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

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In Aplysia, long-term sensitization (LTS) occurs concurrently with a suppression of feeding. At the cellular level, the suppression of feeding is accompanied by decreased excitability of decision-making neuron BSI. We examined the contribution of voltage-gated Na\(^+\) and K\(^+\) channels to BSI decreased excitability. In a pharmacologically isolated Na\(^+\) channels environment, LTS training significantly increased BSI firing threshold, compared with untrained controls. Conversely, in a pharmacologically isolated K\(^+\) channels environment, no differences were observed between trained and untrained animals in either amplitude or area of BSI K\(^+\)-dependent depolarizations. These findings suggest that Na\(^+\) channels contribute to the decrease in BSI excitability induced by LTS training.

The mechanisms by which aversive stimuli modulate the activity of defensive neural circuits have been extensively studied in vertebrates and invertebrates (e.g., Frost et al. 1985; Kandel 2001; Tomchik and David 2009; Ardiel and Rankin 2010; Johansen et al., 2011; LeDoux 2012; Byrne and Hawkins 2015). Conversely, the effects of aversive stimuli on nondefensive neural circuits have been only marginally explored. In Aplysia, aversive electrical stimuli, which mimic a predator attack (Watkins et al. 2010; Mason et al. 2014), induce concurrent sensitization of withdrawal reflexes (e.g., Frost et al. 1985; Cleary et al. 1995; Kandel 2001; Byrne and Hawkins 2015) and suppression of feeding (Acheampong et al. 2012; Shields-Johnson et al. 2013). Despite the extensive knowledge of the mechanisms of sensitization (e.g., Scholz and Byrne 1987; Cleary et al. 1999; Wainwright et al. 2002; Herdegen et al. 2014), only recently have we begun to investigate the cellular underpinnings responsible for the suppression of feeding (Shields-Johnson et al. 2013).

In Aplysia, a 1-day training protocol, which induces long-term sensitization (LTS) of the tail-elicted siphon withdrawal reflex (TSWR), also suppresses feeding for at least 24 h (Acheampong et al. 2012; Shields-Johnson et al. 2013). A cellular correlate of feeding suppression is the decreased excitability of a putative decision-making neuron within the feeding central pattern generator (CPG): neuron BSI (Shields-Johnson et al. 2013). BSI generates an all-or-nothing intrinsic burst of action potentials (i.e., plateau potential) (Plummer and Kirk 1990) that contributes to the selection of ingestive buccal motor programs (BMPs), which are in vitro neurophysiological correlates of biting (Nargeot et al. 1999a,b; Brems et al. 2002; Nargeot and Simmers 2012). Modifications of BSI excitability have been previously associated with changes in feeding induced by nonassociative (Shields-Johnson et al. 2013) and associative forms of learning (Brems et al. 2002; Lorenzetti et al. 2006) as well as by food satiation (Dickinson et al. 2015).

The decrease of BSI excitability, measured 24 h after LTS training, manifests as an increase in the amount of current necessary to drive BSI into producing its plateau potential (i.e., burst threshold) (Nargeot et al. 1999a; Shields-Johnson et al. 2013). Resting membrane potential (V_m) and input resistance (R_in) are not affected by LTS training (Shields-Johnson et al. 2013). Previous work revealed that BSI plateau potential does not depend on external Ca\(^{2+}\) (Plummer and Kirk 1990), suggesting that voltage-gated (VG) Na\(^+\) and K\(^+\) currents are involved in its genesis and maintenance. Therefore, the goal of this study was to identify biophysical changes underlying BSI decreased excitability following LTS training.

To assess learning-induced behavioral modifications, the TSWR and feeding were measured prior to (pre-test) and 24 h after (post-test) treatment (LTS training/no training) (Acheampong et al. 2012; Shields-Johnson et al. 2013; Supplemental Fig. 1A; see also Supplemental Material for details about testing and training procedures). The duration of the siphon withdrawal was used as a measure of the TSWR strength (Scholz and Byrne 1987; Cleary et al. 1998). The number of bites (i.e., rhythmic movements of the radula) (Kupfermann 1974; Susswein et al. 1976), counted during a 5-min test, was used to assess feeding (Brembs et al. 2002; Acheampong et al. 2012). The experimenter performing the behavioral tests was unaware of the treatment history of the animals. Training induced concurrent LTS (change in TSWR duration, trained: 1.7 ± 0.1, n = 30; untrained: 1.2 ± 0.2, n = 33; P < 0.05; U = 188.5; Mann–Whitney U-test; Fig. 1A) and suppression of feeding (difference in bites; trained: −7.9 ± 1.1, n = 34; untrained: 1.1 ± 1.2, n = 43; P < 0.05; U = 263.5; Mann–Whitney U-test; Fig. 1B).

Once behavioral post-tests were completed, the buccal ganglia were removed and BSI properties were measured in artificial seawater (ASW) (Supplemental Fig. 1B; see also Supplemental Material).
Sodium-dependent plasticity of B51 excitability

Figure 1. Training induced LTS of the TSWR, suppressed feeding and decreased the excitability of neuron B51. Training produced concomitant LTS (A) and suppression of feeding (B) 24 h after training. (C) LTS Training also increased B51 burst threshold, resulting in decreased excitability 24 h after training. Sample traces of B51 from trained (C1) and untrained animals (C2) illustrate qualitative differences in burst threshold. (C3) Summary data illustrate that LTS training significantly increased B51 burst threshold. In these and the following figures, values are expressed as mean ± SEM and statistical significance is set at P < 0.05.

Figure 2. LTS training selectively modified the Na\(^+\)-dependent firing threshold of neuron B51. Sample traces of B51 from trained (A1) and untrained animals (A2) illustrate qualitative differences in firing thresholds in the pharmacological environment in which VG Na\(^+\) channels were isolated. The intensity of the injected current in the isolated VG Na\(^+\) channels environment indicates the threshold at which B51 fired action potential (i.e., firing threshold; A1,A2). (B) Summary data illustrate that LTS training significantly increased B51 firing threshold.
scenario has been reported in B31/32 where delayed rectifier and transient A K+ channels participate to the sustained depolarization underlying the plateau potential (Hurwitz et al. 2008). In light of this finding, the firing threshold (i.e., the minimum amount of current necessary to elicit action potentials) was used in lieu of the burst threshold to assess B51 excitability. Mirroring the burst-threshold results obtained in ASW (Fig. 1), trained animals exhibited a firing threshold (13.4 ± 1.1 nA, n = 10; Fig. 2A1,B) greater than that of untrained animals (7.6 ± 0.8 nA, n = 7; P < 0.05; U = 4.5; Mann–Whitney U-test; Fig. 2A2,B). This finding indicates that the training-induced decrease of B51 excitability observed in ASW persisted in the absence of contributions of K+ or Ca2+ channels, suggesting that long-term modifications altered the properties (activation and/or conductance) of VG Na+ channels.

To isolate VG K+ channels, 100 μM tetrodotoxin (TTX) (Trudeau et al. 1993; Dong et al. 2006; Chen et al. 2012) was used to block VG Na+ channels, along with 15 mM Co2+. Ten minutes after channel blockers application, Vm of trained (−50.1 ± 1.9 mV, n = 9) and untrained animals (−52.9 ± 1.8 mV, n = 10) were not significantly different (P = 0.32; U = 32.5; Mann–Whitney U-test). B51 was then clamped at −60 mV for the remaining measurements. As for Va, Vs were not significantly different between trained (3.9 ± 0.4 MΩ, n = 7) and untrained animals (3.6 ± 0.5 MΩ, n = 10; P = 0.31; U = 24.0; Mann–Whitney U-test). In this pharmacological environment, action potentials were no longer evoked in B51. Consequently, together with Va and Vs, we measured the amplitude and area of K+-dependent depolarizations, evoked by 5-sec depolarizing pulses of incremental intensities from 5 to 30 nA, corresponding to the range in which B51 plateau potentials are generated in ASW (Shields-Johnson et al. 2013; Dickinson et al. 2015). For each depolarization, the peak amplitude and the area were measured (Supplemental Fig. 2; Shields-Johnson et al. 2013). The peak amplitudes of the depolarizations evoked in the 5–30 nA range were not significantly different between trained (n = 8) and untrained (n = 9) animals (F1,17 = 0.17; P = 0.69; repeated-measures ANOVA on the factor “training”; Fig. 3A). Similarly, the areas of the depolarizations were not significantly different between trained (n = 8) and untrained (n = 9) animals (F1,17 = 0.02; P = 0.89; repeated-measures ANOVA on the factor “training”; Fig. 3B).

Overall, the above results indicate that the decrease of B51 excitability is due, at least in part, to a learning-induced modulation of VG Na+ channels. Conversely, no differences were observed between trained and untrained animals in either amplitude or area of B51 depolarizations measured in a pharmacologically isolated VG K+ channels environment, suggesting that VG K+ channels are not a target of LTS training.

The pharmacological environment utilized to isolate the contribution of VG Na+ channels and the current-clamp technique used to record the neuron’s voltage responses cannot help fully describe the biophysical properties of the Na+ currents involved in the decrease of excitability. However, our findings indicate that B51 action potentials are sustained by fast-inactivating Na+ channels that are sensitive to TTX (Supplemental Fig. 2), confirming voltage-clamp data previously obtained from B51 in culture (Chen et al. 2012). A possible TTX-insensitive persistent Na+ current might contribute to the depolarization supposedly carried primarily by K+ ions. Nevertheless, we did not observe any change in the curves of peak amplitude and area of the depolarizations, indicating that LTS training did not alter them, regardless of their composition: either K+ channels, or a combination of K+ channels and TTX-resistant Na+ channels. The possibility that the lack of learning-induced changes in depolarization might be due to opposite changes in K+ currents and TTX-resistant Na+ currents cancelling each other is unlikely. In addition, learning-induced changes of persistent Na+ currents have been associated with depolarizations of the resting membrane potential (Kemenes et al. 2006), a correlate that was not observed in B51 following LTS. Therefore, our results provide one of the first pieces of evidence describing learning-induced modifications in the function of VG Na+ channels in a behaviorally relevant decision-making neuron.

Research in the past decades has shown that modifications of excitability can contribute to the memory trace (e.g., Brors and Woody 1980; Crow and Alkon 1980; Nargeot et al. 1993a,b; Schall 2003; Zhang and Linden 2003; Mozczachiodi and Byrne 2010). Furthermore, work conducted on the mammalian hippocampus suggests that knockout of voltage-gated ion channels (e.g., HCN1) results in a robust change in behavioral output that promotes anxiolytic- and antidepressant-like behaviors (Lewis et al. 2011; Kim et al. 2012). Investigations on the biophysical substrates of behavior and memory have focused primarily on modifications of K+, Ca2+, or Ca2+-activated K+ channels in both vertebrates (e.g., Remage-Healey et al. 2011; Lamberton et al. 2012; Mirkovic et al. 2012; Truchet et al. 2012; Lovell et al. 2013) and invertebrates (e.g., Alkon et al. 1985; Scholz and Byrne 1987; Farley 1988; for review, see Brunelli et al. 1997; Mozczachiodi and Byrne 2010). However, Na+ channels have also become prime candidates for a role in mediating neuronal plasticity because of their key contribution in determining the threshold for action potential generation (for review, see Cantrell and Catterall 2001).

A Na+ -dependent reduction in the ability of B51 to fire might occur, for example, through altering the functional kinetics of VG Na+ channels (Srinivasan et al. 1998; for review, see Yu and
Catterall 2003). α-Subunits of B51 VG Na⁺ channels are a likely target of modulation by L Ts training. The positively charged lysine and arginine residues on the S4 segment of the α subunit are key regulators of voltage sensing (Yu and Catterall 2003) and, subsequently, of activation kinetics in many vertebrate and invertebrate species, including Aplysia (Vassilev et al. 1988; Catterall 1995, 2014; Dyer et al. 1997; Ruiz and Kraus 2015). Mutation of arginine residues in the S4 segment reduces the steepness of Na⁺ voltage-dependent gating (Stühmer et al. 1989; Kontis et al. 1997), with a subsequent greater depolarization required to activate VG Na⁺ channels. When arginines are replaced by cysteines in the S4 segment of VG Na⁺ channels, segment movement needed to achieve full activation and influx of Na⁺ during depolarization is suppressed (Yang et al. 1996). Therefore, learning-induced modulation of positively charged residues of the S4 segment of VG Na⁺ channels might underlie B51 decreased excitability after training.

Learning-dependent modulation of Na⁺ channels has been previously reported in the pond snail Lymnaea stagnalis. Changes in currents at the level of the tonically firing cerebral giant cells (CGCs), which play a permissive role in feeding (Staub and Benjamin 2001), altered the activity of command-like interneurons in the feeding neural circuit (Kemenes et al. 2006). Specifically, one-trial chemical classical conditioning induces a depolarization of CGCs via a persistent Na⁺ current that appears involved in the maintenance of the long-term memory (Kemenes et al. 2006; Nikitin et al. 2008).

The diversity in the type of Na⁺ channel modulated by learning paradigms in CGCs and B51 is consistent with the different behaviors of these two neurons and the induced cellular correlates. In CGC, a change in persistent Na⁺ channels leads to modifications of the membrane potential (an increase, in the case of appetitive classical conditioning) that modulate its output to CPG neurons, without altering other CCG properties, including excitability and firing frequency (Kemenes et al. 2006). In B51, a change in VG Na⁺ channels leads to modification of the firing threshold (an increase in the case of sensitization training) that represents an effective mechanism to control the neuron’s excitability and, subsequently, the all-or-nothing expression of the plateau potential.

Further experimentation is required to investigate the mechanisms underlying the above modulation of VG Na⁺ channels induced by sensitization training and its functional relevance in the feeding suppression induced by sensitization training. For example, the biochemical pathway responsible for feeding suppression and the Na⁺-dependent B51 decreased excitability is yet unknown. Despite its well-established modulatory role in sensitization in Aplysia (Byrne and Hawkins 2015), serotonin does not alter feeding or B51 excitability (Shields-Johnson et al. 2013). Dopamine leads to an increase, rather than a decrease of B51 excitability through a cAMP-dependent mechanism when it is released contingently with B51 activity during operant reward learning in Aplysia (Brembs et al. 2002; Lorenzetti et al. 2008). A putative modulator responsible for the Na⁺-dependent decrease of B51 excitability is nitric oxide, which mediates nociceptive sensitization in Aplysia (Lewin and Walters 1999) and plasticity of VG Na⁺ channels in vertebrate neurons (for review, see Ahern et al. 2015).

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