DOE DE-FG02-08ER64652 – Final Scientific Report

Project Title: Physiological, Molecular, and Genetic Mechanisms of Long-Term Habituation

Recipient: Dominican University, River Forest, IL

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Specific Aims:

- Identify the physiological and genetic correlates of long-term habituation
- Identify the mechanisms of experience-dependent transcriptional regulation
- Foster a world-class undergraduate neuroscience major

Executive Summary

Work funded on this grant has explored the mechanisms of long-term habituation, a ubiquitous form of learning that plays a key role in basic cognitive functioning. Specifically, behavioral, physiological, and molecular mechanisms of habituation have been explored using a simple model system, the tail-elicited siphon-withdrawal reflex (T-SWR) in the marine mollusk *Aplysia californica*. Substantial progress has been made on the first and third aims, providing some fundamental insights into the mechanisms by which memories are stored.

We have characterized the physiological correlates of short- and long-term habituation. We found that short-term habituation is accompanied by a robust sensory adaptation, whereas long-term habituation is accompanied by alterations in sensory and interneuron synaptic efficacy. Thus, our data indicates memories can be shifted between different sites in a neural network as they are consolidated from short to long term. At the molecular level, we have accomplished microarray analysis comparing gene expression in both habituated and control ganglia. We have identified a network of putatively regulated transcripts that seems particularly targeted towards synaptic changes (e.g. SNAP25, calmodulin) . We are now beginning additional work to confirm regulation of these transcripts and build a more detailed understanding of the cascade of molecular events leading to the permanent storage of long-term memories.

On the third aim, we have fostered a nascent neuroscience program via a variety of successful initiatives. We have funded over 11 undergraduate neuroscience scholars, several of whom have been recognized at national and regional levels for their research. We have also conducted a pioneering summer research program for community college students which is helping enhance access of under-represented groups to life science careers.

Despite minimal progress on the second aim, this project has provided a) novel insight into the network mechanisms by which short-term memories are permanently stored, and b) a strong foundation for continued growth of an excellent undergraduate neuroscience program.

Detailed Report

We were able to complete a physiological and transcriptional analysis of long-term habituation (first aim), providing what we believe will be meaningful insight into some of the mechanisms of long-term habituation. However, the time available was not sufficient to complete the full scope of proposed research. Specifically, we were unable to begin exploring transcriptional regulation of genes correlated with the expression of long-term habituation memory (second aim).

Background

Our project explored the physiological and transcriptional correlates of long-term habituation in the *Aplysia* tail-elicited siphon-withdrawal reflex (T-SWR).

Our project focused on habituation, a decrease in behavior due to repeated stimulation (Groves & Thompson, 1970). This form of memory is ubiquitous across the animal kingdom (Abramson, 1994) and seems to play an important role in filtering and attention (Dow & Anastasio, 1999; Klamer et al., 2004; Linster et al., 2007). In fact, rates of habituation provide a useful global index of cognitive function in humans (Fagan et al. 1992; 2007).

As a model organism, we focused on *Aplysia,* which has long served as a useful system for linking neural and behavioral phenomena. Specifically, we focused on the tail-elicited siphon-

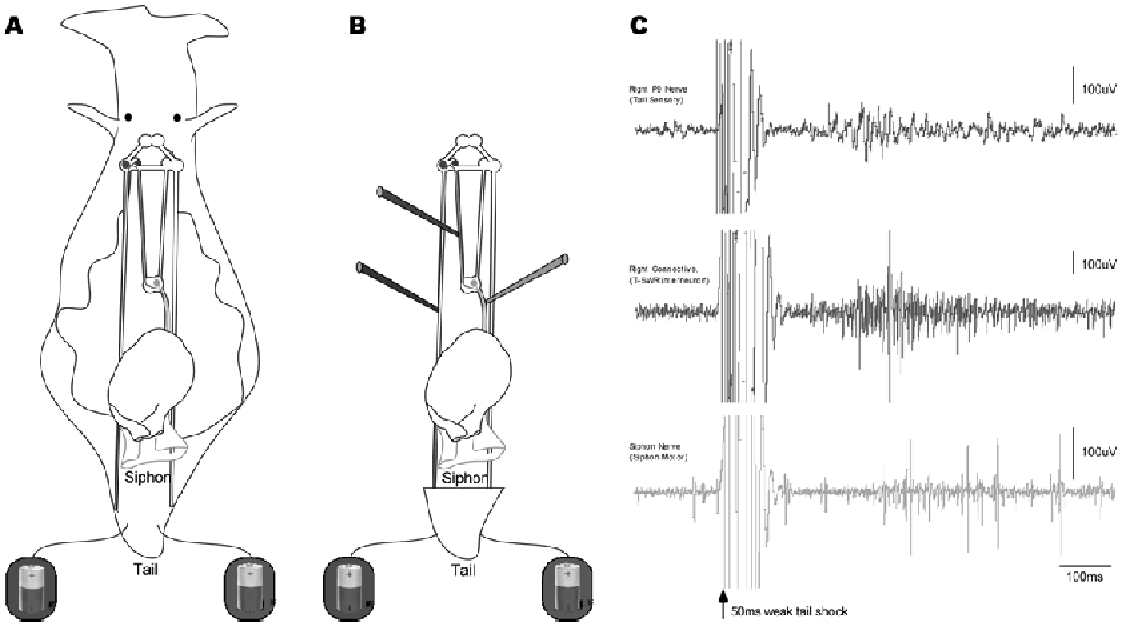


Figure 1: The Tail-Elicited Siphon-Withdrawal Reflex (T-SWR). A) Weak electrical stimulation of the tail produces a transient withdrawal of the siphon (retracts from grey-outline to solid outline). This behavior is mediated by a simple, 3-layer circuit consisting of tail sensory neurons (purple), a population of interneurons (green), and siphon motor neurons (orange). B) Physiological correlates of T-SWR behavior can be studied in the semi-intact preparation which preserves the tail, siphon complex, the entire CNS. In this configuration, activity from each layer of the circuit can be recorded separately via extracellular electrodes. C) Representative extracellular traces during an evoked T-SWR (arrow). Although each nerve also carries activity unrelated to the T-SWR circuit, evoked activity is strong relative to background, providing a sensitive but technically unchallenging means to monitor the entire circuit. The stimulus artifact lasts slightly longer than the stimulus and is clipped in these records. The large spike during the evoked response in the connective has also been clipped (it is from the giant R2 and otherwise dwarfs the record).

withdrawal reflex (T-SWR), a defensive withdrawal of the siphon due to tactile or weak electrical stimulation of the tail (Figure 1A). This behavior is particularly attractive for study because it underlying neural circuitry is relatively well understood: it is mediated by tail sensory neurons, a population of interneurons, and siphon motor neurons (Figure 1A; Cleary et al., 1993, 1995; Walters et al., 1983, 2004). This reflex is maintained in the reduced siphon+tail preparation (Stopfer & Carew, 1996b), a dissection which preserves the tail, siphon, CNS and their interconnections (Figure 1B), enabling simultaneous collection of behavioral and physiological measures. Fortuitously, each layer of the tail-elicited T-SWR network passes through a distinct nerve: the P9 nerves, pleural-abdominal connectives, and siphon nerve, respectively. Thus, the entire network can be monitored via three extracellular suction electrodes (for each side of the animal).

The T-SWR undergoes habituation when stimulated repeatedly by weak electrical current to the tail. This habituation is expressed as a decrease in reflex duration. Interestingly, habituation of the T-SWR is completely lateralized, allowing each animal to serve as its own control (Bristol et al., 2004; Stopfer et al., 1996b). Repeated, spaced training sessions produce long-term habituation (LTH) lasting more than 24 hours (Stopfer et al.,1996b).

Our goal was to make physiological and transcriptional measurements from the T-SWR circuit 1 day after long-term habituation. By comparing measurements from the trained and untrained sides of the animal, we hoped to identify 1) the physiological correlates of long-term habituation memory, 2) the transcriptional correlates of long-term habituation memory, and 3) the relationships between transcriptional and neural plasticity.

Our original goal was to complete this procedure 1, 5, and 15 days post training, allowing examination of the expression, maintenance, and decay of this memory. Unfortunately, we were only able to complete the first third of this plan.

Validation of extracellular measurement of T-SWR circuit activity

Our project required the ability to monitor the physiological changes that accompany habituation across the T-SWR circuit. Although this is normally completed via intracellular physiology, this approach requires CNS microdissection that is known to alter patterns of gene expression (Alberini et al. 1994). Thus, we explored the possibility of using extracellular physiology to monitor the T-SWR circuit. Extracellular physiology is less invasive, but would also include activity unrelated to the T-SWR circuit, raising concern about the sensitivity of this approach for monitoring the circuit.

To ensure good sensitivity of extracellular recordings of the T-SWR circuit, measures were taken in 5 semi-intact preparations. As shown in Figure 2A, sensitivity was excellent in each prep at each layer of the T-SWR circuit. Signal/noise ratios averaged 3.49 in the P9 nerves (min = 2.6, max = 5.3), 3.48 in the pleural-abdominal connectives (min = 0.9, max = 6.1), and 3.04 in the siphon nerve (min = 2.27, max = 3.8). Moreover, evoked-activity correlated well with both observed behavior and stimulus amplitude, with correlations ranging from 0.54 to 0.96 (examples in Figure 2B).

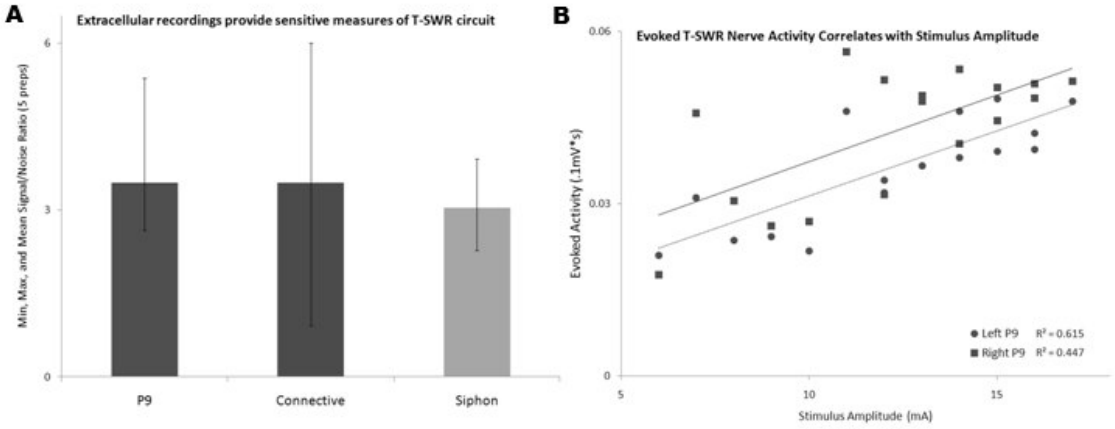


Figure 2: Extracellular recordings provide a sensitive measure of T-SWR circuit activity. A). Signal-noise ratio. To determine the sensitivity of extracellular recordings of the T-SWR circuit, measurements were made from 5 sites in semi-intact preparations: the left and right P9 tail nerves, left and right pleural-abdominal connective, and siphon nerve. These nerves carry activity related to the sensory-, interneuron-, and motor-layers of the T-SWR circuit, respectively (Figure 1B). After recording background activity for 5 minutes, T-SWR behavior was evoked via weak shocks (60Hz AC, 5-20mA, 50ms) applied to the left or right tail via implanted silver wires at a 10 min ISI. Signal/noise ratio was calculated as the ratio of activity evoked for 250ms after shock relative to the background activity recorded for 250ms prior to shock. Shown here is the average signal/noise ratio for each nerve. **Note that error bars indicate the minimum and maximum signal/noise ratios, expressing the entire range over 5 preps. B) Correlation with stimulus amplitude. Stimulus amplitude was systematically varied in 1mA steps. Evoked input at each layer of the T-SWR tracked stimulus amplitude and behavior. Shown here is a representative correlation from the left and right P9 nerves of a single prep.

These observations demonstrate that it is feasible to rely primarily on extracellular physiology to monitor this circuit. It also opens up the possibility of making these recording in intact animals in the future via implanted cuff electrodes (Cobbs & Pinsker, 1978).

Validation of the measurement of gene expression in the *Aplysia* CNS

Our project also required the ability to reliably profile changes in gene expression within the *Aplysia* CNS. As an *Aplysia* genomic microarray is not yet available, we developed and validated a cross-species microarray approach to profiling gene expression. This approach was developed through an ongoing collaboration with Dr. Joe Moskal and Dr. Roger Kroes, director and associate director of the Falk Center for Molecular Therapeutics, respectively.

Our approach was straightforward (Figure 3). Tissue from the *Aplysia* CNS was prepared using standard techniques for cell isolation, RNA extraction, and reverse transcription (e.g. Lee et al., 2008; Moroz et al., 2006). Paired samples were then run in triplicate on a targeted microarray consisting of probes for 1,178 genes from *Rattus norvegicus* and processed relative to a standard sample from rat (Kroes et al., 2006; Moskal et al., 2006). Regulated transcripts were identified via SAM-RS (Significance Analysis of Microarrays using Rank Scores; Van de Wiel, 2004) analysis set for a false-positive level of < 5%. To match regulated transcripts to the *Aplysia* genome, probe sequences from the chip were blasted against both genomic and est databases, and high-match sequences were identified and aligned to produce a consensus sequence for the likely region identified by the array probe. The consensus sequence was then used to design *Aplysia* specific primer sets for confirmation with quantiatative real-time PCR (qPCR). This technique does not require RNA amplification; we can achieve yields sufficient for microarray analysis from 1-50 neurons, depending on their size, and a single ganglion provides enough RNA for multiple rounds of microarray analysis. Although the use of a cross-species microarray may

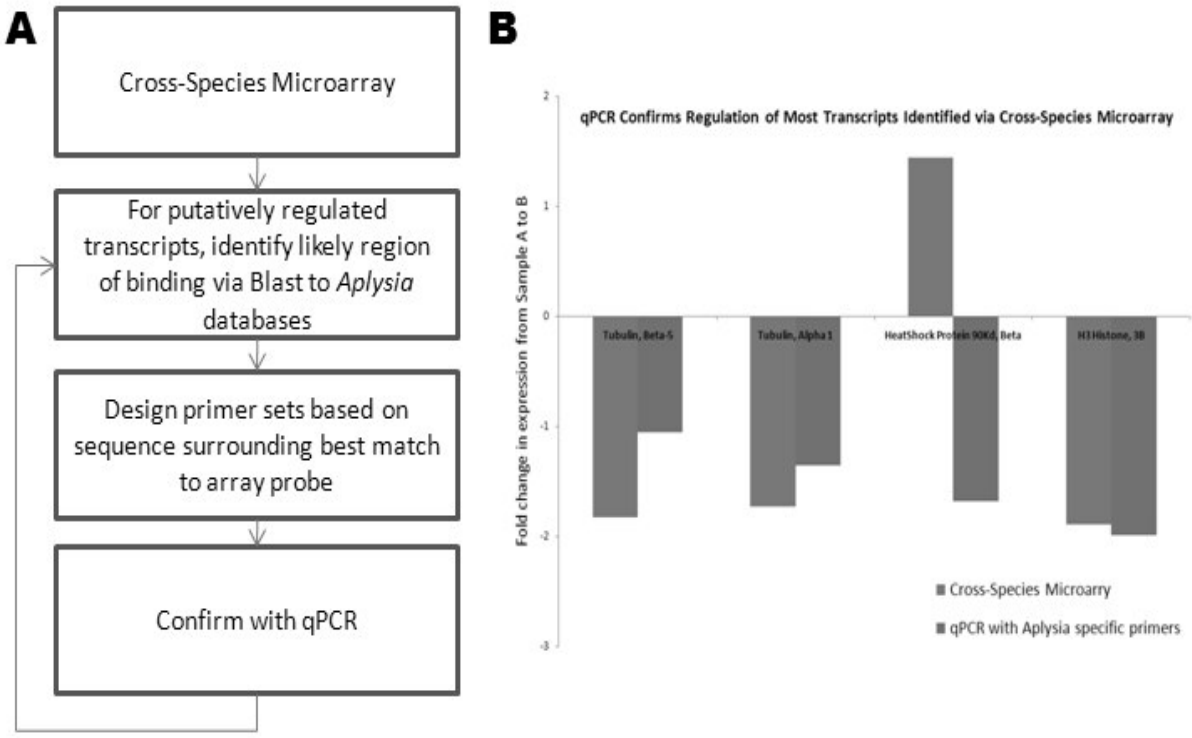


Figure 3: Workflow and validation of cross-species microarray to measure gene expression in the *Aplysia* CNS. A) Workflow for confirming microarray results. See text for details and B) Confirmation of validity. Samples from two *Aplysia* abdominal ganglia were compared via a cross-species microarray (blue). To confirm the validity of this approach, 4 array probes were matched to the *Aplysia* genome and then used to repeat the same analysis with the same samples using qPCR and *Aplysia*-specific primer sets. Shown here are measured levels of expression calculated as the fold-increase from sample A to sample B. In 3 out of the 4 probes tested, qPCR results are similar to that predicted by the cross-species microarray.

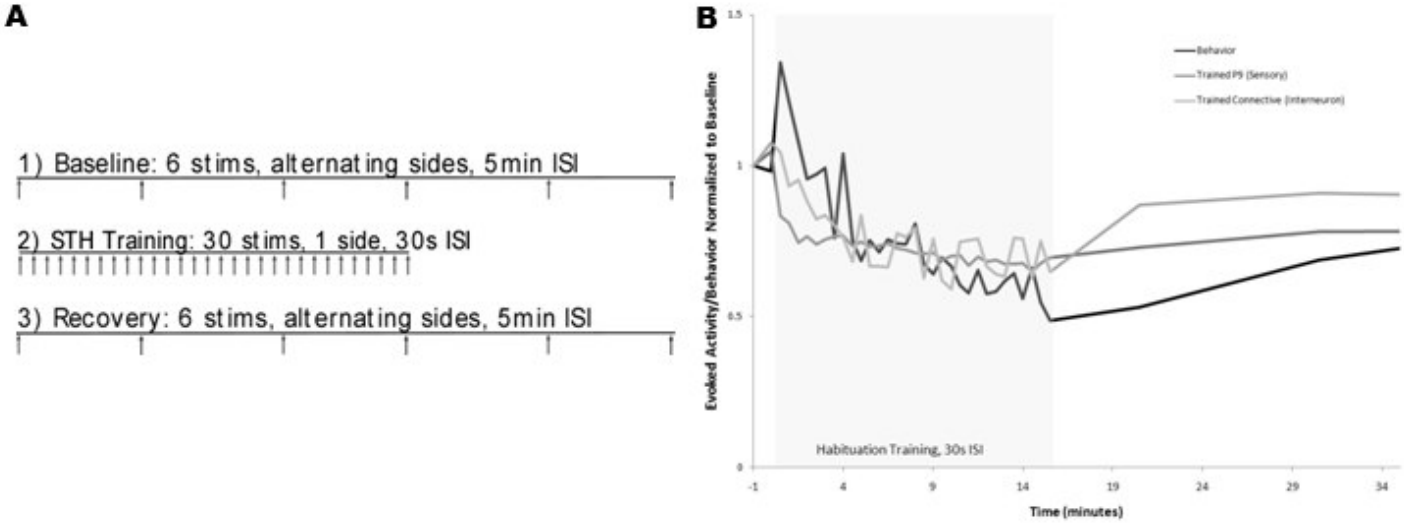


Figure 5: Short-Term Habituation is Accompanied by Multiple Forms of Plasticity in the T-SWR circuit. A). Protocol. Behavioral and physiological measures were monitored in semi-intact preparations before (baseline), during, and after (post-test) short-term habituation training. The T-SWR circuit was monitored via *en passant* suction electrode recordings to detect tail sensory input (P9 tail nerve, purple), interneuron activity (plueral-abdominal connective, green), and siphon motor output (siphon nerve, orange). The complex evoked responses were quantified in each nerve as the integral of the absolute value of the recorded trace for 500ms after the end of the stimulus artifact (Calin-Jageman & Fischer, 2007). B). Summary Data. Mean physiological and behavioral measures are shown normalized to the average baseline. Note that sensory input declines more rapidly than interneuron activity and behavior, suggest both sensory adaptation and a downstream transient facilitation.

seem tenuous, it has been used fruitfully with other species pairs (for a review see Bar-Or, Czosnek, & Koltai, 2007).

To validate our approach, mRNA was isolated from the abdominal ganglia of two animals and then subjected to microarray analysis. Across three replications, strong and consistent hybridization was achieved within the standard treatment conditions for the array. A total of 84 regulated transcripts were identified (< 5% false positives expected). *Aplysia*-specific primers were then designed for 4 of these transcripts, and qPCR on the same samples confirmed significant regulation in 3 of these 4. This 75% success rate at predicating regulated transcripts exceeds the success rate recently published using an EST-based microarray in the related species *Aplysia kurodai* (5 qPCR confirmations out of 12 microarray-identified probes, Lee et al., 2008).

This data suggests that our cross-species approach can be useful in identifying transcriptional correlates of habituation.

Short-Term Habituation Produces Sensory Adaptation and a Transient Downstream Facilitation.

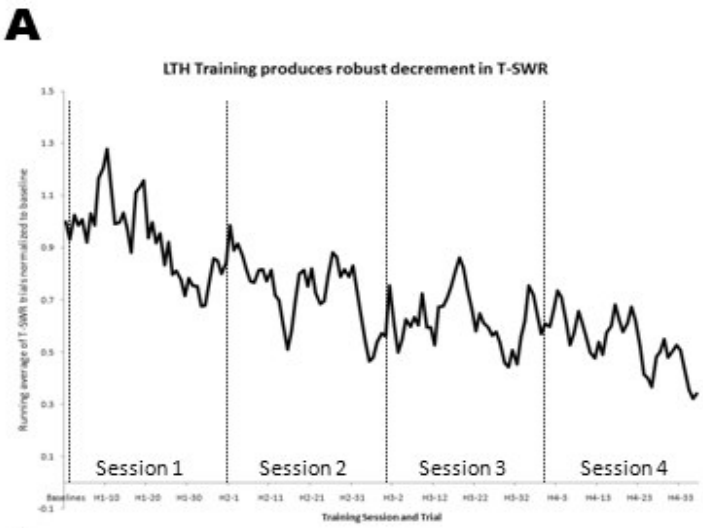
To provide a benchmark for comparison with long-term habituation, we completed an experiment documenting the physiological changes that accompany short-term habituation.

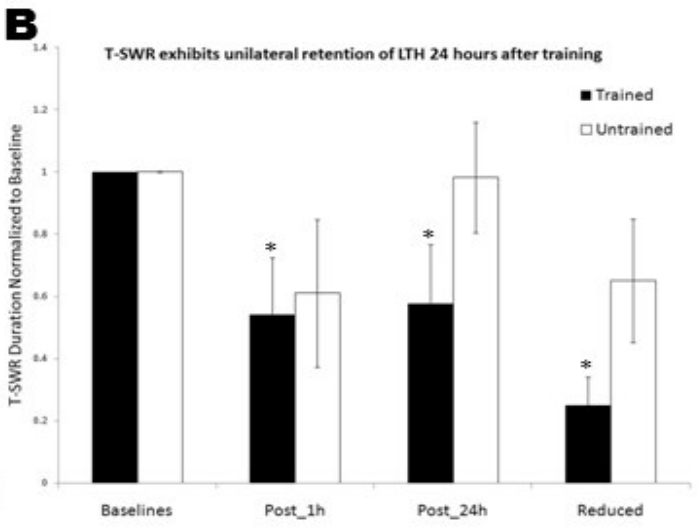
As shown in Figure 5, the training protocol produced a transient increase in T-SWR behavior (black line), followed by a robust decrease that recovered within 20 minutes of training. This behavioral plasticity was accompanied by a rapid decrease in tail sensory activity (P9 nerve, purple line), indicating that short-term habituation is accompanied by a robust sensory adaptation. Interestingly, sensory activity declined much more rapidly than both behavior and interneuron activity (compare purple line to black and green lines during initial training). This suggests the operation of a transient facilitory process operating downstream of sensory input, perhaps at sensory synapses.

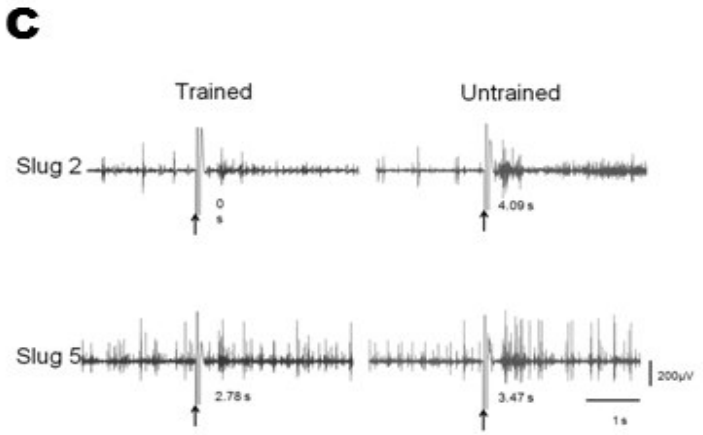
These results again demonstrate the distributed nature of habituation memory (e.g. see Krasne & Teshiba, 1995). They are also consistent with previous studies of short-term habituation of the T-SWR using intracellular techniques designs (Stopfer et al., 1996a, 1996b), which reported, in part, a decrease in sensory neuron excitability and a facilitation of sensory neuron synapses. Thus, this data provides further validation of the use of extracellular techniques to monitor the T-SWR.

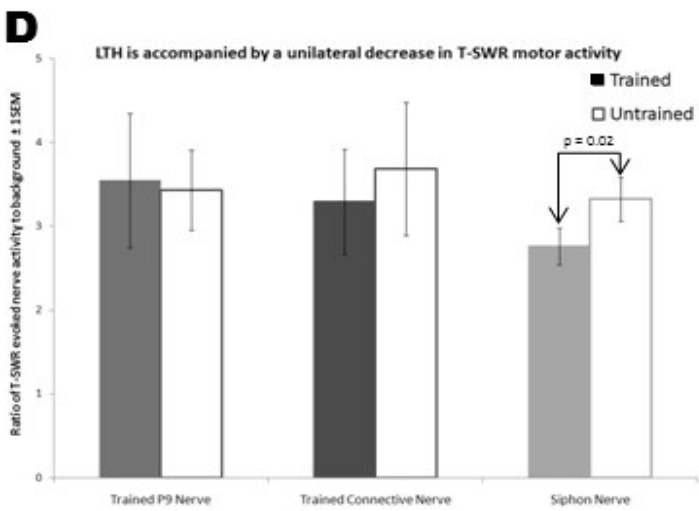
LTH training alters T-SWR motor output and sensory neuron expression of synaptic genes

With our techniques validated and benchmark data collected, we began exploring physiological and transcriptional mechanisms of long-term habituation. Specifically, we have completed behavioral and physiological measurements of 5 animals, and have completed microarray analysis on the pedal ganglia of one of these animals.









E

Animal 4, Pedal Ganglia, Gene Expression Change from Trained to Untrained Side

xChange	Symbo	Name	xCha	Symbol2	Name3
1.62	RCA	PROTEIN AT2G39730	-1.72	LEF1	LYMPHOID ENHANCER BINDING FACTOR 1
1.60	TSHB	THYROID STIMULATING HORMONE, BETA SUBUNIT	-1.47	SNAP25	SYNAPTOSOMAL-ASSOCIATED PROTEIN 25
1.59	NLGN3	NEUROLIGIN 3	-1.46	CCBP2	CHEMOKINE BINDING PROTEIN 2
1.57	PNCK	NON-UBIQUITOUSLY EXPRESSED CAM KINASE	-1.45	GABRA3	GAMMA-AMINOBUTYRIC ACID RECEPTOR, SUBUNIT ALPHA 3
1.43	PRK	PRK	-1.41	WIF1	WNT INHIBITORY FACTOR 1
1.41	IGF1	INSULIN-LIKE GROWTH FACTOR 1	-1.40	NTF5	NEUROTROPHIN 5
1.40	GSTA3	GLUTATHIONE S-TRANSFERASE A3	-1.40	MTAP1A	MICROTUBULE-ASSOCIATED PROTEIN 1 A
1.39	EPHA7	EPH RECEPTOR A7	-1.40	ABCC9	ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 9
1.36	LMNB1	LAMIN B1	-1.40	STAT4	SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 4
1.33	APOE	APOLIPOPROTEIN E	-1.38	CPE	CARBOXYPEPTIDASE E
1.33	HCN4	HYPERPOLARIZATION-ACTIVATED, CNG K+ 4	-1.35	GRIP2	GLUTAMATE RECEPTOR INTERACTING PROTEIN 2
1.30	IL1RL2	INTERLEUKIN 1 RECEPTOR-LIKE 2	-1.34	CALM3	CALMODULIN 1
1.28	AXIN1	AXIN 1	-1.32	IL6	INTERLEUKIN 6
			-1.32	SLC15A4	PEPTIDE/HISTIDINE TRANSPORTER
			-1.30	RGD1562793	SIMILAR TO NEX-1
			-1.28	SMAD1	MAD HOMOLOG 1 (DROSOPHILA)
			-1.25	SYT9	SYNAPTOTAGMIN 9

Figure 6: Behavioral, Physiological, and Transcriptional Changes produced by LTH training. A). Behavior during training. After characterizing baseline responding, animals were subjected to unilateral long-term habituation training (see Figure 7). Shown here is the average T-SWR response normalized to baseline for each of the4 training sessions (n = 5). B) LTH Retention. Retention tests were given 1 hour after training, 24 hours after training, and again after dissection to as semi-intact preparation (~28 hours). Unilateral decreases in T-SWR behavior are evident on the trained side 24 hours after training and in the reduced prep. C) Representative physiology. During retention tests in the semi-intact prep, evoked nerve activity was recorded to measure sensory (P9 nerves, purple), interneuron (pleural-abdominal connectives, green), and motor (siphon nerve, orange) activity in the T-SWR circuit. Shown here are representative recordings of the siphon nerve from the trained and untrained sides of two animals. A decrease in evoked activity on the trained side is evident. D) Summary Physiology. Shown here is mean evoked-activity ± 1 SEM for trained and untrained sides of each layer of the T-SWR circuit. LTH produced a significant decrease in T-SWR motor activity (siphon nerve, trained vs. untrained), but did not alter sensory (P9) nor interneuron activity (Connective). E) Gene Expression. Immediately after physiological recording, pedal ganglia were isolated for microarray analysis and gene expression was compared between the trained and untrained side (n=1). Shown here transcripts that are upr-egulated (left) or down-regulated (right) in trained vs. untrained ganglia (<5% false-discovery rate expected)

habituation. As expected, habituation training produced a long-lasting (24 hour) and unilateral decrease in T-SWR behavior (Figure 6 A and B). This behavioral decrement was accompanied by a decrease in evoked nerve activity in the siphon nerve (Figure 6 C and D), which carries the motor output for the T-SWR circuit. Unexpectedly, however, no change was observed in sensory and interneuron activity. This stands in contrast to short-term habituation, which is accompanied by a rapid and strong decrease in sensory activity (Figure 3C). This suggests a shift in expression mechanisms between short- and long-term habituation, with near-term behavioral changes mediated in part by a reduction in sensory excitability, and long-term changes mediated by changes downstream of sensory input (e.g. synaptic plasticity between sensory and motor components).

Our initial microarray results are also promising. Triplicate microarray runs produced consistent and strong hybridization within the optimal operating conditions for the array. With a 5% false-discovery rate, 13 genes were identified a significantly upregulated on the trained side, and 17 were identified as significantly down-regulated (partial list in Figure 6E). This initial list is truly intriguing and represents many genes relevant to synaptic function despite the wide range of genes present on the chip (NOTE: the rat gene from which the probe was derived does not always provide a good description of the aplysia gene that best matches the probe so caution of interpretation is required). We are now working 1) to process additional samples (other layers of the circuit and the remaining 4 animals), and 2) to begin qPCR confirmation of putatively regulated transcripts. Overall, this approach seems fruitful, yielding novel insights into both the physiological and transcriptional events that accompany long-term habituation, and potentially illuminating how these levels of plasticity inter-relate.

Fostering a world-class undergraduate neuroscience program.

The work presented here was conducted primarily by undergraduate researchers. Over the course of the grant period, 11 different undergraduate researchers were funded to participate in research. This quality of this work is attested not only by the novel data collected but by a remarkable set of national and regional honors: a national travel award (Mary Petrosko), 2nd place in a regional poster competition (Mary Petrosko), and a SOMAS award to both the PI and Benora McBride, an undergraduate research assistant (SOMAS is a program for junior neuroscience faculty and their research assistants; it is funded by HHMI and Davidson College).

To help establish a pipeline of neuroscience researchers, we have also established a Summer Undergraduate Research Experience (SURE). Modeled after the "Neural Systems and Behavior" course that has been offered over the past 30 summers at the Woods Hole Marine Biology Laboratory (the first PI has previously served as a faculty member in this summer course), SURE provides an immersive but engaging laboratory research experience for community college students interested in life science research.

In the summer of 2008, 11 community college sophomores (mostly from the City Colleges of Chicago), 9 of whom are from communities underrepresented in the research sciences, honed their research skills through SURE activities for five intensive days. The participants engaged in hands-on learning to develop their knowledge of basic investigative techniques in neuroscience, cell biology,

inorganic chemistry, social psychology, and nutrition/microbiology. Through team-building, intensive tutoring, and selection of engaging research projects, the program helped prepare a cadre of undergraduate scientists for participation in more extensive independent research projects while advancing the research culture at Dominican University. Participants in SURE 2008 responded very positively to the program overall and most felt that it was an "excellent" experience. Most students found all the activities either "just right" or "challenging." Students also reported that SURE helped them 1) appreciate the wide range of research topics in science; 2) understand scientific techniques; 3) work better with scientific data, and 4) develop their presentation skills.

The SURE program was repeated in the summer 2009, with the curriculum revised to add experiences in scientific computing, medical chemistry and antibiotic efficacy. It was again an extremely successful program with many students in the program going on to successful summer research experiences at universities across the country.

Outcomes

Professional Presentations

- Petrosko M, Calin-Jageman IE & Calin-Jageman RJ (2008). Mechanisms of long-term habituation of the *Aplysia* tail-elicited siphon-withdrawal response. *SFN Abstracts, 880.13*.
- Petrosko M & Calin-Jageman RJ (2008). Behavioral and Physiological Effects of Ginko Biloba Extract EGB761 on *Aplysia Califronica*. FUN Poster Session, *Society for Neuroscience Meeting, Washington, DC*.
- McBride B, Bonnick K, Calin-Jageman IE, Calin-Jageman RJ (2009). Quantitative analysis of changes in gene expression following long-term sensitization of the *Aplysia* tail-elicited siphon-withdrawal reflex. *Society for Neuroscience Abstracts*, 889.11
- McBride B, Salazar L, Bonnick K, Matel K, Kroes R, Moskal J, Calin-Jageman IE, Calin-Jageman RJ (2009). Behavioral, physiological and genetic analysis of habituation of the *Aplysia* tail-elicited siphon withdrawal reflex. *MCC Meeting,* Chicago, IL.
- McBride B, Salazar L, Bonnick K, Matel K, Kroes R, Moskal J, Calin-Jageman IE, Calin-Jageman RJ (2009). Behavioral, physiological and genetic analysis of habituation of the *Aplysia* tail-elicited siphon withdrawal reflex. Fun Poster Session, *Society for Neuroscience Meeting,* Washington , DC.

Honors, Awards

- Petrosko, M. (2008). Faculty for Undergraduate Neuroscience Travel Award
- Petrosko, M. (2008). 2nd Place, Undergraduate Poster Competition, Chicago Chapter of the Society for Neuroscience
- McBride B & Calin-Jageman RJ (2009). SOMAS grant, HHMI and Davidson College.