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Supplemental information

Behavioral and neurophysiological evidence suggests affective pain experience in octopus

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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 lxwxh, 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 though 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 MgCl2 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/CPP: 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

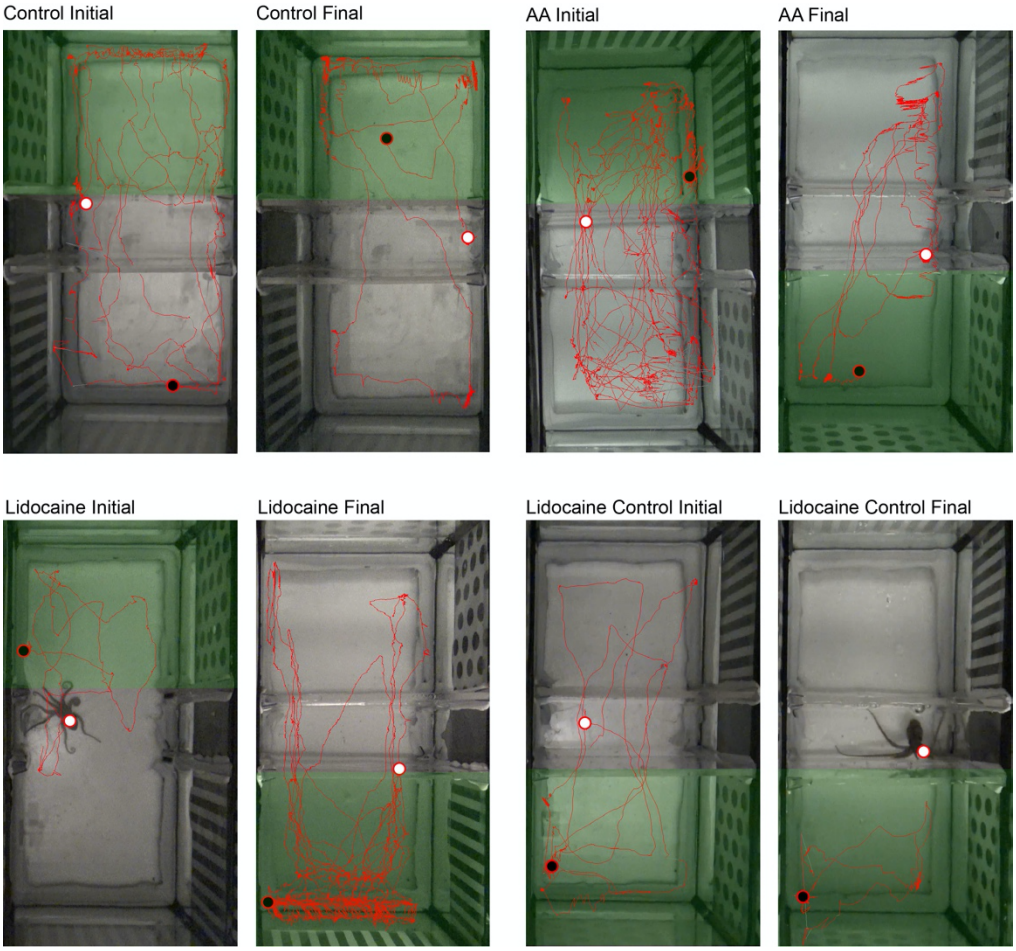
Fisher's exact tests. At the conclusion of training trials, arms were inspected for evidence of skin stripping behavior.

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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