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Research Report

Retrograde labeling reveals extensive distribution of genioglossal motoneurons possessing 5-HT$_{2A}$ receptors throughout the hypoglossal nucleus of adult dogs

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ABSTRACT

Inspiratory hypoglossal motoneurons (IHMNs) innervate the muscles of the tongue and play an important role in maintaining upper airway patency. However, this may be reduced during sleep and by sedatives, potent analgesics, and volatile anesthetics. The genioglossal (GG) muscle is the main protruder and depressor muscle of the tongue and contributes to upper airway patency during inspiration. *In vitro* data suggest that serotonin (5-hydroxytryptamine, 5-HT), via the 5-HT$_{2A}$ receptor (5-HT$_{2A}$R) subtype, plays a key role in controlling the excitability of IHMNs. The distribution of GG motoneurons (GGMNs) within the hypoglossal (XII) nucleus has not been studied in the adult dog. Further, it is uncertain whether the 5-HT$_{2A}$R is located on GGMNs in the adult dog. We therefore used the cholera toxin B (CTB) subunit as a retrograde tracer to map the location of GGMNs in combination with immunofluorescent labeling to determine the presence and colocalization of 5-HT$_{2A}$R within the XII nucleus in adult mongrel dogs. Injection of CTB into the GG muscle resulted in retrogradely labeled cells in a compact column throughout the XII nucleus, extending from 0.75 mm caudal to 3.45 mm rostral to the obex. Fluorescence immunohistochemistry revealed extensive 5-HT$_{2A}$R labeling on CTB-labeled GGMNs. Identification of the 5-HT$_{2A}$R on GGMNs in the XII nucleus of the adult dog supports *in vitro* data and suggests a physiological role for this receptor subtype in controlling the excitability of GGMNs, which contribute to the maintenance of upper airway patency.

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Abbreviations: IV, 4th ventricle; XII, Hypoglossal nucleus; 5-HT, 5-hydroxytryptamine, serotonin; 5-HT$_{2A}$R, Serotonin receptor subtype 2A; CTB, Cholera toxin B subunit; Cy2, Cyanine dye 2; d, depth; DAB, Diaminobenzidine; (DAB-Ni)-H_2O_2, Diaminobenzidine-nickel peroxidase; dfm, distance from midline; DMV, Dorsal motor nucleus of the vagus; GG, Genioglossal; GGMN, Genioglossal motoneuron; IHMN, Inspiratory hypoglossal motoneuron; i.v., intravenous; m, midline; nXII, Hypoglossal nerve; PBS, phosphate-buffered saline; PBST, PBS with Triton X-100; SD, standard deviation

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1. Introduction

Upper airway patency is compromised during sleep, sedation, and by potent analgesics and anesthetics. The genioglossal (GG) muscle is considered the main protruder and depressor muscle of the tongue (Mu and Sanders, 2000) and contributes to upper airway patency during inspiration (Brouillette and Thach, 1980; Jeffries et al., 1984; Miki et al., 1989). A more in depth analysis shows that the genioglossus is co-activated during inspiration with tongue retractor muscles and this seems to reduce airway compliance and promote patency (Mateika et al., 1999). In most mammals, including dogs, it is a symmetrically paired sheet-like muscle that originates from the midline of the inner surface of the mandible (Miller et al., 1979). Genioglossal motoneurons (GGMNs) comprise the largest subgroup of such inspiratory hypoglossal motoneurons (IHMNs) and their activity controls GG muscle tone that leads to protrusion of the tongue, which helps to maintain upper airway patency during inspiration. The location of GGMNs within the hypoglossal (XII) nucleus has been studied in the rat (Krammer et al., 1979; Uemura-Sumi et al., 1988; Altschuler et al., 1994; Aldes, 1995), cat (Uemura et al., 1979; Miyazaki et al., 1981), rabbit (Uemura-Sumi et al., 1988), frog (Matesz et al., 1999), monkey (Uemura-Sumi et al., 1981; Sokoloff and Deacon, 1992), and neonatal dog (Chibuzo and Cummings, 1982; Uemura-Sumi et al., 1988). However, the exact distribution and relative density of GGMNs within the XII nucleus of adult dogs has not been investigated but is of great interest to assess the findings from recent neurophysiological studies of IHMNs as in our decerebrate canine model (Tonkovic-Capin et al., 1998; Brandes et al., 2006). Brandes et al. (2006) have recently provided strong neurophysiological evidence in an in vivo decerebrate dog model that IHMNs receive strong serotonergic modulation via the 5-HT_{2A} receptor subtype under hypercapnic hyperoxia. The selective antagonist ketanserin reduced the baseline activity of these neurons by 68% (Brandes et al., 2006). Therefore, the immunohistochemical demonstration of 5-HT_{2A} receptors on GGMNs will provide important complementary evidence that these receptors are physiologically important.

The primary purpose of this study was to map the exact anatomic location, distribution, and density of GGMNs within the XII nucleus of adult dogs by combining retrograde tracing techniques *in vivo* with postmortem immunohistochemical labeling methods. A second aim was to obtain immunohistochemical evidence that the serotonin (5-hydroxytryptamine, 5-HT) receptor subtype 2A (5-HT_{2A}R) is present on GGMNs since *in vivo* data in decerebrate dogs provided strong neurophysiological evidence of an important endogenous serotonergic contribution via this receptor subtype to the baseline activity of IHMNs (Brandes et al., 2006).

2. Results

Neuroanatomical data on the distribution of GGMNs and immunohistochemical data for 5-HT_{2A} immunoreactivity were obtained in 4 adult mongrel dogs.

2.1. Distribution of GGMNs in the XII nucleus

Injection of cholera toxin B subunit (CTB) into the GG muscle resulted in retrogradely labeled cells confined to the hypoglossal motor nucleus in the brainstem. Whether injections of CTB were placed unilaterally or bilaterally along the full length of the GG muscle just lateral and beneath the lingual frenulum, the experiments yielded CTB-labeled GGMNs bilaterally (Fig. 1, upper) throughout the extent of the hypoglossal motor nuclei in all dogs. The distribution of labeled cells was similar in the unilateral versus the bilaterally injected animals. Bilateral injections yielded an average of 943 cells per nucleus, and unilateral injections resulted in an average of 975 cells per nucleus.

Immunofluorescent labeling for CTB (every other 25 μm transverse section) allowed the visualization of cell profiles, axonal projections and dendritic processes (e.g., Fig. 1, lower and 2). Retrogradely labeled GGMNs were generally multipolar with intense dendritic networks oriented in the mediolateral direction. The cell processes projected laterally but were confined to the hypoglossal nucleus in the dorsal direction, not extending into the neighboring dorsal motor nucleus of the vagus. The axons were evident running in the hypoglossal nerve (Fig. 2).

2.2. Number and location of GGMNs

Diaminobenzidine (DAB)-stained sections (alternate 25-μm sections) were photographed using light microscopy for off-line computer-assisted cell counting. Computer-assisted cell counting and coordinate determination of the DAB-labeled sections revealed that in the adult dog GGMNs are densely distributed in the hypoglossal motor nucleus in a long, compact column extending from 0.75 mm caudal to 3.45 mm rostral to the obex, with cells 0.37 to 2.12 mm below the dorsal surface, and symmetrically centered between 0.66 and 1.33 mm from the midline. Fig. 3 shows the exact distribution of CTB-labeled cells in the left hypoglossal motor nucleus of one dog. The cells were on average (mean (±SD)) 1.43 mm (±0.53) below the dorsal surface and 1.15 mm (±0.14) lateral from the midline. The total number of counted cells on the left side was 881. The corresponding data from the right side were depth 1.49 mm (±0.53), lateral 1.13 mm (±0.13), and total number of counted cells 761. Fig. 4 shows the bilateral distribution of CTB-labeled neurons in the hypoglossal motor nucleus for all four dogs. On the left side, the cells were on average 1.24 mm (±0.46) below the dorsal surface and 0.98 mm (±0.12) lateral from the midline. The corresponding data for right side were 1.24 mm (±0.45) below the dorsal surface, 1.00 mm (±0.12) lateral from the midline.

Based on the number of slices that were studied, we performed a total cell count. The total GGMN count per hypoglossal motor nucleus on the left side was 964 (±142) cells per dog in 62 (±9) slices. On the right side, 61 (±8) slices yielded 954 (±201) cells per dog. On average, we found 16 cells (±2) per slice on each side, ranging from 2 to 40 cells/slice on each side. As described in Experimental procedures, we used a Monte Carlo simulation model to determine the amount of double-counted labeled cells and the number of cells in the alternating non-DAB-stained sections that were not counted.