Minireview

The trappist's approach to pathfinding: elucidating brain wiring using secretory-trap mutagenesis

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Abstract

A key problem in using genetics to dissect the wiring of the mammalian brain lies in discovering which of the billions of neural connections have been disrupted by a particular mutation. A novel gene-trap approach targets the genes involved in brain wiring and labels the axons of neurons expressing those genes, enabling the effects of mutations to be observed directly.

Understanding the wiring of the nervous system, and of disease states that result when this wiring is damaged or degenerates, requires genetic dissection of the molecular mechanisms involved in the guidance of axons and in the establishment and maintenance of specific synaptic connections. To date, such a dissection has only been practicable in invertebrates [1] and, more recently, in zebrafish [2]. Even in such genetically tractable organisms, genetic screens have been limited by the ability to detect the phenotype; the more successful screens have relied either on changes in behavior [3] or on the availability of markers to identify specific axonal tracts [1].

The higher costs of maintaining larger animals with longer generation times means that the relative inefficiencies of classical chemical mutagenesis methods that are tolerated in smaller, short-generation organisms become prohibitive in mammals, unless the goals of the screen are very broad [4,5]. When the search is more specific, the number of relevant mutations isolated falls, increasing the costs proportionately. Moreover, the relative complexity and plasticity of the mammalian brain means that detection of the effect of a mutation may require labor-intensive histological methods and/or sophisticated behavioral tests. Even then, the defect may be due to a disruption of a tissue through which an axon tract passes, rather than to a defect in the axon-guidance process itself. When a phenotype can be detected in one of these ways - and, indeed, large-scale behavioral screens are under way in mice [4,5] - mapping and identification of the gene involved still remains a formidable task, even with the current advances in the mapping and sequencing of the mouse genome. Thus, in mammals, the tendency has been to rely on testing the function of known genes, identified either by biochemical means or by homology to genes found in genetic screens in invertebrates, using targeted mutagenesis in embryonic stem (ES) cells. Although this has yielded some important insights, it is obviously not as systematic or as objective as a genetic screen and is reliant, on the one hand, on the availability of suitable assays and relatively abundant sources of material and, on the other, on the assumption that vertebrate guidance mechanisms will always be similar to those used in invertebrates. The latter assumption is undermined by the fact that key vertebrate guidance molecules, notably the neuropilin family of semaphorin receptors and hepatocyte growth factor, do not have invertebrate orthologs [6].

To the rescue comes 'gene trapping' and, in particular, an elegant modification of the classical gene trap, recently described in two papers from the Skarnes and Tessier-Lavigne labs [6,7], that makes it possible to prescreen for molecules likely to be involved in brain wiring before generating lines of mice and to identify easily axon tracts affected by mutations caused by the gene trap. Developed from 'enhancer traps' used in flies, a gene trap involves introduction into the germline of a DNA construct that can monitor

and potentially disrupt the transcriptional activity of the region of the genome into which it is inserted. In its simplest form [8-11], a gene-trap construct consists of a selectable marker - such as that encoding neomycin phosphotransferase, often fused to the gene encoding β-galactosidase, allowing expression of the marker to be visualized (the fusion is called β-geo [8]) - joined to a suitable RNA splice-acceptor signal upstream and followed by a polyadenylation signal (Figure 1a). When this construct is introduced into cells, like other DNAs it integrates at low frequency into chromosomal DNA at more-or-less random sites. The absence of an endogenous promoter means, however, that

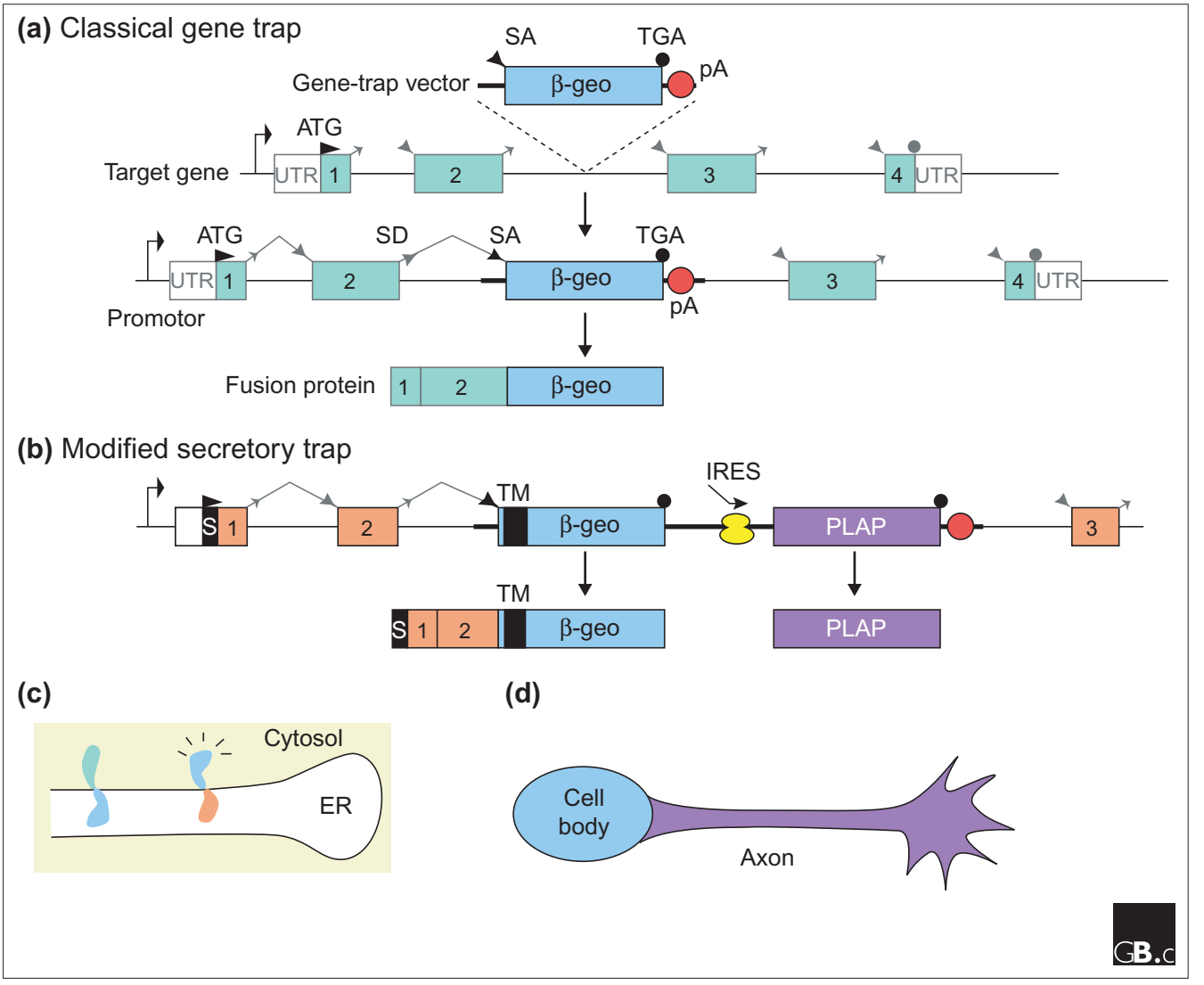

Figure 1
Mechanisms of classical and secretory gene traps. **(a)** In this example, the classical gene-trap vector inserts into a gene (in this case encoding a protein without a leader sequence) between exons 2 and 3. During transcription from the target-gene promoter, the splice-acceptor site (SA) at the 5′ end of β-geo is spliced to the next upstream splice-donor site (SD). The vector polyadenylation signal (pA) stops transcription; a truncated transcript is thus produced that encodes a fusion protein between sequences encoded by exons 1 and 2 of the target gene and β-geo. UTR, untranslated region. **(b)** The modified secretory-trap vector incorporates a transmembrane (TM) domain, an internal ribosome entry site (IRES) and an alkaline phosphatase (PLAP) gene. Only the situation after insertion of this vector into a gene encoding a protein with a hydrophobic leader sequence (S) is shown. A bicistronic transcript is produced, encoding first a fusion between the endogenous protein and β-geo that localizes to the neuronal cell body and second the PLAP protein, which localizes to the entire cell surface, including the axon. **(c)** Classical gene-trap insertions apparently result in fusion proteins that are inserted into membranes in a type I orientation (left) [12], placing the β-galactosidase domain of β-geo (blue) in the cytosol, where it is more active than in fusions with leaderless proteins as in the modified secretory trap (a), which result in a type II orientation (right) with β-galactosidase in the lumen of the endoplasmic reticulum (ER). **(d)** Expression of β-geo (blue) and PLAP (purple) in a neuron.

the selectable marker can be transcribed only when it is inserted into a chromosomal transcription unit (Figure 1a). In principle, as well as conferring drug resistance on the cell, insertion of the construct should also disrupt the function of the gene into which it has inserted, particularly if the insertion is in the coding region. When such traps are performed in ES cells, the mutation induced can be passed into the germ line; this has now been done successfully for a number of genes (see [7] for references). Identification of the trapped gene is then relatively easy, as fusion transcripts contain known pieces of DNA from which 5′ rapid amplification of cDNA ends (5′ RACE) can be performed to clone the adjacent, unknown sequences.

The novelty of the gene trap described by Leighton *et al*. [6] lies in the combination of two important modifications to the basic vector (Figure 1b). The first, previously described by Skarnes *et al*. in 1995 [12], is designed to target the vector to genes encoding secreted or membrane-spanning proteins. The rationale for this modification is that this class of protein will have major roles in axon guidance. Moreover, it is this class of protein that has undergone the biggest expansion in number and diversity in vertebrates compared to invertebrates [13], making it likely that there will be proteins among these molecules that are important for the massively increased complexity of the vertebrate nervous system. In this modification, β-geo has a hydrophobic transmembrane domain included within it, separating the splice-acceptor site from the catalytic domain, forming a so-called 'secretory trap'. If the mutant ES clones are prescreened for β-galactosidase activity, this configuration enriches for fusions to transcripts that encode an amino-terminal hydrophobic leader sequence, because β-galactosidase activity is significantly enhanced when located in the cytoplasm (Figure 1c). The enrichment is particularly high if the clones are screened for β-galactosidase activity that is located subcellularly in the normal secretory pathway [12,14].

The second innovation is the introduction of an additional marker gene, this time placental alkaline phosphatase (PLAP), which is included downstream of β-geo following an internal ribosome entry site (IRES), forming a bicistronic message (Figure 1b). The beauty of including this second marker is that, in neurons in which the trapped gene is expressed, whereas β-geo is normally restricted to intracellular compartments around the cell body, PLAP, a glycosylphosphatidylinositol (GPI)-linked cell-surface protein, covers the entire cell surface, including the axon (Figure 1d). The result is that the markers of the trap can be used both to locate the cell bodies of neurons expressing the trapped gene and the pathways taken by their axons (Figure 1d), thus effectively solving the problem of knowing where to look for the effects of the induced mutation.

How well does this strategy work? Although the secretory trap clearly enriches for secreted and transmembrane proteins - 70% of the traps obtained were obviously of this class - the introduction of a secondary sequencing screen before mouse lines were created was clearly an important and cost-saving step. All 528 lines obtained in the initial screen were sequenced following 5′ RACE. Of these, the 371 that clearly encoded secreted or transmembrane proteins were independent insertions into 187 different genes (some genes were represented by multiple insertions). Gratifyingly, of the 120 known genes trapped, 13 encode known or predicted axon-guidance molecules, for example Eph receptors, semaphorins and netrins, and a similar proportion of the new genes contained domains commonly found in axon-guidance molecules. Moreover, in the initial screen of some 24 lines of mice produced from these clones, 88% showed β-galactosidase and PLAP expression in the nervous system and clearly demonstrated that PLAP consistently allows the axons of neurons that express the trap to be traced effectively. Most impressively, the traps revealed a remarkable variety and specificity of axonal staining patterns (Figure 2), and many of the novel traps defined distinct neuronal pathways, sometimes comprising a very small number of axons.

Are the traps mutagenic? Clearly, the secondary sequencing screen selects for those traps that interrupt the coding region of the trapped gene. In fact, in 10 out of 11 cases where comparison to existing gene knockouts was possible, the gene traps gave identical phenotypes, suggesting that a high proportion of the traps are nulls or extreme hypomorphs [7]. Moreover, among the mutants were some that had neurological phenotypes and some that gave rise to anatomically identifiable axon guidance defects [6]. Most notably, an *EphA4* trap mimicked the published phenotype of an *EphA4* knockout mouse - which showed defects in the guidance of corticospinal tract (CST) axons [15]. In the *EphA4* trap, however, the presence of the PLAP and β-galactosidase markers in the corticospinal neurons themselves forced a reappraisal of the phenotype, which had previously been thought to be due to a deficit of EphA4 in the central nervous system tissues through which CST axons passed; this reappraisal has been supported by more recent detailed studies of the *EphA4* knockout [16]. Thus, it seems that the secretory-trap approach targets genes relevant to the wiring of the brain, in most cases mutates them, and, where relevant, enables the neurons and axons most likely to be affected by the mutation to be identified.

What, then, are the limitations of the approach? Among the genes identified so far there are some notable omissions, for example NCAM and indeed all of the related immunoglobulin-like cell-adhesion molecules such as L1 and contactin, which, as transmembrane proteins, might have been picked up by the screen, and all of which have significant effects on brain wiring [17-19]. This may simply be a statistical anomaly, as the screen was far from saturated in these pilot experiments [7]. There may also be some inherent limitations to the approach, however. First, and most obviously,

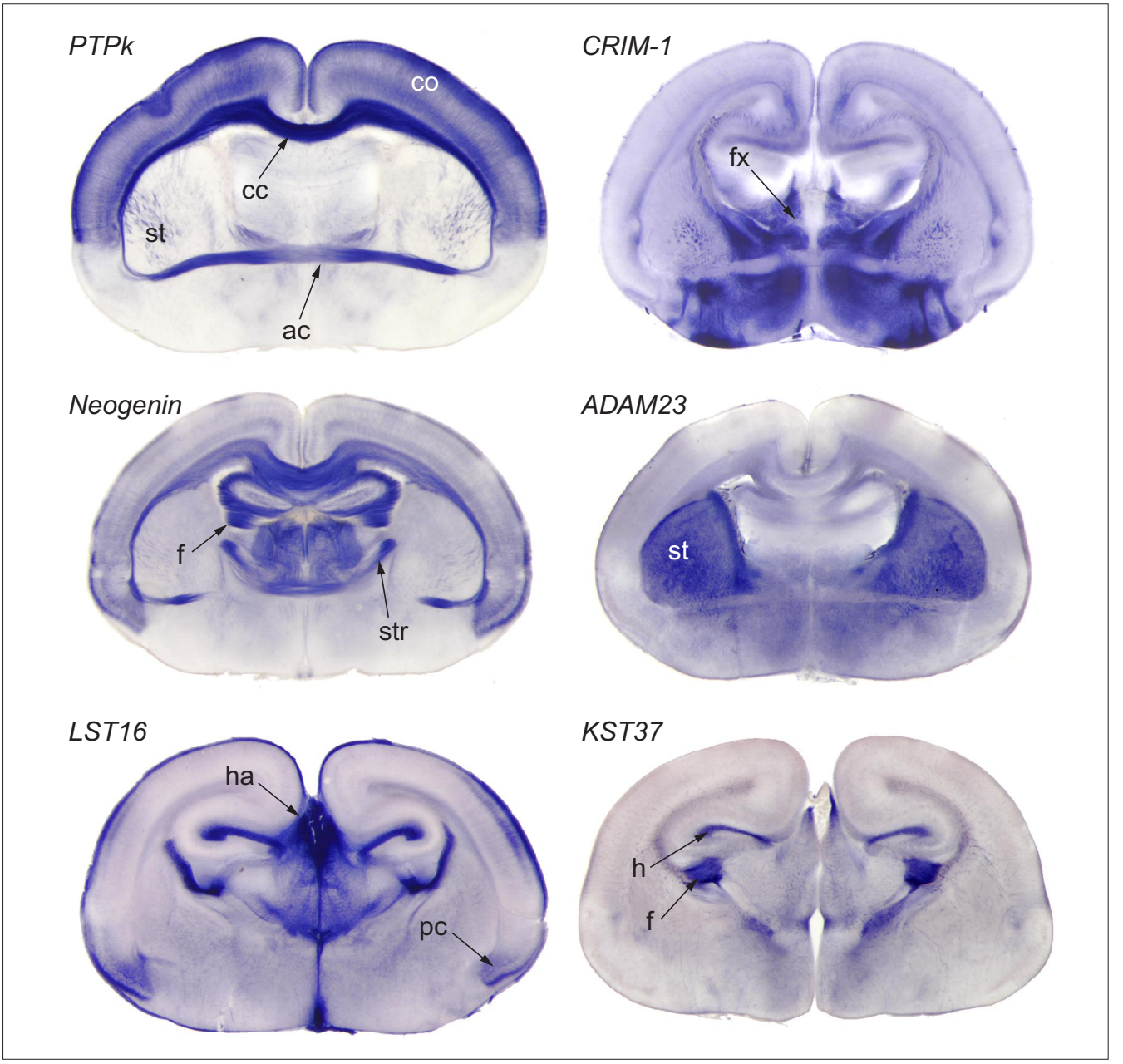

Figure 2
Coronal sections through the forebrains of six different example secretory-trap mouse lines at birth, showing the variety of axonal pathways labeled by the PLAP marker. The genes trapped in each case are indicated; *LST16* and *KST37* are examples of traps in new genes. Abbreviations: ac, anterior commissure; cc, corpus callosum; co, cortex; f, fimbria; fx, fornix; h, hippocampus; ha, habenula; pc, piriform cortex; st, striatum; str, stria terminalis. See Leighton *et al.* [6] for further details. Reproduced with permission from [6].

the initial targeting of the secretory-trap vector relies on the target gene being expressed at some level in ES cells. Despite improvements in the function of the neomycin phosphotransferase activity of β-geo that allow the current generation of gene-trap vectors to target genes that are expressed in ES cells at very low levels [7,20], it is likely that a set of genes - those completely transcriptionally inactive in ES cells - will remain inaccessible to gene trapping. The size of this set has yet to be determined. It might therefore be thought that only embryonic phenotypes would be found, but it is now abundantly clear from these and other recent large-scale gene-trap studies [20,21] that this methodology can give rise to phenotypes at stages other than early embryogenesis. Other limitations are perhaps less insurmountable. For

example, although roughly a third of the genes trapped in the secretory trap were trapped multiple times, the most frequent being 27 integrations into laminin γ 1 [7], analysis of other studies [21] suggests that the frequency of the most commonly trapped genes can be reduced and their identity varied, not apparently by varying the vector or method of DNA introduction but perhaps by varying some other aspect of the method, for example the ES-cell line used.

Despite these limitations, the triumph of the PLAP secretory trap is undoubtedly its ability to mark molecularly specific axon pathways in great variety, even in this pilot study. Although its initial impact is likely to be greatest in the fields of axon guidance and what must now be called 'molecular neuroanatomy', the availability of a library of such marked ES-cell lines and mouse strains (which can be accessed online [22]) will without doubt have important consequences in other fields of neuroscience. For example, in the heterozygous state, many of these strains could have uses in other experimental areas, from electrophysiology to regeneration studies, where knowing the identity of the neurons being studied is important. Indeed, the availability of the markers will also allow specific neuronal populations to be selected with precision, opening up the possibility of comparing identified neurons at specific stages of differentiation, in different states of physiological activity, or in diseased or lesioned animals - for example using microarrays to analyze mRNA isolated from the selected neurons. Traps that mark specific neuronal progenitor cells may also allow isolation of those cells for use in therapeutic models. Finally, viable mutants identified in this and future screens will also provide a substrate for behavioral investigations in which narrowing down the neurons that are likely to be involved in the behavioral defect will put the investigator at a massive advantage. The fruits of the labors that led to these secretory-trap lines seem certain to be harvested for many years to come.

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