

Imaging Neuronal Subsets in Transgenic Mice Expressing Multiple Spectral Variants of GFP

Neurotechnique

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Summary

We generated transgenic mice in which red, green, yellow, or cyan fluorescent proteins (together termed XFPs) were selectively expressed in neurons. All four XFPs labeled neurons in their entirety, including axons, nerve terminals, dendrites, and dendritic spines. Remarkably, each of 25 independently generated transgenic lines expressed XFP in a unique pattern, even though all incorporated identical regulatory elements (from the *thy1* gene). For example, all retinal ganglion cells or many cortical neurons were XFP positive in some lines, whereas only a few ganglion cells or only layer 5 cortical pyramids were labeled in others. In some lines, intense labeling of small neuronal subsets provided a Golgi-like vital stain. In double transgenic mice expressing two different XFPs, it was possible to differentially label 3 neuronal subsets in a single animal.

Introduction

To a remarkable extent, progress in our understanding of neural organization and development has depended on a series of improvements in our ability to image individual neurons—from the Golgi stain to electron microscopy to anterograde and retrograde labeling methods to immunohistochemical labeling protocols to confocal microscopy. An advance of arguably similar magnitude was the introduction of the jellyfish green fluorescent protein (GFP) as a vital stain (Chalfie et al., 1994). Several features have made GFP especially useful. First, GFP is a protein, so cells can be rendered fluorescent by introduction of a cDNA rather than the chromophore itself. Therefore, the label can be rendered stable and heritable. Second, the GFP chromophore is derived *entirely* from the polypeptide chain (by spontaneous cyclization and oxidation), so no exogenous cofactors or substrates need to be delivered to render GFP-expressing cells fluorescent. Therefore, GFP can be used to view living cells with minimal perturbation. Third, GFP can be fused to other proteins without loss of fluorescence, so it can be directed to specific subcellular compartments. Fourth, GFP can be mutated to generate

variants with altered spectral properties and improved translational efficiency, thermostability, and quantum yield. As a result of these favorable properties, GFP and its variants have been used to follow molecules and cells in at least a dozen species, ranging from slime molds to ferrets (reviewed in Chalfie and Kain, 1998; Tsien, 1998; Conn, 1999).

The first report on GFP expression in heterologous cells illustrated its use as a vital stain for neurons (Chalfie et al., 1994). Since that time, neuroscience has been one of the greatest beneficiaries of GFP technology, and GFP has been used to facilitate the study of neuronal development and plasticity in transgenic worms, flies, fish, and mice (for example, Dynes and Ngai, 1998; Murray et al., 1998; van den Pol and Ghosh, 1998; Knobel et al., 1999; Zito et al., 1999; Rodriguez et al., 1999; Higashijima et al., 2000). A few years ago, we began generating transgenic mice that expressed GFP or one of its spectral variants in motoneurons and retinal ganglion cells, to complement our ongoing studies of synaptogenesis in these two cell types (for reviews, see Sanes and Lichtman, 1999; Sanes and Yamagata, 1999). In the course of characterizing these mice, we made several observations that may be generally useful and are therefore documented here. First, we show that each of four spectral variants (green [GFP], yellow [YFP], cyan [CFP], and red [RFP] fluorescent proteins, which we term collectively XFP) satisfactorily labels neurons *in vivo*. Second, we show that XFPs label axons over centimeter-long distances and dendrites over millimeter-long distances, even when they are not fused to peptides designed to facilitate their transport (e.g., GAP-43 [Moriyoshi et al., 1996] or Tau [Rodrigues et al., 1999]). Importantly, both dendritic spines and presynaptic nerve terminals are labeled in their entirety. Third, we demonstrate that expression of XFP for up to 9 months has no discernible effect on synaptic structure and that multiple imaging of XFP-labeled neurons *in vivo* is not detectably toxic. Fourth, we report a remarkable variability in patterns of XFP expression among mice generated from the same construct. Expression is similar among offspring of each transgenic founder but differs among lines, indicating that the variation reflects differences in integration site and/or copy number. Indeed, each of 25 lines examined displayed a unique, heritable pattern of expression. As a result, XFP labels discrete groups of neurons in Golgi-like fashion in some lines, allowing novel analyses of processes such as neuronal migration, axonal guidance, dendritic growth, and synaptogenesis. Finally, we report generation of double transgenic mice that express contrasting spectral variants (e.g., YFP and CFP or GFP and RFP) in overlapping but distinct neuronal subsets. Such multiple labeling enables new studies of neuronal connectivity and competition *in vivo*.

Results

Imaging Synapses in YFP Transgenic Mice

Our original aim was to image the formation of synapses by motoneurons and retinal ganglion cells. To this end,

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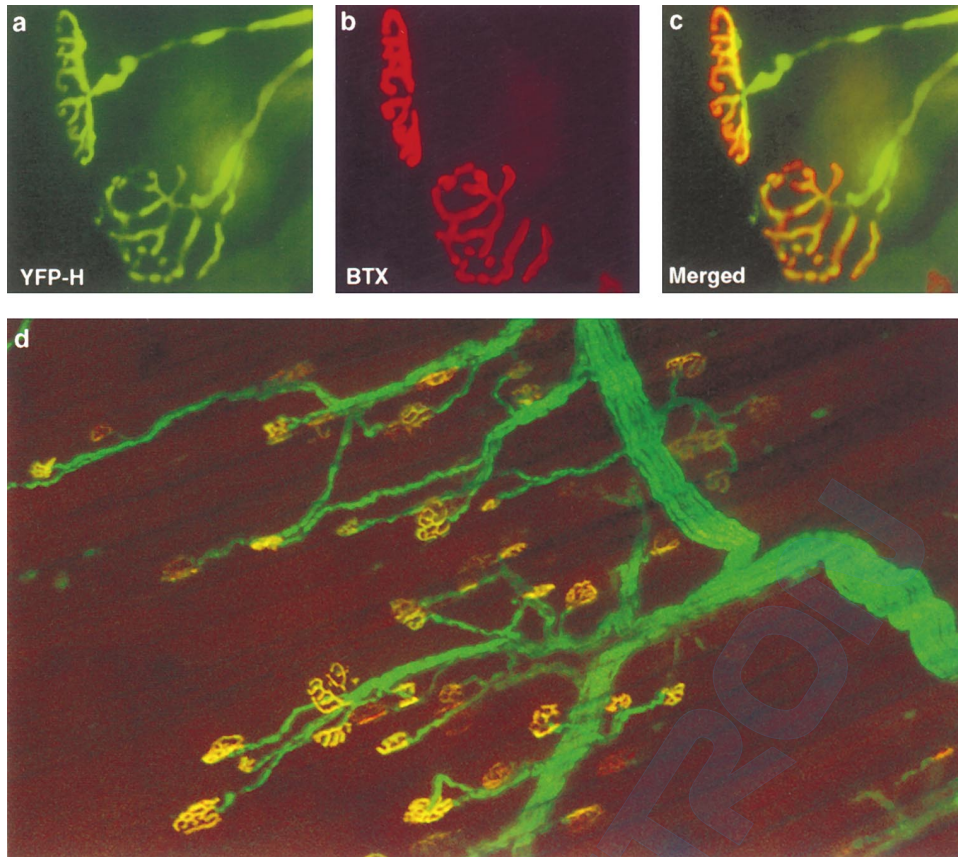


Figure 1. Vital Staining of Adult Neuromuscular Junctions in a *thy1-YFP* Transgenic Mouse

(a–c) The sternomastoid muscle was exposed, incubated briefly with rhodamine- α -bungarotoxin (BTX) (to label postsynaptic AChRs), and viewed with a water immersion lens.

(a) YFP labels two branches of a motor axon and the motor nerve terminals they form.

(b) Same field, viewed with rhodamine optics.

(c) Perfect apposition of pre- and postsynaptic specializations indicates that YFP completely filled the nerve terminal.

(d) Low power view of labeled NMJs in a fixed gluteus muscle from a *thy1-YFP* line 16 mouse. Motor axons run through an intramuscular nerve, then branch and terminate on muscle fibers. All AChR-rich postsynaptic sites are apposed by YFP-positive nerve terminals, indicating that all motoneurons supplying this muscle express the transgene.

we carried out two sets of preliminary studies. In the first, we compared four regulatory elements that had previously been shown to drive expression in one or both of these cell types in transgenic mice. In the second, we compared expression and subcellular localization of four variants of GFP. Based on the results of these studies (see Experimental Procedures for details), we chose mice expressing YFP under the control of neuron-specific elements from the *thy1* gene for detailed characterization. Thy1 is an immunoglobulin superfamily member that is expressed by projection neurons in many parts of the nervous system, as well as by several nonneuronal cell types, including thymocytes (hence its name; Morris, 1985; Gordon et al., 1987). Early transgenic analysis revealed that neural and nonneural expression depend on distinct genomic elements and that deletion of a particular intron selectively abolishes expression in nonneural cells (Vidal et al., 1990). A construct lacking this intron has been successfully used to overexpress β -galactosidase and growth-promoting molecules in neurons with minimal nonneural expression (Kelley et al., 1994; Aigner et al., 1995; Caroni, 1997).

Figures 1a–1c show two neuromuscular junctions

from the sternomastoid muscle of a live *thy1-YFP* mouse. The muscle was counterstained with rhodamine- α -bungarotoxin, which labels acetylcholine receptors (AChRs) in the postsynaptic membrane, then viewed with YFP- or rhodamine-selective filters. Several points are worthy of note. First, the complete overlap of labeled pre- and postsynaptic elements indicates that YFP labeled the entire nerve terminal. Second, no expression of YFP was detectable in muscle fibers or other nonneural cells, confirming the neuron-specific character of the regulatory elements used. (Occasional YFP-positive mononucleated cells were observed in a few transgenic lines, but their number was small and they never obscured neural labeling.) Third, all postsynaptic sites were apposed by YFP-positive nerve terminals, indicating that all motoneurons expressed YFP (Figure 1d). Likewise, all motor axons were labeled in each of 20 muscles examined, including forelimb, hindlimb, and axial muscles. Fourth, axons were labeled in muscles supplied by long nerves (e.g., the diaphragm and tibialis anterior), implying that YFP diffuses or is transported at least a few centimeters along axons. Moreover, there was no

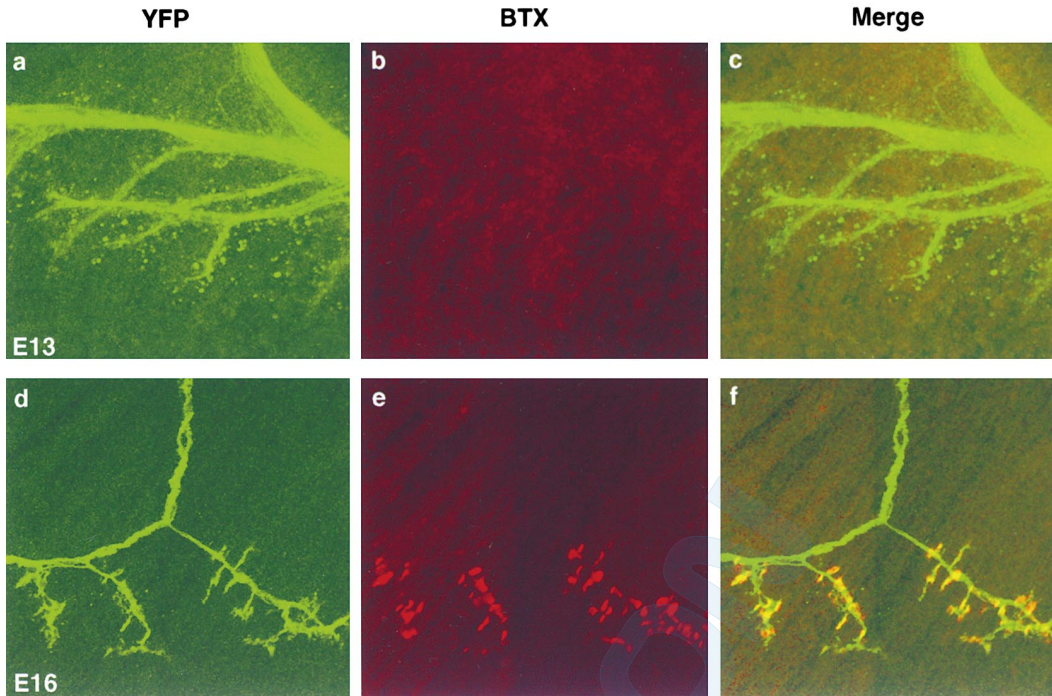


Figure 2. Viewing Initial Stages of Synaptogenesis in *thy1-YFP* Mice

Diaphragm muscles were dissected from *thy1-YFP* line 16 mice at embryonic day (E) 13 (a–c) or E16 (d–f), stained with rhodamine- α -bungarotoxin (BTX), and viewed with selective filters as in Figure 1. At E13, motor axons had formed an intramuscular nerve in the diaphragm, but AChR staining was diffusely distributed on myotubes indicating that postsynaptic differentiation had not yet commenced. By E16, AChRs were clustered in precise apposition to YFP-labeled nerve terminals.

obvious difference in staining intensity between distal and proximal portions of peripheral nerves. Finally, YFP fluorescence was intense in both live and paraformaldehyde-fixed tissue (compare Figures 1c and 1d), permitting reexamination of specimens following live imaging.

Thy1 levels in neurons increase markedly during early postnatal life, and *thy1*-derived transgenes have been reported to exhibit corresponding developmental regulation (Morris, 1985; Kollias et al., 1987; Caroni, 1997). As expected, levels of YFP increased postnatally in *thy1-YFP* mice. Nonetheless, in some lines, YFP expression was readily detectable in motor axons by embryonic day (E) 13, the stage at which neuromuscular junctions are just beginning to form (Noakes et al., 1993; Figure 2). This has made it possible to view the initial stages of synaptogenesis in live embryos exo utero (data not shown).

Assessing Toxicity and Phototoxicity of YFP Expression

GFP has generally been found to be innocuous, but a few reports have raised the possibility that its expression can be toxic in cultured cells (Liu et al. 1999) or transgenic mice (Huang et al., 2000). To address this issue, we imaged identified YFP-labeled neuromuscular junctions at multiple intervals over periods of weeks to months (Lichtman et al., 1987). Figure 3 shows a junction that was first viewed through a small incision in the skin of an anesthetized mouse at 8 weeks of age. Once imaging was complete, the incision was sutured and the mouse was returned to its cage. One week later, the

mouse was reanesthetized and the same junction was identified, using blood vessels and other landmarks, and imaged again. This procedure was repeated after further intervals of 1 and 34 weeks. During this entire 9 month period, the only changes observed in the structure of this and other synapses were those characteristic of normal growth and aging (Balice-Gordon and Lichtman, 1990). Thus, prolonged expression of YFP has no detectable effect on synaptic structure.

This longitudinal study suggested repeated imaging of YFP was negligibly toxic. To address the issue of phototoxicity more directly, we performed two additional experiments. First, we illuminated YFP-labeled neuromuscular junctions for 3 hr without interruption. Seeing no changes, we revived the animal, then viewed the same synapses 1 week later, as described above. No changes attributable to illumination were detected (data not shown). Second, we imaged growing axons 3 days after axotomy, a time at which they were reinnervating synaptic sites. Reinnervation proceeded throughout a 5 hr period, during which the axon was imaged every 2 min (data not shown). Thus, YFP-labeled axons can survive, grow, and even form synapses during illumination.

Labeling of Neuromuscular Junctions with Various Fluorescent Proteins

The spectrum of YFP is red shifted with respect to that of GFP, but the colors of the two fluorophores differ only subtly to the human eye. Other spectral variants have been generated, however, in which the colors are

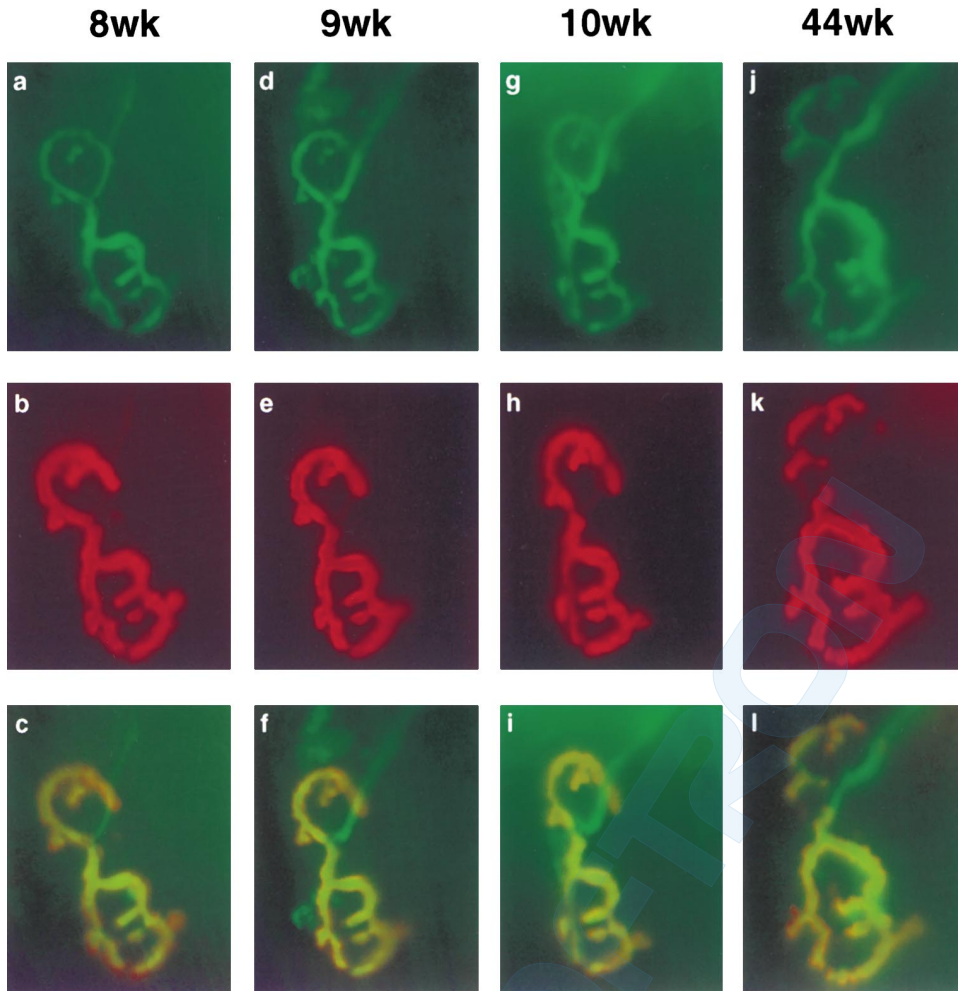


Figure 3. Long-Term Expression of YFP Is Innocuous to Motor Nerve Terminals

(a–c) A neuromuscular junction in the sternomastoid muscle of an 8-week-old *thy1-YFP* mouse was imaged through a small incision in the skin, as in Figure 1. Following imaging, the incision was sutured and the animal returned to its cage. The same synapse was then identified and imaged again 1, 2, and 36 weeks later (d–l). Throughout this 9 month period, synaptic structure showed only the small changes characteristic of growth and aging. These results suggest that neither prolonged expression nor repeated excitation of YFP is toxic to neurons.

markedly different from that of GFP, most notably the blue (BFP) and cyan (CFP) fluorescent proteins (Tsien, 1998). Moreover, a homologous red fluorescent protein (RFP) was recently isolated from *Discosoma sp* (sea anemone relative; Matz, et al., 1999). To pave the way for double-labeling experiments and to maximize the potential for compatibility with other stains, we generated transgenic mice in which GFP, CFP, or RFP was expressed under the control of *thy1* regulatory elements. (BFP was unworkably dim in transfected cultured cells [data not shown], so we did not test it in transgenic mice.)

Labeling of motor axons by GFP was similar to that by YFP, both qualitatively and in intensity (Figures 4a and 4b). CFP was, on average, somewhat less intense than YFP, presumably because it has a lower quantum yield (<http://www.clontech.com>). It was, however, suitable for in vivo labeling (Figure 4c). Like YFP (see above), CFP and GFP are not detectably toxic.

The most recently introduced fluorophore, RFP, also

worked well as a vital label in transgenic mice (Figure 4d). However, we noted two differences between RFP and the other three XFPs. First, RFP accumulated in the nucleus of neurons as well as in the cytoplasm and also formed bright speckles in some cells (data not shown). Second, only 14% of transgenic founders (2/14) tested expressed RFP, compared to 84% (38/45) of founders expressing CFP, GFP, or YFP under the control of identical regulatory elements. Therefore, the distinct properties of RFP make it a useful fluorophore, but it may be a less reliable label than other XFPs.

Distinct Neuronal Cohorts Labeled in Each Transgenic Line

Based on results from the neuromuscular junction, we extended our analysis to other regions of the nervous system. Neurons were XFP positive in many parts of the peripheral and central nervous system, including sympathetic and sensory ganglia, retina, forebrain, mid-brain, cerebellum, and spinal cord (Figures 5–7 and data

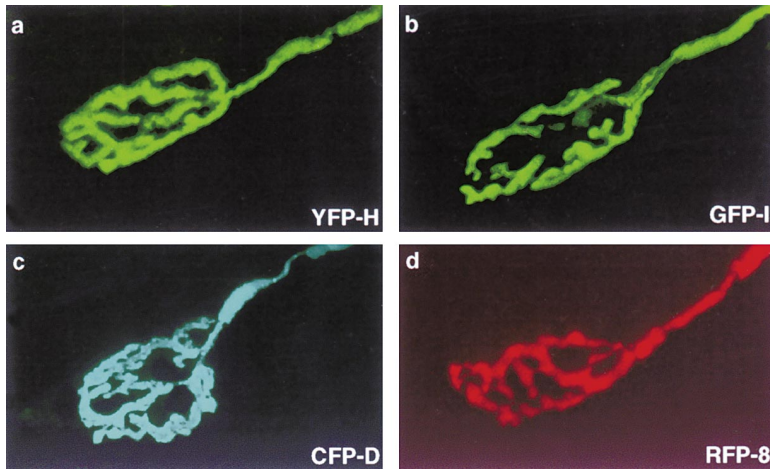


Figure 4. Four Spectrally Distinct XFPs Serve as Vital Stains in Transgenic Mice

Neuromuscular junctions from *thy1-YFP* line H (a), *thy1-GFP* line H (b), *thy1-CFP* line D (c), and *thy1-RFP* line 8 (d) transgenic mice. In each case, muscles were labeled with bungarotoxin, to confirm perfect apposition of pre- and postsynaptic specializations (data not shown).

not shown). In nearly all cases, labeling was confined to neurons, and XFP labeled neurons in their entirety, including somata, nuclei, axons, dendrites, and dendritic spines (e.g., Figures 6D, 7B, and 7C). Remarkably, however, the types and numbers of labeled neurons varied greatly among mice, even though all were generated from identical (for each spectral variant) or nearly identical (when comparing one XFP to another) constructs. To assess the extent and nature of this variability, we established lines from a large number of *thy1-XFP* transgenic mice and performed a detailed analysis of transgene expression in mice of 25 *thy1-CFP*, GFP, and YFP lines. Table 1 summarizes expression in several representative regions, and the following paragraphs summarize some features of the patterns we observed.

Sources of Variation

We first asked whether all descendants of a single founder expressed their transgene in the same pattern or whether animals from the same line differed in expression pattern. In the first case, expression patterns would be genetically determined; in the latter case, they might reflect epigenetic or environmental factors. To distinguish these alternatives, we examined two to five (generally three) adult (>4 weeks of age) animals from each line. Mice were taken from at least two separate litters and, for most lines, from at least two successive generations. In each case, patterns of expression listed in Table 1 were consistent among all descendants of a particular founder. Since the expression patterns are heritable yet all lines were generated from identical or nearly identical transgenes, variation among lines almost certainly reflects differences in the chromosomal integration site and/or copy number of the transgene (see Discussion).

Even though patterns of expression are determined by sequences in or near the transgene, they might be influenced by unlinked genes. For example, numerous strain-dependent variations in transgene expression have been documented (e.g., Allen et al., 1990). Because the transgenic founders were generated in interstrain hybrids (CBA/C57), we considered this possibility. However, three observations suggest that variations we observed were not attributable to unlinked genes. First, most comparisons involved animals that had been backcrossed to C57 mice for multiple generations, so their

genetic background, although not identical, was similar. Second, with few exceptions (see Experimental Procedures), expression patterns varied little between CBA/C57 hybrid founders, and their C57 descendants. Third, in several cases, we crossed mice to the non-Swiss Albino (NSA) outbred strain for three to five generations, then compared expression patterns on C57 and NSA backgrounds. No strain-dependent differences were noted.

Types of Variation

In every region of the nervous system studied, we observed line-to-line variations. Two types of variation were quantitative. First, variable proportions of each neuronal type were labeled. For example, nearly all sensory neurons were labeled in the dorsal root ganglia (DRGs) of some lines, whereas only a few percent of these neurons were labeled in other lines (Figure 5). Second, neurons were intensely XFP positive in some lines, but only dimly stained in others. Importantly, the intensity and extent of staining were not correlated. In some lines, a few neurons were intensely labeled in a particular region, whereas their neighbors were completely unlabeled (e.g., Figures 5C, 6D, and 7B). In other lines, all neurons of a particular type were dimly labeled. Thus, labeling of subsets is not an artifactual consequence of dim labeling. As a further test of this point, we generated homozygotes for five of the lines by crossing heterozygotes. As expected, labeling was stronger in homozygotes than in heterozygotes, but patterns of expression were unaffected by gene dosage.

A third sort of variation was qualitative: different cell types were labeled in different lines. For example, preganglionic axons but no ganglion cells were labeled in the superior cervical ganglia (SCGs) of some lines, whereas ganglion cells but few or no preganglionic axons were labeled in others (e.g., Figures 5E and 5F). Likewise, in the cerebellar cortex, Purkinje cells were labeled in some lines, interneurons (basket and stellate cells) in others, and both in still others (Table 1). Particularly striking in this regard were patterns in the cerebral cortex. In most lines, cells were labeled in multiple laminae, but in two lines, labeling was largely confined to a single lamina (Figures 7D–7F).

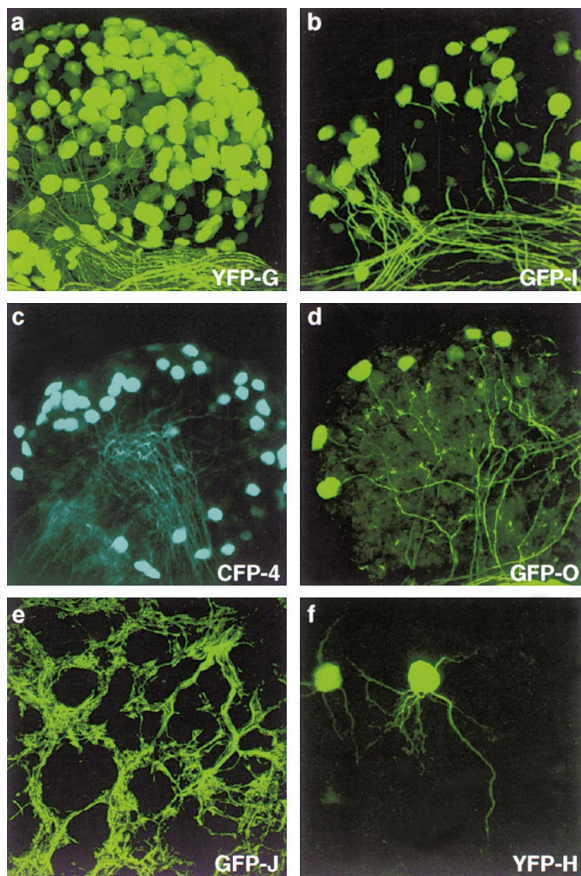


Figure 5. Expression of XFP in Peripheral Ganglia
(a–d) Dorsal root ganglia. Most sensory neurons were YFP-positive in *thy1-YFP* line G (a), about 10%–20% of neurons were labeled in *thy1-GFP* line I (b) and *thy1-CFP* line 4 (c), and only a few percent of neurons were labeled in *thy1-GFP* line O (d). In each case, whole DRGs from a lumbar level were mounted and imaged.
(e and f) Superior cervical ganglia. In *thy1-GFP* line J, preganglionic axons and terminals were labeled, but ganglion cells were not (e). In *thy1-YFP* line H, most preganglionic axons were unlabeled, but a small subset of ganglion cells was transgene positive. Axons and dendrites of the ganglion cells are labeled.

Nature of Subsets

XFP expression in restricted subsets of a particular cell type raised the question of whether the labeled neurons shared some quality. We cannot provide a definitive answer to this question, but several observations indicate that many labeled subsets are heterogeneous. First, the size of DRG neurons correlates with their neurochemical identity and patterns of peripheral and central projection (Lawson, 1992). In *thy1-XFP* lines with labeled subsets of sensory neurons, both large and small neurons were labeled. Moreover, examination of the periphery revealed that labeled sensory axons innervated both muscle spindles and skin in a single animal (data not shown). We were also able to categorize XFP-labeled retinal ganglion cells as α or non- α (based on their soma sizes) or as “ON” or “OFF” (based on their dendritic structures; Wässle and Boycott, 1991). In a single line (*thy1-YFP* 12), only \sim 20% of retinal ganglion cells were labeled, but the labeled cells included repre-

sentatives of all four types (ON- α , OFF- α , ON-non- α , and OFF-non- α ; data not shown). Finally, the precise numbers and types of labeled neurons in a given structure frequently differed between the right and left side of a single animal. Together, these results suggest a stochastic component to the choice of which cells express the transgene.

Extent of Variation

At one extreme, each *thy1-XFP* line might exhibit a unique expression pattern. Alternatively, each line might exhibit one of a modest number of patterns. A third possibility is that most lines are similar, but a few are highly aberrant. To distinguish these alternatives, we tabulated expression patterns in six regions of the adult nervous system of 25 *thy1-XFP* lines (Table 1). To provide a minimal estimate of variation, we omitted differences in labeling intensity or developmental regulation and grouped patterns into as few categories as possible. For example, all lines in which 10%–80% of DRG neurons were labeled are listed in Table 1 as “Many,” even though some lines reproducibly exhibited more labeling than others. Likewise, all lines with labeled somata in multiple cortical layers are listed as “2–6,” even though the number and types of neurons labeled varied greatly among lines. Despite these stringent criteria, no two lines of the 25 listed share an expression pattern. There is a general tendency for lines with robust expression in one structure to exhibit robust expression in others, but patterns in any one region are not reliable predictors of those in other regions. Moreover, if the number of possible patterns were only a few-fold larger than 25, it is likely that the same pattern would have been encountered more than once. Thus, we believe that the *thy1-XFP* transgene can be expressed in many, probably hundreds, of line-specific patterns.

Multicolor Labeling of the Nervous System

YFP can be readily distinguished from CFP using commercially available filters (Miller et al., 1999), and GFP can be distinguished from RFP using conventional fluorescein- and rhodamine-selective filters. The marked variation of XFP expression in different lines made it possible to label subset of neurons with different colored fluorescent proteins in the same mouse. We therefore generated transgenic mice with two different colored fluorescent proteins by crossing *thy1-CFP* to *thy1-YFP* mice or *thy1-GFP* to *thy1-RFP* mice. As shown in Figures 8a and 8c, neurons expressing GFP, RFP, or GFP+RFP form three distinct classes. Likewise, CFP-, YFP-, and CFP+YFP-positive subsets are readily distinguishable (Figure 8b). By the same logic, YFP+CFP+RFP mice, now being constructed, should allow differential labeling of up to seven subsets.

Discussion

Perhaps our most striking finding is that XFP expression patterns vary dramatically among transgenic lines generated from the same construct. In this regard, two observations are particularly pertinent. First, variations are great from line to line but minimal among descendants of a single founder. Lines differ in transgene copy number (which can range from 1 to >50 copies, arranged in

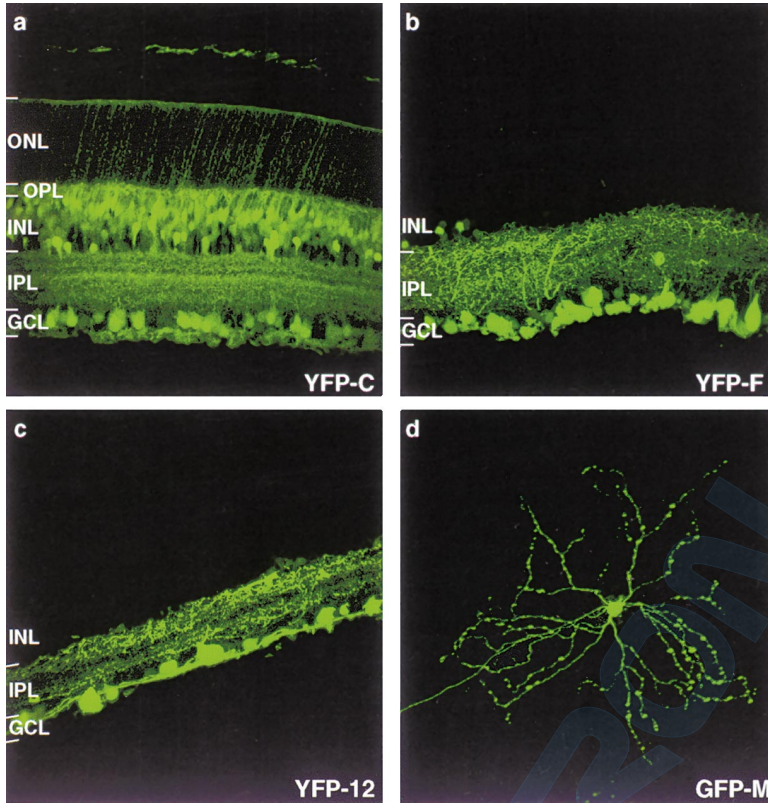


Figure 6. Expression of XFP in Retina

Cross sections of retinæ from *thy1-XFP* mice are shown in (a)–(c); (d) is from a whole mount. Ganglion, amacrine, bipolar, and Muller cells are all labeled in *thy1-YFP* line C (a), most ganglion cells are labeled in *thy1-YFP* line F (b), about 20% of ganglion cells are labeled in *thy1-YFP* line 12 (c), and only a few ganglion cells are labeled in *thy1-GFP* line M (d). XFP fills the dendrites of ganglion cells, as indicated by intense labeling of the inner plexiform layer (IPL) in sections and the elaborate arbor visible in the whole mount. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

tandem) and chromosomal insertion site (which is random and therefore almost certainly different for each line). Therefore, sequences near the insertion site and/

or differences in copy number affect expression pattern. Second, although these factors influence expression, the main determinants are *thy1*-derived sequences. Ex-

Line	Motor Axon	Retina			SCG		DRG	Cortex	Cerebellum		
		RGC	INL	Pre	Post	Mossy			Purk	Molec	
YFP-12	All	Many	A	None	None	Many	5,6	All	Many	Few	
-16	All	All	A+B	All	Few	All	2-6	All	None	Few	
-21	All	All	A	All	Many	All	2-6	All	All	None	
-A	Many	Many	A	Many	None	Many	2-6	All	Many	Few	
-C	All	All	A+B+M	All	Few	All	2-6	All	None	None	
-D	All	All	A	All	None	All	2-6	All	None	Few	
-F	All	Many	A	Many	None	All	2-6	All	Few	Few	
-G	All	Many	A	All	Few	All	2-6	All	None	None	
-H	Few	Few	None	Few	Few	Many	5	Many	None	None	
GFP-F	All	All	A+B+M	All	None	All	2-6	All	Many	Few	
-G	All	All	A+B+M	All	Few	All	2-6	All	Many	Few	
-H	All	Many	None	All	None	Many	2-6	Many	None	None	
-I	All	Few	None	All	None	Many	6	All	Many	None	
-J	All	All	A+B+M	All	None	All	2-6	All	Many	None	
-M	Few	Few	None	None	None	Few	Few	Many	None	None	
-O	All	Few	None	None	None	Few	2-6	All	Many	None	
-S	Few	Few	None	None	None	None	Few	Few	None	None	
CFP-4	All	Many	None	None	Few	Many	5,6	All	Many	None	
-11	All	Many	None	Few	Few	Many	2-6	All	Few	Few	
-23	All	All	A	None	None	Many	2-6	All	None	None	
-D	All	Few	None	None	None	All	5,6	All	All	None	
-F	All	Many	None	All	None	All	2-6	All	None	Few	
-H	All	Many	None	None	None	Many	2-6	Many	None	None	
-I	All	All	None	None	None	All	2-6	All	None	None	
-S	Few	None	None	None	None	Few	Few	Few	None	None	

Table 1. Patterns of Transgene Expression in 25 *thy1-XFP* Lines

Patterns of expression were determined in two to five adult (>4 week) animals of each line. To avoid overestimating variation, patterns were grouped into large categories. “All,” expression in >80% (usually 100%) of neurons of the indicated class (shown in red); “Many,” expression in 10%–80% of neurons (orange); “Few,” expression in <10% of neurons (yellow-orange). Numbers under “cortex” indicate laminae in which somata of labeled neurons were observed. In some cases, a small number of neurons were present in layers other than those indicated (see, for example, Figures 7E and 7F). The great line-to-line variation in the fraction of cortical neurons labeled is not indicated here. A, amacrine cells; B, bipolar cells; DRG, dorsal root ganglion (lumbar ganglia were examined); INL, inner nuclear layer of the retina; M, Muller cells; Mossy, mossy fibers in internal granule layer of the cerebellum; Purk, cerebellar Purkinje cells; Molec, interneurons of the cerebellar molecular layer; RGC, retinal ganglion cells; SCG, superior cervical ganglion. In lines *thy1-GFP* M and O, some labeled nonneuronal cells (fibroblasts or satellite cells) were present in the SCG. In many lines, granule cells of the cerebellum were labeled, but because their labeling was often obscured by that of mossy fibers, this cell type is not listed.

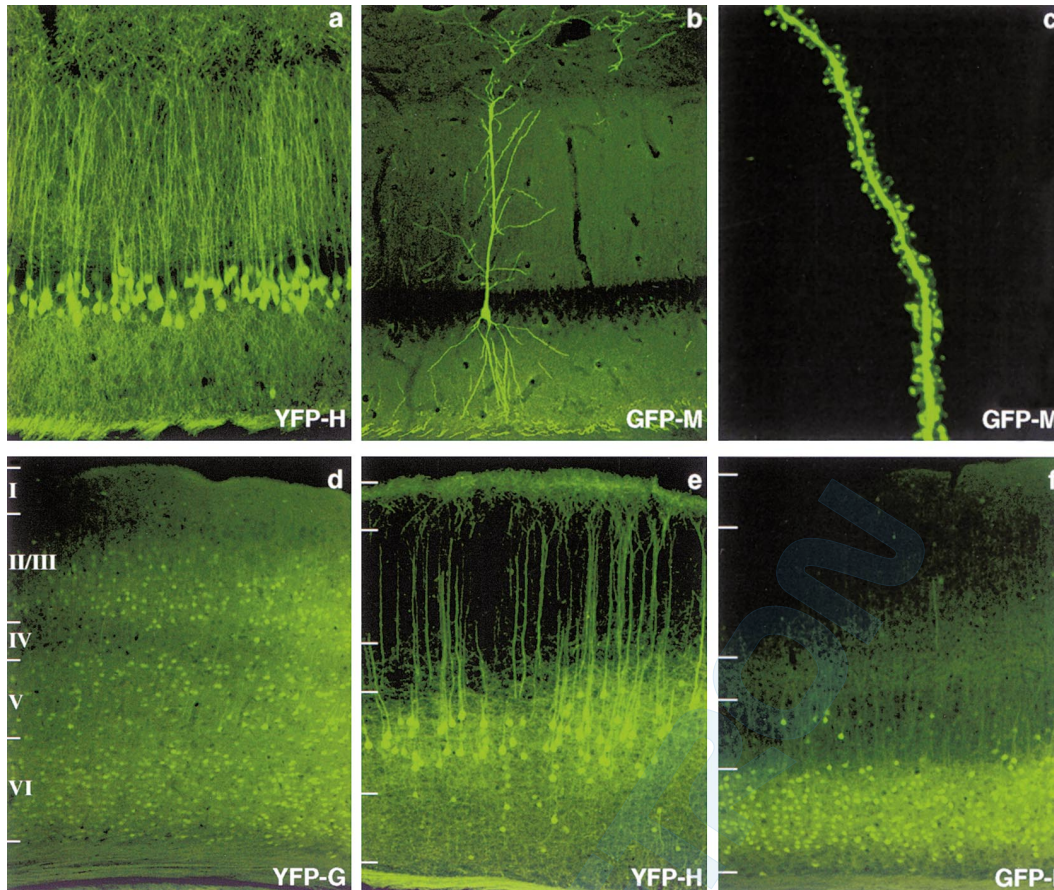


Figure 7. Expression of XFP in the Hippocampus and Cerebral Cortex

(a–c) Hippocampal pyramidal cells express YFP in most lines examined, but most cells of this class are positive in some lines (such as *thy1-YFP* line H, [a]), whereas only a small fraction of cells are positive in others (such as *thy1-GFP* line M, [b]). When only a few cells are labeled, dendrite spines are clearly visible (c).

(d–f) In cerebral cortex, neurons are labeled in multiple laminae of most lines (such as *thy1-YFP-G*, [a]). In a few lines, in contrast, expression is largely restricted to neurons with somata in specific layers—layer V in *thy1-YFP* line H (e) and layer VI in *thy1-GFP* line I (f).

pression is neuron specific and, in general, most prominent in those neurons that are Thy1 positive. For example, Thy1 is present at high levels on all motoneurons and sensory neurons (Raff et al., 1979; Morris, 1985), and XFP is expressed by many or all such neurons in 21/25 lines. Likewise, Thy1 is detectable on only a minority (10%; Raff et al., 1979) of cortical and cerebellar neurons, and the transgene is expressed on a minority of these neurons in most lines. Particularly telling in this respect is the retina: Thy1 is a “specific” marker for retinal ganglion cells in this structure but is also present at low levels on some amacrine, bipolar, and Muller cells (Barnstable and Drager, 1984; Perry et al., 1984; but see Dabin and Barnstable, 1995). Consistent with this pattern, the transgene is expressed in at least some retinal ganglion cells in 24/25 lines, but in amacrine (generally small subsets) of only 12/25, in bipolars of 5/25 and in Muller cells of 4/25 lines. Thus, patterns of transgene expression represent restricted subsets or close relatives of the neurons that normally express *thy1*.

How then does insertion site or copy number influence transgene-directed expression? Previous studies suggest many possibilities, of which we mention four. First,

expression might reflect a combination of elements in the transgene and in flanking DNA. In transgenes with minimal or no promoter sequences, expression patterns are sometimes dictated entirely by elements at the integration site, leading to “enhancer trapping” or “gene trapping,” respectively (O’Kane and Gehring, 1987; Gossler et al., 1989). Such trapping is clearly not the sole determinant of expression in *thy1-XFP* mice, both because of the obvious influence of *thy1* elements and because it is unlikely that transgenes in so many lines would, by chance, have integrated near genes that are themselves expressed in neuronal subsets. Nonetheless, combinatorial specificity has been documented (Russo et al., 1988) and is a clear possibility. Second, conformation of chromatin near the integration site might affect the recognition of the transgene by transcriptional regulators. For example, one integrant might be better recognized by a transcription factor that drives expression in cortical layer 5 than by one that drives expression in layer 6, whereas another integrant would display the reverse pattern. Third, adjacent chromatin could silence the transgene in some cells or cell types. This phenomenon, called position effect variegation, has been best stud-

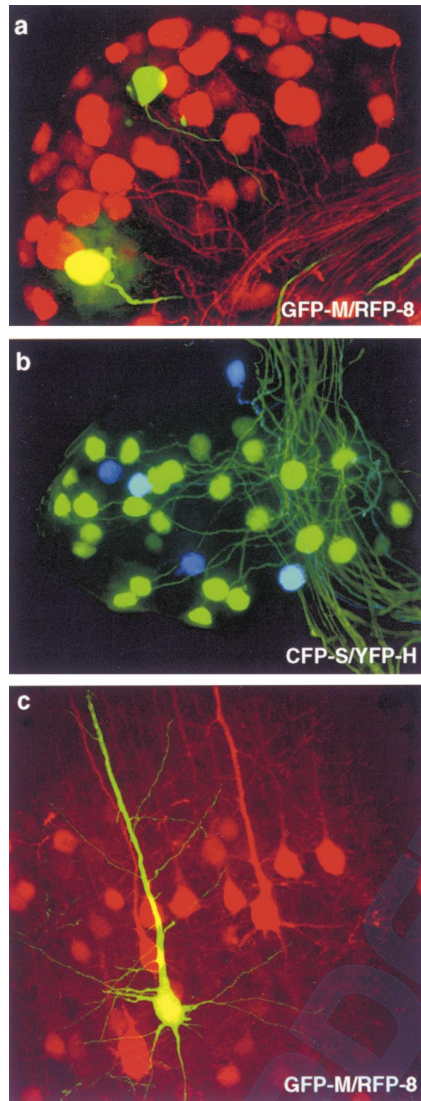


Figure 8. Multiple Neuronal Subsets in *thy1-XFP* Double Transgenic Mice

(a) Dorsal root ganglion from a GFP+RFP double transgenic (*thy1-GFP* line M and *thy1-RFP* line 8). Most neurons are RFP positive, but only a few are GFP positive; rare double-labeled neurons appear yellow.

(b) Dorsal root ganglion from a CFP+YFP double transgenic (*thy1-CFP* line S and *thy1-YFP* line H). Many neurons are YFP positive, and subsets are also YFP or YFP+CFP positive.

(c) Section from the cortex of the mouse shown in (a).

ied in *Drosophila* but has also been amply documented in transgenic mice (Walters et al., 1995; Festenstein et al., 1996; Graubert et al., 1998; Festenstein and Kioussis, 2000). In position effect variegation, the gene is “stochastically” silenced in a proportion of cells that would normally be expected to express it. Interestingly, the decision to activate or silence occurs early in development and is then heritable. Thus, cells that express a variegated transgene may in some cases be clonal relatives. Finally, in a variant of position effect variegation, some tandemly repeated sequences are preferentially silenced because they induce or mimic heterochroma-

tin. As a result, transgenes present in multiple copies are sometimes expressed in smaller subsets of cells than the same transgenes present in few copies (Garrick et al., 1998; Henikoff, 1998).

The observation that XFP is expressed in small subsets of neurons that normally express *thy1* is strikingly reminiscent of position effect variegation and repeat-induced gene silencing. At present, however, we have no data that bear directly on the mechanism of variation. Indeed, although the *thy1-XFP* mice may be useful in elucidating such mechanisms, our interest to date has been in documenting the nature and extent of variation, so that it can be exploited for neuroscientific analyses. In this regard, it is important to emphasize that line-to-line variation in transgene expression is likely to be extremely common. We and others have observed such variation in many transgenes, including but not restricted to those containing *thy1*-derived elements (e.g., Sanes et al., 1991; Carroll et al., 1995; Kelley et al., 1994; Caroni, 1997; and see citations above). The high prevalence of such variation may not be widely appreciated for several reasons. (1) Transgene expression was measured biochemically in many published studies, so that quantitative and qualitative variation could not be distinguished. (2) In most cases, only one or a few lines were examined in detail. (3) When encountered, such variation has more often been viewed as a problem than as an opportunity and has not, therefore, been emphasized. Importantly, in most cases reported to date, variation is of the type documented here for the *thy1-XFP* lines: the transgene is expressed in restricted subsets of those cells that express the endogenous gene, with the main differences being in the number and subtypes of the transgene-positive cells. If this pattern of variation is indeed general, it should be possible to apply the strategy described here to mark other neuronal subsets. In brief, one would choose regulatory elements known to drive expression in cell types that include those of interest, then generate and analyze a small set of transgenic mice from a single construct.

In summary, we have documented three features of XFP transgene expression that may be generally useful. First, multiple spectral variants express well in transgenic mice. Second, long-term expression and repeated excitation of XFP are minimally toxic. Third, lines generated from identical or nearly identical transgenes exhibit distinct patterns of expression. Together, these features make it possible to label multiple neuronal subsets *in vivo* and to image them over protracted periods. These abilities, in turn will facilitate studies of neuronal structure, function, and development in mammals.

Experimental Procedures

Generation of Transgenic Mice

Initially, we tested regulatory elements from four genes, all of which had previously been shown to drive neuron-specific expression in transgenic mice. Using β -galactosidase as a reporter, we generated transgenic founders (as described below) with elements from the neuron-specific enolase (Forss-Petter et al., 1990), $T\alpha 1$ - α -tubulin (Gloster et al., 1994), the low affinity neurotrophin receptor (p75; Carroll et al., 1995), and *thy1* (Vidal et al., 1990) genes. Although these studies were not systematic, expression of the reporter in motoneurons and retinal ganglion cells was strongest and most consistent in mice harboring the *thy1*-derived transgene (see also

Andra et al., 1996). The *thy1* vector has been described by Vidal et al. (1990), Kelley et al. (1994), and Caroni (1997) and was generously provided to us by Pico Caroni (Friedrich Miescher Institute, Switzerland). It contains 6.5 kb of the murine *thy1.2* gene, extending from the promoter to the intron following exon 4, but lacking exon 3 and its flanking introns (see Figure 1 in Caroni, 1997). The deleted sequences are required for expression in nonneural cells but not in neurons, whereas sequences in upstream introns are required for expression in neurons (Vidal et al., 1990). Reporters are cloned into an XhoI site, effectively replacing the deleted sequences.

In a second set of transgenic mice, we used the *thy1* vector to compare four versions of GFP. The first two were widely used, commercially available GFP constructs, called "Green Lantern" (GIBCO, Grand Island, NY) and the enhanced GFP (EGFP, Clontech, Palo Alto, CA). In both transfected cells and transgenic mice, EGFP was brighter than "Green Lantern" (data not shown), although both have their codons optimized for expression in mammalian systems. In the third version, GFP was fused to Tau, a tubulin-associated protein, to facilitate the axonal transport of GFP. Previous work in transgenic flies and mice showed that native LacZ was poorly transported down axons whereas Tau-LacZ fusions were well transported (Callahan et al., 1998). Although both unfused GFP and Tau-GFP labeled axons, unfused GFP labeled presynaptic terminals better. This may reflect the fact that microtubules are abundant in axons but are excluded from the nerve terminals. Finally, we tested a red-shifted EGFP variant, EYFP (Ormo et al., 1996), which was similar in brightness to EGFP.

Based on results from these initial studies, we generated ~60 transgenic mice in which *thy1* sequences drove expression of one of four XFPs: ECFP, EGFP, EYFP, or DsRed1 (Clontech). We refer to them as CFP, GFP, YFP, and RFP, respectively. Transgenic mice were generated by injection of gel-purified DNA into fertilized oocytes, using standard techniques (Hogan et al., 1994). Embryos for injection were obtained from matings between (C57BL6/J and CBA) F1 hybrids. Examination in founders was made possible by our ability to image neuromuscular junctions in live mice (Figure 1); positive animals were kept to establish lines, and negative animals were sacrificed for more extensive examination. The number of founders examined and the number that showed expression in motor axons were 11/13 for *thy1-CFP*, 13/15 for *thy1-GFP*, 14/17 for *thy1-YFP*, and 2/14 for *thy1-RFP*. In no case did we detect extensive staining of other neuronal types in mice with XFP-negative motoneurons. Thus, we discarded few if any founders that might have given rise to interesting lines.

Transgenic founders were backcrossed to C57BL6/J mice for one to four generations before undertaking detailed analysis of expression patterns. In a few cases, expression patterns observed in founders were more limited than those seen in their offspring, perhaps because the founders were mosaics, in which the transgene was integrated into only some of the motoneurons. These cases, which clearly did not represent line-specific expression, are not presented in Results or included in Table 1.

Genotypes were determined by PCR using the following primers: Thy1F1 (TCTGAGTGGCAAAGGACCTTAGG) from *thy1* sequence and EYFPR1 (CGCTGAACCTGTGGCCGTTACG) from pEGFP/pEYFP sequence for *thy1-YFP* and *thy1-GFP* mice; Thy1F1 and ECFPR1 (CCGTCGCCGATGGGGGTGT) for *thy1-CFP* mice; and Thy1F1 and RFP1 (TCACGGTGCCTCCATGCGCACC) for *thy1-RFP* mice. PCR reactions were performed in 25 μ l total volume containing 50 mM Tris-HCl (pH 9.2), 16 mM ammonium sulfate, 3.5 mM MgCl₂, 0.1% Tween-20, 0.2 mM dNTP, 2.5 U KlenTaqLA, and 1 μ l mouse tail DNA. Mouse tail DNA was prepared by digesting 2–3 mm of mouse tails overnight at 55°C in 200 μ l tail digestion buffer (50 mM Tris-HCl [pH 8.0], 1 mM CaCl₂, 1% Tween-20) plus 25 μ l proteinase K (10 mg/ml). Digested tails were boiled for 15 min to inactivate proteinase K.

Histology

For live imaging, mice were anesthetized with ketamine and xylazine, intubated, and respirated with a small animal ventilator. A ventral incision was made in the neck skin to expose the sternomastoid muscle. XFP images were taken with a cooled CCD camera. To label acetylcholine receptors in live animals, tetramethyl rhodamine-

conjugated α -bungarotoxin (1 μ g/ml; Molecular Probes, Eugene, OR) was applied to the surface of the muscle for 3 min then washed with lactated Ringer's solution.

For visualizing XFP expression in fixed tissues, mice were anesthetized and perfused through the heart, first with lactated Ringer's solution, then with 4% paraformaldehyde. Images of muscles, dorsal root ganglia, and retina were taken from whole-mount tissues. For brains, images were taken from 40 μ m vibratome sections. Cross section images of retina were taken from 20 μ m cryosections.

Filters for visualizing YFP and CFP were purchased from Chroma Technology Corp. (Brattleboro, VT). GFP and RFP were visualized with standard fluorescence and rhodamine filters, respectively.

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