Effects of aversive stimuli beyond defensive neural circuits: reduced excitability in an identified neuron critical for feeding in Aplysia

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SUPPLEMENTAL MATERIAL

Material and Methods

General Methods

Adult Aplysia californica (100 – 200 g; Marinus Scientific, CA) were individually housed in two aquaria (Acquatic Enterprises Inc., WA) of continuously circulating 15 °C aquarium seawater (Instant Ocean) on a 12-h light/dark cycle. Animals were fed ~1 g of dried seaweed (Emerald Cove Organic Pacific Nori, NC) three times a week, but were food deprived for 48 h prior to behavioral testing and remained so throughout the remainder of the experiment (Acheampong et al. 2012). A period of acclimation of at least three days upon arrival was allowed before animals were utilized, to minimize the effects of stress caused by shipping and handling (Levenson et al. 1999). In each of the three experiments, treatment and control procedures were performed in parallel (see below). Each experiment was conducted by different experimenters in different periods (first experiment: January 2009 – February 2010; second experiment: March 2010 – December 2011; third experiment: February 2010 – November 2010).

Procedures for behavioral testing

The tail-elicited siphon withdrawal reflex (TSWR) and feeding were measured prior to and after treatment (LTS training/5-HT application), using previously-
described methodologies (Cleary et al. 1998; Acheampong et al. 2012). Briefly, the duration of the siphon withdrawal, from onset of contraction to onset of relaxation, was used as a measure of TSWR strength (Cleary et al. 1998). The TSWR was elicited using 20-ms AC electric stimulation delivered through a pair of Teflon-coated silver-wire electrodes implanted in one randomly-chosen side of the tail. Five test stimuli were delivered at 10-min intervals before (pre-test) and at different time points after treatment (post-tests). The five responses were averaged and the change in TSWR duration (i.e., \([\text{post-test TSWR duration}]/[\text{pre-test TSWR duration}]\)) was calculated to assess modifications in the reflex strength due to treatment (Cleary et al. 1998; Acheampong et al. 2012).

Feeding was assessed by counting the number of bites (Kupfermann 1974) generated during a 5-min exposure to a seaweed extract, which provides a tonic chemical stimulus that reliably elicits bites (biting-test; Brembs et al. 2002; Acheampong et al. 2012). Biting tests were conducted 30 min after the conclusion of the TSWR pre-test and post-tests, respectively, in a glass pedestal bowl containing 1500 mL of 15 °C seaweed extract solution (Acheampong et al. 2012). The 30-min interval between TSWR and biting tests is sufficient to exclude any effect of TSWR elicitation on the subsequent expression of feeding (biting test preceded by TSWR test: 18.23 ± 1.76 bites, n = 30; biting test alone: 21.50 ± 2.52 bites, n = 14; \(P = 0.33; U = 171\)). The difference in bites (i.e., bites during post-test minus bites during pre-test) was used to analyze modifications in feeding due to treatment (Lorenzetti et al. 2006; Acheampong et al. 2012).

Animals were excluded from the study if they had a pre-test TSWR duration greater than 10 s, if they generated less than five bites in the pre-test, or if they inked
and/or secreted opaline before training, as these conditions may indicate that they were already sensitized or unhealthy (Acheampong et al. 2012). Animals were also disqualified if they laid eggs at any time during the experiment (Goldsmith and Byrne 1993). Some animals lost one or both wire electrodes prior to one of the post-tests, preventing TSWR measurements at that time point(s). For these animals, the post-test protocol was followed, but only feeding and B51 properties were measured and included in the statistical analysis. In all of the experiments, the experimenter performing the behavioral tests was unaware of the treatment history of the animals.

LTS-training protocol

In the first and second experiments, a previously established one-day LTS-training protocol was used (Cleary et al. 1998; Khabour et al. 2004; Acheampong et al. 2012). Thirty min after the end of the biting pre-test, animals were randomly assigned to either the trained or untrained (control) group. Trained animals received four trains (10 s, 1Hz) of electric shocks (500 ms, 60 mA AC) repeated at 30-min intervals. Training stimuli were delivered via a hand-held probe to the lateral body wall ipsilateral to the implanted tail electrodes (Cleary et al. 1998). This protocol induces concurrent unilateral LTS of the TSWR and suppression of feeding that persist for at least 24 h (Acheampong et al. 2012). Untrained animals were handled identically, but did not receive any training. In the first experiment, behavioral post-tests and measurements of B51 properties were conducted 24 h after treatment. In the second experiment, behavioral measurements were performed 24 h (to confirm the occurrence of LTS and suppression of feeding) and 72 h after treatment (to examine the recovery of the TSWR and feeding). B51 properties were measured after the completion of the 72-h post-tests.
5-HT treatment

In the third experiment, animals were exposed to an *in vivo* 5-HT treatment that is known to induce LTS (Levenson et al. 2000; Lyons et al. 2006). Thirty min after the end of the biting pre-test, animals were placed, for 1.5 h, in plastic chambers containing either 2L of 500 µM 5-HT (Sigma) dissolved in seawater, or 2L of seawater alone (control; Levenson et al. 2000). Water temperature was maintained at 15 °C during the treatment, which was randomly chosen for each animal. Behavioral post-tests and measurements of B51 properties were conducted 24 h after treatment.

Measurement of membrane and synaptic properties of B51

Immediately after the end of the last post-test, animals were anaesthetized by injecting a volume of isotonic MgCl₂ equivalent to 50% of their body weight. The buccal ganglion and one of the two buccal nerves 2,3 (denoted n.2,3) were removed and pinned on the Sylgard-coated bottom of a Plexiglas recording chamber containing artificial seawater (ASW) with a high concentration of divalent cations. For nerve stimulation, bipolar electrodes were placed on n.2,3 and isolated from the bath with Vaseline (e.g., Mozzachiodi et al. 2008). Ganglia were desheathed on the rostral side to access the soma of neuron B51. After desheathing, the high divalent ASW was exchanged for normal ASW, and ganglia rested for 30 min before measurements were taken. The composition of normal and high-divalent ASW has been published previously (Lorenzetti et al., 2006). The soma of neuron B51 was identified based on its relative size and position and the occurrence of its characteristic intrinsically-generated plateau potential (Plummer and Kirk 1990; Brembs et al. 2002).
Standard two-electrode current-clamp technique was used for intracellular recordings. Fine-tipped glass microelectrodes (resistance 10-12 MΩ) were filled with 3 M potassium acetate. Five min after impalement, resting membrane potential, input resistance and plateau potential threshold were measured. Cells were included in the study only if they displayed a resting membrane potential of at least -45 mV. B51 was current clamped at -60 mV. Input resistance was determined by injecting a 5-s, 5 nA hyperpolarizing current. The threshold to elicit a plateau potential was defined as the minimum amount of depolarizing current necessary to elicit a burst of activity that outlasted the duration of the current pulse (Nargeot et al. 1999). Burst threshold was determined by delivering a series of 5-s depolarizing current pulses of incremental intensity, from 5 to 30 nA, spaced 10-s apart. Cells that did not plateau in the 5–30 nA range were assigned the maximal burst threshold of 30 nA.

In the first and third experiment, we also analyzed the synaptic input in B51 triggered by monotonic electrical stimulation of the peripheral buccal nerve n.2,3 (Nargeot et al. 1999; Mozzachiodi et al. 2008). Two seconds of n.2,3 stimulation (4 Hz, 0.5-ms pulses, 10 V) were used to evoke a complex postsynaptic potential (cPSP) in B51, which is primarily inhibitory (Fig. 1C1). The cPSP was evoked ten min after B51 membrane properties were taken. To quantify the magnitude of the cPSP, we measured both the peak amplitude and the area of the cPSP during the 2-s n.2,3 stimulation. The peak amplitude of the cPSP was defined as the point of maximal hyperpolarization within the 2 s of n.2,3 stimulation (Fig. 1C1). The area of the cPSP over the 2-s n.2,3 stimulation (Fig. 1C1) was analyzed with the ImageJ software (National Institute of Health). More animals were used for the behavioral assessments than for the cellular
analysis. This discrepancy was caused primarily by: 1) the inability to find B51 in every preparation, 2) the inability to collect every measurement from each cell.

**Statistical analysis**

For the behavioral measurements, the change in TSWR duration and the difference in bites were compared between treated and control animals at different time points (Wainwright et al. 2002; Acheampong et al. 2012), using the Mann-Whitney U test. At the cellular level, B51 properties were compared between treated and control groups, also using the Mann-Whitney U test (Lorenzetti et al. 2006). Statistical significance was set at $P < 0.05$. Data analyses were performed using the statistical package of SigmaPlot 11.0 (Jandel Scientific, San Rafael, CA).

**Supplementary References**


