Human brainstem preganglionic parasympathetic neurons localized by markers for nitric oxide synthesis

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Summary
Identification of human parasympathetic preganglionic neurons in pontomedullary regions has been largely based on studies using cholinesterase histochemical procedures, and so far there is no adequate account of the location of these cells. Nitric oxide synthase (NOS) is present in brainstem parasympathetic preganglionic salivatory neurons in the rabbit (Zhu et al., 1996). In the present study we have used histochemical and immunohistochemical staining for NOS to examine possible preganglionic parasympathetic neurons in the human brainstem. We examined, in five human brains, the distribution, through the caudal pons and rostral medulla, of NOS-positive neurons in serial sections stained with NADPH diaphorase for histochemistry, and with antibodies against neuronal NOS peptide for immunohistochemistry. In scattered pontomedullary regions (rostral to the dorsal motor nucleus and the nucleus ambiguus) known to contain parasympathetic preganglionic neurons in animals, we observed groups of NOS-positive neurons which correspond in morphology and distribution with NOS-positive parasympathetic preganglionic neurons in rabbits. These neurons are probably parasympathetic preganglionic neurons in the human brainstem, involved in the control of lacrimation, salivation, oral and nasopharyngeal secretion, as well as the control of the dilation of extra- and intracranial blood vessels.

Keywords: nitric oxide synthase; parasympathetic preganglionic; human brainstem; lacrimation; salivation

Abbreviation: NOS = nitric oxide synthase

Introduction
The most rostral cranial preganglionic parasympathetic neurons are located in the midbrain, in the Edinger–Westphal nucleus (axons in cranial nerve III). The most caudal cranial parasympathetic neurons are located in the medulla, in the dorsal motor nucleus of the vagus and in the nucleus ambiguus (axons in cranial nerve X). Other brainstem parasympathetic preganglionic neurons project via cranial nerves VII and IX to sphenopalatine, submandibular, sublingual, otic and other small unnamed ganglia in various extracranial locations. Postganglionic neurons project to lacrimal and salivary glands, to mucosal glands in the mouth and nasopharynx, as well as to arteries supplying the cranial vasculature, including major cerebral arteries.

In spite of the diverse functions served by the various target tissues, the parent preganglionic parasympathetic neurons are usually lumped together and assigned to the ‘salivatory nuclei’, either to the superior salivatory nucleus (axons in cranial nerve VII) or the inferior salivatory nucleus (axons in cranial nerve IX). Accounts of the location of these salivatory nuclei in the human brainstem are sketchy. Textbooks of human neuroanatomy usually refer to studies utilizing the cholinesterase histochemical procedure as a marker of acetylcholine-synthesizing neurons (Lewis and Shute, 1959). The more critical accounts acknowledge the lack of evidence available for specifying the location of these parasympathetic neurons in the human (Ranson and Clark, 1959).

In the human brainstem Feiling (1913), on the basis of evidence from retrograde neuronal degeneration in a patient who died with injuries to the IX, X, XI and XII nerves, identified salivatory neurons just dorsal to the inferior olivary nucleus. Braak (1972), in a Nissl study, identified the human inferior salivatory nucleus dorsomedial to the rostral portion of the nucleus tractus solitarius. A similar region was also designated as the inferior salivatory nucleus by Paxinos et al. (1990), and by McRitchie and Tork (1993) on the basis of
cholinesterase histochemistry. Neither of the latter studies defines a superior salivatory nucleus, and no inferior salivatory nucleus was identified in the human brainstem in a detailed study using both cholinesterase histochemistry and choline acetyltransferase immunohistochemistry (Mizukawa et al., 1986).

In experimental animals, appropriate retrograde intraxonial tracing studies demonstrate that parasympathetic preganglionic neurons are located in thepons at the level of the facial nucleus, and in rostral portions of the nucleus tractus solitarius. This has been demonstrated in rat (Hiura, 1977; Contreras et al., 1980; Nicholson and Severin, 1981; Mitchell and Templeton, 1981), rabbit (Matsu et al., 1980; Hanamori and Smith, 1989), hamster (Whitehead and Frank, 1983), cat (Satomi et al., 1979; Nomura and Mizuno, 1981; Tramont and Bauer, 1986), dog (Chibuzo and Cummings, 1980) and monkey (Perwaiz and Karim, 1982). The preganglionic neurons do not form discrete cell groups, so that it is difficult to identify them on Nissl appearance alone; nor is there a clear rostrocaudal distinction between neurons with axons exiting the brainstem in VII and those exiting in IX, so that Contreras et al. (1980) question the wisdom of the traditional division of the neurons into superior and inferior salivatory nuclei.

In the rabbit, the parasympathetic preganglionic neurons described by Hanamori and Smith (1989) on the basis of retrograde transport studies from appropriate branches of cranial nerves VII and IX, are very similar, in both distribution and morphology, to neurons containing NOS in the same general region of the brainstem (Gai et al., 1995). We recently confirmed in the rabbit that NOS is present in nearly all neurons retrograde labelled after application of tracer to the chorda tympani nerve or to preganglionic axons travelling along the submandibular duct (Zhu et al., 1996). This close correspondence between identified preganglionic salivatory neurons and NOS-positive neurons in the rabbit suggested that NOS might prove useful for identifying parasympathetic preganglionic neurons in the human. The distribution of NOS-positive neurons in the human brainstem, including the medulla and caudal pons, has been mapped (Kowall and Mueller, 1988), but not in detail sufficient to determine whether the distribution of NOS-positive neurons corresponds to that of the salivatory nuclei defined in animals. We now present, for the human, a detailed map of NOS-positive neurons in those pontomedullary regions which, in the rabbit and in other experimental animals, have been demonstrated to contain parasympathetic preganglionic neurons.

Material and methods

We examined five human brains, obtained at autopsy from patients dying from non-neurological causes (see Table 1). The brains were perfuse-fixed with 4% formaldehyde and 2% picric acid in 0.1 M phosphate buffer at pH 7.4, as previously described in detail (Halliday et al., 1988). The brainstem was sectioned transversely (50 μm) on a freezing microtome, and serial sections collected into 15 or 30 consecutive containers. One set of sections was immunostained using a rabbit polyclonal antiserum, raised against the C-terminal sequence (amino acid residue 1409–1429) of rat cerebellar NOS peptide by Dr Colin Anderson of the Department of Anatomy and Cell Biology, University of Melbourne (available from Auspep Pty Ltd, Parkville, Victoria, Australia). Staining of neural tissue is abolished by preincubation of diluted antiserum with the peptide antigen. The antiserum recognizes a single band corresponding to neural NOS in blots from rat brain. Our previous study in rabbit (Gai et al., 1995) and the present study in humans, demonstrates that the antiserum stains only neurons. Blood vessels known to contain endothelial NOS were not stained. The NOS antiserum was used at 1:2000 dilution. Control sections were incubated with non-immune rabbit serum, and there was no neuronal staining in these sections. Another set of sections was stained with the NADPH diaphorase procedure. Free-floating sections were incubated for 2–3 h at 37°C, in a solution containing 1.25 mg nitroblue tetrazolium, 5.0 mg beta-NADPH (Sigma Chemical, Sydney, Australia), and 0.1% Triton X-100 in 10 ml of 0.1 M Tris buffer (pH 8.0). Control sections were processed identically except NOS antibody or beta-NADPH were omitted from the incubation medium. Sections were mounted, dehydrated and coverslipped. Other section series were stained for Nissl substance (cresyl violet) or myelin (Weil method).

The microscopic image of a section was superimposed onto a Macintosh IIfx computer screen using a camera lucida device. Boundaries of nuclei or fibre bundles were outlined under dark-field illumination, and positive cells marked under bright-field illumination. The information was recorded in the computer using Canvas software (Deneba, Fla., USA). The rostrocaudal distance of a section was calculated in reference to the obex.

Results

Nitric oxide synthetase positive neurons were observed in all brains studied. The NADPH diaphorase reaction and the NOS antibody procedure stained similar sets of neurons throughout the brainstem. No positive staining was observed in control sections. The overall distribution of NOS-positive neurons was similar, but there was a gradual decrease in the number and staining intensity of positive neurons with longer post-mortem delays, for both diaphorase reaction and NOS

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Post-mortem delay (h)</th>
<th>Cause of death</th>
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<tbody>
<tr>
<td>N20</td>
<td>F</td>
<td>84</td>
<td>15</td>
<td>Peritonitis</td>
</tr>
<tr>
<td>N21</td>
<td>M</td>
<td>88</td>
<td>19</td>
<td>Renal failure</td>
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<tr>
<td>N22</td>
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<td>86</td>
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<td>Cancer</td>
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<td>N23</td>
<td>F</td>
<td>86</td>
<td>17</td>
<td>Cancer</td>
</tr>
<tr>
<td>N24</td>
<td>F</td>
<td>71</td>
<td>7</td>
<td>Cardiac infarct</td>
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antibody. In the present study, sections from case N24 (7 h post-mortem delay) were used for mapping the distribution of NOS-positive cells. Nitric oxide synthetase-positive perikarya have been divided into medium sized (long axis >25 μm) and small neurons (long axis <25 μm).

Many NOS-positive neurons were found in pontomedullary regions (Fig. 1) known to contain preganglionic parasympathetic neurons in experimental animals. Medium-sized neurons were located in a region bordered by the spinal trigeminal nucleus laterally, the facial nucleus medially and the medial vestibular nucleus dorsally. Rostrally, these neurons were scattered in a narrow region between the oral spinal trigeminal nucleus and the facial nucleus, medial to the descending intramedullary fibres of the facial nerve (Fig. 1B and C). At the level of the midportion of the facial nucleus, ~12 mm rostral to the obex, a few NOS-positive neurons were found ventrolateral to the facial nucleus (Fig. 1D). At the level ~11 mm rostral to the obex, these ventrally positioned neurons maintained a similar relation to the facial nucleus, whereas dorsally located neurons became more concentrated in a region dorsomedial to the oral spinal trigeminal nucleus (Fig. 1E). Further caudally, at the level ~10 mm rostral to the obex, the ventral cell group was no longer present, whereas the dorsal group was still quite well developed, now situated medial to the nucleus tractus solitarius (Fig. 2F and G). At the level 9 mm rostral to the obex, only a few large NOS-positive neurons were found medial to the nucleus tractus solitarius (Fig. 1H).

Most medium-sized NOS-positive neurons had spindle-shaped or ovoid perikarya with two prominent dendrites emanating from the long ends of the cell body (Fig. 2A–D, G and H). Those NOS-positive neurons located ventral to the facial nucleus were usually round or ovoid (Fig. 2E and F). The dendrites of most medium-sized NOS-positive neurons located rostrally (corresponding to level 11–13 mm rostral to the obex; see Fig. 1) were arranged in a ventrolateral to dorsomedial direction, roughly parallel with the trajectory of descending facial nerve tract (Fig. 2A–F), but many dendrites of caudally situated neurons were horizontally arranged (Fig. 2G and H).

As shown in Fig. 1, small NOS-positive neurons were scattered widely in the pontomedullary region including the region containing medium-sized NOS-positive neurons. The oral spinal trigeminal nucleus contained a discrete NOS-positive neuron group that was apparent with the naked eye. This cell group was located in the centre of the oral spinal trigeminal nucleus, and extended ~5 mm rostrocaudally (Figs 1B–H and 3A and B). Numerous NOS-positive fibres (large and small calibres) were seen in the intramedullary tracts of the facial and glossopharyngeal nerves (Fig. 3C–E).

Discussion
The human pontomedullary region examined in the present study contains many NOS-positive neurons, distributed in a manner quite similar to neurons demonstrated to be parasympathetic preganglionic neurons by appropriate retrograde transport studies in experimental animals (see Introduction). The distribution is also similar to that of NOS-positive neurons in the rabbit, cells which have been shown by double-labelling procedures to include salivatory preganglionic neurons (Zhu et al., 1996). Most of them are medium sized (Zhu et al., 1996). We consider it reasonable to suppose that, in humans, NOS also occurs in parasympathetic preganglionic neurons with axons exiting in VII and IX cranial nerves. Other brainstem parasympathetic preganglionic neurons (with axons exiting in III and X) do not appear to contain markers for NOS (Kowall and Mueller, 1988; W. P. Gai and W. W. Blessing, unpublished observations). The rostrally located NOS-positive neurons (at levels of rostral half of the facial nucleus) are dispersed and not easily distinguished from other neurons in Nissl-stained sections. The caudally located neurons can be identified in Nissl sections, but the neurons are continuous with other neurons of the dorsal motor nucleus of the vagus which are NOS-negative.

In the human oral spinal trigeminal nucleus, Olszewski and Baxter (1982) described a cell group termed the oval nucleus. They state that ‘the nucleus lies along the dorsal or dorsolateral border of the nucleus tractus spinalis trigemini oralis, and extends from the level of the oral pole of the dorsal motor nucleus to the caudal pole of the main sensory trigeminal nucleus, a distance of 5 mm’ (p. 167). In plate XX of Olszewski and Baxter (1982) the oval nucleus is situated in a region corresponding to the more rostral extent of the interstitial nucleus of the nucleus tractus solitarius. However, in plate XVIII of the atlas, the oval nucleus is consigned to the oral spinal trigeminal nucleus, ventrolateral to the true position of the nucleus tractus solitarius at this rostrocaudal level. The NOS-positive cell group we observed in the oral spinal trigeminal nucleus is just ventromedial to the region designated as the oval nucleus in plate XVIII of Olszewski and Baxter (1982).

The inferior salivatory nuclei, described dorsomedial to the rostral portion of the nucleus tractus solitarius by Paxinos et al. (1990), McRitchie and Törk (1993) and Braak (1972), presumably correspond to the NOS-positive cells shown in our Fig. 1D and E. No superior salivatory cells are identified. The cholinesterase procedure may have demonstrated these neurons without being able to distinguish them from the neurons of the accessory facial nucleus and other cholinesterase-positive (but NOS-negative) neurons in the region. Satoh and Fibiger (1985a, b), in a careful histochemical (acetylcholinesterase reaction) and immuno-histochemical (choline acetyltransferase antibodies) study of the baboon brainstem, described parasympathetic preganglionic neurons in regions very similar to those identified in the present study. Our study is the first to identify these more rostral parasympathetic neurons in the human brainstem. The supposed existence of superior and inferior subnuclei arose from the knowledge that the axons were distributed in either the facial or the glossopharyngeal nerves. The excellent
Fig. 1 Computer camera lucida drawings showing the distribution of NOS-positive neurons in the caudal pons and rostral medulla. Sections are arranged from rostral (A) to caudal (D). The numbers in the upper left corner of each drawing indicate the approximate distance to the obex. The diagrams on the left show the distribution of all NOS-positive neurons in a 50 μm thick section at the given level. The enlarged views on the right show the distribution of medium-sized NOS-positive neurons in more detail. Each marker represents one NOS-positive neuron. Large squares represent perikarya with long axis >25 μm. Small squares represent perikarya.
with the long axis <25 µm. Vo = oral spinal trigeminal nucleus; Vp = principal sensory trigeminal nucleus; VI = abducent nucleus; VII = facial nucleus or facial fibre tract; VIII = lateral vestibular nucleus; VIIIIm = medial vestibular nucleus; VIIIIs = superior vestibular nucleus; VIIIsp spinal vestibular nucleus; icp = inferior cerebellar peduncle; IO = inferior olivary nucleus; mcp = middle cerebellar peduncle; ml = medial lemniscus; mlf = medial longitudinal fasciculus; NTS = nucleus tractus solitarius; p = pyramidal tract; PrP = prepositus hypoglossal nucleus; SO = superior olivary nucleus; A = nucleus ambiguus.
Fig. 2 Photomicrographs showing the morphology of medium-sized NOS-positive neurons in lateral pontomedullary regions. The approximate areas from which the photographs were taken are indicated by the horizontal pairs of arrows in Fig. 1. All photomicrographs are presented as the left side of corresponding brainstem region, with medial to the right. (A) Nitric oxide synthase positive neurons medial to the oral trigeminal nucleus ~12 mm rostral to the obex (see Fig. 1C). (B) Enlarged view of A. (C and D) Nitric oxide synthase positive neurons in the region between the facial nucleus and descending facial nerve fibres (see Fig. 1D). (E and F) Nitric oxide synthase positive neurons ventrolateral to the facial nucleus (see Fig. 1E). (G) Nitric oxide synthase positive neurons medial to the oral spinal trigeminal nucleus and the NTS ~10 mm rostral to the obex (refer to Fig. 1G). (H) Enlarged view of G.
Fig. 3 (A) Nitric oxide synthase positive neurons and their dendrites in the oral spinal trigeminal nucleus. (B) Enlarged view of A. (C) Nitric oxide synthase positive fibres in the intramedullary tract of the glossopharyngeal nerve. (D) Nitric oxide synthase positive fibres in the genu of the facial nerve. (E) Nitric oxide synthase positive fibres in the descending intramedullary tract of the facial nerve.

discussion section in a detailed study of the rat (Contreras et al., 1980) questions the utility of the superior/inferior dichotomy, as well as emphasizing the complexity of function of these parasympathetic preganglionic cells. In the species studied, neurons with axons exiting in the glossopharyngeal nerve have a brainstem distribution generally similar to those with axons exiting in the facial nerve.

In rats, neurons in the more dorsal parasympathetic neurons project to otic ganglia, to ganglia associated with the sublingual and submandibular salivary glands, as well as to mucosal glands in the oral cavity and vessels supplying all these structures (Contreras et al., 1980). In addition, postganglionic neurons supply the vertebrobasilar arterial system (Suzuki, 1989; Hara et al., 1993; Suzuki and Hardebo, 1993; Shimizu, 1994). By analogy, the more dorsal NOS-positive neurons identified in the present human study also presumably project to ganglia supplying parasympathetic innervation to the salivary glands, mucosal glands in the oral cavity and to the vertebrobasilar arterial system.

Neurons on the lateral aspect of the facial nucleus can be retrograde labelled from the sphenopalatine ganglion and the greater superficial petrosal nerve, indicating that they are likely to include lacrimal preganglionic cells, neurons innervating the vasculature and mucous glands of the nasal and palatal mucosa, and possibly neurons with postganglionic connections innervating cerebral blood vessels in forebrain regions (Contreras et al., 1980; Hara et al., 1993; Shimizu, 1994). These more ventrally located parasympathetic preganglionic neurons are also observed in retrograde transport studies from the sphenopalatine ganglion in rabbits, and the same more ventral region also contains a dense group of NOS-positive neurons (Zhu et al., 1996). The more ventrally located parasympathetic preganglionic neurons in the human may therefore be parasympathetic preganglionic neurons controlling, via appropriate postganglionic neurons, the functions of lacrimal glands, as well as mucus-secreting cells in the nasopharynx. In addition, blood supply to these structures, as well as to other extracranial vessels and cerebral blood vessels supplying the forebrain may also be regulated by these brainstem parasympathetic neurons.

Acknowledgements
We wish to thank Ms R. A. Flook for excellent technical assistance. This study was supported by the Australian Brain
Foundation (NSW), the Julia Farr Foundation (SA), the National Heart Foundation of Australia and the NH and MRC.

References


Received November 15, 1995. Revised February 3, 1996.
Accepted February 16, 1996