Defining brain wiring patterns and mechanisms through gene trapping in mice

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The search to understand the mechanisms regulating brain wiring has relied on biochemical purification approaches in vertebrates and genetic approaches in invertebrates to identify molecular cues and receptors for axon guidance. Here we describe a phenotype-based gene-trap screen in mice designed for the large-scale identification of genes controlling the formation of the trillions of connections in the mammalian brain. The method incorporates an axonal marker, which helps to identify cell-autonomous mechanisms in axon guidance, and has generated a resource of mouse lines with striking patterns of axonal labelling, which facilitates analysis of the normal wiring diagram of the brain. Studies of two of these mouse lines have identified an *in vivo* guidance function for a vertebrate transmembrane semaphorin, Sema6A, and have helped re-evaluate that of the Eph receptor EphA4.

Biochemical purification strategies in vertebrates and genetic screens in invertebrates have converged on several conserved families of molecular cues and receptors for axon guidance[1]. In addition to these initial discoveries, a thorough understanding of human brain wiring mechanisms is likely to require genetic screens in mammals. Mammals exhibit a greater diversity of axon guidance molecules than invertebrates; for example, the neuropilin family of semaphorin receptors and the chemoattractant hepatocyte growth factor do not have invertebrate orthologues[2,3]. Furthermore, biochemical strategies are not well suited to a systematic dissection of brain wiring, as assays must be tailored to each guidance event and require sufficient expression of the factor(s) being purified. By contrast, genetic approaches are not limited by expression levels and can identify molecules important for guidance of both small and large populations of axons.

Conventional genetic screens for brain wiring defects in mice are, however, daunting. Screening random chemically induced mutations can be inefficient, as only a small fraction of mutants are likely to present defects in a process as specific as axon guidance[4–6]; furthermore, many of these defects will arise only indirectly, for example as a result of mutations in genes regulating the development of tissues through which axons navigate[7]. This problem presents a serious impediment in mice because of cost and the time-consuming task of identifying the mutated gene by positional cloning. Second, many mutants will display subtle wiring defects affecting small numbers of axons, leaving most of the nervous system unaffected (for example, see refs 8, 9). Finding such specific defects is often challenging or impossible, as many axon tracts lack specific markers to visualize them against the background of a mostly normal pattern of projections. Indeed, finding specific wiring defects is tantamount to finding the proverbial needle in a haystack. Finally, little is known about the normal wiring pattern of the brain and its development, often making it difficult to detect or assess wiring defects.

Here we report a modified 'gene-trapping' method, the PLAP secretory trap method, that circumvents these problems by making it possible to pre-screen for candidate axon guidance receptors before generating lines of mutant mice, and to identify wiring defects readily through transgenic labelling of neurons expressing the trapped gene. The method has yielded numerous lines displaying many expression patterns, ranging from labelling throughout the nervous system to labelling of select axon tracts, creating a valuable resource for elucidating the normal pattern of axonal projections in the brain. In addition, screening for defects has revealed an axon guidance role for a vertebrate transmembrane semaphorin, Sema6A, and helped re-evaluate the function of the EphA4 receptor tyrosine kinase. The PLAP secretory trap method thus provides a powerful way to access the biology and mechanisms of axonal guidance in the mammalian brain.

Rationale for a modified gene-trap strategy

To uncover mechanisms directly involved in axon guidance, we modified a gene-trapping procedure. In gene-trapping approaches, mutagenesis is performed in embryonic stem (ES) cells using a DNA construct that integrates at random in the mouse genome[10]. When the vector inserts into an intron, a fusion transcript is created through the splicing of the 'trapped' gene's upstream regions to vector sequences, in our case a 'secretory trap' form of β-geo (a fusion between β-galactosidase and neomycin phosphotransferase[11]) that markedly enriches for insertions in candidate ligands and receptors[12,13] (Fig. 1). The gene is easily identified by 5′ rapid amplification of complementary DNA ends (RACE) and sequencing[14,15].

To help identify subtle wiring defects against a background of normal axonal projections, we added a second marker, human placental alkaline phosphatase (PLAP) and an internal ribosome entry site (IRES)[16,17] (Fig. 1a). PLAP is a glycosyl phosphatidylinositol (GPI)-linked cell-surface protein that, when expressed transgenically in neurons, labels axons completely[18,19]. When a gene is trapped, the endogenous promoter and enhancer elements direct production of a bicistronic transcript that encodes two proteins: the β-geo fusion to the endogenous protein and the PLAP protein, which is translated independently using the IRES (Fig. 1a).

In mice derived from mutant ES cells, neurons expressing the trapped gene are labelled in their cell bodies by β-geo (which is retained within an intracellular compartment[12]) and on their axons by PLAP. A direct anatomical screen for differences in the projection patterns between heterozygotes (taken to be the normal pattern; see Discussion) and homozygous mutants should identify cell-autonomous axon guidance defects. We are directed to the relevant axons by the histochemical marker; the 'needle in a haystack' problem is, in effect, solved through the molecular tagging of the 'needle'.

The PLAP vector traps axon guidance molecules

In initial experiments the PLAP vector performed as designed, effectively mutating candidate axon guidance molecules while simultaneously integrating a useful axonal marker. We sequenced fusion transcripts from 379 X-gal positive clones and found that they include similar numbers and types of genes to those trapped with the original secretory trap vector[13]. Of 120 known genes trapped, 87 (73%) encode secreted or transmembrane proteins (see Table 1). Notably, 13 of these gene products are known or predicted axon guidance molecules, including two Eph receptors and a plexin family member. A similar proportion (~15%) of the new genes is likely to encode receptors or cues used during brain wiring; indeed, 4 of the new genes (of 43 trapped) contain modules that have been found in axon guidance molecules, such as immunoglobulin domains and plexin repeats (Table 1).

We compared X-gal and PLAP staining of adjacent sections from heterozygous mice and found that, as predicted, PLAP reliably labels the axons of neurons stained with X-gal (Fig. 1b, c). Moreover, for all known trapped genes, both the β-gal and PLAP reporters faithfully reflected the reported expression pattern (Fig. 2).

Defining brain wiring patterns and mechanisms

To examine expression patterns and screen for wiring defects, we examine progeny at three ages encompassing many axon guidance events: embryonic day (E)11.5, E15.5 and birth (P0). Alternating coronal/transverse sections through the entire brain and portions of the spinal cord are processed by X-gal or PLAP staining. Axon tracts stained in the heterozygote are compared with the homozygous mutant pattern to find guidance defects. Forty-six lines of mice have been produced, including seventeen with insertions in new genes. To date, we have screened 24 lines of mice and most (88%) exhibit staining in the nervous system. We have observed a remarkable variety and specificity of axonal staining patterns (Fig. 2). For example, whereas *PTPκ* and *neogenin* are both expressed in the corpus callosum and in the anterior commissure, only *neogenin* labels the subcortical commissures (Fig. 2a, c). In contrast, commissural axons are not labelled in *ADAM23* or *CRIM-1* animals, whereas the striatum shows strong expression only for *ADAM23* (Fig. 2a–d). Outside the forebrain, robust staining of specific central and peripheral projections has been observed, for example in olfactory (*LGR4*, Fig. 2e), trigeminal (*Sema6A*, Fig. 2f) and retinal ganglion cell axons (*Sema6A*, Fig. 2g).

Twenty-seven per cent of the trapped genes are new, and many of these also define molecularly distinct tracts (Fig. 3). *LST16*, which encodes a transmembrane protein with leucine-rich and immunoglobulin repeats (Fig. 3a), labels the habenula, piriform cortex and barrel cortex (Fig. 3b, c). *KST37* encodes a nidogen/plexin homology transmembrane protein (Fig. 3d) that is expressed in peripherally projecting axons (Fig. 1c), as well as in the fimbria and stripes of Purkinje cells in the cerebellum (Fig. 3e, f). *KST223* is weakly

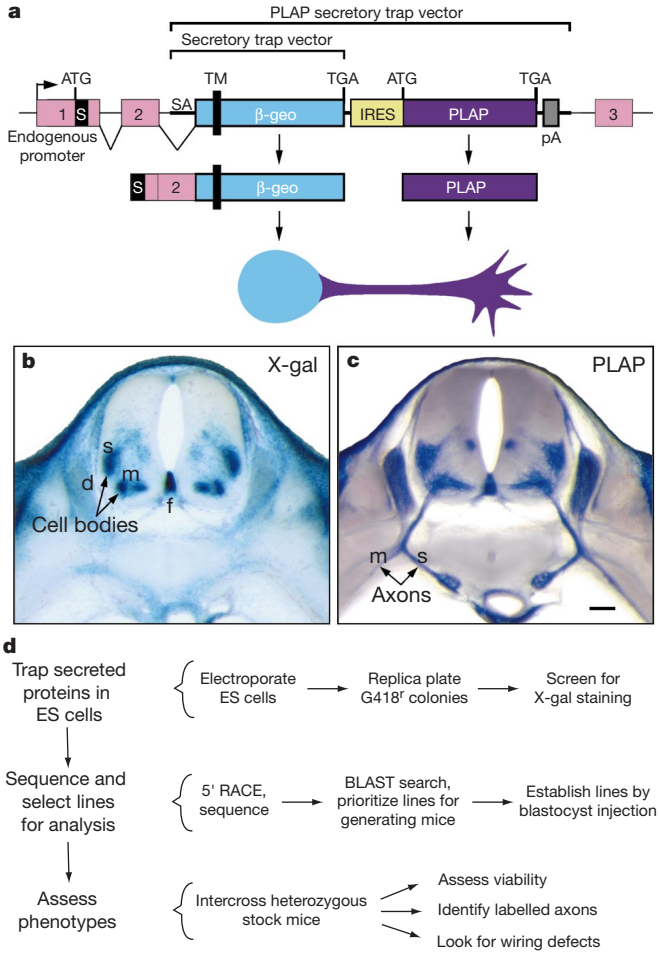

Figure 1 The PLAP secretory trap vector and flow of screen. **a**, An IRES and the PLAP gene were added to the original secretory trap vector[12]. A bicistronic transcript makes two proteins: first, a fusion between the endogenous protein (including its signal sequence, s) and β-geo, localized to cell bodies; and second, PLAP protein, localized to axons. **b**, **c**, Co-expression of β-gal and PLAP in a KST37 heterozygote, E12.5. **b**, X-gal staining. d, dorsal root ganglia; f, floor plate; m, motorneurons; s, sympathetic preganglionic neurons. **c**, PLAP staining. **d**, The three main stages of the screen. Scale bar, 100 μm.

Table 1 Selected genes trapped with the PLAP secretory trap vector

Transmembrane (31)	**Multi-transmembrane (6)**
ADAM23	GABA-BR1a/b
Attractin	N33
CD98	LGR4
CRIM-1	p76
Desmoglein-2	RW1
E-cadherin	TASK-2
EphA2*	
EphA4*	**Secreted (15)**
E selectin ligand 1	Agrin
ICAM1	Fibulin-1
Integrin α5*	Bikunin-related protein
Integrin α6*	Cyr61
Jagged-1	Fibronectin
Jam	Glypican-3
LDL receptor	Glypican-4
Lisch7	GS3786
LRP6	Laminin α1*
LRP2 (megalin)	Laminin α5
mFAT1	Laminin β1*
MFG E8	Laminin γ1*
Mph poliovirus receptor	Netrin-1*
Neogenin*	Perlecan
Notch-1	Protein S
Notch-2-like	
Notch-3	**New genes with axon guidance motifs (4/43)**
Plexin-A1*	KST37 (plexin repeat)
pM5	KST72 (Ig, FNIII repeats)
PTPκ*	KST225 (cadherin domains)
PTPσ*	LST16 (LRRs)
SDR-1	
Sema6A*	

All transmembrane and secreted proteins are listed, as are several new genes with intriguing predicted structures. Ig, immunoglobulin, LRR, leucine-rich repeat. FNIII, fibronectin type III.
*Known or likely axon guidance molecules.

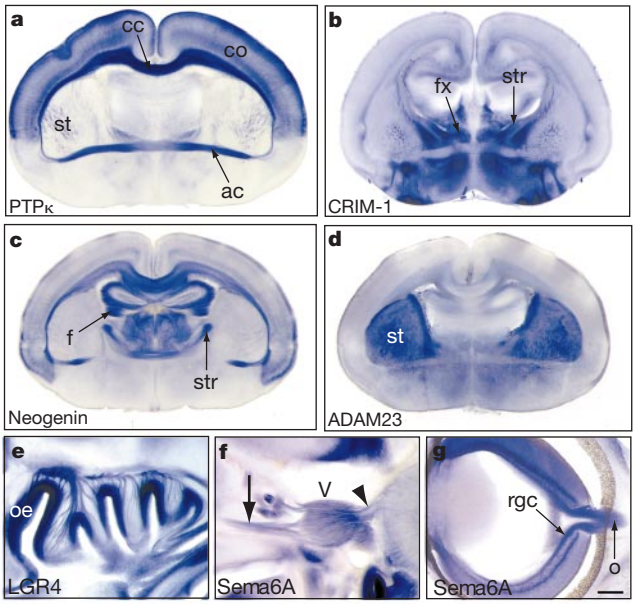

Figure 2 A survey of axonal populations labelled by PLAP. **a**–**d**, P0 brains, coronal sections, dorsal is up. ac, anterior commissure; cc, corpus callosum; co, cortex; f, fimbria; fx, fornix; st, striatum; str, stria terminalis. **a**, PTPκ homozygote; **b**, CRIM-1 heterozygote; **c**, neogenin homozygote; **d**, ADAM23 homozygote. **e**, LGR4 heterozygote, E15.5, sagittal section. oe, olfactory epithelium. **f**, Sema6A homozygote, E12.5, sagittal section. Both peripheral (arrow) and central (arrowhead) projections of the trigeminal ganglion neurons (V) are labelled. **g**, Sema6A heterozygote, E15.5, coronal section. o, optic nerve; rgc, retinal ganglion cells. Scale bars, 500 μm (**a**–**d**); 106 μm (**e**); 225 μm (**f**); 70 μm (**g**).

related over much of its sequence to the interleukin-17 (IL-17) receptor (Fig. 3g), and shows remarkably specific expression in the fasciculus retroflexus (Fig. 3h) and in a restricted region of the inferior colliculus and cerebellum (Fig. 3i).

Axon guidance phenotypes in PLAP gene-trap mutants

Insertion of the PLAP vector induces either null or severe hypomorphic mutations[13] that can be analysed at several levels. First, the mutants are assessed for lethality and gross defects, such as ataxia. Neurological defects have been observed in two mutants: *ADAM23* (ref. 20), in which homozygotes display tremor and ataxia[13]; and *EphA4*, in which homozygotes have an abnormal gait (see below). Second, lines that survive to at least E11.5 are screened systematically for wiring defects, even if the line is fully viable, because axon guidance molecules are not necessarily expected to cause overt phenotypes (for example see refs 8, 9). Eventually, viable lines can also be screened for defects in locomotion or behaviours such as learning and memory.

The anatomical screen has already revealed axon guidance defects in two mutant lines, *Sema6A* and *EphA4*. Preliminary analysis of these two mutants confirms, first, that we are able to identify new axon guidance phenotypes, and second, that characterization of PLAP staining can extend the analysis of genes that have already been knocked out.

Thalamocortical axon defects in *Sema6A* mutants

Semaphorins comprise a large family of transmembrane and secreted proteins that so far have been defined as guidance signals (for review see ref. 3). *Sema6A* belongs to a subfamily characterized by an extracellular semaphorin domain, a transmembrane domain

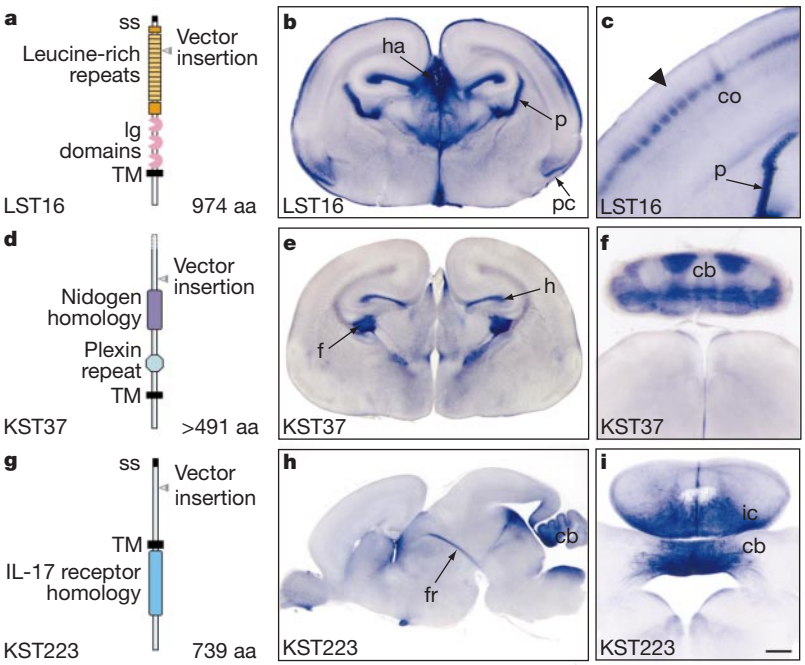

Figure 3 A sample of new predicted transmembrane proteins and their expression. All expression patterns are at P0 except for **c**, which is at P8. **a**, LST16 is a predicted protein of 974 amino acids (aa) comprising an N-terminal signal sequence (ss), 14 leucine-rich repeats (bracketed by N- and C-terminal leucine-rich repeat motifs), 3 immunoglobulin (Ig) domains, a single transmembrane domain and a cytoplasmic domain of 167 amino acids. It is 64% identical to another gene, KIAA0806 (GenBank accession no. XM_001307). **b**, LST16 homozygote, coronal section. ha, habenula; p, pia, pc, piriform cortex. **c**, LST16 heterozygote, coronal section. co, somatosensory cortex; arrowhead indicates barrels. **d**, KST37 is a predicted protein of more than 491 amino acids with a nidogen-related domain, a plexin repeat, a transmembrane domain and a cytoplasmic domain of 54 amino acids (the extreme N terminus has not been determined), and is 55% identical over its length to tumour endothelial marker 7 (ref. 37). **e**, KST37 homozygote, coronal section. f, fimbria; h, hippocampus. **f**, KST37 homozygote, transverse section. cb, cerebellum. **g**, KST223 is a predicted protein of 739 amino acids that is weakly related to the IL-17 receptor, particularly in the cytoplasmic domain. **h**, KST223 heterozygote, sagittal section. fr, fasciculus retroflexus. **i**, KST223 homozygote, transverse section. ic, inferior colliculus. Scale bar, 500 μm (**b**, **e**); 205 μm (**c**); 275 μm (**f**); 700 μm (**h**), and 400 μm (**i**).

and a long cytoplasmic tail[21,22]. Members of this class, including *Sema6A*, can repel sympathetic and dorsal root ganglion axons *in vitro*[22,23], consistent with a traditional role as guidance signals. However, the length of the cytoplasmic tail, which includes an Evl-binding site in Sema6A[24] and a Src-binding site in Sema6B[25], suggests that these semaphorins may also function as receptors.

We isolated an insertion in the *Sema6A* gene at amino-acid 473 in the semaphorin domain that completely abolishes wild-type *Sema6A* transcripts[13]. Homozygous mutant mice are viable and fertile and display no obvious behavioural or morphological phenotypes. Staining for β-gal activity duplicates the published *in situ* hybridization pattern for *Sema6A*[21] (Fig. 4a; and data not shown). PLAP staining labels specific axonal populations including the cranial nerves and optic tract (Fig. 2f, g), dorsal root ganglion axons, and several tracts in the brain including the fasciculus retroflexus, stria medullaris, the anterior commissure and thalamocortical axons (Fig. 4; and data not shown).

Although most of the tracts examined appeared unchanged in homozygotes, proper development of the thalamocortical projection requires *Sema6A* function. PLAP staining in heterozygotes shows thalamocortical axons projecting normally through the thalamus in a well-ordered fan-shaped array, turning sharply through the internal capsule and sweeping up to the cortex[26,27] (Fig. 4c, d). X-gal staining labels cell bodies of these neurons in the thalamus (Fig. 4b). As there is only weak expression in the cortex at this stage, and no strong PLAP stain in cortical layer 6, it is likely that few if any descending corticothalamic axons are stained (Fig. 4c). Strikingly, thalamocortical axons in homozygotes fail to turn up through the internal capsule and instead project down towards the amygdala region (Fig. 4g–i). This defect is fully penetrant ($n = 12$), but is specific to caudal thalamocortical axons; rostral projections appear normal in every animal ($n = 12$) (data not shown). Aberrant thalamocortical axons were confirmed by injection of the lipophilic axonal tracer, DiI, into the dorsal thalamus (Fig. 4f, i).

The fact that *Sema6A* is expressed in thalamocortical neurons and required for their axons to project properly suggests that the guidance defect is cell-autonomous and that Sema6A is acting in these axons as a guidance receptor. *Sema6A* is expressed broadly both in the thalamus and amygdala at the time when these axons are growing (E12.5–E15.5, Fig. 4a), however, which makes it impossible to distinguish fully between autonomous and non-autonomous activities without further experimentation. Alternatively, by analogy to the function of the *Drosophila Sema1a* gene (which has a closely related extracellular, but not intracellular domain)[28], Sema6A may function by promoting defasciculation of thalamocortical axons in a paracrine fashion, thereby enabling them to respond to other cues; misrouting would then be secondary to a failure to defasciculate.

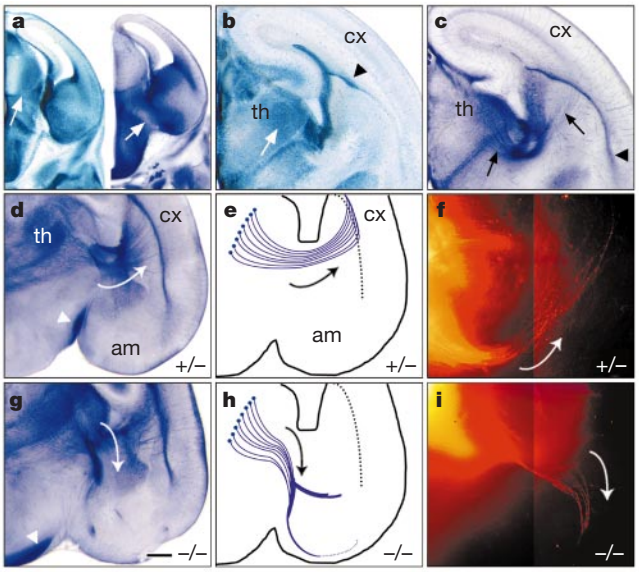

Figure 4 Thalamocortical axon misrouting in Sema6A mutants. Coronal sections, dorsal is up. **a**, Sema6A homozygote, E14.5, X-gal (hemisection on left; midline to left) and PLAP (right) staining. **b**, **c**, Sema6A heterozygote, P0, X-gal (**b**) and PLAP (**c**) staining. cx, cortex; th, thalamus; white arrow, dorsal thalamus. Arrowheads point to staining in a glia palisade. **d**–**i**, Thalamocortical projection (arrows) in Sema6A P0 heterozygotes (**d**–**f**) and homozygotes (**g**–**i**), shown with PLAP staining (**d**, **g**), in schematic form (**e**, **h**), or with DiI labelling (**f**, **i**; contrast increased selectively on right half). The optic tract (arrowheads) is unaffected. Scale bar, 350 μm (**a**–**c**); 400 μm (**d**, **g**); 800 μm (**f**, **i**).

Revisiting defects in *EphA4* mutants

Null mutations generated by the gene targeting of *EphA4* (refs 29, 30) possess defects in the corticospinal tract (CST; see Fig. 5a) and anterior commissure. As *EphaA4* expression was not detected previously in the CST, a model was proposed in which an ephrin ligand on the axons senses EphA4 on spinal cord cells surrounding the CST[29]. This possibility was supported by evidence that transmembrane ephrin ligands can also have a receptor function[31,32].

We have revisited the issue of whether EphA4 must have a ligand function by analysing an *EphA4* mutant generated by inserting the PLAP vector. Like the targeted *EphA4* mutants[29,30], homozygous *EphA4* gene-trap mice exhibit a hopping kangaroo gait and have guidance defects in the CST and anterior commissure (Fig. 5e; and data not shown). Unexpectedly, however, we found evidence for *EphA4* expression in CST neurons themselves. First, X-gal staining was observed in layer 5 of the motor cortex (as well as other layers) (Fig. 5b). Second, CST axons exhibited PLAP staining along their length, including in the internal capsule, at the pyramidal decussation, and in the spinal cord, where the pattern was consistent with expression in CST axons and their collateral branches (Fig. 5c, d; and ref. 33). In homozygotes, PLAP staining revealed an abnormal projection in the dorsal midline at the pyramidal decussation, suggesting crossing defects of CST axons (data not shown). In the spinal cord, the dorsal funiculus appeared smaller and shifted dorsally; there was also abnormal midline crossing of processes, which may consist partially or entirely of collateral branches of the CST axons (Fig. 5e).

The CST defects observed here are identical to those described previously[29]. The fact that CST neurons produce β-gal and PLAP is, however, unexpected. The low level of expression of *EphA4* in the CST was easily detectable using the sensitive X-gal and PLAP staining but might be difficult to detect specifically with antibodies or by *in situ* hybridization, possibly explaining the discrepancy between our results and those of Dottori *et al.*[28] Indeed, *in situ* hybridization analysis has revealed intermediate level expression of *EphA4* in the cortex in a pattern identical to that observed by X-gal staining in the gene-trap line[33]. Thus, EphA4 may indeed act as a receptor in the CST, a model further supported by the analysis of EphA4-kinase-deficient mice[33].

Discussion

We have developed and initiated a large-scale screen for new axon guidance molecules in the developing mouse nervous system. The screen combines two previous methods: the 'secretory trap' technique, used to mutate genes encoding proteins with signal sequences[12,13]; and the use of IRES vectors to drive expression of an axonal marker in transgenic animals, previously used in targeted insertions into known genes[16,34]. Our initial studies show that the PLAP secretory trap vector provides a useful tool for identifying the functions of both known and unknown genes in brain wiring, because it effectively mutates genes of interest and simultaneously introduces transgenic markers, β-geo and PLAP, that faithfully label the cell bodies and the axons of neurons expressing the trapped gene, respectively. This approach makes it possible to scan the

mammalian genome efficiently for axon guidance molecules.

The use of the PLAP marker in conjunction with the gene-trap vector solves, at least for cell-autonomous mechanisms, the most vexing problem facing a genetic screen for brain wiring mechanisms: how to examine only those axons most likely to be affected by the mutation among the many axons that project normally. As shown for *Sema6A* and *EphA4*, one can identify defects in the trajectories of axons expressing the trapped gene simply by comparing the wiring of PLAP-stained axons in heterozygous and homozygous embryos. The analysis of *Sema6A*, in particular, shows how PLAP staining can reveal previously unreported axon guidance phenotypes. The PLAP secretory trap method is likely to be most useful for identifying genes that encode axon guidance receptors or that have other cell-autonomous functions in guidance, as the axons that require the function of the gene are labelled with PLAP. This does not, however, preclude the discovery of non-autonomous functions, as defects in axons that do not express PLAP may sometimes be discernable.

Although we are using as our reference point the pattern of PLAP-stained axons in heterozygous rather than wild-type animals, this pattern will be identical to the wild-type pattern when the mutations are fully recessive (as is expected in most cases), and can be verified by comparison with known anatomy where available. In rare instances of haplo-insufficiency some defective projections may be present in the heterozygotes (for example, see ref. 30), but it is highly unlikely that they would be as severe as in homozygotes; therefore, the involvement of a trapped gene in axon guidance is unlikely to be obscured even in those cases.

Although the PLAP secretory trap screen in this study has already helped to identify a new axon guidance phenotype (in *Sema6A* mutants), and to characterize further a known phenotype (in *EphA4* mutants), its full impact will come from its application on a large scale. Because the method does not rely on advance knowledge of the type of molecules that will be involved, axon guidance mechanisms in mammals can be identified without bias for the kinds of proteins or their expression levels. The PLAP secretory trap screen can also accelerate the enumeration of the various membrane proteins made by particular axonal tracts and therefore help build a molecular map of axonal projections. As the complement of surface proteins made by axons is defined, it might be possible to uncover a code of surface receptors that defines particular axonal trajectories, much as studies have defined a transcriptional code (the LIM code) for specifying motor neuron trajectories[35]. Finally, the bank of ES cells and of mouse lines in which particular axonal populations are labelled with PLAP (which can be accessed through http://www. genetrap.org) provides a resource to help elucidate the normal wiring diagram of the mammalian brain. □

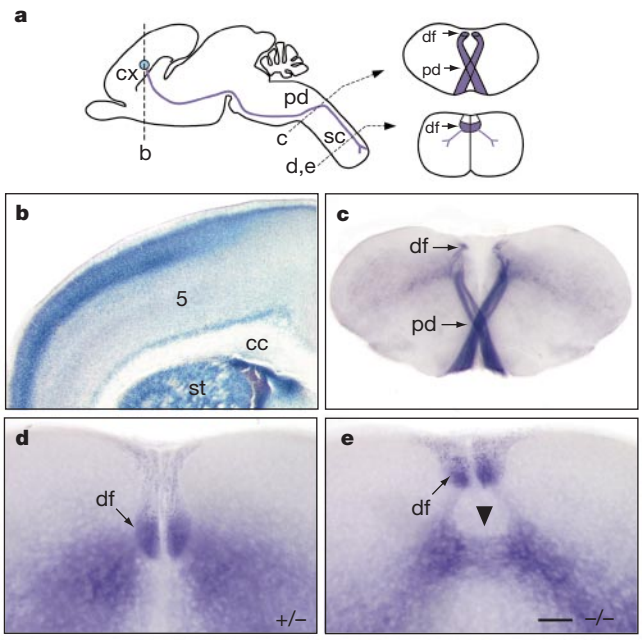

Figure 5 Axon guidance defects in EphA4 mutants. **a**, The mouse corticospinal tract (CST): sagittal (left) and transverse (right) sections. Corticospinal neurons (blue circle) reside in the motor cortex (cx) and send their axons (purple line) across the midline and dorsally in the pyramidal decussation (pd, top right), projecting through the spinal cord in the dorsal funiculus (df, bottom right), with collateral branches extending into the grey matter. Dashed lines indicate coronal (**b**) and transverse (**c**–**e**) planes of section. **b**, EphA4 heterozygote, P4. X-gal staining. 5, layer 5 of motor cortex; cc, corpus callosum; st, striatum. **c**, **d**, EphA4 heterozygote, P4. PLAP staining in pyramidal decussation (**c**) and in dorsal funiculus (**d**). **e**, EphA4 homozygote, P4. Arrowhead indicates abnormal crossing of midline in spinal cord. Scale bar, 315 μm (**b**); 360 μm (**c**), 60 μm (**d**, **e**).

Methods

The PLAP vector

A fusion of the EMCV IRES[16] to PLAP[36] was inserted downstream of β-geo in pGT1tm, a modification of pGT1.8TM[12,13]. We introduced FRT sites flanking β-geo. Vectors in all three reading frames were generated and were named pGTOTMpfs, pGT1TMpfs and pGT2TMpfs.

ES cell culture and sequencing

We cultured and screened E14 ES cells for X-gal staining as described[12]. 5′ RACE of ES cell clones was done as described[13].

Mouse husbandry

F_1 heterozygous progeny (generated from crosses of chimaeras to C57Bl/6J (B6)) were backcrossed to B6. F_2 males and females were intercrossed to generate heterozygous and homozygous progeny for the screen. Genotypes were determined by quantitative dot blot hybridization using a *lacZ* probe[13].

Histology

Tissues were fixed in 4% paraformaldehyde and embedded in 5% low-melt agarose. We collected 100-μm sections using a vibrating microtome and air dried them onto Superfrost Plus slides (Fisher Scientific). β-gal activity was detected with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) under standard conditions, followed by Nuclear Fast Red counterstain (Vector Laboratories). We detected PLAP activity with AP staining buffer (0.1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate, 1 mg ml^{-1} nitroblue tetrazolium in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), after a 1-h incubation in PBS at 65 °C. PLAP stained tissue was dehydrated in methanol, cleared in benzyl alcohol:benzyl benzoate (1:2) and rehydrated.

DiI tracing

We fixed 300-μm thick coronal sections in 4% paraformaldehyde and injected DiI (Molecular Probes) into the dorsal thalamus, before incubating them at 37 °C for 3 d to allow the DiI to diffuse down the axons.

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