Behaviour of raphe cells projecting to the dorsomedial medulla during carbachol-induced atonia in the cat

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1. The activity of most brainstem serotonergic cells is suppressed during sleep, particularly the rapid eye movement (REM) phase. Thus, they may play a major role in state-dependent changes in CNS functioning. Our main goal was to search for medullary raphe cells having axonal branches in the region of the hypoglossal (XII) motor nucleus and assess their behaviour during the atonia produced by microinjections of a cholinergic agonist, carbachol, into the dorsal pontine tegmentum. In chronic animals, such microinjections evoke a desynchronized sleep-like state similar to natural REM sleep; in decerebrate animals, they produce eye movements and a motor suppression similar to the postural atonia of REM sleep.

2. In decerebrate, paralysed, vagotomized and artificially ventilated cats, we recorded extracellularly from medullary raphe cells antidromically activated from the XII nucleus region. Forty-five cells recorded in the raphe obscurus and pallidus nuclei were antidromically activated with latencies characteristic of non-myelinated fibres (4.4–42.0 ms). For thirty-three of the forty-five cells, we found one or more axonal branches within or just below the XII nucleus. The remaining twelve cells, in addition to the XII nucleus, had axonal ramifications in the medial nucleus of the solitary tract (NTS) and/or the dorsal motor nucleus of the vagus (DMV).

3. A subset of fourteen spontaneously active cells with identified axonal projections were held long enough to be recorded during the carbachol-induced atonia, and eight of these also during the subsequent recovery and a systemic administration of the serotonergic 1A receptor agonist (±)-8-hydroxy-2-(di-N-propylamino)tetralin hydrobromide (8-OH-DPAT). All but one were suppressed during the atonia in parallel to the suppression of XII, phrenic and postural nerve activities (firing rate, 1.3 ± 0.7 Hz before and 0.1 ± 0.2 Hz after carbachol (means ± s.d.)). Following the recovery from the atonia, the firing rates of the eight cells increased to the pre-carbachol level (1.6 ± 1.0 Hz). Subsequently, all were silenced by 8-OH-DPAT.

4. These cells fulfil most physiological criteria for serotonergic cells and have the potential to modulate, in a state-dependent manner, activities in the motor XII nucleus, visceral sensory NTS, and DMV. The decrements in serotonergic neuronal activity that occur during the carbachol-induced atonia suggest that a similar withdrawal of serotonergic input may occur during REM sleep and contribute to the characteristic reductions in upper airway motor tone.

The neurones of the medullary raphe (mid-line magnus, pallidus and obscurus nuclei and cells of the parapyramidal region) form a heterogeneous and intermingled population having diverse properties and functions. Subsets of these neurones have axonal projections to the spinal cord dorsal horn, intermediolateral nucleus and ventral horn (Skagerberg & Björklund, 1985). In addition, some medullary raphe cells send their axons to the brainstem orofacial (trigeminal, facial, vagal, and hypoglossal (XII)) motor nuclei (Fort, Luppi, Sakai, Salvet & Jouvet, 1990; Manaker, Tischler & Morrison, 1992; Li, Takada & Mizuno, 1993), as well as to the nucleus of the solitary tract (NTS;
Thor & Helke, 1987; Schaffar, Kessler, Bosler & Jean, 1988), a major termination site of cranial nerve visceral afferents. Immunocytochemical studies combined with retrograde tracing demonstrated that many raphe cells projecting to the dorsomedial medulla are serotonergic (Thor & Helke, 1987; Schaffar et al. 1988; Li et al. 1993; Manaker & Tischler, 1993). In electrophysiological studies, serotonergic neurones can be recognized by their slow, regular discharge, long duration action potentials and slow axonal conduction velocities (West & Wolstencraft, 1977; Heym, Steinfels & Jacobs, 1982; McCall & Clement, 1989; see Jacobs & Azmitia, 1992, for a review). An important characteristic of brainstem serotonergic neurones, including those in the medulla, is the strong state dependence of their activity; they are most active during wakefulness, reduce their activity during slow wave sleep, and reach a minimum during rapid eye movement (REM) sleep (Heym et al. 1982; Trulson & Trulson, 1982).

In a series of recent studies, we investigated the hypothesis that a withdrawal of the serotonergic excitatory drive to upper airway; including XII, motoneurones plays an important role in the sleep-related decrements in their excitability (Kubin, Tojima, Davies & Pack, 1992; Kubin, Reignier, Tojima, Taguchi, Pack & Davies, 1994). The purpose of the present study was to identify cells that fulfill the three criteria required by this hypothesis: they have axonal arborizations within the XII nucleus; they are likely to be serotonergic; and they reduce their activity during sleep, particularly REM sleep, when the activity of upper airway muscles reaches its nadir (e.g. Sauerland, Orr & Hairston, 1981). Since a study of single cells during natural sleep is difficult to combine with electrophysiological investigation of their projections, we used acute decerebrate cats in which a state having important similarities to natural REM sleep (see below) was produced by pontine microinjections of a cholinergic agonist, carbachol.

The rationale for using the carbachol model is based on extensive evidence that pontine carbachol microinjections in both chronically instrumented and acutely decerebrate animals activate at least a subset of the same mechanisms as those activated during natural REM sleep. In brief, in chronically instrumented animals, there is: (1) a remarkable polygraphic similarity between the carbachol-induced state and naturally occurring REM sleep (Silberman, Vivaldi, Garfield, McCrery & Hobson, 1980; Vanni-Mercier, Sakai, Lin & Jouvet, 1989); (2) site specificity of the effect (Vanni-Mercier et al. 1989); (3) an appropriate REM sleep-related change in acetylcholine release at the same pontine sites where carbachol is effective (Kodama, Takahashi & Honda, 1990). In decerebrate animals, where only selected signs characteristic of REM sleep can be recorded, pontine carbachol has the same effective sites as in chronic animals, and its injections result in postural atonia (Morales, Engelhardt, Soja, Pereda & Chase, 1987; Kimura, Kubin, Davies & Pack, 1990; Lai & Siegel, 1990; Takakusaki, Matsuyama, Kobayashi, Kohyama & Mori, 1993), eye movements (Tojima, Kubin, Kimura & Davies, 1992), and a stereotyped depression of the respiratory motor output (Kimura et al. 1960). Importantly, the changes in membrane properties of lumbar motoneurones after the injection of carbachol in decerebrate cats are similar to those observed during natural REM sleep, including discrete, large amplitude, state-specific inhibitory post-synaptic potentials (Morales et al. 1987). Thus, changes in brainstem neuronal activity following pontine carbachol injections, whether in intact or decerebrate animals, are likely to result from activation of at least a subset of those mechanisms and pathways that are also activated during REM sleep.

Use of the acute carbachol model enabled us to determine directly the axonal projections of medullary raphe cells. We identified a population of putative serotonergic raphe cells that reduce their activity during the carbachol-induced atonia and have axonal projections that should allow them to control both the motor output from, and the visceral sensory input to, the dorsomedial medulla. A preliminary report has been published (Woch, Pack, Davies & Kubin, 1994).

METHODS

Animal preparation

The data were obtained from twenty cats of either sex (2.1–2.9 kg), preanaesthetized with ketamine (80 mg, i.m.) and diazepam (2 mg, i.m.), intubated, anaesthetized with halothane (0.2–1.0%) and decerebrated at a precollicular level under halothane (Kirsten & St John, 1978). The entire forebrain cranial to the superior colliculus was removed, thus removing all areas of the brain involved in the conscious perception of pain. Anaesthesia was then discontinued. The procedures used to minimize discomfort, for anaesthesia, for decerebration, and for all subsequent preparation of the animal and recording were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. The surgical methods, techniques of recording from peripheral nerves and pontine microinjections have been described in detail elsewhere (Kubin, Kimura, Tojima, Davies & Pack, 1993). In all experiments, the activities of one phrenic nerve (C5 branch), the genioglossal branches of both XII nerves and, to monitor postural motor tone, a branch of the fourth cervical (C4) nerve were recorded. The caudal medulla was exposed and the cerebellar vermis retracted rostrad to uncover the caudal 3–4 mm of the fourth ventricle. All recordings were done with the animal paralyzed (gallamine triethiodide, 10 mg, i.v., followed by a continuous infusion of 5 mg kg⁻¹ h⁻¹), vagotomized and artificially ventilated at elevated levels of O₂ (30–50% F₁o₂). Carbon dioxide was added to the inspiratory mixture (3–5%) to maintain a regular respiratory modulation of the XII nerve activity (end-tidal CO₂ of 4.5–6.0%; Godard capnograph). Within a given experiment, the CO₂ and O₂ levels were kept constant. Blood pressure was continuously monitored and its mean level was always above 80 mmHg. Rectal temperature was maintained at 37–38.5 °C by a servo-controlled heating pad.
Recording and microinjection procedures

Medullary raphe cell activity was recorded using glass pipettes filled with 2 M sodium acetate and 2% Pontamine Sky Blue dye (tip diameters, 2.5–3.5 μm; resistance, 2–0.35 MΩ) held in a piezoelectrically driven manipulator (6000ULN, Burleigh, Fishers, NY, USA). Cell activity was amplified (NL-100/104, Digitimer Research Instruments, Welwyn Garden City, UK), filtered (50–2000 Hz), and fed to a window discriminator. Peripheral nerve activities were amplified (Grass S511) and fed to analog moving averages (MA-821, CWE Inc., Ardmore, PA, USA) having time constants of 200 ms. The nerve activities and marker signals were recorded on an instrumentation tape recorder (Hewlett Packard 3968A) and monitored on a chart recorder (Gould TA2000).

To induce a state of postural atonia and respiratory depression, carbachol (10 mM in saline and 2% Pontamine Sky Blue) was microinjected into the dorsal pontine tegmentum from glass pipettes (tip diameters, 25–40 μm) using a series of pressure pulses controlled from a NeuroPhore BH-2 injection system (Medical Systems, Greenvane, NY, USA). The injected volumes (30–255 nl; mean, 165 nl) were determined by observing the movement of the meniscus through a pocket microscope with reticle. Typical injection sites were shown in our earlier publications (Kimura et al. 1990; Tojima et al. 1992). The effect of carbachol was recognized from the stereotyped suppression of the XII, phrenic and postural activities, and blood pressure and respiratory rate decreases, as described earlier (ibid.). Unless a complete recovery of neural activities occurred spontaneously, the effects of carbachol were reversed by injecting atropine sulphate (250 nl, 10 mM in saline) into the same pontine site, or i.v. (1 mg).

Antidromic activation of raphe neurones

A tungsten microelectrode (NL-05), held in a hydraulically controlled manipulator (F. Haer, Brunswick, ME, USA) and connected to an AC amplifier (NL-100/104), was inserted into the region of the XII nucleus using the obex as a reference. Typical coordinates were 1–0.1–3 mm lateral and ±0.5 mm rostrocaudal to the obex. At the beginning of the experiment, the electrode was placed at a locus where inspiratory activity could be recorded from large cells (likely to be motoneurones). Proper location was then verified by obtaining a sharp action potential with a latency of about 1 ms in the spike-triggered average (PAR 4202 averager) of the ipsilateral XII nerve activity (50–200 sweeps), using action potentials recorded with the tungsten electrode as triggers (see Kubin et al. 1993). The electrode was then disconnected from the amplifier, and connected to a constant current stimulus isolation unit (Grass S511/6A) controlled from a stimulator (Grass 588). Cells were tested for antidromic activation from the XII nucleus using single rectangular pulses (0.2 ms) of at least 400 μA amplitude applied at a frequency of 0.5 Hz.

Experimental protocol

We searched for cells along the mid-line from the obex to 3 mm rostral, at depths 1–0.5–5 mm below the floor of the fourth ventricle. For each spontaneously active cell found, we attempted to stimulate its axon from within the XII nucleus (original placement of the stimulating electrode ±0.3 mm). For cells that could be activated, a collision test was performed to ascertain the antidromic character of the response. A systematic record of the depth, threshold and latency of the antidromic response was obtained from a wider depth range than in the initial search: 0.7–2.7 mm from the dorsal medullary surface in 50 μm steps. Then, a sufficiently long segment of undisturbed activity of the cell was recorded, to obtain at least 250 spikes, and carbachol injected into the pons. Occasionally a spontaneously active cell tested for its projections to the XII nucleus could not be antidromically activated; instead another, silent cell was activated at the same recording site. Such silent cells were not submitted to carbachol challenge and were studied only to a limited extent. First, based on the stability of the threshold and latency, we assessed whether the cell was likely to be activated antidromically. If it was, we then varied the stimulus intensity and moved the stimulating electrode up and down to a limited extent (±0.3 mm) to find a minimum threshold point and sites at which responses with two or more fixed, yet distinct, latencies could be evoked. Cells having the latter feature were classified as having axons that branched within the stimulated region (cf. Davies & Kubin, 1986; Kubin, Kimura & Davies, 1991).

Following carbachol injection and evocation of the postural atonia and respiratory depression, the activity of most raphe cells studied was abolished. Since each cell could still be activated antidromically during the atonia, we then searched for additional axonal ramifications. Additional penetrations were placed within the dorsomedial medullary region in a 300–400 μm grid around the first penetration from which the cell was initially stimulated. During this period of 1–2 h, a spontaneous recovery from the effect of carbachol occurred in most cases. If the recovery was not complete, atropine was administered i.v. (3 experiments) or into the same pontine site as carbachol (1 experiment) (Kimura et al. 1990). Following the recovery of motor activities, a period of undisturbed spontaneous activity of the cell containing at least 250 spikes was recorded. Subsequently (±3-hydroxy-2-(di-N-propylamino)tetraethyl hydrobromide (8-OH-DPAT; Research Biochemicals International), a serotonin 1A receptor agonist, was administered either s.c. (0.2 mg) or i.v. (10–50 μg). If the cell ceased firing, the stability of the recording was again verified by antidromic activation.

After completion of all procedures, the recording site was marked by Pontamine Sky Blue (2–5 μA, 20 min), and the last stimulation site within the dorsomedial medulla was marked by an electrolytic microlesion (40 μA, 30 s). The animal was perfused intracardially with 100 ml of 10% formalin and the brainstem removed. After postfixation and cryoprotection, the medial region of the medulla was cut serially in the parasagittal plane. The sections (50 μm) were mounted, counterstained with Neutral Red, covered slipped and inspected on a Leitz microscope equipped with a drawing tube at ×55 magnification.

Off-line data analysis

The location of minimum threshold points from which axonal branches of the studied cells were stimulated in the dorsomedial medulla was determined using the analysis fully described previously (Davies & Kubin, 1986; Kubin et al. 1991). In brief, to localize the minimum threshold points, depth–threshold curves were plotted for each response characterized by a distinct latency, and the locations of the penetrations with the stimulating electrode redrawn from the serial sections of the medulla. The minimum threshold sites were plotted on the corresponding cross-sections using the microlesion site and dorsal medullary surface as references. Antidromically activated axons were considered to ramify within the stimulated region if, in a single penetration with the stimulating electrode, we encountered antidromic responses having at least two distinct latencies. Whenever the analysis of the
effective current spread made this likely, responses having the same latencies obtained from several closely spaced penetrations were regarded as representing one and the same branch. Finally, the locations of minimum threshold points were transferred onto a standard parasagittal cross-section of the medulla, so that their position with regard to the obex and the nuclei of the region was preserved. Similarly, the locations of the marked recording sites along the mid-line were transferred to a standard mid-sagittal section, so that their position with regard to the obex and the floor of the 4th ventricle was preserved.

To analyse the spontaneous activity patterns of the cells, the action potentials were converted to standard pulses. Interspike interval histograms and autocorrelation histograms were obtained from the control cell activity using an IBM computer-based data acquisition and analysis system (EGAA system, R-C Electronics, Santa Barbara, CA, USA).

The variability of the means is characterized by standard deviation (s.d.) throughout this report.

**RESULTS**

Our interest was limited to those neurones located along the mid-line of the medulla and having axonal projections to the XII nucleus. Therefore, the first step was to record from raphe neurones and verify that they were antidromically activated from the XII nucleus region. Forty-five cells were found, and these are described first. Twenty-nine of them were spontaneously active and thus suitable for the study of their behaviour following pontine carbachol injections. However, only fourteen of the spontaneously active cells with identified axonal projections were held long enough to be recorded during the carbachol-induced atonia, and eight of those during the subsequent recovery and 8-OH-DPAT administration. No criteria other than the presence of spontaneous activity, antidromic activation from the XII nucleus region and recording stability were used in selecting these cells for further study. In a single experiment, the response to pontine carbachol can be produced in the cat only twice (one microinjection on each side; Kimura et al. 1990). Cells suitable for tests with carbachol often had to be searched for for several hours, and the full testing required at least 3 h. Consequently, with one exception, each of the fourteen cells was recorded in a different animal. Thus, these fourteen extensively studied cells represent a randomly selected subset of those present along the mid-line of the lower medulla and antidromically activated from the XII nucleus region, except for the presence of spontaneous activity. Their properties are described in detail following the general description of all the cells antidromically activated from the XII nucleus.

**Branching of raphe cell axons in the dorsomedial medulla**

Forty-five cells recorded in the mid-line medullary raphe were antidromically activated from the XII nucleus region. The latencies for antidromic activation from points within or just below the XII nucleus ranged from 4.4 to 42.0 ms. The combined filled and open bars in Fig. 1 show the distributions of these latencies. This range corresponds to conduction velocities of 1.1–0.1 m s\(^{-1}\) for an estimated average conduction distance (straight line) between the stimulation and recording sites of 47 mm. For twenty-two of the forty-five cells, we recorded the action potential shape. In fifteen cells, it had a prolonged positive component, so that the total duration was 3–5 ms. In the remaining seven cells, the positive component was not apparent, and the total duration was less than 2 ms.

For twenty-seven of the forty-five cells (17 of the 29 spontaneously active and 10 of the 16 silent cells), two or more branches were identified in the stimulated region, as indicated by the presence of two or more minimal threshold points for antidromic responses having distinct latencies along a single electrode track. In individual cells, the difference between the shortest (5.5–42.0 ms) and the longest (6.0–44.0 ms) antidromic latencies was 0.5–17.0 ms.

Except for the cells described in the next section, antidromic stimulation was performed from only one track. Therefore, the absence of evidence for axonal branching for eighteen of the forty-five cells is not definitive. To estimate the maximal effective current spread for our standard searching stimulus (400 \(\mu\)A, 0.2 ms), we overlaid the widest depth–threshold curves selected from eight cells, whose antidromic latencies covered most of the observed range (7–34 ms), on the basis of their smooth and symmetrical shape around the minimum threshold point, which is indicative of stimulation of a single axonal branch at a single point throughout the penetration (Davies & Kubin, 1986). Based on this estimate, a 400 \(\mu\)A stimulus will excite

![Figure 1. Distribution of antidromic latencies of responses obtained by microstimulation within or adjacent to the XII nucleus from the 45 raphe cells identified as sending axons to the dorsomedial medulla](https://www.jospehphysoc.org)
axonal branches located less than 350 μm from the stimulating electrode tip (cf. depth–threshold curves in Figs 2 and 3). Thus, for these thin fibres, stimulation within a single electrode track covers only a small volume of the dorsomedial medulla (cf. Kubin et al. 1991).

Depth–threshold curves were constructed for all antidromically activated cells. Figures 2 and 3 show examples of such curves for two cells with axonal branches. For the cell shown in Fig. 2, the nine penetrations made within the XII nucleus region covered an area extending 1 mm rostro-caudally and 0.9 mm mediolaterally from the obex. In eight of these penetrations, we found antidromic responses with at least ten different latencies and minimal thresholds ranging from 15 to 550 μA. All axonal branches so identified were localized within, or ventral to, the XII nucleus. However, in this early experiment of this series, more dorsal regions (NTS and dorsal motor nucleus of the vagus (DMV)) were not as thoroughly tested as in subsequent experiments. Figure 2A shows, in a parasagittal section, the location of one of these penetrations and the depth–threshold curves obtained from this track. Responses with four different latencies were found (10.0, 10-7, 11-0 and 11-5 ms). For two of these (10.0 and 11-5 ms), a minimum threshold point could be localized

![Diagram](image_url)

**Figure 2. Example of a cell having axonal arborizations within and just below the XII nucleus**

A, location of one penetration with the stimulating electrode is shown on a parasagittal section of the dorsomedial medulla. Responses with four distinct latencies were found, as shown in the depth–threshold curves on the right. Corresponding symbols on the left show the locations of the minimal threshold points for the four responses along this penetration. Abbreviations: AP, area postrema; DMV, dorsal motor nucleus of the vagus. B, tests for the antidromic character of the response. Stimuli marked by triangles. Ba, the case when an antidromic response occurred in most, but not all, trials. Bb, the stimulus was applied with a slightly shorter interval after the spontaneous spike than in Ba and, due to collision, no antidromic response occurred. Traces in Bc show that the cell could respond antidromically to a pair of stimuli applied 13 ms apart. Bd, the shape of a spontaneous action potential recorded on an expanded time scale to show the characteristic, prolonged positive component. Each record in B contains 3–5 superimposed sweeps. Data are from a different penetration than the one shown in A; this is cell 22/1 in Table 1. In this and Figs 3B and 6C, the resolution of data acquisition (20 μs) was limited by the graphic interface, resulting in the unsmooth appearance of the traces.
because points with a higher threshold were found both above and below the minimum point. For the other two latencies, the location of the minimum could not be determined directly due to the occurrence of a response with another, shorter, latency (presumably evoked in a branch located more proximal to the cell body). To estimate the distance between the minimum threshold point and the stimulated fibre, data points corresponding to each of the four distinct latencies were interpolated by best-fitting second-order polynomials (cf. Davies & Kubin, 1986; Kubin et al. 1991). Figure 2B shows the results of collision tests (B1 and B2), the ability of the cell to follow stimuli applied with a frequency higher than the minimal interspike interval between the spontaneous and the antidromically

**Table 1. Properties of cells studied during the carbachol-induced atonia**

<table>
<thead>
<tr>
<th>Exp. or cell no.</th>
<th>Firing rate (Hz)</th>
<th>Location of branches</th>
<th>Latency (ms)</th>
<th>No. of tracks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con Carb Rec 8-OH-DPAT</td>
<td>Below XII XII Int DMV mNTS</td>
<td>Min Max No. of tracks</td>
<td></td>
</tr>
<tr>
<td>19/2</td>
<td>0·9 0 0 n.d. n.d.</td>
<td>+ + + n.d. n.d.</td>
<td>7·0 7·8 4</td>
<td></td>
</tr>
<tr>
<td>21/1</td>
<td>0·54 0 0 n.d. n.d.</td>
<td>n.d. + - - n.d.</td>
<td>26·0 27·0 5</td>
<td></td>
</tr>
<tr>
<td>22/1</td>
<td>2·4 0 2·6 0</td>
<td>+ + - - n.d.</td>
<td>11·0 14·5 9</td>
<td></td>
</tr>
<tr>
<td>23/5</td>
<td>1·4 0 1·1 0·1</td>
<td>n.d. + - - +</td>
<td>9·0 14·5 13</td>
<td></td>
</tr>
<tr>
<td>24/1</td>
<td>0·9 0·04 n.d. n.d.</td>
<td>n.d. + + n.d. n.d.</td>
<td>19·5 27·0 1</td>
<td></td>
</tr>
<tr>
<td>26/1</td>
<td>2·2 0·83 2·3 0</td>
<td>n.d. + - + +</td>
<td>6·5 15·0 1</td>
<td></td>
</tr>
<tr>
<td>27/1</td>
<td>1·6 0 1·5 0</td>
<td>n.d. + - - +</td>
<td>20·5 23·0 1</td>
<td></td>
</tr>
<tr>
<td>29/2</td>
<td>0·56 0·03 0·68 0</td>
<td>n.d. - + - -</td>
<td>38·0 40·0 1</td>
<td></td>
</tr>
<tr>
<td>33/4</td>
<td>1·2 0·02 2·8 0</td>
<td>n.d. + - - -</td>
<td>8·8 10·0 1</td>
<td></td>
</tr>
<tr>
<td>34/3</td>
<td>0·95 0·01 n.d. n.d.</td>
<td>+ - - - - -</td>
<td>5·8 7·2 1</td>
<td></td>
</tr>
<tr>
<td>37/1</td>
<td>1·2 0 n.d. n.d.</td>
<td>n.d. + - - -</td>
<td>4·8 5·0 4</td>
<td></td>
</tr>
<tr>
<td>39/2</td>
<td>2·6 0 n.d. n.d.</td>
<td>n.d. + - - -</td>
<td>5·6 - 1</td>
<td></td>
</tr>
<tr>
<td>39/7</td>
<td>0·28 0 0·3 0</td>
<td>n.d. + - - -</td>
<td>33·0 44·0 1</td>
<td></td>
</tr>
<tr>
<td>31/1</td>
<td>0·05 0·78 1·07 0·01</td>
<td>+ + - - + +</td>
<td>6·5 11·0 7</td>
<td></td>
</tr>
</tbody>
</table>

Con, control; Carb, carbachol; Rec, recovery; Int, nucleus intercalatus; No. of tracks, number of tracks with stimulating electrode; n.d., not determined; +, present; -, absent.

**Figure 3. Example of a cell having axonal arborizations within the XII, medial NTS and DMV**

(A) depth–threshold curves obtained from one penetration through the NTS, DMV and XII nucleus, as marked to the left of the depth axis. B1, superimposed responses obtained by stimulation at a depth of 1·75 mm and with a stimulus intensity at which, in individual sweeps, a response with either 8 or 14·5 ms latency was evoked in a random fashion. B1c, demonstration that the antidromic response of the cell can follow a pair of stimuli applied with an interval shorter than the 'critical interval' found with the collision test. Stimuli marked by triangles. For further explanation, see text. This is cell 26/1 in Table 1.
evoked spikes \((Bc)\), and the shape of a spontaneous action potential with the characteristic prolonged positive component \((Bd)\).

In contrast to the limited projections found for the cell in Fig. 2, the cell shown in Fig. 3 had axonal branches in the medial NTS, DMV and XII nuclei. Four antidromic latencies were detected in the penetration shown in Fig. 3A, with minimum threshold points located in regions corresponding to the three nuclei. In addition, a branch was found in the reticular formation ventral to the XII nucleus. Figure 3Ba shows superimposed sweeps of antidromic responses produced by stimulation at a depth of 1.75 mm with a stimulus intensity such that the response with either the 8 ms or the 14-5 ms latency occurred at random. Figure 3Bb shows the collision of the antidromic response with a spontaneous spike, and Fig. 3Bc the ability of the cell to follow a pair of stimuli at an interval shorter than that possible between the spontaneous and antidromic action potentials.

In summary, for thirty-three of the forty-five cells, we found one or more axonal branches within, and/or about 300 \(\mu m\) ventral to, the XII nucleus. The remaining twelve cells, in addition to one or more branches in the XII nucleus, had branches in structures dorsal to the XII nucleus.

**Spontaneous activity patterns in the cells studied during the carbachol-induced atonia**

A subset of fourteen spontaneously active and antidromically excited cells were recorded during the atonia produced by carbachol. The major features of these cells are listed in Table 1. For thirteen of the fourteen cells, we obtained evidence of axonal branching within the dorsomedial medulla. For the remaining one, we found only its axon in the XII nucleus, yet the search for branches was limited to one penetration and the negative evidence cannot be regarded as conclusive (cell 39/2 in Table 1). The antidromic latencies of these cells (filled bars in Fig. 1) were representative of those found for the larger group of raphe

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**Figure 4.** Examples of interval histograms (left column) and autocorrelograms (right column) from three cells \((rows A, B and C)\) antidromically activated from the dorsomedial medulla.

Bin widths: 32 ms in \(A\) and 64 ms in \(B\) and \(C\). Note that the interval histograms are skewed to the right and that oscillations on the correlograms are either absent or damped after 3-4 cycles, indicating a relative irregularity of the firing rate. These are cells 22/1, 29/2 and 34/3 of Table 1, respectively.
cells described in the preceding section. Only the presence of spontaneous activity distinguished this group from the remaining cells, of which fifteen of thirty-one were silent.

The spontaneous activity of these fourteen cells was relatively irregular. The middle two quartile ranges of the interval histograms corresponded to 40–200% of the median interspike interval (mean, 85 ± 50%). Most of the histograms were also slightly skewed to the right, so that the mean third-to-second quartile ratio was 1.4 ± 0.4 and significantly different from 1:0 (P < 0.02; range, 0.9–2.0).

Figure 4 (left column) shows three typical examples. The autocorrelograms of the cell activities (Fig. 4, right column) were either flat (part A), or showed moderate oscillations that were damped after 3–4 cycles (parts B and C).

**Behaviour of antidromically activated cells during the carbachol-induced atonia**

Figures 5A and 6A show examples of changes in the activity of two cells following pontine microinjection of carbachol. They were selected because they represent the extremes of the rate of the response to carbachol: a very gradual suppression of motor activities in Fig. 5A, and a rapid suppression in Fig. 6A. In these, and all other cases, the change in cell firing rate paralleled the suppression of motor activities.

Following a successful recording throughout the time course of the carbachol-induced depression of motor activities, eight cells were maintained until a sustained recovery from the depression either occurred spontaneously or was produced by atropine. In all eight cases, the cell firing rate returned to, or to slightly above, that observed before carbachol injection (Table 1). After the recovery from the effect of carbachol, the eight cells were challenged with 8-OH-DPAT administration (0.2 mg s.c. for 3 cells, and 10–60 μg i.v. for 5 cells). In all cases, 8-OH-DPAT abolished the activity or reduced it to less than 0.1 Hz (Table 1). Examples are shown in Figs 5B and 6B.

Figure 7 shows the mean control firing rates and those during the carbachol-induced atonia for the thirteen cells

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**Figure 5. Example of suppressions of the activity of an antidromically activated cell produced by pontine microinjection of carbachol (A) and systemic administration of 8-OH-DPAT (B)**

8-OH-DPAT (0.2 mg s.c.) was injected after the recovery from the effect of carbachol, about 2.5 h after the record in A. Signals from the top are: action potentials of the cell, cell firing rate determined in successive non-overlapping 10 s bins, moving average of the XII nerve activity (XII), moving average of the postural activity in a triangle of the top record (c.p.c. 200%), and record of the injection. This is cell 22/1 in Table 1.
suppressed by carbachol. The average was $1.3 \pm 0.7$ Hz before, and $0.1 \pm 0.2$ Hz after, carbachol ($P < 0.0001$, paired t test). (The one cell not suppressed is not shown in this figure, see below.) For the seven of those thirteen cells that were tested further, the figure also shows their firing rates after the recovery from the carbachol effect and following the administration of 8-OH-DPAT. For these seven cells, the average firing rates before and after carbachol were also different ($1.4 \pm 0.8$ and $0.1 \pm 0.3$ Hz, respectively; $P < 0.003$, paired t test), while there was no difference between the firing rate before carbachol and after the recovery ($1.6 \pm 1.0$ Hz; $P > 0.4$, Wilcoxon signed rank test). The average firing rate after carbachol was not different from that following 8-OH-DPAT ($0.01 \pm 0.04$ Hz; $P > 0.6$, Wilcoxon signed rank test).

One cell (listed at the bottom of Table 1) increased its firing rate following carbachol administration, and a further increase was observed following the recovery from the carbachol-induced atonia. Thus, the cell was classified as not responsive, rather than paradoxically responsive, to the carbachol-induced motor suppression. Otherwise, the cell was not different from the remaining thirteen cells, although it was only partly suppressed by 20 and 40 $\mu$g of 8-OH-DPAT and became silent only after a cumulative dose of $60 \mu$g (i.v.). In contrast, three other cells were silenced by 10–25 $\mu$g of 8-OH-DPAT (cells 27/1, 29/2 and 39/7 in Table 1; see Fig. 6B).

Figure 8A shows the location of the recording sites for the fourteen cells studied with carbachol. The cells were scattered throughout the explored portion of the raphe obscurus and pallidus nuclei rather than localized to one particular locus. All the sites were located not farther than 200 $\mu$m from the mid-line, as identified by the characteristic pattern of blood vessels penetrating this region.

The cells differed with regard to the sites of their axonal projections (Table 1). For all but two, branches were found within the XII nucleus. This is likely to reflect the bias of our searching procedure whereby cells were accepted for

Figure 6. Example of rapid responses to both carbachol (A) and 8-OH-DPAT (B) challenges in an antidromically activated cell

About 70 min elapsed between records in A and B during which time atropine (1 mg i.v.) was administered to produce a recovery from the carbachol-induced suppression. In B, 10 $\mu$g of 8-OH-DPAT, i.v., silenced the cell. Trace labels the same as in Fig. 5. C, individual traces with antidromic responses evoked from the XII nucleus: Ca, response to a single stimulus; Cb, response to a pair of closely spaced stimuli; Cc and Cd, collision test with the antidromic response present in the top, and absent in the bottom trace, when the interval between the spontaneous spikes and the stimulus was reduced. Stimuli marked by triangles. This is cell 29/2 in Table 1.
further testing only when the initial stimulation from within the XII nucleus produced an antidromic response (see Methods). Nevertheless, for one cell, the minimum threshold points were localized only below (within 400–600 μm; cell 34/3, Table 1) and for one cell (29/2, Table 1) only above, the XII nucleus. Two cells had distinct branches with minimum threshold points localized in the XII nucleus, DMV and medial NTS (one shown in Fig. 3). Three other cells had axonal branches only within the XII and medial NTS nuclei. Given that our procedure favoured cells projecting to the XII nucleus, the extent of branching dorsal to the XII nucleus must have been underestimated.

Figure 8B shows, superimposed on a parasagittal section of the dorsomedial medulla about 1 mm lateral to the midline, the location of all the distinct minimum threshold points found for these fourteen cells. Only the points with minimum thresholds less than 500 μA are shown (mean minimal threshold for the fifty-nine points shown was 180 ± 150 μA, with nineteen sites having thresholds of 3-6–50 μA). The 500 μA threshold limit corresponds to a maximal distance from the stimulating electrode to the stimulated fibre of less than 200–400 μm, depending on the thickness of the particular fibre studied. Of these fifty-nine sites, thirty-one were in the XII nucleus, nine in the medial NTS, five in the nucleus intercalatus (a structure interposed between the DMV and XII nuclei but not shown in this figure), twelve in the reticular formation ventral to the XII nucleus, and only two in the DMV. The average antidromic response latencies tended to be longer in dorsal than in ventral locations. The mean for the reticular formation just below the XII nucleus was 10 ± 11 ms; for the XII nucleus, 14 ± 9.6 ms; and for the NTS, 16 ± 10 ms, but the differences between any two locations were not statistically significant.

**DISCUSSION**

In this study, we have identified cells located within the medullary raphe obscurus and pallidus nuclei that: (1) project to the dorsomedial medulla, with axonal branches often present in both the XII (motor) and NTS (visceral sensory) nuclei; (2) have slowly conducting axons (less than 1·1 m s⁻¹); (3) have low firing rates (less than 3 Hz); (4) are suppressed during the carbachol-induced atonia; and (5) are suppressed by a serotonergic 1A receptor agonist. Cells with such features are likely to be serotonergic and, based on this study, may play a role in the sleep state-dependent modulation of the motor output from, and visceral sensory input to, the dorsomedial medulla.

There have been few electrophysiological studies of neurones of the medullary raphe with identified projections within the brainstem and no electrophysiological studies of those caudal raphe cells that project to brainstem orofacial motor nuclei (Fort et al. 1990; Li et al. 1993; Manaker & Tischler, 1993). The presence of medullary collaterals in the region of the rostral ventrolateral medulla (RVLM) involved in cardiovascular regulation was reported for a population of bulbospinal raphe pallidus neurones (Morrison, 1993). More extensive are electrophysiological studies of spinally projecting raphe cells, particularly those proposed to be involved in cardiovascular (e.g. Morrison & Gebber, 1984, 1985; McCall & Clement, 1989; Gilbey, Futuro-Neto & Zhou, 1995) and pain (e.g. Wessendorf & Anderson, 1983; Wilcockson, Gerhart, Cargill & Willis, 1983) control by raphe pallidus/obscurus and magnus nuclei, respectively. There are no electrophysiological studies of the presumably serotonergic cells (axonal conduction velocities < 6 m s⁻¹; West & Wolstencroft, 1977; Wessendorf & Anderson, 1983) with demonstrated axonal ramifications within the spinal cord ventral horn.

![Graph](image-url)
Thus, this is the first study that combines investigation of selected physiological properties of caudal medullary raphe cells with identification of some of their brainstem termination sites, including a motor nucleus.

**Serotonergic nature of the studied cells**

The cells that we studied, and in particular those fourteen that were submitted to extensive testing, share many features with those raphe cells that are likely to be serotonergic: low firing rates, low conduction velocities, action potentials of extended duration (seen in most, but not all, cells), and suppression by the systemic administration of a serotonergic 1A antagonist. The last has often been used to identify putative serotonergic cells in the pons and midbrain (see Jacobs & Azmitia, 1992, for a review). In the medulla, the sensitivity of putative serotonergic cells to inhibition by serotonin and 8-OH-DPAT was reported in one study to be as high as those in the dorsal raphe nucleus (McCall & Clement, 1989), but other studies observed relatively weak inhibitions in raphe magnus (Wessendorf & Anderson, 1983) and pallidus (Heym et al. 1982; Trulson & Frederickson, 1987) nuclei to other serotonin 1A receptor agonists. In this study, all eight cells tested were silenced, or profoundly suppressed, by less than 85 μg kg⁻¹ of 8-OH-DPAT, a dose that silenced putative medullary serotonergic cells, while having little effect on those presumed to be non-serotonergic (McCall & Clement, 1989). The cells in our study were also suppressed during the carbachol-induced atonia, parallel to the decrease in XII, phrenic and postural activities. Such behaviour is analogous to the behaviour of putative serotonergic medullary raphe cells during natural REM sleep (Heym et al. 1982; Trulson & Trulson, 1982).

The only frequently cited characteristic of serotonergic cells that our cells did not fully express is an extremely regular firing rate. Some variability of the firing rate was always present, as documented by the asymmetrical interval histograms and autocorrelograms with no or rapidly damped oscillations. Other studies show that cells with clock-like, regular activities are present in both medullary and pontine raphe nuclei under diverse conditions, including in vitro (Trulson & Frederickson, 1987), and in anaesthetized (McCall & Clement, 1989) and behaving animals (Shima, Nakahama & Yamamoto, 1986; see Jacobs & Azmitia, 1992, for a review), and are often intermingled with less regularly firing cells. In the pontine dorsal raphe nucleus, both regular and irregular cells have minimal firing rates during REM sleep (Shima et al. 1986), whereas in the medullary raphe, only cells with regular firing rates are suppressed during REM sleep and by serotonin agonists (Heym et al. 1982). Analogously, the cells that we studied were suppressed during both the carbachol-induced atonia and by 8-OH-DPAT. Thus, the reason for the relative

![Figure 8. Location of the recorded cells and their projection sites to the dorsomedial medulla](image)

_A, midsagittal section of the medulla with the location of 14 cells antidromically activated from the dorsomedial medulla and studied during carbachol-induced atonia. All cells were recorded within 200 μm from the mid-line. Activities of all but one (cell 31/1 in Table 1, ○) were suppressed during the carbachol-induced atonia. The unhatched area represents the region explored in this study. B, parasagittal section through the dorsomedial medulla showing the location of all minimum threshold points at which distinct branches of the 14 cells submitted to the carbachol challenge were stimulated. AP, area postrema; Gr, gracile nucleus._
irregularity of our cells is unclear, but may be a result of either the decerebrate preparation or a characteristic feature of cells projecting to the dorsomedial medulla. More data from caudal medullary raphe cells are needed to clarify the significance of the diversity in spontaneous firing patterns of medullary raphe cells. Other than the regularity of firing, the features displayed by the cells identified in this study are consistent with their being serotonergic.

Diverging projections of medullary raphe cells

The axonal projection patterns differed among the cells that we studied, and these differences should not be ascribed solely to differences in the extent of antidromic mapping performed in individual cases. While many cells had relatively limited projection sites, twelve of the forty-five cells (and 4 of the 14 extensively studied) had axonal ramifications in both a motor (XII) and visceral sensory (NTS) nucleus. On the other hand, for three cells, in spite of antidromic mapping in at least four tracks, no branches were found dorsal to the XII nucleus. Thus, there appears to be heterogeneity in the projections of individual raphe neurones, with some cells having widespread and others limited projections. This is similar to bulbospinal raphe cells and their spinal targets (Morrison & Gebber, 1985).

The substantial projections to both the XII nucleus and NTS suggest that at least some of our cells have widespread axonal projections within the medulla. This is consistent with anatomical studies that show diverging projections of the raphe system to many levels of the neuraxis (Skagerberg & Björklund, 1985). In particular, many cells of the medullary raphe have both a spinal axon and intramedullary collaterals (Lovick & Robinson, 1983; Manaker et al. 1992; Allen & Cechetto, 1994). Thus, for some cells of the present study, the identified projection sites may represent only a fraction of their efferent projections, and it is conceivable that some of these cells may also have spinal axons. Whether they belong to any of the three categories of caudal bulbospinal raphe neurones distinguished so far remains to be determined. Two of these categories have projections to sympathetic preganglionic neurones, while the spinal targets of the third have not been identified (Morrison & Gebber, 1984, 1985; McCall & Clement, 1989).

Studies of divergent projections of the medullary raphe often focused on functionally related target sites. Thus, arborizations of caudal medullary raphe cells were demonstrated for different combinations of orofacial motor nuclei (Li et al. 1993), or combinations of medullary and spinal sites involved in pain (Lovick & Robinson, 1983) or cardiovascular (Morrison, 1993) control. However, there is also evidence that individual medullary raphe cells can project to diverse targets, such as both autonomic and somatic cell groups (Barman & Gebber, 1988; Allen & Cechetto, 1994), and our study demonstrates diverging projections to motor and visceral sensory nuclei. Thus, at least a subset of medullary raphe cells can have extensive projections to functionally heterogeneous sites. Our study, in addition, shows that such cells change their activity in a predictable manner during the carbachol-induced atonia. Thus, they have properties that would allow them to act as state-dependent modulators of neuronal traffic at many functionally distinct levels of the neuraxis.

Functional significance

The main rationale for this study was our finding that there is a serotonergic excitatory drive to XII motoneurones (Kubin et al. 1992) and that the extracellular level of serotonin decreases in the XII nucleus region during the atonia produced by pontine carbachol injections (Kubin et al. 1994). These data led us to hypothesize that the withdrawal of a serotonergic excitatory input may play an important role in the decrements of activity in upper airway motoneurones that occur during REM sleep. The cells identified in the present study had axon collaterals in the XII nucleus and, importantly, were silenced during the carbachol-induced atonia. Therefore, they may provide the cellular basis for our hypothesis and the results reported in our earlier studies.

The interpretation of our results relies on earlier work, described in the introduction, showing similarities between the carbachol-induced states (in both chronic and acute preparations) and natural REM sleep. A major component of the motor suppression during REM sleep originates in the pontine structures that can be activated by carbachol injections. In this light, the carbachol-induced decrease in the activity of the putative serotonergic cells projecting to the XII nucleus supports the hypothesis, albeit indirectly, that a serotonergic input to XII motoneurones decreases during REM sleep. Since the predominant effect of serotonin on XII motoneurones is excitatory (Berger, Bayliss & Viana, 1992; Kubin et al. 1992), this will reduce the excitability of XII motoneurones and contribute to their characteristic suppression during REM sleep. In addition, our data suggest that a subset of putative serotonergic medullary raphe neurones has the potential to modulate, in a state-dependent manner, not only motor activity, but also visceral afferent input.

Allen, G. V. & Cechetto, D. F. (1994). Serotonergic and nonserotonergic neurons in the medullary raphe system have axon collateral projections to autonomic and somatic cell groups in the medulla and spinal cord. Journal of Comparative Neurology 350, 357–366.


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