CHOLINERGIC MOTOR NEURONES IN THE STOMATOGASTRIC SYSTEM OF THE LOBSTER

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SUMMARY

1. A study of the neurotransmitters used by each of the eleven types of excitatory motor neurones (identified according to the muscle innervated) of the lobster stomatogastric ganglion was undertaken.

2. The dorsal dilator muscle is innervated by the two motor neurones designated ‘PD’. Bath and ionophoretic applications of acetylcholine (ACh) produce contractures and depolarizations respectively in the dorsal dilator muscle.

3. Pharmacological experiments support the cholinergic nature of the excitatory junctional potentials (e.j.p.s) recorded in the dorsal dilator muscle when the PD motor nerve is stimulated.

4. The apparent reversal potentials for the e.j.p.s and the ionophoretic ACh response in the dorsal dilator muscle are the same.

5. On the basis of choline acetyltransferase assays on identified stomatogastric ganglion motor neurone somata and tension measurements on the muscles innervated by each type of stomatogastric ganglion motor neurone, a transmitter candidate was established for each type of motor neurone. Motor neurones named VD, LPG, GM, MG, LG, and DG are putatively cholinergic. l-Glutamate is a transmitter candidate for the motor neurones called LP, PY, IC, and AM.

6. Potential correlations between the distribution of putatively cholinergic and glutaminergic motor neurones and the electrical coupling among the stomatogastric ganglion motor neurones are discussed.

INTRODUCTION

Many low molecular weight compounds are currently thought to be neurotransmitters in both vertebrate and invertebrate nervous systems. At present the functional significance of the diversity among

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neurotransmitters is not understood. Why many different neurotransmitters are used in nervous systems, and why any one neurotransmitter is used by any given neurone are unanswered questions. One approach to these questions is to characterize the neurotransmitters and receptors used by all the neurones within a physiologically complete nervous system. This would determine if there are any correlations between the functional relationships of the neurones within the nervous system and the neurotransmitters used by those neurones. This paper reports the first results of an attempt to characterize completely the neurotransmitters within the stomatogastric system of the lobster, *Panulirus interruptus*.

The stomatogastric ganglion contains the somata of about thirty neurones, twenty-three of which are motor neurones which send axons to innervate thirty-eight pairs of striated muscles which move the lobster’s stomach. Maynard & Dando (1974) provide a complete anatomical description of the pattern of innervation of the stomach, which allows the identification of eleven types of motor neurones in the stomatogastric ganglion according to the muscle they innervate. These include neurones of the pyloric system, designated PD (two in each ganglion), LP (one), PY (eight), VD (one), IC (one) (Maynard & Silverston, 1975), and those of the gastric system, GM (four), LPG (two), LG (one), MG (one), DG (one) and AM (one) (Mulloney & Silverston, 1974a). Since the stomatogastric ganglion is functionally autonomous and the synaptic interactions among the stomatogastric ganglion neurones are well-understood (Maynard, 1972; Mulloney & Silverston, 1974a, b; Silverston & Mulloney, 1974a; Maynard & Silverston, 1975; Hartline & Maynard, 1975; Silverston, King, Russell & Miller, 1976) it seemed an ideal system with which to study the relationship between the transmitters used by neurones and the functional interactions among those neurones. This study establishes a neurotransmitter candidate for each of the stomatogastric ganglion motor neurones, and demonstrates that some but not all of the stomatogastric ganglion motor neurones use acetylcholine (ACh) as an excitatory neuromuscular transmitter.

In contrast to several other crustacean neuromuscular junctions where L-glutamate is believed to be the neurotransmitter (Takeuchi & Takeuchi, 1964; for review see Gershenfeld, 1973), previous work (Marder, 1974) showed that ACh is likely to be the neurotransmitter at the excitatory neuromuscular junction in the stomatogastric system between the PD neurones and the dorsal dilator muscle. The first part of this paper presents further physiological and pharmacological experiments on the PD-dorsal dilator neuromuscular junction which corroborate this conclusion.

The second section of this paper presents the results of bath application
of ACh and l-glutamate on the muscles innervated by each of the stomatogastric ganglion motor neurones. Additionally, choline acetyltrans-
ferase assays were performed on the somata of many of the stomatogastric ganglion motor neurones. On the basis of these experiments the motor neurones are divided into two classes, those putatively cholinergic, and those non-cholinergic and possibly glutaminergic. Correlations between this distinction and the known physiological interactions of these neurones are discussed.

**METHODS**

Animals. *Pandalus interruptus* were bought from San Diego, California, fishermen, and kept in running sea water holding tanks at Scripps Institute of Oceanography until used. The animals weighed between 400 and 1000 g and were of both sexes. All experiments were performed at room temperature unless otherwise noted.

Solutions. A physiological salt solution called 'Pandalus saline' was used for these experiments. *Pandalus* saline has the following composition (in mM): NaCl, 47.9; KCl, 12.8; CaCl₂, 13.7; Na₂SO₄, 3.9; MgSO₄, 10; Trizma base (mono-
tris(hydroxymethyl)aminomethane base maleate), 5.1; and Trizma Base (Tris(hydroxy-
ethyl)aminomethane), 6. Saline was used at pH 7.4-7.5 and aerated before use. In some experiments 2 mM glucose was added to the saline directly before use. In the cell identification experiments 5 ml/l. Gibco Antibiotic-Antimycotic (100 x) solution was added. All drugs used in physiological experiments were made up fresh in *Pandalus* saline just before use. Acetylcholine chloride, monosodium L-glutamate, potassium L-aspartate, dopamine HCl, octopamine HCl, glycine, strychnine, atropine sulphate, picrotoxin, hexamethonium, eserine sulphate, taurine, carbacol (carbachol chloride), Trizma maleate, and Trizma Base were obtained from Sigma Chemical Company. Tubocurarine chloride, and serotonin creatinine sulphate were obtained from CalBiochem. Tension (Edrophonium) was obtained from a local hospital supplier. A-Bungarotoxin was a gift from Dr Hannah Friedman.

Choline acetyltransferease assays on single motor neurones. Physiological identification of stomatogastric ganglion somata has been carefully described by Mulloney & Selverston (1974a), Maynard & Selverston (1975), and Selverston et al. (1976) and was performed as those authors describe using conventional electrophysiological techniques and equipment. A picture was drawn of the position of the identified cell somata, and the cells were dissected by the method of Giller & Schwartz (1971) with modifications by R.E. McCann (personal communication). The ganglion was treated with cold (−20°C) 70% ethylene glycol, 30% *Pandalus* saline for 1 hr followed by dissection with hand-held forceps. The cells were dissected on to a point of a fine glass rod, and under visual control a small test tube was slipped over the rod causing the cells to stick to the bottom of the tube, which was immediately frozen on dry ice and kept at dry ice temperature until the time of the assay. Choline acetyltransferease (EC.2.3.1.6) was assayed as described by Coggeshall, Deslandres, Weinreich & McCann (1972). 10 µl. buffer-substrate (final concentrations: 0.1 M sodium phosphate, pH 7.9: 0.25 M NaCl; 10 mM choline chloride; 1 mM EDTA; 0.1 mM acetylcholine; 16 mM MgCl₂; and 0.4 mM (2°)acetyl CO₄, specific radioactivity 2-10 µCi/mole (New England Nuclear Corporation)) was added to the tubes contain-
ing the cell somata and to empty tubes (blank). Tubes were incubated at 38°C for 20 min. The reaction was stopped with 100 µl. 50 mg/ml sodium tetraphenyl-
boron (K and K Cievelucks) in 3-heptanone (K and K Cievelucks). The tubes were mixed, and 100 µl. 0.02 M sodium phosphate, pH 7.6, was added. The tubes were
mixed again, and then spun in a table top centrifuge for 2-5 min; 50 μL organic phase was removed and counted in 10 mL scintillation fluid. Ten to twenty blanks were run with each assay, and the limit of sensitivity was calculated on the basis of the variation in the blanks and the specific radioactivity of the reaction mixture. Significant activity was judged to be any activity more than three S.D. of the observation higher than the mean of the blanks, and in no case was this value lower than any of the blank values. In general, the blanks varied within a 10 c.p.m. range, and the number of counts above the blanks in single cell tubes was 20-100 c.p.m.

Control experiments were done with choline acetyltransferase in homogenates from stomatogastric ganglia to verify that the activity was not affected by the ethylene glycol treatment and freezing used in the cell dissections, and that the assay conditions were appropriate for the Panulirus enzyme.

Muscle physiology. In all physiological experiments using muscles, the muscle was dissected out of the animal with its origin and insertion intact. The muscle was pinned into a small dish, either flat for intracellular recording, or with one end attached to a Grass FT03 isometric tension transducer for tension measurements. A suction electrode was used to stimulate the motor nerve entering the muscle. A continuously flowing perfusion system (0-8 mL/min) that allowed rapid changing of solutions without disturbing the preparation was used in all experiments. Intracellular recording or current passing was done with 15-50 MΩ, 2.5 mM-KCl-filled micro-electrodes.

Iontophoresis. In order to avoid desensitization due to leakage, high impedance electrodes were used for iontophoresis. These were kindly provided by Dr. N. C. Spitzer, and had impedances of about 100-150 MΩ when filled with 3 M-KCl. For ACh iontophoresis, they were filled with 3 M-ACh, and the ACh was iontophoresed using positive current pulses 5-30 msec in duration and 1-500 nA in amplitude. L-Glutarnato iontophoresis was done with 1 mM monosodium L-glutamate, pH 8-9. The L-glutamate was passed with negative current pulses. In some experiments a bucking voltage was applied to the iontophoretic electrode, but the high impedance electrodes prevented noticeable desensitization even in the absence of a bucking voltage. In all cases the iontophoretic electrode was positioned under visual control and moved over the surface of the muscle membrane to find spots of sensitivity.

Current monitor. Iontophoretic or intracellularly injected current was monitored using a current-to-voltage converter inserted between the bath and system ground. Extracellular junctional potentials. Conventionally, 2-10 μm tip electrodes are used for recording extracellular junctional potentials (Dautel & Kuffler, 1964a; b; Takenoshi & Takenoshi, 1964; Zucker, 1974), but fine-tipped electrodes did not produce satisfactory recordings in this preparation. However, a suction type electrode filled with 2.5 M-NaCl with a tip between 20 and 50 μm in diameter, connected to a Tektronix 122 pre-amplifier gave good reproducible results. The extracellular electrode was moved over the surface of the muscle fibres until an area of neuromuscular junctions was located. The current electrode position was judged to be the spot which gave the largest amplitude negative-going junctional potentials when the motor nerve was stimulated. The necessity for this recording configuration may be explained by the anatomy of the neuromuscular junctions on this muscle. An electron microscope examination of one such extracellularly localized neuromuscular junction (D. KING, unpublished) showed several small (≈ 0.25 μm) neuromuscular junctions in the region under the electrode. It is possible that the current produced at one such terminal is small, and that a large-tipped electrode records the summed response from many of these terminals within a relatively restricted region.

Reversal potentials. All reversal potential measurements were done with two intracellular electrodes placed within a muscle fibre within 100 μm of each other,
one used for current passing to alter the membrane potential, and the other to record the membrane potential. For experiments on the ionophoretic ACh response, the ACh electrode was positioned within 100 μm of the recording electrode, and ionophoretic pulses were given at least 1 min apart. Pulses were given at resting potential every third or fourth pulse to ensure that no desensitization had occurred. Small ionophoretic pulses (giving responses about 1 mV at resting potential) were used. The determination of the apparent reversal potential of the intracellularly recorded e.j.p.s was complicated by the considerable facilitation shown by the e.j.p.s in some muscle fibres, so only fibres showing e.j.p.s of uniform size were used. Trains of about thirty pulses were given, and the average amplitude was plotted as a function of membrane potential. In the case of the reversal potentials obtained using extracellularly recorded e.j.p.s the extracellular focal electrode was within 100 μm of the membrane potential recording electrode. Trains of e.j.p.s were elicited at various membrane potentials and filmed; amplitudes of the potentials were measured, and the mean amplitude was plotted as a function of membrane potential. Because of the quantal variation of these potentials, about 100 extracellularly recorded e.j.p.s were averaged at each membrane potential. In all cases the value of the reversal potential was obtained by extrapolation using a computer programme least squares fit (courtesy of Dr W. A. Witanen).

RESULTS

The PD-dorsal dilator neuromuscular junction

The stomatogastric ganglion contains two PD motor neurones which innervate both the dorsal dilator and ventral dilator muscles of the lobster foregut (Maynard & Dando, 1974). The dorsal dilator muscle is a typical crustacean striated muscle, with muscle fibres about 1 cm long, and 100-300 μm in diameter. When the PD motor nerve is stimulated, small (0.5-2 mV), highly facilitating e.j.p.s can be recorded in the dorsal dilator muscle fibres. Many muscle fibres receive innervation from both PD motor axons.

Earlier work (Marder, 1974) showed that single PD motor neurone somata contained the enzyme choline acetyltransferase, and that the dorsal dilator muscle contracted in the presence of 2 × 10^{-5} M ACh with 10^{-4} g/ml, Tension (also see Fig. 8). In addition, ionophoretic depolarizing responses to ACh were recorded from dorsal dilator muscle fibres and e.j.p.s in the dorsal dilator muscle fibres were blocked by 5 × 10^{-3} M tabun. These data suggested that ACh is the transmitter at this junction. This section presents additional evidence which supports this conclusion.

Pharmacological comparison of the ACh response and the e.j.p. in the dorsal dilator muscle. Typical responses to iontophoretically applied ACh can be seen in both Figs. 1 and 2. In all experiments the amplitude and rise time of the ACh response were dependent on the amplitude and duration of the ionophoretic current pulse and the positioning of the ionophoretic electrode over the muscle fibre membrane.
Fig. 1 shows that $5 \times 10^{-5}$ M tubocurare reversibly blocked the ACh response. This concentration of tubocurare also blocked c.j.p.s in this muscle (Marder, 1974). Hyperpolarizing current pulses were passed into the muscle fibre with a second micro-electrode to determine if any conductance change was associated with the action of the tubocurare, but none was detected.

Fig. 1. The response to iontophoretically applied ACh was reversibly blocked by $5 \times 10^{-4}$ M tubocurare. In all cases the current through the iontophoretic electrode was 150 nA for 25 msec. A, response in normal saline; B, 3 min in $5 \times 10^{-4}$ M tubocurare; C, 6 min in $5 \times 10^{-4}$ M tubocurare; D, 3 min wash in normal saline; E, 6 min wash in normal saline.

Hexamethonium reversibly blocked the iontophoretic ACh response and the c.j.p.s, as can be seen in Fig. 2. Hexamethonium, $5 \times 10^{-4}$ M, attenuated the c.j.p.s to 40% of the control level, and they returned to 80% of the control value after a short wash; $3 \times 10^{-4}$ M hexamethonium
decreased the iontophoretic ACh response 50%, and a complete reversal of the effect was seen after washing. No conductance change due to the hexamethonium was noted.

Similar experiments were performed with several other drugs. Atropine, 10⁻³ M, produced an 87% decrease in the size of the ACh response, with complete reversal after a short wash; 2 x 10⁻⁴ M atropine reduced the amplitude of the e.j.p.s to 30% of their control size. Strychnine, 10⁻³ M, reversibly blocked both responses by 70–90%. Concentrations of picrotoxin as low as 10⁻⁴ M had an appreciable blocking effect on the e.j.p. in this muscle, and 10⁻³ M picrotoxin blocked the ACh response 87%, completely reversibly. No direct effect of the drugs on membrane conductance was noted.

Several experiments were done to determine if the ACh receptor on the dorsal dilator muscle was sensitive to the snake toxin, α-bungarotoxin.
(Chang & Lee, 1963). These experiments were done with tension experiments rather than intracellular recording for technical reasons, but showed that the ACh-contracture and the nerve-elicited contraction in the dorsal dilator muscle were blocked by α-bungarotoxin (approximately $10^{-4}$ M). The response failed to recover after several hours of washing.

Fig. 3. Excitatory junctional potentials, e.j.p.s, in the dorsal dilator muscle were unaffected by $2 \times 10^{-5}$ M γ-methyl ester glutamate. A, e.j.p.s in normal saline; B, e.j.p.s 14 min after the addition of $2 \times 10^{-5}$ M γ-methyl ester glutamate. There is a small decrement in the size of the e.j.p.s owing to a depolarization of the membrane potential from $-64$ to $-52$ mV. C, e.j.p.s after a 12 min wash; they are the same size as in (A), and the membrane potential returned to $-60$ mV.

In contrast to the above drugs, γ-methyl ester glutamate, a suggested N-glutamate antagonist (Lowagie & Gerschenfeld, 1974), had no such effect (Fig. 3). The e.j.p.s in the presence of the drug were a little smaller, but the drug depolarized the membrane from $-61$ to $-52$ mV, and this may account for the slight change in the size of the e.j.p.s. After washing the membrane potential returned to $-60$ mV.

**Potentiation of the e.j.p. with Tension.** Since the early demonstration by Fatt & Katz (1951, 1952) that anticholinesterase drugs enhanced the
amplitude and the duration of the cholinergic e.j.p. at the frog neuromuscular junction, potentiation of a synaptic response with anti-cholinesterase drugs has been used to provide support for its cholinergic nature. Tensilon is a rapidly acting, highly reversible cholinesterase inhibitor (Katz & Thealff, 1957; Koelle, 1970).

![Graphs](image)

Fig. 4. The effect of $10^{-4}$g/ml. Tensilon on the excitatory junctional potentials, e.j.p.s, in the dorsal dilator muscle: A, e.j.p.s in normal saline; B, e.j.p.s after 7 min in $10^{-4}$g/ml. Tensilon; C, e.j.p.s after 8 min washing in normal saline.

It was demonstrated physiologically in two ways that Tensilon blocked the acetylcholinesterase in the dorsal dilator muscle: firstly, Tensilon produced a significant potentiation of the ACh contracture, assayed by monitoring muscle tension during bath application of $2 \times 10^{-4}$M-ACh, $10^{-3}$g/ml. Tensilon produced a maximal contracture; secondly, Tensilon produced large potentiations of the iontophoretic response in this muscle. Concentrations as low as $10^{-2}$g/ml. doubled the amplitude of the iontophoretic response. Higher concentrations caused potentiations that were difficult to quantitate because the iontophoretic responses became large enough to produce muscle contraction. The effects of the Tensilon were rapidly reversible in both experiments.
The effects of Tensilon on the e.j.p. in the dorsal dilator muscle depended on the concentration of Tensilon applied. Fig. 4 shows the effects of low Tensilon concentrations (10⁻⁵ g/ml.) on the e.j.p.s. In this experiment the e.j.p.s were almost doubled in size to 188% of the control value, and after a short wash the effect of the Tensilon was partially reversed. This would be the predicted effect on a cholinergic e.j.p.

Higher Tensilon concentrations (10⁻⁴ g/ml.) potentiated the e.j.p. in the dorsal dilator muscle; the e.j.p.s increased further during the wash period, and then finally decreased after a long wash. High Tensilon concentrations (10⁻³ to 10⁻⁴ g/ml.) produced no apparent potentiation of the e.j.p.s, and sometimes appeared to diminish them, but a slight potentiation was frequently seen some time after the start of the wash in normal saline.

\[ A \]

\begin{figure}[h]
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\includegraphics[width=0.5\textwidth]{figure5}
\caption{Desensitization of the ACh receptor on the dorsal dilator muscle. Iontophoretic pulses of ACh were delivered from a high impedance electrode. The current was 290 nA for 20 msec for all pulses. A, iontophoretic ACh responses once every 10 sec. Note the decrease in amplitude. After a 2.5 min wait the amplitude is larger again. B, iontophoretic responses once every 2 sec. The decrease in amplitude is more dramatic than that in (A). After a 2.5 min wait, the amplitude increases again.}
\end{figure}

Cross-desensitization of the junctional response with ACh. The ACh receptor on the dorsal dilator muscle shows rapid desensitization to repeated or continual applications of ACh. Fig. 5 shows that repeated iontophoretic applications of ACh resulted in successive responses of decremented size. After a rest period the amplitude of the ACh response recovered. The ACh electrode used here had a high impedance which minimized ACh leakage. Lower resistance electrodes gave rapid, uncontrollable desensitization even when a bucking voltage was used.

Both application of ACh reversibly blocked the nerve-evoked contractions in the dorsal dilator muscle. To determine if this effect was due to desensitization of the post-synaptic receptor, or to action on the pre-synaptic release of transmitter, experiments were done using focal
application of ACh at sites of extracellularly recorded junctional potentials. In the experiment shown in Fig. 6 a recording electrode was placed near the surface of the muscle at a point which gave a large nerve-terminal spike followed by negative-going junctional potentials (Fig. 6A). Then a large-tipped pipette filled with 3 M ACh was placed nearby, allowing ACh to diffuse out of the tip on to the junctional area. The negative-going junctional potentials were completely abolished by this treatment, but the size of the nerve terminal spike was also affected (Fig. 6B). After a short wash both the nerve terminal spike and the junctional potential returned to their control levels (Fig. 6C). In similar experiments $5 \times 10^{-4}$ M L-glutamate placed in the bath had no effect on either extracellularly recorded junctional potentials or on intracellularly recorded e.j.p.s.

Fig. 6. The effects of exogenously applied ACh on the nerve terminal spike and extracellularly recorded excitatory junctional potentials, e.j.p.s. A, extracellularly recorded e.j.p.s with the nerve terminal spike preceding them are shown. B, a large-tipped pipette filled with ACh was lowered down next to the focal recording electrode. Leakage of the ACh from the electrode caused the abolition of the e.j.p.s and a diminution of the nerve terminal spike. C, after 8 min of washing with normal saline, both the nerve terminal spike and the e.j.p.s recovered in amplitude.
Reversal potentials. The apparent reversal potential of the iontophoretic ACh response was determined thirteen times. One of these experiments is plotted as the squares in Fig. 7. In this determination the reversal potential determined by extrapolation was \(-18\) mV. The mean reversal potential of the thirteen determinations was \(-18 \pm 8\) mV (s.d. of the observation), and the range was between \(-30\) and \(-6\) mV. If care was not taken to avoid desensitization, or if large amplitude iontophoretic responses were used, misleadingly positive reversal potentials were obtained.

![Graph showing reversal potentials](image)

Fig. 7. Reversal potentials for the iontophoretic ACh response and for intracellularly and extracellularly recorded excitatory junctional potentials, e.j.p.s, in the dorsal dilator muscle; see Methods for full description. Filled circles, extracellularly recorded e.j.p.s; filled squares, iontophoretically applied ACh; open circles, intracellularly recorded e.j.p.s. The resting potentials for the muscle fibres used in the iontophoretic and intracellular e.j.p. determinations were \(-56\) mV. The resting potential for the muscle fibre used for extracellularly recorded e.j.p. determination was \(-61\) mV.

Two different methods were used to determine the apparent reversal potential of the e.j.p. in the dorsal dilator muscle. The open circles on Fig. 7 show a plot of a reversal potential determination using intracellularly recorded e.j.p.s. The reversal potential determined by extrapolation is \(-18\) mV. In five out of seven cases, the reversal potential was in the range \(-1\) to \(-28\) mV with a mean and s.d. of values of \(-15 \pm 9\) mV. In two cases the extrapolated values were \(+60\) and \(+40\) mV.

The closed circles in Fig. 7 are the results of an experiment done to determine the reversal potential of the e.j.p. using extracellularly recorded e.j.p.s. Because of the quantal variation in these potentials (see Fig. 6)
each point on the graph represents the mean of fifty to a hundred potentials at the given membrane potential. In this experiment the extrapolated reversal potential was $-20 \text{ mV}$. In four out of five trials the reversal potentials were between $-8$ and $-20 \text{ mV}$, with a mean and s.d. of observations of $-16 \pm 5 \text{ mV}$. In one case a value of $+30 \text{ mV}$ was obtained.

Fig. 8. The effects of bath application of ACh and L-glutamate on the muscles innervated by the PD and VD neurones. A, the PD-innervated ventral dilator muscle cpv2b, was attached to the tension transducer. In the upper trace, $5 \times 10^{-3} \text{ m ACh}$ with $10^{-3} \text{ g/ml Tension was added to the bath at the downward arrow. The muscle contracted, and was washed at the upward arrow. In the bottom trace, } 10^{-5} \text{ m L-glutamate was added to the bath at the downward arrow, but no response resulted. The wash was started at the upward arrow. B, the same as in (A), for the PD-innervated dorsal dilator, cpv1a,b, except } 2 \times 10^{-5} \text{ m ACh was used. C, as in (A), for the VD-innervated cv1 muscle.\\\\Screening of the other stomatogastric motor neurones\\\\In order to establish a neurotransmitter candidate for each of the stomatogastric ganglion motor neurones two kinds of experiments were done: choline acetyltransferase (E.C. 2.3.1.6), the enzyme responsible for the biosynthesis of ACh in nervous tissue (Hebb, 1972), was assayed in single, physiologically identified motor neurone somata to determine which of these neurones were capable of synthesizing ACh; additionally, experiments were performed to test which putative transmitters were
capable of producing contractures in the muscles innervated by each of the stomachogastric ganglion motor neurones.

**Pyloric group motor neurones**

The neurones included in the pyloric group include the PD, VD, PY, LP, and IC (Maynard & Solerston, 1975).

**PD neurones.** There are two PD neurones in each stomachogastric ganglion, and they innervate two main muscles, the dorsal dilator (epv1a, b) and the ventral dilator (epv2b) (Maynard & Dando, 1974). Bath application of ACh (2–5 x 10^-4 M ACh with 10 g/ml Tension) produced maximal

<table>
<thead>
<tr>
<th>Motor neurone</th>
<th>Mean and s.d. p-mole ACh/cell per hour</th>
<th>No. somata with activity</th>
<th>No. somata assayed</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>12±6±8±1</td>
<td>10/18</td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td>1±1±7±3±6</td>
<td>1/10</td>
<td></td>
</tr>
<tr>
<td>VD</td>
<td>11±3±2</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>IC</td>
<td></td>
<td>0/3</td>
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<tr>
<td>PY</td>
<td>7±2±6</td>
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<td></td>
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<tr>
<td>LPG</td>
<td>9±1±3±6</td>
<td>3/7</td>
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**Ganglion remainders** 2058±1280

This table summarizes ten experiments in which single, physiologically identified stomachogastric motor neurone somata were assayed. The last column shows the ratio of the number of somata which contained significant activity to the number of somata assayed. The means and s.d. of the values obtained for those cell types in which a majority of the cells contained activity are shown in the middle column. The limit of sensitivity was calculated for each individual assay as described in the Methods, and varied between 1-5 and 4-0 p-mole ACh/tube per hour. The mean of the limit of sensitivities of the individual assays was 2.3±0.9 p-mole ACh/tube per hour. Ganglion remainders refer to the piece of neuropile tissue remaining after the cells were dissected.

contractures in both of these muscles (Fig. 8), but 10^-5 M L-glutamate failed to do so. The dorsal dilator muscle also failed to respond to 10^-2 M octopamine, dopamine, serotonin, aspartate, glycine, y-aminobutyric acid (GABA) and taurine. Carbamyl choline (carbachol) and ACh without Tension are effective in producing contractures as well, although higher concentrations are required to produce maximal contractures. Individual PD somata contained choline acetyltransferase activity in sixteen out of eighteen somata assayed (Table 1). These data are presented here for comparison with those of the other motor neurones, since further extensive
work on the PD-dorsal-dilator junction has been presented in the first section of this paper, and the PD neurones are almost certainly cholinergic.

**VD neurone.** There is one VD neurone in each stomatogastric ganglion which innervates muscle cv1 (Maynard & Dando, 1974). Fig. 8 shows that the cv1 muscle responded to $5 \times 10^{-5}$ M ACh with Tensilon, but not to $10^{-3}$ M L-glutamate. Choline acetyltransferase activity was detected in four out of four VD somata assayed (Table 1), so ACh is a transmitter candidate for the VD neurone.

![Diagram](image)

Fig. 9. **A**, effect of ACh and L-glutamate on the pyloric region of the stomach containing muscles innervated by the LP and PY neurones. The nerve innervating the muscle was stimulated at 10/sec for 2 sec to elicit a nerve-evoked contraction to show that the muscles were viable. In the upper trace, $10^{-4}$ M ACh with $10^{-4}$ g/ml Tensilon was added to the bath at the downward arrow. No contraction resulted. NS denotes nerve stimulation. The wash was started at the upward arrow. In the bottom trace, $10^{-3}$ M L-glutamate was added to the bath at the downward arrow. A small contraction resulted. The wash was started at the upward arrow. **B**, the effects of ACh and L-glutamate on muscle cv2, innervated by the IC neurone. In the upper trace $10^{-4}$ M ACh with $10^{-4}$ g/ml Tensilon was added to the bath at the downward arrow. No contraction resulted. The wash was started at the upward arrow. In the lower trace, $10^{-3}$ M L-glutamate was added to the bath at the downward arrow, resulting in a contraction. The wash was started at the upward arrow.
LP and PY neurones. There is one LP and eight PY neurones in each stomatogastric ganglion. The responses to ACh and L-glutamate of the region of the stomach innervated by these neurones (including muscles p1, p2, p8, p10, p12, p13 (Maynard & Dando, 1974)) are shown in Fig. 9.1. In this experiment the nerve innervating these muscles was stimulated to verify the viability of the preparation. ACh, $10^{-4}$ M, with $10^{-3}$ g/ml. Tension failed to produce contractures in these muscles, but $10^{-3}$ M L-glutamate did produce slight contractures. C. Lingle (unpublished) found similar results using the muscles innervated by the LP neurone alone, and has shown that these muscles are sensitive to concentrations of L-glutamate comparable to those active in other crustacean glutamate-sensitive muscles (Robbins, 1959; Van Harreveld & Mendelson, 1959). Neither the LP or PY somata contained detectable choline acetyltransferase activity (Table 1). L-Glutamate is a candidate for the neurotransmitter at these junctions, and ACh can be ruled out.

IC neurone. There is one IC neurone in each ganglion, and it innervates muscle ev2 (Maynard & Dando, 1974). Muscle ev2 contracted in the presence of $10^{-3}$ M L-glutamate, but did not respond to $10^{-3}$ M ACh with Tension (Fig. 9B). Only three IC somata were assayed, but choline acetyltransferase activity was not detected in them (Table 1). L-Glutamate is a transmitter candidate at this junction, and ACh can be ruled out.

Gastric system

The neurones comprising the gastric system of the stomatogastric ganglion include the GM, LPG, LG, MG, DG, and AM neurones (Mulloney & Selverston, 1974a; Selverston & Mulloney, 1974a).

GM neurones. There are four GM neurones in each stomatogastric ganglion. They innervate three main muscles gm1, gm2, and gm3c. Each of these muscles contracted in the presence of $5 \times 10^{-5}$ M ACh with $10^{-3}$ g/ml. Tension, but failed to respond to $10^{-3}$ M L-glutamate. Choline acetyltransferase activity was present in nineteen out of twenty-three GM somata assayed (Table 1). ACh is a transmitter candidate for the GM neurones.

LPG neurones. There are two LPG neurones which innervate gm3a (Maynard & Dando, 1974). Muscle gm3a contracted in response to $5 \times 10^{-5}$ M ACh with Tension, but did not respond to $10^{-3}$ M L-glutamate. Choline acetyltransferase activity was present in five out of seven of the LPG somata assayed (Table 1). ACh is designated the putative transmitter for the LPG neurones.

MG neurone. The MG neurone innervates gm9 (Maynard & Dando, 1974). Muscle gm9 contracted in the presence of $10^{-4}$ M ACh, but failed to respond to $10^{-3}$ M L-glutamate. Choline acetyltransferase assays were not performed
on MG somata, but ACh iontophoresis on to gm9 muscle fibres resulted in depolarizations of the muscle fibres, so ACh is a likely transmitter candidate for the MG neurone.

**LG neurone.** The LG neurone innervates muscle gm6b (Maynard & Dando, 1974), which contracted to bath application of $2 \times 10^{-5}$ M ACh, with $10^{-4}$ g/ml Tension, but not to $10^{-3}$ M L-glutamate, and is putatively cholinergic.

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**Fig. 10.** Iontophoretic response to L-glutamate recorded in a c7 muscle fibre innervated by the AM motor neurone. A, e.j.p.s in the c7 muscle fibre produced by stimulating the AM motor nerve at 30/sec. The horizontal calibration bar is 80 msec. The vertical calibration bar is 0.4 mV. B, Iontophoretic response to L-glutamate. The iontophoretic electrode was filled with 1 mM sodium glutamate, pH 8.0 and glutamate was iontophoresed using negative current pulses. The horizontal calibration bar is 2 sec and the vertical bar is 4 mV. The iontophoretic pulse was 100 nA for 50 msec.

**DG neurone.** The single DG neurone innervates muscle gm4 (Maynard & Dando, 1974), which contracted in response to $10^{-4}$ M ACh, but not in response to $10^{-3}$ M L-glutamate. Iontophoresis of ACh on to this muscle produced depolarizations of the muscle fibres.

**AM neurone.** The AM neurone innervates a diffuse set of muscle fibres running over the surface of the cardiac sacc, designated c7 (Maynard & Dando, 1974). Bath application of L-glutamate ($10^{-3}$ M) produced contractions in the cardiac sacc, but innervation of portions of the sacc by neurones other than the AM is possible. Therefore c7 muscle fibres were impaled with a recording electrode, and a suction electrode was used to
stimulate the A\(\text{M}\) motor nerve to produce e.j.p.s (Fig. 10.4). L-Glutamate was then iontophoresed on to the muscle fibre receiving e.j.p.s from the A\(\text{M}\) (Fig. 10.5). L-Glutamate is a candidate for the neurotransmitter at the A\(\text{M-e}\) neuromuscular junction.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Effect on ACh response</th>
<th>Effect on excitatory junctional potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubocurarine (5 (\times) 10(^{-5}) m)</td>
<td>Reversibly blocks</td>
<td>Reversibly blocks</td>
</tr>
<tr>
<td>Hexamethonium (3 (\times) 10(^{-4}) m)</td>
<td>Partial block (reversible)</td>
<td>Partial block (reversible)</td>
</tr>
<tr>
<td>Atropine (10(^{-3}) m)</td>
<td>Reversibly blocks</td>
<td>Reversibly blocks</td>
</tr>
<tr>
<td>2-Hungarotoxin ((\geq) 10(^{-3}) m)</td>
<td>Irreversibly blocks</td>
<td>Irreversibly blocks</td>
</tr>
<tr>
<td>Strychnine (10(^{-3}) m)</td>
<td>Reversibly blocks</td>
<td>Reversibly blocks</td>
</tr>
<tr>
<td>Pierotoxin (10(^{-4}) – 10(^{-5}) m)</td>
<td>Reversibly blocks</td>
<td>Reversibly blocks</td>
</tr>
<tr>
<td>(\gamma)-Methyl ester glutamate</td>
<td>Not tested</td>
<td>No effect</td>
</tr>
<tr>
<td>(2 (\times) 10(^{-7}) m)</td>
<td>Not tested</td>
<td>No effect</td>
</tr>
<tr>
<td>L-Glutamate (5 (\times) 10(^{-4}) m)</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Pharmacological agents are widely used to provide evidence about the identity of the neurotransmitter at a given synapse. Table 2 is a summary of the effects of pharmacological blocking agents on the e.j.p. and the ACh response in the PD-innervated dorsal dilator muscle, and shows that drugs which blocked the ACh receptor also blocked the junctional receptor at similar concentrations and to a similar extent. These drugs include some widely known for their ability to block cholinergic receptors such as tubocurarine, atropine, hexamethonium, and 2-hungarotoxin, as well as drugs which are not widely known for their ability to block cholinergic receptors, in particular, strychnine (which does, however, block some *Aplysia* ACh receptors (Kehoe, 1972)) and pierotoxin. Thus the junctional receptor and the ACh receptor have a similar pharmacological profile, which is consistent with the junctional receptor being an ACh receptor. The failure of both \(\gamma\)-methyl ester glutamate and L-glutamate to block the e.j.p. in this muscle is further evidence that L-glutamate is not the neurotransmitter at this junction.

Tensilon, a rapidly acting anticholinesterase drug, had concentration-dependent effects on the e.j.p.s recorded in the dorsal dilator muscle, with reversible potentiation seen at low concentrations and a blocking effect at high concentrations. Similar effects were seen by Eccles & MacFarlane (1949) and Boyd & Martin (1956) using other anticholinesterase drugs, and by Dennis, Harris & Kuffler (1971) with Tensilon. The potentiation seen at low (10\(^{-5}\) g/ml.) Tensilon concentrations is evidence that the e.j.p. in the dorsal dilator muscle is cholinergic.
Exogenously applied ACh blocked both nerve-elicited contraction and extracellularly recorded junctional potentials in the dorsal dilator muscle. This is consistent with the interpretation that exogenous ACh reacts with the junctional receptors to desensitize them, thus leaving them incapable of reacting to neurally released ACh. These experiments do not clearly rule out a presynaptic effect of the ACh, since the amplitude of the nerve terminal spike was somewhat affected by the local application of ACh at the nerve terminal region. However, the nerve terminal spike did remain under conditions in which the junctional potentials were completely abolished.

The reversal potentials of the iontophoretic ACh response and the e.j.p. in the dorsal dilator muscle are approximately $-20$ to $-15$ mV. This suggests that the same conductance changes are involved in both responses, which is consistent with the junctional receptor being an ACh receptor. The quantitative accuracy of these measurements is open to some doubt since it was necessary to extrapolate to determine the reversal potential, which is quantitatively accurate only if the muscle fibre has a linear current–voltage relationship, which many crustacean muscles do not show (Reuben & Gainer, 1962; Ozeki, Freeman & Grundfest, 1966; Tarasevich, 1971, 1975). The dorsal dilator muscle showed fairly linear current–voltage plots, with some anomalous rectification, in the range between $-40$ and $-90$ mV, but it is still not possible to judge the extent of the error due to the extrapolation. Several cases of positive reversal potentials for the junctional response were found. These may be evidence of real diversity in the ionic mechanisms involved (Tarasevich, 1975). Alternatively, they may be artifacts due either to the multi-terminal innervations (see Burke & Ginsborg, 1956, for a treatment of this issue) or, in the case of the extracellularly recorded junctional potentials, due to picking up current from a neighbouring fibre.

The evidence that ACh is the neurotransmitter at the PD-dorsal dilator neuromuscular junction is considerable. This includes the biochemical demonstration that choline acetyltransferase is present in the cell somata, that ACh mimics the PD nerve by causing both contraction and depolarization, and physiological and pharmacological evidence of the identity of the receptor for ACh and the neurally released transmitter.

Two additional lines of evidence would be desirable to complete unequivocally the proof that ACh is the neurotransmitter at this junction: the first is the demonstration of stimulus-dependent, Ca$^{2+}$-dependent ACh release; the second is the demonstration that ACh receptors on the dorsal dilator muscle are actually localized at the nerve-terminal regions, in post-junctional membrane. This could be done directly using fluorescent or radioactively labelled α-bungarotoxin.
Table 3 presents a summary of the evidence used to establish a transmitter candidate for each of the stomatogastric ganglion motor neurones. Twelve motor neurones, including the GMs, PDs, LPGs, LG, MG, DG, and VD are putatively cholinergic. Eleven motor neurones, including the LP, PYs, IC, and AM are putatively glutaminergic. These account for all the known motor neurones, and for twenty-three out of the thirty or so neurones in the ganglion.

Selverston & Mulloney (1974b) and Selverston et al. (1976) present a complete diagram of the synaptic interactions among the stomatogastric ganglion motor neurones, based on all currently available physiological

<table>
<thead>
<tr>
<th>Motor neurone</th>
<th>Muscle innervated</th>
<th>Choline acetyltransferase activity</th>
<th>Muscle response to ACh ($10^{-4}$ M)</th>
<th>Muscle response to L-glutamate ($10^{-3}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD (2)</td>
<td>epv1a, b</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>epv2b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP (1)</td>
<td>p1,</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>PY (8)</td>
<td>p8,13,6,11,7, etc.</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>VD (4)</td>
<td>ev1</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>IC (4)</td>
<td>ev2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GM (4)</td>
<td>gn1</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>gn2</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>gn3c</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LPG (2)</td>
<td>gm3a</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DG (1)</td>
<td>gn4</td>
<td>Not tested</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>LG (1)</td>
<td>gn5b</td>
<td>Not tested</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MG (1)</td>
<td>gn9</td>
<td>Not tested</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>AM (1)</td>
<td>c7</td>
<td>Not tested</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

The first column has the names of the stomatogastric motor neurones, according to the terminology of Mulloney & Selverston (1974a), and in parentheses the number of those neurones in each stomatogastric ganglion. The second column contains the muscles innervated by the motor neurones, according to the terminology in Maynard & Dando (1974). The third column contains a (+) in cases where choline acetyltransferase activity was found in the majority of the cells of that type assayed. The fourth column contains a (+) if the muscle contracted in the presence of ACh in concentrations of $10^{-4}$ M or lower. The fifth column indicates (+) when the muscle responded to L-glutamate in concentrations of $10^{-3}$ M or lower.

* In most experiments the ACh-sensitive muscles showed no responses to L-glutamate ($10^{-3}$ M) at the amplifier sensitivities set to give full scale deflections for the ACh responses. However, in several cases very small contractions ($<10\%$ of the ACh responses) were seen in response to $10^{-3}$ M glutamate. These contractions appear to be a non-specific effect on the muscle at high glutamate concentrations. The glutamate-sensitive muscles responded to L-glutamate in the $10^{-3}$ M range, and gave proportionally much larger glutamate responses.
data. There are a number of electrical connexions among the motor neurones, including one series of connexions linking the PDs, VD, LPGs, MG, LG, and GMs. All of these neurones are putatively cholinergic. This suggested the possibility that electrically coupled neurones might be under constraints to use the same neurotransmitter. However, the DG and AM neurones are weakly coupled to each other, although not to any other neurones (Sundlerton & Mulloney, 1974a), and apparently use different transmitters, which argues against the generality of the above hypothesis. Further experiments are in progress to establish more completely the neurotransmitters used by the DG and AM neurones.

There is no apparent difference between either the anatomy or physiology of the stomatogastric system muscles and those of the well-studied claw or walking leg muscles of these animals (Govind, Atwood, & Maynard, 1973). This raises the issue of why certain stomatogastric motor neurones utilize a different excitatory neuromuscular transmitter than the well-studied, presumably glutaminergic systems (Takeuchi & Takeuchi, 1964; Taraskevich, 1971, see Gerschenfeld, 1973, for review). A possible factor is the lack of peripheral inhibition in the stomatogastric system (Maynard & Atwood, 1969; Govind et al., 1975), in contrast to the claw and walking leg muscles which also receive inhibitory innervation, likely GABAnergic (Takeuchi & Takeuchi, 1965, 1966). The superficial flexor muscles of the abdomen of the crayfish and lobster receive innervation from one inhibitor and five excitors (Kennedy & Takeda, 1965). Futamachi (1972) provided suggestive evidence that F6, the largest of the excitatory motor neurones, is cholinergic. Evoy & Beránêk (1972) found localized areas of glutamate and GABA sensitivity on the same muscle, and Otsuka, Kravitz & Potter (1967) found GABA in the cell body of the superficial flexor inhibitor. Thus the superficial flexor muscle fibres appear to receive a mixed cholinergic and glutaminergic excitatory innervation along with a GABAergic inhibitory innervation.

It therefore becomes of interest to examine the neuromuscular transmitters used at hitherto unstudied junctions within the crustacean neuromuscular system, to determine if there are substances other than ACh and glutamate used as transmitters. It may be that the transmitter used by motor neurones is in some way defined by the nature of the physiological interactions the motor neurones make with other neurones in the central nervous system, rather than being an intrinsic property of the excitatory motor neurone.

At present no information is available concerning the neurotransmitter substances released by the stomatogastric motor neurones within the neuropile of the stomatogastric ganglion. Most of these motor neurones make chemical inhibitory synapses within the ganglion (Maynard, 1972;
Mulloney & Selverston, 1974a, b; Selverston & Mulloney, 1974a; Maynard & Selverston, 1975), and it is important to establish whether the neurons which maintain cholinergic excitatory neuromuscular junctions also make cholinergic inhibitory junctions in the neuropile of the stomatogastric ganglion.

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REFERENCES


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