Research

Transcriptional analysis of a whole-body form of long-term habituation in *Aplysia californica*

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Habituation is the simplest form of learning, but we know little about the transcriptional mechanisms that encode long-term habituation memory. A key obstacle is that habituation is relatively stimulus-specific and is thus encoded in small sets of neurons, providing poor signal/noise ratios for transcriptional analysis. To overcome this obstacle, we have developed a protocol for producing whole-body long-term habituation of the siphon-withdrawal reflex (SWR) of *Aplysia californica*. Specifically, we constructed a computer-controlled brushing apparatus to apply low-intensity tactile stimulation over the entire dorsal surface of *Aplysia* at regular intervals. We found that 3 d of training (10 rounds of stimulation/day; each round = 15 min brushing at a 10-sec ISI; 15-min rest between rounds) produces habituation with several characteristics favorable for mechanistic investigation. First, habituation is widespread, with SWR durations reduced whether the reflex is evoked by tactile stimulation to the head, tail, or the siphon. Second, long-term habituation is sensitive to the pattern of training, occurring only when brushing sessions are spaced out over 3 d rather than massed into a single session. Using a custom-designed microarray and quantitative PCR, we show that long-term habituation produces long-term up-regulation of an apparent *Aplysia* homolog of cornichon, a protein important for glutamate receptor trafficking. Our training paradigm provides a promising starting point for characterizing the transcriptional mechanisms of long-term habituation memory.

[Supplemental material is available for this article.]

Habituation is a decline in reflex responsiveness due to repeated stimulation (Thompson and Spencer 1966; Groves and Thompson 1970; Rankin et al. 2009). This form of nonassociative memory is ubiquitous across the animal kingdom (Abramson 1994) and seems to play an important role in filtering and attention (Dow and Anastasio 1999; Linster et al. 2007). In fact, rates of habituation provide a useful global index of cognitive function in humans (Fagan et al. 2007), and deficits in habituation are one of the most reliable neurocognitive markers of schizophrenia (Light et al. 2012).

Long-term habituation has been extensively studied in a number of model organisms. This work has shown that the apparent simplicity of habituation belies complex neural underpinnings. For example, in the crayfish tail-flip reflex, long-term habituation depends not only on homosynaptic depression of sensory synapses but also on complex changes in descending inhibition from the CNS (Krasne and Teshiba 1995; Shirinyan et al. 2006). In nematodes (Lau et al. 2013) and in crustaceans (Hermitte et al. 1999) mechanistically distinct forms of long-term habituation can be induced by subtly different patterns of training.

In addition to physiological changes, long-term habituation has been repeatedly shown to depend on changes in gene expression (Beck and Rankin 1995; Chew et al. 1995; Pedreira et al. 1996; Esdin et al. 2010). In most model systems, however, it remains unclear what specific transcriptional changes are required to enable the CNS to encode long-term habituation memories. In *Caenorhabditis elegans*, long-term habituation of the reversal-response to tap has been associated with decreased synaptic expression of GLR-1, a non-NMDA glutamate receptor (Rose et al. 2003). Furthermore, a selective deficit of long-term habituation is produced in mutants lacking either GLR-1 or a nematode homolog of CREB (CRH-1) (Timbers and Rankin 2011). In zebra finch, microarray analysis has recently been used to show that long-term habituation to a conspecific's song is associated with the persistent regulation of over 3000 transcripts in the auditory lobule (Dong et al. 2009), with a gene-ontology analysis showing particularly strong and distinctive down-regulation of transcripts related to electron transport and translation. Outside of these efforts, little is known about the specific transcriptional correlates of long-term habituation.

The goal of this project is to begin characterizing the transcriptional signature of long-term habituation in the siphon-withdrawal reflex (SWR) of *Aplysia californica*. The *Aplysia* SWR represents an attractive model system because it is controlled by a relatively well-defined neural circuit and there is already a large literature on the physiological mechanisms of SWR habituation (for review, see Glanzman 2009).

One difficulty for studying habituation of the SWR is that the sensory neurons which mediate this reflex have not been fully characterized. Within the CNS, several clusters of nociceptors have been identified that can directly or indirectly activate the motor neurons which generate the SWR. These include the LE nociceptors in the abdominal ganglion which innervate the gill, mantle, and siphon (Castellucci et al. 1970; Byrne et al. 1974), and the VC nociceptors in the pleural ganglia which form a somatotopic map of most of the rest of the body (Walters et al. 1983). The tactile threshold for evoking the SWR, however, is

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considerably below that for evoking activity in the LEs and VCs (e.g., Walters et al. 2004). This indicates the existence of a low-threshold population of mechanoreceptors which have yet to be identified (Dubuc and Castellucci 1991; Frost et al. 1997; Hickie et al. 1997; Illich and Walters 1997). Fortunately, habituation of the SWR can be induced even at stimulus intensities sufficient to activate the VC nociceptors (Stopfer and Carew 1996). Moreover, strong activity may not be necessary to evoke plasticity in these nociceptor clusters, as habituation training with a stream of seawater is sufficient to produce robust and long-lasting decreases in the synaptic contacts of the LE nociceptors (Bailey and Chen 1983).

A second difficulty for analyzing the transcriptional mechanisms of habituation is that behavioral effects are often extremely site-specific. In the *Aplysia* SWR, for example, habituation generalizes to only a few millimeters from the site where the animal is trained (Stopfer et al. 1996; Ezzeddine and Glanzman 2003). This is a problem for transcriptional analysis because it implies changes within a very small number of neurons and therefore poor signal/noise when analyzing volumes of CNS tissue sufficient for measuring gene expression.

A final methodological difficulty is that habituation training involves repeated stimulation, making it difficult to untangle transcriptional changes that are memory-related from those that are merely activity-related. For example, work in several model systems has shown that habituation training produces a rapid increase in the expression of genes in the early growth response (Egr) family of transcription factors (Mello et al. 1992; DeSteno and Schmauss 2008; Harvey-Girard et al. 2010). Experimental manipulations have shown, however, that blocking these training-induced changes does not prevent the development of LTH memory (Dong and Clayton 2008; this work examined ZENK, the avian homolog to Egr-1). Thus, Egr regulation may be a byproduct of training-induced activity that is not related to the formation of habituation memory. Although untangling activity- and memory-related processes may seem daunting, this problem can be solved by leveraging the well-established difference in the effectiveness of massed and spaced training (for review, see Philips et al. 2013). Specifically, massed training can serve as a no-memory control that is matched for the amount of stimulation applied to habituated animals. This clever solution has been used effectively for investigating the mechanisms of a habituation-like associative memory by Romano and associates (e.g., Freudenthal et al. 1998).

To analyze the transcriptional correlates of LTH we thus sought to develop a habituation protocol that would (a) have sufficient intensity to produce at least modest activation of nociceptors in the SWR circuit, (b) have sufficient impact to induce strong habituation over a large surface of the body, and (c) provide controls for mere activity through the comparison of effective (spaced) and noneffective (massed) training protocols. Our solution is a whole-body habituation apparatus in which *Aplysia* are trained via repeated mechanical application of a brush across their entire dorsal surface (Fig. 1). Here we show that our training system produces strong, widespread long-term habituation that is highly sensitive to the pattern of training. We then use microarray and qPCR to show that long-term habituation training produces a persistent regulation of a putative *Aplysia* homolog of cornichon.

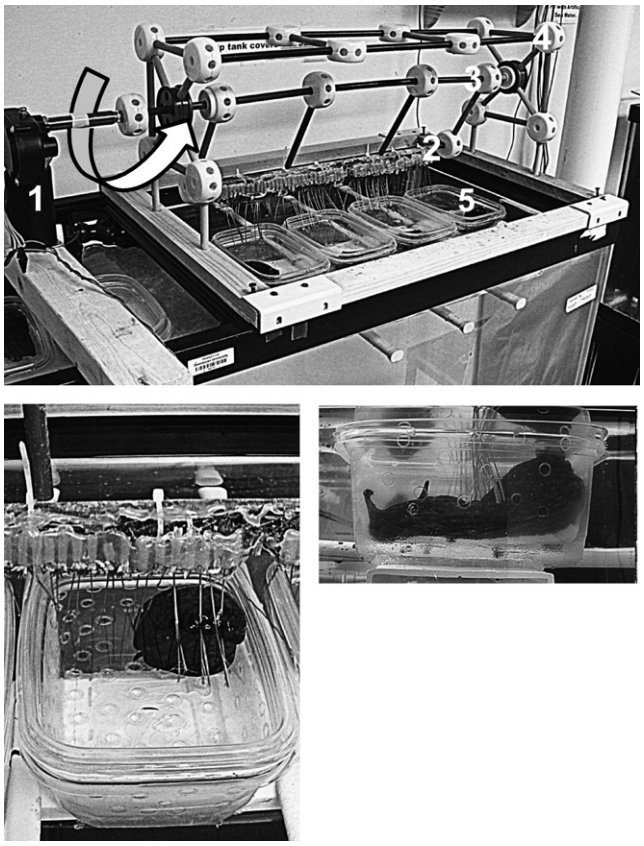

Figure 1. Whole-body habituation training apparatus. (*A*) Photo of the training apparatus. A windshield-wiper motor (1) rotates a long brush (2) attached to a control arm (3) suspended via a frame (4) directly over a rack (5) holding up to four colanders just at the surface of the tank. Each forward-then-back rotation (arrow) drags the bristles on the brush through the colanders, applying tactile stimulation over much of the dorsal surface of the body of each animal. (*B*) *Top-down* view of brush completing a back-stroke through a colander, with bristles spragging along the dorsal surface of an animal (head is oriented *down* and to the *left*). (*C*) *Side*-view as the brush completes a back-stroke through a colander as the animal crawls in the opposite direction. Photos were taken with a digital camera, converted to grayscale, and then processed with an HDR-like filter to enhance contrast.

Results

Brush stimulus produces input to VC nociceptors

One of the requirements for our habituation protocol is at least modest activation of the nociceptive sensory neurons in the central nervous system, as these neurons can be harvested for transcriptional analysis. In contrast, the location of the cell bodies of the mechanoreceptors that mediate innocuous touch remains a mystery.

To ensure our brushing apparatus (Fig. 1) would produce at least some nociceptor activity, we selected bristles that would apply a bending force of 29 mN. In comparison, the median threshold for activation of the VC nociceptors is 6.1 mN, with a range between 1.4 and 43 mN (Walters et al. 2004). These thresholds, however, were determined by direct application of a Von Frey hair, where the force is applied over only the small diameter of the filament. In the brushing apparatus we designed, the bristles are applied from the side, distributing the force over a larger surface area (though, at the point of inflection of the brush arm, the bristles are briefly applied nearly point on). In addition, we applied multiple brushes simultaneously, which may alter response thresholds. Given these considerations, it is difficult to predict from bristle properties alone if the brushing apparatus designed is sufficient to produce the desired activation of CNS nociceptors. Thus, we evaluated the issue empirically, recording from the nociceptors in the VC cluster of the pleural ganglia in reduced preparations that preserved almost all of the posterior

body from just below the head. These VC neurons innervate most of the body.

Upon recording from a VC neuron, a brush stimulus was applied by hand from the anterior to posterior end of the remaining body using a set of 10 bristles identical to those used in the training apparatus. Brushing had to be less brisk than in the training apparatus to avoid disrupting the ongoing recording. In addition, the posterior portion of the body was not pinned, nor was the animal capable of establishing a hold-fast. This meant that resistance to the brush was probably somewhat diminished. Still, the procedure enabled us to roughly characterize the likely response of VC neurons to the stimulus.

We recorded from 20 VC neurons across four preparations. Most neurons (10/20) exhibited transient hyperpolarization (Fig. 2A), as typically occurs in these neurons with off-field stimulation (Walters et al. 1983). Some (5/20) fired single or double action potentials (Fig. 2B), though often mixed with at least some hyperpolarization. Finally, some VCs (5/20) showed no clear response to the brush stimulus.

Whole-body long-term habituation training produces lasting memory after 3 d of training

We next sought to determine if the training apparatus can produce short- and long-term habituation, and if so how much training is required for long-term habituation to develop. To do this, SWR measures were evoked from the siphon, head, and tail in trained animals and matched controls ($n = 12$/group) before and 24-h after four consecutive days of training (Fig. 3A). In addition, S-SWR measures were taken each day to monitor short- and long-term habituation throughout training (short-term measures: ST1-4; long-term measures: LT1-3 and 24 h).

As expected, the matched control design ensured that control and trained animals exhibited similar S-SWR responses at baseline ($M_{control} = 9.2$ sec, SD = 2.6; $M_{trained} = 10.2$, SD = 3.4; $t_{(22)} = 0.74$, $P = 0.47$). Over the course of training, however, S-SWR behavior diverged markedly between groups (Fig. 3B). This was confirmed with a 2 (Condition: control, trained) × 9 (test-phase) mixed-factorial ANOVA on response durations. This showed the expected interaction between condition and testing phase ($F_{(8,176)} = 6.41$, $P < 0.0001$) indicating that the two groups had different behavioral changes over the course of the experiment.

Control animals exhibited a mild decline in responding, with S-SWR durations averaging 80%–90% of baseline responding. During the middle of the experiment, this decrement reached statistical significance relative to baseline ($P = 0.046$, 0.046, and 0.003 for LT1, ST2, and LT3; all comparisons to baseline reported are on raw data using Dunnett's post hoc *t*-tests). This may have been due to repeated testing of the S-SWR group.

Despite the modest decline in control group responding, clear group differences emerged over the course of training. Short-term measures showed that each round of training produced

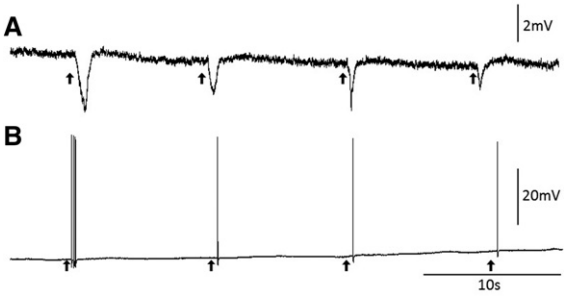

Figure 2. Examples of the response of VC sensory neurons to repetitive brushing stimulation in reduced preparations. Brush stimuli (arrows) were delivered at ~10-sec ISI. Most VC neurons tested exhibited hyperpolarization, a common response to off-field stimulation (*A*), 25% fired 1–2 spikes/stimulus (*B*), and 25% showed no response (data not shown). Note that *A* and *B* are not simultaneous recordings and that stimulus markers are approximate.

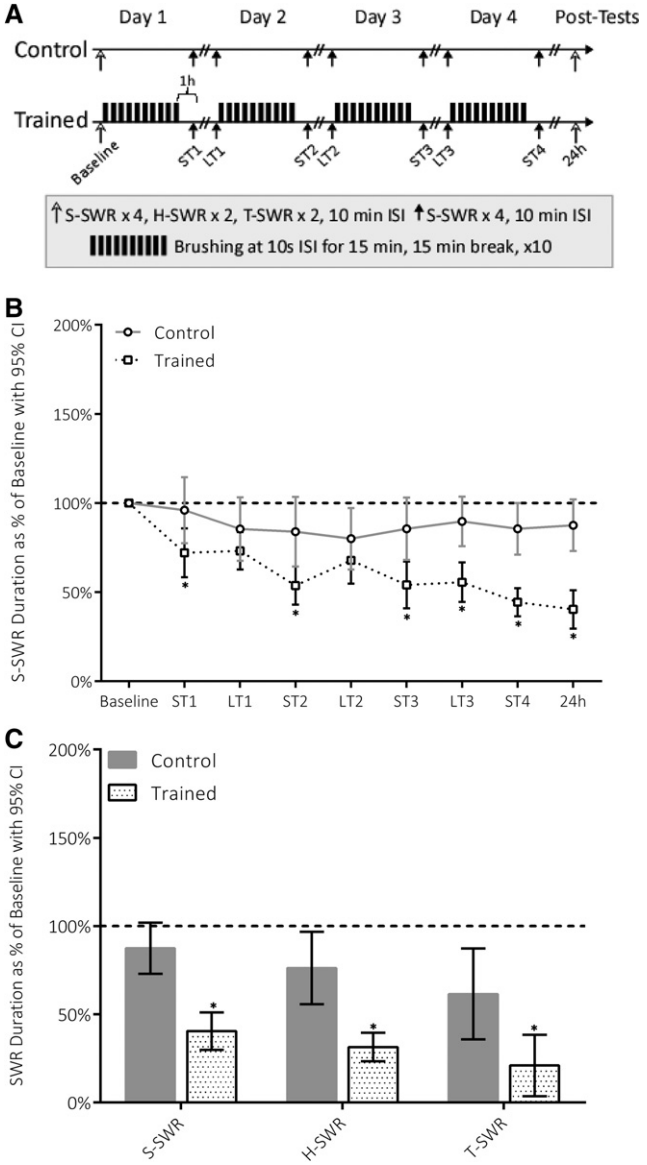

Figure 3. Short- and long-term effects of whole-body habituation training. (*A*) Experimental design. (Open arrows) SWR measures obtained by stimulating the siphon, head, or tail before (baseline) and 24 h after training. (Closed arrows) S-SWR measures obtained 1 h after each round of training to monitor short-term retention (ST1-4) or 1 h before the next round of training to measure long-term retention (LT1-3, ~14 h after the end of prior training). Alternating bars represent the daily habituation sessions given to trained animals. (*B*) Changes in S-SWR responses during and after training ($n = 12$/group). The dotted line at 100% indicates no change in behavior. Group means are shown with error bars representing 95% confidence intervals. Means labeled with (*) indicate a $P < 0.05$ for comparison between trained and control group with Holm–Sidak correction for multiple comparison. (*C*) Long-term habituation by site of stimulation. Shown are changes in S-SWR (siphon), H-SWR (head), and T-SWR (tail) responses 24 h after training by condition (same animals as in *B*). Label for significance same as in *B*.

significant short-term habituation in trained animals relative to both baseline measures ($P < 0.003$ for each comparison) and the control group ($P < 0.03$ for each comparison; all comparisons to controls are on normalized data with Holm–Sidak correction for multiple comparisons). Effect sizes were large and grew across each short-term measure (Cohen's $d = -0.9$, -1.2, -1.3, and -2.3 for ST1-4, respectively).

Early in training, S-SWR habituation faded somewhat overnight, with long-term tests the next morning reaching significance relative to baseline ($P < 0.0003$ for LT1 and LT2) but not relative to the control group ($P = 0.20$ for LT1; $P = 0.23$ for LT2). After 3 d of training, however, strong long-term habituation had fully developed, with responses at LT3 averaging only 56% of baseline. This was a significant decline relative to both baseline measures ($P < 0.0001$) and controls ($P = 0.0003$, $d = -1.7$). Long-term habituation was also retained after a fourth day of training (24 h versus baseline: $P < 0.0001$; 24 h trained versus control: $P < 0.0001$, $d = -2.4$). In the last two long-term tests there was almost no overlap in the behavioral responses of control and trained animals.

As hoped, long-term habituation was evident to stimuli applied to any site on the dorsal surface of the body (Fig. 3C). Specifically, H-SWR measures taken 24 h after training were decreased in trained animals relative to controls ($P < 0.0001$, $d = -1.8$). The same trend was evident in T-SWR responses in trained animals relative to controls ($P = 0.006$, $d = -2.9$).

These data indicate that our whole-body training system produces robust long-term habituation at SWR stimulation sites across the dorsal surface of the body. Because substantial long-term habituation is evident after 3 d of training, we adopted this shorter protocol for subsequent work.

Massed training does not produce long-term habituation

We next sought to develop a training protocol which could serve as a no-memory control for transcriptional analysis. Building on the work of Freudenthal et al. (1998) and others we sought to leverage the well-established differential effectiveness of massed versus spaced training. Specifically, we designed a massed training protocol in which animals receive the same cumulative number of stimuli (2700) as spaced animals do in 3 d of training, but with all stimuli delivered in a single, massed session (Fig. 4A).

To compare the effectiveness of the massed and spaced protocols, we used the same experimental design as in the previous experiment (Fig. 4A), but with only 3 d of consecutive training for the standard spaced condition.

As before, there were no initial differences in S-SWR behavior ($n = 14$/group, $M_{control} = 10.0$, $M_{trained} = 9.7$, $M_{massed} = 9.5$; SD = 1.7 in each group; $F_{(2,39)} = 0.37$, $P = 0.70$) nor in H-SWR ($F_{(2,39)} = 0.24$, $P = 0.78$) and T-SWR responses ($F_{(2,39)} = 1.03$, $P = 0.37$). Once training began, however, the groups diverged substantially in S-SWR responsivness (Fig. 4B), with a mixed-factorial ANOVA showing the expected interaction between condition and experimental phase ($F_{(12,234)} = 30.4$, $P < 0.0001$).

The control group exhibited stable responding, with S-SWR responses ranging between 94% and 99% of baseline over the course of the experiment (all comparisons to baseline nonsignificant).

Standard spaced training produced the same pattern of learning as in the previous experiment, with the progressive development of long-term habituation with each training session ($d = -0.98$, -4.29, -5.42 for LT1, LT2, and 24 h, respectively). In this case, comparisons at each time point reached significance against both baseline measures ($P < 0.0001$ for each comparison) and controls ($P < 0.02$ for each comparison). The effects of spaced training were also evident regardless of stimulation site for the

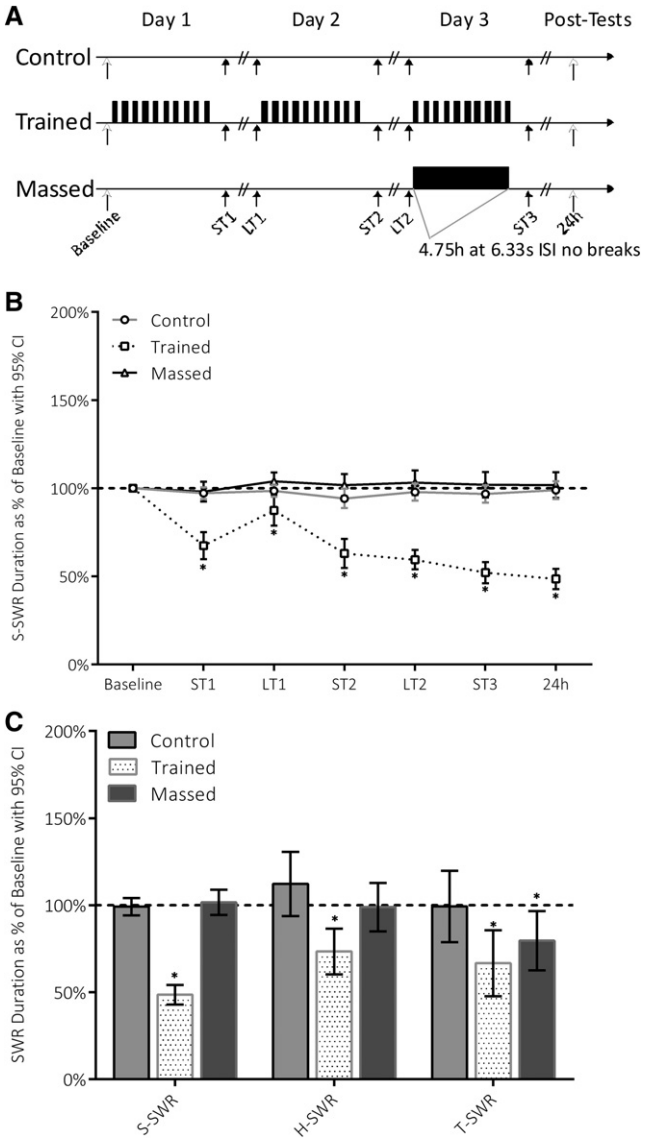

Figure 4. Differential effectiveness of massed and spaced protocols for whole-body habituation training. (*A*) Experimental design. Same as in Figure 2, but with a massed group receiving a single block of whole-body habituation training encompassing the same total number of stimuli as in massed training (2700) condensed into a single training session. (*B*) Changes in S-SWR responses during and after training ($n = 14$/group). Means labeled with (*) indicate a $P < 0.05$ for comparison between trained and control group with Holm–Sidak correction for multiple comparison. Contrasts from control to massed were not significant. (*C*) Long-term habituation by site of stimulation (same animals as in *B*). Shown are changes in S-SWR (siphon), H-SWR (head), and T-SWR (tail) responses 24 h after training by condition. Label for significance same as in *B*.

SWR (Fig. 4C): there was a long-term decline in H-SWR duration to 73% of baseline (to baseline: $P = 0.0007$; to controls: $P = 0.008$, $d = -1.4$) and in T-SWR duration to 67% of baseline (to baseline: $P = 0.001$; to controls: $P = 0.008$, $d = -0.96$).

Remarkably, massed training proved completely ineffective at producing both short-term and long-term habituation of the S-SWR. Mean responses ranged from 98% to 103% of baseline. These modest changes were not significantly different relative to controls ($P > 0.06$ for each comparison) nor in comparison with baseline measures ($P > 0.77$ for each comparison). Massed

training was similarly ineffective at producing long-term changes in the H-SWR, with responsiveness remaining at 99% of baseline levels, a nonsignificant different compared with both baseline ($P = 0.97$) and controls ($P = 0.32$). Bizarrely, we did observe a modest long-term decline in T-SWR responses, to 80% of baseline measures. This decline was statistically significant relative to both baseline measures ($P = 0.03$) and the control group ($P = 0.02$, $d = -0.6$). We did not, however, observe this pattern of response in subsequent experiments (see below).

Taken together, these data indicate that whole-body habituation is sensitive to the pattern of training. With the possible exception of long-term modification of the T-SWR, it seems that massed training could serve as a useful no-memory control for transcriptional analysis.

Massed training does not produce short-term habituation

Although we designed the massed training protocol to serve as a no memory control, it was surprising to see no change in behavior in the short-term measures taken each day after the end of training. This led us to wonder if the timing of the short-term measures missed some transient regulation after massed training.

To find out, we exposed animals to either 1 d of standard spaced training or the single-day massed training protocol ($n = 8$ per group, Fig. 5A). We then measured S-SWR durations starting just 10 min after training and continuing every 10 min for 1 h. In addition, we conducted long-term tests on these animals for the next 3 d to see if there are any late-developing changes in behavior following these training protocols.

Baseline responding was similar between both groups ($M_{spaced} = 5.9$, $M_{massed} = 5.7$, $t_{(14)} = 0.34$, $P = 0.73$), though somewhat less strong than in previous experiments. Training caused responding to diverge between groups, leading to a significant interaction between condition and test phase, $F_{(6,84)} = 2.35$, $P = 0.04$.

Remarkably, there was no short-term habituation evident following massed training (Fig. 5B), with all responses averaging 97%–103% of baseline ($P > 0.98$ for each comparison to baseline). In contrast, standard spaced training produced short-term habituation that was evident within 20 min from the end of training ($P < 0.003$ for each comparison to baseline). This produced a significant group difference at the 30, 40, and 50-min short-term tests ($P < 0.03$ for each comparison).

Long-term tests (Fig. 5C) showed no significant changes in S-SWR duration in the days following this 1-d protocol ($P > 0.97$ for each comparison to baseline; $P > 0.61$ for each comparison between groups).

Overall, these data confirm that the expression of habituation in *Aplysia* is remarkably sensitive to the pattern of training, with spaced training sufficient to produce robust short- and long-term training, but massed training completely ineffective at producing either form of habituation.

Mild noxious stimulation produces dishabituation but not sensitization

One of the key parametric features of habituation is the occurrence of dishabituation (Thompson and Spencer 1966), the rapid rescue of reflex responsiveness due to exposure to another (usually stronger) stimulus. Traditionally, the occurrence of dishabituation has been taken as evidence that the habituated response is not due to peripheral changes in the muscles (though see Giles and Rankin 2009).

To test for dishabituation (Fig. 6A), we exposed animals to either no brushing or 3 d of standard spaced training ($n = 8$/group).

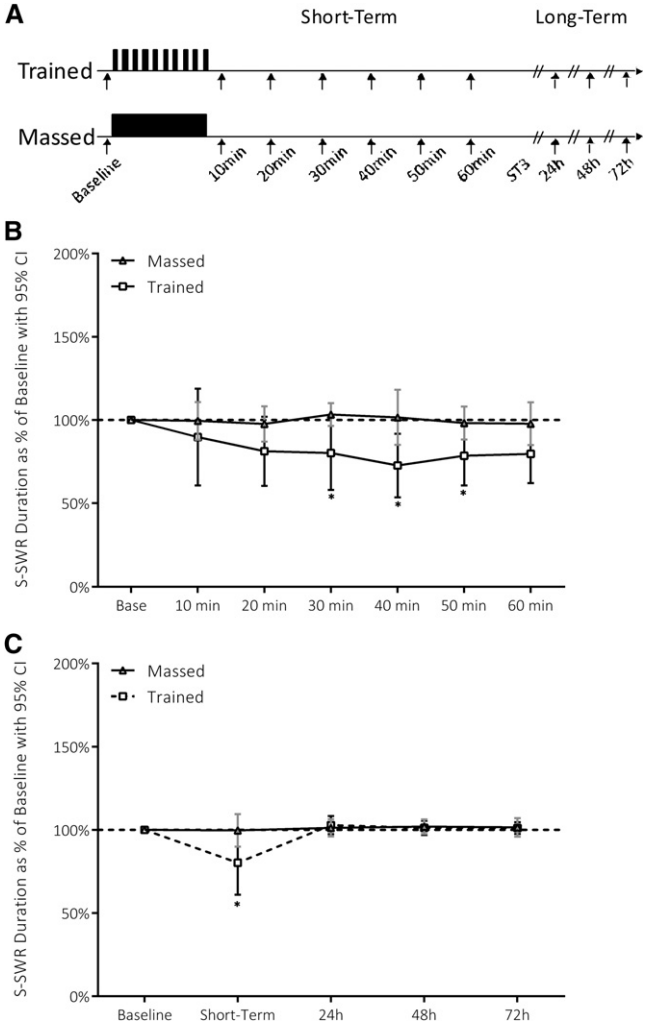

Figure 5. Dynamics of short-term habituation. (*A*) Experimental design. S-SWR durations were measured before and just after 1 d of standard spaced training or standard massed training. (*B*) Short-term changes in S-SWR responses ($n = 8$/group). Means marked (*) indicate a $P < 0.05$ for comparison between trained and massed group with Holm–Sidak correction for multiple comparison. (*C*) Test for delayed effects after a single day of training. Shown are changes in S-SWR responses 24, 48, and 72 h after training by condition. Label for significance same as in *B*.

After long-term S-SWR retention tests, animals in both conditions received a mild electrical shock to the tail (15 mA AC for 500 msec, repeated four times with 500-msec breaks between shocks), a level of stimulation insufficient to produce short-term sensitization of the S-SWR, but sufficient to produce dishabituation (see Hawkins et al. 1998; Marcus et al. 1988). The tail shock was followed by an additional four measurements of the S-SWR (10 min ISI); these were averaged together to represent dishabituated responding. To reduce the complexity of the experiment, H-SWR measures were not made. T-SWR measures were made prior to S-SWR measures to occur prior to the dishabituation test. In addition, at the suggestion of a reviewer, we ran a follow-up study of animals treated exactly as the trained animals but exposed only to a sham dishabituating shock ($n = 8$, training + sham condition).

Results during training replicated those from the previous experiments. Untrained + shock and trained + shock animals began with similar S-SWR ($t_{(14)} = 0.23$, $P = 0.81$) and T-SWR durations ($t_{(14)} = 0.40$, $P = 0.69$). As training progressed, though, the groups

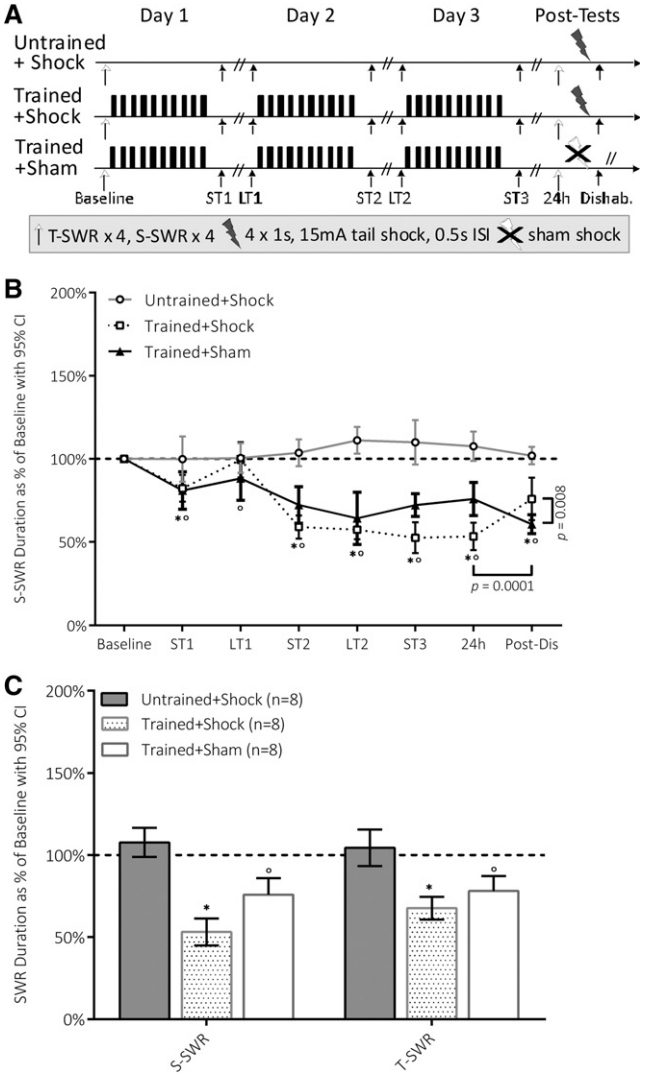

Figure 6. Dishabituation to moderate shock. (*A*) Experimental design. Same as in Figure 2, but with a moderate tail shock (untrained + shock, trained + shock) or sham shock (trained + shock) administered after 24-h tests but just before a set of dishabituation measures. Also, both baseline and 24-h measures consisted of 4 T-SWR measures followed by 4 S-SWR measures. (*B*) Changes in S-SWR responses during and after training ($n = 8$/group). Means labeled (*) or (°) indicate a $P < 0.05$ for comparison with the untrained + shock condition for the trained + shock and trained + sham conditions, respectively, with Holm–Sidak correction for multiple comparison. There was a significant change in "both" trained + shock and trained + sham conditions after the dishabituating/sham shock, but in opposite directions, leading to a significant difference between these groups in the dishabituation measures. (*C*) Long-term habituation by site of stimulation (same animals as in *B*). Shown are changes in S-SWR (siphon), and T-SWR (tail) responses 24 h after training by condition. Label for significance same as in *B*.

diverged markedly in S-SWR durations (Fig. 6B), leading to a significant interaction between group and test phase ($F_{(7,98)} = 25.5$, $P < 0.0001$).

As expected, responding in the untrained + shock condition remained stable throughout the experiment. No responses differed significantly from baseline, even after the dishabituating shock ($P > 0.12$ for each comparison).

In the trained + shock condition, training again produced increasing levels of short- and long-term habituation in the S-SWR. In this case, there was enough forgetting after the first day of training that the first long-term test (LT1) was not significantly different compared with baseline ($P = 0.99$) or controls ($P = 0.86$). All other comparisons, however, were significant ($P < 0.002$ compared with baseline; $P < 0.02$ compared with controls), and there was again a progression of effect sizes in each long-term test ($d = -0.1$, -6.4, -5.3 for LT1, LT2, and 24 h, respectively). In addition, T-SWR responses also showed significant long-term habituation relative to baseline ($P < 0.0001$) and controls ($P < 0.0001$, $d = -3.3$, Fig. 6C).

Critically, the tail shock produced significant dishabituation for previously trained animals, with responses rising from 54% of baseline prior to shock to 76% of baseline afterward (Fig. 6B). This was a significant increase in responsiveness ($P < 0.0001$ comparing 24 h to post-dishabituation measures, adjusted for multiple comparisons), though still significantly lower than initial baseline ($P < 0.0001$) and control measures ($P = 0.0005$, $d = 2.2$).

In the separately run training + sham condition, training again produced robust long-term habituation. S-SWR measures were significantly reduced from baseline ($P < 0.01$) at all time points. Comparisons to the previously run untrained + shock condition were also significant at all times points ($P < 0.02$) except LT1 ($P = 0.09$) and showed a similar, though weaker, progression in long-term effect sizes ($d = -0.9$, -3.1, -2.8 for LT1, LT2, and 24 h). Long-term habituation was also evident in T-SWR responses relative to baseline ($P < 0.0001$) and the untrained + shock condition ($P = 0.0007$, $d = -2.2$). Critically, application of the sham shock did not produce dishabituation of S-SWR responses. On the contrary, sham treatment and repeated S-SWR measurement produced further habituation, with post-treatment measures further reduced relative to 24-h measures ($P < 0.001$ adjusted for multiple comparisons). Although the previously run trained + shock group had expressed stronger S-SWR habituation at the 24 h measure ($P = 0.0001$ corrected for multiple comparisons), after the dishabituating shock the trained + shock group expressed significantly weaker habituation than the trained + sham group ($P = 0.008$ corrected for multiple comparisons). This demonstrates that the dishabituation produced by tail shock is not due to the decay of habituation during testing.

Twenty-one transcripts in the pleural ganglia are persistently regulated by long-term habituation training

We next used microarray analysis to characterize the persistent transcriptional changes evoked by whole-body habituation training.

First, we exposed a new set of animals ($n = 12$/group) to 3 d of standard spaced training, massed training, or no training (controls). To simplify the experiment, we collected only a single short-term measure after the first day of training and a single long-term measure 24 h after the end of all training (Fig. 7A).

We again observed the same pattern of learning (Fig. 7B), with a significant interaction between condition and test phase indicating that group differences emerged over the course of training ($F_{(4,66)} = 36.7$, $P < 0.0001$). Control and massed-trained animals showed no long-term changes in behavior ($P > 0.19$ for each comparison to baseline; $P > 0.12$ for each comparison from massed to control). In contrast, animals given standard spaced exhibited long-lasting decreases in T-SWR, H-SWR, and S-SWR durations ($P < 0.0001$ for each comparison to baseline; $P < 0.02$ for each comparison to control). Effect sizes at 24 h were again quite large, with $d = -2.6$, -3.7, and -4.1 for T-SWR, H-SWR, and S-SWR, measures, respectively (Fig. 7C).

Immediately after the long-term tests, the pleural ganglia were harvested from each animal. We then used two-color

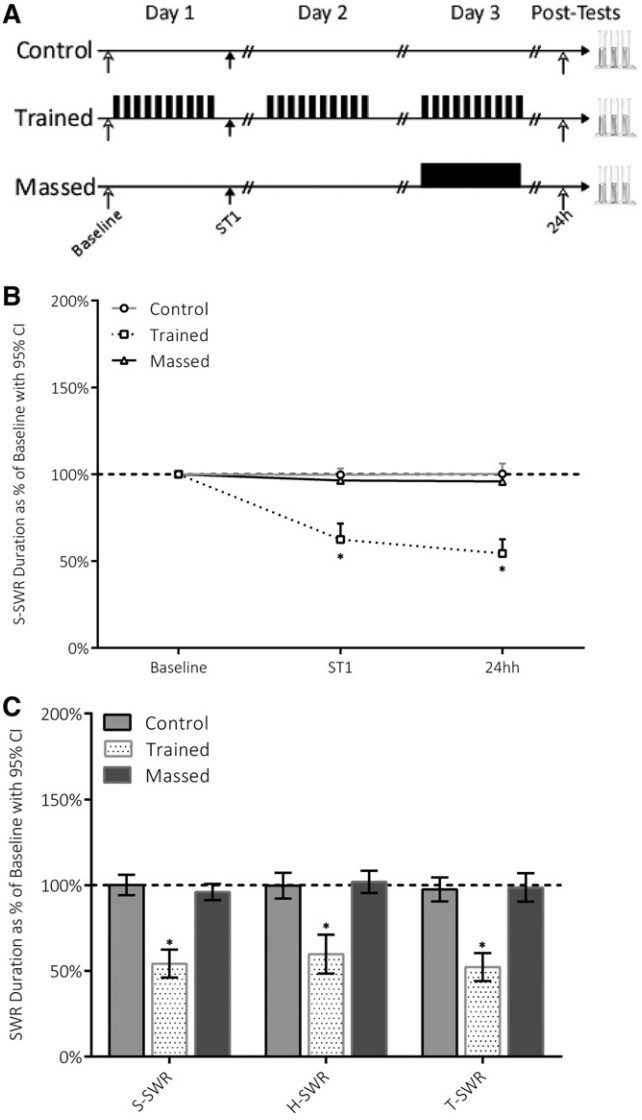

Figure 7. Long-term habituation for microarray experiment. (*A*) Experimental design. Same as in Figure 4, but only ST1 and 24-h measures were made after training. Immediately after 24-h tests, each animal was sacrificed and pleural ganglia were harvested for transcriptional analysis (test tubes). (*B*) Changes in S-SWR responses during and after training ($n = 12$/group). Means labeled (*) indicate a $P < 0.05$ for comparison between trained and control group with Holm–Sidak correction for multiple comparison. Contrasts from control to massed were not significant. (*C*) Long-term habituation by site of stimulation (same animals as in *B*). Shown are changes in S-SWR (siphon), H-SWR (head), and T-SWR (tail) responses 24 h after training by condition. Label for significance same as in *B*.

microarray to directly compare gene expression from eight matched sets of trained and control animals (eight different microarrays, each with one matched set). This left four additional sets of animals to provide a test of generalizability (see below). Massed-trained animals were not analyzed at this stage. Holding back the massed condition allowed us to use a two-color design directly comparing control and trained animals, increasing statistical power while decreasing the number of microarrays needed for the analysis. In addition, this provided an independent set of samples for comparing to trained animals during qPCR validation (see below). The microarray platform used (*Aplysia* Tellabs Array) includes 26,149 distinct probes selected to represent all currently known *Aplysia* ESTs and mRNAs (see Materials and Methods).

Comparing trained to control expression revealed that whole-body habituation training produces persistent regulation of 21 transcripts in the pleural ganglia (see Table 1, 14 up-regulated, seven down-regulated, $P < 0.05$ adjusted for multiple comparisons).

Whole-body habituation training persistently and specifically up-regulates cornichon expression

From the set of 21 regulated transcripts, we focused on EB295883.1, an EST that aligns to a refSeq mRNA annotated as a cornichon homolog (GenBank: XM_005106771.1). We refer to it here as ApCornichon. ApCornichon was the only transcript among those regulated to pass an even more stringent test of regulation (>1.1-fold change in either direction, see Table 1). We have previously found with this microarray platform that transcripts meeting this stringent criterion are very likely to generalize to independent samples (Herdegen et al. 2014). Indeed, in limited testing of 11 other transcripts which did not meet this more stringent criterion, none of them reached statistical significance in qPCR analysis (see Table 1).

To confirm regulation of ApCornichon, we used qPCR, measuring expression in the entire set of 12 animals from all three conditions (control, standard spaced training, and massed training). As expected (Fig. 8), we found that the expression of ApCornichon is significantly higher in trained animals than in controls (mean fold change ("MFC") = 1.33 [1.01, 1.78], $d = 0.65$, $t_{(11)} = 2.3$, $P = 0.046$). Critically, this included up-regulation in all four of the sets of animals which had not been included in microarray analysis.

ApCornichon was not up-regulated by massed training, and instead showed a nonsignificant trend toward down-regulation (MFC = 0.74 [0.53, 1.01], $d = -0.61$, $t_{(11)} = -2.1$, $P = 0.057$). Thus, there was a statistically significant difference in cornichon expression when comparing standard spaced training to massed training ($d = 1.02$, $t_{(11)} = 3.5$, $P = 0.005$).

Taken together, these data indicate that the formation of long-term habituation memory is accompanied by a selective and persistent up-regulation in the expression of ApCornichon.

Discussion

The whole-body training apparatus we developed produces robust habituation of the SWR. Several of the 10 defining parametric features of habituation (Thompson and Spencer 1966; Rankin et al. 2009) are evident with this paradigm: increasing response decrement with increasing repetition of stimulation (characteristic #1), generalization to other stimuli (characteristic #7), the occurrence of dishabituation after presentation with another stimulus (characteristic #8), and the development of long-term habituation with extended training (characteristic #10).

In addition to producing these classic features of habituation, the paradigm we have developed meets our criteria for facilitating transcriptional analysis: modest activation of the VC nociceptors, strong effects across a large portion of the body, and the availability of a no-memory control (massed training). Indeed, we were able to measure consistent and persistent transcriptional changes evoked by this training protocol with both microarray and qPCR. To our knowledge, this is only the second comprehensive analysis of the transcriptional correlates of long-term habituation (see Dong et al. 2009).

Our microarray analysis revealed a relatively small set of putatively regulated transcripts 24-h after long-term habituation

Table 1. Table of transcripts regulated in the pleural ganglia 24 h after standard spaced long-term habituation training EST Accession gives the GenBank Accession number for the transcript used to design the microarray probe

EST accession	Microarray mean fold change	Adjusted *P* value	qPCR validation (if conducted)	Annotation
EB295883.1	1.42	0.0053	Validated	PREDICTED: *Aplysia californica* protein cornichon homolog 4-like (LOC101864174), mRNA
EB259494.1	1.44	0.0260	Not validated	PREDICTED: *Aplysia californica* atrial natriuretic peptide receptor 3-like (LOC101856817), transcript variant X1, mRNA
EB195205.1	0.80	0.0260	Not validated	Transcribed locus
FF062939.1	0.70	0.0260	Not validated	Transcribed locus
EB218640.1	1.43	0.0260	Not validated	Transcribed locus
EB239689.1	1.64	0.0260	Not validated	PREDICTED: *Aplysia californica* *N*-α-acetyltransferase 15, NatA auxiliary subunit-like (LOC101851279), transcribed variant X2, mRNA
FF064783.1	1.44	0.0260	Not validated	Transcribed locus
GD220643.1	0.66	0.0260		Transcribed locus
GD198180.1	1.27	0.0260		Transcribed locus
EB321038.1	1.54	0.0260		Transcribed locus
EB326237.1	0.70	0.0260		Transcribed locus
EB311506.1	1.45	0.0282	Not validated	PREDICTED: *Aplysia californica* neural proliferation differentiation and control protein 1-like (LOC101848620), mRNA
EB255983.1	1.55	0.0282		Transcribed locus
EB239637.1	0.70	0.0294	Not validated	Transcribed locus
EB259771.1	1.59	0.0294		Transcribed locus
EB305488.1	1.50	0.0294	Not validated	PREDICTED: *Aplysia californica* striatin-3-like (LOC101854025), transcript variant X4, mRNA
EB289764.1	0.80	0.0329	Not validated	Transcribed locus
EB216921.1	1.53	0.0440		Transcribed locus
EB298055.1	1.52	0.0485		Transcribed locus
GD232191.1	0.70	0.0485		Transcribed locus
EB196623.1	0.76	0.0490	Not validated	PREDICTED: *Aplysia californica* sodium/ potassium-transporting ATPase subunit α-like (LOC101857391), mRNA

Microarray mean fold change gives the average of the ratio between trained and control animals across all eight biological replicates. The column of *P* values gives statistical significance of the comparison from trained to control. Note that this column reports raw fold change, not a log-transformed change. qPCR validation reports if same result (at $P < 0.05$) was obtained using qPCR on all 12 sets of animals (eight from microarray + four additional generalization samples). No entry in this column indicates that transcript was not tested. Annotation provides the annotation for that transcript drawn from either UniGene or, when available, the RefSeq mRNA for that EST.

training (21 transcripts at $P < 0.05$ after adjustment for multiple comparisons, 26,092 targets tested). This is considerably less than the 1494 transcripts we found regulated immediately after long-term sensitization training using the same array platform, tissue sample, sample size, and statistical criteria (Herdegen et al. 2014). The most likely explanation is a difference in statistical power due to different experimental designs. The whole-body habituation paradigm we developed is a between-subjects experiment (comparing pleural ganglia from separate trained and control animals), whereas long-term sensitization in *Aplysia* is administered as a within-subjects experiment (comparing pleural ganglia from trained and untrained sides of the same animal). It is worth considering, however, that the number of regulated transcripts we observed is not abnormal for learning and memory microarray studies. For example, Levenson et al. (2004) found only 38 regulated transcripts in the CA1 subfield of the hippocampus following contextual fear conditioning ($n = 3$, $P < 0.05$ without correction for multiple comparisons, 12,420 targets tested). In *Aplysia kurodai* (Lee et al. 2008), exposure to serotonin, which mimics long-term sensitization training, is associated with rapid regulation of 27 transcripts ($n = 2$, fold change >2, 6912 targets tested).

In a microarray analysis of habituation in songbird, over 3000 transcripts were found to be up- or down-regulated relative to controls exposed only to silence (Dong et al. 2009). However, the experimental condition was not only habituated, but also exposed to a reminder cue just before sacrifice. Thus, this comparison mixes transcriptional changes due to both the habituation trace and recent activity. This may partly explain why we observed so many fewer regulated transcripts, though differences in species, tissue type, sample size, and statistical criteria also can greatly impact microarray analysis.

We have focused our initial transcriptional analyses on the pleural ganglia, which contain the VC nociceptors activated by our training protocol. In addition, the pleural ganglia contain a variety of other cell types. This includes a set of interneurons which inhibit the VCs (Mackey et al. 1987; Buonomano et al. 1992), motor neurons controlling the opaline-gland (Tritt and Byrne 1980) and mucus secretion (Rayport et al. 1983), and a number of additional cell clusters and types which have not been fully characterized (Fredman and Jahan-Parwar 1979).

Our focus on the pleural ganglia is purely pragmatic. We expect that our long-term habituation protocol produces widespread changes in the SWR circuit (possibly including peripheral components of this circuit), as occurs in other learning paradigms in *Aplysia* (Frost et al. 1988; Falk et al. 1993). The pleural ganglia are an attractive target for transcriptional analysis, however, because the VCs make up a relatively large proportion of these ganglia (in comparison, for example, to the LE nociceptors in the abdominal ganglion), and because we could easily confirm that the training apparatus activates the VCs. We have specifically found that the pleural ganglia provide a strong signal/noise ratio for detecting learning-regulated transcripts (manuscript in prep.).

Within the pleural ganglia, we found that long-term habituation training is accompanied by a long-lasting increase in the expression of a putative *Aplysia* cornichon homolog (ApCornichon). This up-regulation is specific to the formation of long-term habituation memory, as it does not occur following massed training.

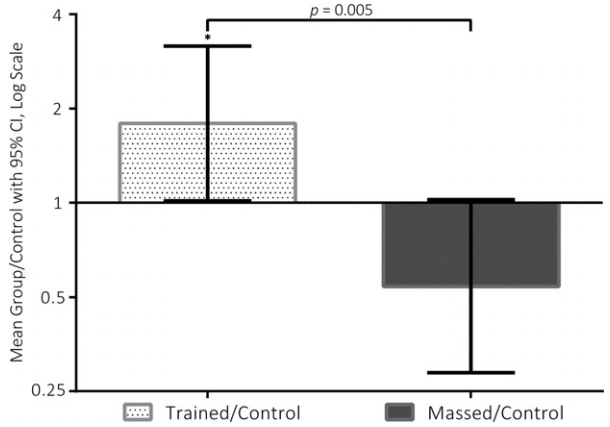

Figure 8. Regulation of cornichon after long-term habituation training. Mean fold change in cornichon expression in standard spaced-trained animals relative to controls (black) and massed-trained animals (gray) relative to controls ($n = 14$/group). Each bar represents a group mean with 95% confidence interval. A $\log_2$ scale is used to give equal weight to up- and down-regulated transcripts. The dotted line at 1 represents no regulation (equal expression relative to controls). (*) indicates expression is significantly different from control at $P < 0.05$. Significance of paired comparisons from spaced to trained animals is marked with brackets.

Moreover, we have confirmed this finding using qPCR, including a test for generalization to an independent data set.

Cornichon was first identified in *Drosophila* as a member of a highly conserved family of proteins that serve critical functions in development (Roth et al. 1995) and in protein transport from the endoplasmic reticulum (Bökel et al. 2006). In the CNS, a proteomic screen has revealed that two vertebrate homologs of cornichon (CNIH2 and CNIH3) can serve as auxiliary subunits of AMPA receptors (Schwenk et al. 2009). While the physiological functions of vertebrate cornichons are complex (e.g., Kato et al. 2010; Herring et al. 2013), in invertebrate models cornichon is proposed to limit the exportation of GLR-1 receptors from the endoplasmic reticulum to the cell surface (Brockie et al. 2013). Specifically, overexpression of cornichon in *C. elegans* decreases synaptic expression of GLR-1 receptors and reduces evoked glutamate currents; loss-of-function mutants exhibit the opposite phenotypes. It is interesting to consider that in *C. elegans*, long-term habituation produces decreased synaptic expression of the GLR-1 glutamate receptor (Rose et al. 2003), an effect that could also be consistent with increased cornichon function. We hypothesize that the *Aplysia* cornichon homolog may function in a similar manner and that an increase in its expression may contribute to a reduction in AMPAR surface expression that could limit evoked SWR output. We are now working to confirm if ApCornichon does indeed function as a regulator of glutamatergic signaling in *Aplysia*, and if so which cell types in the SWR circuit express this protein.

At this point, we have only analyzed transcriptional changes occurring 24 h after training. The acquisition of long-term memory, however, is associated not only with persistent transcriptional changes but also with immediate transcriptional changes that help encode the long-term memory (e.g., Alberini 2009). We are currently working to characterize the rapid transcriptional response to long-term habituation training.

One puzzling aspect of our results is the complete ineffectiveness of massed training, which failed to produce even short-term habituation following 2700 back-and-forth brushes. One possibility is that the effectiveness of massed training was blunted not only by the pattern of training but also by the more rapid ISI (6.33 sec compared with 10 sec). For example, repetitive tactile stimulation of the siphon at short ISIs (1 sec) produces much larger decrements in sensory input than with longer ISIs (30 sec) (Fischer et al. 2011). This could enable shorter ISIs to prevent downstream activation of the SWR circuit during repeated stimulation, perhaps gating plasticity that otherwise could be expressed there (e.g., Calin-Jageman and Fischer 2003a). Another possibility is that the massed training protocol involves so much stimulation that it activates a mixture of habituation and sensitization processes, leading to offsetting neuronal changes and stable behavior.

Materials and Methods

We report how we determined our sample size, all data exclusions (if any), all manipulations, and all measures in these studies. Raw data files, analysis files, a detailed material list, and a protocol video are posted to the Open Science Framework (https://osf.io/6ew4i/). Our microarray data are also posted to GEO (accession number: GSE59448, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59448).

Animals

Animals (75–125 g) were obtained from the RSMAS National Resource for *Aplysia* (Miami, FL) and maintained at 16°C in one of two 90-gallon aquariums with continuously circulating artificial sea water (Instant Ocean, Aquarium Systems Inc.). Animals were separately housed in rectangular colanders (approximate dimensions: 11.5-cm wide, 16.5-cm long, 7.3-cm deep), fed dried seaweed twice a week, and maintained on a 12-h light–dark cycle. Two days prior to any experimental testing, animals were fed to satiation and then food deprived. This was done because feeding can alter reflex responsiveness (Advokat 1980) and also to match procedures for long-term sensitization protocols (Wainwright et al. 2002) so that our results with habituation can be compared with those for sensitization. For the experiment examining the decay of habituation animals were placed back on a regular feeding schedule after the 48-h tests, and were again food deprived starting 2 d prior to the 7-d tests. All other animals were food deprived throughout the experiment. To control for batch/shipment effects, animals from at least two different shipments were used for each experiment.

A whole-body habituation training apparatus

Whole-body habituation training was applied via a "slug car wash" (Fig. 1; Supplemental Video 1). The apparatus consisted of a long brush (41 cm) mounted on a control arm attached to a windshield-wiper motor (MSS41534C02, Schmitt and Ongaro Marine). Rotational force from the motor swept the brush forward and then backward through an arc of 110°, dragging the bristles of the brush through the home colanders of up to four animals. The brush moved at a rotational speed of ~157°/sec (0.7 sec per stroke).

The motor for the apparatus was connected via both the low-speed and return lines to a DC power supply (HY1802, RSR Electronics) providing 2 A of current at 12 V. The connection to low-speed line, however, was gated by a computer-controlled relay board. Making a transient (0.5 sec) connection on this line would thus activate a forward sweep of the brush. When the relay was closed the constant power to the return line would complete any remaining movement on the forward stroke and then complete a backward stroke to return the brush to its home position. We considered a combined forward/backward stroke to constitute a single stimulus for training. The precise timing of training stimuli was controlled by custom software written in QuickBasic running on FreeDOS.

The brush contained a single row of nylon bristles 9-cm long. Each produced a bending force of ~3 g_F (29 mN) when applied length-wise to a laboratory-grade electronic balance. As a reference, we compared the bristles to a set of calibrated Von Frey hairs (Baseline Sensory Evaluation Kit, Fabrication Enterprises). We

found best match in diameter and stiffness to the 26 g_F (255 mN) monofilament from this kit (part #12-1655).

The brush was mounted over the tank so that it was 5-cm above the water surface at the midpoint of each sweep. Colanders were held with their rims ~2-cm above the water surface (see below). Thus, with each stroke the bristles on the brush were dragged through the colanders, reaching at the midpoint of the stroke 4-cm down into the tank water and within 1.3 cm of the colander bottoms. Based on pilot testing, we established a spacing of 1.5–2 bristles/cm along the length of the brush. With home colanders 11.5-cm wide, each animal had 16–23 bristles swept through its home colander on each stroke of the motor.

To expose animals to the training apparatus, their home colanders were placed in a rack suspended below the surface of the water so that each colander top reached just above the surface of the water. This enabled exposure to the brush but prevented animals from crawling out of their colanders. Each rack held four colanders. Identical racks were available for holding sets of animals receiving different training protocols.

Animals remained free to move in their colanders, but generally remained attached via their ventral foot. Thus, each stroke of the brush inevitably caused multiple bristles to drag across the animal, producing tactile stimulation across much of its dorsal surface. The stimulus was gentle: it usually produced some initial withdrawal responses early in training, but these typically faded within 5–10 stimuli. Moreover, the brushing was not strong enough to dislodge animals from their holdfasts. Inking never occurred except in two animals from a shipment which had to be discarded due to poor health (see below). However, brushing often caused lines to appear on the skin of stationary *Aplysia*—depressions following the track where each bristle whisked across the skin. These faded after training.

Animal exposure to the brush stimulus was influenced by their position within their home colander. Some postures and positions could shield portions of the dorsal surface from the brush (e.g., crawling directly below the brush enabled the tail to be out of reach of the bristles). However, training duration was very long, and animals adopted a range of holdfasts across training. Thus, it was inevitable that all portions of the dorsal body surface would receive considerable stimulation.

We built two identical training systems, both powered by the same computer and relay board. This enabled simultaneous training of both massed and spaced animals (see below), with training system counterbalanced across conditions.

Behavioral measurement

Siphon-withdrawal reflexes (SWR) were evoked by brief stimulation (flick) with a stiff nylon bristle glued to the end of a plastic rod (~6 g_F). To determine the spatial extent of habituation, stimuli were applied to three different body sites: the inner lumen of the siphon (evoking the siphon-elicited SWR, S-SWR), the dorsal surface of the tail (evoking the tail-elicited SWR, T-SWR), or the head just anterior to the rhinophores (evoking the head-elicited SWR, H-SWR). Responses were timed from the beginning of siphon contraction to the first visible sign of relaxation by an observer (G.H.) blind to the experimental condition (Supplemental Video 2), except in the trained + sham condition of the dishabituation experiment (see below). SWR measurements were taken at a 10-min interstimulus interval (ISI), an interval which does not typically produce habituation on its own (e.g., Calin-Jageman and Fischer 2003b). Responsiveness at each stage of the experiment was characterized as the average of 4 (S-SWR) or 2 (T-SWR, H-SWR) responses.

To ensure the reliability of SWR measurements, trials from eight animals were videotaped and scored by a second rater also blind to the experimental condition. Recordings were made at four time points, producing 32 independent measurements. Interrater reliability was good ($r = 0.73$), and scores from the second rater also indicated significant habituation in only trained animals. This analysis was conducted after the first experiment reported (Fig. 3), subsequent experiments have a notable decrease in variation in normalized responses due to increased measurement reliability, which is likely due to the training provided by the interrater reliability analysis.

Habituation protocols

Based on pilot testing we developed a standard "spaced" habituation protocol consisting of 10 sessions of brushing a day, with each session consisting of 15 min of stimulation at a 10-sec ISI followed by a 15-min rest (Fig. 2A). Thus, each day's training lasted 285 min and contained 900 back-then-forth brush stimuli. We applied this training protocol for three or four consecutive days. This protocol involves significantly more stimulation than in previous studies of long-term habituation in *Aplysia* (e.g., 20 stimuli for each of 5 d; Castellucci et al. 1978). The intention for our procedure, however, was to produce the strongest and most reliable behavioral impact possible.

Massed training consisted of a single session lasting 285 min at an ISI of 6.33 sec with no rest periods (Fig. 3A). This yielded 2700 stimulations in a single session, the same cumulative number of stimuli delivered over 3 d of spaced training, but compressed into the same amount of time required for one day's spaced training. This massed protocol confounds changes in ISI (10 sec versus 6.33 sec) with pattern of training (breaks versus no breaks). The goal, however, was to provide a no-memory control exposed to the same total amount of stimulation.

Control animals were placed in a holding rack but without a brushing apparatus. Thus, they experienced the same colander handling and the same exposure to the surface of the tank as trained animals.

To monitor the effectiveness of LTH training, siphon-, tail-, and head-elicited SWR responses were measured before (pretest) and after the end of training (1, 2, or 7 d). To monitor the progress in acquiring LTH, the siphon-elicited SWR was also measured 1 h before and 1 h after each training session. Measurements taken 1 h after training provide a measure of short-term retention (labeled ST in figures). Measures taken 1 h before training provide a measure of long-term retention from the previous day's training (~14 h from the end of the prior day's training; labeled LT in figures). Once all training was completed, long-term measures were timed to be precisely 24 h, 48 h, or 7 d from the end of training.

To maintain blinding, different researchers were responsible for SWR measurement and training.

Protocols (including SWR measures before and after training) were always begun early in the light cycle of the animals (within 2 h of light onset).

Dishabituation

To produce dishabituation, a mild but noxious train of tail shocks was applied using a hand-held electrode connected to a constant-current stimulator (WPI Constant-Current Stimulator) triggered to apply a 60-Hz biphasic DC pulse. Each train consisted of four shocks of 15 mA lasting 0.5 sec with a 0.5-sec break between each shock. This stimulus level was selected based on pilot testing because it is too mild to produce robust sensitization in untrained animals, but strong enough to produce dishabituation in habituated animals (see Marcus et al. 1988; Hawkins et al. 1998). The researcher measuring behavior observed the shocks but was blind to the training status (untrained + shock or trained + shock). At the suggestion of a reviewer, a third condition (trained + sham) was run in which animals received standard training followed by a sham shock (4 sec of application of the shock wand, no current passed). This condition was run at a later time, so no matching or blinding was used this condition. In addition, a different researcher (C.C.) collected these data, but was not briefed on expectations for the effects of treatment.

Experimental design

We adopted a matched-control design. Animals from the same shipment were pretested in batches of 8 or 12. After pretesting, animals were ranked by average S-SWR responsiveness and then alternately assigned to experimental conditions. Matching

animals in this way ensures equal responsiveness at the start of training as well as equal representation of different initial response levels across experimental conditions.

Quality controls

It has previously been reported that some shipments of wild-caught animals do not reliably exhibit long-term habituation memory (Ezzeddine and Glanzman 2003). In the 16 shipments of cultured animals used across all these experiments, 2 shipments exhibited health problems which required exclusion from training and analysis. Specifically, in both of these shipments we observed global and substantial decreases in the responses of both trained and control animals ($n = 4$ tested per condition in each shipment). In addition, inking occurred during the first training day in both control (1 of 4) and trained (2 of 4) animals from one of these shipments. We have not included data from these animals here, and did not train further animals from these shipments.

Electrophysiology

To measure the evoked response to the brush stimulus we used a reduced preparation. Briefly, animals were anesthetized with an injection of isotonic 333 mM $MgCl_2$ (50% of body weight). An incision was then made along the ventral midline enabling the removal of the gut. Connective tissue anchoring the anterior CNS ganglia (buccal, cerebral, pedal, and pleural) was trimmed away, as well as the nerves connecting to the anterior portions of the body (e.g., P1, P2, P3, and P5 in the pedal ganglia). Then the anterior body was removed by making a cut across the dorsal surface of the skin just posterior to the ring ganglia. This preserved almost the entire body posterior to the head and its innervation but allowed the anterior ganglia of the CNS to protrude. The prep was then placed in a recording chamber perfused with artificial sea water and the anterior CNS ganglia were pinned to a raised sylgard-coated platform. The anterior edge of the body (which had lost most innervation) was pinned adjacent to the recording platform. The rest of the body (siphon/mantle/gill complex, parapodia, tail) was left free in the dish. This was done to avoid sensitizing effects due to pinning (Illich and Walters 1997), though this allowed the body to move during brushing. The body was cannulated on the left and right side of the cut edge, and continuously perfused with artificial sea water for a 2-h recovery and throughout recording. After recovery, preparations were discarded if they did not show robust tail- and siphon-elicited withdrawal responses as well as spontaneous body movements (1 out of 8 preps discarded).

VC neurons were recorded using standard sharp-electrode intracellular physiology. Glass electrodes were filled with 3 m KCl (resistance 10–15 MΩ). VC neurons were identified by their distinctive location, color, and rapidly adapting response to intracellular current injection. To ensure quality of recording, VC neurons were used for analysis only if their resting membrane potential was under −40 mV and evoked spike amplitude was >70 mV from peak to peak (Liao et al. 1999).

Isolation and processing of RNA

Because dissection can alter the expression of some *Aplysia* immediate-early genes (Alberini et al. 1994) we harvested tissue samples rapidly, usually within <5 min from anesthetization. Briefly, animals were anesthetized with an injection of isotonic 333 mM $MgCl_2$ (50% of body weight). A ventral incision was then made enabling the rapid extraction of the pedal, pleural, and/or abdominal ganglia.

Tissue was rapidly homogenized and RNA extracted using TRIzol (Invitrogen) and Direct-Zol Mini RNA Kit (Zymo). Samples were homogenized using the Bullet Blender (NextAdvance). Quantity and quality of RNA was assessed using the NanoDrop 1000 (Thermo Scientific).

Microarray analysis

We used the Aplysia Tellabs Array (ATA: GEO: GPL18666) to characterize changes in gene expression due to long-term habituation training. The array was designed using the UniGene clustering of *Aplysia* ESTs (build 9, July 2011, ftp://ftp.ncbi.nih.gov/repository/UniGene/Aplysia_californica/) and includes 2–3 probes designed for each of the 24,702 distinct EST clusters identified by Unigene, 15 *Aplysia* mRNAs deposited in GenBank since the latest UniGene build, and 1432 orphaned probes from two previous *Aplysia* microarray designs (the *Aplysia* Discovery Array (GEO:GPL3635) and the updated *Aplysia* Annotated Array (GEO: GPL13815/GPL17112)). Thus the ATA design includes 26,149 distinct probes representing all known sources of *Aplysia californica* ESTs and mRNAs at the time of design (January 2012). Based on estimates from previous microarray designs (Moroz et al. 2006), the ATA should cover >50%–60% of all neurally expressed transcripts. Complete details on the ATA design are in Herdegen et al. (2014).

Microarray processing was completed by Mogene Inc. A two-color approach was used with each array hybridized to a sample from a trained or untrained animal. In half of cases, trained samples were hybridized with Cy3 and controls to Cy5; the other half we dye-swapped.

Sample integrity was determined by Bioanalyzer RNA 6000, Pico total RNA protocol. Three hundred nanograms of total RNA was amplified and labeled with Cy3 or Cy5 using the Agilent Quick Amp Two-Color Labeling Kit. Dye incorporation and yield was determined by Nanodrop. Samples were hybridized to the microarray slide at 65°C and 10 rpm for 17 h. Slides were scanned on an Agilent C scanner at 3 μm resolution. Data were extracted using Agilent Feature Extraction software, v. 11.5. All labeling and post-labeling processing was carried out in an ozone regulated environment, monitored at <5 ppb.

Microarray data were analyzed using limma (Smyth 2005) from the Bioconductor suite of tools (Gentleman et al. 2004) for R (Ihaka and Gentleman 1996). Our processing script is posted on the Open Science Framework. Median expression values were analyzed (Zahurak et al. 2007). These were corrected for background using the normexp + offset algorithm recommended for Agilent microarrays by Ritchie et al. (2007). An offset of 30 was selected based on inspection of MA Plots (Supplemental Fig. 1).

Reverse-transcription quantitative PCR (qPCR)

RNA was reverse transcribed using oligo(dT) primes and RevertAid First Strand cDNA Synthesis Kit (Fermentas).

Quantitative PCR was conducted using Sybr Green and the MyIQ real time PCR system (Bio-Rad). Primers were validated for correct PCR efficiency and are listed in Supplemental Table 1. qPCR samples were analyzed in duplicate, and the relative amounts of each transcript were determined using the ddCT method and the Bio-rad IQ5 gene expression analysis (Bio-Rad). All qPCR expression levels were normalized to levels of histone H4 (Bonnick et al. 2012).

Statistical analysis

Behavioral responses were averaged by time point. Mixed-factorial ANOVAS were used to test for expected interactions between condition and test phase. These were followed up within each experimental group separately with paired comparisons between baseline and each test phase (Dunnett correction for multiple comparisons). To compare across conditions responses were normalized to baseline, so that 100% represents no change in behavior. Comparisons across conditions were conducted at each test phase using Holm–Sidak adjustment for multiple comparisons. Following the convention in the statistical software package we used (GraphPad Prism), *P* values are reported exactly except where they are smaller than 0.0001. Effect sizes are estimated using Cohen's *d* by comparing normalized responses across conditions ($d = [M_{\text{Control}} - M_{\text{Trained}}]/\text{SD}_{\text{pooled}}$); negative values of *d* indicate habituation.

Microarray analysis was conducted exactly as in Herdegen et al. (2014). Briefly, expression was normalized using the loess function (Smyth and Speed 2003). Where multiple probes were used to measure the same EST or mRNA, these were then averaged. Finally, trained and control expression were compared using an empirical Bayes-moderated *t*-test (Smyth 2004). Statistical significance was calculated using Benjamini–Hochberg correction for multiple comparisons to maintain a 5% overall false-discovery rate (Benjamini and Hochberg 1995). We used the treat function from limma (McCarthy and Smyth 2009) to conduct two separate tests of significance: (1) against the standard null hypothesis of no regulation (H_o: mean fold change <1 or >1; note that a ratio of 1 is produced when both trained and untrained samples have the same levels of gene expression) and then (2) against a null of at least 1.1-fold change in either direction. We have found that transcripts regulated by this more stringent criterion are likely to generalize to an independent sample (Herdegen et al. 2014).

For quantitative PCR, a fold-change score was calculated for each trained or massed animal as the ratio of expression to its matched control. For all analyses, fold-change scores were log transformed (base 2). This ensures equal weight to both up- and down-regulated measures and maintains consistency with microarray analysis. Changes from control were tested using a one-sample *t*-test against an expected value of 0 for the null hypothesis (0 represents no change for log-transformed fold-change scores). Differences in regulation between massed and standard spaced training were analyzed with a paired *t*-test (by matched group) comparing log fold change from control. For ease of interpretation, fold-change scores are plotted in raw format on a log scale and are reported in text in raw format as mean fold change (MFC) with 95% confidence intervals in brackets.

Discussion

Cornichon proteins are transmembrane proteins that function as auxiliary subunits of AMPA receptors. They can alter AMPAR surface expression and affect channel gating kinetics (Schwenk et al. 2009). For example, in cornichon knockout mice, AMPAR synaptic transmission is reduced in the hippocampus due in part to alteration in AMPAR surface expression (Herring et al. 2013). Consistent with this, *C. elegans* cornichon mutants exhibit larger glutamate currents and an increase in AMPAR number (Brockie et al. 2013).

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