Expression Pattern of Enkephalinergic Neurons in the Developing Spinal Cord Revealed by Preproenkephalin-Green Fluorescent Protein Transgenic Mouse and Its Colocalization with GABA Immunoreactivity

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Key Words
Enkephalin · Development · Immunofluorescence labeling · Fluorescent in situ hybridization histochemistry

Abstract
To gain better insight into the ontogenic function of enkephalin (ENK) in the spinal cord, it is necessary to have a clear picture of the developing pattern of the ENKergic neurons. To address this question, we used transgenic mice which reveal ENKergic neurons easily by expressing enhanced green fluorescent protein (GFP) under the specific transcriptional control of the preproenkephalin (PPE) gene. GFP-positive neurons first appeared at embryonic day (E) 11.5 in the ventromedial part of the cervical ventral gray matter. At E13, they were mainly present in the intermediate zone. Thereafter, GFP-positive neurons increased progressively in number and extended from ventral to dorsal regions. Quantitative analysis showed that GFP-positive neurons peaked in number at postnatal day (P) 7 at the cervical level. The number of GFP-positive neurons reached a peak at P3 at the lumbar level. At P21, the distribution pattern of GFP immunoreactivity was similar to that in the adult spinal cord. Double-labeling results showed that about one-third of the total γ-aminobutyric acid cell population colocalized with GFP: 34.9 ± 3.5% at E16 and 32.4 ± 3.7% at P3. Double-labeled neurons accounted for nearly half of the GFP-positive neurons: 42.4 ± 2.4% at E16 and 44.1 ± 2.9% at P3. Taken together, the present results suggest that ENKergic neurons develop according to a rostrocaudal and ventrodorsal gradient. These results have broad implications for understanding the functional roles of ENKergic neurotransmission in the developing spinal cord.

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Abbreviations used in this paper

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<th>Abbreviation</th>
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<tr>
<td>ENK</td>
<td>enkephalin</td>
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<td>FISH</td>
<td>fluorescent in situ hybridization histochemistry</td>
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<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>NLS</td>
<td>N-lauroylsarcosine</td>
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<td>PI</td>
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<td>PPE</td>
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Introduction

Opioid peptides, which are derived from distinct precursors – preproenkephalin (PPE), preprodynorphin and preproopiomelanocortin – have a wide distribution in the vertebrate central nervous system [Finley et al., 1981]. Enkephalin (ENK), as one of the important opioid peptides, is involved in the inhibitory modulation of the nociceptive information transmitted from the peripheral regions of the body to the superficial laminae of the spinal dorsal horn [Basbaum and Fields, 1984]. However, ENK has been proposed to play different roles during individual development stages. In early development, it influences central nervous system developmental events, such as cell proliferation, migration and differentiation [Hammer and Hauser, 1992]. While, during later development and adulthood, it acts on opioid receptors as classical neurotransmitters [Basbaum and Fields, 1984]. To better understand the role of ENK, it is necessary to know the evolution of the population of ENKergic neurons during the course of development. A wealth of data pointed out that in adulthood, spinal ENKergic neurons exist mainly in the superficial laminae [Bennett et al., 1982; Huang et al., 2008a] and play an important role in the transmission and modulation of nociceptive information [Basbaum and Fields, 1984]. However, developmental studies of ENKergic neurons in the mouse spinal cord are scant.

Immunohistochemistry and in situ hybridization studies have reported the presence of ENK peptides and PPE messenger RNA (mRNA) in the avian telencephalon [Casini et al., 1995], with a general pattern of distribution that was consistent with their distribution in the telencephalon of other vertebrate species [de Lanerolle et al., 1981; Casini et al., 1995]. The ontogeny of the embryonic ENKergic system has only been partially described in the spinal cord of the rat and chick by using indirect immuno-
antisense single-strand riboprobes with digoxigenin RNA labeling mix (Roche Diagnostics, Basel, Switzerland) and in vitro transcription kit (MEGAscrip T7 and SP6 kits; Ambion, Austin, Tex., USA). Six adult PPE-GFP transgenic mice were perfused transcardially with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde. Lumbar segments 3–5 of the spinal cord were obtained and postfixed with the same fixative for 24 h, placed in diethylpyrocarbonate-treated 30% (weight/volume, w/v) sucrose solution in 0.05 M phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C and cut into 25-μm-thick sections on a freezing microscope. Free-floating sections were acetylated at room temperature (RT) for 10 min by gentle shaking in freshly prepared 0.25% (volume/volume, v/v) acetic anhydride in 0.1 M triethanolamine. After a rinse, the sections were preincubated for 1 h at 60°C with a hybridization buffer, which was composed of 5 × saline-sodium citrate (SSC), 2% (w/v) blocking reagent (Roche Diagnostics), 50% (v/v) formamide, 0.1% (w/v) N-lauroylsarcosine (NLS) and 0.1% (w/v) sodium lauryl sulfate. The sections were then hybridized for 18 h at 60°C with 1 μg/ml sense or antisense digoxigenin-labeled PPE riboprobe in the hybridization buffer. After two washes at 60°C in 50% (v/v) formamide, 2 × SSC and 0.1% (w/v) NLS for 20 min, hybridized sections were treated for 30 min at 37°C with 10 units/μl RNase H (Toyobo, Osaka, Japan) in 10 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid and 0.5 M NaCl. The sections were washed at RT twice for 10 min in 2 × SSC and 0.1% (w/v) NLS, and then twice for 10 min in 0.2 × SSC and 0.1% (w/v) NLS. The following incubations were performed at RT with 1% (w/v) blocking reagent (Roche Diagnostics) in 0.1 M Tris-HCl (pH 7.5) and 0.9% (w/v) acetic anhydride. After a wash, the hybridized sections were incubated overnight with a mixture of 1:3,000-diluted peroxidase-conjugated anti-digoxigenin antibody Fab fragment (Roche Diagnostics), 1 μg/ml affinity-purified guinea pig antibody to heat-denatured GFP [Nakamura et al., 2008]. The sections were then incubated with TSA (tyramide signal amplification)-biotin (1:100; NEN Life Science Products, Mass., USA) in 1× amplification diluent for 10 min at RT. After washing, sections were incubated with a mixture of 4 μg/ml Alexa Fluor 488-conjugated donkey anti-guinea pig IgG (1:500; Molecular Probes) and Alexa Fluor 594-conjugated streptavidin (Invitrogen) and 2 μg/ml Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:500; Molecular Probes). Sections were washed twice in 0.01 M PBS (pH 7.4) and air dried and cover slipped with a mixture of 50% (v/v) glycerol-AquaMount (FV-1000, Olympus). Control experiments were performed in which the primary or secondary antibody was omitted. No labeling was observed under these conditions.

Image Analysis and Quantification

For fluorescent in situ hybridization histochemistry (FISH) and double immunofluorescence-labeling results, the total number of positive neurons in laminae I–III of the spinal dorsal horn was counted from 6 sections randomly selected from 3 animals. For relative quantitative analysis of developmental GFP expression, the number of GFP-positive somata in the spinal cord was counted on each section. Six sections from 3 animals at each stage were randomly selected for cell counting. The profile counts were corrected by using Abercrombie's formula. Abercrombie's formula for the ratio of the 'real' number to the observed number is T/T + h, where T = section thickness and h = mean diameter of the GFP-positive somata along the axis perpendicular to the plane of the section [Abercrombie, 1946; Guillery, 2002]. The values were expressed as means ± SEM.

Results

Specificity of PPE-GFP Transgenic Mice

To detect the specificity of GFP fluorescence, we performed the double-labeling experiment by a combination of immunofluorescence staining for GFP protein and FISH for PPE mRNA. GFP immunoreactive products in the adult spinal dorsal horn were seen in green in cell somata and some axon terminals or dendritic endings (fig. 1A). PPE mRNA appeared as a red hybridization product in cell cytoplasm (fig. 1B). Immunofluorescence and FISH showed good consistency of the expressions of PPE mRNA and GFP immunoreactivities (fig. 1C). Quantitative analysis indicated that more than 97.5 ± 1.7% of GFP immunoreactive neurons showed...
signals for PPE mRNA in the adult spinal cord and about 98.1 ± 1.1% of PPE-expressing neurons were immunoreactive for GFP. Sense RNA displayed no signals in the spinal cord (data not shown). Thus, the above double-labeling results demonstrated that PPE-GFP transgenic mice showed specific and sufficient expression of GFP at least in the spinal cord.

**GFP Distribution in the Developing Mouse Spinal Cord**

Next, we took advantage of the PPE-GFP transgenic mice and analyzed the distribution of ENKergic neurons from E10.5 to adult spinal cord at cervical and lumbar levels.

By E10.5, no GFP-positive cells were detected regardless of the spinal level considered (fig. 2A). GFP-positive cells started to appear at around E11.5 at cervical level (fig. 2B). These stained somata were located in the ventromedial part of the ventral gray matter and lie preferentially in the vicinity of the ventricular zone (fig. 2B). Most GFP-positive neurons were spindle-shaped with fairly long processes (fig. 2C, D). At E12.5, GFP-positive neurons were detected at all considered levels of the spinal cord, but were more numerous at the cervical than at the lumbar level (fig. 2E, I; fig. 7). Numerous ENKergic neurons were found in ventromedial areas of the gray matter close to the ventricular zone (fig. 2E). At this stage, the characteristics of GFP-positive cells were similar to those at E11.5, and these aspects were confirmed by confocal laser scanning microscopy (fig. 2F, G). However, as by E12.5 stage, the whole dorsal part of the spinal cord was devoid of ENK immunoreactivity at both the cervical (fig. 2E) and the lumbar levels (fig. 2I).

Sections were counterstained with propidium iodide (fig. 2F, H).

At E13, GFP-positive neurons were mainly present in the intermediate zone at the cervical and lumbar levels (fig. 3A, B, F). At this period of development, the intermediate zone contained spindle-shaped or triangular GFP-positive cells with extended processes in various directions (fig. 3B–E, G). However, by E13 stage, the whole dorsal part of the spinal cord was still devoid of GFP immunoreactivity at both the cervical and lumbar levels (fig. 3A, F). The first remarkable evolution within the distribution of ENKergic population occurred at around E13.5 stage. A few GFP-positive cells were first observed at the cervical level of the dorsal gray matter (fig. 3H, I, M). In the intermediate region at E13.5, GFP-positive cells increased and appeared to form streams extending long processes (fig. 3H–M, fig. 7). Several GFP-positive cells were spindle-shaped with processes and oriented horizontally between the ventricular and pial surface (fig. 3–I).

As development proceeds, at E14, GFP-positive cells were detected at all considered levels of the dorsal spinal cord but were more numerous at the cervical than at the lumbar level (fig. 4A, E, F). However, ENKergic neurons were primarily located in the intermediate zone (fig. 4A, F) and some of them were spindle-shaped and had vertical or horizontal processes (fig. 4B–D). At the lumbar level, the intermediate lamina exhibited numerous ENKergic cell bodies while fewer ENKergic cells in the dorsal horn were detected (fig. 4F). Thereafter, ENKergic neurons increased in extent and intensity. At E15, rostral and caudal spinal levels exhibited numerous GFP somata, as illustrated in figure 4G, H, whereas cells

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Fig. 2. E10.5, E11.5 and E12.5 stages of development. A At E10.5, no GFP-positive cell was detected at the cervical level. B First evidence of GFP staining at E11.5. At the cervical level, some ENKergic neurons were clearly detected in the ventromedial part of the gray matter at E11.5. C Higher magnification of the spindle-shaped GFP-positive cell (arrow) in B. D Higher magnification of the spindle-shaped GFP-positive cell in the left ventral part of the spinal cord from a section at E11.5. E Distribution of GFP-positive neurons at the cervical level at E12.5. F, G Enlarged view of the area (asterisk) in E. Note the colocalization of GFP-positive neurons with propidium iodide (red). I Distribution of GFP-positive neurons at the lumbar level at E12.5. H Confocal images of the lumbar spinal cord at E12.5 stained with propidium iodide (PI). Arrows indicate some GFP-positive neurons with long processes. dh = Dorsal horn; vh = ventral horn. Scale bars = 100 μm in A, B, E, H, I; 10 μm in C, D, F, G.

Fig. 3. Distribution of GFP-positive ENKergic neurons at E13 and E13.5. A, F Overview of GFP-positive neurons and propidium iodide at E13 at cervical (A) and lumbar (F) levels. B–E, G Note the spindle-shaped or triangular GFP-positive cells which extended processes in various directions. H Overview of GFP-positive neurons and propidium iodide at E13.5 at the cervical level. I–M Higher magnification of some spindle-shaped GFP-positive cells with long processes, oriented horizontally between the ventricular and pial surface. In this figure, as in the following figures, boxed areas indicate enlargements, with the relative lowercase letters in the boxed areas. L Enlarged view of the area (asterisk) in K. Arrows indicate some GFP-positive neurons with long processes. Arrow-heads indicate the processes of the GFP-positive neurons. Scale bars = 100 μm in F, H; 50 μm in A, B, I; and 10 μm in C–E, G, J–M.
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Fig. 4. Expression of GFP at E14, E15 and E16. A, F Distribution of GFP-positive cells in the spinal cord at E14 at the cervical (A) and lumbar (F) levels. B–E Higher magnification views of the boxed areas in A, G, H At E15, the total number of GFP-positive cells increased in the dorsal horn and the intermediate zone both at the cervical (G) and lumbar (H) levels. I, L At E16, the GFP-positive cells were localized within the whole spinal cord in the future gray matter. J, K Higher magnification views of the boxed areas in I. Some GFP-positive neurons around the ventricle extended long processes (K). Arrows indicate some GFP-positive neurons with long processes. Arrowheads indicate the processes of the GFP-positive neurons. Scale bars = 100 μm in A, F, G, I, L; 50 μm in E, H; 10 μm in B, C, D, J, K.

Fig. 5. Overview of ENKergic immunoreactivity at E17 (A), E18 (B–E), P1 (F–H), P3 (I–K) and P7 (L, M). A, B At E17 and E18, the cervical ENKergic immunoreactivity evolved toward dorsal parts of the spinal cord. C Enlarged view of the box in B. D Distribution of GFP-positive cells in the section of spinal cord parallel to the longitudinal direction at E18. E Enlarged view of the box in D. F At P1, GFP labeling increased in the neuronal population of the dorsal horn. G Enlarged view of the box in F showing a few multipolar cells in the intermediate zone which gave rise to dendrites radiating in all directions. H Compared to the cervical level, GFP-positive neurons were less at the lumbar level. I At the cervical level, GFP-positive neurons were primarily located in the superficial layers of the dorsal horn at P3. J Enlarged view of the box in I. K At the lumbar level, more GFP-positive neurons were distributed in the intermediate zone. L, M View at low magnification of GFP-positive neurons at P7. Arrows indicate some GFP-positive neurons with long processes. Scale bars = 100 μm in A, B, D, F, H, I, K–M; 50 μm in C, E, J; 10 μm in G.
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immunoreactive for GFP were less numerous at the lumbar level (fig. 4G, H, fig. 7). A similar pattern was observed at E14 and the total number of GFP-positive cells increased in the dorsal horn and in the intermediate laminae (compare fig. 4G, H with 3A, F). At this stage of development, GFP-positive cells occupied almost the entire area of the section, invading the previously GFP-negative region in the spinal dorsal horn. GFP-positive neurons in the intermediate lamina appeared to be more intensely labeled (fig. 4G, H). At E16, the GFP-positive cells were localized within the whole spinal cord (fig. 4I, L). A few spindle-shaped GFP-positive cells were also detected in the intermediate area (fig. 4J). At this stage, we found some GFP-positive cells surrounding the cen-

Fig. 6. Distribution pattern of ENKergic neurons at P14, P21 and adult spinal cord. A At P14, the majority of immunoreactive cell bodies were found in the superficial layers of the dorsal horn, although some GFP-positive neurons were occasionally seen in the deeper layers of the dorsal horn. B At P21, GFP immunoreactivity appeared as a dense band within lamina II of the dorsal horn with moderate intensities within lamina I and III. The distribution pattern at this stage is similar to that of the adult (D). C Enlarged view of the box in B. Note the presence of some ENKergic neurons with very long processes in the deep layers of the dorsal horn at P21. E, F Higher magnification of the boxed areas in D. Arrows indicate some GFP-positive neurons with long processes. Arrowheads indicate the processes of the GFP-positive neurons. Scale bars = 100 μm in A, B, D; 10 μm in C, E, F.
tral canal and extending processes into the cavity of the central canal (fig. 4K). At E17 and E18, the GFP immunoreactivity evolved toward the dorsal part of the spinal cord. The dorsal horn exhibited numerous GFP-positive cell bodies (fig. 5A–C). A large amount of GFP-positive perikarya was found in the dorsal horn at E18 (fig. 5C, D). As illustrated in the sections of spinal cord parallel to the longitudinal direction, the ventral part of the spinal cord exhibited sparse GFP-positive cell bodies (fig. 5D, E).

The general distribution of GFP immunoreactivity at P1 was not fundamentally changed as compared with E18, although the number and intensity of immunofluorescent cells increased (compare fig. 5F and B). A few multipolar cells in the intermediate zone gave rise to dendrites radiating in all directions (fig. 5F, G). The staining intensity of GFP-positive cells became strong and their dendrites showed relatively developed arborization (fig. 5G). The distribution at the lumbar level (fig. 5H) was similar to that observed at the cervical level (fig. 5F), while with a lower density.

At P3 and P7 and at both levels, we detected (1) some GFP-positive cells in the intermediate zone and around the central canal, (2) a high number of stained cells in the dorsal horn and (3) a few GFP immunoreactive cells in the ventral horn of the spinal cord (fig. 5I–M). In the intermediate zone, several GFP-positive cells displayed long processes (fig. 5J). Quantitative analysis showed that GFP-positive neurons peaked in number at P7 at the cervical level (fig. 7A), while the number of GFP-positive neurons reached a peak at P3 at the lumbar level (fig. 7B). Thereafter, there was a gradual decrease in the number of GFP-positive cells (fig. 7).

Two weeks after birth, the majority of GFP immunoreactive cell bodies and puncta were found in the superficial layers of the dorsal horn, although some GFP-positive neurons were occasionally seen in the deeper layers of the dorsal horn (fig. 6A). By P21, GFP immunoreactivity appeared as a dense band within Rexed’s lamina II of the dorsal horn with moderate intensities within laminae I and III (fig. 6B). In the deep layers of the dorsal horn, we found some ENKergic neurons with very long processes. These processes increased in length up to this date, and were around 2–5 times the size of the cell body (fig. 6C). The general appearance of GFP immunoreactivity at this stage showed an adult-like distribution (fig. 6B, D). In the superficial layer of the adult spinal dorsal horn, ENKergic neurons were rounded or oval (fig. 6E). Some GFP-positive neurons in the deep layer extended long processes (fig. 6F).

The relative quantitative analysis of GFP-positive neurons in the mouse spinal cord during the development is shown in figure 7. Finally, the quantitative analysis indicated that at both spinal levels, a rostrocaudal delay in the appearance of the ENK immunoreactivity exists. ENKergic neurons in the spinal cord appear to undergo develop-
Fig. 8. Double labeling of GFP with GABA in the spinal cord of PPE-GFP transgenic mice at E16 (A–F) and P3 (G–I). In each case, GFP (green) is shown on the left, GABA (red) in the middle, and a merged image on the right. D–F Higher magnification views of the boxed area in C. Arrows indicate some double-labeled neurons. Scale bars = 50 μm in C (applies to A–C); 20 μm in F (applies to D–I).
ment in the form of upregulation, downregulation or distribution changes (fig. 7).

Analysis of Colocalization of GFP Expression and GABA Immunoreactivity

At E16, colocalization of GFP/GABA was mainly observed in the dorsal horn (fig. 8A–F). At P3, the localization pattern of double-stained perikarya remained quite similar to the pattern observed at E16 (fig. 8G–I). Finally, to improve our understanding of the dynamic of the GFP/GABA colocalization evolution during the development period, we analyzed the ratio of GABA immunoreactivity, GFP, and GFP/GABA somata in the spinal cord. Double-stained cells represented about one-third of the total GABA cell population (34.9 ± 3.5% at E16; 32.4 ± 3.7% at P3) and corresponded to nearly half of the ENKergic neurons (42.4 ± 2.4% at E16; 44.1 ± 2.9% at P3).

Discussion

Specificity of GFP Expression in ENKergic Neurons

The validity of GFP in the PPE-GFP transgenic mouse as a marker for ENKergic neurons has been confirmed [Koshimizu et al., 2008; Huang et al., 2010]. In this study, by using FISH and immunofluorescent histology double staining, we observed that PPE mRNA signals in the spinal cord showed GFP immunoreactivity with a colocalization rate of >95%. The above double-staining experiments in the spinal cord and previous study in other brain regions in the PPE-GFP transgenic mice further confirmed that GFP-positive neurons were ENKergic and thus confirmed the reliability of the PPE-GFP transgenic mouse in our studies on the developmental pattern and characteristics of spinal ENKergic neurons.

Comparative Analysis of the Spinal ENKergic Neuron Development

Although it is well established that ENK plays a crucial role during the development of neuronal tissues [Hammer and Hauser, 1992], the ontogenic establishment of ENKergic neurons in the mouse spinal cord has never been fully documented. Previous studies showed that Met-ENK immunoreactivity was first detected in the chick spinal cord at embryonic stages 29–30 (incubation day 6). From stage 40 (incubation day 13 or 14), much more Met-ENK immunoreactivity was observed in several spinal areas. Such a distribution of Met-ENK immunoreactivity remained visible at later embryonic stages, but labeled cells gradually decreased in number and disappeared after hatching [Du and Dubois, 1988]. High-performance liquid chromatography showed that the level of total radioimmunoassayable Met-ENK in the lumbar spinal cord of the chicken embryo increased more than 1,000-fold between day 4.5 and day 18 [Maderdrut et al., 1986]. By using in situ hybridization, two recent studies observed the spatial and temporal expression patterns of some peptide genes, including Penk.1. Their data indicated that Penk1-expressing cells emerged in the ventral spinal cord at E14.5 and then expanded to the dorsal horn from E16.5 to P0. From P0 and on, Penk1-expressing cells were scattered throughout the spinal cord [Xu et al., 2008; Huang et al., 2008b]. However, our data showed that GFP-positive ENKergic neurons first appeared in the ventral region of the spinal cord at E11.5. The first labeled GFP-positive cells were observed in the cervical dorsal gray matter at E13.5, though ENKergic neurons were primarily located in the intermediate lamina. Thereafter, GFP-positive neurons increased progressively in number and extended from the ventral to the dorsal regions. Between E11.5 and E18, a rostrocaudal and ventrodorsal evolution of the ENKergic population occurs, leading to a redistribution of ENK immunoreactivity, from ventrally early in development to dorsally. When compared with embryonic stages, GFP-positive neurons became primarily restricted to the dorsal horn during the postnatal period. We suspect that the difference in these two sets of results might be related to the different research methods, particularly the use of the PPE-GFP transgenic mouse, which most likely accounts for the discrepancy.

Parallels between ENK and GABA Immunoreactivity during Development

Our previous study examined the temporal and spatial developmental changes of GABAergic neurons at the cervical level of the spinal cord by using glutamic acid decarboxylase-67-GFP knock-in mouse [Huang et al., 2007], GFP-positive GABAergic neurons appeared at E11.5 in the ventral region of the spinal cord and became abundant in the future gray matter at E12. Although there was a vast difference in the expression pattern of ENKergic and GABAergic neurons at early developmental stages, the evolution was similar at late developmental and postnatal stages. The majority of ENKergic and GABAergic neurons were found in the superficial layers of the dorsal horn, and the ventral part of the spinal cord exhibited only sparse cell bodies at late developmental and postnatal stages. In view of our data indicating that ENKergic and GABAergic neurons were detected in the same dorsal
area, we performed double staining to reveal possible colocalization of GABA and ENK in the developing spinal cord. Double GFP/GABA staining revealed a significant population of neurons expressing both of them. Interestingly, this proportion remained stable during the course of development. From a functional point of view, ENK together with GABA may exert a trophic influence on secondary events of axonal growth and synapse formation.

Moreover, as studies of the development or electrophysiological characteristics of ENKergic neurons progress, the necessity for visualizing ENKergic neurons in living tissues becomes more urgent. PPE-GFP transgenic mice presented a major advantage in that ENKergic neurons could be easily visualized by GFP. Compared with those previously observed with ENK antibody labeling, this newly generated mouse line can be a reliable tool for the study of specific spinal ENKergic neuronal populations.

In summary, the present study examined the expression pattern of ENKergic neurons and its colocalization with GABA in the developing spinal cord of the PPE-GFP transgenic mouse. The generation timetable and migration of ENKergic neurons still remain to be elucidated and we will study further to clarify the molecular mechanisms that control the fate of the ENKergic neurons. Identifying core transcription factors that coordinate a large set of transmitter phenotypes may help to generate specific types of neurons in vitro for future functional and therapeutic studies.

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