A novel in vitro analog expressing learning-induced cellular correlates in distinct neural circuits

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When presented with noxious stimuli, *Aplysia* exhibits concurrent sensitization of defensive responses, such as the tail-induced siphon withdrawal reflex (TSWR) and suppression of feeding. At the cellular level, sensitization of the TSWR is accompanied by an increase in the excitability of the tail sensory neurons (TSNs) that elicit the reflex, whereas feeding suppression is accompanied by decreased excitability of B51, a decision-making neuron in the feeding neural circuit. The goal of this study was to develop an in vitro analog coexpressing the above cellular correlates. We used a reduced preparation consisting of buccal, cerebral, and pleural-pedal ganglia, which contain the neural circuits controlling feeding and the TSWR, respectively. Sensitizing stimuli were delivered in vitro by electrical stimulation of afferent nerves. When trained with sensitizing stimuli, the in vitro analog expressed concomitant increased excitability in TSNs and decreased excitability in B51, which are consistent with the occurrence of sensitization and feeding suppression induced by in vivo training. This in vitro analog expressed both short-term (15 min) and long-term (24 h) excitability changes in TSNs and B51, depending on the amount of training administered. Finally, in vitro application of serotonin increased TSN excitability without altering B51 excitability, mirroring the in vivo application of the monoamine that induces sensitization, but not feeding suppression.

In the past decades, neuroscientists have greatly benefited from the development of in vitro reduced preparations, obtained from isolated portions of the central and peripheral nervous system of vertebrates and invertebrates, and capable of expressing features of brain functions exhibited in vivo (Kovac and Davis 1977; Lukowiak and Sahley 1981; Hawkins et al. 1983; Byrne 1987; Zhang et al. 1994; Nargeot et al. 1997; Proekt et al. 2004; Marder et al. 2005; Mentel et al. 2008; Keifer and Zheng 2010; Mozzachiodi and Byrne 2010; Crossley et al. 2016). These preparations, commonly termed "in vitro analogs" have been particularly useful to investigate the cellular and molecular mechanisms of learning and memory in identified neural circuits (Hawkins et al. 1983; Walters and Byrne 1983; Kemenes et al. 1997; Nargeot et al. 1997; Burrell et al. 2001; Scuri et al. 2002; Keifer and Zheng 2010).

Several in vitro analogs have been developed using the nervous system of the marine mollusk *Aplysia* to analyze the mechanisms of various forms of learning, including habituation (Frost et al. 1997) and sensitization (Zhang et al. 1994; Antonov et al. 1999; Marinesco and Carew 2002; Philips et al. 2011), both aversive and appetitive forms of classical conditioning (Walters and Byrne 1983; Antonov et al. 2001; Mozzachiodi et al. 2003; Lorenzetti et al. 2006) and appetitive operant conditioning (Nargeot et al. 1999a,b; Mozzachiodi et al. 2008; Sieling et al. 2014). The use of these in vitro analogs has been crucial for characterizing learning-dependent mechanisms because it allows one to conduct experiments that are not otherwise feasible in vivo, such as manipulation of the mechanisms responsible for cellular changes at the level of both neural circuits and single cells (Nargeot et al. 1999b; Antonov et al. 2003, 2007; Mozzachiodi et al. 2008, 2013; Sieling et al. 2014). Up to now, however, one limitation presented by in vitro analogs of learning is that their use has been primarily restricted to the study of individual circuits, examined one at a time (Walters and Byrne 1983; Zhang et al. 1994; Nargeot et al. 1997; Mozzachiodi et al. 2003; Philips et al. 2011). The goal of this study was to expand upon previously established in vitro analogs and develop one capable of coexpressing learning-dependent changes in distinct neural circuits and in different temporal domains.

Recent work from our laboratory revealed that, *in Aplysia*, noxious electrical stimuli produce two concurrent behavioral changes: a well-characterized enhancement of defensive withdrawal reflexes, known as sensitization (for review, see Kandel 2001; Byrne and Hawkins 2015), and suppression of feeding behavior (Acheampong et al. 2012; Shields-Johnson et al. 2013). The above behavioral changes share analogous time courses that depend on the amount of aversive stimulation (Acheampong et al. 2012; Shields-Johnson et al. 2013). Training consisting of a single-trial produces sensitization of the tail-induced siphon withdrawal reflex (TSWR) and suppression of feeding at 15 min, but not 24 h after training (Acheampong et al. 2012). Training consisting of four trials induces sensitization and feeding suppression lasting at least 24 h (Acheampong et al. 2012; Shields-Johnson et al. 2013).

Cellular correlates of sensitization and feeding suppression have been previously identified in the corresponding underlying neural circuits. Within the neural circuit controlling the TSWR, plasticity associated with sensitization has been identified at the level of the sensory neurons in the ventrocaudal (VC) cluster of the pleural ganglion that respond to tail stimulation (Walters et al. 1983a,b, 2004). These tail sensory neurons (TSNs) make monosynaptic contacts onto motor neurons in the pedal ganglion, generating contraction of the tail, and polysynaptic contacts onto motor neurons in the abdominal ganglion, generating contraction

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of the siphon (Cleary et al. 1995). Training with sensitizing stimuli increases the excitability of the TSNs both in the short and long term (Zhang et al. 1994; Cleary et al. 1998; Liao et al. 1999; Marinesco and Carew 2002). In *Aplysia*, serotonin (5-HT) mediates sensitization and its cellular correlates at the level of the sensory-to-motor neuron synapses and can serve as analog of sensitization training (Walters et al. 1983b; Byrne and Kandel 1996; Byrne and Hawkins 2015). In particular, 5-HT treatment increases TSN excitability in an intrinsic manner, as demonstrated by results obtained both in neurons from surgically excised sensory clusters (Baxter and Byrne 1990) and in neurons isolated in culture (Dale et al. 1987; Liu et al. 2011).

Within the neural circuit controlling feeding, sensitization training decreases the excitability of neuron B51 (Shields-Johnson et al. 2013; Hernandez et al. 2017). B51 is a premotor neuron that contributes to the genesis of ingestive buccal motor programs, which are in vitro neurophysiological correlates of a bite/swallow (Plummer and Kirk 1990; Nargeot et al. 1999a,b; Cropper et al. 2004; Sasaki et al. 2013). B51 expresses an intrinsically generated all-or-nothing burst of action potentials (i.e., plateau potential; Plummer and Kirk 1990) that participate in the selection of ingestive buccal motor programs (Nargeot et al. 1999a,b; Brembs et al. 2002; Nargeot and Simmers 2012). Notably, B51 exhibits in vitro decision-making features that enable it to switch in an all-or-nothing fashion from an inactive subthreshold state to an active plateau potential during the occurrence of ingestive buccal motor programs (Nargeot and Simmers 2012; Mozzachiodi et al. 2013; Dickinson et al. 2015). The decrease of B51 excitability induced by in vivo sensitization training is not accompanied by changes in input resistance, resting membrane potential, or synaptic input (Shields-Johnson et al. 2013) and persists when synaptic transmission is pharmacologically blocked (Hernandez et al. 2017).

The above findings provided the framework for the development of an in vitro analog coexpressing excitability changes in TSNs and B51 similar to those induced by in vivo sensitization training. We implemented a previously established in vitro analog that expresses cellular correlates of sensitization in the isolated pleural-pedal ganglia, including increased TSN excitability and facilitation of the sensorimotor neuron synapses (Zhang et al. 1994; Liao et al. 1999; Marinesco and Carew 2002). As an in vitro representation of sensitizing stimuli, we used patterns of simultaneous electrical stimulation of afferent pedal nerves P8 and P9 because they induce cellular correlates of sensitization in the isolated pleural-pedal ganglia (Goldsmith and Byrne 1989; Zhang et al. 1994; Liao et al. 1999; see Materials and Methods for details). In order to retain the critical components of the neural circuits controlling the TSWR and feeding and to capture the learning-induced changes occurring within these circuits, the buccal, cerebral, and pleural-pedal ganglia were isolated in vitro together with their interganglionic connectives (Fig. 1A; Zhang et al. 1994; Cleary et al. 1995; Cropper et al. 2004; Marinesco et al. 2004). We used increase of TSN excitability and decrease of B51 excitability as readouts of sensitization and feeding suppression, respectively (Cleary et al. 1998; Shields-Johnson et al. 2013).
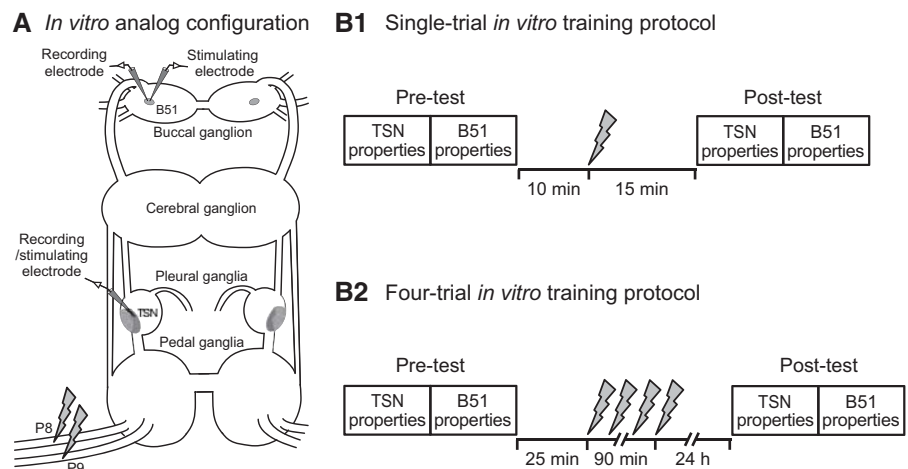
Figure 1. Configuration of the in vitro analog, and testing and training protocols. (*A*) The cartoon illustrates the reduced preparation used for the in vitro analog. The preparation consisted of the buccal, cerebral, and pleural-pedal ganglia. Intracellular recordings are shown from B51 in the buccal ganglion and from one TSN in the pleural ganglion. The dark-gray area in the pleural ganglion represents the portion of the VC cluster that contains the TSNs. Simultaneous electrical stimulation of peripheral nerves P8 and P9 (lightning bolts) was used as the in vitro analog of in vivo sensitization training. (*B*) Testing and training procedures used for the single-trial (*B1*) and four-trial (*B2*) in vitro protocols. In the single-trial protocol, membrane properties of TSNs and B51 were assessed prior to and 15 min after training (*B1*). After a 10-min rest, the single-trial in vitro training was delivered. Training consisted of one 10-sec train of P8/P9 electrical stimulation (Lightning bolt, *B1*). In the four-trial protocol, membrane properties of TSNs and B51 were assessed prior to and 24 h after training (*B2*). After a 25-min rest, the four-trial in vitro training was delivered. Training consisted of four 10-sec trains of P8/P9 electrical stimulation (lightning bolts), spaced 30 min apart (*B2*).

Results

The in vitro analog coexpressed short-term excitability changes in TSNs and B51

The first goal of this study was to examine whether short-term changes in the excitability of TSNs and B51 were concurrently induced by in vitro sensitization training. For the in vitro training, we modified a previously established protocol, consisting of four 10-sec trains of electrical stimuli applied simultaneously to both pedal nerves P8 and P9 (Fig. 1B2; see Materials and Methods for details), which induces synaptic facilitation lasting at least 24 h (Zhang et al. 1994). From this paradigm, we derived a training protocol, comprised of a single train of P8/P9 electrical stimulation (Fig. 1B1), which is analogous to an in vivo paradigm that produces sensitization and suppression of feeding lasting for at least 15 min (Acheampong et al. 2012). We initially tested whether the single-trial in vitro training induced short-term (15 min) increased TSN excitability in the isolated pleural-pedal ganglia. TSN excitability was measured as the number of action potentials evoked by a 1-sec, 2-nA current injection (Cleary et al. 1998). Fifteen minutes after training, TSN excitability was significantly increased in trained ganglia (change in number of spikes: $180.16 \pm 85.10\%$ of pretest; $n = 7$) when compared with untrained ganglia (change in number of spikes: $0.44 \pm 16.10\%$ of pretest; $n = 9$; $P < 0.05$; $U = 9.50$; Mann–Whitney U-test). There was no significant effect of training on the resting membrane potential (V_m) of TSNs (change in V_m, trained: $5.25 \pm 3.50\%$ of pretest; $n = 7$; untrained: $8.13 \pm 2.47\%$ of pretest; $n = 9$; $P = 0.75$; $U = 28.00$; Mann–Whitney U-test). These results indicate that P8/P9 electrical stimulation successfully served as in vitro sensitization training.

We next tested the hypothesis that the single-trial in vitro training induced concurrent excitability changes in both TSNs and B51. The analog configuration consisted of the buccal,

cerebral, and pleural-pedal ganglia (Fig. 1A). B51 excitability was measured as the current threshold to elicit the regenerative burst of action potentials known as a plateau potential (Nargeot et al. 1999a). Fifteen minutes after training (Fig. 1B1), we observed the coexpression of increased TSN excitability (Fig. 2A1,A2) and decreased B51 excitability (Fig. 2B1,B2). The above excitability changes were not accompanied by modifications of V_m in TSNs, or by modifications of V_m or input resistance (R_m) in B51 (Table 1). These findings indicate that the in vitro analog successfully coexpressed short-term changes in intrinsic excitability in both TSNs and B51. In ~77% of the preparations examined, B51 exhibited inhibitory synaptic potentials (IPSPs) originating from presynaptic neurons within the feeing neural circuit, including B52 (Plummer and Kirk 1990; Nargeot et al. 2002). Changes in this inhibitory synaptic input might potentially contribute to the training-induced decrease of B51 excitability. To address this issue, we examined the magnitude of the IPSPs in the trained preparations before and after training (see Materials and Methods for details). The average IPSP amplitude did not change after the single-trial in vitro training (pretest: -2.05 ± 0.46 mV; post-test: -2.61 ± 0.73 mV, $n = 7$; $P = 0.69$; $W = -6.00$; Wilcoxon signed rank test).

In vitro training did not alter the excitability of neuron B4/5

In order to test whether learning-dependent changes were limited to predicted sites of plasticity, such as B51 and TSNs, in the second

Figure 2. Single-trial in vitro training induced a concurrent increase of TSN excitability and a decrease of B51 excitability 15 min after training. (A1) Sample traces of TSN firing from trained and untrained preparations. (A2) Summary data illustrate that the single-trial in vitro training significantly increased TSN excitability (change in number of spikes, trained: $58.97 \pm 20.14\%$ of pretest; $n = 13$; untrained: $-1.68 \pm 7.22\%$ of pretest; $n = 10$; $P < 0.05$; $U = 21.00$; Mann–Whitney U-test). (B1) Sample traces of B51 burst threshold from trained and untrained preparations. (B2) Summary data illustrate that the single-trial in vitro training significantly increased B51 burst threshold (change in burst threshold, trained: $12.72 \pm 5.98\%$ of pretest; $n = 10$; untrained: $-2.93 \pm 3.92\%$ of pretest; $n = 9$; $P < 0.05$; $U = 20.00$; Mann–Whitney U-test). In these and the following figures, values are expressed as mean ± SEM.
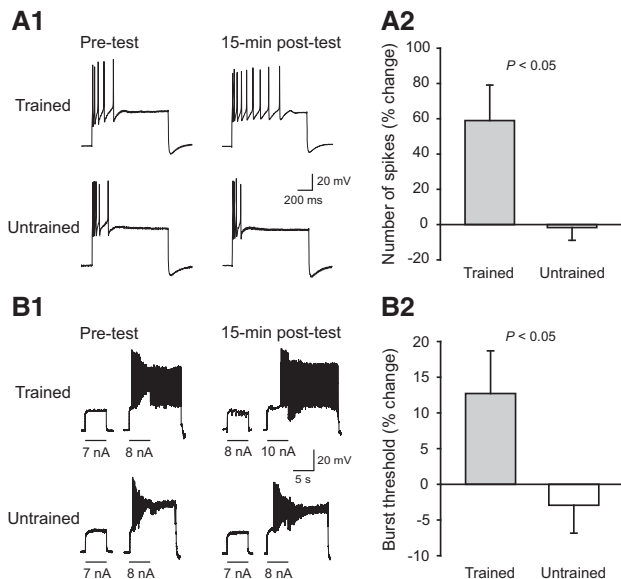
experiment, we used the above in vitro analog to analyze the effects of in vitro training on the membrane properties of another neuron in the feeding neural circuit: B4/5. B4/5 are two pairs of multifunctional neurons, which are involved in the neural mechanisms of feeding motor program switching (Gardner 1977; Church and Lloyd 1994; Warman and Chiel 1995; Jing and Weiss 2001). Based on previous negative results observed following appetitive classical conditioning (Lechner et al. 2000; Mozzachiodi et al. 2003), we hypothesized that B4/5 would not be a site of plasticity following sensitization training. B4/5 excitability was measured as the number of action potentials evoked by a 1-sec, 10-nA current injection. Training procedures were identical to those used in the previous experiment. Although the single-trial in vitro training successfully increased the excitability of TSNs 15 min after training (Fig. 3A1,A2), it did not alter the excitability of B4/5 (Fig. 3B1,B2). The single-trial in vitro training also did not change the V_m, or the R_m of B4/5 (Table 1).

The in vitro analog coexpressed long-term excitability changes in TSNs and B51

In the next experiment, we investigated whether the short-term excitability changes observed in TSNs and B51 could be extended to the long term (24 h). Previous work in *Aplysia* indicates that training with repeated trials of noxious stimuli induces concurrent sensitization and feeding suppression lasting at least 24 h (Acheampong et al. 2012; Shields-Johnson et al. 2013). Therefore, we tested whether multiple in vitro sensitization trials induced concomitant long-term excitability changes in TSNs and B51 persisting for at least 24 h. We used a previously developed in vitro training protocol, consisting of four trials (each identical to that used in the short-term protocol described above) spaced 30 min apart (Fig. 1B2; Goldsmith and Byrne 1989; Zhang et al. 1994). This in vitro training significantly increased the excitability of TSNs (Fig. 4A1,A2) and decreased the excitability of B51 (Fig. 4B1,B2) at the 24-h post-test. Similar to the results obtained with the single-trial in vitro training, the above long-term excitability changes were not accompanied by modifications of resting membrane properties in either TSNs, or B51 (Table 1). Moreover, the analysis of inhibitory synaptic input to B51 revealed that the average IPSP amplitude did not change after the four-trial in vitro training (pretest: -3.93 ± 1.09 mV; post-test: -3.37 ± 0.72 mV, $n = 8$; $P = 0.74$; $W = -6.00$; Wilcoxon signed rank test). These findings indicate that cellular correlates of sensitization and feeding suppression are coexpressed 24 h following the four-trial in vitro training.

Serotonin differentially modulated the excitability of TSNs and B51

In the final experiment, we characterized the effects of in vitro application of serotonin (5-HT) on the excitability of TSNs and B51. Previous work in *Aplysia* indicates that 5-HT mediates sensitization and its underlying cellular underpinnings, including increased TSN excitability (Walters et al. 1983a,b; Kandel 2001; Byrne and Hawkins 2015). However, an in vivo 5-HT bath-application protocol, which induced sensitization of the TSWR, did not induce feeding suppression or changes in B51 excitability (Shields-Johnson et al. 2013). In this experiment, we analyzed whether the differential effects of 5-HT induced by in vivo 5-HT bath application were reproduced in vitro following 5-HT treatment. We used the in vitro configuration in Figure 1A and the testing protocol of Figure 1B1 in which 5-min application of 50 µM 5-HT replaced P8/P9 electrical stimulation (Philips et al. 2011). 5-HT application significantly increased the excitability of TSNs 15 min after treatment (Fig. 5A1, A2), but did not alter B51 excitability (Fig. 5B1,B2). There was no

Table 1. Effects of in vitro training protocols and 5-HT application on the resting properties of TSN, B51 and B4/5

In vitro protocol	Neuron	Preparations (n)	Resting property	Trained/5-HT	Untrained/ASW	P	U
Single-trial training	TSN	Trained = 13; Untrained = 9	V_m	−3.02 ± 3.16%	2.66 ± 3.73%	0.24	45.50
	B51	Trained = 12; Untrained = 9	V_m	6.96 ± 1.92%	6.70 ± 1.32%	0.62	46.50
		Trained = 12; Untrained = 9	R_m	1.24 ± 4.74%	−5.50 ± 1.84%	0.41	42.00
Single-trial training	TSN	Trained = 28; Untrained = 29	V_m	−2.32 ± 2.10%	2.53 ± 2.47%	0.10	300.00
	B4/5	Trained = 34; Untrained = 35	V_m	0.73 ± 1.56%	3.37 ± 1.28%	0.41	526.00
		Trained = 32; Untrained = 34	R_m	0.04 ± 3.06%	2.10 ± 1.92%	0.88	532.00
Four-trial training	TSN	Trained = 14; Untrained = 17	V_m	3.45 ± 4.50%	−0.38 ± 2.26%	0.29	92.00
	B51	Trained = 11; Untrained = 11	V_m	−2.44 ± 3.78%	0.85 ± 1.84%	0.69	54.00
		Trained = 11; Untrained = 9	R_m	3.59 ± 2.32%	16.7 ± 6.08%	0.11	28.00
5-HT application	TSN	5-HT = 8; ASW = 7	V_m	−5.64 ± 1.32%	−3.87 ± 3.99%	0.19	16.50
	B51	5-HT = 9; ASW = 6	V_m	2.13 ± 2.40%	2.21 ± 2.82%	0.82	16.00
		5-HT = 8; ASW = 6	R_m	−16.20 ± 4.50%	6.63 ± 3.86%	<0.05	4.00

Values are expressed as mean ± SEM. The Mann–Whitney U-test was used to compare changes between groups. Statistical significance was set at $P < 0.05$.

effect of 5-HT on V_m in either TSNs, or B51 15 min after treatment (Table 1). We observed a significant decrease of R_m in B51 at 15 min after 5-HT application (Table 1). However, there was no significant correlation between the change in R_m and the change in burst threshold in B51 treated with 5-HT ($r = −0.18$, $P = 0.62$, Spearman Rank Order Correlation). Finally, we analyzed the effect of 5-HT on the inhibitory synaptic input to B51. The average IPSP amplitude did not change after 5-HT bath application (pretest: −6.06 ± 1.10 mV; post-test: −4.72 ± 0.60 mV, $n = 8$; $P = 0.20$; $W = 20.00$; Wilcoxon signed rank test).

Discussion

This study illustrates the successful development of the first in vitro analog that expresses learning-induced cellular correlates in multiple behaviorally relevant neural circuits. In particular, increased TSN excitability in the TSWR circuit, and decreased B51 excitability in the feeding circuit were observed following in vitro training consisting of P8/P9 electrical stimulation.

The configuration of this in vitro analog (Fig. 1A) was obtained by retaining the pleural-pedal ganglia (site of the TSWR circuit and origin of the nerves P8 and P9; Cleary et al. 1995), the buccal ganglion (site of the feeding circuit; Cropper et al. 2004) and the cerebral ganglion that connects them and contains interneurons activated by sensitizing stimuli (Marinesco et al. 2004) as well as command-like interneurons that control the feeding circuit (Cropper et al. 2004). This preparation was derived from a previous in vitro analog of sensitization, consisting exclusively of the pleural-pedal ganglia, which expresses correlates of sensitization (e.g., increased TSN excitability and synaptic facilitation of the sensorimotor neuron synapse) following P8/P9 electrical stimulation (Goldsmith and Byrne 1989; Zhang et al. 1994; Liao et al. 1999).

Learning-dependent changes in excitability were originally identified in TSNs and B51 using a four-trial in vivo sensitization protocol that induces long-term behavioral modifications lasting at least 24 h (Cleary et al. 1998; Shields-Johnson et al. 2013). For this study, we used an in vitro protocol, which closely matched the in vivo sensitization training in number of trials, stimulus intensity, and temporal pattern of nerve stimulation (Fig. 1B2; see Materials and Methods for details). Previous use of this in vitro protocol in the isolated pleural-pedal ganglia revealed cellular correlates of sensitization, including reduction of the net outward current in TSNs (Noel et al. 1991) and synaptic facilitation of the synapse between TSNs and tail motor neurons (Goldsmith and Byrne 1989; Zhang et al. 1994). Our findings indicate that another long-term correlate of sensitization is expressed following in vitro training: increase of TSN excitability (Fig. 4A; Cleary et al. 1998). The increase of TSN excitability was not accompanied by modifica-

tions in resting membrane potential, which is consistent with previous results obtained with both in vivo (Cleary et al. 1998) and in vitro sensitization protocols (Zhang et al. 1994), and with 5-HT treatments used in lieu of training (Dale et al. 1987; Baxter and Byrne 1990; Liu et al. 2011).

In this study, we have also demonstrated that the in vitro training induced modifications outside the TSWR neural circuit, precisely a decreased excitability in B51, an element of the feeding neural circuit (Fig. 4B). The decreased excitability in B51 was not accompanied by modifications of resting membrane potential, input resistance or synaptic input, thus recapitulating the results
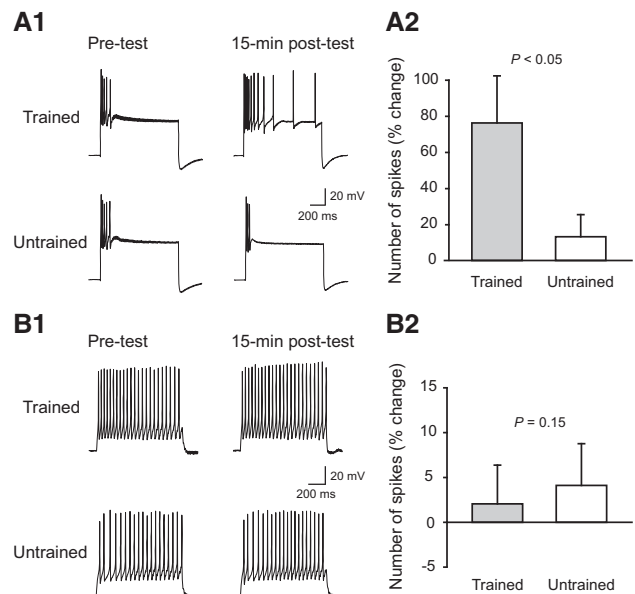
Figure 3. Single-trial in vitro training increased TSN excitability, but did not alter the excitability of neuron B4/5. Using the short-term in vitro analog illustrated in Figure 1A and B1, the effects of in vitro training were examined on B4/5. The excitability of TSN and B4/5 was assessed prior to and 15 min after treatment. (A1) Sample traces of TSN firing from trained and untrained preparations. (A2) Summary data illustrate that the single-trial in vitro training significantly increased TSN excitability (change in number of spikes, trained: 76.42 ± 26.17% of pretest; $n = 28$; untrained: 13.20 ± 12.25% of pretest; $n = 29$; $P < 0.05$; $U = 282.50$; Mann–Whitney U-test). (B1) Sample traces of B4/5 firing from trained and untrained preparations. (B2) Summary data illustrate that B4/5 excitability did not differ between trained and untrained preparations (change in number of spikes, trained: 2.06 ± 4.32% of pretest; $n = 34$; untrained: 4.12 ± 4.66% of pretest; $n = 35$; $P = 0.15$; $U = 474.00$; Mann–Whitney U-test).
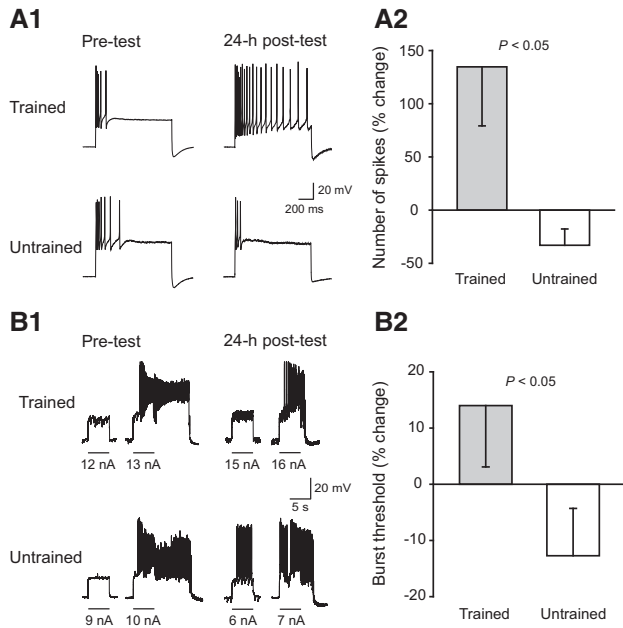
Figure 4. Four-trial in vitro training induced a concurrent increase of TSN excitability and a decrease of B51 excitability 24 h after training. (*A1*) Sample traces of TSN firing from trained and untrained preparations. (*A2*) Summary data illustrate that the four-trial in vitro training significantly increased TSN excitability (change in number of spikes, trained: 134.64 ± 55.47% of pretest; $n = 14$; untrained: −33.07 ± 15.30% of pretest; $n = 17$; $P < 0.05$; $U = 60.00$; Mann–Whitney U-test). (*B1*) Sample traces of B51 burst threshold from trained and untrained preparations. (*B2*) Summary data illustrate that the four-trial in vitro training significantly increased B51 burst threshold (change in burst threshold, trained: 13.99 ± 10.92% of pretest; $n = 11$; untrained: −12.72 ± 8.42% of pretest; $n = 11$; $P < 0.05$; $U = 29.50$; Mann–Whitney U-test).

obtained with a four-trial in vivo sensitization protocol (Shields-Johnson et al. 2013; Hernandez et al. 2017). Several lines of evidence suggest that decreased B51 excitability is intrinsic: (1) the lack of training-induced changes in resting membrane potential and input resistance rules out the possibility that modulation of either tonic synaptic inputs to B51 or electrical coupling between B51 and other cells might contribute to the observed decreased excitability. (2) The lack of training-induced changes in IPSP amplitude (Shields-Johnson et al. 2013; this study) excludes the contribution of synaptic plasticity to decreased B51 excitability. (3) The plateau potential in B51 is known to be an intrinsic feature that is retained in isolated cultured cells (Brembs et al. 2002; Mozzachiodi et al. 2008). (4) Recent findings revealed that B51 decreased excitability induced by in vivo training is sustained by a training-induced nonsynaptic modulation of voltage-gated Na$^+$ channels (Hernandez et al. 2017).

From the four-trial in vitro protocol, we derived a single-trial in vitro training (Fig. 1B1), which is analogous to an in vivo protocol that induces short-term sensitization and feeding suppression (Acheampong et al. 2012). This single-trial in vitro training induced concurrent increased TSN excitability (Fig. 2A) and decreased B51 excitability (Fig. 2B), with no effect on the resting properties of either neurons. Like the long-term modifications described above, these excitability changes are consistent with the coexpression of short-term sensitization and feeding suppression, respectively (Acheampong et al. 2012). Overall, the in vitro analog developed in this study coexpressed excitability changes in TSNs and B51 in two distinct temporal domains: short term (i.e., 15 min after the single-trial training; Fig. 2), and long term (24 h after

the four-trial training; Fig. 4), indicating that the mechanisms of memory storage (i.e., plasticity of intrinsic excitability) are conserved from the short term to the long term.

To expand the application of the in vitro analog, we began to examine to which extent the effects of sensitization training are distributed within the feeding neural circuit. We investigated the role of neurons B4/5, which are involved in motor program switching (Warman and Chiel 1995, Jing and Weiss 2001). Previous results revealed that the membrane properties of B4/5 were not altered following in vivo or in vitro paradigms of appetitive classical conditioning (Lechner et al. 2000; Mozzachiodi et al. 2003). A similar outcome was observed in this study: although in vitro training induced the expected increase of TSN excitability (Fig. 3A), it did not induce changes in B4/5 membrane properties (Fig. 3B). This result indicates that B4/5 is not a locus of plasticity underlying the feeding suppression produced by sensitization training. By providing a "negative control" of neuronal plasticity, these findings suggest that the changes induced by sensitization training are not generalized throughout the feeding neural circuit, but they are restricted to specific neurons, such as B51. It is important to note that B51 is unlikely the sole site of plasticity induced by sensitizing stimuli within the feeding neural circuit. Our developed in vitro analog will allow us to further explore additional sites of plasticity underlying the learning-induced feeding suppression, including pattern-initiating neurons in the buccal ganglion, such as B63 and B31/32
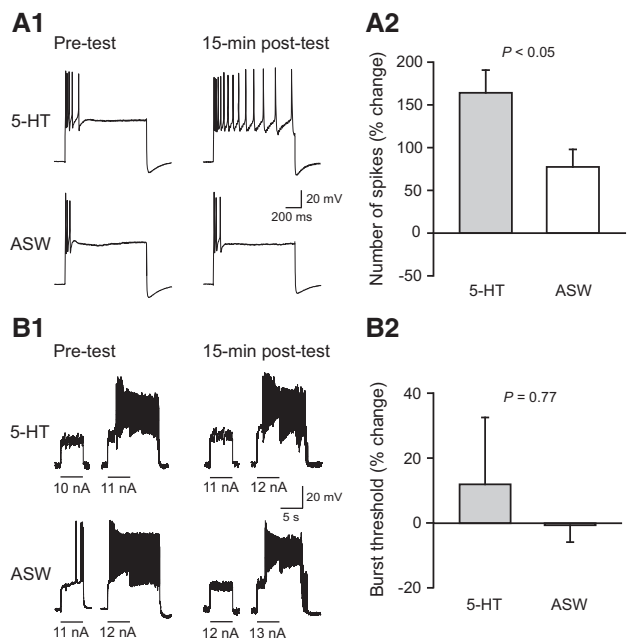
Figure 5. 5-HT differentially modulates excitability of TSNs and B51. The in vitro configuration and the testing protocol were identical to the ones illustrated in Figure 1A and B1 with the exception that treatment with 50 µM 5-HT replaced P8/P9 electrical stimulation. Excitability of TSN and B51 was assessed prior to and 10 min after a 5-min treatment with either 5-HT or ASW. (*A1*) Sample traces of TSN firing from preparations treated with 5-HT or ASW. (*A2*) Summary data illustrate that 5-HT significantly increased TSN excitability change in number of spikes, 5-HT: 164.14 ± 26.55% of pretest; $n = 8$; ASW: 77.36 ± 20.55% of pretest; $n = 7$; $P < 0.05$; $U = 9.00$; Mann–Whitney U-test). (*B1*) Sample traces of B51 burst threshold from preparations treated with 5-HT or ASW. (*B2*) Summary data illustrate that B51 burst threshold did not differ between preparations treated with either 5-HT or ASW (change in burst threshold, 5-HT: 11.95 ± 20.53% of pretest; $n = 9$; ASW: −0.63 ± 5.15% of pretest; $n = 6$; $P = 0.77$; $U = 24.00$; Mann–Whitney U-test).

(Nargeot and Simmers 2012), as well as cerebral command-like interneurons (CBIs), which are capable of driving the feeding neural circuit (Cropper et al. 2004).

Previous work in *Aplysia* has established that 5-HT mediates sensitization of withdrawal reflexes and its underlying cellular correlates (Brunelli et al. 1976; Glanzman et al. 1989; Levenson et al. 2000; Marinesco and Carew 2002; Byrne and Hawkins 2015). Indeed, 5-HT can serve as analog of sensitization training in inducing facilitation of the sensory-to-motor neuron synapses and increased sensory neuron excitability (Walters et al. 1983a,b; Dale et al. 1987; Baxter and Byrne 1990; Byrne and Kandel 1996; Liu et al. 2011, Byrne and Hawkins 2015). However, recent results indicate that 5-HT does not mimic sensitization training in inducing feeding suppression or decreased B51 excitability (Shields-Johnson et al. 2013).

The in vitro analog developed in this study recapitulates the differential effects that 5-HT exerts on sensitization and feeding suppression: 5-HT treatment induced an increase of TSN excitability (Fig. 5A), but did not modify B51 excitability (Fig. 5B), or the inhibitory synaptic input to the cell. 5-HT bath application induced an unexpected significant decrease of R_m in B51, but this change did not correlate with the change in burst threshold and was not sufficient to significantly alter B51 excitability. Current evidence indicates that R_m is not altered in B51 by either in vivo (Shields-Johnson et al. 2013) or in vitro sensitizing stimuli (this study), which are known to trigger 5-HT release from interneurons in the pleural-pedal and cerebral ganglia (Marinesco and Carew 2002; Marinesco et al. 2004; Philips et al. 2011). An explanation for the discrepancy between the effects of sensitization training and 5-HT bath application may reside in the different modes of 5-HT action: in vivo and in vitro sensitizing stimuli lead to the local release of 5-HT in the neuropil (Marinesco and Carew 2002), whereas 5-HT bath application may have more diffused effects within the feeding neural circuit. Indeed, previous findings indicate that 5-HT bath application alters the properties of multiple feeding neurons, such as decreasing the input resistance of neuron B31/32 and the excitability of neuron B4/5 (Kabotyanski et al. 2000). In addition, we cannot rule out the possibility that the decrease of B51 R_m may reflect a modulation of the electrical coupling between B51 and other buccal neurons (Plummer and Kirk 1990; Mozzachiodi et al. 2013; Sasaki et al. 2013) induced by 5-HT bath application.

The different effects of 5-HT on the excitability of TSNs and B51 strengthen the view that distinct biochemical pathways are responsible for the changes occurring in the neural circuits controlling the TSWR and feeding, following sensitizing stimuli. Despite the extensive knowledge that 5-HT release modulates the activity of the withdrawal circuits (Marinesco and Carew 2002; Marinesco et al. 2004; Byrne and Hawkins 2015), the training-induced pathway that leads to the decrease of B51 excitability remains uncharacterized and future investigations are necessary to identify the underlying modulator(s). A putative candidate is nitric oxide (NO), which in *Aplysia* mediates forms of nociceptive sensitization (Lewin and Walters 1999) and aversive classical conditioning (Antonov et al. 2007), and modulates feeding and its neural circuit (Susswein and Chiel 2012). Current findings indicate that NO contributes to the training-induced suppression of feeding. Systemic in vivo injection of the NO synthase inhibitor L-NAME prevented the expression of feeding suppression following either single-trial or four-trial sensitization protocols (Farruggella et al. 2015). Recent results also indicate that, at the cellular level, iontophoretic injections of cGMP decreases B51 excitability in a manner analogous to that produced by sensitization training, with one injection reducing excitability for at least 15 min, and four injections, spaced 30 min apart, reducing excitability for at least 24 h (Goldner et al. 2016). These findings suggest that a NO–cGMP

pathway may mediate the training-induced feeding suppression and the underlying decreased of B51 excitability. Future experiments will use the in vitro analog to characterize the biochemical steps linking sensitizing stimuli to decreased B51 excitability.

The use of in vitro analogs derived from vertebrate and invertebrate preparations has greatly contributed to revealing the occurrence of distributed forms of learning-dependent neuronal plasticity in identified neural circuits. For example, in the mammalian cerebellum, long-term potentiation (LTP) and long-term depression (LTD) can be induced by different learning rules in distinct cerebellar regions (Suvrathan et al. 2016). Similarly, in the leech *Hirudo*, multiple forms of LTP and LTD (Burrell and Sahley 2004) and bidirectional changes in excitability (Burrell et al. 2001) are induced in defensive neural circuits by patterns of electrical stimulation in vitro. In *Aplysia*, bidirectional modifications of synaptic efficacy and neuronal excitability have been identified at the level of sensory neurons and interneurons responsible for defensive withdrawal reflexes of the tail, siphon and gills, following in vitro analogs of sensitization training (Trudeau and Castellucci 1993, Cleary et al., 1995; Cohen et al. 1997). Equivalent findings have been reported in the neural circuit controlling feeding in *Aplysia* in which in vitro analogs of appetitive classical and operant conditioning both express forms of synaptic and intrinsic plasticity occurring at multiple sites (for review, see Nargeot and Simmers 2012; Mozzachiodi et al. 2013). However, in the above in vitro analogs, the learning-induced plasticity is expressed within individual circuits or in multiple circuits with similar functions, such as those controlling defensive withdrawals of tail, siphon, and gills in *Aplysia* (Cleary et al. 1995).

The novelty of the in vitro analog described in this study is that it expresses distributed cellular changes occurring concomitantly in multiple neural circuits of very different nature: defensive (TSWR) and appetitive (feeding), following exposure to aversive stimuli. In particular, in vitro sensitization training induces opposite changes in excitability in key neurons within the TSWR and feeding neural circuits, which are analogous to those measured following in vivo training and are consistent with the observed learning-dependent behavioral modifications.

In conclusion, this in vitro analog, expressing cellular correlates of both sensitization and feeding suppression, offers an unprecedented opportunity to compare and contrast learning-induced changes occurring simultaneously in multiple neural circuits. This goal will be achieved through the use of procedures and techniques that are not feasible in vivo, such as local/global activation or inhibition of individual biochemical pathways, as well as cytosolic/nuclear manipulations of individual neurons in different neural circuits. This approach will be instrumental in exploring important aspects of learning-induced neuronal plasticity, including: (1) the relationship between the cellular and molecular underpinnings underlying modifications occurring in different neural circuits, (2) the comparison between short-term and long-term mechanisms, and (3) the comparison between changes induced by learning paradigms using stimuli of different modalities. The latter aspect is particularly relevant for B51, the excitability of which is modulated in opposite directions by appetitive (Nargeot et al. 1999a,b; Brembs et al. 2002; Mozzachiodi et al. 2008) and aversive stimuli (Shields-Johnson et al. 2013; this study).

Materials and Methods

General methods

Wild-caught *Aplysia californica* (130–180 g) were obtained from Marinus Scientific and South Coast Bio-Marine, and housed individually in perforated plastic cages in two aquaria (Acquatic Enterprises Inc.) of continuously circulating 15°C aquarium seawater (Instant Ocean) on a 12-h light–dark cycle. A period of

acclimation of at least 3 d upon arrival was allowed before animals were utilized, to minimize the effects of stress caused by shipping and handling (Levenson et al. 1999). Animals were fed ~1 g of dried seaweed (Emerald Cove Organic Pacific Nori) three times a week. Animals were food deprived for 2–3 d prior to the experiment.

Dissection

Prior to each experiment, the general health and motivational state of the animal was tested by presenting it with a food stimulus. The animal was included in the study only if it generated bites in response to the food stimulus. *Aplysia* were anesthetized by injecting a volume of isotonic $MgCl_2$ (equivalent to 50% of the weight of the animal) through the foot. The animal was placed ventral side up in a dissecting tray and a longitudinal incision was made, along the midline of the foot to expose the central nervous system. The incision extended approximately one-third the length of the animal to the margin of the lips. The buccal, cerebral, and pleural-pedal ganglia were removed together with their interganglionic connectives to retain the critical components of the neural circuits controlling the TSWR and feeding (Fig. 1A; Cleary et al. 1995; Cropper et al. 2004). A few centimeters of the left or right pedal nerves P8 and P9, which innervate the lateral and posterior body wall and tail (Fig. 1A; Hening et al. 1979; Walters et al. 2004), were retained for extracellular nerve stimulation. The side of nerves P8 and P9 origin (left or right) was randomly selected for each preparation. One of the two buccal nerves 2,3 (denoted n.2,3) was retained for identification of neuron B51 (Nargeot et al. 1999a; Shields-Johnson et al. 2013). All other nerves were cut short.

The isolated ganglia were transferred to a Sylgard-coated petri dish, containing high-divalent artificial seawater (ASW) composed of 210 mM NaCl, 10 mM KCl, 145 mM $MgCl_2$, 20 mM $MgSO_4$, 33 mM $CaCl_2$, and 10 mM HEPES, with the pH adjusted to 7.5 using NaOH (Mozzachiodi et al. 2003). The cerebral and the pleural-pedal ganglia were pinned ventral-side up. In this configuration, the buccal ganglion was flipped to expose the rostral side by bringing the radula nerve up over the ganglion and pinning it between the esophageal nerves. The sheath on the ventral surface of the pleural ganglion on the side of the retained nerves P8 and P9 was surgically removed using fine forceps and scissors to expose the somata of TSNs in the VC cluster (Fig. 1A; Zhang et al. 1994; Cleary et al. 1998). The sheath was also removed from the rostral surface of both buccal hemiganglia to expose the somata of B51 and B4/5 (Nargeot et al. 1999a; Mozzachiodi et al. 2003). Individual bipolar extracellular stimulating electrodes were placed along P8, P9, and n.2,3 and were isolated from the bath with Vaseline (Nargeot et al. 1997; Mozzachiodi et al. 2003). The high-divalent ASW was replaced by normal ASW (composed of 450 mM NaCl, 10 mM KCl, 30 mM $MgCl_2$, 20 mM $MgSO_4$, 10 mM $CaCl_2$, and 10 mM HEPES, with pH adjusted to 7.5 using NaOH; Mozzachiodi et al. 2003) and the preparation was rested for 30 min. The recording chamber was maintained at 15°C throughout the electrophysiological recordings and experimental treatments with a feedback-controlled cooling device (Model BTC-100/BTC-S, Bioscience Tools, CA).

Intracellular recordings

All intracellular recordings were conducted in current-clamp mode using a preamplifier (IX2-700, Dagan Corporation). Fine-tipped glass microelectrodes were filled with 3 M potassium acetate (resistance 10–12 MΩ).

Neuron B51

The soma of neuron B51 was identified based on its relative size and position within the buccal ganglion and the occurrence of its characteristic intrinsically generated all-or-nothing bursts of action potentials, known as plateau potentials (Plummer and Kirk 1990; Nargeot et al. 1999a). If B51 did not fire spontaneously or in response to brief intracellular depolarizing pulses, then n.2,3 was briefly stimulated (1–2 sec of 10V, 0.5 msec pulses at 4 Hz),

and the cell's response was used to aid in identification. Two-electrode current-clamp was used for intracellular recordings from B51. The V_m was recorded 5 min after impalement. Cells were included in the study only if they displayed a V_m of at least −45 mV (Shields-Johnson et al. 2013). B51 was then current clamped at −60 mV, and the input resistance (R_m) and the excitability was measured. R_m was calculated from the voltage drop elicited by a 5-sec, 5-nA hyperpolarizing current pulse (Nargeot et al. 1999a). The excitability of B51 was assessed by measuring the neuron's burst threshold, defined as the minimum amount of depolarizing current necessary to elicit a plateau potential outlasting the duration of the current pulse (Nargeot et al. 1999a). B51 burst threshold was determined by delivering a series of 5-sec depolarizing current pulses, beginning at 5-nA intensity and increasing in intensity in 1-nA increments, at 10-sec intervals (Nargeot et al. 1999a,b). Cells that did not exhibit a plateau potential by 25 nA were excluded from the study. Inhibitory synaptic potentials (IPSPs), originating from presynaptic neurons within the feeing neural circuit, including B52 (Plummer and Kirk 1990; Nargeot et al. 2002), were observed in B51 in about 77% of the preparations used in this study. The contribution of this inhibitory synaptic input to the training-induced decrease of B51 excitability was assessed by analyzing the magnitude of the IPSPs in the preparations that were trained with either the single-trial or the four-trial in vitro training, as well in the preparations that were treated with 5-HT. We measured the amplitude of the IPSPs occurring during the 5-sec depolarizing current pulses, using the current intensity immediately prior to the burst threshold. The IPSP amplitude was averaged before and after training/5-HT treatment.

TSNs

One-electrode current-clamp was used for intracellular recordings from TSNs (Zhang et al. 1994; Cleary et al. 1998; Chin et al. 1999; Liu et al. 2011). We opted for the use of a single electrode for recording and stimulation because of the risk of dislodging electrodes during the delivery of the in vitro training or 5-HT bath application and following washout. The somata of TSNs were identified based on their position in the VC cluster of the pleural ganglion, and the occurrence of action potentials in response to a single 3-msec stimulus delivered to nerve P9 (Cleary et al. 1998; Walters et al. 2004). V_m was recorded 5 min after impalement. Cells were included in the study only if they displayed a resting membrane potential of at least −35 mV (Zhang et al. 1994). TSNs were subsequently clamped at −45 mV (Baxter and Byrne 1990; Chin et al. 1999) and their excitability was assessed as the number of spikes evoked by a 1-sec, 2-nA depolarizing current pulse (Cleary et al. 1998).

Neuron B4/5

The soma of B4/5 was identified in the buccal ganglion based on size, location and the occurrence of spontaneous excitatory synaptic inputs (Gardner 1977; Church and Lloyd 1994). B4/5 membrane properties were measured using two-electrode current clamp. V_m was recorded 5 min after impalement. Cells were included in the study only if they displayed a resting membrane potential of at least −45 mV. B4/5 was subsequently clamped at −55 mV for the assessment of R_m and excitability. R_m was calculated from the voltage drop induced by injecting 5-sec, 5-nA pulse of hyperpolarizing current. Excitability was measured as the number of action potentials elicited by a 1-sec, 10-nA depolarizing current pulse.

Experimental protocols

Single–trial in vitro sensitization training

In *Aplysia*, the effects of sensitization training on TSWR and TSN excitability are restricted to the trained side (Scholz and Byrne 1987; Zhang et al. 1994; Cleary et al., 1998). Therefore, in this study, only TSNs ipsilateral to the retained nerves P8 and P9 were used (Fig. 1A; Zhang et al. 1994). However, because feeding suppression and the subsequent decreased excitability in B51 are not

lateralized (Acheampong et al. 2012; Shields Johnson et al. 2013), B51 and B4/5 were used regardless of whether they were ipsilateral or contralateral to the retained nerves P8 and P9 (Fig. 1A). Membrane properties (V_m, R_m and burst threshold for B51; V_m, R_m and number of action potentials for B4/5; V_m and number of action potentials for TSNs) were initially measured (pretest; Fig. 1B1). The current clamp was then removed and the ganglia were then rested for 10 min before delivery of the single-trial training protocol. The single-trial training consisted of 10-sec, 1 Hz, train of ten 500-msec, 60-Hz, 60-V impulses delivered simultaneously to the retained nerves P8 and P9 (Zhang et al. 1994). Controls consisted of untrained preparations that did not receive any electrical stimulation to P8/P9. Preparations were randomly assigned to receive training or serve as untrained controls. Cells were held throughout training. Fifteen minutes after training, membrane properties were measured again (post-test; Fig. 1B1) to assess changes induced by training.

Four–trial in vitro sensitization training

TSNs and B51 were selected following the same procedure used for the single-trial training. Membrane properties of B51 and TSNs were initially measured as described above (pretest; Fig. 1B2), and then the electrodes were removed. Prior to removing the electrode from the recorded TSN, the cell location was marked by iontophoretically injecting 4–5 cells immediately surrounding it with Fast Green dye (10-nA hyperpolarizing current for ~30 sec, or until the soma was dark green in color; Scholz and Byrne 1987; Zhang et al. 1994; Sherff and Carew 2004). Images of pleural and buccal ganglia were also taken (MD800E microscope-mounted USB camera, AmScope), and the cells were annotated in Microsoft Paint to aid in the identification of B51 and TSN for 24-h post-test measurements of membrane properties (Fig. 1B2). Once TSNs were labeled and images were taken (10–15 min), ASW was exchanged with modified L-15 culture medium consisting of L-15 medium (Sigma-Aldrich) supplemented with (in mM) NaCl 293.1, KCl 4.6, $MgCl_2$ 26, $MgSO_4$ 18.9, $CaCl_2$ 10.9, HEPES 30 and 0.10 g/L of streptomycin (Sigma-Aldrich) and 0.12 g/L of penicillin-G (Sigma-Aldrich), pH adjusted to 7.5 with NaOH (Mozzachiodi et al. 2008). To monitor the efficacy of subsequent training, one of the large neurons in the pleural ganglion adjacent to the medial edge of the VC cluster (that reliably responded to p8/p9 stimulation with fluctuations in the V_m; Liao et al. 1999) was impaled and held throughout the training period. The in vitro training (or no training) protocol began 10 min after the ASW exchange. The four-trial training consisted of four 10-sec trains of P8/P9 electrical stimulation (each identical to that used for the single-trial training) spaced 30 min apart (Goldsmith and Byrne 1989; Zhang et al. 1994). Controls consisted of untrained preparations that did not receive any electrical stimulation to P8/P9. Preparations were randomly assigned to either receive training or serve as untrained controls. Once the training was concluded, the extracellular stimulating electrodes were gently removed and the recording chamber was covered and stored at 15°C for 23.5 h. On the next day, ganglia were rinsed in normal ASW and allowed to rest for 30 min. Post-test measurement of membrane properties commenced 24 h after training (Fig. 1B2).

Effects of 5–HT application on the membrane properties of B51 and TSNs

In the last experiment of this study, we examined whether 5-HT application could successfully replace the single-trial training in inducing concomitant excitability changes in B51 and TSNs. Because previous findings indicate that 5-HT increases the excitability of TSNs (Walters et al. 1983b), we used increased excitability as a "positive control" for the effectiveness of 5-HT treatment. Serotonin hydrochloride (Alfa Aesar) was dissolved in ASW to make a 50-mM stock. Stock solutions were made fresh daily and stored at 4°C. The stock solution was diluted to 50 µM in ASW prior to being bath applied to the ganglia. The experimental design was identical to the one used for the in vitro single-trial training with the exception that 5-HT treatment replaced the training. Membrane properties of B51 and TSNs were measured as described

above (pretest). Five minutes after the measurements were completed, preparations were randomly assigned to receive treatment with either 5-HT or vehicle (ASW). For the 5-HT treated preparations, 50 mL of 50 µM 5-HT was manually exchanged at a rate of ~10 mL/min by slowly adding the 5-HT solution with a transfer pipette to one side of the petri dish while simultaneously removing an equal volume from the opposite side of the petri dish. The application of 5-HT took 5 min. At the end of this period, 5-HT was washed out of with 50 mL of ASW in an identical manner. Controls were handled identically, except that ASW was used in place of 5-HT. Ten minutes after the end of the washout (i.e., 15 min after the beginning of treatment with 5-HT or ASW), membrane properties were measured again (post-test) to assess changes induced by treatment.

Statistical analysis

For each membrane property of each neuron recorded, the percent change was calculated as [(post – pre/pre) × 100] to assess modifications due to training and/or treatment. We were not able to collect every measurement from B51, B4/5 and TSNs in each preparation. This discrepancy was caused primarily by: (1) the inability to find B51, B4/5 and TSNs in every preparation, (2) the inability to maintain recordings until the end of the experiment. The Mann–Whitney U-test was used to compare changes in membrane properties between trained/treated and untrained/control groups (Mozzachiodi et al. 2003; Lorenzetti et al. 2006). The Wilcoxon signed rank test was used to analyze the effect of in vitro training protocols and 5-HT bath application on the amplitude of B51 IPSP. Finally, the Spearman rank correlation coefficient r was used to analyze the correlation between changes in R_m and changes in burst threshold in preparations treated with 5-HT. Statistical significance was set at $P < 0.05$. Data analyses were performed using the statistical package of SigmaPlot 11.0 (Jandel Scientific).

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