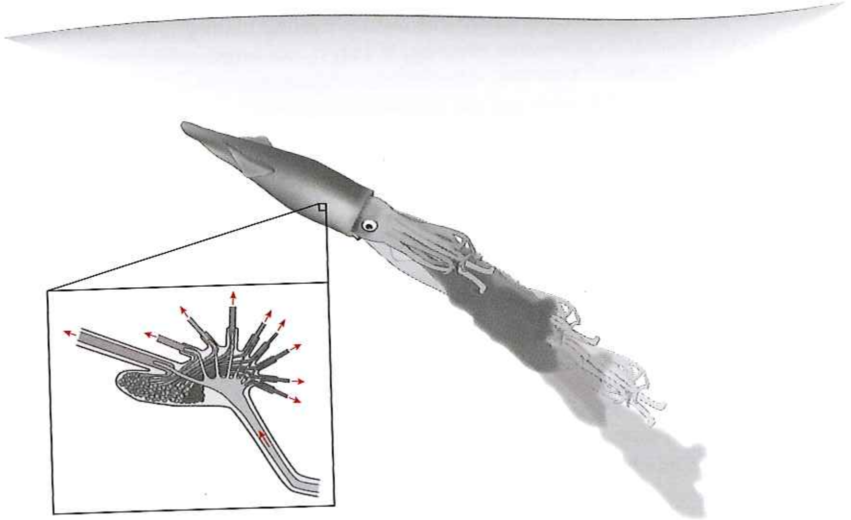
CHAPTER 12

Molluscan Defence and Escape Systems



The Neuroethology of Predation and Escape, First Edition. Keith T. Sillar, Laurence D. Picton and William J. Heitler.

CHAPTER 12

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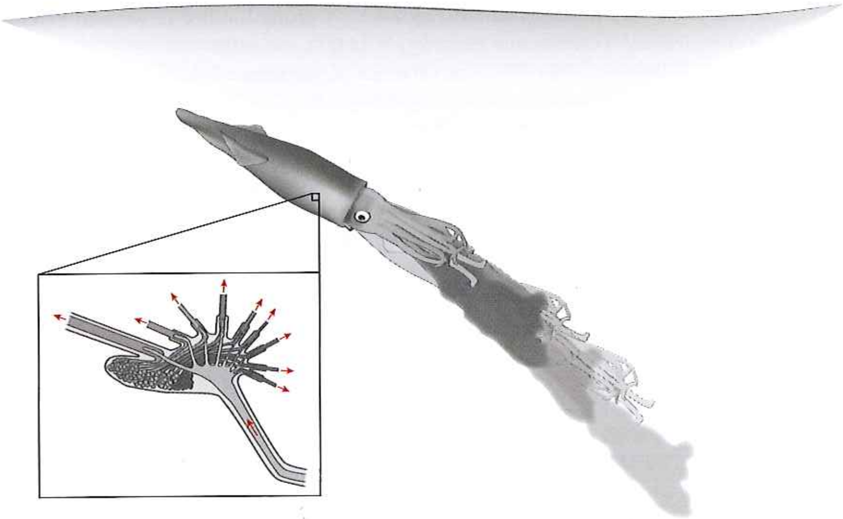

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The humble snail is not often thought of as a star of neuroscience, yet it belongs to an animal group whose study has contributed to many ground-breaking discoveries in the field, including two which eventually led to Nobel Prizes[1]. This group is the **molluscs** – one of the most diverse and ancient phyla in the animal kingdom. There are about ten different classes of mollusc but, arguably, the two most interesting from a neurobiological perspective are the **gastropods** and the **cephalopods** (Figure 12.1).

The gastropod ('stomach-foot') molluscs include the slugs and snails. These, particularly in their marine variants, have contributed to our understanding of: learning and memory (sea slugs); attack using harpoons and venom (cone snails); and escape using swimming and smoke, or more accurately, ink screens (sea slugs again).

The cephalopod ('head-foot') molluscs, which include the squid and the octopus, are thought to have the most advanced nervous system of all invertebrates, and have many complex and sophisticated behaviours[2]. Perhaps their biggest claim to fame in neuroscience, however, is that our modern understanding of how nerve impulses actually work derives from studies originally carried out on squid. Although most molluscs are quite sluggish in their behaviour, some are fast, including the jet-propelled squid. Also, even the slow molluscs may be active predators through the use of a variety of strategies to overcome their speed problem, either by tackling prey which are even slower than themselves or by ambushing their prey, or by utilising some of the most powerful neurotoxins known to science.

Molluscs may have some hard parts, such as a shell or mouthparts, but they are essentially soft-bodied animals with hydrostatic skeletons. This means that their muscles do not act through the rigid levers that we are familiar with in our own arms and legs but, instead, squeeze fluid-filled cavities to transmit pressure through hydraulic linkage. This

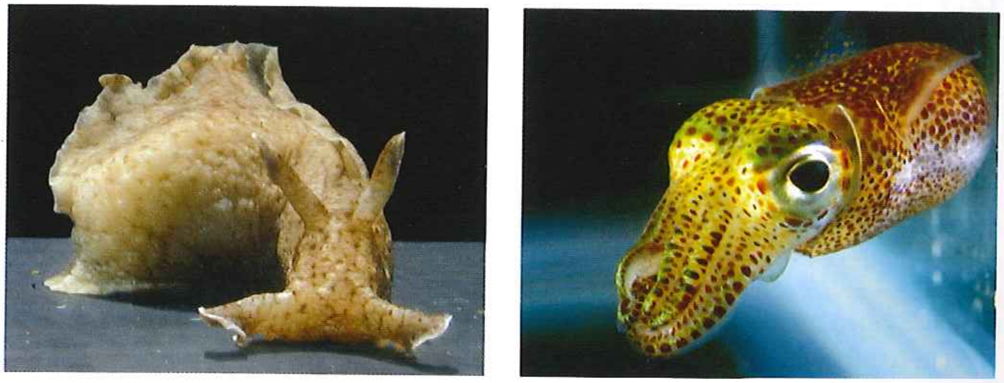

Figure 12.1 Two molluscs. Left: The sea hare: a marine slug-like gastropod mollusc, *Aplysia californica*. Photograph courtesy of Genny Anderson, Santa Barbara City College. Right: The Hawaiian bobtail squid *Euprymna scolopes*. Photograph courtesy of Chris Frazee (photographer) and Margaret McFall-Ngai (research director), University of Wisconsin-Madison.

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[1] Alan Hodgkin and Andrew Huxley got a share of the Nobel Prize for Physiology or Medicine in 1963 for their collaborative work on the mechanism of the action potential, which used giant axons from squid as the experimental preparation. This fabulous work still forms the foundation of our understanding of how nerve impulses actually work. Eric Kandel got a share of the prize in 2000 for his work on memory, which started off studying simple learning circuits in the marine snail *Aplysia*.

[2] In the UK, cephalopod molluscs like the octopus are the only invertebrates currently included in the very strict laws regulating the use of animals in scientific experiments.

can sometimes make it hard for a human investigator to understand the detailed neuromuscular consequences of a particular motor programme, but the mollusc clearly does not have this problem – many of them can control their bodies with superb accuracy. The common octopus is famously able to squeeze through just about any hole big enough to accommodate the only hard bit of its body – its chitinous beak.

Probably the most spectacular example of 'body sculpting' anywhere in the animal kingdom is the mimic octopus *Thaumoctopus mimicus*, which can change its shape and colour to convincingly mimic at least 15 different species, including poisonous fish and snakes. Sometimes, the octopus even seems to invent body forms from its own imagination – one observer described a mimic octopus scuttling along looking like 'a furry turkey with human legs'[3].

In this chapter, we recount three short stories on the neuroethology of defence and escape in molluscs. When threatened, a squid may jet off at high speed to escape, while simultaneously emitting a cloud of ink as a 'smoke screen' to hide behind and, at the same time, lightening its own body colour so as to reduce the contrast of its silhouette relative to the ink. The three components of the **blanch-ink-jet** manoeuvre are driven by very different mechanisms but, since they can all be produced at the same time by the same animal, they seem a suitable collection of topics to select for this chapter (although, in fact, we draw our examples of these behaviours from a variety of mollusc groups). There is a fourth story in which molluscs feature, concerning the use of venom by cone snails but, as this is longer, we have placed it in a separate chapter, along with information about some other animals that use neuroactive venoms (see chapter 13)

12.1 Squid jet propulsion

12.1.1 Biomechanics

Squid belong to the molluscan class Cephalopoda, which also includes cuttlefish, octopus and *Nautilus*. They have two primary modes of locomotion: slow, gentle swimming, driven by rhythmical undulations of the lateral fins; and faster, forceful swimming, generated by **jet propulsion**. When hunting or feeding, adult squid mainly advance head first using fin-driven swimming, capturing fish or crustaceans with their massively extensible hydrostatic tentacles[4]. However, when startled or threatened, they switch to jet propulsion and dart off backwards, using a high-pressure pulse of sea water exhaled from an internal chamber called the **mantle cavity**.

Jet propulsion conforms to the common principle underlying all locomotion, namely Newton's Third Law: 'for every action there is an equal and opposite reaction'. In normal legged locomotion, such as running or jumping, a limb pushes against the ground and the ground pushes back against the limb, but the enormous mass of the earth relative to that of the animal means that the ground is essentially immoveable, so only the animal moves. In the case of squid jet propulsion, the squid pushes the water out of the mantle cavity, and the water pushes back on the squid. The only difference is that the volume of water

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[3] Mark Norman and Erich Hochberg identified this marvellous creature. Apparently, it is sometimes confused with another of their discoveries, the improbably-named but almost equally amazing octopus, *Wunderpus photogenicus*!

[4] Squid, octopus and cuttlefish have eight arms and squid and cuttlefish have two additional limbs known at tentacles. Contrary to common usage, octopuses don't have tentacles.

expelled is quite low, so its mass is certainly not immoveable, and the water squirts forwards, while the squid darts backwards. The water does not have to push against anything solid – the principle is exactly the same as that which enables a rocket to work in the vacuum of space, where the expelled gases also do not have anything to push against. Squid jet propulsion is inherently less energy efficient than swimming[5], but it does allow very rapid acceleration and, in fact, squid can out-accelerate most fish.

Unlike a modern jet engine, the squid cannot generate a continuous through-flow of fluid – it can only generate a series of squirts. The mantle cavity has two sets of openings: inhalant openings, formed by the mantle collar surrounding the body; and a narrow, forward-pointing exhalent tube, known as the **siphon** (Figure 12.2). The mantle cavity houses the gills (as is the case for most molluscs) and, while at rest or moving gently, the squid naturally maintains a flow of water through the cavity, sucking oxygenated water in through the inhalant openings, passing it over the gills to extract the oxygen, and blowing it out through the siphon.

Throughout this process the mantle cavity maintains a relatively high volume of water within it, so essentially it is loaded and ready for jetting. Just prior to jetting, there may be slight hyperinflation due to contraction of radial muscles, which sucks in some extra water, but the main power stroke is generated by the massive and synchronous contraction of circular muscles around the entire length of the mantle (Packard and Trueman, 1974). Simultaneously, the head is pulled back towards the mantle, which closes off the inhalant openings and enhances the mantle constriction. The result is that a high-pressure jet of water is forced out of the siphon. By analogy, it is like slapping a tube of toothpaste with a flattened hand along its length, causing the entire contents to spurt out of the nozzle.

Immediately after the power stroke, the siphon closes, while the inhalant openings relax and open. The mantle circular muscles relax, and the mantle cavity refills through the now-open inhalant ports, due to elastic recoil of the mantle walls (Ward and Wainwright, 1972), aided by radial muscle contraction. The cycle may repeat several times to propel the animal away from danger. The siphon orientation is under fine neuromuscular control during jetting, allowing **thrust vectoring**, so that the escape trajectory can be altered as appropriate to the direction of the threat. The same neuromuscular machinery can be deployed in routine fast swimming, particularly in juvenile squid, as well as in attack and prey capture.

12.1.2 Neural circuitry

The most rapid form of jet propulsion in squid is controlled by three sets of **giant fibres** operating in series (for review, see, for instance, Williamson and Chrachri, 2004).

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[5] Squid gain propulsive force mainly through a simple exchange of momentum with the water they expel, and momentum is linearly proportional to both mass and velocity (momentum = mass × velocity). However, the *energy* the squid must impart to the water rises linearly with the water *mass*, but with the square of the water *velocity* (kinetic energy = 0.5 × mass × velocity × velocity). Thus, it is energetically more efficient to expel a large mass of water at low velocity than vice versa, although the momentum exchange is the same in both situations. The problem for the squid is that it can only expel the water mass it holds in its mantle cavity, which has an upper limit set by its volume. Therefore, to increase speed, it has to increase the velocity of the water it expels. Fin movement, on the other hand, can potentially deflect a larger mass of water at lower velocity, achieving the same momentum exchange with less energy expenditure. (We said the thrust came mainly from *simple* momentum exchange. This is because detailed analysis is complicated by the formation of numerous vortices in the flow (Bartol *et al.*, 2009), but that is beyond the remit of this book!).

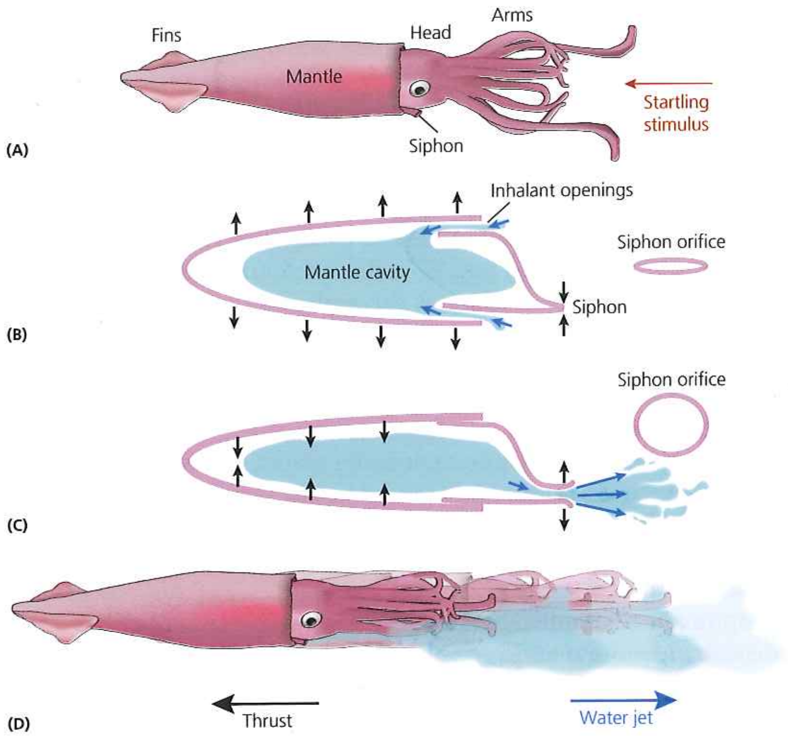

Figure 12.2 Jet propulsion in the squid. **A**: The general anatomy of a squid. **B**: Prior to jetting, water is drawn into the mantle cavity through inhalant openings, while constriction of the siphon orifice prevents the water from leaving. **C**: Jetting is achieved through closure of the inhalant openings, immediately followed by massive contraction of circular muscles throughout the mantle. This reduces the mantle volume and expels a jet of water through the siphon. **D**: As the water shoots forwards, the squid shoots backwards.

First and second order giants in the brain

The *initiation* of escape relies on activation of a set of first order giant interneurons located on either side of the brain, in a region known as the ventral **magnocellular** (MC) lobe (Figure 12.3). Their job is to integrate information from a large range of sensory modalities, including visual, vestibular, olfactory and tactile inputs – essentially any sensory information that might signal an imminent threat converges on these two large command neurons in the brain. They also receive numerous inputs from higher brain centres, including cerebral ganglia. If – and only if – these first order giants cross the spike threshold will a sequence of escape jetting take place. In this sense, the first order giant fibres serve as what has been described as a **central integrating manifold** (Llinas, 1999), to allow the squid to respond to startling inputs signalling threat or danger.

For an optimal jet thrust to occur, both sides of the mantle must contract simultaneously, with the funnel direction controlling escape trajectory. To ensure that this takes place, the first order giants on either side of the brain meet near the midline of the **palliovisceral** (PV) lobe of the brain, where in the squid *Loligo*, they fuse together at a cytoplasmic bridge (Figure 12.3), which establishes electrical continuity to ensure that

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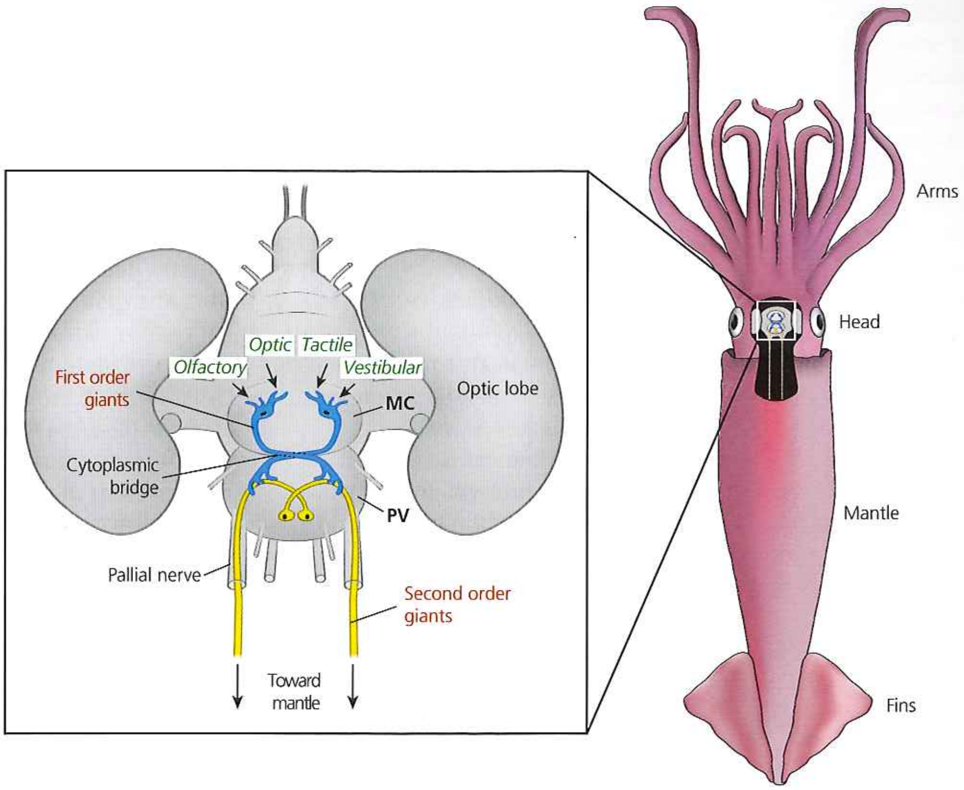

Figure 12.3 Primary and secondary giant fibres in the squid brain. Sensory inputs, including optic, tactile, vestibular and olfactory information, converge onto first order giant fibres, whose cell bodies are located in the magnocellular (MC) lobe of the squid brain. The axons of the first order neurons cross over, fusing at a chiasmatic bridge. The first order neurons synapse with second order interneurons in the palliovisceral (PV) lobe. The axons of the largest second order interneurons then leave the brain, and project to third order motorneurons in the wall of the mantle.

when one fires, both fire[6]. The first order command is relayed via powerful electrical synapses onto the **second order giants**, whose somata are located within in the PV lobe (Figure 12.3). Each first order neuron makes contact with seven second order neurons on each side. The axon of the largest of these second order giants exits the brain via the **pallial** nerve towards the **stellate ganglia**. The axons of the other six second order giants project directly to muscles controlling siphon aiming, ink ejection and head retraction (Young, 1939; Gilly *et al.*, 1991).

Third order giant axons and synapses in the stellate ganglia

The bilaterally paired stellate ganglia are located within the mantle and each contains an array of 9–11 (depending on species) **third order giant motorneurons**, whose axons innervate the circular muscles (Young and Keynes, 2005), the contraction of which causes

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[6] In other squid groups there is no cytoplasmic fusion, but there is electrical coupling through gap junctions. Note the similarity with the crayfish lateral giant fibre network in which electrical connections cause bilateral pairs of neurons to function as one, and the difference compared with the fish Mauthner cell circuit in which it is essential that the two giant fibres do *not* fire together.

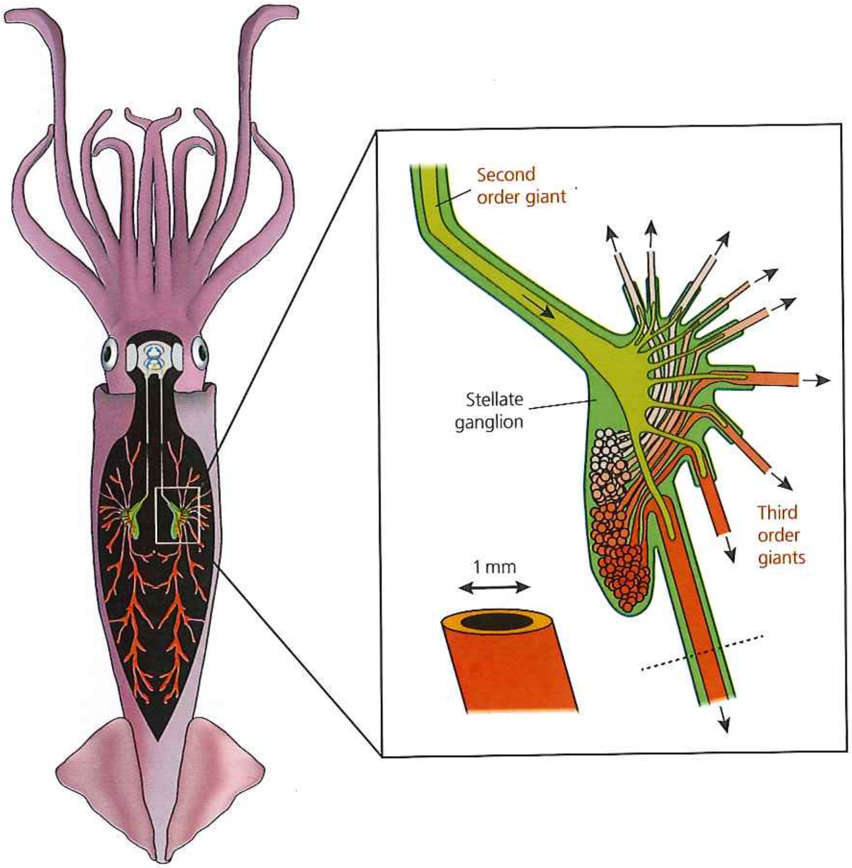

Figure 12.4 Giant motorneuron axons emanate from the stellate ganglion. The largest of the second order giant interneurons enters the stellate ganglion through the pallial nerve, where it makes contact with 9–11 third order giant motorneurons. These third order giants innervate circular muscle throughout the mantle. The third order giants vary in diameter, with the largest and, thus, fastest-conducting axons innervating the most distal regions of the circular muscle. This mechanism ensures that electrical activity arrives at all regions of the mantle at the same time, producing synchronous contraction of circular muscle throughout the mantle.

the mantle to expel the water jet (Figure 12.4). The largest of these axons can be more than 1 mm in diameter, which makes them amongst the largest known to biology, and wonderfully amenable to experimental manipulation. It was these that Hodgkin and Huxley chose to study in their seminal work on action potential mechanisms, which led to them being awarded the Nobel Prize in 1963. Each giant motorneuron axon is, in fact, a syncytium formed by the fusion of axons from some hundreds of smaller neurons, which probably helps to explain how they could grow so large (Young, 1939)[7]. As well as this

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[7] This is a fascinating example of how large cell size can be achieved, and it is not unique (for instance, the large standard vertebrate striated muscle fibres are syncytial, formed by the fusion of several developmental precursors). However, neither is it a general principle. In other giant fibre systems described in this book, such as those in the crayfish or Mauthner system, there is no suggestion of a syncytial origin – individual neurons just grow very large.

limited number of giant motorneurons, there is also a parallel array containing an indeterminate, but much larger, number of non-giant motorneurons, which also innervate the circular mantle muscles, although possibly a different sub-set of muscle fibres (see below).

The giant motorneurons are specialised for speed, with a conduction velocity that can exceed 25 m s^{-1}. This is, perhaps, not very impressive compared to a myelinated mammalian spinal motorneuron with a conduction velocity of up to 120 m s^{-1}, but it is pretty impressive for an animal closely related to a slug! The large diameter is certainly part of the explanation for the high speed of these axons but they are, in fact, specialised right down to individual ion channel kinetics. For instance, the sodium channels in squid giant axons have an activation time constant in the same range as mammalian spinal motorneurons (≈0.4 ms at resting potential; Miles *et al.*, 2005), despite the fact that the latter operates at much higher temperature. This is much faster than those of more typical molluscan neurons such as the ink motorneurons (Byrne, 1980b).

Within each stellate ganglion, the giant motorneurons are driven by excitatory chemical synapses from the second order giant interneuron. Perhaps not surprisingly, these synapses are themselves giant[8]. The largest is around 0.7 mm in length (Figure 12.4 B), which makes it visible to the (young) naked eye. Like the giant axons, the giant synapse of the squid has been a hugely important model system in experimental neuroscience. Its enormous size means that both the pre- and postsynaptic sides can be impaled with several microelectrodes, to control both the membrane voltage and the internal chemical milieu. This accessibility enabled Rodolfo Llinás and his colleagues to elucidate such fundamental properties of synaptic transmission as calcium dynamics, vesicle fusion and transmitter release mechanisms. Not many individual synapses have had an entire book written about them, but this one has (Llinás, 1999)!

Now let us return to the jet propulsion mechanism. As already mentioned, the circular muscles that are innervated by the giant motorneurons must contract simultaneously in order to maximise the power of the jet and facilitate rapid propulsion. However, the squid's mantle is an elongated mass of tissue, which means that the axon length of motorneurons innervating muscle near to the stellate ganglia is much shorter than those innervating the more distal part of the muscle. This presents a potential problem, given that the efficiency of the jet relies on the synchronised contraction of the entire circular muscle. The solution found in the squid is for the distal part to be innervated by the largest diameter and, therefore, fastest-conducting axons, and for the proximal part to be supplied by the smaller diameter and, hence, slower of the giant axons. This is a simple, effective solution to the need to equalise conduction delays along the length of animal, and thereby to ensure the simultaneous contraction of the circular muscle along the length of the body.

12.1.3 Jetting behaviour

Squid have two types of jetting response (Otis and Gilly, 1990). The first is a rapid (for a mollusc – latency ≈ 50 ms) reaction to a sudden visual stimulus such as a light flash. This triggers a single action potential in the set of giant motorneurons described above, and produces a vigorous, stereotyped pulse of water. This is the classic escape response. The second type has much longer latency (≈200 ms), and primarily uses bursts of spikes in

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[8] It perhaps *is* slightly surprising that this giant synapse is chemical rather than electrical, given that electrical synapses are faster. It is pure speculation, but perhaps it is because the gigantic post-synaptic neurons would impose an intolerable current drain on the pre-synaptic neuron if the connections were electrical.

the non-giant motorneurons. This can produce a more varied type of reaction, and can be used for prey capture in juveniles, or for longer periods of sustained swimming at all ages.

There is some evidence that the giant motorneurons innervate glycolytic mantle muscle fibres with large sodium currents specialised for strong but brief contractions, while the non-giant motorneurons innervate mitochondria-rich muscle fibres that are used for repeated contractions (Gilly *et al.*, 1996). The giant motorneurons are sometimes also activated in the repeated-cycle type of jetting, but they always spike *after* the non-giants have started the muscle contraction, so they are reinforcing, rather than initiating, the behaviour. In principle, this is very similar to the giant vs. non-giant reactions we have already seen in crayfish and fish escape circuits (see Chapters 7 and 8).

Squid are, of course, primarily aquatic animals, but their jet propulsion mechanism works in air as well as in water, and some species (commonly known as **flying squid**) take advantage of this (Muramatsu *et al.*, 2013). They propel themselves out of the ocean with their jet, and spread both their fins and their arms to form lift-generating aerofoils at either end of the body. They can continue to jet, and hence to accelerate, while airborne, and have been observed to travel as much as 25 m before flattening their 'wings' against their mantle and diving back into the ocean (Figure 12.5). Aerial jetting is probably a highly effective escape manoeuvre, since the squid moves about four times faster through air than it does through water, due to the lower resistance from friction. Also, any predator in visual pursuit of the squid would have to deal with the problem of the refractive index change at the air-water interface. The squid would not actually be where the fish's eye imaged it, and so the fish would be likely to misjudge the squid's re-entry point[9].

Jetting is great for escape, and it is acceptable for general swimming purposes, but it is not a good *attack* strategy if the prey itself has a rapid escape system. In this case, an approach by jetting is just too obvious – the prey is usually alerted in time to evade capture. Thus, squid need to learn when, and when not, to jet. In a series of experiments in which young captive squid were fed exclusively on slow-moving prey, they learned to capture them efficiently by jetting, but failed to develop the alternative feeding strategies which would be needed to tackle more challenging meal prospects (Preuss and Gilly, 2000). However, if the young squid were fed a diet that included fast-responding prey that could elude the direct-jetting approach, then the squid learned stealth tactics, swimming slowly and surreptitiously up to the prey with its fins, before grabbing it at the last minute with its tentacles! It seems that, in squid at least, youthful failure is an important motivator for developing alternative sneaky strategies which can lead to success in later life.

12.2 Inking

Many molluscs within the cephalopod and marine gastropod groups discharge ink if attacked or threatened. Ink is probably rather expensive to produce in energy terms, and yet it has evolved convergently in these two classes – so what are its advantages? The most obvious is the simplest; the ink acts like a smoke screen that can obscure the emitter from view and allow it to escape. In this case, the ink forms a large, diffuse haze, much like a cloud.

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[9] The archer fish (Chapter 10) is one fish species that has solved this problem, but it is a freshwater fish and its prey are insects and other small creatures, not oceanic squid.

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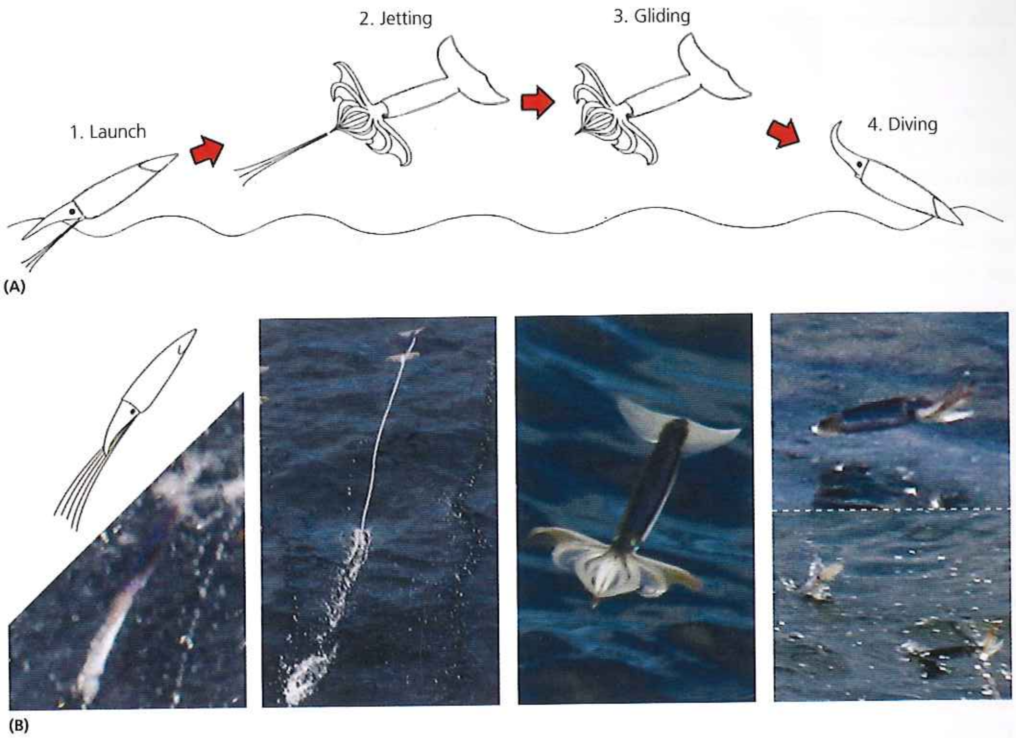

Figure 12.5 Flying squid. **A**: A schematic illustrating the four phases of squid flight. **1**. Launch: the squid uses its jet propulsion to launch itself forward and out of the water at a shallow angle, keeping its fins against the mantle and arms folded to minimise drag. **2**. Jetting: once airborne, the squid continues to expel a jet of water, but now with its arms and fins spread out, travelling at speeds of up to 11.2 m s^{-1}. **3**. Gliding: jetting stops, but the squid continues to glide through the air, using its spread arms and fins like a wing, aided by the thin membrane that connects the arms. **4**. Diving: on approach back into the ocean, the fins are retracted against the mantle and the arms folded, to produce a streamlined posture that reduces disturbance upon re-entry into the water. **B**: Photographs of ommastrephid squid, showing the different phases of the behaviour. Adapted from Muramatsu *et al.* (2013) with permission of Springer Science + Business Media.

However, in another type of ink release used by some squid, the ink is viscous and hangs together in dense blobs, which have the approximate size and shape of the releaser. These **pseudomorphs** act as decoys; the production of the dark pseudomorph is often accompanied by a simultaneous paling of the squid's body in such a way that the high-contrast morph is more interesting to the predator than the real meal. In deeper, darker environments, where black ink would be a less useful form of defence, some squid species secrete ink that contains light-emitting bacteria, which effectively generates a bioluminescent invisibility cloak (Robison *et al.*, 2003). These creatures really do take us into the realms of science fiction!

For a long time, these visual deceptions were the only well-documented function of mollusc ink but, in recent years, it has become apparent that, in addition to pigment, the ink also contains a rather complex and varied soup of neuroactive chemicals (Derby, 2007). Because these are released into the environment and can affect all creatures in the vicinity, the term **neuroecology** has been adopted for their study (Zimmer and Derby, 2007).

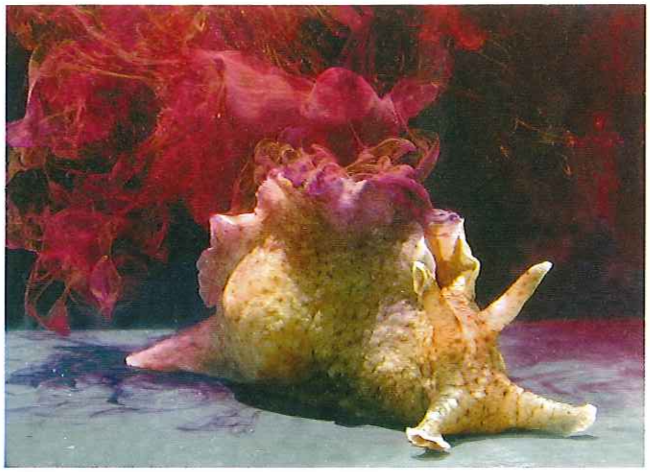

Figure 12.6 The sea hare, *Aplysia californica*, emits purple ink. The colour comes from its diet of red algae. Photograph courtesy of Genny Anderson, Santa Barbara City College.

12.2.1 Neuroecology of inking

There seem to be at least four separate neurochemical anti-predator functions of molluscan ink: **aversion**, **phagomimicry**, **sensory interference** and **conspecific alarm** (Derby and Aggio, 2011). Most research has been done on the gastropod sea hare (*Aplysia* sp.; Figure 12.6), but equivalent effects may well exist for cephalopod ink, too.

Sea hare ink contains several compounds which seem to taste repulsive to some predators. Some of these (e.g. aplysioviolin and phycoerythrobilin) are derived from the sea hare's own diet of red algae (which also gives the ink its red-purple colour), while others are synthesised by the sea hare *de novo*. The latter includes an enzyme called **escapin**, that produces a noxious mixture of hydrogen peroxide and ammonia. These ink components can be detected by predators such as the sea catfish, and deter attack and feeding (Nusnbaum *et al.*, 2012). They also deter attack by sea anemones, which are major sea hare predators (Nolen *et al.*, 1995). Since anemones are sessile, presumably the sea hare just blunders into them by accident!

In direct contrast to aversion, some crustacean predators, such as the spiny lobster, find the ink highly attractive. The ink contains high concentrations of free amino acids, and these activate the taste receptors on the lobster and make it concentrate on the ink, rather than the sea hare producing it. This phagomimicry is something of a Barmecide feast[10], since the ink quickly disperses and provides little or no nutrition, while the meaty sea hare makes its slow but effective escape.

Sea hare ink is actually the product of two separate glands, with one producing the ink, while the other produces a white, milky substance called **opaline**. On contact with the external sea water, opaline polymerises into a highly viscous gloop that sticks to the body surface of a would-be predator. The opaline can have two opposite, but equally disruptive, effects: it can coat the surface of olfactory/gustatory receptors and block the reception of appetitive chemicals; or it can lock chemicals that have already been detected onto the

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[10] The Arabian Nights: The Story of the Barber's Sixth Brother: http://www.sacred-texts.com/neu/lang1k1/tale25.htm – although, in the story, the lobster-equivalent actually gets food in the end.

receptors, causing overstimulation and eventual desensitisation. The former seems the most common mechanism, but the end result is the same – the predator gets confused and cannot smell or taste its prey (Love-Chezem *et al.*, 2013).

Finally, both sea hare and squid ink contain alarm pheromones, which can cause aversive reactions and escape in conspecifics. In sea hare ink, the alarm chemicals include nucleic acids and diet-derived amino acids whereas, in squid ink, the primary components are the neurotransmitter dopamine and its precursor L-DOPA (Lucero *et al.*, 1994). Squid ink also contains a high concentration of the pigment melanin which, as well as providing colour for the ink, also acts as an antioxidant, which may stabilise the labile dopamine in sea water and prevent its degradation.

12.2.2 Neural circuitry of inking

Ink is a valuable commodity, and it takes a sea hare about two days of grazing to obtain enough ingredients to replenish a completely empty ink gland (Nolen and Johnson, 2001). An ink-exhausted sea hare is much more vulnerable to predation, so they only ink in response to a serious threat – they just ignore transient or weak stimuli. How is this selectivity achieved?

Inking is controlled by a small group of electrically-coupled ink motorneurons (Carew and Kandel, 1977). The motorneurons receive direct and indirect excitatory synaptic input from sense organs that detect noxious stimuli, but only generate spikes if the sensory activation is strong and long-lasting. They are, thus, selective for sensory input that is likely to indicate a serious threat.

Four key cellular properties underlie these characteristics. First, the motorneurons have a high spike threshold, so they do not fire in response to small depolarisations. Second, they have a low input resistance, which limits the initial depolarisation produced by excitatory synaptic current. Third, the synaptic input includes a slow, late-developing conductance *decrease* component that increases membrane resistance, and so enhances both the response to coincident excitatory inputs and the electrical coupling – but only after a delay (Carew and Kandel, 1976). Finally, the motorneurons all possess a type of voltage-dependent potassium current known as the transient, fast, or **A-current**. This is similar to the standard Hodgkin-Huxley potassium current, except that it activates more rapidly and, importantly, it *inactivates* with maintained depolarisation.

What this means is that, when an ink motorneuron is first depolarised by a stimulus, the outward potassium-mediated A-current activates on the same time scale as the inward sodium current. This 'holds down' the membrane potential, so that it does not depolarise as much as it otherwise would, thus preventing the neuron from reaching threshold (Figure 12.7). However, the A-current inactivates with time so, if the stimulus is maintained, the motorneuron is released from this restraining influence and spikes, thus triggering the release of ink (Byrne, 1980a). The A-current is similar in its characteristics to that found in many mammalian neurons (e.g. Huguenard *et al.*, 1991), but it operates on a much slower timescale.

Ink is synthesised and stored in cell-derived vesicles embedded in layers of collagen and smooth muscle within the ink gland (Prince *et al.*, 1998). Acetylcholine causes the muscle to contract and squeeze the vesicles, which extrude their contents through pores at the gland surface. However, a sea hare does not normally release all its ink in one go. Instead, activation of the ink motorneurons causes release of 30–50% of the *remaining* ink in the gland (Nolen and Johnson, 2001). This means that an aggravated sea hare can normally deliver 4–6 salvos of ink from a full gland, but with each salvo having about half the ink

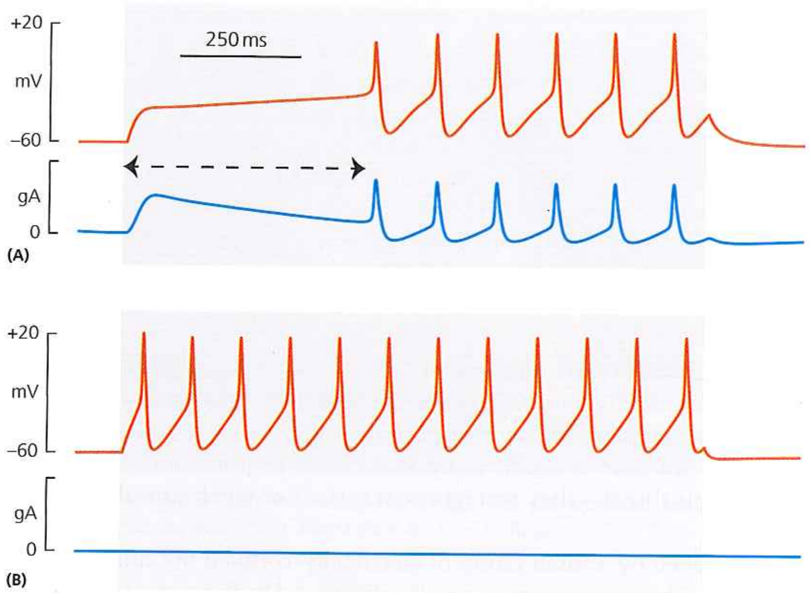

Figure 12.7 The A-type potassium current helps ensure that ink motorneurons only spike to sustained stimuli. **A**: A long-duration excitatory stimulus (shaded region) does not induce spiking in an inking motorneuron (upper trace, red) until the conductance mediating the A-current (lower trace, blue) has substantially inactivated (dashed line). **B**: With the A-conductance blocked, the same stimulus causes immediate spiking in the motorneuron. Data simulated in Neurosim (Biosoft), using kinetic parameters from Byrne (1980b). The conductance units are arbitrary.

content of the previous one. It seems that the sea hare likes to keep some ink in reserve for as long as possible.

12.3 Cephalopod colour and shape control

Many animals gain a selective advantage by changing their appearance to suit the situation. Chameleons are so famous for this that their name has become a metaphor for an unprincipled but advantageous change in human behaviour. However, cephalopod molluscs are arguably the champions of shape and colour change. The speed and coordination of the changes shown by octopuses, cuttlefish and squid can be simply staggering.

Often, the purpose of colour change is intra-specific signalling, either for attracting mates or intimidating rivals, but colour change is also part of the ongoing battle between predators and prey. Many octopuses can almost instantly adopt the colour, and sometimes the shape, of their background as they settle on the sea bed, rendering them virtually invisible both to their would-be predators, and their hoped-for prey. Even more spectacular to the human observer is when an octopus suddenly emerges from its background by changing away from the ambient – it was there all along, but avoids detection until it reveals itself.

The change need not always be for crypsis; it can be startling (deimatic) in the form of suddenly-appearing stripes or eyespots, or warning (aposematic), with more stable bright colours to deter predators. Sometimes it may simply be for distraction; some species of hunting squid display rapidly moving stripes along their arms and tentacles while

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approaching their prey. Nobody knows for sure why they do this, but perhaps the prey is so mesmerised by the moving colours that it fails to pay attention to the approaching animal generating them.

There are two main ways that cephalopods are able to change their colour rapidly. One uses pigments in specialised structures called **chromatophores**; the other uses iridescence generated by reflecting surfaces in structures called **iridophores**. There are usually many thousands, or even millions, of these structures scattered across the body surface, in an organised, complex and species-specific pattern. Rapid and coordinated changes in colour are possible because both types of structure can be under direct neural control. There are also longer-term colour-generating structures known as **leucophores** and **photophores**.

12.3.1 Chromatophores

Chromatophores use pigment-based colour. Pigments appear coloured to the observer because they reflect light at the spectral wavelength(s) of the colour they display, while absorbing light of all other wavelengths (see Messenger, 2001, for a detailed review of chromatophores). In cephalopods, each chromatophore is a small, multicellular neuromuscular organ, covered by several layers of transparent cells (Figure 12.8).

At the centre of each chromatophore is a single large cell with an internal elastic cytoplasmic sac, which contains pigment granules in the yellow-red-brown-black colour range (different colours for different chromatophores and species, but no blue or green pigments anywhere in cephalopods). In the relaxed state, this sac is very small – less than 0.1 mm in diameter – and barely visible to the naked eye. However, the central cell is surrounded by 15–20 radial muscle fibres and, when these contract, they pull the sac outwards, expanding it to as much as 1.5 mm in diameter. This expanded surface area makes the pigment content highly visible.

Each muscle fibre is innervated by one or more motorneurons originating in the chromatophore lobe of the brain, but each motorneuron is a branched structure which may innervate a group of chromatophores, a little like a motor unit in a vertebrate striated muscle. This combination means that a very fine level of motor control can be achieved, both at the level of the individual chromatophore, and at the level of groups of chromatophores forming patterns on the skin.

The mode of operation is quite straightforward in principle. If an octopus wants to show a yellow stripe on its body, it simply activates the motorneurons to the yellow-pigment chromatophores in the appropriate region. This may even be just a single motorneuron, if that motorneuron innervates a stripe-shaped group of chromatophores in the correct place. There are two excitatory neurotransmitters: glutamate, acting on non-NMDA-type receptors, mediates rapid and transient contractions to produce a yellow flash; while FMRFamide-related peptides (FaRPs) mediate sustained contractions to keep the yellow stripe visible for a longer period of time.

If the octopus wants its yellow stripe to disappear, it simply stops activating the motorneurons. The muscle fibres then relax and the pigment sac contracts, due to its natural elasticity. It is perhaps worth pointing out that this is a completely different control mechanism from that of chromatophores in vertebrates, where colour change is achieved by migrating pigment granules within a cell with a fixed shape. This process is controlled by hormones, and is relatively slow in contrast to the fast neuromuscular control of chromatophore cell shape in cephalopods.

The value of the rapid neural control of chromatophores is evident in the **countershading reflex** shown by many species of squid. A squid normally expands chromatophores on

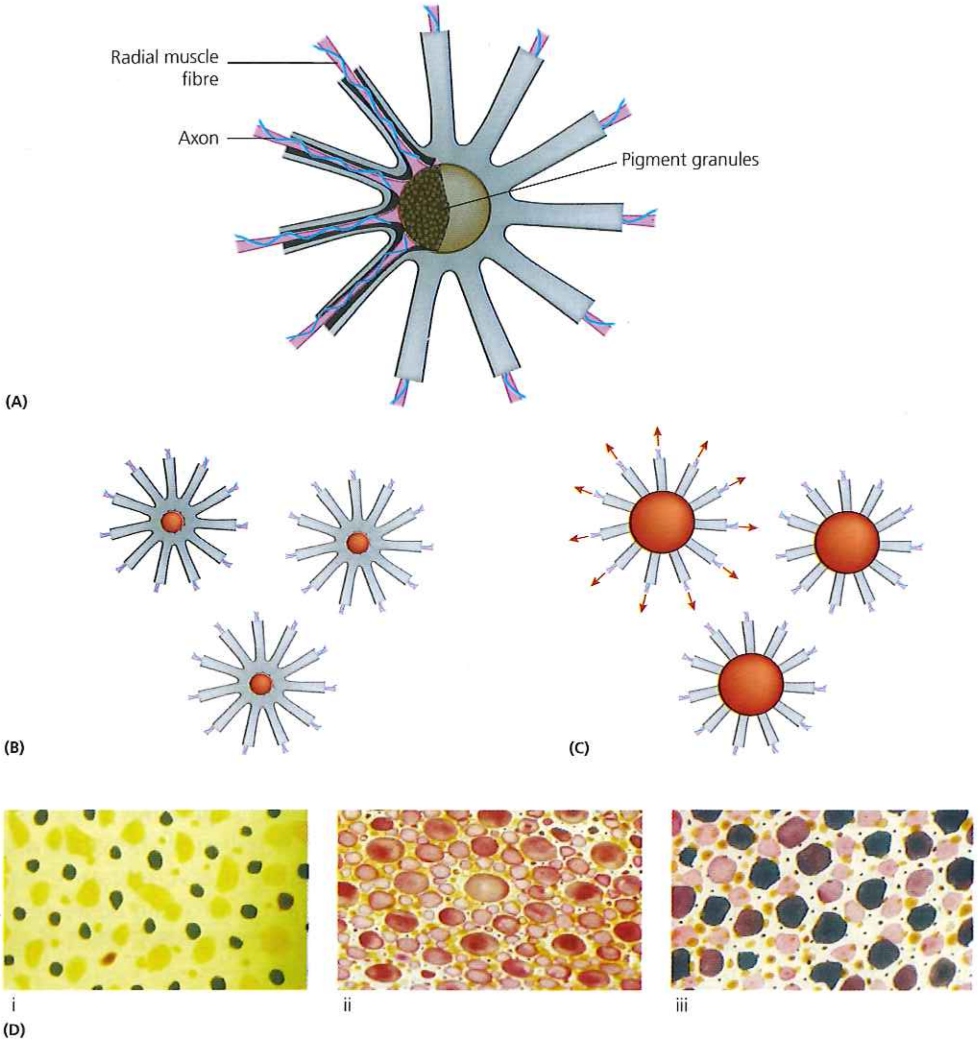

Figure 12.8 Chromatophore expansion in cephalopods. **A**: General anatomy of a chromatophore. At the centre is a cyto-elastic sacculus containing pigment granules, whose colour varies between chromatophores and different species of cephalopod. Several radial muscle fibres (pink) converge onto this elastic sac, and motorneurons (blue) control the contraction of these muscles. **B**: Retracted chromatophores. When the radial muscles are relaxed, the central sac of pigment is small (<0.1 mm) and the pigmentation is not visible to the eye. **C**: When the radial muscles contract, this expands the sac and the pigmentation becomes visible, changing the perceived colour of the surface of the animal. **D**: Images of chromatophores from three different species of cephalopod: i: *Sepia officinalis*. ii: *Loligo plei*. iii: *Sepioteuthis sepioidea*. Magnification = i: × 80; ii: × 4; iii: × 5. Photographs courtesy of Roger Hanlon, Woods Hole Oceanographic Institution.

the dorsal surface of its body to keep it at a much darker colour than the ventral surface, which reduces its visibility to predators from above by reducing contrast with the background. However, if the squid is suddenly rolled over, it very rapidly adjusts its colour, so that the now-uppermost ventral body becomes dark, and the dorsal body light. This reflex is mediated by the cephalopod's organs of balance (the **statocysts**), and is thought to increase protection if the squid inadvertently rolls itself over while pursuing its own prey, or while jetting to escape from predators.

The question of how cephalopods regulate the motor control of chromatophores is still largely unsolved. There is a limited level of local control; the skin of cephalopods contains opsin phototransduction proteins, similar to those found in the eyes, and chromatophores in isolated patches of octopus skin expand in response to illumination (Kingston *et al.*, 2015; Ramirez and Oakley, 2015).

However, the dominant influence undoubtedly comes from the central nervous system. There seem to be a limited number of chromatophore motor programmes hardwired into the brain from birth – perhaps 10–12 – and these operate under open-loop control (i.e. without needing sensory feedback). However, we do not know how they are generated. No obvious chromatophore topographic map has been found in the brain, but there is a hierarchy of regions that mediate control, the highest level of which comprises the **optic lobes**.

Since the chromatophores produce a visual display, it makes sense that ultimate control lies within a region dedicated to the processing of visual information. The optic lobes project to the **lateral basal lobes**, which integrate information from both eyes, which in turn project to the **chromatophore lobes**, containing the output motorneurons themselves. However, there are numerous reciprocal connections and more long-range connections with other brain regions, making for a very complex overall control network.

12.3.2 Iridophores

Cephalopod iridophores contain cells which are completely colourless, but have multiple layers of reflecting surfaces within them (Mäthger *et al.*, 2009). The effect of this is that a light wave bouncing off one layer may *interfere* with the light wave bouncing off the next layer in the stack. For certain light frequencies (and, hence, colours) and angles of view, the peaks of these two waves will coincide, leading to constructive interference and amplification of that colour while, for other frequencies and angles, the peak of one wave will coincide with the trough of the other, leading to cancellation and the disappearance of that colour from the reflection. For this to happen, the separation between the surfaces has to be in the order of the wavelength of light (400–700 nm) while, within that range, a narrow separation of layers leads to a bluish reflection and a wider separation leads to reddish reflection (although the exact colour depends on the angle of view).

The overall mechanism is known as **thin-film interference**, and it is exactly the same in principle as that which gives a dirty puddle on a city street a rainbow appearance when viewed from certain angles. In the case of the puddle, a thin film of motor oil on the surface provides the upper and lower reflecting layer which generates the interference. In cephalopod iridophores, the reflecting layers are formed from a stack of deep cell-membrane invaginations (lamellae), interspersed with layers of a protein called **reflectin** – which, as its name suggests, is highly reflective. Together, these constitute what physicists call a **Bragg reflector**[11]. Colours generated by interference have a metallic, shimmering appearance, and can be very spectacular.

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[11] William Henry Bragg and William Lawrence Bragg were a father-and-son team who jointly won the Nobel Prize for Physics in 1915 for their work on X-ray diffraction. Bragg the son was also an ardent shell collector, and has a species of cuttlefish named after him – *Sepia braggi* – which makes it apposite to mention him here!

Iridophores occur in a distinct layer of the cephalopod skin under the chromatophores, so one way in which iridophore reflectance is controlled is simply by expanding or contracting the overlying chromatophores. This can occlude (black chromatophores) or filter (coloured types) the reflected light. However, in some species, some of the iridophores themselves are under direct neural control, and can change the colour that they reflect. Recent studies have revealed the fascinating molecular mechanism by which this works (Figure 12.9; DeMartini *et al.*, 2013).

Iridophore colour change is under the control of neurons releasing acetylcholine and this, when applied to isolated iridophores, causes them to change from transparent to red, then green and, finally, blue. The acetylcholine acts through G-protein-linked muscarinic receptors, which activate a second-messenger cascade with two sequential effects.

First, it causes the reflectin proteins between the membrane lamellae to condense, which increases their refractive index and causes them to switch from being transparent to being reflective. This is a necessary pre-condition for thin-film interference.

Second, the change in protein structure also causes the expulsion of water from the cell and consequent shrinkage. This changes the spacing between the lamellae and, therefore, the optical tuning of the reflector. The greater the shrinkage, the narrower the spacing and the bluer the light generated by the interference.

The process is graded and fully reversible, and its discovery has increased interest in biophotonics as a possible source of material with tuneable and switchable optical properties. The possibility of a 'cloak of invisibility' may not remain in the realm of science fiction for much longer!

The iridophore motorneurons originate from the stellate ganglion (the same one containing the giant neurons and synapse described in jet propulsion), and are separate from the motorneurons controlling chromatophores (Gonzalez-Bellido *et al.*, 2014). When the iridophore motorneurons are silent, the iridophores are transparent; when they are active, the iridophores reflect a colour which can change from red to blue as the concentration of

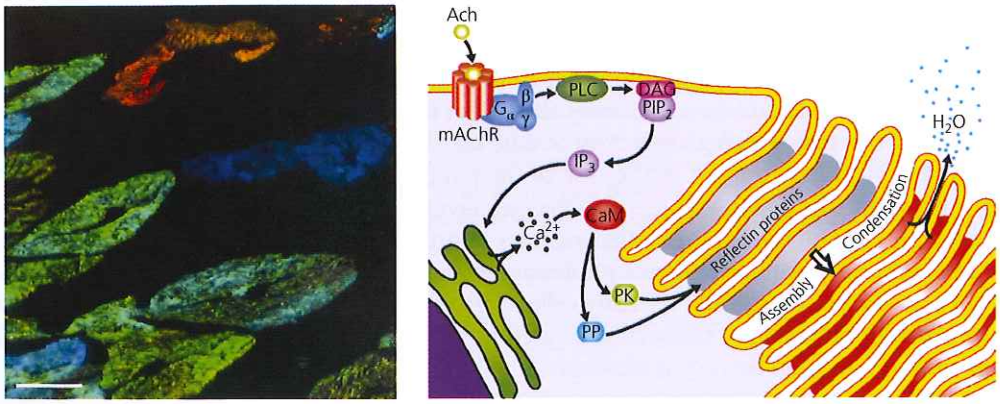

Figure 12.9 Tuneable iridescence in the squid *Doryteuthis opalescens*. Left: Iridophores from the skin of the squid, viewed with dark-field illumination (scale bar: 50 μm). Right: Diagram showing the proposed mechanism for tuneable iridescence. Acetylcholine activates an intracellular signalling cascade which increases the reflectivity of the interlamellar proteins and narrows the lamella spacing by extruding water. As the spacing narrows, the iridescence shifts from red to blue. Adapted from DeMartini *et al.* (2013). Reproduced with permission from PNAS.

acetylcholine increases. However, how these neurons are controlled by the brain remains a complete mystery.

12.3.3 Leucophores

Leucophores are structures that simply reflect the ambient light, so their colour depends on the colour of the light impinging on them. However, they reflect that light very efficiently indeed. Furthermore, they are not mirrors but, rather, almost perfect diffusers, so they appear as a bright constant colour, whatever the angle they are viewed from. The leucophore cells get this property because their surface is covered with numerous spherical proteinaceous globules, ranging in size from 250 nm to over 1 μm in diameter. The presence of leucophores creates light patches of skin, which can aid camouflage by acting as a highlight to the contrasting colours generated by chromatophores and iridophores. Unlike those two structures, though, they are not under any direct active control. However, leucophores sometimes occur directly under chromatophores, and can thus be effectively switched on or off by masking, through contracting or expanding the chromatophore.

12.3.4 Photophores

Photophores are the light-emitting organs found in many deep-water animals, including the cephalopods. The light is generated by an enzymatic reaction (the luciferin-luciferase mechanism), and the necessary chemicals may either be generated by the organ itself, or by symbiotic bacteria within it. Photophores vary in structure, from a simple collection of cells to a whole complex organ, complete with reflecting layers and lenses to focus the light. They can be used both as lures for predation and distractors for anti-predation, and they are sometimes found on the very tips of arms and tentacles, where they may act as a 'sacrificial diversion' that can be bitten off by predators without doing serious harm to the owner (Robison *et al.*, 2003)[12]. However, a very common use of photophores in cephalopods is to improve countershading by lightening the under-surface so that, when viewed from below, the cephalopod merges with the paler background generated by light filtering down from the surface.

One of the most fascinating examples of cephalopod countershading is found in the Hawaiian bobtail squid, *Euprymna scolopes* (Figure 12.1 right). The light-generating photophore is a complex organ embedded within the squid's ink gland in the mantle cavity, and the light itself is generated by symbiotic bacteria (*Vibrio fischeri*) which live within the photophore. The organ is roofed by reflecting cells similar to iridophores, while a transparent muscle-derived lens covers the ventral surface so that the light is focused downwards (McFall-Ngai and Montgomery, 1990). The squid can adjust the light output of its photophore to match the ambient down-welling light which, since the squid hunts at night, is starlight or moonlight (Jones and Nishiguchi, 2004).

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[12] One of the most bizarre species to use this defence is *Vampyroteuthis infernalis* (literally 'vampire squid from hell'). Vampire squid are covered in photophores, but the largest and most complex are found on the tips of its arms. In a manoeuvre known as 'the pineapple', a startled vampire squid will curl its webbed arms backwards over the mantle, essentially turning itself inside out and exposing rows of spiny projections called cirri on the inside of the cloak, resembling the spikes on a pineapple. The tips of the arms, which are now behind the cloaked squid, can glow and pulse to divert attention away from critical areas of the body and, if the tip of an arm is severed, it can be re-grown by the animal. Sticky clouds of bioluminescent mucus can also be released from the tips of the arms, to add to the anti-predator light show (Robison *et al.*, 2003).

The mechanism for controlling light output is not entirely clear, but it is thought that the ink gland surrounding the photophore acts as an adjustable iris, so that the whole structure acts rather like an eye in reverse. Furthermore, the photophore tissue expresses genes encoding for proteins associated with visual transduction and, on exposure to light, the whole structure generates extracellular field potentials similar to those generated by the eyes themselves (Tong *et al.*, 2009). Thus, the photophores may be sensing their own bioluminescence and/or ambient light levels, which may be part of a feedback control system.

Baby squid do not contain bacterial symbionts at birth, but have to acquire them in the immediate post-natal period, and they are very fussy about getting the correct species. The photophore rejects interlopers using a species-specific bacteriocide (Kremer *et al.*, 2014), and even non-bioluminescent mutants of the correct species are rejected. It is a two-way interaction, since the photophore itself does not develop properly into its mature form unless fully-functioning bacteria of the correct species are acquired.

One might think that, having taken so much care to acquire the correct symbionts, the squid would hoard them carefully. However, this is not the case. Bobtail squid are nocturnal, and spend the daylight hours quietly buried in the sand on the sea floor. When a squid settles down to sleep each morning, it expels about 95% of the bacteria from its photophore. Then, while it is quiescent during the day, the remaining 5% repopulates the whole organ, so that it is fully operational and ready to go by evening when the squid starts to hunt again (Nyholm and McFall-Ngai, 2004). The expulsion may be an act of 'generosity', ensuring a plentiful supply of symbionts for newly-hatched squid in the area – or, perhaps, if the expulsion is selective (which has not been demonstrated but is not completely implausible), it could be a part of a sifting process that reseeds the squid's bacterial population from its healthiest members.

12.3.5 Body shape and dermal papillae

As part of their anti-predator camouflage, cephalopods may not only take on the colour of their background, but its shape as well. An octopus hiding in seaweed may extend its arms and allow them to sway in the current, while the same animal resting on a rock may 'sit on its hands' to keep them out of sight. Such postural change is largely under visual control. In a laboratory setting, a cuttlefish kept in a chamber surrounded by vertical stripes kept at least one pair of its arms held upright, so that they aligned perfectly with the background stripes but, when the stripes were changed to an oblique angle, the arms rapidly followed suit (Barbosa *et al.*, 2012). These postural adjustments could be maintained for at least 20 minutes.

Octopuses and cuttlefish (although, apparently, not squid) also have an ability which seems to be unique in the animal kingdom, which is to change the 3D texture of their skin surface by raising variously-shaped lumps and bumps across their body (Allen *et al.*, 2014). Each protrusion is formed from an individual dermal papilla, and there may be many hundreds of these arranged in groups across the skin surface.

Each papilla contains a set of three muscles. There are circular erector muscles, which squeeze the base of the papilla and, thus, raise it above the body surface, due to hydrostatic pressure. There are also horizontal erectors, which radiate from the centre to the edge, and which help to determine the shape of the erect papilla. Finally, there are retractors, which help the papilla to subside when the animal decides it would rather have smooth skin.

The end result is that an octopus or cuttlefish can almost instantly change from having a streamlined, smooth body, suitable for rapid movement through the water, to having a hairy, or spiky, or lumpy surface that can render it almost indistinguishable from its immediate background. The whole process is under visual control; the animal will morph its body as readily and convincingly in response to a background consisting of a laminated photograph of seaweed as it will to the real thing (Allen *et al.*, 2009)!

12.4 Summary

Some aspects of molluscan defence show convergence with other escape systems – in particular, the use of giant fibres in rapid-response locomotion. Here, the large-diameter axons of the neurons enables the high-speed transfer of information which, undoubtedly, is useful for escape, but variation in diameter is also used, to ensure that signals arrive synchronously along the length of the mantle. We have seen similar mechanisms in other sensory contexts (e.g. the auditory system, electroreception), where the same coordination is required. Another convergence is the existence of parallel giant and non-giant systems driving rather similar behaviours; the former is reserved for emergency use, while the latter is used for more routine situations.

In the inking section, we noted a dual-function protection system; a strong visual signal (one intended to confuse or obscure, but a signal nonetheless) is combined with a strong chemical signal that interferes with the perceptive mechanisms of the recipient. We also saw how, to a certain extent at least, a clear behavioural characteristic – the need for a strong, sustained triggering stimulus – can be traced down to the kinetic properties of individual types of ion channels.

In discussing the control of colour and shape change in cephalopods, we have touched on some of the most spectacular morphing capabilities in the animal kingdom. Little is known about how the brain controls colour and shape, but we do know how it is controlled by neurons at the level of individual cells in chromatophores and iridophores, with the latter involving a novel biophotonic effector system that results in tuneable iridescence. Finally, we discussed a remarkable example of symbiosis in the photophores of squid, in which acquiring and maintaining the correct species of bioluminescent bacteria is essential. The squid exploits this relationship to produce anti-predator counter-shading to protect itself while it is hunting prey of its own.

Abbreviations

MC	magnocellular
PV	palliovisceral
FaRPs	FMRFamide-related peptides

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