

1-GLUTAMATE AND SEROTONIN ARE ENDOGENOUS IN SQUID CHROMATOPHORE NERVES

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Summary

Colour changes in cephalopods are controlled by complex organs termed chromatophores whose radial muscles are directly innervated from the brain. In the squids Alloteuthis subulata and Loligo vulgaris, light microscopy of silver- or Methylene-Blue-stained preparations shows that each muscle is innervated by 2–6 nerves running along its length. An electron microscope (EM) study shows that most of these nerves contain 50 nm diameter electron-lucent vesicles organised into numerous synapses along the muscle. Their size and appearance is consistent with their containing l-glutamate (l-Glu). Usually there is one nerve on each muscle containing 95 nm diameter electron-dense vesicles that are not organised into synapses. Such vesicles, whose appearance is consistent with their containing serotonin (5-HT), are never found co-localised with the small, clear vesicles.

Topically applied l-Glu causes the radial muscles to contract (and the chromatophore to expand), even after chronic denervation; this effect is blocked by the glutamate antagonists CNQX and DNQX. In contrast, topically applied 5-HT (or its agonists 8-OH-DOPAT and α-methyl 5-HT) induces relaxation of precontracted muscle. Incubation with antibodies to l-Glu (Lg-A), using peroxidase anti-peroxidase/diaminobenzidine visualisation, produces specific staining along the radial muscles like that seen with silver. Antibodies to 5-HT produce similar specific staining. When sections of skin that had stained positively with Lg-A in the light microscope are examined at the EM level, it is seen that such staining is confined to nerve axons.

These results, showing that l-Glu and 5-HT are endogenous in the nerves innervating squid chromatophores and that the radial muscles contain receptors for both substances, suggest that l-Glu is an excitatory transmitter at squid chromatophore muscles. The way in which 5-HT acts to relax the muscles, however, remains to be established.

Key words: cephalopods, squids, Alloteuthis subulata, Loligo vulgaris, Lolliguncula brevis, chromatophores, glutamate, serotonin (5-HT), neurotransmitters.

Introduction

Naturalists have known about the colour changes of cephalopods since antiquity, and physiologists have been studying their chromatophores for well over 150 years. Our current understanding of chromatophore structure and function, however, is due almost entirely to Florey and his collaborators (Florey, 1966, 1969; Cloney and Florey, 1968; Florey and Kriebel, 1969), who used ultrastructural and electrophysiological techniques in their study of the squid Loligo opalescens. Cephalopod chromatophores are quite different from those of other animals, and Florey’s work is so fundamental that it is essential to summarise it here.

First, the chromatophores are organs, composed of five different cell types: the chromatophore proper (pigment granules in an elastic ‘sacculus’); a set of 15–25 radial muscles; axons; glial cells associated with the axons; and chromatophore sheath cells. Contraction of the muscles expands the chromatophore; when they relax, elastic forces stored in the sacculus cause it to retract (Fig. 1). Secondly, the radial muscles receive only excitatory innervation: there is no electrophysiological evidence for an inhibitory innervation. Single stimulation of the motor nerves causes twitch-like contractions; summation of individual contractions occurs above 2 Hz and smooth tetanus occurs above 12 Hz. Intracellular recordings show that the muscle fibres respond to motor nerve stimulation with non-propagating excitatory postsynaptic potentials (EPSPs) of fluctuating amplitude. There are several size classes of EPSP, indicating polynuclear innervation. Thirdly, although acetylcholine (ACh) expands the chromatophores and serotonin (5-HT) retracts them, neither substance affects the muscle membrane. Finally, neighbouring
radial muscles on a chromatophore are electrically coupled, via gap junctions.

Bone and Howarth (1980) demonstrated that l-glutamate (l-Glu) expands the chromatophores of Sepia officinalis, Alloteuthis subulata and Loligo vulgaris, and Florey et al. (1985) showed that l-Glu (and its agonists, kainate or quisqualate) elicits chromatophore expansion in another squid, Lolliguncula brevis. Subsequently, the advent of a battery of specific agonists and antagonists for l-Glu in mammals and the development of immunohistochemical techniques prompted us to re-examine the nature of the transmitters of squid chromatophore nerves. We have been able to confirm and extend Florey’s findings, principally by demonstrating for the first time that l-Glu and 5-HT are endogenous in the nerves supplying the chromatophore muscles. A very brief preliminary account of some of these findings has appeared elsewhere (Cornwell and Messenger, 1995).

Materials and methods

The results were obtained from three loliginid squids, Alloteuthis subulata Lamarck, Loligo vulgaris Lamarck, caught off Plymouth, England, and Lolliguncula brevis Blainville, from Galveston, TX, USA. The animals were caught in shallow water and transferred to holding tanks where they were maintained in circulating sea water. They were killed by decapitation and small pieces of skin (approximately 10 mm²) were covered with sea water to allow the chromatophore nerves to degenerate. The tissue was used for this experiment because it is relatively robust. After MgCl₂ anaesthesia (Messenger et al., 1985), the nerves dorsal to the stellate ganglion on one side of the mantle were cut with fine scissors. The animals recovered well from this brief operation: locomotion was unimpaired, the fin nerves remained intact and the animals fed normally and survived for up to 16 days at 21°C.

Electron microscopy

To study the ultrastructure of the chromatophore nerve–muscle junction, pieces of skin were covered with Ca²⁺-free artificial sea water (in mmol l⁻¹: NaCl, 450; KCl, 9; MgCl₂, 30; Hapes, 10; pH 7.8) and then fixed in 2.5% glutaraldehyde at pH 7.4 for 1 h at room temperature. After post-fixation in 2% OsO₄ in 1.25% sodium bicarbonate for 1 h at 4°C (for details, see Reed, 1995a), the tissue was dehydrated and embedded in hard resin (Taab). Ultra-thin sections (90 nm) were cut and stained in a saturated solution of uranyl acetate in methanol for 20 min, followed by lead citrate for 2 min.

Pharmacology

Experiments were performed at room temperature on pieces of fresh skin mounted on Sylgard, dermis uppermost. A window was cut in the dermis to allow solutions ready access to the chromatophore layers, and the preparation was stored under chilled filtered sea water. Immediately prior to testing a substance, the sea water was poured off the skin and 50–100 μl of the test solution was pipetted into the window. Drugs were first applied at a concentration of 10⁻³ mol l⁻¹, then in a series of tenfold dilutions, until there was no response. When levels close to threshold were reached, intermediate concentrations were tested. Subsequently, the experiments were repeated beginning with the most dilute solution. A seawater wash was given between each test. L-glutamate, kainic acid and 5-HT were from Sigma; 5-HT agonists were from Research Biochemicals International (Natick, MA, USA) and all other drugs were from Tocris Cookson (Bristol, UK). Stock solutions were made up in distilled water and diluted as required with filtered sea water; where necessary, the pH was adjusted to approximately 7.5 with NaOH or HCl.

In one series of experiments, we cut the nerves to an area of skin to allow the chromatophore nerves to degenerate. The squid Lolliguncula brevis was used for this experiment because it is relatively robust. After MgCl₂ anaesthesia (Messenger et al., 1985), the nerves dorsal to the stellate ganglion on one side of the mantle were cut with fine scissors. The animals recovered well from this brief operation: locomotion was unimpaired, the fin nerves remained intact and the animals fed normally and survived for up to 16 days at 21°C.

Immunohistochemistry

For immunohistochemistry, the stripped skin was fixed for 1 h in a 1% paraformaldehyde/1% glutaraldehyde solution and stored in 0.1 mol l⁻¹ Tris/NaCl buffer (pH 7.5) with 0.2% sodium azide as a preservative. For light microscopy, we used an L-glutamate antiserum (Lg-A) given to us by I. Duce and T. Budd (University of Nottingham). This was raised against L-glutamate coupled to bovine serum albumin with glutaraldehyde. The 5-HT antiserum (5-HT-A) was purchased from Immunonuclear Corporation, USA. We used the standard unlabelled peroxidase–antiperoxidase (PAP) method of Sternberger (1979), visualised using diaminobenzidine tetrachloride (DAB) as the chromogen. The swine anti-rabbit secondary antibody and the rabbit PAP were from Dakopatts Denmark Ltd. All other chemicals were from Sigma. Sections were examined and photographed using a Zeiss Ultraphot or a Nikon Optiphot microscope.

To localise l-Glu in the chromatophore organ, skin that had stained positively in the light microscope (LM) with commercially available Lg-A (Sigma) was cut into 2 mm² pieces, rehydrated through a series of alcohols, then post-fixed in 2% OsO₄ in distilled water for 30 min at 4°C. The pieces were
Fig. 1. Light micrographs of skin from *Alloteuthis subulata*. (A) Some of the radial muscles (r) of three chromatophores, attached to the pigment sacculus (s) proximally. Two muscles are seen branching and anchoring themselves in connective tissue distally (arrowheads). Scale bar, 0.1 mm. Methylene Blue staining. (B) Part of a single chromatophore after incubation with l-glutamate antiserum and PAP/DAB staining. Note intense brown staining (arrowheads) running along the radial muscles (r). Scale bar, 1 mm. (C) Part of an expanded red chromatophore (s) with its radial muscles (r) contracted. The nerves are folded at the most contractile portion of the muscle (arrowheads). Scale bar, 0.1 mm. Methylene Blue staining. (D) Partially retracted red chromatophore (s) with muscle relaxed. The nerves are no longer folded (between arrowheads). Scale bar, 0.1 mm. Methylene Blue staining. (E) Low-power view of a piece of skin (dermal side uppermost) that has just been flooded with l-glutamate (5x10^{-4} mol\text{l}^{-1}). Note how the chromatophores are expanded only in, or at the edge of, the window cut in the dermis. Scale bar, 1 mm. (F) A similar preparation in which a piece of skin with partially expanded chromatophores has just been flooded with 5-HT (10^{-5} mol\text{l}^{-1}). Scale bar, 1 mm.
dehydrated in alcohol, embedded in resin and semi-thin sections cut and stained in Toluidine Blue to locate the chromatophore muscles. Finally, ultra-thin sections (80 nm) were cut onto copper mesh grids and stained as above. Sections were examined under a Philips EM 300 electron microscope operated at 60 kV.

**Immunohistochemical controls**

Four types of control were carried out to test the specificity of the immunohistochemical results. (1) Omission of the primary antibody from the incubation medium (which left the skin unstained). (2) Incubation of skin samples in 0.3% hydrogen peroxide for 30 min prior to staining to ensure there were no peroxidase-active sites in the tissue that could bind to the PAP complex (resulting in unaltered staining). (3) Incubations with whole rabbit serum, which contains a multitude of antibodies, though presumably none to L-Glu or 5-HT (which never produced intense staining, although the background was similar to that obtained with Lg-A or 5-HT-A). (4) For the Lg-A, which was not a commercial product with a published protocol, a primary antibody absorption control was made. The antibody was exposed to the original immunogen [L-glutamate/bovine serum albumin (BSA) complex] in excess to remove all antibodies in the primary antiserum able to bind to the L-glutamate/BSA complex. Incubations of this preabsorbed antiserum with the tissue showed only a pale background staining similar to that seen using whole rabbit serum, strongly suggesting that it is a component of the primary antiserum specific to the L-glutamate/BSA complex that causes the intense staining in the tissue. Cross-reactivity controls showed that the antiserum was specific for L-glutamate (T. Budd, personal communication).

**Results**

**Light microscopy**

Staining the skin of a squid with silver or Methylene Blue revealed a rich innervation, with numerous nerve fibres running in all directions and at different levels in the tissue (Fig. 2A,C). Some fibres run to the chromatophore muscles, but the destination of others is unknown. Bundles of chromatophore nerves could be identified leaving the mantle muscle surface and entering the dermis. These bundles are typically composed of 5–15 fibres, but as they approach the chromatophore layers they split into smaller bundles, consisting of 2–6 fibres of varying thickness (Fig. 2A). These join the muscles at some point along their length; sometimes they run closely alongside the muscle towards the proximal region next to the pigmented sacculus (Fig. 2A). Some fibres terminate here, turning towards the nucleus as described by Weber (1968). Usually there was more than one nerve fibre on each chromatophore muscle (Fig. 2B) and as many as six were sometimes seen. Nerves closely associated with the radial muscles were seen in all colour classes of chromatophores.

An interesting feature of the nerve fibres is that they are much folded in the medio-proximal region of the radial muscle, which is the contractile region. Such an arrangement allows the nerves to increase in length when the chromatophore retracts and the muscle is stretched (Fig. 1C,D). With Methylene Blue vital staining, it is possible to see the nerves folding and straightening as the chromatophores expand and retract.

**Electron microscopy**

Nerve bundles were found closely associated with a lateral extension of myofilament bundles from each chromatophore muscle fibre (Fig. 3A). These bundles contain 2–4 axons. One side of the nerve bundle runs directly alongside the myofilaments, while the other side of the bundle is surrounded by glial cells.

Axons contain two kinds of synaptic vesicles: electron-lucent vesicles, with a mean diameter of 50.4±1.62 nm (S.E.M., N=50) and electron-dense vesicles, mean diameter 95.5±1.81 nm (Fig. 3B). The sizes of these two populations of vesicles were significantly different (t=1.6, d.f.=99, P<0.05). The different types of vesicle were never seen in the same axon, and only the electron-lucent vesicles were organised into synapses (see also Reed, 1995a).

**Pharmacology**

Preliminary tests with a large range of putative transmitter substances made it clear that the chromatophores were especially sensitive to L-Glu and 5-HT, and we therefore concentrated on these two substances, although some experiments were carried out with acetylcholine (ACh) (see Discussion).

**L-Glutamate**

Simple qualitative experiments were carried out to test the effects on the chromatophores of topical application of L-Glu and its agonists and the results are summarised in Table 1.

L-glutamate and certain L-Glu agonists unequivocally elicit expansion of all colour classes of squid chromatophores (Fig. 1E). The final ‘threshold’ figures given in Table 1 must be regarded as approximate because of the varying amounts of connective tissue present in different preparations, but the differences between the thresholds of the various drugs were consistent. In essence, Table 1 shows that the chromatophore muscles respond to L-Glu and its non-NMDA agonists. At the time of our first experiments, there were thought to be three types of glutamate receptor in mammals ‘N-methyl-D-aspartate’ (NMDA), ‘kainate’ and ‘quisqualate’ (Monaghan et al. 1989). We tested all of these substances and found that NMDA had no effect on the chromatophores while kainate and quisqualate expanded them. Subsequently, mammalian pharmacologists have reclassified glutamate receptors as ‘NMDA’, ‘kainate’ and ‘AMPA’ (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) (Keinanen et al. 1990, Table 1). We found that AMPA was not particularly effective at the chromatophore radial muscles, certainly less so than quisqualate, although its newly synthesised agonists, such as the halogenated willardiines, were quite active. The most effective of all the L-Glu agonists is domoate, thought to be active at kainate receptors in mammals (see Discussion).
Fig. 2. Light micrographs of skin from *Alloteuthis subulata*. (A) Chromatophore nerve (arrow) arising from a small bundle of nerves (n) running across the radial muscles (r). Scale bar, 0.1 mm. Bielschowsky silver staining. (B) Two nerve fibres (arrowheads) running in parallel along a radial muscle (r). Scale bar, 1 mm. Silver staining. (C) L-glutamate antiserum (Lg-A) (1:1000) and PAP/DAB staining. Note the staining along the radial nerves (arrowheads) of a chromatophore (s) and elsewhere in the skin (see text). Scale bar, 0.1 mm. (D) Lg-A (1:1000) and PAP/DAB staining of a nerve running along a single radial muscle fibre (r). Note the dotted appearance. Scale bar, 0.1 mm. (E) 5-HT antiserum (5-HT-A) (1:250) and PAP/DAB staining (arrowheads) along two radial nerves (r). Note the relatively high level of background staining. Scale bar, 0.1 mm. (F) 5-HT-A (1:250) and PAP/DAB staining (arrowheads) showing that at least two nerves on two radial muscles (r) contain 5-HT. Scale bar, 0.1 mm.
Fig. 3. Electron micrographs of sections through the skin of *Loligo vulgaris*. (A) Transverse section of the proximal region of a chromatophore muscle fibre. Note the two mitochondrial cores (c) surrounded by myofilaments (mf) and the lateral nerve bundle, containing two axons (ax) surrounded by glia (g). Scale bar, 1 mm. (B) Transverse section of the distal region of a muscle fibre, where the myofilaments (mf) have begun to diverge and no longer enclose a mitochondrial core. Note the large nerve bundle with axons containing either electron-dense (edv) or electron-lucent (elv) vesicles. Scale bar, 1 mm.
Neurotransmitters of squid chromatophores

Table 1. Effects on the chromatophores of some glutamate agonists

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Effect</th>
<th>Threshold (mol l⁻¹)</th>
<th>Comments</th>
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<tr>
<td>l-Glutamate</td>
<td>Expands</td>
<td>10⁻⁴</td>
<td>Inactive even at 10⁻² mol l⁻¹</td>
</tr>
<tr>
<td>d-Glutamate</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-, d-Aspartate</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMDA</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPA</td>
<td>Expands</td>
<td>5×10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Kainate</td>
<td>Expands</td>
<td>10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>C-, F-, I-Willardiine</td>
<td>Expands</td>
<td>10⁻⁴</td>
<td></td>
</tr>
<tr>
<td>Bromowillardiine</td>
<td>Expands</td>
<td>5×10⁻⁵</td>
<td></td>
</tr>
<tr>
<td>Quisqualate</td>
<td>Expands</td>
<td>2×10⁻⁵</td>
<td></td>
</tr>
<tr>
<td>l-ODAP</td>
<td>Expands</td>
<td>2×10⁻⁵</td>
<td></td>
</tr>
<tr>
<td>Domoate</td>
<td>Expands</td>
<td>5×10⁻⁷</td>
<td></td>
</tr>
</tbody>
</table>

AMPA, (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; C-, F-, I-willardiine, chloro-, fluoro-, iodo-willardiine; l-ODAP, β-N-oxalyl-d-α,β-diaminopropionic acid; NMDA, N-methyl-d-aspartic acid.

Threshold values, based on at least six measurements, are approximate.

Experiments carried out with Alloteuthis subulata.

Bath application of the quinoxalainediones, CNQX and DNQX (6-cyano-7-nitroquinoxaline; 6,7-dinitroquinoxaline), which in mammals are selective antagonists of AMPA and kainate receptors (Honoré et al. 1988), was carried out with Alloteuthis subulata. After 5 min of exposure to either of these substances (5×10⁻² mol l⁻¹), topically applied L-Glu failed to elicit chromatophore expansion, and neurally induced chromatophore expansion was also blocked. Both effects were reversible after a brief wash-out. However, application of CPP [3-[(RS)-2-carboxypiperazine-4-yl]-propyl-1-phosphonic acid], an antagonist of NMDA receptors, did not block the effects of topically applied L-Glu.

To obviate the possibility that L-Glu or its agonists were acting presynaptically, we tested the effects of L-Glu on the chromatophores after chronic denervation. For this, we used the squid Loliguncula brevis, a robust species that can survive a portion of normal skin it elicited expansion at 5×10⁻⁴ mol l⁻¹. In another animal, tested 11 days after lesioning, ACh was active on the intact ones. In both these animals, the denervated chromatophores, even at 10⁻³ mol l⁻¹, although on a portion of normal skin it elicited expansion at 5×10⁻⁵ mol l⁻¹.

In another animal, tested 11 days after lesioning, ACh was again ineffective on the denervated chromatophores; these all caused rapid expansion of the pigment sac, sometimes to a size greater than the ‘resting’ state. Taken together, these preliminary results strongly suggest the presence on or in the chromatophore radial muscles of 5-HT receptors (see Discussion).

Acetylcholine

Topically applied ACh is known to expand the chromatophores in several cephalopods but there are reasons to believe that it acts presynaptically, i.e. on the chromatophore nerves (see Discussion). If this is true, then removal of the nerves should prevent ACh from acting on the chromatophore muscles. We tested this hypothesis by taking pieces of chronically denervated skin from the squid Loliguncula brevis and comparing the effects of topically applied ACh and L-Glu on denervated and normal chromatophores. In one animal, tested 4 days (at 24 °C) after lesioning, ACh had no effect on denervated chromatophores, even at 10⁻³ mol l⁻¹, although on a portion of normal skin it elicited expansion at 5×10⁻⁵ mol l⁻¹.

In another animal, tested 11 days after lesioning, ACh was again ineffective on the denervated chromatophores, although it was active on the intact ones. In both these animals, the denervated (as well as the intact) chromatophores expanded in response to L-Glu (see Table 2).

Table 2. Increase of the sensitivity of chromatophores to glutamate after denervation

<table>
<thead>
<tr>
<th>Animal</th>
<th>Time after lesion (days)</th>
<th>Threshold (mol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>92-1</td>
<td>2</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>92-2</td>
<td>8</td>
<td>2×10⁻⁴</td>
</tr>
</tbody>
</table>

Experiment carried out with Loliguncula brevis.

Serotonin (5-HT)

Topical application of 5-HT to squid skin leads to rapid retraction of the chromatophores (Fig. 1F). The threshold for this is approximately 10⁻⁸ mol l⁻¹ in the three loliginids (Alloteuthis subulata, Loligo vulgaris and Loliguncula brevis) and in the cuttlefish Sepia officinalis. We also tested the effects on the chromatophores of three 5-HT agonists (Table 3). They all induced retraction, although only 8-OH-DPAT acts as quickly as 5-HT itself. Four 5-HT antagonists were tested on Loligo vulgaris skin after 5-HT had been bath-applied to retract the chromatophores; these all caused rapid expansion of the pigment sac, sometimes to a size greater than the ‘resting’ state. Taken together, these preliminary results strongly suggest the presence on or in the chromatophore radial muscles of 5-HT receptors (see Discussion).
Immunohistochemistry

Because of the cumulative evidence that l-Glu may be the natural transmitter substance at the chromatophore nerve–muscle junction, we employed standard immunohistochemical techniques to establish whether l-Glu is actually present in the chromatophore. Staining squid skin with Lg-A, using the standard PAP/DAB visualisation method, produces dark brown reaction products visible against a pale brown background. The pictures produced are so similar to those obtained with silver or Methylene Blue (Figs 1B, 2C) that it is assumed we are staining the chromatophore nerves. The Lg-A (1:1000) produces particularly intense staining along the course of the radial nerves, but the staining is uneven (Fig. 2D). It is more intense proximally than distally and the staining is dotted rather than continuous (see Discussion). It was not possible at the LM level to recognise individual fibres. It is interesting that the dotted appearance is absent from fibres running in bundles, i.e. before they have separated; these look much more like silver-stained fibres, being stained evenly. Other fibres in the skin, not demonstrably related to the radial muscles, also stain with Lg-A (Fig. 2C).

We then examined in the EM material that had stained positively at the LM level with Lg-A, using PAP/DAB visualisation. The quality of this material was poor because of the low glutaraldehyde concentration in the fixative and the rehydration procedure, but the major features of the chromatophore muscle and its associated nerve bundle could still be distinguished in the EM. In sections transverse to the radial muscles, the axons innervating the muscle fibres were stained dense black. Particularly striking was the fact that these densely staining axons, which could be followed through serial sections, were always surrounded by unstained glial cells (Fig. 4). This is evidence that the positive Lg-A staining seen in the LM represents a specific location of l-glutamate within the axons innervating the chromatophore muscles.

Pieces of skin treated with 5-HT-A (1:250) were also examined by light microscopy. These showed weaker staining than with Lg-A despite the much higher concentration of antibody used, and the background staining was heavier (Fig. 2E,F). Nevertheless, a dotted distribution along the chromatophore muscles was again present, similar to that seen after silver staining (Fig. 2A). Again the inference is that 5-HT is present in the axons running along the muscle.

Discussion

Multiple innervation of the chromatophores

Silver and Methylene Blue staining of squid skin confirm that squid chromatophores receive multiple innervation (Florey, 1966; Cloney and Florey, 1968; Weber, 1968; Mirow, 1972). These stains also show that the skin of loliginids is richly innervated, even though they have no musculature to produce papillae, as in Octopus vulgaris or Sepia officinalis (Packard and Hochberg, 1977; Hanlon and Messenger, 1988). The destination or origin of the numerous nerve fibres is at present unknown; some may innervate blood vessels (Schipp, 1987).

The EM investigations reported here and elsewhere (Reed, 1995a) have also shown, by following serial sections, that the radial muscles are innervated by more than one axon. However, although individual axons do not branch as they run...
along the radial muscle, they could branch before reaching it, so we cannot be certain that each axon derives from a different motoneuron in the brain, although the physiological data suggest that they do (Florey, 1969).

We have also established that the majority of axons on the radial muscle contain small (50 nm) clear vesicles organised into synapses at frequent intervals along the muscle. The size and appearance of these vesicles is consistent with their containing L-Glu (Attwood, 1982) although this awaits confirmation, for example by immuno-gold staining. The other novel finding shown by our EM studies is that there is generally one axon per radial muscle that lacks clear vesicles but has large (90 nm) electron-dense vesicles. We followed serial EM sections for the entire length of the muscle but never saw vesicles of this type organised into synapses (see below). The size and appearance of these vesicles is consistent with their containing 5-HT but, again, further evidence is required.

L-Glutamate as an excitatory transmitter at the chromatophores

The immunohistochemical evidence reported here confirms the reports of Bone and Howarth (1980), Florey et al. (1985) and Messenger et al. (1991) suggesting that L-glutamate may be the excitatory transmitter expanding squid chromatophores. However, our findings extend this earlier work in one important detail: L-glutamate is now shown to be endogenous in axons innervating squid chromatophore muscles.

Staining the chromatophores with Lg-A produces images strikingly like those seen with silver or with Methylene Blue (first pictured by Hofmann as long ago as 1907). The staining is dotted and uneven as if the concentration of L-glutamate varies along the nerve fibre, and it is tempting to speculate that high concentrations may represent groups of synapses. The electron micrographs of tissue that had stained positively for L-Glu in the LM also show unequivocally that it is the axons, not the glia, that have stained (Fig. 4).

We are aware that all immunohistochemical evidence, especially for such a ubiquitous substance as L-glutamate, needs to be treated with caution. However, we performed four standard controls that suggest it is those antibodies in the primary antisera raised against L-glutamate that are binding to the antigens in the stained squid skin. When such antibodies were removed by specific adsorption, no staining was obtained. Because the Lg-A was a novel preparation, we performed further controls to ensure that the antiserum reacted with the original immunogen and that there was no cross-reactivity with any similar substance that may have been present in the tissue. We are confident, therefore, that the staining obtained at the LM level represents endogenous L-glutamate in the chromatophore nerves.

Little can be said at present about the nature of the L-Glu receptors on the chromatophore muscles. They appear to be of the non-NMDA type and, since they are exquisitely sensitive to domoate, they seem to be most like the vertebrate kainate type. Yet they are more sensitive to quisqualate than to kainate, although relatively insensitive to AMPA. These findings agree with data for the squid giant synapse (Messenger et al. 1995) and make it clear that cephalopod glutamate receptors are not the same as mammalian ones. Yet the fact that so many of the new mammalian L-Glu agonists are active on squid chromatophore muscles reminds us that receptor molecules are very ancient and have been conserved throughout the evolution and radiation of the major phyla (Walker et al. 1996).

Serotonin at the chromatophores

We have presented immunohistochemical evidence at the LM level that 5-HT is endogenous in some of the nerve fibres innervating the chromatophore muscles. Again, a series of controls for the specificity of such staining were unequivocal, although we did not ourselves test for cross-reactivity, accepting the details supplied by the manufacturer (Sigma). Localisation of 5-HT at the EM level has yet to be made, and although the 90 nm diameter electron-dense vesicles in some nerves could well contain 5-HT (Pelletier et al. 1981; Beaudet and Descarries, 1987), more evidence is needed on this point.

We have good evidence, however, that the radial muscles bear 5-HT receptors since the chromatophores respond to 5-HT antagonists such as ketanserin and mianserin and to agonists such as 8-OH-DPAT (Table 3), even in moribund preparations in which the nerves may be presumed dead. These substances act selectively at 5-HT1 and 5-HT2 receptors in mammals and in many invertebrates (Walker et al. 1996) and, although further experiments are clearly needed, our preliminary data suggest that the 5-HT receptor in squid chromatophores is an orthodox one.

More unusual is the fact that the presumed serotonergic vesicles are not organised into synapses, suggesting that 5-HT may not be acting conventionally at the nerve–muscle junction. This is currently under investigation.

Are there other neuroactive agents at squid chromatophores?

In preliminary experiments, a range of transmitters was tested on squid skin. Apart from those already discussed, the only other transmitter to have any effect was dopamine but, since this only induced partial chromatophore expansion at concentrations in excess of 5×10⁻³ mol litre⁻¹ and since glyoxylic-acid–fluorescence staining (De La Torre and Surgeon, 1976) of the skin was negative, we concluded that the chromatophores receive no aminergic innervation.

However, in the cuttlefish Sepia officinalis, it has recently been shown that the chromatophore nerves stain positively with an antibody to FMRFamide and that the chromatophores are expanded by this peptide when it is topically applied to the skin (Loi et al. 1996). We have not stained squid skin with an antibody to FMRFamide but we have applied FMRFamide to the skin of Allocuteus subulata and Loligo vulgaris. In neither was there an effect, even at concentrations as high as 10⁻⁴ mol litre⁻¹; using the same stock solution, however, we obtained positive responses from Sepia officinalis skin, even at dilutions of 10⁻⁸ or 10⁻⁷ mol litre⁻¹, levels that agree with the findings of Loi et al. (1996). We also found that FMRFamide
was excitatory in *Octopus vulgaris*, so we conclude that there may be important differences among cephalopods in the way the chromatophores are regulated. On present evidence, it seems that loliginid squids may rely only on l-Glu and 5-HT to regulate their chromatophores.

It is worth clarifying here the status of ACh as a putative transmitter at cephalopod chromatophores, partly because Florey tested this ester extensively in his early experiments (Florey, 1966; Florey and Kriebel, 1969) and partly because there have been several recent reports in which nicotine was used to expand chromatophores (Packard, 1999a,b, 1995). Although the author made no such claim, this could be taken to imply that ACh is the natural transmitter. There is no doubt that ACh or nicotine, topically applied to the skin, expands the chromatophores in *Octopus vulgaris* (Andrews et al. 1983) and often, although not always, in loliginids (see Florey et al. 1985). There is good evidence, however, that ACh is acting presynaptically to achieve this effect. In *Loligo opalescens*, Florey and Kriebel (1969), recording from chromatophore muscles with intracellular electrodes, found that ACh had no effect on the membrane potential. However, ACh undoubtedly increased the frequency of the miniature postsynaptic potentials, an effect prevented by cholinergic blocking agents (Florey, 1966), so that ACh must have been acting presynaptically to release the (as then unidentified) natural transmitter substance. As Florey and Kriebel (1969) put it, ‘there is no question........that the presynaptic terminals are cholinceptive’.

Our denervation experiments with *Lolliguncula brevis* confirm Florey’s findings. Cephalopod nerves degenerate rapidly (Sereni and Young, 1932) and at 21 °C it can be safely assumed that by 11 days post-operation no trace of the nerves will survive. In these circumstances, ACh no longer expands the chromatophores, while l-Glu not only expands them but is effective at a lower threshold. The implications are clear: it is the nerves not the muscles that bear ACh receptors, and it is the muscles that bear l-Glu receptors.

**How are squid chromatophores controlled?**

We are now in a position to discuss the way in which squids control their chromatophores. All the evidence suggests that the radial muscles, which are obliquely striated in typical molluscan fashion (Cloney and Florey, 1968), receive only an excitatory innervation. l-Glu, a common excitatory amino acid in many phyla (Walker et al. 1996), would seem a very strong candidate to be the natural excitatory transmitter at these muscles. The innervating axons stain positively with Lg-A and the frequent synapses that they make with the radial muscle contain clear 50 nm vesicles.

These axons derive from cell bodies situated in the chromatophore lobes in the brain (Sereni and Young, 1932; Boycott, 1961; Young, 1976). When these motoneurons are activated, l-Glu will be released at numerous sites along the radial muscle, causing it to contract. Chromatophore expansion can thus be thought of as a conventional neuromuscular event, although the fact that the postsynaptic potentials are non-propagating is unusual. It is almost certainly an adaptation to allow fine control of the radial muscles by recruitment (Florey, 1969), although it is not clear why there are so many synapses (Reed, 1995a).

In *Alloteuthis subulata* and *Loligo vulgaris*, there is no evidence that any other substances are involved in expanding the chromatophores or in maintaining their expansion. In *Sepia officinalis*, however, at least some of the chromatophore nerves contain FMRFamide, which has been shown to expand the chromatophores (Loi et al. 1996). FMRFamide also expands *Octopus vulgaris* chromatophores (J. B. Messenger, unpublished observations). These important differences between cephalopods may have functional, rather than phylogenetic, significance. Both *Sepia officinalis* and *Octopus vulgaris* have to maintain the chromatophores in an expanded state for hours at a time as they show one of the chronic body patterns suitable for concealment (Hanlon and Messenger, 1988). The loliginids have a simple repertoire of body patterns (Hanlon and Messenger, 1996) and for much of the time they may conceal themselves by being transparent (i.e. chromatophores retracted).

Retraction of the chromatophores in cephalopods is thought to be a property of the cytoelastic sacculus (Cloney and Florey, 1968; Mirow, 1972). When the chromatophore nerves cease firing, energy stored in the sacculus will cause it to contract, stretching the radial muscles to their resting state. Theoretically, a single excitatory transmitter is all that is needed in such a system, but we have now shown that there is a second putative transmitter substance present in at least some of the nerves running to the radial muscle: 5-HT. When topically applied, this relaxes the radial muscles, causing the chromatophores to retract not only in loliginids but also in *Sepia officinalis* and *Octopus vulgaris* (Andrews et al. 1983; Packard, 1988), and it seems that 5-HT may play a fundamental part in the regulation of chromatophore tone. There is a substantial body of evidence that 5-HT functions as a relaxing neurotransmitter in other molluscs, both gastropods and bivalves (Muneoka and Twarog, 1983) so that this is not too surprising. Moreover, the finding of Florey and Kriebel (1969), in *Loligo opalescens*, that 5-HT has no effect on the radial muscle membrane potential agrees with data from other molluscan systems in which 5-HT acts as a neuromodulator rather than a neurotransmitter (e.g. *Mytilus edulis* anterior retractor byssus muscle, Twarog, 1954, and *Aplysia californica* accessory radula closer muscle, Weiss et al. 1978). Certainly the fact that the dense vesicles presumed to contain 5-HT are not organised into synapses militates against 5-HT being a neurotransmitter.

Florey (1969) hypothesised long ago that 5-HT might be acting intracellularly at the chromatophore muscles to initiate Ca²⁺ binding. To this extent, 5-HT could be thought of as a modulator of the excitatory action of l-Glu on the muscle. To test this and other hypotheses, we are beginning to study the Ca²⁺ dynamics of the radial muscles using modern Ca²⁺-sensitive dyes (Lima et al. 1997). Preliminary results suggest that 5-HT may be acting to suppress release of Ca²⁺ from internal stores in the muscle (Lima et al. 1997). This could also
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References


Note added in proof

Ernst Florey, whose beautiful work on chromatophores forms the starting point for this paper, died in Konstanz, Germany, on 26 September. We wish to dedicate this paper to the memory of a friend and a most distinguished physiologist.