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Phenotypic Diversity and Expression of GABAergic Inhibitory Interneurons During Postnatal Development in Lumbar Spinal Cord of GAD67-GFP Mice

Kimberly J. Dougherty[#], Michael A. Sawchuk, and Shawn Hochman^{*}

Department of Physiology, Emory University School of Medicine, Atlanta GA, 30322

Abstract

The synthesis enzyme glutamic acid decarboxylase (GAD65 or GAD67) identifies neurons as GABAergic. Recent studies have characterized the physiological properties of spinal cord GABAergic interneurons using lines of GAD67-GFP transgenic mice. A more complete characterization of their phenotype is required to better understand the role of this population of inhibitory neurons in spinal cord function. Here, we characterize the distribution of lumbar spinal cord lumbar GAD67-GFP neurons at postnatal days (P) 0, 7, 14, and adult based on their co-expression with GABA and determine the molecular phenotype of GAD67-GFP neurons at P14 based on the expression of various neuropeptides, calcium binding proteins, and other markers.

At all ages >67% of GFP⁺ neurons were also GABA⁺. With increasing age; (i) GFP⁺ and GABA⁺ cell numbers declined, (ii) ventral horn GFP⁺ and GABA⁺ neurons vanished, and (iii) somatic labeling was reduced while terminal labeling increased. At P14, vasoactive intestinal peptide and bombesin were expressed in ~63% and ~35% of GFP⁺ cells, respectively. Somatostatin was found in a small number of neurons, whereas calcitonin gene-related peptide never co-localized with GFP. Moderate co-expression was found for all the Ca²⁺ binding proteins examined. Notably, most laminae I-II parvalbumin⁺ neurons were also GFP⁺. Neurogranin, a protein kinase C substrate, was found in ~1/2 of GFP⁺ cells. Lastly, while only 7% of GFP⁺ cells contain nitric oxide synthase (NOS), these cells represent a large fraction of all NOS⁺ cells.

We conclude that GAD67-GFP neurons represent the majority of spinal GABAergic neurons and that mouse dorsal horn GAD67-GFP⁺ neurons comprise a phenotypically diverse population.

Keywords

Neuropeptides; Ca²⁺ binding proteins; dorsal horn; nociception; sensory; inhibition

Spinal cord neurons have been molecularly-tagged to associate reporter molecules to developmentally-expressed genes that identify distinct neural progenitors (eg. Jessell, 2000;

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^{*} Correspondence: Shawn Hochman, Ph.D., Whitehead Biomedical Research Building, Room 644, Emory University School of Medicine, 615 Michael St., Atlanta GA 30322 U.S.A., Ph: (404) 712-3131 Fax (404) 727-2648, shawn.hochman@emory.edu

[#]Present address for K.J.D.: Department of Neuroscience, Karolinska Institute, 17177 Stockholm, Sweden

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Department of Anesthesiology, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0818, USA

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Helms and Johnson, 2003) and more recently to expressed genes identifying transmitter phenotypes (e.g. Oliva, Jr. et al., 2000; Zeilhofer et al., 2005). The ability to visually target, then characterize these molecularly distinct neurons has significantly advanced studies on spinal cord function (Heinke et al., 2004; Dougherty et al., 2005; Hinckley et al., 2005; Wilson et al., 2005; Zeilhofer et al., 2005; Gosgnach et al., 2006). However, the extent to which reporter-based neuronal targeting accurately defines a target population is uncertain. For example, in hippocampus and neocortex, Oliva et al. (2000) demonstrated that GFP-expressing neurons comprised only a small and phenotypically-specific subpopulation of GABAergic neurons (somatostatin⁺).

In spinal cord, GABAergic interneurons are found throughout the gray matter but are concentrated in the superficial laminae (I-III), where they depress excitability by both pre- and postsynaptic mechanisms. Axoaxonic GABAergic synapses onto primary afferent terminals produce presynaptic inhibition (Alvarez et al., 1992; Rudomin and Schmidt, 1999) while postsynaptically, GABAergic neurons reduce the excitability of both projection neurons (Alvarez et al., 1992) and interneurons (Jankowska, 1992).

Spinal cord GABAergic neurons constitute a phenotypically heterogeneous population (Todd and Spike, 1993; Laing et al., 1994). A neuron is GABAergic if it contains either or both of the glutamic acid decarboxylase (GAD) synthesis enzymes GAD65 and GAD67 (Soghomonian and Martin, 1998). Both are found in cell bodies throughout the spinal cord, except lamina IX (Barber et al., 1982; Ma et al., 1994) and almost all GAD⁺ boutons are labeled with both isoforms (Mackie et al., 2003). Recently, using the GAD67-GFP mice created by Oliva et al. (2000), GFP expression has been shown in 68% of lamina I GABAergic neurons at P14 (Dougherty et al., 2005) and 35% of lamina II GABAergic neurons in the adult (Heinke et al., 2004), demonstrating that somatic GAD67-GFP expression does not report for all spinal GABAergic interneurons (Heinke et al., 2004; Dougherty et al., 2005).

The present study characterizes lumbar spinal cord GAD67-GFP⁺ neurons in relation to developmental expression patterns, topographical location, and co-expression with various neuropeptides and Ca²⁺ binding proteins. The goal is to provide essential information on the phenotypic properties of these GABAergic neurons in the lumbar spinal cord. Using a different line of GAD67-GFP generated mice, Huang et al (2007) studied developmental expression patterns in the cervical spinal cord and confirmed their GABAergic phenotype. As detailed below, we similarly found that spinal GAD67-GFP expression reports the identity of most GABAergic neurons in lumbar cord. Moreover, we undertook more detailed quantitative analyses, including the degree of co-labeling of GFP with GABA, neuropeptides, and calcium binding proteins to demonstrate that these neurons constitute a population as phenotypically diverse as those observed using GABA immunolabeling (GABA-IR). Hence, physiological and anatomical studies on GAD67-GFP neurons can be used to study many aspects of GABAergic neuron function in the mouse lumbar spinal cord.

Some results have been presented in abstract form (Dougherty et al., 2003; Dougherty et al., 2004).

Experimental procedures

All experimental procedures complied with the NIH guidelines for animal care and the Emory Institutional Animal Care and Use Committee. Homozygotic GAD67-GFP mice (Oliva et al., 2000) were obtained from The Jackson Laboratory (Bar Harbor, ME).

GABA/EGFP labeling comparisons

Three mice each at 3 postnatal (P) ages, P0, P7, P14, and adult (P45) were anesthetized with urethane (2mg/kg ip), perfused with 1:3 volume/body weight ice cold 0.9% NaCl, 0.1% NaNO₃, 1 unit/ml heparin, followed by equal volume/body weight of modified Lana's fixative (4% paraformaldehyde, 0.2% picric Acid, 0.16 M PO₃); pH 6.9. Spinal cords were isolated and post-fixed 1hr in modified Lana's fixative, cryoprotected in 10% sucrose, 0.1M PO₃, pH 7.4 until sectioning in 10µm-thick slices on a cryostat (Leitz 1720).

Colchicine experiments

For colchicine experiments, three mice (P14) were anesthetized with urethane (2mg/kg ip), decapitated, and the spinal cord was carefully dissected out of the body cavity and placed in an artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 25 glucose, 1.25 NaH₂PO₄, and 26 NaHCO₃ at a pH of 7.4. Isolated cords were incubated for 12 hrs in aCSF continuously oxygenated with 95% O₂-5% CO₂ containing 1µM colchicine prior to being fixed for 1 hr in modified Lana's fixative, cryoprotected in 10% sucrose, 0.1M PO₃, pH 7.4, and cryostat sectioned (10µm-thick slices).

Stereology

Two 14 day old GAD67-EGFP mice were perfused with modified Lana's Fixative (4% paraformaldehyde, 0.2% picric acid, 0.16 M PO₃, pH 6.9). The L2 region of the spinal cord was isolated and frozen onto a cryostat chuck. Sections, 50µm thick, were cut and thaw mounted onto microscope slides sequentially, yielding 16 slices for each animal. Tissue was processed for GABA immunohistochemistry as previously described. Laminae I and II were outlined at 10x objective magnification in Stereoinvestigator (Microbrightfield). Optical fractionator probes with a 5µm guard and 30µm dissector height were performed at 100x objective magnification. A sampling grid area of 6677µm and a section periodicity of 1 were used. Section thickness was determined at each sampling grid and yielded a mean measured section thickness of 42.6µm in one animal and 43.2µm in the other. Some variation in thickness was found from sampling site to sampling site suggesting that the estimated total by number weighted section thickness was more appropriate for cell counts.

Antibody labeling

All incubations and washes for immunohistological processing were performed in 0.1M PO₃ buffered saline containing 0.3% triton X-100 (PBS-T). Tissue was washed overnight in PBS-T at 4°C followed by incubation in various primary antibodies for 48-72 hours [rabbit anti-GFP (AB3080P), 1:100; rabbit anti-GABA (A2052), 1:1000; rabbit anti-somatostatin (AB3080P), 1:250; rabbit anti-met-enkephalin (AB1975), 1:1000; rabbit anti-neuropeptide Y (N9528), 1:2500; rabbit anti-CCK-8 (PEPA25), 1:100; mouse anti-parvalbumin (MAB1572), 1:50; rabbit anti-calbindin 28K (AB1778), 1:1000; mouse anti-calcitonin gene-related peptide (CGRP; MAB317), 1:50; rabbit anti-choline acetyltransferase (ChAT; AB143), 1:200; rabbit anti-dynorphin (AHP373), 1:50; rabbit anti-nitric oxide synthase (NOS; AB5380), 1:250; guinea pig anti-Substance P (AB5060), 1:1000; rabbit anti-vasoactive intestinal polypeptide (VIP; AB982), 1:250; rabbit anti-bombesin (AB905), 1:250; goat anti-calretinin (AB1550), 1:2500; rabbit anti-neurogranin (AB5620), 1:500]. GABA and neuropeptide Y antibodies were purchased from Sigma, CCK-8 and dynorphin from Serotec, and all others from Chemicon. Slides were then washed 3x 30 min in PBS-T and incubated in Cy3 conjugated secondary antibodies [from Jackson Immunoresearch: anti-rabbit (711-165-152), anti-mouse (715-165-151), anti-guinea pig (706-165-148), anti-goat (705-165-147)] at 1:250.

Cell counting

Stereology counts were performed as described above. For the comparison of eGFP and GABA counts at P0, P7, P14, and adult (n=3 mice at each age), sections (10 μ m) from spinal segments L2-L4 were collected 100 μ m apart. These sections were mapped and counted using the Neurolucida image analysis system (Microbrightfield). For all colchicine studies, 10 μ m sections from spinal segments L1-L6 were collected 200 μ m apart were visualized on a Nikon E-800 microscope. The Neurolucida image analysis system was used for the mapping of sections and counting of immunopositive cells. All cells within 70-100 μ m (depending on age) of dorsal edge of the grey matter were considered laminae I-II. Cells in the next 100 μ m were counted as laminae III-IV. Cell counts of deep dorsal horn were defined as laminae III-VI. The ventral border of lamina VI was approximated by a horizontal line extending at the midline through the central canal. Hence, dorsal lamina X is also included in these cell counts. The ventral horn includes laminae VII-IX and the ventral portion of lamina X.

Spinal cord growth with age was taken into account by estimating the total number of cells in each spinal segment. The cell counts above were multiplied by a factor to correct for the increasing segment size with development. Since one spinal segment at P0 is approximately 400 μ m in length, the number of cells counted in 10 sections that were 10 μ m thick (100 μ m) was multiplied by 4 to estimate the total number of cells per segment. For P7, P14, and adult, correction factor values were 6, 8, and 10 respectively to account for corresponding increases in segment size. Cell count estimates are presented as mean $\pm$ S.E. Other statistical tests are described in their associated Tables.

Images of transverse sections were taken with a Nikon digital camera through a Nikon E800 microscope. Confocal images were visualized on an Olympus FV1000 fluoview confocal microscope using GFP and Cy3 filter sets and an optical slice thickness of 0.254 μ m. Images were pseudo-colored, overlaid, and contrast adjusted in CorelDraw 12.0 (Ottawa, Ontario).

RESULTS

Expression of GFP and GABA at various postnatal ages

The number of cells expressing endogenous GFP (eGFP) was compared to the number of cells labeled with a GABA antibody in the same section of lumbar cord at 4 different ages - P0, P7, P14, and adult (Figure 1). Both eGFP fluorescence (Figure 1, top row) and GABA immunolabeling (Figure 1, second row) were observed predominantly in the dorsal horn. An overlay of eGFP and GABA labeling shows that many eGFP⁺ cells were also GABA⁺ in the dorsal horn (Figure 1, third and bottom rows). Note that somatic eGFP labeling is more prominent than GABA at all ages compared. Both eGFP and GABA labeling becomes progressively restricted with age to the superficial laminae and overt somatic labeling is greatly reduced. Also, in the adult, while there is little somatic labeling, the spinal distribution of eGFP and GABA is nearly identical. As seen in the confocal images, GABA labeling is strongest at the cell membrane and, therefore, appears as a ring around the cell interior.

A representative section reconstructed to more clearly show the distribution of eGFP⁺ only, GABA⁺ only, and double labeled neurons is shown in Figure 2A. While, the number of GABA⁺ and eGFP⁺ cells per 10 μ m section declines with age in all regions (Figure 2A), this is at least partly due to reduced cell densities as the spinal cord grows considerably between P0 and adult. This was taken into account by estimating the total number of cells in each spinal segment. Cell counts per 10 μ m section were multiplied by a factor to correct for the increasing segment size with development. Since one spinal segment at P0 is approximately 400 μ m in length, the number of cells counted in 10 sections that were 10 μ m thick (100 μ m) was multiplied by 4 to estimate the total number of cells per segment. For P7, P14, and adult, correction factor

values were 6, 8, and 10 respectively to account for corresponding increases in segment size. Following this correction, the estimated number of somatically-labeled GABA⁺ and eGFP⁺ cell counts are approximately stable from P0 to P14 but decline from P14 to adult (Table 1). The reduced number of somatically-labeled cells in the adult may reflect a trend toward preferential terminal labeling (Figure 1). Counts of double labeled cells in laminae I and II were verified using stereology. Results were very comparable (2751±17 cells using stereology and 2602±386 without).

Figure 2B and Table 1 also show that, at P0, there is a high degree of correspondence between eGFP and GABA somatic labeling. Seventy-six percent of GABA⁺ cells are eGFP⁺. The correspondence is strongest in laminae I-II where 80% of GABA⁺ cells are eGFP⁺. The incidence of double labeled cells does not change much after P0 (61% of GABA⁺ cells are also eGFP⁺ cells at P7, 74% at P14, and 63% in the adult; c.f. Heinke et al., 2004).

At all ages eGFP labeling is evident in both somata and neuropil (Figure 3). Labeling dominates in the dorsal horn and somatic labeling is completely absent in the ventral horn of the adult (c.f. Heinke et al., 2004). In deeper dorsal horn laminae, there is a preferential medial labeling directed towards lamina X dorsal to the central canal. Interestingly, there is also strong midline labeling of a presumed axon fascicle from the dorsal central canal to the dorsal column surface that is particularly apparent in P0 but disappears in the adult (Figure 3, A₁ vs. A₄). Somatic labeling becomes replaced by extensive neuropilar labeling dorsal to the central canal (Figure 3D). The loss of somatic labeling with age is seen in all regions. Ventral horn neurons seen in younger animals are not seen in adult. Labeling is observed in the ventral-most part of the dorsal column at all ages (Figure 3A) and is consistent with location of the corticospinal tract (Brown, 1971; Hughes et al., 2005).

Expression of GABA and eGFP following colchicine treatment

One explanation for the reduction in labeled eGFP and GABA with age is the trend toward loss of somatic expression and an increase in expression in terminals (Figure 1). To examine this, we blocked anterograde transport with colchicine for 12 hours prior to fixation and observed a greater number of both eGFP⁺ and GABA⁺ cells (examined at P14; Figure 4). Only the cells in the dorsal horn laminae I-IV were compared because the core of the colchicine-treated cord became anoxic during the incubation (Wilson et al., 2003). The number of double-labeled cells in the dorsal horn increased by ~35% (4334 to 5809) after colchicine pretreatment (Table 2). This was due to both an increase in the total number of GABA⁺ cells (by 76%) and of total eGFP⁺ cells (by 65%). Thus, given the strong correspondence between somatic GABA and eGFP labeling at P0, the near-identical terminal field labeling in adult (Figure 1), and the increased incidence of somatic labeling with colchicine, it is likely that GABA is expressed in the large majority, and perhaps all, eGFP⁺ neurons and eGFP is expressed in at least 2/3 of GABAergic neurons in the dorsal horn.

Neurochemical heterogeneity in GAD67-GFP neurons

We next undertook studies to examine the phenotypic diversity of GAD67-GFP neurons by immunostaining for various neuropeptides, Ca²⁺-binding proteins, and other neural markers.

(i) Expression of various neuropeptides in GAD67-GFP neurons—Antibodies to various neuropeptides were tested in P14 mice to determine if they co-localized with eGFP. Colchicine-treated cords were used as there was very little neuropeptide somatic labeling otherwise (not illustrated). Labeled somata were evident with all antibodies tested on colchicine-treated cords. While eGFP⁺ cells were previously identified in hippocampus and neocortex as GABAergic cells expressing somatostatin (Oliva, et al., 2000; Ma et al., 2006), this is not the case in the spinal cord. Very few eGFP⁺ cells were somatostatin⁺ in the dorsal

horn (<10%; Figure 5). Conversely, >25% of somatostatin⁺ cells were eGFP⁺ (28%, laminae I-II; 29% laminae III-IV). No eGFP⁺ dorsal horn cells were found to co-express CGRP.

As shown in Figure 5B, bombesin and VIP both stained a very high number of dorsal horn cells and were co-expressed in the highest percentages with eGFP⁺ cells (bombesin: 33% in laminae I-II, 38% in laminae III-IV; VIP, 61% in laminae I-II and 65% in laminae III-IV). Neither of these peptides are specific to GABAergic neurons in that eGFP⁺ cells made up ~1/5 of bombesin⁺ and VIP⁺ cells in laminae I-II and even less (bombesin, 11%; VIP, 15%) in laminae III-IV.

(ii) Expression of calcium-binding proteins in GAD67-GFP neurons—Calcium binding proteins have been used to classify GABAergic neurons in other CNS regions (see Hendry et al., 1989). Antibodies to the calcium binding proteins calbindin, calretinin, and parvalbumin were tested to determine if they co-localize with the eGFP⁺ cells in colchicine-treated cords (Figure 5). A small percentage of eGFP⁺ cells contained calbindin (9% in laminae I-II, 17% in laminae III-IV). While a moderate percentage of lamina I-II eGFP⁺ cells expressed parvalbumin (26%) and calretinin (21%), the incidence of double labeled cells was lower in laminae III-IV (parvalbumin, 8%; calretinin, 11%). Although parvalbumin was found in only ~1/4 of eGFP⁺ laminae I-II cells, most (64%) parvalbumin⁺ cells were eGFP⁺. There were less parvalbumin⁺ cells in laminae III-IV, only 29% of which were also eGFP⁺.

(iii) Expression of other neural markers in GAD67-GFP neurons—In addition to the neuropeptides and calcium binding proteins, other enzymes and substrates are commonly found in dorsal horn cells. NOS and ChAT, both of which have been shown to be expressed in GABAergic neurons in the dorsal horn, and neurogranin, a PKC substrate found in the dorsal horn, were also examined in colchicine-treated cords (Figure 5). Both NOS and ChAT were co-expressed with eGFP cells scattered throughout the dorsal horn. Diffuse ChAT labeling in the dorsal horn prevented ChAT⁺ cell numbers to be accurately quantified. NOS⁺ cells accounted for <10% of eGFP⁺ cells, however 45% and 33% of NOS⁺ cells were eGFP⁺ in laminae I-II and III-IV, respectively. Many eGFP⁺ cells (59% in laminae I-II and 42% in laminae III-IV) were neurogranin⁺. Neurogranin⁺ somata were mainly in a band in the dorsal horn and almost half (52% in laminae I-II and 43% in laminae III-IV) of these cells also expressed eGFP.

DISCUSSION

We chose to undertake our analyses in the lumbar spinal cord to best integrate findings with the dominance of spinal cord research studying functional systems related to hindlimb sensorimotor function. The primary finding of this study is that transgenic mice expressing GFP fluorescence under the control of a GAD67 regulatory element can be used to identify a significant fraction of lumbar spinal GABAergic neurons from birth through adulthood. These neurons represent a phenotypically-diverse population, as these cells displayed different co-expression patterns for various neuropeptides, Ca²⁺-binding proteins, and other protein substrates. Overall, these observations support the use of the Oliva et al. (2000) GAD67-GFP transgenic mice to identify and characterize the cellular and synaptic properties of most spinal GAD67-expressing GABAergic interneurons.

We noted a progressive loss of somatic eGFP and GABA labeling with age in the deep dorsal horn and ventral horn. This apparent loss may not represent an actual reduction in cell numbers since, particularly in the adult, most labeling seems to be preferentially located within the neuropil, presumably at nerve terminals. In support of this possibility, blocking axonal transport in colchicine-treated cords resulted in substantially increased numbers of both eGFP⁺ and GABA⁺ cells detected somatically.

Extensive immunocytochemical studies undertaken to identify co-expression pattern with neuropeptides and Ca^{2+} binding proteins identified several subpopulations of GABAergic neurons. As described in detail below, these phenotypes are consistent with those observed in the rat (Proudlock et al., 1993; Todd and Spike, 1993; Laing et al., 1994) and support recent observations in lamina II of mouse (Heinke et al., 2004).

Both GAD67-GFP and GABA cell counts decrease with age

In the paper originally describing this line of GAD67-GFP mice, it was reported that GFP was expressed in a small subpopulation of GABAergic neurons in the hippocampus; 7% in CA1, and 22% in CA2 (Oliva et al., 2000). Although it appears that only a subpopulation of GABAergic neurons express GFP in the lumbar spinal cord also, it is a much more substantial proportion of GABAergic neurons. In spinal cord, there are several possible reasons for the expression of GFP in subsets of the total GABAergic neuron population. First, GAD65 and GAD67 are expressed in different but overlapping neuronal populations in the spinal cord with more GAD67, relative to GAD65, in both the dorsal horn and the ventral horn (Feldblum et al., 1995). Therefore, $\text{GABA}^+/\text{eGFP}^-$ neurons may be GAD65^+ . Another possibility is that eGFP is only expressed or detected in neurons with high levels of GAD67. Feldblum et al. (1995) distinguished three different populations of GABAergic neurons in the dorsal horn based on mRNA levels. It has also been suggested that GAD67 levels are lower in neurons containing glycine (Feldblum et al., 1995), which is the case for at least half of GABAergic neurons in the dorsal horn (Laing et al., 1994; Keller et al., 2001).

Throughout development, 80% to 94% of eGFP^+ neurons in laminae I-II are also GABA^+ . This correspondence is lower in other laminae since 67% to 80% of all eGFP^+ neurons are GABA^+ in the lumbar spinal cord. It has been shown that GABA moves towards terminals with development (Tran et al., 2003) and, therefore, can no longer be detected in the soma by antibody labeling. This may contribute to our report of reduced numbers of eGFP^+ and GABA^+ neurons with age. In order to determine that this was the case, spinal cords were pretreated with colchicine to block axonal transport, thereby sequestering proteins in the cell soma. Only laminae I-IV of the dorsal horn could be compared in colchicine-treated cords maintained in vitro because the center of the cord was anoxic (Wilson et al., 2003). Colchicine-treated cords had increased eGFP and GABA detected in the dorsal horn. This increased incidence of GABA^+ neurons suggests that GABA was transported to the terminals. Additionally, colchicine treatment concentrated eGFP in the somata so that a greater number of cells were above detection threshold. Consistent with Ma, et al. (1994) in rat and Huang et al. (2007) in mouse, GAD67 has a similar expression pattern at all ages studied. While overall cell density for GABA and eGFP expression decreased throughout development, when estimates of neuronal labeling per segment were taken into account, the decrease in labeled somata was only seen from P14 to adulthood. Similarly, GAD67 mRNA levels have been reported to be higher at P7-P14 than in the adult (Ma et al., 1994). Where all levels throughout the dorsal-ventral cord decreased, the most dramatic reduction was in the ventral horn (Ma et al., 1994). Consistent with this, we report the greatest decrease in the ventral horn. This loss in ventral horn somatic labeling is likely a consequence of developmental change in phenotype from GABAergic to glycinergic neurons (Gao et al., 2001).

Colocalization of GFP with neuropeptides and calcium binding proteins

Neuropeptides potently modulate neural function and the spinal cord contains a rich repertoire, particularly in dorsal horn, with heterogeneous cell expression patterns (Todd and Spike, 1993). In order to further identify the subpopulation of GABAergic neurons expressing eGFP in the spinal cord, the extent to which neuropeptides, calcium binding proteins, and other factors co-localized with eGFP in colchicine-treated cords was examined. Previous studies have used antibodies to GABA, GAD65, and GAD67 in addition to antibodies to neuropeptides and

calcium binding proteins (Lima et al., 1993; Spike et al., 1993; Laing et al., 1994). Here, we used endogenous fluorescence of GAD67-GFP to identify the somata of GABAergic neurons. We chose to compare peptide labeling in cells expressing endogenous GFP as this is an important population to appraise in relation to electrophysiological studies in these neurons (Heinke et al., 2004; Dougherty et al., 2005).

Colchicine was used so that peptides, predominantly found in axon terminals, could be detected in somata and expression could be compared to eGFP. Colchicine has been shown to induce the expression mRNA of certain peptides (Kiyama and Emson, 1991; Rethelyi et al., 1991). Therefore, in the following sections, we compare our results with those obtained in other studies.

Previous studies for somatostatin immunolabeling report little expression in GABAergic neurons with only some cells co-labeled in laminae III-IV in rat (Proudlock et al., 1993) and no eGFP⁺/somatostatin⁺ cells in lamina II in mouse (Heinke et al., 2004). Similarly, here, somatostatin was expressed in a small number of eGFP⁺ neurons in the deep dorsal horn. Therefore, unlike the clear association of GFP⁺ neurons with somatostatin in hippocampus and neocortex (Oliva et al., 2000), labeling in spinal cord is unrelated to somatostatin co-expression.

The expression of VIP and bombesin in eGFP⁺ cells was most striking. Large numbers of VIP⁺ and bombesin⁺ somata were found throughout the dorsal horn of the lumbar spinal cord and many of these were also eGFP⁺. However, both VIP and bombesin labeling is likely evenly distributed, regardless of transmitter phenotype, in that only ~20% of cells labeled by either antibody are eGFP⁺. Bombesin⁺ cells have been shown to be numerous in the dorsal horn of adult rats, particularly in laminae I-III (Leah et al., 1988; Todd and Spike, 1993). VIP⁺ fibers have been reported to be dense in the superficial dorsal horn, including somata localized to the lateral spinal nucleus and lamina X in the lumbar and sacral spinal cord of adult rats (Fuji et al., 1983; Gibson et al., 1984a; Leah et al., 1988; Todd and Spike, 1993). Our distribution appears more widespread than in previous studies. Consistent with previous studies (Gibson et al., 1984b), CGRP was not found in any neurons in the dorsal horn.

Calcium binding proteins subclassify GABAergic neurons in other CNS regions, such as the amygdala (Kempainen and Pitkanen, 2000) and cortex (Tamamaki et al., 2003). Calbindin, calretinin, and parvalbumin antibodies were used to see if GFP⁺ cells could be distinguished by calcium binding proteins. As in hippocampus (Oliva et al., 2000), most GFP⁺ neurons in the spinal cord did not express calbindin, calretinin, or parvalbumin. Also in these mice, it had been reported that 23% of GFP⁺ cells in lamina II were parvalbumin⁺ (Heinke et al., 2004). Similarly, we show that 26% of eGFP⁺ cells in laminae I-II are parvalbumin⁺. In rat, parvalbumin expressing cells are known to be found in laminae II-III, where 70% of these are GABAergic (Laing et al., 1994). Consistent with this, 64% of parvalbumin⁺ cells in laminae I-II here are eGFP⁺ (see Figure 5). These comparable observations support the accuracy of GFP as a marker of the GABA phenotype.

NOS⁺ cells have been shown to be mostly in lamina I and II (Dun et al., 1993), most of which are GABAergic (Bernardi et al., 1995). Our results show that 45% of NOS⁺ cells are eGFP⁺ in laminae I-II and 33% in laminae III-IV. Additionally, Bernardi, et al (1995) report 1/3 of GABAergic neurons in lamina I and II are NOS⁺ while 14% of eGFP cells in lamina II of these mice are NOS⁺ (Heinke et al., 2004). Our results are more consistent with Heinke et al. However, less than 10% of eGFP cells in laminae I-IV are NOS⁺.

In addition to motoneurons, ChAT has been shown in laminae III-V (Barber et al., 1984), and more specifically in GABAergic neurons in lamina III (Laing et al., 1994). Here, very few ChAT⁺/eGFP⁺ cells were found in laminae III-IV.

Neurogranin is a PKC substrate with a spinal distribution localized primarily in the dorsal horn (Houben et al., 2000). Neurogranin has been implicated in dendritic plasticity in other CNS regions but its function in the spinal cord is unknown (Houben et al., 2000). Since the distribution of neurogranin is similar to GFP, we looked for overlap. A large number of eGFP⁺ cells are neurogranin⁺ (59% in laminae I-II, 42% in laminae III-IV) and approximately 1/2 of the neurogranin⁺ cells in the dorsal horn are eGFP⁺.

Conclusion

The most obvious strength of GAD67-GFP mice is the ability to identify neurons as GABAergic by somatic fluorescence for electrophysiological recordings. The present results, demonstrating that endogenous GFP is expressed in most GABAergic neurons with heterogeneous expression patterns, comparable to those previously seen with GABA antibody labeling, supports reliable use of this animal to study the functional properties of most populations of GABAergic interneurons in the spinal cord.

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ABBREVIATIONS

CCK-8, cholecystokinin-8
 CGRP, calcitonin gene related peptide
 ChAT, choline acetyltransferase
 eGFP, endogenous green fluorescent protein
 GABA, gamma-aminobutyric acid
 GAD, glutamic acid decarboxylase
 GFP, green fluorescent protein
 IR, immuno reactive
 L, lumbar
 NOS, nitric oxide synthase
 P, postnatal
 PBS-T, phosphate buffered saline with triton
 VIP, vasoactive intestinal peptide

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Figure 1.

Comparison of endogenous GFP to GABA-IR in P0, P7, P14, and adult mice. Shown are transverse lumbar spinal slices (10µm) at four different developmental stages - day 0, day 7, day 14, and adult. eGFP⁺ cells (top row) in GAD67-GFP mice are compared to cells immunoreactive to GABA (second row). An overlay of the two (third row) shows that most, but not all, eGFP⁺ cells are also GABA⁺. This is more clearly seen in confocal pictures from the dorsal horn (bottom row). Scale bars are 100µm in the first three rows and 10µm in the bottom row. Overlay was set at 50% transparency.

Figure 2. Comparison of cell labeling distributions at all ages examined. **A.** Neurolucida reconstructions of a representative section of L2 at each age. Scale bars are 100μm. **B.** The histograms show the average number of cells per estimated segment that are eGFP⁺ only (green), GABA⁺ only (red), and double labeled (black). Insets show the ventral horn values on an expanded scale.

Figure 3.

Age-related changes in the distribution of GAD67-GFP. Images were obtained from the L2 spinal segment and images are presented as grayscale negatives. Endogenous GFP fluorescence in P0, P7, P14, and adult are compared in dorsal horn (A) and ventral horn (B). Arrowheads in A for P0 (A₁) and P14 (A₃) indicate labeling of presumed axon fascicle from the dorsal central canal to dorsal column surface. Dashed lines in A identify eGFP labeling in the ventral-most aspect of the dorsal column, at a location consistent with the corticospinal tract. In B, arrows at P7 (B₂) identify ventral horn neurons and arrowheads at P14 (B₃) and adult (B₄) outline presumed axonal and terminal labeling. C-E represent higher power images of dorsal horn (C), central gray (D) and ventral horn (E). For dorsal horn (C), note the decline in the

number of somatic labeling with age. For the central gray region (**D**), note that somatic labeling becomes replaced with extensive neuropilar labeling dorsal to the central canal. White ovals in the middle row outline neuroepithelial cells of the central canal. While there are neurons in the ventral horn (**E**) in younger animals, these are not seen in the adult. Arrowheads in **D** and **E** point out neuropilar punctate labeling, seen most intensively in the adult. Scale bars are 50 μm . Scale bar in **B**₄ applies to panels **A** and **B** and in **E**₄ to **C-E**.

Figure 4. P14 spinal cords treated with colchicine have an increase in eGFP and GABA somatic labeling. **A.** eGFP (green), GABA (red), and the extent to which they are co-expressed (yellow/orange) in the dorsal horn of a 10 μ m thick slice from a lumbar spinal segment. Overlay was set at 50% transparency. **B.** Histograms show the quantification of the mean labeling per estimated segment: eGFP⁺ only (green), GABA⁺ only (red), and double labeled (black). Scale bar is 10 μ m.

Figure 5.

Several neuropeptides are expressed in a small number of eGFP⁺ cells. **A.** Co-expression of eGFP (green) and antibody-labeling (red) is shown in yellow in the dorsal horn of colchicine-treated sections from spinal segments L1-L6. Solid arrows identify examples of cells expressing eGFP⁺ only, examples of antibody⁺ cells are shown with open arrows, and stick arrows point to examples of double labeled cells. **B.** Quantification of cells in laminae I-II (**B₁**) and III-IV (**B₂**). Black bars represent % of eGFP⁺ cells that are also positive for the listed antibody. Grey bars on the bottom half of the graphs show the % of antibody⁺ cells that are eGFP⁺. Error bars are S.E. (n=3).

Table 1

Number of cells in an estimated segment (see Methods) throughout development that are eGFP⁺ only, GABA⁺ only, and double labeled. P values resulting from ANOVAs are in the last column

	P0	P7	P14	adult	P
eGFP only	2032±160	2831±809	1834±237	1255±284	0.18
GABA only	2553±768	3816±1091	2554±371	1920±506	0.38
Double	7835±1190	5795±677	7354±783	3380±799	0.03*
% eGFP also GABA	79%±2%	67%±7%	80%±2%	71%±9%	0.35
% GABA also eGFP	76%±3%	61%±7%	74%±1%	63%±6%	0.13

Table 2

Comparison of estimated number of cells in a spinal segment (see Methods) at P14 in control and colchicine-treated cords. P values resulting from Student's t-tests are in the last column

	no colchicine	colchicine	p value
eGFP only	637±47	2406±272	0.003*
GABA only	1564±82	4593±965	0.04*
Double	4334±285	5809±427	0.045*