



Associative learning in the cnidarian *Nematostella vectensis*

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The ability to learn and form memories allows animals to adapt their behavior based on previous experiences. Associative learning, the process through which organisms learn about the relationship between two distinct events, has been extensively studied in various animal taxa. However, the existence of associative learning, prior to the emergence of centralized nervous systems in bilaterian animals, remains unclear. Cnidarians such as sea anemones or jellyfish possess a nerve net, which lacks centralization. As the sister group to bilaterians, they are particularly well suited for studying the evolution of nervous system functions. Here, we probe the capacity of the starlet sea anemone *Nematostella vectensis* to form associative memories by using a classical conditioning approach. We developed a protocol combining light as the conditioned stimulus with an electric shock as the aversive unconditioned stimulus. After repetitive training, animals exhibited a conditioned response to light alone—indicating that they learned the association. In contrast, all control conditions did not form associative memories. Besides shedding light on an aspect of cnidarian behavior, these results root associative learning before the emergence of NS centralization in the metazoan lineage and raise fundamental questions about the origin and evolution of cognition in brainless animals.

nervous system evolution | learning | cnidaria | associative learning | cognition

Learning and memory provide adaptive capacities allowing animals to adjust their behavior rapidly in a changing environment, based on previous experiences (1–4). Habituation and sensitization result in the modulation of the behavior response strength upon repeated presentation of a single stimulus. In contrast, during associative learning, organisms form a predictive association between different event occurrences. Classical (or Pavlovian) conditioning relies on the pairing of a biologically significant outcome, the unconditioned stimulus (US), usually appetitive or aversive, with an initially neutral event, the conditioned stimulus (CS). While the ability of unicellular organisms to form associative memories remains debated (5, 6), it is generally assumed that the evolution of nervous systems (NS) provides the ground for memory formation, based on the modulation of synaptic strength and plasticity (7). However, while associative learning has been studied in numerous bilaterian phyla, studies on nonbilaterian clades, such as sponges, ctenophores, placozoans, and cnidarians, remain sparse (8).

Cnidaria, the sister group of Bilateria, include corals, jellyfish, and sea anemones. They possess neurons, which are organized in a diffuse nerve net. It is generally agreed that cnidarian and bilaterian nervous systems originate from a common nerve net-like ancestral NS (9). To date, only two early studies, which were never replicated, have shown partial evidence for classical conditioning in sea anemones (10, 11). Such scarcity could be the consequence of unpublished negative results or could simply reflect the focus of the scientific community on selected model organisms. Therefore, studying cnidarians can answer crucial questions about the evolutionary origin and function of associative learning.

Here, we performed Pavlovian aversive classical conditioning experiments, pairing light as the conditioned stimulus (CS) with an electric shock as the unconditioned stimulus (US) in the anthozoan model *Nematostella vectensis*. Indeed, despite the absence of eyes as dedicated structures to sense light, *Nematostella* rely on light input to modulate circadian rhythms and spawning behaviors (12). We predicted that if animals learned the association between both stimuli, they would exhibit a conditioned response (i.e., body retraction), in anticipation of the shock, when stimulated with light (CS alone) after conditioning.

Results

As aversive US, we delivered an electric shock (ES) of small amplitude (6 V) to animals without directly touching them, using a pair of needles connected to a power supply (details in *SI Appendix*). Positioned for a short time (≈ 1 s) around the oral end of the animal, the shock triggers a strong, fast, and reversible retraction of the oral region and tentacles (Fig. 1A). We initially recorded the response of the animals to the light pulse (CS) alone, prior to conditioning (pretest in Fig. 1B and D). Then, training consisted of a massed conditioning session

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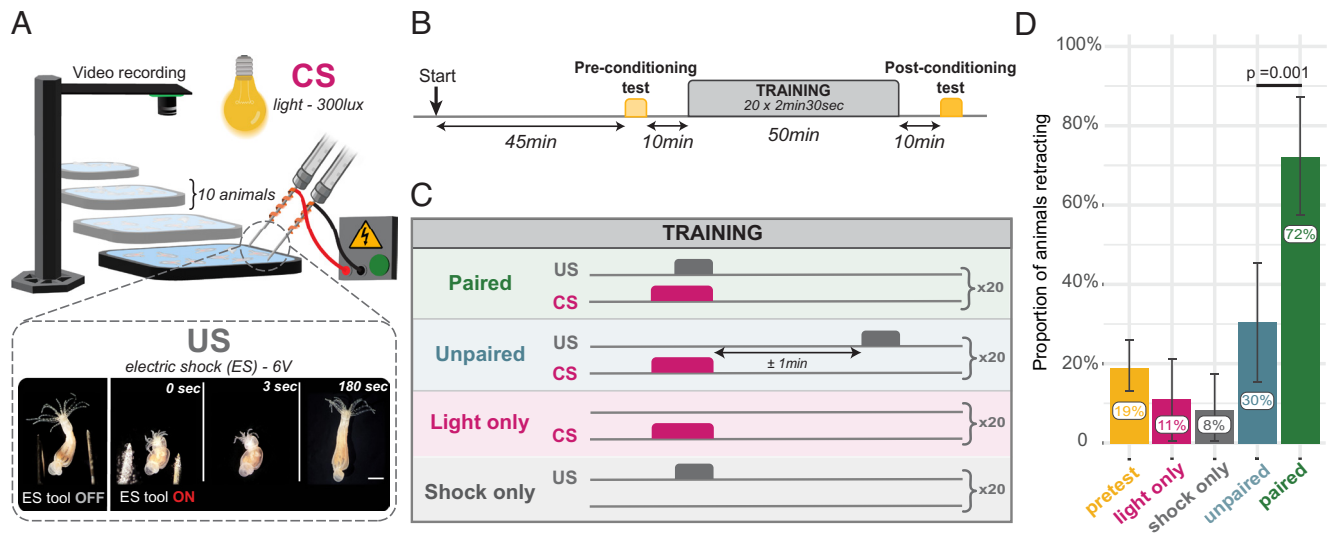


Fig. 1. (A) Experimental setup for aversive classical conditioning experiments. A white light (CS, measured illuminance \approx 300 lux) is paired with an electric shock (ES, voltage of \approx 6 V) as US. When the ES is applied, the animal retracts immediately (screenshots at 0 s and 3 s) and extends to its initial state in less than 3 min (screenshot at 180 s). (Scale bar: 2 mm.) (B) Timeline of conditioning experiment protocol. A 50-min training session consists of 20 rounds of training (2.5 min each). (C) Stimuli presentation during training for each condition: CS and US presented simultaneously (paired condition, green), CS and US explicitly unpaired (unpaired, blue), CS alone (light only, pink), and US alone (shock only, grey). (D) Manual scoring of animals retracting before (pretest, yellow) and after conditioning for each condition. Error bars: 95% CI. Chi square test, $\chi^2 = 10.897$, $P = 0.001$, $n_{\text{subj}} = 36$ animals/condition from $n = 2$ independent experiments.

of 50 min, where light (CS) was presented simultaneously with electric shock (US) for the paired condition or in a desynchronized fashion for the unpaired condition (Fig. 1C, details in *SI Appendix*). Then, 10 min after the end of the training protocol, reactions of the animals to the CS were assessed during a 4-min video recording, encompassing 1 min of CS application in the middle (Fig. 1B). Recordings were blindly analyzed, and the number of animals retracting was manually scored (Fig. 1D). The percentage of animals retracting was more than twofold higher in the paired group (72%) compared to the unpaired group (unpaired = 30%, $\chi^2 = 10.897$, $P = 0.001$) and lower in the other control groups (pretest = 19%, light only = 11% and shock only = 8%).

To quantitatively assess the behavioral changes elicited by the conditioned response (CR) and to monitor dynamic variations, we established an automated analysis workflow using the pose-estimation software DeepLabCut (13). We tracked three points along the body axis of 10 animals recorded simultaneously in each experimental group (Fig. 2A and *SI Appendix*). Then, we calculated the variation of the body length of each animal during the testing phase: prior, during, and after CS application. Our observations revealed that animals subjected to paired conditioning retracted during the CS application, in contrast to animals from the unpaired group (Fig. 2B). Next, we scored significantly more animals retracting during CS

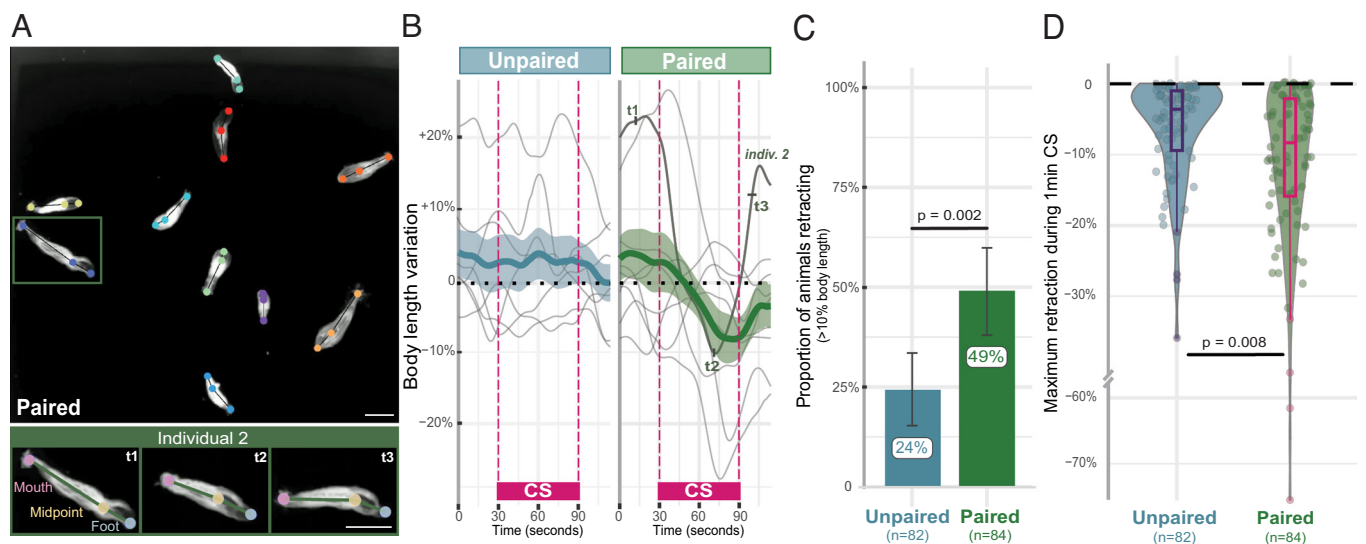


Fig. 2. (A) Screenshot of a video after conditioning, showing three points per individual. Body length as sum of the mouth–midpoint and midpoint–foot segments' lengths (see *SI Appendix* for details). The lower panels show screenshots of individual 2 before (t1), during (t2), and after (t3) CS application. (Scale bars: 5 mm.) (B) Plots of body length variation during video recordings after conditioning. Curves from individual animals (thin grey lines), mean (thick line), and SE of the mean (shaded area around the mean) for the paired and unpaired conditions. The right panel (paired condition) corresponds to the video depicted in (A), and the body length variation of the individual 2 is highlighted [thin dark green line, time points corresponding to the screenshots in the lower panel in (A)]. (C) Proportion of animals retracting more than 10% of their initial body length during CS application. Error bars: 95% CI. Chi-square test, $\chi^2 = 9.6204$, $P = 0.002$, animals from $n = 5$ independent experiments. (D) Maximum retraction measured during CS application in unpaired (blue) and paired (green) conditions. Mann-Whitney U test, $W = 2,620$, $P = 0.008$, $n = 5$ independent experiments.

application (Fig. 2C) in the paired group (retraction_{paired} = 49%) compared to the unpaired group (retraction_{unpaired} = 24%, $\chi^2 = 9.6204$, $P = 0.002$). Finally, we measured the maximum retraction recorded during CS application in all animals of both groups, showing that animals in the paired group were also quantitatively retracting more than unpaired control animals (Fig. 2D, median_{unpaired} = -3.59%, median_{paired} = -8.30%, $W = 2,620$, $P = 0.008$).

Discussion

Taken together, our results suggest that sea anemones can form associative memories in a classical conditioning paradigm, a phenomenon unreported—to our knowledge—in a nonbilaterian species since the end of the 20th century (8). Animals subjected to classical conditioning, receiving simultaneously light (CS) and an electric shock (US) during training, adapted their behavior and reacted to the CS alone after conditioning. The difference in the percentage of animals retracting between the manual scoring (72%, Fig. 1D) and the automated analysis (49%, Fig. 2C) is likely due to the threshold fixed at 10% of body length reduction in the automated analysis. Indeed, the latter only accounts for clear longitudinal contractions, whilst some subtle retractions visible to the trained eye may not be distinguishable by analyzing only the body length variation. Overall, these animals displayed a quantitatively and qualitatively different behavioral response compared to control animals, that received unpaired stimuli (Figs. 1D and 2 C and D). Moreover, the light-only and shock-only controls allow us to rule out a potential effect of sensitization or pseudoconditioning induced by the repeated presentation of the CS or US alone, respectively (14). Our observations in all control conditions (Figs. 1D and 2 C and D) suggest that the potential effect of repeated CS or US stimulation is rather limited. It therefore does not explain the difference in behavior observed in the paired condition and strongly argues for associative learning.

In most model organisms, defined neural circuits and molecular mechanisms responsible for specific forms of memories have been identified. They appear to be remarkably conserved within bilaterians (7), raising the question of whether they are also present in cnidarians. In particular, the presence and potential role of classic neurotransmitters or neuromodulators, such as serotonin and dopamine, is still debated (15). Likewise, the role of cyclic adenosine monophosphate and its downstream effectors such as Protein kinase A (PKA) or cAMP response element-binding protein (CREB) remains a crucial point to be elucidated. Conversely, it is also possible that associative learning evolved independently in cnidarians and relies on different mechanisms (16).

Finally, learning in cnidarians could be seen as a compelling example of “embodied cognition,” i.e., cognition without a central brain (1, 3, 4), and raises fascinating questions on the processing, storage, and recall of learned associations in these animals.

Materials and Methods

Unsorted juvenile male and female animals were kept at 19 °C, and a 12:12 light cycle was followed. For learning experiments, 10 or 18 animals per group (for automated or manually analyzed experiments, respectively) were transferred in the morning into square petri dishes in a room kept at 19 °C. Four groups of animals/dishes were conditioned and tested simultaneously for each experiment: either 1 group/condition, i.e., paired, unpaired, light only and shock only groups (Fig. 1C), either 2 groups of paired and 2 groups of unpaired (Fig. 2). Animals' body parts were tracked using DeepLabCut 2.2.0.2 (13), and the data were analyzed in R (17).

Data, Materials, and Software Availability. All study materials, detailed protocols, and methods are described in *SI Appendix*. Data and code have been deposited in a Zenodo repository (<https://doi.org/10.5281/zenodo.7568367>) (18).

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