Demonstration of Thyrotropin-Releasing Hormone Immunoreactivity in Neurons of the Mouse Spinal Dorsal Horn

J. A. Coffield,* V. Miletic,* E. Zimmermann,† M. J. Hoffert,† and B. R. Brooks‡

*Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706, †Neurology Department, University of Wisconsin Medical School, Madison, Wisconsin 53792, and ‡Neurology Department, University of Wisconsin Medical School, and Neurology Service, William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin 53792

Thyrotropin-releasing hormone-like immunoreactivity was demonstrated in neurons within the superficial laminae (I, II, III) of the mouse spinal dorsal horn by light-microscopic peroxidase immunocytochemistry. The immunoreactivity was distributed in a narrow dorsoventral band that enclosed the substantia gelatinosa (lamina II), with a higher concentration along the lamina II/III border. At present, the functional significance of these neurons is unknown. Their existence within the substantia gelatinosa suggests a role in sensory information processing.

Thyrotropin-releasing hormone (TRH) is a neuropeptide originally isolated from bovine and porcine hypothalamic tissue. Its characterization as a hypothalamic releasing hormone is well documented (Vale and Rivier, 1975). Within the last decade, however, numerous studies have revealed that two-thirds of the total CNS content of TRH is contained within extrahypothalamic tissue (Brownstein et al., 1974; Jackson and Reichlin, 1974; Oliver et al., 1974; Winokur and Utiger, 1974). Indirect immunofluorescence and peroxidase–antiperoxidase (PAP) immunocytochemical techniques have been used to localize TRH-like immunoreactivity (TRH-LI) in neuronal cell bodies, processes, and vesicle-containing varicosities (Hökfelt et al., 1975; Johansson and Hökfelt, 1980; Johansson et al., 1980). These immunoreactive profiles have been seen in axoaxonic, axodendritic, and axosomatic synaptic specializations (Shioda and Nakai, 1983), suggesting a role for TRH in synaptic transmission in addition to its established role as a hypothalamic releasing hormone. Iontophoretic application of TRH onto rodent motoneurons indicates that the peptide may more accurately be described as a modulator since it does not evoke neuronal activity *per se,* but rather potentiates glutamate excitation (White, 1983).

Until recently, reports on the distribution of TRH-LI within the spinal cord have described its presence as limited to varicose fibers within the ventral horn in close association with motoneurons (Hökfelt et al., 1975; Johansson and Hökfelt, 1980; Johansson et al., 1981; Kardon et al., 1977). These fibers are

thought to originate in certain brain stem nuclei and to contain 5-HT and/or substance P in addition to TRH (Johansson et al., 1981). Recent autoradiographic studies, however, have revealed that the highest concentration of TRH receptors is within the dorsal, rather than the ventral, horn (Manaker et al., 1985; Mantyh and Hunt, 1985; Sharif and Burt, 1983).

While little is known about a functional role for TRH in the dorsal horn, several other neuropeptides have been localized to this region, and ongoing investigations of their functional significance focus on their involvement in the synaptic transmission of sensory, especially nociceptive, input and its modulation (Hunt et al., 1981). TRH demonstrates no direct analgesic effect (Martin et al., 1977; Nemeroff et al., 1979); however, it has been shown to antagonize neurotensin-induced analgesia (Osbahr et al., 1981). The effects of TRH on opiate analgesia are inconclusive. Intracerebroventricular administration of TRH is reported to potentiate some forms of stress-induced analgesia, i.e., foot-shock analgesia (opiate), and swim analgesia (Butler and Bodnar, 1984; Butler et al., 1984). Intrathecal administration of TRH yields differential effects on morphine analgesia, i.e., inhibiting at high (2.5 µg) and low (0.25 ng) doses, while potentiating at middle doses (2.5 ng) (Watkins et al., 1984).

In order to study the role of TRH in the dorsal horn we have employed the PAP immunocytochemical technique to examine the distribution of TRH within the spinal cord. Here, we provide evidence for the existence of TRH-LI within neurons in the mouse superficial dorsal horn.

Materials and Methods

Immunocytochemical experiments were conducted on 21 mouse spinal cords (NIH:N strain). Under sodium pentobarbital anesthesia, the mice were heparinized and perfused transcardially with physiological saline (25°C) followed by 4% paraformaldehyde containing 0.05–0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C). The spinal cord and brain stem were removed intact and stored in fresh fixative for an additional 2–4 hr at 4°C. Fifty micrometer transverse or parasagittal sections of the cervical, thoracic, lumbar, and sacral spinal segments were cut with a vibratome. The sections were rinsed in 0.1 M PBS. To identify areas of TRH localization we employed the PAP immunocytochemical technique (Ruda et al., 1982) using a rabbit antibody raised against TRH conjugated to bovine serum albumin (TRH-BSA, Institut Pasteur). Free-floating sections were incubated in TRH antisera dilutions of 1:250–1:600 at 4°C for 48–72 hr, with gentle agitation. The sections were subsequently incubated in goat anti-rabbit (GAR) IgG serum (1:50) followed by incubation in PAP complex (1:80). The primary antibody (anti-TRH), link antibody (GAR), and PAP complex were diluted in 1% normal goat serum containing 0.75% Triton X-100 to ensure maximum antibody penetration throughout the 50 µm tissue section thickness (Ruda et al., 1982). All three antibody incubations were preceded by PBS washes, and a 30 min blocking wash in 3% normal
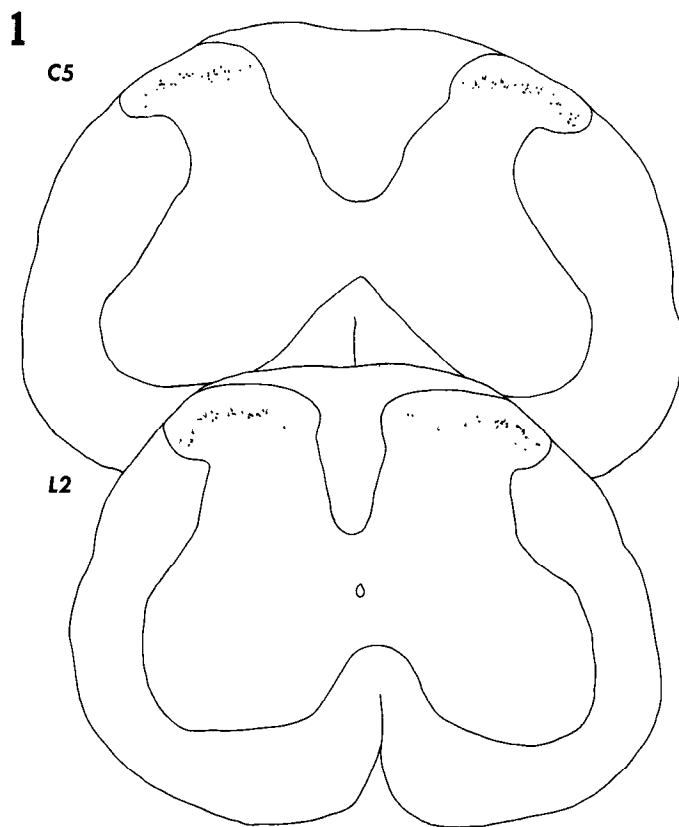
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Figure 1. Schematic representation demonstrating approximate size, shape, and distribution of the mouse dorsal horn neurons stained for TRH-LI. Their shape is oval. They are located within the superficial laminae (40–200 μm from the dorsal white–gray matter border) and are concentrated along the laminae II/III border (100–180 μm). To better illustrate the distinctive banding of these cells, each drawing represents a composite of three semiserial 50 μm transverse sections taken from segments C5 and L2 of the cervical and lumbar cord, respectively. Scale bar, 100 μm.

goat serum to minimize nonspecific background staining. After the PAP incubation, the sections were washed in PBS and incubated for 7–10 min in 0.05% diaminobenzidine HCl in 0.1 M PBS reacted with 0.01% hydrogen peroxide to obtain the distinctive reddish-brown label distinguishable with the light microscope. The sections were mounted on gelatin-coated slides, dehydrated in ethanol, cleared in xylene, and coverslipped using Permount. Sections were examined for TRH-LI under the light microscope. Immunoreactive cell bodies were drawn with a 20× objective using the camera lucida technique.

In initial specificity control experiments, 50 μm transverse sections of the hypothalamus (containing the median eminence) were similarly processed for TRH-LI using this antibody. These areas are known to be positive for TRH localization. In addition, antibody specificity and cross-reactivity were determined by preabsorption of the TRH antisera with 100 mg TRH or with 100 mg of the following putative neurotransmitters: cholecystokinin (CCK), leucine-enkephalin (L-ENK), neurotensin (NT), 5-hydroxytryptamine (5-HT), somatostatin (SS), substance P (SP), and vasoactive intestinal polypeptide (VIP). Radioimmunoassay studies by the Institute Pasteur show this antibody to be highly specific for TRH, with a cross-reactivity of less than 0.01% to TRH analogs TRH-OH, L-Pyroglu-L-His-OH, and L-Pyroglu-L-His-NH2, and to luteinizing hormone-releasing hormone. Using this antiserum, localization of TRH-LI within hypothalamic regions known to contain TRH and in the ventral horn is consistent with previous findings (not shown). The TRH-LI in the spinal dorsal horn is almost completely blocked by preabsorption with TRH, but is neither blocked nor reduced by preabsorption with other putative CNS transmitters (CCK, NT, 5-HT, SS, SP, VIP).

Table 1. Location, morphology, numbers, and sizes of neurons demonstrating TRH-LI in the mouse spinal dorsal horn

Cells examined (n)	Lamina location (%)	
	IIb	IIa and III
659	70	30

	Somata shape and orientation (%)		
	Fusiform; rostro-caudal processes	Round; dorso-ventral processes	Round/oval; no visible processes
Sagittal plane, 263	81	10	9
Transverse plane, 396	0	9	91

	Mean number and size of cells[a]	
	Number	Soma size (μm)
Cervical level, 171	10.1 ($\pm$4.5)	8.1 ($\pm$1.6)
Lumbar level, 196	9.1 ($\pm$3.8)	8.7 ($\pm$1.2)

[a] Cell numbers and somata sizes are based on counts and measurements per single dorsal horn taken from 50 μm transverse sections.

Results

Light-microscopic examination of the mouse spinal cord reveals TRH-LI in neurons of the dorsal horn at all levels of the spinal cord. Direct measurements place these neurons within the superficial laminae, 40–200 μm from the dorsal gray–white matter border. Superimposition of camera lucida drawings taken from three to four serial transverse sections illustrates that the immunoreactive neurons form a dorsoventral band that encompasses the substantia gelatinosa (lamina II) with a selective concentration along the lamina II/III border, approximately 100–180 μm from the dorsal white–gray matter border (Fig. 1). Approximately 70% of these neurons can be found in the ventral two-thirds of lamina II, lamina IIb. The remaining 30% are found in lamina IIa and dorsal lamina III. The immunoreactivity is confined to the cell body and proximal 30 μm of their primary dendrites. In the parasagittal plane, 81% of the total immunoreactive neurons ($n = 263$) have oval or fusiform cell bodies with primary processes arising from their rostrocaudal poles (Fig. 2). They exist in clusters with no apparent periodicity. A much smaller percentage (10%) have more rounded or triangularly shaped cell bodies with primary processes projecting dorsoventrally (not shown). The remaining 9% do not demonstrate immunoreactive processes. In the transverse plane, all of the cell bodies tend to be more rounded. Approximately 9% of these neurons ($n = 396$) demonstrate dorsoventrally projecting proximal dendrites. These data, and the numbers and sizes of immunoreactive TRH neurons in lumbar and cervical sections of the dorsal horn, are summarized in Table 1.

Discussion

Using PAP immunocytochemistry we have localized TRH-LI to neuronal cell bodies of the superficial dorsal horn (laminae I–III). Our immunocytochemical technique enables us to visualize the immunoreactive cell body and the most proximal 30 μm of the primary dendrites, but not the full extent of their dendritic arbors. Consequently, unequivocal identification of morphological cell type is not possible. Nevertheless, our data suggest that the TRH-LI is contained in at least two morphologically distinct interneuronal cell types. We base this morphological
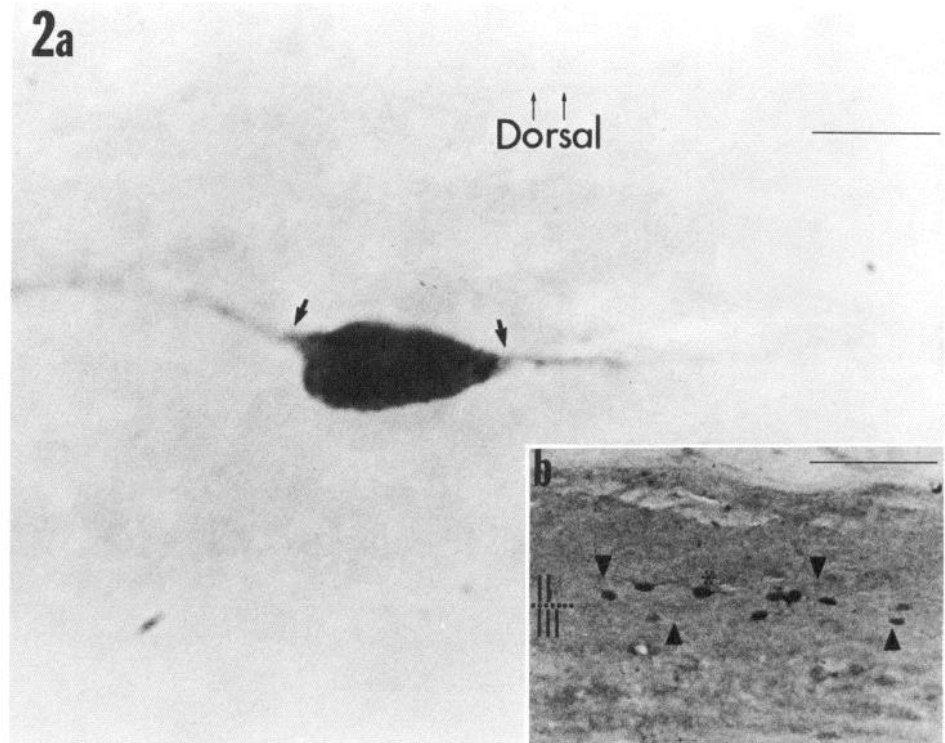
Figure 2. a, High-magnification photomicrograph of a TRH-immunoreactive neuron in a 50 μm parasagittal cervical section of the mouse spinal dorsal horn. The oval cell has proximal processes (*arrows*) arising from each pole of the cell body extending in the rostrocaudal direction (rostral at left). Scale bar, 10 μm. *b,* Low-magnification photomicrograph of same cervical section shown in *a.* The *asterisk* indicates the neuron shown under high magnification in *a.* The tight dorsoventral banding of the neurons is clearly evident and is demarcated by the *arrowheads. Dotted line* at left indicates laminar borders. Scale bar, 100 μm.

distinction on the location of the neuron within the dorsal horn, the shape of the cell body, and the orientation of its primary dendrites. The majority of the TRH-immunoreactive neurons are located within lamina IIb. Eighty-one percent of these neurons exist in clusters and have oval or fusiform cell bodies with rostrocaudally projecting primary processes. The predominant cell type encountered in both anatomical and physiological studies of lamina IIb is the islet cell (Bennett et al., 1980; Gobel, 1975, 1978; Light et al., 1979; Miletic et al., 1984). Lamina IIb islet cells have oval or fusiform cell bodies with primary dendrites projecting from their rostrocaudal poles, and they typically exist in clusters. This suggests that most of the TRH-LI seen in the ventral substantia gelatinosa is contained in IIb islet cells. It is noteworthy that previous studies have reported the existence of several other neurons containing peptide-like substances within the substantia gelatinosa. These include avian pancreatic polypeptide, L-ENK, NT, SS, and SP (Hunt et al., 1981). Some of these neurons were also thought to be IIb islet cells (Bennett et al., 1982; Seybold and Elde, 1982). The possibility that TRH and/or any of these other peptides coexist within the same neuron remains to be established.

Approximately 10% of the TRH-immunoreactive neurons have a more rounded or triangularly shaped cell body with proximal processes projecting dorsoventrally. The majority of these neurons are found deep in lamina IIb, near the lamina II/III border. These neurons bear a morphological similarity to the spiny cell (Gobel, 1975, 1978) in location, cell body shape, and orientation of their primary dendrites. The spiny cell has a polygonal cell body and dorsoventrally projecting primary dendrites, and is found between islet cell clusters in the substantia gelatinosa, frequently in lamina IIb near the lamina II/III border.

A smaller percentage of TRH-immunoreactive neurons is found in lamina IIa, and in the outermost region of lamina III. These neurons may represent any of the other interneuronal cell types described in this area, i.e., stalked, IIa islet, arboreal, or IIa/IIb border cells (Gobel, 1975, 1978).

The presence of TRH-immunoreactive neurons on both sides

of the lamina II/III border indicates a possible role for TRH as a neurotransmitter or neuromodulator in the processing of cutaneous sensory information. This region is the main termination site of primary afferent fibers signaling innocuous, rather than noxious, information (Dubner and Bennett, 1983). Physiological studies of lamina IIb islet cells indicate that they are exclusively low-threshold mechanoreceptive neurons, i.e., maximally activated by innocuous cutaneous stimulation (Bennett et al., 1980; Light et al., 1979; Miletic et al., 1984).

We present immunocytochemical evidence for the existence of TRH in dorsal horn neurons of the mouse spinal cord. This finding is in agreement with recent studies establishing that the highest concentration of spinal cord TRH receptors is in the substantia gelatinosa (Manaker et al., 1985; Mantyh and Hunt, 1985; Sharif and Burt, 1983). Additionally, TRH-immunoreactive neurons have also been seen in the rat superficial dorsal horn (Harkness and Brownfield, personal communication). The functional significance of TRH-containing neurons in the rodent dorsal horn is presently unknown. Their exclusive existence within the superficial laminae and their high density within the substantia gelatinosa suggest a role in the modulation of sensory input. Additional studies, however, are needed to establish the precise role of TRH in the neural circuitry of the dorsal horn.

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