

Dual Roles of Transcription Factors in Forebrain Morphogenesis and Development of Axonal Pathways

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Introduction

During its development the brain must generate a variety of neural structures and organise the correct axonal connections between and within them. In this Chapter we concentrate on how transcription factors specify both these processes in the developing eye and forebrain. It is now well-established that regionally expressed transcription factors regulate the morphogenesis of each region of the brain. More recently, many of these same transcription factors have been implicated in regulating the development of axonal pathways including those providing sensory inputs to the cerebral cortex. In some cases there is evidence that the effects of transcription factors on axonal development involve direct, cell autonomous actions.

The recent sequencing of the mouse and human genomes has allowed estimates of the number of protein coding genes required to generate a mouse and a human. It appears that about 30,000 proteins are sufficient to generate a mammal. Given the enormous complexity of the finished product, the construction of the animal during development would seem to demand that the available genes are used efficiently. One way of doing this would be to allow a particular gene to participate in several developmental processes. The use of the same transcription factors for both tissue morphogenesis and axonal growth and guidance may be an example of the efficient use of available genetic resources.

In this Chapter we consider three possible mechanisms of gene action. The first regulates morphogenesis, the second and third regulate axon guidance. (1) A gene may coordinate the proliferation, differentiation, migration and death of cells required to generate tissue shape or cell type composition, for example the cup-shaped retina with its six cell types organised in their characteristic laminated pattern. (2) A gene may control the properties of a cell projecting an axon, for example by regulating the expression of proteins on the navigating growth cone of

a thalamocortical axon. (3) A gene may influence axon navigation by regulating the properties of the environment through which the growth cone must navigate, for example by regulating the proteins expressed at the optic chiasm where retinal axons are sorted into the optic tract.

Transcription factors are proteins that bind to DNA and regulate the transcription of genes into messenger RNA (mRNA) and control the amount available to translate into protein. A given transcription factor may regulate the expression of many target genes. Mouse genetics have allowed the importance of transcription factors in eye and forebrain development to be tested by examining the consequences of perturbing their expression. An emerging theme is that many transcription factors have dual roles in forebrain morphogenesis and development of axonal pathways and the next section examines the roles of the transcription factors *Foxd1*, *Foxg1*, *Islet2*, *Pax2*, *Pax6*, *Vax1*, *Vax2* and *Zic2* in these processes. We examine the behaviour of RGC axons at the optic chiasm in particular detail. The final section examines the several roles of *Pax6* in specifying the morphology and connectivity of the forebrain.

Untangling the Roles of Transcription Factors in Regulating Both Tissue Morphogenesis and Axonal Development

In some ways, examining a mutant phenotype can be likened to a crash investigation where the aim is to identify the cause of the crash from a mangled pile of wreckage. Tissue morphogenesis generally precedes axon navigation and so disrupting a gene with a role in both morphogenesis and axon navigation may produce a mutant animal with an abnormally shaped brain and with axon pathfinding errors. It is not always obvious whether the axon pathfinding errors are a mechanical consequence of a change in brain shape, or whether they reflect a subsequent direct [and in this context more interesting] alteration in the adhesive or other properties of the navigating growth cone and the cells through which it navigates. As in the case of the crash investigation, identifying the primary cause of observed defects is a vital concern.

There are several experimental approaches available to dissect the causality of axon guidance mistakes in mutant mice where the (1) the gene is expressed in both the cells projecting axons and in the tissues through which they navigate or (2) in which disrupted brain shape precedes axon navigation and can complicate the analysis of axon guidance phenotypes. Mouse mosaics comprising mixtures of wild-type and mutant cells are powerful tools for determining the site of action of a particular gene. These can be in the form of chimeras produced by the fusion of a wild-type and a mutant embryo or conditional gene knockouts in which the gene of interest is mutated in a genetically defined subset of cells at a specific time point in their differentiation. Because they contain wild-type cells, mosaics also have the potential to minimise any alterations in brain shape that might complicate the analysis of unconditional

mutants. Another approach is to combine wild-type and mutant tissues in culture. Both *in vivo* and *in vitro* approaches provide the opportunity to observe the behaviour of axons projected by mutant cells into a wild-type environment and *vice versa*. If axons projected by mutant cells make navigation errors when navigating a wild-type environment, or wild-type axons are able to navigate correctly through a mutant environment, this shows that the gene is required to program the responsiveness of the growth cone to its environment. Finding mutant axons navigate a wild-type environment correctly shows that the gene is required outwith the growth cone to supply it with guidance cues. Another possibility is that both wild-type and mutant axons navigate correctly through both wild-type and mutant environments, in which case the navigation errors seen in the unconditional mutant are in fact secondary to other factors such as aberrant morphogenesis.

Transcription Factors and the Development of the Visual Pathway

Normal Development of the Eye and Visual Pathway

During normal development, at around embryonic day 9 (E9) in the mouse, the retina, retinal pigment epithelium, and optic stalk are formed from an out-bulging of the ventral diencephalic neuroepithelium that undergoes a series of folding manoeuvres in concert with ectodermal tissue that will form the cornea and lens (reviewed by Smith et al., 2002). The retina and retinal pigment epithelium form distally. The retina is initially open at its ventral surface (the choroid fissure) but this soon closes to complete the familiar eye ball shape. The optic stalk is formed from more proximal diencephalic tissue. The retina then differentiates to generate several cell types including retinal ganglion cells (RGCs) that project axons to the brain (Cepko et al., 1996). The first RGC axons exit the retina at the optic nerve head at E12 and navigate along the optic stalk to form the optic nerve, which connects to the ventral surface of the brain at the optic chiasm. In mice the vast majority of retinal axons cross the ventral midline at the optic chiasm and join the contralateral optic tract whereas a minority do not cross and join the ipsilateral tract (Fig. 1A). The optic tract then grows over the surface of the thalamus and onto the superior colliculus.

The following sections examine the consequences of mutating transcription factors in transgenic mice for the formation of the structures of the eye and chiasm and the navigation of RGC axons along the optic nerves, through the chiasm, and into the optic tract. The transcription factors are dealt with in pairs reflecting functional relationships revealed by complementary expression domains (Fig. 1) and defective axon navigation phenotypes in mutants. These examples serve to illustrate the dual roles of transcription factors in tissue morphogenesis and axon guidance, the experimental approaches used to dissect these processes, and the challenges posed by these types of experiment.

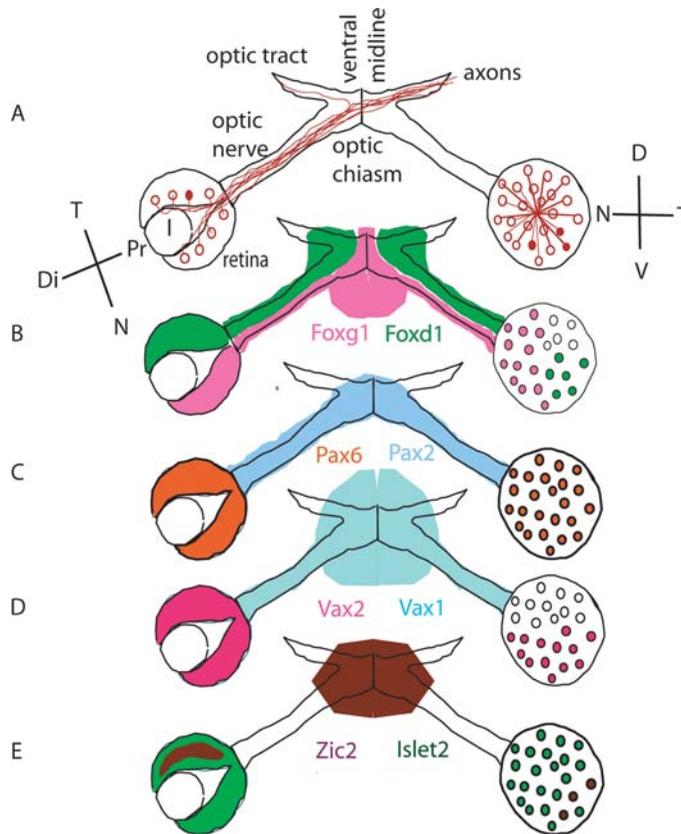


Figure 1 Diagram showing the relationship between the structures of the developing visual system in the eye and ventral forebrain, the trajectory of its axons, and the expression of transcription factors regulating its formation. (A) Retinal ganglion cells (RGCs) project axons along the inner surface of the retina to the optic nerve head where they exit the eye to form the optic nerve. The optic nerve contacts the ventral surface of the hypothalamus at the optic chiasm where axons are sorted into the optic tracts. The retina on the left is viewed in horizontal section, the retina on the right is viewed head on, parallel to the optic nerve. Ipsilaterally and contralaterally projecting RGC bodies are represented by filled and open ovals respectively. (B–E) The expression of transcription factors is mapped onto the RGCs and the structures of the developing visual system through which their axons navigate: (B) *Foxg1* and *Foxd1*; (C) *Pax2* and *Pax6*; (D) *Vax1* and *Vax2*; (E) *Zic2* and *Islet2*. Abbreviations: D, dorsal; Di, distal; N, nasal; l, lens; Pr, proximal. Literature on which this diagram is based is cited in the text.

Foxg1 and Foxd1

Foxg1 (formerly called BF1) and *Foxd1* (formerly called BF2) are fork-head box winged helix transcription factors expressed throughout the development of the eye and optic chiasm. The expression of these genes is strikingly complementary with *Foxg1* expressed in the nasal retina and anterior optic chiasm and *Foxd1* being restricted to the temporal retina and posterior chiasm (Fig. 1B; Hatini et al., 1994; Xuan et al.,

1995; Huh et al., 1999; Marcus et al., 1999). Experiments in the chick have shown that forced expression of *Foxd1* and *Foxg1* in the retina directly controls the retinotectal mapping of RGC axons (Yuasa et al., 1996; Takahashi et al., 2003), indicating that these genes are capable of directly influencing the properties of the navigating RGC growth cone. Mice lacking these genes exhibit defects in several aspects of eye and forebrain morphogenesis and retinal axon guidance. Careful examination of their mutant phenotypes reveals that these genes may well be involved in simultaneously regulating the properties of the navigating retinal growth cone and in defining the properties of the environment through which it navigates.

The most obvious consequences of depriving the embryo of *Foxg1* are the abnormal shape of the eyes and forebrain (Xuan et al., 1996). The abnormal shape of the forebrain is due mainly to an extremely hypoplastic telencephalon. The eye develops an elongated retina which fails to close properly, resulting in coloboma, and the lens is small (Huh et al., 1999). These morphological defects are not restricted to nasal territory which normally expresses *Foxg1*, suggesting a non-autonomous role for *Foxg1* in morphogenesis of temporal eye structures. The eye lacks an optic stalk and the retina connects directly to the base of the brain at the optic chiasm. Loss of *Foxg1* does not dramatically affect the dorso-ventral patterning of the eye, as evidenced by the fact that the reciprocal gradients of the receptor tyrosine kinase *EphB2* and its ligand *ephrinB2* are maintained in the mutant. Naso-temporal polarity is not abolished in the mutant: *Foxg1* gene activation remains predominantly nasal and ipsilaterally projecting RGCs are located predominantly in temporal retina, as in wild-types (Pratt et al., 2004). The expression domain of *Foxd1* does, however, encroach upon nasal territory, which normally expresses *Foxg1* but not *Foxd1* (Huh et al., 1999). In spite of this the mutant generates retinal ganglion cells (RGCs) which project axons along the inner surface of the retina, where they fasciculate and enter the optic tract via the optic chiasm. Although the overall trajectory of retinal axons in the mutant strongly resembles that seen during normal development (Pratt et al., 2002), *Foxg1* is required for at least one important aspect of axon pathfinding. In the *Foxg1*^{-/-} mutant the ipsilateral projection is massively increased and matches the size of the contralateral projection. *Foxg1* therefore normally suppresses the ipsilateral projection of RGC axons. In the nasal retina RGCs normally express *Foxg1* and so might repress the expression of proteins required for ipsilateral projection or might activate the expression of proteins required for contralateral projection. In the temporal retina it is more likely that *Foxg1* assists the contralateral projection of RGCs, which never express *Foxg1*, by regulating the expression of navigational instructions supplied to RGC growth cones by cells at the optic chiasm and other points along their journey (Pratt et al., 2004). It remains an open question as to whether the expression of *Foxg1* by nasal RGCs is directly involved in the midline crossing behaviour of these axons.

Foxd1 is normally expressed in temporal retina and optic stalk and in the posterior chiasm. Its complementary expression to Foxg1 might suggest that these related genes perform similar functions in their respective domains of the developing visual pathway, but comparison of the Foxg1 and Foxd1 mutant phenotypes shows it is not that simple. The morphology of the Foxd1 mutant eye is not greatly disturbed, but there are alterations to the expression of genes whose expression normally coincides with Foxd1. These include a loss of the ipsilateral determinants Zic2 (a transcription factor, see below) and EphB1 (Williams et al., 2003) from the ventral-temporal retina and an invasion of Foxg1 expression into temporal territory normally occupied by Foxd1. Perhaps surprisingly, in light of the loss of ipsilateral determinants from the ventro-temporal retina, the Foxd1 mutant exhibits an increased ipsilateral projection. Closer examination shows that the ipsilateral projection from the ventro-temporal retina is indeed reduced consistent with a cell autonomous role for Foxd1 in these RGCs. The increased ipsilateral projection arises mostly from RGCs located outside the normal domain of Foxd1 expression in the ventro-temporal retina. RGCs located outside the ventro-temporal retina would not normally express Foxd1 and would normally cross the midline at the optic chiasm to join the contralateral optic tract. This increased ipsilateral projection is attributed to alterations of the molecular properties of the *Foxd1*^{-/-} chiasm including a reduction in expression of Zic2 and Islet1 (both transcription factors) and an expansion of the expression domain of Slit2 (Herrera et al., 2004). Slit family members Slit1 and Slit2 are expressed around the optic chiasm as it develops (Erskine et al., 2000) and their mutant phenotypes indicate a repulsive role for these proteins in preventing RGC axons from wandering from their normal path (Plump et al., 2002).

Foxg1 and Foxd1 mutually repress each other's expression, either directly or indirectly, but it is at present unknown whether Foxd1 and Foxg1 each regulate the expression of the same target genes in the retina and optic chiasm or whether the presence of different cofactors in these two structures allows participation in distinct molecular programs. It is also unknown whether their target genes involved in regulating morphogenesis are the same as those engaged in axon navigation.

Pax6 and Pax2

Pax2 and Pax6 are dynamically expressed during the early development of the eye. As morphogenesis proceeds Pax6 becomes restricted to more distal structures including the lens, retinal pigment epithelium, and retina. Pax2 is expressed in the optic fissure as it closes, in the optic stalk, and in the preoptic area of the ventral diencephalon, where contralaterally projecting RGC axons will cross the midline at the optic chiasm (Fig. 1C). A combination of elegant transgenic and *in vitro* experiments demonstrated that Pax2 and Pax6 bind to regulatory elements in each other's promoters to mutually repress transcription (Schwarz et al., 2000). Pax2 and Pax6 are required for the formation of optic stalk and optic cup respectively, as shown by the lack of optic cup in Pax6

mutant embryos (Hill et al., 1991) and optic stalk in Pax2 mutant embryos (Torres et al., 1996).

The *Pax6* gene has retained its ability to specify the formation of an eye in species as diverse as *Drosophila*, *Xenopus*, mouse and humans. Loss of Pax6 results in a failure of the eye to form. Although Pax6 has not yet been shown to have a role in the navigation of retinal axons, *Pax6* is expressed by projecting RGCs (Baumer et al., 2002) and so is poised to fulfil this function. Certainly, in other parts of the developing brain Pax6 has functions in axon guidance as well as in tissue morphogenesis and regulates genes implicated in axon guidance (see below).

Pax6 is expressed in both surface ectoderm and optic vesicle tissues, which integrate to generate the structures of the eye. These fail to progress past their very early development in embryos completely lacking Pax6. This complicates the examination of the functions of Pax6 in subsequent events in eye formation, including its roles in morphogenesis and axon guidance. This problem has recently been addressed by the use of Cre-lox technology to selectively disrupt Pax6 in discrete parts of the developing eye. The studies have shown that removing Pax6 from the developing surface ectoderm produces an eye lacking a lens but possessing a retina with RGCs able to project axons (Ashery-Padan et al., 2000). Removing Pax6 function after the retina forms results in a retina comprising mainly amacrine cells at the expense of other retinal cell types including RGCs (Marquardt et al., 2001). Examination of *Pax6*^{+/+} ↔ *Pax6*^{-/-} mouse chimeras has shown that Pax6 is required in the optic vesicle for maintenance of contact with the overlying lens epithelium, a necessary event in eye formation, providing a clue that Pax6 may be involved in defining the adhesive properties of these cells. Pax6 appears to act in a cell autonomous manner in these aspects of eye development (Collinson et al., 2000; Quinn et al., 1996). The dosage of Pax6 is important as increasing (Schedl et al., 1996) or decreasing (Hill et al., 1991) *Pax6* gene dosage in the eye both result in aberrant eye development.

As discussed above, an important aspect of the developing retina with consequences for the trajectory of its axons is the establishment of naso-temporal and dorso-ventral polarity defined by the expression of proteins including the transcription factors Foxg1 and Foxd1 and the EphB2 receptor tyrosine kinase. In embryos where Pax6 has been conditionally ablated from the retina, expression of both Foxg1 and Foxd1 is lost indicating that Pax6 may be required in the generation of nasal-temporal polarity (Baumer et al., 2002). In the chick retina Pax6 is expressed in a ventral^{High} to dorsal^{Low} gradient coincident with the gradient of EphB2 expression (Ziman et al., 2003). Although no such retinal Pax6 gradient has been reported in the mouse, Pax6 may be involved in specifying the dorso-ventral polarity as in the absence of Pax6 the optic vesicle loses its dorsal expression of the transcription factor Tbx5 while the ventral expression domain of Vax1 is expanded (Baumer et al., 2002). Genetic dissection of the *Pax6* locus has revealed that Pax6 expression is controlled independently in different parts of the

developing eye. For example, although Pax6 is expressed throughout the developing retina, expression in its distal regions is specifically driven by an ' α element'. Furthermore this element continues to direct Pax6 expression in a subset of RGCs as they project axons into the brain (Baumer et al., 2002).

The expression of Pax2 is complementary to that of Pax6. Whereas Pax6 expression is restricted to the structures of the developing eyeball (lens, retina, retinal pigmented epithelium), Pax2 is expressed in the developing optic stalk and optic chiasm. Mice lacking Pax2 produce elongated retinas, probably at the expense of optic stalk tissue, reminiscent of those seen in Foxg1 mutant embryos described above. The Pax2 mutant retinas are able to project RGC axons which form an optic nerve. The optic nerves from the two eyes do not converge to form the optic chiasm as in wild-types but instead project ipsilaterally to their targets in the brain. Pax2 mutants are therefore classed as achiasmatic (Torres et al., 1996). As Pax2 is not expressed by RGCs but is expressed at the location where the chiasm normally forms it is likely that Pax2 is needed to specify the formation of the preoptic area, whose cells normally support the contralateral projection of RGC axons (Torres et al., 1996).

Vax1 and Vax2

Vax1 and Vax2 are homeodomain containing transcription factors that exhibit complementary expression patterns in the developing visual system. Vax2 is restricted to the developing retina and Vax1 is expressed by cells at the midline where RGC axons form the optic chiasm (Fig. 1D; Hallonet et al., 1998; Bertuzzi et al., 1999; Hallonet et al., 1999). Vax1 is required for morphogenesis of the eye as the optic cup fails to close properly resulting in coloboma in embryos lacking Vax1. The boundary between mutant optic cup and optic stalk is poorly defined with regions normally occupied by optic stalk exhibiting retinal features including retinal pigment epithelium. Although RGCs form in these mutants, their axons navigate abnormally and, instead of approaching the midline to form the optic chiasm, become stalled shortly after leaving the eye (Bertuzzi et al., 1999; Hallonet et al., 1999). As Vax1 is not expressed by RGCs this defect is most likely to reflect a requirement for Vax1 in producing the correct environment for navigating axons. Indeed, Netrin-1, that is normally expressed at the optic nerve head and at the point where the optic nerve connects to the brain and is believed to guide axons along their path (Deiner et al., 1997; Deiner et al., 1999), is missing in Vax1 null-mutants. This provides a plausible molecular mechanism for the inability of RGC axons to reach the chiasm (Bertuzzi et al., 1999).

Vax2 expression is restricted to the ventral region of the prospective neural retina. In embryos lacking Vax2 the optic cup fails to close resulting in coloboma. Vax2 appears to specify ventral character. Its absence causes loss of the expression of EphB2, which is normally present in ventral retina, and expansion of ephrinB2 expression, which is normally

restricted to dorsal retina, throughout the mutant retina. *Vax2* mutant mice generate RGCs which, unlike those in *Vax1* mutants, are able to navigate to the optic chiasm and into the brain. As ipsilaterally projecting RGCs are present in ventral retina and in *Vax2* mutants the ventral retina acquires a dorsal character, it might be predicted that the ipsilateral projection would be lost in these mutants. This was reported to be the case in one line of *Vax2* null-mutant mice (Barbieri et al, 2002) but in a different line of *Vax2* null-mutant mice produced by another group (Mui et al., 2002) the dorsalisation of retina produced an increased ipsilateral projection. This discrepancy may reflect differences in the mutant *Vax2* alleles or in their genetic backgrounds.

Zic2 and Islet2

Zic2 is a zinc finger protein homologous to the *Drosophila* gene *odd-paired* that is widely expressed in neural and non-neural tissues in the mouse. In the developing visual system *Zic2* is restricted to ventrotemporal retina and cells around the chiasm (Fig. 1E). In the E15 retina at the time RGC axons are sorted into ipsilateral and contralateral optic tracts, *Zic2* expression is restricted to the ventro-temporal quadrant of the retina from which the ipsilateral projection arises. *Zic2* is also expressed at the optic chiasm. Targeted disruption of the *Zic2* gene produced a *Zic2^{kd}* allele (*kd* indicates a 'knockdown' allele in which *Zic2* function is reduced rather than completely abolished as in a 'knockout' allele). *Zic2^{kd/kd}* embryos have profound morphological brain defects including hypoplasia of the dorsal telencephalon, disruption to midline structures, and eye defects. In contrast *Zic2^{kd/+}* embryos have morphologically normal eyes and brains (Nagai et al., 2000). In addition to this early role in specifying the morphology of brain structures associated with the optic tract, *Zic2* also appears to directly control the trajectory of retinal axons. The size of the ipsilateral projection is reduced in *Zic2^{kd/+}* embryos and *in vitro* experiments showed that RGCs forced to express *Zic2* produce axons that are repelled by the optic chiasm. A comparison of *Zic2* expression across species with different degrees of binocular vision shows a positive correlation between the number of RGCs expressing *Zic2* and the size of the ipsilateral projection (Herrera et al., 2003). Although these experiments are consistent with *Zic2* regulating the navigation properties of RGC growth cones, *Zic2* is also expressed at the optic chiasm so it is conceivable that *Zic2* also influences the navigation of RGC axons by regulating the expression of guidance cues at the optic chiasm. In fact, in the *Foxd1^{-/-}* mutant described above (Herrera et al., 2004) reduced expression of *Zic2* at the chiasm is associated with an increased ipsilateral projection.

Islet2 is a LIM homeodomain containing transcription factor. *Islet2* expressing RGCs are located throughout the retina and project contralaterally (Fig. 1E). In embryos lacking *Islet2* the ipsilateral projection is increased with the increased projection mapping exclusively to the ventrotemporal retina, coincident with an increase in the number of *Zic2* expressing RGCs. This suggests that in the ventrotemporal quadrant,

Islet2 represses *Zic2* expression by RGCs and therefore prevents them from projecting ipsilaterally (Pak et al., 2004). Ipsilaterally projecting RGCs express the receptor tyrosine kinase EphB1 which causes their axons to be repelled by its ligand ephrinB2 expressed on cells at the optic chiasm (Nakagawa et al., 2000, Williams et al. 2003). It remains to be determined whether *Zic2* specifies ipsilateral projections by directly positively regulating the transcription of EphB1 and whether *Zic2* transcription is in turn negatively regulated by Islet2.

One feature of the above genes is that they are needed to regulate the structures of the eye and forebrain and the degree of ipsilateral and contralateral projection by RGCs. This is intriguing since, whereas the physical structure of the eye and the developing visual pathway is highly conserved between vertebrates, the fine details of axon organisation within the ubiquitous X-shape formed by the optic nerves, chiasm, and tract varies considerably. For example, there is considerable variation between species in the proportion of axons sorted into the ipsilateral and contralateral optic tracts. It might seem a risky strategy to employ the same gene to regulate the shape of the eye, that is relatively fixed in evolution, and the fine tuning of its RGC projections, that is far more plastic. Perhaps these different aspects come under the control of distinct regulatory genetic elements that can evolve independently. Further diversity can be achieved by the production of several functionally distinct isoforms with distinct transcriptional properties from a single gene, for example by differential splicing.

Transcription Factors that Regulate the Development of the Thalamocortical Tract

The thalamus can be thought of as a 'relay station' for sensory information from the periphery (sight, touch, taste, and hearing) passing through the thalamus *en route* to the cerebral cortex for processing and interpretation. In the mouse, axons exit the dorsal thalamus at E12.5 and grow through the ventral thalamus. They make a sharp lateral turn at the hypothalamus and enter the ventral telencephalon through the internal capsule (Braisted et al., 1999, Tuttle et al., 1999, Auladell et al., 2000). The thalamic axons then grow into the cerebral cortex where they form synapses with layer 4 neurons. The basic thalamocortical circuitry is complete at this point. The navigation of the thalamocortical axons has complex spatial (as the tract describes a three dimensional geometry) and temporal (as all thalamic axons do not navigate synchronously) dimensions. The section below concentrates on how the complex spatial and temporal expression of the transcription factor Pax6 contribute to several aspects of the formation of the structures of the thalamocortical tract and the navigation of its axons.

Several transcription factors have been implicated in the control of thalamocortical development on the basis of defects in this pathway in mice with null mutations in the corresponding genes (reviewed recently

in Lopez-Bendito and Molnar, 2003). These factors include *Emx2*, *Tbr1*, *Gbx2*, *Mash1*, *Ebf1*, *Foxg1* and *Pax6*. Loss of *Gbx2*, *Mash1*, *Foxg1* or *Pax6* results in failure of thalamic axons to innervate the cortex (Miyashita-Lin et al., 1999; Tuttle et al., 1999; Pratt et al., 2000 & 2002); loss of other transcription factors cause more subtle targeting defects. Loss of these factors also cause morphological defects of the thalamus and/or the tissues through which thalamocortical axons normally grow. Expression of *Gbx2* is normally restricted to the thalamus and loss of this factor causes defects of thalamic differentiation (Miyashita-Lin et al., 1999); it is likely, therefore, that thalamic cells have an intrinsic requirement for *Gbx2* to allow their innervation of the cortex. *Foxg1*, on the other hand, is not expressed by thalamic cells but is expressed by ventral telencephalic territory through which thalamic axons normally grow. Failure of thalamic axons to enter the telencephalon in *Foxg1*^{-/-} mouse embryos is, therefore, most likely secondary to defects in the ventral telencephalon (Pratt et al., 2002). For other factors, the likely mechanisms are less clear since, in many cases, they are expressed in the thalamus and at other sites along the route taken by thalamocortical axons. In the case of *Pax6*, experiments outlined in the next sections have been carried out to test whether there might be a thalamic requirement for it to allow axons to navigate correctly.

How Pax6 Regulates the Morphogenesis of Thalamus and Cortex

Pax6 is expressed in the developing diencephalon. Up until about E12 in the mouse, *Pax6* is expressed in diencephalic regions that will become both the major elements of the thalamus. These elements are known traditionally as the dorsal and ventral thalamus, although they are probably better renamed as thalamus and prethalamus respectively. The thalamus is the major recipient of afferents from the sensory periphery and sends its thalamocortical efferents to the cerebral cortex. After E12, *Pax6* expression in the diencephalon becomes more restricted, mainly to the prethalamus, that lies rostral to the zona limitans intrathalamica (zli), although expression persists in the proliferating ventricular zone of the thalamus. In mice lacking *Pax6*, there are major defects in the development of these regions of the diencephalon. Their structure appears abnormal, with a reduction in the size and distortion in the shape of particularly the prethalamus. This is most likely due to a reduction of cell proliferation throughout the diencephalon in the absence of *Pax6* (Warren and Price, 1997). The major components of the diencephalon are present in mutants, but there are changes in the patterns of gene expression. These include changes in the expression of other regionally-expressed transcription factors (Grindley et al., 1997; Stoykova et al., 1996; Warren and Price, 1997; Pratt et al., 2000). For example, the expression domains of *Nkx2.2* and *Lim1* (also known as *Lhx1*) are expanded throughout the diencephalon, suggesting that a primary action of *Pax6* is to generate correct patterning in this region of the brain (Pratt et al.,

2000). *Pax6*^{-/-} cells do not intermingle freely with their wild-type counterparts in the thalamus of *Pax6*^{+/+} ↔ *Pax6*^{-/-} mouse chimeras indicating that Pax6 defines the adhesive properties of thalamic cells (Pratt et al., 2002). Thalamocortical axons start to grow at about E13-4 in both wild-type mice and in mice lacking Pax6 but, in mutants, they fail to navigate correctly through the ventral telencephalon and, even by the time of birth, when these mutants die, there is no cortical innervation from the thalamus (Auladell et al., 2000; Kawano et al., 1999; Pratt et al., 2000).

Pax6 is also expressed in the developing telencephalon. It is expressed dorsally in the developing cortex and hippocampus and also in some ventral regions, mainly in the region of the amygdala, through which thalamocortical axons normally grow. In the developing cortex and hippocampus Pax6 is expressed in proliferating progenitor cells but is downregulated in differentiating neurons. It is expressed from before the folding of the neural plate throughout neurogenesis. Recent work has shown that radial glial cells, which have been known for decades to guide the migration of neuronal precursor cells, are in fact neuronal progenitor cells and that they express Pax6 (Heins et al., 2002). Loss of Pax6 causes numerous defects in the morphology of the developing cerebral cortex. The cortex is smaller than normal, and cells become densely packed into numerous dense clusters in the intermediate zone (Schmahl et al., 1993; Caric et al., 1997). This has been ascribed to changes in the cell-surface properties of the mutant cells (Warren et al., 1999; Talamillo et al., 2003; Tyas et al., 2003). There is a failure of late-born cells to migrate into the cortical plate. This defect can be corrected by transplanting late-born cells into wild-type cortex, indicating that it is not a cell-autonomous defect but more likely secondary to defects of other cells (Caric et al., 1997). There are two main contenders for the primary source of this migration defect. First, the radial glial cells, which produce and provide guidance for migrating neuronal precursors, show defective morphology in the absence of Pax6 (Gotz et al., 1998). Second, thalamocortical axons can stimulate migration of cortical precursors and so loss of these inputs might impair migration in mutants (Edgar and Price, 2001).

How Forebrain Axon Pathways are Altered in Mutants Lacking Pax6

The early brain contains a primitive network of axonal tracts and there have been many studies of the development of these pathways in a variety of species. The first major longitudinal (i.e. coursing rostrocaudally) tract to form is the tract of the postoptic commissure (TPOC) which runs along the ventrolateral diencephalic surface and continues into the midbrain as the ventral longitudinal tract. Mouse embryos lacking Pax6 show pathfinding defects in the developing TPOC (Mastick et al., 1997; Andrews and Mastick, 2003; Nural and Mastick, 2004). Whereas in wild-type embryos TPOC axons spread out when they contact Pax6-expressing diencephalic neurons, in mutants they make errors

indicating that Pax6 is required for local cues guiding the navigational behaviour of TPOC axons as they enter its expression domain.

It has been shown that the cell adhesion molecule R-cadherin (Cdh4) is lost from the region in which TPOC navigational errors occur in mice lacking Pax6 and that axonal growth through this region can be restored by replacing R-cadherin. This indicates that the action of Pax6 in regulating early TPOC tract formation is mediated by the regulation of a cell adhesion molecule in the region through which the axons would grow. Expression of R-cadherin is also lost in the embryonic cerebral cortex of mice lacking Pax6 (Stoykova et al, 1997). In the cortex, this loss is thought to explain changes in the tangential and possibly radial migratory properties of neuronal precursors and hence the cellular constitution and morphology of this tissue. It seems, therefore, that the regulation of cell adhesion molecules by Pax6 is not only necessary for the correct development of tissues but also the subsequent navigation of axons through those structures. In the case of the TPOC, the transcription factor Pax6 is not expressed by the projecting neurons (Mastick et al., 1997; Andrews and Mastick, 2003) so its regulation of axonal navigation appears to be secondary to actions on regional expression of cell adhesion molecules by cells encountered by navigating axons.

Similarly, there is a cell non-autonomous role for Pax6 in regulating the guidance of the catecholaminergic neurons of the substantia nigra (SN) and the ventral tegmental area (VTA) (Vitalis et al., 2000). This is known to be cell non-autonomous since SN-VTA neurons do not express Pax6. Mice lacking Pax6 show defective pathfinding by SN-VTA projections as they cross regions that do express Pax6. It has been suggested that this can be attributed to an expansion of the expression domain of the axon guidance molecule Netrin-1. Jones et al. (2002) suggested that Pax6 is required for the normal development of thalamocortical axonal connections by regulating expression of surface molecules including Sema5A and Sema3C in the regions through which the axons grow.

There is also evidence that PAX6 is essential for the development of axon tracts in the human brain. It is well-known that humans heterozygous for mutations in PAX6 suffer from congenital aniridia but more recent work using magnetic resonance imaging (MRI) has revealed either the absence or hypoplasia of the anterior commissure of the brain in a large proportion of aniridia cases (Sisodiya et al., 2001).

The thalamus and cortex form at similar stages of gestation. Thalamic axons grow through the diencephalon, turn sharply laterally to enter the ventral telencephalon, cross the medial and lateral ganglionic eminences and then turn dorsally to penetrate the cortex. The mechanisms thought to direct thalamocortical axons to the cortex include guidance from (i) pioneering axons growing from cortex towards thalamus and (ii) a transient set of axons growing from the ventral telencephalon to the thalamus (Metin and Godement 1996; Molnar et al., 1998; Molnar 1998; Braisted et al 1999). In *Pax6*^{-/-} mutants, neither of these form correctly (Kawano et al., 1999; Hevner et al., 2002; Jones et al., 2002; Pratt et al., 2002) and so it is possible that defects of thalamocortical axons are secondary to the absence of normal descending projections. Jones

et al. (2002) examined the corticofugal projections in mice lacking Pax6 and described abnormalities of these axons at the corticostriatal junction. Jones et al. (2002) and Pratt et al. (2002) showed defects of ventral telencephalic cells within the internal capsule associated with altered early thalamic growth.

Is there any evidence that Pax6 plays a primary role in the projecting thalamic cells themselves, allowing them to navigate to their cortical targets? Evidence that this is the case has come from co-culture studies (Pratt et al., 2000). Explants from either wild-type or *Pax6*^{-/-} mutant embryonic thalamus were co-cultured with wild-type ventral telencephalon and it was found that while axons from wild-type thalamus navigated correctly through wild-type ventral telencephalon, axons from mutant thalamus did not (Fig. 2). This indicates that the

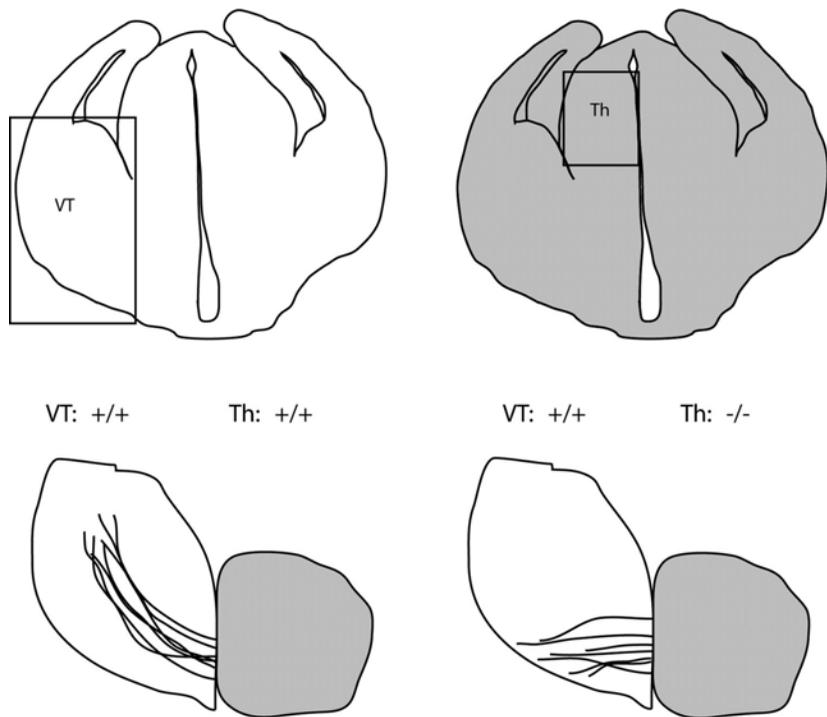


Figure 2 Experiments showing an intrinsic requirement for Pax6 in the thalamus for thalamocortical development (Pratt et al., 2000). Explants of ventral telencephalon (VT) were taken from wild-type mice. Thalamic explants were taken from mice in which all cells express green fluorescent protein linked to tau (tauGFP); these mice were either wild-type or *Pax6*^{-/-}. Explants of wild-type ventral telencephalon were placed with explants of tauGFP-expressing wild-type or mutant thalamus and axons labelled with tauGFP could be seen growing into the ventral telencephalon. If the thalamus was wild-type, then these axons navigated through the ventral telencephalon in the direction of the cortex. If the thalamus was *Pax6*^{-/-}, then these axons failed to navigate correctly. Since the ventral telencephalon is wild-type in both cases, there must be a defect in the *Pax6*^{-/-} dorsal thalamus.

navigational defects of *Pax6*^{-/-} thalamic cells are not corrected if they are confronted with a normal environment through which to grow—the gene must be needed by the thalamus itself for normal development of its cortical projections.

Does Pax6 Regulate Separate Sets of Genes in Morphogenesis and Guidance?

To regulate morphogenetic processes of cell proliferation, migration and fate determination, Pax6 controls the expression of a wide range of molecules, including transcription factors, cell adhesion and cell-cell signalling molecules, hormones and structural proteins (Simpson and Price, 2002). At present, too little is known about the targets of Pax6 to know whether or not Pax6 might regulate the same, overlapping or distinct sets of target genes during early morphogenesis and later axon guidance. As discussed above, there is strong evidence that Pax6 regulates cell-cell adhesion during brain morphogenesis and this control is likely to be equally important during axon pathfinding. Further work is needed to discover what the targets of Pax6 are and whether they change during development.

Regulation of Genes that Might be Involved in Guidance

It is most likely that this involves regulating the transcription of members of the molecular network that connects guidance cues with the cytoskeleton to control growth cone behaviour. It is possible that a lack of Pax6 alters the expression of a number of members of the network and that the combined effect causes a failure of thalamic responsiveness. A simplified list of many known members of the network is given in Fig. 3; the top rows include guidance cues shown to play or likely to play important roles in thalamocortical development. There is evidence from other systems that the expression of some of these molecules is affected by Pax6.

- (i) Semaphorins are a large family of secreted and membrane-associated proteins that are chemorepellant or chemoattractant. They are grouped into 8 classes; vertebrate semaphorins are in classes 3–7 (Semaphorin Nomenclature Committee, 1999). Semaphorins are expressed in and around the developing thalamocortical pathway (Skaliora et al., 1998). *In vitro*, thalamocortical axons are responsive to at least one of these, secreted Sema3A (Bagnard et al., 2001). Mice lacking the transmembrane Sema6A, which is proposed to act in thalamocortical axons as a guidance receptor, have thalamocortical defects similar to (although less severe than) those in *Pax6*^{-/-} embryos (Leighton et al., 2001). Thus, Sema6A is a good candidate as one potential direct or indirect target of Pax6 in dorsal thalamus. In addition, expression of Sema5A and Sema3C are altered in the telencephalon of mice lacking Pax6 and this has been suggested to contribute to thalamocortical defects in these mutants (Jones et al., 2002). Neuropilins are receptors

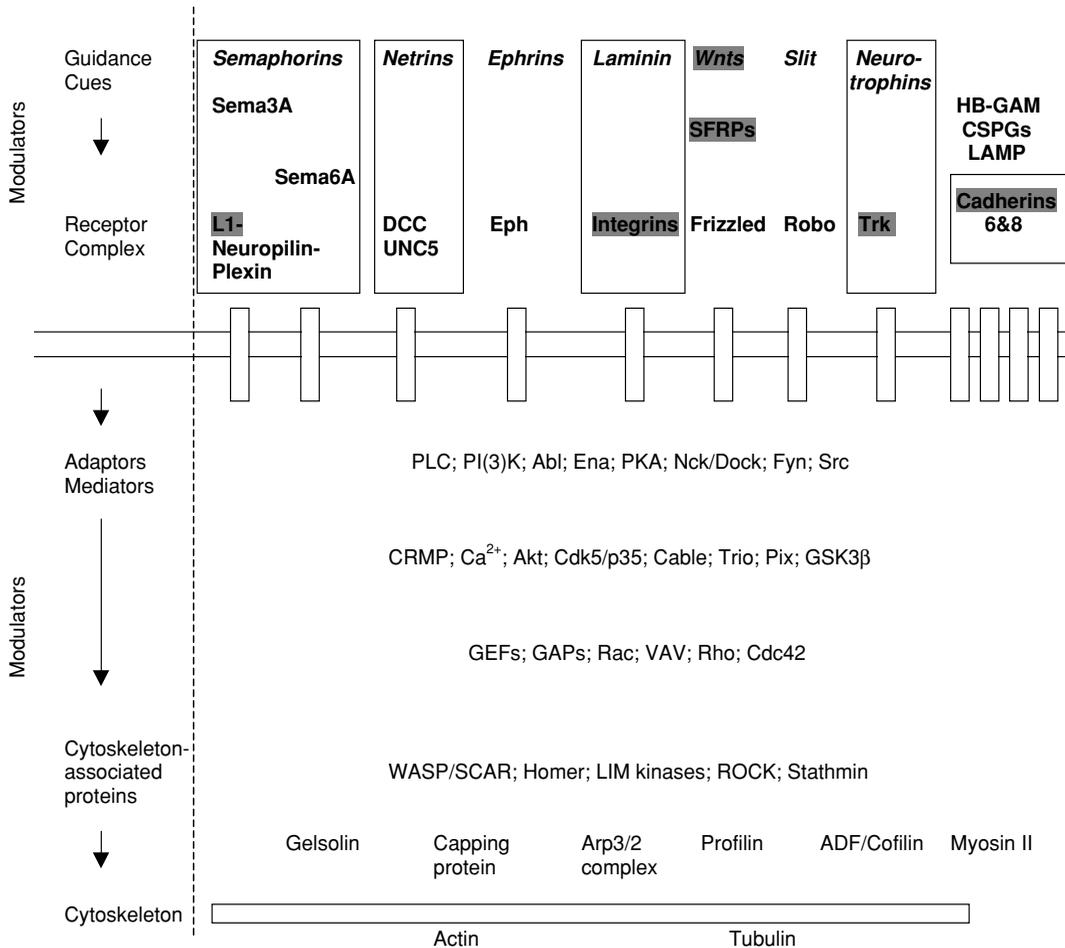


Figure 3 This diagram shows many of the molecules or classes of molecule that are likely to be involved in directing axonal growth, from extracellular cues to cytoskeletal rearrangement. Types of molecule are listed to the left of the broken vertical line; individual molecules or families of molecule are listed to the right. Molecules that are outside the cell or are components of the cell membrane are in bold at the top of the diagram. In many cases the cues interact with receptors, which are lined up below the corresponding ligands. Highlighting is used to identify molecules whose expression is regulated by Pax6. Families of molecule enclosed by boxes are those which include members that are prime candidates for being regulated in the thalamus, directly or indirectly, by Pax6. Many molecules inside the cell might have their expression affected by Pax6, but evidence is lacking at present: for simplicity, the pathways that may link these molecules are not drawn. Many of the families of molecule indicated are very large. Literature on which this diagram is based is cited in Song and Poo (2001) and in the text.

for class 3 secreted semaphorins; they complex with plexin and neural cell adhesion molecule L1 to form Sema3A receptors (Rohm et al., 2000; Castellani et al., 2000). *In vitro* experiments have indicated that Pax6 can bind to specific sequences in the L1 promoter (Chalepakakis et al., 1994), although L1 is still expressed at high level in Pax6^{-/-} embryos (Vitalis et al., 2000). Nevertheless, defects of

- L1-neuropilin-plexin receptors may also contribute to thalamocortical defects in *Pax6*^{-/-} embryos.
- (ii) Netrins include (a) diffusible proteins, whose attractive effects are mediated *via* receptors of the DCC (Deleted in colorectal cancer) family (DCC and neogenin) and whose repulsive effects require members of the UNC5 family, and (b) a membrane-linked member expressed at sites that include embryonic thalamus (Nakashiba et al., 2000). Diffusible Netrin1 is present in ventral telencephalon and, acting via DCC and neogenin receptors on dorsal thalamic axons, it may play a role in guiding thalamocortical axons through the ventral telencephalon (Braisted et al., 2000). Defects of thalamocortical axons are much less severe in loss-of-function mutation of Netrin1 than in *Pax6*^{-/-} embryos. One intriguing possibility is that *Pax6*^{-/-} dorsal thalamic neurons upregulate UNC5 receptors thereby converting a normally chemoattractive effect of Netrin1 into a chemorepulsive effect and so preventing thalamocortical development.
 - (iii) Ephrins and Eph receptor tyrosine kinases are involved in processes including growth cone guidance (Wilkinson, 2001) and Eph receptors and ephrins are expressed in the developing thalamocortical system. In particular, a role for ephrin-A5 in thalamocortical development has been suggested on the basis of expression and *in vitro* data (Gao et al., 1998; Mackarehtschian et al., 1999), although thalamocortical axons do form in mice lacking ephrin-A5 these do exhibit subtle mapping errors in their synaptic connections with the cerebral cortex (Vanderhaeghen et al., 2000). Interestingly, work in other systems has shown that the actions of Eph receptors and ephrin-A5 involve activation of integrins including β 1-integrin, which may be directly regulated by Pax6 at least in the lens (Duncan et al., 2000; Davy and Robbins, 2000). Integrins have been shown to play an important role in growth cone motility (Condic and Letourneau, 1997).
 - (iv) Neurotrophins, which act *via* Trk receptors, have been implicated as chemoattractants in the developing nervous system (Gallo and Letourneau, 2000) and thalamic axons do respond to members of this family (Lotto and Price, 1995). Furthermore, there is evidence that Pax6 directly or indirectly regulates the expression of Trk receptors in the developing cortex, although the thalamus was not investigated (Warren et al., 1999).
 - (v) Other diffusible molecules that need to be considered include members of the Wnt family, which signal through Frizzled receptors and whose actions are modulated by secreted frizzled related proteins (SFRPs). Their possible involvement is suggested by findings that Wnt7a regulates axonal development in cerebellum (Lucas and Salinas, 1997) and that Pax6 regulates forebrain expression of Wnt7b and SFRP-2 (Kim et al., 2001). It is possible that expression of Frizzled receptors or SFRPs in thalamus may be disrupted in *Pax6*^{-/-} embryos. Robo receptors, highly conserved molecules that mediate

- the chemorepulsive activity of secreted Slits (Erskine et al., 2000), are expressed in dorsal thalamus and so may be involved in thalamocortical axon guidance (Nakagawa and O'Leary, 2001).
- (vi) Cell adhesion and extracellular matrix molecules (ECMs) are essential in axon guidance. Pax6 is required for normal cortical expression of members of the cadherin family of calcium-dependent cell adhesion glycoproteins (Stoykova et al., 1997; Bishop et al., 2000). Cadherins present in the developing thalamocortical system include cadherin-6 and cadherin-8 (Rubenstein et al., 1999); their expression in the thalamus of *Pax6*^{-/-}embryos remains to be investigated. Chondroitin sulphate proteoglycans (CSPGs), heparin-binding growth-associated molecule (HB-GAM) and limbic system-associated membrane protein (LAMP) are suggested to play roles in thalamocortical development (Mann et al., 1998; Kinnunen et al., 1999); the possibility that their expression is regulated by Pax6 has yet to be tested.

Conclusion

Mutant mice show that the transcription factors discussed here are required for the morphogenesis of forebrain structures projecting and receiving axons and for axon navigation in the forebrain. This efficient use of genes may explain the massive biological diversity delivered by a relatively small number of genes. We predict that the list of transcription factors playing multiple roles in tissue morphogenesis and axon guidance will increase. The next challenge will be the comprehensive identification of their transcriptional targets and a molecular biological dissection of their various functions.

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