Nerve Growth Factor Promoter Activity Revealed in Mice Expressing Enhanced Green Fluorescent Protein

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Abstract

Nerve growth factor (NGF) and its precursor proNGF are perhaps the best described growth factors of the mammalian nervous system. There remains, however, a paucity of information regarding the precise cellular sites of proNGF/NGF synthesis. Here we report the generation of transgenic mice in which the NGF promoter controls the ectopic synthesis of enhanced green fluorescent protein (EGFP). These transgenic mice provide an unprecedented resolution of both neural cells (e.g., neocortical and hippocampal neurons) and non-neural cells (e.g., renal interstitial cells and thymic reticular cells) that display NGF promoter activity from postnatal development to adulthood. Moreover, the transgene is inducible by injury. At 2 days after sciatic nerve ligation, a robust population of EGFP-positive cells is seen in the proximal nerve stump. These transgenic mice offer novel insights into the cellular sites of NGF promoter activity and can be used as models for investigating the regulation of proNGF/NGF expression after injury.

INDEXING TERMS

neurotrophin; nerve growth factor; transgene; brain; peripheral nerve; injury

Nerve growth factor (NGF), one of several neurotrophins of the vertebrate nervous system, was first localized in the male mouse submaxillary gland (Levi-Montalcini and Cohen, 1960). Later studies found that sympathetic ganglia, as well as target sites of sympathetic innervation (e.g., irides, heart atria, and prostate), have detectable levels of NGF mRNA and protein (Korsching and Thoenen, 1983a; Heumann et al., 1984; Shelton and Reichardt, 1984). The presence of NGF protein in sympathetic neurons has been linked to the binding and retrograde transport of this neurotrophin from target tissues, rather than to endogenous

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synthesis by ganglionic neurons (or adjacent glia) (Hendry et al., 1974; Stöckel et al., 1976; Johnson et al., 1978).

Intracerebral injections of iodinated NGF into selected sites, including the neocortex and hippocampus, revealed that the basal forebrain cholinergic neurons are capable of binding and retrogradely transporting NGF (Seiler and Schwab, 1984). This observation suggested that endogenous NGF might be utilized by these central projection neurons, in a manner similar to how NGF is acquired by sympathetic neurons from their peripheral target tissues. In the rat brain, levels of NGF mRNA are, in fact, highest in the neocortex and hippocampus, and select neuronal populations in both these central sites display NGF mRNA expression and protein synthesis (Korsching et al., 1985; Shelton and Reichardt, 1986; Whittemore et al., 1986; Ernfors et al., 1990; Lauterborn et al., 1991; Conner and Varon, 1992). Detection of NGF mRNA by in situ hybridization and NGF protein by immunostaining elsewhere in the mammalian brain led to both complementary (e.g., in the basal forebrain) and conflicting (e.g., in the striatum) observations. Other methods are needed to provide further insight into the central sources of NGF synthesis (during normal development and as a consequence of disease or injury).

To date, only one study by Heinrich and colleagues (1989) has documented the generation of reporter mice for NGF promoter activity; the reporter gene used was human growth hormone. Although tubular cells of the submaxillary gland in these transgenic mice were immunopositive for human growth hormone, no specific staining was detected in the brain (or in any other organ system). This absence may have been the result of limited resolution of human growth hormone by immunostaining.

Here we report the generation of new lines of transgenic mice in which the reporter gene encoding enhanced green fluorescent protein (EGFP) is driven by the mouse NGF promoter (Zheng and Heinrich, 1988). This promoter regulates the transcription for the entire NGF gene, which includes proNGF and mature NGF, as well as a variety of splice variants from the pre-proNGF gene (Dicou, 2007). In this first description of these reporter mice, we provide a comprehensive assessment of the spatial and temporal patterns of EGFP localization at postnatal and adult ages. In addition, we provide evidence that transgene expression can be successfully induced in response to nerve injury in adult mice. The clear visualization of EGFP in these transgenic mice offers new evidence regarding the many cellular populations that display detectable NGF promoter activity, both centrally and peripherally.

MATERIALS AND METHODS

All animal procedures (i.e., collection of tissues and injury models) were approved by the Queen's University Animal Care Committee, in accordance with the guidelines set forth by the Canadian Council on Animal Care. All efforts were made to minimize the number of animals used and their suffering.

Generation of founder mice and establishment of mouse lines

To produce a reporter for NGF promoter activity, we first removed the cytomegalovirus immediate-early gene promoter from the plasmid EGFP-N1 (ClonTech, Palo Alto, CA) and then inserted the -2100 bp 5' mouse NGF promoter (NGFpr), including 120 bp of sequence following the transcriptional start site, which has a functional AP-1 transcription factor binding site within the first intron (D'Melo and Heinrich, 1991; Cowie et al., 1994; Veenstra et al., 1998). This same promoter sequence has been used successfully to drive human growth hormone expression in transfected cells (Cowie et al., 1994; Veenstra et al., 1998) and in transgenic mice (Alexander et al., 1989).

EGFP was chosen as the reporter gene for NGF promoter activity because the translated fluorophore displays both excellent brightness and photostability, thereby facilitating its detection in transgenic mice. The new construct was designated NGFpr-EGFP (Fig. 1A). An EGFP-N primer (ClonTech) was used to sequence the construct DNA (Cortec, Kingston, ON, Canada). The construct was shown to align with the NGF promoter sequence of Zheng and Heinrich (1988). SCA-9 clone 15 cells (ATCC #CRL-1734), which normally express NGF, displayed green fluorescence when stably transfected with the plasmid containing the NGFpr-EGFP construct, thus demonstrating that the promoter was biologically active (data not shown).

Our first set of transgenic mouse lines (named SR1-6; two males and four females) were established from founders generated by zygote microinjection of the linearized NGFpr-EGFP construct into C3H $\times$ C57Bl/6 mice; we received these mice from Transgenesis (Montreal, QC, Canada) in May 2008. Our second set of transgenic mouse lines (named SR7-11; three males and two females) were established from founders generated by zygote microinjection of the same linearized NGFpr-EGFP construct into C3H $\times$ C57Bl/6 mice; we received these mice from the Quebec Transgenic Research Network (Montreal, QC, Canada) in May 2010. DNA from all potential founders was screened for the NGFpr-EGFP construct by polymerase chain reaction (PCR). The primers spanned NGFpr (5' - tcggtgagtcaggcttct-3') and EGFP (5' - tgagtttgacaaccacaac-3'). The PCR protocol was 35 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1.5 minutes, yielding a 1069-bp product.

All transgenic founder mice were crossed with C57Bl/6 mice; all progeny of the subsequent generations were genotyped (as per the founders). Successive generations of transgenic mice were obtained by breeding positive male and positive female mice. To ensure the sustainability of the lines on the C57Bl/6 background, transgenic male mice were backcrossed with C57Bl/6 females at two different times over a 2-year period. DNA for genotyping was obtained from ear punches. All microscopic data reported here were obtained from the second to tenth generation progeny from founder SR3: postnatal day 1 (PD1; n = 20), postnatal day 10 (PD10; n = 24), 3-month-old male and female mice (n = 80), and 8-month-old male mice (n = 24). Ten postnatal and adult wild-type (non-transgenic) male and female siblings (n = 24) from the same generations were used as controls.

Preparation of tissues for microscopic examination

Under deep anesthesia (sodium pentobarbital at 32.5 mg/kg), all mice were perfused transcardially with 2% paraformaldehyde in phosphate buffer (pH 7.4). Tissues were removed and stored in 30% phosphate-buffered sucrose (pH 7.4). The following tissues from both male and female mice were cut on a cryostat at a thickness of 30 μ m: brain and spinal cord (cervical), eyes (for irides), sensory ganglia (i.e., trigeminal), sympathetic ganglia (i.e., superior cervical), peripheral nerves (i.e., sciatic and hypoglossal), pituitary gland, pineal gland, salivary glands, lacrimal gland, thymus, heart (and aorta), lung, liver, stomach, pancreas, intestines (i.e., duodenum and descending colon), kidney, urinary bladder, and skin (i.e., external auditory meatus, whisker pads, and footpads). From male mice, the prostate gland and preputial gland were isolated and sectioned; from female mice, the uteri were isolated and sectioned. Sections from some tissues were subsequently processed for p75 neurotrophin receptor (p75NTR) and/or growth-associated protein (GAP-43) immunostaining.

Antibody characterization

Information regarding the primary antibodies used in this study (summarized in Table 1), includes:

1. Anti- β -actin IgG (Sigma-Aldrich, St. Louis, MO, cat. no. A5441). This monoclonal antibody, developed in mice, was raised against a synthetic peptide (Ac-DDDIAALVIDNGSGK), which corresponds to the N-terminal peptide of the mammalian β -actin; this peptide was conjugated to keyhole limpet hemocyanin (KLH). The isotype was determined by a double diffusion immunoassay using the mouse monoclonal antibody isotyping reagents (Sigma, St. Louis, MO, cat. no. ISO2). According to Liao et al. (2000) this mouse monoclonal antibody (Clone AC-150, ascites fluid) identified a single band of roughly 50 kDa on a Western blot of the rat liver. In addition, because this antibody was raised against the N-terminus, which has low sequence (50–60%) homology among actin isoforms (Sigma), this antibody shows specificity to β -actin (non-muscle) and not α -actin (in skeletal and cardiac muscle; absence of binding of protein isolated from the adult mouse heart; see Fig. 13).
2. Anti-growth-associated protein 43 IgG (GAP-43, Millipore, Bedford, MA, cat. no. AB5220). This purified rabbit polyclonal antibody was raised against the complete sequence of the recombinant rat GAP-43. We (and the manufacturer's datasheet using mouse brain lysates) have previously shown that this GAP-43 antibody stains a band at 43 kDa on Western blot (Hannila and Kawaja, 2005).
3. Anti-p75NTR IgG (Millipore, cat. no. AB1554). This polyclonal rabbit antibody was raised against a GST-tagged NGF receptor p75 corresponding to the cell membrane. We have previously shown that although this antibody stains other smaller bands in addition to the 75-kDa form of this molecule on Western blots, specific immunostaining with this antibody was absent in sympathetic ganglia of mice lacking either exonIII or exonIV of this receptor (Dhanoa et al., 2006).

4. Anti-NeuN IgG (Millipore, cat. no. MAB377). This monoclonal mouse antibody (clone A60) was raised against purified cell nuclei from mouse brain, reacts with most neuronal cell types, and recognizes two to three bands in the 46–48-kDa range on Western blots.
5. Anti-NGF, β -subunit IgG (MC-51, a gift from Dr. M Coughlin, also available from Cedarlane Laboratories, Burlington, Canada, as CLMCNET-011). This polyclonal antibody, developed in rabbit, was raised against high-performance liquid chromatography (HPLC)-purified whole mouse 2.5S NGF β -subunit. This antibody recognizes both purified proNGF (~36 kDa) and purified 2.5S β -subunit NGF (~13 kDa) on Western blots. In addition, the antibody was specific for NGF, with no cross-reactivity with brain-derived neurotrophic factor (BDNF) or NT-3 (Fahnestock et al., 2001).

Injury models

To investigate whether the transgene is inducible by peripheral nerve injury, adult transgenic male mice (i.e., 3-month-old progeny from founder SR3) were anesthetized and underwent a tight ligation of the left sciatic nerve. Completeness of the injury was assessed by paralysis in the affected limb. After recovery periods of 1, 2, 4, 6, 7, and 14 days ($n = 4$ –6 mice per endpoint), the mice were deeply anesthetized and sacrificed by transcardial perfusion, as described above. The injured and uninjured sciatic nerves, as well as the spinal cord, were removed and sectioned at a thickness of 30 μ m for microscopic examination. Under observer-blind conditions, the tissues were assessed for the induction of the transgene.

To investigate whether the transgene expression in the pineal gland may be influenced by a partial loss of sympathetic inputs, adult transgenic male mice (i.e., 3-month progeny from founder SR3) were anesthetized and underwent the removal of the left superior cervical ganglion. Completeness of the injury was assessed by ipsilateral ptosis. After recovery periods of 3 and 8 days ($n = 4$ per endpoint), the mice were deeply anesthetized and sacrificed by transcardial perfusion, as described above. The pineal glands were removed and sectioned at 10 μ m for microscopic examination. Under observer-blind conditions, the tissues were assessed for induction of the transgene.

Photography

Photomicrographic images of immunostained tissues were obtained by using a Zeiss AxioCam high-resolution scanning digital camera, mounted on a Zeiss microscope (Carl Zeiss Vision, München-Hallbergmoos, Germany). The Carl Zeiss software package AxioVision (version 4.2) was used to capture 48-bit RGB color images at $1,300 \times 1,030$ (scanning) pixels. Magenta-green images are available as supplemental figures. Images were adjusted as uncompressed .zvi files (the intrinsic AxioVision file format) with respect to brightness and contrast and saved as uncompressed .tif files; the figures were then generated by using Adobe Illustrator CS5 and Photoshop 6.0 (Adobe Systems, San Jose, CA). Once the images were converted from the 48-bit .zvi format, no other manipulations were performed.

RT-PCR

Tissues for RT-PCR were harvested from PD1 and 3-month-old transgenic mice sacrificed by decapitation and cervical dislocation, respectively. All tissues were placed in coded tubes and flash-frozen in liquid nitrogen. Total RNA was isolated with Ambion (Austin, TX) TRI Reagent Solution. Only RNA with OD 260/280 of 1.8–2.0 was used, and any contaminating DNA was removed with DNase (DNA-free Kit, Ambion). The Ambion RETROscript Kit was used to produce cDNA, and an Eppendorf (Hamburg, Germany) Mastercycler was used for PCR. The primers NGF (sense) 5′-ggcatgctggaccaagctc-3′ and NGF (antisense) 5′-gcgcttgctccggtgagtc-3′ (Giordano et al., 1992), yielded a 460-base product after 35 cycles of 1 minute at 94°C, 1 minute at 58°C, and 1.5 minutes at 72°C; these primers span the 5′ end of the murine NGF gene. The PCR amplification mix contained 1.5 mM Mg²⁺. Control samples (e.g., DNA-free RNA with no RT) produced no cDNA products under these amplification conditions.

Western blotting

Tissues for immunoblotting were harvested from 3-month-old transgenic mice sacrificed by cervical dislocation. All tissues were placed in coded tubes and flash-frozen in liquid nitrogen. Western immunoblotting was carried out as described in Fahnestock et al. (2001), with minor modifications. Tissues were homogenized in 0.05 M Tris buffer (pH 7.5) supplemented with 0.05% Tween-20, 10 mM EDTA, 2 µg/ml pepstatin, and EDTA-free complete protease inhibitor cocktail tablet (Roche, Laval, Canada). Then the homogenates were incubated on ice for 10 minutes and centrifuged. The supernatants were collected. A DC protein assay (BioRad, Hercules, CA) was used to determine protein concentrations. All samples were aliquotted and stored at –80°C. Total protein samples (60 µg) were separated on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred onto PVDF Immobilon-FL membranes (Millipore, Billerica, MA). The membranes were first blocked for 1 hour at room temperature in Odyssey Blocking Buffer (Cedarlane Laboratories) diluted 1:1 in phosphate-buffered saline (PBS; pH 7.4), and then incubated sequentially overnight at 4°C in affinity-purified rabbit anti-NGF IgG (MC-51, gift from Dr. Michael Coughlin, McMaster University; 1.25 µg/ml dilution), and mouse anti-β-actin IgG (Sigma; 1:5,000 dilution).

Details for each antibody, including the characterization of specificity and the controls that ensure appropriate immunostaining, are given above in the “Antibody characterization” section.

For immunodetection on Western blots, the primary IgGs were diluted in PBS plus 0.05% Tween-20 (v/v). After incubation, the membranes were washed in PBS plus 0.01% Tween-20 at room temperature and incubated in the secondary antibodies IRDye 680-conjugated goat anti-rabbit and IRDye 800CW-conjugated goat anti-mouse (1:7,000; Li-Cor Biosciences, Lincoln, NE) in PBS plus 0.05% Tween-20 for 1 hour at room temperature. After rinsing, the membranes were scanned at 700 and 800 nm by using an Odyssey Infrared Imaging System (Li-Cor Biosciences). Color and pseudo-gray-scale digital images were generated by using Odyssey software v. 2.0 (Li-Cor Biosciences).

RESULTS

Establishing lines of NGFpr-EGFP mice

Six lines of NGFpr-EGFP mice were initially established from founder mice that were generated in May 2008; the presence of the transgene was determined by PCR genotyping of ear punches. In the first generation of progeny, an initial screening of 14 transgenic offspring from founder female SR3 revealed that four adult males and seven adult females displayed robust expression of EGFP in the tubular cells of the submaxillary gland (Fig. 1B). The adjacent sublingual and parotid glands, which were identified by histological criteria (Gude et al., 1982), lacked EGFP-positive cells. Founder male SR2 had 84 progeny, of which 43 carried the transgene (51%), and 22 of these adult mice displayed EGFP expression in the submaxillary gland. The fluorescence intensity in the tubular cells of SR2 offspring was substantially weaker than that observed in the tubular cells of SR3 offspring. Subsequent investigation showed that SR2 offspring had no EGFP expression in the brain. As for the remaining lines, founder female SR1 had 8 transgenic progeny from a total of 21 (38%), founder male SR4 had 5 transgenic progeny from a total of 33 (15%), founder female SR5 had 3 transgenic progeny from a total of 10 (30%), and founder female SR6 had 3 transgenic progeny from a total of 6 (50%). None of the adult transgenic offspring of founders SR1, SR4, SR5, and SR6 had EGFP staining in the submaxillary glands.

A line of NGFpr-EGFP mice was therefore established from the first-generation transgenic progeny of founder SR3. Of the 129 second-generation mice obtained from 15 litters, 99 (77%) of these carried the NGFpr-EGFP transgene. Comparable intensities and patterns of EGFP-positive tubular cells were seen in the submaxillary glands of both adult male and female mice from this second generation. Ear punches taken from the external auditory meatus of adult transgenic mice also displayed robust EGFP expression, much of which appeared to be localized to cells surrounding the bulb of each hair follicle (Figs. 1C, D; red filter shows autofluorescence of the hair shafts). Both the submaxillary glands and external auditory meatus of 1- and 10-day-old transgenic mouse pups lacked EGFP, showing that transgene expression in these tissues begins after the first 10 days of life. These results are consistent with the known developmental expression pattern of NGF in the submaxillary gland (Ishii and Shooter, 1975; Aloe and Levi-Montalcini, 1980). Since the phenotype of the SR3 progeny (generated between May 2008 and May 2010) has remained unchanged as described herein, it would appear that transgene transmission over this 2-year period of breeding has remained stable.

To provide direct confirmation that the EGFP phenotype seen in the progeny founder SR3 (one of the first six founders) was not due to possible epigenetic mechanisms, an additional five NGFpr-EGFP founder mice were generated in May 2010. Again, the presence of the transgene was determined by PCR genotyping of ear punches. In the first generation of progeny, an initial screening of five transgenic offspring (of eight siblings) from founder female SR7 revealed that three adult females displayed dense populations of EGFP-positive thymic reticular cells, renal interstitial cells, and lacrimal serous cells, as well as sparse populations of submaxillary tubular cells and neocortical neurons. Two adult transgenic mice (of 47 siblings) from founder male SR11 had a distribution of EGFP-positive cells

similar to that of the three adult females from founder female SR7. No transgenic progeny having EGFP expression were detected from the lines generated from founder female SR8 (of 5 offspring), founder male SR9 (of 33 offspring), and founder male SR10 (of 18 offspring).

EGFP-positive cells in the brain and spinal cord

The complete neuraxis of adult male and female transgenic mice was studied in coronal, horizontal, and sagittal planes of sectioning. It is important to note that the localization of EGFP was consistent among all the brains examined and that wild-type (sibling) mice lacked EGFP-positive cells. The most prominent central population of EGFP-expressing cells was found throughout the cortical areas including the neocortex and piriform and entorhinal cortices. Two distinct subpopulations of EGFP-positive neurons were evident in the piriform cortex of adult transgenic mice: the majority of these neurons displayed large somata with dendritic processes, whereas the remaining neurons were smaller in size (Fig. 2A,B). Robust neuronal EGFP expression was seen in layer III of the piriform and entorhinal cortices (and a sparse number scattered in layers I/II), as well as in layer V of the neocortex. In the developing cortices of PD1 pups (Fig. 2C,D [higher magnification of C]) and PD10 pups (Fig. 3A–D), bands of EGFP-positive neurons were found in the same layers (i.e., predominantly in layer III of the piriform and entorhinal cortices, and in layer V of the neocortex) as the adult transgenic neocortex. In the rostral forebrain, EGFP- and NeuN-positive neurons were observed in the dorsal endopiriform nucleus, which lies between the piriform cortex and the caudate putamen (Fig. 3B). In these cortical regions of PD10 mice, both large- and small-sized EGFP-positive neurons were evident.

In contrast to the robust populations of EGFP-positive neurons in the neocortex, the hippocampal formation of adult transgenic mice had only a sparse population of EGFP-positive neurons confined to the hilar region. No detectable EGFP was found in the granular neurons of the dentate gyrus or in the pyramidal neurons of the CA1–3 fields of these adult mice. A similar pattern of EGFP localization was seen in PD1 transgenic mice (Figs. 4, 5). At day 10 of postnatal development, transgenic pups displayed EGFP- and NeuN-positive pyramidal neurons in the CA1–3 fields (Fig. 5C,D); the dentate granular layer had comparatively fewer EGFP-positive neurons (Figs. 4A,B, 5A; autofluorescent mossy fibers are seen in the outer layer of the dentate gyrus). These NeuN-positive pyramidal neurons display dense nuclear staining similar to those reported by Lind et al. (2005). The pyramidal neurons of PD10 mice also had prominent EGFP staining in the somata that radiated into the proximal dendritic processes. These data suggest that neuronal levels of NGF promoter activity change during the postnatal development of the mouse hippocampus, whereas levels among subpopulations of the neocortical neurons remain relatively stable from PD1 to adulthood.

In the remaining portions of the adult telencephalon, the only neural areas showing EGFP-positive cells were the nucleus basalis (Fig. 6A–C; a higher magnification of EGFP-positive neurons in the nucleus basalis is in B), the anterior olfactory nucleus, the nucleus accumbens, and the basolateral nucleus of the amygdala. All of these neurons had small somata with several dendritic processes (Fig. 6B). EGFP-positive neurons were detected in

these nuclei at PD1 and PD10. To assess whether EGFP-positive basalis neurons were those cholinergic projection neurons that innervate the neocortex, we immunostained sections of the transgenic brains for p75NTR, a reliable phenotypic marker of these NGF-sensitive neurons. No p75NTR-immunopositive basalis neurons had EGFP, revealing that these were two distinct neuronal populations in the same nucleus (Fig. 6C). Moreover, the cell bodies of the p75NTR-immunopositive projection neurons were generally larger than those of the adjacent EGFP-positive neurons. Somal diameters for p75NTR-immunopositive neurons were $27.0 \pm 3.5 \mu\text{m}$ ($n = 54$ neurons from 4 mice), and for EGFP-positive neurons they were $15.2 \pm 1.9 \mu\text{m}$ ($n = 41$ neurons from 4 mice).

In the diencephalon, two nuclei displayed robust populations of EGFP-positive neurons: the reticular thalamic nuclei (Fig. 6A) and lateral habenula nuclei (data not shown). Sparse populations of EGFP-positive neurons were also seen scattered throughout the thalamic and hypothalamic nuclei. In the brainstem, a similar pattern of dispersed EGFP-positive neurons was evident in the reticular formation, as well as in the superior and inferior collicular nuclei. The cerebellum of adult transgenic mice (but not PD1 or PD10 transgenic pups) displayed subpopulations of molecular and granular neurons expressing EGFP (Fig. 7A). Although no EGFP-positive neurons were detected below the level of the medullocervical junction in adult transgenic mice, a widespread distribution of positive neurons was seen in the spinal gray matter of 1-day-old transgenic pups (Fig. 7C). At 10 days of postnatal development, only sparse clusters of EGFP-positive spinal neurons were found in the superficial lamina of the dorsal horn and immediately adjacent to the central canal (Fig. 7D).

Regarding the brains and spinal cords of postnatal and adult transgenic mice, it is worth noting that EGFP was localized specifically to neuronal populations and not in central glial cells (astrocytes, oligodendrocytes, or ependymal cells). The only exception to this observation was the appearance of EGFP-positive processes of astrocytes (or Fañanas cells) that ensheath the Purkinje somata in the cerebellum (Fig. 7B). EGFP expression by Fañanas astrocytes was detected in the cerebella of adult transgenic mice but not at PD1 or PD10.

EGFP-positive cells in hollow organs

In adult male and female transgenic mice, several hollow organs were examined microscopically, including those in the cardiovascular, gastrointestinal, and urogenital systems. The first general observation made across all of these organ systems was the absence of detectable EGFP in smooth muscle cells. No EGFP was found in the tunica media of arteries and arterioles, in the muscular layers of the alimentary canal, in the wall of the urinary bladder, or in the walls of both male (e.g., prostate) and female (e.g., uteri) reproductive organs. The same was true of PD1 and PD10 transgenic pups. This absence of EGFP in smooth muscle cells was unexpected, in light of reports showing NGF synthesis by vascular and bladder smooth muscle cells *in vivo* and *in vitro* (Bandtlow et al., 1987; Ueyama et al., 1991, 1993; Spitsbergen et al., 1995; Clemow et al., 2000). Furthermore, atrial and ventricular cardiac muscle cells in postnatal and adult transgenic mice also lacked EGFP.

There were three interesting sites of EGFP-positive cells in the gastrointestinal system. First, the stomach of PD1 transgenic pups had a robust population of round EGFP-positive cells

(Fig. 8A). By PD10, it was evident that these cells were localized in the developing epithelial layer and not in the underlying external muscle layer (Fig. 8B). In adult mice, these gastric EGFP-positive cells were clearly an epithelial subpopulation in the cardiac glands (Fig. 8C,E). No other positive cells were found in the remaining areas of the stomach (e.g., fundus, pyloric, and body). Sections of the adult mouse stomach were immunostained for GAP-43 to assess whether these EGFP-positive cardiac glands were innervated by peripheral axons. GAP-43 is a commonly used biomarker of axons, which peripherally include the NGF-sensitive sympathetic efferents and/or sensory afferents; these GAP-43-positive axons innervate organs such as the gastrointestinal tract and kidneys (Belai et al., 1995; Yamada et al., 2006). Little to no overlap was seen between the localization of these EGFP-positive epithelial cells and immunostained peripheral fibers (data not shown).

Second, pyramidal EGFP-positive cells were scattered through the epithelial lining of the small and large intestines of postnatal and adult transgenic mice. These cells resemble those endocrine cells in the intestinal villi and crypts that stain positively for LIP1 (i.e., a biologically active 29-amino acid protein derived from proNGF), as previously described (Dicou et al., 1997; Dicou, 2007).

Third, a prominent population of EGFP-positive cells was detected in the external muscle layer in the intestines of PD1 transgenic pups (Fig. 8D). Unlike the round EGFP-positive cells seen in the gastric epithelia and the pyramidal EGFP-positive cells seen in the intestinal epithelia, these EGFP-positive cells had two or three elongated processes radiating away from the cell body, thus resembling neurons. These EGFP-positive cells, likely a subtype of enteric neurons in the developing myenteric plexus, were scattered throughout the entire intestinal tube at this developmental age. Both the incidence and intensity of EGFP-positive cells in the external muscle layer of the gastrointestinal tract of PD10 and adult mice were similar (Table 2).

EGFP-positive cells in exocrine glands

Ear punches taken from the external auditory meatus of adult transgenic mice displayed EGFP-positive cells associated with each hair follicle (Fig. 9A; also see Fig. 1C). Confocal microscopy revealed that these EGFP-positive cells were associated with the paired sebaceous glands of each follicle (Fig. 9B). No EGFP-positive cells were detected in the dermis surrounding the whiskers of adult mice, whereas the hind footpads showed a sparse population of EGFP-positive cells at the base of small gland-like structures (Fig. 9C). By comparison, PD1 and PD10 transgenic pups lacked EGFP-positive cells associated with the skin, whiskers, and footpad glands (see Table 2).

Both adult male and female transgenic mice displayed robust expression of EGFP in the tubular cells of the submaxillary gland (Fig. 10A; also see Fig. 1B) but not in the adjacent sublingual and parotid glands. (The three salivary glands were identified by histological criteria.) In contrast to the localization of EGFP in the tubular cells of the submaxillary glands, the lacrimal glands had dispersed populations of EGFP-positive serous cells in the secretory acini (Fig. 10D). Unlike adult animals, PD1 and PD10 transgenic mice lacked EGFP-positive cells in the submaxillary and lacrimal glands (Table 2). In adult male transgenic mice, the prostate gland displayed EGFP-positive cells; these columnar-shaped

epithelial cells were sparsely scattered throughout the lining of the secretory tubules (Fig. 10C). The preputial glands of adult mice had numerous EGFP-positive cells (Fig. 10B).

EGFP-positive cells in other organ systems

The kidneys of transgenic mice, from PD1 to adulthood, displayed a robust population of EGFP-positive interstitial cells. In the renal cortex, these cells form columns between the tubular and vascular components, whereas in the renal medulla their distribution appears to be scattered (Fig. 11A–D; also see Table 2). EGFP was not localized to either the urinary tubules or arterioles of the kidney (Fig. 11B, red filter showing the urinary tubules). To assess whether these clusters of renal interstitial cells having NGF promoter activity were innervated by NGF-sensitive sympathetic efferents and/or sensory afferents, we immunostained sections of the adult kidneys for GAP-43. (This marker labels both types of peripheral axons.) Little to no overlap was seen between the localization of EGFP-positive interstitial cells and immunostained peripheral fibers. Instead, GAP-43-immunopositive axons were usually found adjacent to tubular and/or vascular components of the kidney (Fig. 11D).

From PD1 to adulthood, the medullary portions of the transgenic mouse thymus displayed clusters of cells having intense EGFP (Fig. 12A). These thymic cells had a stellate appearance with several cytoplasmic processes radiating from the cell body. This description best fits thymic reticular cells. In contrast to the kidneys, the thymus was devoid of GAP-43-immunopositive peripheral axons (data not shown), and no NGF-sensitive fibers were seen in close proximity to the medullary clusters of EGFP-positive reticular cells. Reticular cells are a putative population of NGF-producing cells.

Other tissues also displayed specific temporal patterns of EGFP expression. For instance, very small numbers of EGFP-positive cells (putatively smooth muscle cells) were found at the openings of the pulmonary alveolar ducts in 1-day-old transgenic pups (Fig. 12B) but were not seen in adult tissue. A similar temporal pattern of EGFP expression was detected in the pituitary gland, where EGFP-positive cells were seen in PD1 pups (Fig. 12C) but not in adult transgenic mice.

Several tissues, including the eyes (for assessment of the irides), sensory ganglia, sympathetic ganglia, and peripheral nerves all lacked EGFP-positive cells in PD1, PD10, and adult transgenic mice (data not shown).

Assessment of NGF mRNA and protein levels in various organ systems of adult mice

It is recognized that many of the tissues (e.g., gastrointestinal tract, kidney, and thymus) displaying EGFP-positive cells in these lines of transgenic mice have yet to be examined for NGF mRNA and/or protein in many mammalian species, including mice. To corroborate our microscopic observations in NGFpr-EGFP mice, RT-PCR was undertaken to determine the presence of NGF mRNA levels in a variety of murine tissues taken from postnatal and adult male transgenic mice. NGF mRNA was found at detectable levels in selected areas of the brain, submaxillary gland, lacrimal gland, thymus, and kidney of adult transgenic mice (Table 3); these same tissues have EGFP-positive cells. It is also worth noting that NGF

mRNA was not detected in those tissues of adult transgenic mice that lacked EGFP-positive cells, including the heart and urinary bladder.

Western immunoblotting for proNGF/NGF was also carried out in a variety of murine tissues taken from adult male wild-type mice (Fig. 13A,B). Proteins isolated from the mouse submaxillary gland yielded an intensely stained doublet at ~13–14 kDa; this doublet pattern is indicative of the presence of β -NGF endopeptidase activity in this salivary gland (Fahnestock et al., 1991). Purified 2.5S NGF, consisting primarily of des(1-8)NGF (Mobley et al., 1976), yielded intensely stained bands at ~13 kDa on the same gel. Weakly to moderately stained bands at ~32 kDa (i.e., the molecular weight of proNGF, as detected in lane 1) were seen in the protein preparations of the mouse thymus, liver, colon, kidney, and lacrimal gland. Intensely stained bands at ~14 kDa (i.e., the molecular weight of the upper [mature NGF]doublet, as detected in lane 2) were seen in the protein preparations of the mouse lung, thymus, heart, liver, colon, and kidney; weakly stained bands at ~14 kDa were seen in the protein preparations of mouse lacrimal gland. Detection of bands at ~14 kDa (but not ~13 kDa) may indicate insufficient levels of β -NGF endopeptidase activity for the cleavage of NGF to des(1-8)NGF in these tissues (Fahnestock et al., 1991). Bands at slightly higher molecular weight (~35 kDa), possibly more glycosylated forms of proNGF, were seen in protein preparations of the mouse hippocampus and neocortex.

Transgene detection after sciatic nerve injury

It has been previously reported that, whereas uninjured peripheral nerves (e.g., sciatic) of adult rats have little to no detectable NGF mRNA and/or protein, there is a rapid upregulation of NGF synthesis at the site of nerve injury. Although the precise resolution of these NGF-producing cells was difficult, it is generally agreed that Schwann cells, macrophages, and/or other cell types (i.e., smooth muscle cells, fibroblasts) are all possible sources of this injury-induced NGF production (Marcinkiewicz et al., 1991). To provide evidence that the NGF promoter in our transgenic mice is inducible by nerve injury, we performed a tight ligation of the left sciatic nerve of adult transgenic mice. As early as 2 days after injury, a robust population of EGFP-positive cells was seen in the proximal stump at the site of the nerve ligation (Fig. 14A–C, C; red filter showing a lack of autofluorescence), with fewer EGFP-positive cells evident in the distal stump. The uninjured (contralateral) sciatic nerve lacked EGFP-positive cells (data not shown).

At 6 days after the sciatic nerve ligation, a small number of EGFP-positive neurons was detected in the ipsilateral (but not in the contralateral) dorsal horn of the lumbar spinal cord (Fig. 14D–F). The presence of these EGFP-positive cells in the injured nerve and EGFP-positive neurons in the spinal cord did not persist; at 7 and 14 days after injury, no EGFP-positive cells were seen in these tissues.

No transgene detection in the partially deafferented pineal gland

Previous studies have shown detectable levels of NGF in the pineal gland of adult rats (García-Mauriño et al., 1992; Kuchel et al., 1999). Although the pineal glands of PD1, PD10, and 3-month-old NGFpr-EGFP mice lacked EGFP-positive cells, a robust population of EGFP-positive cells (likely developing pinealocytes) was found in embryonic transgenic

mice at 20 days of gestation (Fig. 15A,B). To assess whether a partial sympathetic deafferentation might activate NGF promoter expression, we performed a unilateral sympathectomy in adult transgenic mice. At 3 and 8 days after removal of the left superior cervical ganglia, no EGFP-positive cells were seen in the partially deafferented pineal gland at either endpoint (Fig. 15C).

DISCUSSION

Functional considerations

To our knowledge, these NGFpr-EGFP mice are only the second documented line of reporter mice generated that allow for the microscopic and/or biochemical resolution of the cellular sites of NGF promoter activity. The first study by Heinrich and colleagues (1989) described the generation of reporter mice in which NGF promoter activity controlled the ectopic expression of human growth hormone. Although positive staining for the transgene was seen in submaxillary tubular cells, the transgene was not detected in any other organ system of these adult mice, including the brain. Our use of the EGFP transgene has addressed possible issues of limited resolution of certain transgenes (such as human growth hormone) and has allowed for the ready detection of the transgene protein without using antibodies.

The murine NGF promoter drives the transcription of NGF mRNA, which when translated yields proNGF species of 32 and 25 kDa after cleavage of the signal sequence. Further post-translational processing of proNGF yields mature NGF (~13 kDa), as well as two biologically active peptides (i.e., 29-amino acid LIP1 and 38-amino acid LIP2) (Dicou et al., 1997; Dicou, 2007). It is, therefore, important to note that detection of EGFP-positive cells in our lines of transgenic mice provides evidence of NGF promoter activity (not the localization of proNGF/NGF), and that determination of proNGF versus mature NGF abundance in these EGFP-positive cells must be achieved by other methods (e.g., immunostaining).

Comparing the localization of NGF mRNA/protein and EGFP in central neurons

To date, several studies have documented the sites of NGF mRNA production (by either Northern blot or in situ hybridization analyses) in the adult rat brain (Korsching et al., 1985; Shelton and Reichardt, 1986; Whittemore et al., 1986; Ernfors et al., 1990; Gall et al., 1991; Lauterborn et al., 1991). Together, these investigations have shown that the neocortex and hippocampus possess the highest levels of NGF mRNA centrally; in agreement with these previous studies, we offer supporting evidence that NGF mRNA is detected in the neocortex and hippocampus of PD1 and adult transgenic mice (Table 3). Specifically, positive hybridization has been detected in cortical neurons of layers II, III, and V, as well as in both hippocampal pyramidal and granular neurons. This distribution of NGF mRNA production correlates well with the central immunolocalization of NGF protein in adult rats treated with cholchicine, which results in the detection of NGF-like immunoreactivity in both granular and pyramidal hippocampal neurons (Conner et al., 1992; Conner and Varon, 1992).

When comparing these previous data with our present observations in NGFpr-EGFP transgenic mice, there are two notable similarities. First, the widespread distribution of EGFP-positive neurons in layer V of the neocortex, as well as in layer III of the piriform and entorhinal cortices, parallels that seen with NGF mRNA in situ hybridization in the adult rat. In our transgenic mice, NGF promoter activity is present in both large- and small-sized cortical neurons, which likely constitute subtypes of cortical projection neurons and interneurons, respectively. Second, the production of NGF in the neocortex has been linked to its innervation by NGF-dependent cholinergic neurons that originate in the nucleus basalis. The EGFP-positive cells in the nucleus basalis are not the p75NTR-immunopositive cholinergic projection neurons, which are expected to obtain NGF from the neocortex. Instead, these EGFP-positive cells are possibly a population of interneurons that provide the NGF-sensitive cell bodies with a local source of NGF (in addition to their target-derived source of NGF from the neocortex). Our observations concerning these two neuronal populations in the nucleus basalis are in agreement with those of previous studies (Conner and Varon, 1992; Lauterborn et al., 1995).

The hippocampal formation is one important site where our current data differ from those of previous investigations. We found no EGFP-positive granular or pyramidal neurons in the hippocampus of adult transgenic mice. This observation is in contrast to the localization of NGF mRNA and protein in these same neuronal populations of adult rats (Gall et al., 1991; Lauterborn et al., 1991; Conner and Varon, 1992). One possible explanation is that the –2100-bp promoter does not contain all the regulatory sequences required for proper NGF expression in the adult murine hippocampus. There appears to be a developmental window, around PD10, when pyramidal hippocampal neurons of developing mice display NGF promoter activity, as shown by prominent EGFP expression. Our observations in transgenic mice do agree with those of Auburger et al. (1987), in which these authors noted that rats display a rapid increase in hippocampal NGF levels between PD12 and PD14. Although cholinergic septal axons reach the hippocampus soon after birth, increases in NGF promoter activity (as seen in EGFP transgenic mice) and in NGF mRNA/protein synthesis (as seen in rats) likely coincide with functional innervation of the postnatal hippocampal neurons by NGF-sensitive septal efferents.

Comparing the localization of NGF mRNA/protein and EGFP in peripheral tissues

Since the early experiments of Levi-Montalcini and colleagues (Levi-Montalcini and Cohen, 1960; Caramia et al., 1962; Levi-Montalcini and Angeletti, 1964), the mouse submaxillary gland has been recognized as a major source of biologically active NGF. In agreement with these pioneering investigations and other studies showing NGF immunostaining in the submaxillary tubular cells (Schwab et al., 1976; Conner et al., 1992), EGFP is specifically localized to the tubular cells of the submaxillary gland of adult transgenic mice; similar expression, however, is not seen in either 1- or 10-day-old transgenic mouse pups. Although male mice are known to have significantly higher levels of submaxillary NGF than female mice (Levi-Montalcini and Angeletti, 1964), the appearance of similar intensities of EGFP in both sexes reflects the robust signal of this reporter protein. The presence of EGFP in the submaxillary tubular cells, a recognized source of endogenous NGF, is proof of the

successful transmission and expression of the NGFpr-EGFP construct in these transgenic mice.

One tenet regarding site-specific NGF production is that mRNA expression and/or protein synthesis of this neurotrophin in peripheral tissues correlates with robust sympathetic innervation in mammalian species. Certain data gathered from the NGFpr-EGFP transgenic mice do not completely concur with this principle of neurotrophin biology. First, although several studies have shown that the rat heart atria and irides have detectable levels of NGF and robust densities of sympathetic axons (Korsching and Thoenen, 1983a; Heumann et al., 1984; Shelton and Reichardt, 1984, 1986), these two tissues in post-natal and adult transgenic mice lack EGFP-positive cells. One possible explanation may be that the heart atria and irides express much higher levels of NGF in rats than these same tissues in mice.

Second, the kidneys and thymus of rats have low levels of NGF production (Harper et al., 1980) and negligible to sparse densities of sympathetic efferents (personal observations); we detected NGF mRNA in both the thymus and kidney of adult transgenic mice (Table 3). Yet these organs in PD1, PD10, and adult transgenic mice display prominent populations of EGFP-positive cells. Regardless of the precise cellular mechanism, it is clear that further research is warranted to determine whether site-specific levels of NGF are significantly different among related mammalian species, such as rats and mice.

Whereas the kidneys, thymus, stomach, and intestines of the transgenic mice display EGFP-positive cells from early postnatal development into adulthood, other tissues exhibit a temporal expression of the transgene. For instance, EGFP-positive cells in the pituitary and lungs are seen in postnatal but not adult animals. In contrast, EGFP-positive cells are seen in the adult (but not post-natal) submaxillary glands, skin, and footpad glands. These patterns of tissue-specific expression may reflect differing requirements for proper axonal innervation that are responsive to local levels of NGF.

Transgene expression after sciatic nerve injury

Previous studies have detected NGF-like bioactivity, NGF mRNA, and NGF immunostaining in the proximal and/or distal nerve segments after sciatic nerve injury (Richardson and Ebendal, 1982; Bandtlow et al., 1987; Heumann et al., 1987; Marcinkiewicz et al., 1991). In agreement with these data, we found a robust EGFP signal in the proximal nerve stump as early as 2 days after nerve injury in adult transgenic mice. These observations provide direct evidence that the transgene can be successfully induced by nerve injury. That no EGFP-positive cells were found in the distal nerve stump following injury is consistent with the idea that any increases in local mRNA synthesis are likely to be very low or below the limits of detectability (Korsching and Thoenen, 1983b; Michalski et al., 2008). Although we have yet to determine the precise cellular sources of EGFP expression in the injured sciatic nerve of transgenic mice, NGF mRNA activity is putatively localized to Schwann cells, macrophages, and smooth muscle cells in the injured nerve segments of adult rats (Marcinkiewicz et al., 1991).

Given the activation of the EGFP transgene in response to injury, we foresee the future use of these mice to define cellular sites of NGF synthesis in experimental models of seizures,

head trauma and spinal injury, partial sympathetic denervation of peripheral tissues such as skin, and peripheral nerve injury, as well as autonomic responses to inflammation of the lungs, bowel, and synovial joints.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EGFP	Enhanced green fluorescent protein
GAP-43	Growth-associated protein of 43 kDa
NGF	Nerve growth factor
NGFpr	Nerve growth factor promoter
p75NTR	p75 neurotrophin receptor
RT-PCR	reverse transcriptase-polymerase chain reaction

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Figure 1.

A: The linear map of the construct of the mouse nerve growth factor (NGF) promoter driving expression of the reporter gene enhanced green fluorescent protein (EGFP; this construct is designated as NGFpr-EGFP). The $-2,100$ bp fragment of the mouse NGF promoter, which includes an AP-1 transcriptional factor binding site (black box), was cloned upstream of the EGFP cDNA and the adjacent SV40 early polyadenylation signal sequence (Poly A; forward arrow: transcription start site; ATG: translation start site). **B:** Examination of sections of the salivary glands from adult NGFpr-EGFP transgenic mice (as determined by PCR genotyping) revealed a robust population of EGFP-positive tubular cells in the submaxillary glands (but not in the adjacent sublingual or parotid glands). This section was stained with DAPI (blue) to reveal nuclei. **C,D:** Skin taken from the external auditory meatus of adult transgenic mice displayed EGFP-positive cells associated with the roots of each hair follicle. (D shows autofluorescence by the hair shafts under the rhodamine filter.) (See Supplemental Fig. 1 for images converted to magenta-green.) Scale bar = $200\ \mu\text{m}$ in B; $500\ \mu\text{m}$ in D (also applies to C).

Figure 2.

Distribution of EGFP-positive neurons in the cortices of transgenic mice (horizontal sections). **A:** In the piriform cortex of adult animals, a prominent population of EGFP-positive neurons is evident in layer III (arrows), as well as other neurons in the underlying dorsal endopiriform nucleus (arrowheads). **B:** At a high magnification, the population of piriform cortical neurons resolves into two subtypes: larger neurons with dendritic processes extending from the somata, and smaller neurons with shorter dendritic processes. **C,D:** In the neo-cortex of PD1 pups, a robust population of EGFP-positive neurons is seen in layer V (D, higher magnification of cortical neurons). Scale bar = 200 μm in A; 100 μm in B,C.

Figure 3.

In the entorhinal cortex from PD10 pups, numerous EGFP-positive neurons, which are also immunopositive for NeuN (B) are present in layer III (arrows, with a sparse scattering of neurons in layers I/II), as seen at low (A), intermediate (C), and high (D) magnifications. As observed elsewhere in the cortices, there appear to be both large- and small-sized EGFP-positive neurons. (See Supplemental Fig. 2 for images converted to magenta-green). Scale bar = 500 μ m in A; 100 μ m in B,D; 200 μ m in C.

Figure 4. Distribution of EGFP-positive neurons in the hippocampal formation of PD10 transgenic mice (horizontal sections). At both low (**A**) and high (**B**) magnifications, a prominent population of EGFP-positive neurons is evident in the CA fields of the hippocampal formation. By comparison, the dentate gyrus (DG) has only a sparse population of EGFP-positive hilar neurons. Autofluorescence is observed in the outer layer of the DG (*). EC, entorhinal cortex. Scale bar = 500 μ m in A; 100 μ m in B.

Figure 5. Distribution of EGFP-positive neurons in the hippocampal formation of PD10 transgenic mice pups. **A,B:** Robust populations of EGFP-positive neurons are seen in the CA1–3 regions (A) in the adjacent subiculum (B). **C,D:** The majority of EGFP-positive neurons (C, arrows) display immunostaining for the neuronal marker, NeuN (D) in the hippocampal formation. (See Supplemental Fig. 3 for images converted to magenta-green). Scale bar = 200 μ m in A; 100 μ m in B, D (also applies to C).

Figure 6. Distribution of EGFP-positive neurons in other neural areas of transgenic mice (horizontal sections in A–C). **A:** The nucleus basalis (NB) and reticular thalamic nucleus (RT) in the forebrain both show robust populations of EGFP-positive neurons in adult mice. **B,C:** EGFP-positive neurons in the nucleus basalis, having two or three dendritic processes, are smaller in size than the p75NTR-immunopositive projection neurons (red). (See Supplemental Fig. 4 for images converted to magenta-green). Scale bar = 200 μ m in A; 50 μ m in B; 100 μ m in C.

Figure 7.

Distribution of EGFP-positive cells in the cerebellum and spinal cord of adult transgenic mice. **A:** The cerebellum (horizontal section) of adult mice displays numerous EGFP-positive neurons in the molecular layer (ML), with fewer neurons seen in the granular layer (GL). The adult cerebellum was the only central tissue in which a population of EGFP-expressing glial cells is clearly visible. **B:** Astrocytic Fañanas cells (arrows) are seen enveloping the somata of EGFP-negative Purkinje neurons (*). **C:** In the spinal cord of PD1 transgenic pups, numerous EGFP neurons are seen throughout the central gray matter. **D:** By PD10, the incidence of these neurons is drastically diminished, with only sparse populations found in the superficial laminae and adjacent to the central canal. Scale bar = 50 μ m in A; 200 μ m in C,D.

Figure 8.

Distribution of EGFP-positive cells in the gastrointestinal tracts of postnatal and adult transgenic mice. **A–C,E:** The stomach of PD1 pups (A), PD10 pups (B), and adult mice (C,E) showed robust populations of EGFP-positive cells in the mucosa of the cardiac region. By adulthood, it is apparent that these are a subpopulation of epithelial cells in the cardiac glands. Note that the smooth muscle cells of the underlying external muscle layer (Mus Ext) lack EGFP expression. **D:** Compared with the stomach of PD1 pups, the intestines have EGFP-positive cells in the external muscle layer (arrows) but not in the developing epithelia of the mucosa. Scale bar = 200 μ m in A; 100 μ m in B,D; 20 μ m in C,E.

Figure 9.

A: Skin, such as the external auditory meatus used for genotyping (shown here), has an abundance of EGFP-positive cells found in association with individual hair follicles. **B:** By using confocal microscopy, it was revealed that these EGFP-positive cells are found in the sebaceous glands. **C:** The footpads show a sparse population of EGFP-positive cells at the base of small gland-like structures. (Rhodamine filter shows the presence of autofluorescence.) (See Supplemental Fig. 5 for images converted to magenta-green). Scale bar = 200 μm in A; 8 μm in B; 100 μm in C.

Figure 10.

Distribution of EGFP-positive cells in exocrine glands of adult transgenic mice. **A:** The submaxillary gland of both male and female mice have scattered populations of EGFP-positive tubular cells. **B:** The preputial glands of male transgenic mice have numerous EGFP-positive cells. **C:** The only EGFP-positive cells seen in the reproductive tracts of male and female adult mice are a scant population of columnar epithelial cells in secretory tubules of the prostate. **D:** Lacrimal glands have a dispersed population of EGFP-positive serous cells in the secretory acini. (Sections in A and C were stained with DAPI to reveal nuclei.) Scale bar = 50 μ m in A; 100 μ m in B–D.

Figure 11.

Distribution of EGFP-positive cells in the kidneys of postnatal and/or adult transgenic mice.

A–D: Both cortical and medullary regions of the adult kidney have robust populations EGFP-positive interstitial cells. In the renal cortex, these cells often appear as columns adjacent to the tubular and vascular structures, whereas in the renal medulla these cells have a scattered organization. Sections of adult kidneys immunostained for GAP-43 (a marker of peripheral axons) reveal no obvious association between EGFP-positive interstitial cells and GAP-43-immunopositive fibers (red axons in D). (Section in B shows autofluorescence of the urinary tubules with the rhodamine filter.) (See Supplemental Fig. 6 for images converted to magenta-green). Scale bar = 200 μ m in A,C; 50 μ m in B,D.

Figure 12.

A: Dense clusters of EGFP-positive cells are seen in the thymus of adult transgenic mice (primarily in the medullary portion). **B:** The only examples of EGFP-positive smooth muscle cells appear to be those at the openings of the pulmonary alveoli in PD1 pups (arrowheads). **C:** The developing pituitary also has EGFP-positive cells in PD1 mice. Scale bar = 100 μm in A,C; 200 μm in B.

Figure 13.

Immunoblotting for proNGF and mature NGF in a variety of tissues isolated from adult transgenic and wild-type (C57Bl/6) mice. Membranes were immunoblotted by using the MC-51 NGF IgG (A) and MC-51 NGF IgG plus β -actin IgG (B). Purified samples of proNGF were loaded in lane 1, and purified samples of 2.5S NGF were loaded in lanes 13 and 14. Protein samples of tissues from transgenic mice were loaded as follows: submaxillary gland in lane 2, lung in lane 3, thymus in lane 4, heart in lane 5, liver in lane 6, descending colon in lane 7, kidney in lane 8, lacrimal gland in lane 9, hippocampus in lane 10, and neocortex in lane 11; protein samples of neocortical tissue from wild-type mice were loaded in lane 12. By using the MC-51 NGF IgG, intensely stained bands at ~13 and ~14 kDa were detected in the submaxillary gland, and weakly to moderately stained bands at ~32 kDa were detected in the thymus, liver, colon, kidney, and lacrimal gland; weakly stained bands at ~32 kDa were found in the murine brain samples. Digital pseudo-coloring of the same gel (B) reveals positive immunostaining with the NGF IgG (green) and positive immunostaining with the β -actin IgG (red). (See Supplemental Fig. 7 for images converted to magenta-green).

Figure 14.

Localization of EGFP-positive cells after sciatic nerve injury. **A–C:** At 2 days post injury, EGFP-positive cells are seen in the proximal nerve stumps (at low [A] and high [B,C] magnifications). The distal nerve stumps, as well as the uninjured sciatic nerves, do not display EGFP-positive cells (data not shown). **D–F:** At 6 days after injury, a small number of EGFP-positive neurons (E, higher magnification of EGFP-positive neurons) are detected in the ipsilateral cervical spinal cord. EGFP is not detected under other filters for fluorescence microscopy (rhodamine filter shown in C and F). (See Supplemental Fig. 8 for images converted to magenta-green). Scale bar = 200 μm in A, C (also applies to B); 100 μm in D,F.

Figure 15.

Localization of EGFP-positive cells in the pineal gland. **A,B:** At embryonic day 20, the pineal gland is clearly identified in horizontal sections through the neuroaxis, as it is situated between the two cerebral cortices rostrally and the two superior colliculi caudally (A, low magnification; B, higher magnification). **C:** No EGFP-positive cells are found either in the pineal glands of postnatal and adult mice or following a unilateral sympathetic deafferentation. Scale bar = 200 μ m in A; 50 μ m in B; 100 μ m in C.

TABLE 1

Primary Antibodies Used

Antigen	Immunogen	Source	Host species	Dilution	Cat. No.
β -Actin	N-terminal peptide of the β isoform of actin (Ac-DDDIAALVIDNGSGK conjugated to KLH)	Sigma	Mouse monoclonal	1:5,000	A5441
GAP-43	Recombinant rat GAP-43 (complete sequence)	Millipore	Rabbit polyclonal	1:500	AB5220
P75NTR	GST-tagged NGF receptor p75 (mouse) corresponding to the cell membrane	Millipore	Rabbit polyclonal	1:1,000	AB1554
NeuN	Purified cell nuclei from mouse brain	Millipore	Mouse monoclonal	1:1,000	MAB377, clone A60
NGF (MC-51)	HPPLC-purified mouse 2.5S NGF (complete sequence)	Gift from Dr. Michael Coughlin (same as Cedarlane)	Rabbit polyclonal	1.25 μ g/ml	CLMCNET-011

KLH, keyhole limpet hemocyanin; HPPLC, high-performance liquid chromatography. For other abbreviations, see list.

TABLE 2

Relative Intensities of EGFP in Central and Peripheral Tissues of NGFpr-EGFP Transgenic mice¹

	PD1	PD10	Adult (3 mo)	Adult (8 mo)
Brain				
Neocortices ¹	+++	+++	+++	+++
Hippocampus ¹	–	+++	+	+
Nucleus basalis ¹	++	+++	+++	+++
Reticular thalamic ¹	++	+++	+++	++
Cerebellum ^{1,2}	–	–	+++	–
Spinal cord ¹	+++	++	–	–
Irides	–	–	–	–
Sensory ganglia	–	–	–	–
Sympathetic ganglia	–	–	–	nt
Peripheral nerves	–	–	–	–
Pituitary	++	–	–	nt
Salivary glands				
Submaxillary gland ³	–	–	+++	++
Sublingual gland	–	–	–	–
Parotid gland	–	–	–	–
Lacrimal gland ⁴	nt	nt	++	++
Pineal gland	–	–	–	–
Thymus ⁵	+++	+++	+++	–
Heart	–	–	–	–
Lung ⁶	+	–	–	–
Liver	–	–	–	–
Stomach ⁷	++	++	+++	+++
Pancreas	–	–	–	–
Intestine ⁸	++	++	++	++
Kidney ⁹	++	++	+++	++
Urinary bladder	–	–	–	–
Prostate ¹⁰	–	–	+	nt
Prepubertal gland	nt	nt	++	+++
Uterus	nt	–	–	–
Skin ¹¹	–	–	+++	variable
Footpad gland	–	–	+	nt

+++ , very strong; ++ , moderate; + , weak; – , absent; nt , not tested. EGFP-positive cells include (1) neurons, (2) astrocytes, (3) tubular cells, (4) serous cells, (5) reticular cells, (6) smooth muscle cells, (7) mucosal epithelia, (8) enteric neurons, (9) interstitial cells, (10) columnar epithelia, and (11) sebaceous gland cells. PD, postnatal day. For other abbreviations, see list.

TABLE 3Detection of NGF mRNA by RT-PCR in Central and Peripheral Tissues of NGFpr-EGFP Transgenic Mice¹

	Postnatal day 1	Adult
Brain		
Neocortices	+	+
Hippocampus	+	+
Cerebellum	+	+
Submaxillary gland	nt	+
Lacrimal gland	nt	+
Thymus	nt	+
Heart	nt	–
Lung	nt	+
Colon	nt	–
Kidney	nt	+
Urinary bladder	nt	–

+, present; –, absent; nt, not tested. For abbreviations, see list.