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# SPATIAL ORGANIZATION OF RESPIRATORY NEURONES IN THE MEDULLA OF TENCH AND GOLDFISH

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## INTRODUCTION

The respiratory pump in teleost fish is mechanically complex, but the activity in motor neurones which control this pump has a relatively simple pattern (Ballintijn & Hughes, 1965; Ballintijn, 1969). Despite the complex hinging of the skeletal system which mediates the muscle action, most motor neurones are active either only during opening of the mouth and operculum or only during closing. Thus the basic motor output pattern is a series of rhythmically alternating bursts of activity in 'opener' and 'closer' motor neurones.

The respiratory neurones in the medulla of the tench and the goldfish have also been reported to constitute two relatively distinct groups: those active during opercular opening, and those active during closing (Shelton, 1961; Hukuhara & Okada, 1956; Baumgarten & Salmoiraghi, 1962). Several observations suggest that the respiratory motor output pattern is generated by interactions among these respiratory neurones in the medulla. Transections of other parts of the brain do not abolish the breathing rhythm in the tench (Shelton, 1959). In the goldfish, respiratory activity has been shown to persist in medullary neurones after elimination of sensory feedback and apparently after isolation of the medulla from the rest of the brain (Hukuhara & Okada, 1956; Baumgarten & Salmoiraghi, 1962). Since local destruction of respiratory neurones in the tench never abolished the respiratory rhythm, it seems likely that no single cell is a crucial pacemaker for breathing (Shelton, 1961).

There is little direct evidence about how neurones in the teleost medulla interact to produce the respiratory rhythm, but several lines of evidence suggest that the pattern may be generated by mutually excitatory interactions among opener neurones and among closer neurones, and mutually inhibitory interactions between opener and closer neurones. Such a 'reciprocal inhibition' network will tend to produce alternating bursts of activity in opener and closer neurones, provided that the neurones in the network show fatigue (i.e. become increasingly unresponsive to excitation after each successive action potential they produce). In such a network, if activity begins in opener neurones it would at first tend to excite further activity in the opener neurones and inhibit any activity in closer neurones. With repeated activity, however, the opener neurones would become increasingly unresponsive, their activity would slow down, and they would inhibit the closer neurones less. Meanwhile the closer neurones would

have recovered full responsiveness during their inactive period, and, at some point, the decreasing inhibition from the opener neurones would no longer be sufficient to prevent closer activity. Once closer activity began, it would inhibit any further opener activity and reinforce further activity in closer neurones. Closer activity would continue until these neurones in turn became relatively unresponsive and inactive, when a new burst of opener activity would begin. Cyclic repetition of this process would result in alternating bursts of activity in the two groups of neurones.

A reciprocal inhibition network like this probably generates the motor output rhythm for breathing in the cat (Baumgarten & Nakayama, 1964). We would expect the respiratory pattern generator in teleosts to resemble the respiratory pattern generator in cats because (1) both networks have a similar function (to produce a similar motor output pattern), and (2) the evidence from comparative physiology suggests that during the evolution of the vertebrate respiratory pattern generator no fundamental reorganization has occurred. The evolutionary progression from the ancestral water-pump to the modern air-pump probably involved relatively little change in pumping mechanism at the time of the switch from water to air. Slight modifications of the same basic buccal force-pump mechanism are used to pump water in elasmobranch and teleost fishes, lungfish and amphibia, and to pump air in lungfish and amphibia (McMahon, 1969). Rib and diaphragm pumps appear to have evolved gradually as supplementary pumps which eventually replace the buccal pump. Other data also suggest evolutionary continuity of the mammalian network generating the breathing rhythm with that in the common ancestors shared by mammals and teleosts. In all vertebrates studied thus far, the neural network which controls the respiratory pump is located in and around the motor nuclei of the cranial nerves which innervate the breathing muscles in fish. A layer of spinal neurones has been added in the cat, and the main group of respiratory neurones is located in a slightly more caudal region of the medulla, but the basic location of the respiratory neurones is much the same in the tench and in the cat. (Compare for example Shelton, 1961, and Waldron, 1970.) Even the number of respiratory neurones which can be recorded in the medulla of cat and tench is very similar. (Compare Waldron, 1970, and this paper.) These observations all suggest that the respiratory pattern generators in teleosts and cats should resemble each other on grounds of evolutionary homology as well as on grounds of analogy. These evolutionary arguments lend support to the hypothesis that teleost breathing rhythms are generated by a reciprocal inhibition network of the kind believed to be acting in the cat. The data to be presented in this paper are compatible with this hypothesis, but are also compatible with alternative hypotheses.

Earlier work suggested that the network which generates the breathing rhythm in teleosts might be composed of anatomically localized subgroups of neurones which interact primarily with other neurones within the subgroup. Shelton (1961) observed that 'when several insertions [of the electrode] were made within a radius of about 300 $\mu$m of a successful site it was quite common to find no respiratory discharges at all, even though multi-unit discharges occurred at the active region'. Baumgarten & Salmoiraghi (1962) also reported that respiratory neurones occurred in 'active patches'. A suggestion that interactions may be stronger between neurones within an anatomically localized cluster than between neurones in different clusters came from the following preliminary observation: for any pair of neurones physically close

enough for their activity to be recorded simultaneously with a single electrode the times of activity in the respiratory cycle usually show total overlap or no overlap at all, whereas for distant neurones the times of activity may show any degree of partial overlap (C. M. Ballintijn, personal communication regarding the carp). These observations suggest that respiratory neurones are organized into anatomically localized functional subgroups of a size similar to that described in somatosensory cortex (Powell & Mountcastle, 1959) and visual cortex (Hubel & Wiesel, 1962). The data to be presented, however, do not support the notion of small functional subgroups for the respiratory system, although they do indicate some anatomical localization of function on a slightly larger scale.

## METHODS

Experiments were begun in England with tench (*Tinca tinca*), and continued in the U.S. with goldfish (*Carassius auratus*) since tench are not available in the U.S. Systematic records of neural activity were made from six tench and ten goldfish, with additional fish used for exploratory observations. The goldfish were 9–16 cm long from the tip of the head to the base of the tail, and tench sizes were in the same range. Basic technique was very similar to that used by Shelton (1961). The fish were anaesthetized with urethane at a concentration of about 0·5% during the operation and roughly half that thereafter. In most cases the olfactory lobes were severed from the optic lobes.

The breathing rhythm is quite regular in fish that are anaesthetized and restrained. At least for the goldfish, this contrasts sharply with the irregular amplitude and frequency of breathing in their home tank. In that situation, bouts of breathing are often separated by pauses of up to 10 sec with the mouth held closed or almost closed. Regular breathing in unanaesthetized goldfish has only been observed when the fish are placed in a very small container. Thus some aspect of the experimental procedure, perhaps immobilization, alters the regulation of the respiratory rhythm. However, it seems probable that the mechanism which generates the basic breathing rhythm remains the same.

A thread sewn through the lip was attached to a strain-gauge (for the tench) or a lever that moved a flag between a photocell and a light (for the goldfish). The strain-gauge offered more mechanical resistance to mouth motion than did the lever, which was balanced on a jewelled pivot. Both devices produced electrical records which were displayed together with the neural activity on an oscilloscope and recorded on film. Neural activity was recorded with tungsten electrodes insulated with glass micropipettes except for a tip about 8 $\mu$m long and 2 $\mu$m in diameter.

The neurones used in the analysis of activity patterns were continuously active for some part of the respiratory cycle and inactive during the rest of the cycle. A few neurones had more than one burst of activity during each cycle, and others were continuously active but frequency-modulated in time with the respiratory cycle. These were not included in the analysis of activity patterns since they could not be characterized by the most convenient measures of activity pattern, namely the time when activity began and the time when activity ended. These two times were measured relative to the time when the mouth was maximally opened, or maximally closed, in several respiratory cycles for each neurone. Each time was converted to a

phase measure by dividing it by the length of the respiratory period. Activity patterns were characterized by the average phase when activity began and the average phase when activity ended. For most neurones the activity pattern was nearly the same in every cycle recorded, but for some the time when activity began or ended varied by as much as one-tenth of the cycle. Occasionally the fish coughed. In these cough cycles many of the neurones became more active, and previously quiescent neurones became active. These cough cycles were not included in the averages.

The anatomical location of recorded units was generally determined by measuring stereotactic position relative to various features of the surface topography. Like Shelton (1961) and Baumgarten & Salmoiraghi (1962), I found that neither the presence of activity nor the type of activity was exactly the same for every penetration in a given stereotactic region. This was presumably due to variation in the anatomical region reached for given stereotactic co-ordinates, and to variation in the physiological condition of the animal caused, for example, by differences in depth of anaesthesia. Variation in physiological condition is suggested by the observations that the respiratory period varied between 0·5 and 2·0 sec and, for goldfish, maximum closing of the mouth occurred from 0·4 to 0·6 of a cycle after maximum opening of the mouth.

Precise information on the relative position of many units in the same animal was obtained by using the same electrode to make repeated penetrations at 100 or 150 $\mu$m intervals in either a parasagittal or an approximately frontal plane. Data from these series of penetrations provide the basis for making a minimum estimate of the number of respiratory neurones in the tench medulla. The method is described under Results.

## RESULTS

### *Activity patterns and locations of respiratory neurones*

Shelton (1961) found respiratory neurones in a region of the brainstem which, in the tench, lies mainly ventral to the cerebellum and facial lobe. Baumgarten & Salmoiraghi (1962) found respiratory neurones in a similar region which, in the goldfish, lies mainly ventral to the cerebellum and vagal lobes. My observations generally confirm their results, but extend the region of respiratory neurones in both cases. In the tench, in the part of the brainstem which lies under the cerebellum, I recorded respiratory neurones as far lateral as 2 mm from the midline, which is near to the edge of the brainstem (Fig. 3). In the goldfish, respiratory neurones were found in penetrations all the way from the rostral edge of the cerebellum to the caudal edge of the vagal lobes.

As reported by Baumgarten & Salmoiraghi (1962), many of the respiratory neurones in the goldfish medulla were active exclusively or predominantly during the part of the respiratory cycle when the mouth was opening, and many were active exclusively or predominantly during the part of the respiratory cycle when the mouth was closing (Fig. 1). However, other neurones were active at intermediate times, partly during mouth opening and partly during mouth closing, and could not be classified as either opener or closer neurones. These neurones with intermediate activity patterns were not reported by Baumgarten & Salmoiraghi (1962) or by Hukuhara & Okada (1956), perhaps because neither group made a systematic study of the phases of activity for

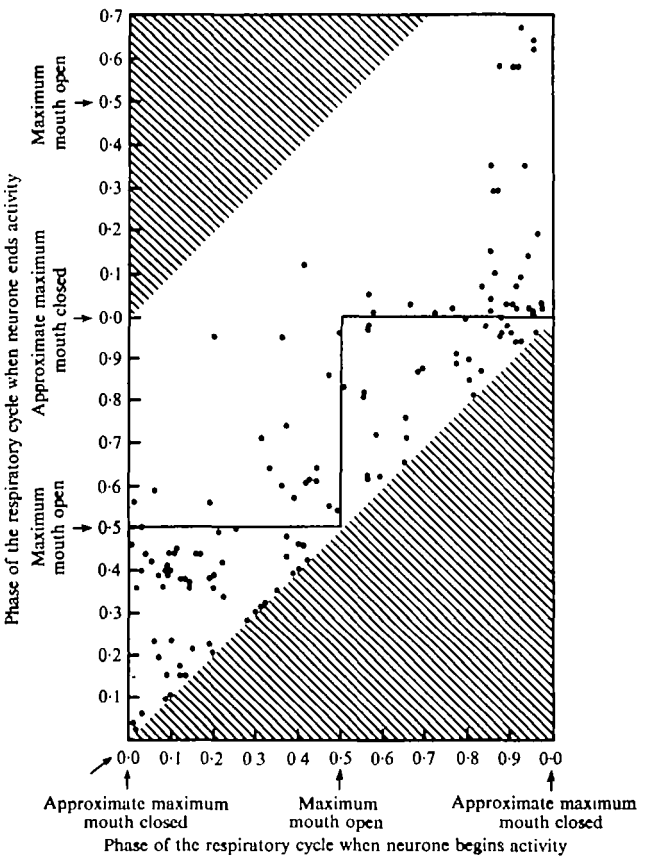


Fig. 1. Times of activity of respiratory neurones in the medulla of the goldfish. Each point represents the average activity pattern of one neurone. The horizontal position indicates phase of the respiratory cycle when activity begins; vertical position indicates phase when activity ends; and distance above the diagonal indicates duration of activity. Each neurone graphed was active for only a part of each respiratory cycle; the point representing the activity of any such unit must fall somewhere in the diagonal band between the shaded triangles. The upper unshaded triangle includes all neurones which were active only during the part of the respiratory cycle when the mouth was closing, and the lower unshaded triangle includes all neurones which were active only during the part of the respiratory cycle when the mouth was opening. Neurones which began activity slightly before the mouth began to open fall in the upper right-hand corner. Although many of the neurones can be classified as 'openers' or 'closers', quite a few neurones have intermediate activity patterns.

recorded neurones. A wide range of intermediate activity patterns has been found for carp respiratory neurones (C. M. Ballintijn, personal communication).

The patterns of activity are somewhat different in different regions of the goldfish brainstem. For example, the most caudal part of the respiratory region (under the caudal half of the vagal lobes) contained almost no neurones that had brief activity at the time of maximal closing of the mouth.

Activity patterns of respiratory neurones in the tench do not seem to fall into distinct closer and opener groups (Fig. 2*a*). A rather large proportion of the units begin activity during mouth opening and end activity during mouth closing. Such units seem to be more common in my recordings for the tench than in Shelton's

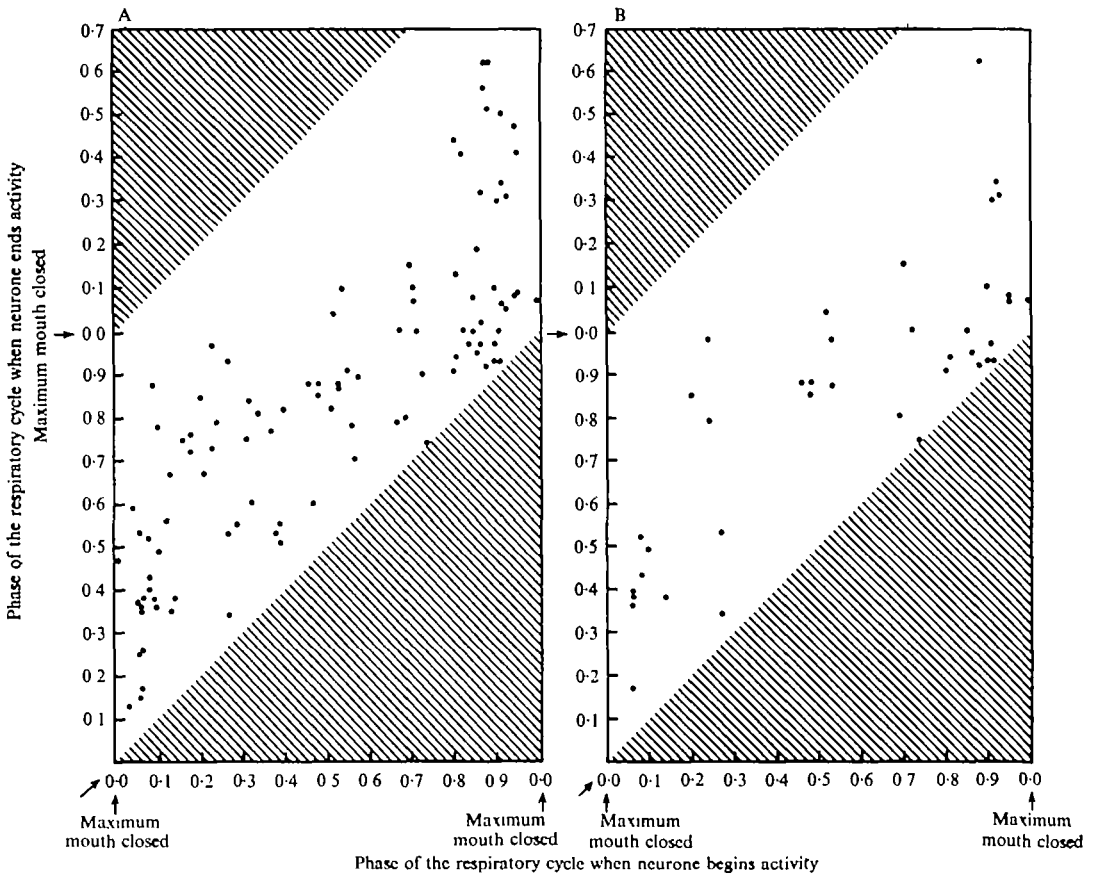


Fig. 2. Times of activity of respiratory neurones in the medulla of the tench. Each point in graph (A) represents the average activity pattern of one neurone recorded in the tench medulla. Different types of activity patterns were recorded in different regions of the medulla. This is illustrated by graph (*B*) which shows the activity patterns for neurones recorded in a region near the anterior and posterior facial (VIIth) motor nuclei (more than 1·8 mm caudal to the rostral tip of the cerebellum, less than 0·5 mm caudal to the caudal tip of the cerebellum, and less than 1·0 mm lateral to the midline). Almost none of the neurones in this region end their activity at phases 0·1–0·3 or 0·55–0·75; i.e. these neurones generally ended their activity either when the mouth had almost finished closing or when the mouth had almost finished opening.

recordings for the tench (Shelton, 1961) or in my recordings for the goldfish. Perhaps this is because the strain-gauge that I used with the tench provided a slight resistance to mouth motion which may have altered sensory feedback and thus altered activity in medullary neurones.

Regional differences in activity patterns were somewhat more marked than in the case of the goldfish. Fig. 2(*b*) illustrates that in the region near the facial (VIIth) motor nuclei the activity patterns fell into two distinct groups. Neurones in the first group ended activity just before the end of mouth closing. Neurones in the second group ended activity near the end of mouth opening.

Table 1. *Phase relationships for pairs of neurones separated by different distances in goldfish medulla*

(If the mid-point of the time of activity of one unit differed from the mid-point of the time of activity of another unit by less than 0·1 cycle, then that pair of units was designated a 'synchronous' pair of units. If the mid-times of activity of the two units differed by 0·4–0·6 cycle, then the pair of units was designated an 'anti-synchronous' pair. If the relative timing of the activity in different units were random, then 40% of the pairs would be synchronous or anti-synchronous. Pairs of units separated by less than 0·2 mm or by 1·0–2·0 mm appear to be either synchronous or anti-synchronous more often than expected by chance, and pairs of units separated by 0·5–1·0 mm appear to be synchronous or anti-synchronous less often than expected by chance ($P < 0{\cdot}1$, $\chi^2$ test). Pairs of units separated by less than 0·2 mm or by 1·0–2·0 mm are clearly more often synchronous or anti-synchronous than pairs of units separated by 0·5–1·0 mm ($P < 0{\cdot}01$ and 0·02 respectively, $\chi^2$ test).)

| Distance between the two neurones (mm) | Total no. of pairs | No. of synchronous or anti-synchronous pairs | Proportion of pairs which were synchronous or anti-synchronous (%) |
|---|---|---|---|
| < 0·05 | 31 | 16 | 52 |
| 0·05–0·2 | 53 | 28 | 53 |
| 0·2–0·5 | 87 | 37 | 43 |
| 0·5–1·0 | 139 | 46 | 33 |
| 1·0–2·0 | 131 | 62 | 47 |
| 2·0–3·0 | 64 | 26 | 41 |

### *Spatial organization*

The graphical method illustrated in Figs. 1 and 2 was adequate to reveal differences in activity patterns recorded in different large regions of the respiratory area in the medulla. A statistical analysis has revealed spatial segregation of activity patterns on a somewhat finer scale. Table 1 gives a summary of this statistical analysis of the phase relationships between pairs of neurones separated by different anatomical distances. Pairs of neurones separated by a distance of less than 0·2 mm or of 1·0–2·0 mm are more often either synchronous or anti-synchronous than are pairs of units separated by a distance of 0·5–1·0 mm ($P < 0{\cdot}01$ and 0·02 respectively, $\chi^2$ test; no other differences were statistically significant). Inspection of plots of activity pattern *v.* anatomical location suggests the following interpretation of this statistical difference: nearby neurones or neurones in symmetrical positions with respect to the midline tend to have activity of similar or opposite timing; for a pair of neurones separated by 0·5–1·0 mm distance, one tends to have long-duration activity and the other tends to have short-duration activity which occurs either at the beginning or the end of the long-duration activity of the other.

The statistical analysis has revealed different types of phase relationship at closer distances, but neither this statistical analysis nor the graphical analyses have revealed any evidence of more precise phase relationships at very close distances. Thus the activity-pattern analysis does not provide support for the hypothesis that there are anatomically localized functional subgroups, with stronger neural interactions or greater shared input for nearby neurones within a subgroup than for distant neurones in different groups.

Furthermore, systematic search of the tench medulla has not revealed the small anatomically localized groups of respiratory neurones that Shelton hypothesized.


Fig. 3. Locations of respiratory neurones recorded under the caudal cerebellum in the left half of the medulla of one tench. Successive penetrations were made at 150 $\mu$m intervals in a plane about 2·5 mm caudal to the rostral tip of the cerebellum. Depths and lateral locations were determined stereotactically. The separation into dorso-lateral and ventro-medial groups of neurones is illustrated rather clearly. The gap in the ventro-medial group and the two spatially isolated neurones are somewhat atypical.

Repeated penetrations at 100–150 $\mu$m intervals in either an approximately frontal plane or a parasagittal plane generally show a more or less solid cluster of respiratory neurones, thinning out towards the edges. There was a gap with no respiratory neurones near the midline in most series of penetrations in the approximately frontal plane. Also, under the middle portion of the cerebellum, each bilateral group tends to be separated into a larger dorso-lateral subgroup and a smaller ventro-medial subgroup (Fig. 3). The bilateral group or, more rostrally, each subgroup ranges in size from 300 $\mu$m deep and 500 $\mu$m wide to 950 $\mu$m deep and 1300 $\mu$m wide. Both bilateral groups seem to be continuous in the rostro-caudal direction. Spatial distribution of neurones within each group is not entirely uniform. Isolated respiratory neurones are occasionally observed at some distance from the main clusters. In conclusion, most respiratory neurones in the tench are found within two long bilateral groups and not in anatomically isolated clusters with a diameter of 600 $\mu$m or less.

### *How many respiratory neurones?*

A minimum estimate of the total number of respiratory neurones in the tench medulla has been calculated on the basis of the total number of cyclically active respiratory neurones recorded in three series of penetrations in parasagittal planes, and

five series of penetrations from midline to the lateral edge. For each series, all respiratory neurones were counted, except those that had very short action potentials and were therefore probably fibres (Cooper, Robson & Waldron, 1969). To estimate the total number of respiratory neurones in the medulla, I have used the observation that activity of most respiratory neurones could be detected over a vertical distance of 120 $\mu$m or less, and the assumption that electrical spread of action potentials was similar in horizontal and vertical directions. This implies that a grid of penetrations at 120 $\mu$m intervals would make it possible to record most respiratory neurones while avoiding duplicate recordings. Therefore, to estimate the total number of respiratory neurones, the number of neurones recorded in each series of penetrations is multiplied by the length of the region for which that series is representative divided by 120 $\mu$m, and these products are summed for the whole respiratory region. Using the series of penetrations in frontal planes, this sum gives an estimate of 988 neurones on a side. The estimate from the series of penetrations in parasagittal planes is 1115 neurones on a side. These estimates must be considered minimum estimates, since some neurones may well give potentials too small to be recorded by my techniques; some units in a given plane were probably too far from any penetration to be detected, or were missed because they could not be distinguished from near-neighbours; and some respiratory neurones are inactive under light anaesthesia. On this basis I conclude that there are a minimum of roughly 2000 respiratory neurones in the tench medulla.

A very crude cross-check suggests that the minimum estimate of 2000 may not be too far below the actual number of respiratory neurones. About 30% of the respiratory neurones that Shelton recorded in the tench were in the cranial motor nuclei from which originate the motor neurones that innervate respiratory muscles (Shelton, 1961, fig. 2). If there are at least 2000 respiratory neurones, then Shelton's observation implies that there are at least 600 respiratory neurones in the motor nuclei. The number of respiratory neurones in the motor nuclei must be at least as large as the number of active motor neurones to the respiratory muscles. During normal breathing five muscles on each side are active in the carp (Ballintijn, 1969), and during shallow breathing three muscles on each side are active in the trout (Ballintijn & Hughes, 1965). (Both the muscle recordings and Shelton's neural recordings did not include motor neurones with axons in the tenth cranial nerve.) I have no direct evidence on the number of active motor neurones to each respiratory muscle, but a muscle of similar size, the extensor longus digitorum IV muscle in *Rana temporaria*, receives about ten motor neurones (Katz, 1949). Very different estimates of 200 motor neurones to the soleus and 270 motor neurones to the extensor longus digitorum muscles in the cat have been given (Clark, 1931), but both of these muscles are much larger than the respiratory muscles in teleosts. Therefore a rough estimate of the total number of motor neurones to active respiratory muscles in the tench is $5 \times 10 \times 2 = 100$. Thus the number of active motor neurones is apparently less than the 600 respiratory neurones estimated to be in the motor nuclei. This suggests that the initial estimate of 2000 respiratory neurones, while it is a minimum estimate, may perhaps be not too serious an underestimate.

## DISCUSSION

Several differences in technique probably explain why I have failed to confirm Shelton's suggestion that respiratory neurones occur in anatomically localized clusters with a diameter of 0·6 mm or less. Shelton used electrodes with tips at least three times as large as those used here, and reports 'difficulty...in locating...respiratory neurones'. This suggests that he recorded from a smaller proportion of the respiratory neurones than I did. My technique apparently differed also in that I counted a penetration as revealing an absence of respiratory neurones only if respiratory neurones were subsequently recorded with the same electrode. Also I included in the analysis respiratory neurones whose action potentials were too small to be seen on film, but were audible, and visible on the oscilloscope. The technique used seems adequate to establish that anatomically localized clusters of respiratory neurones are certainly not common, if they occur at all.

On the other hand, respiratory neurones are clearly not uniformly distributed through the general brainstem region where they occur. The neurones tend to fall into two long bilateral groups which may merge together in the middle and which, in more rostral regions, tend to be split into dorso-lateral and ventro-medial subgroups. There may be other more complex patterns of localization within the respiratory region, but more reliable techniques of location, especially histological identification of recording sites, would be needed to give consistent evidence for these.

Neurones with similar patterns of activity show some tendency to cluster in particular parts of the respiratory region. The spatial variations in patterns of activity cannot yet be reliably correlated with anatomical locations. Nevertheless a highly speculative model of spatial and neuronal organization can be proposed that is compatible with the available data, though certainly not uniquely implied by that data. There may be a reciprocal inhibition oscillator that generates the basic breathing rhythm and is centred in the medial group of respiratory neurones near the facial (VIIth) motor nuclei. At a distance of about 1 mm or less from this oscillator network there may be neurones that are active just at the transition from mouth closing to mouth opening and vice versa. These neurones may be 'read-out' neurones, i.e. motor neurones that innervate muscles which are active at these transition times. Alternatively, they may be sensory neurones or central neurones which contribute to the transition from mouth opening to mouth closing by inhibiting opener neurones and stimulating closers (or vice versa for the transition from closing to opening). If this is the case, the pattern-generating network would be a modified reciprocal-inhibition oscillator of the kind proposed by Cohen (1970) for the cat. The possibility that the basic mechanism of pattern generation is entirely different from a reciprocal inhibition network cannot be excluded as yet.

## SUMMARY

1. A minimum of 2000 neurones in the medulla of the tench have cyclic activity that is phase-locked to the respiratory cycle.

2. These respiratory neurones are not uniformly distributed throughout the medullary region where they occur. They tend to occur in two bilateral groups, each of which, toward its rostral end, tends to be split into a dorso-lateral and ventro-

medial group. Specific patterns of activity are more common in some regions than in others.

3. No evidence was found for anatomically localized groups of neurones with interactions primarily within the group. Neurones within 0·2 mm of each other are more often either synchronous or anti-synchronous than are neurones separated by a distance of 0·5–1·0 mm, but so are neurones separated by 1·0–2·0 mm. Contrary to a speculation by Shelton, respiratory neurones are not bunched into anatomical clusters with a diameter of 0·6 mm or less.

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