Neuroanatomical characteristics of the human pre-Bötzinger complex and its involvement in neurodegenerative brainstem diseases

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The pre-Bötzinger complex has been identified as an essential part of the medullary respiratory network in mammals. Although well described in experimental animals, its localization in the human brain has remained elusive. Using serially sectioned brainstems from 19 normal individuals and patients suffering from neurodegenerative diseases (multiple system atrophy, n = 10; spinocerebellar ataxia type 3, n = 8), we have identified a circumscribed area of the ventrolateral medulla that represents the human homologue of the pre-Bötzinger complex and have mapped its longitudinal and horizontal extents. The presumed human pre-Bötzinger complex is characterized by an aggregation of loosely scattered, small and lipofuscin-rich neurons, which contain neurokinin 1 receptor as well as somatostatin, but are negative for markers of monoaminergic neurons and of motoneurons. In brains of patients suffering from multiple systems atrophy (with central respiratory deficits but without swallowing problems), pre-Bötzinger complex neurons were reduced, whereas pharyngeal motoneurons of the ambiguus nucleus were not affected. In contrast, in brains of patients with spinocerebellar ataxia 3 (no reported central respiratory deficits but with dysphagia), pre-Bötzinger complex neurons were preserved, whereas ambiguus motoneurons, which control swallowing, were diminished. These pathoanatomical findings support the view, that affection of the central respiratory network, including the pre-Bötzinger complex, contributes to breathing disorders in multiple system atrophy, whereas damage to ambiguus motoneurons is important for pathogenesis of breathing disturbances and dysphagia in patients with spinocerebellar ataxia type 3. On the basis of these findings, the putative human homologue of the pre-Bötzinger complex can now be reliably delineated on pigment-Nissl-stained sections, making neuropathological investigations of central respiratory disturbances feasible.

Keywords: pre-Bötzinger complex; brainstem; respiratory control; multiple system atrophy; spinocerebellar ataxia

Abbreviations: ChAT = choline-acetyltransferase; MSA = multiple system atrophy; NK1R = neurokinin 1 receptor; PH8 = anti-tryptophan hydroxylase/tyrosine hydroxylase/phenylalanine hydroxylase, clone PH8; SCA3 = spinocerebellar ataxia type 3
Introduction

The pre-Bötzinger complex was identified as an essential part of the medullary rhythm-generating network in experimental animals almost two decades ago (Smith et al., 1991). Subsequently, it has been shown that the pre-Bötzinger complex is critical for breathing under in vivo conditions (Ramirez et al., 1998; Gray et al., 2001; Feldman and Del Negro, 2006). In mammals, the pre-Bötzinger complex is well characterized as part of the group of ventral medullary respiratory neurons that form a rostrocaudal column throughout the medulla oblongata from the facial nucleus (VII) to the pyramidal decussation. This column can be subdivided into four distinct groups: the rostral Bötzinger complex, followed by the pre-Bötzinger complex, the rostral ventral respiratory group and the caudal ventral respiratory group (for review see Feldman and Del Negro, 2006). In contrast to the many detailed structural and functional analyses of central respiratory control in experimental animals (for review see Smith et al., 2009), there is still little information concerning central respiratory control in the human brain (Blessing, 2004; Pattinson et al., 2009). As the pre-Bötzinger complex and other medullary respiratory areas were primarily defined by functional criteria in experimental animals, any analysis in human tissue will depend on cytoarchitectonic and/or neurochemical homologies. In addition, the complex nature of the medulla oblongata and the strong interconnectivity in the so-called ‘reticular formation’ hinders the histological localization of circumscribed respiratory nuclei. Analogies between experimental animals, such as rats, mice or cats, and human tissue are further hampered by the enlargement of the human inferior olive that is accompanied by a rearrangement of all other areas of the ventrolateral medulla (Blessing, 2004).

In spite of these difficulties, a number of morphological criteria originating from animal studies can be employed to localize the human pre-Bötzinger complex. In particular, the distinct cytoarchitectonic characteristics of neighbouring nuclei and fibre tracts (Schwarzacher et al., 1995; Ruangkittisakul et al., 2006) as well as markers for interneurons in the area of the pre-Bötzinger complex can be utilized. It has previously been demonstrated that interneurons belonging to the pre-Bötzinger complex express high levels of neurokinin 1 receptor (NK1R) (Gray et al., 1999) and somatostatin (Sornetta et al., 2003). These interneuron populations are of functional relevance for central respiratory control, since bilateral silencing of somatostatin expressing pre-Bötzinger complex neurons induced persistent apnoea in awake rats (Tan et al., 2008), while selective bilateral ablation of pre-Bötzinger complex NK1R-expressing neurons resulted in an irreversible deterioration of the breathing pattern, initially during sleep, later progressing to an ataxic breathing pattern during wakefulness (Gray et al., 2001).

To identify the elusive human pre-Bötzinger complex, we used a combination of three morphological approaches, which make us confident that we have correctly identified the area of human brain wherein it is contained. First, we stained unconventionally thick (100μm) serial transverse sections of human brainstems with the pigment-Nissl method (Braak and Braak, 1991; Braak et al., 2003). This technique is highly advantageous for the analysis of human brain tissue, since neuronal subtypes can be distinguished based on their pigment content. Secondly, we used immunocytochemistry to identify accumulations of NK1R and somatostatin neurons in the human ventrolateral medulla. We then analysed the ventrolateral medulla of normal individuals and of patients suffering from two different neurodegenerative diseases associated with breathing disturbances: multiple system atrophy (MSA) and spinocerebellar ataxia type 3 (SCA3, also known as Machado–Joseph disease), and have searched for pathoanatomical changes in the putative pre-Bötzinger complex. Using these approaches in combination, we identified a circumscribed region of the ventrolateral medulla that contains a high number of NK-1R and somatostatin immunoreactive neurons as the presumptive human homologue of the pre-Bötzinger complex region in experimental animals.

Materials and methods

Subjects

Brains were obtained at autopsy from 19 individuals with no history of neurological disease (six females and 13 males, age 59±17 years), 10 individuals that suffered from MSA (five females and five males, age 68±12 years), and eight individuals that suffered from SCA3 (three females and five males, age 58.5±19.4 years). Examination of these brains was approved by the ethical board of the Faculty of Medicine of the Goethe University of Frankfurt/Main. Brains were immersion fixed in 4% buffered paraformaldehyde for at least 24 h. After removal of the cerebellum, the lower brainstem was separated and stored in 0.2 M phosphate buffer at pH 7.2, with 0.01% thimerosal added.

Histology

Brainstems were embedded in gelatine or polyethylene glycol (PEG 1000, Merck, Darmstadt, Germany) with the rostral end down and strictly perpendicular to their longitudinal axis. Starting at the pyramidal decussation and ending at the pontomedullary junction, they were cut into sets of 60 or 100μm thick transverse sections with a vibrotome or tetrander. For purposes of anatomical orientation, cytoarchitectonic investigation and nerve cell-counting tissue sections of all brains (including nine series of 100μm transversal sections of control individuals) were stained for lipofuscin pigment (aldehyde-fuchsin) and Nissl material (Darrow red).

Immunocytochemistry

Immunocytochemistry was performed on 60μm transverse sections. In each case, we first tested different immunocytochemical protocols with all marker antibodies on caudal medullary sections (caudal to the obex). Preincubation included 0.05M sodium citrate (pH 6, 30 min at 4°C) followed by 10% methanol and 3% Perhydrol® (pH 7.2, 30 min at room temperature). Successful protocols were then used to stain serial sections of the medulla between the obex and +14 mm rostral to the obex (+14 mm obex), with reference to the atlas of Paxinos and Huang (1995). If more than one antibody was applied, antibodies were used sequentially to stain a series of brain sections, i.e. in the case of three markers the first marker was processed on the first, fourth, seventh section and so on. Sections were incubated for 24 h with the following primary antibodies: NK1R (1:1000, guinea pig, Chemicon), NK1R (1:1000, rabbit, Chemicon); somatostatin (1:2000, rabbit, Immunostar), choline-acetyltransferase
could be delineated. The rostral and the caudal borders of the brain region of interest are defined by characteristic brainstem nuclei (cytoarchitectonic landmarks). The rostral border of the ventral reticular formation (VII) is the rostral landmark. It is located in human brain at +14 mm rostral to the obex (this location corresponds to the pontomedullary junction). The rostral end of the undivided lateral reticular nucleus serves as caudal landmark. In human brain, it is located at +7 mm rostral to the obex. Of note, both nuclei are located in a similar ventrolateral position as the pre-Bötziinger complex (Fig. 1A).

Although the rostral subnuclei of the lateral reticular nucleus correspond to the level of the pre-Bötziinger complex, their diffuse and inconstant appearance in humans makes it difficult to use them for orientation (Braak, 1970). A similar problem precludes the use of the inferior olive for orientation, which can be used to identify the rostrocaudal level throughout the subfacial medulla in rodents (Ruangkittisakul et al., 2006). In humans, the different subnuclei of the inferior olive show considerable cytoarchitectonic variations (Braak, 1970) and, thus, these nuclei cannot be used as reliable landmarks. For these reasons, other brainstem nuclei had to be considered. Additional spatial information was obtained by using the rostrocaudal changes of dorsal medullary nuclei, such as the 12th nucleus, and the extensions of the fourth ventricle at the dorsal medullary surface, as reference points. By using these dorsal structures in combination with the ventral areas, the rostral and the caudal borders of the brain region of interest could be delineated.

Finally, the intermedullary courses of the rostral hypoglossal rootlets were employed, as they serve as valuable markers of the rostrocaudal level of the pre-Bötziinger complex in experimental animals (Ruangkittisakul et al., 2006). The most rostral bundles of the hypoglossal nerve run at +9 to +11 mm to the obex and their presence facilitated orientation at these levels. In summary, the pre-Bötziinger complex was expected within the ventrolateral medulla, at rostrocaudal levels of +7 to +14 mm of the obex. Accordingly, transversal human brainstem sections were analysed in detail from the obex to levels +14 mm rostral to the obex (pontomedullary fissure, caudal end of VII).

**Mapping, cell counting and statistical analysis**

A light microscope (magnification: ×10 to ×1000) with a drawing tube was used for the examination of tissue sections. The atlas of Paxinos and Huang (1995) was used to identify rostrocaudal levels and the outlines of the ventrolateral area of the medulla. Outlines and major anatomical landmarks of each tissue section investigated were compared and fitted to the anatomical structures found at the corresponding level of the atlas.

Somata of interest were counted on each pigment-Nissl- and immuno-nostained section. Cells were counted in the ventrolateral medulla in an area defined by the following borders: ventral (ventrolateral surface), medial (inferior olive), lateral (spinal trigeminal tract) and dorsal borders (a line perpendicular to the midline through the dorsal edge of the dorsal compact formation of the ambiguous nucleus). This roughly rectangular area was subdivided into three square areas (dorsal, intermediate and ventral). Mean and standard deviation of cell numbers in corresponding areas of the same rostrocaudal level (grouped in steps of 0.5 mm from obex to +14 mm obex, 1–5 sections per group and brainstem site) from the left and right brainstem sites were calculated using standard software (Excel, Microsoft).

**Clinical background of spinocerebellar ataxia type 3 and multiple system atrophy patients**

The clinically diagnosed and genetically confirmed patients with SCA3 were descended from SCA3 families in The Netherlands (age at disease onset: 30 to 75 years, mean age at onset: 48 years, range: 21 to 75 years).

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**Figure 1** Localization of the human pre-Bötziinger complex by cytoarchitectonic criteria. (A) Based on cytoarchitectonic criteria, the putative human homologue of the pre-Bötziinger complex (preBoTC) in experimental animals can be localized in the ventrolateral medulla, caudal to the facial nucleus (VII) and rostral to the lateral reticular nucleus (LRN). (B) On a transversal section at +9 mm to the obex, the pre-Bötziinger complex is located lateral to the inferior olive (IO), ventral to the compact part of the ambiguous nucleus (Amb) and medial to the spinal trigeminal tract (Sp5). (C) At the ventrolateral surface of the brainstem, the pre-Bötziinger complex is positioned in the retro-olivary sulcus lateral to the inferior olive and to the rostral rootlets of the hypoglossal nerve (XII) and medial to the ventral rootlets of the vagal nerve (X). ICP = inferior cerebellar peduncle.

(ChAT: 1:500, goat, Chemicon), anti-tryptophan hydroxylase/tyrosine hydroxylase/phenylalanine hydroxylase, clone PH8-1 (PH8: 1:1000, mouse, monoclonal, Chemicon). This was followed by ABC-staining with horse radish peroxidase and diaminobenzidine as chromogen. For fluorescence double-labelling, sections were stained with Alexa 488 and 568 secondary antibodies and counterstained with 0.3% Sudan Black to reduce autofluorescence. For anatomical orientation, tissue sections were, in part, counterstained for lipofuscin pigment (aldehyde-fuchsin) and Nissl material (Darrow red).
Co-localization of NK1R and somatostatin immunoreactive cells in the rostral ventrolateral medulla

To further delineate the putative pre-Bötzing complex region, we looked for the presence of pre-Bötzing complex neurons within the ventrolateral medulla using immunocytochemistry. Although NK1R and somatostatin have been proven to be valuable markers for pre-Bötzing complex neurons, the widespread distribution of NK1R and somatostatin-positive neurons throughout the medulla precludes their use as exclusive markers for pre-Bötzing complex neurons (Gray et al., 1999; Benarroch et al., 2003; Stornetta et al., 2003). Since both NK1R and somatostatin-positive neurons accumulate in the pre-Bötzing complex area in experimental animals (Gray et al., 1999; Stornetta et al., 2003), we searched for neurons co-expressing NK1R and somatostatin in the putative pre-Bötzing complex region. An accumulation of NK1R and somatostatin neurons was found in the rostral ventrolateral medulla oblongata at the rostrocaudal level of the presumed pre-Bötzing complex. Fluorescence double-labelling revealed the co-localization of NK1R and somatostatin (Fig. 2). NK1R and somatostatin co-labelled neurons exhibited small- to medium-sized ovoid cell bodies, in agreement with the interneuron phenotype of pre-Bötzing complex neurons (Gray et al., 2001). These neurons were most abundant at +9 to +10 mm obex in an area delineated dorsally by the compact part of the ambient nucleus, ventrally by the ventral surface (retro-olivary sulcus), and medially and laterally by the medial and lateral rostral subnuclei of the lateral reticular nucleus, respectively (Fig. 3). This area closely corresponded to the presumed pre-Bötzing complex region, as suggested by our comparative cytoarchitectonic analysis.

To further investigate the distribution of NK1R and somatostatin neurons in the medulla, their rostrocaudal and regional distribution was determined. The number of ventrolateral medullary NK1R and somatostatin neurons was low at +0 to +5 mm obex, began to increase around +6 mm, and was highest at +9 mm rostral to the obex. The number of NK1R and somatostatin neurons declined after the maximum and decreased towards the rostral end of the medulla (+14 mm obex; Fig. 3; for details see Supplementary Figs 1A and 2A). NK1R and somatostatin-immunostained neurons were clustered in ventral parts of the ventrolateral medulla where cells were abundant at the level of +9 mm to the obex, with some neurons positioned close to the ventral surface between myelinated fibres of the spinothalamic tract.

As rostral ventral respiratory neurons can intermingle with vagal motoneurons and adrenergic neurons of the cardiovascular C1 group (Fig. 3; Benarroch et al., 2003; Blessing, 2004), we also used markers for vagal motoneurons (ChAT) and for monoaminergic neurons (PH8) to distinguish these neuronal populations. By using this approach, NK1R and somatostatin-positive neurons could be readily distinguished from motoneurons since they were immunonegative for ChAT (n = 3, data not shown). Although NK1R- and somatostatin-positive neurons accumulated ventrally, they were also found dorsomedially in the area of the adrenergic neurons of the cardiovascular C1 group (Fig. 3; Supplementary Fig. 3A and B).

Results

For localization of the putative pre-Bötzing complex region, unconventionally thick (100 μm) serial transverse sections of human brainstems with no history of neurological disease were stained with the pigment-Nissl method (n = 9; Braak et al., 2003). As outlined in the ‘Materials and methods’ section, we expected the pre-Bötzing complex to be between +7 and +14 mm rostral to the obex (Fig. 1A). In each transverse section, we focused on the ventrolateral medulla, where we expected rostral ventral medullary respiratory neurons in the area between the inferior olive medially, the spinal trigeminal nucleus laterally and the compact part of the medial nuclei laterally and the compact part of the ambiguus nucleus dorsally (Fig. 1B).

As described above, the putative region of the human pre-Bötzing complex was delineated on the basis of cytoarchitectonic criteria that have also been used in cat (Schwarzacher et al., 1995) and rat (Ruangkittisakul et al., 2006). Using this comparative technique, the rostrocaudal level of the putative human pre-Bötzing complex was characterized by the rostral end of the lateral reticular nucleus, and the origin of the most rostral rootlets of the hypoglossal nerve (Fig. 1B). This corresponded to approximately +9 mm rostral to the obex. On transverse sections, the area of the putative pre-Bötzing complex is located in the ventrolateral medulla, dorsal to the rostral rootlets of the hypoglossal nerve and the retro-olivary sulcus, and directly medial to the ventral rootlets of the vagal nerve (Fig. 1C). To provide additional evidence for these conclusions, we performed further immunocytochemical investigations and a detailed analysis of brains of patients suffering from neurodegenerative diseases.

onset: 36.5 ± 15.6 years; duration of disease: 22 ± 5.4 years). Disease onset was determined as the time that the patient or a close relative noticed, without a doubt, the first neurological symptoms of SCA3 (i.e. dystonia, diplopia or ataxia). All of the patients with SCA3 suffered from progressive gait, stance and limb ataxia, dysarthria and dysphagia and were eventually wheelchair bound. Respiratory disorders or signs of abnormal respiration were not reported in the medical records of these patients. Six of the eight patients with SCA3 died from dysphagia-related complications (i.e. aspiration pneumonia or dehydration). Clinical diagnosis was confirmed by neuropathological investigation. All SCA3 brains exhibited widespread astrogliosis. Neuronal loss was detected in the cerebellar dentate nucleus, red nucleus, substantia nigra, pallidum, vestibular nuclei and external cuneate nucleus. Ataxin-3 immunopositive neuronal intranuclear inclusions were present in affected and spared brain areas (Rübs et al., 2008).

MSA represents an adult-onset progressive degenerative disease of the nervous system with average disease duration of ∼6 years. The core clinical features of this sporadic disease of unknown cause comprise autonomic failure with cardiovascular dysfunctions and sleep apnoea, parkinsonism, cerebellar ataxia and pyramidal dysfunctions (Braak et al., 2003; Benarroch, 2007). Clinical diagnosis of the brains in this study was confirmed neuropathologically using immunoreactions against alpha-synuclein protein, in addition to the Campbell–Switzer and Gallyas silver staining methods. In all MSA cases, argyrophilic and/or α-synuclein immunopositive neuronal and oligodendroglial inclusions characteristic of MSA were present. MSA cases showed no or very low levels of Parkinson’s and Alzheimer’s disease-related pathology.
However, as described previously in human material (Benarroch et al., 2003), neurons positive for both NK1R and somatostatin showed no immunostaining for PH8, and were therefore clearly distinct from the monoaminergic medullary neurons (n = 3, data not shown). In summary, double-labelling for NK1R and somatostatin is helpful to identify putative pre-Boëtzinger complex neurons in human brain tissue and to distinguish them from other brainstem neurons.

Distribution of ambigual vagal motoneurons

Vagal motoneurons of the ventrolateral medulla form the ambigual complex, which is composed of several subnuclei representing groups of neurons innervating different pharyngeal and laryngeal muscles (Bieger and Hopkins, 1987). In human control tissue, we mapped the distribution of vagal ambigual motoneurons using ChAT-immunocytochemistry (Supplementary Fig. 3C). Within the ventrolateral medulla, ChAT neurons aggregated in a dorsal group of neurons (d-ChAT), that corresponded to the compact and semicom pact ambigual nucleus in rats (Fig. 3C–E; Bieger and Hopkins, 1987), whereas ventral ChAT neurons (v-ChAT) were more loosely scattered, forming the ventral ambigual formation (external ambigual formation in rats; Fig. 3A, B, E and F; Supplementary Fig. 2C). Serial sections (n = 5) revealed large numbers of dorsal ChAT neurons at + 8.5 to + 11.0 mm from the obex corresponding to the compact and semicom pact ambigual motoneurons of pharyngeal and oesophageal muscles in rats (Bieger and Hopkins, 1987). Ventral ChAT neurons accumulated caudal and rostral to this level, again in analogy to the distribution of laryngeal and pharyngeal motoneurons forming the external ambigual formation in rats (Bieger and Hopkins, 1987).
In experimental animals, the rostrocaudal distribution of ambiguous motoneurons closely corresponds to the different subgroups of ventral respiratory neurons, including the pre-Bötzinger complex (Feldman and Del Negro, 2006). Therefore, in analogy to data from experimental animals, the caudal ventral ChAT accumulation that we found in human brains at +6.5 to +8.5 mm obex corresponds to the level of the inspiratory rostral ventral respiratory group (Fig. 3A), whereas the rostral ventral ChAT accumulation at
degeneration in the area of the putative pre-Bo¨ tzinger complex, predicted that brains of patients with MSA should show neuronal central respiratory dysfunctions in their medical records. We disturbances (Benarroch in patients with MSA suffering from sleep-related respiratory more or less left intact.

To study the possible pathophysiological role of the presumed pre-Bo¨tzinger complex cells in neurodegenerative diseases

Delineation of the putative pre-Bo¨tzinger complex region in pigment-Nissl-stained human tissue

Diaminobenzidine-immunostained sections were counterstained with the pigment-Nissl method. Importantly, NK1R- and somatostatin-positive pre-Bo¨tzinger complex cells exhibited small- to medium-sized ovoid cell bodies and harboured many lipofuscin granules, making it possible to reliably identify these cells in unconventionally thick (100 µm) pigment-Nissl-stained sections of human brainstem (Fig. 4A1, B1, C1 and D1). In addition, ChAT-positive vagal motoneurons exhibited characteristic medium-to-large unpigmented multipolar cell bodies with a dense Nissl stain, which made it possible to identify the different subnuclei of the ambigual complex in this material (Fig. 4A1 and B1). Using the larger ambigual motoneurons for orientation, we could precisely identify the rostrocaudal level of the presumptive pre-Bo¨tzinger complex in human brainstem sections stained with pigment-Nissl only. Furthermore, we could identify the group of small- to medium-sized ovoid cell bodies that represent the NK1R- and somatostatin-positive cell population. Taken together, the level of the presumptive pre-Bo¨tzinger complex could be localized in pigment-Nissl-stained sections to an area located directly rostral to the rostral end of the caudal accumulation of ventral ambigual motoneurons (at +8.5 mm obex), and directly caudal to the maximal accumulation of dorsal ambigual motoneurons forming the compact ambigual nucleus (at +10.0 mm to the obex; Fig. 5A and B).

Pre-Bötzinger complex cells in neurodegenerative diseases

To study the possible pathophysiological role of the presumed human pre-Bötzinger complex area in the neuronal control of breathing, we took advantage of the fact that some neurodegenerative diseases involve the brainstem. We investigated brains of patients suffering from MSA (n = 10), a disease known to cause central respiratory deficits (Nogue’s and Benarroch, 2008), and brains of patients with SCA3 (n = 8), suffering from dysphagia, but without central respiratory dysfunctions in their medical records. We predicted that brains of patients with MSA should show neuronal degeneration in the area of the pre-Bötzinger complex, whereas in brains of patients with SCA3, the same area should be more or less left intact.

Since depletion of medullary NK1R neurons has been described in patients with MSA suffering from sleep-related respiratory disturbances (Benarroch et al., 2003), we performed NK1R-immunostaining in tissue of patients with MSA. In these brains, the number of NK1R cells consistently underwent a strong reduction at the rostrocaudal level of the pre-Bötzinger complex, especially in ventral areas (Fig. 5C; Supplementary Fig. 1A and B). In contrast, in the brainstems of our patients with SCA3, the number of NK1R-positive cells was only slightly diminished (Fig. 5D; Supplementary Fig. 1C). A very similar pattern was observed for somatostatin cells in MSA brains, while SCA3 brains were largely unaffected (Fig. 5C and D; Supplementary Fig. 2). In line with these results, in pigment-Nissl-stained tissue of the same brains, the number of small- to medium-sized medullary pigmented cells was severely reduced in MSA but not in SCA3 brainstem sections (Fig. 4D). Taken together, these data demonstrate a severe loss of neurons in the presumptive pre-Bötzinger complex region of MSA brains but not in the pre-Bötzinger complex region of patients with SCA3.

We further examined MSA and SCA3 brains for neurons of the ambigual complex. As described above, vagal motoneurons were identified by ChAT-immunoreactivity, by their characteristic large multipolar size, and by their strong Nissl staining in pigment-Nissl material. Remarkably, the dorsal compact ambigual nucleus, which innervates swallowing muscles, was unaffected in MSA brains (Supplementary Fig. 4A and B). In contrast, in brains of patients with SCA3 with severe swallowing problems (Rüb et al., 2008), ambigual motoneurons were strongly reduced (Supplementary Fig. 4C), consistent with the clinical picture of dysphagia.

Discussion

The localization of the presumed pre-Bötzinger complex was studied in human brain using three complementary approaches: (i) comparison of cytoarchitectonic landmarks within the medulla oblongata (regions and nuclei) between experimental mammals and humans; (ii) immunocytochemical staining of putative pre-Bötzinger complex neurons in human material based on data from laboratory animals; and (iii) systematic comparison between brainstems of normal human individuals and of patients suffering from neurodegenerative diseases associated with the occurrence of breathing deficits of different pathological origin (i.e. MSA and SCA3).

Using these strategies in combination, we have localized the putative human pre-Bötzinger complex in the ventrolateral area of the medullary reticular formation at the rostrocaudal level of +9 mm from the obex. The pre-Bötzinger complex is characterized by an aggregation of neurons that co-localize NK1R and somatostatin, two established markers for pre-Bötzinger complex neurons in experimental animals. These cells were heavily reduced in numbers in brains of patients with MSA suffering from central respiratory deficits but not in brains of patients with SCA3, who did not suffer from central respiratory problems. Importantly, after the presumed human pre-Bötzinger complex region was identified using the above-mentioned techniques, we could reliably detect presumptive pre-Bötzinger complex neurons in pigment-Nissl-stained sections. Thus, routine neuropathological investigations of the pre-Bötzinger complex region in neurological diseases with central respiratory problems will be feasible in the future.
The putative pre-Bötzinger complex region can be localized in human brainstem sections

Several studies in the past provided descriptions of human brainstem respiratory groups including the pre-Bötzinger complex based on correlations with animal studies (Blessing, 2004; Nogués and Benaroch, 2008). Here, we extend these reports and compile a detailed description of the putative human pre-Bötzinger complex using the Braak method (Braak and Braak, 1991; Braak et al., 2003), which is most suitable to identify cytoarchitectonic landmarks (areas and nuclei) in human post-mortem material. The

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**Figure 4** Localization of the human pre-Bötzinger complex in pigment-Nissl-stained sections. The region of the human pre-Bötzinger complex can be characterized by an accumulation of densely pigment-Nissl-stained small- to medium-sized ovoid cell bodies (black arrows), within the ventrolateral medulla, in an area lateral to the inferior olive (IO), and ventral to the dorsal compact part of the ambiguous nucleus (Amb), in control material (A1, B1, C1 and D1). In MSA brains (A2, B2, C2 and D2), these pre-Bötzinger complex cells (preBötC) are strongly reduced (C2 and D2), whereas the Amb cells are not affected (B2). In contrast, in SCA3 brains (A3, B3, C3 and D3), pre-Bötzinger complex cells are only slightly diminished (C3 and D3), whereas Amb cells are heavily reduced (B2). Note the punctate staining of reactive gliosis that is typical for SCA3 brains in C3. Pigment-Nissl-stained cells were counted in serial transverse sections in the area between the dorsal edge of the ambiguous nucleus, the ventral surface, the spinal trigeminal tract and the inferior olive. Mean and standard deviation of cell numbers in corresponding areas of the same rostrocaudal level from the left and right sites from nine control (D1), eight MSA (D2) and eight SCA3 (D3) brains. Scale bars = 1 mm in A1, B1 and C1; 500 µm in A2, B2 and C2; 80 µm in A3, B3 and C3.
Figure 5. The human pre-Bötzing complex in control and neurodegenerative diseases. (A) The putative human pre-Bötzing complex (preBötC) is localized within the ventrolateral medulla oblongata, rostral to the lateral reticular nucleus (LRN), and caudal to the facial nucleus (VII). At the level of the pre-Bötzing complex (+9 mm from obex), the dorsal ambiguous nucleus (Amb dorsal) exhibits its maximal expansion, whereas the ventral nuclei of the ambiguous formation (Amb ventral) show prominent expansions rostral and caudal to the pre-Bötzing complex. (B) On a transverse section at +9 mm from obex, the pre-Bötzing complex is positioned lateral to the inferior olive (IO), and medial to the rostral vagal nerve rootlets (X). At this rostrocaudal level the highest number of somatostatin (SOM) and neurokinin-receptor 1 (NK1R)-positive neurons is found. Ventral (ChAT-v) and dorsal (ChAT-d; Amb, ambiguous nucleus) cholinergic vagal motoneurons serve as landmarks. (C) In humans with multiple systemic atrophy (MSA) somatostatin- and NK1R-positive neurons are diminished whereas the dorsal ChAT group of neurons is unaffected. (D) In spinocerebellar ataxia type 3 (SCA3), somatostatin- and NK1R-positive neurons are preserved, whereas the ventral and dorsal cholinergic motoneurons (ChAT-v and ChAT-d) are reduced in numbers. Patients with SCA3 had swallowing problems, but no reported respiratory disorders. ICP = inferior cerebellar peduncle; XII = hypoglossal nucleus.
During mammalian phylogenesis (Schwarzacher et al., 1999, 2001; Stornetta et al., 2003), correlation of human medullary levels with those of laboratory animals such as cats, rats or mice—the major animals for respiratory research—is confirmed by the unusual size of the human inferior olive (Blessing, 2004). In addition, the human dorsal lateral medulla including the spinal trigeminal tract and nucleus (laterally) appears to undergo fewer distortions between the inferior olive, (medially) and the spinal trigeminal peduncle, are enlarged. However, the ventrolateral medullary area between the inferior olive, (medially) and the spinal trigeminal tract and nucleus (laterally) appears to undergo fewer distortions during mammalian phylogenesis (Schwarzacher et al., 1999; Ruangkitissakul et al., 2006). Thus, it can be used as a reference for comparative neuroanatomical studies. In addition, branchiomeric motoneurons, including the ambigual complex, are arranged in a rostrocaudal order that follows the ontogenetic segmentation of the rhombomeres. Recent evidence from genetic mouse studies suggests that the adult respiratory neuronal network is functionally organized according to the rhombomeric segmentation of the brainstem in embryos (Thoby-Brisson et al., 2009). Thus, the rostrocaudal organization of the ambigual subgroups can serve as a cytoarchitectonic template for the identification of human medullary respiratory groups.

Within the ventrolateral medulla, the ambigual complex is composed of subnuclei representing groups of neurons innervating different pharyngeal and laryngeal muscles (Bieger and Hopkins, 1987). We identified dorsal and ventral ambigual neurons with ChAT in human material that correspond to the dorsal compact and semi-compact ambigual nucleus, and the ventral external ambigual formation, respectively. In addition, ChAT-positive vagal motoneurons exhibited characteristic pigment-Nissl cell staining making it possible to reliably identify the different subnuclei of the ambigual complex in human transverse sections. In analogy to data from experimental animals, the rostral ventral ChAT accumulation corresponds to the level of rostral expiratory neurons forming the Bötzinger complex. Directly caudal to the Bötzinger complex, the presumed pre-Bötzinger complex is located in the ventrolateral medullary area at +9 mm from the obex, followed by a caudal accumulation of ventral ChAT neurons that corresponds to the level of the inspiratory rostral ventral respiratory group (Feldman and Del Negro, 2006). Thus, the characteristic of ambigual subnuclei is another approach that can be employed to find the rostrocaudal sequence of medullary respiratory groups in pigment-Nissl-stained human brain sections.

NK1R and somatostatin label neurons of the human pre-Bötzinger complex region

In experimental mammals, neurons of the pre-Bötzinger complex area can be labelled with antibodies against NK1R and somatostatin (Gray et al., 1999, 2001; Stornetta et al., 2003). Here, we systematically investigated serial transverse sections of human brainstem tissue without neuropathological alterations (‘control tissue’). We found a characteristic distribution of NK1R and somatostatin-positive neurons in the rostral ventrolateral medulla oblongata with a strong accumulation of cells expressing both markers at the level of +9 mm from the obex, in the area of the pre-Bötzinger complex, as assumed based on our cytoarchitectonic deliberations (see previous paragraph).

Benarroch et al. (2003) previously reported abundant NK1R neurons in the human ventrolateral medulla. These neurons were found to be distinct from cholinergic or catecholaminergic neurons, as also shown in the present study. They also discussed possible localizations of the human pre-Bötzinger complex but primarily focused on the distribution of NK1R neurons at brainstem levels caudal to the pre-Bötzinger complex level. In our study, the entire rostral medulla from the pontomedullary fissure (+14 mm obex) to obex levels was systematically analysed in serial sections. In addition, NK1R labelling was combined with somatostatin labelling. Furthermore, NK1R/somatostatin-positive cells were found to exhibit a characteristic size and staining in pigment-Nissl preparations, making it possible to reliably identify these cell populations in fixed human material. Using pigment-Nissl staining alone, the area of the ventrolateral medulla containing the presumptive pre-Bötzinger complex neurons can be sufficiently circumscribed to detect a gross involvement of the pre-Bötzinger complex in neurological diseases. However, for more detailed analyses and the detection of discrete neuronal alterations, the use of neurotransmitter markers remains crucial. In addition to NK1R and somatostatin, which have been used as markers in our study, other neurochemical markers for pre-Bötzinger complex neurons, such as glutamate receptors (Paarmann et al., 2000; Morgado-Valle and Feldman, 2007) or the calcium-binding protein parvalbumin (Alheid et al., 2002) could also be helpful.

**Cells of the pre-Bötzinger complex region are severely reduced in multiple system atrophy but not in spinocerebellar ataxia type 3 brains**

Functional studies in rats and mice have provided robust evidence for an important role of NK1R and/or somatostatin-positive pre-Bötzinger complex neurons in regular respiratory control (Gray et al., 2001; McKay and Feldman, 2008; Tan et al., 2008). Experimental reduction of NK1R or of somatostatin neurons led to severe breathing disturbances, including apnoea. Here, we found a strong reduction of NK1R and somatostatin as well as pigment-Nissl-stained neurons in the presumed pre-Bötzinger complex region in patients suffering from MSA, a severe brainstem neurodegenerative disease commonly associated with central respiratory deficits (Lantos and Quinn, 2003; Paulson, 2007; Nougues and Benarroch, 2008). Central respiratory dysfunctions of patients with MSA include sleep-related breathing disorders, particularly laryngeal stridor and sleep apnoea, but also dyssrhythmic breathing and arrhythmic respiration (Nougues and Benarroch, 2008). Concurring with the results of our study, Benarroch et al. (2003) found a
depletion of medullary NK1R neurons in patients with MSA suffering from sleep-related respiratory disorders. In addition, Tsuboi et al. (2008) reported a case of a man with Perry syndrome (autosomal dominant parkinsonism associated with depression, weight loss and central hypoventilation), who died from respiratory failure and sepsis. The authors found a loss of NK1R and tyrosine hydroxylase immunoreactive neurons in the ventrolateral medulla.

Interestingly, a number of breathing disorders, including sleep disturbances, have also been reported in patients with SCA3 (Iranzo et al., 2003; Rüb et al., 2008). While the brainstems of our patients with SCA3 showed widespread neurodegeneration with severe reduction of ambigual motoneurons and only slightly diminished pre-Bötzinger complex neurons, ambigual motoneurons were preserved and the presumed pre-Bötzinger complex area considerably degenerated in our patients with MSA. These different distribution patterns of medullary neuronal loss support the view that involvement of the central respiratory network, including the pre-Bötzinger complex, contributes to the occurrence of breathing disorders in MSA (Nogués and Benarroch, 2008), while damage to respiratory motoneurons, especially ambigual motoneurons innervating laryngeal and pharyngeal muscles, represents the main cause of the breathing disturbances occurring in patients with SCA3 (Iranzo et al., 2003; Rüb et al., 2008).

Ambigual motoneurons are severely reduced in spinocerebellar ataxia type 3 but not in multiple system atrophy

Found in our patients with SCA3, but absent in patients with MSA, loss of pharyngeal motoneurons in the medullary ambigual nucleus offers an appropriate explanation for the impairments of upper airway muscle control in patients with SCA3, and may contribute substantially to dysphagia in patients with SCA3 (Iranzo et al., 2003; Rüb et al., 2008). Indeed, dysphagia, which can lead to nutritional deficiencies, weight loss and dehydration, represents an important risk factor for aspiration pneumonia in patients with SCA. Rüb et al. (2008) found neurodegeneration of ingestion-related brainstem nuclei including ambigual motoneurons in dysphagic patients with SCA3, who died of aspiration pneumonia in the majority of cases. Based on these findings, a rehabilitative swallowing therapy was proposed that is better adapted to the needs of patients with brainstem lesions.

A number of diverse neurological diseases have been described that affect respiratory control (Nogués and Benarroch, 2008). These include disorders of the respiratory motor unit (e.g. motor neuron diseases and peripheral neuropathies) as well as disorders affecting the central respiratory network. The latter imply brainstem and spinal cord lesions, degenerative disorders (e.g. MSA, parkinsonism or Perry syndrome), and putative developmental disorders (e.g. sudden infant death syndrome). Sudden infant death syndrome is postulated to result from abnormalities in brainstem control of autonomic function and breathing during a critical developmental period (Kinney et al., 2009; Paterson et al., 2009). Lavezzi and Matturi (2008) examined NK1R and somatostatin immunoreactivity in brains from foetal and newborn victims of unexplained sudden death. Interestingly, these authors found developmental brainstem defects, including hypoplasia and reduced immunoreactivity, within the brainstem reticular formation. In view of the topographical, architectonic and immunocytochemical data of our study, the localization of the human pre-Bötzinger complex suggested by Lavezzi and Matturi (2008), i.e. caudal to the compact division of the ambigual complex, and in close association to the dorsal subnucleus of the inferior olive, is questionable, however. Sudden death is also the leading cause of death in MSA. In a meticulous study of human autopic material, Tada et al. (2009) found a depletion of medullary serotonergic neurons in patients with MSA who died of sudden death within 3.5 years of disease onset. These results indicate that the serotonergic medullary system is involved in early stages of MSA. A decline of serotonergic excitatory drive to the pre-Bötzinger complex, together with a depletion of pre-Bötzinger complex neurons, as described in the present study in MSA cases, could well be responsible for respiratory failure and sudden death in patients with MSA. The delineation of the putative human pre-Bötzinger complex reported here will help to address these and other urgent questions in the future.

In summary, a number of neuropathological studies provided correlations between structural deficits in the ventrolateral medulla and respiratory failure. The identification and precise localization of the human homologue of the pre-Bötzinger complex is an essential step towards a better understanding of human central respiratory disturbances. Since systematic polysomnographic investigations have now become more widespread and numerous patients have been identified who suffer from impaired respiratory control during sleep (Banno and Kryger, 2007; McKay et al., 2008; Nogués and Benarroch, 2008), clinical data will emerge that can be correlated with pre-Bötzinger complex pathology found upon autopsy. These data may help to establish new therapeutic guidelines, as has been the case with patients with SCA3 and treatment recommendation for dysphagia (Rüb et al., 2008).

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Supplementary material

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