SENTIENCE, PAIN, AND ANESTHESIA IN ADVANCED INVERTEBRATES

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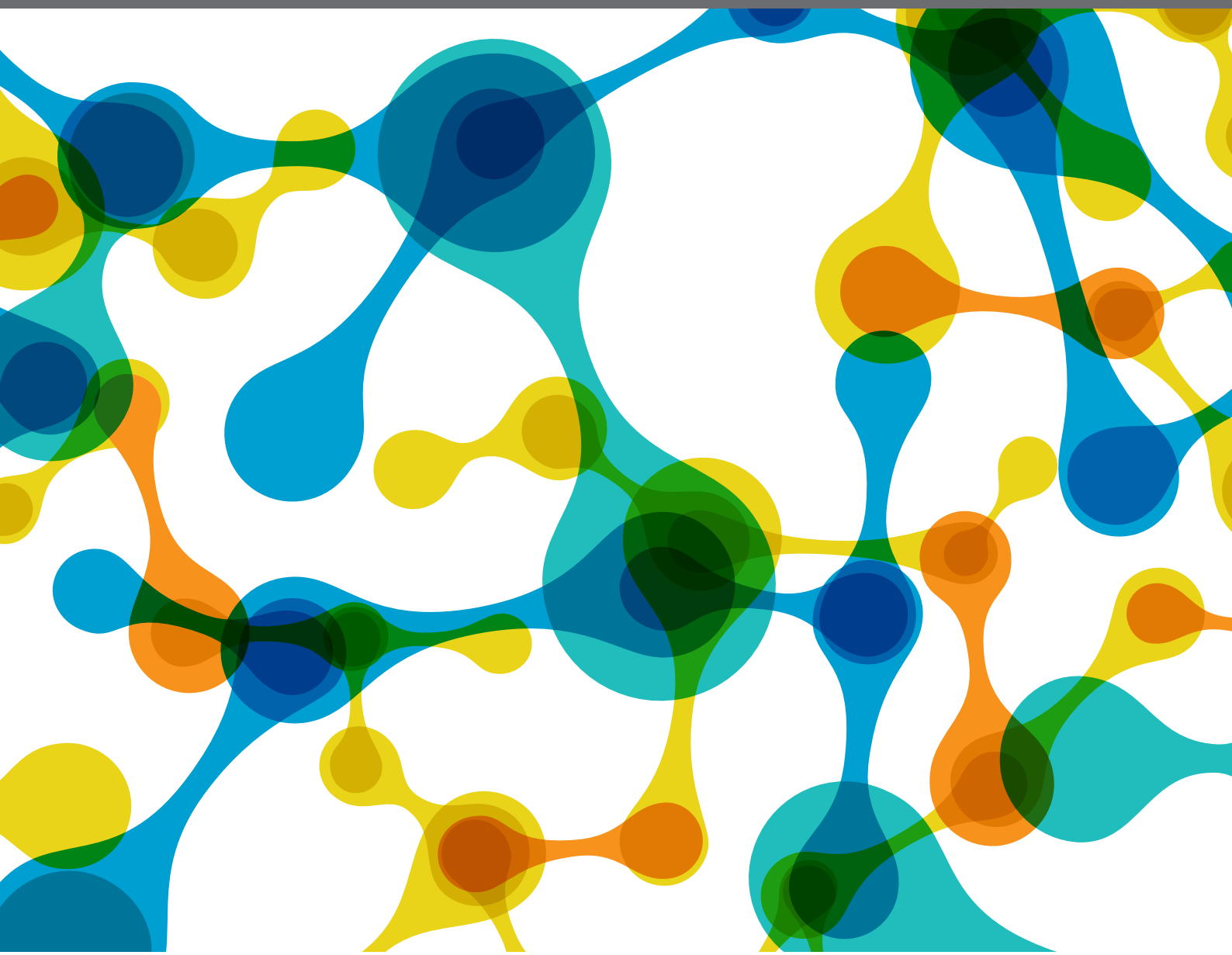

frontiers Research Topics

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SENTIENCE, PAIN, AND ANESTHESIA IN ADVANCED INVERTEBRATES

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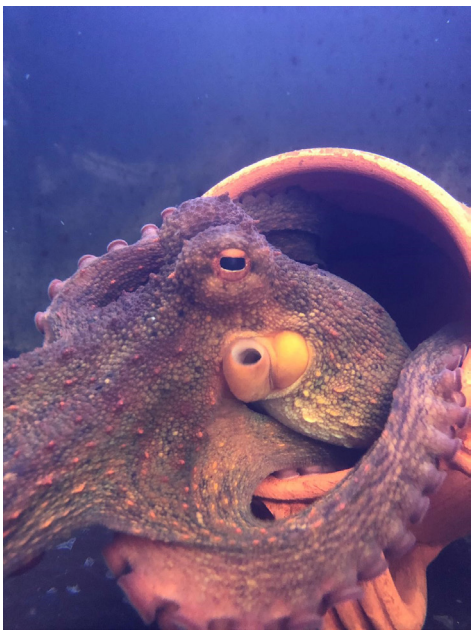

Octopus Vulgaris. Photograph provided from the archive of Professor Ann Di Cosmo, department of Biology, University of Naples, Italy.

There is an emerging view, supported by animal welfare legislation in a number of countries, that some advanced invertebrates are self-aware, sentient beings with the ability to feel pain. Sentience must encompass elements of time and neural complexity, including memory and learning, which leads us to ask: At what convergent point in the evolution of nervous systems does the subjective sensation of pain arise? Here we start to grapple with this issue, particularly with regard to arthropods and cephalopod molluscs, and to consider the most appropriate ways of anesthetizing them to minimize pain wherever possible. We also report on the development of cell culture techniques to understand the actions of the anesthetics being used. A better understanding of sentient creatures, other than ourselves, may eventually assist future development of artificial intelligence, particularly if we are able to perceive whatever common neural features underlie sentience in those animals that possess it.

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EDITORIAL
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Editorial: Sentience, Pain, and Anesthesia in Advanced Invertebrates

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Editorial on the Research Topic

Sentience, Pain, and Anesthesia in Advanced Invertebrates

Sentience may be thought of as the ability to perceive events in the context of previous or future events, resulting in conscious non-reflex behavioral modification(s) and is dependent on self-awareness. The ability to perceive pain, not just nociceptive stimuli, is thus a consequence of self-awareness, which then imparts the ability to avoid potentially damaging and painful future encounters. Thus, sentience must encompass elements of both time and complexity, including learning and memory, and is dependent upon individual experiences, but at what evolutionarily convergent points in the evolution of nervous systems does the subjective sensation of pain arise? If advanced invertebrates are conscious, sentient and self-aware, then there must be numerous different neural systems with which consciousness is associated, so what sort of neural matrix is required for a creature to become sentient?

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ARE ADVANCED INVERTEBRATES SENTIENT AND CAN THEY FEEL PAIN?

Cephalopod molluscs and decapod crustaceans are believed to the most developed and intelligent invertebrates and may be sentient animals. They are legally protected by a number of national and trans-national organizations, such as the European Union, but it is difficult for us to understand sentience in these creatures given the major structural differences between us and them, particularly since we last shared a common ancestor about 550 million years ago (Walters).

In a theoretical article Key and Brown discuss what they describe as anecdotal evidence for sentience and sensory awareness in cephalopod molluscs. They have developed an algorithm that generates multiple levels of awareness and show that the interconnectivity of the human brain is consistent with the algorithm in generating pain awareness, but that the cephalopod brain lacks the circuitry to do this, implying that cephalopods cannot feel pain. In a separate and very extensive review, Walters considers the evolutionary nociceptive biology of a wide range of molluscs and arthropods. He points out that both molluscs and arthropods have systems that suppress nociceptive responses, developed through convergent evolution. He then considers pain-like states in cephalopod molluscs, crustaceans, and insects and points out their similarities in response to damaging or potentially damaging stimulation to the body, which implies functional awareness of injury induced vulnerability. However, he concludes that it is not yet possible to say that "any molluscs or arthropods have evolved a capacity for conscious emotion and for suffering after noxious experience."

DEVELOPMENT OF ANESTHETIC METHODS FOR CEPHALOPOD MOLLUSCS

It is very difficult to assess pain in invertebrates, because a withdrawal response to a noxious stimulus could be a simple reflex reaction in simpler animals. This leads to questions as to how the more advanced invertebrates should be anesthetized. Two differing approaches have been outlined in this Research Topic. Butler-Struben et al. have carried out *in vivo* recording of neural and behavioral correlates of anesthesia in cephalopods and conclude that magnesium chloride and ethanol are suitable anesthetic agents for these animals. In their review Winlow et al. conclude that anesthesia in *Octopus Vulgaris* is best achieved with clinical anesthetics, but agree that pre-treatment with a muscle relaxant such as magnesium chloride, acting as an anesthetic adjuvant, might be the best future approach. Much of the work on clinical anesthetic techniques has been developed using simpler molluscs such as the pond snail *Lymnaea stagnalis* as outlined by Moghadam et al., who have discovered significant differences between the responses of identified motor neurons and interneurons to both applied systemic and volatile anesthetics, *in situ* and in single cell culture.

USEFULNESS OF CELL CULTURE TECHNIQUES

Although general anesthetics are considered to be safe and effective, care must be taken in their use as they can have cytotoxic effects particularly during peak periods of neurodevelopment. Armstrong et al. have reviewed this issue and have demonstrated the usefulness of synaptically connected identified neurons from *Lymnaea* when grown in cell culture. They also demonstrated some novel preliminary data on the newer anesthetic agent dexmedetomidine on synaptic transmission using these techniques. Such techniques have proved difficult to apply to cephalopods, which have much smaller neurons than do pulmonate molluscs such as *Lymnaea.* However, this problems is being resolved by the studies of Maselli et al. and Maselli et al., who have now developed suitable techniques for primary cell culture of dissociated neurons from two specific brain regions of *Octopus vulgaris*, the vertical superior frontal system and the optic lobes. These regions of the brain are involved in memory, learning, sensory integration, and adult neurogenesis. The data obtained from this work opens the prospect of more detailed studies of injury-induced neuronal regeneration in *Octopus* brain regions, which may be equivalent to those of vertebrates.

GETTING THE BASICS RIGHT

One of the key points in our understanding of the functioning of nervous systems in general was the discovery of the ionic mechanisms underlying the action potential using the squid giant axon by Hodgkin and Huxley (1952). However, Johnson and Winlow indicate that this is by no means the whole story. More recent findings suggest that action potentials are accompanied by a soliton pressure wave which may instigate channel opening. What is more, it is suggested that that the action potential should be considered as a ternary rather than a binary event, thus including the refractory period in computational models of interactions between colliding action potentials. If action potentials are not yet fully understood then one of the key elements in understanding sentience is imperfect, with potential negative consequences for our understanding of sentience and also for the development of artificial intelligence.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Designing Brains for Pain: Human to Mollusc

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There is compelling evidence that the "what it feels like" subjective experience of sensory stimuli arises in the cerebral cortex in both humans as well as mammalian experimental animal models. Humans are alone in their ability to verbally communicate their experience of the external environment. In other species, sensory awareness is extrapolated on the basis of behavioral indicators. For instance, cephalopods have been claimed to be sentient on the basis of their complex behavior and anecdotal reports of human-like intelligence. We have interrogated the findings of avoidance learning behavioral paradigms and classical brain lesion studies and conclude that there is no evidence for cephalopods feeling pain. This analysis highlighted the questionable nature of anthropometric assumptions about sensory experience with increased phylogenetic distance from humans. We contend that understanding whether invertebrates such as molluscs are sentient should first begin with defining the computational processes and neural circuitries underpinning subjective awareness. Using fundamental design principles, we advance the notion that subjective awareness is dependent on observer neural networks (networks that in some sense introspect the neural processing generating neural representations of sensory stimuli). This introspective process allows the observer network to create an internal model that predicts the neural processing taking place in the network being surveyed. Predictions arising from the internal model form the basis of a rudimentary form of awareness. We develop an algorithm built on parallel observer networks that generates multiple levels of sensory awareness. A network of cortical regions in the human brain has the appropriate functional properties and neural interconnectivity that is consistent with the predicted circuitry of the algorithm generating pain awareness. By contrast, the cephalopod brain lacks the necessary neural circuitry to implement such an algorithm. In conclusion, we find no compelling behavioral, functional, or neuroanatomical evidence to indicate that cephalopods feel pain.

Keywords: pain, consciousness, feeling, noxious stimuli, cortex, awareness, qualia

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INTRODUCTION

Why has the question of whether and which animals experience pain become so vexed? Among research topics, consciousness is unique in being private, first-personal and subjectively known. This makes theorizing about it a "hard problem" (Chalmers, 1995) because the subjective nature of feelings can only be definitively known by first-person experience and verbal report, i.e., by

those creatures capable of both thought and language, namely, humans. As yet, there is no clear understanding of how feelings emerge from organic tissue. How is it that firing of nerve impulses in the human brain can generate either pain or pleasure or alternatively remain non-conscious? In the absence of both verbal reports and a neurobiological basis of feelings, researchers tend to rely overly on behavioral observations and "benefit of the doubt" assumptions (i.e., the precautionary principle) to decide whether certain species of animals are capable of feeling.

The classical approach to determining whether an animal feels pain is to observe its behavioral response to a noxious (harmful) stimulus. Behavioral studies are based on the premise that the behavior reflects some qualitative feature of the experience (e.g., an avoidance response reflects unpleasantness). The difficulty here of course is distinguishing whether the behavior truly demonstrates an underlying experience of pain. Analyses can be supported by ablation studies that remove portions of the nervous system that are believed to be involved in conscious rather than non-conscious behaviors. This approach then becomes laden with assumptions about which neural regions are involved in conscious behaviors in humans and whether these same regions and their functions are phylogenetically conserved.

To begin to address the question of whether an animal can, or cannot, experience pain requires a working definition of pain that is broadly applicable across phylogenetically distant species. We simply describe pain here as an unpleasant feeling. This definition indicates that a feeling that is not unpleasant is not pain. Consequently lobotomized patients who claim that they are experiencing pain that is not unpleasant (Bain, 2014) cannot therefore be experiencing pain. Because pain is a feeling, it is then not possible to have an unfelt pain as some have argued (Palmer, 1975). Agreeing on what a "feeling" is has been notoriously difficult (Searle, 2000; Carruthers and Schier, 2017). Feelings have been variously referred to as "conscious awareness," "inner awareness," (Farrell and McClelland, 2017) "subjective experience," (Tye, 1986; Sytsma and Machery, 2010) "something-it-is-like" for the subject (Nagel, 1974), sentience (Harnad, 2016), "phenomenal consciousness" (Block, 1995) and "qualia" (Tye, 1994).

It is widely acknowledged that feelings share a close relationship with awareness (Natsoulas, 1983, 1999; Berger, 2014; Carruthers, 2015; McClelland, 2015; Farrell and McClelland, 2017; Kouider and Faivre, 2017; LeDoux and Brown, 2017). Given that awareness in any system is dependent on detection of change in the state of the system, then a brain must be able to selectively monitor internal changes in its neural information processing in order to be subsequently aware of them. Feelings, however, are more than detection of state change – there needs to be some implicit knowledge (Schacter, 1992) about the nature of what the brain is currently processing (Cleeremans, 2011). How does a creature generate such implicit understanding? If the internal monitor was a model of sensory processing that could accurately predict the future state of the processing, then that model would possess implicit knowledge or understanding of its internal operations. By way of analogy, if an artificial neural network was trained to predict the outcome of a chess match between two chess champions on the basis of the opening sequence of moves it would then possess some implicit knowledge (contained within the synaptic weights and connectivity of the network) of the players strategies. In comparison, a naïve observer network that merely monitored the game so as to report the outcome would lack any awareness of player strategy.

Returning to an animal nervous system, if a model network was monitoring sensory processing arising from a noxious stimulus then it would contain implicit knowledge about the type of stimulus (e.g., burning, freezing, stabbing, or cutting) as well as its intensity and location. Thus, establishing whether an animal's nervous system has the capacity to observe and predict an outcome of its sensory processing following a noxious stimulus is a reasonable test of the animal's capacity to feel pain. This strategy is not burdened by any need to explain the hard problem of how a conscious experience might be expected to feel qualitatively. We contend that an internal model of sensory processing is necessary for implicit awareness but not sufficient for the explicit qualitative feeling of pain. While the necessity of predictive modeling of sensory processing following a noxious stimulus is a significant first hurdle in assessing whether an animal is considered at least a possible candidate for experiencing pain, it is considerably less stringent then requiring an understanding of how the nervous system generates the qualitative nature of the pain experience itself.

Recently it has been claimed that some species of mollusca can experience pain (Mather, 2008, 2016; Mather and Carere, 2016; Godfrey-Smith, 2017). In the following sections, we briefly describe the molluscan nervous system before critically evaluating evidence purportedly supporting feeling in these creatures. This analysis will reveal that molluscs clearly exhibit non-conscious nocifensive behaviors in response to certain types of noxious stimuli. However, behavioral studies have been found wanting with regards to pain in molluscs. In an attempt to move away from weak inferences about pain based on behavioral studies we instead adopt the necessity test for animal pain based on a neuroanatomical framework containing model prediction networks. After discussing this framework in detail, we conclude that molluscs are incapable of feeling pain since the nervous system of molluscs (unlike humans) lacks the neural architecture required to implement the requisite computations defined within this framework.

MOLLUSC NERVOUS SYSTEM

Mollusca consist of over 74,000 species that inhabit marine, freshwater, and terrestrial environments (Dunn et al., 2014). They have diverse body plans and encompass bivalvia, gastropods, and cephalopods which include animals such as clams, mussels, snails, squid, and octopi. The most basal lineages in this group possess two bilateral symmetrical longitudinal nerve cords embedded in a plexus of neural cell bodies, that coalesce and form a ring in the head (Faller et al., 2012). This nerve ring is referred to as the brain and contains an uncompartmentalized neuropil surrounded by neuronal perikarya. In more differentiated nervous systems, neurons cluster and form distinct ganglia interconnected by nerve fibers called connectives. Cephalopods (nautilus, cuttlefish,

squid, and octopus) have the most morphologically complex nervous system in mollusca (Budelmann, 1995) and also display remarkably sophisticated behavioral repertoires and cognition (Zarrella et al., 2015; Mather and Dickel, 2017). Given these properties and recent claims that cephalopods are the best candidates for invertebrate consciousness (Mather, 2008; Mather and Carere, 2016), we have restricted our discussion regarding sensory awareness to this group and only to those few (of ∼700) species (Kröger et al., 2011) that have been experimentally investigated.

The octopus nervous system is partitioned into three principal regions: the brain (40 million neurons within a cartilaginous capsule); optic lobes beneath the eyes (130 million neurons) and the associated small peduncle and olfactory lobes; and the peripheral ganglia of the arms (350 million neurons) (Young, 1963). The arm nervous system consists of both sensory and motor neurons controlling simple movements that contribute to goal directed behaviors even when the arm is severed from the body (Sumbre et al., 2001). The brain consists of two principal regions: a supraesophageal complex of lobes (enlarged ganglia), which lie above the esophagus, and a subesophageal complex, which lies below the esophagus (**Figure 1**). Together these regions consist of about 25 major lobes with each comprising an outer layer of neuronal cell bodies and an inner neuropil. The axon connectives between these lobes are short and contribute to making the lobes appear fused as a single large mass.

The cephalopod nervous system, like that of vertebrates, is hierarchically organized into levels that sequentially control behaviors (Boycott, 1961; Young, 1976, 1988; Sumbre et al., 2001; Zullo et al., 2009; Zullo and Hochner, 2011; Kobayashi et al., 2013). Sensory and memory brain centers such as the optic lobes for vision, the inferior frontal lobes for tactile discrimination and the vertical and median superior frontal lobes for memory and learning are important in regulating elaborate behaviors such as camouflage patterning, navigation, attack and evasive planning (Mather and Dickel, 2017). Each of these brain centers project directly or indirectly to the higher motor centers located in the basal and peduncle lobes (Ba, **Figure 1**). The higher-order centers coordinate complex motor action like swimming and walking. Following ablation of the higher-order motor centers octopi are no longer able to perform spontaneous movements. The higher motor centers project to and regulate the intermediate and lower motor centers (in the subesophageal region). The intermediate motor centers coordinate simple movements such as arms working in synchrony. The intermediate motor centers control lower motor centers (which are motor neuron clusters present in both the subesophageal region and arms). The lower motor centers regulate select muscle groups such as those involved in eye and single arm movements.

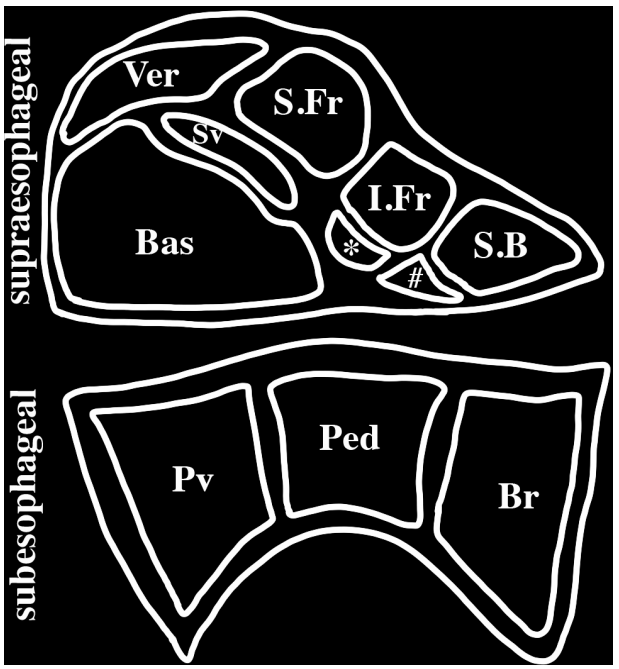

FIGURE 1 | Schematic representation of a mid-sagittal section of an octopus brain. The brain is encased in a cartilaginous capsule and consists of two principal regions: the supraesophageal region lying above the esophagus and the subesophageal region lying below the esophagus. The principal lobes of the midline supraesophageal region consists of the vertical (V), subvertical (Sv), superior frontal (S.Fr), inferior frontal (I.frontal), superior buccal (S.B), posterior buccal (#), subfrontal (*) and basal (Bas) lobes. The inferior frontal consists of a median lobe (shown here) and a lateral lobe (not shown here) that is located lateral to the midline. The subesophageal brain is partitioned into three lobes: brachial (Br) which gives rise to the brachial nerves to the arms, pedal (Ped) and palliovisceral (Pv) lobes.

BRAIN REGIONS RESPONSIBLE FOR BEHAVIORAL RESPONSES TO NOXIOUS STIMULI IN MOLLUSCA

Octopus blinded by sectioning of the optic nerves can use tactile information arising from a single arm to discriminate between two texturally distinct objects (Wells, 1964). This tactile discrimination is achieved through a reward and punishment training regime involving a "positive" object whose selection is rewarded by food (i.e., sardine), and a "negative" object, whose selection is punished with a mild electric shock. This shock also elicits an escape response involving the animal swimming away to another place in the aquarium (Wells, 1959b). Within a few trials, the negative object is pushed away or rejected while the positive object is accepted and passed toward the mouth. Such learning behavior is commonly interpreted as evidence that the animal consciously feels pain following electric shock (Andrews et al., 2013). If this premise is true, then it should be possible to localize the site of pain in the octopus brain by assessing the effects of specific brain lobe ablations on the performance of tactile discrimination during operant conditioning.

Removal of the entire supraesophageal brain completely destroys the ability of octopus to learn to reject objects associated with electric shock (Wells, 1959a). When presented with negative objects these brain-ablated individuals repetitively accept them despite the shock punishment. This result seems consistent with the idea that pain is generated in this part of the brain. If the inferior frontal system (posterior buccal, inferior frontal, and

subfrontal lobes) are selectively spared from the surgical ablation of the supraesophageal brain, animals regain their ability to learn to discriminate. Accordingly "pain" must be arising somewhere in this brain region.

However, the gross motor behavior of these brain-ablated animals is severely compromised. Animals can no longer walk or swim and instead sit on the bottom of the holding tank with arms in disarray (Wells, 1959a). By instead selectively removing only the inferior frontal system, while leaving the rest of the supraesophageal brain intact, animals display normal gross motor behaviors (Wells, 1978). In the absence of these lobes animals do not reject negative objects. Although these confirmatory results support the idea that "pain" arises in this very specific region of the supraesophageal brain, these animals now strongly react to electric shock. After receiving an electric shock for failing to reject the negative object, an animal lacking the inferior frontal system rapidly swims away while dragging the tightly grasped object in its arm (Wells, 1961). This escape behavior demonstrates that the animal is capable of responding to an electric shock (supposedly it can still feel "pain") and yet it doesn't release the object. These results expose a dissociation between learning and any so-called "pain" felt by the animal. Thus "pain" is not the driver for octopus learning to respond to negative objects. Avoidance learning is therefore not evidence of pain. Rather, these results are consistent with the inferior frontal system directly regulating arm motor behaviors. When present, the inferior frontal system activates a reject motor program (or inhibits an accept motor program) in response to a noxious stimulus. When ablated, the reject response is not activated (or the accept program is inhibited) and a default accept program dominates.

It could be argued that "pain" was generated in the brachial lobe of the subesophageal brain (an arm motor center) and then relayed to the inferior frontal system where it regulated learning. Thus, when only the inferior frontal system was spared ablation in the supraesophageal brain, "pain" could still drive learning since it arose from the lower subesophageal brain. However, while afferent sensory fibers arising from the arms project via the brachial nerves and innervate the brachial lobe of the subesophageal brain, they terminate on motoneurons (Young, 1978). No second-order sensory fibers subsequently project from the brachial lobe to the inferior frontal system. Rather, sensory afferents from the arm enter the cerebrobrachial connective and terminate directly in the inferior frontal system (Budelmann and Young, 1985). Thus "pain" is not generated in the subesophageal brain and is not relayed to the inferior frontal system.

To continue to accept that an octopus feels pain during operant conditioning it is necessary to suppose that this pain must be multiply realized throughout the brain. Multiple realization is the hypothesis that a mental state (e.g., pain) can occur in many different organisms with vastly different neural morphologies (such as humans and molluscs) (Kim, 1992). Here, we apply the term to include the possibility that pain would also need to arise in many different independent regions within the same nervous system in order to account for the ablation data in molluscs. To adhere to the idea that molluscs feel pain one needs to propose that pain is generated locally in the inferior frontal system and also in all other brain regions associated with behaviors elicited by noxious stimuli. Pain associated with an escape swim response must be generated outside of the inferior frontal system since this escape behavior continues in the absence of this system. Likewise pain from electric shocks used during operant conditioning involving visual stimuli must also be generated elsewhere since this learning occurs in the absence of the inferior frontal system (Wells, 1961). Since this visual learning is dependent on the superior frontal and vertical lobes (Boycott and Young, 1955) pain must also be generated locally within these lobes. However, multiple realization of pain in the inferior frontal system and the vertical-superior frontal lobes is unlikely given that the known circuitry in these regions specifically supports neural processing associated with learning and memory formation (**Figure 2**) (Young, 1991; Shomrat et al., 2015). These brain regions have wiring patterns that share strong structural and functional similarities with the human hippocampus (Young, 1991; Shomrat et al., 2015). While the hippocampus is important for learning and memory involving pain in humans, it is not involved in the neural computations proposed to underlie the sensation of pain (Bastuji et al., 2016; Tseng et al., 2017; Garcia-Larrea and Bastuji, 2018). This neural processing underlying pain in humans will be examined in more detail below.

This discussion started with the premise that octopus feels pain. We have now shown that this assumption creates some conceptual difficulties and leads to the conclusion that discrete isolated regions of the brain such as the inferior frontal system generate pain. Interestingly, the median inferior frontal lobe within the inferior frontal system is not essential for tactile learning during operant conditioning (Wells, 1959a; Wells and Young, 1975; Wells, 1978). While selective removal of the subfrontal lobe leads to markedly reduced tactile discrimination, it is still possible to produce some learning using extended reward and punishment training (Wells and Young, 1975; Wells, 1978). This residual learning in response to electric shock punishment can now only be achieved by circuitry in the remaining undamaged lateral inferior frontal lobes within the inferior frontal system. The simple circuitry in this lobe is similar in principal to that underlying classical conditioning of gill and siphon withdrawal reflexes in Aplysia in response to electric shock (Kupfermann et al., 1974; Carew et al., 1983; Benjamin and Kemenes, 2008; Hochner and Glanzman, 2016). As noted above it does not resemble the global, integrative neural network matrices considered to generate pain in vertebrates (Garcia-Larrea and Peyron, 2013; Garcia-Larrea and Bastuji, 2018). Rather than pain driving tactile discrimination learning in octopus, the data are more consistent with noxious sensory information autonomously regulating local neural circuits at multiple hierarchical levels in the octopus nervous system. This conclusion is further supported by the isolated arm experiments described below.

Altman (1971) revealed that isolated amputated arms of octopus are able to either accept a sardine or reject a sardine soaked in noxious quinine hydrochloride. Thus, accept and reject responses are reflex behaviors generated by local sensory and motor circuitry in the arm and are not contingent upon the animal consciously feeling pain. However, Altman (1971) showed that different levels of the brain exhibited hierarchical control

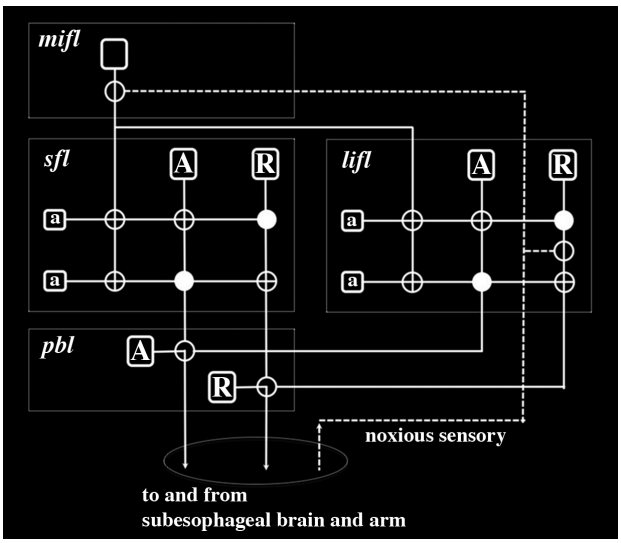

FIGURE 2 | Wiring diagram for the inferior frontal system controlling tactile discrimination behaviors in Octopus as proposed by Young (1983, 1991). This representation neglects positive sensory input associated with acceptance behaviors and instead depicts input from noxious sensory fibers from the arms that enter the inferior frontal lobes from the cerebro-brachial connective (Budelmann and Young, 1985). The stylistic circuit is drawn to highlight similarities in neural connectivity as previously described for learning and memory circuits of the superior frontal and vertical lobes (Hochner and Shomrat, 2013; Shomrat et al., 2015). Large projection neurons are depicted as unfilled rounded rectangles and in the subfrontal lobe (*sfl*), lateral inferior frontal lobe (*lifl*) and posterior buccal lobe (pbl) there are two principal types – acceptance (A) and rejection (R) – which selectively project to one another. The projection neurons associated with tactile discrimination in the median inferior frontal lobe (*mifl*) project to both the *sfl* and *lifl* where they terminate on small amacrine interneurons (a). Although this projection neuron is represented as having axon branches this is simply diagrammatic and it is most likely that there are multiple types of these neurons that have different innervation densities in the *sfl* and *lifl*. Synapses are represented as filled circles (inhibitory) or unfilled circles (excitatory). Noxious sensory fibers selectively innervate projection neurons in the *lifl* and *mifl*. Electric shock would be expected to activate R projection neurons in the *lifl* and depending on their state of activation by local amacrine interneurons, these R neurons would activate R projection neurons in the *pbl* and lead to a reject behavior. Noxious sensory fibers projecting to the *mifl* would cause a cascade of modulatory activity in the *sfl* and *lifl* that would robustly regulate motor behavior. With tactile discrimination training (using either or both positive and negative reinforcements) this circuitry outlined here would enable behavioral learning and memory formation that could readily increase efficacy of motor actions.

of the reject reflex. When only the supraesophageal brain was removed (leaving only the subesophageal brain intact), animals could no longer reject objects. The reflex was regained when the inferior frontal system was spared from this ablation. This result revealed that the subesophageal brain inhibited the reflex while the inferior frontal system facilitated this reflex. Recent isolated octopus arm experiments have further demonstrated a classic withdrawal reflex response of arms to either pinches or noxious chemical applied to the tip of the arm (Hague et al., 2013). The reject arm reflex in octopus has some similarity to the spinal control of leg withdrawal reflexes of humans in response to peripheral noxious stimuli (Hagbarth, 1960). Taken together, the brain lesion experiments and the isolated arm preparations reveal that nocifensive behavior in response to noxious stimuli are stereotyped movements executed principally by local arm circuitry but regulated hierarchically in the brain and as such provide no evidence that octopi feel pain.

Arm injury in some octopi causes arm autotomy distal to the injury site. Following autotomy, animals initially display wound grooming followed by guarding behavior where the injured limb is shielded by other arms (Alupay et al., 2014). This behavior is accompanied by reduced local mechanosensory threshold for arm withdrawal and escape responses. Removal of all the supraesophageal brain except for some optic lobe stalk and partial basal lobes (containing the higher motor centers) did not abolish the grooming and guarding behaviors. These behaviors were only lost with complete supraesophageal brain removal, which is consistent with the known role of basal lobes in controlling general body movement (Wells, 1959a). These results further demonstrate the autonomous nature of the behavioral responses to short term noxious stimuli and chronic injury. These nocifensive behaviors do not provide any evidence that octopi feel pain and that pain is driving these motor actions (Crook and Walters, 2011; Crook et al., 2013; Butler-Struben et al., 2018).

Such behaviors are instead adequately accounted for by non-conscious, feedforward neural circuits executing hierarchically controlled motor actions (Hochner and Shomrat, 2013; Shomrat et al., 2015; Hochner and Glanzman, 2016; Levy and Hochner, 2017). Numerous studies indicate that goal-directed movements are predominantly under control of autonomous motor programs in the peripheral nervous system and that the central brain is involved in activating these programs (Sumbre et al., 2001, 2006; Levy et al., 2015; Levy and Hochner, 2017). The ability of complex behaviors to be executed using non-conscious hierarchical control systems in octopus has been convincingly demonstrated by progressively increasing external microstimulation of the basal motor lobes (Zullo et al., 2009). A variety of elementary motor actions can be recruited in various combinations leading to the production of complex behavioral responses as a result of simply increasing electrical stimulation to these lobes.

BEHAVIOR IS NOT SUFFICIENT TO INFER CONSCIOUS AWARENESS

Despite known difficulties in inferring sentience from behavior, stimulus-response paradigms continue to be widely used in animal studies to assess the presence of feelings such as pain (King and Porreca, 2014; Barrett, 2015). This is particularly problematic in molluscs when the anatomy and physiology are so divergent from mammals (Crook and Walters, 2011). A clear distinction needs to be drawn between nociception and pain (Crook and Walters, 2011) and importantly, nociception in molluscs should not be confused with evidence for pain-like states (Crook et al., 2013). Similar arguments have been countenanced for insects (Adamo, 2016a,b).

While many animal studies still rely on non-conscious action responses, others have embraced an idea that complex

behavior involving goal pursuit is a better indicator of conscious awareness. However, the obligatory association between goal pursuit and conscious processing is challenged even in humans (Custers and Aarts, 2010). Many complex and goal-oriented behaviors, such as the *Drosophila* male courtship ritual, can be deconstructed into a series of innately driven and genetically determined stereotyped subroutines (Manoli et al., 2006). Awareness of a goal or the presence of feelings clearly plays no role in the courtship ritual, since this complex behavior can be performed by headless flies (Pan et al., 2011). There is no evidence that complex learning in insects involves sentience (Giurfa, 2013; Chittka, 2017). There is no need to assume conscious awareness in either insects or molluscs in order to explain complex behaviors when non-conscious neural networks can effectively account for such abilities (Ardin et al., 2016; Faghihi et al., 2017; Goldschmidt et al., 2017; Müller et al., 2017; Peng and Chittka, 2017; Perry et al., 2017; Roper et al., 2017).

Given the specious relationship between complex behaviors and conscious awareness, there is some support for the idea that "flexible behavior" (i.e., the ability of an animal to adapt its behavior in response to changing environments or novel challenges; Griffin, 1976) is a better indicator of conscious awareness (Bekoff, 2003; Edelman and Seth, 2009; Seth, 2009; Droege and Braithwaite, 2014; Mather and Carere, 2016). However, conflating flexible behavior with feelings remains problematic, since even innate, stereotyped behaviors are known to exhibit considerable plasticity. For instance, spinal central pattern generators (CPGs) controlling limb movements during vertebrate locomotion (Frigon, 2017) can easily adapt to changing environments to allow an animal to locomote in both water (swimming) and on land (stepping) using vastly different gait kinematics (Ryczko et al., 2015). The non-conscious nature of this flexible motor behavior is supported by evidence that distinct, behavior-specific CPG outputs can be achieved even in the isolated vertebrate spinal cord. The autonomous decentralized and flexible nature of the CPG is exemplified in the millipede, which is able to regulate kinematics of each leg in response to local environmental cues (Kano et al., 2017).

It has been suggested that some animals (e.g., fish) are sentient because they appear to display declarative memory, conditioned place preference, trace conditioning and transitive inference. However, none of these behaviors necessarily rely on subjective awareness (Reber et al., 2012; Mudrik et al., 2014); and so embracing these criteria will lead to erroneous inferences concerning sentience (Key, 2015, 2016). In summary, relying on behavior alone is not sufficient to justify claims of conscious awareness in an animal.

Despite inherent problems with using behavior as a yardstick for consciousness it has been argued that cephalopods possess a simple form of consciousness referred to as "primary consciousness" (Mather, 2008). Mather (2008) seems to associate this type of consciousness with the ability of some cephalopods to display complex behaviors, to learn and to learn using simple concepts. While we have already dismissed complex and flexible behaviors as a measure of feeling, Mather's adoption of learning and use of simple concepts as a measure of primary consciousness is mistaken given that such behaviors could be either implicit or explicit (Schacter, 1992), and only the latter could be argued to depend upon the availability of concepts. In many instances, anthropomorphic claims are used to defend conscious awareness in cephalopods. For instance, Mather (2016) claims that octopi adopt "cautious" approaches to stinging sea anemones and even blow jets of water at the anemone and hence do not just respond reflexively to noxious stimuli. These anthropomorphic descriptions based on anecdotal observations need to be critically assessed within the context of innate behaviors and implicit learning (LeDoux and Daw, 2018).

Mather (2008) suggests that play behavior exhibited by octopi is consistent with these animals having consciousness. Mather defines behavior as play-like if any of the following actions were performed with novel plastic objects: pushing or pulling of the object in one coherent action; dragging an object by an arm across the surface of the water in more than one direction; or passing the object between the arms more than six times (Kuba et al., 2006). Using these criteria 9/14 octopi in her study were reported to engage in play-like behavior. While no evidence is provided that such behavior actually represents any form of play there is the underlying assumption that it involves conscious awareness of "fun" since it is labeled as "play-like." However, recent optogenetic experiments in mice have revealed that craving, selective attention and so called play-like activity toward novel objects is automatically induced by simply activating a single neural pathway between the medial preoptic area and the ventral periaqueductal gray area (Park et al., 2018). The take-home lesson here is that causes of behavior may not be extrapolated from observation of the behavior alone and that describing animal behavior (e.g., as play-like) based on anthropometric measures is question-begging.

Bronfman et al. (2016) have proposed that animals capable of specific types of associative learning (referred to as "unlimited associative learning") must be sentient. Unlimited associative learning is considered to involve complex behaviors rather than simpler forms of associative learning. This hypothesis is again built on the false premise that complex behavior is dependent on sentience. For instance, Bronfman et al. (2016) consider that an animal can feel if it is capable of learning to associate an object by a combination of its properties (e.g., color, shape, and texture) with the future presentation of food, whereas each property alone is not sufficient for eliciting a behavioral response. Unfortunately no evidence is provided that this form of associative learning necessarily involves sentience. Bronfman et al. (2016) refer to compound operant conditioning in octopus (as in Hochner and Shomrat, 2013) as evidence of sentience.

Hochner and Shomrat (2013) showed that octopus could be trained not to attack a red ball (containing the integrated properties of brightness and shape) by negative reinforcement with electric shocks. According to Bronfman et al. (2016) this learning was evidence of unlimited associative learning since the animals continued to approach balls (same shape) that were white instead of red. However, Shomrat et al. (2008) describe the neural circuitry underlying this associative learning and conclude that "our results fit a simple feed-forward model of octopus avoidance-learning systems." There is no evidence, in short, that such behavior demands sentience (LeDoux and Daw, 2018). Of

interest is recent research demonstrating that non-conscious (i.e., subliminal) sensory stimuli such as novel pairs of visual and spoken presentations of words can mediate complex associative learning in humans (Scott et al., 2018). This builds on earlier research demonstrating that awareness of conditioning stimuli is not needed for instrumental conditioning in humans (Pessiglione et al., 2008). Such findings argue against the necessity of sentience for unlimited associative learning in cephalopods.

A WAY FORWARD IN ADDRESSING CONSCIOUS AWARENESS

A foundational principle of evolutionary biology is that structure determines function. Call this the "SDF principle." According to the SDF principle, the morphology of any biological tissue is the key to its physiological function. The structure of a nervous system imposes fundamental limitations of what it can and cannot do. For instance, the ability of an animal to perform non-gliding flight is determined by the structure (i.e., anatomy) of the animal's wing or wing-like appendage. While the shape and form of these appendages varies considerably across winged species, there is a common design plan that enables the necessary aerodynamic force of lift to be generated (Lindhe Norberg, 2002). The anatomy of a wing explains how it can be used for flight. Consequently, any animal lacking the common design feature of the wing will lack the potential to perform non-gliding flight. Why suppose that the SDF principle does not also apply in explaining the capacity for feeling, that there is not some common design or structural features that explain the capacity for feeling across different species? It is this question that frames our current approach to the design of a nervous system that is capable of conscious awareness.

Once the properties of neural tissue deemed both necessary and sufficient for feeling sensory stimuli have been identified, then the assessment of whether any particular animal is likely or has the potential to feel or not can be reduced to the identification of those relevant properties in the animal's nervous system. Given that there does not appear to be any solution to this problem in the near future, one way forward is to define the basic underlying design principles and use this knowledge to create a minimal neural architecture necessary (but not sufficient) to support pain. Two important questions provide a framework for addressing this problem. First, what sorts of algorithms need to be executed by a nervous system to generate pain? Second, how are those algorithms implemented in a nervous system? An answer(s) to the latter question would begin to expose some of the necessary neural architectural prerequisites for pain.

An argument against trying to identify the necessary neural architectures is that the solution to the algorithms may be multiply realized in different animals (Weiskopf, 2011). That is, different neural circuits may be able to implement the algorithms. This is not reason enough to disregard this approach since all that is needed is to identify all possible circuits in extant creatures. Given that this is likely to prove a formidable task, a better approach would be to define instead the generic architecture that enables multiple realization to be captured since multiple realization does not necessarily apply to basic computations (Keeley, 2000).

As proof of principle, we have tested this strategy by characterizing the necessary circuitry underlying rhythmic motor movements during locomotion of bilaterally symmetrical animals. The basic algorithm generating left-right rhythmical motor activity is an alternating left-right rhythmical muscle activity occurring at the same segmental or anterioposterior level. That is, there is sequential contraction and relaxation of the same muscles on the left and right sides of the body, respectively. If these muscles fail to exhibit this cyclic activity, then the animal no longer engages in left-right phased rhythmical locomotion. In order for left-right phase activity to be rhythmical, left muscles must be activated while those controlling the same muscles on the right must be simultaneously inhibited.

Given such an algorithm, what then is the circuitry that implements it? Reciprocal inhibition (i.e., activated neurons on one side cause muscle contraction and also inhibit the same muscles on the opposite side) is an essential component of left–right rhythmical locomotion since independent pacemakers on either side spontaneously drift in and out of phase (Friesen, 1995). While the specific interconnectivity of neurons (i.e., microcircuitry) that leads to reciprocal inhibition can be multiply realized between different species, all species possess neurons that project across the midline to reciprocally inhibit the other side so that left muscles are activated while right muscles are simultaneously inhibited. This crossed inhibitory circuitry involves activation and inhibition of excitatory motor neurons in almost all animal models, including: leeches, fish, and mammals. To date, only nematodes achieve simultaneous contraction and inhibition of muscles using a combination of both excitatory and inhibitory motor neurons (Wen et al., 2012). A left excitatory motor neuron activates right muscles and simultaneously excites a right inhibitory motor neuron that causes right muscles to relax. This crossed excitation of inhibitory motor neurons produces the alternating rhythmical muscle activity. Thus, by knowing whether an animal possesses the necessary neural architecture required to perform cyclic inhibition of left-right muscle activity (i.e., crossed connections that lead to simultaneous contraction and inhibition of the same muscles on left and right sides), it is possible to predict whether an animal is, at least, capable of performing locomotion based on left-right rhythmical contractions. If an animal lacks this fundamental neural architecture, then one can confidently conclude that it cannot perform this type of locomotion.

While we have concentrated on CPGs, there are numerous examples of conserved circuitry that subserve similar functions both within and across phyla (Loesel et al., 2013; Farris, 2015). For instance, basic circuitry for associative learning is conserved in the vertical lobe in octopi, mushroom bodies in insects and hippocampus in vertebrates (Katz, 2016). Olfactory glomerular-like structures are also involved in processing of olfactory sensory information in molluscs, insects and vertebrates (Strausfeld and Hildebrand, 1999; Eisthen, 2002; Farris, 2015). Likewise, the loss of either olfactory or visual neural circuitries within some species in a phyla correlates with the absence of behavioral responses to these sensory

stimuli (Ramm and Scholtz, 2017). Interestingly, Scaros et al. (2018) have recently failed to morphologically identify olfactory glomeruli in embryonic and hatchling stages of development in the cuttlefish *Sepia officinalis*. However, olfactory glomeruli are not morphologically defined in developing late embryonic mice (Royal and Key, 1999). At this stage of development visualization of glomeruli formation relies on the expression of odorant receptor genes (Royal and Key, 1999). Similarly, olfactory glomeruli emerge slowly in developing *Xenopus* and never achieve the morphological definition of those in mammals (Byrd and Burd, 1991). Olfactory glomeruli are also not easily discernible by immunohistochemical staining in the adult frog *Rana catesbeiana* (Key and Akeson, 1990) and most glomeruli in adult zebrafish are anatomically indistinguishable (Braubach et al., 2012). The question as to whether *Sepia officinalis* possesses glomeruli and its implications for olfaction must await further more detailed investigation.

A NEURAL ARCHITECTURE NECESSARY FOR FEELINGS

We hypothesize that one of the fundamental organizational principles of feeling nervous systems is that they must be able to internally monitor their own neural processing (i.e., internal states). Such internal monitoring is critical for any system to achieve a level of awareness of its own processes and to use that awareness to execute functions (Kwon and Choe, 2008; Jeremy, 2014). Air conditioning systems in buildings must monitor the internal temperatures of rooms in order to adjust air flow accordingly. Similarly, nervous systems must possess specialized neural circuitry to monitor their internal sensory processing of noxious stimuli in order to become aware and feel pain. We contend that there are at least three hierarchical levels of a system that are diagnostic for assessing whether that system has the potential to be aware. First, there must be a change in the internal state of the system ("internal state" in **Figure 3**) caused by the stimulus. This internal state is equivalent to the sensory processing pathways leading to some output (e.g., behavior). Second, the system needs to be able to monitor for changes in those internal states ("state observer" in **Figure 3**). Internal monitoring has a long history in consciousness studies (Lycan, 1995). Third, the system needs to become aware of those internal state changes ("system awareness" in **Figure 3**).

The role of awareness in consciousness and its independence from report and self-reflection is well debated in the literature (Farrell and McClelland, 2017). One of the design constraints of this framework is that the state observer and state awareness subsystems need to be external to (i.e., independent of) the sensory processing pathways (internal state) so that their processes do not mutually corrupt each other and to ensure that the prediction (i.e., awareness) is available for the function of the whole system (Cleeremans, 2011; Dehaene et al., 2017).

How could such an algorithm be implemented in an animal nervous system? We propose that when a system can predict the outcome of its current internal processes, then it must be capable of having a level of awareness of its internal state.

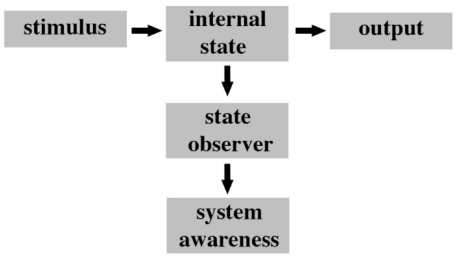

FIGURE 3 | Fundamental organization of a system with awareness potency. The ground state of the system represents a stimulus response module whereby a stimulus (i.e., input) produces a change in the internal state of the system and this in turn leads to a specific output. In order for the system to begin to be aware of what it is doing there must be some independent process of observing changes in its own internal state. This process is performed by a state observer module that detects changes in the internal state that are responsible for the output. The state observer has a low level of awareness (e.g., a temperature gauge in a car engine is a state observer). An additional system awareness module needs to subsequently monitor changes in the state observer and to interpret this information in context of the overall functioning of the system. This second layer of monitoring represents a higher level of awareness (i.e., awareness of awareness). For instance, in the example of an engine temperature this module would detect rises in temperature and determine their significance to the car in respect to overall performance of the engine.

Such predictions can be generated by an internal model of its processing that makes outcome predictions about ongoing neural activity. Modeling internal states and using the resultant predictions to rapidly adjust ongoing processing has been central to the field of plant engineering since the 1960s (Luenberger, 1971). For instance, the state of internal processes of a coal-fired power station can be indirectly monitored by a "state observer" (e.g., an artificial neural network; Paton, 2008) that continuously receives information about the current input (coal) and output (energy) of the station. The state observer is an "internal model" of the ongoing processing that makes predictions about a future output given a particular input. This prediction is then compared with the subsequent real output of the station and the error margin is used to adjust the processing of the coal and modify the state observer so that it becomes a more accurate predictor of the current processing state of the coal station (**Figure 4**).

The internal model ("internal model 1" in **Figure 4**) is a very efficient and rapid process because it does not need to monitor all the stages of processing in the plant. In plant engineering, human operators oversee the function of the internal model 1 and ensure overall fidelity of the system. In doing so, human operators also make use of information not available to the internal model (e.g., transport delivery systems, end-users, and global financial markets). This same algorithm can be applied to the processing of sensory information in a nervous system. In this case, human operators are replaced by a second level internal model ("internal model 2" in **Figure 4**). This second model and its prediction is equivalent to "system awareness" in **Figure 3**. It uses a copy of the input to a sensory system as well as "global input" (**Figure 4**) from other sensory systems to create its prediction. This second prediction ("prediction 2" in **Figure 4**) is a prediction of the

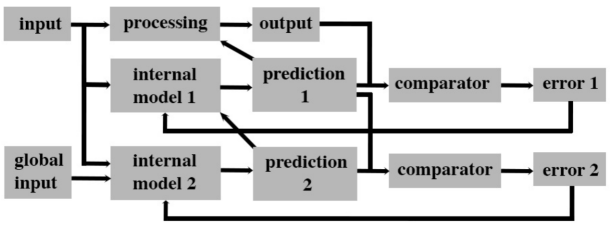

FIGURE 4 | A parallel forward models algorithm that generates levels of awareness within a system. This scheme is based on the concept that a system that can accurately predict its future state based on its current state has awareness that is functionally significant for the system. This form of awareness is postulated to be necessary but not sufficient for subjective experiences such as pain. This algorithm processes input simultaneously along three parallel streams. The base level is the processing module that creates an output for the system. A second level consists of a module that creates a simplified model of the base level processing that predicts (predictions 1) the future output of the system. That prediction is then compared to the actual output in a separate module (comparator) and the error generated is used to adjust the internal model 1 to better predict future states (by attempting to reduce error 1 to zero). A second internal model (internal model 2) uses both the input to the system and available inputs from other systems to predict the outputs of internal model 1. Prediction 2 is feedback into internal model 1 to facilitate a rapid and accurate prediction 1. Prediction 1 likewise feeds back on to base level processing to control its output.

output of the first internal model since it uses a comparator to determine the error ("error 2" in **Figure 4**) between prediction 2 and prediction 1. That error is then used to adjust the internal model 2 so as to reduce error 2. Predictions 1 and 2 both control (directly and indirectly, respectively) the ongoing processing so that the system produces an appropriate output.

Multiple internal models are fundamental building blocks of self-aware computing systems and computational "feelings" in agents (Lewis, 2016; Kounev et al., 2017; Lewis et al., 2017; Sánchez-Escribano, 2018). First-order internal models are restricted to monitoring specific processing events in order to efficiently control their behavior and to reduce overall processing time. Second-order internal models then correct errors in these first-order models arising from the noisy environment, track the consequences of the outcomes of the first-order models, and determine their relevance to and suitability for the whole functioning system. Second-order models enable the system to learn the consequences of its internal processing and to act for the benefit of the whole system. As such, they may be considered as displaying a rudimentary form of subjective awareness (i.e., they can predict the future on the basis of the system's past experience via prediction error feedback). In the proposed algorithm, it is prediction 2's (**Figure 4**) higher-level of awareness through its integration of global and local information that endows it with greater functional significance for the system, a proposed defining feature of conscious awareness (Dehaene et al., 2017). We argue that prediction 2 is a necessary (but not sufficient) condition for feelings. Even though such an "awareness" might become conscious, it is not sufficient to explain why it should feel like something, rather than nothing. As noted above, the aim here is not to provide a reductive analysis of what feeling is, but only to establish the legitimacy of demarcating boundaries between species of animals that are candidates for attributions of feeling and those that are not.

RELATIONSHIP TO HIGH-ORDER THEORIES OF AWARENESS

Our algorithm (**Figure 4**) is distinct from Rosenthal's higher-order theory of awareness (Rosenthal, 1993) in that we recognize the existence of multiple levels of awareness without asserting that awareness becomes immediately conscious through a higher-order representations of awareness. At present, our proposed algorithm is neutral on the necessity of higher-order thoughts. Its explanatory power can, however, be extended by exploring contributions made by other types of neural processing. Internal models are just one type of state observer (**Figure 3**). Working memory can temporarily store a copy of a sensory stimulus that can be compared subsequently with new incoming sensory information to assess changes in neural states. Such assessments are a form of internal monitoring and hence represent a level of awareness of internal state changes. Although we plan to examine how frameworks involving working memory could explain feeling states in future, this is a controversial topic given the importance of the prefrontal cortex to working memory and suggestions that this cortical region is not necessary for consciousness (Boly et al., 2017). How other types of memory (short-term, long term and associative), attention, ensemble coding, saliency, and executive control networks interact with and might further strengthen the framework also need to be explored.

RELATIONSHIP TO HIERARCHICAL PREDICTIVE CODING

We refer to our proposed framework as a parallel forward models algorithm (**Figure 4**). By definition forward internal models use inputs (e.g., sensory data) to predict outputs (i.e., motor behaviors). In our framework, the forward models run in parallel whereas in hierarchical predictive coding the internal generative models run in series and are interconnected by feedforward and feedback connections (Rao and Ballard, 1999). The feedforward ascending connections constitute an inverse model (i.e., using outputs to predict inputs) (Harth et al., 1987; Kawato et al., 1993; Friston, 2005). The difference between the output of a higher-level model and its input from a lower level model creates an error signal that is then used to modify the next input (which approximates using outputs to predict inputs). In contrast, the feedback descending connections represent a forward model (i.e., inputs from higher levels are used to generate output predictions of what caused the lower level inputs). Consequently, in hierarchical predictive coding, top-down predictions modulate bottom up processing. In our algorithm, bottom-up predictions lie outside of the causal chain of processing and hence are able to contribute to an inner (implicit) sense of awareness of what is being processing.

Forward models as used in our algorithm have been empirically tested and confirmed in movement performance by robots (Tani, 2016) and *in silico* models of artificial self-awareness (i.e., gambling; Cleeremans, 2011). These forward models differ from those used in hierarchical predictive coding frameworks to explain visual recognition (Rao and Ballard, 1999; Friston, 2005). These latter models rely on top-down inputs in a linear hierarchy to infer what is currently being processed whereas the models in our algorithm use bottom-up inputs that uniquely feed into parallel models. While the hierarchical top-down models can explain visual recognition and categorization (Friston and Kiebel, 2009), these models are an integral component of the processing pipeline and do not act as external state observers (**Figure 3**) of the type needed for the system to develop a sense of awareness of its neural processing states (Cleeremans, 2011; Dehaene et al., 2017).

Seth (2013) uses a hierarchy of top-down-driven forward models to explain emotional responses with the ultimate driver being higher-order goals. While his proposed framework is specifically aimed at accounting for motor responses he postulates that a conscious emotion (i.e., emotional awareness) arises from the integration of sensory predictions across multiple levels. Unfortunately this idea is not further interrogated in later explorations of the role of predictive coding and active interoceptive inference in emotions (Seth and Friston, 2016). Nonetheless, higher level integration (lying outside of the causal chain of emotional responses) is consistent with our idea that integration of predictions with other pertinent system inputs forms the basis of a higher-level of awareness necessary, but not sufficient, for the feeling of pain.

Like Seth (2013), Barrett (2017) also believes that hierarchical predictive coding uses internal, generative models to anticipate and make inferences about ongoing sensory stimuli and, hence, drive motor and visceromotor actions. In the theory of constructed emotion, Barrett (2017) proposes that when predictive coding is used to meaningfully categorize or conceptualize sensations (e.g., as happiness), then one consciously experiences that sensation (i.e., as happiness). For Barrett (2017) an affective conscious state somehow emerges when predictions are given conceptual meaning. Barrett (2017) does not make it clear, however, why conceptual meaning should feel like something rather than nothing. One could imagine that predictions could lead to inferences about a particular mental state, but there remains an explanatory gap with respects to how that state could possibly feel like something.

LOCALIZATION OF PAIN AWARENESS IN THE HUMAN BRAIN

Our proposed algorithm involves at least three hierarchical levels (rather than two as proposed by the higher-order thought theory; cf. Rosenthal, 1993). The first level has the external stimulus as the object of intent (referred to as "sensory processing"). The second level has "sensory processing" as the object of intent (referred to as "sensory awareness"). This level is proposed to recognize that a particular type of sensory information is being processed. The third level has "sensory awareness" as its object of intent (referred to as "inner awareness"). By its ability to recognize that it is aware of some sensory stimulus this level has created an inner awareness of its internal processing.

There is some disagreement with respect to the cortical localization of conscious awareness in the human brain. First-order theorists contend that awareness directly arises in the earliest stages of cortical processing of sensory input (Dretske, 1993). Higher-order theorists instead subscribe to the idea that conscious experience only occurs when a higher-order of neural processing becomes aware of first-order sensory processing (Rosenthal, 1993). There is little neuroanatomical and neurophysiological support for conscious experience arising directly from first-order sensory processing. For instance, in the visual system, conscious awareness of color is dependent on processing occurring in V4, a higher-order cortical region (Gegenfurtner, 2003). Lesions in this cortical region lead to achromatopsia. Moreover, visual awareness is still present following direct stimulation of higher visual cortices in cortically blind subjects (due to lesions in their first-order V1 cortex) (Mazzi et al., 2014; Bagattini et al., 2015). Feedback from cortical areas higher than V1 is considered essential for visual awareness in normal sighted individuals (Lamme and Roelfsema, 2000; Pascual-Leone and Walsh, 2001; Hurme et al., 2017). However, the role of feedback from higher visual cortices remains unclear and continues to be investigated (Klink et al., 2017). It is important to note that these higher-order visual cortices might be necessary but not sufficient for visual awareness. Higher cortical non-visual posterior areas are also likely to be necessary for visual awareness (Koch et al., 2016).

Although there are problems with trying to correlate subjective experience of visual stimuli with evoked cortical potentials (Aru et al., 2012; Koch et al., 2016), it remains a valuable approach for providing some mechanistic insights into the localization of awareness (Koch et al., 2016). Visual awareness consistently correlates with a negative potential with onset at ∼200 ms and then a positive potential arising at ∼300 ms after stimulus presentation (Koivisto and Revonsuo, 2010; Rutiku et al., 2016; Rutiku and Bachmann, 2017). There is considerable variability between studies in the reported timing of these potentials but there is agreement that the negative potential (referred to as the visual awareness negativity) that occurs over the occipital-temporal-posterior parietal cortices is a signature of visual awareness (Koch et al., 2016; Schelonka et al., 2017).

Some of the temporal variability is associated with technical differences between studies. For instance, Schelonka et al. (2017) recorded the largest amplitude of the negative potential between 320 and 380 ms post-stimulus, whereas Rutiku et al. (2016) reported that the mean amplitude occurred at ∼240 ms and Shafto and Pitts (2015) timed the negative potential at ∼260–300 ms. Notwithstanding these differences, the timing of this potential is relatively late and not consistent with awareness arising solely from within the early visual cortex. The role of the occipital and posterior parietal cortices in

visual awareness have also been highlighted by functional magnetic imaging studies during binocular rivalry with no-report paradigms (Frässle et al., 2014). We have avoided the controversial discussion of whether or not the prefrontal cortex is necessary for visual awareness (Safavi et al., 2014; Boly et al., 2017; Odegaard et al., 2017) since it is enough here to conclude that the primary visual cortex is not sufficient for awareness.

Unlike the visual system, there is no primary cortical region for pain (Treede et al., 1999; Mano and Seymour, 2015; Thomas, 2017). Neuroimaging has revealed that a network of first-order and higher-order cortical regions including somatosensory areas I (SI) and II (SII), and insular and cingulate cortices is specifically activated during pain awareness in humans (**Figures 5A–C**; Rolls et al., 2003; Vogt, 2005; Wager et al., 2013; Vogt et al., 2016). This result is consistent with transneuronal pathway tracing experiments that demonstrated the terminations of the spinothalamic tract, the main ascending tract transmitting noxious information to the cortex, principally within the posterior insular, SII and cingulate cortices in monkeys (Dum et al., 2009). Similar pathways have been demonstrated by neuroimaging studies in humans (Brooks and Tracey, 2005; Omori et al., 2013). Human neurophysiological studies have also revealed nociceptive responses within SI, SII, insular and cingulate cortices (Frot et al., 2001, 2013, 2014; Liberati et al., 2017). Lesions and electrical stimulation in these cortical regions as well as strokes involving the spinothalamic tract in the internal capsule have together confirmed a role of this cortical network in human pain awareness (Ballantine et al., 1967; Berthier et al., 1988; Kim, 1992; Cereda et al., 2002; Torta et al., 2013; Boccard et al., 2014, 2017; Hirayama et al., 2014; Russo and Sheth, 2015; Agarwal et al., 2016; Denis et al., 2016; Wang et al., 2017).

Most human neuroimaging studies using functional magnetic resonance imaging are insensitive to dynamic temporal changes in neural activity across the cortical networks (Kucyi and Davis, 2015; Morton et al., 2016) and, hence, do not adequately reflect activity correlating with instances of pain awareness. Intracortical electroencephalograph recordings have instead provided more precise temporal resolution of cortical activation following noxious stimulation (although early studies were limited by the small number of electrodes). SII responses contralateral to the side of noxious heat stimulation to the wrist and hand occur initially at a peak latency of 140 ms (Frot et al., 1999; Frot et al., 2001). These SII responses were specifically associated with stimuli that elicited pain and were not recorded from other sites including hippocampus, amygdala, temporal pole, temporal neocortex, cingulate gyrus, and orbitofrontal cortex (Frot et al., 2001). The insular cortex responds to noxious stimuli ∼40 ms after the SII with an initial peak at 180 ms (Frot and Mauguière, 2003). This analysis was not able to resolve any differences in latency of these potentials along the posterior-anterior axis of the insula. Subsequent analyses revealed that the SII responses were more selective for stimuli that were below pain threshold or only mildly painful, whereas posterior insular cortex responses more fully reflected thermal noxious stimuli clearly above pain threshold (Frot et al., 2006). These studies suggested that the posterior insular cortex and SII play different roles in pain awareness.

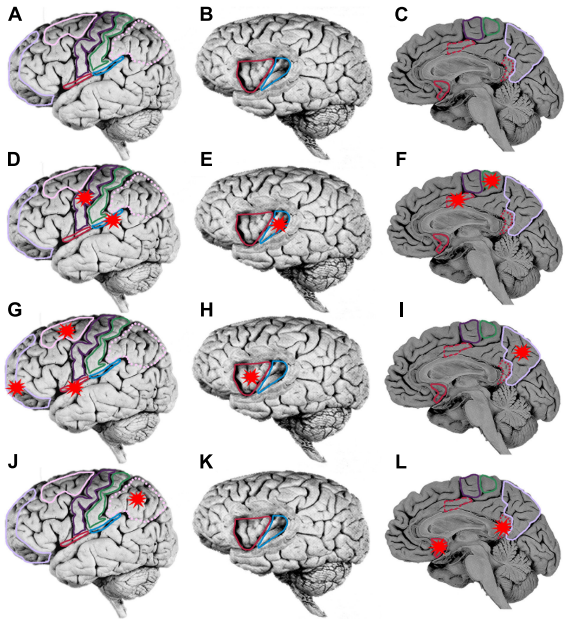

FIGURE 5 | Localization of human brain regions. **(A,D,G,J)** Lateral view of brain, orbitofrontal cortex (mauve), dorsolateral prefrontal cortex (pink), motor cortex (purple), somatosensory cortex (green), posterior parietal cortex (dotted pink), lateral margin of frontal operculum (red), lateral margin of parietal operculum (blue). On a lateral view only the lateral margins of the operculum are exposed. The operculum forms the roof of the lateral sulcus and is only accessible by removal of the temporal lobe (which forms the ventral floor of the lateral sulcus). **(B,E,H,K)** Lateral view of brain with partial frontoparietal lobe removed to reveal underlying gyri of insula, anterior insula (red), posterior insula (green). **(C,F,I,L)** Medial wall of brain, perigenual anterior cingulate cortex (red), posterior mid cingulate cortex (dashed red), ventral posterior cingulate cortex (dotted red), supplementary motor area (purple), somatosensory cortex (green), and precuneus (pink). Each of the three rows starting at **(D,G,J)** represent a temporal sequence of cortical activation (red stars). **(D–F)** After initial activation of the somatosensory area there is co-activation of five cortical areas **(G–I)**. A third wave of co-activation of five cortical area. **(J–L)** A fourth wave of co-activation of three cortical areas.

Intracortical electroencephalograph recordings from multiple sites simultaneously in the cingulate cortex and SII has begun to provide a clearer understanding of the spatiotemporal relationships of neural activity during pain perception (Frot et al., 2008). SII and posterior middle cingulate cortex (pMCC) co-activate initially at around 120–140 ms and this is followed by later activity in the posterior insula at ∼180 ms post-stimulus. In a subsequent study, it was revealed that areas 1 and 2 in SI consistently elicited intracortically recorded neural responses consisting of four components at ∼102, 129, 140, and 190 ms latency to noxious stimuli (Frot et al., 2013). Simultaneous recordings revealed a 126 ms response in the supra-sylvian operculum (which included SII) and a ∼218 ms latency biphasic potential in the insular cortex. The primary motor cortex I (M1) also responded with a distinctive triphasic potential beginning at ∼116 ms post-stimulus. A picture was

emerging whereby noxious stimuli leads to initial processing in the SI, followed by activity in the parietal operculum (containing SII) and pMCC and then slightly later in the insular cortex.

Temporal analysis of processing in the insular cortex elicited by a noxious stimulus revealed that biphasic evoked potentials occurred first in the two posterior insular gyri (at 212–221 ms) and then slightly later in the three anterior insular gyri (237–309 ms) (Frot et al., 2014). Given that the anterior insula only receives a very minor (if any) direct projection from the spinothalamic noxious pathway (Dum et al., 2009), it appears that the shortest latency evoked potentials are serially processed in the insula, passing from the posterior to the anterior insula (Frot et al., 2014). This interpretation is consistent with known direct connections between the posterior and anterior insula (Mesulam and Mufson, 1982; Almashaikhi et al., 2014).

Pain awareness following a noxious laser irradiation of the hand occurs in a broad window of 260–422 ms (mean 349 ms) post-stimulus (Bastuji et al., 2016). This latency was measured as the time from noxious stimulation to a voluntary motor response (finger lift) that signified sensation of pain. However, given that cortical stimulation of the primary motor cortex elicits a wrist motor response within 20 ms (Calancie et al., 1987; Amassian et al., 1989), cortical activity leading up to the experience of pain must occur before a 240–400 ms temporal window (with a mean of 329 ms). Bastuji et al. (2016) found three consistent waves of onset of cortical activity: first, activity (at ∼120 ms) was detected in the posterior insula, parietal operculum (SII), MCC and supplementary motor area; second, activity was followed shortly later (beginning at ∼140 ms) in the frontal operculum, precuneus (part of superior parietal lobe), anterior insula, orbitofrontal cortex and dorsolateral prefrontal cortex; and third, activity beginning at 149 ms in the posterior parietal cortex, ventral posterior cingulate cortex (vPCC) and perigenual anterior cingulate cortex (pACC).

It should be noted that Bastuji et al. (2016) did not distinguish between regions of the MCC although in an earlier study they demonstrated that it was the pMCC that was selectively activated by noxious stimuli (Frot et al., 2008). The highest peak activity in most of these regions was reached by 319 ms, which is prior to the time of awareness (at 329 ms). Only peak activities in vPCC and pACC occurred after pain onset (at ∼350 and 398 ms respectively). Despite differences in electrode placement and noxious stimulation between the various electroencephalographic studies described above, it is apparent that there is a specific temporal pattern of activation of cortical regions leading up to pain. First, there is early activity in SI; second, co-activation of the SII, posterior insular, pMCC, M1 and supplementary motor area (**Figures 5D–F**); third, co-activation of anterior insular, precuneus, frontal operculum, orbitofrontal cortex and dorsolateral prefrontal cortex (**Figures 5G–I**); and finally, activity in the posterior parietal cortex, vPCC and pACC (**Figures 5J–L**). Onset of activity in all of these regions is temporally consistent with them contributing to neural processing leading to pain awareness.

CORTICAL NEURAL CIRCUITS SUPPORTING A PARALLEL FORWARD MODELS ALGORITHM

The proposed parallel forward models algorithm (**Figure 4**) imposes structural restrictions on the types of nervous systems that could implement it. Is the structural connectivity captured by the algorithm neuroanatomically plausible? In the primate somatosensory system, the spinothalamic axon tract delivers sensory input from noxious stimuli into the cerebral cortex where it splits and terminates in three principal regions: cingulate cortex, SII and posterior insular cortex (PIC) (Dum et al., 2009). In humans, a main target in the cingulate cortex regulating motor responses (e.g., facial expressions, Kunz et al., 2011) to noxious stimuli is the pMCC (Perini and Bergstrand, 2013; Vogt et al., 2016). Each of these three regions were shown in electroencephalographic studies (discussed above) to be co-activated via noxious stimuli (**Figures 5D–F**) and by neural pathway tracing experiments to feed forward to the AIC (pMCC to AIC, Mufson and Mesulam, 1982; Mesulam and Mufson, 1982; SII to AIC, Mufson and Mesulam, 1982; Mesulam and Mufson, 1982; PIC to AIC, Almashaikhi et al., 2014). Both SII and PIC receive strong, reciprocal feedback from AIC (Mesulam and Mufson, 1982; Morecraft et al., 2015). PIC projects to SII (Mesulam and Mufson, 1985;

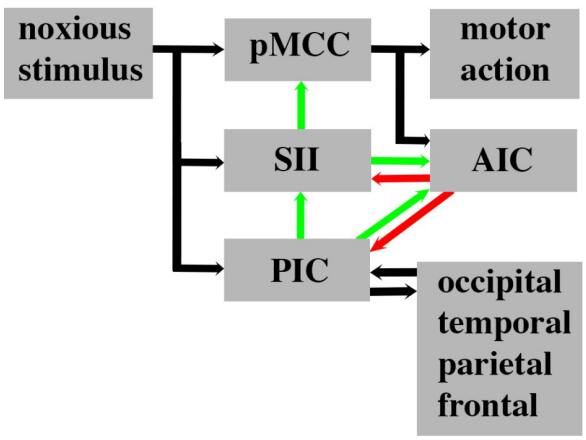

FIGURE 6 | Human cortical circuitry consistent with the parallel forward models algorithm as presented in **Figure 4**. Parallel streams of noxious sensory input enter the posterior middle cingulate cortex (pMCC), secondary somatosensory area (SII), and posterior insula cortex. The SII and PIC contain internal models of pMCC and SII respectively. The outputs of these models are predictions (green arrows) and they are relayed to a comparator module in the anterior insular cortex (AIC). The AIC compares the output of pMCC and SII and the error (red arrow) is fed back to the SII to adjust its internal model of pMCC. The prediction of SII is fed back to the pMCC where it is used to refine its response to the noxious stimulus. The prediction of the internal model in PIC is fed forward to the AIC where it is compared with the prediction of SII. The error is then relayed back to the PIC to fine tune its internal model of SII. The prediction of the PIC is also fed back to the SII to refine its prediction. The internal model in the PIC is also adjusted in response to widespread feedback from across the cortex and the PIC has reciprocal connections with these regions that contribute to the feeling of pain.

Augustine, 1996; Cerliani et al., 2012), and SII in turn projects to pMCC (Morecraft et al., 2004; Vogt et al., 2005). This connectivity (**Figure 6**) is consistent with the pMCC processing sensory information to produce an output while putative internal models in SII and PIC create predictions that are compared in the AIC with the outputs of the pMCC and SII, respectively.

Somatosensory area II has previously been reported to play an important role in conscious awareness of somatosensory stimuli (Weisz et al., 2014), which is consistent with it participating in awareness of sensory processing in our algorithm. Strength of activity in SII has also been shown to be predictive for subsequent somatosensory awareness (Hirvonen and Palva, 2016), which also aligns to our framework. SII is additionally involved in distinguishing awareness of self-generated touch from external tactile stimuli (Blakemore et al., 1998). Taken together, the awareness arising in SII is consistent with this region generating predictions that are relayed to both AIC and pMCC where they are then available for modulating ongoing processing within this neural circuitry.

The prediction errors created in AIC are proposed to feed back to fine tune the performance of the internal models in PIC and SII (**Figure 6**). Functional neuroimaging studies (Metereau and Dreher, 2013; Allen et al., 2016; Meder et al., 2016; Bastin et al., 2017; Geuter et al., 2017) have confirmed a long-held view (Singer et al., 2009; Bossaerts, 2010; Ullsperger et al., 2010; Klein et al., 2013) that AIC is involved in error monitoring and awareness in humans. According to our algorithm, PIC is a higher order internal monitor that generates the awareness of awareness of sensory processing leading to motor behavior. Neuroimaging evidence and lesion data support such a role for PIC in awareness of limb position (Karnath et al., 2005) and self-awareness of motor actions (Baier and Karnath, 2008). We do not contend that predictions (awareness) and prediction error related to pain do not occur elsewhere in the brain (Ploghaus et al., 2000; Roy et al., 2014) but rather that such processing is important in modulating PIC predictions. The widespread connectivity of PIC (**Figure 6**) (Nomi et al., 2017) and its multimodal processing of visual, tactile, nociceptive, and vestibular information (zu Eulenburg et al., 2013; Frank et al., 2016) strengthen the proposal that its predictions need to be modulated by global input in order to ensure awareness functions for the whole system.

The proposed cortical neural circuitry underlying our algorithm (**Figure 6**) is supported by considerable evidence that pain is elicited by electrical stimulation and perturbed by lesions to the operculoinsular region (i.e., SII, AIC, and PIC; Bassetti et al., 1993; Cereda et al., 2002; Bowsher et al., 2004; Birklein et al., 2005; Cattaneo et al., 2007; Afif et al., 2008, 2010; Garcia-Larrea et al., 2010; Isnard et al., 2011; Mazzola et al., 2011, 2017; Hirayama et al., 2014; Montavont et al., 2015; Denis et al., 2016; Maesawa et al., 2016; Bouthillier and Nguyen, 2017; Garcia-Larrea and Bastuji, 2018; Garcia-Larrea and Mauguière, 2018). An example of one patient (referred to as Roger) with extensive bilateral damage to the insula who exhibited no deficits in pain sensation has been used to argue against a role of this brain region in pain (Feinstein et al., 2016).

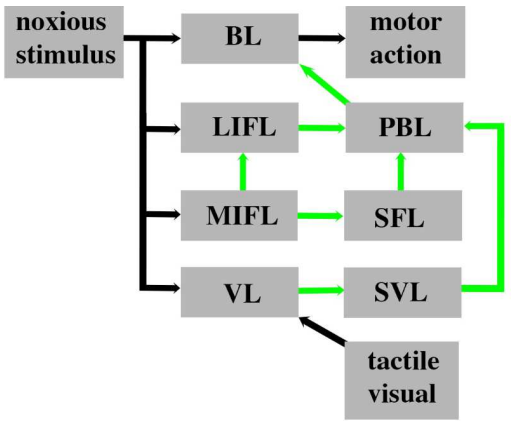

FIGURE 7 | Octopus brain circuitry underlying processing of noxious stimuli based on Young (1991). The circuitry as described in **Figure 2** is schematized here to fit the parallel forward models algorithm. The circuitry can be compared with the human cortical circuitry presented in **Figure 6**. Noxious information is relayed in parallel to the brachial lobe (BL), lateral inferior frontal lobe (LIFL), medial inferior frontal lobe (MIFL), and the vertical lobe (VL). The VL feeds forward to the subvertical lobe (SVL). The MIFL feeds forward to the subfrontal lobe (SFL) and the LIFL. The SVL, SFL, and LIFL all feed forward to the posterior buccal lobe (PBL) which then feeds forward to the brachial lobe. The brachial lobe controls motor actions. The VL also receives input from the tactile and visual system.

However, this case report needs to be considered in the context of the overwhelming evidence that the insula does play a key role in human pain. Negative results are difficult to interpret and can be explained by inter-subject variability in lesion site and size and post-lesion cortical plasticity (Key, 2016). For instance, Roger still had 22% of his left insula intact as well as the entire left SII and both left and right PCC (whether this included the pMCC was not assessed) and his lesion occurred about 30 years prior to sensory testing (Feinstein et al., 2010; Philippi et al., 2012).

Damasio et al. (2012) discuss another patient (patient B) with extensive cortical lesions involving the insula whom continued to experience pain. However, patient B had normal right and left SII, normal right MCC and only partially damaged left MCC. Unfortunately there was no high resolution or quantitative analysis of the lesion sites, no quantitative nociceptive sensory testing and no functional magnetic resonance imaging of patient B in response to nociceptive stimuli which makes interpretation of this case report problematic. Damasio et al. (2012) acknowledged themselves that pain could have been generated by a combination of undamaged cortical regions and cortical plasticity of function in patient B. This is an important point given that we are not contending the necessity of insula for pain but merely using evidence of the role of a neural circuit involving the insula to support the implementation of our algorithm in the human brain. It is entirely feasible that other cortical regions have internal models and comparator modules that can implement our algorithm (provided they possess the necessary neural interconnectivity; **Figure 6**).

CEPHALOPODS LACK THE NEURAL CIRCUITRY FOR PAIN

Given that avoidance learning and neural lesioning approaches involving noxious stimuli are not evidence for pain, we next examined whether the cephalopod brain possessed the prerequisite neural circuitry as outlined in the parallel forward models algorithm (**Figures 5**, **6**). The basic circuitry in the supraesophageal brain underlying avoidance learning using noxious electrical shock during tactile discrimination was presented in **Figure 2**. When the subesophageal brachial lobe is included in this circuitry (**Figure 7**), it reveals that the noxious stimuli enter four parallel streams (which is consistent with our algorithm). The basal stream is from the arms into the brachial lobe and its output drives motor behavior. Noxious input also directly enters both the lateral and median inferior frontal lobes as well as the vertical lobe. Each of these lobes feeds forward to the posterior buccal lobe, which, in turn feeds forward to the brachial lobe to modulate motor actions. The vertical lobe receives global input from the tactile and visual systems. While the circuitry associated with noxious stimuli has some of the components as detailed in the parallel forward models algorithm, it critically lacks the necessary feedforward and feedback pathways between the lobes (cf. **Figure 6**). If the lateral and median inferior frontal lobes and vertical lobes were to generate predictions that were fed forward to the posterior buccal lobe, this latter lobe lacks the ability to feedback prediction errors to these lobes so as to regulate their models. The overall circuitry is instead consistent with a simple feedforward model that modulates motor outputs from the brachial lobe.

CONCLUSION

We have argued here that behavioral responses to noxious stimuli in animals cannot be used to assess whether an animal feels pain. Attempting to reconcile behavioral responses to noxious stimuli with brain lesioning approaches leads to paradoxical conclusions about the origins of pain in the octopus brain. The experiment findings are instead congruous with octopi responding non-consciously to noxious stimuli. We contend that for any animal to feel pain it must possess the appropriate neural circuitry to perform the neural processing necessary for pain. Most extant models of consciousness have not specifically addressed the neural basis of feelings such as pain and have rather concentrated on recognition and discrimination in sensory perception. We have postulated here that feeling states are dependent on specific neural computations. Rather than reverse engineer the human brain in order to define these computations we have instead used basic design principles to construct an algorithm that forms a necessary although not sufficient basis for pain. Our framework is built on the premise that for any nervous system to be capable of feeling it must have the potential to be aware of changes in its own neural states. Although awareness begins first with detection of those changes it must also involve higher levels of awareness whereby the system becomes aware that it has detected those changes. We suggest that dedicated neural circuits (called state observers) must initially monitor the noxious sensory processing that generate motor behaviors. These state observers are themselves further monitored by additional state observers and this tiered circuitry leads to awareness of awareness of sensory processing – the fundamental neural basis of a feeling state.

Our algorithm is consistent with the widely held view that feelings have a functionally significant role for the organism. Such a role emerges naturally when the state observer creates an internal forward model that predicts the output of the neural processing based on its current input. That prediction is a cogent test of the system's awareness of its ongoing processing. It also plays a functional role by its ability to modulate that same processing. We utilize an algorithm that incorporates parallel forward models that make predictions which are subsequently compared with future outputs. Differences between predictions and real outputs (prediction errors) are then used to train the forward models to become more accurate in their predictions. Those predictions are also used to bias sensory neural processing toward the predictions of the model and hence enhance efficacy of the neural processing. We show that the human brain possesses the necessary cortical circuitry to implement the algorithm. Further, we find that the octopus brain cannot execute this algorithm since it lacks the necessary feedforward and feedback pathways between brain regions associated with sensory processing of noxious stimuli. This inability to create an awareness of awareness of sensory processing is not consistent with the octopus feeling pain.

AUTHOR CONTRIBUTIONS

BK and DB contributed to the research and writing of this manuscript.

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Nociceptive Biology of Molluscs and Arthropods: Evolutionary Clues About Functions and Mechanisms Potentially Related to Pain

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Important insights into the selection pressures and core molecular modules contributing to the evolution of pain-related processes have come from studies of nociceptive systems in several molluscan and arthropod species. These phyla, and the chordates that include humans, last shared a common ancestor approximately 550 million years ago. Since then, animals in these phyla have continued to be subject to traumatic injury, often from predators, which has led to similar adaptive behaviors (e.g., withdrawal, escape, recuperative behavior) and physiological responses to injury in each group. Comparisons across these taxa provide clues about the contributions of convergent evolution and of conservation of ancient adaptive mechanisms to general nociceptive and pain-related functions. Primary nociceptors have been investigated extensively in a few molluscan and arthropod species, with studies of long-lasting nociceptive sensitization in the gastropod, *Aplysia*, and the insect, *Drosophila*, being especially fruitful. In *Aplysia,* nociceptive sensitization has been investigated as a model for aversive memory and for hyperalgesia. Neuromodulator-induced, activity-dependent, and axotomy-induced plasticity mechanisms have been defined in synapses, cell bodies, and axons of *Aplysia* primary nociceptors. Studies of nociceptive sensitization in *Drosophila* larvae have revealed numerous molecular contributors in primary nociceptors and interacting cells. Interestingly, molecular contributors examined thus far in *Aplysia* and *Drosophila* are largely different, but both sets overlap extensively with those in mammalian pain-related pathways. In contrast to results from *Aplysia* and *Drosophila*, nociceptive sensitization examined in moth larvae (*Manduca*) disclosed central hyperactivity but no obvious peripheral sensitization of nociceptive responses. Squid (*Doryteuthis*) show injury-induced sensitization manifested as behavioral hypersensitivity to tactile and especially visual stimuli, and as hypersensitivity and spontaneous activity in nociceptor terminals. Temporary blockade of nociceptor activity during injury subsequently increased mortality when injured squid were exposed to fish predators, providing the first demonstration in any animal of the adaptiveness of nociceptive sensitization. Immediate responses to noxious stimulation and nociceptive sensitization

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have also been examined behaviorally and physiologically in a snail (*Helix*), octopus (*Adopus*), crayfish (*Astacus*), hermit crab (*Pagurus*), and shore crab (*Hemigrapsus*). Molluscs and arthropods have systems that suppress nociceptive responses, but whether opioid systems play antinociceptive roles in these phyla is uncertain.

Keywords: nociceptive sensitization, nociceptor, hyperalgesia, allodynia, nerve injury, synaptic potentiation, anxiety, aversive learning

INTRODUCTION

Darwin (1871) considered pain an emotion that evolved by natural selection and is shared by many species. While most research addressing pain has focused on humans and a few mammalian species, findings shedding light on pain-related functions have also been made in invertebrate taxa. Many of these findings came from studies of species in Mollusca and Arthropoda. By species number, these are by far the two largest metazoan phyla, and they contain species with the most complex nervous systems and most sophisticated behavior of any invertebrates.

If human pain is a product of evolution, its neural and molecular mechanisms are unlikely to have arisen *de novo* in our species, and thus at least some processes important for human pain should also occur in other taxa. Informative comparisons and contrasts of pain-related phenomena across taxa require a clear definition of pain. Having primarily been investigated within a clinical/preclinical tradition, the most frequently cited definition of pain is from the International Association for the Study of Pain[1]: pain is "an unpleasant sensory and emotional experience associated with actual or potential tissue damage." This definition has three distinctive features: (1) pain sensation is usually produced by noxious events that produce or threaten to produce injury, (2) the sensation includes sensory information about the noxious event (quality, location, intensity, etc.), and (3) the sensation is tied to a negative emotion that motivates immediate and future avoidance of the apparent source of the sensation (Walters, 2018). Aspects of each of these features can appear in responses to noxious stimuli in non-human species, including molluscan and arthropod species. One property of pain-like states that cannot be assessed conclusively in non-human animals is their emotional content, at least when emotion is defined in terms of conscious experience, as it often is (Izard, 2009). That is because subjective feeling is not directly accessible to observers of non-verbal organisms (Allen, 2004). However, the objective motivational effects that pain-like states have on behavior can be determined experimentally. It is likely that the behavioral consequences of pain-like motivational states were the major selection pressures for the evolution of pain mechanisms.

Pain-like states are inferred in animals from animal behavior and from neural activity in nociceptive systems that process information related to actual or imminent bodily injury. Noxious stimuli are detected by sensory neurons called primary nociceptors, and their activation (nociception) evokes defensive responses (Walters, 1994; Tobin and Bargmann, 2004; Smith and Lewin, 2009; Sneddon, 2015; Burrell, 2017). Because of the potency of nociceptors in driving both human pain and pain-like responses in animals (discussed in Odem et al., 2018), including selected molluscs and arthropods, and because enhanced function of nociceptors contributes substantially to various persistent pain states in mammals (Gold and Gebhart, 2010; Walters, 2012), a major focus of this article is on primary nociceptors.

[1]http://www.iasp-pain.org/terminology?navItemNumber=576#Pain

IMMEDIATE RESPONSES TO NOXIOUS STIMULATION IN GASTROPOD MOLLUSCS

To protect their soft bodies, most molluscs produce a hard shell, but many lack a shell or enough of a shell for adequate protection and must rely on other defenses. Among the seven extant taxonomic classes of molluscs, only two have been studied extensively by behavioral scientists and neurobiologists: Gastropoda and Cephalopoda, both of which include many species possessing little or no shell. The gastropods represent 80% of molluscan species and occupy an enormous range of marine, freshwater, and terrestrial habitats. Within Mollusca, only the coleoid cephalopods (octopus, cuttlefish, and squid) have more complex nervous systems and behaviors. Selected cephalopods and gastropods first attracted the attention of neuroscientists because their giant axons and neuronal somata permitted cellular studies that, until a few decades ago, were impossible in mammals. From the 1960s through the 1990s numerous laboratories exploited the experimental advantages of uniquely identifiable neurons in central neural circuits of gastropods to directly relate cellular and synaptic properties to the organization and mediation of defensive, feeding, and reproductive behaviors (Kandel, 1979; Chase, 2002). Unusual advantages include large neuronal somata (cell bodies) that (1) exhibit overshooting action potentials, (2) allow high-fidelity somal monitoring of synaptic potentials, and (3) display exceptional tolerance for prolonged or repeated impalement by micropipettes.

Behavioral Responses to Noxious Stimulation in Gastropod Molluscs

Many mechanistic studies have focused on synaptic alterations underlying aversive learning and memory in the large marine snail, *Aplysia californica* (Kandel, 2001), which possesses only a rudimentary, internal shell that provides little or no protection. Associated behavioral studies of learning in *Aplysia* utilized electric shock to the soft body surface to modify behavior.

Such shock was considered aversive because it evoked the same immediate defensive responses as produced either by strong mechanical pinch delivered to the body by an experimenter (which produced signs of tissue injury), by bites during staged attacks from a predatory gastropod, *Pleurobranchaea californica,* or by application of a chemical stimulus, NaCl crystals, to the skin (Walters and Erickson, 1986; Walters, 1994; Gasull et al., 2005). Noxious stimuli produced local withdrawal, directed release of ink and other defensive secretions, and escape locomotion directed away from the point of "attack" (Walters and Erickson, 1986). These responses are examples of active defenses that are common throughout the animal kingdom (Edmunds, 1974; Kavaliers, 1988; Walters, 1994): most notably, withdrawal, retaliation (in this case by directed ejection of offending chemicals) (Kicklighter et al., 2005; Love-Chezem et al., 2013), and flight. Production of defensive responses in *Aplysia* is accompanied by inhibition of competing behavioral responses (Walters et al., 1981; Illich et al., 1994; Acheampong et al., 2012).

Nociceptors That Detect Noxious Stimulation in Gastropod Molluscs

Although electric shock is an artificial stimulus, shock delivered to the body surface of *Aplysia* evokes strong defensive responses indistinguishable from those activated by natural stimuli because the shock activates peripheral axons of the same primary nociceptors that are activated by noxious mechanical pressures (Walters et al., 1983a; Illich and Walters, 1997). Important functional properties of identified nociceptors in *Aplysia* (Walters et al., 1983a, 2004; Frost et al., 1997; Illich and Walters, 1997) – especially a relatively high threshold for activation by mechanical stimuli, and silence in the absence of noxious stimulation – are typical of mechano-nociceptors described in diverse animals, including leech (Nicholls and Baylor, 1968), lamprey (Martin and Wickelgren, 1971), teleost fish (Ashley et al., 2007); frog (Hamamoto and Simone, 2003), snake (Liang and Terashima, 1993), chicken (Koltzenburg and Lewin, 1997), mouse (Koltzenburg et al., 1997), rat (Handwerker et al., 1987), cat (Burgess and Perl, 1967), and monkey (Perl, 1968).

The nociceptors identified in *Aplysia* have coiled peripheral terminals embedded in the muscle layer rather than the skin (Steffensen and Morris, 1996), which can explain why sharp poking or pinching stimuli produce optimal activation, and light, brushing stimuli are ineffective. Unlike the nociceptors in insects discussed below, these neurons have somata located within central ganglia, far from their more vulnerable peripheral terminals. These nociceptors show functional properties (Walters et al., 1983a, 2004; Illich and Walters, 1997) more similar to mechanosensitive nociceptors in mammals that are myelinated, rapidly conducting, and rapidly adapting (Aδ- and Aβ-nociceptors) than to unmyelinated, slowly conducting and slowly adapting, often polymodal (chemosensitive) C-nociceptors (Light et al., 1992; Djouhri and Lawson, 2004). Myelin does not occur in molluscs (Roots, 2008), so increased conduction velocity depends upon increased axonal diameter. *Aplysia* nociceptors have central cell bodies and axonal diameters that, while not large compared to axons of truly giant neurons in *Aplysia* (Rayport et al., 1983; Steffensen et al., 1995), are much larger than the small axons coming from the far more numerous afferent neurons of unknown function that possess peripheral cell bodies (Xin et al., 1995). Relatively rapid conduction in *Aplysia* nociceptors and rapid adaptation are functionally consistent with rapid detection of the onset of threatening peripheral stimulation rather than provision of continuing information to the CNS about ongoing (e.g., inflammatory) noxious states, which in mammals is primarily provided by C-nociceptors (Odem et al., 2018). It is not known whether any of the small-diameter afferents or other sensory neurons in *Aplysia* have functions equivalent to those of mammalian C-fiber nociceptors – especially, the non-accommodating activity continuously induced by persistent states of injury and/or inflammation. Among all invertebrates, the leech N lateral neurons are the only nociceptors shown to have non-accommodating, polymodal properties (as well as weak capsaicin sensitivity) resembling the properties of mammalian C-fiber nociceptors (Pastor et al., 1996).

NOCICEPTIVE SENSITIZATION AND PAIN-LIKE STATES IN GASTROPOD MOLLUSCS

In mammals, an unusual property of nociceptive systems is a propensity to sensitize rather than adapt to repeated stimulation (Light et al., 1992; Walters, 1994). Nociceptive sensitization in mammals can also be produced by a single noxious event, which is often manifested as a pain-like response to a subsequent stimulus that normally would not be painful (allodynia) or as an enhanced response to a normally painful stimulus (hyperalgesia). As illustrated by the examples from gastropod molluscs discussed below, nociceptive sensitization can be central or peripheral and short-term or long-term, it includes both general sensitization and site-specific sensitization, and it can refer either to sensitized behavior or to sensitized neurons (for definitions, see Walters, 1994). Long-term sensitization probably protects animals made more vulnerable by serious injury during prolonged recuperative periods (Walters, 1994, 2012).

General Sensitization in Gastropod Molluscs

Nociceptive sensitization has been studied more extensively in *Aplysia* than any other invertebrate. Most mechanistic studies have used a general sensitization paradigm, where noxious shock applied to one part of the body produces sensitization of withdrawal reflexes evoked by test stimuli applied to another body part (the sensitization occurs generally across the body). A single shock produces short-term general sensitization lasting hours, whereas multiple shocks spaced over hours or days produce long-term general sensitization lasting days or weeks (Carew et al., 1971; Pinsker et al., 1973). General sensitization can be induced by extrinsic neuromodulators (notably serotonin, 5-HT) released during noxious stimulation (Brunelli et al., 1976; Glanzman et al., 1989; Marinesco et al., 2004b). General sensitization in *Aplysia* was modeled at the cellular level by culturing nociceptors with motor neurons known to produce

withdrawal responses *in vivo* and stimulating the culture with repeated application of the neuromodulator, 5-HT, to induce long-lasting (24–48 h or longer) facilitation of the synapses between these neurons. This simple model enabled the discovery of basic memory mechanisms that helped Eric Kandel win a Nobel Prize in 2000. The mechanisms by which 5-HT induces long-term presynaptic facilitation include highly conserved cell signaling pathways that also are involved in persistent pain in mammals (Walters and Moroz, 2009; Rahn et al., 2013; Byrne and Hawkins, 2015). Prominent among these are requirements during the induction or maintenance of long-term facilitation for signaling by cAMP and PKA (Brunelli et al., 1976; Castellucci et al., 1980; Ocorr et al., 1986; Bergold et al., 1992), PKCs (Sossin et al., 1994; Sutton et al., 2004; Cai et al., 2011), MAPK (Martin et al., 1997; Sharma et al., 2003), and tyrosine kinases (Purcell et al., 2003; Pu et al., 2014), as well as activation of the transcription factors CREB (Dash et al., 1990; Kaang et al., 1993; Liu et al., 2011) and C/EBP (Alberini et al., 1994; Herdegen et al., 2014), and regulation of local protein synthesis at the synapse by yet another protein kinase, target of rapamycin (TOR) (Casadio et al., 1999; Weatherill et al., 2010) and by cytoplasmic polyadenylation element binding protein (CPEB) (Miniaci et al., 2008).

The cellular model of general, long-term sensitization in *Aplysia* (5-HT applied to cultured nociceptors and motor neurons) has revealed important roles for non-coding RNAs in the regulation of gene expression in nociceptors. These include 5-HT-induced downregulation of micro RNAs (miR-124 and miR-22) (Rajasethupathy et al., 2009; Fiumara et al., 2015) and upregulation of Piwi-associated RNAs (piRNAs) (Rajasethupathy et al., 2012), which alter gene transcription, mRNA translation, and enzyme expression (e.g., increased presynaptic expression of atypical PKC) to enhance synaptic transmission from nociceptors.

Non-coding RNAs were suggested recently to mediate the transfer of sensitization from *Aplysia* receiving repeated noxious electric shock to unshocked recipients by injection of RNA extracted from central ganglia of shocked donors into the recipients (Bédécarrats et al., 2018). This surprising study is notable for several reasons. First, it suggests that extracellular RNAs (presumably non-coding RNAs) can promote behavioral and neuronal sensitization and thus might be yet another of the myriad extracellular signals that produce nociceptive sensitization (Walters, 2014; Ji et al., 2016). Second, the presumed non-coding RNAs are specific to the noxious event; RNAs extracted from the ganglia of unshocked *Aplysia* did not produce sensitization. Third, the donor RNA extract modestly increased the excitability of dissociated nociceptors, suggesting that extracellularly transported non-coding RNAs can directly sensitize nociceptors (perhaps by epigenetic alteration of gene expression by appropriate DNA methylation, as suggested by the authors' finding that the RNA transfer effects were blocked by a DNA methylation inhibitor). The authors assume that the transferred non-coding RNA is part of the memory ("engram") of sensitization and they imply that this RNA comes from the population of nociceptors they studied plus their downstream neural circuits, thus storing a central neural memory of the shock. However, the sensitization-specific RNA might be produced within any neurons (or other cell types) strongly activated (directly or indirectly) by the noxious shock, including numerous unidentified afferents with peripheral cell bodies that send their axons (which might transport RNA) to central ganglia (Xin et al., 1995). Injected RNAs would have access to central and peripheral neurons. Furthermore, the RNA-induced hyperexcitability of dissociated nociceptors they describe is unlikely to explain the observed enhancement of defensive siphon withdrawal because the siphon responses were elicited by weak tactile stimuli that are unlikely to activate this family of nociceptors (see above and Illich and Walters, 1997; Walters et al., 2004). Thus, their test stimuli, like the weak tactile test stimuli used in many *Aplysia* sensitization studies (e.g., Pinsker et al., 1973; Hawkins et al., 1998; Sutton et al., 2001; Cai et al., 2011), reveal an *allodynia*-like effect that is more likely to involve enhanced responsiveness of low-threshold mechanoreceptors than sensitization of the nociceptors that have been examined electrophysiologically. On the other hand, the interesting nociceptor hyperexcitability observed by Bédécarrats et al. (2018) suggests that use of an additional test stimulus that activates the nociceptors might reveal RNA transfer of a *hyperalgesia*-like effect (see also Walters, 1987a,b).

Site-Specific Sensitization in Gastropod Molluscs

Most pain research in mammals is more concerned with the localized sensitization that occurs near a site of noxious stimulation or injury than with general sensitization expressed at distant sites (Woolf and Walters, 1991). Sensitization of *Aplysia* tail and siphon withdrawal responses elicited at a site that had received a brief series of electric shocks was found to be much stronger and longer lasting than general sensitization produced by the same shocks (Walters et al., 1983b; Walters, 1987b). This site-specific sensitization also occurred at a site of tissue injury, and thus appears functionally similar to primary hyperalgesia in mammals (Walters, 1987b). Both general sensitization and site-specific sensitization were linked to concurrent enhancement of synaptic connections from primary nociceptors to motor neurons mediating defensive reflexes, and to hyperexcitability of the nociceptor soma (Brunelli et al., 1976; Walters et al., 1983b; Frost et al., 1985; Scholz and Byrne, 1987; Cleary et al., 1998). These electrophysiological alterations were especially pronounced in tests of site-specific sensitization (Walters, 1987a). Importantly, short-term and long-term behavioral sensitization were found after staged attacks on *Aplysia* by lobsters, showing that both forms of sensitization can be induced by trauma resulting from interaction with a natural predator (Watkins et al., 2010; Mason et al., 2014). Lobster attacks produced long-term hyperexcitability (LTH) of nociceptor somata, but synaptic facilitation was not observed (Watkins et al., 2010; Mason et al., 2014).

Site-specific sensitization is produced by activity-dependent enhancement of the modulatory effects of extrinsic neuromodulators including 5-HT (Carew et al., 1983; Hawkins et al., 1983; Walters and Byrne, 1983; Walters,

1987a,b; Billy and Walters, 1989a; Lin et al., 2010). Other likely contributors to site-specific sensitization in *Aplysia* are NMDA-receptor-dependent long-term synaptic potentiation (LTP) of the activated synapses between nociceptors and motor neurons (Lin and Glanzman, 1994; Murphy and Glanzman, 1997; Antonov et al., 2003) and activity-dependent, proteolytic generation of several active PKC fragments (PKMs) in nociceptors and their postsynaptic targets (Sutton et al., 2004; Farah et al., 2017; Hu J. et al., 2017).

Nociceptive sensitization has also been investigated in the snail, *Helix lucorum*. An aversive chemical stimulus, quinine solution, applied to the head evokes head withdrawal and enhances subsequent withdrawal responses to mechanical and chemical test stimuli for several days, paralleled by potentiation of synapses onto withdrawal command neurons from mechanosensory and chemosensory neurons, along with hyperexcitability of the command neurons (Nikitin and Kozyrev, 1996). This sensitization involves many of the mechanisms described for general and site-specific sensitization in *Aplysia*. These include potentiating roles for 5-HT, cAMP, PKC, and C/EBP (Shevelkin et al., 1998; Nikitin and Kozyrev, 2000, 2005b; Nikitin et al., 2005; Tagirova et al., 2009) and NMDA receptor-dependent LTP of synapses from sensory neurons activated by the noxious stimulus (Nikitin et al., 2002; Nikitin and Kozyrev, 2003).

Peripheral Sensitization in Gastropod Molluscs

In mammals, one component of site-specific sensitization contributing to primary hyperalgesia is localized hypersensitivity of the peripheral receptive fields of primary nociceptors (Gold and Gebhart, 2010; Smith et al., 2013). In *Aplysia,* short-term sensitization of nociceptor fields occurs after peripheral injection of 5-HT into the same fields (Billy and Walters, 1989b). Sensitization was recognized by a reduction in the force threshold for eliciting a response during application of a series of increasingly stiff nylon (von Frey) filaments to the skin. Either tissue injury (deep incision through half of the tail) or strong shock applied to the tail produced a persistent decrease in threshold for mechanical activation of nociceptors with receptive fields bordering a site traumatized 1–3 weeks earlier, but no sensitization in nociceptors with distant receptive fields (Billy and Walters, 1989a). This study also found a long-term expansion of the receptive fields of nociceptors innervating the traumatized region, and evidence for collateral sprouting from neighboring fields. The receptive field alterations are likely to involve injury-induced growth of peripheral fibers, given that nociceptor axons are capable of impressive regenerative growth after injury produced by crushing the nerve innervating the tail of *Aplysia* (Steffensen et al., 1995). Importantly, prior to complete regeneration (before receptive fields are restored to their normal size), the regenerating nociceptors exhibit peripheral sensitization, which was manifested as reduced threshold for activation by von Frey filaments, and hyperexcitability expressed as afterdischarge of action potentials in response to these brief mechanical stimuli (Dulin et al., 1995). In addition, nociceptor sprouting was observed within central ganglia (Steffensen et al., 1995), perhaps contributing to the enhancement of synaptic transmission observed after peripheral neural injury (see below). Peripheral regenerative growth and collateral sprouting can increase the density of nociceptive terminals near the injury and thereby increase nociceptive sensitivity, which also should be increased by hyperexcitability occurring in individual peripheral processes. These complementary alterations may function to compensate for loss of peripheral innervation caused by traumatic injury (Billy and Walters, 1989a; Dulin et al., 1995), and to protect the animal by increasing somatosensory vigilance (especially to mechanical stimulation in the injured region) after the animal is made more vulnerable by the injury (Walters, 1991, 1994).

Axotomy-Induced Alterations of the Nociceptor Soma in *Aplysia* Resembling Alterations Linked to Neuropathic Pain in Mammals

Deep tissue injury is likely to sever nociceptor axons. In mammals, peripheral axotomy of a sufficient number of somatosensory neurons leads to neuropathic pain, which has been associated with hyperexcitability of primary afferent neurons at both the site of axonal injury (the neuroma) and the distant soma (Baron, 2009; Devor, 2009; Gold and Gebhart, 2010; Walters, 2012; Ellis and Bennett, 2013; Smith et al., 2013). The tail incision that was first used to study peripheral sensitization in *Aplysia* nociceptors cut through the entire depth of the mid-tail region to the midline, transecting ∼100% of the axons innervating a distal quarter of the tail (Billy and Walters, 1989a). A less severe incision transecting ∼50% of the axons innervating this tail quadrant was used to investigate effects of deep tissue injury on the excitability of the nociceptor soma (located in a central ganglion ∼10 cm away). One to 2 weeks after partial tail cut, somata of nociceptors likely to have been axotomized exhibited LTH compared to nociceptors with uninjured receptive fields, which showed little difference from nociceptors tested from uninjured animals (Gasull et al., 2005). LTH was similar when Ca^{2+}-dependent exocytosis of neuromodulators was blocked during testing, suggesting that *maintenance* of the LTH was independent of ongoing modulation by extrinsic neuromodulators and instead represented long-lasting intrinsic alterations. On the other hand, *induction* of somal LTH by the injury could have been caused by activity-dependent extrinsic modulation (because no local anesthetic was present during the incision to reduce neuromodulator release), which as discussed above is known to induce LTH of the nociceptor soma and hypersensitivity of peripheral terminals after tail shock (Scholz and Byrne, 1987; Walters, 1987a; Billy and Walters, 1989b).

Evidence that somal LTH also can be induced directly by injury to nociceptor axons came from studies in anesthetized *Aplysia* utilizing an *in vivo* nerve crush injury. Crushing the nerve with forceps transected all axons in the peripheral nerve that innervates the tail without severing the nerve sheath (Walters et al., 1991; Steffensen et al., 1995). This injury produced, after a

delay of 1–2 days, LTH of the nociceptor soma and an increase in amplitude of EPSPs from axotomized nociceptors onto tail motor neurons (Walters et al., 1991). The delay was caused by retrograde axonal transport of molecular signals from the injury site to the ganglion (Gunstream et al., 1995), a conclusion supported by showing that injection of axoplasm collected from crushed nerves into the somata of nociceptors from uninjured animals also produced somal LTH (Ambron et al., 1995). Furthermore, somal LTH could be induced by transecting the neurites of isolated nociceptors growing in culture, showing that extrinsic signals such as 5-HT released at the time of nociceptor injury are not required to induce LTH (Ambron et al., 1996; Bedi et al., 1998). At least two of the axonally transported induction signals are protein kinases; one an unidentified kinase that phosphorylates the transcription factor, Elk1 (Lin et al., 2003), and the other the cGMP-activated kinase, PKG (Sung et al., 2004). While injection into the soma of high concentrations of cAMP (a major downstream signal of 5-HT in *Aplysia* nociceptors) can induce somal LTH (Scholz and Byrne, 1988), somal injection of cGMP was much more potent than cAMP. NO-cGMP-PKG signaling was found to be required for *induction* of LTH by a damaging stimulation sequence applied to the body wall (Lewin and Walters, 1999). On the other hand, continuing activity of PKA in the nociceptor soma was required for the *maintenance* of somal hyperexcitability after nerve crush (Liao et al., 1999b). Crush-induced somal LTH lasted as long as 41 days, but decreased significantly in animals showing recovery of a tail-evoked, centrally mediated siphon response when nociceptors regenerated into the tail, with some recovery of the reflex and normal excitability evident within 2 weeks of the nerve crush (Gasull et al., 2005). LTH of the nociceptor soma after nerve injury (Ungless et al., 2002), like somal hyperexcitability and perhaps action potential broadening in the presynaptic terminal observed acutely or persistently by 5-HT or cAMP (Klein et al., 1982, 1986; Scholz and Byrne, 1988; Goldsmith and Abrams, 1992), involves the closing of "S-type" K^+ channels that are open at resting potential and may be members of the 2-pore domain K^+ (leak) channel family (Patel et al., 1998).

Axotomy-Induced Alterations in Axons of *Aplysia* Nociceptors Similar to Persistent Somal Alterations, and Their Surprising Ca^{2+}-Independent Induction

Long-term hyperexcitability lasting at least 1 day is also exhibited by *Aplysia* nociceptor *axons* following nerve crush in an excised ganglion-nerve preparation (Weragoda et al., 2004). This hyperexcitability, manifested as a decrease in both axonal action potential threshold and accommodation, is highly localized, extending <2 mm along the proximal side of the crush site. Axonal LTH was not reduced when the nerve was crushed in saline containing 1% of the normal extracellular [Ca^{2+}], which blocked detectable effects of any released neuromodulators, suggesting that axonal LTH is a direct effect of axotomy. Because transection can depolarize *Aplysia* axons for several minutes (Berdan et al., 1993; Spira et al., 1993), an interesting question was whether similar depolarization (to ∼0 mV, produced by 2-min exposure of a 1.5 cm nerve segment to elevated [K^+]) might by itself induce axonal LTH. Depolarization-induced axonal LTH was produced in 1% [Ca^{2+}] saline and, like the induction of long-term synaptic facilitation by 5-HT (Montarolo et al., 1986; Casadio et al., 1999), induction of axonal LTH by either depolarization or nerve crush required rapamycin-sensitive (TOR-dependent) protein synthesis in the same nerve segment (Weragoda et al., 2004). Unexpectedly, given the general importance of Ca^{2+} as a cellular transducer of depolarization, the depolarization-induced LTH, as well as short-term (minutes) and intermediate-term (hours) axonal hyperexcitability induced by 2 min of strong depolarization occurred equally well when all detectable Ca^{2+} signaling was prevented by chelation of virtually all extracellular and intracellular Ca^{2+} (Kunjilwar et al., 2009). These results suggest that axotomy directly induces localized axonal LTH by mechanisms involving local rapamycin-sensitive protein synthesis (see Price and Inyang, 2015, for discussion of similar signaling in mammalian nociceptors) and, at least in part, a surprising depolarization-activated pathway that does not require Ca^{2+} signaling. The same Ca^{2+}-independent depolarization procedure applied to the ganglion containing nociceptor-motor neuron synapses potentiated EPSPs from 15 min to >24 h, indicating that this unconventional depolarization-activated pathway can induce synaptic LTP as well as axonal LTH (Reyes and Walters, 2010).

Axonal LTH, unlike somal LTH (Liao et al., 1999a) can also be induced by prolonged or repeated exposure of a nerve segment to 5-HT (which modulates but does not activate nociceptor axons) in the absence of injury to the segment (Weragoda and Walters, 2007). Axonal LTH was induced by 5-HT in 1% or 0.02% [Ca^{2+}] saline, suggesting a direct, Ca^{2+}-independent effect on the axons. This neuromodulator-induced LTH, like injury-induced and depolarization-induced axonal LTH, requires local rapamycin-sensitive protein synthesis. It thus seems likely that natural injuries severe enough to transect nociceptor axons produce LTH in injured and nearby uninjured nociceptor axons by multiple mechanisms, including depolarization-induced and possibly 5-HT-induced signaling within the axons. Sources of peripheral 5-HT after injury could be central neuroendocrine release into the hemolymph (Cooper et al., 1989; Levenson et al., 1999), release by peripheral axons from central serotonergic axons at the site of injury (Marinesco et al., 2004a; Jhala et al., 2011), and/or local release from hemocytes mediating inflammatory-like responses at the injury site (Clatworthy et al., 1994; Farr et al., 1999). As in mammalian neuropathic pain models (Ellis and Bennett, 2013; Walters, 2014), not only injury signals intrinsic to damaged axons but also multiple extrinsic (inflammatory and damage-related) signals may contribute to persistent sensitizing effects of peripheral nerve injury in gastropod molluscs.

Inhibition of Nociceptive Responses in Gastropod Molluscs

Nociception elicits and sensitizes some defensive responses in *Aplysia* but at the same time inhibits competing behavioral responses, including defensive responses incompatible with those directly elicited by the noxious stimulus (Walters et al., 1981;

Walters and Erickson, 1986; Illich et al., 1994; Acheampong et al., 2012). The strongest evidence for an endogenous chemical inhibitor of nociceptive behavior and nociception in *Aplysia* has been found for FMRFamide, a short neuropeptide that is found in several phyla. In *Aplysia,* FMRFamide suppresses responses of primary nociceptors and their downstream targets centrally (Belardetti et al., 1987; Mackey et al., 1987; Montarolo et al., 1988; Schacher and Montarolo, 1991) and peripherally (Billy and Walters, 1989b; Cooper et al., 1989), at least in part by decreasing the excitability and synaptic strength of the nociceptors.

Opioid systems have been claimed to exist in gastropod molluscs on the basis of numerous behavioral-pharmacological and immunohistochemical studies, as well as on some biochemical and molecular evidence (e.g., Kavaliers et al., 1983; Leung et al., 1986; Kavaliers, 1987; Carpenter et al., 1995; Cadet and Stefano, 1999; Achaval et al., 2005; Nikitin and Kozyrev, 2005a; Miller-Pérez et al., 2008). In *Aplysia*, application of met-enkephalin at low doses suppressed the gill-withdrawal reflex (Lukowiak et al., 1982; Cooper et al., 1989). However, the existence in invertebrates of opioid systems that are homologous and functionally similar to opioid systems in vertebrates is controversial (Dores et al., 2002; Dreborg et al., 2008; Mills et al., 2016). Opioids and FMRFamide-related neuropeptides have been suggested to originate from a common ancestral gene (Taussig and Scheller, 1986). Alternatively, the weak molecular similarities between opioids and FMRFamide-related neuropeptides (and other families) might reflect convergent evolution because of fundamental constraints on binding between peptides and certain types of receptor proteins rather than homology across neuropeptide families (Greenberg et al., 1988).

IMMEDIATE RESPONSES TO NOXIOUS STIMULATION IN CEPHALOPOD MOLLUSCS

Cephalopods (squid, cuttlefish, octopuses, and nautiloids) comprise far fewer extant species than do gastropods or bivalves. However, they boast the largest living invertebrate (the colossal squid, weighing half a ton) as well as the largest nervous systems of any animal except some species of mammals and birds. While today's cephalopods are far less common than fish, which currently represent the majority of large marine predators, during the Paleozoic and Mesozoic eras cephalopods were dominant marine predators (Kröger et al., 2011). For neuroscientists, the squid giant axon is famous because it enabled the discovery of the basic mechanisms of the action potential (e.g., Hodgkin and Huxley, 1952), and fundamental discoveries were also made about mechanisms of neurotransmitter release at the squid giant synapse (e.g., Katz and Miledi, 1967). However, few scientists working in pain-related fields have investigated squid.

Defensive functions, usually related to visual stimuli, have been investigated extensively in cephalopods, especially camouflage (Langridge et al., 2007; Allen et al., 2010; Staudinger et al., 2013; Bedore et al., 2015; Panetta et al., 2017), escape jetting (Otis and Gilly, 1990; Preuss and Gilly, 2000; Huffard, 2006), and chemical defense (Derby et al., 2007, 2013). In contrast, little attention has been paid to cephalopod responses to noxious stimulation or injury of the body, although the squid giant axon has been used to study cellular reactions to injury (Fishman et al., 1990; Godell et al., 1997). Behavioral responses to noxious stimulation were first described systematically for the squid *Doryteuthis pealeii*. Minor injury produced by amputation of the distal third of one of the 10 arms of an unanesthetized squid immediately evoked escape jetting and ink release, followed by display of cryptic body patterns and settling of the body onto the substrate (Crook et al., 2011). Grooming of the injured arm (which occurs after similar injuries in mammals) was never observed. Recordings of afferent electrical activity from the fin nerve in an excised fin preparation during mechanical stimulation revealed a population of nociceptive fibers that fire preferentially in response to high intensity mechanical stimuli (Crook et al., 2013). Because neuronal somata are not present in the fin, these mechanosensory neurons appear to be primary nociceptors, with somata located somewhere within the CNS. Nociceptive behavioral and neuronal responses were also described in a small octopus, *Abdopus aculeatus,* which sometimes uses self-amputation (autotomy) of an arm as a defense. Crushing the middle of an arm with forceps usually induced immediate autotomy, and always evoked escape jetting and ink release (Alupay et al., 2014). Interestingly, unlike squid with injured arms, all the octopuses showed wound-grooming behavior, holding the injured arm in the animal's beak for at least 10 min. Nociceptive afferent units were found in recordings from the proximal end of the axial nerve cord, but these might have been second- or third-order neurons from ganglia within more distal parts of the arms. Direct evidence for primary nociceptors was found in units recorded from the mantle nerve that were activated selectively by strong mechanical stimuli applied to the mantle (Alupay et al., 2014).

NOCICEPTIVE SENSITIZATION AND PAIN-LIKE STATES IN CEPHALOPOD MOLLUSCS

Nociceptive sensitization was not described in any cephalopod until recently. Crook et al. (2011) showed that amputation of the distal third of one arm of an unanesthetized squid (*D. pealeii*) sensitized defensive responses (escape jetting, ink ejection) for at least 2 days after injury. Some site-specific cutaneous sensitization was evident near the injury site in blindfolded squid. However, equally strong general sensitization was revealed by similarly enhanced responses to the tactile test stimulus applied to a contralateral arm, the head, or mantle of both freely swimming and partially restrained squid. Squid are highly visual species, and the largest enhancement of defensive responses occurred in freely swimming squid without blindfolds *before* contact with the von Frey filament, showing that the arm injury produced long-lasting sensitization (hypervigilance) to visual stimuli (Crook et al., 2011). Injured squid also became more likely to join schools of other squid when exposed to predator cues (Oshima et al., 2016). The hypervigilance and increased tendency to "seek safety in numbers" are consistent

with an injury-induced, anxiety-like state. A somewhat different pattern of behavioral sensitization was reported after arm injury in the octopus, *Abdopus aculeatus*. Crushing the middle of an arm produced site-specific sensitization to von Frey stimulation, but little general sensitization of defensive behavior was found and no hypervigilance to visual stimuli was reported (Alupay et al., 2014).

Peripheral injury in cephalopods can sensitize primary nociceptors to mechanical stimulation of their peripheral receptive fields. A crush injury to one fin produced both immediate and long-term (lasting at least 24 h) sensitization, observed as a decrease in mechanical threshold and an increase in the number of afferent action potentials evoked by stimulation with a moderate intensity von Frey stimulus (Crook et al., 2013). Like the behavioral sensitization found after arm injury (Crook et al., 2011), nociceptor sensitization was not specific to the injured region; similar sensitization was found in nociceptors innervating the contralateral fin, suggesting that widespread nociceptor sensitization contributes to general behavioral sensitization in squid. Long-term sensitization of nociceptor responses was also found after natural injuries produced by attacks from other squid. Unexpectedly, fin injury produced significant ongoing (apparently spontaneous) electrical activity in fin nociceptors both ipsilateral and contralateral to the injured fin (Crook et al., 2013). Spontaneous activity in probable nociceptors has not been reported previously in invertebrates, although it is not uncommon in persistent pain models in mammals (Djouhri et al., 2006; Devor, 2009; Walters, 2012; Odem et al., 2018). This persistent spontaneous activity in the periphery may drive continuing activity in the brain that produces hypervigilance. At the same time, spontaneous activity generated in widespread nociceptors can provide little or no information about the location of the injury. Knowing the injury location may be less important for squid than the basic information that they have sustained a significant injury and are in a dangerous environment (Crook et al., 2013). In the octopus, *Adopus*, arm injury also produced widespread activity in peripheral neurons, increasing evoked and spontaneous activity recorded from the axial nerve cord at the base of both the previously injured and uninjured arms excised from injured animals compared to those excised from uninjured animals (Alupay et al., 2014). Given the large number of neuronal cell types in the axial nervous system, this afferent activity could represent activity in interneurons and/or primary sensory neurons.

Experiments on squid nociceptors led to the first direct demonstration in any species of the adaptiveness of nociceptive sensitization. Local and general nociceptor sensitization were found to be prevented by locally blocking all neural activity during fin crush, which was accomplished by injecting isotonic $MgCl_2$ into the site to be injured (Crook et al., 2013; see also Butler-Struben et al., 2018). This non-specific local block effectively anesthetized the squid at the injured site while also locally blocking motor function (for similar effects and mechanisms in *Aplysia,* see Walters, 1987a,b and discussion in Liao and Walters, 2002). The localized motor block was experimentally useful because local relaxation of the chromatophores indicated the very limited spread and rapid reversal of the effects of the injected $MgCl_2$. To test the adaptiveness of nociceptive sensitization, isotonic $MgCl_2$ was injected into an arm just before distal amputation, 6 h before exposing the squid to a natural predator – black sea bass – for 30 min while confined in a relatively large tank (Crook et al., 2014). Direct effects of the $MgCl_2$ remained localized to the injected arm, dissipated long before introduction to the fish, and by itself failed to alter camouflage, escape jetting, or inking during the encounter, or the likelihood of pursuit, attack, and capture by the fish. Interestingly, although human observers could not discern any difference in the general appearance or behavior of injured and uninjured squid (regardless of whether the neural block had been given earlier), the injured squid were selectively targeted by the fish and captured more often than uninjured squid. The adaptiveness of nociceptive sensitization was revealed by greater mortality during the 30-min encounter in squid that had been anesthetized during injury (81%) compared to squid sensitized by injury without anesthesia (55%), or to uninjured squid given sham procedures with anesthesia (25%) or without anesthesia (20%). Thus, persistent nociceptive sensitization can be evolutionarily adaptive by enhancing survival of a previously injured animal during predatory attack.

IMMEDIATE RESPONSES TO NOXIOUS STIMULATION IN CRUSTACEANS

The phylum Euarthropoda contains over 80% of living animal species, most of which are terrestrial insects, but also includes crustaceans, arachnids (spiders, ticks, and mites), and myriapods (millipedes, centipedes). Most marine arthropods are in the crustacean subphylum, which includes both the most massive arthropod (the American lobster, weighing over 40 pounds) and tiny copepods that may have the greatest biomass of any animal group on the planet. All arthropods have a hard, chitinous, often mineralized cuticle that provides protection and a firm exoskeleton for attachment of muscles. Defensive behaviors have been investigated extensively in arthropods. In crustaceans, these include neurobiological studies of escape behavior, especially in crayfish (e.g., Edwards et al., 1999), and ecological studies of inducible defenses, often using water fleas (Daphnia) (Tollrian and Leese, 2010; Herzog et al., 2016). Across all arthropods, far less research has been conducted on behavioral and neural responses to noxious somatosensory stimuli than on responses to visual, auditory, and chemosensory stimuli (e.g., Joseph and Carlson, 2015; Göpfert and Hennig, 2016; Knaden and Graham, 2016; Ter Hofstede and Ratcliffe, 2016; Tomsic, 2016).

Surprisingly, primary nociceptors have yet to be identified in any crustacean, although indirect evidence supports their existence. Earlier suggestions that crustaceans have sensory neurons that detect noxious stimuli came from the elicitation of vigorous defensive responses by electric shock applied to the hard exoskeleton of crayfish and crabs (Krasne and Glanzman, 1986; Lozada et al., 1988; Fossat et al., 2014). The aversiveness of the shock was suggested by learning experiments, in which crayfish or crabs would escape or avoid a chamber paired

with shock (Denti et al., 1988; Kawai et al., 2004; Magee and Elwood, 2013), and by anxiety-like effects produced by shock (Fossat et al., 2014). Somewhat similarly, shock delivered within a shell of a hermit crab stimulated evacuation of the shell and promoted switching to a new shell (Appel and Elwood, 2009a,b). A possible caveat in some of these studies, however, was a lack of controls for the possibility that avoidance was produced by long-lasting repellent chemicals released from animals shocked in a conditioning chamber or shell. A more general caveat for all studies of aversive learning (including mammalian studies) is that aversion, although arguably the most important feature of human pain, is not equivalent to pain; electric shock might produce non-painful sensations an animal seeks to avoid, such as itch, or unpleasant but non-painful feelings such as the pins and needles sensation that low-intensity shock can evoke in humans.

Other noxious stimuli investigated in crustaceans include the injection of formalin (sometimes used to model inflammatory or arthritic pain in rodents) into the joint of the claw of a crab, which produced brief freezing, rubbing of the claw, autotomy of the claw, and guarding-like postures (Dyuizen et al., 2012). These responses lasted less than 10 min, and tests for persistent sensitizing effects of the formalin injection were not reported. A few studies have described the elicitation of defensive responses in crustaceans by more natural noxious stimuli, providing indirect evidence for functional nociceptors. These include grooming by prawns of antennae stimulated with low- or high-pH saline (Barr et al., 2008) (although another study failed to find such responses in three decapod crustaceans) (Puri and Faulkes, 2010); grooming-like responses, escape, and withdrawal after stimulation of the mouth or eyes of crabs with acetic acid (Elwood et al., 2017); and defensive responses to touching crayfish claws or antennae with a hot probe (Puri and Faulkes, 2015). Tentative electrophysiological evidence for crustacean nociceptors came from recordings of increased ongoing afferent activity in crayfish antennal nerves during application of hot saline (Puri and Faulkes, 2015). However, the observed increase in activity was modest and the small volume applied in the bath might not have been sufficient to heat antennal receptors to noxious levels. Thus, the observed neural responses might have been to warmth rather than intense heat that threatens imminent tissue damage. An interesting question is whether peripheral nociceptors homologous with, or functionally equivalent to, the class IV multidendritic nociceptors in insects (see below) are found underneath the exoskeleton of crustaceans.

NOCICEPTIVE SENSITIZATION AND PAIN-LIKE STATES IN CRUSTACEANS

Few crustacean studies have addressed sensitizing effects of noxious stimulation that persist for hours, days, or longer. An early study showed that amputation of both claws of crayfish sensitized tail-flip escape behavior elicited by tactile or visual stimuli for at least several days (Krasne and Wine, 1975). Long-lasting nociceptive sensitization produced by aversive shock has been implicated in hermit crabs which, 24 h after being shocked in their shell, approached and occupied new shells more rapidly than did previously unshocked crabs (Appel and Elwood, 2009a). Sensitization lasting hours was found for the crayfish lateral-giant-fiber-mediated tail flip response after repeated electric shock (Krasne and Glanzman, 1986), which was associated with long-term synaptic potentiation of chemical and electrical synapses onto the lateral giant command neuron (Edwards et al., 2002). Long-lasting sensitization of the crayfish escape system, like sensitization of defensive responses in *Aplysia* (see above), may involve actions of 5-HT (Schnorr et al., 2014). Another similarity to *Aplysia* (see Lewin and Walters, 1999; Sung et al., 2004) is potential involvement of NO, with increased activity of NO synthase being reported in the crab nervous system for at least 1 h after injection of formalin into the joint of a claw (Dyuizen et al., 2012). Extensive knowledge of the neural circuitry controlling escape behavior in crayfish (Edwards et al., 1999) should facilitate investigation of nociceptive alterations in crustaceans, but very little is known about mechanisms of short- or long-term nociceptive sensitization in any crustacean species. Similar to the earlier finding with squid (Crook et al., 2014), the evolutionary adaptiveness of nociceptive sensitization in a crustacean was indicated recently by demonstrating that noxious shock applied to small amphipods (*Gammarus fossarum*) increased anxiety-like sheltering behavior and reduced capture by predatory goldfish (Perrot-Minnot et al., 2017).

Evidence for opioid inhibition of nociceptive behavioral responses in crustaceans, based on injection of morphine, has been reported for a mantis shrimp (Maldonado and Miralto, 1982) and crab (Lozada et al., 1988; Maldonado et al., 1989; Valeggia et al., 1989). However, a later study found that the high concentrations of morphine used in these crustacean studies did not reduce pain-like responses to shock in crabs, and may instead have produced a transient general impairment of motor function (Barr and Elwood, 2011). Although opioid systems are reported in crustaceans (Leung et al., 1987; Martinez et al., 1988), controversy about the existence of opioid systems in any invertebrate taxa (Dores et al., 2002; Dreborg et al., 2008) suggests that more study is needed to establish whether opioid-mediated anti-nociceptive function occurs in this sub-phylum. On the other hand, potent neuromodulatory systems that strongly suppress nociceptive responses have been found in crustaceans (e.g., Krasne and Wine, 1975; Vu and Krasne, 1993), so it will be of interest to further define the neuromodulatory mechanisms involved and their relationships to those described for anti-nociceptive systems in other animal groups.

IMMEDIATE RESPONSES TO NOXIOUS STIMULATION IN INSECTS

Insects have been the subject of numerous neurobiological studies of escape behavior (Camhi and Levy, 1988; Hoy et al., 1989; Allen et al., 2006; Card, 2012; Yager, 2012) and of chemical defenses (Sobotník et al., 2010; Nouvian et al., 2016; Touchard et al., 2016). Until recently, little attention was paid to injury-related behavior

or to primary nociceptors in insects. Because they are relatively small, often very short-lived, and are reported to continue normal activities such as feeding or mating without interruption even as they sustain mortal injury, it has often been assumed that nociceptive systems are rudimentary and that pain-like states are absent in insects (Eisemann et al., 1984).

As in molluscs and crustaceans, some of the early experimental evidence for nociceptive responses came from experiments on learning in which electric shock was observed to elicit immediate withdrawal and escape responses as well as aversive learning (Horridge, 1962; Pritchatt, 1968; Booker and Quinn, 1981; Eisenstein et al., 1985). An early report described defensive responses of several lepidopteran larvae (caterpillars) to sharp mechanical stimuli, which included withdrawal, striking at the stimulation site with the head and mandibles, and non-directed thrashing of the body (Frings, 1945). The same responses, as well as cocking before striking (a preparatory posture to increase the force of the strike), regurgitation, and grooming-like behavior directed at a wound were analyzed in detail in larvae of the large moth, *Manduca sexta,* in response to stimulation with stiff filaments and sharp pinch (Walters et al., 2001). Striking and prolonged thrashing were described in the field during natural attacks by an avian predator, and similar striking responses were noted in wild lepidopteran larvae during egg-deposition attempts by parasitoid wasps (Walters et al., 2001). Tiny *Drosophila* larvae also show a well-studied nocifensive response – vigorous corkscrew-like rolling elicited by sharp mechanical stimuli or noxious heat (Tracey et al., 2003). Like the strike response of lepidopteran larvae, the rolling response of fruit fly larvae is evoked by attacks from parasitoid wasps, especially when the cuticle is penetrated, and this response was demonstrated to be adaptive by promoting escape from attacking wasps (Hwang et al., 2007; Robertson et al., 2013).

Primary nociceptors have been identified in both *Manduca* and *Drosophila*. A subset of sensory neurons with peripheral cell bodies and profuse multidendritic arbors beneath the epidermis and cuticle was discovered and shown to respond preferentially to strong mechanical stimuli in *Manduca* larvae (Grueber et al., 2001). The vast set of experimental genetic tools available for research on *Drosophila* has encouraged intensive research on apparently homologous nociceptors in fruit fly larvae (Grueber et al., 2002). These multidendritic class IV nociceptors were shown to be required for rolling responses to heat, sharp mechanical stimuli, and attacks by parasitoid wasps, and also for aversion to dry substrates (Tracey et al., 2003; Hwang et al., 2007; Johnson and Carder, 2012). Extracellular recordings showed heat-evoked activity in nerves containing axons of the nociceptors (Tracey et al., 2003). Optogenetic activation of this class of multidendritic sensory neurons was sufficient to trigger rolling behavior, and genetically targeted RNA interference (RNAi) silenced the nociceptors and prevented rolling responses (Hwang et al., 2007). The nociceptors express an ion channel in the TRPA family, "Painless," that is distantly related to TRPA1 in vertebrates, and this channel is necessary for the defensive responses evoked by heat, harsh mechanical stimuli, and wasp attacks, and for aversion to dry substrates (Tracey et al., 2003; Hwang et al., 2007; Johnson and Carder, 2012). Class IV nociceptors also express TRPA1 (a close homolog of mammalian TRPA1), which contributes to noxious heat detection (Neely et al., 2011; Zhong et al., 2012), and they express at least two widely conserved channel types associated with mechanical nociception: degenerin/epithelial sodium channels (Zhong et al., 2010; Gorczyca et al., 2014; Mauthner et al., 2014) and a mechanosensitive piezo channel (Kim et al., 2012). Targeted silencing, optogenetic activation, and electron microscopy have revealed some of the downstream neural circuitry of interneurons and motor neurons that mediate rolling and other defensive responses in *Drosophila* in response to activation of identified nociceptors (Hu C. et al., 2017; Yoshino et al., 2017; Burgos et al., 2018). A separate class (III) of multidendritic sensory neurons was found to mediate cold nociception, involving three different TRP channels (Turner et al., 2016).

A mammalian transcription factor, PRDM12, known to control the developmental specification of primary somatosensory neurons and linked to nociceptive function in humans (Nagy et al., 2015), has a homolog, Hamlet, in *Drosophila* that specifies the fly multidendritic sensory neurons embryonically (Moore et al., 2002). This intriguing finding indicates that the development of insect and human nociceptive sensory neurons involves a shared regulatory gene inherited from an extremely ancient metazoan ancestor. RNAi knockdown of Hamlet in *Drosophila* or knockdown of some of its target genes reduced the sensitivity of larvae to noxious heat and decreased dendritic branching of the Class IV nociceptors (Nagy et al., 2015). Remarkably, overexpression in *Drosophila* nociceptors of PRDM12 mutants associated with impaired pain function in humans also impaired the larval response to noxious heat (Nagy et al., 2015). Another example of a conserved protein that was found to have similar functions related to noxious heat sensitivity in *Drosophila* and mammals is an auxiliary subunit, α2δ3, of voltage-gated Ca^{2+} channels (Neely et al., 2010). These results point to the conservation of some very basic molecular mechanisms tied to nociceptive function over at least 550 million years of evolution.

NOCICEPTIVE SENSITIZATION AND PAIN-LIKE STATES IN INSECTS

The first description of nociceptive sensitization produced by a stimulus other than artificial electric shock in any arthropod came from studies of *M. sexta* larvae (Walters et al., 2001). Incremental sensitization of directed strike responses occurred during repeated sharp pinch but not gentle pokes delivered to one or more prolegs. General sensitization was also seen, lasting for up to an hour and expressed by an increased number of strikes during a series of gentle pokes applied to prolegs ipsilateral or contralateral to prolegs previously stimulated by multiple pinches. General sensitization after just a single noxious pinch of a proleg was later shown to be expressed as a marked decrease in strike threshold to mechanical stimulation, which persisted for at least 19 h (McMackin et al., 2016). This robust sensitization survived dissection after *in vivo* pinch, which allowed neural correlates of the sensitization to be examined (Tabuena et al.,

2017). In contrast to nociceptive sensitization in *Aplysia* and mammals, neural sensitization did not include enhancement of primary afferent activity evoked by test stimulation of the previously pinched region, but it was expressed as increased evoked activity recorded from an interganglionic connective. This shows a form of central sensitization, perhaps with some functional and mechanistic similarities to the central sensitization that contributes to pain states in mammals (Woolf, 2011). Clues about the mechanisms of pinch-induced central sensitization came from blocking the induction of sensitization of strike responses (and central hyperactivity) by pre-treatment with NMDA receptor blockers, and reversal of sensitized strike responses by post-treatment with a blocker of cAMP-activated HCN channels (Tabuena et al., 2017). This pattern is interesting because, like some forms of nociceptive sensitization in *Aplysia* and mammals, it suggests that NMDA receptor-dependent LTP is involved in the induction of sensitization, and ongoing generation of cAMP may be involved in the maintenance of sensitization (e.g., Bavencoffe et al., 2016).

In insects (especially *Drosophila,* but also honeybees), as with gastropods and crabs, early indirect evidence for long-lasting effects of noxious stimulation came from studies of aversive learning and memory (e.g., Booker and Quinn, 1981; Busto et al., 2010; Diegelmann et al., 2013; Tedjakumala and Giurfa, 2013), including "pain relief learning" in which flies learned that a stimulus predicts safety from shock (Gerber et al., 2014). Intriguingly, a form of aversive operant learning potentially similar to conditioned place aversion was found in honeybees, in which flight of harnessed bees toward a salient landmark was punished by focused heat (Heisenberg et al., 2001). Direct studies of nociceptive sensitization have already yielded rich molecular insights. Epidermal damage and apoptosis caused by UV radiation without apparent injury to underlying nociceptors were associated with a long-lasting (∼1 day) sensitization of heat-evoked rolling responses (Babcock et al., 2009). The sensitization was expressed as both an enhanced incidence of rolling to focal contact with a probe heated to a noxious temperature ("hyperalgesia") and by a decrease in the threshold temperature to elicit rolling ("allodynia"). Genetically targeted RNAi manipulations indicated that the allodynia required activation of a caspase in the epidermis with consequent signaling via *Drosophila* homologs of TNFα in epidermal cells and TNF receptor in adjacent nociceptors. Epidermal apoptosis turned out not to be necessary for sensitization, but downstream targets of TNF binding in nociceptors were, including a pathway involving p38 MAPK, NFκB, and a nuclear transcriptional regulator, enhancer of zeste (Jo et al., 2017).

A surprising discovery was that both thermal allodynia and hyperalgesia in larvae required a developmental signaling protein, the morphogen Hedgehog (Hh), a finding that inspired experiments by these authors using rats that provided the first evidence that the vertebrate morphogen homolog, sonic hedgehog, contributes to inflammatory and neuropathic pain in mammals (Babcock et al., 2011). In *Drosophila*, the thermal allodynia was found to depend upon TRPA (Painless) function, whereas thermal hyperalgesia depended on TRPA1 function in the same nociceptors (Babcock et al., 2011). Heat allodynia was found to require signaling by a tachykinin neuropeptide which, unlike substance P in mammals, is not produced by primary nociceptors (Im et al., 2015). Instead, UV radiation appears to stimulate the release of tachykinin from central neurons, which then binds to G protein-coupled tachykinin receptors in nociceptors, where it stimulates release of Hh, which by autocrine actions increases the expression and/or function of TRPA (Painless) channels and thereby causes allodynia. A critical pathway downstream from Hh in nociceptors is the bone morphogenetic pathway (BMP), which is required for nociceptive sensitization but not for normal nociception or nociceptor development in *Drosophila* (Follansbee et al., 2017).

Little or no evidence is available for endogenous antinociceptive systems in insects. Genes that are clearly homologous to opioid or opioid receptor genes in humans were not found in the *Drosophila* genome (Kreienkamp et al., 2002). While FMRFamide, which is antinociceptive in *Aplysia*, also occurs in insects, no links of this neuropeptide to inhibition of nociceptive responses have yet been implicated in arthropods (e.g., Merte and Nichols, 2002).

IMPLICATIONS FOR THE EVOLUTION OF FUNCTIONS AND MECHANISMS IMPORTANT FOR NOCICEPTIVE SENSITIZATION AND PAIN

Paraphrasing the widely accepted definition of pain stated in the Introduction, pain is an aversive emotional experience related to actual or imminent tissue damage. A premise of this article is that mechanisms important for pain evolved from mechanisms that (1) have functioned to detect and evaluate tissue damage (nociception) and (2) to motivate adaptive behavior that would help avoid or minimize probable tissue damage (nociceptive sensitization). Molluscs and arthropods have provided abundant information about general functions and mechanisms of nociception and especially of nociceptive sensitization. Before discussing the implications of these findings, it should be noted that these animal groups are not optimal for answering all basic questions about the biology of pain. For example, questions related to the emotional content of pain can be addressed more clearly with mammalian species in which the expression of pain-related emotions appears similar to human expression (Darwin, 1886; Williams, 2002; Damasio and Carvalho, 2013). As another example, more has been learned about fundamental molecular mechanisms of nociceptive sensory transduction from the extremely simple, genetically tractable nematode, *Caenorhabditis elegans*, than from any other species, even genetically tractable *Drosophila* (Tobin and Bargmann, 2004; Venkatachalam et al., 2014; Katta et al., 2015). However, the molluscan and arthropod species discussed here have many analytic advantages, and in terms of numbers of neurons and some prominent physiological properties (notably, a reliance on classical overshooting action potentials for neural communication) (Lockery and Goodman, 2009) their nervous systems appear more similar to mammals than to *C. elegans*.

Evolutionary and Functional Considerations

The large taxonomic separation between chordate, molluscan, and arthropod phyla means that similar functions and mechanisms found across these taxa represent either convergent evolution (homoplasy) or highly conserved descent from the last common ancestor of these groups (homology). This common ancestor is now thought to have lived more than 550 million years ago, near the end of the Ediacaran Period, when the complexity of animals, their nervous systems, and behavior began to increase quickly in response to substantial changes in the marine environment and the appearance of predators (Telford et al., 2015; Kristan, 2016; Budd and Jensen, 2017). It seems likely that the evolution of nociception, nociceptive sensitization, and pain-like responses, like other defensive responses, has been shaped by strong selection pressures exerted by predation (Vermeij, 1987; Walters, 1994; Huntley and Kowalewski, 2007; Crook et al., 2014; Monk and Paulin, 2014; Budd, 2015; Kristan, 2016). Similarities in nociception- and pain-related processes across all three phyla may point to common, independently derived solutions to general problems related to avoiding and surviving traumatic injury in a hostile environment. If effective solutions (and/or molecular building blocks that later proved effective for these solutions) had already evolved in the last common ancestor of chordates, molluscs, and arthropods, then some of these early solutions might have been conserved to function in today's species. A fascinating finding consistent with this possibility is the common involvement in *Drosophila* and mammals of the PRDM family of transcription factors in both the embryonic development of nociceptive sensory neurons and in nociceptive responsiveness or pain (Nagy et al., 2015).

Many similarities in immediate responses to noxious stimulation are obvious in molluscs, arthropods, and chordates. Damaging or potentially damaging stimulation of the body (or electric shock that is likely to activate the fibers of primary nociceptors) usually elicits rapid defensive responses in nearly all animals tested across the animal kingdom, including all the molluscs and arthropods discussed here. The types of defensive responses vary enormously, depending upon the size, mobility, structure (including armor), developmental stage (larval versus adult), habitat, and life style of the species.

The prevalence of defensive responses to intense stimulation of the body surface supports the universal and obvious presumption that acute activation of nociceptive systems is an adaptive response to somatosensory stimuli threatening the integrity of the body (Sherrington, 1906; Kavaliers, 1988; Walters, 1994; Tobin and Bargmann, 2004; Costigan et al., 2009; Smith and Lewin, 2009; Burrell, 2017; Tracey, 2017; Sneddon, 2018). However, evolutionary adaptiveness is defined by reproductive success, not by survival; avoiding mortal danger is only adaptive to the extent that it enhances successful reproduction (Stearns and Medzhitov, 2015). In certain physiological states or at some stages of life it can be adaptive to lack, suppress, or ignore nociceptive sensation, an idea familiar to pain researchers because of the powerful suppression of nociceptive responses and pain by opioid and non-opioid systems during human fight-or-flight situations (Wall, 2002). Indeed, possession of opioid systems that strongly inhibit nociceptive responses has often been considered evidence for the possible existence of pain-like states in non-human species (Bateson, 1991; Sneddon et al., 2014). While the activation of opioid systems plays a large role in suppression of pain-related responses in mammals, it remains unclear whether homologous opioid systems function similarly or even exist in molluscs and arthropods (Dores et al., 2002; Kreienkamp et al., 2002; Dreborg et al., 2008).

Trade-offs between survival and reproductive success are found in all animal groups but seem especially striking in insects. It is well known that nociceptive responses fail to deter male mantids from mating with females that practice sexual cannibalism (Schwartz et al., 2016), and there are innumerable observations of adult insects showing no obvious changes in behavior after severe injury, e.g., continuing to use badly damaged limbs, copulating or eating while being eaten, or even eating their own innards spilled by abdominal rupture (Eisemann et al., 1984; Adamo, 2016). In contrast to these observations on adults, examination of larvae of *Drosophila* and *Manduca* has revealed specialized nociceptors that cover the entire body wall, and these larvae show strong, relatively long-lasting (hours or days) nociceptive sensitization of defensive behaviors evoked by mechanical or heat stimulation.

It would not be surprising for adults of short-lived species like most insects to maximize reproductive activities at the expense of behavior (such as nociceptive sensitization) that promotes survival of the adult but diverts energy and time away from mating and reproduction (Stearns and Medzhitov, 2015). On the other hand, some arthropods, such as lobsters, can live for several decades or longer. Lobsters have not been tested explicitly for nociceptive sensitization, and it is possible that the strong armor of large adults might reduce the need for such sensitization. However, claw amputation sensitizes escape behavior in crayfish (Krasne and Wine, 1975) – a much smaller but closely related decapod crustacean – and adult lobsters are reported to change their preferred defensive response from retaliation to escape after loss of their claws (Lang et al., 1977). It will be interesting to test lobsters and other long-lived crustaceans for long-lasting nociceptive sensitization after actual injury or events threatening damage that would increase vulnerability to predators and decrease future reproductive success.

Long-lasting nociceptive sensitization (lasting hours, days, or weeks) is expressed readily in molluscs such as *Aplysia* and squid. With lifespans of 1–2 years, these animals are not long lived, but their adult lives are substantially longer than many insects, and they grow much larger than insects. The high susceptibility to traumatic injury of their soft bodies and their well-developed capacities for repair and regeneration after injury sustained as adults (Dulin et al., 1995; Moffett, 2000; Shaw et al., 2016; Imperadore et al., 2017) coupled with documented risks of injury from predators (and in the case of squid, from conspecifics) (Nolen et al., 1995; Kicklighter et al., 2005; Watkins et al., 2010; Crook et al., 2013; Hanlon and Messenger, 2018) seems likely to make long-lasting nociceptive sensitization evolutionarily adaptive in these species. This

prediction was confirmed in experiments on squid that provided the first demonstration that a procedure preventing nociceptive sensitization during injury reduces survival during subsequent exposure to a natural predator (Crook et al., 2014). Similar evidence for the adaptiveness of nociceptive sensitization came from showing that noxious shock delivered to an amphipod crustacean increased anxiety-like sheltering behavior and thereby reduced capture by a fish predator (Perrot-Minnot et al., 2017).

Comparative Mechanistic Considerations

At physiological and molecular levels, as well as behavioral levels, the similarities of nociceptive sensitization across molluscs, arthropods, and chordates is striking. Each group shows enhanced defensive responses after noxious stimulation, and the behavioral sensitization is often associated with enhanced function (hyperexcitability, hypersensitivity, synaptic potentiation, growth) in primary nociceptors. Possible exceptions are crustaceans, where nociceptors have not yet been identified, and perhaps *Manduca* larvae, where prominent sensitizing effects of pinch have been found in central neural activity but not primary afferent activity (Tabuena et al., 2017). The neurophysiological alterations in nociceptors and their synaptic targets that have been identified in gastropods and insects involve signaling pathways known to contribute to the induction and maintenance of persistent pain in mammals.

Interestingly, the pathways identified in *Aplysia* and *Helix* are largely different from those identified in *Drosophila*, even though both sets are important in mammalian pain models. In addition to differences in focus between fields with different experimental traditions, this difference in pathways may reflect differences in the noxious stimuli employed in each model: primarily shock, nerve injury, or 5-HT application in *Aplysia* versus UV irradiation in *Drosophila*. Major pathways identified in *Aplysia* (and to a large extent in *Helix*) include G protein-coupled receptor- and NMDA receptor-driven signaling involving cAMP, PKA, PKC, and other protein kinases, regulation of gene expression by transcription factors such as CREB and C/EBP, regulation of mRNA translation by kinases such as TOR, and epigenetic regulation by non-coding RNAs. Major pathways identified in *Drosophila* include TNFα-p38 MAPK-NFκB, enhancer of zeste, Hedgehog, and BMP. In contrast, nociceptive sensitization in another insect, *Manduca*, induced by a different noxious stimulus, sharp pinch, was blocked by inhibitors of cAMP-activated ion channels and NMDA receptors (resembling effects in *Aplysia*). This suggests that differences between gastropods and insects in the pathways found thus far to be involved in nociceptive sensitization reflect at least in part differences in the noxious stimulation models employed rather than fundamental differences between the phyla in the cellular signaling underlying nociception-related neuronal plasticity.

Many of the pathways discussed here and others (e.g., growth factor-regulated pathways) implicated in nociceptive sensitization across major phyla are also important both for pain and for learning and memory (Walters and Moroz, 2009; Rahn et al., 2013; Géranton and Tochiki, 2015; Price and Inyang, 2015), although some features of epigenetic mechanisms in *Drosophila* have been noted to differ from mammals (and implicitly from molluscs) (Deobagkar, 2018). Widely shared molecular contributors to neural plasticity might represent conservation of fundamental mechanisms that originally were selected for adaptive responses to bodily injury (including nociceptive sensitization) and were later co-opted for learning and memory (Walters, 1991; Walters and Moroz, 2009; Price and Dussor, 2014). Alternatively, the original mechanisms could have evolved for learning and memory, later being co-opted for adaptive responses to injury. A possibility not mutually exclusive with the preceding two is that a set of core signaling modules evolved earlier for other functions and were pre-adapted for later use in plasticity underlying both injury/pain-related functions and learning-related functions (an example of what some evolutionary biologists call exaptation).

Motivational and Emotional Considerations

During evolution, physiological and molecular mechanisms driving nociceptive functions became linked not only to sensory and discriminative processes that elicit immediate defensive responses, but also to motivational and cognitive processes that enable an animal to avoid ongoing and future threats related to a noxious experience. This requires an ability to maintain functional "awareness" of injury-induced vulnerability until the vulnerability subsides (perhaps until adequate repair of damaged body parts has been achieved). The phylogenetically widespread occurrence of memory of injury that may drive defensive motivational states is indicated by the examples of nociceptive sensitization described above in several molluscs and arthropods. Important support for this idea comes from the studies described in squid and amphipods in which nociception-induced, hypervigilant (anxiety-like) states reduce mortality from predators (Crook et al., 2014; Perrot-Minnot et al., 2017).

Interesting examples of nociception-induced hypervigilance also come from *Aplysia*. The general sensitization to tactile stimuli produced in *Aplysia* by unpredictable noxious shocks typically used to induce long-term general sensitization can be considered a hypervigilant state resembling the anxiety states in humans that motivate avoidance of general threats (Walters, 1980; Kandel, 1983). Interestingly, when repeated shock to *Aplysia* was predicted by pairing it with a non-threatening chemical cue, a conditioned fear-like state was triggered by subsequent exposure to the previously paired cue (Walters et al., 1981). This state was expressed as enhanced defensive responses (head withdrawal, tail withdrawal, inking, escape locomotion) and a suppressed appetitive response (feeding) in the presence of the chemical cue. Unlike the anxiety-like motivational state (ongoing general sensitization), the conditioned fear-like motivational state produced by similar amounts of shock was only expressed in the presence of the conditioned chemical cue, indicating that the same motivational state in *Aplysia* either can be persistently active if predictive cues are unavailable during noxious experience, or it can be activated selectively and transiently by subsequent detection of predictors

of the noxious event (Walters et al., 1979, 1981; Walters, 1980; Colwill et al., 1988a,b). This indicates that gastropods have a capacity for cognitive processing of predictive information available during noxious experience. A similar capacity is suggested by higher-order aspects of aversive conditioning in *Drosophila*, such as learning about signals predicting safety from shock (Gerber et al., 2014).

Important questions remain about how invertebrates employ information from nociception and from nociception-associated stimuli to avoid further noxious stimulation. The ability to make adaptive choices on the basis of cognitive and motivational processing of information from noxious experience appears likely in many invertebrates, given that operant paradigms in which animals can freely choose among alternative responses reveal that noxious shock can produce diverse examples of avoidance. These include conditioned food aversion in gastropods (Mpitsos and Davis, 1973; Maximova and Balaban, 1984), conditioned avoidance of odors in *Drosophila* (Quinn et al., 1974), and avoidance learning in crabs (Fernandez-Duque et al., 1992; Magee and Elwood, 2013) and cockroaches (Pritchatt, 1968; Eisenstein et al., 1985) that may involve alterations in motor as well as sensory systems. Moreover, avoidance learning similar to conditioned place aversion produced by noxious heat in honeybees has been reported (Heisenberg et al., 2001). Conditioning of aversion to a place in which nociception had occurred previously and of preference to a place in which pain relief was experienced may be the clearest methods available for demonstrating the aversiveness of states hypothesized to be painful in animals (Minami, 2009; Navratilova et al., 2013). Increasingly used in mammalian pain studies (although the tests are still uncommon), conditioned place aversion and conditioned place preference tests could also help fill a large gap in the evidence implicating pain-like features of nociceptive sensitization states in invertebrates.

It seems likely that behavioral consequences of pain-like motivational states were major selection pressures for the evolution of mechanisms important for human pain. At some point(s) in evolution, physiological and molecular mechanisms driving the motivational and cognitive responses to actual or likely tissue damage became linked in some species to conscious emotional experience of pain. Building on the results reviewed here, it is likely that much more will be learned about mechanisms that not only detect and remember noxious experience, but also motivate a mollusc or arthropod to avoid further injury after significant nociception. Sensory and motivational mechanisms involved in nociceptive sensitization may also help to drive potentially separate processes that generate pain-like emotions in those animals that are capable of emotion. Whether any molluscs or arthropods have evolved a capacity for conscious emotion and for suffering after noxious experience remain major questions (see also Walters, 2018). While these questions are probably impossible to answer conclusively in species that are distantly related to humans (Allen, 2004), additional study of the nociceptive biology of molluscs and arthropods should point to commonalities and differences across these major phyla in selected biological characteristics that are important for producing pain and suffering in people.

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In Vivo Recording of Neural and Behavioral Correlates of Anesthesia Induction, Reversal, and Euthanasia in Cephalopod Molluscs

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Cephalopod molluscs are among the most behaviorally and neurologically complex invertebrates. As they are now included in research animal welfare regulations in many countries, humane and effective anesthesia is required during invasive procedures. However, currently there is no evidence that agents believed to act as anesthetics produce effects beyond immobility. In this study we demonstrate, for the first time, that two of the most commonly used agents in cephalopod general anesthesia, magnesium chloride and ethanol, are capable of producing strong and reversible blockade of afferent and efferent neural signal; thus they are genuine anesthetics, rather than simply sedating agents that render animals immobile but not insensible. Additionally, we demonstrate that injected magnesium chloride and lidocaine are effective local anesthetic agents. This represents a considerable advance for cephalopod welfare. Using a reversible, minimally invasive recording procedure, we measured activity in the pallial nerve of cuttlefish (*Sepia bandensis*) and octopus (*Abdopus aculeatus, Octopus bocki*), during induction and reversal for five putative general anesthetic and two local anesthetic agents. We describe the temporal relationship between loss of behavioral responses (immobility), loss of efferent neural signal (loss of "consciousness") and loss of afferent neural signal (anesthesia) for general anesthesia, and loss of afferent signal for local anesthesia. Both ethanol and magnesium chloride were effective as bath-applied general anesthetics, causing immobility, complete loss of behavioral responsiveness and complete loss of afferent and efferent neural signal. Cold seawater, diethyl ether, and MS-222 (tricaine) were ineffective. Subcutaneous injection of either lidocaine or magnesium chloride blocked behavioral and neural responses to pinch in the injected area, and we conclude that both are effective local anesthetic agents for cephalopods. Lastly, we demonstrate that a standard euthanasia protocol—immersion in isotonic magnesium chloride followed by surgical decerebration—produced no behavioral response and no neural activity during surgical euthanasia. Based on these data, we conclude that both magnesium chloride and ethanol can function as general anesthetic agents, and lidocaine and magnesium chloride can function as local anesthetic agents for cephalopod molluscs.

Keywords: general anesthesia, local anesthesia, analgesia, immobilization, cephalopoda, welfare impact, neurophysiology

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INTRODUCTION

Cephalopods are used frequently in studies of camouflage, motor control, cognition, visual processing, environmental toxicology, and microbiology (Hanlon and Messenger, 1996; Dickel et al., 2001; Sumbre et al., 2001; Darmaillacq et al., 2004; Lee et al., 2009; Mäthger et al., 2009; Allen et al., 2014; Chiao et al., 2015; Zepeda et al., 2017). In the course of such studies, it may be necessary to anesthetize, immobilize or euthanize cephalopods. Although there is no direct evidence that cephalopods experience pain or distress as a result of noxious sensory input (Crook and Walters, 2011; Crook, 2013; Crook et al., 2013; Hague et al., 2013; Alupay et al., 2014), in many nations cephalopods are included in regulations that govern the ethical use of vertebrate animals in research, and such legislation [for example, in the UK Scientific Procedures Act (1986), EU Directive 2010/63/EU, and the Canadian Council on Animal Care] requires that anesthesia be provided during potentially harmful procedures. Thus, a major compliance challenge in cephalopod research is the lack of data on the efficacy of commonly used "anesthetic" agents on sensation, perception and states of consciousness or unconsciousness.

A recent review article (Fiorito et al., 2015) compiled a list of some 48 studies in which procedures for "anesthetizing" cephalopods were described. Of these, 17 used high concentrations of magnesium chloride, 23 used some concentration of ethanol in seawater, 3 used a combination of these two agents, and 5 used a different agent. Notably, none of these studies used "modern" volatile or injectable anesthetic agents that are standard in the fields of vertebrate animal anesthesia (e.g., volatiles such as Isoflourane or Sevoflourane, or injectables such as Ketamine or Propofol). Instead, the field relies overwhelmingly on magnesium chloride and ethanol, which are generally considered effective for immobilization and from which cephalopods recover reliably.

A striking feature of all these studies is the lack of evidence for anesthetic efficacy that goes beyond immobility; all the measures of "anesthesia" depth reported in previous studies are behavioral indicators that cannot discriminate between paralysis and lack of sensation or loss of consciousness. Typically, anesthesia is expected to achieve four main effects: to block movement in response to stimulation (immobility), to block noxious sensation and pain (anti-nociception and analgesia) to render the subject "unrousable" (unconsciousness), and to prevent memory of the event (anteriograde amnesia; Villars et al., 2004). To date, there is no evidence that either magnesium chloride or ethanol have effects on three of these four components of anesthesia. Neural recordings of the response of the cephalopod nervous system to these two agents have never been attempted, and as such, there is currently no empirical support for the belief that their use during invasive and potentially painful procedures enhances animal welfare.

Effects of anesthetic substances have been examined in some detail in isolated neuronal cells of other molluscs (Winlow et al., 1991, 1995; Yar et al., 1993). In some gastropods and the sea-slug Aplysia, large, identified sensory and motor neurons can be co-cultured and synapses between the two can be formed *in vitro* (Carew et al., 1981; Kandel and Schwartz, 1982; Spencer et al., 1995; Winlow et al., 1995), allowing for detailed mechanistic studies of effects of various anesthetics on sensory neurons, motor neurons and their synapses (Girdlestone et al., 1989; Qazzaz and Winlow, 1999; Onizuka et al., 2005). Cephalopod neurons are not amenable to such studies; somata of most neurons are small (<10 um) (Novicki et al., 1990; Wollesen et al., 2010; Bellier et al., 2017), and isolated cells do not form synapses in culture. There are no identified sensory neurons in the cephalopod nervous system (and very limited numbers of identified motor neurons; Saidel and Monsell, 1986). The location of sensory neuron cell bodies in cephalopods is unknown, and the circuits that underlie sensation and perception remain completely undescribed (Crook and Walters, 2011; Crook, 2013). Although there have been a number of studies of synaptic mechanisms in slice preparations of the octopus brain, only one or two identified synapses have been studied, and both are interneuron-interneuron (Hochner et al., 2003; Shomrat et al., 2008; Hochner and Shomrat, 2013).

Despite these considerable limitations, laboratories in regulated countries that use cephalopods in research are charged with providing "safe, effective, and humane" anesthesia and euthanasia (Andrews et al., 2013; Smith et al., 2013; Fiorito et al., 2015). The difficulties associated with measuring sensation at the behavioral level in an immobilized animal, the lack of information about neural effects, and the field-standard use of putative anesthetic substances that are not typically in use in any other taxon, have resulted in the imposition of a regulatory requirement that is currently impossible for researchers to satisfy; without evidence that these field-standard methods produce desirable effects of anesthesia beyond immobility, it is impossible to conclude that the animal's welfare is being protected.

In this study, we aimed to investigate the effect of the two most commonly used "anesthetic" substances in cephalopod research, (magnesium chloride and ethanol) on afferent and efferent neural activity in cephalopods. The explicit goal of this work is to provide cephalopod researchers with empirical support for the assumption that these two agents protect animal welfare. Secondarily, we aim to provide a set of behavioral criteria that reliably correlate with loss of afferent neural signal (loss of sensation) and loss of efferent signal (loss of consciousness), such that researchers who lack equipment to monitor neural effects can make reasonable assumptions about anesthesia efficacy based on behavioral indicators. We do not suggest that cephalopods are potential models for mechanistic anesthesia studies, nor do we rule out that other, more modern drugs may be equally or more effective at achieving anesthesia in cephalopods. Instead, we present the first strong evidence that bath applications of either magnesium chloride or ethanol are effective at blocking afferent signal to the CNS, blocking efferent signal from the CNS, and blocking reflex and voluntary movements. In addition, we demonstrate that local anesthesia using either injected lidocaine or high concentrations of magnesium chloride is effective at blocking afferent signal and behavioral responses to noxious stimulation over the injected area;

We demonstrate that these agents, although not commonly accepted as anesthetics in other taxa (with the exception of

lidocaine), achieve the primary goal of anesthesia—protecting the welfare of the animal when subject to invasive and potentially painful procedures. We also make empirically supported recommendations (see section Recommendations) for anesthesia and euthanasia during scientific procedures involving cephalopods.

Definitions

Throughout the remainder of this paper we use the following definitions:

Anesthesia: "Anesthesia" is the collective term used commonly to refer to a multi-faceted effect on an animal: anterograde amnesia, anti-nociception, immobility, and loss of consciousness. Because we focus heavily in this study on blockade of afferent (sensory) signal, hereafter, we use the term "anesthesia" in its most literal and narrow sense; referring solely to the loss of sensation, which is represented experimentally as the absence of afferent neural signal produced in response to external stimuli.

Loss of efferent signal: Loss of conscious awareness during anesthesia is difficult to measure, even for human and mammalian patients. There is evidence that loss of consciousness is associated with decreased communication of neural signal originating in different regions of the brain (Purdon et al., 2013). Here, we use the operational definition that loss of consciousness may be inferred by the absence of organized, efferent neural signal. We note that "loss of consciousness" in human studies is often ascertained by behavioral measures such as absent responses to shouting at or shaking the patient (Villars et al., 2004); these are impractical for cephalopods, and complicated by the hypothesis that immobility in cephalopods occurs separately from loss of consciousness. Hence, we use loss of efferent signal as a proxy for loss of organized within-brain, and brain-body, communication.

Immobility: The complete absence of reflex or voluntary movements of the skin, arms, or body of the animal, not including respiration.

METHODS

Animals

Stumpy-spined cuttlefish (*Sepia bandensis,* **Figure 1A**) were reared from eggs purchased from a commercial vendor. Animals were between 3 and 5 months old (mantle length 25–40 mm) at the time of experiments. Cuttlefish were fed daily on live grass shrimp (*Palaemontes* spp.) and were housed in a recirculating, artificial seawater (Instant Ocean) system at 24.5°C. After experiments concluded, animals were returned to the housing system and remained in the lab until they died of natural causes. One cuttlefish was euthanized as part of the study. Cuttlefish were used in anesthesia trials once only.

Octopuses (*Abdopus aculeatus* and *Octopus bocki*, **Figures 1B,C**) were obtained as adults from commercial vendors, and were housed in individual enclosures visually isolated from other animals. Octopuses were fed 2–4 live shrimp on alternate days. Octopuses were either euthanized as part of the study, or remained in the lab at the conclusion of experiments and were later euthanized (according to the protocol described in this study). All animals were monitored daily for at least 3 days after experiments.

Ethical Note

In the USA, cephalopods are not considered animals for the purposes of animal welfare regulation, thus no protocol or approval number was required for this study. We followed the general guidelines for care and husbandry of cephalopods in the EU.

Equipment

A hook electrode was made from insulated silver wire (0.15 inch diameter, Item AGTI510, World Precision Instruments, Sarasota, FL, USA), and the Teflon coating was stripped from the inner side of the hook. A ground of the same wire was wrapped around the shaft of the hook, and insulation stripped from the last 2 mm. The electrode was connected to an extracellular amplifier (A-M Systems model 2100), and electrical signal was sampled at 20 kHz and digitized by a PowerLab 4/35, while synchronized video footage of the experimental chamber was recorded using the "Video Capture" module of LabChart Pro software (AD Instruments). The recording chamber was a 125 mm diameter evaporation dish filled with 450 mL of artificial seawater taken from the home tank system. Equipment was identical for each of the species in the study.

General Procedure

Placing the hook electrode required animals to be sedated for handling, which was always done using ethanol in seawater. For cuttlefish, animals were placed immediately into 3% v/v EtoH in seawater, and remained undisturbed until they were behaviorally unresponsive to gentle disturbance. We did not observe any adverse reactions to the ethanol bath, which has been reported in some other studies (García-Franco, 1992). Once the subject was behaviorally unresponsive (typically within 3–5 min), the animal was held in a vertical positon and the mantle flap on the rostral edge of the mantle was moved aside, revealing the pallial nerve visible against the inner dorsal aspect of the mantle. Fine forceps were used to gently separate the nerve from the mantle, and the connective tissue was cut below the nerve with fine scissors. The hook electrode was passed behind the nerve, toward the caudal aspect of the mantle, then rotated and lifted to hook the nerve (**Figure S1A**). The hook was clamped loosely onto the nerve to provide good stability and contact between the exposed metal of the hook and the nerve. Desheathing the nerve was not necessary. The ground wire was positioned to sit freely from the nerve and muscle. No sutures or bonding material were needed to secure the electrode in place. At the conclusion of experiments, animals were re-sedated in ethanol and the hook was removed from the nerve by hand.

For octopuses, sedation in ethanol was conducted progressively, starting at 0.5% and increasing to 1, 2, then 3% in 5 min intervals. In octopuses, the pallial nerve is more anterior than in cuttlefish and is not connected to the mantle wall, thus the hook electrode was placed and clamped in

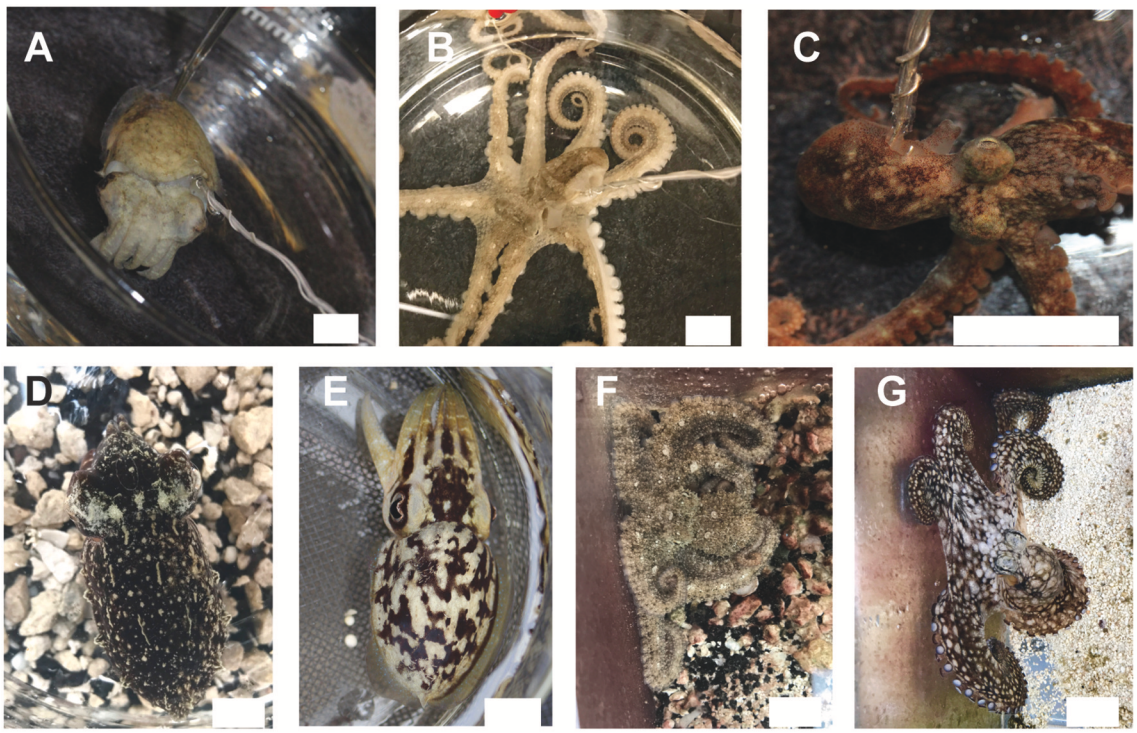

FIGURE 1 | We studied effects of anesthesia on three tropical, commercially available cephalopod species. **(A)** *Sepia bandensis*. **(B)** *Abdopus aculeatus*. **(C)** *Octopus bocki*. Recordings were conducted *in vivo* using a minimally-invasive hook electrode, attached to one pallial nerve. Twenty-four hours after experiments, animals showed normal camouflage and signaling behavior **(D,E)** the same specimen of *S. bandensis* shown in **(A)**; **(F,G)** the same specimen of *A. aculaeatus* shown in **(B)**, demonstrating that neither the anesthesia nor the experimental procedure itself caused any long-term damage to the animal's nervous systems or behaviors. Scale bar: 15 mm.

position without the need to dissect away any connective tissues (**Figure S1B**).

Experienced experimenters were typically able to place the electrode in a cuttlefish in 2–3 min. In octopuses electrode placement took <1 min.

Experimental Procedure

As soon as the electrode was secured, animals were placed in the recording chamber, which contained artificial seawater taken from the main housing system (24.5°C) and recording of neural signal and behavior began. Gills and mantles were not irrigated with positive flow, so substances were exchanged from the gills by normal respiratory movements of the animals' mantles.

Every minute during anesthesia induction or reversal, we used grooved forceps to pinch the skin on the mantle in two locations ("medial," located approximately over the stellate ganglion, and "distal," on the ipsilateral rear tip of the animal's mantle), until the animal showed both behavioral and neural responses to pinch in both locations. Animals were judged to be completely recovered when they were upright, made coordinated swimming or jetting movements, showed normal chromatophore patterns, and showed normal responses to visual or other stimulation. Once animals had recovered from the initial sedation in ethanol, an experimental "general anesthetic" solution was added to the seawater bath, and the animal's induction process was monitored with distal and medial pinches each minute. In addition, we monitored changes in righting response, changes in spontaneous behaviors, changes in chromatophore patterning and loss of response to visual or other stimuli. We recorded the time between anesthetic induction and loss of spontaneous and evoked behavior responses, and latency to complete cessation of afferent and efferent neural signal evoked by pinch. At this point the solution was changed completely to normal seawater, and we monitored the recovery process using the same measures as described above. Most animals received two cycles of induction/recovery in the experimental anesthetic solution.

Once anesthesia was achieved on the second induction, one of two putative "local anesthetic" substances was injected subcutaneously on the distal mantle. A single bolus of 0.1 mL was injected with a 30G needle. We monitored behavioral and neural responses to pinch over the injected area until the animal was completely awake from the general anesthetic, then monitored animals' responses in their home tanks after the recording concluded.

Finally, we tested a standard euthanasia protocol. Once a single induction/recovery cycle of general anesthesia had concluded, animals were immersed in 330 mM $MgCl_2$. Responses were monitored each minute, until 5 min after visible signs of respiration had ceased. While the recording continued, we incised the skin between the eyes, exposed the cranium, then used

a scalpel blade to make multiple cuts through the central brain mass.

Data Analysis and Statistical Procedures

Electrophysiological signals were sampled at 20 kHz, amplified by a model 2100 extracellular amplifier (A-M Systems) and digitized using a PowerLab 4/35 (AD Instruments). Simultaneous recording of the neurophysiological signal and video recording of the behaving animal was obtained using the Video Capture model of LabChart Pro software (AD Instruments). Video was recorded at 60 Hz.

Files were scored by multiple experimenters, to check for agreement on scoring and to ensure impartial evaluation of data. From video data we recorded the latency from anesthetic substance introduction to the first behavioral sign of an effect, noted the time of other behavioral effect such breathing changes, loss of chromatophore control and loss of righting response, and latency until behavior had completely ceased. From neurophysiological traces, we recorded the latency to the complete absence of neural signal (i.e., only background electrical noise on the recording) evoked by pinch on the ipsilateral and contralateral side relative to the electrode.

Identification of afferent vs. efferent signal: On the ipsilateral side to the electrode, signal recorded was likely a combination of afferent and efferent spikes, and as such we did not always make determinations of signal direction. Pinches on the contralateral side produced signal on the recorded trace that were considered to be purely efferent, as signal leaves the brain through the paired pallial nerves to produce coordinated responses on both sides of the mantle, and afferent signal does not cross the midline of the body before entering the brain. In cases where there were no spikes evoked by contralateral pinch, but there were spikes evoked by ipsilateral pinch, we considered that this activity was most likely to be afferent signal only. Loss of efferent signal is indicated in the temporal sequences of neural activity for cuttlefish (**Figures 5A,B**) and octopuses (**Figures 6A,B**).

Statistical Analyses

Latencies were normally distributed and were analyzed with parametric statistics. We used paired *t*-tests to test for significant differences between the latency from drug onset to loss of evoked behavioral response, and from drug onset to loss of afferent signal (anesthesia) upon induction, and between return of neural signal and return of behavioral responses upon reversal. Independent sample *t*-tests were used to compare behavioral or neural metrics between the two anesthetic substances, within species. Lags and advances (time difference between neural and behavioral signal loss or recovery) were tested with one-sample *t*-tests with expected values of zero. We used a critical α of 0.05, and all *p*-values reported are two-tailed.

RESULTS

In Vivo Recording of Peripheral Nervous System Activity

All animals in the study tolerated the hook electrode well (**Figures 1A–C**), with only one cuttlefish showing signs of nerve damage after hook removal (ipsilateral mantle paling). Twenty-four hours after testing, all other animals showed normal cryptic and signaling behavior (**Figures 1D–G**) indicating that the procedure produced no adverse effects on the nervous system or skin. Signal recorded by the electrode included afferent and efferent spikes ranging in amplitude from 0.2 to $\sim$3 μV, and was qualitatively similar among the three species. Variation in electrode-nerve contact caused substantial between-trial variation in spike amplitudes, although overall patterns were similar. Results from the two species of octopus were pooled. In addition to capturing evoked responses to touch, we also recorded spontaneous activity in the nerve associated with chromatophore activity, mantle contraction during swimming, and ventilation. Example signal from respiratory motor neurons in fully awake cuttlefish and octopus is shown in **Figures 2A,B**, respectively. Respiration rates were not significantly slower during periods of complete anesthesia (absence of evoked afferent signal) compared with awake periods, or with rates of unrestrained animals in home tanks.

Effective Doses

Effective doses (those that clearly affected both behavior and neural signal in fewer than 5 min from introduction) varied from 1 to 4% for cuttlefish and octopuses exposed to ethanol, and from 1:3 to 1:1 for $MgCl_2$:ASW (**Figures 3A,B**). Effective doses were determined on the first induction of each experimental trial by progressively increasing the dose (in 1% increments for ethanol and from 1:3 to 1:1 mixes for $MgCl_2$) until an effect was apparent. On the second induction for each trial, the effective dose was administered directly, without progression, and the data averaged within animal. In general, effective dose was weakly positively correlated with induction times for cuttlefish under ethanol anesthesia (Pearson $r^2 = 0.89$, $p = 0.02$), suggesting that some animals were more resistant to anesthesia than others, requiring both higher doses and longer times to anesthesia. There was no pattern for octopuses. Nearly all animals tested were effectively anesthetized by a 1:3 mix of $MgCl_2$ in ASW.

Induction

Patterns of induction were similar in cuttlefish exposed to magnesium chloride ($n = 7$, **Figure 4A** top, **Figure S2**) and ethanol ($n = 7$, **Figure 4A** bottom, **Figure S3**), although there was more spread in latencies for magnesium chloride. Behavioral and neural indicators of induction had highly similar latencies from the introduction of ethanol, with no significant differences between any of the measures. Latencies from introduction of magnesium chloride to behavioral signs of induction (immobility, evoked responses to pinch) did not differ from those in ethanol, but loss of neural signal took significantly longer compared with ethanol (medial: unpaired *t*-test, $p = 0.049$, distal: $p = 0.02$). There was also a significant delay between the loss of behavioral response to distal pinch and the loss of neural signal in response to distal pinch (paired *t*-test, $p = 0.015$). Examples of traces from a single sequence of induction and recovery from ethanol anesthesia are shown in **Figure 5A**. Induction was characterized by a progressive decline in signal, with a clear decrement observed at the point where

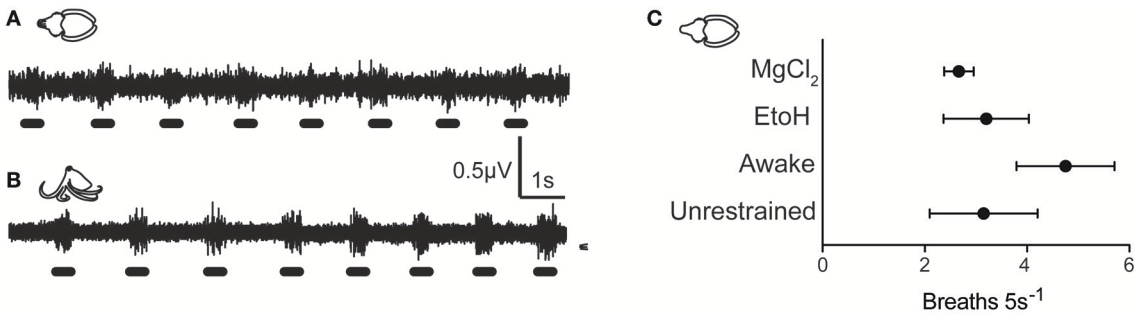

FIGURE 2 | Background neural activity during quiescent, awake periods of cuttlefish **(A)** and Octopus (*O. bocki,* **B)**, showing rhythmic bursts associated with mantle contractions during respiration. Respiration rates during anesthesia can be computed from electrophysiological traces, and respiration signal was a good indicator that the electrode was well-positioned, the nerve was healthy, and that animal was physiologically stable. **(C)** Respiration rates of animals in various procedure stages did not differ significantly. Awake animals showed slightly elevated rates of respiration which was likely due to handling stress. Respiration rates from cuttlefish in the EtoH and $MgCl_2$ treatment groups were measured during periods in which no afferent neural signal was detected during mantle pinch. Breathing rates were not significantly slower than during awake periods, and were within the range of normal respiration rates of unrestrained animals observed in home tanks.

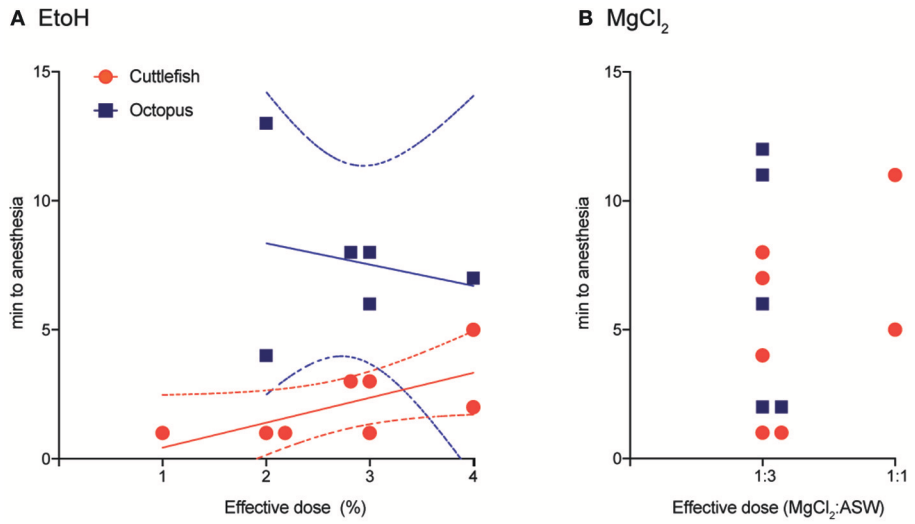

FIGURE 3 | Effective doses and the time to anesthesia (the loss of neural signal at both the medial and distal stimulation sites). **(A)** Effective doses of ethanol ranges from 1 to 4% in ASW. There was a weakly positive correlation between does and time-to-induction for cuttlefish (Pearson $r^2 = 0.89$, $p = 0.02$), line shows best fit from linear regression, dotted lines are 95% CI), but no relationship for octopus. **(B)** Nearly all subjects were effectively anesthetized in a solution of 1:3 $MgCl_2$: ASW, but times to anesthesia varied considerably. There was insufficient variation in dose to test dose/time relationships.

spontaneous behavior and efferent signal ceased. Anesthesia (complete loss of afferent signal) followed rapidly thereafter. In contrast, traces from a single induction and recovery cycle for a cuttlefish exposed to magnesium chloride (**Figure 5B**) show a prolonged period where afferent and efferent signal persist with minimal progressive deficit (from 4 to 8 min), after cessation of behavioral responses and prior to complete anesthesia.

For octopuses, (**Figure 4B**, **Figures S4**, **S5**), patterns were similar to those seen in cuttlefish, although octopuses generally took longer to show effects. There were no significant differences in any behavioral or neural measure either within or between substances. Examples of traces from a single sequence of induction and recovery from ethanol anesthesia are shown in **Figure 6A**. Similar to cuttlefish, octopuses in ethanol showed a rapid loss of neural signal that was associated with cessation of spontaneous behavior, and reached full anesthesia at the same time as evoked behavioral responses ceased. Traces from octopuses in $MgCl_2$ (**Figure 6B**) showed a similar induction process, except that loss of evoked behavioral response preceded anesthesia.

Reversal

Reversal of anesthesia for cuttlefish in ethanol ($n = 7$, **Figure 7A** bottom) was characterized by rapid, highly consistent and statistically simultaneous return of spontaneous behavior, neural responses to pinch and evoked behavior in response to pinch. There was a brief but significant delay between the first incidence of evoked neural signal and the animals showing a "fully awake"

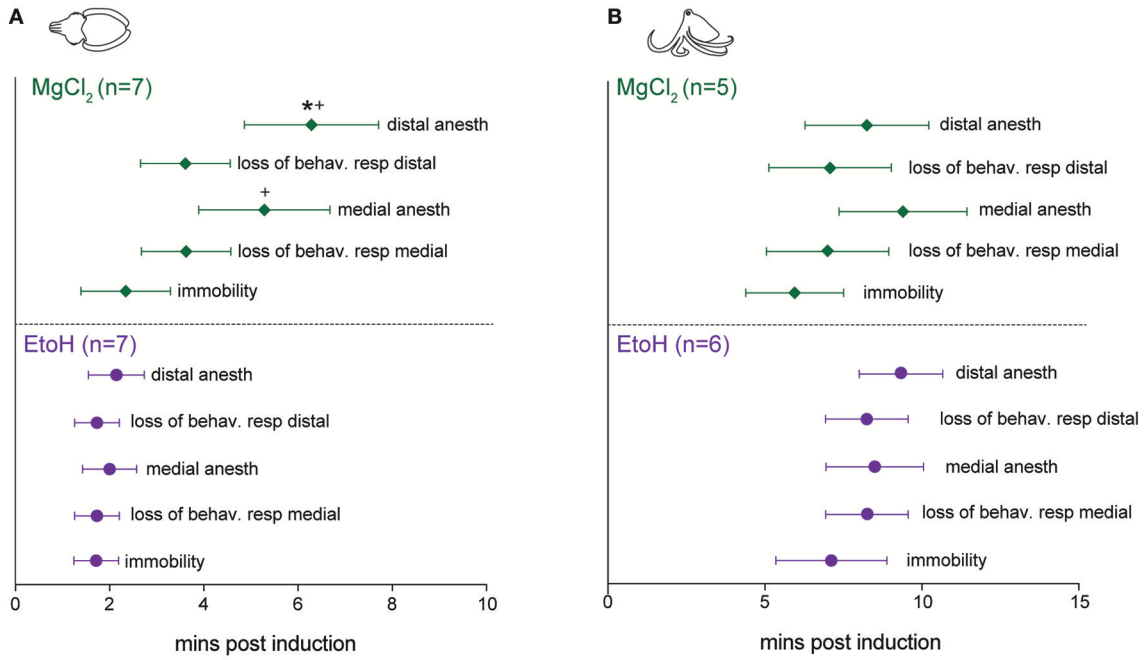

FIGURE 4 | Latencies to behavioral and neural indicators of tri-partite anesthesia induction for cuttlefish and octopus (*O. bocki* and *A. aculeatus* pooled). **(A)** Ethanol anesthesia (effective doses pooled) showed tight temporal coupling of immobility, loss of evoked behavioral responses and loss of evoked neural responses. While magnesium chloride achieved the three requirements of anesthesia—immobility, loss of afferent signal and loss of efferent signal (not shown on figures, see Results for details)—there was a significant delay between the loss of behavioral response and the loss of neural response at the distal stimulation site (denoted by *). Latencies to medial and distal anesthesia were significantly delayed compared with the same measures for ethanol anesthesia (denoted by +). **(B)** The same variables shown for octopuses. Octopus sample sizes were smaller, and octopuses were inherently more variable, thus although patterns are similar to those of cuttlefish there are no significant differences. Within-treatment comparisons: paired *t*-tests. Between treatment comparisons: unpaired *t*-tests. All *p*-values are two-tailed. $*p < 0.05$.

state (paired *t*-test, $p = 0.013$). Cuttlefish in magnesium chloride ($n = 7$, **Figure 7A** top) showed return of neural signal at similar times to those animals exposed to ethanol, but behavioral responses were significantly delayed both in comparison to neural signal in the same animals (paired *t*-tests: medial $p = 0.032$, distal $p = 0.03$, and in comparison to behavioral signs of recovery after exposure to ethanol (unpaired *t*-test: medial, $p = 0.005$, distal, $p = 0.0015$). There was also a significant delay from the first incidence of evoked neural signal and the animals showing a "fully awake" state (paired *t*-test, $p = 0.0035$), and between the latency to show fully awake behavior compared with animals in the ethanol group (unpaired *t*-test, $p = 0.0009$).

For octopuses in the ethanol group ($n = 6$, **Figure 7B**, bottom), trends and latencies were similar to those seen in cuttlefish, except that there was no significant delay between the first incidence of evoked neural signal and fully awake behaviors. An example of electrophysiological traces from an octopus undergoing reversal of ethanol anesthesia are shown in **Figure 6A**. In the magnesium chloride group ($n = 5$, **Figure 7B** top), latencies were highly variable for all measures, with no significant differences among any of the measures within the group. Return of behavioral responses to medial and distal pinches, return of evoked neural activity in response to medial pinch, and latency to being fully awake were all significantly delayed compared with measures for the ethanol-treated octopuses (unpaired *t*-tests, medial behavior: $p = 0.004$, distal behavior: $p = 0.002$, medial neural: $p = 0.02$, fully awake: $p = 0.003$). Although not significant, octopuses treated with magnesium chloride tended to recover spontaneous behaviors later than evoked behaviors, a pattern not seen under ethanol treatment for either cuttlefish or octopuses, or in the magnesium chloride group for cuttlefish.

The latencies between the loss of evoked behavioral responses and evoked neural responses, we hereafter term "neural lag." This lag is the interval where an animal may appear to have entered a true anesthetized state (that is, where afferent signal is absent), but is in fact simply immobilized. The inverse relationship upon reversal (i.e., where neural signal returns prior to behavioral responses), we term "neural advance." This advance is the interval where the animal appears to still be anesthetized, but is not. These two periods represent critical points during invasive procedures where the animal's welfare may be compromised.

For cuttlefish induced with ethanol (**Figure 8A**), neural lags upon induction were negligible, but were slightly positive (i.e., a positive value indicates behavioral signal is lost earlier than neural signal) for magnesium chloride. There was a significant lag in anesthesia onset at the distal pinch location (one-sample *t*-test vs. 0, $p = 0.014$). During reversal of ethanol anesthesia,

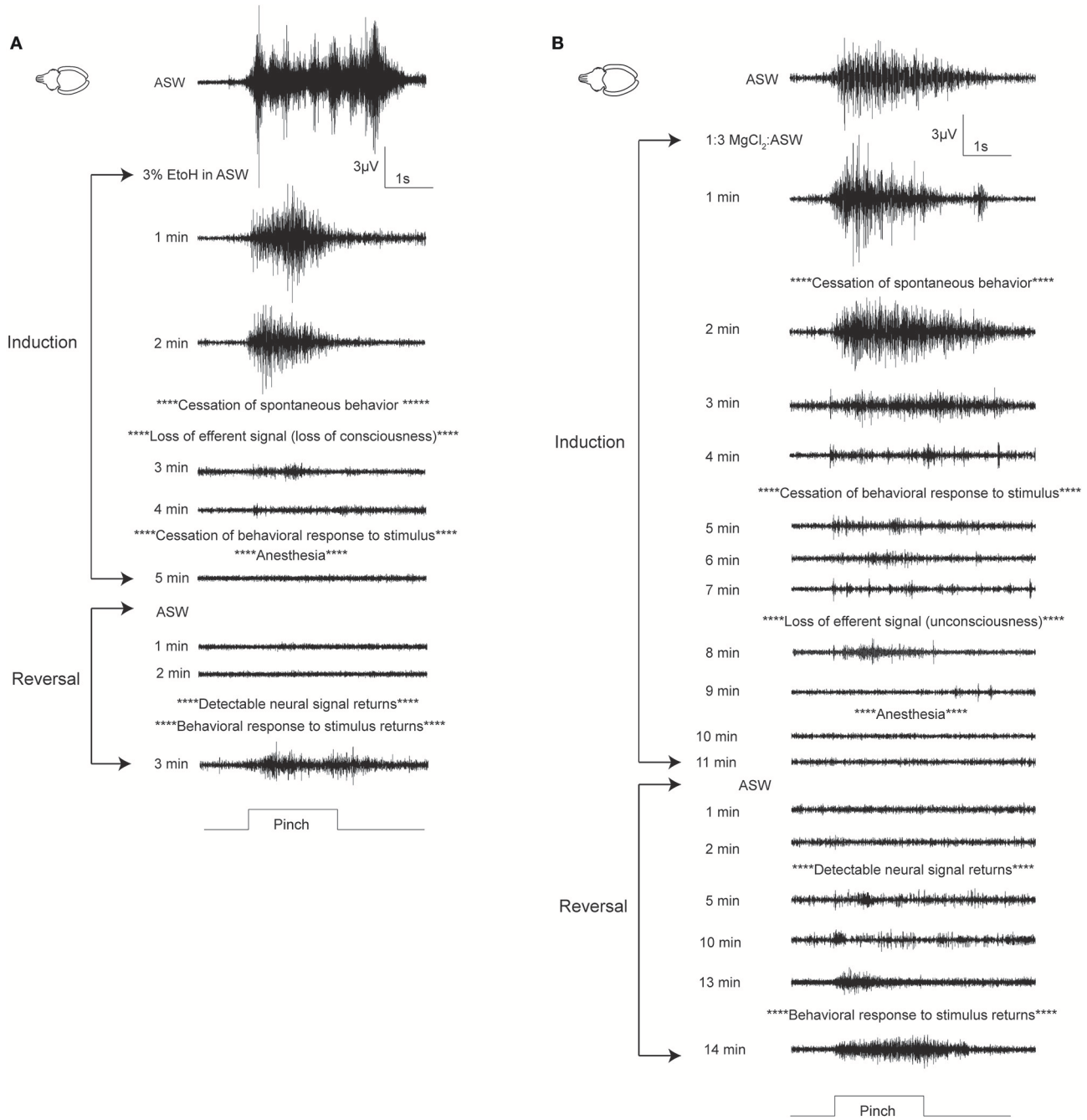

FIGURE 5 | Examples of electrophysiological traces recorded during pinch on the distal stimulations site, for cuttlefish in ethanol **(A)** and $MgCl_2$ **(B)**. **(A)** A fully-awake cuttlefish has a strong response to pinch prior to anesthesia induction. This trace shows motor efferent signal associated with escape jetting (this animal makes 7 jets in rapid succession, coinciding with peaks in the signal). After 2 min in 3% ethanol, the animal ceases spontaneous behavior, and there is no neural signal in response to pinch on the contralateral side to the electrode (trace not shown). Evoked behavior (which may be reflexive or intentional) ceases after 4 min, at the same time that neural afferent signal ceases. Reversal in ASW is rapid, with simultaneous return of evoked behavioral and neural signals. **(B)** Cuttlefish undergoing $MgCl_2$ anesthesia showed a different pattern. While spontaneous and evoked behavior ceased at a similar time to the animal in ethanol in these examples, afferent and efferent neural signal persisted for much longer, and their loss was temporally more separated than for ethanol. Evoked afferent signal in response to pinch was present for more than 10 min after the cessation of evoked behavioral responses; this period is of great concern for welfare. Upon reversal, neural signal returned rapidly, but there was a length period in which behavioral responses remained absent; again, the existence of this extended period is likely to be problematic for researchers using behavioral measures to ascertain anesthesia. Fully awake behavior usually lagged behind return of evoked behavioral responses by more than 5 min.

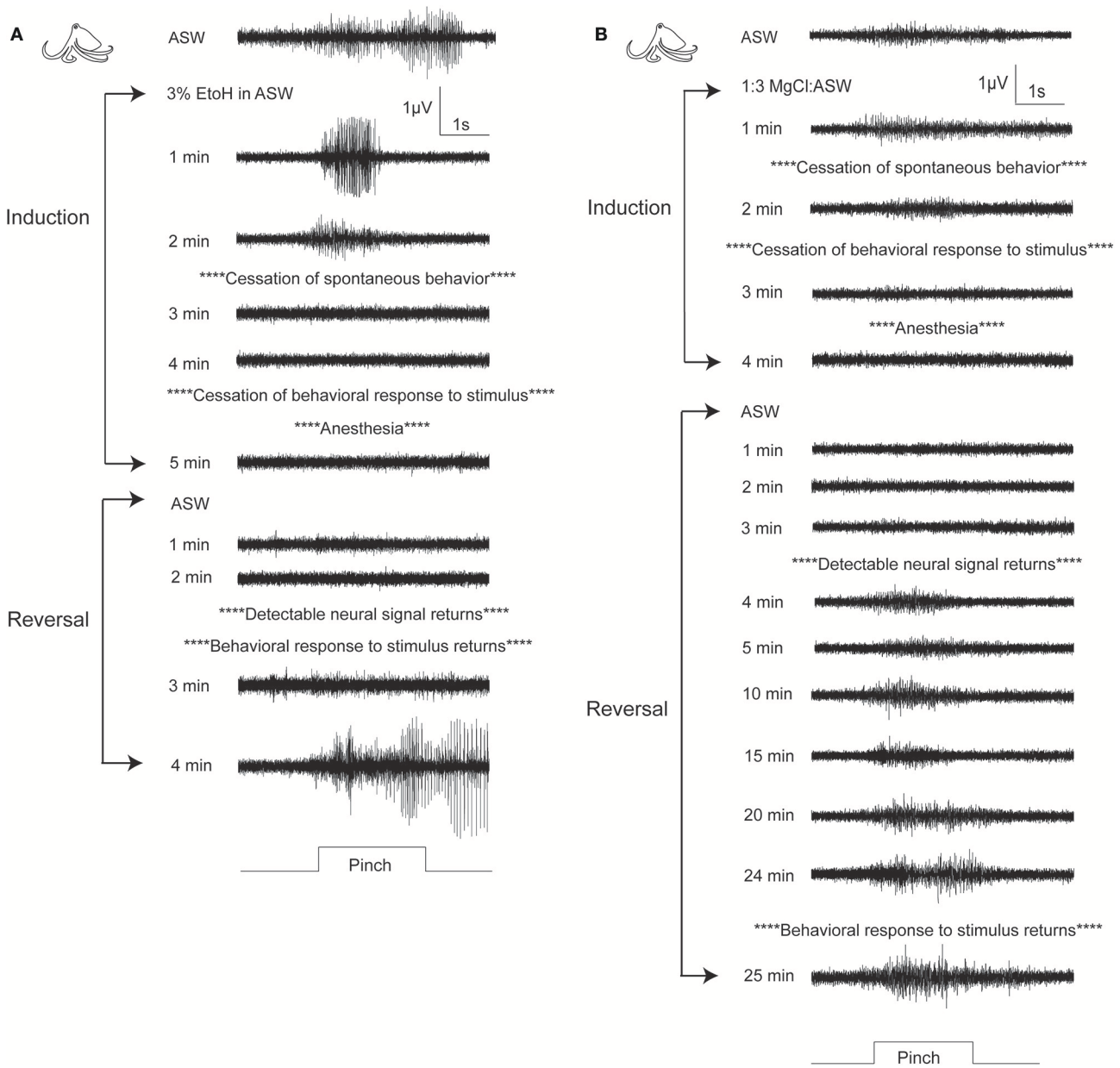

FIGURE 6 | Examples of electrophysiological traces recorded during pinch on the distal stimulation site, for one octopus undergoing ethanol **(A)** or $MgCl_2$ **(B)** anesthesia. **(A)** A fully awake octopus shows strong neural response to pinch. Within 2 min of induction in ethanol, spontaneous behaviors have ceased and neural signal is almost completely abolished. By 5 min there is no neural and no behavioral responses to pinch. Similar to cuttlefish under ethanol anesthesia, reversal is rapid with simultaneous return of neural and behavioral responses to pinch. **(B)** In this example of an octopus in $MgCl_2$, anesthesia induction is uncharacteristically rapid (compare with means shown in **Figure 4**), with minimal delay between loss of behavioral responses and loss of neural responses. We selected this example to show the very prolonged recovery process that was typical of octopuses anesthetized in $MgCl_2$. By 4 min after reversal begins, neural signal is apparent, and it remains strong and mostly unchanging for the next 20 min. There is a slight increase in signal amplitude and duration at 24 min, and the first behavioral response to pinch is recorded at 25 min after reversal begins. During this period there is no behavioral indication that the animal's nervous system is functional. Behaviors indicating that animal is fully awake typically returned 5–10 min after evoked behaviors.

neural signal return was not advanced compared with behavioral signal (**Figure 8B**). In contrast, advances were significantly different from zero for magnesium chloride anesthesia in both test locations (a negative value on the graph indicates neural signal returns earlier than the behavioral signal; medial, $p = 0.032$, distal, $p = 0.03$). Lastly, we plotted the advance of neural signal compared with the re-appearance of spontaneous behaviors (**Figure 8C**), as a measure of the period between when afferents in either location regain their function and when the nervous system, as a whole, regains its function.

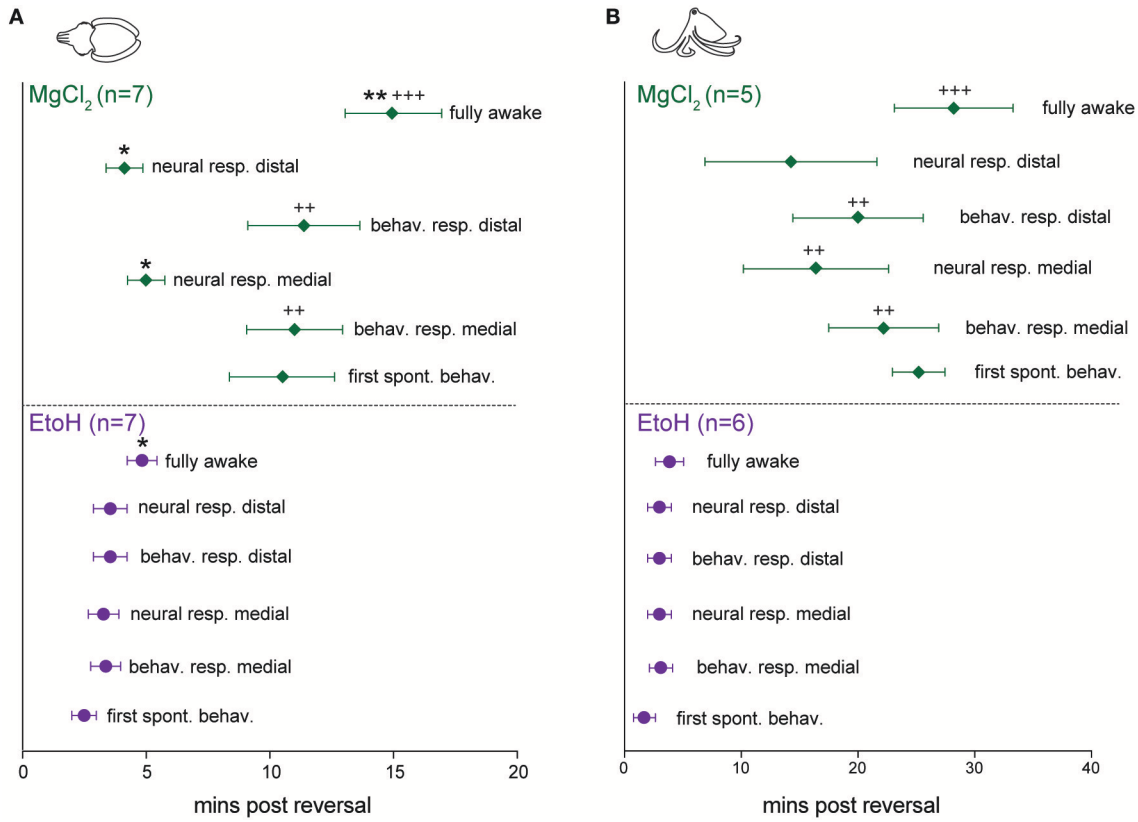

FIGURE 7 | Latencies to behavioral and neural indicators of anesthesia reversal for cuttlefish and octopus (*O. bocki* and *A. aculeatus* pooled). **(A)** Cuttlefish undergoing reversal of ethanol anesthesia (**A**, bottom) showed tight temporal coupling of return of spontaneous behaviors, return of evoked behavioral responses and return of evoked neural responses. Return of neural signal in cuttlefish under magnesium chloride anesthesia (**A**, top) occurred on a similar timescale to that for ethanol, but return of behavioral responses was significantly delayed, both with respect to neural signal in the animals (denoted by *), and with respect to behavioral recovery for ethanol (denoted by +). **(B)** The same variables shown for octopuses. **(B)**, bottom: Octopuses undergoing reversal from ethanol anesthesia showed tight temporal coupling of all reversal indicators. Octopuses undergoing reversal of $MgCl_2$ anesthesia showed significant delays of neural responses at the medial stimulation site, behavioral responses at both sites, and latency to full recovery compared with ethanol-treated octopuses, but within-group, data were highly variable and not significantly different. Within-treatment comparisons: paired *t*-tests, marked with *. Between treatment comparisons: unpaired *t*-tests, marked with +. All *p*-values are two-tailed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

For ethanol this was almost simultaneous; deviations from zero are an artifact of the test procedure because we noted spontaneous behaviors at any time, but only tested neural signal each minute. In contrast, there was a significant advance of evoked afferent signal in comparison to the re-appearance of spontaneous behavior for cuttlefish in the magnesium chloride group ($p = 0.048$).

Patterns were similar for octopuses. During induction (**Figure 8D**), there were non-significant lags between disappearance of evoked behaviors and evoke neural activity for ethanol, but both medial ($p = 0.006$) and distal ($p = 0.012$) stimulation sites showed significant lags in the magnesium chloride group. Upon reversal (**Figure 8E**), there was no advance in the reappearance of neural signal compared with reappearance of behavioral responses to pinch, at either location, for ethanol anesthesia. In the magnesium chloride group, there were substantial but non-significant advances in the return of neural signal compared with the return of evoked behavioral responses. Similarly, there was a substantial but non-significant advance of evoked afferent signal in comparison to the re-appearance of spontaneous behavior for cuttlefish in the magnesium chloride group (**Figure 8F**).

Behavioral Signifiers of Induction and Reversal

We categorized behaviors that were most closely linked temporally (i.e., the last noted behavioral change before neural signal ceased) with the onset of anesthesia and of anesthesia reversal (the behavioral that first occurred either right before or after return of neural signal), for each substance and for each taxon (**Figure 9**). For cuttlefish undergoing ethanol anesthesia (**Figure 9A**), sudden, whole-body paling (**Figure 9E**) was the most frequently occurring behavior in the minute before loss of neural signal was noted. Less frequent was an all-yellow color (**Figure 9F**) In general, onset of ethanol anesthesia was reliably indicated by relaxation of chromatophores. For cuttlefish undergoing

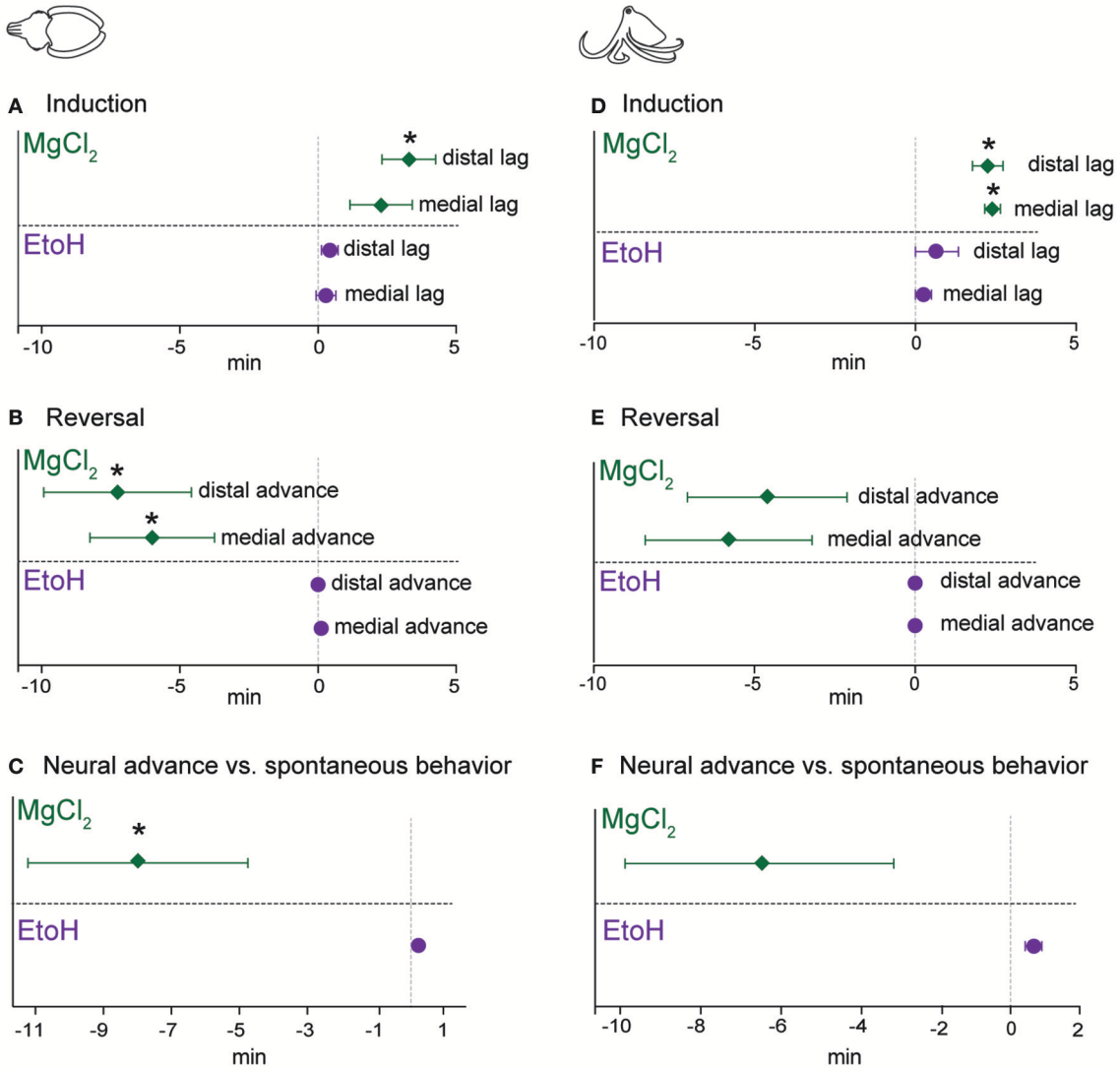

FIGURE 8 | Lag of neural signal loss (compared with behavior) upon induction, and advance of its return (compared with behavior) upon reversal. For each experimental trial, we used the following formula to compute "neural lag" (for induction) and "neural advance" (for recovery). Lag or Advance = (latency to loss/return of neural signal—latency to loss/return of behavioral signal). Thus, a positive value upon induction shows that neural signal persisted longer than behavioral responses did, and a negative value upon reversal shows that neural signal returned sooner than behavioral responses returned. Each outcome is tested with a one-sample *t*-test against an expected value of zero (i.e., neural and behavioral responses are lost or return simultaneously) **(A)** Cuttlefish induced with ethanol had very little very lag, while for those in magnesium chloride group, there was a significant lag at the distal site. Upon reversal **(B)**, cuttlefish in the ethanol group had advances of zero, while both stimulation sites showed significant neural advances for cuttlefish in the magnesium chloride group. We also compared the advance of neural recovery compared with the return of spontaneous behavior **(C)**, here also there was effectively zero advance for ethanol but a significant advance for magnesium chloride. Patterns were largely similar for octopuses. **(D)** Both the medial and distal stimulation sites showed significant neural lags for octopuses in the magnesium chloride group, and although there was some slight lags for the ethanol group, neither was significantly different from zero. **(E)** Upon reversal, there were noticeable but non-significant advances (medial; $p = 0.08$, distal, $p = 0.13$) at both stimulation sites for the magnesium chloride group, but effectively zero advances for the ethanol group. **(F)** Advance of neural recovery compared with the return of spontaneous behavior for octopuses was significant ($p = 0.002$, note that this is in the positive direction, i.e., spontaneous behavior occurred sooner than neural recovery, see results for explanation). There was a long advance for octopuses in the magnesium chloride group, but this was not significantly different from zero ($p = 0.12$), most likely due to the highly variable advances across trials. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

magnesium chloride anesthesia (**Figure 9A**), all-pale was also the most frequent indicator of anesthesia. Occasionally, we noted chromatophore flickering, waving or pulsing instead of paling; we group these various changes under "other color change."

Behavioral indicators of reversal for cuttlefish (**Figure 9B**) were more variable than for induction. The most frequent behavior in both the ethanol and magnesium chloride groups was yellowing (**Figure 9F**). We also observed some animals show a sudden and pronounced change in respiratory rate, or show

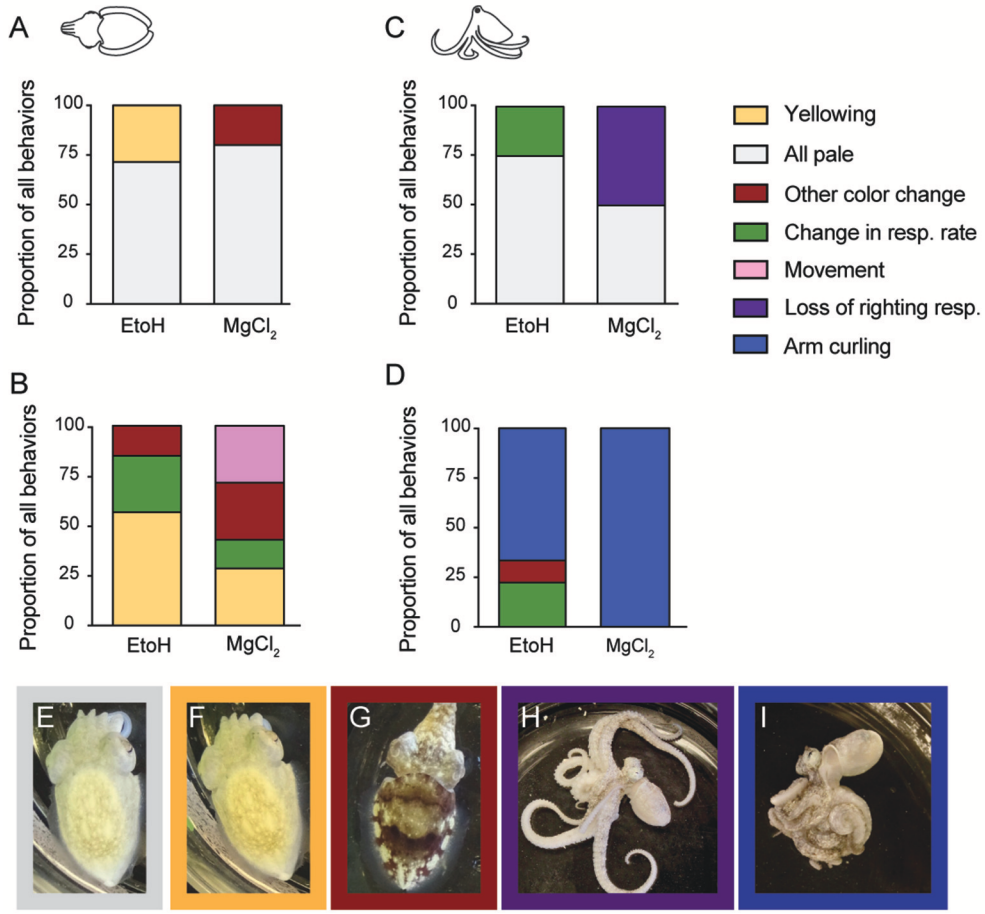

FIGURE 9 | Behavioral signifiers of anesthesia induction and reversal. **(A)** Cuttlefish undergoing ethanol induction most frequently showed an all-pale **(E)** body color as their last spontaneous behavior before loss of neural signal. All-yellow **(F)** was also common for ethanol, and chromtophore pulsing, flickering, or waving occurred in some animals in the $MgCl_2$ group. Upon reversal of anesthesia **(B)**, cuttlefish in the ethanol groups showed all-yellow most frequently as the behavior occurring closest to re-emergence of neural responses to pinch. Other behaviors included increased respiration rate (see **Figure 2** for example), and other color changes **(G)**. In the $MgCl_2$ group, behavioral indicators were more varied, with all-yellow, other color change, movement, and increased respiration noted as the behavior occurring closest to return of neural signal. For octopus inductions **(C)**, all-pale was the most common behavioral sing of anesthesia in both ethanol and magnesium chloride groups. Respiration rate change in the ethanol group and loss of righting response **(H)** in the magnesium chloride group were also frequent indicators. Reversal for octopuses **(D)** was reliably signaled in both groups by a sudden coiling or curling of the arms **(I)**. In the ethanol group, respiration rate changes and color change were also noted.

a strong mottle color pattern (**Figure 9G**). In the magnesium chloride group, spontaneous arm or fin movement was noted in some animals. However, we note that in most cases in the magnesium chloride group, the first behavioral indicator of reversal appeared *after* return of neural signal.

For octopuses, induction with either agent was most frequently signaled by relaxation of chromatophores (paling) (**Figure 9C**). In the ethanol group, the other indicator was a sudden decrease in respiration rate. For magnesium chloride, loss of righting response (**Figure 9H**) was the best indicator of induction in about half of all trials. Reversal of anesthesia in octopus was indicated in almost all trials by a very distinct and abrupt tight coiling of all the arms (**Figures 9D,I**). This was the universal indicator of reversal for magnesium chloride, but as for cuttlefish, we note that this first behavioral indicator of reversal appeared *after* return of neural signal. For the ethanol group, a sudden increase in respiratory rate or other color change (sudden reappearance of strong mottled pattern) were the other indicators.

Unsuccessful General Anesthetic Agents

We tested a small number of animals with other candidate anesthetic agents: chilled (4°C) ASW ($n = 2$ cuttlefish), 1% diethyl ether in ASW ($n = 2$ octopus, $n = 1$ cuttlefish), and 500 mg/L MS-222 (tricaine) in ASW ($n = 1$ cuttlefish). In chilled seawater, initial exposure produced sustained, high frequency firing (**Figure S6**) and behavioral signs of aversion, including strong withdrawal of the head into the mantle and escape jetting. Although neural signal was lost after around 5 min and animals recovered well in warmed seawater, we concluded that the procedure was likely aversive.

Animals tested in 1% ether showed rapid induction, with all losing neural signal within 2 min of exposure. No animals showed signs of aversion or stress upon induction, however, reversal times were extremely lengthy, characterized by relatively rapid return of afferent signal and evoked behavior, but no further recovery toward normal, spontaneous behavior. One animal showed impaired vestibular function for multiple hours after return to the home tank, but thereafter appeared normal.

We attempted MS-222 general anesthesia on one cuttlefish only. There is no suggested dose for cephalopods, so we first tried 100 mg/L, the standard dose for general anesthesia of fish. This was ineffective, and the animal produced strong escape jetting in response to its introduction. After 2 min, we increased the dose to 500 mg/L, half the effective dose recommended for amphibians. The resulted in respiratory arrest 7 min after introduction, and no apparent effect on afferent neural signal. Despite a lengthy period of manual respiration in aerated, flowing seawater, the animal did not recover.

Local Anesthesia

We tested two candidate local anesthetic drugs on cuttlefish; 0.1 mL lidocaine (0.5%, $n = 5$), and 0.1 mL isotonic magnesium chloride ($n = 4$). Both drugs provided highly effective blockade of sensation, with average time to complete absence of evoked neural signal of 3 min 20 $\pm$ 46 s for lidocaine, and 2 min 15 $\pm$ 38 s for isotonic magnesium chloride (unpaired t-test, $p = 0.34$). Absence of neural signal persisted until recordings were completed (11 $\pm$ 4 min after injections, $n = 9$), but we did not test durations beyond. Both drugs produced changes in chromatophore activity over the area of infiltration (**Figure 10**).

Euthanasia

We tested four octopuses and one cuttlefish using a standard euthanasia protocol—immersion in 330 mM $MgCl_2$ followed by decerebration (**Figure 11**). Octopuses took around 5 min until complete respiratory arrest, but this was often preceded by 1–2 min of extremely slow respiration of around 3–4 breaths per minute. Five minutes after breathing had ceased (and an average of 7 min after neural signal had ceased), we began decerebration while monitoring signal on the pallial nerve. We observed no behavioral, reflexive or neural responses to skin incision or to surgical destruction of the brain.

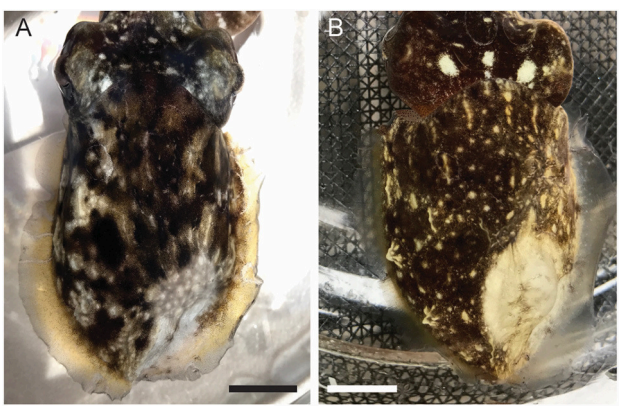

FIGURE 10 | Representative images of the distal mantle of a cuttlefish injected with **(A)**. 0.1 mL of 0.5% lidocaine in ASW, and **(B)**. 0.1 mL of isotonic (330 mM) magnesium chloride. Images taken 20 min after injection. Scale bars 10 mm.

DISCUSSION

Here, for the first time, we provide conclusive evidence that both ethanol and magnesium chloride are effective anesthetic agents for three species of tropical cephalopods. We expect that these results should be applicable to many other cephalopod species, thus providing new assurance to researchers, institutional animal care bodies and legislative agencies charged with protecting the welfare of cephalopods in research. Although the species that we tested were tropical, we anticipate that effective anesthesia in temperate species should be readily achievable using these methods.

The two substances differed markedly in the temporal characteristics of their effects. Induction and reversal in ethanol was rapid and was indicated reliably by behavioral changes that were tightly temporally coupled with loss and return of afferent neural signal. In contrast, anesthesia in magnesium chloride was considerably delayed compared with immobility. Even though neural signal was blocked reliably in all trials, there were no behavioral indicators that were tightly coupled with anesthesia. Thus, unless researchers are prepared to monitor neural signal directly, ascertaining anesthesia depth with magnesium chloride anesthesia is likely to be almost impossible.

Magnesium chloride and ethanol are generally not accepted anesthetic substances for any taxon other than cephalopods; as such, there is limited knowledge of their mechanism of action in promoting anesthesia-like states. Magnesium chloride is generally considered to be a muscle relaxant or sedative in vertebrates (Sung et al., 2017), but there is some evidence that is also anti-nociceptive (Kroin et al., 2000; Albrecht et al., 2013; De Oliveira et al., 2013). Ethanol also has analgesic properties and can certainly affect consciousness and produce amnesia in vertebrates (Tamerin et al., 1971; Messing, 2014; Thompson et al., 2017), but its molecular targets or mechanisms in cephalopods are not clear. Despite their differing effects and uses in more typical vertebrate models of anesthesia, for both substances we show conclusively that while they are certainly effective at producing immobility and sedation, they are also effective at blocking both afferent and efferent neural signal. This effect does not appear to be due to hypoxia resulting from respiratory depression (although we note that we did not monitor hemolymph oxygen content, and this cannot completely exclude the possibility that hypoxia contributes to the anesthetic effects we observed) or from damage to the neurons themselves; thus, in cephalopods at least, these agents act as "functional" anesthetics that are readily reversible and from which animals recover with no apparent ill effects.

We also show that several other agents are unsuitable as general anesthetics for cephalopods, although they have utility in other species. We had hypothesized that diethyl ether,

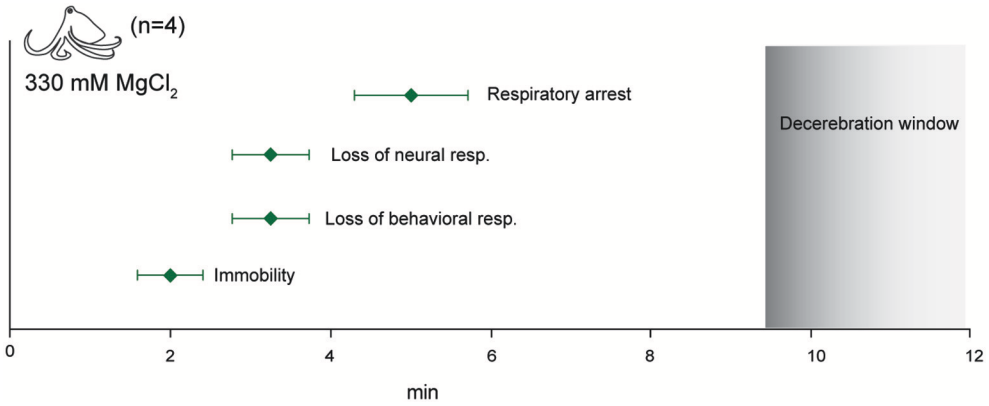

FIGURE 11 | Euthanasia. Immersion in isotonic (330 mM) magnesium chloride resulted in rapid loss of afferent and efferent signal and respiratory arrest. No neural signal was recorded upon skin and cranial incision 5 min after respiratory arrest. This interval is shown as the "decerebration window" where the animal is deeply anesthetized and insensible during destruction of the CNS.

a volatile anesthetic with many shared molecular targets to ethanol (Solt and Forman, 2007), would show induction and reversal characteristics similar to ethanol. While the induction times for ether were similar, reversal was protracted; evidently the unbinding of ether molecules from one of its molecular targets is significantly delayed in cephalopods. In addition to producing delayed reversal times, ether can be hazardous to laboratory personnel and requires special handling, making it impractical as well as unsuitable. We also hypothesized that tricaine methanesulphonate (MS-222), a blocker of voltage-gated sodium channels (Attili and Hughes, 2014; Ramlochansingh et al., 2014), would act similarly in cephalopods as in vertebrates due to the ubiquity of voltage-gated sodium channels in nervous tissue throughout the animal kingdom. Instead, we found no anesthetic effect at a low dose and a lethal effect at an intermediate dose, although this was tested only on one animal.

We tested cold water as an anesthetic agent because it had been reported to be an effective adjuvant to other substances in some studies (Andrews and Tansey, 1981), and because it has been used as a euthanizing method in others (Staudinger et al., 2011). The strong behavioral and neural evidence we observed for its aversiveness (**Figure S5**) indicate that it is not suitable for either anesthesia or euthanasia in tropical species, although we do not rule out that it may be suitable for temperate cephalopods. Further work is needed to identify the precise molecular targets of each of these substances in cephalopods, and to determine their binding kinematics.

While we were successful at demonstrating three aspects of general anesthesia, we did not tests the fourth component, anterograde amnesia (Villars et al., 2004). Cephalopods are highly capable of many different learning and memory tasks, and multiple species show excellent performance in habituation (Agin et al., 2006a; Kuba et al., 2006), sensitization (Crook et al., 2011; Alupay et al., 2014), classical conditioning (Cole and Adamo, 2005; Crook and Basil, 2008) and operant conditioning (Darmaillacq et al., 2004, 2008; Agin et al., 2006a,b; Alves et al., 2007; Zepeda et al., 2017) procedures. We suggest that these validated learning procedures that produce short-term memory after a single training trial could be modified for such a test.

We also tested local anesthesia, which we used in conjunction with general anesthesia in this study for ease of application and to reduce handling stress. While it is possible that some interaction between general and local anesthetics occurred, it is clear that local anesthesia is readily achievable by subcutaneous or intramuscular injection in cephalopods. Local anesthesia use has been reported infrequently in cephalopods, although unpublished observations of the efficacy of several candidate substances (xylocaine, mepivicaine) have been reported (Fiorito et al., 2015), and complete nerve block by isotonic magnesium chloride has been shown previously (Crook et al., 2013, 2014). Lidocaine has been shown to have adverse effects on cultured neurons of gastropods (Onizuka et al., 2005, 2012a,b), but we observed no obvious long-term effects in the animals we tested, however, the injection location in our study would have affected only the peripheral terminals of sensory neurons, and different or adverse results may be observed by applying lidocaine to ganglia.

Cephalopods with localized tissue injury show both local and generalized sensory neuron hyperexcitability after injury (Crook et al., 2013; Alupay et al., 2014; Perez et al., 2017) that is activity dependent; silencing of sensory afferents during injury with any effective local anesthetic agent should therefore produce both local anesthesia and some measure of generalized or systemic analgesia. Although we did not measure the duration of local anesthetic effects in the study, we are confident that they extend beyond the period of general anesthetic reversal. Thus, an animal that has undergone a surgical procedure under general anesthesia without local anesthesia may experience immediate, strong activation of now-sensitized (or spontaneously active) nociceptive afferents (Crook et al., 2013; Perez et al., 2017) immediately upon reversal of general anesthesia. In contrast, cephalopods that received an injectable local anesthetic at the surgical site may have a considerably prolonged period of relief from sensitized nociceptive input. In the absence of validated analgesic agents for cephalopods, applying local anesthetic agents

at the time of issue injury is likely to be a meaningful way of promoting welfare.

We tested one method of euthanasia only, although multiple methods have been reported (Andrews et al., 2013). The procedure we used produced rapid anesthesia and loss of consciousness (no efferent signal), and respiratory arrest that followed rapidly thereafter. We did not monitor heart function directly, therefore it is possible that the heart continued to beat for some period after respiration ceased. We also monitored neural signal only in the peripheral nervous system. Although we observed no neural signal in the pallial nerve during the decerebration procedure, we cannot exclude the possibility that some residual neural activity in the central brain was ongoing at the time of decerebration, or that incision into the brain produced a burst of activity that was not represented in efferent output. We hypothesized that if the animal were consciously aware of the incision either into the skin of the head or into the brain itself, we would have observed activity in the pallial nerve, which carries motor neuron signals to muscles of the mantle that generate escape behavior. However, further study of central brain activity during euthanasia is needed.

Recommendations

General Anesthesia

Both ethanol and magnesium chloride are "functional" anesthetics in cephalopods. Ethanol anesthesia is likely to be easiest to manage; the tight and reliable relationship between behavioral responses and peripheral, afferent neural signal should allow researchers clear indications of depth of anesthesia without the need to monitor neural signal directly. Induction with ethanol was rapid and appeared to cause no adverse behavioral effects. Reliable behavioral indications of sufficient depth are easily observed. At the point where these external signifiers of awareness cease, surgery or other invasive procedures may occur with assurance that the animal's welfare is protected. Upon reversal of ethanol anesthesia, changes in efferent signal typically lagged behind changes in afferent signal and coincided with behavioral responses, thus monitoring behavioral responses such as increased respiration depth, change in color from uniform gray to uniform yellow, and observing the whole body carefully for movement responses to stimulation, should provide good indication of reversal.

We make special note that in octopuses in particular, reversal of ethanol anesthesia may be spontaneous, with breakthrough behavior occurring without changes in the anesthetic concentration; but we found that this did not occur in cuttlefish. Careful monitoring of octopus is necessary, and we recommend that local anesthetic be employed around surgical sites in combination with general anesthesia, prior to incisions being made.

While this relationship is less reliable under magnesium chloride anesthesia we suggest that for some procedures, the long recovery times and better muscle relaxation we observed in this study might allow for extended surgical procedures without the need for additional anesthesia dosing. In no circumstances do we recommend cold seawater, ether or tricaine be used as anesthetic or sedating agents for tropical species.

Local Anesthesia

Both local anesthetics we tested showed very good reliability and efficacy, with no apparent adverse effects on tissue integrity or normal function after recovery. Magnesium chloride was effective at blocking sensation, and the strong muscle relaxant effect provides an easily observed read-out of the infiltration's extent; this paling was less clearly defined, although still present, with lidocaine. However, we caution that the muscle relaxant properties of magnesium chloride make it unsuitable for use at surgical sites. Lidocaine also produced good blockade of sensation, however its infiltration margins were less apparent than for magnesium chloride. Wide injection margins around surgical sites should be used wherever possible. Its less pronounced interference with muscle contraction and its ready availability make it the most suitable of the two agents we tested for regional surgical anesthesia.

Euthanasia

Euthanasia protocols are often dictated by the need for unadulterated tissue harvest post-mortem. We recognize that in such cases, the use of a drug agent for initial anesthesia is undesirable. Likewise, we recognize that intact pieces of CNS tissue are a common requirement of cephalopod research. However, euthanasia that does not involve these restrictions can be humanely and rapidly achieved by immersion of the animal in isotonic magnesium chloride followed by the secondary method of surgical decerebration, as long as at least 5 min have elapsed from the cessation of respiration until the onset of the surgical method of killing. We caution that the animals on which we validated this procedure are small, and that a longer period be used for larger animals.

CONCLUSIONS AND FURTHER WORK NEEDED

We have demonstrated that non-invasive monitoring of neural signal during anesthesia is a highly reliable indicator of anesthetic efficacy. We show conclusively that two commonly-used substances are effective as general, anesthetic agents in cephalopods, and two substances are effective as local anesthetics, but there is considerable work still needed to validate other sedating substances, which may or may not have anesthetic properties (clove oil, Seol et al., 2007; Gonçalves et al., 2012; and isoflourane, Polese et al., 2014, for example), and determine the full duration of local anesthetic effects. A major ongoing challenge for cephalopod research in nations where approval is required for procedures, is the use of long-lasting analgesics whose effects have been thoroughly tested. We expect that with the minimally-invasive and reversible neural recording procedures such as those described here, a range of candidate analgesics could be tested and approved relatively easily.

Magnesium chloride and ethanol are cheap, readily available, require no special equipment to use, and can be used by any experimenter without the need for extensive training. Given that cephalopods in research laboratories

may not be under the direct care of veterinarians, obtaining prescription anesthesia drugs can be challenging for researchers. Thus, the evidence we supply here, that these readily available agents are effective at achieving immobility, loss of consciousness, and blockade of afferent sensory signal, represents a vitally important advance for the field of cephalopod research.

ETHICS STATEMENT

This study was conducted in the USA. It is exempt from US animal welfare regulations, as all animals used were invertebrates.

AUTHOR CONTRIBUTIONS

RC: Designed study, conducted experiments, analyzed data, wrote the paper; HB-S, SB and NJ: Conducted experiments, analyzed data, wrote the paper; HB-S and SB: Contributed equally to this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00109/full#supplementary-material

Figure S1 | Hook electrodes in place, showing position on the pallial nerve. **(A)** Ventral view of cuttlefish (post-mortem), with ventral mantle incised to expose the nerve. In experimental animals no incisions were made. **(B)** In octopuses the pallial nerve is rostral, large, and easily identified close to the mantle margin. This animal is alive and fully anesthetized in ethanol.

Figure S2 | Still image sequence of an unrestrained cuttlefish undergoing progressive ethanol anesthesia and reversal. Timer shows the total time elapsed.

Figure S3 | Still image sequence of an unrestrained cuttlefish undergoing magnesium chloride anesthesia and reversal. Timer shows the total time elapsed.

Figure S4 | Still image sequence of an unrestrained octopus undergoing progressive ethanol anesthesia and reversal. Timer shows the total time elapsed.

Figure S5 | Still image sequence of an unrestrained octopus undergoing magnesium chloride anesthesia and reversal. Timer shows the total time elapsed.

Figure S6 | Electrophysiological traces from a cuttlefish undergoing chilled (4°C) seawater induction. There was an immediate burst of high-frequency firing in response to the cold water, which persisted for at least 5 min after immersion. This experiment was terminated at 5 min.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sense and Insensibility – An Appraisal of the Effects of Clinical Anesthetics on Gastropod and Cephalopod Molluscs as a Step to Improved Welfare of Cephalopods

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Recent progress in animal welfare legislation stresses the need to treat cephalopod molluscs, such as *Octopus vulgaris*, humanely, to have regard for their wellbeing and to reduce their pain and suffering resulting from experimental procedures. Thus, appropriate measures for their sedation and analgesia are being introduced. Clinical anesthetics are renowned for their ability to produce unconsciousness in vertebrate species, but their exact mechanisms of action still elude investigators. In vertebrates it can prove difficult to specify the differences of response of particular neuron types given the multiplicity of neurons in the CNS. However, gastropod molluscs such as *Aplysia*, *Lymnaea,* or *Helix*, with their large uniquely identifiable nerve cells, make studies on the cellular, subcellular, network and behavioral actions of anesthetics much more feasible, particularly as identified cells may also be studied in culture, isolated from the rest of the nervous system. To date, the sorts of study outlined above have never been performed on cephalopods in the same way as on gastropods. However, criteria previously applied to gastropods and vertebrates have proved successful in developing a method for humanely anesthetizing Octopus with clinical doses of isoflurane, i.e., changes in respiratory rate, color pattern and withdrawal responses. However, in the long term, further refinements will be needed, including recordings from the CNS of intact animals in the presence of a variety of different anesthetic agents and their adjuvants. Clues as to their likely responsiveness to other appropriate anesthetic agents and muscle relaxants can be gained from background studies on gastropods such as *Lymnaea,* given their evolutionary history.

Keywords: clinical anesthetics, gastropods, cephalopods, identified neurons, behavior

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INTRODUCTION

Under normal circumstances animals are sensitive to their environment and to changes within it, particularly damaging changes which will elicit fight or flight or withdrawal responses. These capabilities are shared among distant related lineages supporting the proposal that the fundamental mechanisms evolved as adaptive responses to noxious stimuli (Walters and Moroz, 2009;

De Lisa et al., 2012). The role of anesthetics is to render subjects insensible to surgical interventions which would normally be perceived as damaging noxious stimuli. Clinical anesthetics are renowned for their ability to produce unconsciousness in vertebrate species, but their exact mechanisms of action still elude investigators (Herold and Hemmings, 2012; Herold et al., 2017b), for which reason a number of model systems have been studied over the years e.g., lipid bilayers (Herold et al., 2017a,b) and invertebrate systems (Winlow, 1984; Franks and Lieb, 1988). However, on the basis of strong evidence, it is now assumed that general anesthetics have limited effects on the properties of lipid bilayers (Herold et al., 2017b) and that anesthetics have specific actions on membrane proteins, both at the cell membrane (Lopes et al., 1998) and intracellularly (Harris, 1981; Winlow et al., 1995; Moghadam and Winlow, 2000). Ideally anesthetics should have no side effects, act as muscle relaxants, as analgesics and as amnesics as well as rendering the individual unconscious. In practice this is difficult to achieve with a single molecule and a cocktail of molecules (Donohue et al., 2013), including hypnotics for sedation (Meerts and Absalom, 2013) and muscle relaxants (Khirwadkar and Hunter, 2012; Torensma et al., 2016), is used in practice. The muscle relaxants used in the induction phase of clinical or veterinary anesthesia minimize the voluntary and involuntary excitation and struggling that would otherwise be observed during the excitement phase of anesthesia (Guedel, 1937).

Human beings are conscious and sentient beings and the same is assumed to be true in other advanced vertebrates. Thus, it is often assumed that the clinical anesthetics used to diminish human pain and suffering have similar effects on other vertebrates, mammals in particular, and assumptions about their level of anesthesia are based on their behavioral responses (Guedel, 1937). This is not so easy to verify in invertebrate animals which vary enormously in both physical and behavioral complexity and Varner (2012) proposed that invertebrates, apart from cephalopods, do not feel pain. However, there is substantial evidence that advanced crustaceans may feel pain (Elwood, 2012) based on behavioral and physiological responses to noxious stimuli (McGee and Elwood, 2013) and an electrophysiological investigation of methods of complete anesthesia in lobsters and crayfish was recently carried out (Fregin and Bickmeyer, 2016). It may well be that the precautionary principle should be adopted when evidence for sentience is inconclusive for a particular species (Birch, 2017) and under those circumstances the animal should be considered as sentient until proved otherwise.

The presence of nociceptors does not necessarily mean that animals feel pain because pain is presumed to be an emergent property of the conscious brain (Mather, 2008; Fiorito et al., 2014). However, if advanced invertebrates feel pain this suggests that they must have a degree of consciousness and some appreciation of self. This leads us to the question of what is consciousness? At what level of neural complexity do creatures become conscious of themselves as individuals? We cannot yet answer the first of these questions, but clues are beginning to emerge about consciousness in bilaterians other than in deuterostomes such as advanced chordates, and also in the lophotrochozoan cephalopod molluscs (Godfrey-Smith, 2016; Carls-Diamante, 2017) and in the ecdysozoan decapod crustaceans (McGee and Elwood, 2013). If members of these three disparate animal groups are demonstrably conscious and sentient, but with different neurological structures, we need to determine how they converge to generate self-awareness, but we have not yet reached that position. If we are to do so we will need to determine the common characteristics in the brains of advanced molluscs, arthropods and ourselves. To do this it will be necessary to combine elements of neurophysiology and neuroethology with those from cognitive science and to attempt to understand the emergent properties of neural networks at many "levels above the single neuron" (Bullock, 1958). In common with vertebrates, cephalopod molluscs and arthropods in particular exhibit the following common characteristics:

- condensed central ganglia often organized into a central brain centers due to cephalization during evolution. Thus their nervous systems are hierarchically organized with localization of function (Bullock et al., 1977, chapter 1; Sidorov, 2012), but it should be pointed out that the arms of *Octopus vulgaris* contain about two thirds of the 500 million neurons in the nervous system (Sumbre et al., 2001, 2005, 2006) and are semi-autonomous (Yekutieli et al., 2005a,b; Hochner, 2012)
- complex behaviors, problem solving abilities, play like behavior, learning and memory capabilities, and adult neurogenesis (Wells, 1978; Mather, 1991; Boal, 1996; Kuba et al., 2003; Mather, 2008; Gutnick et al., 2011; Hochner, 2013; Di Cosmo and Polese, 2014; Godfrey-Smith, 2016; Bertapelle et al., 2017)
- possess nociceptors and may have a capacity to feel pain (Crook et al., 2013; Alupay et al., 2014; Burrell, 2017).

The implication is that such animals are all likely to feel pain and recent progress in animal welfare legislation reflects this situation with an increased interest in invertebrate welfare (UK Statutory Instruments, 1993; Sharman, 2004; Moltschaniwskyj et al., 2007; European Parliament and European Union, 2010; Elwood, 2011; De Lisa et al., 2012; Andrews et al., 2013; Horvath et al., 2013; Magee and Elwood, 2013; Fiorito et al., 2014; Polese et al., 2014). Thus, it is now imperative that experimental biologists should pay attention to reducing pain and suffering at least in cephalopod molluscs and decapod crustaceans. For this reason we present this review of the actions of local and general anesthetics, mainly on gastropod molluscs, and also the limited available data on cephalopods, with a view to developing improved anesthetic techniques for cephalopods in the future.

WHAT IS AN ANESTHETIC?

As can be seen from **Table 1**, a wide variety of substances have been used as "anesthetics" on gastropod molluscs in the past, largely prior to the introduction of the more common, non-flammable halogenated ethers, and modern systemic anesthetics and analgesics used clinically and in veterinary care. Thus, many substances have been used as "anesthetics," but the most appropriate should have relatively few side effects, rapid actions and be rapidly reversible.

TABLE 1 | A selection of substances used to relax gastropod species prior to surgery or fixation.

Species used	Type of preparation	Substances used as "anesthetics" or "analgesics"	Investigation	Reference
Muricidae (predatory marine gastropods)	Intact animals	Cocaine	Methods for maximal relaxation before fixation or dissection	Lo Bianco and Hovey, 1899
Amnicolidae, freshwater gastropods	Intact animals	Menthol crystals	Method for full relaxation of animals, followed by morphological studies and histological fixation	Berry, 1943
Pomatiopsis spp.	Intact animals	Nembutal for general use but menthol was only partially successful	Relaxing agent for molluscs	Van Der Schalie, 1953
Physa spp, *Bulinus* spp.	Intact animals	Menthol and Chlorohydrate (Gray's mixture)	Relaxation of snails before fixation or dissection	Van Eeden, 1958
Lymnaea palustris, Physa gyrina, Lymnaea humilus, Pomatiopsis lapidaria, Pomatiopsis cincinnatiensis	Intact animals	Menthol for small snails; Nembutal for large snails	Relaxation of snails before fixation or dissection	McGraw, 1958
Muricidae	Intact animals	CO2, tetraethyl monothionpyrophosphate, Sevin (1-naphtyl *N*-methylcarbamate)	Method for full relaxation of animals, followed by morphological studies and histological fixation	Carriker and Blake, 1959
Lymnaea stagnalis	Intact animal	Nitrogen (to remove oxygen from solution), Nembutal (sodium pentobarbitone), MS222, carbon dioxide	A rapid method for anesthetization and recovery	Lever et al., 1964; updates a paper by Joose and Lever, 1959
Various marine and freshwater gastropods including *Lymnaea stagnalis*	Intact animals	Urethane, ether, MgCl2, propylene phenoxetol (1-phenoxy-2-propanalol), a mixture of Nembutal and MS222 were recommended for internal operations, particularly on *Lymnaea*	Methods for anesthetizing gastropods	Runham et al., 1965
Agriolimax reticulatus	Intact animal	Carbon dioxide	Explantation of organs	Bailey, 1969
Helisoma trivolvis	Intact animal	Menthol	Preparation for surgical implantation to study functional regeneration of identified neurons	Murphy and Kater, 1980
Lymnaea stagnalis	Intact animal and semi-intact preparation	Menthol	Anesthesia of animals prior to dissection. Neurons become quiescent, blockage of chemical synaptic transmission, but not electrical synapses. Probable blockage of Ca^{2+} components of action potential.	Haydon et al., 1982
Snails from the genera: *Biomphalaria, Helisoma, Bulinus, Lymnaea, Gyraulis, Anisus,* but not including *Lymnaea stagnalis*	Intact animals	Two variants of the combined use of Nembutal and MS222	Anesthesia prior to injections or transplantation, implantation of various organs	Mutani, 1982
Helisoma duryi	Intact animal	Sodium pentobarbital (Nembutal)	Preparation of animal for surgery	Kunigelis and Saleuddin, 1984

ACTIONS OF CLINICAL ANESTHETICS ON GASTROPOD MOLLUSCS

Studies on gastropods may well give clues as to the effects of anesthetics on cephalopods, such as *Octopus vulgaris,* given their possible evolution from a common ancestor (Morton, 1958; Moroz, 2009; Shigeno et al., 2010), probably during the Cambrian period (Boyle and Rodhouse, 2005). This being so, findings on the actions of anesthetics on gastropods should give some clues as to their likely effects on cephalopods.

Early studies on sedation of gastropods used a variety of compounds (see examples in **Table 1**), but it is noticeable that menthol (Berry, 1943; McGraw, 1958; Van Eeden, 1958) and Nembutal (sodium pentobarbitone) (Van Der Schalie, 1953; Lever et al., 1964; Runham et al., 1965; Mutani, 1982; Kunigelis and Saleuddin, 1984) have been used alone or in combination with other substances for quite some time. Most of these studies were concerned with ensuring that the animals were sufficiently relaxed for fixation or dissection rather than with animal welfare. In later studies, the actions of anesthetics/analgesics on central nervous function were being considered (e.g., Haydon et al., 1982).

It can prove difficult to specify the differences of response to anesthetics of particular neuron types in vertebrates, given the multiplicity of neurons in the CNS, although modern techniques in electrophysiology, particularly patch clamp, applied to brain slices and isolated neurons have gone some way to alleviating the problem. However, gastropod nervous systems such as those of *Aplysia* (e.g., Chalazonitis and Takeuchi, 1966; Chalazonitis et al., 1966; Ascher et al., 1976; Just and Hoyer, 1977; Marty,

1978; Cote and Wilson, 1980; Arimura and Ikemoto, 1986; Ikemoto, 1986; Ikemoto et al., 1988; Komatsu et al., 1996; Winegar et al., 1996; Winegar and Yost, 1998), *Helix* (e.g., Chalazonitis et al., 1966; Chalazonitis, 1967; Judge and Norman, 1982; Akaike et al., 1982) and *Lymnaea* provide us with excellent models for studies on anesthesia given their large identifiable nerve cells and well-studied behavioral repertoires (e.g., Kandel, 1976; Benjamin, 2012; Winlow and Polese, 2014). Since the middle 1980s a substantial body of work has accrued on the pond-snail *Lymnaea stagnalis* (L.) (e.g., Cruickshank et al., 1985b,c; Franks and Lieb, 1988; Girdlestone et al., 1989a,b; Winlow et al., 1992, 1998; McKenzie et al., 1995; Spencer et al., 1995, 1996; Lopes et al., 1998; Hamakawa et al., 1999; Onizuka et al., 2005b; Browning and Lukowiak, 2008; Onizuka et al., 2008a,b, 2012a,b; Yar and Winlow, 2016; Qazzaz and Winlow, 2017; Armstrong et al., 2018) and on related molluscs (*Euhadra* - Onozuka et al., 1993; *Bulla* – Khalsa et al., 1995; *Achatina fullica* – Lin and Tsai, 2005; Lin et al., 2010; *Tritonia diomedea* –Wyeth et al., 2009; *Elysia viridis* – Cruz et al., 2012). Cephalopod molluscs are of course more complex animals than gastropods and have not easily lent themselves to the sorts of study outlined above. However, a recent major breakthrough has established a new neuronal cell culture protocol for *Octopus vulgaris* (Maselli et al., 2018) and promises to allow more detailed studies at a cellular and subcellular level, including direct studies on the actions of anesthetics on Octopus neurons.

Gastropods as Model Systems for Anesthetics Research

In common with many other gastropods, *Lymnaea stagnalis* (L.), with its large uniquely identifiable nerve cells (Winlow and Benjamin, 1976; Benjamin and Winlow, 1981; Slade et al., 1981) (**Figure 1**), makes studies on the cellular, subcellular, network and behavioral actions of anesthetics more feasible than in either vertebrates or cephalopods. Given that *Lymnaea* has a wide range of neurotransmitters similar to those in cephalopods (Tansey, 1979; Di Cosmo et al., 2004, 2006; D'Aniello et al., 2005; Moccia et al., 2009) this makes it a useful testbed for preliminary studies on more advanced molluscs. What is more, *Lymnaea* has a well-researched behavioral repertoire (Winlow and Polese, 2014) so that both the cellular and behavioral actions of anesthetics can be studied. Central pattern generators (CPGs) for feeding (Benjamin, 2012) respiration (Syed et al., 1990; Syed and Winlow, 1991), locomotion and control of pedal cilia via the Pedal A (PeA) cluster neurons (Syed et al., 1988; Syed and Winlow, 1989) and control of rhythmic shell movements (Haydon and Winlow, 1986) have been reviewed elsewhere (Winlow and Polese, 2014). Application of volatile anesthetics can reveal the properties of neurons within the CPG and can relate those changes to behavioral outputs (McCrohan et al., 1987; Woodall et al., 2001). In *Aplysia californica*, enflurane was found to exert both facilitatory and suppressive control over the rhythmic contractions of the gill and siphon when superfused over the abdominal ganglion and a depolarizing shift in gill withdrawal motor neurons was observed (Komatsu et al., 1993). Similar data has been revealed in vertebrate preparations using isoflurane (Jinks et al., 2005) and opioids (Blivis et al., 2007). Further studies by Komatsu et al. (1996), using a semi-isolated preparation showed that enflurane could have a dual effect, most usually suppressing the gill withdrawal reflex, but in other cases facilitating it, suggesting greater complexity in the underlying neural network than previously reported.

Identifiable, isolated neurons in culture are suitable models for studying the cellular and molecular mechanisms of anesthesia under strictly controlled conditions without the intervention of other neuronal elements (Spencer et al., 1995, 1996; Hamakawa et al., 1999). Cultured *Lymnaea* neurons retain their normal action potential types (Yar and Winlow, 1991; Winlow et al., 1991), transmitter identity (Syed et al., 1990; Spencer et al., 1995; Naruo et al., 2005), responsiveness to applied transmitters (Haydon, 1989; Syed et al., 1990) and responsiveness to applied general anesthetics (Spencer et al., 1995, 1996). Substantial research on the effect of anesthetics on neurotransmission in *Lymnaea* was accrued in Syed's laboratory in Calgary.

Pathways to Silence

Halothane, menthol, ketamine, and also sodium pentobarbitone (Nembutal), which is a sedative often used as a veterinary anesthetic, had differential effects on identifiable neurons in *Lymnaea* (McCrohan et al., 1987; Franks and Lieb, 1988; Winlow et al., 1992). In *Lymnaea*, some neurons gradually became quiescent, whilst others exhibited a series of paroxysmal depolarizing shifts (PDS) (**Figure 2** and **Table 2**) prior to quiescence. Eventually all neurons became quiescent (Winlow et al., 1992) except for the tightly electrically coupled neurons VD1 and RPD2 which become silent in halothane and isoflurane, but exhibit PDS after administration of sodium pentobarbitone. (Qazzaz and Winlow, 2015) (**Table 2**). Evidence now suggests that PDS may be due to suppression of calcium activated potassium currents as a consequence of blockade of voltage gated potassium currents which then unmask persistent sodium currents (Pathak, 2017). Such calcium and potassium currents are known to be affected by general anesthetics in *Lymnaea* (see Systemic General Anesthetics below). Differential effects of Propofol and sevoflurane have recently been demonstrated in the ventrobasal thalamus of mice (Kratzer et al., 2017) suggesting that the loss of consciousness associated with different anesthetics are drug and pathway specific in mammals.

Menthol as an Anesthetic

In preliminary behavioral studies on *Lymnaea* a saturated solution of menthol (which is only slightly water soluble) in snail saline (Benjamin and Winlow, 1981) was successfully used as an anesthetic. It had previously been used by Murphy and Kater (1980) to anesthetize the related freshwater snail *Helisoma trivolvis* prior to surgical manipulations, but for experimental work on the central control of behavior it was important to know when the animal had fully recovered from its effects on central identified neurons. This occurred within 40–60 min of washing in normal snail saline (Haydon et al., 1982). In many

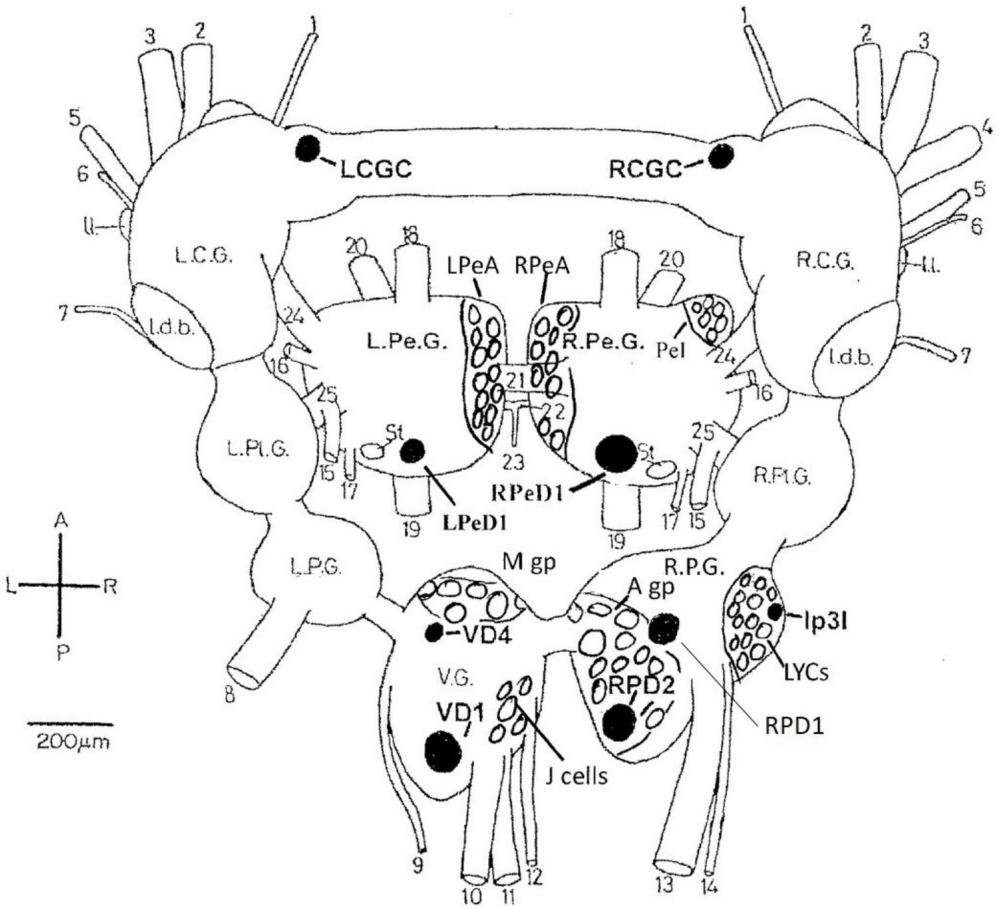

FIGURE 1 | Dorsal view of the central nervous system of *Lymnaea stagnalis* (L.) with the exception of the paired buccal ganglia. The diagram shows locations of individual neurons (black), cell groups and clusters (clear) identified in the text. LCGC and RCGC, left and right cerebral giant cells; RPeD1, right pedal dorsal cell 1; LPeD1, left pedal dorsal cell 1 VD1 and VD4, visceral dorsal cells 1 and 4; RPD1 and RPD2, right parietal; dorsal cells 1 and 2; Ip3I, input 3 interneuron; A gp, right parietal A group; M gp, visceral M group; LPeA and RPeA, left and right pedal A group cells; Pel, pedal I group cells. A, anterior; P, posterior; L, left; R. right. L.C.G. and R.C.G, left and right cerebral ganglia; L.Pe.G. and R.Pe.G., left and right pedal ganglia; L.Pl.G. and R.Pl.G., left and right pleural ganglia; L.P.G. and R.P.G., left and right parietal ganglia: V.G., median visceral ganglion; ldb., lateral dorsal body; ll., lateral lobe; St, statocyst. (1) cerebro-buccal connective: (2) superior labial nerve; (3) median labial nerve; (4) penis nerve; (5) tentacle nerve; (6) optic nerve; (7) nuchal nerve; (8) left parietal nerve; (9) cutaneous pallial nerve; (10) intestinal nerve; (11) anal nerve; (12) genital nerve; (13) right internal parietal nerve; (14) right external parietal nerve; (15) inferior cervical nerve; (16) superior cervical nerve; (17) columellar nerve; (18) superior pedal nerve; (19) inferior pedal nerve; (20) medial pedal nerve; (21) dorsal pedal commissure; (22) ventral pedal commissure; (23) medial columellar nerve; (24) cerebro-pedal connective: (25) pedal-pleural connective.

molluscan neuronal somata inward currents are carried by both sodium and calcium ions (Type 2 action potentials) and menthol suppressed the pseudoplateau during repolarization in these cells. The pseudoplateau is known to be generated by and inward calcium current and appeared to be blocked by menthol. Menthol is now known to have analgesic properties mediated by K-opioid receptors (Galeotti et al., 2002) in mice. It has also been shown to block voltage-dependent sodium channels in rat neurons and human skeletal muscle (Haeseler et al., 2002), where it is said to be as potent as the local anesthetic lidocaine. According to Watt et al. (2008) it shares anesthetic properties with propofol, by its action on $GABA_A$ receptors and more recently Lau et al. (2014) have shown it to modulate $GABA_A$ – mediated tonic currents and associated inhibitory postsynaptic currents (IPSCs) in rat periaqueductal gray neurons.

Volatile General Anesthetics in Clinical Use

Over short period from 1956, when halothane was clinically introduced, the non-flammable volatile anesthetics (Terrell, 2008), which are halogenated ethers, gradually replaced flammable volatiles such as trichloroethylene, diethyl ether and cyclopropane previously in clinical use. Volatile anesthetics are toxic, relatively insoluble, with a low therapeutic index and it is essential to do experimental work in the clinically useful range. For example, halothane is much more soluble in blood with a blood/gas partition coefficient of 2.3 compared with isoflurane, 1.4, and so induces anesthesia less quickly, because, in vertebrates, inhalational agents with low solubility in blood diffuse from the alveoli into the circulation more quickly using smaller quantities of the anesthetic (Harvey

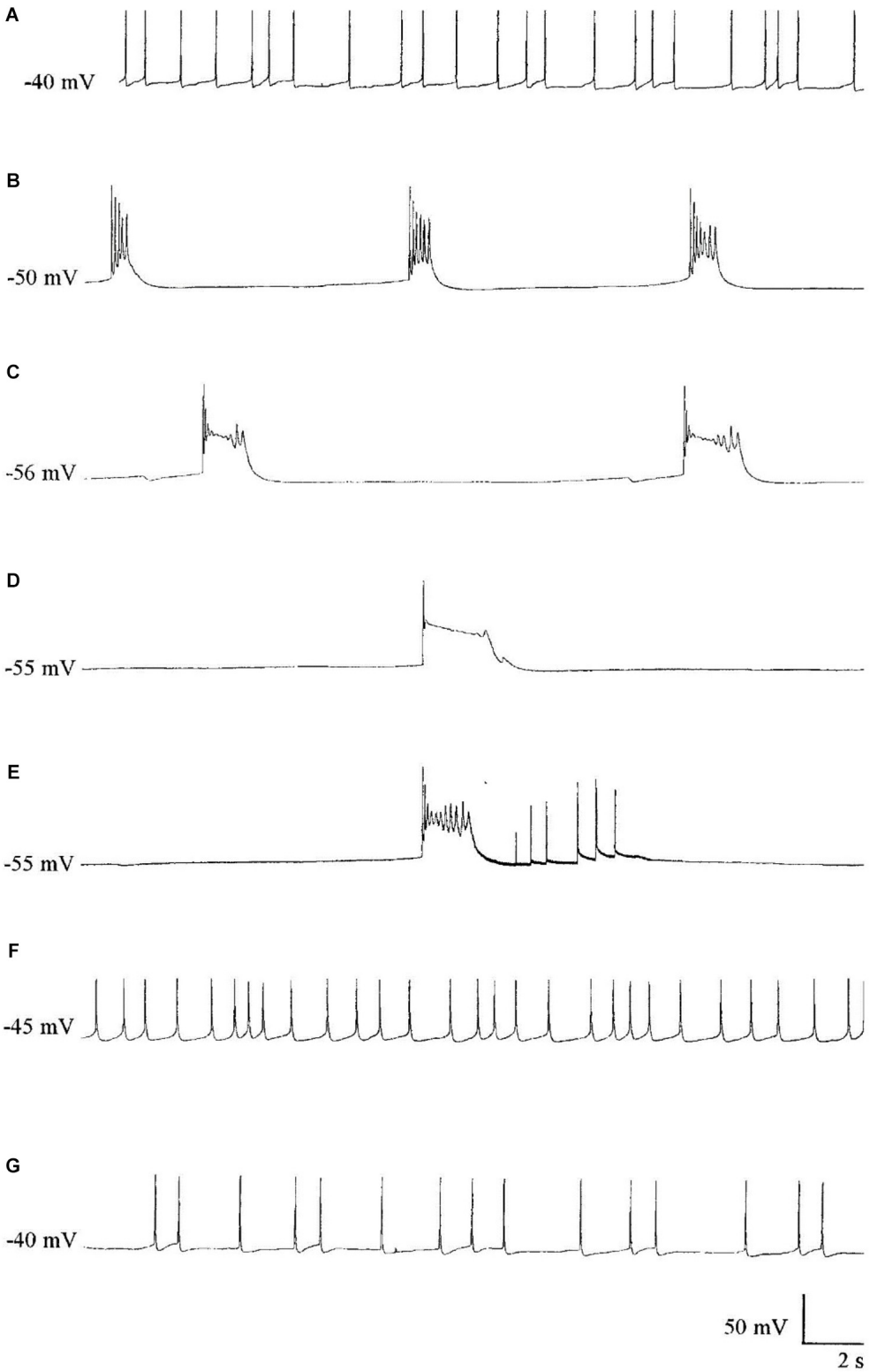

FIGURE 2 | The effect of Pentobarbital on the spontaneous firing pattern and frequency of an A group neuron. **(A)** control; **(B)** 1 mM pentobarbital after 6 min; **(C)** 1 mM pentobarbital after 30 min; **(D)** 2 mM pentobarbital after 6 min; **(E)** 10 min wash out prior to quiescence and **(F)** 30 min wash out and **(G)** 60 min wash out. Membrane potential increased in response to pentobarbital, and decreased as continuous wash out of pentobarbital proceeded. (From Moghadam, 1996 – Reproduced under the Creative Commons License).

TABLE 2 | Differential actions of halothane and sodium pentobarbitone on specific cells and cell groups (see **Figure 1**) in the isolated brain of *Lymnaea*.

	PDS				Quiescence		
Cell type	**A gp**	**J cells**	**M gp**	**VD1/ RPD2**	**RPeD1**	**RPD1**	**VD1/RPD2**
Halothane (n)	8	2	4		4	2	8
Na Pentobarbitone (n)	10	8	13	10	13	11	

Data from Girdlestone (1986) and Moghadam (1996). n, number of cells studied in each case. With the exception of VD1/RPD2, isoflurane did not cause PDS in the other cell types listed above.

and Champe, 1992). However, the effects of volatile agents are more easily controllable than systemically applied anesthetics.

Prior to consideration of the actions of volatile anesthetics on isolated brains (Girdlestone et al., 1989a) and cultured identified neurons (Spencer et al., 1995, 1996), it should be noted that volatile anesthetics are usually applied at body temperature in the operating theater. How then does their application at room temperature affect their concentration, since these volatile compounds are more soluble at lower temperatures than at body temperature? Experiments in Winlow's laboratory were all carried out in the clinical concentration range by careful adjustment of the delivery system combined with measurement of anesthetic concentrations in the experimental dish. A detailed discussion of this issue has been published elsewhere (Qazzaz and Winlow, 2015; Yar and Winlow, 2016), but in short, the work on volatile anesthetics indicates that *Lymnaea* is anesthetized within the clinical range at room temperature.

In order to test whether *Lymnaea* was responsive to modern volatile anesthetics in the clinical range, the whole animal withdrawal response was chosen for study because, as with other animals, protective and escape responses sit at the top of the behavioral hierarchy (Winlow et al., 1992; Winlow and Polese, 2014). Volatile anesthetics are regularly used surgically and their methods of application are well tried and tested. Although in mammals they are delivered via the respiratory system, they are eventually dissolved in body fluids in the lungs and blood stream and therefore the volatile agents were delivered at room temperature direct to the bathing medium of the pond snail. *Lymnaea* respires through a primitive lung via the pneumostome and also across its entire body surface (Mill, 1972) and anesthetics are directly absorbed into the animal from the bathing medium. Experiments with halothane, enflurane (introduced in 1966) and isoflurane (introduced in 1972) showed that the whole-body withdrawal response of *Lymnaea* was dose-dependent within the same clinical range of anesthetic concentrations as man, other mammals, toads and goldfish (Girdlestone, 1986; Girdlestone et al., 1989b). Further experiments on *Lymnaea* have since been carried out successfully with sevoflurane (introduced in 1990) by Morris (1997) and by Syed and his co-workers Thus modern volatile anesthetics are known to be effective in *Lymnaea*, within the clinical range, suggesting that their actions can be generalized to invertebrates.

Effects of Volatile Anesthetics on Membrane Currents

The general findings on the actions of volatile anesthetics on *Lymnaea* and other gastropods are similar to those in other organisms.

Calcium Currents

Using the whole cell clamp technique halothane has been shown to depress high-voltage activated calcium currents and potassium currents in cultured identified *Lymnaea* neurons which are known to retain their normal action potential types (Yar and Winlow, 1991; Winlow et al., 1992). Intracellular recordings from these neurons showed that 1% halothane diminished action potential amplitude, pseudoplateau and after hyperpolarization all of which are calcium dependent phenomena. The effects of four concentrations of halothane were studied on the macroscopic, high-voltage activated calcium channel currents of cultured neurons of the pedal I (PeI) cluster of *Lymnaea stagnalis* (Yar and Winlow, 2016). Following application of increasing concentrations of halothane, in the clinical range, a rapid, reversible and dose-dependent depression of both the peak and end-pulse Ca^{2+} channel currents was observed (**Figure 3**). The rate of inactivation of the calcium current was significantly accelerated by halothane in a dose-dependent manner suggesting that halothane affects the channels both in the open and the closed state. The observations of depression of chemical synaptic transmission, alteration of rate of firing, and alteration in the action potential amplitude, duration and after-hyperpolarization can all be partially explained on the basis of effects of halothane on calcium channels, but volatile anesthetics appear to have multiple targets at the cellular and subcellular levels. Further experiments with isoflurane indicated that low concentrations (0.5%) of isoflurane enhanced the high voltage activated (HVA) current whereas 2% isoflurane significantly decreased it (Moghadam, 1996). In addition, halothane is more potent than isoflurane in decreasing Ca^{2+} currents (Yar and Winlow, 1993). In experiments on the neurosecretory light yellow cells (LYC) (Wendelaar Bonga, 1970; Benjamin and Swindale, 1975) of *Lymnaea,* the effects of sevoflurane were compared with those of halothane and isoflurane on HVA calcium currents (Morris, 1997), revealing that halothane had the most potent effect on peak and end-pulse currents compared with isoflurane and sevoflurane was the least effective.

Potassium Currents

Molluscan neurons have a complicated pattern of outward currents (Reuter and Stevens, 1980) which can be separated

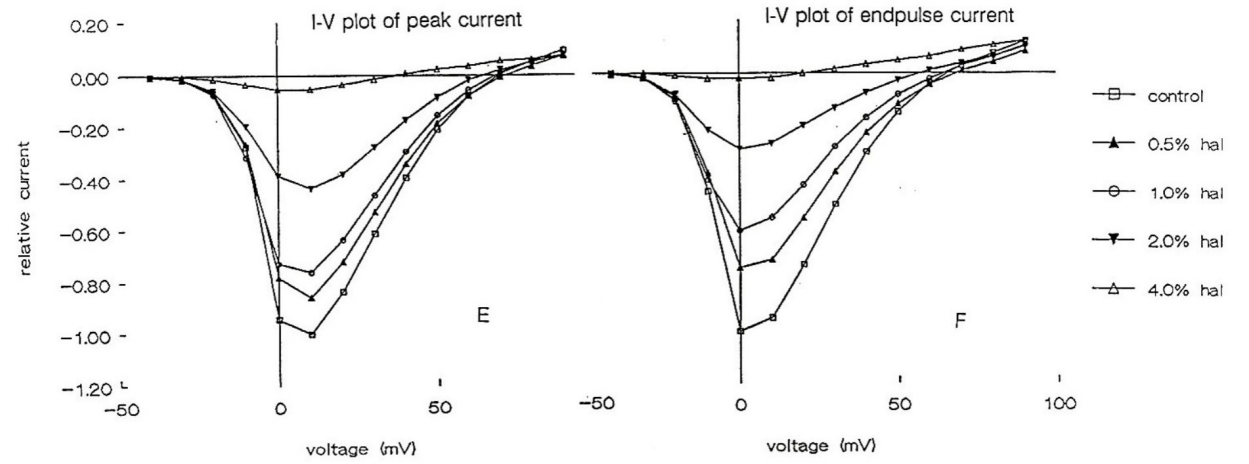

FIGURE 3 | Dose dependent depression of peak current and endpulse current in a PeI cluster cell of *Lymnaea stagnalis*. The Cell was held at a holding potential of –50 mV and 180 ms long depolarizing pulses were applied in 10 mV increments in control (snail saline), 0.5, 1.0, 2.0, and 4.0% halothane. There is a very clear reduction of calcium channel current which is concentration-dependent. The peaks of the I-V curves are not shifted by halothane. (From Yar and Winlow, 2016 – Reproduced under the Creative Commons License).

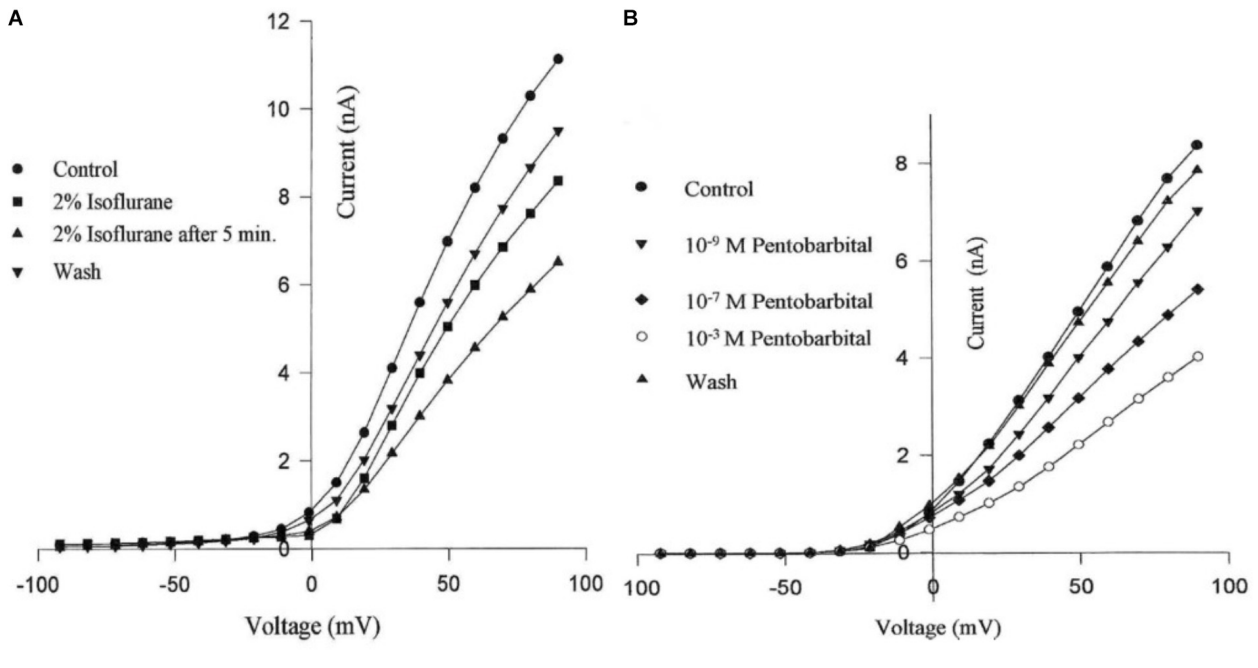

FIGURE 4 | Suppression of potassium currents by both isoflurane **(A)** and sodium pentobarbital **(B)** in cultured pedal I cluster neurons of *Lymnaea*, using the whole cell voltage clamp technique. (From Moghadam, 1996 - Reproduced under the Creative Commons License).

either kinetically or pharmacologically (Meech and Standen, 1974; Heyer and Lux, 1976; Thompson, 1982). K^+ channels are divided into two categories; those activated by the membrane potential and those activated by various modulators (Schwarz and Passow, 1983). Pharmacological separation of K^+ currents on isolated identified PeI cluster cells of *Lymnaea* in culture using the whole cell clamp technique demonstrated that several potassium currents could be identified in PeI cluster neurons: transient current (A current), K_{ATP}, Ca^{2+} dependent K^+ current (I_{KCa}) and the delayed rectifier current (I_k). The ATP dependent current, K_{ATP}, has a low open state probability and is regulated by intracellular ATP and other metabolites (Nelson et al., 1990; Larach and Schuler, 1993). Pentobarbital significantly decreased K_{ATP}, I_{KCa} and I_k in a dose dependent manner (**Figure 4B**) (Moghadam and Winlow, 1995b; Winlow et al., 1995; Moghadam, 1996). Both halothane and isoflurane (**Figure 4A**) depressed gross K^+ currents (Moghadam and Winlow, 1995a), but halothane is more effective than isoflurane in depressing the

gross K^+ current of PeI cluster neurons. Furthermore, halothane partially depressed I_{KCa} as do other anesthetics (Moghadam, 1996). However, many different cell types exist within the nervous system of *Lymnaea* and Lopes et al. (1998) demonstrated the unexpected, but significant finding, that volatile anesthetics activated the novel potassium current $I_{K(AN)}$ first described by Franks and Lieb (1988). This current was not demonstrable in the surrounding Light Yellow Cells (LYCs) (Wendelaar Bonga, 1970; van Swigchem, 1979) in the right parietal ganglion. Similar K^+channels - the serotonin activated S channels - have been observed in isolated, cultured neurons from *Aplysia californica* using patch clamp techniques (Winegar et al., 1996) when treated with halothane or isoflurane. In addition $I_{K(AN)}$ is thought to be activated by certain cytrochrome P450 isoforms (Lopes et al., 1998).

Sodium and Chloride Currents

Many gastropod action potentials have both sodium and calcium components (Type 2 action potentials) as in the giant *Aplysia* neuron R2 from the abdominal ganglion (Geduldig and Junge, 1968) and various neurons from *Helix* (Gerasimov et al., 1964, 1965) and *Lymnaea* (Haydon et al., 1982; Winlow et al., 1982). Chalazonitis (1967) considered the depressive and selective effects of halothane, ether and chloroform on identifiable neurons of *Helix* and *Aplysia* and showed that their somatic action potentials were much more sensitive to volatile anesthetics than were axonal action potentials. Later, Geduldig and Junge clearly demonstrated that both sodium and calcium currents contributed to somatic action potentials of the Aplysia R2 but that the axon spike was only sodium dependent (Junge and Miller, 1974).

Systemic General Anesthetics

Although volatile anesthetics are easier to control than systemic anesthetics, systemic anesthetics may, under some circumstances, be easier to administer. Some of the intravenous anesthetics cause irritation and pain on injection or hangover after recovery or have the potential to produce allergic reactions and histamine release or several effects on the cardiopulmonary system (Reilly, 1994). The ideal intravenous anesthetic would of course induce hypnosis, amnesia and analgesia (Reves and Glass, 1990).

Barbiturates are not stable in solution for more than 24 h (Reilly, 1994) and have relatively short actions (ultrashort acting barbiturates) (Goth, 1976). In addition to anesthesia some of the barbiturates also induce convulsions (Daniell, 1994). Pentobarbital was used for many years as a soporific and anesthetic agent (O'Beirne et al., 1987) and has been suggested to be suitable for anesthesia of *Biomphalaria tenagophila* and *B. glabrata* (Martins-Sousa et al., 2001). Thiopentone is a potent anesthetic which induces anesthesia in seconds, but postanesthetic care is required. Large doses of thiopentone given clinically induce circulatory depression and even small doses cause central respiratory depression (Goth, 1976). Phenobarbitone and barbitone are long-acting barbiturates and are less lipophilic than ultra-short and short-acting barbiturates such as thiopentone and pentobarbital (Bush, 1963). Some of the effects of anesthetics can be accounted for by their solubility in the membrane matrix which causes conformational changes to ionic channels, in accord with the observations of Judge et al. (1979) on identified neurons in the brain of *Helix aspersa*, that thiopentone may have a non-selective action on receptor-coupled ionophores rather than on specific receptors for acetylcholine, glutamate and dopamine and Somei (1981).

Frequency-dependent spike broadening is a characteristic feature of molluscan neurons with mixed sodium/calcium dependent somatic action potentials and pentobarbital and phenobarbitone reduce action potential duration with sedative and anesthetic doses in large multipolar spinal cord neurons (Heyer and Macdonald, 1982). Pentobarbital can reduce L and N type Ca^{2+} currents in a dose dependent manner in cultured mouse neurons (Gross and Macdonald, 1988), and thus might reduce frequency, width and AHP of the action potential. Furthermore, the anticonvulsant/hypnotic agent, phenobarbital, appears to act through depression of frequency-dependent spike broadening on neurons of *Helix aspersa* (Eagan et al., 1987) which could in turn depress excitatory transmission at nerve terminals, possibly a general mechanism for barbiturate actions, and supported by observations of the effects of pentobarbital on *Aplysia* neurons (Ikemoto et al., 1986). It has been suggested that the sodium and calcium currents underlying the spikes are equally sensitive to pentobarbital (Goldring and Blaustein, 1982) in the giant R2 neuron of *Aplysia.* Thiopentone, pentobarbitone, phenobarbitone and barbitone, all accelerated the decay phase of the I_{Ca} in *Helix aspersa* neurons (Nishi and Oyama, 1983a), and pentobarbitone also inhibited its maximum peak amplitude (Nishi and Oyama, 1983b). In *Aplysia* neurons in excised ganglia, both pentobarbital and phenobarbital enhanced spike frequency adaptation via a slowly developing outward current unique to neurons of this type (Cote et al., 1978; Zbicz and Wilson, 1981) and in other neurons they depressed chloride-dependent inhibitory responses to either iontophoretically applied acetylcholine or GABA (Cote and Wilson, 1980) and attenuated excitatory responses while potassium-dependent inhibitory responses were minimally affected. However, patch clamp recordings from the circadian pacemaker cells of the *Bulla* eye showed that pentobarbital reduces the calcium dependent potassium current, probably by reducing an inward calcium current (Khalsa et al., 1995).

The after hyperpolarization (AHP – also termed: SK channels, slow AHP, K_{Ca}, I_{AHP} and the apamin sensitive calcium-activated potassium channel after the bee venom neurotoxin (Moczydlowski et al., 1988; Strong, 1990a,b), which is a Ca^{2+} dependent phenomenon, was reversibly decreased or abolished with pentobarbital (Moghadam, 1996). Furthermore, different AHPs exist in different cell types in *Lymnaea* with respect to their sensitivity to pentobarbital and time dependency (onset of action). The AHP, which occurs after an action potential and is due to a Ca^{2+} activated K^+ conductance (Hoston and Prince, 1980; Schwartzkroin and Stafstrom, 1980), decreased significantly in those *Lymnaea* neurons exhibiting PDS in response to pentobarbital applications (Moghadam, 1996) (**Table 2**). For example, the AHP in M group

neurons disappeared after 24 min, and then PDSs appeared during continuous pentobarbital application. This phenomenon suggests that the response of components of the AHP in these neurons is time dependent with respect to pentobarbital application.

1-phenoxy-2-propanol (PP) (aka: propylene phenoxitol) is a glycol ester that has been shown to have fully reversible anesthetic properties on gastropods including *Hermissenda crassicornis, Tritonia diomedea* and *Lymnaea stagnalis* (Wyeth et al., 2009). PP can be bath applied and is biodegradable. It has been used relax or anesthetize molluscs and other groups since 1955 (Owen, 1955; Runham et al., 1965 - see **Table 1**), but its anesthetic actions have only recently been investigated and Wyeth et al. (2009) have demonstrated that it reversibly eliminates neural activity, acts as a muscle relaxant and eliminates behavior. They suggest that it "is a useful candidate for gastropod anesthesia."

General Anesthetics Raise Intracellular Calcium Concentration

In addition to suppressing both calcium and potassium currents in a dose dependent manner, both halothane and pentobarbitone raise intracellular calcium concentration, $[Ca^{2+}]_I$ (**Figure 5**), from intracellular sources (Ahmed, 1995; Winlow et al., 1995; Moghadam and Winlow, 2000) also in a dose dependent manner, even in the presence of zero external calcium (**Figure 6**). The increase in $[Ca^{2+}]_I$ is unlikely to be due to any sodium-calcium exchange effect as it occurs in the absence of external calcium. Because of their lipid solubility anesthetics must have numerous effects both on the plasma membrane and on intracellular sites. Since calcium stores are likely to vary in extent from one neuron type to the next the resulting levels of free $[Ca^{2+}]_I$ will most likely have effects on membrane permeability as well on other metabolic processes. Similar effects have also been demonstrated in squid axons (Vassort et al., 1986), hippocampal cells (Mody et al., 1991), and mouse whole brain synaptosomes (Daniell and Harris, 1988).

Actions of General Anesthetics on Synaptic Transmission

Chemical Transmission

At chemical synapses neurotransmitter substances are released from nerve cells when a nerve impulse arrives to trigger calcium entry into the synapse, through calcium channels. Experiments on identified cells in the abdominal ganglion of *Aplysia oculifera* suggest that enflurane depresses excitatory and inhibitory cholinergic transmission by reducing postsynaptic currents (Arimura and Ikemoto, 1986), but the postsynaptic responses to ACh were complex: it evoked three non-competitive postsynaptic responses under voltage clamp, a depolarizing response due to increased Na^+ and K^+ conductances, a delayed Cl^- conductance increase and a slow increase K^+ conductance, both causing hyperpolarization. Further research showed that halothane had differential actions on glutamate and acetylcholine induced chloride currents in single mechanically isolated, but unidentified, neurons of *Aplysia kurodai*, suggesting that they had different specific binding sites in the receptor-channel complexes (Ikemoto et al., 1988). The anesthetic also resulted in the decay of epsps and ipsps evoked by stimulation of the pleuro-abdominal connective.

Such studies as those set out above have been invaluable, but it can be very difficult to have unambiguous proof that the experimenter is working with monosynaptically connected neurons within the nervous system. Synaptically connected pairs of neurons in culture resolve this problem and are unequivocally known to be coupled together. The original work in this area was carried out in Puschino (Russia) by Kostenko (1972) and Kostenko et al. (1974) and continued in Kater's laboratory at the State University of Iowa (e.g. Haydon and Kater, 1987) using the pond snail *Helisoma trivolvis*. The neuronal strategies for the formation of chemical synapses were clearly set out by Haydon (1989) where pairs of spherical cell bodies effectively give access to the synaptic terminal. This approach has subsequently been further developed in Syed's laboratory at the University of Calgary using identified, cultured neurons from *Lymnaea stagnalis*. Using these techniques, it has been shown that VD4 can form peptidergic monosynaptic connections cells of the PeA clusters, first demonstrated in the intact brain and then in culture. In both cases halothane induced synaptic depression of the IPSP between the monosynaptically connected cells and enhanced the postsynaptic inhibitory response to exogenously applied FMRFamide in cell culture (Spencer et al., 1995, 1996). This latter finding indicates that depression of transmission probably occurred presynaptically. PeA cells may receive either inhibitory or excitatory connections from VD4 and excitatory peptidergic transmission between cultured *Lymnaea* neurons is more sensitive to depression by halothane than is inhibitory transmission (Spencer et al., 1996). Halothane induces a novel dose-dependent depolarizing response to met-enkephalin on isolated PeA neurons in place of the hyperpolarization, but after washout there was no further response to applied met-enkephalin (Spencer et al., 1998; Winlow et al., 1998, 2000). The mechanisms underlying these modifications are as yet unknown, but peptidergic transmission is clearly susceptible to the effects of volatile anesthetics.

Clinically relevant concentrations of sevoflurane have (1–4%) also been tested on dopaminergic inhibitory synapses between the soma-soma paired *Lymnaea* neurons RPeD1 and VD4 (**Figure 7**) (Hamakawa et al., 1999). RPeD1 ipsps on VD4 were found to be reversibly blocked by 4% sevoflurane and action potentials in both cells were suppressed. Dopamine activated a voltage-insensitive K^+ current in VD4 which was also induced by sevoflurane and was probably analogous to IK(An) (Franks and Lieb, 1991). Since HVA calcium currents were not significantly depressed in RPeD1, it is probable that the K^+ induced presynaptic hyperpolarization reduced the presynaptic transmitter release. Sevoflurane also blocks cholinergic epsps between cultured VD4 and LPeD1 reversibly. In this case presynaptic transmitter release was unaffected, but postsynaptic nicotinic receptors were blocked in a dose dependent manner. However post-tetanic potentiation (PTP), a form of working memory, established in the absence of the anesthetic was not eliminated by it (Naruo et al., 2005).

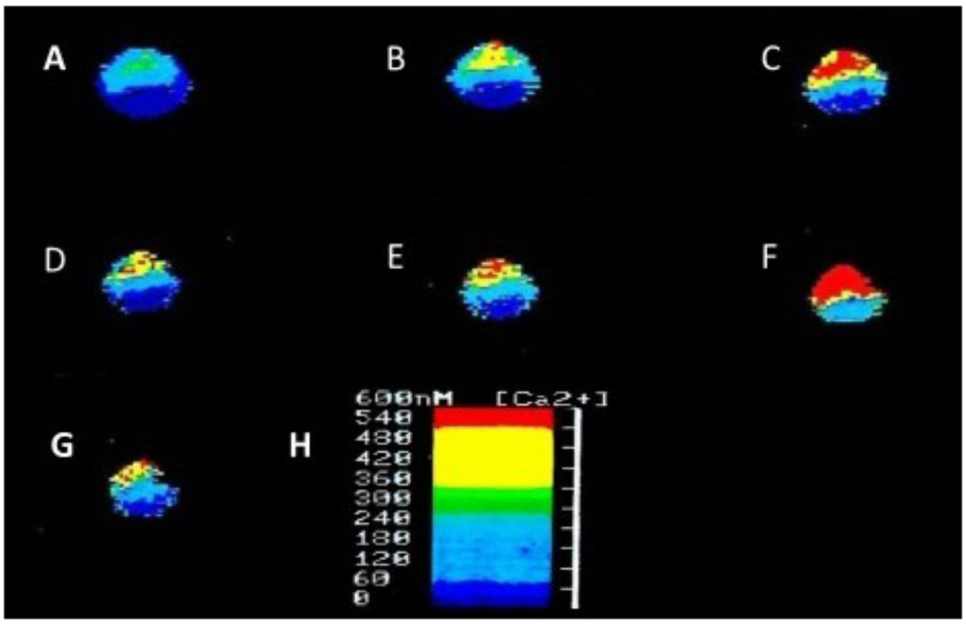

FIGURE 5 | Ratiofluorimetric images showing that Halothane raises intracellular calcium concentration in the cultured *Lymnaea* neurone RPD2 loaded with the fluorescent Ca^{2+} indicator Fura 2. **(A)** Control in normal saline (101.18 nM $[Ca^{2+}]_i$); **(B)** 1 min after addition of 2% halothane (174.12 nM $[Ca^{2+}]_i$); **(C)** 5 min after 2% halothane (287.06 nM $[Ca^{2+}]_i$); **(D)** 10 min after washout of 2% halothane with normal saline (183.53 nM $[Ca^{2+}]_i$); **(E)** 1 min after 4% halothane (232.94 nM $[Ca^{2+}]_i$); **(F)** 5 min after 4% halothane (414.12 nM $[Ca^{2+}]_i$); **(G)** 15 min after washout of 4% halothane with normal saline (211.76 nM $[Ca^{2+}]_i$); **(H)** Fluorescence scale bar (From Ahmed, 1995 – Reproduced under the Creative Commons License).

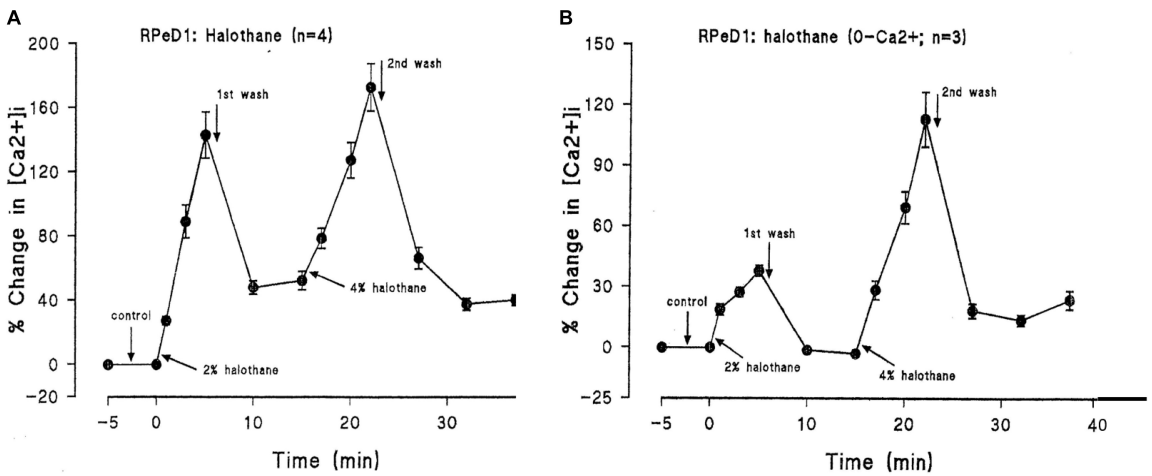

FIGURE 6 | Halothane modifies intracellular calcium concentration in RPeD1, **(A)** when vaporized into normal saline, **(B)** when vaporized into zero calcium saline (From Ahmed, 1995 – Reproduced under the Creative Commons License).

A series of systemic general anesthetics has been applied to identified neurons of *Helix aspersa* to test their action on ACh-induced inhibition using the voltage clamp technique. Thiopentone, etomidate, minaxolone and ketamine all reversibly depressed a chloride-dependent inhibitory response to ACh in a dose-dependent manner, supporting the view that these anesthetics act postsynaptically on receptor coupled ionophores (Judge et al., 1979; Judge, 1980; Judge and Norman, 1980). A group of three or four neurons isolated from the right parietal ganglion of *Lymnaea*, lateral to the light-yellow cells have nicotinic acetylcholine receptors against which McKenzie et al. (1995) tested 31 general anesthetics, observing their half maximum inhibitory concentrations (IC_{50}). The inhalational anesthetics were the most potent inhibitors of the ACh receptor and not dissimilar to their anesthetic potencies on tadpoles.

Behavioral studies following injection of propofol and ketamine caused excitation of intact *Lymnaea stagnalis* (Woodall and McCrohan, 2000). In *Aplysia* neurons it has been suggested

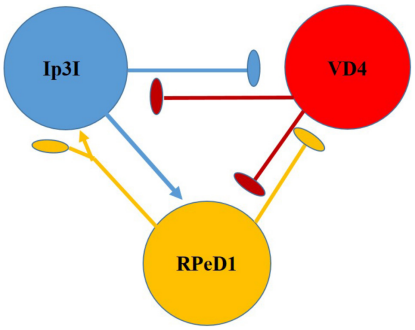

FIGURE 7 | Diagram of the neurons making up the respiratory CPG of *Lymnaea stagnalis*. RPeD1 initiates the RCPG rhythm, VD4 drives inspiration and pneumostome closure, while the input 3 interneuron (Ip3I) drives expiration and pneumostome opening. Ovals are inhibitory synapses and arrows are excitatory synapses. RPeD1 has a biphasic synaptic connection to Ip3I (For further detail, see Syed et al., 1990).

that the dissociative anesthetic, ketamine, depresses cholinergic responses by affecting gating at postsynaptic membranes (Ikemoto, 1986). Ketamine started to be developed in the 1950s and proved potent in analgesia (Mion, 2017), but prolonged exposure may induce neurotoxicity and neuroapoptosis via the PKC/ERK pathway in cultured hippocampal neurons, while activation of excitatory NMDA receptors reverses these effects (Jiang et al., 2018). In some neural stem cell derived neurons, ketamine neurotoxicity appears to be due to NMDA-receptor mediated calcium influx (Wang et al., 2017). Alcohol is also known to amplify ketamine induced apoptosis in cultured cortical neurons and PC 12 cells by down regulation of CREB-related signaling pathways (Zuo et al., 2017). Given the drawbacks of ketamine as a rapid and sustained anti-depressant, its metabolites and other modulatory agents of NMDA receptors are now being studied with a view to the development of less psychoactive substances (Chaki, 2017).

Ketamine has been shown to inhibit long-term memory formation in *Lymnaea*, probably due to its effects on underlying molecular processes, while leaving intermediate-term memory intact (Browning and Lukowiak, 2008). More recently acutely applied ketamine has been shown to significantly decrease synaptic transmission between paired VD4 and LPeD1as well as electrical transmission between PeA neurons *in vitro*, although not in a concentration dependent manner, but it did not significantly affect short-term synaptic plasticity which is thought to underlying short-term, working memory (Woodruff et al., 2015). Earlier experiments on paired VD4 and LPeD1 *in vivo* with propofol showed that the anesthetic blocked synaptogenesis, but not neuronal regeneration. However, synaptogenesis developed several hours after washout of propofol (Woodall et al., 2003).

Electrical Transmission

Electrical synapses are characterized as gap junctions which allow current to flow between cells, but whose conductances may be modulated by chemical synapses or by anesthetics, resulting in modulation of coupling between cells (Winlow et al., 2017). Comparative studies with halothane, isoflurane and pentobarbitone on the isolated brain of *Lymnaea* show that they have very different effects from one another (Winlow et al., 1998; Qazzaz and Winlow, 1999, 2015, 2017). Volatile anesthetics decreased coupling between cells in a dose dependent manner with one notable exception, which requires further research: the very strong electrical connection between the left and right cerebral giant cells whose connection is maintained even in 8% halothane (Girdlestone et al., 1989a). Sodium-pentobarbitone had markedly different effects from the volatile anesthetics. Clinical concentrations of pentobarbitone (100 μM) caused VD1 and RPD2 to become quiescent with no significant change in either their resting membrane potential or the coupling coefficient between them, but at higher concentrations of pentobarbitone both cells exhibited PDS during which the coupling coefficient increased significantly, before quiescence during which the coupling coefficient fell to control values (Qazzaz and Winlow, 2015). A further study on the passive properties of VD1 and RPD2 (Qazzaz and Winlow, 2017) revealed that the differential changes in coupling coefficient were underlain by modifications to input resistance [R_m], membrane time constant [τ_m] and capacitance [C_m] (Qazzaz and Winlow, 2017), presumably due to membrane conductance changes of the type set out in Sections "Systemic General Anesthetics" and "General Anesthetics Raise Intracellular Calcium Concentration" above.

Local Anesthetics

Research into the actions of local and volatile anesthetics on identified, cultured *Lymnaea* neurons demonstrated that clinical concentrations of lidocaine and bupivacaine suppressed neurite outgrowth and drastically reduced synapse formation between soma-soma paired neurons (VD4 and LPeD1) whereas the inhalation anesthetics sevoflurane and isoflurane allowed both neurite regeneration and synapse formation between these cells (Onizuka et al., 2005a).

Cocaine and Its Structural Relations

Local anesthetics are structurally related to cocaine and block voltage-sensitive sodium channels. Most molluscan studies on local anesthetics have been carried out using cocaine itself and procaine, norcocaine, lidocaine, bupivacaine and dubicaine. Norcocaine is a minor metabolite of cocaine and its only confirmed pharmacologically active metabolite. It has been found to have greater potency than cocaine itself when tested against neurons of the visceral ganglion of *Aplysia californica* (Just and Hoyer, 1977) using conventional microelectrode recordings and the phase-plane technique (Winlow et al., 1982; Holden and Winlow, 1984). However, cocaine is still more commonly used. Furthermore, cocaine has been shown to activate an inward calcium current at low concentrations and to inhibit it at higher concentrations in internally perfused neurons of *Lymnaea stagnalis* (Vislobokov et al., 1993). In *Achatina fulica* (Ferussac) cocaine was found to elicit action potential bursts in the identifiable neuron RP4 probably due to its inhibitory effects on the delayed rectifying K^+ current, in addition to

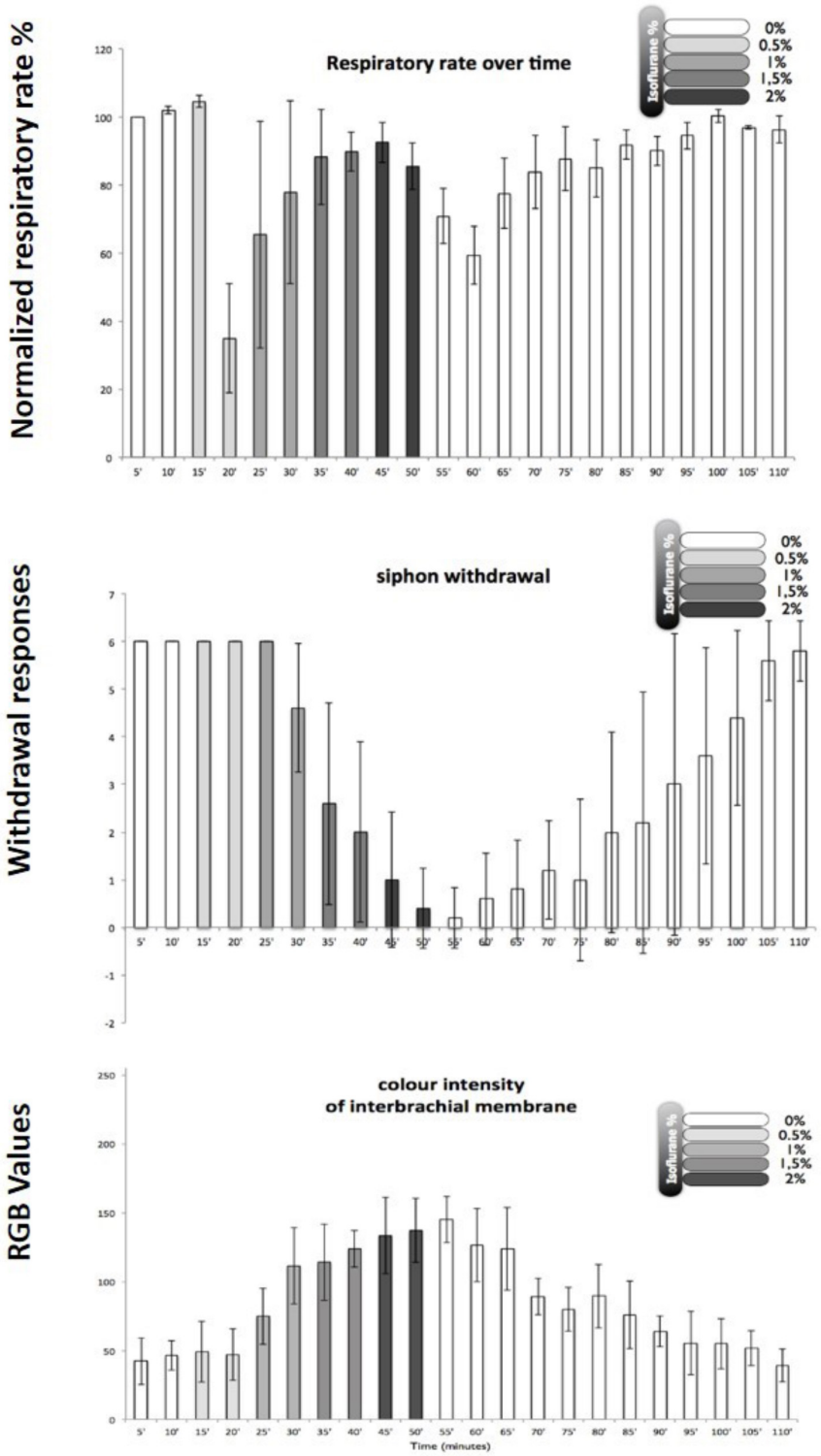

FIGURE 8 | Illustration of behavioral criteria for anesthesia in *Octopus vulgaris*. Effects of isoflurane on respiratory rate **(upper)**, siphon withdrawal **(middle)** and color intensity of the interbrachial membrane **(lower)** in *Octopus vulgaris.* Normalized respiratory rate as determined by the number of mantle contractions in 10 animals at 5 min intervals. The relative strength of siphon withdrawal in response to a touch stimulus (6 = strong, 4 = medium, 2 = low and 0 = none). The color intensity was measured using imageJ software and the RGB model, whereby a zero intensity (value 0) for each component gives the darkest color (no light = black) and full intensity for each component gives white (value 255). Thus the higher recorded value, the paler the interbrachial membrane and *vice versa*. After 50 min the seawater tank was refreshed and the isoflurane flow was switched off. Error bars are standard deviations. (Modified from Polese et al., 2014 – with permission from Journal of Aquatic Animal Health, 2014, Publisher John Wiley and Sons).

which it decreased I_{Ca}, the fast inactivating K^+ current and I_{KCa} (Chen et al., 2006). In intact *Lymnaea*, cocaine was found to inhibit dopamine uptake in isolated snail brains and to impair the extinction of memory, an active learning process, during reinstatement of operant conditioning of the intact animal (Carter et al., 2006).

Lidocaine

Lidocaine (also known as xylocaine and lignocaine) is widely used to block sodium channels to impart pain relief in regional anesthesia and postoperatively, but there are many reports of neurotoxicity following its use in mammals and molluscs, where it can cause an extreme increase in intracellular calcium concentration (Gold et al., 1998; Johnson et al., 2002; Armstrong et al., 2018) and/or damage to plasma membranes (Kanai et al., 2000). Dibucaine and bupivacaine have damaging effects on growth cones (Kasaba et al., 2001). Of seven local anesthetics tested on the morphology of growth cones and neurites of developing, cultured *Lymnaea* neurons, lidocaine was found to be the most toxic (Kasaba, 2007; Kasaba et al., 2003, 2007). Lidocaine has also been found to increase sodium concentration and to promote depolarization and excitation through voltage–dependent sodium channels (Onizuka et al., 2004) of the giant dopamine-containing neuron RPeD1 (Winlow and Benjamin, 1977; Cottrell et al., 1979; Haydon and Winlow, 1981; Winlow et al., 1981), which is part of the respiratory central pattern generator (rCPG) in *Lymnaea* (Syed et al., 1990) (**Figure 7**). Soma –soma pairing of two neurons from the rCPG, RPeD1 and VD4 allowed Onizuka et al. (2008b) to consider the effects of lidocaine on synaptic transmission between mutually inhibitory neurons, both of which were depolarized in a dose dependent manner, resulting in increased spike frequency and action potential broadening as well as dose-dependent decreases in outward potassium currents and inward calcium currents. IPSPs between the two neurons were suppressed at high lidocaine concentrations, promoting excitation of the two neurons. However, lidocaine has also been shown to raise intracellular calcium concentration and to cause morphological damage and shrinkage to cells even in the presence of a calcium chelating agent to prevent the rise in intracellular calcium concentration (Kasaba et al., 2006). This suggests that lidocaine may have a direct effect on cell morphology and shrinkage. Although lidocaine blocks voltage-dependent sodium channels, in common with other local anesthetics such as mepivacaine and prilocaine, it also increases intracellular sodium concentration, $[Na^+]$in, via a Na^+- H^+ exchanger activated by intracellular acidification (Onizuka et al., 2008a). It is suggested that entry of the base form of lidocaine enters the cell thus causing acidification by trapping protons and causing cellular toxicity. Furthermore, Onozuka et al. (1993) have demonstrated that lidocaine suppression of Na^+ current in *Euhadra* neurons is mediated by cyclic AMP dependent protein phosphorylation. Clinical doses of lidocaine have been found to cause necrosis and apoptosis in the cultured *Lymnaea* LPeD1 neuron with a dose-dependent decrease in membrane resistance and an increase in membrane capacitance (Onizuka et al., 2012b).

Using reconstructed *Lymnaea* cholinergic synapses from VD4 to LPeD1 it has been possible to demonstrate that lidocaine suppresses excitatory transmission in a dose dependent manner: presynaptically by voltage-dependent inactivation of the presynaptic membrane due to depolarization and postsynaptically by reducing the response to iontophoretically applied acetylcholine (Onizuka et al., 2008b). By pairing VD4 with neurons from the left pedal E (LPeE) cluster it has also been possible to show that lidocaine treatment during synapse formation permanently suppresses nerve growth factor (NGF) induced excitation by suppressing both axonal growth and neurotransmitter release from presynaptic neurons (Onizuka et al., 2012a) probably by suppressing voltage-dependent calcium currents (Kasaba et al., 2006).

Procaine

Experiments on small neurons of the pleural ganglia of *Aplysia californica*, have shown that procaine blockade of acetylcholine induced depolarizations, similar to those at the frog neuromuscular junction (Katz and Miledi, 1975), and resembles the effects of curare and hexamethonium. Its actions can be accounted for by assuming that procaine binds preferentially to the activated receptor-channel complex and converts it into a non-conducting state (Ascher et al., 1976; Marty, 1978). In *Achatina fulica* (Ferussac), procaine initiated paroxysmal depolarizing shifts (PDSs) in the RP1 neuron, which were decreased if lithium replaced Na^+ in the bathing medium or in a high magnesium solution (Lin and Tsai, 2005), suggesting similar effects to those observed with lidocaine by Onizuka et al. (2012a) (see above). In addition, PDS was associated with phospholipase activity and calcium mobilization in the neuron (Lin et al., 2010).

Opioids and Opiates

In gastropod molluscs endogenous opioid systems are involved in modulation of nociception and analgesic responses (Kavaliers, 1991; Liu, 2008; Miller-Pérez et al., 2008). At a behavioral level the terrestrial pulmonate *Cepaea nemoralis* has been shown to develop tolerance to morphine induced analgesia (Kavaliers and Hirst, 1986), but required specific environmental cues for the tolerance to be expressed, consistent with a classical conditioning of habituation model. The activity of the serotonergic ciliomotor neurons (PeA clusters) of *Lymnaea stagnalis* (Syed and Winlow, 1989; McKenzie et al., 1998) is suppressed in the presence of the opiate antagonist naloxone resulting in a decrease in the rate of ciliary locomotion, whereas opiate agonists such as morphine accelerated the synaptically driven firing of the PeA neurons *in vivo* (D'iakonova, 1998). Voltage clamp studies by Vislobokov and Savos'kin (2000) on the opioid analgesics morphine, promedol, tramadol, and butorphanol indicate that sodium and potassium currents in *Lymnaea* neurons *in vivo* are reversibly inhibited and non-specific leak currents are reduced, thus stabilizing the cell membrane. It has been suggested that morphine and related compounds decrease the efficacy of a presumed cholinergic epsp on cell R15 in the intact abdominal ganglion of *Aplysia californica* by reduction of transmitter release (Tremblay et al., 1974). However, the vertebrate morphine antagonist naloxone did not inhibit the actions of morphine

but decreased the epsp amplitude suggesting that the opiate receptor was not stereospecific. More specific experiments by Waziri (1976) on the identified multiaction neuron L10 and its identified follower cells, some of which it excites and some of which it inhibits, yielded rather different results. The effects of morphine were demonstrated to be postsynaptic by receptor blockade, rather than presynaptic. In *Lymnaea* there is substantial evidence for an enkephalinergic system (Leung et al., 1990), for example the opioid peptide met-enkephalin can modulate membrane potentials and rhythmic activity in its central neurons (Moroz and Winlow, 1993). Met-enkephalin can also enhance electrical coupling between identified neurons in both *Helix pomatia* and *Lymnaea* (Dyakonova et al., 1993) by increasing their input resistance, but these effects of met-enkephalin on the weakly electrically PeA cluster neurons are reversibly abolished by halothane or isoflurane (Qazzaz and Winlow, 1999). Furthermore, the presence of opioid receptors has been proposed in cephalopods by Stefano et al. (1981), and they are believed to play a prominent role in regulation of transmitter release in most invertebrates (Sha et al., 2013).

ACTIONS OF ANESTHETICS ON CEPHALOPOD MOLLUSCS

Given that so much of the early work on the understanding the mechanism of the action potential was carried on the giant axons of the squid *Loligo forbesi* (see review by Hille, 1992, chapter 2) and several closely related species and on synaptic transmission at the squid giant synapse (reviewed by Llinás, 1999), it is most likely that effects of clinical general and local anesthetics on cephalopods are similar to those found in vertebrate and gastropod preparations. The actions of general anesthetics on *Loligo forbesi* have recently been reviewed by Armstrong et al. (2018), but briefly all anesthetics were found to diminish axonal potassium and sodium currents (Shrivastav et al., 1976; Haydon and Urban, 1983, 1986), with a resultant slight depolarization of the membrane (Haydon and Simon, 1988) and recognition of a highly anesthetic sensitive, voltage independent K^+ conductance in the resting squid axon (Haydon et al., 1988; Elliott et al., 1989; Hendry and Haydon, 1991). The actions of clinical concentrations of different anesthetics had variable effects on action potential threshold (Haydon and Simon, 1988). Experiments with barbiturate anesthetics revealed that both thiopentone and pentobarbital also suppress Na^+ and K^+ conductances (Sevcik, 1980) and ketamine appears to suppress both peak transient and steady state Na^+ conductances and is suggested to induce Na^+ accumulation inside the cell which might account for reduction of peak transient current (Shrivastav, 1977). With regard to local anesthetic actions on the squid giant axon, lidocaine and its derivatives appear to reduce the Na^+ current by inactivating a receptor within the Na^+ channel (Narahashi and Frazier, 1975; Cahalan and Almers, 1979a,b; Bekkers et al., 1984) and the drug molecule is trapped by the activation gate of the channel (Yeh and Tanguy, 1985), in accord with Judge et al. (1979), Judge and Norman, 1980 on gastropods.

The giant fiber system of *Loligo* was first described by Young (1939). Although the system is not found in Octopods, the giant fiber system has proved to be such a good model for the basic working of neural signaling and synaptic communication, there is little doubt that it is also a good basic model for the functioning of the Octopus nervous system, particularly as cephalopods in general provide good models for understanding the neural substrates underlying behavior (Young, 1964; Williamson and Chrachi, 2004).

Anesthetizing Cephalopods

Ever since 2013 700 species of cephalopods have had the same legal protections as vertebrates under the European Union Directive 2010/63/EU which means, among other things, that "*objective criteria need to be developed to identify signs of suffering, distress and lasting harm particularly in the context of their induction by an experimental procedure*" (Fiorito et al., 2014). Previous attempts to anesthetize cephalopods have used a variety of substances (see Gleadall, 2013) including muscle relaxants such as magnesium chloride (Messenger et al., 1985; Mooney et al., 2010; Gonçalves et al., 2012; Gleadall, 2013; Butler-Struben et al., 2018) and low temperatures (Andrews and Tansey, 1981), resulting in paralysis of the animals rather than anesthesia. Even ethanol has been used for short duration tagging of the oval squid (Ikeda et al., 2009). A recent study by Gonçalves et al. (2012) compares several different agents commonly used to immobilize *Sepia officinalis* and indicates that hypothermia causes severe stress reactions during the recovery phase while recommending that $MgCl_2$ is an appropriate "anesthetic" agent causing little stress to the animal under these conditions. However, $MgCl_2$ is not an anesthetic agent, and neither of these treatments is acceptable as a form of anesthesia because a loss of consciousness cannot be assumed in the absence movement without detailed analysis of behavioral signs of anesthesia. Additionally, cephalopods are relatively large animals and it is unlikely that immersion in magnesium chloride would have rapid central effects as previously asserted by Messenger et al. (1985). Other muscle relaxants used in cephalopods include gallamine (Flaxedil), and MS-222 (tricaine methanesulfonate or mesylate) (Frazier and Narahashi, 1975; Mooney et al., 2010). MS-222 is used as a muscle relaxant in cephalopods and as an anesthetic and sedative in fish (Palmer and Mensinger, 2004), but has only been shown to act in a similar way to local anesthetics (Frazier and Narahashi, 1975), depressing peak sodium current and steady state potassium currents, but tending to depolarize the axon. While muscle relaxants may have a role in anesthetic induction procedures, none of these substances have anesthetic actions in themselves. Gallamine application to the squid *Doryteuthis peleii* resulted in death of the animals as did the local anesthetic, benzocaine, and the analgesic, clove oil (Mooney et al., 2010). The intravenous anesthetic urethane (ethyl carbamate) was used to anesthetize cephalopods in the past (Messenger, 1968; Young, 1971), but fell into disfavor when found to be carcinogenic and endangered laboratory personnel (Field and Lang, 1988). Ethanol is also unsuitable as it tends to cause the escape behaviors of jetting and inking on initial immersion of the animal (Andrews and Tansey, 1981; Lewbart and Mosley, 2012) although this

phenomenon is not always reported (Froesch and Marthy, 1975; Harms et al., 2006). Mooney et al. (2010) described similar phenomena when *D. peleii* were placed in ethanol as well as dramatic color changes not normally seen in the animal.

Magnesium Chloride as a Likely Adjuvant to Anesthesia

Although $MgCl_2$ has been suggested as an "anesthetic" for *Octopus vulgaris* by several authors (García-Franco, 1992; Pugliese et al., 2016; Butler-Struben et al., 2018), it is a muscle relaxant which renders the preparation immobile but cannot ensure that the animal is pain free and there has been substantial controversy on this issue (Lewbart and Mosley, 2012). In support its action as a neuromuscular blocking agent (NMBA), it is now clear that pre-treatment of isolated rat phrenic nerve-hemidiaphragm preparations with $MgCl_2$ prior to blockade with rocuronium reduces the efficacy of potent NMBAs such as sugammadex (Sung et al., 2017), while also decreasing the time to onset of neuromuscular blockade induced by rocuronium (Rotava et al., 2013). It should, however, be noted that magnesium sulfate proved inadequate for procedural sedation when combined with ketamine in a recent randomized clinical trial (Azizkhani et al., 2018). Magnesium salts have been used as an adjunct to clinical anesthesia (Elsersy et al., 2017; Elsonbaty and Isonbaty, 2017), and may attenuate vincristine-induced neuropathic pain (Bujalska et al., 2009) and chronic diabetic neuropathic pain (Rondon et al., 2010) in rat models, where they have also been found to reduce inflammatory pain (Srebro et al., 2018), probably due to the blocking action of Mg^{2+} on NMDA receptors. Such receptors are well known both in cephalopods (Di Cosmo et al., 2004, 2006) and gastropods (Moccia et al., 2009). Thus, it is probable that magnesium chloride acts as a muscle relaxant and as a mild central sedative analgesic (James, 1992; De Oliveira et al., 2013). External application of $MgCl_2$ should diminish the early excitatory phase of anesthesia, well known in mammals, humans (Guedel, 1937), in *Lymnaea* (McCrohan et al., 1987) and *Octopus vulgaris* (Polese et al., 2014), but not normally observable in current clinical practice due to the use of muscle relaxants. Thus, $MgCl_2$ may be useful as an adjunct to anesthesia.

Anesthetizing *Octopus vulgaris*

As described above, the gastropod *Lymnaea stagnalis* has proved to be a useful model for the study of anesthetics and the clinical inhalational anesthetics halothane, enflurane and isoflurane have all been used to induce anesthesia in *Lymnaea* (Cruickshank et al., 1985a,b,c; Girdlestone et al., 1989b; Winlow et al., 1995, 1998). In a recent paper the inhalational anesthetic isoflurane was chosen to develop an appropriate protocol for anesthetization of *Octopus vulgaris* (Polese et al., 2014) because it is very stable (Ebert, 2006) reliable, easily available, relatively inexpensive, and "can produce adequate muscle relaxation for any surgical procedure" (Eger, 1981). The first response to applied anesthetic in *Octopus* is the defense posture and the animal becomes hyperexcitable (Polese et al., 2014), but preliminary experiments in Di Cosmo's Laboratory suggest that $MgCl_2$ limits hyperexcitability thereby enhancing the actions of isoflurane.

Behavioral Criteria for Anesthesia in *Octopus vulgaris*

In order to develop a method for induction of isoflurane anesthesia of *Octopus vulgaris*, it was necessary to set appropriate practical criteria for observing changes in the animal's physiology and behavior. The most obvious physiological criterion to observe was the respiratory rate as judged by the frequency of respiratory pumping particularly as most clinical anesthetics depress minute ventilation in mammals (Berge and Warner, 2000). In addition, two behavioral tests were applied: (i) the withdrawal response to stimulation of the arms and siphon because withdrawal responses are used to test for depth of anesthesia in humans and other animals and are diminished in a dose-dependent manner by inhalational anesthetics in the pulmonate mollusc *Lymnaea stagnalis* (Girdlestone et al., 1989b); (ii) color change, which is common in cephalopods and is known to be under central motor control. Using these criteria, it was found that in octopuses the best approach to induction of anesthesia was to slowly increase the isoflurane concentration from 0.5% v/v to 2.0% v/v over a period of about 40 min. Using these criteria, *Octopus* were anesthetized with isoflurane, again in the clinical range. Different animals of the same size responded with similar behavioral changes as the isoflurane concentration was gradually increased (**Figure 8**). After gradual application of 2% isoflurane when all the responses indicated deep anesthesia (i.e., minimal withdrawal responses, pale color, but maintained respiratory rate after an initial reduction – see **Figure 8**)[1], the animals recovered within 45–60 min in fresh aerated sea water. Based on previous findings in gastropods, we believe that the process of anesthesia induced by isoflurane is similar to that previously observed in *Lymnaea*.

Further studies on anesthetic mechanisms in a range of cephalopods are necessary and clues as to their likely responsiveness to other anesthetic agents might be gained from background studies on other molluscs such as *Lymnaea*, where probable cellular targets for anesthetic actions on behavior can be identified (Rozsa, 2000; Winlow and Polese, 2014). It is likely that the best and most easily controllable anesthetic agents are likely to be volatile anesthetics which have been shown to work well on gastropod molluscs as well as on vertebrates.

Inappropriate Anesthetic Agents for *Octopus vulgaris*

Preliminary experiments indicate that neither midazolam nor propofol are suitable agents for anesthetizing *Octopus vulgaris* when bath applied. It may be possible to administer them systemically by injection after use of a muscle relaxant, but this approach is much less controllable than the use of volatile anesthetics which are easily removed from the bathing medium. Nor do we recommend the use of local anesthetics without further detailed study. Midazolam, like other benzodiazepines, is believed to interact with the gamma-aminobutyric acid (GABA)-benzodiazepine receptor complex to prolong its inhibitory actions. It may be used as an anesthetic, particularly in

[1] http://www.sciencemag.org/news/2014/11/video-how-anesthetize-octopus

children, and also acts as an amnesic. It may also be used to pre-medicate patients before general anesthesia. However preliminary experiments indicate that midazolam does not anesthetize *Octopus vulgaris*, even at high concentrations, but on the contrary causes a general increase in excitability, including escape behavior, chromatophore flashing and eventually inking. Thus, midazolam is inappropriate as either an anesthetic or for pre-anesthesia of *Octopus*. Propofol immersion is inappropriate because with a gradual increase in propofol concentration over about 20 min, the respiratory rate at first stayed constant, then declined, became very unstable and dropped to zero at which point the animals ($n = 2$) inverted themselves and died.

CONCLUSION

Clinical volatile anesthetics are effective on both gastropods and cephalopods as well as vertebrates and are more easily controllable than systemic general anesthetics. However, further work is required to find the optimal way to anesthetize *Octopus vulgaris* and related cephalopods and there is compelling data to indicate that pretreatment with a muscle relaxant followed by a volatile anesthetic will be the best approach. Elucidation of the neuronal circuits underlying the behavioral response to anesthetic agents are also required and, in conjunction with primary neuronal cultures, recently developed from Octopus brain (Maselli et al., 2018), they present us with opportunities to study anesthetic effects at the cellular level in cephalopods for the first time.

AUTHOR'S NOTE

With apologies to Jane Austen, 1811, for the title.

AUTHOR CONTRIBUTIONS

WW wrote the review in consultation with ADC and with critiques from GP, H-FM, and IA.

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A Comparative Study of Cell Specific Effects of Systemic and Volatile Anesthetics on Identified Motor Neurons and Interneurons of *Lymnaea stagnalis* (L.), Both in the Isolated Brain and in Single Cell Culture

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1. A comparative descriptive analysis of systemic (sodium pentobarbital, sodium thiopentone, ketamine) and volatile (halothane, isoflurane, enflurane) general anesthetics revealed important differences in the neuronal responses of identified motor neurons and interneurons in the isolated central nervous system (CNS) and cultured identified neurons in single cell culture of Lymnaea stagnalis (L.).

2. At high enough concentrations all anesthetics eventually caused cessation of spontaneous or evoked action potentials, but volatile anesthetics were much faster acting. Halothane at low concentrations caused excitation, thought to be equivalent to the early excitatory phase of anesthesia. Strong synaptic inputs were not always abolished by pentobarbital.

3. There were cell specific concentration-dependent responses to halothane and pentobarbital in terms of membrane potential, action potential characteristics, the after hyperpolarization and patterned activity. Individual neurons generated specific responses to the applied anesthetics.

4. The inhalation anesthetics, enflurane, and isoflurane, showed little concentration dependence of effect, in contrast to results obtained with halothane. Enflurane was faster acting than halothane and isoflurane was particularly different, producing quiescence in all cells types studied at all concentrations studied.

5. Halothane, enflurane, the barbiturate general anesthetics, pentobarbital, and sodium thiopentone and the dissociative anesthetic ketamine, produced two distinctly different effects which could be correlated with cell type and their location in the isolated brain:

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either a decline in spontaneous and evoked activity prior to quiescence in interneurons or paroxysmal depolarizing shifts (PDS) in motor neurons, again prior to quiescence, which were reversed when the anesthetic was eliminated from the bath. In the strongly electrically coupled motor neurons, VD1 and RPD2, both types of response were observed, depending on the anesthetic used. Thus, with the exception isoflurane, all the motor neurons subjected to the anesthetic agents studied here were capable of generating PDS *in situ*, but the interneurons did not do so.

6. The effects of halothane on isolated cultured neurons indicates that PDS can be generated by single identified neurons in the absence of synaptic inputs. Further, many instances of PDS in neurons that do not generate it *in situ* have been found in cultured neurons. The nature of PDS is discussed.

Keywords: systemic anesthetics, volatile anesthetics, *Lymnaea stagnalis*, identified motor neurons and interneurons, paroxysmal depolarization shifts, action potentials

INTRODUCTION

In a recent review (Winlow et al., 2018) we suggested that clues to the likely effects of anesthetics on cephalopods such as *Octopus vulgaris* might be gained from related studies on gastropod molluscs such as *Lymnaea stagnalis* which has proved to be an excellent model system for studies of the cellular actions of general anesthetics on individual identified neurons (e.g., McCrohan et al., 1987; Winlow et al., 1987, 1995; Franks and Lieb, 1988; McKenzie et al., 1995; Spencer et al., 1995, 1996) many of which have known functions. The utility of such a preparation is that it allows us to study of the modes of action of general anesthetics, since behavioral, cell physiological, and biophysical experiments can all be performed on this preparation (e.g., McCrohan et al., 1987; Girdlestone et al., 1989a,b; Winlow et al., 2018) at clinical concentrations (Girdlestone et al., 1989c; Yar and Winlow, 2016b). Such studies have provided good basic information on the modes of action of general anesthetics and are also being used to underpin related studies on the actions of volatile anesthetics on cephalopod molluscs such as *Octopus vulgaris* (Polese et al., 2014; Winlow et al., 2018). Whole cell patch clamp studies on the effects of halothane on calcium currents have been reported elsewhere (Yar and Winlow, 2016a,b) as have studies on passive properties of the electrically coupled giant neurons VD1 and RPD2 (Qazzaz and Winlow, 2015, 2017) in response to halothane, isoflurane, and pentobarbitone.

General anesthetics vary widely in structure and physico-chemical properties, but in spite of this diversity they all have the same eventual physiological effect, depression of neuronal activities in the central nervous system culminating in loss of perception and consciousness, due to either axonal conduction block, or diminution of both excitatory (Richards, 1983), and inhibitory (Spencer et al., 1996) synaptic transmission. In addition, neurons have very diverse morphologies, physiologies, biochemical, and pharmacological properties so they might be expected to have differing responses to applied agents and this is the case in relation to anesthetic substances applied to identified neurons of the pond snail *Lymnaea stagnalis* (L.) (Winlow et al., 1991, 1992, 1995, 2018; Qazzaz and Winlow, 2015, 2017).

Previous experiments on *Lymnaea* with menthol demonstrated that some neurons became quiescent and that others generated paroxysmal depolarizing shifts (PDS) in its presence (Haydon et al., 1982) and comparable actions have previously been described in preliminary reports on isolated identified neurons in culture (Winlow et al., 1992; Yar, 1992). Some neurons became quiescent immediately, whilst others only do so after exhibiting PDS as demonstrated for halothane (Girdlestone, 1986; Winlow et al., 1992). PDS is generated by a large depolarizing wave (Jefferys, 2010) which may be endogenous or synaptically driven, and which may trigger a series of damped action potentials. The original experiments on *Lymnaea* anesthesia were carried out using menthol (Haydon et al.) which is slow acting and causes distress to the animal. Since 1982 further studies have been carried out on clinical analgesics and anesthetics on various molluscan preparations (see Winlow et al., 2018 for review). The neurons studied here may be divided into motor neurons and interneurons (see Discussion) and the actions of the anesthetics have rather different effects on these neuron types. Their functions are reviewed in detail elsewhere (Winlow and Polese, 2014).

This is the first report to collate, compare, and contrast all our previously unpublished data on the electrophysiological actions of both systemic and volatile inhalational anesthetic agents on the discharges and patterning of identified *Lymnaea* motor neurons and interneurons, both *in situ* in the intact isolated brain and in single cell culture. Here, we present a descriptive comparative analysis of the actions of the anesthetics on neural discharges. We also present statistical comparison of the modifications of spike width and amplitude of neurons grown in culture as compared with those *in situ* and demonstrate their responses to applied anesthetics.

MATERIALS AND METHODS

Experiments were carried out on *Lymnaea stagnalis* obtained from suppliers (Blades Biological, Kent), maintained in circulating aerated tap water at room temperature and fed on

lettuce supplemented with fish food. Snails of 1–4 g in weight were chosen for dissection and were transferred to a dissecting dish coated with Sylgard resin (Dow Corning, GmbH, USA). Dissection was carried out at 20°C in HEPES buffered saline (HBS) buffered to pH 7.8 with NaOH as previously described (Benjamin and Winlow, 1981).

Experiments on Neurons in the Intact Brain

The recording chamber was regularly flushed with fresh aerated HBS, barbiturate saline or saline equilibrated with volatile anesthetics during the course of the experiment. The neurons were identified under binocular magnification according to their color, locations and electrical activity (Winlow and Benjamin, 1976; Benjamin and Winlow, 1981; Winlow et al., 1981). Intracellular recordings were made from individual motor neurons and interneurons (see **Figure 1**) using filamented glass borosilicate glass microelectrodes (Clark ElectroMedical Instruments), filled with saturated K_2SO_4, with a resistance of 10–30 MΩ. Results were obtained using standard electrophysiological techniques, including Neurolog NL 101 bridge-balanced amplifiers and appropriate stimulators. Signals were monitored on a Tektronix oscilloscope and via a CED interface connected to an EPSON PCV computer for subsequent analysis. The CED SPIKE2 programme was used to capture and process the data.

Data Analysis

Data analysis of the discharges of individual cultured neurons were performed according to Yar et al. (1993). Statistical analysis of significance using Student's *t*-tests were performed as appropriate using the "Oxstat" program (Oxford Logic from Wallingford Computer Services, Oxfordshire, OX10 9BJ). Where possible, data were expressed as mean ± standard deviation (SD) and values were considered statistically significant at a confidence level of 95%. This was not appropriate with the patterned activity presented in most of the Figures, where typical traces, based on long-term recordings are presented. Subgroups of the total number of neurons investigated were studied to reveal changes in their discharge patterns in the presence of applied anesthetics. Temporal changes in discharge patterns associated with anesthetics were clear and the total number of observations of this type of activity are indicated in both the text and figure legends.

Systemic Anesthetics

The standard protocol used for standard electrophysiology with pentobarbital (a relatively slow onset and relatively long duration barbiturate−28) was to:

1) record the normal activity of the cell in normal HEPES saline;
2) perfuse the preparation with the low concentration of pentobarbital (1 mM) for at least 30 min (maximum 1 h), and make recordings at 6 min intervals;
3) perfuse the preparation with the high concentration of pentobarbital (2 mM) for another 30 min (maximum 1 h), and make recordings every 6 min;
4) wash the preparation continuously with saline for 1 h and make recordings at 10 min intervals.

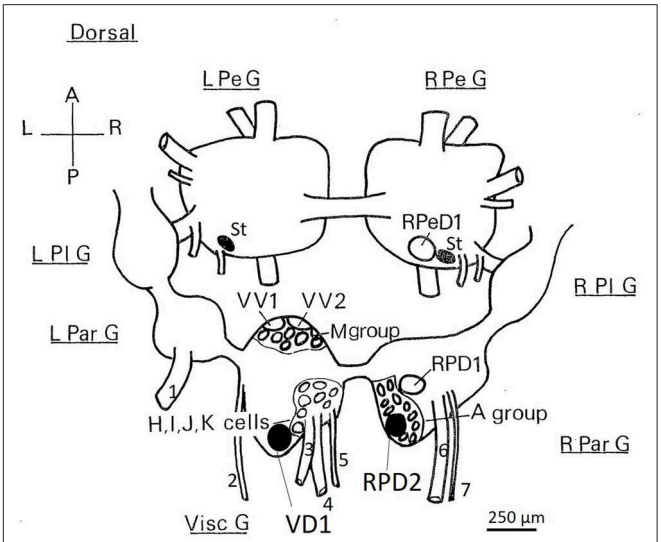

FIGURE 1 | Dorsal view of the location of the somata of *Lymnaea* neurons utilized in this study. RPeD1, RPD1 are interneurons and VV1/VV2 is a putative interneuron, while VD1/RPD2, A group, M group, and the J cells are motor neurons—neither soma size nor color can be used to discriminate these neuronal types. Shaded cell bodies are white, unshaded cell bodies are orange. The diagram is of the dorsal surface of the left parietal ganglion (L Par G, from which the left parietal nerve−1, emerges), the median visceral ganglion (Visc G from which the cutaneous pallial nerve−2, the intestinal nerve−3, the anal nerve−4, and the genital nerve−5 emerge), the right parietal ganglion (R Par G from which the right internal parietal nerve−6 and the right external parietal nerve−7 emerge), the pleural ganglia (L Pl G and R Pl G) and the pedal ganglia (L Pe G and R Pe G). For further details see Slade et al., 1981. L, left; R, right; A, anterior; P, posterior, St: statocysts.

Variations on this protocol were used as required, usually in the form of square depolarizing current pulses in order to stimulate otherwise quiescent cells. The effects of Thiopentone sodium 0.5 mM (May and Baker), a rapid onset, brief duration barbiturate (Bowman and Rand, 1980), and Ketamine hydrochloride (Parke-Davis), 0.2 mM were also studied on small numbers of individual neurons using similar methods.

Volatile Anesthetics

The volatile anesthetics were delivered to the isolated CNS preparation through a specially designed and carefully controlled anesthetic delivery system as previously described for ED_{50} determinations (Girdlestone et al., 1989c) or using standard anesthetic vaporizers (Ohmeda). Concentrations of vaporized halothane, enflurane, and isoflurane, between 0.25 and 4.00% v.v., were monitored using a commercial anesthetic monitor (Datex Normac AA-102) and equilibrated with HEPES buffered saline (HBS) which was then gravity fed to superfuse the preparation at a flow rate of 60 $ml.min^{-1}$.

Experiments on Identified, Isolated Neurons in Culture

Preparation and incubation of neurons as rounded cells without neuritic extensions in short term culture, for 1 to

2 days, was carried out according to the methods of Yar (Yar, 1992), Walcourt-Ambakederemo and Winlow (Walcourt-Ambakederemo and Winlow, 1994), Ahmed et al. (Ahmed et al., 1997), and Yar and Winlow (Yar and Winlow, 2016a) and anesthetics were applied as above.

RESULTS

All the neurons tested with the various anesthetics used in this study eventually became quiescent if an adequate concentration of anesthetic was applied to the brain or to isolated, cultured, identified neurons for an appropriate length time, which varies from one cell type to another. The pathways to silence differ in that some neurons gradually become quiescent, while others exhibit a series of paroxysmal shifts (PDS) prior to quiescence and yet others exhibit both types of response depending on the anesthetic used.

Experiments on Neurons in the Intact, Isolated Brain

Cell Specific Effects of Systemic Anesthetics

a) **Pentobarbital** induces PDS in the motor neurons studied here ($n = 109$), but not in interneurons ($n = 41$). Every cell tested had remained stable for at least 10 min following penetration. Pentobarbital, at both the concentrations used, caused the membrane to either hyperpolarize or depolarize and either reduced or abolished the after hyperpolarization (AHP) (**Table 1**) when first added to the bath and then usually caused PDS (**Figures 2**, **3**), quiescence or significant suppression of normal patterned activity (**Figure 4A**), depending on the cell type (as summarized in **Table 2**.

Motor Neurons Usually Exhibiting PDS

The J cells ($n = 13$) of the right parietal ganglion always exhibited PDS in the presence of pentobarbital, as do the neurons of A group ($n = 13$) (**Figure 2**) and M group ($n = 18$) of the visceral ganglion (**Figure 3A**). The underlying depolarizing wave associated with PDS is particularly clear in **Figure 2C** and occurred spontaneously for several minutes in J cells before quiescence in the presence of 2 mM pentobarbital (**Figure 5B**). In addition the strongly electrically coupled motor neurons VD1 and RPD2 also exhibited PDS in the presence of pentobarbital (**Figure 3B**) ($n = 8$) and were different from other neurons reported here in that they became quiescent in halothane as well as in isoflurane (14 and see below). VD1 and RPD2 eventually become quiescent in 0.5 mM pentobarbital and are strongly hyperpolarized under these circumstances. Such major shifts in membrane potential (E_m) have not been observed in the other identified neurons studied here.

Interneurons Usually Exhibiting a Tendency to Quiescence

RPeD1 ($n = 7$): Application of pentobarbital (1 mM) to RPeD1 causes a gradual reduction in spontaneous firing (**Figure 4**) as the cell hyperpolarizes. The data showed RPeD1 to fire spontaneously and irregularly under normal circumstances; because it receives powerful synaptic input from the input 3 interneuron (see **Figure 4**). Continuous application of 1 mM pentobarbital always induced hyperpolarization and suppressed the spontaneous pattern of firing but could not block EPSPs in this cell. The data showed that in 2 mM pentobarbital, the membrane potential increased, and excitability decreased (**Figure 4F**). Continuous wash out of pentobarbital increased the frequency of firing and the pattern of neuronal activation started to return to normal (**Figures 4A,I**). After 40–60 min continuous washing in normal saline, the spontaneous pattern of firing always returned to normal (**Figure 4J**).

VV1/VV2 ($n = 6$) showed a gradual decline to quiescence in pentobarbital, which slowly returned to normal after washout.

RPD1 ($n = 5$) is much more sensitive to pentobarbital as compared with the other cells studied here. Application of 1 mM pentobarbital induced immediate and long- lasting quiescence (over 30 min) with a clear hyperpolarization and a long recovery period of over 1 h following washout.

b) **Thiopentone** Only 6 experiments were carried out with 0.5 mM thiopentone, but the cells studied responded in a similar way to those in pentobarbital (**Figure 5A** and **Table 2**), except that VV1/2 exhibited PDS. Recovery was much more rapid than in pentobarbital as would be expected from this rapid onset brief duration anesthetic (Rang et al., 2007).

c) **Ketamine** A further 6 cells were observed (2 × A group, 2 × RPD1 , an M group cell and a J cell) after addition of ketamine, but again cell specific effects were observed as summarized in **Table 2**. Unusually the J cell did not exhibit PDS. Again recovery was more rapid than in thiopentone.

Cell Specific Effects of Volatile Anesthetics

The effects of halothane, isoflurane, and enflurane on identified neurons in the isolated CNS of *Lymnaea* ($n = 151$) were characterized at concentrations between 0.5 and 4.0% v/v.

Actions of Halothane on Interneurons and the Giant Motor Neurons VD1 and RPD2

Experiments on input resistance were carried out on the putative interneurons VV1/VV2 ($n = 6$) and on the strongly electrically coupled motor neurons VD1 and RPD2 ($n = 18$) to determine the effects of halothane on membrane conductance. There was a clear concentration-dependent increase in conductance due to a decline in input resistance both in halothane (**Figure 6**) and isoflurane.

Different concentrations of halothane, produced dramatic, concentration-dependent effects which can be generalized for the following cell types ($n = 85$). At low concentrations (0.25–1%) the interneurons RPeD1 ($n = 11$), VV1/2 ($n = 6$), RPD1 ($n = 20$) and the giant motor neurons VD1 ($n = 22$) and RPD2 ($n = 20$) share certain common responses, in spite of variability between neuron types. Sub populations of these neurons were therefore studied in detail to reveal the changes in their discharge patterns to applied anesthetics. Here the responses of RPD2 are used as the exemplar of the common responses to 0.5 and 1.0% halothane (**Figure 7**) which are characterized by an initial increase in the patterned discharge activity, perhaps the

TABLE 1 | Characterization of effects of 1–2 mM pentobarbital Increases due to fall in conductance and 1–2% halothane on spontaneous activity of identified *Lymnaea* neurons ($n = 110$).

	Dose Dep.	**Motor neurones exhibiting PDS before quiescence**			**Interneurons with dual responses**		**Interneurons usually tending to quiescence**		
Cell type		**J cells**	**M group**	**A group**	**VD1**	**RPD2**	**VV1/2**	**RPeD1**	**RPD1**
A P type		1	2	2	2	2	2	Usually 2	2
E_m-Pentobarbital	X	Hyp	Dep	Hyp	Substantial Hyp ca. 30–35 mV after PDS		Hyp	Hyp, ca 10–12 mV	Hyp
E_m-Halothane	X	Minimal Hyp (a few mV)			Substantial Hyp ca. 30–35 mV (see Qazzaz and Winlow, 2015)		Minimal variable effects (a few mV)–Hyp, Dep or no change		
APA-Pentobarbital	√	Declining amplitude during PDS bursts, then quiescence			Normal amplitude gradually declining with onset of PDS		Gradual decline	No change	Declines, slow recovery
APA-Halothane	√	APA largely maintained prior to PDS			Normal APA, then sudden quiescence		Reduced APA		
AP Duration-Pentobarbital and Halothane	√	Decreases	Generally decreased, mainly due to diminution of the calcium dependent pseudoplateau in neurons with type 2 action potentials						
AP Frequency-Pentobarbital	√	Cells become quiescent and then PDS is induced	AP frequency initially increases and is eventually replaced by PDS, which reduces to doublets and triplets at higher doses	Frequency declines and APs replaced by irregular dose-dependent PDS. Long recovery time	Gradual decline and then onset of doublet firing prior to PDS		Gradual decline	Gradual decline, but strong input 3 often apparent	Rapid, long-lasting quiescence. Long recovery time
AP Frequency-Halothane	√	Gradual decline of spontaneous activity into quiescence			Immediate quiescence accompanied by strong epsps. Recovers quickly		Gradual reduction in discharge frequency	Prolonged inhibition of APs but with attendant strong epsp inputs	Strong and immediate early excitation and then quiescence
		Early excitatory response of variable duration depending on neuron type, then frequency declines							
AHP-Pentobarbital	√	Abolished			Markedly reduced		Reduced	Minimal reduction	Reduced
AHP-Halothane	√	Reduced			Minimal change		Markedly reduced		
R_m-Pentobarbital	X	—			Increases due to fall in dose -independent $\mathbf{g_m}$ (see Qazzaz and Winlow, 2015)		—		
R_m-Halothane	√	—			Declines due to rise in dose –dependent g_m (and see Qazzaz and Winlow, 2015, re VD1/RPD2)			—	

E_m, membrane potential; AHP, afterhyperpolarization; AP, action potential; APA, action potential amplitude; Hyp, hyperpolarization; Dep, depolarization; Dose dep, dose dependency, X = no dose-dependency. Data on input resistance (R_m) of neurons is incomplete, but relevant to the present study; g_m , membrane conductance.

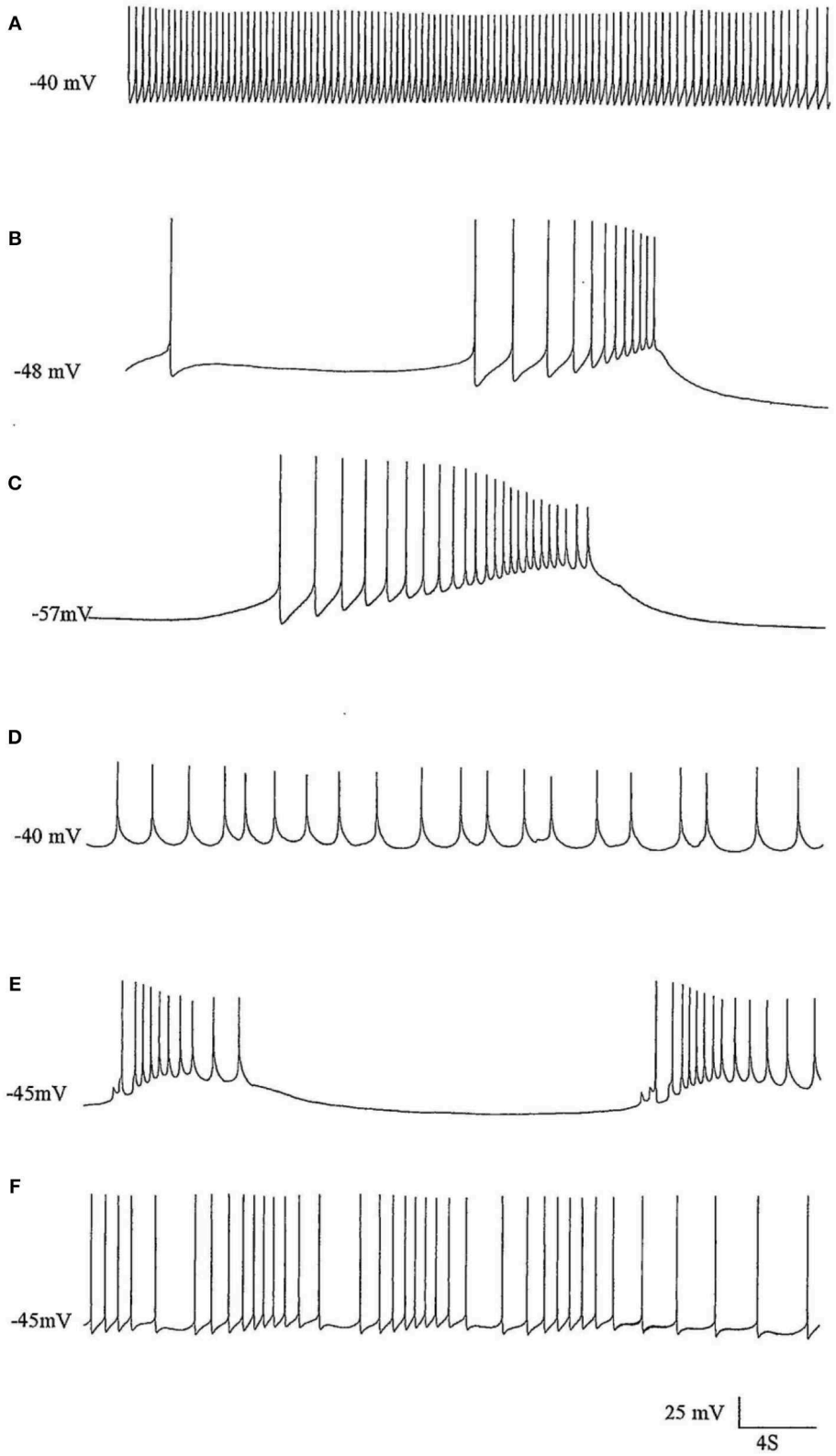

FIGURE 2 | The effects of pentobarbital on the spike pattern of a J cell motor neuron ($n = 4$). **(A)** Control; **(B)** 30 min after application of 1 mM pentobarbital; **(C)** 2 mM pentobabbital after 6 min; **(D)** 2 mM pentobarbital after 18 min; **(E)** 2 mM pentobarbital after 30 min; **(F)** 20 min after continuous washout with fresh HBS. Note that pentobarbital induces hyperpolarisation which is reversible with continuous washing and the large depolarisations induce damped action potentials associated with 1 PDS in B,C, and E and that the cell is depolarised back to control levels in D, but with reduced spike amplitude and ferquency and loss of AHP.

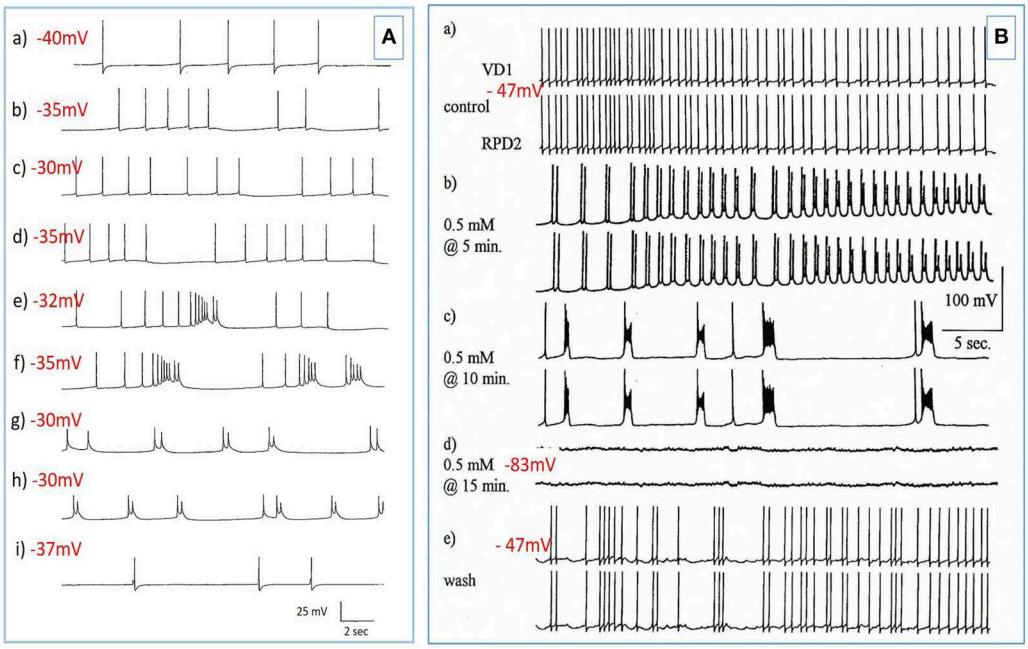

FIGURE 3 | PDS induced by superfusion of the brain with Pentobarbital. **(A)** The effect of Pentobarbital on the pattern and frequency of an M group motor neuron ($n = 4$). **(a)** Control; **(b)** after 6 min in 1 mM Pentobarbital; **(c)** after 12 min; **(d)** after 18 min; **(e)** after 24 min; **(f)** after 30 min; 2 mM pentobarbital **(g)** after 6 min; **(h)** after 30 min; **(i)** 40 min continuous wash in normal saline. Membrane potential decreased with application of both concentrations of pentobarbital and was partially reversible with continuous wash after 40 min. **(B)** Effect of 0.5 mM pentobarbital on the spontaneous discharge of the strongly electrically coupled motor neurons VD1 and RPD2 ($n = 8$). **(a)** Normal electrical activity in VD1 and RPD2 at a resting potential of −47 mV. **(b)** 5 min after perfusing the brain with 0.5 mM pentobarbital in HBS the neurons started to fire in doublets and then **(c)** gradually hyperpolarized and developed PDS, after which **(d)** they became quiescent at a membrane potential of −83 mV. **(e)** The effect of pentobarbital was completely reversed after washing in clean HBS and the cells returned to their normal discharge.

cellular equivalent of the early excitatory phase of anesthesia observed by Guedel (1937). This is followed by a period of diminished spontaneous action potential frequency and/or their complete cessation. Also, there was a marked reduction in the action potential amplitude and the amplitude of the AHP (**Figures 7B–D,G,H**). Higher concentrations of halothane (0.75-2.00%), i.e., those causing loss of the withdrawal response in 68-100% of animals) (Girdlestone et al., 1989c), caused a general depressant effect on the spontaneous activity of all the neurons examined.

In the VV1/2 ($n = 6$) and RPD1 ($n = 5$) neurons, the excitatory period was found to persist as long as the preparation was continuously perfused with low (0.5–0.75%) concentrations of halothane until the beginning of washing, whereas, in RPeD1($n = 7$) and RPD2($n = 5$) neurons, the excitatory period tended to be much briefer, lasting only a few minutes before quiescence. For example, in 1.0% halothane RPeD1 neurons became quiescent in just over 3 min (**Figure 8C**) as did RPD1. All the studied neurons showed a substantial reduction in the action potential amplitude and the amplitude of the AHP after perfusion with 0.5% halothane, but some neurons seem to be more affected than others. For instance, the AHP of RPD1 neurons ($n = 5$) was completely abolished in 1% halothane, whereas, RPeD1 neurons (**Figure 8**) were the least affected in this respect, in common with the effects of pentobarbital upon them(**Figure 4**). The effects of 0.5–1.0% halothane on the different neurons were found to be reversible following a 10–15 min wash in the majority of cases, except that all examples of RPD1, took over an hour to recover. The resting membrane potential (E_m) of these neurons was in the range of −40 to −50 mV. Application of halothane was found to produce a variable effect on the E_m in the studied neurons ($n = 23$), depending on their initial E_m. Sometimes, halothane depolarized the neuron ($n = 8$), or hyperpolarized the neuron ($n = 11$) by a few mV and sometimes there was no marked effect on the E_m ($n = 4$).

Actions of Halothane on the Smaller Motor Neurons

The actions of halothane on the smaller motor neurons (A group, $n = 8$, M group, $n = 4$, and J cells, $n = 6$) are exemplified in **Figure 9** where the patterned activity of an a group neuron is enhanced in 0.5% halothane (**Figure 9A**) and remained so until washout after which normal activity in all these cells resumed within 15 min. In 2% halothane, all cells initially generated PDS, but eventually all became silent, although PDS could be evoked by depolarizing current pulses (**Figure 9B**).

Concentration-Dependent Effects of Halothane on Evoked Responses

Since all types of neurons eventually become silent at concentrations of volatile anesthetics >1.0%, an investigation of the comparative responses of interneurons and motor neurons to injected depolarizing currents in their presence was undertaken

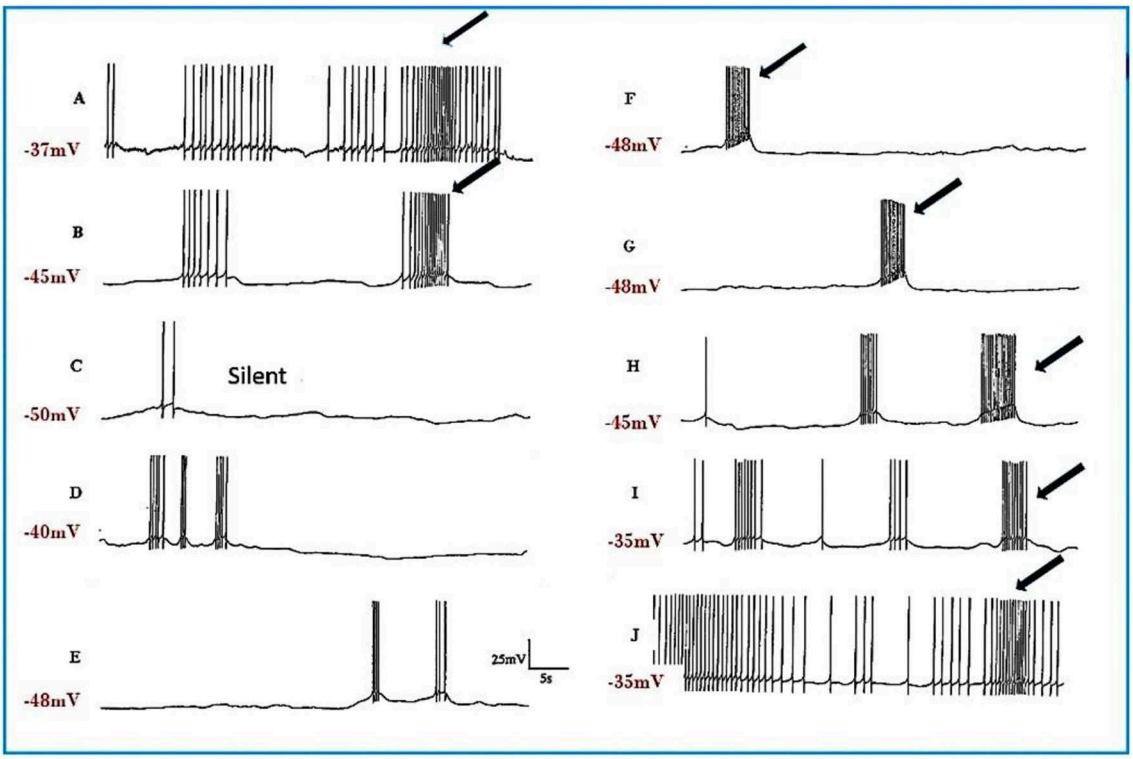

FIGURE 4 | Pentobarbital suppresses the spontaneous patterned discharge of interneuron RPeD1 ($n = 6$), but does not suppress the powerful spontaneous synaptic input from the input 3 interneuron (arrows), which is also part of the respiratory central pattern generator and is located in the right parietal ganglion (Syed et al., 1990; Winlow and Polese, 2014). **(A)** Normal; **(B)** 1 mM pentobarbital after 6 min; **(C)** after 12 min which was silent until **(D)** after 30 min; **(E)** 2 mM pentobarbital after 18 min; **(F)** after 24 min; **(G)** after 30 min; **(H)** after 10 min washout; **(I)** after 20 min; **(J)** after 30 min; (M) after 40 min. Application of pentobarbital hyperpolarized RPeD1. Continuous wash out of pentobarbital decreased membrane potential of RPeD1 to normal with a resumption of normal patterned activity.

to further establish the concentration-dependent effects of halothane on evoked activity. Depolarizing current pulses of 1-2 nA amplitude and 5 s duration were injected into the soma of neurons. In normal HBS these evoked a response which comprised a discharge of rapidly adapting action potentials in all cells irrespective of whether the cell was normally spontaneously active or silent. Peak to peak amplitude of action potentials was between 80 and 100 mV and frequency between 2 and 4 Hz. In the presence of halothane, however, it soon became apparent that the effect of the anesthetic on evoked activity was concentration–dependent (**Figures 9**, **10**), but also the evoked responses could be characterized on the basis of cell type. The small motor neurons (A group, M group, and J cells) did not generate PDS at 0.5% halothane, but often generated more intense patterned activity (**Figure 9A**). However, the cells responded with PDS at 1% halothane and became silent at 2.0% halothane, but PDS-like damped action potentials could be evoked by depolarizing stimuli (**Figure 9B**) whereas this was not the case for the large motor neurons VD1/RPD2, or for the interneurons VV1/2, RPD1, or RPeD1 all of which tended to quiescence and in which no action potentials could be generated.

Enflurane

Enflurane had an overall dramatic effect on spontaneous activity of neurons. In 8 different experiments, with concentrations between 0.5 and 4.0% v.v. enflurane, all cells showed a response to the anesthetic within 20 s after the start of superfusion. Unlike results obtained with halothane, neuronal responses to enflurane were not concentration-dependent. Neither the spontaneous activity, nor synaptic patterning showed any trends with concentration. The responses fell into two clear categories in the 8 cells studied. In the presence of enflurane, the spontaneous activity, of 5 of the 8 cells (interneurons RPeD1 x 2, VV1, VV2, and large motor neuron RPD1) became quiescent with no evidence of PDS and (**Figure 10A**). In the other 3 cells, which were all small motor neurons (A cell, M cell, J cell), in the presence of 1.5-2.0% v.v. enflurane, evoked activity was characterized by the occurrence of PDSs (**Figure 10B** and spontaneous large amplitude depolarizations ca. 20 mV) were sporadically observed.

Isoflurane

The action of isoflurane on spontaneous and evoked neuronal activity was markedly in contrast with those of enflurane ($n = 57$). Within a relatively broad spectrum of concentration (0.5-3.0% v.v.), isoflurane had similar actions at each concentration, i.e., as with enflurane, no concentration-dependence of the neuronal responses was revealed. Furthermore, there was little apparent cell type-dependence in the 6 different cell types examined. In all cells studied isoflurane caused a gradual decline

TABLE 2 | Differential actions of volatile and systemic anesthetics on specific cells and cell groups (see **Figure 1**) in the isolated brain of *Lymnaea*.

	Motor neurons usually exhibiting PDS					Motor and interneurons usually tending to Quiescence					
Cell type	**A gp**	**J cells**	**M gp**	**RPD2**	**VD1**	**VD1**	**RPD2**	**VV1/2**	**RPeD1**	**RPD1**	**Total *n* by agent**
AP type (1 or 2)	2	1	2	2	2	2	2	2	Mostly 2	2	
Halothane	8	6	4			22	20	6	11	7(1)	85
Enflurane	1	1	1					2(1)	2	1	9
Isoflurane	(2)	(2)	(1)			22	25	1	2	2	57
Pentobarbital	14	13	18	32	32			10	16	15	150
Thiopentone	2		2					(1)		1	6
Ketamine	2	(1)	1							2	6
Total n by cell type	27(2)	20 (3)	26(1)	32	32	44	45	19 (2)	31	28 (1)	**313**
% cells with PDS or quiescence	**93.1**	**87.0**	**96.3**	**100**	**100**	**100**	**100**	**90.5**	**100**	**96.6**	

n = number of cells studied in each case. The green box at bottom right shows the total number of individual neurons studied. In isoflurane (orange row) all the cells tested (n = 57) became quiescent. Numbers in brackets indicate that some cells exhibited either PDS or quiescence, unlike the majority of that cell type. Unlike other cells the motor neurons VD1 and RPD2 (blue columns) always exhibited PDS in Na pentobarbital, but did not do so in halothane.

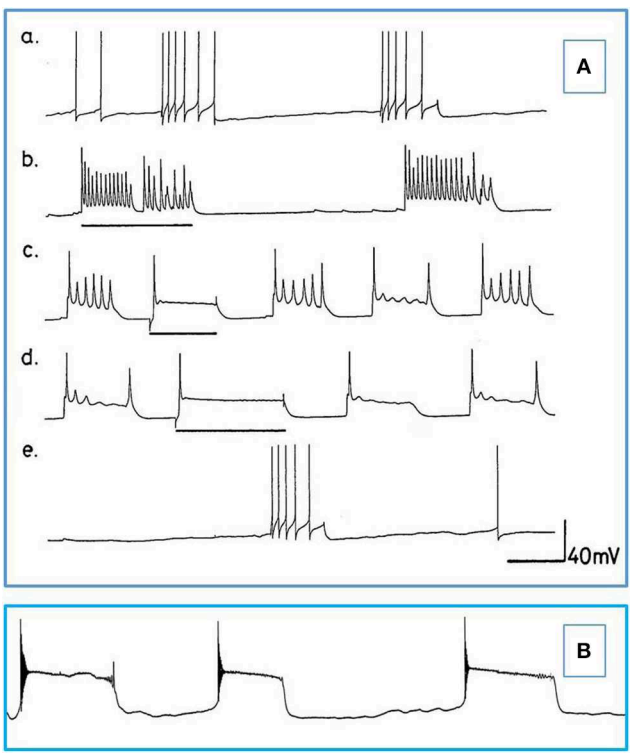

FIGURE 5 | Actions of barbiturates on motor neurons **(A)** Effects of thiopentone on spontaneous and evoked activity of A group motor neurons (*n* = 2 and 2 M group cells exhibited similar activity). Depolarizing current pulses are indicated by horizontal bars. **(a)** Normal activity. **(b–d)** Evoked and spontaneous PDS at 1, 4, and 7 min, respectively after addition of thiopentone. **(e)** Recovery after 15 min in fresh HBS. Time calibrations: a, b, c and e 5 s; d 10 s. **(B)** An example of spontaneous, repetitive PDS in a J cell motor neuron in the presence of 2 mM pentobarbital. The cell had not been artificially hyperpolarized. Time calibration 5 s.

in frequency of spontaneous action potentials during which time, action potential amplitude and AHP amplitude decreased. There was no evidence of PDS-like activity in any of the neurons studied (**Figure 10C** and see **Table 2**). Again large amplitude, sporadic depolarizations were observed.

Comparison of the Electrophysiological Characteristics of Neurons *in situ* and in Culture

The Nature of Paroxysmal Depolarizing Shifts

PDS could be generated in a number of different cell types by each of the anesthetics used (**Table 2**), but it was unclear whether it was synaptically generated or whether it was an endogenous property of the neurons themselves as has been indicated in a preliminary study on the cerebral giant cells of *Lymnaea* (Walcourt-Ambakederemo and Winlow, 1993). Furthermore, it was unclear whether cells identified as exhibiting PDS or tending to quiescence would behave the same way in isolation. We therefore carried out experiments on isolated, identified neurons in culture to determine their action potential characteristics, and their responses to applied halothane.

Here we concentrated on cultured *Lymnaea* interneurons, VV1/2, RPD1, RPeD1, and the motor neurons RPD2 and M group to compare their electrophysiological properties with those *in situ* and to also examine their responses to halothane *in situ* and in culture. The interneurons and giant motor neuron RPD2, are very large in size (100–150 μM in diameter) while the M group neurons form a distinct group with cell bodies about 80 μM in diameter. Hence all were easy to identify and select for cell culture. The electrophysiological activities of these neurons were first recorded in normal saline experiments for each neuron type. The results were then pooled together for the purpose of comparison between the key electrophysiological parameters of resting membrane potential, action potential shape, amplitude and duration *in situ* and in culture. The comparison between these parameters can be seen in **Table 3**.

Resting Membrane Potential

In the intact brain all the cultured neurons were characterized by a slow regular or irregular pattern of firing whose frequency

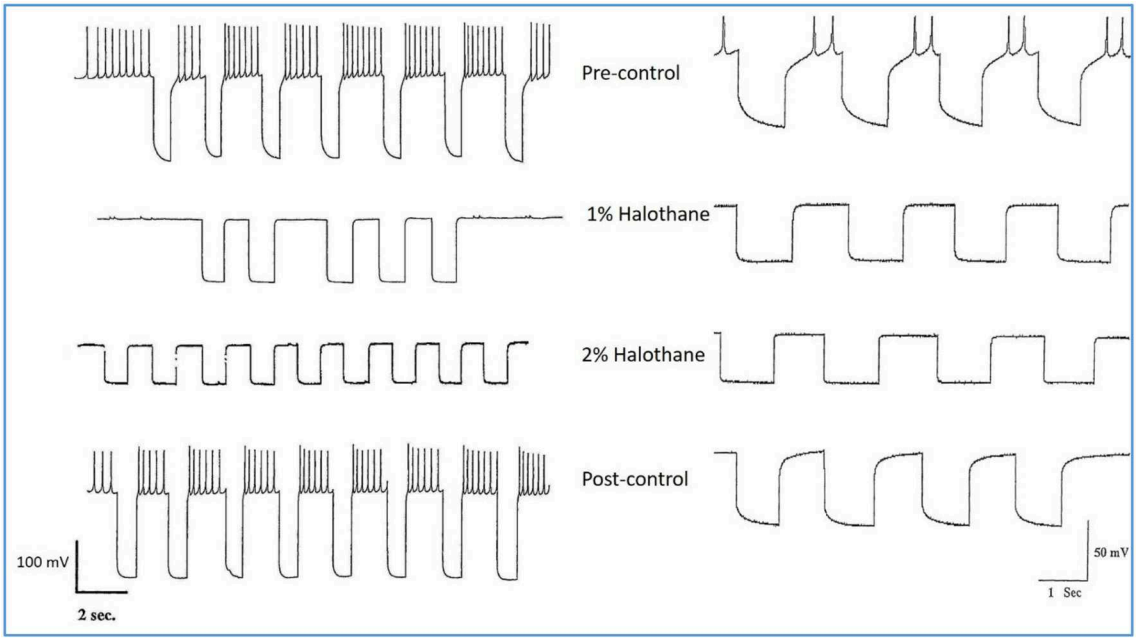

FIGURE 6 | Action of halothane on the input resistance on two examples of motor neuron VD1. 0.1 nA pulses of 1.0 S duration were injected into the cells via the bridge-balanced recording electrode. In both cases the pulse was seen to diminish in a dose dependent manner in halothane, indicating a fall in membrane input resistance (R_m) of the cells and hence an increase in conductance as halothane concentration increased. The left hand cell was maintained at normal resting potential, while the right hand cell had been hyperpolarized by a few millivolts, very close to threshold. Similar data were obtained from RPD1 and VV1/VV2. N.B. A detailed statistical analysis of the effects of anesthetics on the passive membrane properties of VD1/RPD2 appears elsewhere (Qazzaz and Winlow, 2017).

varied among the neuron types from 0.5 to 1.5 spikes/s Also, in intact preparations, they exhibited a resting membrane potential in the range of −40 to −50 mV. *In situ*, they usually fired spontaneously but sometimes they required the injection of depolarizing current (0.5–1.0 nA) in order to elicit action potentials. In culture, their resting membrane potentials varied between −60 and −80 mV, and hence they were unable to fire unless stimulated with the injection of DC current. All the intracellular recordings made from them in culture were obtained after a continuous injection of a small amount of depolarizing current (0.01–0.06 nA). The only exception to this was in RPD2, where it was found to fire spontaneously in 2 out of 8 experiments (25%) with a relatively lower resting membrane potential (about −40 mV).

Action Potential Shape

All this group of neurons exhibited type 2 action potentials *in situ* with the exception of RPeD1, where type 1 action potentials were also observed on some occasions, which is a normal characteristic of this neuron, as mentioned above. In culture, most of the neurons maintained type 2 action potential shapes (53 out of 63, 84.1%) and only a few exhibited type 1 action potentials (10 cells, 15.9%; **Table 3**).

Action Potential Amplitude and Duration

Action potential amplitude (APA) was measured from the peak of depolarization to the peak of the repolarization (i.e., from peak to peak). The action potential duration was determined at half of its amplitude, known as half-width (HW). The method used for the determination of both APA and HW was as previously described (Ahmed et al., 1993). With the exception of M group neurons, **Table 3** shows that the action potential amplitudes of all cultured neurons tend to be reduced compared with those *in situ* while the action potential durations were longer compared with those *in situ*. For all the neurons studied, again with the exception of M group cells, there appears to be an inverse relationship between spike height and its duration. The APA of RPeD1 was found to vary between 99.64 ± 5.38 to 82.44 ± 6.46 mV *in situ* and in culture, respectively, which is highly significant at the 1% level (unpaired *t*-test). In the case of HW, both VV1/2 and RPD2 neurons showed a very significant difference between their action potential durations *in situ* and in culture at 1% level.

Effects of Halothane on Cultured Neurons

The main emphasis of this part of our study was to determine whether isolated neurons could sustain PDS and this turned out to be the case as shown in **Table 4**. Because most of the cultured cells (92.3%) did not fire spontaneously PDS was usually evoked by intracellular stimulation. Interestingly, PDS could be evoked by 1% halothane in some examples of all the cell types, rather than just M group neurons, as demonstrated in the intact brain, although examples of PDS had sometimes occurred in VV1/2 and RPD1 (**Table 2**). In addition the responses of individual neurons could be quite variable as shown by the two examples of the neurons RPeD1 in **Figure 11**.

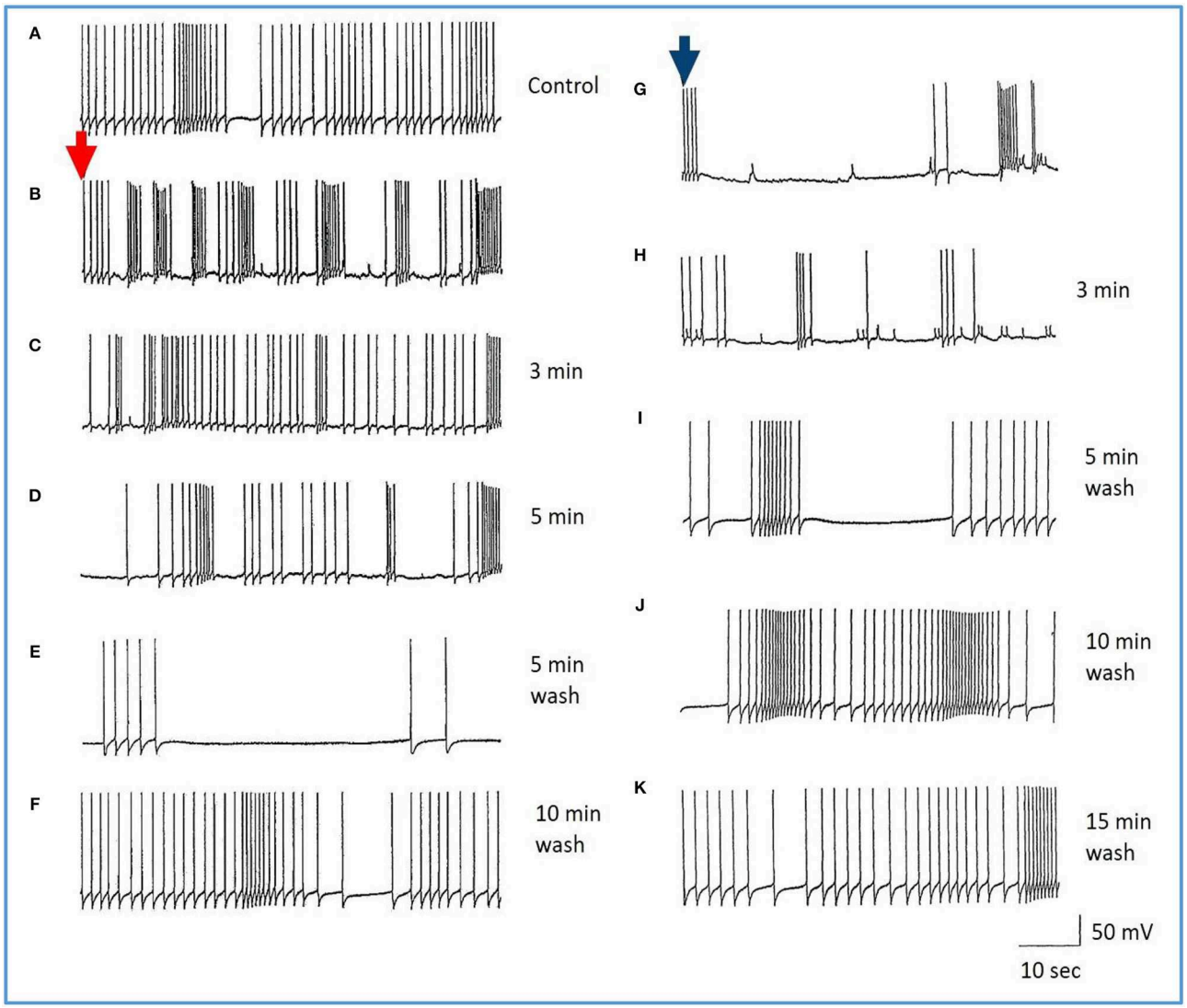

FIGURE 7 | Common responses and concentration dependence of neurons at low and elevated halothane concentrations demonstrated in motor neuron RPD2 (n = 5). **(A)** Irregular spontaneous discharge; **(B)** Addition of 0.5% halothane (red arrow) generated enhanced patterning of the discharge and then a gradual decline of the discharge rate with time **(C,D)** after which the preparation was washed in clean saline **(E,F)** and 1% halothane was added at **(G)** (blue arrow). The neuron eventually became quiescent at the end of trace **(H)** and was then washed **(I–K)** returning to a normal discharge pattern after 15 min in HBS **(K)**. The spike amplitude and AHP were diminished in both 0.5% **(C,D)** and 1% halothane **(G,H)**.

DISCUSSION

Differential Responses of Neurons to Anesthetics

The response of neurons to anesthetics is most often a change in transmembrane voltage and hence in neuronal excitability (Maze, 1990), but as has been demonstrated in this study, these responses are not stereotypical (**Tables 1**, **2**). Results from all the agents used in this report show that specific cells studied in the intact brain have specific responses to anesthetics and their pathways to silence differ profoundly from one cell type to another (**Table 1**). However, it is clear that some cells exhibit PDS in all the anesthetics tested here, e.g., an A group or M group neurons and J cells, all of which are motor neurons, will exhibit either spontaneous or evoked PDS with the exception of isoflurane in which all neurons become quiescent. The giant interneurons VV1, VV2, RPD1, and RPeD1 tend to quiescence in all the anesthetics tested. It is noteworthy that the strongly electrically coupled neurons motor neurones VD1 and RPD2 fall into both categories, exhibiting PDS in pentobarbital and quiescence in both isoflurane and halothane (see also Qazzaz and Winlow, 2015, 2017). Furthermore, in VD1 and RPD2, both pentobarbital and halothane cause a substantial increase non-dose-dependent E_m, but apparently by different mechanisms. In VD1/RPD2 the increase in E_m is not concentration-dependent in pentobarbital and is accompanied by a non-dose-dependent increase in R_m (Qazzaz and Winlow, 2017) indicating a decrease in conductance (g_m). This is not the case halothane where the large increase in

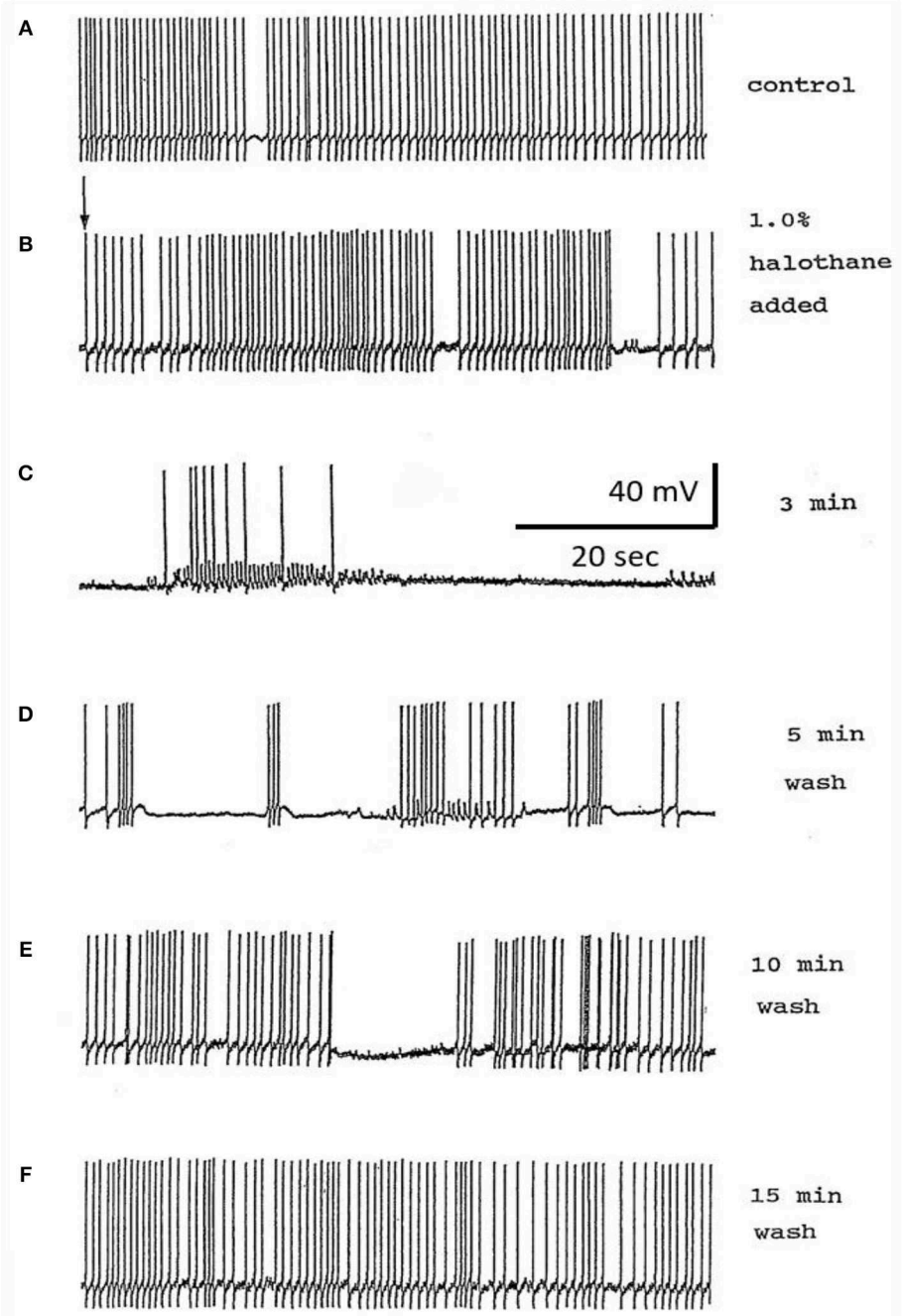

FIGURE 8 | Effects of 1.0% halothane on the interneuron RPeD1 ($n = 7$). **(A)** Control; **(B)** 1.0% halothane was added at arrow. Generally there was a slight reduction in the discharge frequency as well as in the depth of AHP upon perfusion with halothane **(C)** shows the response of the cell after 3 min of application of halothane. The cell was largely inhibited and became quiescent as the cell hyperpolarized after the last spike shown here, but with a large number of e.p.s.ps., due to many synaptic inputs which failed to develop into full action potentials. **(D–F)** show the effects of rinsing the preparation continuously with normal saline for 5, 10, and 15 min, respectively. The cell partially recovered to control levels, in terms of the spontaneous discharge rate and the amplitudes of the AP and AHP.

E_m is accompanied by a concentration-dependent decrease in R_m and thus an increase in gross g_m (**Figure 6**) as previously demonstrated in VV1/VV2 (Winlow et al., 1987) and which also occurs in menthol (Haydon et al., 1982). The underlying mechanisms of these differences remain to be discerned.

Anesthetics Have Distinctive Effects on Motor Neurons and Interneurons

Many of the neurons we have studied here are involved in the control of the cardiorespiratory system via the respiratory central pattern generator (rCPG) of *Lymnaea* (Syed and Winlow, 1991),

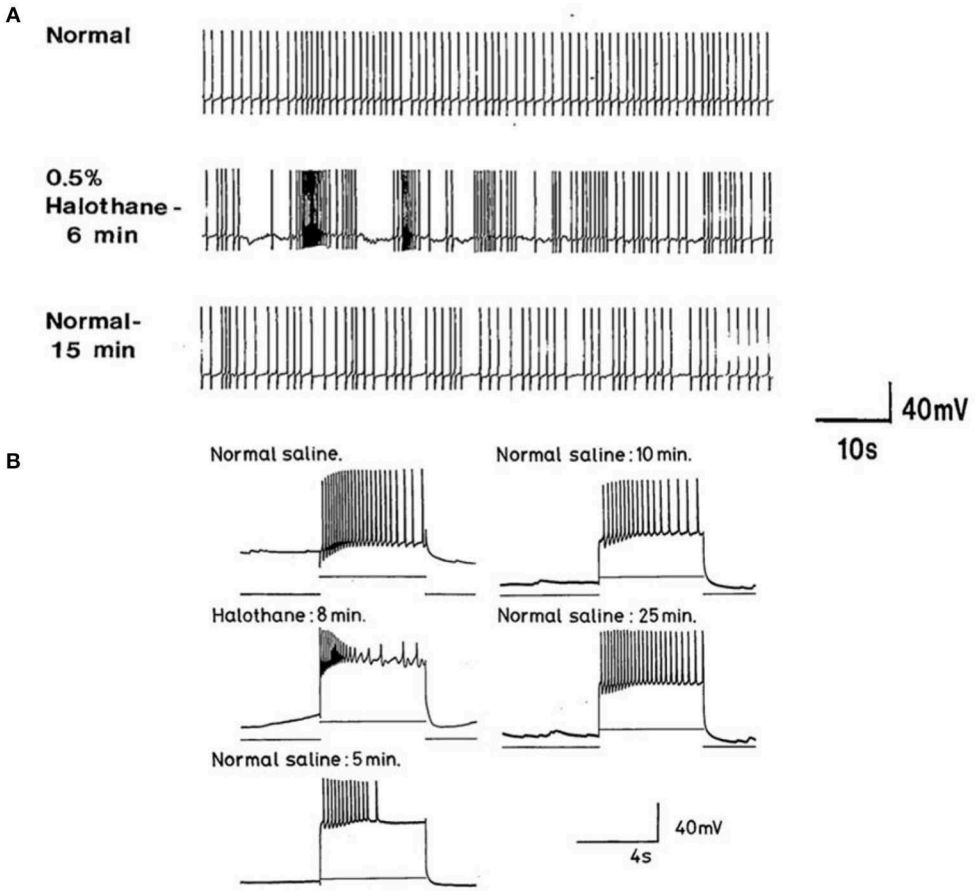

FIGURE 9 | Concentration dependence of the effects of halothane on A group motor neurons ($n = 8$). **(A)** a low dose of halothane (0.5 %) caused patterning of an A cell spontaneous discharge, demonstrated by the occurrence of PSPs and high frequency bursting activity. **(B)** In 2% halothane neuronal activity of another A cell was completely suppressed, but PDS could still be elicited by depolarizing stimuli (1–2 nA for 5 S) into the neuronal soma. In both cases there was complete recovery on rinsing in HBS.

which has been reconstructed *in vitro* (Syed et al., 1990). The rCPG also controls cardiac functions (Benjamin and Kemenes, 2013). RPeD1 projects directly to the osphradial ganglion and receives direct excitatory, monosynaptic, cholinergic inputs from osphradial neurons (Bell et al., 2007) which are responsive to hypoxia (Janes and Syed, 2012). The parietal A group neurons innervate the musculature of the mantle cavity, while the visceral J cells are pneumostome opener motor neurones and some pneumostome closer motor neurons are thought to be located within visceral M group (Moroz, 1991). The strongly electrically coupled neurons VD1 and RPD2 are hypoxia and osmo-sensitive, peptidergic motor neurons which directly innervate the auricle (Kerkhoven et al., 1991) and are thought to modulate heart rate (Benjamin and Kemenes, 2013), but which also project to the skin near the pneumostome, osphradium, and lips. They are unlikely to have a direct sensory function since they were not labeled after back filling the osphradial nerve with either Ni-lysine or biocytin (Nezlin, 1995). The giant neurons VV1 and VV2 are responsive to osmotic stimulation of the osphradium (Kamardin, 1995), but are unlikely to be primary sensory neurones as they did not backfill from the osphradial nerve (Nezlin, 1995). They currently have no known motor function. Finally, the neuron RPD1 is in all likelihood an interneuron which branches into peripheral nerves and which receives polymodal sensory inputs from the skin of the tentacles lips and mantle (Zaitsev and Shuvalova, 1988) including inhibitory non-ocular photo-responses from the foot through the pedal nerves to the pedal ganglia (Chono et al., 1992).

Motor Neurons Exhibit PDS, but Interneurons Do Not Do So in the Intact Brain

A comparison of the responses of these different neuron types to applied anesthetics *in situ* indicate that, except in isoflurane, the four types of motor neurons studied here, A group, J cells, M group VD1/RPD2, are all capable of generating PDS while the interneurons RPeD1 (Syed et al., 1991) and RPD1 as well as the presumed interneurons VV1/VV2 all tend to quiescence in the presence of applied anesthetics in the intact brain (**Table 2**). Similar effects have

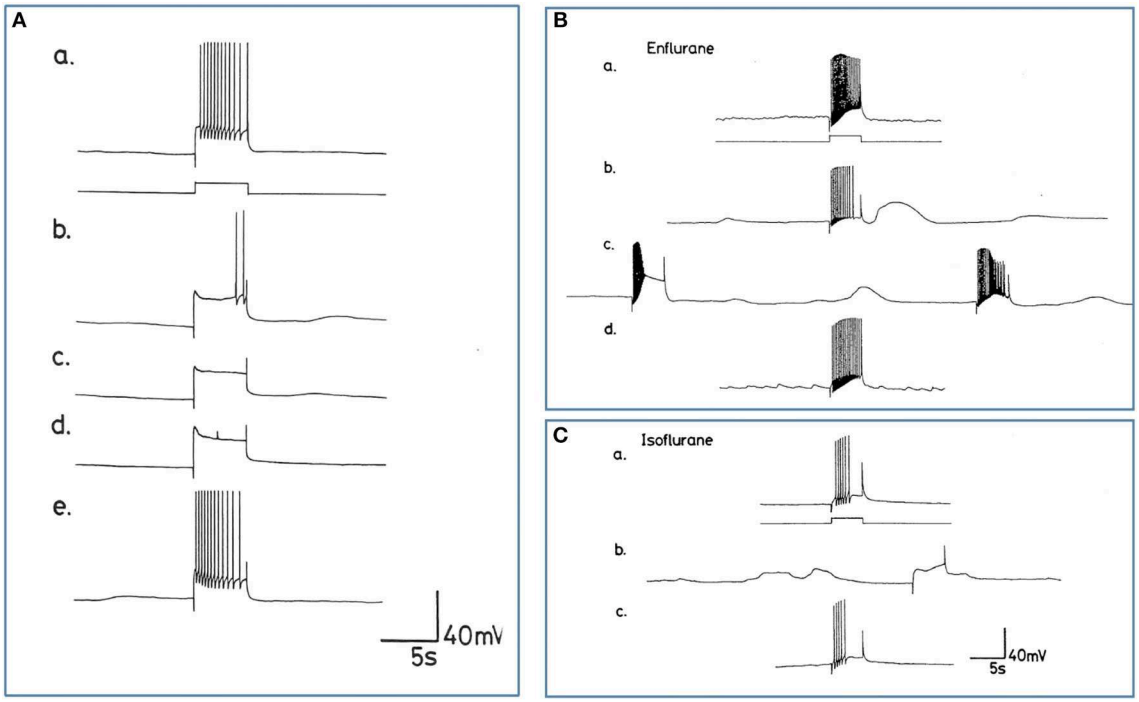

FIGURE 10 | Differential actions of enflurane and isoflurane on individual identified neurons. **(A)** Effect of 1% enflurane on evoked activity in interneuron RPD1. **(a)** Depolarizing current pulses of 1 nA and 5 s duration evoked a train of spikes in normal saline. **(b)** After 30 s in enflurane, the frequency of evoked action potentials decreased, until **(e)** the normal evoked activity was restored 10 min after rinsing. **(B,C)** Large amplitude depolarization's were seen in the presence of enflurane and isoflurane when action potentials were evoked as in box 1. In **(Ba)** a train of action potentials was evoked in an M group motor neuron in HBS. **(b)** After 3 min in 2.0% enflurane, action potentials could still be evoked and a large amplitude, subthreshold depolarization occurred spontaneously. **(c)** After 4 min of superfusion the large amplitude depolarization was still present, but each of the depolarizing current pulses now triggered PDS, rather than a normal spike train. **(d)** There was full recovery of the evoked response after rinsing in HBS. In **(C)**, another example of the RPD1 interneuron is shown, in which both spontaneous and evoked effects are seen in the presence of isoflurane **(b)**, again accompanied by large depolarizing potentials, but no action potentials. **(a,c)** are pre and post c-controls.

also been observed in 1% halothane in the paired buccal 3-cells (McCrohan et al., 1987; Girdlestone et al., 1989b) and in cultured 4-cells (Walcourt and Winlow, both of which are motor neurons. Thus, the motor neurons and interneurons discussed here may be distinguished by their responses to anesthetics, but further work will be required to see if this hypothesis can be generalized, particularly as it is unclear whether other buccal interneurons exhibit PDS. The reasons for the differences in responses are not simply due to cell size (see **Figure 1**) and in isolated cell culture the interneurons were more capable of generating PDS than in the intact brain (**Table 4**), suggesting that PDS may be an endogenous property of all types of neurons (see below), but modified by their interactions with other cells, in terms of the channels expressed by individual neurons or activated by neurotransmitters, either in the intact nervous system or when grown in culture with synaptically connected cells. Interestingly menthol has effects on motor neurons and interneurons (Haydon et al., 1982) similar to those described here. Menthol is now known to block voltage-dependent sodium channels in rat neurons and human skeletal muscle and shares analgesic (Galeotti et al., 2002) and anesthetic properties with propofol (Watt et al., 2008) by its modulatory action on $GABA_A$ receptors (Lau et al., 2014). On the basis of this information it is conceivable that PDS may have a role in the early excitatory phase of anesthesia when uncoordinated movements in *Lymnaea* and other animals can be observed in the absence of sedation or neuromuscular blocking agents.

Effects of Anesthetics on Membrane Potential and Spontaneous Activity

Apart from VD1/RPD2, pentobarbital, and halothane at all concentrations used in the present study caused membrane hyperpolarization, usually of only a few millivolts (**Table 1**), except in M group neurons (**Figure 3A**) which depolarized in pentobarbital. The reasons for this unexplained depolarization remain to be investigated using appropriate patch clamp methodology. Nicoll and Madison (Nicoll and Madison, 1982) have reported that general anesthetics hyperpolarize neurons in the vertebrate central nervous system, due to an increase in potassium, and not chloride, permeability. O'Beirne et al. (1987) have reported neuronal hyperpolarization, decreased spontaneous activity, and sometimes decreased input resistance after administration of different concentrations of pentobarbital in albino guinea pig hippocampal slices. They proposed that pentobarbital caused neuronal inhibition, particularly at

TABLE 3 | Comparison of the action potential parameters of the different neurons both *in situ* and culture.

	Action potential shape		Action potential amplitude (mV)		Action potential half-width (ms) at specified frequency (spikes/s)	
Cell type	***in situ***	**in culture**	***in situ***	**in culture**	***in situ***	**in culture**
M Group Motor neurons	Type 2 $n = 7$	Type 2 (4) $n = 12$	78.42 ± 7.69	80.52 ± 7.37 (NS)	21.10 ± 7.71 Freq ≤ 1.5	16.54 ± 8.19 (S) Freq ≤ 1.5
RPD2 Motor neuron	Type 2 $n = 8$	Type 2 (1) $n = 8$	94.04 ± 5.74	87.10 ± 7.73 (NS)	14.68 ± 2.41 ($n = 8$) Freq = 1.23 ± 0.29	23.37 ± 4.10 ($n = 8$) (S) Freq = 1.20 ± 0.27 (NS)
VV1/2 Putative interneurons	Type 2 $n = 13$	Type 2 (2) $n = 12$	93.79 ± 7.45	85.83 ± 11.94 (S)	8.44 ± 2.59 ($n = 8$) Freq = 0.57 ± 0.16	16.05 ± 4.37 ($n = 8$) (S) Freq = 0.60 ± 0.14 (NS)
RPD1 Interneuron	Type 2 $n = 13$	Type 2 $n = 14$	93.40 ± 7.88	86.89 ± 6.03 (S)	18.25 ± 3.39 ($n = 8$) Freq = 0.85 ± 0.23	22.15 ± 5.03 ($n = 8$)(NS) Freq = 0.82 ± 021 (NS)
RPeD1 Interneuron	Type 2 (1) $n = 21$	Type 2 (3) $n = 17$	95.36± 6.33	81.95 ± 7.32 (S)	7.95 ± 1.65 ($n = 8$) Freq = 1.09 ± 0.30	9.75 ± 1.82 $n = 8$)(NS) Freq = 1.03 ± 0.27 (NS)
Total "n"	62	63				

This Table compares the action potential (AP) shape, APA, and half-width (HW) at the stated frequencies of examples of motor neurons and interneurons both in situ and in culture. Most neurons retained their AP shapes in culture. The number of times the neurons were found to exhibit type 1 action potentials is given in brackets. The neuron RPeD1 exhibited type 2 and type 1 action potentials both in situ and in culture, whereas, in M group, VV1/2 and RPD2, type 1 action potentials were only expressed in culture, indicating modifications to somatic ion channels. The data are expressed as mean ± S.D. In most cell types APA is significantly (S) less in culture than in situ, with the exception of M group neurons whose amplitudes are not significantly (NS) different from one another. Furthermore M group neurons tend not to fire in culture and were stimulated to fire at frequencies of <2 spikes/s, exhibiting a reduction in HW in comparison to cells in situ. All other cells exhibited an increase in HW in culture when AP frequency was maintained both in situ and in culture by injecting appropriate current through the stimulating electrode, although this did not reach significance RPD1 or RPeD1. Thus, with the exception of M group, APs in cultured neurons tend to be shorter and broader compared with those recorded in situ. Freq, firing frequency.

low doses, due to an increase in potassium conductance (O'Beirne et al., 1987). Blaustein (1968) has demonstrated that pentobarbital and thiopentone blocked sodium and potassium conductances under voltage clamp in the lobster. The issue of how barbiturates and especially pentobarbital affect potassium currents, thus remains controversial. We have seen partial blockage of gross K^+ currents (Moghadam and Winlow, 1995; Moghadam, 1996) when different concentrations of pentobarbital are applied to the neurons. The differences in outcome may arise from different preparations and different methods employed, but may also be due to different K^+ channels responding in different ways. In addition, halothane has been shown to block L-type calcium current in cultured *Lymnaea* neurons in a concentration-dependent manner (Yar and Winlow, 2016b), but of course the precise mix of calcium and potassium channels is likely to vary from one neuron type to another.

TABLE 4 | Effect of 1.0% halothane on various cell types—M group cells are motor neurons, the other cells are interneurons.

Cell type	*n*	PDS spont	PDS evok	PDS total	Quiescence
M group	7	0	4	4	3
VV1/2	5	1	3	4	1
RPeD1	10	1	2	3	7
RPD1	4	0	1	1	3
Total "n"	26	2	10	12	14
% of total		7.7	38.4	46.1	53.8

More than 50% of the 26 cells studied went into quiescence on exposure to halothane. The majority of the cells showing PDS were stimulated by injecting a small amount of current. This current injection could alter the membrane potential and predispose a cell to PDS as certain currents are activated or inactivated at particular membrane potentials. See text for details. key: spon, spontaneous; evok, evoked; PDS, paroxysmal depolarising shifts; n, number of cells studied.

Effects of Anesthetics on the After Hyperpolarization and Pseudoplateau

The AHP, which is a calcium-dependent phenomenon, was reversibly reduced or abolished by pentobarbital in all cell types except RPeD1 (see **Table 1**) and was markedly reduced in halothane (**Figures 7**–**9**). Reversibility was clearer and more rapid in halothane than in pentobarbital. Three different AHPs with different time courses exist in hippocampal neurons which are sensitive to the volatile anesthetic isoflurane and it has been reported that isoflurane decreased the AHP in rat hippocampal and human neocortical neurons (Berg-Johnsen and Langmoen, 1990). The AHP, which occurs after an action potential and is due to a Ca^{2+} activated K^+ conductance (Hoston and Prince, 1980; Schwartz-Kroin and Stafstrom, 1980) decreased significantly in the PDS group of neurons in response to pentobarbital applications (**Figures 2**, **3**) and it was slow to recover after washout of the drug (**Figure 3Aa**) even though the membrane potential (E_m) had recovered. This phenomenon suggests that the response of components of the AHP in these neurons is time dependent with respect to pentobarbital application. Different form of PDS **Figures 3Ag,Bb** doublet spiking) compared with earlier or later responses in the same neurons, which suggests that different ionic channels with different kinetics and time-dependencies may be involved in the generation of the different types of PDS.

Lymnaea neurons generate two different types of action potentials categorized as type 1 (typical neuronal action potential) and type 2 (action potential with a calcium-dependent pseudoplateau) (Gardner and Kerkut, 1968; Winlow

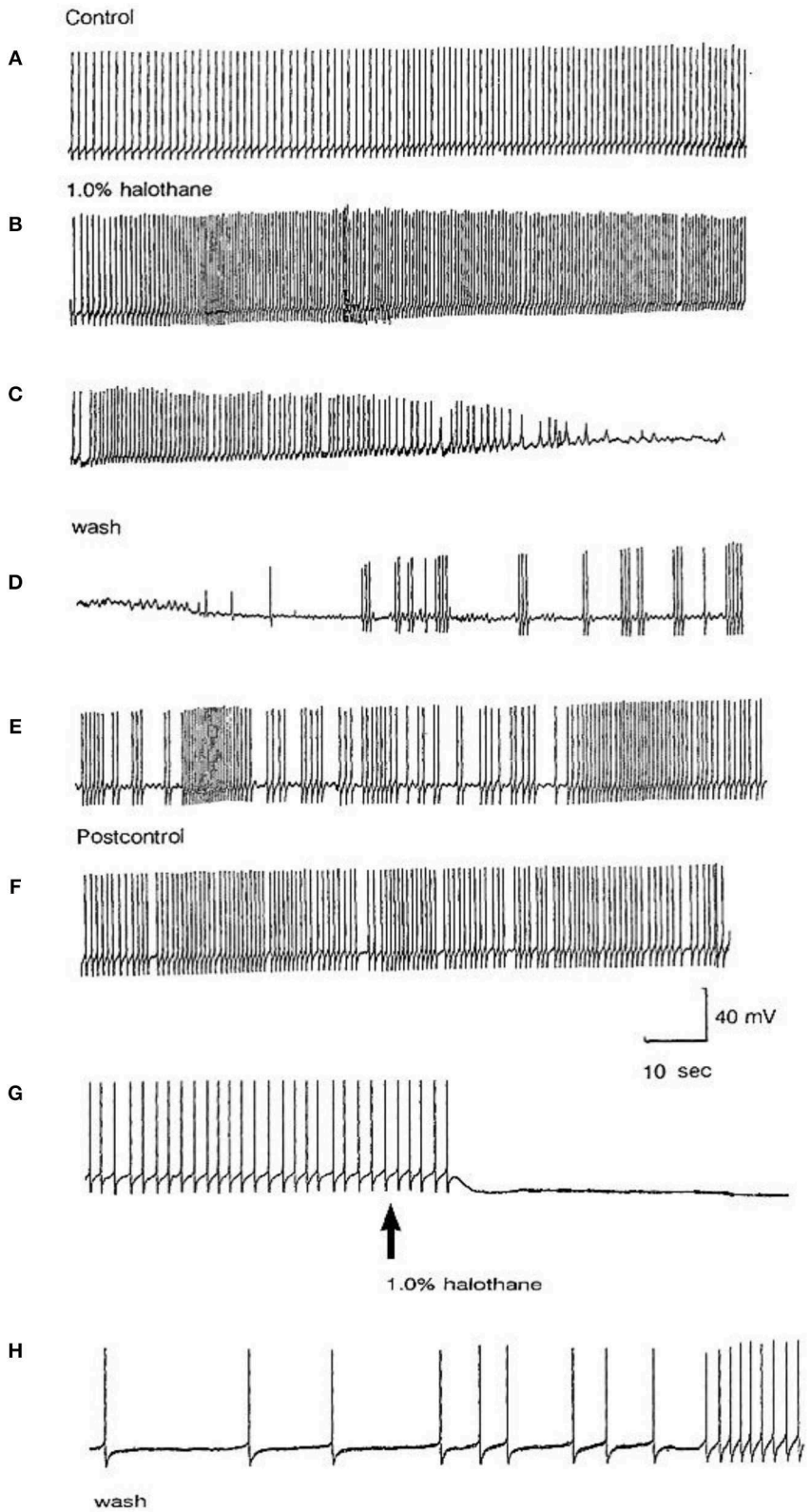

FIGURE 11 | Effect of 1% halothane on two cultured RPeD1 interneurons. The responses of individual cultured neurones are not completely stereotyped as these examples of RPeD1 demonstrate, but all were capable of generating spontaneous action potentials and quickly recovered from the applied anesthetic. **(A)** Under

(Continued)

FIGURE 11 | control conditions, the cell was firing regularly **(a)**. Introduction of halothane-containing saline to the chamber led to an increase in the frequency of firing within seconds **(b)**. In less than a minute, the amplitude of the action potential started decreasing along with a diminution of the AHP and depolarization of the cell **(c)**. The cell ultimately stopped firing in a depolarized state, and only slight oscillations could be seen. When the cell was washed with HBS, the membrane potential gradually returned to normal and the oscillations increased **(d)**. Within a couple of minutes, the cell started firing small action potentials which regained their full amplitude in the next 3–4 min **(e)** and the cell returned to almost normal activity in about 5–6 min **(f)**. In another RPeD1 neuron cultured for 2 days, the neuron was firing spontaneously **(g)** until the introduction of halothane, which led to an immediate cessation of spiking activity. The cell hyperpolarized and remained so until washout commenced, during which the cell slowly recovered **(h)** and normal activity resumed in 3–4 min.

and Benjamin, 1976; Girdlestone, 1986). The action potential type of RPeD1 is variable (Kyriakides et al., 1989), but is most usually type 2 and this variability may be due to seasonal factors (Wood and Winlow, 1996; Copping et al., 2000). The influx of Ca^{2+} during action potentials is supposed to be sufficient to activate Ca^{2+} dependent potassium channels (Meech and Standen, 1974). We have previously demonstrated that pentobarbital (1 and 2 mM) reversibly decreased action potential half width (Moghadam and Winlow, 1993), which is a good measure of the reduction of the width of the pseudoplateau in type 2 molluscan action potentials. This plateau is generated by an L-type calcium current (Yar and Winlow, 2016a) and pentobarbital can also reduce L and N (not T) type Ca^{2+} currents in cultured, vertebrate, sensory neurons in a dose dependent manner (Gross and Macdonald, 1988). The effects on discharge would be to reduce frequency, spike width and AHP of the action potential. This is similar to previous findings, which have shown that the calcium components of action potentials are decreased by barbiturates (Morgan and Bryant, 1977; Heyer and Macdonald, 1982). Pentobarbital (at 25–600 mM) and phenobarbitone (100–5,000 mM) reduce action potential duration with sedative and anesthetic doses in large multipolar spinal cord neurons (Heyer and Macdonald, 1982). In contrast, in the M group neurons we have found pentobarbital at low doses increases activity (**Figure 3**) as previously demonstrated by Larrabee and Posternak (1952) who reported that pentobarbital enhances transmission at lower concentrations.

Concentration Dependence of Anesthetics

There is no obvious trend between volatile anesthetic concentrations and their effects at a cellular level with the two inhalation anesthetics, enflurane, and isoflurane, which is in sharp contrast to results obtained with halothane. Despite the use of a relatively wide concentration range, i.e., 0.25–4.00%, there was little difference in the response produced by each. Both the anesthetics did however, have immediately apparent effects on neuronal activity. Enflurane caused various dramatic changes in neuronal behavior very soon after superfusion, which are discussed in more detail below, and isoflurane caused immediate quiescence irrespective of the concentration. The absence of a concentration dependent effect with these two agents at a neuronal level is difficult to explain. However, in these experiments concentration dependence was judged by observing concentration related changes in spontaneous and evoked neuronal activity. It is possible that concentration dependence is not revealed at this level, and because these two agents are less prone than halothane to cause an excitatory phase. However, concentration dependent changes have been noted in terms of conductance changes, induced by halothane and isoflurane in VD1/RPD2 (Qazzaz and Winlow, 2015, 2017) and patch clamp studies reveal that both calcium currents (Yar and Winlow, 2016b) and potassium currents (Winlow et al., 1995; Moghadam, 1996) are depressed in a concentration-dependent manner by halothane in cultured, identified neurons.

Is PDS an Endogenous or a Synaptically Driven Phenomenon?

PDS-like activity can be triggered synaptically in *Lymnaea* by the action of the input 3 interneuron on the J cell motor neurons (Benjamin and Winlow, 1981) and has been described elsewhere, especially in relation to epilepsy (Prince, 1968, 1978; Speckmann and Caspers, 1973; Lux, 1984; Jefferys, 2010). PDS has been investigated to determine whether it is an endogenous or synaptically mediated phenomenon (Speckmann and Caspers, 1973). Epilepsy is probably driven collectively by large numbers of interconnected neurons, many with intrinsic membrane properties that allow the emergence of PDS (Jefferys, 2010). A PDS, which leads to damped burst of spikes, is a large Pathak (2017), abnormal Kubista et al. (2019) depolarizing wave caused by suppression of calcium-activated potassium currents due to blockade of voltage-gated potassium currents that then unmask persistent sodium currents (Pathak, 2017) Such calcium-activated potassium currents are found in *Lymnaea* neurons (Moghadam, 1996).

There is a wide range of potassium currents in *Lymnaea* neurons (see Winlow et al., 2018 for review) and preliminary papers have demonstrated that halothane, isoflurane and pentobarbital depress gross potassium currents in a dose-dependent manner (Moghadam and Winlow, 1995; Winlow et al., 1995; Moghadam, 1996). In addition, potassium channel blockers such as tetraethylammonium and 4-aminopyridine have been shown to induce PDS in *Lymnaea* neurons (Holden et al., 1982b, 1983) including doublet and triplet spiking (Holden and Winlow, 1982, 1983; Holden et al., 1982a, 1983) similar to that seen in **Figure 3Bb**. Zero calcium saline can also have the same effect (Holden et al., 1983). It should be noted that although PDS does not normally occur in the cerebral giant cells (CGCs), which are interneurons innervating the buccal ganglia, either in the whole brain or in culture, it can be elicited in exceptional, experimental circumstances when cultured CGCs are subjected not only to 1% halothane, but to 1% halothane in zero Ca2+/high Mg2+/1 mM EGTA saline (Walcourt-Ambakederemo and Winlow, 1993), supporting the view that PDS may be an endogenous property of most neurons, given the right conditions. Calcium currents are involved in the pseudoplateau and the AHP of *Lymnaea* neurons as mentioned

above. They have recently been characterized as high voltage activated L-type calcium currents in pedal I cluster neurons (Yar and Winlow, 2016a), which have type 2 action potentials. Their calcium currents are suppressed in a concentration-dependent manner by halothane (Yar and Winlow, 2016b). Thus, there is a panoply of potassium and calcium channels likely to be involved in the generation of PDS within individual *Lymnaea* neurons [for more detail, see (Winlow et al., 2018)]. Within the vertebrate nervous system, both synaptic (Cisse et al., 2004) and endogenous membrane currents are thought to be involved in the generation of PDS (Feher et al., 1982). A further suggestion is that PDS may be due to Cl^- -dependent depolarizing postsynaptic potentials in pyramidal neurons (Timofeev et al., 2002a,b). Whether both mechanisms can occur in the same neuron remains to be seen. Finally several authors suggest that perturbations of intracellular calcium concentration $[Ca]_i$ may be associated with PDS (Raza et al., 2004; Kubista et al., 2019) and that raised $[Ca]_i$ is associated with acquired epilepsy in hippocampal neurons. Both halothane and pentobarbitone are known to raise $[Ca]_i$ in *Lymnaea* neurons (Winlow et al., 2018) in common with vertebrate neurons (Mody et al., 1991). Thus, the rise in $[Ca]_i$ may be sufficient to trigger PDS in susceptible cells even though halothane is known to block L-type calcium currents (Yar and Winlow, 2016b).

Here, we have demonstrated that PDS can be generated by halothane in isolated, cultured neurons and this finding is supported in a preliminary study on the cerebral giant cells of *Lymnaea* (Walcourt-Ambakederemo and Winlow, 1993). In the cultured neurons described in the current report, PDS can sometimes be evoked in all the cell types studied (**Table 4**), particularly in RPeD1 in which PDS has not been observed in the intact brain (**Table 2**). This implies that the neurons are plastic and that machinery to generate PDS may be present in all neuron types, but that the membrane currents generating it are modulated depending on the synaptic and network milieu in which an individual cell finds itself *in vivo*. Given that the cultured *Lymnaea* neurons retain their basic action potential shapes (**Table 3** and see Winlow et al., 1991, 1992, 2018; Yar and Winlow, 1991), their transmitter identity (Syed et al., 1990; Spencer et al., 1995; Naruo et al., 2005), their responsiveness to applied transmitters Haydon, 1989; Syed et al., 1990 and can be reconstructed into meaningful circuits *in vitro* Syed et al., 1990, they are powerful tools for cellular studies of the action of anesthetic agents. It is of interest that the dampened action potential bursts, characteristic of PDS were never observed in isoflurane, but spontaneous and evoked large depolarizing potentials still occurred in its presence in RPD1 (**Figure 10Cb**) which may be the source of PDS, but this remains to be elucidated. However, similar depolarizations are also found in M group cells in enflurane where evoked PDS could be generated (**Figures 10Bbc**) and similar depolarizing events were also noted in VV1/VV2 in enflurane. Such events are unlikely to be synaptic in origin as they still occur at high concentration of enflurane (3%−21) which would be expected to cause complete anesthesia in most animals and would be expected to block synaptic transmission between cultured *Lymnaea* neurons (Spencer et al., 1995, 1996). Thus, the depressant effects of anesthetics on calcium and potassium currents, accompanied by rising $[Ca^{2+}]_i$, may well trigger the depolarizing wave underlying PDS in many types of neuron, although this is often not observed due to suppression of action potentials in these cells.

Relevance of Anesthetic Studies on *Lymnaea* to Studies of Anesthetic Actions on Cephalopod Molluscs

The Cephalopod mollusc *Octopus vulgaris* is now classed as a sentient being (UK Statutory Instruments, 1993; European Parliament European Union, 2010) and should be treated as such when it is used for experimental purposes in the same way as vertebrates so as to reduce pain and suffering. A recent report (Polese et al., 2014), based on previous studies on *Lymnaea* (Girdlestone et al., 1989a,c) has shown that *Octopuses* can be anesthetized with isoflurane and recover well from this procedure. Further studies are now required to refine these procedures. Given that gastropod molluscs are evolutionarily closer relatives to cephalopods than are vertebrates, it is probable that the responsiveness of gastropods to anesthetic agents will yield clues for the future development of cephalopod anesthesia.

CONCLUSION

Neuronal responses to anesthetics are not stereotyped and are characteristic of motor neurons and interneurons in *Lymnaea stagnalis in situ*. The small motor neurons studied here typically generated paroxysmal depolarizing shifts during the application of halothane, enflurane, pentobarbital, thiopentone, and ketamine, but the giant motor neurones only did so in pentobarbital. The interneurons did not usually generate PDS. No cells exhibited PDS in isoflurane and all became quiescent. Examples of motor neurons and interneurons, when isolated in short term culture, were shown to be capable of generating PDS.

ETHICS STATEMENT

This work was carried out on specimens of the pond-snail *Lymnaea stagnalis* which are exempt from this type of approval, but which were maintained in humane conditions in any case.

AUTHOR CONTRIBUTIONS

The basic concept behind this work was generated by WW in whose laboratory (originally in the Department of Physiology, University of Leeds, UK) all the co-authors worked as graduate students completing their PhDs in a timely manner. WW constructed the present paper from their combined their data. All the co-authors contributed equally.

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Mechanisms of Anesthetic Action and Neurotoxicity: Lessons from Molluscs

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Anesthesia is a prerequisite for most surgical procedures in both animals and humans. Significant strides have been made in search of effective and safer compounds that elicit rapid induction and recovery from anesthesia. However, recent studies have highlighted possible negative effects of several anesthetic agents on the developing brain. The precise nature of this cytotoxicity remains to be determined mainly due to the complexity and the intricacies of the mammalian brain. Various invertebrates have contributed significantly toward our understanding of how both local and general anesthetics affect intrinsic membrane and synaptic properties. Moreover, the ability to reconstruct *in vitro* synapses between individually identifiable pre- and postsynaptic neurons is a unique characteristic of molluscan neurons allowing us to ask fundamental questions vis-à-vis the long-term effects of anesthetics on neuronal viability and synaptic connectivity. Here, we highlight some of the salient aspects of various molluscan organisms and their contributions toward our understanding of the fundamental mechanisms underlying the actions of anesthetic agents as well as their potential detrimental effects on neuronal growth and synaptic connectivity. We also present some novel preliminary data regarding a newer anesthetic agent, dexmedetomidine, and its effects on synaptic transmission between *Lymnaea* neurons. The findings presented here underscore the importance of invertebrates for research in the field of anesthesiology while highlighting their relevance to both vertebrates and humans.

Keywords: anesthesia, neurons, synapses, Lymnaea, molluscs, synaptic transmission, cytotoxicity

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INTRODUCTION

A wide variety of anesthetic compounds are safely administered to patients of all ages every year. General anesthetic agents can be broadly classified as either volatile inhaled compounds, or intravenously administered compounds. Structurally, there is a wide range of volatile anesthetic compounds, from simple diatomic compounds such as nitrous oxide, to fluorinated ethers including the modern volatile anesthetics isoflurane, sevoflurane, and desflurane. Intravenous anesthetics are even more varied, from simple hydrocarbons to steroid compounds (Harrison et al., 1987). Common intravenous anesthetics include propofol and ketamine. Since there is a broad spectrum of compounds exhibiting anesthetic effects, there remains significant uncertainty as to how and where these compounds act at the molecular and cellular levels.

Although anesthetic agents are typically regarded as safe to administer to patients with proper monitoring, in recent years, evidence has emerged regarding potential anesthetic-related neurotoxicity. Specifically, studies have shown that general anesthetics may impair nervous system development in animals exposed to these agents during periods of peak neurodevelopment and this has raised concern for young children (Ikonomidou et al., 1999; Flick et al., 2011; Armstrong, 2016; Vutskits and Davidson, 2017; Walters and Paule, 2017). As such, there remains the possibility that general anesthetic exposure in children may lead to long-term cognitive impairment and learning and memory deficits. However, a clear consensus on the precise impact that general anesthetics have on the developing human nervous system and on the kind of protective strategies that may be used to mitigate this risk is lacking. In addition, despite their widespread use, the specific mechanisms of action of general anesthetics also remain unclear (Armstrong, 2016). Molluscs have proven invaluable for teasing out some of the direct effects of general anesthetics, which we report here.

This current review provides a brief overview of molluscan studies that have helped to shape our understanding of both the actions of anesthetic agents as well as their possible detrimental effects on neurons. Broadly speaking, these mollusc studies were the first fundamental investigations into anesthetic mechanisms of action that predated and laid the groundwork for future mammalian studies. We highlight the important roles that molluscs continue to play in the examination of anesthetic-induced neurotoxicity, currently a very active research area in both vertebrates and the clinical setting. In addition, we systematically evaluate the subcellular impact of modern general anesthetics on neuronal excitability, viability, and connectivity between developing neurons. While many of the findings reviewed here emanate from research conducted on various molluscs, particularly the fresh water snail *Lymnaea*, these data have nevertheless stood the test of time as it pertains to vertebrates (Xu et al., 2016).

MOLLUSCS ARE VALUABLE TOOLS FOR FUNDAMENTAL RESEARCH ON GENERAL ANESTHETICS

Notwithstanding considerable efforts, the underlying mechanisms of anesthetic actions, and their potential long-term side effects remain largely unknown. These limitations are due, in part, to the complexity of mammalian models, which are comprised of complex, highly interconnected neuronal networks. Molluscs, on the other hand, are well-suited for researching the fundamental mechanisms of action and neurodegenerative effects of anesthetic agents due to their relatively simple neuronal networks mediating well-defined, simple, behaviors. Several molluscs have been used, including the squid *Loligo forbesi* and the pond snail *Lymnaea stagnalis*.

Some of the earliest work to understand the fundamentals of neuronal function was performed in the squid giant axon. Similarly, early work to understand the mechanisms of action of anesthetic agents also used this easy to manipulate system. Shrivastav et al. performed some of the initial work on *L. forbesi* (Shrivastav et al., 1976). They exposed a giant squid axon to the volatile anesthetic halothane and recorded membrane depolarization at low anesthetic concentrations. They observed a similar depolarizing effect with the volatile anesthetic trichloroethylene, which also increased the threshold potential for action potential firing, and reduced the amplitude of resulting action potentials (Shrivastav et al., 1976). This resulted in the build-up of sodium ions within the cell and an inhibition of potassium permeability across the membrane, creating an intracellular environment that permitted only action potentials with suppressed peak amplitude. These early studies suggested that general anesthetics may transiently block action potential conduction in axons. After the removal of the anesthetic, the action potentials returned to control amplitude, highlighting the transient nature of general anesthetic effect (Shrivastav et al., 1976). Shrivastav later tested the effects of the intravenous anesthetic drug ketamine on squid giant axons (Shrivastav, 1977). Exogenous application of the drug suppressed the peak ion conductance during action potentials similar to that of volatile anesthetics, further validating a hypothesis of anesthetic-induced sodium ion build-up inside the cell (Shrivastav, 1977).

Several years later in 1983, a study by Haydon and Urban expanded on Shrivastav group's work by testing the effects of a range of volatile anesthetics (methoxyflurane, halothane, dichloromethane, and chloroform) on the sodium currents of squid giant axons (Haydon and Urban, 1983). Exposure to the anesthetic agents depolarized the neurons and reduced peak sodium ion conductance, impairing action potential generation and propagation. Interestingly, higher lipid solubility of anesthetic agents correlated with a more rapid onset of effect, suggesting that the lipid solubility of a compound may play an important role in its anesthetic action (Haydon and Urban, 1983). This correlated well with very early observations summarized in the Meyer-Overton hypothesis, which states that the potency of an anesthetic agent is directly related to its lipid solubility (Meyer, 1899; Overton, 1901). Expanding on this work, Haydon and Urban tested many other anesthetic agents, including non-polar molecules, alkanols of various lengths, and a range of volatile anesthetics, on squid giant axons and measured ion currents. They observed that all anesthetics diminished both the potassium and sodium currents of the axons significantly in a manner that correlated broadly with the agent's lipid solubility (Haydon and Urban, 1986). Furthermore, this finding supported Shrivastav et al.'s earlier suggestion that potassium permeability was suppressed after exposure to general anesthetics and generalized it to a wider range of compounds.

Furthering previous work on ion currents under the influence of anesthetics, Haydon and Simon published a study in 1988 where they measured the threshold potential of a squid giant axon under the influence of clinically relevant concentrations of many general anesthetics (Haydon and Simon, 1988). The action potential threshold was changed variably: certain agents reduced the threshold, while others increased it. General anesthetic agents all reversibly depolarized the resting membrane potential slightly by roughly 1–5 mV. The actions of all agents were fully reversible,

although some subsided immediately whereas others required extensive washout (Haydon and Simon, 1988).

There were several key parallels between the experiments of Shirvastav et al., Haydon and Urban, and the study performed by Haydon and Simon in *L. forbesi*. Specifically, all of these studies demonstrated that the giant squid axons were slightly depolarized due to general anesthetic exposure. Shirvastav et al. hypothesized that this was achieved due to the accumulation of sodium ions inside the tested neurons concurrent with the inhibition of potassium ion movement (Shrivastav et al., 1976). This meant that the cell remained depolarized and unable to repolarize completely until the complete removal of the anesthetic agent from the bathing solution. Haydon and Urban then confirmed this observation by measuring these sodium and potassium currents directly (Haydon and Urban, 1983, 1986).

Another mollusc, the fresh water pond snail *L. stagnalis,* rose to prominence in the early to mid 1980s when Winlow's laboratory conducted a series of behavioral studies on the effects of inhalational anesthetics on freely behaving animals. Specifically, Girdlestone et al. demonstrated that halothane and isoflurane, when used in clinically relevant concentrations, brought about a state of anesthesia in freely behaving *Lymnaea* (McCrohan et al., 1987; Girdlestone et al., 1989). Similarly, Franks and Lieb reinforced *Lymnaea*'s effectiveness in anesthesia research in 1990, when they used this invertebrate to demonstrate that general anesthetics act on proteins rather than on the lipid bilayer as their primary target (Franks and Lieb, 1991). Previous research by Harris and Groh had shown that a temperature change of merely 1°C caused a change in membrane potential across the lipid bilayer roughly equal to that caused by general anesthetics, thus challenging the notion that general anesthetic agents solely act on the lipid bilayer as their potential target site (Harris and Groh, 1985). Correspondingly, Franks and Lieb noted that different general anesthetics competitively inhibited an anesthetic-sensitive protein, Luciferase, at the same ED_{50} concentrations required to put a patient into the state of general anesthesia (Franks and Lieb, 1990). This result strongly suggested that the most likely primary sites of action for general anesthetics are protein molecules rather than the lipid bilayer (Franks and Lieb, 1981).

Franks and Lieb further demonstrated the cellular specificity of general anesthetics by exposing several *Lymnaea* neurons to halothane, and observing that only a single cell had its electrophysiological properties altered after exposure, while the other cells remained unaffected (Franks and Lieb, 1988). Interestingly, Franks and Lieb reported that the affected cell exhibited an anesthetic-activated potassium channel, which the neighboring anesthetic-insensitive cells did not possess (Franks and Lieb, 1988, 1990). Activation of this channel resulted in hyperpolarization of the cell. These results stand in contrast to those observed in the squid, whereby potassium conductance was reduced after anesthetic exposure (Shrivastav et al., 1976). This discrepancy could perhaps be attributed to cell specific differences in *Lymnaea* channel proteins, which may be absent in the squid.

Franks and Lieb then went on to determine where exactly on the neuron the halothane-activated potassium conductance [$I_{K(An)}$] was activated (Franks and Lieb, 1991). They suggested that since halothane reversibly inhibited the action potentials (and by extension synaptic transmission in the isolated cell), then the halothane sensitivity may likely be greatest at an area rich with synaptic connections (Franks and Lieb, 1991). Thus, synaptic stimulation could possibly modulate the $I_{K(An)}$ conductance *in vivo*. Concurrently, the neuropeptide FMRFamide, which acts as a neuromodulator in molluscs, inhibited the $I_{K(An)}$ conductance in isolated neurons, suggesting a certain measure of control over potassium flow (Franks and Lieb, 1991). Neuropeptides such as FMRFamide act both presynaptically and postsynaptically, and modulate communication between neurons by altering acetylcholine release or response. Later in 2007, Andres-Enguix et al. advanced these observations regarding the halothane-activated potassium conductance made by Franks and Lieb (Franks and Lieb, 1988, 1990, 1991; Andres-Enguix et al., 2007). Andres-Enguix et al. cloned the two-pore domain potassium (K+) channel, which exhibited significant similarities to the anesthetic-activated potassium channel identified in *Lymnaea* and was found to have an amino acid necessary for anesthetic binding (Franks and Lieb, 1988, 1991; Andres-Enguix et al., 2007). This suggested that the identified two-pore domain potassium channel may be a potential target for general inhalational anesthetics (Andres-Enguix et al., 2007). Furthermore, these results strongly suggested that there are specific moieties on proteins that are responsible for directly binding anesthetics and modulating their actions.

Focusing on synapses, which play fundamental roles in neuronal function, learning, and neuroplasticity, McKenzie et al. documented the effects of several different general anesthetics on *Lymnaea* nicotinic acetylcholine receptors (nAChR), which are the postsynaptic receptors for acetylcholine (ACh). All of the anesthetic agents suppressed the cells' ion currents through the nAChR, further suggesting that anesthetics target proteins. In support of this, they also mapped potential general anesthetic binding sites on the neuronal nicotinic AChR (McKenzie et al., 1995). These correlations strongly suggested that neuronal nicotinic AChRs might play a role in the molecular mechanism of general anesthetics—with specific impact on neuronal networks involved in learning and memory.

In 1996, Spencer et al. shifted the focus of general anesthesia research by using *L. stagnalis* to test the effects of halothane on inhibitory and excitatory peptidergic synapses. Because it is difficult, if not impossible, even in relatively simple molluscs, to decipher the direct vs. indirect effects of anesthetic at the level of single or synaptic paired cells *in vivo,* Spencer et al. employed the unique ability of the *Lymnaea* nervous system to reconstitute functional synaptic networks *in vitro*. As a result, they were able to compare and contrast the effects of clinically relevant concentrations of halothane on reconstructed synaptic networks (Spencer et al., 1995). Halothane suppressed excitatory synapses at lower concentrations than those that were required to suppress inhibitory synaptic transmission, in effect causing a net increase in inhibitory synaptic transmission at lower concentrations of halothane (Spencer et al., 1996). This study was important because it showed that halothane likely affected not only presynaptic, but also postsynaptic responses (Spencer et al.,

1996). Most significantly, this study demonstrated for the first time that general anesthetic agents may also exert their effects on peptidergic modulated synapses—in addition to classical neurotransmitter synapses. These studies thus broadened the scope of anesthetic actions beyond classical transmitters to include peptidergic communication in the brain.

With the emergence of sevoflurane as a modern volatile anesthetic agent, Hamakawa et al. provided the first direct evidence regarding the fast acting actions of this inhalational agent on the inhibition of synaptic transmission between *in vitro* reconstructed *Lymnaea* synapses followed by a rapid recovery. This study also demonstrated that like halothane, sevoflurane likely suppresses synaptic transmission between identified *Lymnaea* neurons at both presynaptic and postsynaptic sites (Hamakawa et al., 1999).

Intravenous anesthetic agents have also been studied in *Lymnaea*. Woodall and McCrohan compared the effects of the intravenous anesthetics propofol and ketamine on the neuronal and behavioral activity of *L. stagnalis* (Woodall and McCrohan, 2000). Unlike inhalational anesthetic agents (Franks and Lieb, 1988), these intravenous compounds did not induce anesthesia at the tested concentrations in freely moving *Lymnaea* nor did they affect the resting membrane potential of neurons (Woodall and McCrohan, 2000). Rather, both agents suppressed the amplitude of the first action potential occurring after hyperpolarization of only a single specific identified neuron, which acts as part of the respiratory pattern generator in *Lymnaea* (Syed et al., 1990). Interestingly, in other identified neurons, elicited burst spike activity was increased by propofol, which suggested a possible transient excitatory effect on the neurons and by extension, a possible explanation for the snail's hyperactivity observed in the study (Woodall and McCrohan, 2000). Since these compounds are highly effective at inducing anesthesia in humans, this finding highlights that these specific agents may target different neuronal structures than the volatile agents.

In addition to using *Lymnaea* as a system for studying the mechanism of action of anesthetic agents, these molluscs have been used to help elucidate possible detrimental effects of exposure to anesthetic medications. Furthering their initial work, Woodall et al. asked whether the chronic application of the intravenous general anesthetic agent propofol would be detrimental to the growth of injured neurons and if it would also block synaptogenesis between *Lymnaea* neurons. They noted that only the postsynaptic acetylcholine response was inhibited as a result of propofol exposure (Woodall et al., 2003). Taken together with their previous work (Woodall and McCrohan, 2000), this indicated that propofol may not have an impact on a cell's intrinsic electrophysiological properties but rather affects its ability to communicate with other neurons. Furthermore, propofol did not inhibit neurite outgrowth; however, long-term exposure reversibly blocked the initial formation of synapses, perhaps by blunting the postsynaptic acetylcholine responses (Woodall et al., 2003). Similarly, Naruo et al. studied the effects of a commonly used volatile anesthetic, sevoflurane, on a well-characterized *Lymnaea stagnalis* synapse pairing [visceral dorsal 4 (VD4)–left pedal dorsal 1 (LPeD1)] (Naruo et al., 2005). They observed that the agent's effects on synaptic transmission involved a blunting of the postsynaptic nicotinic acetylcholine receptor response, similar to the findings with propofol by Woodall et al. This study also asked whether volatile anesthetics affect short-term synaptic plasticity. Specifically, by pairing neurons they were able to consistently test the synapse's post-tetanic potentiation, which is thought to form the basis of working memory (Luk et al., 2011). Naruo's study was the first to demonstrate that whereas sevoflurane blocked synaptic transmission in clinically relevant concentrations, it did not affect post-tetanic potentiation (Naruo et al., 2005).

Later, Onizuka et al. exposed *Lymnaea* neurons to commonly used general and local anesthetics to observe their effects on synaptogenesis, neurite growth and synaptic transmission (Onizuka et al., 2005b). Similar to previous studies (Woodall et al., 2003), they showed that general anesthetic agents depressed excitatory cholinergic synaptic transmission postsynaptically, but did not inhibit neurite outgrowth or synaptogenesis. Conversely, the local anesthetic agents depressed excitatory cholinergic synaptic transmission presynaptically, and inhibited both neurite outgrowth and synaptogenesis (Onizuka et al., 2005b). However, the exact reason as to why the local anesthetic agents exhibited such dramatic effects is yet to be attributed to any specific inherent neurotoxicity, or to the inhibition of presynaptic mechanisms. Finally, a 2008 study by Browning and Lukowiak shed light on further possible detrimental effects that general anesthetics might have on memory formation. In this study, they determined that ketamine only inhibited long-term memory formation, and not its shorter-term counterparts (Browning and Lukowiak, 2008), which they attributed to be due to ketamine's interference with mRNA transcription required for the formation and storage of memories. This study highlights the potential for further studies that can be conducted to examine transcriptome changes after exposure to anesthetic compounds.

Taken together, these molluscan studies provide direct evidence that in the absence of network complexity and glia cells, anesthetics cause a net reduction in excitatory neurotransmission between neurons. Early work in *Loligo* showed that general anesthetics transiently block action potential amplitude and propagation down axons, likely by trapping sodium ions within cells. Further work in *Lymnaea* fleshed out these findings. Specifically, work showed that volatile general anesthetic compounds directly interact with ion channel proteins, in particular a potassium channel that hyperpolarized neurons, preventing neurotransmitter release. Subsequent work then showed that general anesthetics also directly target and suppress ion movement through the excitatory acetylcholine receptor. These studies (Franks and Lieb, 1991; McKenzie et al., 1995) thus show that some general anesthetics target both presynaptic and postsynaptic sites on neurons. These studies also highlight the potential target sites of anesthetic actions that include classical and peptidergic neurotransmitter synapses. Finally, the data from various studies on *Lymnaea* demonstrate that chronic exposure of cultured neurons to anesthetic compounds might render neuronal growth and synaptic connectivity dysfunctional. This work laid valuable foundations for potential molecular targets of anesthetics. Indeed, anesthetic agents are now believed to target many more channels than merely the

nAChR, including gamma-aminobutyric acid $(GABA)_A$, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and serotonin receptors, in a manner that preferentially enhances inhibitory transmission (Harris et al., 1995; Campagna et al., 2003; Hemmings et al., 2005). **Figure 1** shows a simplified diagram of where general anesthetics may act on the *Lymnaea* synapse.

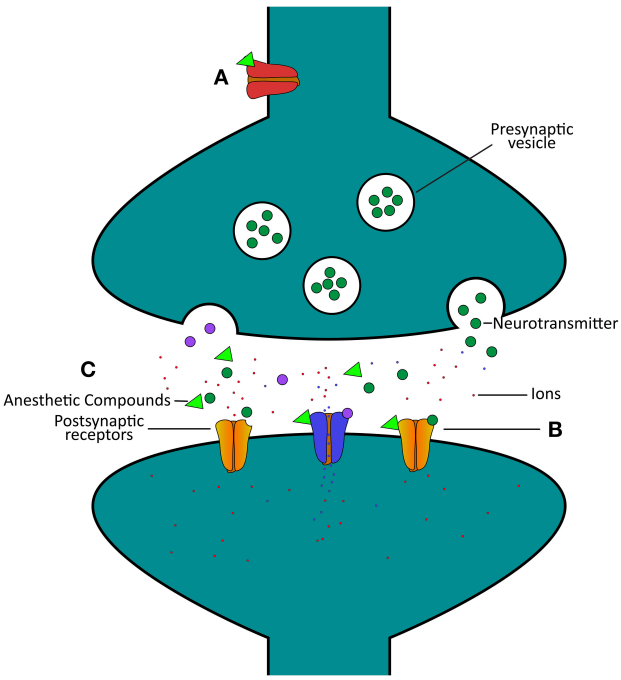

FIGURE 1 | Simplified schematic highlighting certain actions of general anesthetics on the *Lymnaea* synapse. **(A)** The anesthetic (green triangle)-activated potassium channel [red channel, $I_{K(An)}$] hyperpolarizes neurons, and thus will cause a reduction in presynaptic neurotransmitter release (Franks and Lieb, 1991). **(B)** A wide range of general anesthetics have been found to inhibit the response of the acetylcholine receptor to neurotransmitter, thus inhibiting synaptic communication (McKenzie et al., 1995). **(C)** Represents various potential target sites of anesthetic actions on neurons and their synaptic communication, from presynaptic release of neurotransmitter to postsynaptic response. Excitatory postsynaptic receptors for acetylcholine (yellow, excitatory, closed channels) are closed by anesthetic (green triangle) exposure, but at similar concentrations inhibitory postsynaptic channels (blue, inhibitory, open channels) remain open. As such, synaptic communication is preferentially suppressed in excitatory synapses as compared to inhibitory synapses at similar anesthetic concentrations (Spencer et al., 1996).

MOLLUSCS ARE VALUABLE TOOLS FOR LOCAL ANESTHESIA RESEARCH

Similar to general anesthetics, local anesthetic agents have been extensively tested in molluscs to help elucidate their specific mechanisms of action. Interestingly, recent studies have also shown that these anesthetic agents may have detrimental side effects on neuron function in invertebrates.

The first of such studies to elucidate the mechanisms of action of local anesthetics in invertebrates was in 1978 when Yeh exposed a *Loligo pealei* axon to various sodium channel blocking local anesthetics (Yeh, 1978). Yeh showed that the anesthetics produced a blockade of the sodium channel that was dependent on both the membrane potential and the frequency of any action potentials currently passing through the axon using the sodium channels, thus explaining how local anesthetics may perturb transmission of the action potential through nerve fibers. Later, in 1981, Ohki et al. showed that the relative solubility of local anesthetics in the lipid bilayer of axon membranes played an important role in their action (Ohki et al., 1981). This study was further confirmed by Wang et al. who showed that the hydrophobicity of local anesthetics was needed to interact with their binding domains on the sodium channel (Wang et al., 1982). Furthermore, they noted that the greater the hydrophobic characteristic of the local anesthetic, the greater the blockade of the sodium channel. Interestingly, this observation matches that seen with general anesthetics, according to the Meyer-Overton hypothesis.

Starmer et al. subsequently tested whether local anesthetic-mediated sodium channel blockade was the result of local anesthetic compounds being trapped within a cell and then binding receptors from within (Starmer et al., 1986). In the giant squid axon, they observed that local anesthetics bound to the interior of the ion channel, with binding being dependent on the membrane potential. As such, the degree of sodium ion blockade varied with the membrane voltages.

Other molluscs, such as *L. stagnalis*, have allowed further insights into local anesthetic mechanisms of action and possible toxic side effects on neurons. In 2003, Kasaba et al. showed impairments in neuronal growth cones and changes in neurite morphology as a result of local anesthetic exposure. The group showed that mepivacaine and procaine resulted in the least harmful effects, whereas lidocaine was the most toxic (Kasaba et al., 2003). Further studies also noted a significant increase in intracellular calcium (Ca^{2+}) concentration after local anesthetic exposure (Kasaba et al., 2006; Kasaba, 2007). Interestingly, the morphological damage and calcium concentration were not well-correlated suggesting that there was yet another mechanism of action underlying the compromised neuronal growth (Kasaba et al., 2006). To further test this link between calcium and neurite growth, Kasaba et al. exposed *Lymnaea* neurons to lidocaine in the presence or absence of a calcium chelator. They found no morphological damage despite increased intracellular Ca^{2+} concentration at low lidocaine concentrations. However, as the lidocaine concentration increased so too did the calcium concentration, causing an increase in the observed morphological damage. When combined with a calcium chelator, the resulting damage was not reduced, indicating that the damaging effects were likely not mediated solely through calcium ion accumulation (Kasaba, 2007).

In 2007, Kasaba et al. focused on another aspect of lidocaine exposure in *Lymnaea* by examining the burst action potential spikes of neurons. As the drug's concentration increased, the bursts of action potential spikes got weaker indicating an inhibition in synaptic transmission (Kasaba et al., 2007). Further studies have been used to dissect this phenomenon. Onizuka's

group demonstrated that exposing a *L. stagnalis* neuron to lidocaine resulted in a transient increase in intracellular sodium concentration, which might explain the initial excitatory phase observed following the anesthetic exposure (Onizuka et al., 2004). With the addition of the potent sodium channel blocker tetrodotoxin (TTX), there was no change in intracellular sodium concentrations, indicating that the very sodium channels ultimately blocked by lidocaine may underlie the initial rise in sodium concentration. They subsequently noted that lidocaine had a dose-dependent effect (Onizuka et al., 2005a). Further fleshing out these findings, Onizuka et al. demonstrated that lidocaine decreased the excitatory postsynaptic potential response to exogenously applied acetylcholine (Onizuka et al., 2008).

In a study designed to investigate whether or not local anesthetics alter neuron morphology, Onizuka et al. reported the formation of bullae and blebs, as well as increased cell size—which indicates cells undergoing either apoptosis or necrosis—in neurons exposed to lidocaine in a dose-dependent manner (Onizuka et al., 2012b). This may be due to the irreversible depolarization of the resting membrane potential they noted as a result of the lidocaine exposure, which may cause irreversible damage to the neurons (Onizuka et al., 2012b). In addition, Onizuka et al. demonstrated that neuron exposure to lidocaine inhibited neurotrophic growth factor (NGF)-induced axon growth and synaptic excitation (Onizuka et al., 2012a).

In summary, molluscs have proven useful to determine the mechanism of action of local anesthetics. Molluscs have allowed us to precisely identify the intracellular portion of the voltage-gated sodium channel as the primary target of local anesthetics. These findings have been borne out in other studies (Butterworth and Strichartz, 1990; Scholz, 2002). Further work has demonstrated that local anesthetics may be neurotoxic. These more recent studies collectively provide evidence for possible detrimental effects of local anesthetics on snail neurons. Lidocaine has been shown to have substantial effects on neuronal development. Lidocaine may be directly neurotoxic by suppressing synaptic function or by blocking the sodium channels that allow action potential propagation to the synapse. Exposure to lidocaine and its derivatives appears to affect the cell to a point where it is unable to self-regulate its intrinsic membrane properties. The interference in sodium channel flux may thus cause growth abnormalities in both the neurite and growth cones.

CLINICAL RESEARCH RELATED TO ANESTHETIC-INDUCED NEUROTOXICITY

Research conducted in various molluscs has established many of the mechanisms of action of anesthetic agents. In addition, molluscs provide the ability to study relatively simple neuronal networks and to reconstruct individual synapses, which may allow further understanding of the mechanisms of potential anesthetic neurotoxicity. Recent work has shown that there exists robust evidence from vertebrate animals that both intravenous and inhalational anesthetic agents may be neurotoxic and lead to deficits in behavior, cognition, learning, and memory (Walters and Paule, 2017). Results have been consistent across multiple species, using various drugs and dosing paradigms. Early studies in neonatal rats suggested that exposure to anesthetic agents that are NMDA receptor antagonists or $GABA_A$ receptor agonists resulted in cellular apoptosis and neurodegeneration in young brains (Ikonomidou et al., 1999, 2000; Olney et al., 2000). Similar findings were described when postnatal day 7 rats were given a combination of midazolam, nitrous oxide and isoflurane for 6 h, causing neuronal degeneration, deficits in synaptic function and persistent learning and memory impairments (Jevtovic-Todorovic et al., 2003). Numerous subsequent studies have corroborated these findings. For example, young rodents exposed to volatile anesthetics including sevoflurane and isoflurane were found to have deficits in long-term memory (Ramage et al., 2013). Rat cortical neurons exposed to either sevoflurane or desflurane demonstrated increased cell death, decreased neurite outgrowth and compromised mitochondrial integrity and synaptic function (Xu et al., 2016). Studies involving neonatal rodent exposure to intravenous anesthetics such as ketamine and propofol have also described detrimental effects on neuronal survival, dendritic spine density, and memory (Cattano et al., 2008; Pesić et al., 2009; Briner et al., 2011; Huang et al., 2012; Yu et al., 2013).

Findings in non-human primates have strengthened the animal literature in the field. Rhesus monkeys exposed *in utero* or as young neonates to various intravenous or inhalational anesthetics showed increased neuronal and glial apoptosis (Brambrink et al., 2010, 2012; Creeley et al., 2013, 2014). Five to six day old rhesus monkeys exposed to 24 h of ketamine continued to demonstrate cognitive impairments until at least three and one half years of age (Paule et al., 2011). The period of greatest vulnerability to the neurotoxic effects of anesthetic agents appears to correlate with the developmental stage of synaptogenesis, which in humans is thought to occur during the third trimester of pregnancy and the first 2 years of life (Hansen, 2015; Jevtovic-Todorovic and Brambrick, 2017; Walters and Paule, 2017).

Given the preponderance of evidence supporting anesthetic neurotoxicity in the animal literature, concerns have arisen regarding the exposure of human pediatric patients to these agents. However, despite convincing findings in the animal literature, human studies to date have provided limited and inconsistent results with respect to the effects of anesthetic agents on the developing brain (Bartels et al., 2009; DiMaggio et al., 2009; Kalkman et al., 2009; Wilder et al., 2009; Flick et al., 2011; Ing et al., 2012; Davidson et al., 2016; O'Leary et al., 2016; Sun et al., 2016). The majority of published human studies have been retrospective and observational in nature and there is some suggestion of small deficits in language and cognition associated with anesthetic exposure at a young age; however, the results need to be interpreted with caution (DiMaggio et al., 2009; Flick et al., 2011; Ing et al., 2012; O'Leary et al., 2016).

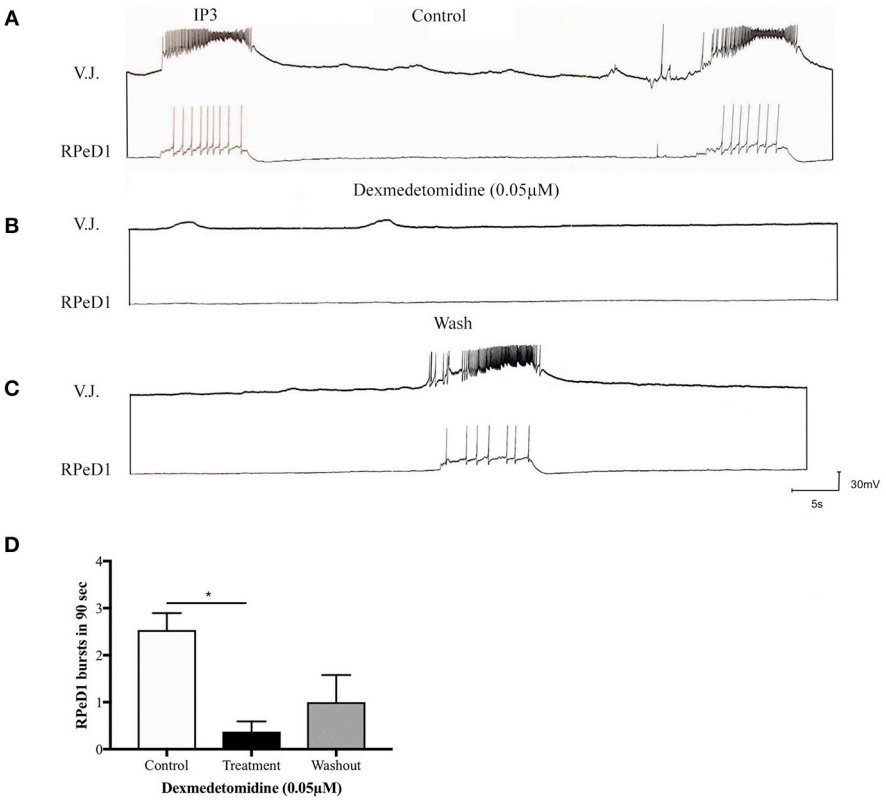

FIGURE 2 | Dexmedetomidine (0.05 μM) blocks spontaneously occurring, respiratory central pattern generator (CPG) activity in isolated *Lymnaea* brain preparation. **(A)** Isolated central ring ganglia of *Lymnaea* exhibits spontaneous, fictive respiratory patterned activity in identified neurons RPeD1 and the VJ cell when recorded intracellularly. These rhythmical discharges are generated by the IP3 interneuron, and have been well-characterized previously. **(B)** When the preparation was bathed in dexmedetomidine (0.05 μM), our preliminary data suggests that the spontaneous rhythmical bursting was blocked. **(C)** The patterned activity began to return upon wash out with normal saline. **(D)** The bar graph presents summary preliminary data suggesting a significant inhibition of respiratory rhythm through cessation of RPeD1 bursting activity during dexmedetomidine treatment. The bursting activity partially restarts upon immediate washout with normal saline. Repeated measure ANOVA was performed with data from three sample preparations. $^*p < 0.05$, error bars ± SEM.

Several large human studies have been published recently. A multicenter prospective randomized controlled trial comparing neurodevelopmental outcomes at 2 years of age after general anesthesia or awake regional anesthesia found no evidence that less than 1 h of sevoflurane anesthesia in infancy for inguinal hernia repair increases the risk of adverse neurodevelopmental outcome when compared with awake regional anesthesia (Davidson et al., 2016). The 5 year outcome data for this study is still pending. The PANDA study was a sibling matched cohort study of 105 sibling pairs between the ages of 8 and 15 years (Sun et al., 2016). One sibling had a single general anesthetic exposure at less than 36 months of age for a hernia repair and the other sibling had no exposure. There was no statistically significant difference in global cognitive function, measured by IQ, between the two groups (Sun et al., 2016). Three recent population based studies have found only subtle differences in developmental outcomes in children exposed to anesthetics at a young age (Graham et al., 2016; O'Leary et al., 2016; Glatz et al., 2017). An Ontario group matched children who had surgery under general anesthesia before Early Development Index (EDI) testing was performed around the age of 5 with control children who had no general anesthesia exposure (O'Leary et al., 2016). There was a very small difference between the two groups in incidence of developmental vulnerability (any EDI in the lowest 10%), with 25.6% in the exposed group and 25.0% in the unexposed group (O'Leary et al., 2016). A Manitoba study matched children who had surgery prior to age 4 with control children (Graham et al., 2016). There was a small difference between the two groups with exposed children doing worse in communication/general knowledge and language/cognition domains. The risk was greater in older children at the time of general anesthetic exposure and there was no difference between single and multiple exposures. The third study compared children in Sweden who had undergone one anesthetic at less than 4 years of age with controls (Glatz et al., 2017). There was a very small difference between the groups with an anesthetic exposure before age 4 associated with a mean difference of 0.41% lower school grades and 0.97% lower IQ test scores. There has been some suggestion that children exposed to multiple general anesthetics at a young age may have an increased risk of learning difficulties, but additional studies are needed to provide conclusive evidence (Wilder et al.,

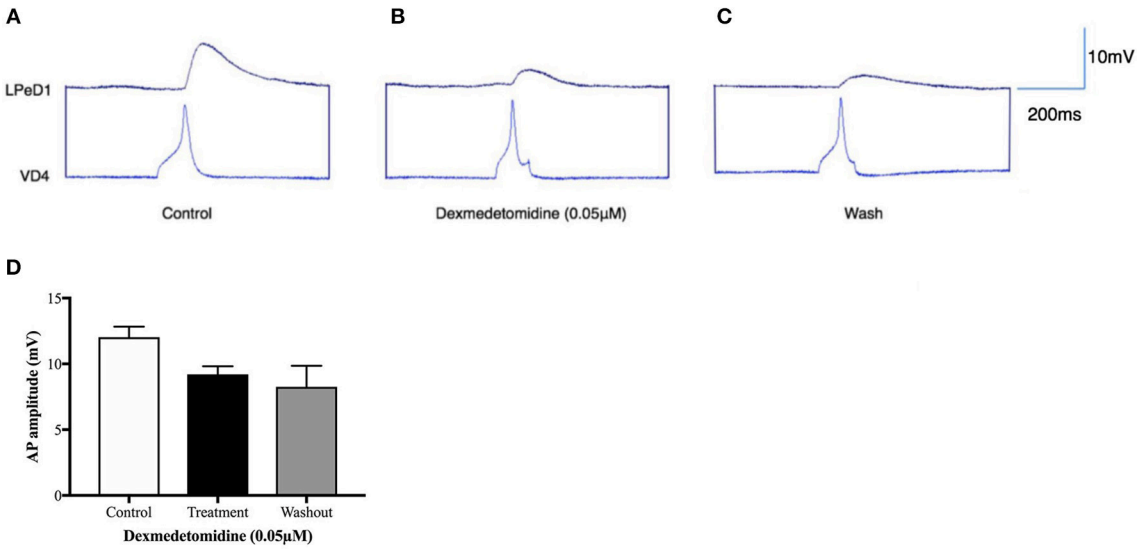

FIGURE 3 | Dexmedetomidine alters synaptic transmission between soma-soma paired *Lymnaea* neurons. Identified neurons were cultured overnight in a soma-soma configuration and simultaneous intracellular recordings were made between presynaptic neuron VD4 and its postsynaptic partner LPeD1 on day two. Induced action potentials in VD4 generated 1:1 excitatory postsynaptic potentials (EPSPs) in LPeD1 (control, **A**). Our preliminary data suggests that the amplitude of the EPSPs in LPeD1 may be reduced in the presence of dexmedetomidine **(B)**. The synaptic transmission did not return to its base line level after 15 min of wash out with normal saline **(C)**. Bar graphs **(D)** present summary preliminary data. Repeated measure ANOVA test on a sample size of three preparations. Error bars ± SEM. AP is an abbreviation of action potential.

2009; Flick et al., 2011; Graham et al., 2016; Glatz et al., 2017).

At this point, it remains difficult to make meaningful conclusions from the current human data. Future studies in both humans and animal models are urgently needed to better understand the actual clinical impact in pediatric patients and to further elucidate the anesthetic effects on neurons and glia with respect to synaptic plasticity, neuronal circuitry, and cytotoxicity. Understanding the specific mechanisms of anesthetic-induced injury will help to delineate ways to mitigate this damage and to develop potential protective strategies.

THE SEARCH CONTINUES FOR BETTER AND LESS TOXIC ANESTHETIC AGENTS

Recent studies are being conducted to identify medications or strategies to reduce any negative effects of exposure to the existing anesthetic agents. Dexmedetomidine, a selective alpha$_2$-adrenergic receptor agonist with sedative and analgesic properties, is a newer agent that has recently shown some promise in this regard. Preliminary studies have suggested that dexmedetomidine provides neuroprotection and can reverse the cytotoxic effects of volatile anesthetic agents (Su et al., 2015; Zhou et al., 2015; Alam et al., 2017). Although this agent shows promising effects, additional studies are needed to confirm its neuroprotective effects in the clinical setting.

When tested at the presynaptic level, dexmedetomidine acts as an alpha$_2$-adrenergic receptor agonist (Tachibana et al., 2012), and prevents the subsequent release of neurotransmitters like glutamate, noradrenaline, and norepinephrine (Ihalainen and Tanila, 2002; Zhou et al., 2015). The activation of alpha$_2$-adrenergic receptors by dexmedetomidine inhibits transmitter release through the suppression of voltage-gated Ca^{2+} channels (Chiu et al., 2011). Dexmedetomidine also results in an overall hyperpolarization of the membrane potential due to activation of K^+ channels by G-protein coupled receptors (Shirasaka et al., 2007; Ishii et al., 2008). However, dexmedetomidine's neuroprotective role at the level of single synapses remains unknown, as intracellular recordings from synaptically paired neurons exposed to dexmedetomidine cannot be obtained directly in mammalian models.

Our group thus conducted experiments using *Lymnaea* to examine the effects of dexmedetomidine at the level of single synapses. The following conclusions are speculative and are based on preliminary data. Firstly, our group asked whether dexmedetomidine blocks spontaneous rhythmical bursting of respiratory central pattern generating (CPG) neurons *in vitro* (unpublished observations). Central ring ganglia of *Lymnaea* were isolated *in vitro* and concurrent intracellular recordings were made from the respiratory CPG neurons Right Pedal Dorsal 1 (RPeD1) and the motor neuron Visceral J Cell (VJ) as described previously (Kyriakides et al., 1989; Syed and Winlow, 1989). Our preliminary data indicates that the isolated ganglia exhibited spontaneous respiratory rhythm, which is blocked by dexmedetomidine within minutes of exposure (**Figures 2A,B**). These effects were reversible and the spontaneous rhythmical pattern returned within minutes of washout with normal saline (**Figure 2C**). **Figure 2D** shows quantification of these results. Next, we sought to determine whether dexmedetomidine also

blocks synaptic transmission between the cardiorespiratory neurons Left Pedal Dorsal 1 (LPeD1) and Visceral Dorsal 4 (VD4) when paired in a soma-soma configuration. Neurons were paired overnight in a soma-soma configuration (**Figure 3**) and simultaneous intracellular recordings were made after 18–24 h. Induced action potentials in VD4 (presynaptic) generated 1:1 excitatory potentials in its cholinergic, postsynaptic partner (**Figure 3A**). This preliminary data allowed us to hypothesize that the cholinergic, excitatory synaptic transmission between the paired *Lymnaea* neurons may be altered within minutes of dexmedetomidine exposure of the preparation (**Figures 3B–D**).

To further determine whether the dexmedetomidine-induced effects may have involved the postsynaptic cholinergic receptor, single LPeD1 were maintained *in vitro* overnight and acetylcholine (ACh) was applied exogenously via pressure pulses (Luk et al., 2015). When cultured in brain conditioned medium (medium containing trophic factors), ACh excited LPeD1 neurons (**Figure 4A**). These non-synaptic responses appear to be reversibly blocked by dexmedetomidine (**Figures 4B–F**). However, when cultured in defined medium (medium with no added trophic factors), the LPeD1 neurons predominantly exhibited an inhibitory response. This response appeared to remain unperturbed by dexmedetomidine (**Figure 5**), although this finding requires further study.

Taken together, our preliminary data from *Lymnaea* allows us to speculate that dexmedetomidine blocks excitatory, cholinergic synaptic transmission between paired neurons directly and that these effects likely involve excitatory postsynaptic, but not the inhibitory postsynaptic, receptors. Due to the preliminary nature of this data, additional studies are required to validate these

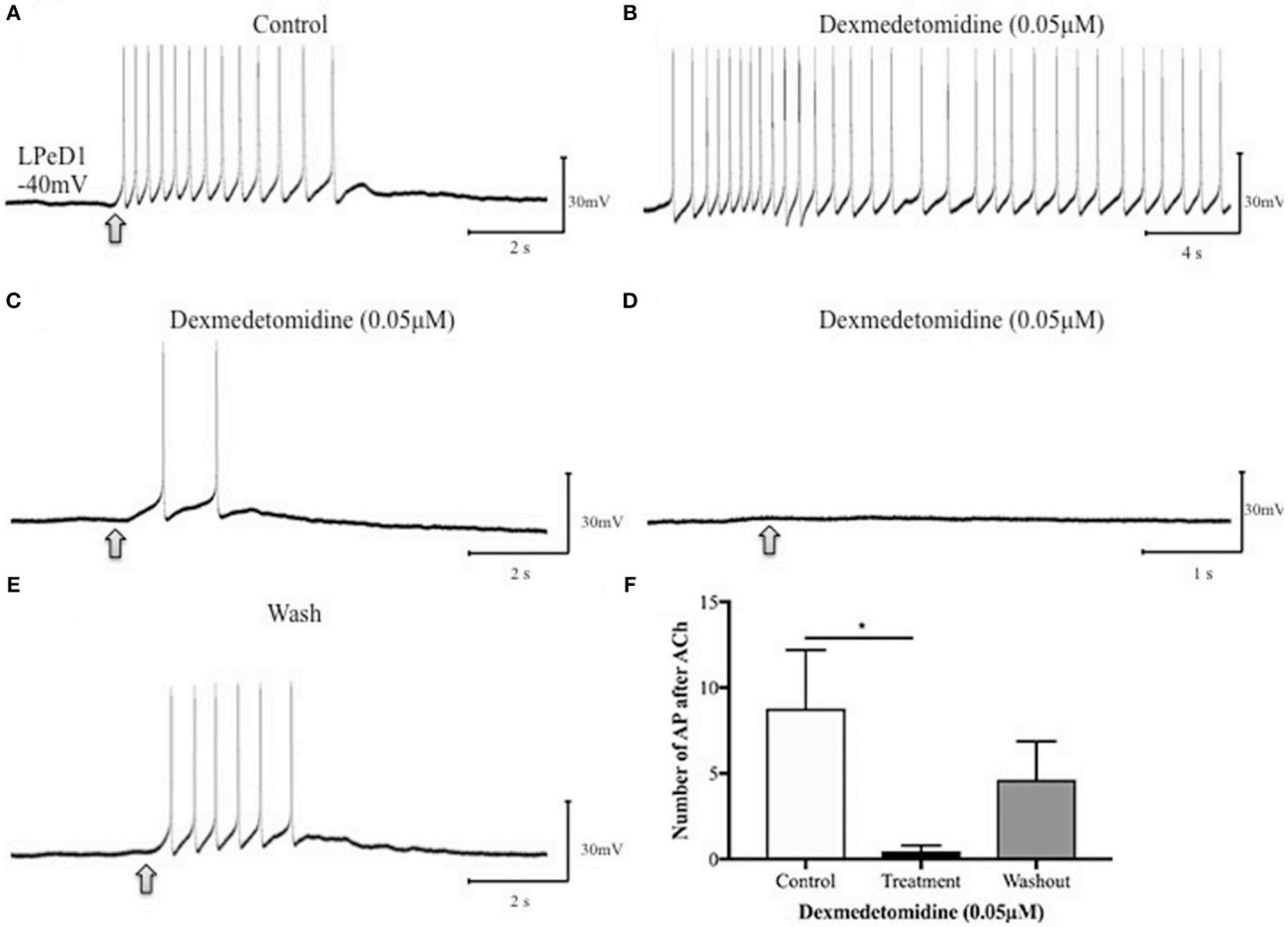

FIGURE 4 | Dexmedetomidine (0.05 μM) blocks extra-synaptic, excitatory cholinergic receptors in the identified *Lymnaea* neuron LPeD1. LPeD1 neurons were isolated *in vitro* and maintained in culture for 18–24h in the presence of brain-conditioned medium (CM). On day two, neurons were impaled with intracellular sharp electrodes and ACh was pressure applied onto the somata, either in the absence or presence of dexmedetomidine. **(A)** LPeD1 neurons exhibited an excitatory response to exogenously applied ACh (1 μM, ∼3–6 s, 15-PSI—at arrow) in CM ($n = 5$) held below the firing threshold at −40mV. **(B)** Our preliminary data suggests induction of spontaneous activity in LPeD1 neurons immediately after their exposure to dexmedetomidine (0.05 μM). **(C)** ACh application to LPeD1 after 5 min of dexmedetomidine exposure significantly reduced the excitability to ACh. **(D)** Excitatory cholinergic response in LPeD1 was completely blocked by dexmedetomidine within 10–15min of exposure. **(E)** Upon wash out of the dexmedetomidine with normal saline, the cholinergic response recovered. **(F)** The bar graph presents summary preliminary data based on the number of action potentials occurring after ACh application before returning to baseline before dexmedetomidine exposure, during exposure (after 5 min of dexmedetomidine exposure, as in **C**), and after washout. These preliminary data suggest that dexmedetomidine may block excitatory nAChRs in LPeD1. Repeated measure ANOVA test on a sample size of five preparations. *$p < 0.05$, error bars ± SEM. AP is an abbreviation of action potential.

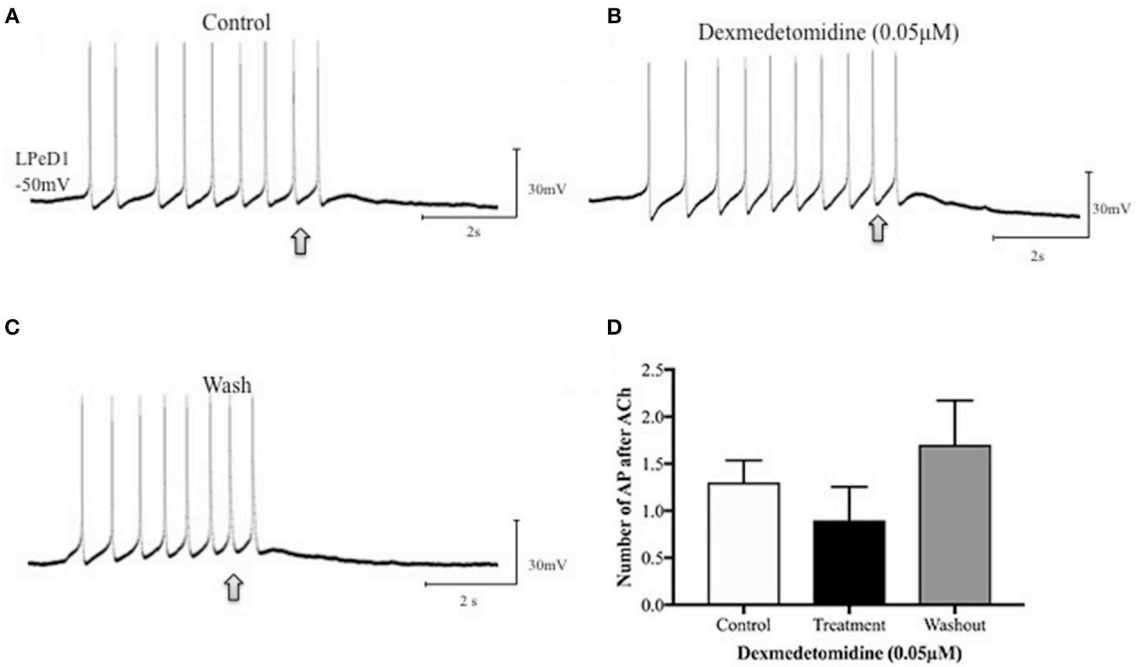

FIGURE 5 | Dexmedetomidine (0.05 μM) does not block inhibitory cholinergic receptors in the identified *Lymnaea* neuron LPeD1. When cultured in defined medium alone (DM—does not contain trophic factors), the LPeD1 neurons exhibit an inhibitory response to exogenously applied ACh. Neurons were isolated in culture and intracellular recordings were made from LPeD1 after 18–24h. All neurons, when held near or below their firing threshold (−40mV), exhibited an inhibitory response to exogenously applied ACh, which prevented continued neuronal spiking. **(A)** Spontaneous firing in LPeD1 was blocked by a single puff of ACh (at arrow). **(B)** Neurons were then exposed to dexmedetomidine for 10–15 min and ACh was applied again (at arrow). Our preliminary data suggests that dexmedetomidine failed to block the inhibitory response of LPeD1 neurons to ACh. **(C)** After several minutes of washout with normal saline, neurons continued to exhibit an inhibitory response to exogenously applied ACh. **(D)** The bar graph shows summary preliminary data of the number of spontaneous action potentials occurring after ACh application until returning to baseline, indicating that dexmedetomidine may not significantly impair inhibitory cholinergic receptors. Repeated measure ANOVA test performed using three preparations. Error bars ± SEM. AP is an abbreviation of action potential.

findings and to further examine how dexmedetomidine, or other similar agents, could perhaps serve a neuroprotective role, when used alone or in combination with other anesthetic agents. Invertebrate models are uniquely suited to help guide these future, fundamental, experiments.

CONCLUSIONS

From initial studies on the effects of anesthetics in *L. forbesi*, to more recent work with other mollusc species, including the highly versatile *L. stagnalis*, it is clear that molluscs continue to serve as valuable organisms for fundamental basic science anesthetic research. Molluscs offer a solution to the anatomical and experimental limitations posed by mammals as they better enable researchers to isolate various factors (e.g., network complexity, glial influences) in simplified neuronal systems. As such, researchers are able to define the effects of anesthetic compounds on brain function—from single ion channels, to neuronal intrinsic and synaptic properties at a resolution not approachable elsewhere. For instance, the giant squid axon allowed researchers to directly observe possible mechanisms of anesthetic agents that underlie nervous system suppression by inhibiting action potential generation and propagation. In addition, the large, easy to manipulate, and individually identified neurons of the *L. stagnalis* allowed for the discovery of proteins (specifically the nAChR) as a potential site of action for general anesthetic agents rather than solely the cell membrane. In particular, studies in *Lymnaea* have shown that anesthetics induced the inhibition of synaptic transmission in both cholinergic and peptidergic synapses at both excitatory and inhibitory synapses. In terms of anesthetic-induced neurodegeneration and neurotoxicity, results from *Lymnaea* are equivocal. Studies have reported reductions in cholinergic synapse formation, yet only with chronic exposure. In addition, general anesthetics have no effect on post-tetanic potentiation or neurite growth in *Lymnaea* neurons. However, despite this lack of documented effect of anesthetic agents on short-term memory, ketamine has been shown to impair long-term memory in *Lymnaea*. In addition, local anesthetics have been shown to cause neuron apoptosis and arrest neurite growth.

A wealth of work in mammals and in the clinical setting has highlighted that possible negative effects of anesthetics can be neither ignored nor ruled out. As such, research into other compounds that can be used either alone or as adjuvants to general anesthetics that may offer less potential for neurotoxicity is key to moving this field forward. Here, we presented

preliminary data on the mechanism of action of one such compound, dexmedetomidine. Additional research is needed to determine if and how this compound might be used to mitigate anesthetic-related neurotoxicity.

AUTHOR CONTRIBUTIONS

All authors wrote and edited the manuscript. SR, SH, and NS performed experiments.

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A Novel Approach to Primary Cell Culture for *Octopus vulgaris* Neurons

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Octopus vulgaris is a unique model system for studying complex behaviors in animals. It has a large and centralized nervous system made up of lobes that are involved in controlling various sophisticated behaviors. As such, it may be considered as a model organism for untangling the neuronal mechanisms underlying behaviors—including learning and memory. However, despite considerable efforts, Octopus lags behind its other counterparts vis-à-vis its utility in deciphering the cellular, molecular and synaptic mechanisms underlying various behaviors. This study represents a novel approach designed to establish a neuronal cell culture protocol that makes this species amenable to further exploitation as a model system. Here we developed a protocol that enables dissociation of neurons from two specific Octopus' brain regions, the vertical-superior frontal system and the optic lobes, which are involved in memory, learning, sensory integration and adult neurogenesis. In particular, cells dissociated with enzyme papain and cultured on Poly-D-Lysine-coated dishes with L15-medium and fetal bovine serum yielded high neuronal survival, axon growth, and re-growth after injury. This model was also explored to define optimal culture conditions and to demonstrate the regenerative capabilities of adult Octopus neurons after axotomy. This study thus further underscores the importance of Octopus neurons as a model system for deciphering fundamental molecular and cellular mechanism of complex brain function and underlying behaviors.

Keywords: primary neuron cell culture, *Octopus vulgaris*, cephalopods, marine invertebrates, central nervous system, vertical-superior frontal system, optic lobes, axon regeneration

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INTRODUCTION

Due mainly to the complexity of both vertebrate and invertebrate nervous systems, our understanding of the fundamental mechanisms ranging from simple reflexes to complex motor patterns and learning and memory has remained limited. Specifically, it is often difficult to decipher how individual or networks of neurons generate patterned activity underlying any given rhythmic behavior or even invoke mechanisms underlying cognition and learning and memory. *In vitro* cell culture technique represents an important tool in a variety of studies with many applications ranging from biological to medical sciences. *In vitro* cultured cells enable a reductionist approach, which is used as alternative tools instead of animal experimentation, for biotechnological applications and pathological investigations. Such studies have played pivotal roles in deciphering mechanisms of cellular excitability to rhythmogenesis at a resolution not approachable in the intact brain (Schmold and Syed, 2012).

In vitro studies on neurons derived from the nervous system of vertebrates such as the chick (Hammarback et al., 1985), frog (Lohof et al., 1992), mouse (Lumsden and Davies, 1986), and rat (Tessier-Lavigne et al., 1988) have been essential to our understanding of neuronal cell biology

and the molecular mechanisms underlying chemotropic guidance of growing axons and network (Gordon et al., 2013; Zhang and Hu, 2013; Eberwine et al., 2014; Mergenthaler et al., 2014; Bardy et al., 2015; Gawad et al., 2016). Alternatively, invertebrates comprise more than 95% of the animal species (Rinkevich, 1999) and may be considered a major source for cell culture applications. In fact, attempts to maintain and grow invertebrate cells *in vitro* were made quite early in the history of tissue culture, nearly 100 years ago (Gomot, 1971; Rannou, 1971). Currently, there have been more than 200 cell lines established from tissues of insects and ticks(Bayne, 1998), in particular *Drosophila melanogaster* (Gonzalez et al., 2011) and *Caenorhabditis elegans* (Christensen et al., 2002; Strange and Morrison, 2006).

In marine invertebrates, there are only limited primary cell cultures/cell lines developed from a few species within six invertebrate phyla (Porifera, Cnidaria, Crustacea, Mollusca, Echinodermata, Urochordata) out of more than 30 invertebrate phyla available, even though they represent a rich source of cell and tissue types and they significantly differ from one group to another (Rinkevich, 1999). Molluscs are probably the most intensively studied group of marine invertebrates as it comes to cell culture techniques (Syed et al., 1999; Schmold and Syed, 2012). During the last 20 years, a variety of organs and cells from molluscs have been cultured, including epithelial cells from embryos, gills and mantles (Cornet, 1995), nervous system (Berdan et al., 1990; Tamse et al., 1995), digestive glands (Odintsova et al., 1994), cardiac muscles (Kleinschuster et al., 1996), giant fiber lobe neurons of the squid (Gilly et al., 1990), and the hematopoietic systems (Davids and Yoshino, 1998; Troncone et al., 2015). In particular, primary cultures of neurons from molluscs have been extensively used for studies on neural growth, axon pathfinding, synapse formation, and nerve regeneration (Syed et al., 1990). Primary cultures of several types of crustacean neurons have also been developed previously (Toullec, 1999), among which the most developed culture conditions are for olfactory sensory neurons and stomatogastric neurons (Graf and Cooke, 1990; Fadool et al., 1991; Zhao et al., 2009).

Our major objective was to develop a neuron cell culture protocol since there were no such techniques available for octopus neurons and all previous attempts were unsuccessful.

Inspired by cell culture work on other invertebrates including molluscs and crustaceans, we set out here to develop a protocol for primary culture of neurons from the nervous system of the cephalopod *Octopus vulgaris*—a powerful model system, that could serve us well in deciphering the behavioral, cellular, and molecular mechanisms at the basis of brain function.

Octopus vulgaris lives an active life, it has a closed vascular system, a vertebrate like blood-brain barrier, possesses complex and centralized nervous system, exhibiting sophisticated behaviors (Young, 1971; Nixon and Young, 2003). Its "intelligence" coupled to some intriguing characteristics of its brain, such as the presence of nervous districts comparable to vertebrates' specific areas, and the absence of myelination in neurons indicated that octopus may be an excellent animal model characterized by a separate evolutionary lineage, which achieved many neuronal complexities independently (Hochner, 2010; De Lisa et al., 2012a,b).

Despite extensive studies and the advanced knowledge that we have gained vis-à-vis octopus's high cognitive abilities *in vivo,* learning and memory skills (Byrne et al., 2006; Kuba et al., 2006a,b, 2010; Gutnick et al., 2011; Tramacere et al., 2013; Richter et al., 2015, 2016) and the recent sequencing of *O. bimaculoides* genome and *O. bimaculoides* and *O. vulgaris* transcriptomes (Albertin et al., 2012; Zhang et al., 2012; Liscovitch-Brauer et al., 2017), almost nothing is known about the cellular mechanisms underlying behaviors at the associative neuronal network level, due mainly to the lack of various cell culture techniques, except for white body cells in *O. vulgaris* (Necco and Martin, 1963) and the stellate ganglion of *O. rubescens* (Gilly et al., 1997).

In view of the above need and motivation, here we developed a novel technique to isolate and culture primary neurons from the *O. vulgaris* brain, accomplishing the following aims: (1) developed a protocol to obtain dissociated neurons from two specific brain areas, the vertical-superior frontal system (VSFS) and the optic lobes (OL), two brain regions are involved in memory, learning, sensory integration and adult neurogenesis in *Octopus vulgaris* (Bertapelle et al., 2017); (2) compared different cell culture coating reagents and culture medium supplements for cell adherence, survival, and growth; (3) conducted immunocytochemistry analysis of neuronal markers; lastly (4) demonstrated, for the first time, that *in vitro* VSFS neurons exhibit robust regenerative behavior after axotomy and cell isolation.

METHODS

Animals

Specimen of *O. vulgaris* ($n = 3$ male, weight ~400 g), collected in Bay of Naples, were maintained in aquarium tanks (Polese et al., 2014; Di Cosmo et al., 2015). This study was carried out in accordance with the recommendations of European Directive 2010/63 EU L276, the Italian DL. 4 /03/ 2014, n. 26 and the ethical principles of Reduction, Refinement and Replacement (protocol n. 0124283-08/11/2012). Octopuses were anesthetized with isoflurane insufflation (Polese et al., 2014) and brains were dissected under sterile conditions. The protocol was approved by the "Centro Servizi Veterinari" of University of Naples Federico II and Ministero della Salute, Ufficio Tutela del Benessere Animale (Prot. 608/2016-PR).

Hemolymph Collection

When octopuses were completely relaxed, the ventral mantle was partially folded backwards to expose the branchial hearts for blood sampling (Malham et al., 1998). The hemolymph was collected using a 2.5 ml sterile syringe with 30G needle. 20 ml of hemolymph was typically obtained and was immediately diluted in an equal volume of marine anticoagulant solution composed

Abbreviations: DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; FBS, Fetal Bovine Serum; HEMO, Hemolymph; L15-ASW: L15-supplemented artificial sea water; OL, optic lobes; PARP1, poli (ADP-ribose) polymerase; PBS, Phosphate Buffered Saline; PDL: Poly - D - lysine; Pen/Strep, Penicillin-Streptomycin solution; PLL: Poly – L - lysine; VSFS, vertical-superior frontal system mass.

of 0.1 M Glucose, 15 mM trisodium citrate, 13 mM citric acid, 10 mM ethylene diaminetetraacetic acid, 0.45 M NaCl, at pH 7.0 and 1,000 mOsm (Barcia et al., 1999). The hemolymph solution was centrifuged at room temperature (21–22°C) for at least 10 min at 2,000 RPM to pellet hemocyte. The supernatant was sterilized through a 0.22 μm pore filter and stored at −20°C.

Neuronal Cell Culture

The central nervous system was removed and the vertical-superior frontal system mass (VSFS) and optic lobes (OL) were dissected. They were then separately cut into small pieces with a scalpel and incubated with 1 mg/ml papain enzyme for 30 min at room temperature. For comparison, tissues were also exposed to first collagenase P (4 mg/ml) in L15-supplemented artificial sea water (L15-ASW, containing 400 mM NaCl, 10 mM KCl, 15 mM HEPES, and Phenol Red, pH 7.8) for 30 min at room temperature (21–22°C) and then with 1 mg/ml trypsin in ASW for 20 min at room temperature (all chemicals and enzymes are from Sigma-Aldrich, St. Louis, MO, USA). VSFS and OL were extensively washed in Leibovitz-15 medium (ThermoFisher Scientific, Waltham, MA, USA) to stop the enzyme function. Tissues were triturated with fire polished glass pipette as well as 1 ml and 0.2 ml pipette tips to yield single cells for 2–5 min or until no cell clusters were visable. After VSFS and OL cell dissociation, cells were diluted to an appropriate density in culture media and plated on glass coverslips which were pre-coated with Poly – L - lysine (100 μg/ml, PLL, Sigma Aldrich) or Poly – D - lysine (100 μg/ml, PDL, Sigma Aldrich) in sterile water. Cells were allowed to attach to the coating surface for 30 min prior to the addition of 2 ml of the Leibovitz-15 medium and cells were maintained in an incubator at saturation humidity at 18°C. For comparison study, additive components to the culture medium including either Fetal Bovine Serum (at 4%, FBS) or Hemolymph (at 10%, HEMO) were applied after plating and maintained through subsequent feeding with media. Half of the culture medium was substituted on the third day.

Trypan Blue Cell Viability Assay

Trypan Blue is a staining dye recommended for cell viability evaluation. The medium was gently removed from the dish and Trypan blue (0.04%) in Penicillin-Streptomycin solution (Pen/Strep 1%) was added to the culture for 10 min. The solution was then removed and the culture was washed two times with Pen/Strep solution. The live (non-stained) and dead (Trypan blue-stained) cells were counted under the inverted phase contrast microscopy, and the cell viability percentage was calculated.

Immunocytochemistry and Neuronal Regeneration Study

Neurons, cultured for 4 days in L15-medium plus 10% HEMO in PLL-coated dishes, were fixed for 20 min with 4% Paraformaldehide in 1X PBS, washed 3 times with 1X PBS, and incubated with blocking solution containing 0.1% Triton-X 100 and 5% Goat Serum in 1X PBS for 1 h at room temperature. Cells were then incubated for 1 h with mouse polyclonal Anti-β III Tubulin antibody (1:500, abcam) and rabbit polyclonal poli (ADP-ribose) polymerase (PARP1) primary antibody (De Lisa et al., 2012a) (1:500, Santa Cruz Biotechnology Inc.) in blocking solution overnight at 4°C. After washing off the primary antibodies with 1X PBS for three times, cells were incubated in dark for 1 h with incubation medium containing secondary antibodies, the FITC-conjugated goat anti-mouse IgG (1:200, Thermo Fisher Scientific) and Rhodamine conjugated goat anti-rabbit IgG (1:200, Thermo Fisher Scientific). Cells were washed three times with 1X PBS and coverslips were mounted with mounting media containing 5% Glycerol and 0.2% DAPI. Fluorescent images were acquired using an A1R MP microscope under a CFI Plan Fluor 20X/0.75 MI objective with NIS Elements v4.13.00 software (Nikon). Fluorophores were excited with 488 nm and 561 nm lasers and emissions collected through 525/50 and 595/50 filter cubes.

In order to test the effectiveness of optimal cell culture conditions for VSFS cells under our culture condition, a neurite transection of a selected neuron was performed on an inverted microscope (Nikon Ti Eclipse E400) using the sharp tip of a pulled glass pipette mounted on a micromanipulator. The neurite re-growth was monitored using the same inverted microscopy for 0–3 h.

Parameters for the Evaluation of Cell Culture Conditions

Phase contrast images of cells under different culture conditions were taken using an inverted microscope (60X magnification, Nikon Ti Eclipse E400). The number of adherent cells with or without neuritic processes in 10 fields of view for each treatment was counted and measured using imageJ software. Neurite exhibiting at least half of the cell body length was counted as a neuronal process. Specifically, the "NeuronJ" plug-in (version 1.4.3) of NIH-ImageJ software (version 1.50 Meijering et al., 2004) was used to quantify the average neurite length of each neuron at different time points (day 1 and day 4). Eight-bit grayscale images of neurons with identifiable neurites were loaded into the software and calibrated according to the image magnification. The average length of the neurites was obtained by manually tracing the length of all neurites from one single neuron's cell body and divided by the total number of neurites per neuron. The lengths and the total number of neurites were averaged across all neurons in each treatment.

Boxplots resulted from 10 images per treatment for the following four parameters: the number of cells attached, the number of cells exhibited neurites outgrowth, the number of neurites, and the neuritic length. The top and bottom of the boxes mark the 25th and 75th centiles and the inner line marks the median value; 25% of the data above the 75th centile and 25% of the data below the 25th centile are marked as "whiskers" limited by the maximum or minimum values. Outliers are displayed as points.

Data Analysis and Significance Test

The experimental data were collected from four separate experiments with the examination of different culture conditions. Statistical analyses were performed with (R Developmental Core Team, 2011). Differences in distribution were tested using

Kruskal-Wallis test, the difference was deemed to be significant at the level of $p < 0.05$.

RESULTS

Our dissociation approach yielded different cell types (**Figure S1**) which were selected on the basis of the morphology and dimension. In particular, we selected amacrine cells of 10 μm diameter from VSFS and 10 μm diameter from OL.

The Effects of Dissociation Enzymes, Coating Regents, and Culture Media on Neuronal Culture Efficacy

To establish an optimal cell culture protocol, we sought to test the efficiency of commonly used dissociation enzymes, papain or collagenase in combination with trypsin, on VSFS cells plated on either PLL or PDL pre-coated dishes in L15 medium with the addition of FBS 4% or HEMO 10% (**Figures 1**, **2**). After 1 day of cell culture, phase contrast images were taken and four parameters were evaluated: the number of cells attached and those that exhibited neurites outgrowth, and the number of neurites and neuritic length (**Figures 1**, **2**).

The choice of these enzymes are based on the fact that Octopus nervous system is rich in both collagen based connective tissues and adhesive proteins surrounding the neurons (Kier and Stella, 2007). Interestingly, we found that both papain and collagenase-trypsin were equally potent in breaking down the octopus VSFS neural tissue into single neurons (**Figure 1**). These neurons exhibited round shaped cell bodies, whose diameters ranged between 5 and 10 μm. After 1 day in culture, the majority of cells, in all culture conditions, sent out fine and branched processes (**Figure 1**). Although both enzymes showed strong dissociation efficiency, interestingly, our statistical data revealed that neurons dissociated with papain demonstrated better performances than those dissociated with collagenase, in all coating and culture conditions (**Figure 2** and **Table 1**).

Specifically, neurons dissociated with papain enzyme exhibited a significantly higher degree of attachment (reflected by a higher cell count per image area of 12,000 μm^2) and more extensive neurite outgrowth (reflected by a larger number of cells developing neurites, more neuritic branches, and longer neuritic processes). Note that papain culture showed less cellular debris as compared to collagenase culture and contained more neurons, which had bright, phase contrast membrane boundaries (**Figure 1**). Furthermore, our results demonstrate that cells cultured on PLL-coated coverslips (the left panel of **Figures 2A–D**) performed significantly better than neurons grown on PDL-coated surfaces (right panel of **Figures 2A–D**) in all parameters measured (**Table 1**). These data indicate that papain dissociation in couple with PLL-coating is a good combination procedure for promoting neuronal attachment and subsequent growth during *in vitro* culture of VSFS neurons.

Because neuronal endogenous factors such as HEMO and exogenous molecules like FBS, could provide growth factors essential for cell survival, migration, differentiation, as well as neurite initiation, process extension, and nerve regenerations both *in vivo* and *in vitro* (Gordon et al., 2013), we tested the effects of these factors by including them in the L-15 culture media. Our results demonstrate that in PLL coated dishes, neurons exposed to HEMO and FBS exhibited a similar cell count and growth when compared with neurons in the absence of these growth factors. Interestingly, both HEMO and FBS significantly promoted the growth of neurons (more cells have growth and each neuron has more branches and longer neurites) cultured in PDL-coated dishes as shown in **Figures 2B–D**, despite the fact that the number of cells attached to the substrate was not affected (**Figure 2A**). It is important to note that overall the cells cultured in PLL dishes in the absence or presence of HEMO or FBS (the left six columns) exhibited similar or better cell count and growth as compared to neurons cultured in PDL dishes with the addition of HEMO or FBS (the right four columns). These results indicate that the coating agent PLL itself is more effective for octopus cell culture and the endogenous or exogenous factors are beneficial to neurons that are cultured on PDL-coated substrates. In summary, these data indicate that the optimal cell culture of octopus VSFS neurons could be achieved by culturing cells on PLL subtract coated dishes either with or without HEMO or FBS or on PDL-coated dishes with the addition of HEMO or FBS to the media.

Cell Viability and Growth of VSFS Cells in Culture

To test the cell health condition in our culture, we next performed the live/dead cell assay using the standard Trypan Blue staining method. Cells dissociated with papain were cultured on PLL coated dishes overnight and stained with Trypan blue solution the next day (see section Method). **Figure 3** shows that the majority of cells (94.60%, ±1.78) did not show any staining with the blue dye indicating that they were all alive. Note that only very few unhealthy cells were stained with the dye. These data clearly demonstrated that cells under our culture procedure are very healthy.

To further confirm that these neurons were indeed healthy, we monitored their growth and tested their ability to develop neuritic contacts. Cells treated with papain were plated on PLL or PDL coated dishes cultured in L15 medium with the addition of FBS 4% or HEMO 10% for up to 4 days, images were acquired from cultures in both day 1 and day 4 (**Figure 4**).

We chose these two-time points as they guaranteed to clearly differentiate among different treatments, and based on our observations that neurons start to show physical contact within 3–4 days. We found that the majority of the cells, in all treatments, send out fine and branched processes from day one of culture as described earlier (**Figure 4**). Specifically, we observed round shaped cells in PLL coated dishes after the 1st day and some of them spread out either one or two neurites or in clusters (**Figure 4A**). At the 4th day in culture, cells exhibited round shapes, showing branched neurites and more of these cells developed either "soma-soma" clusters, soma-neurite or neurite-neurite contacts. It is important to note that cells cultured in the presence of FBS in both PLL and PDL coated dishes exhibited more extensive branches and cell contact after 4 days in culture. Our data indicate that neurons continue to grow up nicely and develop physical contacts with each other within 4 days of culture.

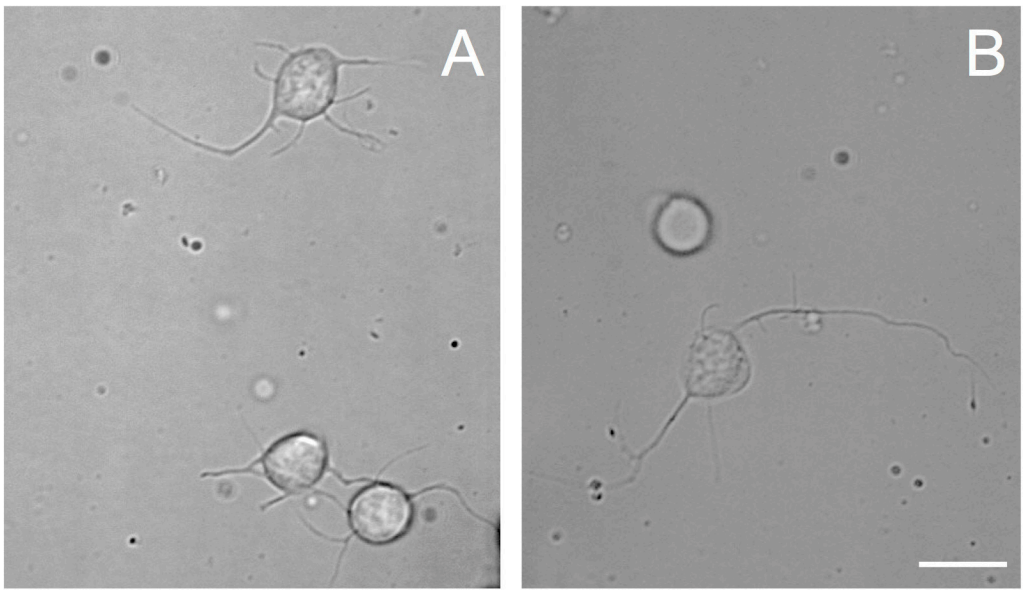

FIGURE 1 | Phase contrast images of cells from VSFS dissociated with papain **(A)** or collagenase **(B)** enzyme, plated on PDL coated glass coverslips and cultured in L15 medium; white scale bar indicates 10 μm.

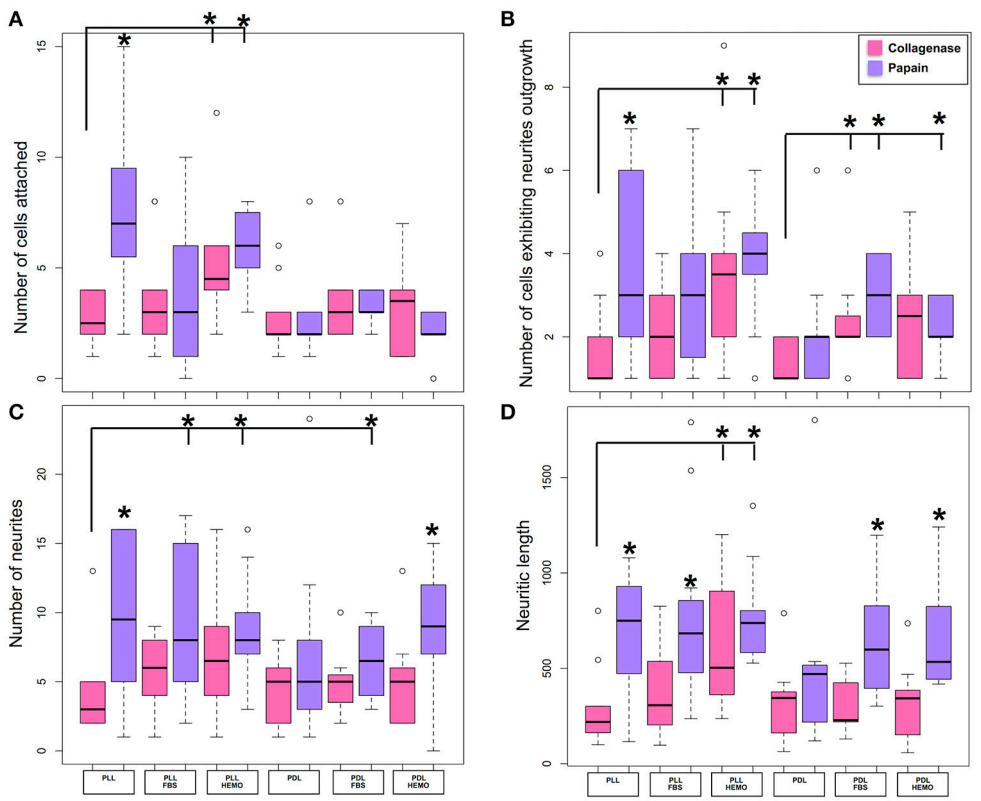

FIGURE 2 | Number of cells attached and exhibited neurites outgrowth and number of neurites and neuritic length of VSFS cells when dissociated with collagenase (pink) and papain (purple), plated on PLL and PDL coated glass coverslips, and cultured in L15 medium with the addition of FBS or HEMO; $^*p < 0.05$.

TABLE 1 | Mean ± standard error of four quantitative parameters for evaluating the effectiveness of cell culture conditions with collagenase (C) or papain (P) enzymes.

Parameters evaluated	Number of cells attached		Number of cells exhibiting neurites outgrowth		Numbers of neurites		Neuritic length	
Enzyme	**C**	**P**	**C**	**P**	**C**	**P**	**C**	**P**
PLL	2.50 ± 0.40	**7.00 ± 1.10***	1.00 ± 0.34	**2.50 ± 0.67***	3.00 ± 1.06	**7.50 ± 1.78***	219.02 ± 68.22	**742.85 ± 113.98***
PLL+FBS	3.00 ± 0.78	3.00 ± 0.89	2.00 ± 0.31	2.00 ± 0.58	6.00 ± 0.83	**7.00 ± 1.75***	306.45 ± 79.18	**657.40 ± 148.23***
PLL+HEMO	**4.50 ± 0.90***	**6.00 ± 0.48***	**3.50 ± 0.72***	**4.00 ± 0.42***	6.50 ± 1.45	**8.00 ± 1.13***	**503.07 ± 111.20***	**737.58 ± 75.81***
PDL	2.00 ± 0.58	2.00 ± 0.63	1.00 ± 0.17	2.00 ± 0.48	5.00 ± 0.91	5.00 ± 2.16	344.34 ± 74.57	470.02 ± 150.10
PDL+FBS	3.00 ± 0.81	3.00 ± 0.25	**2.00 ± 0.61***	**3.00 ± 0.26***	5.00 ± 0.98	6.50 ± 0.76	228.62 ± 59.32	**598.20 ± 98.37***
PDL+HEMO	3.50 ± 0.67	2.00 ± 0.35	2.50 ± 0.50	**2.00 ± 0.36***	5.00 ± 1.05	**8.00 ± 1.70***	342.60 ± 60.95	**473.80 ± 123.05***

Statistical differences are in bold and asterisks indicate positive statistical significance (*).

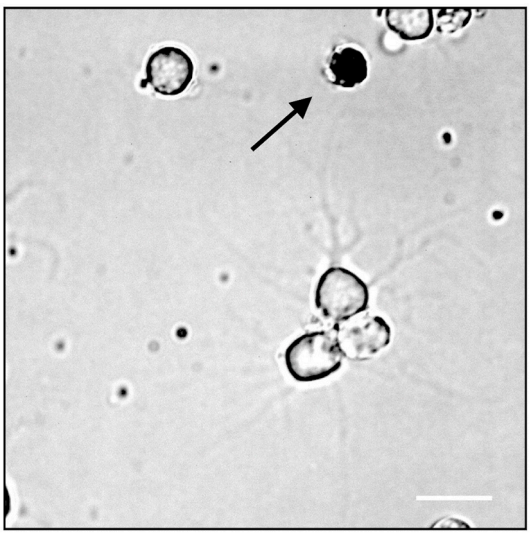

FIGURE 3 | Trypan Blue assay, the arrow indicates a dead neuron cell; white scale bar indicates 10 μm.

Comparing PLL + FBS at 1st and 4th day of culture (**Figures 4C,D**) we observed respectively cells showing morphology quite similar to that of PLL at 1st day (**Figure 4A**) and only at the 4th day of culture axo-somatic contacts were observed (**Figure 4D**).

In PLL + HEMO, the 1st day was dominated by the presence of round cells with few branching neurites (**Figure 4E**), whereas on the 4th day small clusters and axo-somatic contacts were observed (**Figure 4F**). In PDL plated culture at 1st and 4th day, neurons exhibited round shapes with many branched neurites involved in axo-somatic and axo-axonic contacts (**Figures 4G,H**).

In PDL + FBS at 1st day, neurons exhibited long neurites (**Figure 4J**) and on 4th day (**Figure 4K**), they formed networks. In PDL + HEMO at 1st day, the round shaped neurons showed long branched neurites (**Figure 4L**), at 4th day the neurons showed more shorter neurites (**Figure 4M**).

Our statistical analysis revealed that VSFS neurons cultured on PDL-coated dishes with the addition of FBS, and not HEMO, show significant increase in all parameters evaluated after 4 days in culture compared to neurons at 1 day culture (**Figure 5**, **Table 2**). It is important to note that the number of adhered cells and their growth in PLL-coated dishes (with or without the addition of FBS and HEMO) and PDL + HEMO did not show an increase from the 1st to the 4th day of culture, meanwhile in PDL and PDL + FBS exhibited an increase from the 1st to the 4th day of culture (**Figure 5**). Specifically, a similar trend was observed considering the number of cells with neurites outgrowth from 1st to 4th day of cell culture in PLL, PLL enriched (both FBS and HEMO) and PDL + HEMO, meanwhile in PDL and PDL + FBS demonstrated a positive trend (**Figure 5B**). The number of neurites increased only in PDL + FBS treatment from the 1st to 4th day of culture (**Figure 5C**). The length of neurite mirrored the same trend, showing an increase only in PDL + FBS treatment from the 1st to 4th day of culture (**Figure 5D**).

Optical Lobe Cell Culture

We next tested the effectiveness of our protocol for VSFS neurons on other important lobes of interest, the octopus Optic Lobe (OL, integrative lobes for visual sensations). After OL dissection, we collected a mix of different cell types, neurons from the outer and inner granular layers and neurons from the central part (medulla). We dissociated neurons using papain enzyme and cultured them on PLL or PDL coated dishes in culture medium containing either HEMO or FBS enrichment (**Figure 6**). We found that all neurons in culture showed similar morphology to that of VSFS neurons, round shaped cell bodies with a cell diameter ranging between 5 and 10 μm and most cells developed fine and branched processes since the first day in culture (**Figures 6A–D**). Neurons showed an increased number of neurites, branches, and contacts (**Figures 6E,F**). It is interesting that FBS increased branching and cell-cell contacts when compared with HEMO (**Figure 6**).

Our statistical analysis showed that FBS is more robust as compared to HEMO in promoting neuronal adhesion and neurite outgrowth in 4 days (**Figure 7A**, **Table 3**). Specifically, the number of cell count and neurites increased significantly in all treatments from day 1 to day 4 by the addition of FBS. There were no significant differences in cultures of PDL+HEMO between 1st and 4th day of cell culture (**Figures 7A,C**, **Table 3**).

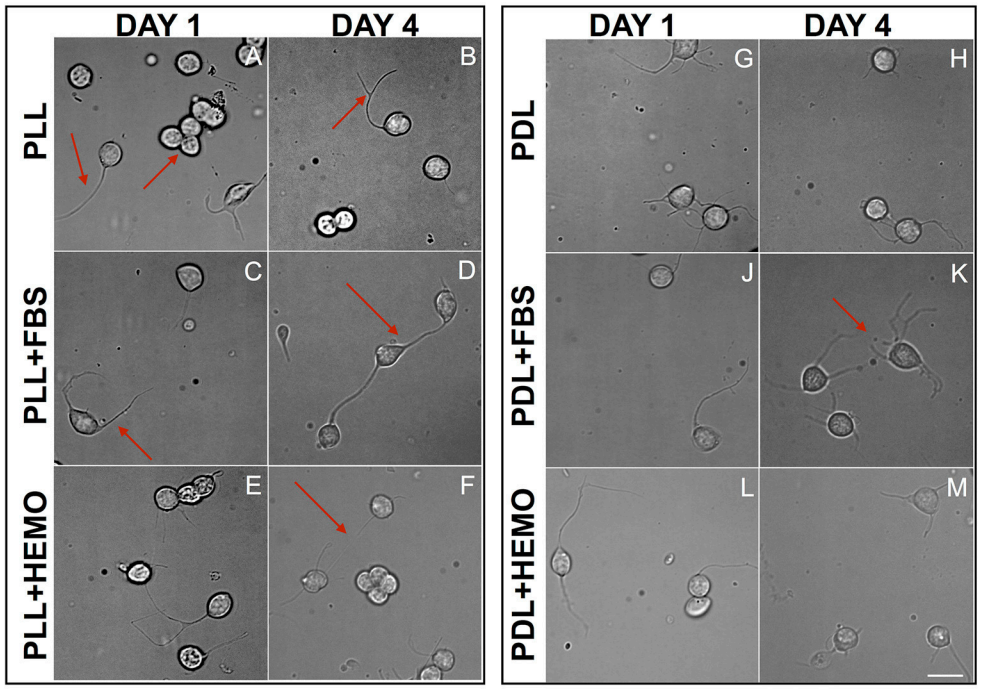

FIGURE 4 | Cells from VSFS plated on PLL **(A–F)** and PDL **(G–M)** coated dishes, cultured for 4 days in L15 medium with the addition of FBS **(C,D,J,K)** or HEMO **(E,F,L,M)**; Red arrows indicate: **(A)** neurite and neurons cluster; **(B)** branched neurite; **(C)** neurite; **(D,F)** axo-somatic contacts; **(K)** neurite networks; white scale bar indicates 10 μm.

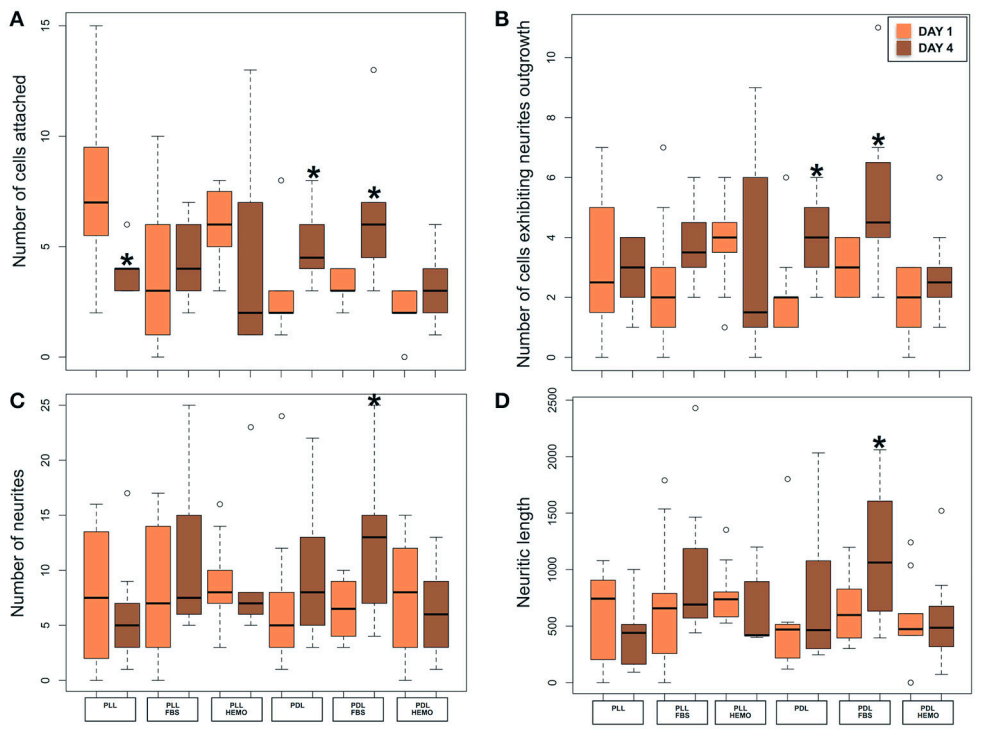

FIGURE 5 | Number of cells attached **(A)** and exhibited neurites outgrowth **(B)**; number of neurites **(C)** and neuritic length **(D)** from VSFS cells in *Octopus vulgaris* plated on PLL and PDL coated dishes, cultured for 4 days in L15 medium with the addition of FBS or HEMO; $^*p < 0.05$.

TABLE 2 | Mean ± standard error of four quantitative parameters used to evaluate the effectiveness of VSFS culture condition the 1st day (1) and the 4th day (4).

Parameters evaluated	Number of cells attached		Number of cells exhibiting neurites outgrowth		Numbers of neurites		Neuritic length	
DAY	**1**	**4**	**1**	**4**	**1**	**4**	**1**	**4**
PLL	7.00 ± 1.10	**4.00 ± 0.32–**	2.50 ± 0.67	3.00 ± 0.41	7.50 ± 1.78	5.00 ± 1.59	742.85 ± 113.98	440.75 ± 98.55
PLL+FBS	3.00 ± 0.89	4.00 ± 0.48	2.00 ± 0.58	3.50 ± 0.39	7.00 ± 1.75	7.50 ± 1.84	657.40 ± 148.23	691.11 ± 165.01
PLL+HEMO	6.00 ± 0.48	2.00 ± 1.96	4.00 ± 0.42	1.50 ± 1.45	8.00 ± 1.13	6.50 ± 3.18	737.58 ± 75.81	415.98 ± 173.30
PDL	2.00 ± 0.63	**4.50 ± 0.53***	2.00 ± 0.48	**4.00 ± 0.42***	5.00 ± 2.16	8.00 ± 1.84	470.02 ± 150.10	464.91 ± 179.26
PDL+FBS	3.00 ± 0.25	**6.00 ± 0.73***	3.00 ± 0.26	**4.50 ± 0.69***	6.50 ± 0.76	**13.00 ± 1.93***	598.20 ± 98.37	**1,062.70 ± 155.01***
PDL+HEMO	2.00 ± 0.35	3.00 ± 0.49	2.00 ± 0.36	2.50 ± 0.47	8.00 ± 1.70	6.00 ± 1.26	473.80 ± 123.05	485.88 ± 128.57

In bold statistical differences, asterisks for positive statistical significance (*), minus for negative statistical significance (–).

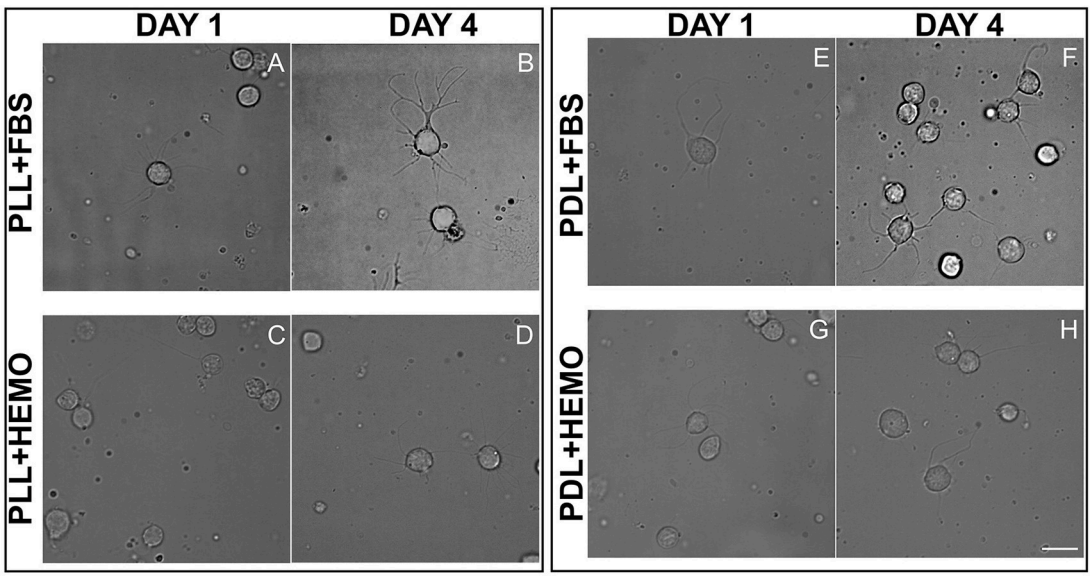

FIGURE 6 | Cells from OL plated on PLL **(A–D)** and PDL **(E–H)** and coated dishes, cultured for 4 days in L15 medium with the addition of FBS **(A,B,E,F)** or HEMO **(C,D,G,H)**; white scale bar indicates 10 μm.

Immunocytochemistry Studies of Neurons in Culture

Because neuronal survival and development depend critically on the presence of certain proteins such as the dynamic cytoskeletal proteins and enzymes, we next tested whether these neurons *in vitro* express such elements in their cell bodies and processes. Specifically, we tested the expression of the cytoskeletal protein β-tubulin and the enzyme poly ADP-ribose polymerase (PARP1, cytosol form). Our data showed that both β-tubulin and PARP1 were strongly expressed in all cultured OL neurons (**Figure 8**). The majority of PARP1 (red) was distributed in the cytosol of neurons and neuronal processes and a few magenta spot surrounding the nuclear area due to a weak cross-reactivity to PARP1 nuclear isoform. Interestingly, β-tubulin (green) is exclusively located in the neuronal cytosol and selectively co-localized with PARP1 in the soma-soma contact sites. In neurons that did not form contact with other cells, β-tubulin appeared to exhibit a non-localized distribution in the entire cytosol. These results are in agreement with our previously published results on the PARP1 immunohistochemistry in the central nervous system (CNS) of *Octopus vulgaris* (De Lisa et al., 2012a).

Regeneration Experiment on VSFS Neurons

In order to test the effectiveness of PDL substrate with 4% FBS to induce regeneration of injured axons, we performed a regeneration experiment on VSFS neurons. We first cultured neurons in the presence of FBS and allowed them to develop active growing processes. The selected long neurite was then severed by a sharp glass pipette mounted on a micromanipulator. Interestingly, we found that the axotomized axon regenerated its severed process within a short time period (**Figure 9**). Time-lapse phase contrast images revealed that neurons after axotomy exhibited a faster re-growth in the first hour (0–1 h) after cut (**Figure 9C**) and slightly slower rate in the second hour after axotomy (**Figure 9D**). These results thus underscore the regenerative capacity of octopus neurons at the level of single neurons and their axons, and demonstrate that the intrinsic regeneration capacity of axons is preserved in cell culture.

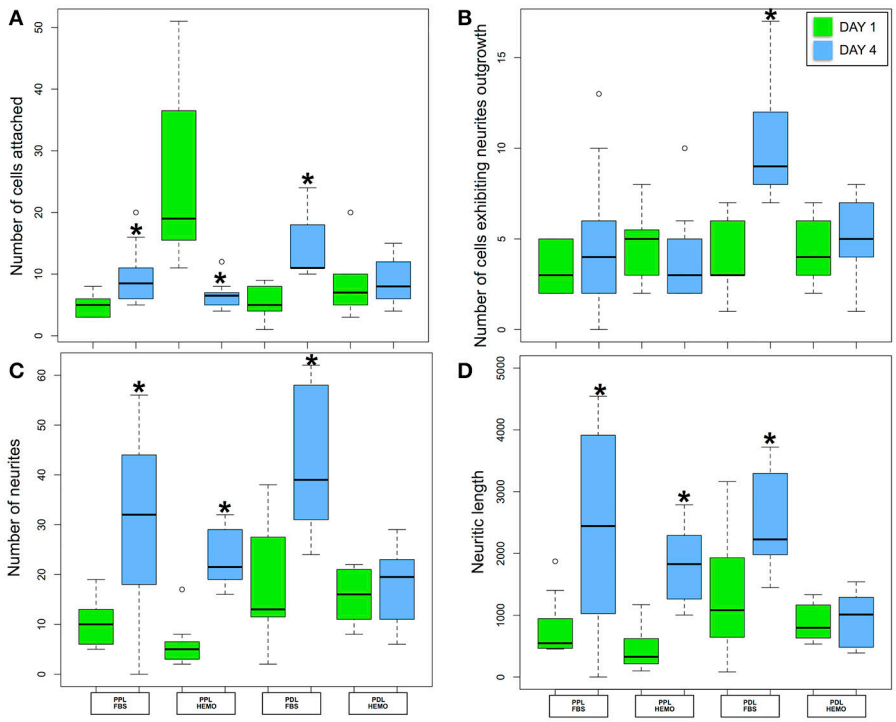

FIGURE 7 | Number of cells attached **(A)** and exhibited neurites outgrowth **(B)**; number of neurites **(C)** and neuritic length **(D)** from OL cells in *Octopus vulgaris* plated on PLL and PDL coated dishes, cultured for 4 days in L15 medium with the addition of FBS or HEMO; $^*p < 0.05$.

TABLE 3 | Mean ± standard error of four quantitative parameters used to evaluate the effectiveness of OL culture condition the 1st day (1) and the 4th day (4).

Parameters evaluated	Number of cells attached		Number of cells exhibiting neurites outgrowth		Numbers of neurites		Neuritic length	
DAY	**1**	**4**	**1**	**4**	**1**	**4**	**1**	**4**
PLL+FBS	5.00 ± 0.55	**8.50 ± 1.16***	3.00 ± 0.40	4.00 ± 0.95	10.00 ± 1.58	**32.00 ± 4.66***	546.47 ± 152.86	**2,443.55 ± 1,239.76***
PLL+HEMO	19.00 ± 4.26	**6.50 ± 0.73–**	5.00 ± 0.59	3.00 ± 0.81	5.00 ± 1.26	**21.50 ± 1.91***	327.12 ± 96.60	**1,827.71 ± 194.49***
PDL+FBS	5.00 ± 0.82	**11.00 ± 2.71***	3.00 ± 0.59	**9.00 ± 1.81***	13.00 ± 3.33	**39.00 ± 7.44***	1,081.93 ± 271.03	**2,227.70 ± 422.83***
PDL+HEMO	7.00 ± 1.57	8.00 ± 1.12	5.00 ± 0.58	5.00 ± 0.77	16.00 ± 1.56	19.50 ± 2.35	797.21 ± 91.32	1,010.25 ± 129.32

In bold statistical differences, asterisks for positive statistical significance (*), minus for negative statistical significance (–).

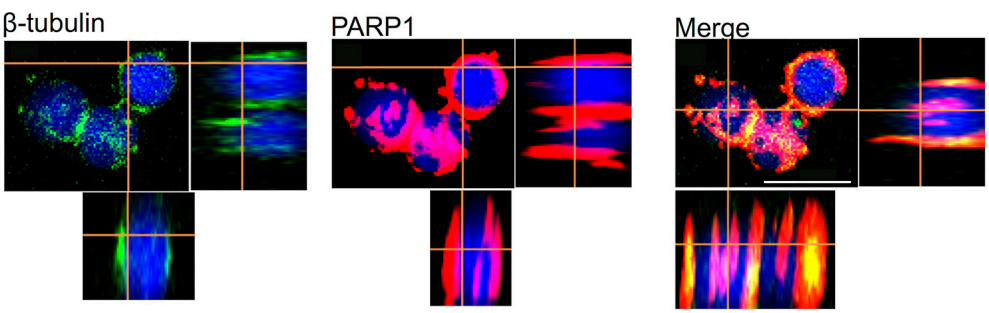

FIGURE 8 | Merging of immunofluorescence images obtained from OL Neurons, β-Tubulin (Green), PARP1 (Red), nucleus (Blue). PARP1 and β-Tubulin are co-localized in cell cytosol, which exhibits yellow stain. Cytoplasmic PARP is also located in nuclei indicated by the overlap of red and blue, which displays in magenta; white scale bar indicates 10 μm.

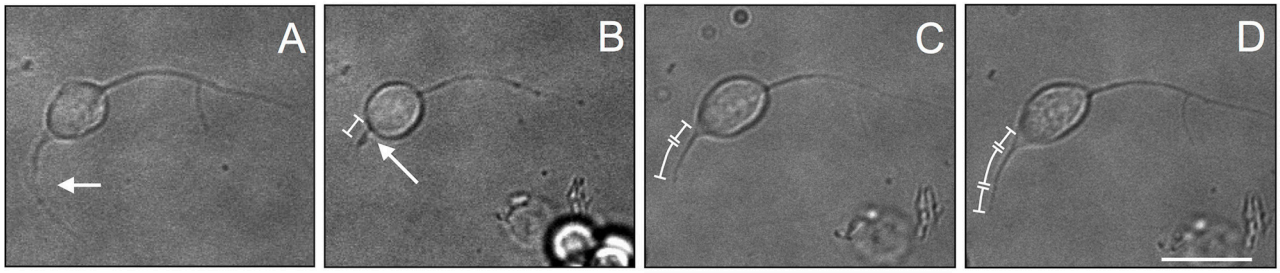

FIGURE 9 | Re-growth of neurite after cut experiment in VSFS neuronal cell cultured in a PDL coated dishes with L15 enriched with FBS; Figures neurite growth in culture **(A)**, neurite after the axotomy **(B)**, neurite re-growth after 1 **(C)** and 2 **(D)** h; white scale bar indicates 10 μm.

DISCUSSION

In the present study, we are the first to develop a protocol for primary culture of neurons from *Octopus vulgaris* VSFS and OL lobes. Previously, white body cells from *O. vulgaris* (Necco and Martin, 1963) and neurons from the stellate ganglion of *O. rubescens* (Gilly et al., 1997) were cultured.

Specifically, we examined the efficacy of commonly used enzymes (papain vs. collagenase/trypsin) for tissue dissociation, adhesive molecules (PLL vs. PDL) for dish coating, and supportive factors (HEMO vs. FBS) for promoting neuronal survival and growth. Our study showed that papain dissociation and PDL coating was a good combination for promoting cell yield and neuronal attachment. Adding growth permissive factors such as HEMO or FBS to the defined medium facilitated neuronal growth in culture, in which FBS was found to be more potent than HEMO in promoting neuritic branches and elongation. The octopus brain is characterized by highly heterogeneous population of neurons and other cell types, and the protocol developed here allowed us to have high cell culture efficiency for both VSFS and OL neurons.

In addition, we also provided interesting data showing that these neurons express cytoskeletal proteins like β-tubulin and enzyme PARP1. The latter is involved in octopus adult neurogenesis, differentiation, and synaptic plasticity after learning and sensory integration (De Lisa et al., 2012a; De Maio et al., 2013; Bertapelle et al., 2017). Lastly, we provided the first direct evidence that the axotomized octopus neurons exhibit robust regenerative ability *in vitro*.

Octopuses are one of the most intelligent invertebrates with an exceptional ability to sense environmental stimuli, to escape from predators, and to learn and execute new tasks (Mather, 1991, 2008; Kuba et al., 2006b, 2010; De Lisa et al., 2012a; Bertapelle et al., 2017). The octopus is phylogenetically remote from vertebrates, and much more sophisticated in its behavior than other mollusc, making it an ideal model for a comparative analysis of brain mechanisms selected during evolution for the mediation of complex behaviors. In addition, cephalopods are protected animals so the development of neural cell culture could be considered as an "alternative method" to study selected lobes of central brain, such as VSFS and OL which are involved in memory and learning and in the integration of sensory stimuli, as well as in adult neurogenesis (Bertapelle et al., 2017).

In molluscs such as the *Aplysia californica* and *Lymnaea stagnalis,* the central nervous system is arranged into a ring of 10–11 ganglia, which comprises about 20,000 large neurons (soma diameters in the range of 20–150 μm) and are wrapped with visible outer and inner sheath. In contrast, the octopus nervous system is organized into three parts, controlling specific functions: (1) the highly developed central nervous system (CNS), which wraps around the esophagus, is situated inside a cartilaginous capsule and composed of ∼45 million neurons, packed in fused ganglia, forming a supraoesophageal and suboesophageal masses; (2) the two optic lobes, which are connected with CNS by a distinct optic traits (on which the olfactory lobes and optic glands are localized), contain ∼180 million neurons; and (3) the Peripheral Nervous System (PNS) of the arms (and body) which is composed of ∼350 million neurons (Young, 1971; Budelmann, 1995). The soma diameters of octopus central neurons are in the range of 5–20 μm (Young, 1971), which are comparable to mammalian central neurons and are much smaller than mollusc neurons. Because of the above similarities with mammalian brain tissue, we adopted a similar dissociation enzyme approach for octopus neurons.

To test the optimal dissociation enzymes, we evaluate the effects of two commonly used dissociation enzymes in mammalian cell culture system (papain vs. collagenase) on octopus cell dissociation efficiency, cell adhesion, survival, and neurite growth. We discovered that papain and collagenase both were potent in breaking the connective tissue of octopus brain, although neurons treated with papain enzyme attached better in culture. These data indicate that the connective tissue for maintaining the structural integrity of octopus neurons is more comparable to mammalian brain tissue. Our study is thus in agreement with several other pioneering studies showing that papain dissociation provides higher yields of viable, morphologically intact neurons than other proteases such as collagenase and/or trypsin in a variety of other cell culture system (Huettner and Baughman, 1986; Finkbeiner and Stevens, 1988; Dreyfus and Black, 1990). However, we observed a difference in the effect of coating reagents (PLL and PDL) in cell adhesion and growth in papain treated neurons. We found that neurons cultured on PLL-coated glass coverslips attached better and

grew well—even in the absence of endogenous and exogenous growth factors (**Figure 2** and **Table 1**). Cell cultured on PDL coated surface had a lower density of attached cells and their growth required the addition of HEMO and FBS, in which FBS had a significant effect on promoting neural growth than HEMO (**Figures 2**, **7**). Although both polymers of PLL and PDL provided positive charges to the glass surface for promoting cell attachment, PLL papered to be a more favorable coating agent for culturing neurons from invertebrates (Syed et al., 1993; Saver et al., 1999), while PDL is more commonly used for vertebrate cell culture surfaces (Xu et al., 2010; Getz et al., 2016). It is important to note that concanavalin A (ConA) was used in the cell culture of giant fiber lobe neurons of the squid (Gilly et al., 1990). Previous studies in cell cultures of *Lymnaea* and *Hirudo medicinalis* neurons have, however, demonstrated that neurons grown on ConA substrates tend to primarily form improper electrical, but not the proper chemical synapses (Syed et al., 1999). Nevertheless, it is very interesting to test the effects of ConA pre-coating on the extent of neurite outgrowth of Octopus neurons in our future experiments.

All invertebrate neuronal cell cultures require either hemolymph as demonstrated in Aplysia (Schacher and Proshansky, 1983; Ghirardi et al., 1996; Schmold and Syed, 2012) or brain conditioned medium as in *Lymnea* (Ridgway et al., 1991; Munno et al., 2000) or *Helisoma* (Cohan et al., 2003), which is not feasible with *O. vulgaris,* we sought to examine the effects of growth factors such as HEMO and FBS (**Figures 5**, **6**) on cell survival and neural growth after day 1 and 4 in culture. On day 1, the survival of VSFS neurons dissociated with papain enzyme treatment was better on the PLL substrate when HEMO was added. The neurons typically grew extensive neurites and exhibited complex branching patterns (**Figures 4E,L**), probably due to the presence of some unknown endogenous growth factor, which may have accompanied the cells during the dissociation (Hyland et al., 2014).

Nevertheless, after 4 days, this growth-promotion stopped and an inversion of the trend took place (**Figures 4F,M**)—suggesting that the neurons may require extrinsic factors for their continued growth/viability. On the contrary, over a culture period of 4 days VSFS neurons, plated on PDL+FBS, responded positively to all selected parameters (**Table 2**). In the same way, OL neurons cultured with PDL+FBS for 4 days provided efficient and satisfying results, inducing cell growth.

These results suggest that the trophic factors present in the blood of these animals may likely be evolutionary conserved across vertebrate and invertebrate species (Ridgway et al., 1991; Munno et al., 2000; Castellanos-Martinez et al., 2014).

Octopus vulgaris has a closed vascular system and a CNS expressing a larger number of proteins involved in specific junctions, transporters and enzymes, than observed in other mollusc model organisms, such as *A. californica*, characterized by an open vascular system(Zhang et al., 2012). Moreover, *Octopus vulgaris* has specific gene expression indicative of a vertebrate-like Blood-Brain-Barrier (Zhang et al., 2012). This issue is supported by the relatively abundant amount of the Acetylcholinesterase (ACHE) in the FBS (Doctor et al., 1990). Recently ACHE, well known for its key role in synaptic and extrasynaptic locations in the nervous system, has been cloned and characterized in *O. vulgaris* (Fossati et al., 2015). The *O. vulgaris* ACHE sequence showed 43–46% identity with vertebrate ACHEs and 43–47% with invertebrate deuterostomes ACHEs and possesses main catalytic and non-catalytic functional sites (Fossati et al., 2015). It will be very interesting to test whether the presence of ACHE in the FBS plays any role in cell survival and growth in our culture in the future.

These data indicated that *Octopus vulgaris* neuronal surface proteins may share some similarity with vertebrate neurons (Cardone and Roots, 1990; Phan et al., 2016), as happens for Protocadherins gene families, involved in neuronal process outgrowth and prevent neurite self-entanglement, that in *O. bimaculoide*s shared features with the mammalian clustered Protocadherins (Liscovitch-Brauer et al., 2017; Wang and Ragsdale, 2017).

In the present study, we have demonstrated that FBS neurotrophic effects improve cell culture in both VSFS and OL neurons. When neurons were cultured for 4 days in PDL coating with the presence of FBS, many of them formed physical contacts with adjacent cells either in soma-soma, soma-neurites and neurite-neurite configurations. To study further these connections, we performed an immunofluorescence experiment on FBS cultured OL neurons using the antibodies against the cytoskeletal protein, β-tubulin, and the PARP1, an enzyme was previously localized in OL and VSFS of *O. vulgaris* CNS, involved in neuronal plasticity (De Lisa et al., 2012a; De Maio et al., 2013; Bertapelle et al., 2017). As expected, the immunoreactivity of both antibodies was widespread throughout the cytoplasm of the cell somata and only few magenta spots were visible in the nuclei due to a weak cross-reactivity to PARP nuclear isoform. Interestingly, the cytoskeletal protein and PARP enzyme tend to be co-localized (spot yellow) in the cell contact sites between two contacted neurons (**Figure 8**), indicating that these sites were likely undergoing active assembly and functional development. A functional evidence for potential synaptic contact between cells however awaits further electrophysiological experimentation.

In summary, the results highlighted here demonstrate that an exogenous factor (FBS) improved cell survival, neural growth, and probably network formation in a more effective manner than an endogenous factor (HEMO). We hypothesize that a growth factor/s present in HEMO provide an initial push along with other factors, such as neurosteroids (De Lisa et al., 2012b), working synergistically in promoting neuronal growth. In support of this notion, studies have shown that the expression of oestradiol receptor in the lobes of *O. vulgaris* CNS has been recently associated neural plasticity (De Lisa et al., 2012b). The effectiveness of VSFS culture conditions (PDL+FBS) was supported by the re-growth of an injured neuron as demonstrated in the regeneration experiment. This result could open a new scenario in which the CNS of *O. vulgaris*, characterized by the presence of nervous districts comparable to vertebrates' specific areas, could be a useful model to study the neurites regeneration after injury (Koert et al., 2001; Lee and Syed, 2004; Onizuka et al., 2005, 2012; Walters and Moroz, 2009).

AUTHOR CONTRIBUTIONS

The authors have made the following declarations about their contributions: Conceived and designed the experiments: VM, FX, NS, and AD. Performed the experiments: VM and FX. Analyzed the data: VM, FX, NS, and AD. Contributed reagents, materials, analysis tools: AD. Wrote the paper: VM and AD. Revised the manuscript: VM, FX, NS, GP, and AD.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00220/full#supplementary-material

Figure S1 | Different cell types from selected lobes (VSFS and OL): neurons from medulla of the OL **(A)**; glial cells from plexiform zone of the OL **(B)**; glial cell (1), large cell (2) and amacrine cell (3) from Vertical lobe **(C)**; amacrine cell (4), and bipolar neuron (5) from frontal system lobe **(D)**; white scale bar indicates 10 μm.

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Conflict of Interest Statement: The handling Editor declared a shared affiliation, though no other collaboration, with the authors VM, GP, and AD.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CATALOG NUMBERS FOR ALL CHEMICALS:

Anti-β III Tubulin antibody, Cat. No. ab78078, abcam;
Collagenase P, Cat. No. 11 213 857 001, Sigma Aldrich, St. Louis, MO, USA;
DAPI, Cat. No. D9542, Sigma Aldrich, St. Louis, MO, USA;
Fetal Bovine Serum, Cat. No. F4135 Sigma Aldrich, St. Louis, MO, USA;
FITC-conjugated goat anti-mouse IgG, Cat. No. 62-6511, Thermo Fisher Scientific;
Glycerol, Cat. No. G2025, Sigma Aldrich, St. Louis, MO, USA;
Goat Serum, Cat. No. G6767, Sigma Aldrich, St. Louis, MO, USA;
HEPES, Cat. No. H4034 Sigma Aldrich, St. Louis, MO, USA;
KCl, Cat. No. P5405 Sigma Aldrich, St. Louis, MO, USA;
Leibovitz's L-15 Medium, Cat. No. 11415064, ThermoFisher Scientific, Waltham, MA, USA;
Papain, Cat. No. P4762 Sigma Aldrich, St. Louis, MO, USA;
Paraformaldehyde, Cat. No. 158127, Sigma Aldrich, St. Louis, MO, USA;
Penicillin-Streptomycin solution, Cat. No. P4333, Sigma Aldrich, St. Louis, MO, USA;
Phenol red solution, Cat. No. P0290 Sigma Aldrich, St. Louis, MO, USA;
Phosphate Buffered Saline, Cat. No. P5493, Sigma Aldrich, St. Louis, MO, USA;
Poly-D-lysine solution, Cat. No. A-003-M Sigma Aldrich, St. Louis, MO, USA;
Poly-L-lysine solution, Cat. No. P4707 Sigma Aldrich, St. Louis, MO, USA;
polyclonal poli (ADP-ribose) polymerase (PARP1) primary antibody, Cat. No. sc-8007, Santa Cruz Biotechnology Inc;
Rhodamine conjugated goat anti-rabbit IgG, Cat. No. 31670, Thermo Fisher Scientific;
Sodium chloride, Cat. No. S5886 Sigma Aldrich, St. Louis, MO, USA;
Triton-X 100, Cat. No. T8787, Sigma Aldrich, St. Louis, MO, USA;
Trypan Blue solution, Cat. No. T8154, Sigma Aldrich, St. Louis, MO, USA;
Trypsin, Cat. No. T2601 Sigma Aldrich, St. Louis, MO, USA.

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REVIEW
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The Soliton and the Action Potential – Primary Elements Underlying Sentience

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At present the neurological basis of sentience is poorly understood and this problem is exacerbated by only a partial knowledge of how one of the primary elements of sentience, the action potential, actually works. This has consequences for our understanding of how communication within the brain and in artificial brain neural networks (BNNs). Reverse engineering models of brain activity assume processing works like a conventional binary computer and neglects speed of cognition, latencies, error in nerve conduction and the true dynamic structure of neural networks in the brain. Any model of nerve conduction that claims inspiration from nature must include these prerequisite parameters, but current western computer modeling of artificial BNNs assumes that the action potential is binary and binary mathematics has been assumed by force of popular acceptance to mediate computation in the brain. Here we present evidence that the action potential is a temporal compound ternary structure, described as the computational action potential (CAP). The CAP contains the refractory period, an analog third phase capable of phase-ternary computation via colliding action potentials. This would best fit a realistic BNN and provides a plausible mechanism to explain transmission, in preference to Cable Theory. The action potential pulse (APPulse), is made up of the action potential combined with a coupled synchronized soliton pressure pulse in the cell membrane. We describe a model of an ion channel in a membrane where a soliton deforms the channel sufficiently to destroy the electrostatic insulation thereby instigating a mechanical contraction across the membrane by electrostatic forces. Such a contraction has the effect of redistributing the force lengthways thereby increasing the volume of the ion channel in the membrane. Na ions, once attracted to the interior, balance the forces and the channel reforms to its original shape. A refractory period then occurs until the Na ions diffuse from the adjacent interior space. Finally, a computational model of the action potential (the CAP) is proposed with single action potentials significantly including the refractory period as a computational element capable of computation between colliding action potentials.

Keywords: sentience, action potentials, soliton, phase ternary computation, brain neural networks

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INTRODUCTION

Sentience may be thought of as the highest ability to perceive events in the context of previous or future events, resulting in conscious non-reflex behavioral modification(s) and is dependent on self-awareness. Sentience must encompass elements of both time and complexity and is dependent upon individual experiences. The generation of sentience and other behavior must depend upon the brain's ability at the level of neurons to compute nerve impulses according to timing defined by the biological processes present.

To understand how we compute sentience we must first understand how action potentials compute in temporal space within the brain neural network (BNN). These computational mechanisms are traditionally described by the action potential (Hodgkin and Huxley, 1952). The Hodgkin Huxley equation describes the potential across the membrane of a neuron in terms of ion exchange changing over a period of time. The timing of the charging and thus the speed of propagation is defined by Cable Theory. It was assumed in 1952 that excitable membranes contained sufficient ion channels close enough together that the spread of charge from one channel could affect another. We now know this is not the case and an alternative method of propagation must be taking place to account for the speed of propagation. A problem is the lack of knowledge about the fundamental and computational mechanisms that underlie the generation and propagation of action potentials in single neurons and neuronal networks. A mechanical pulse known as a soliton always travels with the action potential at the same speed which has been considered ancillary, in this paper we show that it is this pulse that defines the speed and thus the computational mechanisms that form the basis of behavior.

Neurons are diverse and have many shapes, sizes and functions (Bullock et al., 1977). They may have evolved from secretory cells in the early metazoa. We can envisage that as animal size increased the action potential evolved to control secretions at a distance (Grundfest, 1959; Winlow, 1990) although many local circuit neurons in both vertebrates and invertebrates do not conduct nerve impulses (Dowling, 1975, 1992, chapter 13; Shepherd, 1975, 1988, chapter 10; Roberts and Bush, 1981). However, the discovery of the nature of the action potential, which is used to signal over distance, was critical to the development of modern neurophysiology. Unfortunately it has been modeled as a binary event in computational brain networks (Johnson and Winlow, 2017b). We believe this assumption to have been unnecessary and to be the wrong premise for computation both within nervous systems and in the development of artificial intelligence (AI). Furthermore, the advantages of ternary computing over binary computation are that it requires less hardware and contains more information in a shorter code. Phase ternary computing results from phase addition of a ternary pulse. Here we discuss evidence that the action potential is a temporal compound ternary structure, described as the computational action potential (CAP).

The CAP contains the refractory period, an analog third phase capable of phase-ternary computation via colliding action potentials. This would best fit a realistic BNN and provides a plausible mechanism to explain transmission, in preference to Cable Theory. The action potential pulse (APPulse), is made up of the action potential combined with a coupled synchronized soliton pressure pulse in the cell membrane. We describe a model of an ion channel in a membrane where a soliton deforms the channel sufficiently to destroy the electrostatic insulation thereby instigating a mechanical contraction across the membrane by electrostatic forces. Such a contraction has the effect of redistributing the force lengthways thereby increasing the volume of the ion channel in the membrane. Na ions, once attracted to the interior, balance the forces and the channel reforms to its original shape. A refractory period then occurs until the Na ions diffuse from the adjacent interior space.

Finally, a computational model of the action potential (the CAP) is proposed with single action potentials significantly including the refractory period as a computational element and capable of computation between colliding action potentials.

MODELING THE ACTION POTENTIAL

A Lesson From Cephalopods

It is appropriate to this report that the physiology of action potentials was first modeled using the giant axon of the squid, *Loligo forbesi* (Hodgkin and Huxley, 1952). This model predicted the ionic currents crossing cell membranes to create a potential difference and changing over time due to the modulation of currents. However, one of the major problems in AI is how to code accurately for the action potential. Action potentials are critical to the operation of the brain and computation and timing of the action potential is important in considering any possible computational requirements. Thus, the mechanisms that define the speed of the action potential and its temporal accuracy will directly affect the methods of reliable computation available to the neural network. Thus changes in accuracy of action potential timing would make any form of computation unreliable.

The action potential can be divided into three computational phases, resting, threshold and refractory, the specific details of which are discussed elsewhere (Johnson and Winlow, 2017a). The first two phases may be modeled digitally, while the refractory phase is an analog event. Thus the action potential can be considered to be a phase ternary event. Phase ternary computation is an unexplored field in computation.

Action potentials travel at a speed commensurate with the membrane dynamics of the axon and have been shown to be accurate to at least 1 millisecond over its length in small neurons (Diesmann et al., 1999). The transmission dynamic of any axon or part of an axon may be different depending upon the membrane components such as the ion channel spacing (Hodgkin, 1975; Holden and Yoda, 1981; Hille, 1992) and the physical formation of the membrane.

The Macroscopic Point of View

Measurements of the action potential are taken from both sides of the membrane and measure the potential difference across a wide area reflecting the measurement of the H&H model (Hodgkin and Huxley, 1952). An action potential travels not

through the cytoplasm – where it is measured with intracellular microelectrodes – but is a product of the ion changes at the surface of the membrane. Small diameter axons (0.2 μm) have ion channels widely spread with low concentrations of ion channels (Holden and Yoda, 1981; Hille, 1992; Marban et al., 1998). All measured action potentials have been recorded at some distance from the membrane. As the action potential progresses, the micro-pipette measures current not from a point on the membrane, but from an area including multiple ion channels, and may not reflect the mechanisms of propagation from a single point. The same is true for the loose patch clamp method, where rather large (15–30 μm) (Marrero and Lemos, 2007) external patch electrodes are used and are unable to measure changes at a single point (Walz, 2007).

The Hodgkin Huxley Equation and Cable Theory

The H&H equation describes ionic flow across the membrane in mathematical terms over the period when the membrane reaches threshold until the end of the refractory period. The membrane can be considered polarized immediately the threshold takes effect, as after that point there is no return. After threshold the membrane is in the refractory state as no further action potential can be created. The action potential has a maximum speed of about 1 ms^{-1} in unmyelinated the axons (Waxman and Bennett, 1972). Thus, there is a 'leading edge' just before depolarization and as the action potential is self-propagating this leading edge must have the innate property of self-propagation. In effect it also instigates the refractory period once the depolarizing wave has passed. Patch clamping on single ion channels has demonstrated that the existence of threshold, spike and refractory period can be explained by modulation of the Na^+ channel alone (Holden and Yoda, 1981; Marban et al., 1998; Catterall, 2012). Thus the physical origins of the potential changes associated with the action potential can be directly attributed to the ion channel mechanisms, with the activity of the sodium channels defining its progression. This implies that the only element that is responsible for the live propagation of an action potential is whatever mechanism causes the threshold at the leading edge.

The Threshold Alone Is the Initiator of the Action Potential

It is also the rate limiter to the velocity of the action potential along the axon. The timing of the spike is therefore directly related only to the threshold. The threshold may be better defined temporally so that it is not a potential difference but a change over time in terms of action at the level of the membrane, i.e., the equilibrium point at which each subsequent ion channel opens. The predominantly voltage gated Na^+ channels (Yu and Catterall, 2003) open when positive charge approaches their s4 units. This all-or-none point is assumed to be the point of threshold. Conventionally, in the H&H model, threshold is assumed to be a direct result of Na ions arriving from a neighboring channel. For the APPulse structure **Figure 1**, the mechanical pulse opens the channel enough for the electrostatic insulation to break causing electrostatic attraction between the positive Na^+ ions passing through the s4 units and the negative parts of the ion gate structure. A mechanical contraction occurs transferring entropy (energy dispersal) directly to the soliton. Na^+ ions continue to be attracted to the negative intracellular space until the negative charge is balanced, at which point the gate is closed. The remaining positive ions adjacent to the negative regions of the ion channel prevent any further opening, and remain deactivated until the Na^+ charges diffuse into the intracellular space. This is explained in **Figure 2**, where a voltage–gated Na^+ channel is illustrated. The ion channel is therefore reactive both to mechanical and charge stimulus. Entropy from the soliton is only required to break the electrostatic-insulation of the ion channel for membrane contraction to occur, thus producing the next entropy charge for the soliton to proceed. The threshold is defined therefore as the time for the s4 units to fully open the channels. The rest of the action potential is only concerned with the refractory period and stabilization to resting potential. It is irrelevant to speed of transmission, although of course the refractory period can affect the frequency of transmission.

The Microscopic Point of View

At a microscopic level the mechanism of the threshold can be investigated knowing the properties of just the Na^+ channels and the membrane, for example using the patch clamp technique. The Na^+ channel has been isolated and its properties understood (Catterall, 2013; Shen et al., 2017). In addition, the speed and flow of an action potential is directly dependent upon the speed of activation of the threshold potential corresponding to opening of the Na^+ gates which may be achieved mechanically (see below), the resulting entropy causing a soliton. The H&H model is a proven advantageous means for the demonstration of ion transport and passage through a membrane over a large area, but because it cannot explain charge flow along the length of the membrane it must be allied with a mechanical model. However, it should be understood that because of the physical properties of Na^+ ions, threshold charge cannot flow from one ion channel to the next in the time available. There must therefore be another mechanism present and a 'soliton' mechanical pulse is known to be present. This is the force-from-lipid mechanical energy (Brohawn et al., 2014) that opens the Na^+ channels. This coupled APPulse fulfills the requirement for a continuing action potential where Cable Theory fails.

Cable Theory defines the potential arising from ion disparity across the membrane as a capacitance. Historically Cable Theory (Rall, 1962, 1995) comes from a direct analogy from capacitance theory and considers charge over the whole surface of an insulator (Poznanski, 2013). Thus, Cable Theory is a mathematical construct to predict observations on large areas of membrane, in which the depolarization of the membrane during the action potential is analogous to the discharge of a charged capacitor. Experimentally, the mathematics of cross current resistances appear to work correctly over a large part of the membrane in the macroscopic view and this is the basis for the H&H model. Cable Theory itself has recently been revisited to demonstrate that both neuronal morphology (Bestel et al., 2017) and the extracellular medium (Bédard and Destexhe, 2013, 2016) exert significant influences on neuronal cable properties as might

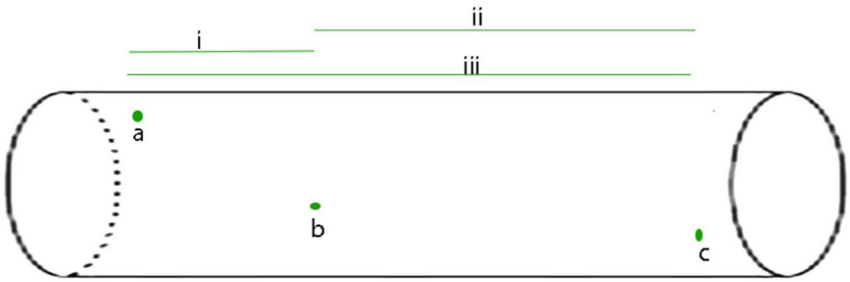

FIGURE 1 | The APPulse - an illustrative, uniform axon containing three widely spaced ion protein channels is shown. For the benefit of a clear description the axon has been standardized as follows: 1 the axon is uniform such that speed along the axon by the lipid pulse is constant; 2 - the protein channels (in green) are gates that reach threshold at a voltage of V and produce a digit of Entropy E; 3 - in this axon there are no lipid channels or other proteins except the three ion channel proteins, a, b, c. The diagram illustrates the events that take place for the APPulse to continue between ion channels. On depolarization at a, an action potential digit of entropy E is created. A Lipid pulse is subsequently created by Entropy E that continues along the axon. Entropy loss e from E causes a proportionate decrease in amplitude but the velocity of the soliton is constant where the membrane components are uniform. This causes the entropy to decrease by dissipation over distance d such that Ed(b) = E-e. This residual entropy Ed (b) is enough to mechanically distort the ion channel at b breaking the electrostatic insulation. Contraction of the ion channel ensues completing the mechanism. In this model the entropy of electrostatic forces produced by the ion channel at (a) must be sufficient to produce a lipid pulse of such entropy E to arrive at (b) with sufficient entropy to break the electrostatic insulation of (b) and repeat to c, where the process again repeats itself. In smaller diameter neurons dissipation will be greater due to entropy being a function of membrane area. i, ii, and iii represent different timings between separated ion channels along the membrane. From Johnson (2015) – reproduced under the Creative Commons License.

be expected. Such effects require considerable mathematical modifications to the original theory.

When H&H were conducting their experiments accurate depictions of the membrane, ion channels and specifically inter-channel distances were not available, but were assumed to be "separated by an infinitesimal distance" (Hodgkin, 1975). It was not until single patch clamping became available (Hille, 1992) that accurate measurements of channel density became known (Hamill et al., 1981; Goodman et al., 2005). At the time that H&H described longitudinal flow, the assumption was that there was a mechanism by which activation of an ion channel during threshold would activate adjacent ion channels. This was thought to produce a cumulative and on-going propagation – just as the charging electrons distribute evenly and almost instantaneously. However the mechanism of propagation between channels was not identified then and has still not been identified. Thus, the action potential takes place at the level of the membrane, which means that the resulting observation is due to all the combined ionic changes in a very wide portion of that membrane.

Mechanisms must exist in the membrane to produce an on-going pulse. Intra-Ion channel distances have been measured accurately although this distance is variable. Single patch clamping a membrane will usually only detect a single active ion channel (or possibly two) if the pipette is sized about 1.5 μm. As the membranes examined contain many different ion channels we can confidently say they are spaced at least 1.5 μm apart. Thus "Channels are not crowded" together (Hille, 1992, pp. 334–335) and the distance over which a single Na ion can affect another channel is limited by the time taken to travel through the ion channel and to the next channel. The ionic radius of Na^+ is about 116 pm (Shannon, 1976) indicating that a Na ion has to lie adjacent or in very close proximity to the Na^+ channel to activate it and the channels are ion specific. The time required for charge to spread from one ion channel to the next can be calculated from the ionic radii and the diffusion coefficient (Goodman et al., 2005). A conservative simple Speed-Time calculation suggests that the maximum speed that charged Na ions can travel between channels is less than 1/1000 of what is necessary for propagation (Johnson, 2015). Cable Theory only models the ion flows of the action potential under conditions of voltage clamp, but as yet there is no known mechanism for propagation of the action potential provided by Cable Theory. Thus, the H&H model very clearly demonstrates the electrically measured activity surrounding the underlying mechanism of propagation from one channel to the next, but not the mechanism itself.

IS THERE EVIDENCE FOR A MECHANICAL COMPONENT IN ACTION POTENTIAL PROPAGATION?

Two supposedly incompatible models for action potential propagation have been proposed and compared (Appali et al., 2012), the H&H model and soliton theory. However evidence at the level of the membrane structure suggests the two models are compatible and are synchronized; the H&H model at the macroscopic level and the soliton model at the microscopic level. In the H&H model of the membrane, for propagation to occur positive ions would have to behave as electrons but positive charges are a part of the atom and so physically a sodium atom must move for the charge to move. Without a mechanical component the H&H action potential cannot propagate. Recent evidence suggests that a "force-from-lipid" model (Brohawn et al., 2014) could transmit pulses into mechanosensitive ion channels in the absence of other cellular components and might also explain propagation through the membrane lipid. We envisage that it would take the form of a soliton known as an APPulse which would be the precursor of ion channel opening.

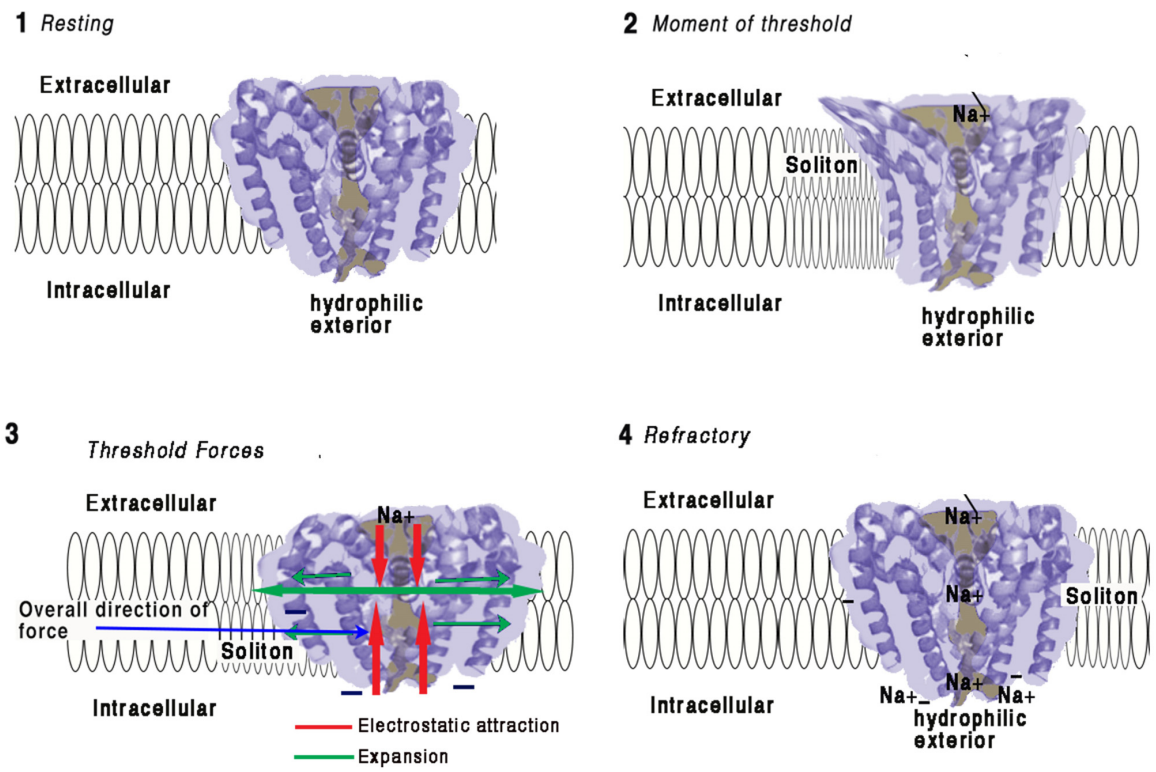

FIGURE 2 | Illustration of how force from lipid may act on a eukaryotic voltage-gated sodium ion channel from the American cockroach (adapted from McCusker et al., 2012 and Shen et al., 2017).

(1) Resting:

Ion channel embedded in a membrane at resting state. The main structure of a channel is four coiled helices. One of these helices is connected to a hydrophilic negatively charged spiral placed intracellularly. The structures of the four main spirals are loosely bonded with the intracellular surface of the structure and are hydrophilic. The intracellular portion of the structure is negatively charged and polarized toward the hydrophilic spiral.

Any proximate connection between extracellular positive charges and the negative hydrophilic portion will have the electrostatic effect of attraction thus drawing the intracellular side of the structure toward the extracellular surface and deforming the structure. The ion channel is surrounded by a membrane approximately 10 nm thick with the hydrophilic spiral toward the interior of the cell. The pore is effectively formed as a valve blocking ions and importantly insulating against charge, thus preventing electrostatic interaction. Any opening of the pore therefore causes entry Na ions and immediate availability of their charges for interaction. The ionic radius of Na is about 16 nm. The surface of the ion protein channel is hydrophilic, a vertical contraction would be expected and this is in fact what happens (McCusker et al., 2012).

(2) Moment of Threshold:

- As a soliton approaches the ion channel pressure will develop on the sides of the ion channel displacing the helices and removing the electrostatic insulating seal. This produces immediate access for electrostatic attraction between the Na and the negative interior surfaces of the ion channel. Constriction of the central helix produces a mechanical invagination of the four helixes at the pore and simultaneously opening the pore sufficiently to destroy its electrical insulating capacity.
- Positive charges are attracted to the intracellular space but also to the hydrophilic surfaces of the pore. The Na^+ ions are in electrostatic proximity to the negatively charged portions of the structure the moment the electrostatic insulation is broken and are drawn inward.

(3) Threshold Forces:

The incoming Na^+ ions are electrostatically bound to the selector causing an opposing force to the hydrophilic parts of the structure which causes the s6 spiral (for detail see Shen et al., 2017) to be drawn toward the Na^+ receptor. Movement of the hydrophilic s6 portion will undo the main spiral causing the pore to open mechanically in an iris movement (McCusker et al., 2012), which causes a mechanical contraction of the structure. At threshold, the action of contracting the structure from external to internal during pore opening creates forces along the surface of the membrane as the helices are pressured outward. This action is synchronized to the arrival of the soliton and the mechanical energy will be transferred to the soliton, thereby reducing entropy.

(4) Refractory:

When sufficient positive charges have reached the interior equalizing charge adjacent to the hydrophilic spiral, the forces equalize and the pore will close. The pore and structure of the ion protein channel is now at rest. There is now a large concentration of Na^+ ions in the vicinity of the hydrophilic regions and the activating spiral. Any further soliton or disturbance to the membrane will not cause activation of the ion channel pump until the excess Na^+ ions are removed by diffusion. The ion channel is refractory until this charge is cleared allowing for further electrostatic attraction. Synchronization is achieved between the ion channel pump and the soliton and electrostatic force is transferred to mechanical force and then to the soliton.

This work is a derivative of Figure 2A,D | Crystal structure of the NavMs pore by McCusker et al. (2012), used under Creative Commons BY-NC-SA 3.0. This work is licensed under CC BY-NC-SA 4.0 by Andrew Johnson and William Winlow.

The APPulse

There is now a large body of evidence showing that:

- a 'soliton' mechanical pulse accompanies an action potential and is stable propagating at constant velocity (Tasaki and Iwasa, 1982; Heimburg and Jackson, 2005; El Hady and Machta, 2015)
- ion channel separation is too great to allow for ion channel interference from adjacent channels caused by ionic charge (Holden and Yoda, 1981; Marban et al., 1998; Catterall, 2012; Johnson, 2015)
- ion channels can be opened by mechanical stimulus (Martinac, 2012; Anishkina et al., 2014; Takahashi et al., 2016; Zhang et al., 2016)
- there is deformation of the membrane by activation of ion channels (Tasaki and Iwasa, 1982; El Hady and Machta, 2015)
- entropy (thermodynamic) measurements do not follow the H&H action potential but do follow the APPulse. (Abbott, 1958; Howarth, 1975; Ritchie and Keynes, 1985; Tasaki and Byrne, 1992; Moujahid et al., 2011).

The action potential measured by H&H is a measure of the sum of all the potential changes of all charges across the membrane over a wide area. The result is always a combination of effects from many ion channels, some open, some closed and some refractory. However, direct mechanical stimuli of axons can elicit action potentials (Howe et al., 1977) suggesting the involvement of a mechanical component (Appali et al., 2012).

The Na^+ Channel Is an Electro-Mechanical Soliton Pump

Positive ions do not behave as electrons and require time and the correct diffusion coefficient to move. Calculation of ion channel distribution from single channel studies, demonstrates that Cable Theory can only account for the action potential in its stable states (resting or maintained by voltage clamp) (Johnson, 2015). The ion channels are spread too far apart and the entropy changes do not match those predicted by the model. The longitudinal resistance in the H&H arrangement is always infinite as there is no mechanism that provides surface spread depolarization. Surface spread and thus speed of action potential must therefore occur by another mechanism. We suggest that this is by a mechanical 'soliton' coupled and synchronized to entropy provided by the ion exchangers described (**Figure 2**). The soliton pulse mechanically opens ion channels leading to further depolarization. Thus a soliton always occurs when a nerve impulse is generated. The membrane soliton is powered by direct mechanical forces from the opening of the pore. These forces originate from charged particles of Na attracted by the hydrophilic units and hydrophilic parts of the structure with electrostatic forces between the Na ions and the negatively charged parts of the ion channel structure. This model accounts for the threshold, spike and refractory period on its own. It produces the correct entropy/time profile if it is assumed transfer of entropy is an almost adiabatic process (in which energy is directly transferred without loss of matter or heat). Furthermore, it explains the ion changes across the membrane. Communication occurs not at the level of the action potential but at the level of ion-channel-pump to ion-channel-pump. In effect computation takes place within the membrane at the rate of transmission. Propagation of membrane pulses is well supported by Mussel and Schneider (2018) who suggest that action potentials may be better described as non-linear acoustic pulses propagating along lipid interfaces and which annihilate on collision, a well-known property of colliding action potentials (Follmann et al., 2015).

Myelinated Fibers

This model also explains action potential transmission in a myelinated fiber where a soliton pulse created at the node of Ranvier, due to a high concentration of ion channels, is then attenuated by the rigidity of myelin. Insulating the movement of the axon membrane has the effect of reducing entropy loss and thus increases efficiency. In a small cylindrical axon, sleeved in myelin, movement is restricted and the entropy created at the nodes of Ranvier cannot be transferred along the axon membrane. Entropy is therefore directionally guided on entry into the myelin sheath creating a pulse-wave within the cytoplasm of the axon. This is similar to the action of pulse wave velocity (PWV), the velocity at which the arterial pulse propagates through the circulatory system. The mathematics is similar to the Moens–Korteweg equation (Moens, 1878) that states that PWV is proportional to the square root of the incremental elastic modulus of the vessel wall given a constant ratio of wall thickness – the myelin sheath. On exiting the myelin sheath, entropy will be transferred back to the axon membrane restarting the APPulse.

Unmyelinated Fibers

The APPulse velocity is a result of the factors contributing to the fluid dynamic qualities of the axon membrane at any point. However, if there are insufficient ion channels producing insufficient energy, then the pulse will not reach subsequent channels and will fail. Thus, in unmyelinated fibers, the action potential travels at a speed commensurate with the membrane dynamics in each part of the axon or neurite. Speed of axonal transmission, and therefore the time impulses take to reach their destination, is variable and depends upon the axon type and diameter (**Figure 2**). Thus, the action potential as expressed by H&H is a measurement of the progression of ionic charge over the axon membrane, it cannot represent the mechanism of propagation.

COMPUTATION BY PHASE DIFFRACTION

In both vertebrates and invertebrates many neurons are multibranched and some have more than one spike initiation zone (Haydon and Winlow, 1982; Ledergerber and Larkum, 2012). This indicates that back-propagation of action

potentials (Stuart and Sakman, 1994) can occur under natural circumstances, allowing action potentials to collide. Deconstruction of the computational variables that can be attributable to the action potential is shown in **Figure 3**: the CAP. Examination of the CAP reveals an inherent ability to compute in a realistic artificial BNN by action of the analog time component of the refractory phase, which is effectively able to reroute (diffract) action potentials along different pathways through the neural network; i.e., the refractory period is capable of interference at axon bifurcations and the axon hillock of cell bodies to produce effective deflection of action potentials along different axonal pathways in the neural network or to cause mutual occlusion.

Annihilation will occur between two action potentials if the refractory period of one action potential interferes with the threshold potential of another (Follmann et al., 2015; Berg et al., 2017; Johnson and Winlow, 2017a). The result of this is that as parallel action potentials meet within a neural network they diffract at branching or junctional points and may be diverted along new pathways. This diffraction occurs due to the timing arrival of the refractory phase in from the point of entry of the action potential into the neural network. The action potential then enters the neural network with a specific refractory period on a timescale defined by the membrane dynamics at that point (**Figure 4**). As the action potential flows along the neurites to the soma it may, at branching points, encounter other action potentials from parallel entry points similarly identifiable by refractory period.

A major advantage of computation with the refractory phase is that along parallel pathways with similar dynamic transmission timing, error is redacted and the pulses synchronize. Over many iterations of nodes this makes the network error and noise free (Johnson and Winlow, 2017a). This is unique to the phase ternary computation and is the first time an error redaction mechanism has been described for a physiological neural network. Error redaction is critical in the smaller neurites of the neural network where signal to noise ratios are of the same order of magnitude as each other (**Figure 5**).

COMPUTATION BY THRESHOLD AND REFRACTORY PERIOD

The threshold is the point at which, either by mechanical (Johnson, 2015) or electrical means (Hodgkin and Huxley, 1952), the Na^+ gates open at a distinct site along the axon membrane. The accuracy of timing in the APPulse can be thought of as the time taken for the threshold to pass between adjacent Na^+ ion channels small neurons where ion channels are separated along the axon as determined by the velocity of the action potential. This distance is from 1 to 5 μm corresponding to a maximum temporal accuracy of about 1 μs if an action potential is traveling at 1 ms^{-1}. The figure of 1 μs can be thought of as the maximum point of accuracy for computation and is a variable figure according to the dynamics of the membrane.

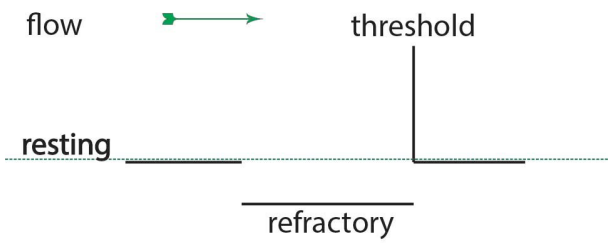

FIGURE 3 | Representation of the CAP, showing the digital resting phase, digital threshold and the analog refractory phase. In this view, the resting potential serves as the ternary 0 of a ternary action potential. Any rise above threshold is the digit or +1 and the refractory period acts as phase -1 refracting any digit+1 during collision to 0. The refractory period is analog and is a result of the specific dynamic of the membrane at that point where the action potential exists. From Johnson and Winlow (2017a) – reproduced under the Creative Commons License.

Inhibition of Incoming Signals by Phase Ternary Annulment

Given that the ternary analog refractory phase often far outlasts the action potential, it is clear that synaptic inputs to a neuron will be obliterated during the refractory period, although powerful inhibitory postsynaptic potentials could prolong it, particularly if delivered during the relative refractory period. The refractory period is a result of protein formation changes and its duration is highly variable. In CAP dependent computation it is the refractory period (defined by the transmission dynamics of the point of interaction, and thus a non-linear time variable) that defines computation. In both the H&H model and the APPulse, the ion channels are inactivated during the refractory period. The refractory period cancels adjacent, out of phase, action potentials when they collide annihilating one of them to produce a change in phase. A neuron is thus capable of taking distinct all-or-none action potential inputs in specific temporal phase and, by interference, changing the phase to create distinct all-or-none action potential outputs. Each neuron is therefore capable of fulfilling computation independently.

NOISE REDUCTION

The usefulness of artificial-networks to model activity is limited by the amount of noise and spontaneous activity in biological systems and synaptic studies give little insight into conduction in more highly evolved neural-networks, where axon conduction is diverse and seemingly unreliable with an alarming amount of noise (Bullock, 1958), which must be reduced for any neural

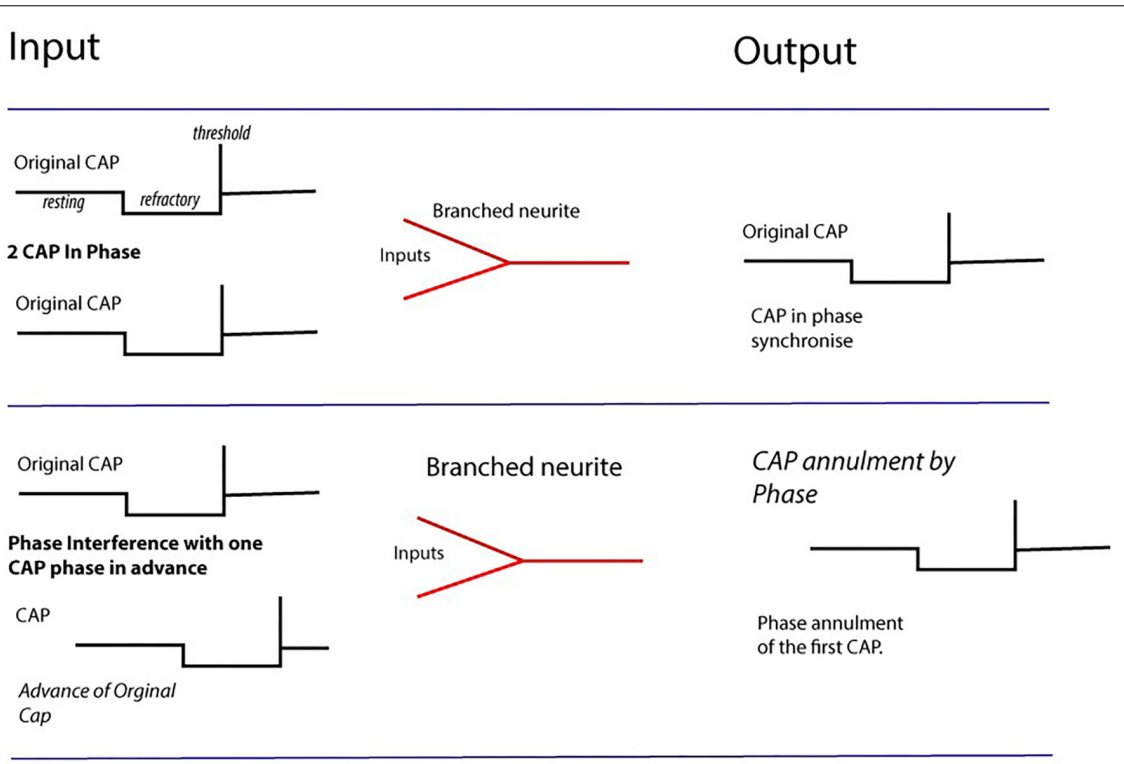

FIGURE 4 | Computation by phase ternary interactions – Collisions from CAPs may result in nullification of CAP (second row) depending upon phase and the dynamics of the membrane at the point of collision. Multiple collisions form patterns navigating pathways through the network. Action potentials firing in-phase map together, whilst those with +1 and –1t overlapping cancel to 0.

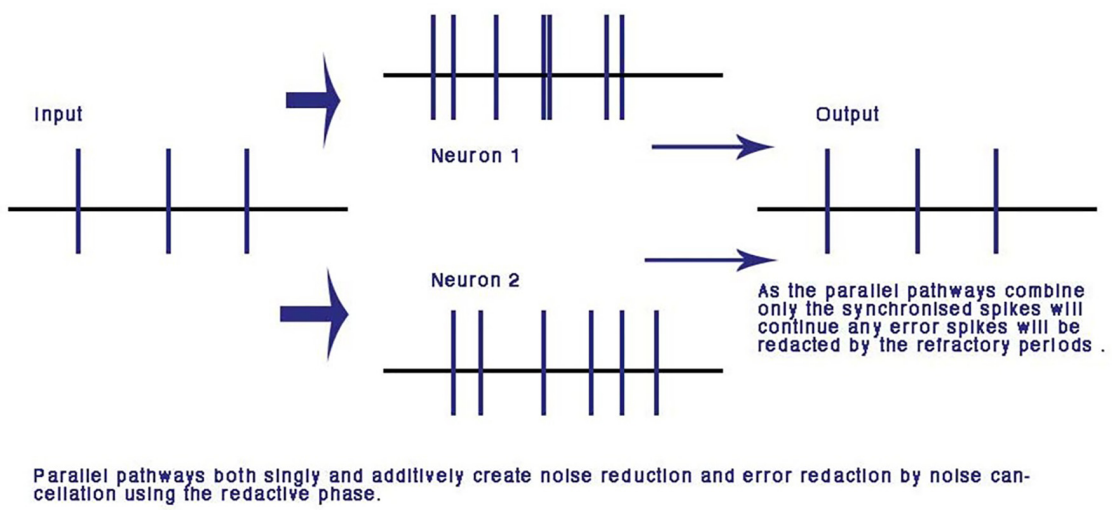

FIGURE 5 | Error and noise redaction by phase ternary computation. In parallel connections, any out of phase errors and spontaneous spikes are automatically redacted by the refractory periods of neighboring spikes: using the CAP any phase shift or spontaneous spike will be reduced to the resting potential by the colliding neighbor CAP. From Johnson and Winlow (2017a) – reproduced under the Creative Commons License.

network of depth. The parallel processing of axons leading to the same neuron reduces variability in the CAP neural network where connections are randomly formed and neurons are connected by more than one pathway and may reduce signal to noise ratio (**Figure 5**). In a concatenated balanced phase system with many interfering CAPs, noise is automatically negated at each parallel point of processing by interference. Error redaction occurs where pulse trains travel along adjacent parallel pathways

whether through one or more neurons or points of contact. This is a mathematical certainty where two CAPs collide from parallel pathways to a common node the trailing refractory period will annul the second threshold. This error negation is particular to phase ternary and depends upon the logic of interference where two spikes are compared from different sources and tested automatically for a match. Error in this system is negated at the point of balanced phase computation and activity within the network becomes synchronized.

CONCLUSION

- We have combined the Hodgkin-Huxley model of the action potential with the soliton theory to produce a unified model of action potential propagation, the APPulse, which also applies to cardiac action potentials. This model is not restricted to spiking neurites and can be applied to non-spiking neurons and any active membrane consisting of ion channels.
- The Hodgkin Huxley model informs only the level of progression at the macroscopic level, but not the underlying mechanical processes. In actuality action potential propagation is due to a combination of separate *microscopic* elements: the APPulse, an electromechanical mechanism incorporating a soliton-ion channel pump, which produces a phase ternary CAP from the separately identifiable resting, threshold and refractive phases. Recovery occurs when the Na^+, K^+ and other ions re-establish membrane stability.
- Phase-ternary computation within physiological neural networks is fast, accurate to microseconds, and efficient. It diffracts parallel inputs within a network along pathways defined by the phase in which action potentials arrive at the neural network in temporal synchrony. In contemporary computing parlance: phase-ternary computation is the brain's machine language and is capable of storing information regardless of any other memory storage or retrieval processes within that network.
- In the H&H action potential the temporal accuracy of the point of computation is variable, restricted to accuracy estimated from the H&H curve. Computation is only accurate to calculated milliseconds. By contrast computation with the action potential pulse is accurate to the exact threshold distance between specific ion channels in microseconds along an unrestricted neurite giving 1000 times greater computational precision.

AUTHOR CONTRIBUTIONS

AJ: the original concept. AJ and WW: split the writing of the review about 50/50 with much discussion between us about the way to finalize the article.

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