocortex may seem like a daunting task. However, recent technical advances have greatly improved our ability to (1) profile gene expression of neuronal populations isolated based on their connectional properties, (2) manipulate gene expression in specific neuronal populations, and (3) visualize their axonal projections in vivo. These new tools are revolutionizing our ability to identify the molecular mechanisms patterning afferent and efferent cortical projections.

Introduction

If Santiago Ramon y Cajal or Roger Sperry were alive today, they would undoubtedly be amazed by the technical and conceptual advances made toward understanding the developmental mechanisms patterning neuronal connectivity. It is truly an exciting time for developmental neurobiologists. Over the past decade, dozens of axon guidance cues, their receptors, and some of their downstream signaling components have been identified (Huber et al., 2003), allowing the deterministic exploration of the molecular mechanisms patterning neuronal connectivity. Furthermore, the techniques available to probe gene expression of specific classes of neurons at a genome-wide scale, to interfere with gene function, and to visualize neuronal projections in vivo have revolutionized our approaches to study the development of neuronal connectivity in both invertebrates and vertebrates (for recent review, see Callaway [2005]). Large-scale-mutagenic and -expression screens are producing (1) expression mapping in the developing brain for thousands of genes and (2) hundreds of transgenic mice expressing genetically encoded reporters, illuminating neuronal projections in vivo (Feng et al., 2000; Gong et al., 2003; Gray et al., 2004; Hatten and Heintz, 2004; Leighton et al., 2001). These transgenic reporter mice crossed to standard, conditional, and inducible knockout mice are now allowing the assessment of defects in the projection of well-defined populations of neurons in intact developing mice. In utero electroporation techniques have been used for years in chick embryos but only recently applied successfully to mouse embryos and now enable us to interfere with gene expression with an unprecedented spatial and temporal accuracy during embryonic mouse development (for example, see Bai et al. [2003], Borrell et al. [2005], and Fukuchi-Shimogori and Grove [2001]). Multiphoton confocal microscopy allows the study of the structural and functional dynamics of neuronal connectivity in vivo in intact embryos or postnatal animals (Ang et al., 2003; Lendvai et al., 2000; Ohki et al., 2005). Until a few years ago, most of these techniques were mainly applied by laboratories studying relatively simple model systems such as *C. elegans*, *Drosophila*, zebrafish, or *Xenopus*. However, one problem remains: these nonmammalian species don't have a neocortex. Today, these techniques are beginning to be applied to mammals and allow the exploration of the cellular and the molecular mechanisms patterning cortical connectivity. This review will highlight some of the recent progress made in this field and illustrate the emerging concepts as well as the challenges ahead.

Cortical Connectivity: Early Specificity Followed by Activity-Dependent Refinement

The nervous system of invertebrates such as the *Drosophila* visual system has provided a powerful model to study the genetic mechanisms specifying neuronal connectivity. The concept emerging from these investigations is that the nervous system of invertebrates is "hardwired" during development and that genetic information is sufficient to pattern the relatively simple connectivity characterizing their functional neural networks (Cutforth and Gaul, 1997; Tayler and Garrity, 2003). In contrast, it is commonly accepted that during early stages of development, neuronal connectivity is more diffuse in complex mammalian systems and that the adult pattern of connectivity is largely shaped by activity-dependent mechanisms pruning nonrelevant connections (Katz and Shatz, 1996). This conceptual framework relied heavily on the developmental anatomy of cortical columns as visualized by qualitative techniques like intraocular injections of transneuronally transported anterograde tracers, such as tritiated proline (Wiesel et al., 1974), whose interpretation can be complicated by "spillover" of label in the LGN. As early studies made clear, this was more severe in younger animals where synaptic contacts are in the process of being formed and could complicate developmental studies (LeVay et al., 1978). This view has changed dramatically over the past two decades with our ability to perform quantitative assessment of the connectivity between two structures with more reliable axon tracing techniques allowing the visualization of single axons (for example, see Crowley and Katz, [2000]). This is especially true in the cerebral cortex where several studies in model systems such as the thalamoco-cortical projections onto the barrel field of rodents (Agmon et al., 1993, 1995), the geniculo-cortical projections of ferret, (Crowley and Katz, 1999, 2000), as well as the feed-forward and feed-back cortico-cortical connections between visual areas of primates (Barone et al., 1996; Batardiere et al., 2002) have revealed an unexpected degree of accuracy early during development—

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in fact, as soon as the axons first reach their target structures. There is overall strong evidence that for most cortical projections, activity-dependent remodeling is not acting on a tabula rasa but rather on a nonuniform prepatterned distribution of axonal projections (Crowley and Katz, 2002).

Taken together, these results suggest that early molecular-axon-guidance mechanisms play an important role in the establishment of the topography of afferent and efferent cortical connectivity. What are these molecular cues guiding axons to specific cortical areas or to specific cortical modules within a given area? What controls the patterned expression of these molecular cues? What are the transcriptional, translational, and posttranslational mechanisms specifying the temporal and spatial responsiveness of a specific axon population to these cues? We are only beginning to answer some of these questions, but some of the answers are quite exciting.

From GENSAT to Gene Trap: Large-Scale Screens to Visualize Neuronal Connectivity in Mice

Several large-scale genetic screens in mice are currently in progress to produce transgenic animals expressing different genetically encoded markers visualizing neuronal connectivity in the cortex and the rest of the CNS. The first large-scale project called the Gene Expression in the Nervous System Atlas (GENSAT) project piloted by the Heintz and the Hatten laboratories takes advantage of the Bacterial Artificial Chromosome (BAC)-recombineering technology in order to insert a fluorescent protein (EGFP) downstream from large portions of a genomic locus controlling expression of a given gene of interest. Multiple copies of this recombined BAC-expressing EGFP are then inserted randomly in the mouse genome by pronuclei injection of fertilized oocytes (Gong et al., 2003). The advantage of this technique is that the large genomic locus where EGFP is inserted usually ensures that EGFP expression reports faithfully the pattern of expression of the targeted gene because of the presence of most of the 5′ and 3′ regulatory sequences. The authors have shown that this approach is relatively insensitive to the sites of genomic insertions (Gong et al., 2003), although this constitutes one of the potential drawbacks of this technology when it is not used for homologous recombination (Copeland et al., 2001).

The GENSAT project has currently produced close to a hundred BAC-transgenic mice in which EGFP highlights selected populations of neurons expressing a gene of interest. Interestingly, even though the authors did not use an axonal-targeted version of EGFP, both axons and dendrites are filled, allowing the visualization of the long-range projections of specific set of neurons in the CNS (for a striking example, see the projections from Dopamine Receptor 4-expressing neurons in the frontal cortex in Hatten and Heintz [2004]).

The second approach is based on a modified version of the gene-trap technique, allowing the targeting of genes encoding secreted or transmembrane proteins with a β-galactosidase reporter (Friedrich and Soriano, 1991; Skarnes et al., 1995; Wurst et al., 1995). The groups of Marc Tessier-Lavigne and Bill Skarnes teamed up to modify this technique with a different targeting vector that encodes both β-geo (a fusion of β-galactosidase and neomycin phosphotransferase) in order to label cell bodies expressing the trapped gene but also a human placental alkaline phosphatase gene (PLAP) that is specifically targeted in axons (but not dendrites) and therefore allows the specific visualization of axonal projections in those neurons expressing the trapped gene (Leighton et al., 2001). This strategy has been shown to have the tendency to produce insertions in the 5′ end of genes and leading to the production of a short truncated fusion protein of the first exons of the gene and the reporter. Therefore, this technique allows the visualization of the position of the cell bodies and the axonal projections of the neurons in heterozygous and homozygous mice. Several of the trapped genes revealed a strikingly specific pattern of axonal projections in the cortex such as LST16 that only labels axonal projections from the dorsal thalamus onto layer 4 in the early postnatal cortex including the barrel field (Leighton et al., 2001). The analysis of some of the trapped genes in homozygous knockout mice unraveled the power of this approach to identify the function of novel genes (Sema6A in thalamocortical projections) or reanalyze the functions of previously identified genes (EphA4 in corticospinal tract crossing) (Leighton et al., 2001). These mice and targeted ES cells are publicly available through the Gene Trap Consortium (Skarnes et al., 2004).

These two large-scale projects as well as several others (see Selected Resources on the Web below) provide the neuroscience community with unprecedented resources to visualize the projections of specific classes of neurons expressing a given gene.

Recent Identification of the Molecular Mechanisms Patterning Some of the Major Efferent and Afferent Cortical Projections

Thalamocortical Projections. Most sensory information coming from the periphery is relayed in individual dorsal thalamic nuclei and projected topographically onto specific cortical areas (Lopez-Bendito and Molnar, 2003). Once thalamic axons from a given nucleus reach a given cortical area, they also project topographically within this area. For example, neurons from different parts of the ventro-basal nucleus relaying somato-sensory information are projecting topographically within the primary somato-sensory area to represent all areas of the body map (the “homunculus” in human). The developmental mechanisms specifying the interareal and intraareal topography of thalamo-cortical projections in mammals are still poorly understood, but data accumulated over the past decade clearly suggested the existence of unidentified cortical and extracortical cues (Vanderhaeghen and Polleux, 2004). Several recent studies have significantly improved our understanding of how the interareal topography of projections is initiated during early development by cues located in their main intermediate target, the ventral telencephalon (Figure 1). First, Garel et al. have shown that knockout mice for the transcription factors mainly expressed in the ventral telencephalon (Ebf1 and Dlx1/2) display a defective topography of thalamocortical projections (Garel et al., 2002). These results suggested that cues present in the ventral telencephalon could initiate thalamocortical topography, but their interpretation is limited by the fact that both *Ebf1* and *Dlx1/2* are expressed not only

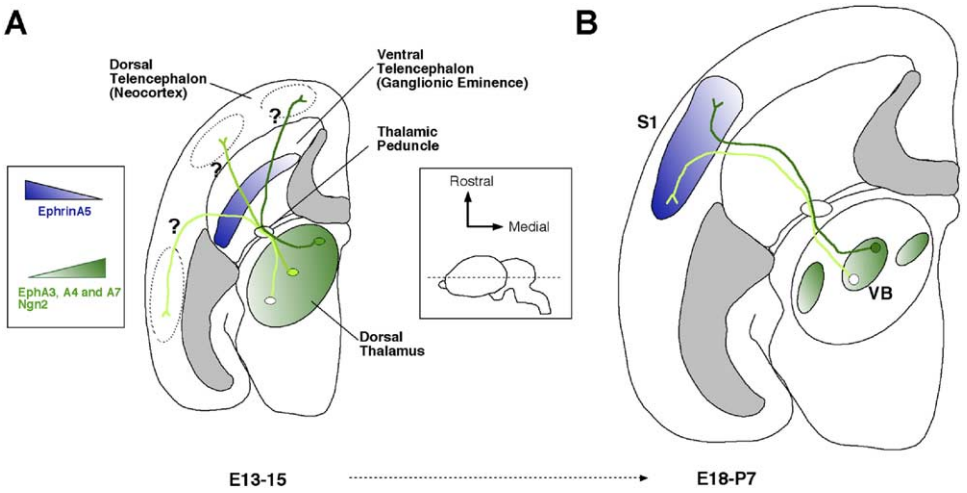

Figure 1. Sequential Specification of the Topography of Thalamocortical Projections in Mammals

(A) During early embryonic murine development (E13-15), thalamocortical axons (green) exit the dorsal thalamus through the thalamic peduncle to pioneer the ventral telencephalon. Thalamic axons express distinct levels of EphA receptors (EphA3, A4, and A7), which are all expressed from high rostro-medially to low caudo-laterally (green gradient). These graded levels of EphA receptors render thalamic axons differentially sensitive to a gradient of the repulsive ligand ephrin-A5 in the ventral telencephalon, which is expressed at high level caudally and lower level rostrally (blue gradient). Therefore, thalamic axons are sorted along the rostro-caudal axis of the ventral telencephalon according to the relative level of EphA receptors they express: axons emerging from rostro-medial domain of the thalamus avoid caudal territories expressing high levels of ephrin-A5, whereas axons emerging from caudo-medial territories project to more caudal parts of the ventral telencephalon being less sensitive to ephrin-A5 repulsion. Interestingly, a gradient of the transcription factor Ngn2 is observed in the early thalamus and Ngn2 plays a cell-autonomous role in the specification of the responsiveness of rostral thalamic axons to ventral telencephalic cues through an unknown mechanism. The question marks refer to unknown cortical attractive cues that are likely to play a role in the final areal targeting of thalamic axons once they enter the dorsal telencephalon (evidence reviewed in Vanderhaeghen and Polleux, [2004]).
(B) Interestingly, the pattern of EphAs receptors expression are drastically changing from whole dorsal thalamus gradient (prior to E15) to a thalamic nucleus specific set of gradients (after E15). Indeed, EphA4/ephrin-A5 are reused to control intraareal mapping of a specific subset of thalamic axons—in this example, axons from the ventro-basal nucleus (VB) projecting to the primary somato-sensory areas (S1). Based on data from Dufour et al. (2003) and Seibt et al. (2003). Adapted from (Marin, 2003).

in the ventral telencephalon but also in the thalamus itself. What was clearly lacking at this point to this field was a solid in vitro assay that could recapitulate some of key aspects of the topography of thalamocortical projections. This was achieved by Seibt et al. (2003) who designed a simple "telencephalic wholemount" assay where an explant of EGFP-expressing dorsal thalamus is cocultured in vitro with a whole telencephalic vesicle flattened on a membrane support. The authors used this almost bidimensional assay to show that axons originating from different portions of the dorsal thalamus (DT) respond differentially to cues located in the ventral telencephalon: axons from the rostral DT tend to grow more rostrally in the ventral telencephalon than axons from the caudal DT. This assay also enables mismatching of genotypically distinct thalami and telencephalons in order to test the cell-autonomous and cell-nonautonomous function of a gene. Seibt et al. demonstrated that Neurogenin2 (Ngn2), a bHLH transcription factor expressed specifically by a subset of rostral thalamic neurons, specifies cell autonomously the response of thalamic axons to cues encountered in the ventral telencephalon that guide these axons toward the rostral portion of this intermediate target and as a consequence to the frontal cortex (Seibt et al., 2003) (Figure 1A). This assay was also used in a collaborative study to identify the role of the repulsive axon-guidance molecule ephrin-A5 in the establishment of the topography of projections of thalamic axons at the level of the ventral telencephalon (Dufour et al., 2003) (Figure 1A). Interestingly, in the same study, the authors also demonstrated that ephrin-A5 is reused later at the level of the primary somatosensory cortex in which a gradient of ephrinA5 expression is controlling the intraareal mapping of ventro-basal axons (Figure 1B).

These recent results have changed the way we view how the interareal topography of thalamocortical projections is established during development and points to the general importance of intermediate target guidance cues not only for simple axon pathfinding decision but also for patterning the topography of axon projections. Future investigations will further explore the role of the ventral telencephalon as an intermediate target focusing on the identification of other guidance cues, but many other questions remain: previous evidence has suggested the existence of cortical cues necessary for the final areal targeting of a given subset of thalamic axons (reviewed in Vanderhaeghen and Polleux [2004]) (question marks in Figure 1A). However, to date, in vitro evidence has failed to demonstrate responsiveness of specific thalamic axons to these cues. Is the ventral telencephalon playing a role in priming the response of thalamic axons to these cues? Are some of the same mechanisms playing a role in the establishment of the topography of corticofugal axons?

Corticospinal Projections. Neurons located in layers 5 and 6 of the cortex project onto subcortical targets such as the tectum, the dorsal thalamus, the basal gan-

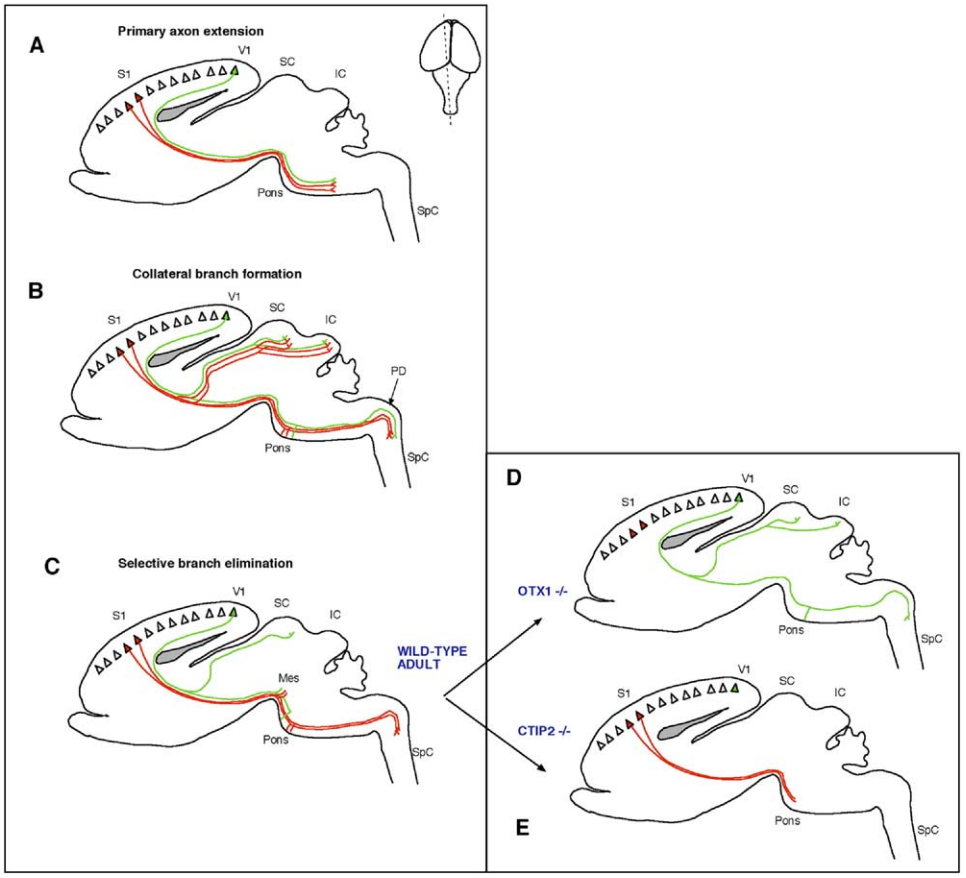

Figure 2. Genes Involved in Distinct Stages of Area-Specific Development of Layer 5 Projections

(A–D) Axons from layer 5 pyramidal neurons of nearly all cortical areas are initially projecting subcortically toward the spinal cord (A), and then axon collaterals emerge at specific sites along these axons, for example, toward the tectum or the pons (B). Finally, axons branches are selectively eliminated in an area-specific manner (C). Several genes expressed in layer 5 subcortically projecting neurons have been involved in some of these processes: first, in *Otx1*$^{-/-}$ mice (D) layer 5 pyramidal neurons from the visual cortex fail to prune their spinal cord collateral and continue to project both to the tectum and the spinal cord (Weimann et al. 1999). In a recent study, Arlotta et al. (2005) have shown that axons from layer 5 pyramidal neurons of the somato-motor cortex of *Ctip2*$^{-/-}$ mice fail to reach the spinal cord, suggesting a cell-autonomous function of this gene in specifying the initial projection pattern of a specific subpopulation of layer 5 neurons. IC, inferior colliculus; Mes, mesencephalon; SC, superior coliculus; SpC, Spinal Cord; S1, primary somatosensory cortex; V1, primary visual cortex. Adapted from O'Leary and Koester (1993) and Weimann et al. (1999).

glia, the spinal cord, the mesencephalon, etc. These efferent projections are area specific in the adult: for example, corticospinal neurons are only found in layer 5 of the sensori-motor cortex, whereas corticotectal projections are only found in layer 5 of the visual cortex projecting to the superior colliculus and auditory cortex projecting to the inferior colliculus (O'Leary and Koester, 1993). How does this area-specific pattern of corticofugal projections emerge during development? In rodents for example, layer 5 neurons from all cortical areas initially project toward the spinal cord (Figure 2A), and then collateral axon branches form at specific positions along these axons to invade mesencephalic territories (Figure 2B) (O'Leary and Koester, 1993). Therefore, soon after birth in rodents, there is a fairly uniform pattern of connectivity where, for example, layer 5 axons from the visual cortex not only project to the tectum but also to the spinal cord. During early postnatal development, there is an area-specific selective elimination of axon branches so that layer 5 pyramidal neurons from the visual cortex retract their axon branch from the spinal cord, whereas layer 5 neurons from the somato-motor cortex selectively eliminate their collateral from the tectum. The molecular mechanisms patterning this complex series of choices (initial axon guidance, axon branching, and selective branch elimination) are poorly understood at the molecular level. This is partially because of the lack of systematic molecular characterization of gene expression for a specific subpopulation of layer 5 neurons (in a given area) at different times corresponding to these three partially overlapping steps (roughly in mice, E18-P1 for initial guidance, P0-P6 for branch formation, and P6-P14 for selective branch elimination) (O'Leary and Koester, 1993). One pioneering study from the group of Susan McConnell showed several years ago that the homeodomain-containing transcription factor Otx1, which is expressed by all subcortically projecting layer 5 neurons (but not layer 5 callosal neurons), played an important role in the last step of selective branch elimination.

In fact, in adult Otx1 knockout mice, layer 5 visual neurons project both to the tectum (aberrantly to both superior and inferior colliculi) and to the spinal cord (Weimann et al., 1999). However, our understanding of the molecular control of these processes is still very poor.

A recent study by the group of Jeff Macklis is clearly changing this perspective (Arlotta et al., 2005). This group has combined gene profiling technology with retrograde axon tracing techniques and Fluorescence-based Activated Cell Sorting (FACS) technique in order to identify genes expressed by three classes neurons: corticospinal (CSN), corticotectal (CT), and callosal neurons projecting to the contralateral hemisphere (CN). The two important technical improvements of this study are that the authors isolated well-defined subpopulations of neurons based on their projections and their cortical area of origin (somato-motor versus visual) at four time points corresponding to critical stages of the development of these projections (E18, P3, P6, and P14) (see above). The fluorescently labeled neurons from different cortical regions were then dissociated and enriched by FACS sorting, and their mRNA was harvested for later hybridization to gene-chip microarrays. The results are very impressive: the authors isolated many genes that were confirmed by in situ hybridization to be expressed in layer 5 neurons. Many of the genes encode transcription regulators, axon guidance receptors, signaling molecules, etc. The authors did not stop there but rather chose one of the CSN-specific genes called *Ctip2* (for COUP-TF1 interacting protein 2) and tested its function in vivo in the development of CSN projections. They produced a *Ctip2* knockout mouse and demonstrated that these mice have a strong disorganization of the internal capsule and importantly that axons originating from the sensori-motor cortex do not reach the spinal cord but are stalled at the level of the pons (Figure 2E). This study opens up a new perspective with regard to our ability to perform gene expression profiling of selected classes of neurons defined by their projection patterns.

Callosal Projections and Intracortical Connections. These two important projections have been the focus of less attention than the thalamocortical and corticofugal projections. Interestingly, dozens of genes have been found incidentally to affect the formation of callosal projections resulting in "acallosal" brain and, in fact, Agenesis of the Corpus Callosum (ACC) is a birth defect that occurs in approximately 50 different congenital syndromes (reviewed by Richards et al. [2004]). It is likely that many of the gene disrupted in mice or congenital syndromes in humans are detected more efficiently than other defects of cortical projections because the absence or reduction of the corpus callosum is relatively easy to detect and typically nonlethal. Recent progress has been made in the identification of some of the key cellular and molecular cues that mediate attraction toward the midline and then midline crossing by callosal axons (Shu et al., 2003a, 2003b).

Finally, the molecular mechanisms patterning cortico-cortical projections are probably the least studied of all cortical projections. In the visual cortex, these long-range cortico-cortical projections mediate most of information processing, and their anatomy and function is studied rather intensely (Salin and Bullier, 1995). They can be categorized in two broad classes, feed-forward and feed-back projections, based on the fact that they link two cortical areas of increasing (feed-forward) or decreasing (feed-back) rank in the cortical "hierarchy" (Batardiere et al., 2002; Felleman and Van Essen, 1991). Interestingly, these two types of projections in primates originate from different layers and mature at a very different tempo (Barone et al., 1996; Batardiere et al., 2002). Given the early specificity of these projections but also the pronounced degree of remodeling during postnatal development, cooperation between axon-guidance mechanisms and activity-dependent remodeling is likely occurring. However, the molecular and cellular mechanisms underlying the early specificity of cortico-cortical connections in mammals is poorly understood and only few laboratories study this at the molecular levels. One of the reason is that these connections are not well developed in rodents and are actually a landmark of mammalian evolution increasing in relative importance and specificity with the large increase of the total cortex size and the total number of cortical areas characterizing carnivores and primates (Salin and Bullier, 1995). Recently, however, a study by the group of John Rubenstein has defined the framework for more studies in rodents (Huffman et al., 2004). In this study, the authors have used dual-fluorescent-retrograde tracing to label the cortical regions connected to a given "point" in the occipital and frontal cortex of early postnatal mice. The authors found that there is a clear bias for neurons in the occipital pole to receive projections from neurons located in the caudal half of the cortex, whereas neurons in the frontal cortex tend to receive projections from neurons located in the rostral half of the cortex. They also show that this mutually exclusive level of convergence is lost in *Fgf8* hypomorphic mice (Huffman et al., 2004). In fact, the authors had shown previously that neurons in the rostral cortex of Fgf8 hypomorphs acquire a new molecular identity characteristic of more caudal cortical neurons (Garel et al., 2003). These observations show that Fgf8 acting as a rostral organizer not only specifies the expression of area-specific molecular markers (Fukuchi-Shimogori and Grove, 2001) but also specifies the topography of cortico-cortical projections characterizing frontal cortical neurons (Huffman et al., 2004).

Still numerous questions remain. What are the axon guidance mechanisms restricting the outgrowth of cortico-cortical projections? Are there area-specific stop signals for a given class of cortico-cortical axons? Is the expression of these putative stop signals dependent on pattern of spontaneous neuronal activity?

Conclusion

There is clearly a lot of work ahead in order to identify the molecular mechanisms underlying the specificity of afferent and efferent as well as local cortical projections and how these early mechanisms are interfaced with activity-dependent mechanisms underlying connectional remodeling. However, we are clearly entering a new era in which the technologies we have at hand will undoubtedly enable us to start deciphering how the complex pattern of cortical connectivity emerges during normal and abnormal development.

Selected Resources on the Web

Gene Trap Projects. Gene Trap Core (J. Rossant and W. Stanford), http://www.cmhd.ca/sub/genetrap.asp; Gene Trap ES cells database (P. Soriano), http://www.fhcrc.org/labs/soriano/trap.html; BayGenomics site, http://baygenomics.ucsf.edu/; Gene Trap Consortium, http://www.genetrap.org/. This Consortium includes different gene-trapped ES cells and mice, including those produced by the Tessier-Lavigne and Skarnes groups.

Other Useful Public Resources. Mutagenic Insertion and Chromosome Engineering Resources (MICER), http://www.sanger.ac.uk/micer/. This project, directed by Allan Bradley at the Wellcome Trust Sanger Institute, has produced 93,960 ready-made gene-targeting vectors that can be easily modified to express EGFP, LacZ, Cre-, or Flp-recombinases or any other cDNA of interest and then used for homologous recombination in preidentified sites of the mouse genome. The potential genomic insertion sites are available directly from the Ensembl database (http://www.ensembl.org/).

Developing and Adult Brain Gene-Expression Screens. Functional Genomic Atlas of the mouse brain, http://mahoney.chip.org/mahoney/database.html; Allen Brain Atlas, http://www.brainatlas.com/; Genepaint http://www.genepaint.org/; Emage, http://genex.hgu.mrc.ac.uk/Emage/; BGEM (Brain Gene Expression Map), http://www.stjudebgem.org/; GENSAT, http://www.gensat.org/.

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