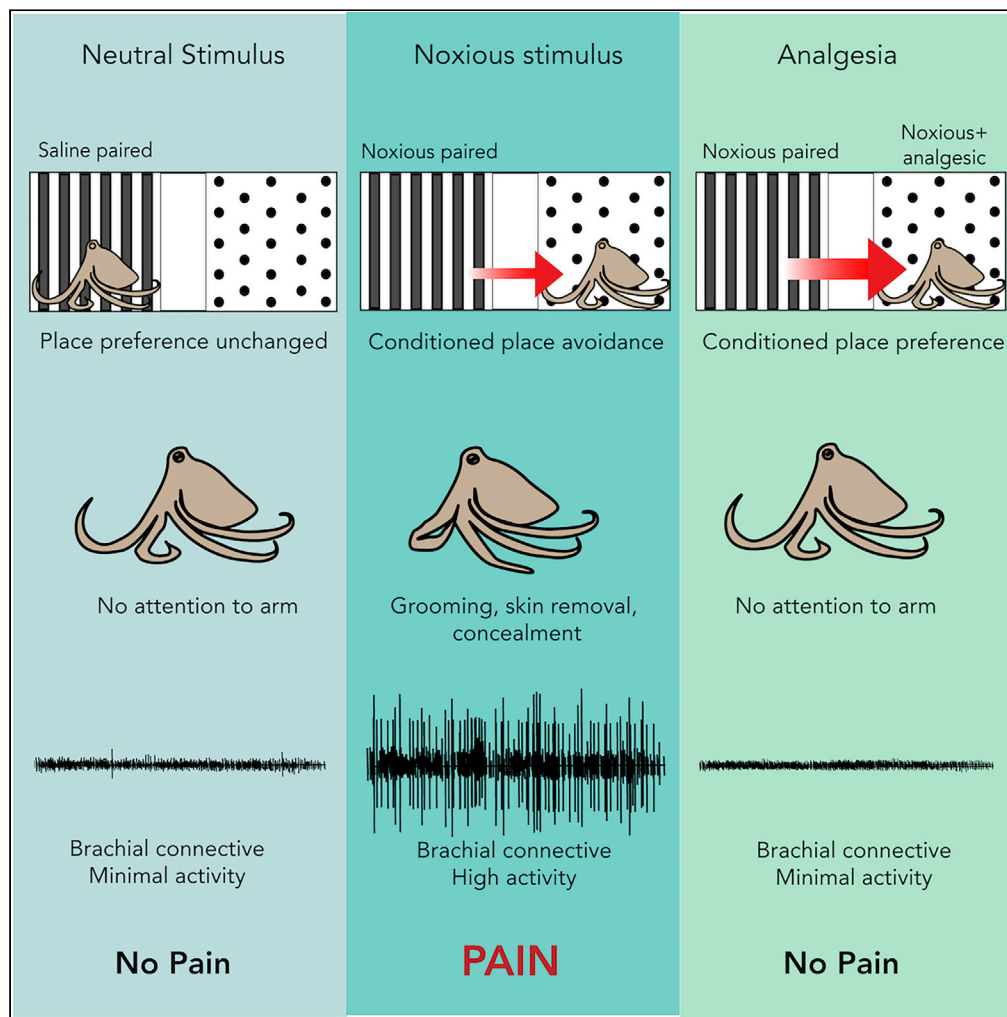


Article

# Behavioral and neurophysiological evidence suggests affective pain experience in octopus



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**HIGHLIGHTS**

Octopuses avoid a location after it is associated with a noxious stimulus

Injection of dilute acetic acid induces lasting, location-specific grooming

Nerve recordings show central processing of noxious sensory input

Octopuses are capable both of discriminative and affective pain experience

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## Article

## Behavioral and neurophysiological evidence suggests affective pain experience in octopus

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## SUMMARY

**Pain is a negative affective state arising from tissue damage or inflammation. Because pain is aversive and its relief is innately rewarding, animals may learn to avoid a context in which pain is experienced and prefer one where pain relief occurs. It is generally accepted that vertebrate animals experience pain; however, there is currently inconclusive evidence that the affective component of pain occurs in any invertebrate. Here, we show that octopuses, the most neurologically complex invertebrates, exhibit cognitive and spontaneous behaviors indicative of affective pain experience. In conditioned place preference assays, octopuses avoided contexts in which pain was experienced, preferred a location in which they experienced relief from pain, and showed no conditioned preference in the absence of pain. Injection site grooming occurred in all animals receiving acetic acid injections, but this was abolished by local anesthesia. Thus, octopuses are likely to experience the affective component of pain.**

## INTRODUCTION

Whether invertebrate animals are capable of experiencing pain is the subject of ongoing debate (Elwood, 2019a; Groening et al., 2017; King and Marino, 2019; Walters et al., 2019). Unlike nociception, which is a simple reflex response, pain is a complex emotional state encompassing distress and suffering and is generally considered to require a highly complex nervous system (Treede et al., 1999). Discrete pain circuits within the central brain produce two distinct aspects of pain experience: the “discriminative” component, encompassing the location, quality, and intensity of pain, and the “affective” component, encompassing the negative emotional state (Auvray et al., 2010). Pain is accepted to occur in all vertebrate animals, although pain experience that is persistent and ongoing (tonic pain) has to date only been demonstrated in mammals (King et al., 2009; Mogil, 2019). Although the evolutionary origins of pain remain unresolved, there are indications in several invertebrate taxa of at least some of the requirements for pain experience (Elwood, 2019b; Kavaliers, 1988; Key and Brown, 2018). It is generally accepted that there are specific requirements for a nervous system to be capable of producing a negative affective state (i.e., pain experience) in response to noxious sensory input, including the presence of nociceptive sensory neurons, which then must connect to integrative brain regions capable of complex processing (Sneddon, 2019). Nociceptors are present and well characterized in multiple invertebrate taxa, including cephalopods ((Crook et al., 2013), other molluscs (Illich and Walters, 1997), insects (Tracey, 2017), nematodes (Chatzigeorgiou et al., 2010), crustaceans (Barr et al., 2008). There are also accepted behavioral criteria that are used to suggest the presence of affective state going beyond simple nociceptive reflex, such as complex behavioral responses that can be modulated by analgesia, motivational trade-offs that balance potential pain against other, usually protective or positively valenced experiences, and associative learning about contexts that signal noxious sensations (Appel and Elwood, 2009; Elwood, 2019b; Walters, 2018). Behavioral studies showing evidence for these capabilities have been conducted most extensively in crustaceans (Elwood, 2019b). Interestingly, there is also accumulating evidence for the existence of positive affective state in some invertebrates as well (Perry et al., 2016), suggesting emotional processing of sensory experiences in invertebrates may be both complex and widespread.

A common argument against the possibility of affective state in invertebrates is that their brains are insufficiently complex to encompass circuits that produce emotional valence (Broom, 2007; Sømme, 2005). A similar argument has also been used to suggest that fish are incapable of pain experience (Derbyshire, 2016; Key, 2016; Rose, 2016), indicating the ongoing controversy over the question of pain in non-mammalian species. Cephalopod molluscs are extreme outliers in the realm of invertebrate brains; unlike all other

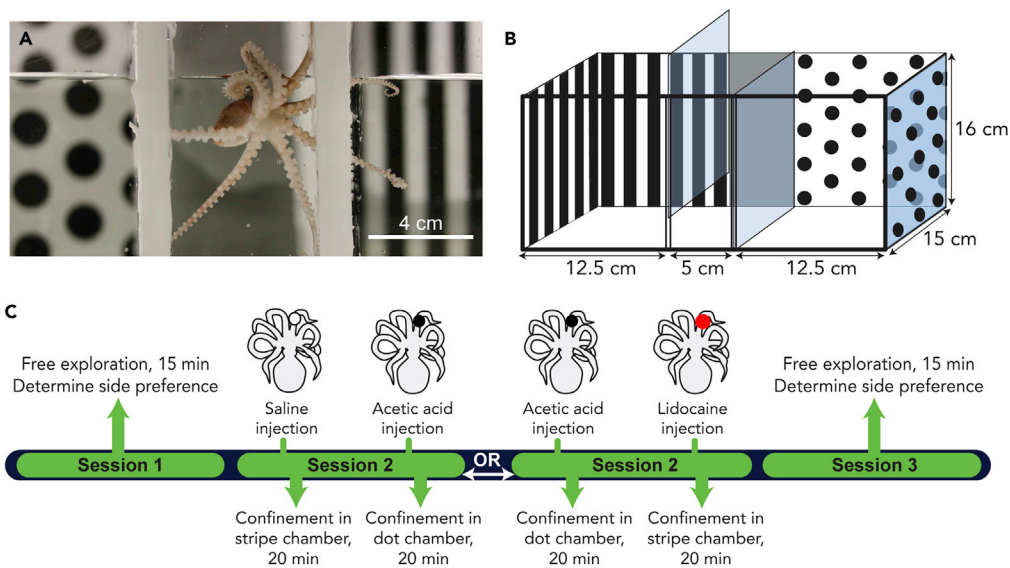
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**Figure 1. CPP design and timeline**

(A) *Octopus bocki* in the start chamber of the CPP box.

(B) Diagram of the apparatus, with pattern shown on the back and sides only for clarity. In experimental trials, visual cues covered all four walls.

(C) Timeline of an experiment showing sequences for CPA and CPP procedures. In this example, an octopus showed an initial preference in session 1 for the dot chamber and is thus trained against initial preference (i.e., the octopus is given AA injection prior to confinement in the dot chamber or lidocaine prior to the stripe chamber). Different animals were used to test each of the four conditions (see Figure 2). Control sequences (saline/saline and saline/lidocaine) not shown.

invertebrates, their brain size, cognitive ability and behavioral flexibility surpass those of some smaller-brained vertebrates, including amphibians and reptiles (Hochner et al., 2006; Schnell et al., 2020). Their nervous system is organized fundamentally differently from that of vertebrates, with extensive peripheral control of sensing and movement which seems to occur largely independently of the central brain (Gutfreund et al., 2006, but see Gutnick et al., 2020; Hooper, 2020). Their large brains and complex behaviors have led to increasing concern for their welfare, and efforts to regulate invasive procedures performed on cephalopods in research laboratories are now established in many nations (Ponte et al., 2019). These rules are informed by the “precautionary principle” (Birch, 2017), which posits that that neural and cognitive complexity is sufficient to suggest that an animal can experience pain, even where no conclusive evidence exists.

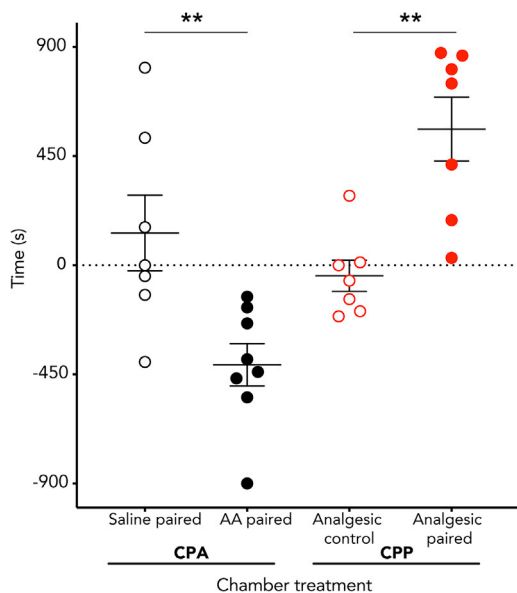
Somewhat surprisingly, there have been few experimental studies focusing on potential for pain experience in cephalopod molluscs. In this study, a well-established assay for demonstrating the affective component of pain in mammals (Navratilova et al., 2013; Sufka, 1994) was applied to octopus, along with detailed measurements of spontaneous pain-associated behaviors and neural activity in centripetal pathways. All three lines of evidence indicate that octopuses are capable of experiencing pain.

## RESULTS

### Octopuses experience the affective component of pain

After a single training session in a three-chamber conditioned place preference (CPP) box (Figure 1), octopuses that received a subcutaneous injection of dilute (0.5%) acetic acid (AA) into one arm ( $n = 8$ ) showed clear avoidance of their initially preferred chamber, in which they were confined after injection (Figure 2A, one-sample  $t$  test,  $p = 0.003$ ). Saline-injected animals ( $n = 7$ ) showed no change in their chamber preference before and after training trials ( $p = 0.19$ ). The change in time spent in the initially preferred chamber also differed between the two groups (Bonferroni post-hoc test,  $p = 0.006$ , Figure 2).

Relief from tonic pain is rewarding, and thus, a drug that provides pain relief provides a strong training signal in the presence of tonic pain but no signal in its absence. CPP for a location associated with an



**Figure 2. Conditioned place avoidance (CPA) and conditioned place preference (CPP) assays reveal the affective component of pain in octopus**

In trials where initially preferred chambers were paired with 0.5% acetic acid (AA) injection, octopuses spent less time in their initially preferred chamber in a post-training period of free exploration, compared with octopuses receiving saline. In trials where octopuses received lidocaine over an area of prior injection (either saline or AA), octopuses preferred the chamber paired with lidocaine only if they had previously been given AA injection. Points show individual change in time values for each subject. Different animals were used to test each condition. Central, wide bars show the mean and whiskers show standard error of the mean. Asterisks indicate significant between-group differences (Bonferroni post-hoc test,  $**p < 0.01$ ).

analgesic is considered strong evidence for pain in vertebrate animals (Navratilova et al., 2013; Roughan et al., 2014). Here, octopuses with AA-induced tonic pain received a localized injection of lidocaine (Butler-Struben et al., 2018) immediately prior to being confined to the chamber they least preferred in initial preference tests. Lidocaine injection induced strong preference for that chamber in test trials for AA-injected animals (Figure 2A, one-sample t test,  $p = 0.005$ ), but there was no preference for the lidocaine-paired chamber in animals that received saline injection instead of AA ( $p = 0.51$ ), and chamber preference also differed between the two groups (Bonferroni post-hoc test,  $p = 0.003$ ). This demonstrates that lidocaine injection was rewarding to animals only if they were experiencing ongoing pain and that lidocaine alone is not innately rewarding for octopuses.

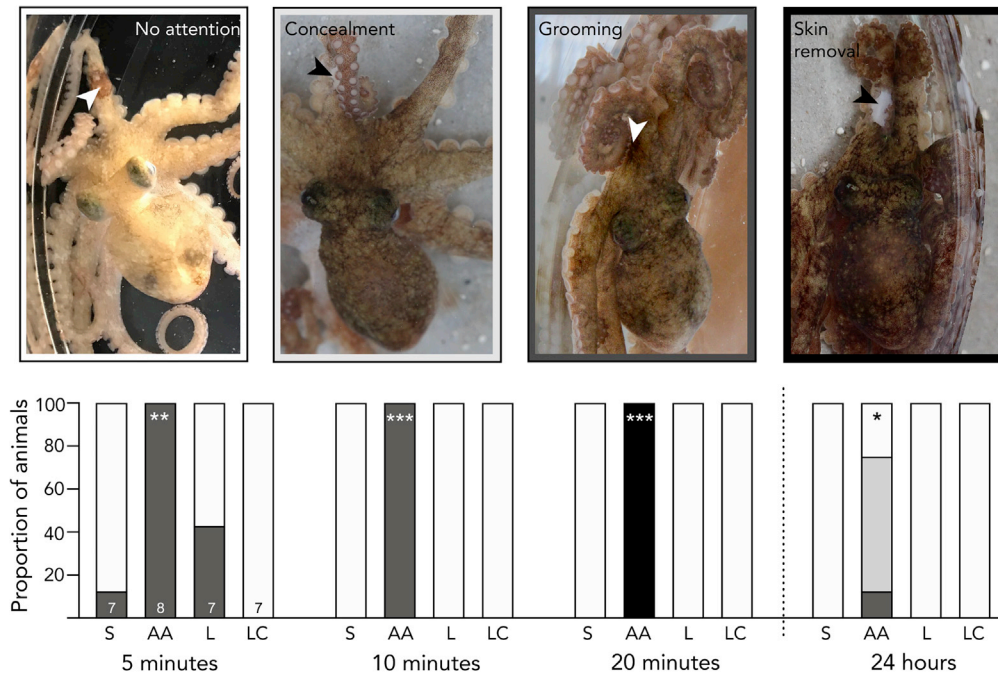
### Octopuses show discriminative pain experience

While CPP is useful for testing the affective component of pain, it does not necessarily reveal the discriminatory aspect, which includes awareness of the location, quality, and intensity of pain (Auvray et al., 2010; Treede et al., 1999). Point observations of potential pain-associated behaviors (grooming, guarding, and concealment) were made at 5-min intervals during conditioning trials (session 2) and again 24 h after conditioning trials. All octopuses injected with AA groomed the injection site with the beak for the full 20-min training trial (Figure 3A), but this behavior was either brief or completely absent in the other groups (Figure 3B). While wound-directed behavior has been reported previously in octopuses (Alupay et al., 2014) and other invertebrates (Elwood, 2011), the behaviors observed here appear to be specific to acid injection. In all animals receiving AA injection, beak grooming resulted in the removal of a small area of skin over the injection site, which was apparent at the conclusion of the 20-min conditioning trial that followed the injection. This behavior was never observed in animals receiving saline injection or after injection of lidocaine.

### Noxious sensory information is processed in the central brain

Cephalopods are highly unusual in the degree to which higher-order sensory information processing occurs in the peripheral nervous system (Gutfreund et al., 2006; Sumbre et al., 2001). Ongoing pain in mammals is driven by sustained activity in primary nociceptors that then drives long-term changes within higher-order, central circuits (Davoody et al., 2011; King et al., 2011). Spontaneous nociceptor firing after tissue injury has also been shown in cephalopods, to date the only invertebrate taxon where this mammalian-like pattern has been recorded (Crook et al., 2013). Whether spontaneous activity in nociceptors drives ongoing excitation of central circuits in the cephalopod brain has not been clear, raising questions of if and how the central brain perceives noxious sensations in peripheral tissues.

To assess what information the central brain receives about nociceptive stimuli in the arms, electrophysiological recordings were taken from the brachial connectives, which connect the arm nerve cords to the



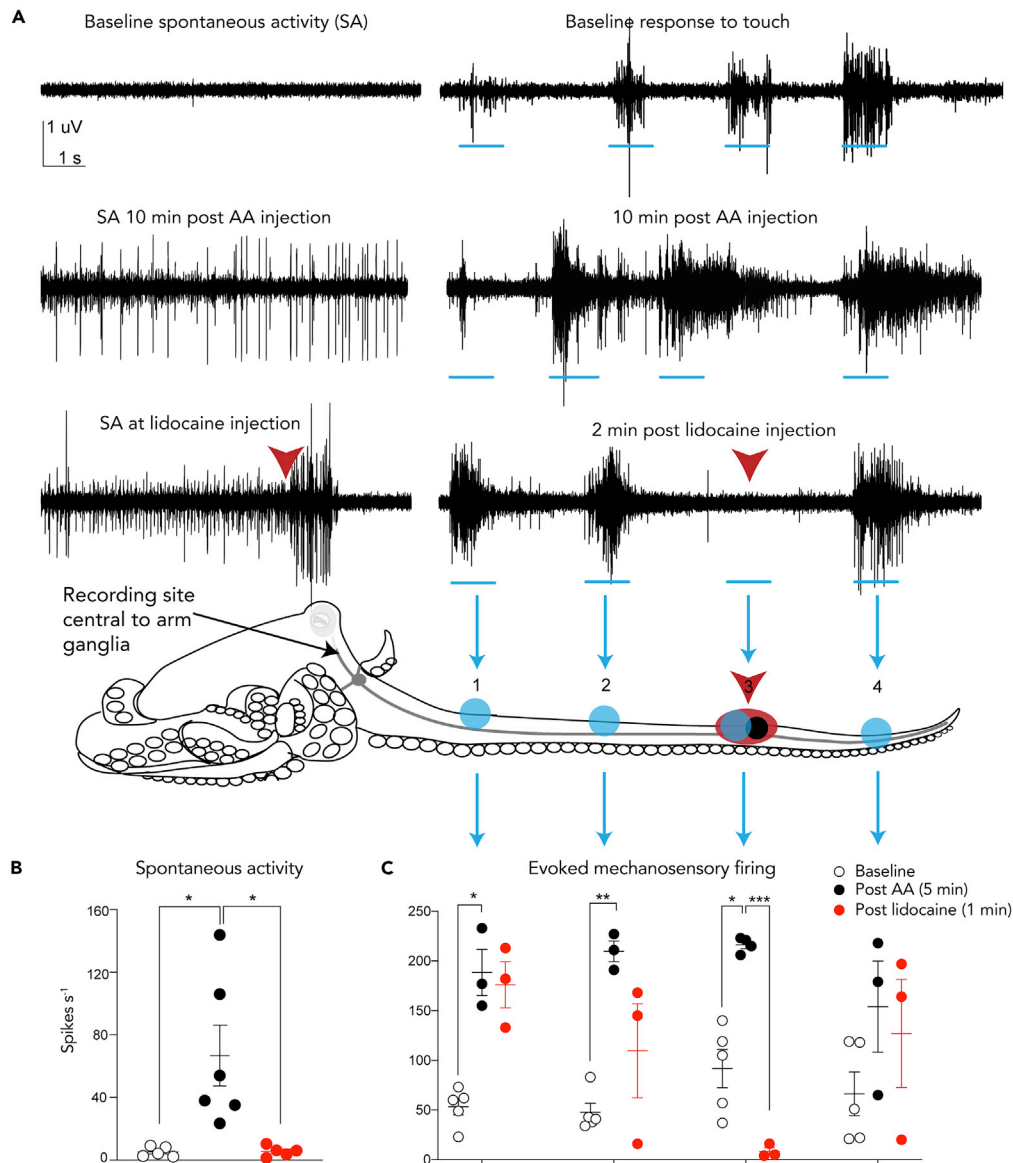
**Figure 3. Precise and specific wound-directed grooming behaviors show discriminative pain experience in octopus**

Top panel shows examples of wound-directed behaviors, and colors surrounding each image correspond to shaded frequencies in stacked bars, below. Arrowheads indicate the location of AA injection on the arm. Behaviors were observed during training trials and 24 h later. AA-injected octopuses showed sustained wound attention and concealment that persisted for at least 24 hr after AA injection. Skin removal behavior was observed in all AA-injected animals, suggesting a specific representation of acid-induced pain that elicits a highly specific behavioral response. Bar acronyms: AA, acetic acid injection; S, saline injection; L, lidocaine injection after earlier AA injection; LC, lidocaine control (lidocaine injected after earlier saline injection). Numbers inside bars in first panel (5 min) are group sample sizes, which are the same for each subsequent interval. Asterisks indicate significant difference in proportions of animals performing each behavior, relative to saline-injected controls (fishers exact tests, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

brain and are central to the major arm ganglia situated in the interbrachial commissure. In a reduced preparation where the connective were severed central to the interbrachial commissure and the Central Nervous System (CNS) removed, injection of a bolus of AA subcutaneously into one arm resulted in a prolonged period ( $>30$  min) of ongoing activity in numerous recorded units, which was silenced rapidly by injection of 2% lidocaine overlying the site of AA injection (Figures 4A and 4B, baseline vs. post-AA injection,  $p = 0.046$ , post-AA vs. post-lidocaine,  $p = 0.045$ ). This activity generated within the area of AA infiltration likely provides information to the brain about the location of the painful stimulus. Lidocaine injection into the infiltration site also reversed the sensitization of afferent activity evoked by strong mechanical stimulation at and proximal to the injection site (baseline vs. post-AA,  $p = 0.02$ , post-AA vs. post-lidocaine,  $p = 0.001$ , Figure 4C).

## DISCUSSION

Together, these data provide strong support for the existence of a lasting, negative affective state in octopuses: the first evidence for pain experience in this neurologically complex invertebrate clade. Although a number of previous studies in cephalopods and other invertebrates have shown avoidance learning of a context in which a brief noxious stimulus was delivered (Budelmann and Young, 1987; Elwood and Appel, 2009; Magee and Elwood, 2013; Sanders, 1970), here, octopuses were able to learn to avoid a visually specific location that was explicitly unlinked both in time and space from the injection procedure that initiated nociceptor activation. Thus, the most plausible explanation for the strong place avoidance behavior observed here is that octopuses experience a state of ongoing (tonic) pain and negative affect after AA injection. Tonic pain has to date been demonstrated only in mammals (Davoody et al., 2011; King et al., 2009; Uhelski and Fuchs, 2010), and this study provides the first example of



**Figure 4. Examples of electrophysiology recordings and summary data showing that nociceptive signal from the arms is available to the octopus CNS**

(A) Examples of spontaneous (ongoing) and evoked activity in the brachial connective before and after injection of acetic acid (AA, shown as a black circle at arm stimulation position 3) and at the point where lidocaine is injected locally over the region of prior AA injection (shown as a red overlay of the black circle on the arm at position 3). Note the almost immediate cessation of ongoing activity after lidocaine injection, and the complete suppression of evoked activity in the region where lidocaine was injected at position 3 on the arm of the octopus.

(B) Ongoing, spontaneous firing in the brachial connective is increased after AA injection and blocked by injection of lidocaine into the same position on the arm.

(C) Summary data showing responses to touch on the arm at four locations (indicated by shaded blue circles on the octopus body outline). There is clear enhancement of evoked activity after injection that is suppressed by injection of a local anesthetic. Points show individual values for each subject. Central, wide bars show the mean and whiskers show S.E.M.

Asterisks above linking bars indicate significant differences between groups (paired, Holm-Bonferroni corrected t-tests, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

probable ongoing pain in any non-mammalian animal. While there is now considerable evidence for transient pain experience in crustaceans (Elwood, 2019b) and both positive and negative affective states in other invertebrates (Baracchi et al., 2017; Bateson et al., 2011; Gibson et al., 2015; Perry et al., 2016), the presence of lasting pain experience in cephalopods (and possibly other invertebrate species) as a result of tissue injury raises both significant concerns for their welfare and interesting new questions about the evolutionary origins of pain experience.

Pain experience in cephalopods has long been considered likely based on their exceptional neural and behavioral complexity, and this probability has informed various regulatory efforts aimed at protecting their welfare in research and other settings (Fiorito et al., 2015). With this study, we not only provide the first strong evidence supporting the existence of a pain state in cephalopods but also suggest that CPP may be an effective screening tool for assessing analgesic drug candidates and other welfare-promoting interventions.

In addition to affective pain experience as demonstrated by conditioned place avoidance (CPA) and CPP, spontaneous pain-associated behaviors in octopuses reveal the presence also of discriminative pain experience. The nervous system of octopuses is unusual in its high degree of distributed processing, which has led to conjecture that the arms are at least partially autonomous and the central brain does not necessarily process all information gathered by the sense organs within them (Alupay et al., 2014; Fouke and Rhodes, 2020; Gutfreund et al., 2006; Gutnick et al., 2011, 2020; Rowell, 1966; Sumbre et al., 2001). In this study, we show clearly that the central brain receives location-specific information about noxious sensation in the arms and that the central brain coordinates location-specific beak grooming behavior. The specific quality of the pain also appears to be represented in the octopus CNS. In other studies of nociception in octopus, arm compression, skin pinch, and skin incision induced prolonged beak grooming and concealment but never skin removal (Alupay et al., 2014), suggesting that AA injection produced a central representation of pain that was quite different to other injury modalities. Noxious stings, which AA injection likely approximated, are likely encountered by octopuses as they hunt venomous prey (Brooks, 1988; Ross, 1971). It is plausible that skin stripping is an injury-induced behavior that has evolved to release injected venom from the skin. This distinct behavior suggests that the octopus central brain is capable of encoding not only the location but also the specifics of pain quality. In this study, central representation of intensity—the third component of discriminative pain experience—was not tested directly; however, previous research has shown that intensity is encoded and transmitted faithfully in the peripheral nerves and central projections from the mantle of cephalopods (Crook et al., 2013; Howard et al., 2019); thus, it is likely that intensity is also an aspect of pain experience in octopus.

Reversal of pain-associated spontaneous and cognitive behaviors by analgesics is considered to be strong evidence for the presence of pain. Among invertebrates, the use of analgesics to demonstrate potential pain experience has had mixed results (Barr et al., 2008; Barr and Elwood, 2011; Groening et al., 2017; Puri and Faulkes, 2010). Analgesia-induced place preference in mammals is widely accepted to signal pain experience; however, a common criticism of such studies is that the chosen analgesic drug is innately rewarding, and its hedonic quality is sufficient to create place preference even in animals in neutral affective states (Sufka, 1994; Tzschentke, 2007). The use of lidocaine in this experiment precludes this alternative explanation; lidocaine had no central effect when injected locally and produced no place preference in octopuses who had not previously received AA injection (and thus were not in pain).

The functionality of pain experience—rather than nociception alone—in cephalopods is not yet clear. In evolutionary terms, pain has been hypothesized to be adaptive primarily among social species where injured individuals can recruit help from in-group members while ongoing pain reinforces resting and recuperative behaviors (Walters et al., 2019). Additionally, the strong negative affect produced by injury is cited as an adaptive mechanism for reinforcing contextual memory of danger that lasts throughout life. Although the octopus is often described as being “vertebrate like” in cognitive ability and intelligence, its asocial habits, short life span, and severe nutritional costs of recuperative inactivity (Wells and Clarke, 1996) argue against the prevailing evolutionary hypotheses cited for the evolution of pain in vertebrates and instead suggest a wider, more ecological perspective on the evolution of pain and affective state. Indeed, the evolution of exceptional neural complexity in cephalopods is typically attributed to their ecological association with complex habitats, niche competition with fish, and their reliance on complex camouflage and signaling behaviors (Birch et al., 2020; Hanlon and Messenger, 2018; Mather et al., 2014;

O'Dor and Webber, 1986). How and why pain experience has evolved in cephalopods remains to be understood, and further, careful investigations of the molecular, genetic, and anatomical bases of pain in cephalopods will be necessary to shed light on the extraordinary parallel evolution of pain experience in this unique invertebrate clade.

### Limitations of the study

Reversal of place preference by lidocaine was variable, which may have been due to incomplete or off-target lidocaine infiltration of the acetic-acid-affected region. More generally, there is ongoing debate about the relationship between sentience, consciousness, and affect that can complicate links between behavioral experimental readout and internal state. If an animal must necessarily be conscious and sentient to experience negative affect, it must therefore be necessary for octopuses to be conscious to experience pain; a controversial proposition, but one which has received considerable attention (Birch et al., 2020; Low et al., 2012; Mikhalevich and Powell, 2020; Turnbull and Bär, 2020). Even in the absence of proof on conscious awareness or sentience in cephalopods, it remains clear that the responses demonstrated by octopuses in this study are so similar to those that would be expressed by mammals experiencing pain that a reasonable, cautionary argument can be made that internal state of these disparate species is likely also similar.

### Resource availability

#### Lead contact

Robyn J Crook, [rcrook@sfsu.edu](mailto:rcrook@sfsu.edu).

#### Material availability

Raw video electrophysiological files are available upon reasonable request from the lead contact.

#### Data and code availability

Data associated with each figure are available for download from Open Science Forum under the Project Name "Conditioned Place Preference reveals tonic pain in octopus", <https://osf.io/3fmuy/>.

## METHODS

All methods can be found in the accompanying [Transparent methods supplemental file](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2021.102229>.

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## AUTHOR CONTRIBUTIONS

This study was conceived, designed, conducted and written by R.J.C.

## DECLARATIONS OF INTERESTS

The author declared no competing interests.

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**iScience, Volume 24**

**Supplemental information**

**Behavioral and neurophysiological evidence  
suggests affective pain experience in octopus**

**Robyn J. Crook**

## Supplemental Information

### Transparent Methods:

Animals: Adult *Octopus bocki* (Bock's pygmy octopus, N=29, sex undetermined, average mantle length 12 mm) were obtained from a commercial vendor (Sea Dwelling Creatures, Los Angeles, CA, USA), and housed individually in rectangular tubs (23 x 15 x 15.8 cm l x w x h, capacity 1900 mL), providing physical, visual and chemical isolation from neighbors. Individual inflow pipes circulated artificial seawater (Instant Ocean, S.G. 1.023, pH 8.1-8.2, 24 Deg C) through each enclosure at a rate of 500 mL/min. Full turnover of water volume occurred every four minutes. Enclosures were located within larger recirculating seawater systems, where water was filtered constantly through physical, biological and charcoal filters. Water quality was monitored daily; ammonia and nitrite were 0 ppm and nitrates ranged up to 20 ppm. Each octopus enclosure contained a bed of crushed coral chips 2 cm deep, three PVC elbow joints of either ½ or ¾ inch, two plastic plants, at least six empty snail and clam shells and two pieces of coral rubble.

Octopuses were fed once per day on a 5 mm cube of thawed, frozen, uncooked shrimp (Trader Joe's brand, CA, USA). Uneaten food was siphoned from the tank once per day during routine tank maintenance. During daily husbandry, octopuses were habituated to being moved from their home tank by being guided into a glass beaker to allow tank siphoning, and this habituation step also facilitated later movement into the conditioning chamber during experiments, using the same procedure. Animals were maintained in the laboratory for at least one week prior to being used in experiments, and only animals that were readily accepting food, sheltering normally and were habituated to daily husbandry were used in behavioral experiments.

At the conclusion of behavioral studies, animals that had received painful stimuli were euthanized 24 hours after conclusion of testing. The delay was to ensure that the drugs did not induce toxicity or cause death in the acute post-injection period. Octopuses were killed according to established methods (Butler-Struben et al., 2018), and tissue was fixed for later use. Control animals were maintained for up to two weeks prior to being used in electrophysiology experiments. Two females in the control group laid eggs within the two weeks and were left to brood their eggs until they died of natural senescence-induced decline.

Ethical note: In the United States octopuses are not included in vertebrate animal regulations that govern the use of animals in research. Although no formal approval process occurred, all animal procedures were conducted in accordance with EU Directive 63/2010/EU (Fiorito et al., 2015), which contains the most stringent requirements for cephalopod research globally. Because the study necessarily involved the use of painful stimuli, sample sizes were calculated to capture moderate and large effect sizes only at a power of 0.8. Post-hoc power analysis indicated 86% power in the CPA experiment and 98% power in

the CPP experiment. Procedures, record keeping and reporting were conducted using ARRIVE guidelines (Percie du Sert et al., 2020).

#### Experiment 1, Conditioned place avoidance (CPA) experiments

Apparatus: The CPP/CPA arena was made from a modified 9.5 L glass aquarium (Carolina Biological, Item 671226). Two flexible, PVC channels were glued to the sides and bottom of the tank to create holders for two removeable, clear, plexiglass dividers, which when inserted created a three-chamber box (see Fig. 1A&B) with a narrow central start box and two equal-sized end chambers. Visual cues on the tank walls were either black spots (diameter 12 mm, spaced edge-to-edge 6 mm apart) on a white background, or equally spaced black and white, vertical bars (8 mm wide). Walls in the central start box were uniform, 50 % grey, and the floor in all three chambers was white. Chamber dividers were clear, but were covered with same-chamber patterns during conditioning confinements in each chamber. The arena was filled with 3 L of home tank water, which was not circulated or aerated during trials. Between trials, the water was discarded and tanks were washed inside and out with hot, soapy water to remove any olfactory cues, then rinsed three times with Milli-Q filtered water, sprayed with 70% ethanol solution, and left to dry in bright sunlight. Trials were conducted in an isolated, black-walled room with limited external visual cues. Supplemental, controlled light was provided by a fiber-optic light reflecting diffuse light from the ceiling, which was white. Light level at the water surface was measured with a digital light meter (Dr. Meter LX1010B) at 11 lux. Trials were recorded by a camcorder (Sony FDR-AX33) fitted with a polarized light filter and positioned directly overhead.

Drugs. Glacial acetic acid (Sigma-Aldrich, A6283) was diluted in filtered, artificial seawater to produce a final concentration of 0.5 % v/v. Sham injections were fASW only. Injectable lidocaine solution (2 % HCl) was obtained from A-to-Z Vet Supply (item 515-510212).

Procedure: On Day 1, (Session 1, or “Initial Preference Test”), octopuses were allocated randomly to either treatment or control groups by card draw. Each animal was moved from its home tank and placed into the central start box of the CPP arena. After a two-minute acclimation period, the clear dividers were lifted and octopuses explored freely for 15 minutes. At the conclusion of exploration, octopuses were removed from the CPP chamber in small transfer beakers and returned to their home tanks. Routes taken by each subject were analyzed by Ethovision animal tracking software (Noldus), and end-chamber in which each animal spent the most time (i.e., its initial preference) was recorded. In three cases the octopus did not leave the start box in the first trial. These animals were assigned an initial preference randomly.

The following day, Session 2 (“Training”) comprised two conditioning trials, with the animal confined first in one chamber and then the other. Training was against initial preference, meaning that painful stimuli were experienced in the chamber the animal preferred initially, and neutral or pain-relieving treatments were given prior to confinement in the initially non-preferred chamber.

Prior to the first conditioning trial, animals were removed from their home tank and lightly sedated in 1% EtOH in ASW for handling. Once animals were unresponsive to touch (5-10 minutes after EtOH introduction), one arm was selected for drug treatment. 1-2uL of saline was injected about 1/3 along the length of the arm under the dorsal skin, using a 10uL Hamilton syringe and a 30g needle, fitted with a 0.2 micron filter.

Immediately after injection the sedation bath was replaced by running fASW. Animals typically recovered normal behavior within 5-10 minutes. Fifteen minutes after recovery from sedation, octopuses were confined in their initially non-preferred chamber.

At the conclusion of the first 20-minute training trial animals were removed using the standard transfer procedure and allowed to rest undisturbed in small holding tanks for 30 minutes while tanks were washed, dried and refilled with fresh home tank water. After 30 minutes, octopuses were re-sedated for the second injection procedure. Half of the subjects received 0.5% acetic acid ("AA") into the arm adjacent to that used for the first injection, while the other half received a second saline injection. Recovery from sedation followed the same procedure as above, and then animals were confined in their initially-preferred chamber.

During training, the clear plexiglass divider was replaced with an opaque panel showing the same pattern as the other three chamber walls, thus the pattern in the opposite chamber was completely out of sight during each training. After the second training trial, animals were returned to home tanks. Animal movements were not tracked during single-chamber confinements in the training sessions.

Test trials (Session 3, or "Final Preference Test") occurred between 5 and 6 hours after the conclusion of the second training trial, on the same day. The procedure was identical to the initial preference test on the preceding day. No drugs or sedation were administered prior to the final training trial.

#### Experiment 2, Conditioned Place Preference (CPP)

All apparatus, handling and timing of trials was identical to the CPA experiment as described above. Prior to the first conditioning trial, half of the animals received 0.5% AA solution, and half received saline. After recovery from sedation, octopuses were confined in their initially preferred chamber. For the second conditioning trial, all the animals received a single, subcutaneous injection of 3uL of 2% lidocaine hydrochloride at the same site as the first injection. After recovery from sedation, octopuses were confined to their initially non-preferred chamber for 20 minutes. All other procedures were identical to those described above.

#### Electrophysiology

To ascertain what information the central brain receives about noxious events in the arms, activity was recorded from the brachial connectives, which run between the CNS and the first major ganglion at the top of the arm nerve cord (see Fig. 4). The major arm ganglion lies within the inter-arm commissure, which is a ring linking all the arm that sends signals from one arm to the other. Because there is extensive

peripheral processing and sensorimotor integration at the level of the individual brachial ganglia along the arm, and again at the level of the major arm ganglia in the inter-arm commissure, afferent signals recorded from the brachial connectives represent highly pre-processed input into the central brain (Gutfreund et al., 2006). Previous studies have shown that relatively little non-nociceptive mechanosensory information is transmitted centrally from distal arm regions (Alupay et al., 2014; Fouke and Rhodes, 2020; Rowell, 1966), raising the possibility that noxious sensory information is processed entirely in the periphery, without involvement of the central brain.

Octopuses were killed by immersion in isotonic magnesium chloride solution (330mM in Milli-Q filtered water). Ten minutes after respiration stopped, the arm crown was cut from the head and mantle with a scalpel and the brachial connectives exposed by microdissection of overlying tissues. The preparation was pinned tightly into a Sylgard-coated petri dish and the MgCl<sub>2</sub> solution was washed off with fASW. One brachial connective was drawn into a suction electrode and the preparation was allowed to rest for 15 minutes. Background firing was recorded for one minute, then a stiff (potentially noxious) von Frey filament (number 5.07, applying 10 g of tip force) was applied to four positions on the arm, moving distally. The stimulation sequence was repeated three times, then the same volume of 0.5% AA used in behavioral experiments was injected into the arm. Background firing and response to the mechanical stimuli were recorded at 1, 5, 10, 30 and 120 minutes in two preparations (data not shown). In six other preparations, 2% lidocaine HCl was injected into the arm at 20 minutes, and background firing and evoked responses recorded 2 minutes thereafter.

Signals were amplified by an A-M Systems differential extracellular amplifier (model 1700), then digitized and recorded at 10kHz with a PowerLab 4/35 running LabChart Pro software.

#### Data analysis and statistics

CPA/CP: Octopus movements were tracked from recorded video files using Ethovision 13 (Noldus). Examples of tracks and associated data are shown in Fig S1. Time spent per chamber in Session 3 was subtracted from pre-conditioning times spent in Session 1, and all data are expressed as changes from baseline chamber preferences recorded in Session 1. All statistical procedures were conducted in Prism 8.0 (GraphPad). Data distribution was tested with the Kolmogorov Smirnov test and met the assumptions of normality. A single-factor ANOVA followed by planned, post-hoc Bonferroni tests was used to identify between-group differences. To assess whether individual groups' change in time-per-chamber differed from zero, (a zero value would indicate no change in preference) a one-sample t-test was conducted with an expected value of zero.

Pain-associated behavior: Point observations of pain-related behavior were taken every 5 minutes from recorded video footage of training trials. At each point, beak grooming and concealment of the treated area were noted, and frequency per treatment group (proportion of total animals) was compared using

Fisher's exact tests. At the conclusion of training trials, arms were inspected for evidence of skin stripping behavior.

Electrophysiology: Spikes above noise threshold were counted using the automated "Spike Histogram" module in LabChart Pro. For each touch, spikes were counted for a 1s period of maximal firing.

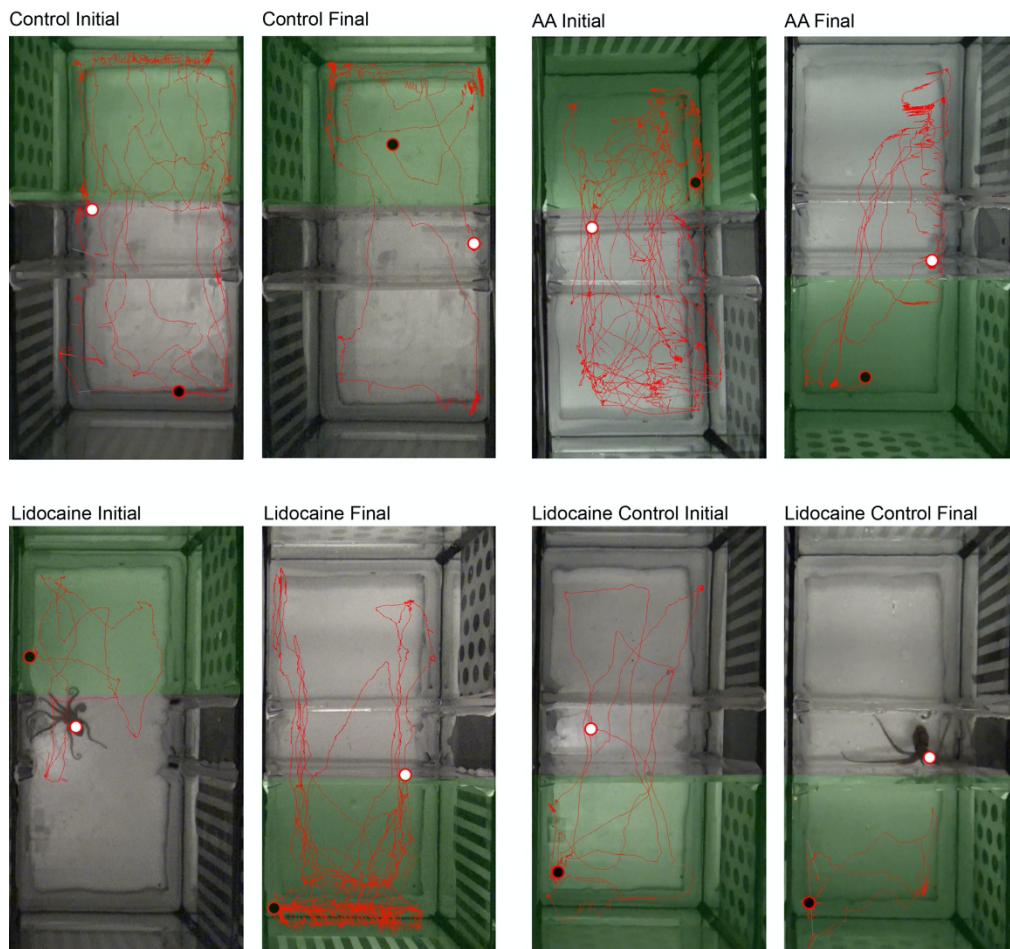
Mechanical stimuli were repeated at the same location and timepoint, averaged, and compared at baseline, after AA injection and after lidocaine injection with a repeated-measures ANOVA followed by post-hoc, paired t-tests corrected using the Holm-Bonferroni method (Holm, 1979).

All reported p-values are two-tailed.  $p < 0.05$  was considered significant.



Supplemental Figure:

Figure S1. Route maps of representative animals from each treatment group in CPA/CPP assays, related to Figure 2. Tracks generated by Ethovision 13.0 tracking software (Noldus Inc). Routes (red lines) are shown overlaid on a reference image of the chamber for each trial. Start position in the middle chamber is shown by a filled, white circle. Final position is shown with a filled, black circle. The chamber where the octopus spent more time is shaded in green. This chamber is defined as the “preferred” chamber, even when the final position of the animal was in the opposite chamber (as seen in the Control Initial example). Octopuses were tracked via center point marker, which was subject to considerable position “jitter” caused by the shift in the computed midpoint as the outline of the animal changed from extended and curled body postures (most notable here in the Control Initial and Lidocaine Final routes). Because this typically occurred along chamber edges it did not affect automatic detection of chamber occupancy.



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