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Synaptic relationships between induced neuropeptide Y-like immunoreactive terminals and cuneothalamic projection neurons in the rat cuneate nucleus following median nerve transection

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ABSTRACT

Previous studies have demonstrated that following complete median nerve transection (CMNT), neuropeptide Y-like immunoreactive (NPY-LI) fibers were dramatically increased and predominantly expressed in the ventral portion of the middle cuneate nucleus (CN), reaching maximum numbers at four weeks. Ultrastructurally, NPY-LI terminals made axodendritic synapses, but the postsynaptic elements are unknown. In the present study, using retrograde tract-tracing of wheat germ agglutinin conjugated with horseradish peroxidase (WGA-HRP) and NPY immunocytochemistry we examined the synaptic relationships between cuneothalamic projection neurons (CTNs) and NPY-LI terminals in the rat CN following CMNT. The injury-induced NPY-LI fibers were distributed throughout the rostrocaudal extent of the CN. Further, the greatest number of HRP-labeled CTNs was observed in the ventral portion of the middle CN. Ultrastructurally, the NPY-LI terminals made asymmetric axodendritic synaptic contact with the HRP-labeling CTN dendrites. These data suggest that injury-induced NPY may modulate the excitability of CTNs, and thus, play a role in the transmission of neuropathic sensation following median nerve injury.

1. Introduction

The cuneate nucleus (CN) receives information related to innocuous tactile and proprioceptive sensations from the forelimb areas through primary Aβ afferent fibers, and relays the information to the contralateral thalamus (Andersen et al., 1964; Berkley et al., 1986; Lue et al., 1994, 1996; Day et al., 2001). Previous studies have shown that sciatic nerve injury induced profound changes in neuropeptide expression, including calcitonin gene-related peptide (CGRP) (Miki et al., 1997; Ma and Bisby, 1999), galanin (Zhang et al., 1993; Ma and Bisby, 1997), neuropeptide Y (NPY) (Zhang et al., 1993; Ohara et al., 1994; Ma and Bisby, 1998), and substance P (SP) (Noguchi et al., 1995) in the gracile nucleus. However, after complete median nerve transection (CMNT) only NPY was significantly induced in the injured side CN (Tsai et al., 2004). Ultrastructurally, NPY-like immunoreactive (NPY-LI) terminals made axodendritic synapses (Tsai et al., 2004); however, the nature of the postsynaptic elements is unknown.

Our previous study demonstrated that median nerve injury along with electrical stimulation caused a marked increase of numbers of c-Fos-LI cells in the CN (Lue et al., 2002). After CMNT, the distribution and expression time course of the induced NPY-LI fibers in the injured side CN were similar to those of the c-Fos-LI cells. Further, according to previous studies approximately 70% of c-Fos-LI cells in the CN were cuneothalamic projection neurons (CTNs) (Day et al., 2001; Lue et al., 2002). Based on these data, we postulated that the postsynaptic elements are derived from CTNs. Thus, the aim of the present study was to investigate the existence of direct synaptic contact between injury-induced NPY-LI terminals and CTNs.

2. Materials and methods

Adult albino male rats (Wistar strain) weighing between 180 and 250 g were used. Under anesthesia achieved by an intraperitoneal injection of 7% chloral

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hydrate (0.45 ml/100 g body weight), the right median nerve was cleaned of its surrounding tissue at the level of the elbow immediately proximal to where it entered between the two heads of the pronator teres muscle. A tight ligature (5.0 silk) was made around the nerve, and an approximate 2 mm segment of the distal end was removed to prevent possible regeneration (Lue et al., 2002; Tsai et al., 2004, 2007). After the operation, the skin was sutured and the animals were allowed to survive for four weeks.

Animals with ($n = 7$) or without ($n = 3$) right median nerve transection were given stereotaxic injection of WGA-HRP into the left thalamus under re-anesthesia. For this, a small part of the left parietal bone was removed with a dental drill. The underlying dura was then exposed and incised. Using a 10 μl Hamilton microsyringe mounted on an electrode carrier, 0.25 μl of 2% WGA-HRP (Sigma, St. Louis, MO, USA) was injected into three sites of the left ventrobasal nucleus of the thalamus (coordinates: AP, ML, H: 4, 3, −0.5; 4, 3, 0; 4, 2.5, −1; System A) (Pellegrino et al., 1979; Day et al., 2001; Lue et al., 2002). The animals were allowed to survive for 48–72 h before being sacrificed by perfusion. Three naive rats were also sacrificed and used as normal controls. The present experiments and animal care were approved by the National Science Council Committee as well as the Animal Center Committee, College of Medicine, National Taiwan University, Taiwan.

Experimental animals were further divided into two groups. Group 1 was prepared for light microscopy, and included rats with CMNT along with WGA-HRP injection ($n = 3$), injection of WGA-HRP without CMNT ($n = 3$), and normal control rats ($n = 3$). The animals were re-anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Tissue blocks of the medulla containing the CN were removed and stored in PB containing 30% sucrose overnight, and cut transversely with a cryostat (Bright, Cambridge, England) at 30 μm thickness. The floating medulla sections, except for those of normal control rats, were collected and processed for HRP-tetramethylbenzidine (HRP-TMB) enzymatic histochemistry, and then enhanced with diaminobenzidine (DAB; Sigma) and cobalt acetate (Co) (Lue et al., 1994, 2000). Next, the sections, including those of normal control rats, were treated with 0.5% H_2O_2, blocked with 5% normal goat serum (NGS; GibcoBRL, NY, USA) in PB for 2 h, and incubated in rabbit polyclonal anti-NPY antibody (1:2000; DiaSorin, MN, USA) for 16 h at 4 °C. The primary antibody was diluted in 0.01 M phosphate buffer saline (PBS, pH 7.4) containing 0.2% Triton X-100 and 5% NGS. After rinsing with PBS, the sections were incubated in biotinylated goat anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) for 2 h, and processed with avidin-biotin-HRP complex (ABC; ABC Kit, Vector) for 1 h at room temperature. The peroxidase activity was subsequently visualized by treating with Vector® SG Substrate Kit (Vector). Finally, the sections were mounted onto gelatin-coated slides and examined with a light microscope (Zeiss, Axiophot, Germany). Immunolabeling was absent in all sections incubated in normal rabbit serum without anti-NPY antibody or with the omission of the same antibody as control.

For quantitative analysis, tissue sections were collected from the entire rostrocaudal extent of the CN. The nucleus at the level of about 0.3–0.7 mm caudal to obex was defined as the middle region (Maslany et al., 1991; Day et al., 2001; Lue et al., 2002; Tsai et al., 2004). In the present study, the number of sections collected from the caudal, middle, and rostral regions was 19, 7, and 5, respectively. To determine the changes in NPY-LI fibers and HRP-labeled CTNs in the three subregions of the CN, sections were examined with a Zeiss light microscope and images were captured with a digital camera (Nikon, Xillix MicroImager 1400, Japan) at a magnification of 100×. Pictures were processed and evaluated with a computer-based image analysis system (MGDS) and Image Pro-Plus software (Media Cybernetics, MD, USA). The area occupied by NPY-LI fibers and the area of outlined CN was measured (Tsai et al., 2004). The former divided by the latter was defined as the percentage of area occupied by NPY-LI fibers in the ipsilateral CN. The density of HRP-labeled CTNs in the three subregions of the CN was defined as the number of HRP-labeled CTNs divided by the number of respective tissue sections. The mean percentage of area occupied by NPY-LI fibers and the density of HRP-labeled CTNs in the three subregions were calculated and compared statistically with one-way ANOVA.

Group 2 was prepared for electron microscopy and included rats at four weeks after CMNT with WGA-HRP injection ($n = 4$). The animals were re-anesthetized and perfused with 3% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M PB for 30 min. The brainstems including the CN were removed and cut into 50 μm-thick slices with a vibratome (TPI, Series 1000, Portland, USA). The sections were processed for the HRP-TMB-DAB-Co protocol and NPY immunocytochemistry as described above, except that following ABC treatment the sections were reacted with DAB to reveal the peroxidase activity. Subsequently, the cuneate region was trimmed, osmicated, and dehydrated in ascending ethanol, and infiltrated with propylene oxide before being embedded in Araldite mixture. Ultrathin sections were cut on Reichert-Jung Ultracut E Ultramicrotome and collected onto grids. The sections, without staining with uranyl acetate and lead citrate, were viewed under a JEOL JEM-2000 electron microscope (JEOL, Tokyo, Japan).

3. Results

The CTNs and NPY-LI fibers in the CN were identified with WGA-HRP retrograde labeling and NPY immunocytochemistry, respectively. NPY-LI fibers were absent or hardly detected in the CN of the naive rats (Fig. 1A, C and E), but numerous NPY-LI fibers were detected throughout the rostrocaudal extent of the ipsilateral CN at four weeks after CMNT (Fig. 1B, D and F). In the caudal region of the CN only a few NPY-LI fibers were identified, which were localized in the laterodorsal portion (Fig. 1B). When traced rostrally the majority of NPY-LI fibers were distributed in the ventral portion of the middle level of the nucleus (Fig. 1D). At the rostral level of the nucleus the density of the NPY-LI fibers decreased, and the fibers were located in the mid-ventral area of this region (Fig. 1F). The quantitative analysis of the three regions revealed that the percentage of the area occupied by NPY-LI fibers in the middle CN was 48.2 ± 1.3%, which was significantly higher than that in rostral and caudal regions (34.5 ± 2.1 and 19.3 ± 0.7%, respectively; both $P < 0.05$). Using stereotaxic injection of WGA-HRP into the contralateral thalamus of the rats without CMNT, the labeled CTNs were recognized by their contents of HRP-TMB-DAB crystal reaction products, and numerous HRP-labeled CTNs were found throughout the rostrocaudal extent of the CN (Fig. 2A, C and E). However, none of these neurons expressed NPY immunoreactivity (Fig. 2A, C and E). Quantitative analysis showed that the density of HRP-labeled CTNs in the middle CN was 95.6 ± 4.1 cells/section, which was significantly higher than that in rostral and caudal regions (57.7 ± 3.0 and 34.3 ± 2.8 cells/section, respectively; both $P < 0.05$). The rats with CMNT were also subjected to stereotaxic injection of WGA-HRP into the contralateral thalamus, and numerous HRP-labeled CTNs intermingled with the injury-induced NPY-LI fibers throughout the rostrocaudal extent of the CN (Fig. 2B, D and F). Moreover, the majority of NPY-LI fibers and the largest number of HRP-labeled CTNs were identified in the ventral portion of the middle CN (Fig. 2D).

Ultrastructural observations were based on material taken from the ventral portion of CN at the middle level. The labeled CTNs were identified by the needle- or bar-shaped HRP-TMB-DAB crystals in the soma, proximal dendrites, and distal dendrites, but not in axonal terminals (Figs. 3 and 4). In contrast, the homogeneous electron-dense DAB immunoperoxidase reaction products representing NPY immunoreactivity were only localized in axon terminals ($n = 311$) (Figs. 3 and 4). Furthermore, the injury-induced NPY immunoreactivity was not present in glial cells. Of note, the immunoperoxidase-labeled NPY-LI terminals made asymmetric axodendritic synaptic contacts with HRP-labeled CTNs ($n = 44$) (Figs. 3 and 4). At least two types of synaptic relationships between NPY-LI terminals and HRP-labeled CTN dendrites were identified randomly: (1) a single NPY-LI terminal was presynaptic to an HRP-labeled CTN dendrite (Fig. 3) and (2) a centrally located HRP-labeled CTN dendrite was postsynaptic to both an immunoperoxidase-labeled NPY-LI terminal and an unlabeled axon terminal (Fig. 4).

4. Discussion

Following peripheral nerve injury, an increase in NPY-LI fibers in the dorsal column nuclei has been previously reported (Zhang et al., 1993; Ohara et al., 1994; Ma and Bisby, 1998, 1999; Tsai et al., 2004, 2007). The results of the present study support those reports, and by using retrograde transport of WGA-HRP and NPY immunocytochemistry, demonstrate the direct synaptic contacts between the NPY-LI terminals and CTNs in the CN at four weeks after the median nerve transection.

Numerous HRP-labeled CTNs were observed in the CN after the retrograde transport of WGA-HRP. Previous studies demonstrated that approximately 74% of cuneate neurons in the CN were CTNs, and the HRP-labeled CTNs were characterized ultrastructurally by needle- or bar-shaped reaction products in the soma and their