Nociceptive Behavior and Physiology of Molluscs: Animal Welfare Implications

Robyn J. Crook and Edgar T. Walters

Abstract

Molluscs have proven to be invaluable models for basic neuroscience research, yielding fundamental insights into a range of biological processes involved in action potential generation, synaptic transmission, learning, memory, and, more recently, nociceptive biology. Evidence suggests that nociceptive processes in primary nociceptors are highly conserved across diverse taxa, making molluscs attractive models for biomedical studies of mechanisms that may contribute to pain in humans but also exposing them to procedures that might produce painlike sensations. We review the physiology of nociceptors and behavioral responses to noxious stimulation in several molluscan taxa, and discuss the possibility that nociception may result in painlike states in at least some molluscs that possess more complex nervous systems. Few studies have directly addressed possible emotion-like concomitants of nociceptive responses in molluscs. Because the definition of pain includes a subjective component that may be impossible to gauge in animals quite different from humans, firm conclusions about the possible existence of pain in molluscs may be unattainable. Evolutionary divergence and differences in lifestyle, physiology, and neuroanatomy suggest that painlike experiences in molluscs, if they exist, should differ from those in mammals. But reports indicate that some molluscs exhibit motivational states and cognitive capabilities that may be consistent with a capacity for states with functional parallels to pain. We therefore recommend that investigators attempt to minimize the potential for nociceptor activation and painlike sensations in experimental invertebrates by reducing the number of animals subjected to stressful manipulations and by administering appropriate anesthetic agents whenever practicable, welfare practices similar to those for vertebrate subjects.

Key Words: *Aplysia*; cephalopod; ethics; invertebrate; mollusc; nociception; pain; sensitization

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Nociceptive Biology and Evolution

Nearly all animals that have been studied display marked behavioral responses to stimuli that cause tissue damage—the ability to sense and respond to noxious stimuli is an almost universal trait (Kavaliers 1988; Smith and Lewin 2009; Sneddon 2004; Walters 1994). Nociception, defined as the detection of stimuli that are injurious or would be if sustained or repeated, has clear adaptive advantages because it triggers withdrawal and escape during injury or in the face of impending injury.

Nociception and Nociceptive Sensitization

The first stage of nociception occurs with the activation of nociceptors, primary sensory neurons preferentially sensitive to noxious stimuli or to stimuli that would become noxious if prolonged (Sherrington 1906). Nociceptors were first demonstrated in Chordata by Burgess and Perl (1967), in Annelida by Nicholls and Baylor (1968), in Mollusca by Walters and colleagues (1983a), in Nematoda by Kaplan and Horvitz (1993), and in Arthropoda by Tracey and colleagues (2003). Preliminary studies indicate that nociception in these phyla involves many conserved sensory transduction processes (Smith and Lewin 2009; Tobin and Bargmann 2004; Tracey et al. 2003), although differences have also been found. It is not yet known whether specialized nociceptors also occur in other phyla, although this seems likely.

Many nociceptors exhibit a property rare among primary sensory neurons: enhanced sensitivity produced by intense stimulation of the sensory neuron's peripheral terminals (Campbell and Meyer 1983; Gold and Gebhart 2010; Hucho and Levine 2007; Illich and Walters 1997; Light et al. 1992). Enhancement of the sensitivity of a nociceptor, nociceptive network, or behavioral response after noxious stimulation is called nociceptive sensitization (Walters 1994; Woolf and Walters 1991).

Nociceptive sensitization can be measured directly at the neuronal and behavioral levels and has been investigated extensively in mammals because it is thought to represent concrete, quantifiable effects that in humans are related to increased pain sensitivity. As defined by the International Association for the Study of Pain (IASP; Merskey and Bogduk 1994), increased pain sensitivity occurs in the form of hyperalgesia (greater pain in response to a normally painful stimulus) and allodynia (pain evoked by a stimulus that is not normally painful).

In a few molluscs and other invertebrates, mechanisms of nociceptive sensitization have been investigated intensively in hopes of discovering fundamental processes that contribute to hyperalgesia and allodynia in humans and other animals. Indeed, behavioral and neurophysiological alterations in nociceptors during nociceptive sensitization appear remarkably similar in snails and rats (Walters 1994, 2008; Woolf and Walters 1991), and these alterations involve many intra- and extracellular biological signaling pathways that are highly conserved (Walters and Moroz 2009).

Interpreting Nociceptive Differences across Phyla

There are numerous differences between the neuronal systems that mediate nociceptive sensitization in molluscs and in mammals (as well as presumably enhanced pain in the latter). At the axonal level, molluscs and other invertebrates lack myelination, so conduction of information from one part of the nervous system to another is usually much slower than in vertebrates. At the synaptic level, an evolutionarily recent expansion of the synaptic proteome in vertebrates may underlie unique cognitive capabilities of this group (Ryan and Grant 2009) and in principle also contribute to a potentially unique capacity of vertebrates to experience pain.

Furthermore, at the neuroanatomical and, presumably, neural network levels, little homology exists across any of the phyla, which diverged before most of the evolution of neuroanatomical structures in contemporary animals (Farris 2008). Thus noxious information in vertebrates is relayed from primary nociceptors via neurons in the dorsal horn of the spinal cord to brain structures including the thalamus and the somatosensory, insular, and anterior cingulate cortices (Peyron et al. 2000), but homologues to these brain structures do not exist in invertebrates. The fact that these areas are not present in the invertebrate central nervous system (CNS[1]) does not prove that invertebrates cannot feel pain; independently derived neural structures might, in principle, have evolved the capacity to mediate the same functions. For example, some invertebrates (many cephalopods and some insects) can process highly complex visual information even though they lack a structure homologous to the mammalian visual cortex. While it is plausible that the more elaborate neural structures of mammals confer a capacity for the experience of pain, analogous processing in other phyla might mediate painlike experiences using neural structures unrelated to and quite different from those in the mammalian brain.

Similarity across phyla of nociceptive behavior, mechanisms of nociception, and nociceptive sensitization implies either deep homology of underlying mechanisms or convergence arising from similar selection pressures among diverse and distantly related taxa. Either of these possibilities may permit insights into mechanisms important for pain and its alleviation in vertebrates from studies of animals with far simpler nervous systems. Invertebrates thus present two considerable advantages: (1) research on a complex process in a simpler system often permits a clearer picture of what is really important, and (2) the study of states bearing similarities to pain or unpleasantness in an animal that appears to have less capacity to interpret and "feel" such sensations is more palatable to scientists, to the public, and to animal welfare committees.

The presence in invertebrates of some nociceptive mechanisms that are homologous and/or convergent with those important for pain in humans presents a question of ethics: If scientists are willing to appeal to evolutionary conservatism to support the use of "lower" animals to study physiological building blocks that in humans contribute to pain and suffering (assuming that the information revealed will translate to humans and other "higher" vertebrates), is it acceptable to ignore possible implications of evolutionary conservation that similar processing of nociceptive information by higher and lower animals might in each case produce suffering? Are there reasons to think that experimental unpleasantness is less keenly felt by a snail or fly than by a mouse, monkey, or human, and if so where should one draw the line when conducting experiments that might cause suffering?

Nociception Versus Pain

Humans tend to experience nociception and pain as a single phenomenon, but for the study of animals it is important to draw a distinction between sensory activation and emotional perception (see Braithwaite 2010 for an excellent discussion of the separable nature of the two processes).

Defining Nociception and Pain

Nociception is a capacity to react to tissue damage or impending damage with activation of sensory pathways, with or without conscious sensation. Activity in nociceptive sensory pathways usually results in reflexive behavioral responses and may or may not result in other responses. Reflexive withdrawal responses tend to be mediated by very simple sensorimotor circuits optimized for speed and reliability and can occur without input from higher processing centers, although more complex escape and avoidance behaviors involve more complex neural circuits (Chase 2002; Walters 1994). Even in humans the initial reflexive response to a noxious stimulus is sometimes faster than can be consciously perceived, and nociceptors can sometimes be activated without conscious sensation (Adriaensen et al. 1980). Invertebrates that lack appropriate processing centers may be capable of only this rapid, unconscious processing.

The definition of pain widely accepted by scientific investigators is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey and Bogduk 1994). The emotional component required by this definition of pain makes its identification in other species, and especially in species quite different from humans, extremely difficult, if

[1]Abbreviation that appears ≥3x throughout this article: CNS, central nervous system

not impossible. This is because emotion is usually defined in terms of conscious experience (e.g., Izard 2009), and while evidence of consciousness in some animals is available, proof of consciousness is not (e.g., Allen 2004).

It is therefore important to distinguish between nociception as detection of a noxious stimulus (which can be recognized scientifically by unambiguous behavioral and neural responses), and pain as the unpleasant feeling associated with that stimulus (and inferred by behavioral and neural responses of uncertain relation to consciousness).

Moreover, the emotional response during pain may be linked to cognition, "knowing" in some sense that the sensation is negative and involves a threat to the body. Whereas nociception leading to a nociceptive response can be mediated by the simplest of neural circuits (in principle just a single nociceptor connected to an effector system—e.g., a muscle), pain requires neural circuitry that incorporates additional functions, some of which might entail highly complex processing by very large numbers of neurons.

Identifying Nociception and Pain in Animals

In invertebrates, like mammals, responses to noxious stimulation can be complex. Indeed, immediate defensive responses to injury or noxious stimulation are followed by a longer-term phase (sometimes lasting weeks or months) where damaged regions are hypersensitive (e.g., Walters 1987b) and some invertebrates remember associations of the noxious event with its context (e.g., Colwill et al. 1988; Walters et al. 1981).

To date, long-term nociceptive sensitization in invertebrates has been explained by long-term alterations of primary sensory neurons (especially nociceptors) (e.g., Montarolo et al. 1986; Scholz and Byrne 1987; Walters 1987a) and motor neurons (e.g., Cleary et al. 1998; Glanzman 2008; Weragoda et al. 2004); there is little evidence for electrical activity or alterations in other types of neurons that outlast a noxious stimulus for more than tens of minutes (see Cleary et al. 1998; Marinesco et al. 2004). Interneurons and modulatory neurons have received far less experimental attention because they are much more difficult to identify and sample with the intracellular recording methods used for neurophysiological experiments on invertebrates. Truly systematic investigations of the distribution and duration of enhanced activity (possible neural correlates of pain) across an entire nervous system will benefit from the development of functional imaging methods for invertebrates (for a start, see Frost et al. 2007; Zecevic et al. 2003) equivalent to those used to examine patterns of activity in the mammalian brain after noxious stimulation (e.g., Peyron et al. 2000; Tracey and Bushnell 2009).

Several considerations suggest that pain may be absent in at least some invertebrates. Capacities for processing all types of information, including that involved in pain or painlike phenomena, increase with the size and complexity of a nervous system, and mammals with the most complex brains have, thus far, shown the most evidence for a capacity for humanlike pain, although this might reflect a far greater experimental effort directed at vertebrates rather than genuine differences in the invertebrate pain experience.

Nociceptive reflexes and nociceptive plasticity can occur without conscious, emotional experience because these responses are expressed not only in the simplest animals but also in reduced preparations, such as spinalized animals (Clarke and Harris 2001; Egger 1978) and snail ganglia (Walters et al. 1983b). Similarly, in human patients nociceptive reflexes can occur without conscious awareness below a level of complete spinal transection (Finnerup and Jensen 2004).

But conservation or convergence of physiological and molecular mechanisms of nociception and nociceptive sensitization across distant phyla does not necessarily imply that higher-order phenomena (such as pain) that can be supported by these mechanisms are equivalent. Even if the critical mechanisms turn out not to be equivalent, it is not possible to be certain that an animal does not feel pain, and thus the ethical questions remain (Allen 2004).

How can researchers balance the opportunities some invertebrates offer for discovering mechanisms that, for example, may alleviate chronic pain or slow dementia in humans, with the possibility that the invertebrate subjects might suffer? This is a fundamental problem for all of biomedical research, but has received very little consideration in invertebrate studies. We refer readers to other articles in this issue (Elwood 2011; Mather 2011) dealing with ethical and philosophical considerations of invertebrate use.

Nociception and Painlike Phenomena in Mollusca

Molluscs have provided invaluable models for neuroscience, yielding a wealth of basic information applicable to humans and other vertebrates. A consideration of their nociceptive behavior and physiology will inform choices about their optimal use for biomedical research and improve the welfare of molluscs used in the laboratory.

Mollusca is a highly diverse and successful metazoan phylum with over 100,000 species (Ponder and Lindberg 2008) distributed among terrestrial, aquatic, and marine environments and divided into seven classes: Aplacophora, Polyplacophora, Monoplacophora, Scaphopoda, Bivalvia, Gastropoda, and Cephalopoda. Along with great diversity in body plan, lifestyle, and ecology, Mollusca encompasses enormous intraphylum variation in sensory organs and neuroanatomy (Bullock and Horridge 1965). Because the capacity for sensing and integrating information about the environment and an individual's own body is probably important for the ability to feel pain or experience distress, this large variation in neural and sensory complexity suggests that welfare concerns may differ among groups.

The primary model species considered here are from the gastropod and cephalopod clades; members of the other classes are discussed briefly.

Aplacophora, Polyplacophora, Monoplacophora, and Scaphopoda

The classes less commonly used in neurobiological research tend to be the primitive, sedentary, or deepwater-dwelling groups.

Aplacophora contains small wormlike molluscs that burrow into the substrate (Salvini-Plawen 1981) and have paired ventral and dorsal nerve cords running along the body as well as paired cerebral and buccal ganglia in the head. Visual and vestibular organs are absent but putative mechanosensory neurons innervate the oral surface (Shigeno et al. 2007). Little is known about their behavior and nothing about the physiology of sensory neurons.

Polyplacophora contains the chitons, generally intertidal marine animals with multiple platelike shells covering the dorsal surface. They have a simple nervous system without pronounced cephalization. There are rows of primitive photoreceptors along the dorsal surface and mechanosensory organs around the mouth.

Monoplacophora is also an exclusively marine taxon of limpetlike, conically shelled molluscs. Scaphopoda have a single, tusklike shell through which water is pumped while the animal remains mostly buried in the substrate. In both of these groups the neural networks are simple and apparently unspecialized and the ganglia are small. The simplicity of their nervous systems and their behavior suggest that the possibility of these animals experiencing painlike responses to tissue insult is remote.

Bivalvia

The Bivalvia (e.g., oysters, clams, mussels, and scallops) are abundant in both marine and freshwater environments. Their nervous system includes two pairs of nerve cords and three pairs of ganglia (Brusca and Brusca 2003). There is no obvious cephalization and the nervous system appears quite simple. A population of mechanosensory neurons is activated during the foot withdrawal reflex in a razor clam, but it is not known if these are nociceptors (Olivo 1970).

Clams and scallops have simple eyes and chemosensory organs located along the periphery of the mantle and they initiate escape swimming if a threat is detected, thus some integration of information and basic decision making occurs. Escape swimming in scallops is driven by a motor pattern generator in the cerebral ganglion and usually occurs after chemosensory detection or contact with a starfish predator that would normally precede tissue destruction (Wilkins 1981), suggesting that nociception may be involved. However, to our knowledge there are no published descriptions of behavioral or neurophysiological responses to tissue injury in bivalves.

Although a number of studies have claimed that endogenous opioids (e.g., Stefano and Salzet 1999) and opioid receptors (e.g., Cadet and Stefano 1999) are expressed in the mussel *Mytilus edilus*, particularly in immunocytes, neither genes for proopiomelanocortin (POMC) nor opioid receptors are found in *Drosophila melanogaster* or *Caenorhabditus elegans*, and their reported existence in other invertebrates, including molluscs, is controversial (Dores et al. 2002; Li et al. 1996).

Gastropoda

This class includes terrestrial, freshwater, and marine species (e.g., snails, slugs, limpets, whelks, and many others). Typically the shell and body are coiled, although in some taxa (e.g., terrestrial slugs, sea hares, nudibranchs) the shell is absent.

Gastropods have more diverse and specialized sensory organs than the groups above and are typically motile and active foragers. Along with increased range of habitats and associated morphologies, their behavioral range is greater and this is reflected in increased neural complexity. The basic molluscan nervous system is present (Bullock and Horridge 1965) but is expanded in terms of both the number of cells and their specialization. Many gastropods have giant neuronal somata, which have been used to advantage for neuronal analyses of behavioral mechanisms (Chase 2002; Kandel 1976) and perhaps most prominently for investigations of learning and memory mechanisms (Kandel 2001). Gastropods have also provided the largest number of studies of nociceptive behavior and sensitization in invertebrates, in part because noxious mechanical and electrical stimuli have been used in many learning and memory studies. Some of these studies have used pulmonate (air-breathing) snails but most have used opisthobranch (rear-positioned gills) snails. We know of no studies of nociceptive behavior or physiology in the remaining group of gastropods—the prosobranchs.

Pulmonates

Pulmonates descended from gastropod molluscs that moved from the sea to terrestrial and freshwater habitats. The most extensively studied pulmonate is *Helix* (several different species on different continents), which is prey to many generalist predators such as birds and frogs. Thus the well-developed defensive and aversive behaviors elicited by noxious sensory input during failed predation attempts in this genus should be subject to continuing selection.

When presented with a potentially threatening tactile stimulus to soft tissue, *Helix*, like other pulmonates, withdraws reflexively, a behavior mediated by simple neural circuitry including sensory neurons that appear to be nociceptors (Balaban 2002; Ierusalimsky and Balaban 2007). Noxious stimulation can sensitize this behavior. Repeated electric shocks to the foot (which probably activate nociceptors) result in a reduced response threshold to an innocuous mechanical stimulus that persists several days after the shocks, and this long-term behavioral sensitization is associated with several alterations in the circuit that mediates the withdrawal response (Balaban 1983, 1993; Prescott and Chase 1999).

Other pulmonates used to investigate responses to noxious stimuli include *Lymnaea stagnalis* (Sakharov and Rozsa 1989) and *Megalobulimus abbreviatus* (Kalil-Gaspar et al. 2007). The latter study is of interest because it provided pharmacological evidence for nociceptive actions of an ion channel highly expressed in mammalian nociceptors (the transient receptor potential cation channel subfamily V member 1, or TRPV1, the capsaicin receptor).

As explained above, the ability to perceive noxious information as emotionally unpleasant is a delineating point between simple nociception and pain. *Helix*, with a brain containing only about 20,000 neurons in 11 ganglia, might be presumed to be incapable of higher-order processing necessary for emotional responses. Interestingly, however, some evidence suggests an emotionlike reaction in *Helix*.

Experiments using electrodes implanted into two different ganglia allowed animals to "self-stimulate" either of the two neural regions by pressing a bar (Balaban 1993; Balaban and Chase 1991). Results from this experiment suggested that different CNS regions of snails have some rudimentary "emotional coloration": snails self-stimulated more frequently when the electrodes were placed in the mesocerebral region of the cerebral ganglion (containing some neurons involved in sexual behavior) and less frequently when they were placed in the rostral portion of the parietal ganglion, in an area where electrical stimulation of putative nociceptive sensory neurons underlying reflexive withdrawal behavior was likely (Balaban 2002). But relatively little is known about the anatomical organization and actual functions of most neurons in either of these ganglionic regions. A lack of follow-up reports of snail self-stimulation raises questions about the robustness of this finding.

An apparent ability to remember and choose to avoid a negative stimulus would suggest that a snail can selectively modify its behavior on the basis of aversive experience in ways similar to mammals. This would not show that *Helix* can experience pain but it would suggest that fundamental components of painlike information processing in vertebrates might be present in a rudimentary fashion in some molluscs.

Opisthobranchs

The marine opisthobranch *Aplysia californica* is the leading invertebrate model system for analyzing cellular bases of behavioral and neural plasticity in many contexts (Kandel 1976, 2001). *Aplysia* has nine central ganglia containing only about 10,000 neurons (Cash and Carew 1989), of which several hundred have been identified by soma size and location, electrophysiological properties, synaptic connections, and behavioral effects. *Aplysia* and many other molluscs also have numerous neuronal cell bodies in peripheral nerve nets (Bullock and Horridge 1965; Moroz 2006).

Most studies of learning and memory mechanisms in *Aplysia* have used known mechanosensory neurons. Initial studies used these neurons in the abdominal ganglion that innervate the siphon (Byrne et al. 1974), but later studies used homologous sensory neurons in each pleural ganglion that innervate most of the rest of the body surface (Walters et al. 1983a, 2004). Moreover, both sets of neurons function as nociceptors (Illich and Walters 1997; Walters et al. 1983a) and thus their rich plasticity is highly relevant to nociceptive functions. Both sets of nociceptors display prominent sensitizing effects, associated with short- and long-term sensitization of defensive behavior (gill and siphon withdrawal, tail withdrawal, head withdrawal) after noxious stimulation. These effects include enhancement of synaptic transmission (reviewed by Kandel 2001; Walters 1994) and hyperexcitability of the nociceptor expressed in its peripheral terminals (especially near a site of injury), neuronal cell body, axons, and near its presynaptic terminals (Billy and Walters 1989; Gasull et al. 2005; Reyes and Walters 2010; Weragoda et al. 2004). Such alterations are similar to those described in studies of nociceptive sensitization in rodents and humans, suggesting that some mechanisms that promote pain in vertebrates are also present in molluscs and, most likely, in other invertebrate taxa as well (Walters and Moroz 2009).

Interesting similarities also exist in the behavioral responses of *Aplysia* and mammals to noxious stimulation. *Aplysia* displays the nearly ubiquitous pattern of immediate withdrawal reflexes, rapid escape, and prolonged recuperative behaviors exhibited across all major phyla (reviewed by Kavaliers 1988; Walters 1994; see also Babcock et al. 2009; Walters et al. 2001).

In addition, *Aplysia* responds with a motivational state resembling conditioned fear to previously neutral chemosensory stimuli associated with noxious electric shock (Walters et al. 1981). For example, after pairing with shock, the smell of shrimp evoked a state that was not obvious unless combined with other stimuli—indeed, when only the shrimp extract was presented, the animal exhibited a response reminiscent of the freezing exhibited by rats to a conditioned fear stimulus (Walters et al. 1981). When tested in combination with weak tactile stimulation, the shrimp extract greatly facilitated head and siphon withdrawal responses, defensive inking, and escape locomotion. Moreover, when delivered to a feeding animal, the conditioned smell inhibited the feeding.

These extensive and motivationally consistent associative alterations (see also Colwill et al. 1988) suggest that memory of a noxious event in snails can be linked to a fear-like motivational state that can dramatically alter the animal's response to other biologically significant stimuli. Unfortunately, almost nothing is known about the neuroanatomical loci and specific neurons involved in this "higher-order" processing of nociceptive information in opisthobranch ganglia.

Nociceptive sensitization has also been reported in the marine nudibranch *Tritonia diomedea* (Frost et al. 1998), and associatively conditioned avoidance behavior after noxious conditioning stimulation has been investigated in the notaspid *Pleurobranchaea californica* (Jing and Gillette 2003; Mpitsos and Davis 1973). Both of these species offer many advantages for cellular analyses, but they are difficult to obtain commercially and have been investigated much less extensively than has *Aplysia*.

In most animals, including *Aplysia*, nociceptive pathways show a period of inhibition after strong noxious stimulation as the animal engages in escape and active defense (Mackey et al. 1987; Walters 1994). Opioids contribute substantially to nociceptive inhibition in vertebrates but, despite indirect evidence for opioid signaling—such as pharmacological actions of opioids (e.g., met-enkephalin) and opioid antagonists (e.g., naloxone) in molluscs (Kavaliers 1988; Leung et al. 1986)—molecular evidence does not yet provide firm support for true opioid signaling in invertebrates (Dores et al. 2002). Moreover, application of enkephalins fails to produce inhibitory effects on the gill withdrawal reflex (Cooper et al. 1989) or known nociceptors (Brezina et al. 1987) in *Aplysia*. Instead another peptide, FMRFamide, may be a major transmitter that produces immediate and long-term suppressive effects on nociceptor excitability, synaptic transmission, and defensive reflexes in *Aplysia* (Belardetti et al. 1987; Mackey et al. 1987; Montarolo et al. 1988).

Cephalopoda

Cephalopoda is an exclusively marine class that comprises squid, cuttlefish, octopus, and nautilus. The cephalopod shell is chambered and external in nautilus, internal in cuttlefish, and reduced or absent in squid and octopuses. Cephalopods are the most neurologically complex invertebrates, with centralized brains divided into specialized lobes capable of processing and integrating complex visual, chemical, and tactile sensory inputs. The octopus CNS has around 500 million cells (Young 1963), vastly more than that of gastropods. The octopus also has an enormous peripheral nervous system, separate from the CNS (Rowell 1966; Young 1963). Indeed, the number of neuronal cell bodies in the arms of the octopus is close to that of the central brain.

Cephalopods show complex behavior and are excellent learners, with reports of sensitization, habituation, associative learning, spatial learning, and even (although controversial) observational learning (see reviews by Hanlon and Messenger 1998; Hochner et al. 2006). Despite the extensive literature on cephalopod behavior, there have been no systematic behavioral or physiological investigations into nociception and nociceptive plasticity.

Cephalopods are also less commonly used for studies of neurophysiology due to the complex neuroanatomy of the cephalopod CNS, the small size of the neuronal cell bodies, and the lack of overshooting action potentials or large synaptic potentials that can be recorded from the cell bodies (Hochner et al. 2006). Nothing is known about where nociceptive information is processed in the cephalopod brain. Evidence for nociception in cephalopods is therefore exclusively behavioral.

In various learning paradigms electric shock to the arms of an octopus has been used effectively as a negative reinforcement (Boycott and Young 1955; Darmaillacq et al. 2004; Shomrat et al. 2008; Young 1961), indicating that the animal finds this stimulation aversive, but nociceptors have not yet been described in any cephalopod. Octopuses are capable of regenerating damaged or amputated arms, but there are no published studies of behavioral adaptations to damaged or healing tissue.

Although electric shock elicits defensive responses, an intriguing anecdote from Jacques Cousteau (1973, 23-24) describing an octopus' behavior during exposure to damaging heat suggests the absence of a response to intense thermal stimulation:

> One day at Octopus City, in the Bay of Alicaster, Dumas dived with an underwater rocket and began waving it in front of an octopus' house. Nothing happened. The animal reacted not at all. He did not try to hide, or to escape. Dumas then turned the beam directly onto the octopus, which did not even draw [sic] its arms. The game was called off, however, when Dumas saw that it was becoming cruel. The octopus showed signs of having been burned. But even then it had not tried to escape from it.… This surprising insensitivity to fire has been confirmed by Guy Hilpatric, one of the pioneers of diving, who told us that he has seen an octopus, which had been brought onto shore, cross through a fire to get back into the water.

Although it is potentially valuable for an animal (particularly an intertidal species) to sense when environmental temperatures are approaching dangerous levels, selection pressure for nociceptors tuned to extreme heat is unlikely in most aquatic animals. It would certainly be interesting to examine this question in marine invertebrates adapted for life around hydrothermal vents where extreme heat exposure may be a hazard. An interesting question following from Cousteau's anecdote is whether the burned octopus would eventually sense and respond to signals released by the damaged flesh or to inflammatory mediators released during repair of the tissue, as occurs in mammals.

Apart from the nautilus, which retains its external shell, cephalopods have traded the protection of the molluscan shell for an increase in locomotive efficiency and speed. Their soft bodies, almost completely unprotected, should be subject to occasional survivable injuries during failed predation attempts, food contests, and mating competitions. It thus seems probable that nociceptors exist in cephalopods. We have begun our own investigations into this possibility by examining behavioral responses of the squid (*Loligo pealei*) to peripheral, sublethal injury to an arm (R. Crook, T. Lewis, R. Hanlon, and E.T. Walters, unpublished observations). Sensitization of defensive responses to tactile and visual stimuli occurs and persists for at least 48 hours after injury, suggesting that basic behavioral responses to injury in cephalopods may be similar to those in gastropods and vertebrates, but further study is needed.

Notwithstanding the lack of evidence for pain perception in cephalopods, in the United Kingdom octopus are covered by the same welfare act that governs procedures on vertebrates (UK Animals [Scientific Procedures] Act 1986)[2] and

[2]Available online (www.legislation.gov.uk/ukpga/1986/14/contents), accessed on April 4, 2011.

in Canada and Europe an approved welfare protocol is required for the use of cephalopod species in research (CCAC 1993; EU directive 86/609/EEC, updated September 2010[3]).

Cephalopods have far more complex brains and behaviors than any other invertebrate and are capable of impressive cognitive tasks (see Hochner et al. 2006, for review). For example, both the brainy octopus (Boal et al. 2000) and "primitive" nautilus (Crook et al. 2009) are capable of vertebratelike spatial learning. Given these sophisticated cognitive abilities, an important question is whether nociceptive responses in cephalopods are accompanied by affective and cognitive processing functionally similar to some of the processing that is important for pain states in mammals.

Anesthesia in Molluscs

Until the early 1970s physiological and biochemical studies of molluscs dispensed with any anesthetics. This practice reflected the widespread assumption that invertebrates cannot feel pain, a view that is still common (and used to condone, for example, the preparation of living cephalopods, snails, and lobsters for human meals by methods that would cause intense, prolonged stimulation of nociceptors if applied to mammals).

Several studies of the mechanisms of action of widely used mammalian anesthetics exploited the experimental advantages of the giant axon of the squid and the giant cell bodies in gastropod molluscs such as *Aplysia* (e.g., Frazier et al. 1975; Winlow et al. 1992). But these anesthetics were not used by researchers during their dissections, in part because many of the mammalian anesthetics and analgesics, including opioids (Cooper et al. 1989), were not very effective in molluscs (although there were interesting exceptions, including volatile general anesthetics that potently open potassium channels that hyperpolarize and reduce excitability in *Aplysia* nociceptors; Winegar et al. 1996; Winegar and Yost 1998). The widespread use of anesthesia during dissection of *Aplysia* began when investigators interested in learning and memory realized that dissection without anesthesia could cause sensitizing effects that would interfere with the neuronal plasticity they were studying.

The anesthetic of choice for both gastropods (Pinsker et al. 1973) and cephalopods (Messenger et al. 1985; Mooney et al. 2010) is isotonic magnesium chloride solution, typically applied by injection for gastropods and immersion for cephalopods. Magnesium ions provide an ideal anesthetic (and muscle relaxant) because they are normally present at relatively high concentrations (especially in marine molluscs) and thus are relatively nontoxic, their effects are rapidly reversible, and the agent is both inexpensive and highly effective (Walters 1987b). The effectiveness of magnesium chloride in gastropods is a result of its inhibition of neurotransmitter release at chemical synapses, its depressive effect on voltage-gated sodium channels, reducing membrane excitability (e.g., Liao and Walters 2002), and the fact that it is used at high concentrations and injection volumes (>25% of the animal volume) in combination with a balanced reduction of sodium ions (a reduction that by itself reduces excitability). Effectiveness probably also depends on leakiness of the primitive blood-ganglion or blood-nerve barriers of most molluscs (Abbott 1987). Applied magnesium chloride does not penetrate these barriers in vertebrates, precluding its use as an anesthetic in mammals. Given that cephalopods also have a highly effective blood-brain barrier (ibid.) and relatively impermeable skin, the mechanisms underlying the anesthesia that occurs during the animal's immersion in isotonic magnesium chloride are an interesting mystery.

We refer readers to Cooper (2011, in this issue) for further discussion of analgesia and anesthesia in invertebrates.

Evolutionary Selection Pressures and Painlike Phenomena in Molluscs

Insight into the extent to which nociception may lead to pain in molluscs can come from considering possible selective advantages of pain during evolution.

Nociception, like any prominent trait in animals, has been selected and refined over millions of years by evolutionary processes that act to enhance survival and reproductive success. The adaptive value of nociception is obvious and probably universal: it permits rapid avoidance of a damaging stimulus and escape from the context in which such damage occurs. Escape may be from predators, aggressive conspecifics, or threatening environmental features (e.g., rough surf).

The adaptive value of experiencing pain is more difficult to identify, although clues are available from likely consequences of nociceptive responses for survival under natural conditions. A strong negative emotion motivates rapid avoidance learning, decreasing the chances of reexposure to a noxious stimulus. This interpretation raises the question of how much neural processing power is required for emotional responses. Behaviorally expressed motivational states (defined without reference to consciousness) mediated by the actions of neuromodulators on relatively simple circuits in *Aplysia* have been suggested to have functional similarities to emotional states in mammals (Kupfermann 1979), and this is consistent with properties of the conditioned fearlike state in *Aplysia* described above. However, from a functional and evolutionary point of view, there is no need for these states to involve conscious experience by the animal.

In social animals, awareness and communication of injury can be advantageous, allowing not only the behavioral alterations that guard or rest an injured body part (at the expense of other behaviors such as foraging) but also the recruitment of caregivers to help protect and provide for an incapacitated individual during recovery. In nonsocial animals (which may include all molluscs, as even those that aggregate do so opportunistically for exploitation of a local resource, not for social interactions), obvious behavioral

[3]Available online (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm), accessed on April 4, 2011.

changes resulting from injury may be neutral or maladaptive (attracting the attention of predators or aggressive conspecifics). Thus it is unlikely that painlike states would have evolved in molluscs to promote communication with potential caregivers.

Animals with high metabolic rates, such as squid, must forage frequently to survive and thus cannot suppress active behavior for very long during recuperation (when pain is often worst in mammals), so this common behavioral correlate of pain would be maladaptive in such animals, although ongoing pain that promotes minor behavioral changes to protect an injured appendage as a tradeoff against small reductions in foraging success may be highly adaptive. Animals under predation pressure (which includes almost all molluscs, and particularly those lacking primary defenses such as a hard shell or aposematic signals) probably derive minimal value from sustained painful "feelings" if their expression renders them more vulnerable to predators. Thus, while an acute response to injury promoting escape and survival should be strongly selected, and long-term increases in sensitivity to potentially threatening stimuli are likely to be adaptive (Walters 1994), persistent painlike states that profoundly alter ongoing behavior (e.g., decreasing foraging or mating activity) may not be adaptive in molluscs.

Conclusions

All molluscs examined have shown a capacity for nociception as demonstrated by behavioral responses and/or by direct recording from nociceptors and other neurons. Nociception and nociceptive sensitization at the level of primary nociceptors make use of neuronal mechanisms that appear to be highly conserved and widespread throughout the animal kingdom.

But not all mechanisms related to nociceptive biology are widely shared. For example, analgesiclike effects mediated by true opioids and opioid receptors may be absent in invertebrates (Dores et al. 2002), and vertebrates may possess some synaptic mechanisms that are absent in invertebrates (Ryan and Grant 2009). Moreover, the sharing of many basic molecular building blocks does not imply sharing of higher-order processes that depend on those building blocks. For example, nearly all known brain functions in most phyla depend on action potentials generated by the operation of highly conserved sodium channels, but only a few species have brains with the capacity to learn a spoken language or do arithmetic—thus voltage-gated sodium channels are essential for learning German or solving equations, but their presence does not imply the capacity for proficiency in German or algebra. At some level this must also be true for the capacity to experience pain.

Immediate and longer-term neuronal and behavioral responses (including nociceptive memory and simple associative learning) can be mediated entirely by a single small ganglion, such as the abdominal ganglion in *Aplysia*, proving that these effects do not need complex neural structures (e.g., Antonov et al. 2003; Illich and Walters 1997). Some gastropods, which have simple nervous systems compared to cephalopods, exhibit changes in state with apparent functional similarities to emotional states, as illustrated by "conditioned fear" and "self-stimulation" in *Aplysia* and *Helix*, respectively (Balaban and Chase 1991; Walters et al. 1981).

Given the capabilities of relatively simple molluscan nervous systems, and if a key to the experience of pain is the size and complexity of the nervous system, one must seriously consider the possibility that cephalopods can experience some form of pain. The most complex and behaviorally sophisticated of molluscs, cephalopods have vastly more complex central nervous systems, with up to 500 million neurons, distinctive internal divisions of the brain, specialized integrative regions where diverse sensory inputs are processed (Boycott and Young 1955), and dense, specialized innervation of the periphery (Hochner et al. 2006). If subjective pain experience requires a minimal level of network complexity and processing power, these animals' brains might approach that level.

Scientifically accepted definitions of pain and nociception neatly distinguish these concepts (e.g., Merskey and Bogduk 1994), but drawing a line between the two can be difficult in practice. Furthermore, no experimental observation of nonverbal animals (nonhumans) can demonstrate conclusively whether a subject experiences conscious pain (Allen 2004). Suggestive evidence for painlike experiences in some animals is available, and nociceptive responses measured at the neural and behavioral levels in molluscs have provided evidence that is both consistent and inconsistent with painlike states and functions. Unfortunately, inferences drawn from the relatively small body of relevant data in molluscs are limited and prone to anthropocentrism. Identifying signs of pain becomes increasingly difficult as the behavior and associated neural structures and physiology diverge from familiar mammalian patterns of behavior, physiology, and anatomy, making interpretation of responses in molluscs particularly difficult.

In the laboratory, molluscs are often subjected to manipulations that produce nociceptive responses, either as the aim of an experiment or as a byproduct. Profound differences between molluscs and mammals in the size, complexity, and structure of their nervous systems, as well as their lifestyles and evolutionary history, suggest that painlike phenomena, if they exist in some molluscs, are likely to be quite different from pain in mammals, although it does not follow that molluscs are incapable of experiencing pain. Gastropod and cephalopod molluscs have shown long-lasting behavioral alterations induced by noxious experience, which probably involve motivational states that can be used flexibly to alter defensive and appetitive responses. This suggests that some molluscs may be capable not only of nociception and nociceptive sensitization but also of neural states that have some functional similarities to emotional states associated with pain in humans.

While it seems improbable that any mollusc has a capacity to feel pain equivalent to that evident in social mammals,

the existence of some similarities in nociceptive physiology between molluscs and mammals, the paucity of systemic investigations into painlike behavior in molluscs, and the logical impossibility of disproving the occurrence of conscious experience in other animals all suggest that it is appropriate to treat molluscs as if they are susceptible to some form of pain during experimental procedures.

In conclusion, we recommend that the design of experiments using molluscs, particularly those with larger and more complex ganglia or brains (especially cephalopods but also gastropods), take into account the possibility of a capacity for painlike experience in these animals. Effective anesthetics (e.g., magnesium chloride) should be used during dissections and, to the extent possible, during any procedure that produces tissue damage or possible stress. Investigators whose experiments unavoidably produce noxious stimulation should employ efforts similar to those required for vertebrate subjects to reduce both the number of animals and the potential for suffering to the minimum needed to test their hypotheses.

Balancing the benefits from knowledge gained in molluscan experiments with the potential for inflicting pain and distress in the experimental subjects should be an explicit consideration in molluscan studies. (For related guidance to IACUC members, we refer readers to Harvey-Clark 2011, in this issue.)

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