Preliminary Studies on GABA-immunoreactive Neurons in the Rat Trigeminal Ganglion

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– Received for Publication, February 22, 2003 –

Key Words: GABA, Immunocytochemistry, Trigeminal ganglion, Primary sensory neuron, Rat

Summary: GABA-immunoreactive (ir) primary sensory neurons have been reported in many studies. However, the role of GABA in the primary sensory neurons and their targets is quite open to question. The present study aimed to examine the GABA-ir neurons in the rat trigeminal ganglion (TG), for the first step of functional study on them. Some neurons in the TG showed GABA-ir, which were ranged from large to small size. The total number of examined TG neurons from 6 ganglia was 2,531, of which 462 neurons (18.3%) showed GABA-ir. The large subpopulation of GABA-ir TG neurons is likely to involve in nerve-muscle functions, whereas medium and small subpopulations might participate in cutaneous nociceptive sensory function. The present findings demonstrated a considerable number of sensory neurons containing GABA in the rat TG. Functional studies on GABA-ir neurons in the TG would be an interesting and important issues in future studies. The next aim of our study is to examine the size distribution of GABAergic neurons and the coexistence with other neurotransmitters in the rat TG.

GABA (γ-aminobutyric acid) is well known as one of the main inhibitory neuro-transmitters or neuromodulators in the mammalian central nervous system (CNS). The regional and wide distribution of GABA was reported in the brain of some mammals, i.e., substantia nigra, globus pallidus and hypothalamus (Okada et al., 1971). Moreover, there have been many evidences that GABA is present in the superficial dorsal horn of the rat and the mouse spinal cord (Magoul et al., 1987; Todd et al., 1989; Hiura et al., 1991; Willis et al., 1991).

GABA-immunoreactive (ir) primary sensory neurons have been reported in many studies (Roy et al., 1991; Szabat et al., 1992; Zhou et al., 1997; Stoyanova et al., 1998). Roy et al. (1991) provided an evidence that GABA is contained within cells of the chick dorsal root ganglia (DRG). Since immunoreactivity was also found within a few large myelinated and frequently unmyelinated axons, it was suggested that GABA is transported to the peripheral and/or central nerve endings (Roy et al., 1991). Szabat et al. (1992) also reported the presence of GABA in a population of sensory neurons in DRG, trigeminal ganglion (TG), and nodose ganglia of the rat by using monoclonal anti-GABA. Thus, the presence of GABA in the peripheral nervous system (primary sensory neurons) appears to be established as well as in the CNS. But the quantification of GABA-ir neurons in TG have been very few until now (Stoyanova et al., 1998).

A few nerve endings in the Herbst corpuscle of subcutaneous connective tissue and some nerve endings in the muscle spindles of skeletal muscle express GABA-ir (Roy et al., 1991). Zhou et al. (1997) investigated the influence of peripheral target tissues, i.e., skeletal muscle, on the expression of GABA in vitro and in vivo. They postulated that GABA in the chick DRG neurons participates in the regulation of skeletal muscle tension by innervating muscle spindle. However, the role of GABA in the primary sensory neurons and their targets is quite open to question. The present study aimed to quantitatively examine the GABA-
ir neurons in the rat TG, for the first step of functional study on them.

Materials and Methods

Three adult male Sprague-Dawley rats (330–550 g) were anesthetized with sodium pentobarbital (Nembutal; 50 mg/kg) and perfused through the left ventricle of the heart with 100 ml of 0.9% NaCl, followed by 400 ml of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium phosphate buffered saline (PBS, pH 7.3). After perfusion both sides of the TG and the medulla oblongata (MO) were removed, and they were refixed in the same fixative for 2 h at 4°C.

Immunohistochemical procedure

The fixed tissues were cryoprotected in 30% sucrose in PBS at 4°C for overnight. The frozen tissues were cut at 20 μm with a cryostat and floated into the PBS. To diminish nonspecific staining, unsaturated double bonds of the glutaraldehyde were blocked with 0.5% sodium borohydride. Floating sections were incubated in a pretreatment solution (3% normal bovine serum and 0.3% Triton X-100 in 0.1 M phosphate buffer: PB) at room temperature (RT), followed by the monoclonal mouse anti-GABA (Chemicon International Inc., 1:500) at 4°C for overnight. Subsequently, the sections were washed in PB and incubated in the fluorescein isothiocyanate (FITC)-coupled goat anti-mouse IgG (Cappel Laboratories, 1:100) at RT for 1 h. After washing the specimens in PB, they were rinsed in 4 mM sodium carbonate for 10 min to reduce non-specific background staining. Finally, the specimens washed in PB were mounted in a glycerin-paraphenylendiamine mixture (0.1% para-phenylendiamine and 10% PBS in glycerin) and observed under a fluorescence microscope.

The specificity of the immunocytochemical reactions was confirmed by the followings: i) The positive control: the MO was reacted with the monoclonal anti-GABA, because immunohistochemical studies demonstrated GABA-ir in the superficial dorsal horn (laminae I–II) of MO (Magoul et al., 1987; Todd et al., 1989). ii) The negative control: the primary antibody to GABA was pre-absorbed with 50 mM GABA before the reaction with the antibody.

Quantification of the number of GABA-ir neurons

The photographs of entire TG neurons in a section were taken on color reversal film (Fujichrome Provia 400) with a fluorescence microscope. The developed film was inserted into 35-mm slide scanner (POLASCAN 4000; Polaroid corporation). GABA-ir and non-ir neurons on the film were scanned, and their images were changed to digital images. Then, the digital image was manipulated with image manipulative software (Adobe Photoshop 5.0), picking up TG neurons from the other tissues. Afterwards, the manipulated image was analyzed with image analysis software (NIH Image 1.61). The degree of immunofluorescence for GABA-ir varied from weak and intense in density. Then, the density gradient of green fluorescence within TG neurons showing GABA-ir was measured with the software. The density gradient was changed to the numeral exhibiting brightness. Many numerals (figures) indicate a weak immunofluorescence. The density of the most weak GABA-ir TG neurons was arbitrarily decided as the value of 108 (Fig. 4). Therefore, the density value representing GABA-ir neurons was estimated as less than 108. The TG neurons were automatically classified into GABA-ir neurons and non-ir ones and the number of them were similarly calculated by the software.

Results and Discussion

Previous studies (Magoul et al., 1987; Todd et al., 1989; Hiura et al., 1991) demonstrated the presence of GABA in cell bodies and axons in the superficial dorsal horn (laminae I–II). The superficial dorsal horns of MO were GABA-ir in the positive control (Fig. 1), whereas those of MO were not reactive in the negative control (Fig. 2). Accordingly, GABA-ir in the superficial dorsal horn of MO was thought to be accurate as the positive control and the specificity of the monoclonal anti-GABA was believed to be highly verified in this study.

Some neurons in the TG showed GABA-ir (Fig. 3), but the neurons were never reactive in the TG of negative control (Fig. 5). GABA-ir primary sensory neurons were ranged from large to small size (Fig. 3). The total number of examined TG neurons from 6 ganglia was 2,531, of which 462 neurons (18.3%) showed GABA-ir (Fig. 6). Wolff et al. (1989) documented the GABA-ir neurons composing of a subpopulation (5%) of ganglion cells in the superior cervical ganglion (SCG) of rats, and their relative small sizes. Roy et al. (1991) reported that the percentage of GABA-ir neurons was 1.4% in the chick DRG at embryonic day 12. The immunostained neurons gradually increased along with development and their percentage reached 7.3% after hatching. In addition, Stoyanova et al. (1998) showed that the ratio of GABA-ir neurons in the TG of cats was 22.7%, and these were equally dis-
tributed from large- to small-sizes. Although, the present finding of 18.3% of GABA-ir neurons in the rat TG was different from the chick DRG (Szabat et al., 1992) and the rat SCG (Wolff et al., 1989), it is very similar to the cat TG (Stoyanova et al., 1998). The cause of difference between the present results and Roy’s (Roy et al., 1991) may be due to different populations of GABA-ir neurons between mammalia and birds (species difference). Moreover, the different results between ours and Wolff’s (Wolff et al., 1989) implicate a different GABA-ir population between sensory and sympathetic ganglia (characteristic difference).

Zhou et al. (1997) demonstrated that the initiation of GABA expression in sensory neurons requires an interaction with skeletal muscle but not with the spinal cord. Retrograde labeling studies indicated that majority of large subpopulated neurons of TG correspond to muscle afferents and medium and small subpopulated neurons almost correspond to cutaneous nociceptive afferents, respectively (Honmou et al., 1994; Lee et al., 1986; Peyronnard et al., 1982). Thus, large subpopulation of GABA-ir TG neurons is likely to involve in nerve-muscle functions, whereas medium and small subpopulations might participate in cutaneous nociceptive sensory function (Honmou et al., 1994; Lee et al., 1986; Peyronnard et al., 1982). The present findings demonstrated a considerable number of sensory neurons containing GABA in the rat TG. Functional studies on GABA-ir neurons in the TG would be an interesting and important issues in future studies. The next aim of our study is to examine the size distribution of GABAergic neurons and the coexistence with other neurotransmitters in the rat TG.

References

Fig. 1. Superficial dorsal horn (SDH) of the medulla oblongata showing GABA-ir. DF: dorsal funiculus, DH: dorsal horn, L: lateral side. ×46.

Fig. 2. No immunoreactivity can be seen in the superficial dorsal horn of the rat medulla oblongata in the negative control, pre-absorbed with GABA before reaction to the primary antibody. DF: dorsal funiculus, DH: dorsal horn. L: lateral side. ×46.
Fig. 3. GABA-ir neurons (yellow arrows) and non-ir ones (red arrows) in the trigeminal ganglion. Note that the sizes are from large- to small-size. GABA-ir neuronal fibers (blue arrows) are seen among the neurons. ×185.
Fig. 4. An enlargement of the square part of Fig. 3. The arabic numerals on the neurons represent the density values. The density values less than 108 were estimated as the GABA-ir neuron (yellow numerals), and the density values greater than 109 as the non-GABA-ir (red numerals). The validity for the above classification is reasonable from the photographic images. \( \times370 \).

Fig. 5. No-immunoreactive neurons (red arrows, dark configurations) for anti-GABA can be seen in the trigeminal ganglion of the negative control. \( \times46 \).
Fig. 6. Quantitative estimation of GABA-ir neurons in the rat trigeminal ganglion. About 20% of the neurons are GABA-ir.