Transgenic Approaches to the Study of Neural Development in Mice

by

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ABSTRACT

Transgenic mice were used as an assay system for the isolation and characterization of the promoter for cerebroglycan, a neural-specific cell surface proteoglycan expressed during the period of axon extension and guidance. A nuclear-localized β-galactosidase reporter gene was fused to a segment of upstream cerebroglycan genomic DNA and used to create several transgenic mice through pronuclear injection. These mice were stained for transgene expression patterns using X-gal, a chromogenic substrate for β-galactosidase. Nine out of eleven transgenic mice possessed nearly identical staining patterns which were neural-specific and similar, but not identical to the pattern of cerebroglycan expression. The promoter fragment was sequenced and was found to have significant homologies to the promoter for another neural-specific gene, Tα1. The isolated promoter may be useful for the study of neural-specific gene regulation and for the study of neural development by providing the means of expressing exogenous genes in a unique pattern during axon growth and guidance.

Several independent strains of transgenic mice were created which greatly facilitate the rapid visualization of axon pathways during neural development. The promoter for Tα1, an α-tubulin gene expressed specifically in neurons during axon growth, was fused to the gene for tau-β-gal, an axonally-targeted β-galactosidase reporter. This DNA construct was then used to create four independent strains of transgenic mice through pronuclear injection. Embryonic and early postnatal mice from these strains were stained for transgene expression using X-gal and were shown to possess several clearly-visible axon pathways. The transgene from one of the strains was placed on the genetic background of a mutant mouse null for the cerebroglycan gene in order to analyze the null mutants for possible axon guidance errors. Embryonic and early postnatal mice from this new strain, which possessed both the reporter transgene and cerebroglycan null alleles, were stained with X-gal and their axon patterns analyzed. No deviations in axon patterns were visible in cerebroglycan null animals, showing that the absence of cerebroglycan expression does not affect the establishment of major axon pathways.

Thesis Supervisor: Arthur Lander
Introduction

The nervous system with its intricate network of highly interconnected neurons is arguably the most complex object of study within biology. To a great extent this interconnectivity is established by a bewildering number of axons which extend from neuronal cell bodies, sometimes reaching a distance of several thousand cell diameters away, and which then ramify within their respective target fields. Although refinement of these synaptic connections occurs throughout the life of an organism in response to ongoing neuronal activity, a more or less accurate pattern of connectivity is established very early by the processes of axon extension and guidance during embryogenesis (Cline, 1991; Shatz, 1990). Fundamental to the study of the nervous system, then, is the investigation of the molecular signals by which these axons, often traversing complex paths, finally reach their targets.

The number of putative axon growth and guidance molecules has been increasing at a quickening pace during the past two decades, largely as the result of the successes of various in vitro experiments. In vitro systems have proven to be useful tools for these studies in that they provide more or less controlled experimental conditions within which the behavior of defined neuronal cell types can be studied. However, it may be argued that these strategies have been so prolific that, in fact, the field of molecular neurobiology now faces somewhat of an embarrassment of riches having now uncovered several families of proteins, and many distinct variants within those families, whose actions in vitro seem to be quite dependent on cell type, differentiation state, and other factors, and whose particular functions in vivo have yet to be demonstrated (Hynes, 1996).

More recently, several investigators working with invertebrates and lower vertebrates have begun to explore the functions of novel guidance molecules within an in vivo context, through large-scale anatomical or behavioral mutant screens (Seeger et. al.,
Invertebrate systems still offer a distinct advantage here due to the relatively short generation time of these animals, allowing rapid characterization of mutant phenotypes, and ultimately, their associated genes. Researchers in the laboratory of Corey Goodman (Seeger et. al., 1993), for example, have successfully carried out mutant screens in flies focusing on axon guidance errors during development, and have identified interesting anatomical phenotypes which may point to gene products involved directly in axon guidance \textit{in vivo}. There is, no doubt, great potential here for the expansion of our basic knowledge of axon guidance mechanisms, and it is probable that many of these fundamental processes will be found to be commonly utilized even among phylogenetically distant species. However, there are substantial differences between the nervous system of flies and that of mammals and other vertebrates, and it is still an open question as to how far we can justifiably extrapolate from one to the other. For this reason, many investigators have decided to trade ease of experimentation for what many consider to be a more medically relevant experimental system than either \textit{in vitro} strategies or invertebrate models - the mouse.

The creation of transgenic mice and "knockout" mice has already become a common method for the study of gene functions \textit{in vivo} whereby gene functions are added or removed, respectively. The study of axon guidance in mice, however, comes with certain technical problems. One major obstacle is the complexity and magnitude of mammalian neuronal populations which makes axon visualization much more difficult than in invertebrates. One result of this size difference is that whereas the entire network of axons within a fly larva can often be immunostained and visualized all at once, a mouse brain must first be processed into thin sections before similar immunostaining. These manipulations, unless they are done using time-consuming and technically demanding embedding procedures, often cause distortions in the tissue making it difficult to accurately follow the extent of even relatively short axons. In addition, and perhaps more importantly, since large regions of the mouse nervous system
cannot be visualized simultaneously in this manner without extensive serial sectioning, it then becomes necessary to predict, often without adequate knowledge, where and when a particular phenotype will surface when analyzing mutant animals for axon guidance defects.

Other techniques than immunostaining, of course, are routinely utilized for axon visualization but all of them involve a substantial commitment of time and effort, and often are in other ways not ideal methods for extensive mutant analyses. Golgi stains, for example, give an unequaled level of visual detail of cellular morphology including axon shape, however, this procedure, in addition to necessitating thin section preparation and being notoriously difficult, stains randomly distributed cells within a field. This makes it better suited for studying widespread changes in individual neurons such as in axon length or extent of ramification rather than for visualizing nerve fiber bundles as a whole, or for the investigation of guidance errors per se. Staining for retrogradely or anterogradely transported horseradish peroxidase (HRP) is another widely used method which gives detailed visualization of axons, but which has the drawback of working only in live tissue where axonal transport is active. By far the most popular method in recent years is the use of DiI and other axon-filling fluorescent dyes. These dyes can be used in fixed as well as living tissue, however the time for sufficient dye diffusion in fixed tissue takes on the order of weeks, making quick analyses impossible. In addition, both HRP and DiI staining are implemented by carefully embedding a small amount of the substance into a particular area of tissue. This tends to limit the nervous system regions which can be easily studied in this manner, since not all of them are equally accessible to dye placement. Once again, this also makes it necessary to know in advance where a particular phenotype will occur, a precondition which reduces the usefulness of these and similar techniques for thorough surveys of potential axon guidance defects in mutant animals.
It is a reasonable assertion, then, that these technical difficulties are what lies behind a
general lack of axon pattern data within recently published studies of neural
phenotypes in knockout mice. (Tomasiewicz et. al., 1993; Snider, 1994). Instead,
analysis is often limited to phenotypes which can readily be observed either
macroscopically or by microscopic examination of tissue anatomy. Notable exceptions
are transgenic studies in which the affected region within the mutant animal is
predictable based on transgene expression patterns, and where axon visualization is
particularly straightforward (Davis et. al., 1996). It is possible, then, that with the
introduction of more easily utilized axon visualization techniques, currently undetected
axon guidance errors might be uncovered within the growing population of knockout
and transgenic mice.

To this end, plans were laid for the creation of a transgenic mouse which would
express an axonally-targeted reporter enzyme over a wide range of neurons, specifically
during their period of axon extension and guidance. This mouse could then be crossed
with any number of mutant mouse strains in order to place the reporter transgene on
these mutant backgrounds. These mouse strains, in turn, could be maintained
indefinitely, providing a virtually unlimited supply of embryos for study which possess
the axon marker along with the mutant phenotype of interest. The enzymatic nature of
the reporter protein would provide a rapid assay for axon visualization, which, owing to
the relative ease of neural tissue penetration of most enzyme substrates, could be
implemented in relatively thick tissue sections (100-300µm). This would obviate the
necessity for extensive thin sectioning necessary for several other methods of axon
staining, making analysis easier and maintaining the integrity of the three dimensionality
of the axon pathways. An appropriate promoter sequence driving expression of the
reporter would ensure that axon patterns could be analyzed within several nervous
system regions, making it unnecessary to predict the particular location where a
particular phenotype might occur. In addition, selective expression of the reporter
during the period of axon extension would allow direct observation of axons during the
dynamic process of guidance, whereas axon filling dyes and similar reagents are not
selective for these growing axons. This can be important when analyzing potential
axon guidance errors, since these errors often self-correct given enough time, and since
misrouted axons which cannot form stable synapses with their appropriate target fields
are often selectively removed during later development (Oppenheim, 1991).

However, in July 1994 when these plans were made, there were no available cloned
promoter sequences which would drive expression of a reporter in neurons specifically
during the period of axon extension. In fact, very few neuron-specific promoters had
been cloned at all, and most of these provided expression only within mature neurons,
after their axons had most likely reached their targets (Sasaoka et. al., 1992; Forss-Petter
et. al., 1990). Since no appropriate cloned promoters as yet existed, it was decided upon
to attempt the isolation of a promoter for a gene whose expression pattern matched the
desired reporter expression. The most convenient candidate was cerebroglycan, a
proteoglycan which is expressed solely on the surfaces of neurons during the period of
neuronal migration and axon guidance (Stipp et. al., 1994; Ivins et. al., 1997). The gene
for cerebroglycan (hereafter denoted \( cbg \)) had recently been cloned in our lab by Chris
Stipp, in the form of cDNA (Stipp et. al., 1994). In addition, a series of partial genomic
clones had become available, from which a sizable region of upstream sequence could be
readily obtained. A proximal fragment from this upstream sequence was isolated and
analyzed for its ability to drive the expression of a reporter gene within the context of a
transgenic mouse. The results of these studies, presented in the first half of this thesis,
describe an overall temporal and spatial pattern of transgene expression which is similar
but not identical to the endogenous \( cbg \) pattern of expression.

A promoter sufficient for the purposes of axon analysis as outlined above was
therefore successfully generated. However, before this promoter characterization had
been completed, a promoter fragment from the \( \alpha 1 \)-Tubulin gene which possessed the
desired activity was isolated in the laboratory of Freda Miller (Gloster et. al., 1994). Attention was then focused on the production of a transgenic mouse using this promoter coupled with an axonally-targeted reporter construct, which at the time had recently been demonstrated only in flies, but has since been used in mice (Callahan et. al., 1994; Mombaerts et. al., 1996). The second half of this thesis describes the generation of a series of transgenic mouse lines using this construct, along with a brief summary of their use in the analysis of a cbg null mutant mouse strain.
Materials and Methods

pCBGXX:nlacF and pCBGPX:nlacF transgene construction and transgenic mice

The source of the cerebroglycan genomic sequence used in the construction of both pCBGXX:nlacF and pCBGPX:nlacF was a λ library of genomic DNA from the emryonic stem cell line D3, derived from the 129SV mouse strain (Saunders and Lander, unpublished). A 4Kb XbaI fragment from a region 276bp from the 3' terminus of a 10Kb λ clone, cbgλ1, was subcloned into the XbaI polylinker site of pBlueScript, creating a 7Kb plasmid, pCBGXX, which was then used for further subcloning as described below. The cbg genomic fragment contained in this plasmid corresponds to a region within the cerebroglycan gene which extends 5' from an XbaI site positioned 70 bp upstream of the cerebroglycan start codon and 166 bp downstream of a reference point of +1bp corresponding to the 5' terminus of a primer-extended rat cDNA library (see arrowhead in fig. 1). The nuclear-localized lacZ sequence was provided by pnlacF (obtained from Nancy Hopkins at MIT), a pUC18 based plasmid containing an SV40 nuclear transport signal fused to the N-terminus of lacZ and a mouse protamine gene fragment containing intronic sequence and a polyadenylation signal at the 3' end of the construct following the lacZ stop codon (Mercer, et. al. 1991). nlacF was excised from pnlacF using XbaI/PstI and ligated into the polylinker of pBluescript at these sites using T4 DNA ligase, giving the new plasmid pBSnlacF. pCBGXX:nlacF was created by excising the XbaI fragment from pCBGXX and ligation it in the appropriate orientation into the XbaI site of pBSnlacF. pCBGPX:nlacF was created by removing the most 5' 2.5 Kb of the XbaI promoter fragment, leaving 1.5Kb of proximal promoter sequence. This was done by partially digesting pCBGXX:nlacF with XbaI and digesting fully with PacI (which cuts uniquely at position -1362 bp within the promoter), isolating the appropriate fragment, blunt-ending both ends with T4 DNA polymerase and recircularizing the plasmid with ligase.
Bacterial DNA sequences were removed from both constructs prior to pronuclear microinjection by excising the transgene using NotI and XhoI sites within the pBlueScript polylinker and isolating the resultant fragment through agarose gel electrophoresis. The DNA was then purified using the Qiagen Qiaex II gel extraction kit (cat. # 20021), and diluted into injection buffer (10mM Tris, pH 7.4; 0.25 mM EDTA) to a concentration of 2-5μg/ml. The solution was then filter sterilized and used for pronuclear injection into ova from either FVB mice (mice XX1 and XX2; injections done at MIT Transgenic Facility), or C57Bl/6 x DBA2 F1 hybrid mice (mice XX3-6 and PX1-6; injections done at UCI Transgenic Facility).

\textit{pT\alpha1:tau-lacZ transgene construction and transgenic mice}

The T\alpha1 promoter (Gloster et. al., 1994) was obtained from Daniel Belliveau from McGill University in the form of plasmid p253 (hereafter denoted pBS II T\alpha1), a pBlueScript II based Ta1 subcloning cassette containing an SV40 splice site and poly(A) addition sequence. The tau-lacZ fusion gene (Callahan et. al., 1994) was obtained from John Thomas of The Salk Institute in the form of pBS tau-lacZ, an 8.5Kb pBlueScript based construct containing an SV40 splice site and poly(A) addition sequence. The pBlueScript II vector backbone of pBS II T\alpha1 was first replaced with pNEB193 by excising the T\alpha1 promoter and associated SV40 sequence in the form of an XbaI/blunted BssHII fragment and ligating this fragment into the XbaI and blunted BamHI sites of the pNEB193 polylinker, giving a new plamid, pNEB T\alpha1. A XhoI/SpeI fragment from pBS tau-lacZ containing the tau-lacZ fusion gene and associated SV40 sequence was then ligated into the XhoI and SpeI polylinker sites of pNEB T\alpha1, placing the fusion gene downstream of the T\alpha1 promoter and replacing the SV40 sequence from pNEB T\alpha1 with those of pBS tau-lacZ, creating the construct pT\alpha1:tau-lacZ.

The T\alpha1:tau-lacZ transgene was isolated from bacterial sequences by digestion with AscI and PmeI. DNA for the first round of pronuclear injection (tattler-1) was purified
by sucrose gradient: 25μg of digested DNA was loaded onto the top of 6ml of a "bottom up" 10-40% linear sucrose gradient (sucrose solutions in 1M NaCl, 10mM Tris pH 8.0, 1mM EDTA), spun in an ultracentrifuge for 16hrs at high speed (equivalent of 35,000RPM in an SW41 tube), and collected in 40 fractions of 0.3ml each. Fractions containing the greatest amount of DNA, as assayed by gel electrophoresis, were pooled and dialyzed against injection buffer (5mM Tris pH 7.5, 0.1 mM EDTA) at 4°C. DNA was diluted to 2μg/ml final concentration in injection buffer and filter sterilized before pronuclear injection into ova from C57Bl/6 x BalbC F1 hybrids by the Transgenic Facility at UCSD. DNA used for subsequent rounds (tattler-2-4) was isolated by gel electrophoresis and purified using the Qiagen Qiaex II gel extraction kit (cat. # 20021), then diluted into injection buffer (10mM Tris, pH 7.4; 0.25 mM EDTA) to a concentration of 5μg/ml and filter sterilized before pronuclear injection by the UCSF Transgenic Facility.

X-gal staining procedure

Except in the case of E12 frozen sections, which were made following whole-embryo fixation and X-gal staining, any tissue sections were done on unfixed, unstained specimens which were fixed and X-gal stained as described below.

Frozen sections were prepared by fresh-freezing tissue samples in O.C.T. embedding medium (Tissue-Tek) on dry ice, followed by sectioning tissue onto microscope slides using a Jung CM3000 cryostat to a thickness of 20μm. Thawed sections were immediately fixed for 5 minutes in 0.5% glutaraldehyde in phosphate-buffered saline (PBS; 137mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄•7H₂O, 1.4 mM KH₂PO₄; pH 7.2), washed in PBS several times, and assayed for β-gal expression by incubating sections in X-gal staining solution for 24hrs at 37°C(X-gal staining solution in PBS: 1mg/ml X-gal [5-bromo, 3-indolyl, β-D-galactopyranoside], 5 mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 2mM
MgCl$_2$, 0.01% deoxycholate, 0.02% NP-40). Stained sections were then mounted in sucrose-saturated PBS with 0.01% sodium azide, coverslipped, and photographed.

Thick sections were made by embedding fresh tissue in 2% low melting point agarose (Gibco cat.# 15517-022) in PBS and then sectioning at 300µm using a Series 1000 Vibratome. Fresh sections were immediately placed in fixative solution for 15 minutes at 25°C, washed three times for 30 minutes each in PBS, and stained with X-gal as described above (fixative solution in PBS: 1% formaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, pH 8.0, 0.02% NP-40, 2 mM MgCl$_2$).

Whole brains and E12 embryos were fixed for 20-30 min in fixative solution, washed three times for 30 minutes each in PBS, and incubated in X-gal staining solution for 16 hrs at 37°C (E12 embryos XX3-6 were stained for 6 hrs in an effort to reduce background staining).

**Genotyping of transgenic mice using the polymerase chain reaction (PCR)**

PCR was used to genotype mice for the purpose of initial identification of transgenic animals following pronuclear injection (DNA isolated from placental tissue), as well as for transgenic strain maintenance (DNA isolated from adult tail tissue) and determination of homozygous $cbg$ null vs. heterozygous embryos for use in staining analysis (DNA isolated from embryonic tail tissue). DNA was prepared in all cases using the Qiagen QIAamp tissue kit (cat. # 29306).

Primers complementary to sequences internal to the bacterial lacZ gene were used for the analysis of both nlacF expressing transgenic mice (XX and PX strains) and tau-lacZ expressing mice (tattler-1-4), and gave a positive band at 217bp. The forward primer spans nucleotides 387-405 of lacZ (5'-TGATGAAAGCTGGCTACAG-3'), and the reverse primer spans nucleotides 603-582 (5'-ATCCTGATCTTCCAGATAACTG-3'). Two pairs of primers were used for the identification of $cbg$ null mutants. One pair, denoted $cbg$neo1, was used to amplify a 126bp fragment from the neo gene present only
within the null \textit{cbg} allele, and the other, \textit{cbgwt2}, amplified a 206bp fragment from within a region of the \textit{cbg} gene which was deleted in the null allele, and was therefore present only within the wild-type allele (the amplification of both fragments indicates a heterozygous condition). The forward \textit{cbgneo1} primer spans nucleotides 332-350 of the \textit{neo} gene used (5'-CTCCTGCCGAGAAAGTATC-3'), and the reverse primer spans nucleotides 457-440 (5'-CTTCCATCCCGAGTACGTG-3'). The forward \textit{cbgwt2} primer spans nucleotides +366+-387 of the \textit{cbg} genomic sequence, using the reference point illustrated in fig. 1 and 4, (5'-ATAGCTTAAACCTAATCCCTCC-3'), and the reverse primer spans nucleotides +572-+550 (5'-TATATGAGCAGCAGGTTTATTG-3'). Note: the exon I-intron I junction is at +402bp and amplification can therefore only occur when genomic DNA is used as a template.

Amplification conditions were identical for all three primer pairs and were as follows: PCR buffer (20 mM Tris*HCl, pH 8.4; 50 mM KCl), 3 mM MgCl$_2$, 10% glycerol, 0.2 mM each dNTP, 0.5 μm each primer, ~10ng template DNA (10% v/v), 5U Taq). Cycle profile: 30 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. Reactions were analyzed using gel electrophoresis in 4% NuSieve agarose (FMC cat. # 50084).

\textbf{Sequencing of the 1.5Kb cerebroglycan promoter fragment}

The 1.5Kb promoter fragment was sequenced from within the pCBGPX:nlacF construct by the dideoxy chain termination method using a modified t7 DNA polymerase (Sequenase kit 2.0, United States Biochemical). Only dGTP was used in the sequencing reaction and double-stranded DNA was used as the template. Sequencing from the 5' end was initiated from the T3 primer and was continued with subsequent custom primers. Sequencing from the 3' end was initiated from a custom primer which spans nucleotides +1 to -18, and continued with other custom primers. Approximately 58% of the promoter's length has been sequenced with two or more passes (see fig. 4B).
**Tattler-4 thalamic axon outgrowth in tissue culture**

E15 embryos from a cross between a tattler-4 heterozygous mouse and a wild-type FVB strain mouse were decapitated, their heads kept separately in ice-cold PBS, and their tails incubated in X-gal solution at 37°C for 1 hour to assay for tau-lacZ expression within the peripheral nerves. Meanwhile, their brains were removed and placed into sterile, ice-cold dissection medium (PBS supplemented with 4% calf serum, 5.6mg/ml glucose, 25 i.u./ml penicillin, 25 μg/ml streptomycin). Thalami were dissected from the brains of β-gal expressing embryos, and the entire region of the cortex not in direct contact with the underlying corpus striatum was removed from the brains of wild-type embryos. The thalami were pooled and chopped into fine pieces, then returned to ice-cold dissection media while the intact sheets of cortex from the wild-type embryos were positioned pial surface down on the semipermeable membranes of Millipore 30mm Millicell-CM well inserts (cat. # PICM 030 50). The chopped thalami were equally divided and placed on the exposed ventricular surfaces of the cortical tissue, and any excess dissection medium was aspirated to allow surface tension to flatten the tissues onto the membrane surface. The well inserts were then placed into 6-well tissue culture plates (Falcon, cat. #3046), and 1.5 ml of culture medium was added underneath the Millicell membrane (culture medium: Dulbecco's modified Eagle's medium [glutamine free, 4.5 g/L glucose] supplemented with 10 μg/ml transferrin, 5 mg/ml crystalline grade bovine serum albumin [ICN Biochemicals], 20 nM progesterone, 30 nM sodium selenite, 100 μM putrescine, 10 μg/ml bovine insulin, 1 mM sodium pyruvate, 25 i.u./ml penicillin, 25 μg/ml streptomycin). Plates were placed in a humidified 8% CO₂ incubator for 40 hrs at 37°C. Tissue was then fixed in 0.5% glutaraldehyde for 5 minutes at room temperature, washed three times in PBS, 30 minutes each, and then incubated in X-gal solution for 24 hrs at 37°C for staining of thalamic axons. Tissue was mounted on microscope slides in sucrose-saturated PBS with 0.01% sodium azide, coverslipped, and photographed.
Results

Isolation and characterization of the cerebroglycan promoter using transgenic mice

Cerebroglycan is a recently characterized neural specific proteoglycan found on the cell surfaces of neurons during their period of cell migration and/or axon extension (Stipp et. al., 1994; Ivins et. al., 1997). In an effort to characterize the promoter for cerebroglycan (cbg) for use in driving expression of heterologous genes in the study of axon guidance mechanisms, transgenic mice were created which contain mouse genomic DNA fragments from this gene fused to the lacZ gene which codes for the reporter enzyme β-galactosidase (β-gal). The first group of transgenic mice are denoted XX and contain the transgene CBGXX:nlacF, a 4Kb stretch of cbg genomic sequence isolated from a λ genomic library (Saunders and Lander, unpublished) positioned upstream of a nuclear localized lacZ construct which includes a termination codon, intron sequence, and poly(A) addition sequence (fig. 1B). The 3' end of the 4Kb genomic fragment terminates downstream of the transcriptional initiation site and 70bp upstream of the cbg start codon (fig. 1A; Note: since the precise location of the transcriptional start site for cbg is still unknown, the most 5' terminus of a primer extended cDNA library will be used as the reference point of +1bp [small arrowhead in fig. 1A]). A nuclear localized lacZ was chosen for the convenience of comparison of transgenic β-gal expression with in situ hybridization data of cbg expression patterns in the rat (Stipp et. al., 1994).

The first series of pronuclear injections yielded two independent transgenic mouse strains, XX1 and XX2, whose embryonic pattern of expression of β-gal were virtually identical to each other, as assayed by whole-body staining of E12 embryos with X-gal. Blue regions in the figures represent the presence of β-gal unless otherwise noted. Expression of β-gal was neural specific and extended along the entire aspect of the embryonic neuraxis with strongest staining in the developing spinal cord, medulla, and pons and cerebellar primordia. Less intense staining was found in the dorsal root
ganglia (DRG's; fig 3B), midbrain, especially ventrally at the midbrain/diencephalon border, diencephalon and telencephalon, with the vast majority of telencephalic staining restricted to the ventral and lateral aspect of the corpus striatum. (fig 2A,B, fig 2D,E).

This overall pattern of staining corresponds roughly to the mRNA expression pattern of cbg in the rat at a similar stage of development (~E15), especially with regard to the restricted expression of the transgene within the ventral and lateral spinal cord (fig 3A). However, the pattern of transgene expression at E12 deviated from the endogenous cbg expression in the absence of staining in the vast majority of the forebrain, in the neural retina, and in the olfactory epithelium (Note: blue staining in the eye in fig. 2 represents a staining artefact within the lens, which is also present in wild-type animals). Although the vast majority of transgene expression was found to be neural, there was also a small area of non neural expression found in the center of the developing limb bud which is not represented in the cbg in situ hybridization analysis (fig. 1A).

Transgenic line XX1 (fig. 2A,B) was chosen for further analysis at later stages of development using a similar X-gal staining protocol. By E17 the staining pattern has become more complex and various discrete areas of the brain show staining. The heaviest staining by far at this age is in the pons and medullar regions, but less intense staining is also seen in the deep nuclei of the developing cerebellum, the hypothalamus, the pretectal region, and in discrete layers of the midbrain (fig. 3C). There is also a faint layer of staining seen in the cortex at this stage (fig. 3D), though cortical staining remains low in the XX1 mouse up through the time of birth (fig. 3E). The overall level of staining within the brain decreases somewhat with the age of the embryo, consistent with the temporal regulation of endogenous cbg. By the time of birth, high levels of staining are seen in only a few discrete regions including the hippocampus, a region which is similarly positive for cbg mRNA at this stage (fig. 3F). Some ectopic areas of staining are seen at this stage, however, most notably in the anteroverntral nucleus of the
thalamus, an area which does not express the endogenous gene in P0 rats (fig. 3F; Stipp et. al., 1994).

In order to quickly gather more evidence that this putative cbg promoter is responsible for the observed transgene expression pattern, several more transgenic animals were created using CBGXX:nlacF and then directly analyzed instead of establishing stable transgenic strains from each mouse. Four more independent transgenic animals were obtained in this manner and X-gal stained at E12 (denoted XX3-6). Mouse XX3 had a β-gal expression pattern which was divergent from those of XX1 and XX2, with several ectopic regions expressing the transgene, including intensely stained portions of the branchial arches (not shown), the posterior half of the proximal forelimb buds, and the anterior half of the proximal hindlimb buds, as well as central limb bud staining more intense than that seen in XX1 (fig. 2F). Much of the staining in XX3 was neural, however, with the DRG's, the olfactory bulbs, the entire corpus striatum, the diencephalon and the anterior midbrain all staining at a much greater intensity than XX1 and XX2 (fig. 2C). However, staining intensity along the neuraxis of XX3 dropped off abruptly at a location midway along the anterior-posterior axis of the midbrain, and all CNS regions posterior to that point possessed very little staining (see arrow in fig. 2C). XX4 had a transgene expression pattern which was also divergent from XX1 and XX2. Its staining pattern, with the exception of a small region within the proximal limb bud, consisted almost entirely of neural staining within the forebrain, midbrain, medial portions of the cerebellar primordium, and the DRG's. The forebrain staining included cortical staining as well a significant level of staining in the olfactory epithelium, regions which did not stain at all in XX1-3 (fig. 2I).

The last two transgenics produced, XX5 and XX6, were identical to XX1 and XX2 in all regions observed, with a possible exception of lower posterior midbrain expression and an absence of limb bud expression (fig. 2G,H; Note: this may be due to the shorter X-gal processing times used for XX3-6 utilized in order to reduce substrate-induced
yellowing of tissues and high background staining, since both these areas were readily visible in XX1 and XX2 only after longer reaction times). Since 4/6 independent insertions of transgene CBGXX:nlacF yielded identical patterns of E12 expression (XX1,2,5,6) and since 5/6 insertions yielded patterns with a high degree of neural specificity (all but XX3), it was concluded that the 4Kb putative promoter fragment was responsible for the observed patterns of transgene expression.

In order to help characterize which regions within this 4Kb fragment contain essential transcriptional control elements, the most 5' 2.5Kb of the promoter was removed from the transgene construct by taking advantage of a unique internal PacI site, leaving 1.5Kb of proximal promoter sequence within the new construct pCBGPX:nlacF (fig. 1C). This construct was then used to create more transgenic animals for direct analysis as before, through X-gal staining of E12 embryos. Six transgenics were analyzed from this series of pronuclear injections (PX1-6). All but PX2, which only expressed a patch of staining unilaterally in a portion of the midbrain, had expression patterns identical to XX1,2,5, and 6 (fig. 2J,K). Variations were mostly due to intensity of overall staining, which, in two of these five PX embryos, resulted in extremely faint staining within DRG's and corpus striatum, regions which regularly showed lower staining levels in XX transgenics as well. In 3/5 PX embryo's however, there was a distinct unevenness in the spinal cord staining, not seen in any of the XX transgenics, which varied in severity among these embryos. Figure 2L shows this effect in PX1 which lies between the other two PX embryos in terms of the severity of this uneven staining pattern. The two other PX transgenics did not show this type of pattern and appeared identical to the XX embryos. Apart from this uneven spinal cord staining, then, the staining patterns achieved with the PX promoter fragment were identical to those of the much larger XX fragment, indicating that most or all of the necessary promoter elements were present in this 1.5Kb stretch of proximal upstream cbg sequence.
**Sequencing and characterization of the PX promoter**

The entire sequence of the PX promoter was determined in order to uncover known transcription factor binding sites present within the sequence, and also to find similarities between this sequence and those of known neural specific promoters, since any extensively homologous regions could then be studied for their function through deletional mutagenesis and further transgenic mouse analysis. Figure 4A displays the extent of the overlap between sequencing runs (regions shown in black have been verified by at least a second round of sequencing). A BLAST search (Altschul et. al., 1990) was done in order to uncover any large regions of the PX promoter which were homologous to known sequences in the eukaryotic promoter database, however no such matches were uncovered using this method. Another approach was to compare the PX sequence in a more thorough manner against the sequence of a promoter with similar expression patterns to those of the PX promoter. By this time, the To 1 promoter discussed in the introduction had become available, and since this promoter most closely resembled the PX promoter in its ability to drive transgene expression in a neural specific and temporally regulated manner, it was chosen for this sequence comparison. The MEME algorithm (Bailey et. al., 1994) was used to search for relatively large regions of homology between these two sequences in both plus and minus strand orientations. Several homologous regions of considerable identity were discovered in this manner and are displayed above the appropriate regions within the PX promoter sequence shown in figure 4B. Most of the displayed homologous sequences have high levels of identity (≥80%), however, one particularly strong homology is a 24bp stretch of DNA at -880bp within the PX promoter sequence which is 92% identical to the opposite orientation To 1 sequence, differing by only two adjacent T/C nucleotides.
Creation of the tattler mouse strains

For the purpose of facilitating the study axon guidance mechanisms, a transgenic mouse was desired which would express a reporter enzyme within axons specifically during their period of extension and guidance. Although the \( cbg \) promoter was initially planned for use in driving the expression of such a reporter construct, a promoter for a similarly regulated gene, \( T\alpha 1 \), became readily available during the course of the \( cbg \) promoter characterization. \( T\alpha 1 \) is an \( \alpha \)-tubulin gene which is expressed specifically during axon growth, making up >95% of the embryonic \( \alpha \)-tubulin (Miller et. al., 1987). A 1.1Kb fragment of the endogenous promoter for this gene has been isolated and used to express a nuclear localized \( lacZ \) gene in transgenic animals in a pattern consistent with endogenous \( T\alpha 1 \) gene expression (Gloster et. al., 1994). The axon-targeted reporter gene chosen was \( tau-lacZ \), a fusion gene which consists of a 5' fragment of the bovine gene for the microtubule associated protein tau, fused to the bacterial \( \beta \)-gal gene \( lacZ \) (fig. 5). The tau protein fragment consists of the first 383 amino acids of tau, which includes the microtubule binding domain presumed to be responsible for the translocation of the reporter protein to the microtubule rich axon (Callahan et. al., 1994). The resultant construct pT\( \alpha 1 \):tau-lacZ was used to create a series of four independent strains, denoted tattler-1 through tattler-4, which all express \( \beta \)-gal in their axons, but which possess different levels of transgene expression within overlapping nervous system regions.

A large proportion of the tattler-1 staining pattern at E12 is due to expression of tau-lacZ in the DRG's and their associated axons (fig. 6A,G). At E12 the DRG central projections are visible extending medially into the spinal cord through the dorsal roots and then rostrally via an ascending dorsal spinal cord tract (fig. 6A). The DRG peripheral projections are also stained throughout the body, most noticeably in the large intercostal nerves and major limb nerves (fig. 6C). Intense staining is also seen in the midbrain at this stage (fig. 6A-D), in the trigeminal ganglion with its associated sensory...
nerve projections into the face, and in the retina (fig. 6D). Little staining is seen in the spinal cord neurons themselves, or in any other central nervous system region with the exception of the midbrain and retina, inconsistent with extensive neuraxis staining in transgenic mice used for the original characterization of the Tal promoter (Gloster et. al., 1994). The X-gal staining pattern of the E16 brain is dominated by the optic nerve and tract with the associated collicular innervation (fig. 6E,F; Godement et. al., 1984), the vomeronasal organ and vomeronasal nerve (not shown at E16), the habenula and its associated habenulopeduncular tract (fig. 6E; Contestabile et. al., 1987), and the brain stem staining which includes several intensely stained cranial nerve ganglia and their projections (not shown). Staining of the retina at P0, at a time when the various retinal layers have segregated, indicates that transgene expression there is restricted to the retinal ganglion cells which form a layer of axon fibers along the interior surface of the retina and project outward to form the optic nerve (fig. 6H). P10 brain staining patterns corroborate the E16 CNS expression pattern (fig. A), with small regions of intense staining restricted to discrete areas in the brain stem, in the optic tract including the collicular target region staining (fig. 7B), in the habenula and habenulopeduncular tract and its target, the interpeduncular nucleus (fig. 7A; Contestabile et. al., 1987), and in the vomeronasal organ and nerve (fig. C), which runs medially up the olfactory bulbs to innervate the accessory olfactory bulb (Farbman, 1991). Also visible in fig. 7C is a small projection of the vomeronasal nerve which extends towards the ventral forebrain where it innervates targets in the basal ganglia (Farbman, 1991).

Tattler-2 was analyzed only at E12 and has an expression pattern somewhat different from tattler-1. DRG’s are also stained in tattler-2, but at a much lower intensity, which makes it difficult to visualize their central and especially their peripheral projections (fig 8A). The midbrain is stained in tattler-2, but mostly in its ventral aspect, and much more lightly than in tattler-1 (fig 8B). The majority of the dorsal midbrain staining takes the form of several thick fiber bundles which course ventrally from the dorsal midline (not
shown). Also different from tattler-1 is the presence of light staining in the spinal cord neurons themselves, including the motor neurons which extend very lightly stained axon bundles through the ventral roots (not shown). The intensity of the spinal cord staining increases rostrally into the medulla, and continues into the pons and cerebellar primordia, through to the ventral midbrain (fig. 8B). Very light staining is also seen in the corpus striatum (fig. 8B). The retina is stained, though more lightly than in tattler-1 (not shown). Tattler-2 shows some non-neuronal ectopic staining within the proximal limb buds (fig. 8A).

Tattler-3 shows a staining pattern which is much more similar to the original Ta1 transgenic mouse than either tattler-1 or tattler-2, with the DRG, retinal, and midbrain staining of tattler-1, but with extensive neuraxis staining as well. Spinal cord staining is intense (fig. 8C), and the ventral roots are clearly visible in cross section (not shown), however, the peripheral projections of the DRG's and trigeminal ganglion are not as intensely stained as in tattler-1. The pons and cerebellar primordia are only lightly stained, as is the cortex, however, the olfactory epithelium, olfactory bulbs, corpus striatum and diencephalon are stained well (fig. 8D). Some non-neuronal ectopic expression is seen in tattler-3, but is largely restricted to a small area within the central limb buds (similar to the cbg transgenic E12 mice), and some limited staining in the umbilicus (fig. 8D). Sections from E15 tattler-3 brain show stained habenular and retinal pathways identical to tattler-1, however extensive hypothalamic staining is also present, as well as intense staining within various thalamic nuclei and their cortical projections, though the vast majority of cortical staining seems restricted to thalamic axons themselves (not shown).

Tattler-4 has the most widespread expression of all the tattler strains and the E12 mouse has all the staining patterns of tattler-3 in addition to intensely stained peripheral nerves. Staining of extensively branched cutaneous nerves gives the mouse a diffuse blue staining (fig. 8E,F) which only under highly magnified views reveals itself as a
reticular network of axons (not shown). Various cranial nerves are also stained, which do not appear in tattler1-3 (not shown). There seem to be non neuronal ectopic areas of expression within tattler-4 including intense cutaneous staining in the umbilicus and whisker pads, but further analysis is necessary before ruling out cutaneous nerve expression as the cause of the staining. The E15 brain staining pattern of tattler-4 is extremely widespread, and in addition to tattler-3 regions includes all of the hypothalamus, epithalamus including the habenula and its tract, and all of the thalamic nuclei and their associated fiber tracts which can be seen to innervate the largely negative cortex in a discrete layer, presumably the intermediate zone (fig. 9A). Stains of sagittal sections of intact brain at P2 show widespread staining of the entire brain below the level of the telencephalon (fig. 9B), including strong staining within the external layers of the cerebellum (fig. 9D). However several areas in the forebrain are stained as well, including the hippocampus and fimbria, the thalamic axons in the corpus striatum emanating from the internal capsule (fig. 9C), as well as the olfactory bulb plexiform layers which receive input from the stained olfactory epithelium neurons (9E).

**Use of tattler-1 in the analysis of the cerebroglycan knockout mutant**

As mentioned in the introduction, one major utility of the tattler strains would be in their use for the analysis of mutant mice with regard to axon guidance defects. Since a mutant mouse made null for the cerebroglycan gene through homologous recombination had recently been generated in our lab (Saunders and Lander, unpublished), it was decided to use the tattler-1 mouse to look for altered staining patterns against a cbg null background. Tattler-1-expressing progeny from an initial cross between a homozygous null cbg animal and a tattler-1 heterozygote were crossed in turn to homozygous cbg null mice in order to produce mixed litters of progeny consisting of heterozygous and homozygous null siblings (as determined by PCR of
embryonic tail DNA), half of which expressed tattler-1 and could be stained for axon patterns. Several ages were used for the analysis, with some examples of heterozygous vs. homozygous null animals shown in figure 10 (top photo of each pair is heterozygous null; bottom is homozygous null). In E12 embryos special attention was given to whole-body peripheral nerve patterns including intercostals, limb innervation, ascending DRG central projections, retinal staining, and trigeminal projections (fig. 10A,D,G,J). In later embryos, special attention was given to the optic tract projection pattern, including the extent of collicular innervation (fig. 10B,E,H,K,I,L), and also to the pattern of cranial nerve and cranial nerve ganglion staining (fig. 10C,F). No differences were seen in any of these areas even after frozen sectioning for more detailed analysis (Note: sections compared in fig. 10C and 10F were chosen for identical staining patterns within the medullar region, and apparent differences in cerebellar staining intensity between 10C and 10F [see arrows] are due to a slightly different plane of section between samples. The section adjacent to the one shown in 10F displays identical cerebellar staining to the section displayed in 10C).

**Demonstration of tattler-4 thalamic axon staining in culture**

Besides their usefulness in the analysis of in vivo axon staining patterns within a mutant context, tattler strains may also be used in tissue culture systems such as slice culture or co-culture, where visualizing axons from a particular transplanted tissue is often complicated by the complexity of the tissue itself, and by any other tissues used as substrates for axon growth and guidance (Wu et. al., 1995; Molnar et. al., 1991). In order to use tattler neurons in these systems, it is first necessary to ascertain the ability of transplanted tattler strain neurons to maintain transgene expression over extended periods in culture. For this purpose, a pilot co-culture experiment was done using transplanted E15 thalamus from tattler-4 on same age cortex from wild-type siblings.
Axons were allowed to grow over a two day period, and the tissue was then X-gal stained. Figure 11 demonstrates that either the transgene expression was in fact actively maintained, or that residual β-gal was sufficient to allow axon visualization after an extended co-culture.
Discussion

**Characterization of the cerebroglycan promoter fragment**

Genomic sequence directly upstream from the cerebroglycan (cbg) coding region was shown to be sufficient to drive the expression of a nuclear-localized lacZ reporter construct in transgenic mice in the form of two different sized promoter fragments, the 4 Kb XX promoter and the 1.5 Kb PX promoter. The pattern of the transgene expression in these mice taken as a whole was similar to, but not identical to that of cbg itself. Early expression of the transgene at E12 revealed a widespread neural staining pattern which generally coincided with the expression of cbg in rats of the same developmental stage, but which lacked a great deal of the forebrain and some of the midbrain staining seen in *in situ* hybridizations of cbg mRNA (fig. 2; Stipp et. al., 1994). This lack of forebrain staining was especially dramatic in the cortex of mouse XX1 (the only stable transgenic line produced which could then be used for extensive analysis), which was negative through to the later embryonic and early postnatal stages, with only minimal staining of small cortical areas, generally within the posterior region (fig. 3D,E). Discrepancies were seen in other regions as well, including a lack of staining in the retina and the folia of the cerebellum, though the cerebellar primordium was stained very intensely in E12 embryos (fig. 2) and deep cerebellar nuclei were moderately stained well into postnatal life (data not shown).

Some regions, however, possessed staining patterns strikingly similar to the pattern of cbg expression. Transverse sections through the spinal cord, for example, revealed staining which was restricted almost entirely to the ventral and lateral regions in a manner almost identical to the *in situ* hybridization patterns (Stipp et. al., 1994). Sections of P0 brains also revealed a strong similarity between the XX1 strain expression pattern and the cbg *in situ* data in the relatively heavy staining of the hippocampal cell layers, which could be seen prominently against the diffuse and lighter staining of
surrounding tissue (fig. 3E). One notable exception to this lighter background staining, which did not match any in situ data, was the staining of a few isolated thalamic nuclei at this age, including the anteroventral nucleus (fig. 3F).

Six XX transgenic mice and five PX mice were produced, and out of these eleven independent transgenics nine showed patterns of staining which were identical to each other at E12 (with the exception of the uneven spinal cord staining seen in some PX embryos). This indicates that the cloned promoter fragments were responsible for the transgene expression, and that the regions within the genome into which these transgenes were randomly inserted had a minimal affect on this expression. However, this so-called "position effect" of altered transgene expression (Russo et al., 1988) may explain the divergent nature of the staining patterns seen in mice XX3 and XX4. XX3, in particular, possessed a curious staining pattern characterized by sharply defined transition points between staining and non-staining regions along the anterior-posterior axis of the embryo, as well as within the branchial arch regions. This type of staining pattern is much more characteristic of the pattern-forming genes which organize the body plan of developing embryos (Bang et al., 1996), than that of the cbg expression pattern, and suggests the possibility that the XX promoter activity was masked in this mouse by local regulatory elements for this gene type near the region of transgene insertion. On the other hand, even XX3 had a largely neural expression pattern, with a great amount of forebrain expression and heavy staining in the DRG's, and the staining pattern of XX4 was almost entirely neural with a high level of expression within the cortex, a region which expresses high levels of cbg message and whose lack of staining in the other transgenics was unexpected. Therefore, it seems likely that at least some degree of neural specificity was provided by the promoter fragment in mice XX3 and XX4, even within the context of transgene expression altered by position effects.

The great similarity of E12 staining patterns between the majority of transgenic mice produced not only verifies the functionality of the introduced promoter fragment, it also
points to likely promoter deficiencies as well. This is true because the observed discrepancies between the cbg expression data and the staining patterns from later embryos of the XX1 transgenic line now become more difficult to explain away as possible position effects, and instead have to be viewed within the context of a stable and consistent expression pattern. It is clear, then, that if a transgene promoter fragment is desired which accurately mimics the endogenous cbg promoter, more genomic sequence must be added to future constructs. Even so, the isolated promoter fragments confer a transgene expression pattern which may be similar enough to those of cbg that they could be used to gain interesting information concerning the unique features of cbg gene regulation through the use of future in vivo and in vitro experiments. For instance, molecules known to influence neuronal gene expression, such as various growth factors and second messengers, could be modulated either in the XX1 animal itself or in culture, and their effects on gene expression monitored quickly with a simple lacZ assay. The XX1 mouse could also be used in combination with other transgenic animals for the purpose of studying cbg gene regulation. For instance, the null mutant mice for other proteoglycans such as syndecan or glypican could be crossed to the XX1 strain in order to see if there is a common feedback mechanism among this family of genes which results in a compensatory upregulation of the cbg gene during embryonic development, as assayed by β-gal staining.

Finally, since the full sequence of the PX promoter is now known, mutagenesis of various regions of the promoter could be combined with β-gal staining in order to characterize more fully the promoter elements which are responsible for the neural-specific expression seen with this promoter. New transgenic mice could be created with the mutated promoter fragments and directly assayed at E12 as before, or a quicker, though less accurate, analysis could be done in vitro, provided that transgene expression in XX1 mice can be supported in culture. Several candidate sites within the PX promoter fragment already exist based on their high degree of homology to
sequences within the Tα1 promoter, most notably a particular region with greater than 90% identity over a stretch of 24 nucleotides (see fig. 4B, position -880bp).

Creation of the tattler mouse strains

As stated in the introduction, the characterization of the cerebroglycan promoter was undertaken with the hope that its unique characteristics of tight spatial and temporal control over gene expression could be harnessed for the purpose of studying axon guidance within an \textit{in vivo} context. The appearance of the Tα1 promoter shifted attention toward its use in conjunction with an axonally-targeted lacZ fusion gene, tau-lacZ, for the creation of a mouse strain which would possess axon pathways visible by β-gal staining. Four strains were produced, and of the four strains, tattler-3 and tattler-4 had the most widespread neural expression. Tattler-4 had an almost panneuronal expression pattern, with the only exception being a general lack of staining within the cerebral cortex (fig. 9A-C). None of the tattler mice, in fact, show positive staining in the cortex, despite the presence of cortical staining in the original Tα1 mice (Callahan et. al., 1994). It is not clear what the cause of this could be, however when it is considered that the mice from the cbg promoter group of transgenics, with the exception of mouse XX4, demonstrate an equally unexpected lack of cortical staining, the possibility emerges that extensive transgene expression in cortical neurons is somehow difficult to achieve.

The lack of cortical staining in tattler-3 and 4, however, allowed for the visualization of the thalamic innervation of the cortex (fig. 9A), a feature which could prove extremely useful for future studies of thalamocortical axon guidance. It was noticed, in fact, during the course of the analysis of tattler staining patterns, that the incomplete expression of the transgene could prove useful in many ways. For example, since the cbg gene is highly expressed in retinal neurons during the formation of the visual system (Stipp et. al., 1994; Ivins et. al., 1997), analysis of the optic tract in the cbg null mutant mouse was desired for the purpose of detecting any possible guidance errors. Since the
tattler-1 mouse used for the analysis of the cbg null mutant possessed very little staining within the forebrain, the intensely stained optic tract could then be analyzed in great detail against an almost completely negative background (fig. 10H,K,I,L). Various other regions in the tattler-1 mouse have intensely stained axon pathways which also appear against negative backgrounds, and could be studied in a similar manner with a high degree of detail. The vomeronasal nerve can be seen clearly from at least E15 up to P10, the latest age analyzed, and is a good candidate for such study, in that the regions through which the vomeronasal nerve traverses, namely, the area surrounding the olfactory epithelium and the olfactory bulbs, are both completely free of staining (fig. 7A,C; Farbman, 1991). This is also the case for the habenula and its associated habenulopeduncular tract (fig. 7A), and for the innervation of the non-expressing spinal cord neurons from the dorsal root ganglia (fig. 6A). Finally, though the staining of the entire thalamus in tattler-4 makes it useful for the study of cortical innervation in general, the staining of discrete nuclei in the thalami of tattler-3 (not shown) makes it an especially appropriate system for the study of regional specificity of cortical innervation by different thalamic regions.

Though emphasis so far has been placed on the potential usefulness of the tattler mice for the purpose of studying axon guidance within the transgenic strains themselves, they could also be utilized simply as a ready source of tau-β-gal expressing neurons which may be used in other contexts. The persistence of transgene expression in culture (fig. 11), offers evidence that this is possible. The tattler-4 strain becomes especially useful here, since its wide expression pattern allows it to act as a source for a variety of distinct neuronal cell types. For example, mice chimeric for the transgene may be produced through blastomere aggregation for the purpose of studying competition of neural target innervation between distinct neuronal populations, or simply for the study of the development of such innervation patterns. Another alternative might be the use of tattler mice in transplantation experiments between tattler mouse donors and non-
expressing hosts. Tattler-derived neurons may also be especially useful in several in vitro systems which involve growing dissociated neurons on intact brain tissue, since the impermanence of the available cellular dyes makes extended labeling of axons in such systems problematic.

In summary, then, four independent strains of tattler mice were created and shown to provide axon visualization in vivo. Each strain possesses its own distinct expression pattern and its own particular utility, however taken together, in conjunction with relatively simple and rapid β-gal staining techniques, they provide a powerful tool for the study of axon guidance processes in vitro and in vivo,
Figure 1: Cerebroglycan promoter construct design

A. Schematic of the cerebroglycan translational start site in relation to restriction sites used for the subcloning of both promoter constructs. Arrowhead represents the most 5' terminus of a primer-extended cDNA library (Stipp et al., 1994), which is also used as the reference point of +1bp in the absence of a known transcriptional start site.

B. Organization of promoter construct CBGXX:nlacF containing the 4 Kb cbg promoter fragment aligned with the genomic sequence from which it was derived. lacZ, gene for bacterial β-galactosidase; TAA, stop codon for lacZ; nuc, SV40 nuclear localization sequence; mP1, mouse protamine gene fragment (Peschon et al., 1987) containing an intron (shown as a thin black line) and polyadenylation site (poly A).

C. Organization of promoter construct CBGPX:nlacF containing the 1.5 Kb cbg promoter fragment also aligned with figure 1A.
a) Cerebroglycan Genomic Sequence

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<tr>
<td>Pac I</td>
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<td>+236 bp ATG</td>
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b) pCBGXX:nlacF

- CBG Promoter XX
- lacZ

mP1

TAA poly A

c) pCBGPX:nlacF

- CBG Promoter PX
- lacZ

mP1

TAA poly A
Figure 2: cbg promoter XX and PX transgenic mice

Whole-body X-gal stains of E12 cbg promoter mice XX1 (panels A,B), XX2 (panels D,E), XX3 (panels C,F), XX4 (panel I), XX5 (panels G,H), and PX1 (panels J-L). Embryo views are from a side (panels A,D,G,J), dorsal (panels B,E,H,K,F,L), or frontal (panels C,I) perspective. Panel L shows a magnified view of panel K. Arrow in panel C indicates transition from transgene-expressing to non-expressing region along anterior/posterior extent of CNS in mouse XX3. Arrows in panels F and I indicate regions of non-neuronal transgene expression. mid, midbrain; str, corpus striatum; cb, cerebellar primordium; p, pons; med, medulla; drg, dorsal root ganglia; sc, spinal cord; di, diencephalon; ob, olfactory bulb; oe, olfactory epithelium; ctx, cerebral cortex
Figure 3: Developmental expression of nlacF transgene in XX1 mouse

A. Thick tissue cross-section of spinal cord in an E12 embryo showing restricted transgene expression in the ventral spinal cord (vsc).

B. Frozen section of whole-body X-gal stained E12 embryo showing individual expressing neurons within the dorsal root ganglia (drg).

C. Thick sagittal section of an E17 brain indicating several discretely stained regions, including layered staining in the midbrain (mid), heavy pons region staining (p), staining within the medulla (med), the cerebellum (cb), and the pretectum (pt).

D. Thick sagittal section of E17 cortex (arrows indicate faintly-stained discrete layer)

E. Thick sagittal section of a P0 brain showing largely negative cortex (ctx), as well as diffuse staining within the midbrain (mid) and pretectal region (pt), and more intense staining in the hippocampus. Thalamus (th) is largely negative with the exception of intense staining in the anteroventral nucleus (see panel F).

F. Magnified image of panel E. av, ectopic staining of the anteroventral nucleus of the thalamus; dg, dentate gyrus of the hippocampus (an area which expresses cbg mRNA at this time (Stipp et. al., 1994)); cp, choroid plexus (this is a staining artefact and does not represent transgene expression)
**Figure 4A: Sequencing history the cbg PX promoter fragment**

Arrows indicate individual sequencing reactions and their associated directionality. Black areas indicate sequenced regions of the promoter which have been verified by a second or third sequencing run, whereas gray areas represent regions sequenced only once.
**Figure 4B: Complete sequence of the cbg PX promoter fragment**

The complete sequence of the 1.5 Kb *cbg* promoter fragment is listed, using the most 5' terminus from a primer extended cDNA library as the reference point (arrow labeled 5' cDNA indicates +1bp). Sequence appearing above the underlined regions are taken from either the plus strand (normal type) or minus strand (italics) of the 1.1 Kb Tα1 promoter, and have been identified by the MEME algorithm (Bailey et. al., 1994) as being highly homologous to the *cbg* promoter (location of the Tα1 sequence within its promoter is shown in brackets).
Figure 5: Construct design for Tα1:tau-lacZ

Tα1 is a 1.1 Kb promoter fragment (Gloster et. al., 1994) placed upstream of the tau-lacZ fusion gene (separated by a polylinker; short black line), which contains the first 1.1 Kb of tau (including the microtubule binding region in lighter gray) fused to the full-length lacZ sequence (Callahan et. al., 1994). Poly (A) addition sites are included in the SV40 DNA appended onto the construct (shown in black).
pT\(\tau_1\):tau-lacZ

T\(\tau_1\)  tau  lacZ  poly A

microtubule binding domain
Figure 6: Embryonic β-gal expression pattern of tattler-1 transgenic mouse

Panels A-D show E12 whole-body staining of tattler-1 (dorsal view in panel A; side views in panels B-D). Panels E and F show the dorsal and ventral view, respectively, of fixed and stained intact brains from an E18 tattler-1 mouse. Panel G shows frozen section of a whole-body stained E12 tattler-1, with the dorsal root ganglia (drg), and an associated DRG peripheral nerve projection (pn). Figure H shows a fresh-frozen section of a P0 retina, fixed and X-gal stained, indicating the outline of the retina with a dotted line. The inner surface of the retina is stained blue from axons extending from retinal ganglion cells (rgc), which then merge to form the optic nerve (on). Arrow in panel F shows the chiasm, the region at which optic nerve fibers (on) cross and enter the optic tract (ot). Arrow in panel C shows stained axons ascending rostrally from the dorsal root ganglia (drg) through the dorsal spinal cord toward the medulla. mid, midbrain; dr, dorsal root; drg, dorsal root ganglia; ic, intercostal nerves; tri, trigeminal ganglion; max, maxillary branch of the sensory trigeminal pathway; ot, optic tract; hab, habenula; col, superior and inferior colliculi; on, optic nerve; pn, peripheral nerve; rgc, retinal ganglion cell layer.
Figure 7: Postnatal staining pattern of tattler-1

Panel A is a low mag view of thick tissue sagittal section from P10 tattler-1 brain. The vast majority of the brain is negative, including the cortex (ctx) and the cerebellum (cb). One notable exception is the habenula and its associated fiber tract, the habenulopeduncular tract (hp). The collicular targets (col) of the retinal ganglion cells are intensely stained (high mag view in panel B), as is the vomeronasal nerve (vn) seen close up in panel C, along with a minor pathway taken by this nerve into the basal forebrain (small arrows in panel C).
Figure 8: β-gal expression pattern of tattler-2 through tattler-4

Shown are E12 whole-body stains of tattler-2 (panels A and B; dorsal and rear views, respectively), tattler-3 (panels C and D; dorsal and frontal views, respectively), and tattler-4 (panels E and F; dorsal and frontal views, respectively). Arrows in panel F indicate possible non-neuronal ectopic transgene expression in whisker pads and in the umbilicus. sc, spinal cord; drg, dorsal root ganglion; ect, non-neuronal ectopic expression (limb bud in panel A, umbilicus in panel D); vmid, ventral midbrain; dmid, dorsal midbrain; cb, cerebellum; med, medulla; di, diencephalon; ob, olfactory bulb; oe, olfactory epithelium.
Figure 9: Developmental transgene expression in tattler-4

Panel A shows the widespread expression pattern of tattler-4 in an E15 fresh frozen horizontal section, fixed and stained for the tau-lacZ gene. The vast majority of the brain with the exception of the cortex expresses the transgene at high levels. The staining within the cortex is most likely due to thalamic innervation of the developing intermediate zone (shown by arrows). th, thalamus; h, habenula; hp, habenulopeduncular tract. Panels B-E show P2 whole head fresh-frozen sections which have been fixed and stained for β-gal. Panels D and E are high mag views of the same section which was mounted immediately after fixing, and panel C is a high mag view of panel B, which shows a section which has been counterstained in neutral red saturated solution for 10 minutes prior to mounting. ctx, cerebral cortex; hip, hippocampus; fx, fornix; ic, internal capsule; cb, cerebellum; ob, olfactory bulb; g, glomeruli within the olfactory bulb which are targets for olfactory receptor axon input (arrows in panel E).
Figure 10: Analysis of null cbg mutant mice using tattler-1

Tattler-1 mice which were also either heterozygous (panels A-C,G-I), or homozygous (panels D-F,J-L) for the cbg null gene were analyzed side by side in order to detect any differences in axon staining patterns. Staining patterns are shown here for E12 whole-body stain (panels A,D,G,J), E14 whole-brain stains (panels B,E,H,K), or stains of thick coronal sections from E14 brain (panels C,F,I,L). Orientation of stained specimens is either dorsal (panels A,D,B,E), ventral (panels H,K), or side view (panels G,J). Sections shown in panels C,F,I, and L are shown with the dorsal side toward the top and ventral toward the bottom. As discussed in the results section, arrows in panels C and F point to cerebellar staining patterns which seem to differ between heterozygotes and homozygotes, but are actually due to differences in the plane of section. Arrows in panels I and L point to the optic tract just above the optic chiasm, which along with the other stained axon pathways, look identical in sections between the null mutant mice and the heterozygotes.
Figure 11: Staining of thalamic axons from tattler-4 thalamus

Panels A and B show images of E15 thalamic tissue (th) isolated from tattler-4 embryos and grown for a period of two days on the ventricular surface of E15 cortex (ctx) from wild-type siblings. Arrows in panel A display an example of a long, single axon which can be visualized using X-gal staining.
REFERENCES


