Long-term sensitization training in *Aplysia* decreases the excitability of a decision-making neuron through a sodium-dependent mechanism

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SUPPLEMENTAL MATERIAL

Material and Methods

*General Methods*

Adult *Aplysia californica* (100 – 200 g; Marinus Scientific, CA; South Coast Bio-Marine, 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).

*Procedures for behavioral testing*

The tail-elicited siphon withdrawal reflex (TSWR) and feeding were measured prior to and 24 h after long-term sensitization (LTS) training, using previously-described methodologies (Cleary et al. 1998; Acheampong et al. 2012; Shields-Johnson et al. 2013; Suppl. Fig. 1A). Briefly, the duration of the siphon withdrawal 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. The intensities of the stimuli used in the trained (1.60 ± 0.12 mA, n = 33) and untrained groups of animals (1.35 ± 0.09 mA, n = 43) were not significantly different (P = 0.10; U = 551.0; Mann-Whitney U Test). Five test stimuli were delivered at 10-min intervals before (pre-test) and 24 h after treatment (post-tests). The five responses were averaged and the change in TSWR duration (i.e., [post-test TSWR duration]/[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 (biting test), which provides a tonic chemical stimulus that reliably elicits bites (Brembs et al. 2002; Acheampong et al. 2012). Feeding tests were conducted 30 min after the conclusion of the TSWR pre-test and post-tests, respectively (Suppl. Fig. 1A), in a glass pedestal bowl containing 1500 mL of 15 °C seaweed extract solution (Acheampong et al. 2012). 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 (n=5), if they generated less than five bites in the pre-test (n= 9), or if they inked and/or secreted opaline before training (n=3), as these conditions may indicate that they were already sensitized or unhealthy (Cleary et al. 1998; Acheampong et al. 2012). Animals were also excluded if they laid eggs at any time during the experiment (n=1; Goldsmith and Byrne 1993). Some animals lost one or both wire electrodes prior to the 24 h post-test, preventing TSWR measurements at that time.
point. For these animals \( n = 14 \), the post-test protocol was followed, but only feeding and B51 properties were measured and included in the statistical analysis.

**LTS-training protocol**

A previously established one-day LTS-training protocol was used (Cleary et al. 1998; Khabour et al. 2004; Acheampong et al. 2012). Thirty minutes 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 (Suppl. Fig. 1A). 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; Shields-Johnson et al. 2013). Untrained animals were handled identically, but did not receive any training.

**Measurement of B51 membrane properties**

Immediately after the end of the behavioral post-test, animals were anaesthetized by injecting a volume of isotonic MgCl\(_2\) equivalent to 50\% of their body weight. The buccal ganglion was removed and pinned on the Sylgard-coated bottom of a Plexiglas recording chamber containing 2 mL of artificial seawater (ASW) with a high concentration of divalent cations (composition in mM: NaCl 210, KCl 10, MgCl\(_2\) 145, MgSO\(_4\) 20, CaCl\(_2\) 33 and HEPES 10; pH adjusted to 7.5 with NaOH; Lorenzetti et al. 2006). Ganglia were desheathed on the rostral side to access the soma of neuron B51 (Suppl. Fig. 1B). The procedures for anesthesia, animal dissection and desheathing
lasted on average 30 min. After desheathing, the high divalent ASW was exchanged for normal ASW (composition in mM: NaCl 450, KCl 10, MgCl$_2$ 30, MgSO$_4$ 20, CaCl$_2$ 10, and HEPES 10; pH adjusted to 7.5 with NaOH; Lorenzetti et al., 2006), and ganglia rested for 30 min before measurements were taken. 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; Suppl. Fig. 1B).

Standard two-electrode current-clamp technique was used for intracellular recordings. Fine-tipped glass microelectrodes (resistance 10-12 MΩ) were filled 2 M cesium methanosulfate so that voltage responses were not contaminated by K$^+$ in the microelectrodes in B51 (e.g., McGinley and Westbrook, 2012; Madison and Nicoll, 1986; Dong et al. 2006; Malyshev and Balaban, 2014). Five minutes after impalement, resting membrane potential ($V_m$), input resistance ($R_m$) and burst threshold were measured in ASW (Suppl. Fig. 1B). Cells were included in the study only if they displayed a resting membrane potential of at least $-45$ mV (Shields-Johnson et al. 2013). B51 was current clamped at $-60$ mV. Input resistance was determined by injecting a 5-s, 5-nA hyperpolarizing current. The burst threshold was defined as the minimum amount of depolarizing current necessary to elicit a discharge of action potentials the outlasted the duration of the current pulse (i.e., plateau potential; Nargeot et al. 1999). The 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 (Shields-Johnson et al. 2013; Dickinson et al. 2015).
Although we have previously shown that LTS training decreases B51 excitability (Shields-Johnson et al. 2013), the importance of measuring B51 membrane properties in this study was to ensure that the above cellular correlate occurred in B51 from a cohort of animals that expressed sensitization following training. This approach was used to ensure correct interpretation of the subsequent results obtained in the presence of channel blockers (see below), particularly in case of negative results, which could not be predicted a priori.

**Approach to measuring voltage-gated ion channels in B51**

The goal of this study was to analyze which voltage-gated (VG) ionic channel(s) contributes to the LTS training-induced decrease in B51 excitability. Ideally, this analysis would be conducted using voltage clamp. However, effective voltage-clamp recordings rely on the ability to control and clamp the membrane voltage across the neuron’s neuritic tree (i.e., space clamp; e.g., Watanabe et al. 1998; Williams and Mitchell 2008; Poleg-Polsky and Diamond 2011). This issue becomes problematic in situ with molluscan neurons like B51 because the presence of fast-inactivating Na$^+$ currents (e.g., Gilly et al. 1997; Chen et al. 2012) together with the cell’s morphology, consisting of a large axon and several neurites emanating from both the cell body and the axon (e.g., Weiss et al. 1986; Plummer and Kirk 1990; Staras et al. 2002) may impede space clamp of certain regions of the axon (e.g., Scholz et al. 1988; Staras et al. 2002). Consequently, voltage-clamp recordings in molluscan neurons, have been performed primarily under two conditions: 1) focus on slow-inactivating/non-inactivating currents (e.g., Scholz et al. 1988; Hurwitz et al. 2008;) and 2) reduction of the neuron’s arborizations by severing axon/neurites in situ (e.g., Weiss et al. 1986;
Scholz et al. 1988; Staras et al. 2002; Nikitin et al. 2008), or by excision of the cell/clusters (e.g. Kaczmarek et al. 1979; Walsh and Byrne 1989; Baxter and Byrne 1989; Gilly et al. 1997; Chen et al. 2012). The latter condition was applied to improve the spatial control of the membrane potential and to reduce the generation of spikes from uncontrolled (i.e., unclamped) regions of the axon (e.g., Scholz et al. 1988; Staras et al. 2002).

As the first study to investigate the biophysical underpinnings of learning-induced changes in B51 excitability following *in vivo* sensitization training, neither above condition appeared applicable to our experimental design because: 1) the effects of learning needed to be investigated on both VG K\(^+\) and fast-inactivating Na\(^+\) channels, and 2) the learning-induced changes in channel activity needed to be examined in a cell-wide fashion in intact neurons, which must include channel contributions from both the cells body, the axon and the neurites. By limiting *a priori* the analysis to B51 cell body and selected branches, axotomy and/or severing of neurites may potentially prevent the capture of learning-induced contributions from the neurites to channel changes. Consequently, we decided to employ current clamp of intact B51 in two distinct pharmacological environments (see next paragraph) in lieu of voltage clamp.

*Preparation of pharmacological environments to isolate ion-channel components in B51*

Two-electrode current-clamp recordings were performed under pharmacological conditions in which Na\(^+\) or K\(^+\) conductances dominated B51 voltage responses to current injections, respectively (Dong et al., 2006). The following previously-used channels blockers were employed: tetrodotoxin (TTX) to block VG Na\(^+\) channels,
tetraethylammonium (TEA) and 4-aminopyridine (4-AP) to block delayed-rectifier VG and transient K+ channels, cobalt (Co2+) to block VG Ca2+-channels and Ca2+-dependent K+ channels (e.g., Shuster and Siegelbaum, 1987; Walsh and Byrne 1989; Trudeau et al. 1993; Sugita et al., 1997; Mitterdorfer and Bean, 2002; Buss et al. 2003; Pennec et al. 2004; Dong et al. 2006; Leung, 2012; Chen et al. 2012). Two pharmacological environments were designed: one to isolate VG Na+ channels, containing: TEA, 4-AP and Co2+ (Dong et al. 2006), and one to isolate VG K+ channels, containing: TTX and Co2+ (Dong et al. 2006).

For the pharmacological environments used to isolate the contribution of VG Na+ channels, TEA and 4-AP were prepared in ultra-purified water into a stock solution at concentrations of 40 mM for 4-AP and 250 mM for TEA, and were buffered with 25 mM HCl. Co2+ was prepared in ultra-purified water as a separate stock solution at a concentration of 30 mM, and was buffered with 35 mM HEPES. The solution in the recording chamber had a pH of 7.5, after the addition of 4-AP, TEA and Co2+ (see below).

For the pharmacological environments used to isolate the contribution of VG K+ channels, Co2+ was prepared in ultra-purified water as a stock solution at a concentration of 30 mM, and was buffered with 1 M HEPES. TTX was prepared in ultra-purified water as a stock solution at a concentration of 1 mM, added to a solution of 1M HEPES, and buffered with 1M NaOH. The solution in the recording chamber had a pH of 7.6, after the addition of TTX and Co2+.

*Measurement of the effects of LTS training on the contributions of Na+ and K+ channels in B51*
After B51 membrane properties were recorded in ASW, preparations were randomly assigned to one of the two pharmacological environments described above (Suppl. Fig. 1C1,C2). To isolate VG Na⁺ channels contributions in B51, 300 µL of ASW was taken out of the recording chamber. Then, a bolus of 100 µL of the Co²⁺ solution was applied the buccal ganglion. Thirty seconds later, a bolus of 200 µL of the TEA and 4-AP solution was also added. In this way, the final bath concentrations were: 25 mM TEA (Trudeau et al., 1993; Jacklet et al., 2006); 4 mM 4-AP (Trudeau et al., 1993) and 15 mM Co²⁺ (Jacklet and Tieman, 2004; Jacklet et al., 2006). The application of the channel blockers was completed in about 5 min. Ten min after the blockers were added to the recording chamber, Vm was recorded. Then B51 was current clamped at -60 mV and Rm and the burst threshold were measured in a manner identical to that used in ASW (Suppl. Fig. 1C1).

We discovered that in preparations from untrained animals B51 still fired a burst of action potentials in response to a depolarizing current, when the pharmacological environment isolating Na⁺ channels was applied, but the burst did not outlast the duration of the current impulse, thus no longer conforming to the definition of plateau potential (e.g., Nargeot et al. 1999). Because the plateau potential in B51 is still elicited in the presence of Co²⁺ (Plummer and Kirk 1990), it is the addition of K⁺-channel blockers TEA and 4-AP that likely prevented the occurrence of plateau potentials. Although the burst threshold could not be measured in this pharmacological environment, the firing threshold (i.e., the minimum amount of current necessary to elicit a discharge of action potentials) was used to assess the neuron’s excitability. In preparations from untrained animals, B51 firing threshold often coincided with its burst threshold in ASW. The lack of statistical significance in the comparison between burst
threshold and firing threshold (P = 0.13, Wilcoxon signed rank test) supported the above observation and strengthened the rationale of using the firing threshold as an assessment of neuronal excitability in an experimental condition (i.e., Na⁺ channels environment) in which plateau potentials were no longer expressed. In this pharmacological environment, B₅₁ firing thresholds appeared lower than the corresponding burst thresholds in both trained and untrained animals, presumably because the blockade of 4-AP and TEA sensitive K⁺ channels, which are activated at relatively low membrane potentials (i.e., -20 to -40 mV; Martínez-Padrón et al. 1992), shifted the balance between VG outward and inward currents in the depolarizing direction (Chen et al. 2012).

To isolate VG K⁺ channels in B₅₁, 300 µL of artificial seawater was taken out of the recording dish. Next, 100 µL of Co²⁺ solution was applied to the buccal ganglion. Thirty seconds later, 200 µL of TTX was added. In this way, the final bath concentrations were: 100 µM TTX (Trudeau et al., 1993; Jacklet et al., 2006) and 15 mM Co²⁺ (Jacklet and Tieman, 2004; Jacklet et al., 2006). The application of the channel blockers was completed in about 5 min. Ten min after the blockers were added to the recording chamber, Vm was recorded. Then, B₅₁ was current clamped at -60 mV and Rm was measured in a manner identical to that used in ASW. The pharmacological environments isolating K⁺ channels prevented the occurrence of action potentials. Pilot data showed that the employed bath concentrations of TTX and Co²⁺ were effective in isolating VG K⁺ channels as evident by the failure to elicit action potential in the large buccal motor neuron B₃ in the presence of the above blockers (data not shown). To assess the contribution of VG K⁺ channels to the LTS training-induced decreased excitability in B₅₁, we analyzed the peak amplitudes and areas of the K⁺-dominated
voltage responses to 5-s current injections of incremental intensities from 5 to 30 nA (Suppl. Fig. 1C2). Each peak amplitude was calculated as the point of maximal depolarization within the 5-s current injection (Suppl. Fig. 2). Each depolarization area was measured by integrating the depolarization over the 5-s current injection. Areas were calculated using the Image Tool software (University of Texas Health Science Center in San Antonio, TX; grey region underneath the depolarization trace in Suppl. Fig. 2). Current-voltage relationships were generated for peak amplitudes in trained and untrained preparations (Dong et al. 2006). Similarly, current-area relationships were generated for depolarization areas peak in trained and untrained preparations.

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 and 3) the application of the pharmacological blockers, which occasionally caused displacement of the cell and subsequent damage to B51 that precluded recording.

Statistical analysis

For the behavioral measurements, the change in TSWR duration and the difference in bites were compared between trained and untrained animals 24 h after training, using the Mann-Whitney U test (Acheampong et al. 2012; Shields-Johnson et al. 2013). At the cellular level, Vm, Rm, burst threshold and firing threshold were compared between trained and untrained groups, also using the Mann-Whitney U test (Lorenzetti et al. 2006; Mozzachiodi et al. 2008). Current-peak amplitude and current-area relationships were compared between trained and untrained groups using repeated measures ANOVA with two factors: training and current intensity (e.g., Roberts and
Russo 1999; Chin et al. 2002). 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**


Supplementary Figure 1. Protocol for behavioral testing and training, and subsequent measurement of B51 properties in two different pharmacological environments. TSWR and feeding were measured prior to and 24 h after the delivery of either the LTS training or untrained-control protocol (A). After the conclusion of post-tests, B51 membrane properties were measured in normal ASW (B). B51 neurons were then randomly selected to be examined in one of the following pharmacological environments: 1) isolated VG Na⁺ channels (C1) or 2) isolated VG K⁺ channels (C2).

Supplementary Figure 2. Sample trace of an individual depolarization evoked in the pharmacological environment in which VG K⁺ channels were isolated. Both the peak amplitude and the area of the depolarization (grey region) were measured.