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Project Title:

A program for undergraduate research into the mechanisms of sensory coding and memory decay

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Specific Aims:

- Identify the physiological and genetic correlates of the decay of long-term habituation.
- Mechanisms of neural coding in the fly olfactory system.
- Foster a world-class undergraduate neuroscience program.

Executive Summary

For the first aim, we made substantial progress in understanding the mechanisms of long-term memory, but shifted focus from habituation to sensitization, a ubiquitous form of learning that plays a key role in basic cognitive functioning. Specifically, we explored the transcriptional changes that accompany long-term sensitization in a simple model system, the tail-elicited siphon withdrawal reflex (T-SWR). We compared transcriptional changes across different induction paradigms (tail shock vs. 5HT) and across two closely related species (*Aplysia californica* vs. prior publications on *Aplysia kurodia*) using both rtPCR and microarray analysis. We found that a very small set of transcripts show regulation across species and induction paradigms (CEB/P and CREB), but that most transcripts regulated in one condition are not similarly regulated in different contexts. This work suggests that the 'moecular alphabet' for learning may show considerable variability across learning paradigms and species, with only a small set of key transcripts having global relevance to learning and memory. This work may also help develop our limited understanding of the evolutionary pathways by which long-term memory evolved. This work was recently presented at a national-level conference, and two manuscripts are in preparation.

For the second aim, our work has far revealed the complexity present within a "simple" animal's olfactory system. Our systematic analysis of mutations of both Or42a and Or42b has allowed us to conclude that odor receptors contribute to behaviors in a manner that is not fully predictable based on current knowledge of odor receptor electrophysiological activity and that these results are not fully consistent with the combinatorial coding hypothesis. Obvious molecular features of odor molecules are insufficient to predict odor perception and an empirical examination of the relationship between electrophysiological response and behavioral output is absolutely necessary to deduce the rules by which olfactory systems operate. Finally, we are examining how variation in odor receptors may allow variation in sensitivity to odors. We have reproduced an important behavioral experiment and are now examining how molecular level variation may explain behavioral variation. The implications of these questions speaks directly to the important questions of how olfactory systems evolved and allow variation to the odors within a species.

On the third aim, we have fostered a nascent neuroscience program via a variety of successful initiatives. We funded 14 undergraduate neuroscience scholars, several of whom have been recognized at national and regional levels for their research. We have also continued a pioneering summer research program for community college students which is helping enhance access of under-represented groups to life science careers.

Detailed Report:

AIM1: IDENTIFY THE PHYSIOLOGICAL AND GENETIC CORRELATES OF THE DECAY OF LONG-TERM HABITUATION

BACKGROUND AND SIGNIFICANCE

LONG-TERM HABITUATION

Habituation is a decline in reflex responsiveness 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). The universality of habituation has helped maintain steady research interest in this form of learning (Rankin et al., 2009), but it nevertheless remains one of the least-well understood forms of learning (Glanzman, 2006, 2009).

The neural mechanisms of habituation have been studied in a number of model organisms. This work has shown that the apparent simplicity of habituation belies complex neural underpinnings. Specifically, habituation memory seems to be encoded via multiple forms of neural plasticity expressed across a neural circuit (Bristol et al., 2004, 2005; Stopfer et al., 1996a, 1996b). For example, in the crayfish tail-flip reflex, habituation depends not only on homosynaptic depression of sensory synapses but also on complex changes in descending inhibition from the CNS (Krasne & Teshiba, 1995). Similarly, in the *Aplysia* tail-elicited siphon withdrawal reflex, habituation produces a decrease in sensory neuron excitability, an increase in sensory neuron latencies, an increase in sensory neuron synaptic efficacy, and additional changes in interneuron populations (Stopfer et al., 1996). Thus, habituation memories seem to have distributed neural representations.

Some progress has also been made in understanding the genetic mechanisms of long-term habituation. In a wide range of model organisms it has now been established that long-lasting habituation memory requires alterations in gene transcription (Barco, Pittenger, & Kandel, 2003; Korzus, 2003; Watanabe et al., 2005). These experiments, however, largely rely on the use of transcription inhibitors to show a global dependence on transcription. There has been little progress identifying the specific transcriptional changes that support habituation (though see Rose et al., 2003; Salomons et al., 2009).

Genetic analysis of habituation has stalled due to a number of technical challenges. First, the distributed nature of habituation memory suggests that different transcriptional changes may be taking place at different levels of the reflex circuit. This implies a need to separately survey each component of the circuit. Second, habituation is likely to alter only a small subset of neurons, and this subset is further dwarfed by non-neuronal cells (e.g. glia). This implies that the signal-noise ratio for transcriptional analysis will be very poor.

As described below, both of these challenges can be overcome through the study of the *Aplysia* tail-elicited siphon-withdrawal response (T-SWR). The behavior is controlled by a simple circuit of large neurons, providing the ability to analyze gene expression with single-cell resolution across each layer of the network. Moreover, this transcriptional analysis can easily be integrated with behavioral and physiological techniques. We are leveraging these advantages to provide fundamental insights into the neurobiology of habituation.

APLYSIA CALIFORNICA AS A MODEL SYSTEM

Aplysia have long served as an important model system for linking neural and behavioral phenomena. In addition to habituation (Pinsker et al., 1970), *Aplysia* exhibit several important forms of learning, including sensitization (Carew et al., 1971), classical conditioning (Walters, Carew, & Kandel, 1979), and context conditioning (Colwill et al., 1988). Furthermore, different training protocols can produce memories lasting over a number of time-scales, from tens of seconds (Fischer & Carew, 1993) to tens of minutes (Fischer & Carew, 1995) to days (Sutton et al., 2001). In analyzing the physiological mechanisms of learning, *Aplysia* offer several advantages: 1) their CNS consists of a relatively small number of neurons (~20,000); 2) the neural circuitry underlying several ethologically-relevant behaviors has been mapped at the single-cell level, and 3) dissections are available enabling simultaneous behavioral and physiological observation (Figure 1B).

Despite their advantages for physiological techniques, *Aplysia* have not traditionally been amenable for molecular biology techniques. Several recent developments, however, have opened up powerful new opportunities for molecular research with *Aplysia:* 1) The *Aplysia* genome has now been sequenced and published (Moroz et al., 2006; http://www.ncbi.nlm.nih.gov/nuccore/225542573), 2) customized microarrays have become available (Moroz et al., 2006; Affymatrix), 3) protocols for *RNAi* have now been perfected (Lee et al., 2003), and 4) cell culture techniques have become routine, enabling reconstruction of circuit elements in culture (Lovell et al., 2006), Thus, the full complement of modern molecular biology techniques can now be leveraged to dissect the molecular mechanisms of memory in *Aplysia*. Given the ability to directly relate these results to both physiological and behavioral data, *Aplysia* now represent a uniquely potent model organism for understanding long-term memory.

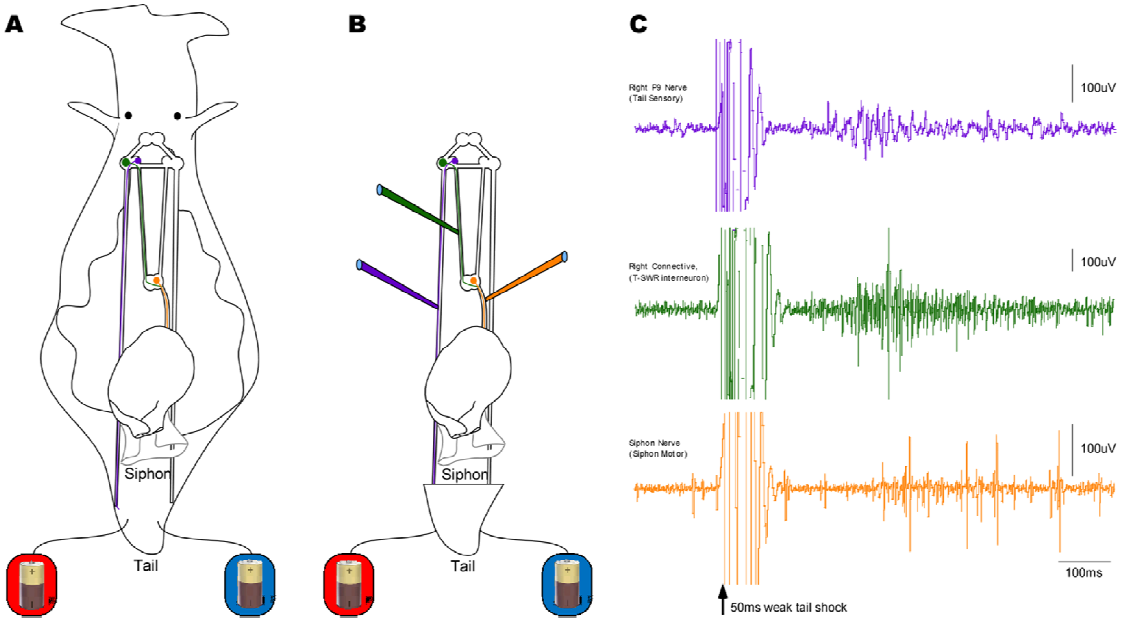


Figure 1: The Tail-Elicited Siphon-Withdrawal Reflex (T-SWR). A) Weak electrical stimulation of the tail produces a transient withdrawal of the siphon (retracts from grey-outline to solid outline). This behavior is mediated by a simple, 3-layer circuit consisting of tail sensory neurons (purple), a population 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).

THE TAIL-ELICITED SIPHON WITHDRAWAL REFLEX

The siphon-withdrawal reflex is a defensive retraction of the siphon elicited by stimulation of the head, siphon, or tail. The tail-elicited siphon-withdrawal reflex (T-SWR) 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).

CURRENT PROJECT

As the simplest and most universal form of learning (Abramson, 1994), habituation is an extremely important area of inquiry, with implications for many fundamental topics in brain function and dysfunction (for a review see Rankin et al., 2009). To date, however, habituation has resisted clear physiological and transcriptional analyses even in simple model systems. With the sequencing of the *Aplysia* genome, however, the *Aplysia* T-SWR offers a promising system for finally resolving fundamental issues in the expression and decay of long-term habituation memory.

In this year of funding, we have conducted a relatively simple set of exploratory experiments aimed at connecting behavioral, physiological, and transcriptional changes that occur during long-term habituation. As a point of contrast, we have also conducted the same experiments but with long-term sensitization memory.

RESEARCH DESIGN AND METHODS

BEHAVIORAL METHODS

Animals. Animals (100-200g) were obtained from the RSMAS National Resource for *Aplysia* (Miami, FL) and maintained at 16^{o} C in one of two 90-gallon aquariums with continuously circulating artificial sea water (Instant Ocean, Aquarium Systems Inc.). Animals were separately housed in floating colanders, fed dried seaweed twice a week, and maintained on a 12 hr light -dark cycle. 2 days prior to any experimental testing, animals were fed to satiation and then food deprived for the remainder of the experiment. Food deprivation helps reduce variability in the T-SWR response.

Behavioral Measurement. To evoke T-SWR behavior, animals were anesthetized in ice-cold sea-water, implanted with wire electrodes to the left and right tail, and then parapodectimized (to ease viewing the siphon). T-SWR behavior was evoked via mild electrical shock (60Hz AC,50ms, 5-

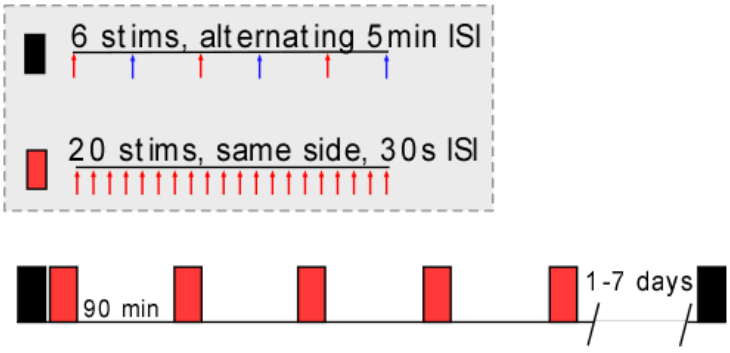


Figure 7: Long-Term Habituation Training. After characterizing baseline T-SWR responding to both the left and right tail (black), habituation is induced by 5 blocks of 20 stimulu (red) to one side of the animal. 1-7 days later, retention is test at both the trained and untrained side (black). Immediately following retention, animals are reduced to a siphon+tail preparation and re-tested while monitoring evoked activity in the T-SWR circuit. Finally, representative neurons from the T-SWR circuit are isolated for microarray analysis.

20mA set at 2x threshold for evoking a T-SWR) delivered by a WPI Constant-Current Stimulator (SYS-A385R). T-SWR behavior was measured as the duration of withdrawal, from the moment of stimulation to the first sign of siphon relaxation.

Habituation Protocol. Standard LTH protocols were be used (Figure 7). First, baseline T-SWR responsiveness was characterized as the average of 3 responses (10 min ISI, alternating sides). Next, unilateral LTH training was administered: 5 blocks of 30 stimuli to the same side, delivered at a 30s ISI, with 90 min between blocks. Immediately after training, a short-term retention will be measured (same as baseline). Long-term retention tests occured the next day (24 hour test). Control animals followed the same procedure, but no current was delivered during training. To ensure high throughput data collection, training was automated through a computer-controlled stimulator that enabled training of up to 4 animals at a time.

PHYSIOLOGICAL METHODS

Dissection to Semi-Intact Preparation. Immediately after retention testing, animals were dissected to a siphon+tail+CNS preparation using standard techniques (Calin-Jageman & Fischer, 2007; Fischer & Carew, 1995). Briefly, animals were anesthetized by injection of isotonic $MgCl_2$ (50% of body weight) into the body cavity. The siphon and mantle, tail, and central nervous system, along with the siphon and tail (P9) nerves, were then removed from the animal. The preparation was then transferred to a Sylgard-coated, two-chambered recording dish, and the mantle and tail was secured, dorsal side up with hypodermic needles. Throughout these experiments, the siphon and tail was continuously perfused with ASW through separate perfusion lines. The wires implanted in the tail are preserved in this dissection, enabling the same exact stimuli to be used to evoke T-SWR behavior. At least 1.5 hours of post-dissection recovery time was allowed before beginning physiological recordings. At this time, test shocks to the tail were be delivered (same parameters and stimulator as in intact animals) to ensure the ability to evoke T-SWR behavior in the preparation. If behavior could not be elicited, the preparation was discarded.

Extracellular Physiology. T-SWR circuit activity was monitored extracellularly via *en passant* suction electrodes (Calin-Jageman & Fischer, 2007). Electrodes were placed on the left and right P9 nerves, left and right pleural-abdominal connectives, and siphon motor neuron. These nerves carry tail sensory, T-SWR interneuron, and siphon motor neuron activity, respectively. Extracellular signals were filtered and amplified (Model 1700, AM-Systems) and then digitized for analysis (Powerlab 8/30, ADIstruments). Shock-evoked nerve activity was quantified as the integral of nerve activity recorded 250 ms after stimulation normalized to activity recorded 250 ms prior to stimulation (Calin-Jageman & Fischer, 2007).

Intracellular Physiology. In some cases, we also conducted intracellular physiology on selected components of the T-SWR circuit. Standard intracellular recording techniques were used. The ganglionic sheath over targeted neurons was microdissected away, and neurons were then impaled with glass microelectrodes (resistance 10-15 mΩ) containing either 3M KCl, 3M KAc, or 0.6m K_2SO_4 · 20 mM KCl. Tail sensory neurons were identified by their size, color, location, and responsiveness to tactile stimulation of the tail (Walters et al., 1983, 2004). Siphon motor neurons were identified by visually monitoring siphon movements that are characteristically produced by intracellular activation of these MNs.

ANALYSIS OF GENE EXPRESSION

Cell Isolation. Upon completion of behavioral or physiological testing, neurons from the tail-elicited SWR circuit were harvested for RNA isolation and microarray analysis using standard protocols (Lovell et al., 2006; Moroz et al., 2006). For tail sensory neurons, the entire sensory neuron cluster was trimmed away from the pleural ganglion. For more precise

isolations, the ganglia were briefly fixed, the ganglionic sheath was micro-dissected away, and single neurons were teased away from the ganglion with a microelectrode.

RNA isolation and reverse transcription. Tissue was homogenized and RNA was isolated using a standard isolation kit (RNeasy mini kit, Qiagen). Quantity and quality of RNA was assessed using the NanoDrop 2000 (Thermo Scientific). RNA was reverse transcribed using 200-400 ng of input material and oligo dT primes with First Strand cDNA kit (Fermentas). Our extraction technique regularly yields very high-quality RNA (260/280nm ratios between 1.9 and 2.1).

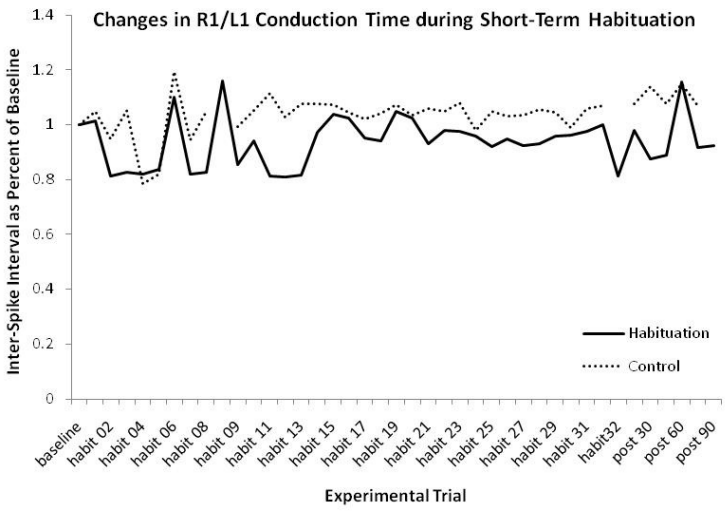
Real-Time PCR. Specific transcript levels of expression were analyzed by qPCR using the IQ real time PCR system (Bio-Rad). Briefly, cDNA was combined with SYBR Green supermix (Bio-Rad) and specific primers that have previously been validated for correct PCR efficiency. Samples were analyzed in triplicate and run in parallel with standard curves generated for all transcripts using serial dilutions of RNA input. Relative amounts of each transcript were determined by comparison to the standard curve and normalized to a housekeeping gene (histone H4 transcript).

RESULTS AND DISCUSSION

SHORT-TERM HABITUATION DOES NOT ALTER CONDUCTION VELOCITY

During the previous year of funding, we tracked the physiological changes that occur during a short-term habituation protocol. We found that there is a rapid and substantial decline in the evoked input traveling through the P9 nerves, which carry the sensory input into the T-SWR circuit. This data is consistent with sensory adaptation contributing to the behavioral decrements that occur during short-term habituation. Another possibility, though, is that the sensory action potentials simply travel more slowly, decreasing the peak activity measured in the nerves.

To rule out this possibility, we measured the conduction velocities of the spikes from L1 and R1 as they traveled through the P9 nerves. L1 and R1 are giant mechanoreceptors with receptive fields that include the left and right tail, respectively. We have repeatedly noted that eliciting the T-SWR evokes a number of L1 or R1 spikes (depending on the side of stimulation). Fortunately, these large neurons produce very large and easily identifiable spikes in the P9 and connective nerves. Thus, we measured their conduction velocity during a short-term habituation protocol by placing two extracellular electrodes on the corresponding connective nerves and determining the interval between the peak of the spike reaching each electrode.



Results are shown in the figure on the right for 4 control and 4 habituation experiments. These results show no clear trend in conduction velocity during habituation training, and in all trials, there were no significant differences relative to baselines nor to controls.

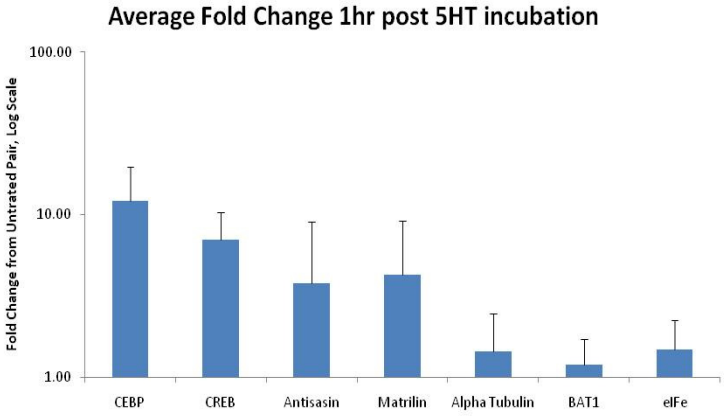
Overall, these results suggest that the decrease in sensory activity that occurs during short-term habituation reflects sensory adaptation, not a change in the rate at which sensory information enters the T-SWR circuit.

TRANSCRIPTIONAL CHANGES FOLLOWING LONG-TERM SENSITIZATION

During the previous year of funding, we completed a microarray experiment analyzing the transcriptional changes that occur 1 hour after a long-term habituation protocol. To provide a point of comparison, we decided to contrast these results with another learning paradigm: long-term sensitization. Sensitization is similar to habituation, in that both are ubiquitous, non-associative, and unilaterally expressed in the T-SWR circuit. Sensitization, however, is produced by repeated exposure to a *noxious* stimulus and yields an *increase* in behavioral responsiveness. Thus, it provides an interesting contrast to help identify transcripts that are involved in plasticity *per se* rather than a specific direction of change.

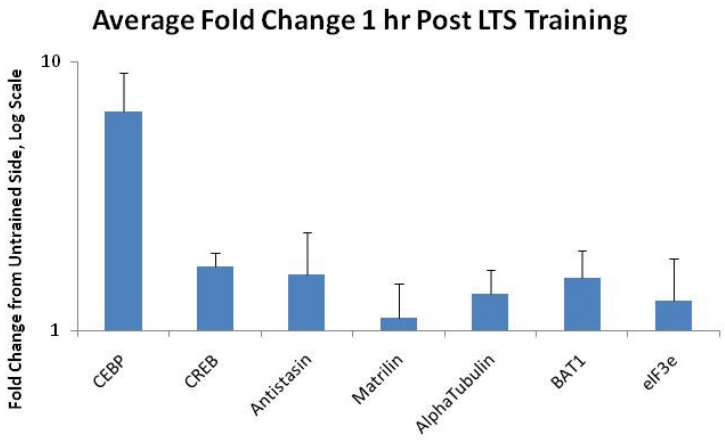
We began by using a pharmacological form of sensitization, exposure to serotonin (5HT). When *Aplysia* encounter painful stimulation, they are known to have CNS release of 5HT, and this release in known to be the critical step in initiating sensitization. Simple exposure to 5HT (dissolved in the tank) produces the same effects.

We exposed 6 animals to 5HT for 2 hours and then dissected them 1 hour later for RNA isolation and qPCR analysis. We specifically examined 6 transcripts previously reported to be regulated by this treatment in *Aplysia Kurodai*, a closely related species of gastropod (Lee et al., 2008).



Results are shown in the figure on the top right. We observed relatively strong and consistent of C/EBP, CREB, Antistasin-related transcript, and Matrilin. On the other hand, we did not observe consistent regulation of BAT1 homolog, nor eIF3e. We included Alpha-tubulin as a control known not to be regulated by sensitization learning.

Next, we repeated the same experiment, but using a slightly different learning context: true long-term sensitization training. This training consisted of 4 noxious shocks to the body wall, given at 30 minute intervals (each shock was 60mA, 500ms on / 500ms off for 10s). Only one side of the body was shocked, allowing comparison of gene expression with the untrained side of the body. 1 hour after the end of training, animals were harvested an the pleural ganglia were removed for analysis via qPCR. Results are show in the figure at bottom right. In this case, we observed robust regulation of C/EBP, modest changes in CREB and Antistasin, and no significant changes in Matrilin, BAT1, and eIF3e. It is important to note that all of these genes (except Alpha Tubulin) are regulated 1 hour after 5HT exposure in *Aplysia kurodai* (Lee et al., 2009).



Our results are quite striking—C/EBP stands out as strongly regulated across learning paradigms and two closely-related species. CREB and Antistasin also seem modestly regulated in all the conditions tested. Outside of these transcripts, however, we observed very little consistency in the changes in gene expression that accompany long-term memory. Although Matrilin, Bat1, and eIF3e are all regulated after 5HT exposure in *A. kurodai*, we see that only Matrilin is regulated after similar treatment in *A. californica*, and that none of these transcripts are regulated after LTS training, which is thought to be roughly equivalent to 5HT exposure. Taken together, these results suggest that there may be considerable variability in the 'molecular alphabet' of long-term memory, with only a few key transcripts showing consistent regulation across species and learning contexts.

AIM 2: MECHANISMS OF NEURAL CODING IN THE FLY OLFACTORY SYSTEM

The second aim is to better understand the neural coding of sensory inputs, using the olfactory system of the fruit fly, *Drosophila melanogaster*. The main set of experiments will allow a direct test of the combinatorial coding of odors hypothesis: are olfactory sensory neurons read by higher brains centers as an ensemble or can odors mediate behaviors by individual sensory receptors and circuits directly? In the fruit fly, the response to a single odor, ethyl acetate, is distributed between two sensory neurons, whose odor receptors respond with different affinities to ethyl acetate. The odor receptors will be mutated and these flies will be examined in chemotaxis behavioral assays in response to structurally similar odor molecules. This component of the project will help provide a better understanding of how the nervous system represents complex inputs for further processing and behavioral outputs; it also has implications for developing more effective insect repellents.

BACKGROUND AND SIGNIFICANCE:

One of the great challenges of neuroscience is to understand the rules by which sensory input is translated into behavioral output. Enormous progress has been made in elucidating principles by which sensory information is encoded by receptors and cells of the peripheral nervous system. Less is known, however, about the internal representations of sensory stimuli at each successive level of circuitry. Anatomical studies have traced synaptic connections in early stages of some circuits, and electrophysiological or imaging studies have revealed how representations of stimuli are transformed at some of these stages. However, the overall logic by which sensory information dictates a particular behavioral response in an organism remains largely unknown. An important long-term goal is the ability to predict the behavior of an organism from the activity of its sensory receptors.

In the case of olfaction, a major impediment to understanding how sensory input is translated into behavior is the numerical complexity of olfactory systems. A full molecular description of the sensory input into the system requires a functional analysis of the entire receptor repertoire. Olfactory systems of mammals and of *C. elegans* contain hundreds of different receptors (Ache and Young, 2005), and a functional analysis of an entire repertoire has not been possible with the available technology.

A fundamental hypothesis in olfaction is that odor identity and concentration are coded by odor receptors in a combinatorial manner. This hypothesis is based on observations that a single odor receptor can respond to multiple odors and that a single odor can activate multiple odor receptors (Malnic et al., 1999; Wilson and Mainen, 2006). The hypothesis is that odor identity is coded by differentially active subsets of odor receptors; odor concentration is coded by subsets of receptors that are recruited as odor concentration is increased. In general, any given olfactory sensory neuron expresses a single class of odor receptor (Mombaerts, 2004) thus odor receptor can be generally conceptualized as a proxy for sensory neuron. Testing the behavioral implications of the hypothesis has been difficult due to the large size of odor receptor repertoires in most species. The olfactory system of fruit flies has been comprehensively characterized at both the adult

and larval stages (Hallem and Carlson, 2006; Kreher et al., 2008). Due to the low number of odor receptors encoded in the genome, powerful genetic tools and robust behavioral assays, the fruit fly is an ideal model in which to test key hypotheses on the relationship between sensory input and behavior. The larval stage in particular is a particularly tractable model. Larvae have only 21 olfactory sensory neurons and a small brain (only 10,000 neurons) but can respond behaviorally to many odors (Cobb, 1999). The larval olfactory system is arranged in a phylogentically conserved manner, similarly to both adult insects and mammals (Kreher et al., 2005). Also, larvae can demonstrate olfactory-based learning, which allows examination of plasticity (Scherer et al., 2003). Finally, all odor receptors expressed in the larval stage have been characterized as well as the anatomy of the olfactory system (Couto et al., 2005; Fishilevich et al., 2005; Kreher et al., 2008).

Current research suggests that the combinatorial hypothesis may not be adequate. The behavioral response of the *Drosophila* larva to a single odor, ethyl acetate, is mediated by two odor receptors, *Or42a* and *Or42b* (Figure 1) (from Kreher et al., 2008). Each receptor is expressed in a distinct sensory neuron and is sensitive to different concentrations of ethyl acetate (Figure 1B); when each receptor is mutated, larvae have behavioral defects only in the odor sensitivity range of the receptors (Figure 1C). An unexpected observation was that when the low affinity receptor is mutated, leaving the high affinity receptor intact, larvae are repelled by ethyl acetate at high concentrations. These results suggest that ethyl acetate concentration is not coded in an additive manner, and thus may not be coded combinatorially. Each individual sensory neuron may relay specific information about the odor to the brain and there may be interaction between the circuits at various levels to allow behavioral responses across odor concentrations.

Figure 1

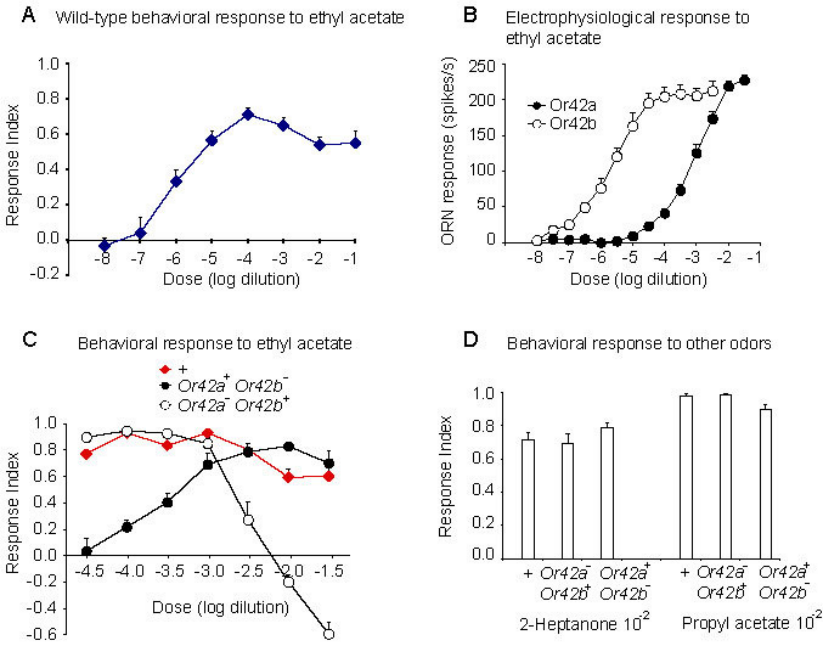


Figure 1. The response to one odorant depends on two receptors

(A) Behavioral response of wild-type to ethyl acetate. For entire figure, error bars=SEM; n=10. (B) Electrophysiological responses to ethyl acetate conferred by Or42a and Or42b in the empty neuron system; n=6 (C) Behavioral responses of larvae with mutant alleles of *Or42a* or *Or42b* to ethyl acetate; $6 \leq n \leq 10$. (D) Behavioral responses to 2-heptanone and propyl acetate, at 10^{-2} dilutions; n=6. Responses are not statistically different from wild-type controls

The outstanding questions are : 1) what is the nature of the code created by these receptors in response to odor molecules which are structurally similar? The exact nature of combinatorial coding has been difficult to examine, however with our panel of mutants and robust behavioral assay, we can use inductive logic to examine how two odor receptors contribute to behavioral responses to various odor molecules. Furthermore, with our comprehensive data set on electrophysiological responses, can we make predictions of how odor receptors underlie behavioral responses. 2) How can variation in behavioral responses be explained at the gene level? We know that robust variation in response to single odors

exists in wild-type strains. Since we have identified necessary odor receptors that respond to odors, can we detect polymorphisms in these receptor genes which may explain behavioral variation.

RESEARCH DESIGN AND METHODS

FRUIT FLY GENETICS

Fruit flies will be housed on standard cornmeal-dextrose medium. Crosses will be established and monitored using standard genetic methods. All mutants will be phenotypically scored before their use in experiments and genotyped using standard PCR screening of alleles. All transgenic flies have already been constructed according to standard procedures. All mutant alleles will be out-crossed to a standard stock to allow comparisons between lines.

BEHAVIORAL EXPERIMENTS

Olfactory behaviors in *Drosophila* larvae are robust and easy to measure. A standard population assay will be used to measure responses to the odor panel (Kreher et al., 2008; Monte et al., 1989). Briefly, a population of flies will be allowed to lay eggs for 24 hours in a bottle of medium and then discarded; 4-5 days later, larvae will be harvested with 15% sucrose and washed three times with water. Approximately 50 larvae will be transferred to a plate containing 1% agarose. The larvae will be presented with an odor diluted in solvent or solvent alone and given 5 minutes to migrate. After 5 minutes, a response index will be scored by counting the number of larvae near the odor. The response index ranges from +1, which indicates total attraction, to -1, which indicates total repulsion.

RESULTS AND DISCUSSION

THE ODOR RECEPTOR PROTEINS OR42A AND OR42B GENERATE DISTINCT SIGNALS ALLOWING DETECTION OF SIMILAR ODOR MOLECULES

To understand how *Or42a* and *Or42b* generate signals allowing odor detection, we extended our examination of behavioral responses to a wider range of odor dilutions of structurally similar odors: methyl acetate, propyl acetate, and ethyl butyrate (Figure 2). In the case of methyl and propyl acetate, these odor molecules differ from ethyl acetate in a series by a -CH_2 group added to the alcohol moiety. Ethyl butyrate differs from ethyl acetate by addition of CH_2-CH_2 to the acid moiety.

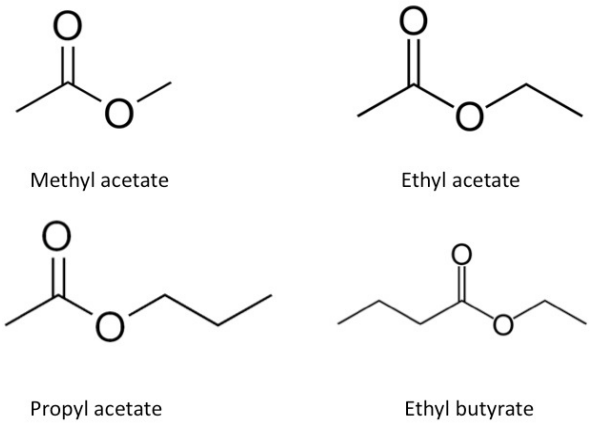


Figure 2 Odorant molecules used in this study

Chemical structures of the esters:

A. Behavioral responses to ethyl acetate across odor concentrations

As an initial experiment and as a control, the responses of the *Or42a* and *Or42b* mutants were examined in response to a dilution series of ethyl acetate. In agreement with previous findings, each mutant displayed complementary phenotypes in response to this odor (Figure 1; Figure 3; Kreher et al., 2008). Additionally, the responses of all strains were examined at lower odor dilutions. The wild-type control and the Or42a mutant displayed similar reductions in attraction to ethyl acetate on a log-linear scale as the odor was reduced in concentration. Finally, a negative control was added, an Or83b mutant. Or83b is an essential co-receptor for all insect odor receptors; mutations of this receptor render animals anosmic (Larsson et al., 2004). As expected, Or83b mutants displayed a response index value of 0 across concentrations, indicating random movement on the plate. The inclusion of this negative control allows confident conclusions about determination of chemotaxis and also the range of response indices which indicate anosmia.

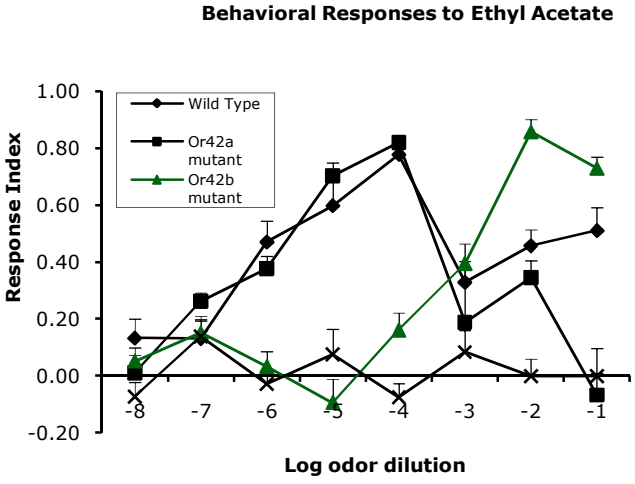


Figure 3

Behavioral responses to ethyl acetate

Responses indices were examined for Or42a and Or42b mutants.

BEHAVIORAL RESPONSES TO METHYL ACETATE ACROSS ODOR CONCENTRATIONS

Responses to methyl acetate were next examined, across odor concentrations. Methyl acetate is also an ester, which differs from ethyl acetate by a methylene (CH_2) group.

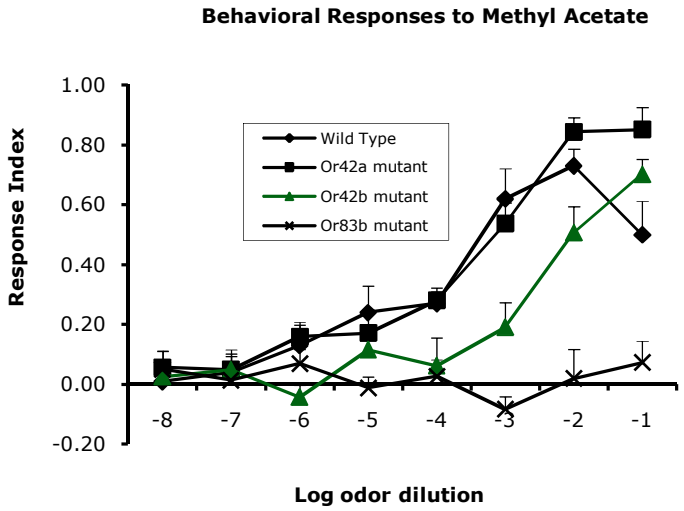


Figure 4

Behavioral responses to methyl acetate

Responses indices were examined for Or42a and Or42b mutants.

The responses of the wild-type strain to methyl acetate were generally similar to ethyl acetate: behavioral response to the highest concentration (10^{-1}) were at a local minimum which rose to the maximal response around 10^{-2} to 10^{-3}. As odor concentration was reduced, behavioral responses declined in a log-linear manner (Figure 4).

The Or42a mutant displayed mean behavioral responses which were very similar to wild-type from 10^{-8} to 10^{-3}. Since the mutation leads to a loss-of-function of Or42a, this receptor is not playing a role at these concentrations of methyl acetate. However, behavioral responses were elevated at 10^{-2} and 10^{-1}, where the typical local minimum response was not observed. From these data, we conclude that Or42a is contributing to the dampened behavioral response at highest odor concentrations.

By contrast, responses of the Or42b mutant are dampened across most odor concentrations. We would then conclude that Or42b is at least partially necessary for response to methyl acetate. There are two possible further explanations that arise from these data. First, Or42b is not fully essential for detection of methyl acetate since behavioral responses do not drop to zero. A second explanation, which is not mutually exclusive with the first, is that Or42a may be partially redundant with Or42b in response to this odor. However, another receptor or receptors may also be playing a role.

Electrophysiological responses of the odor receptors to methyl acetate were not examined, thus we cannot make any conclusions regarding predictions of behavior.

BEHAVIORAL RESPONSES TO PROPYL ACETATE

A third odor, propyl acetate was examined. Similarly to methyl acetate, wild-type responses show a local minimum at the highest odor concentrations (10^{-1}), show a global maximum when odor concentration is slightly reduced (10^{-2}), and then decrease in a log-linear manner as odor concentration is reduced (Figure 5).

Or42 mutants show behavioral responses to propyl acetate that are generally similar to wild-type, indicating that this receptor is not necessary for odor response to propyl acetate (Figure 5). These behavioral responses are inconsistent with electrophysiologial data; Or42a confers a high rate of action potentials (>200 action potentials/second) in response to propyl acetate (Kreher et al., 2008). A more encompassing explanation is that Or42a may allow detection of propyl acetate, but may be redundant with other receptors. An extension of this explanation is that many odor receptors confer high electrophysiological responses to various odors, yet these odors are either only weak attractants or larvae are indifferent to them (Kreher et al., 2008)

In contrast, responses of the Or42b mutant are reduced at lower odor concentration ($<10^{-2}$), similar at 10^{-2}, and elevated at 10^{-1} (Figure 5). The best conclusion here is that Or42b is contributing a distinct signal regarding propyl acetate which depends on concentration, that may be negated by the activity of other odor receptors.

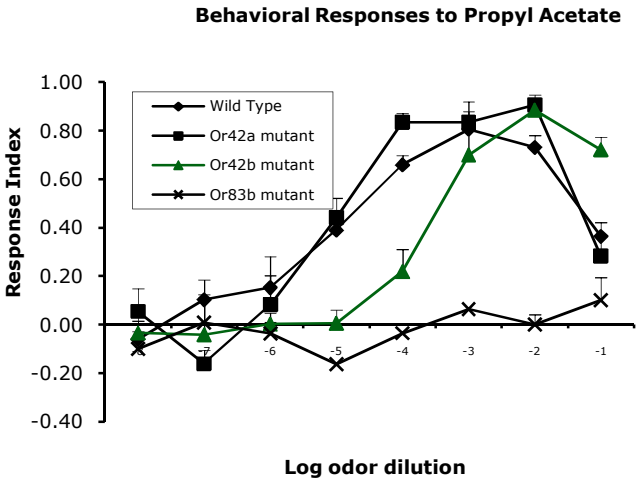


Figure 5

Behavioral responses to propyl acetate

Responses indices were examined for Or42a and Or42b mutants.

BEHAVIORAL RESPONSES TO ETHYL BUTYRATE

A fourth similar odor was examined, ethyl butyrate. Unlike the previous three odors, both mutants display similar alterations in behavior relative to the wild-type control (Figure 6). The wild-type strain again displays responses which are local minima at the highest odor concentration, which rise as odor concentration decreases. Both Or42a and Or42b mutants, however, display reduced responses at 10^{-1}; behavioral responses increase as odor concentration is reduced. Currently, we lack data on responses to lower odor concentrations.

These behavioral data are consistent with electrophysiological data: both Or42a and Or42b show strong responses to ethyl butyrate at high odor doses and they both decrease similarly in response to lower odor doses. The best explanation is that receptors with similar electrophysiological dose response curves may contribute similarly to reception and perception of odors.

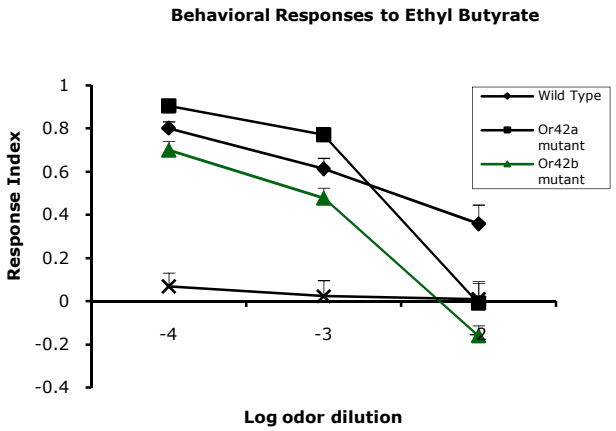


Figure 6

Behavioral responses to ethyl butyrate

Responses indices were examined for Or42a and Or42b mutants.

II. Two wild-type strains vary in their sensitivity to a single odor

As a related but distinct research question, we examined how standard wild-type strains vary in their response to a single odor. Previous researchers concluded that two standard wild-type strains, Canton-S and Oregon-R, varied in their sensitivity to the odor ethyl acetate (Monte et al., 1989).

Our first experiment was to repeat this experiment using the wild-type strains. As had been previously found, Canton-S and Oregon-R had consistent variation in attraction to ethyl acetate. At higher odor concentrations, the two strains display similar levels of attraction to ethyl acetate (Figure 7). However, at the dose is reduced ($<10^{-4}$), Oregon-R responds more strongly to ethyl acetate. At the lowest odor doses, both strains show little response.

Based on our previous findings that Or42a and Or42b are largely explanatory for response to ethyl acetate, we would predict that variation in these two genes would explain behavioral differences. Furthermore, since variation is seen only at low to midlevel odor doses, we would predict to find variation in the high-affinity receptor, Or42b, and the lack of consistent variation in the low-affinity receptor, Or42a.

To answer this question, we have isolated DNA from multiple individual flies from both strains and are sequencing the entire coding region of Or42a and Or42b. We would predict that we would find consistent polymorphisms in Or42b that may lead to amino acid differences in the receptor protein; we would predict that the Or42a coding regions are similar or vary in an inconsistent manner.

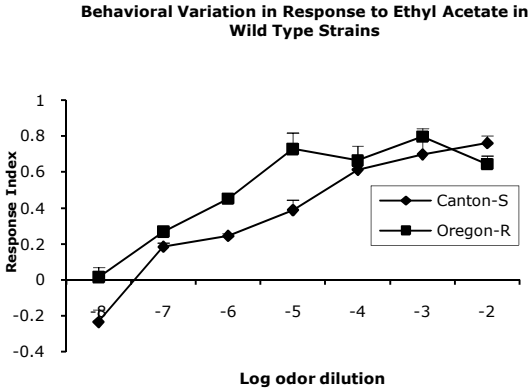


Figure 7

Variation in behavioral responses to ethyl acetate

Responses indices were examined for the standard wild-type strains, Canton-S and Oregon-R.

CONCLUSION

Our work has thus far revealed the complexity present within a "simple" animal's olfactory system. Our systematic analysis of mutations of both Or42a and Or42b has allowed us to conclude that odor receptors contribute to behaviors in a manner that is not fully predictable based on current knowledge of odor receptor electrophysiological activity and that these results are not fully consistent with the combinatorial coding hypothesis.

Responses to four structurally similar odor molecules can be classified into multiple groups: 1. Both receptors contribute in a similar manner (ethyl butyrate) 2. Both receptors contribute in a complementary manner (ethyl acetate) 3. One receptor, but not both, are at least partially necessary for behavioral responses (methyl acetate and propyl acetate). Furthermore, in the case of propyl acetate, Or42b contributes a different signal quality depending on odor concentration.

These data, taken together, demonstrate the complexity of how olfactory systems may allow discrimination of both odor quality and concentration. Obvious molecular features of odor molecules are insufficient to predict odor perception and an empirical examination of the relationship between electrophysiological response and behavioral output is absolutely necessary to deduce the rules by which olfactory systems operate.

Finally, we are examining how variation in odor receptors may allow variation in sensitivity to odors. We have reproduced an important behavioral experiment and are now examining how molecular level variation may explain behavioral variation. The implications of these questions speaks directly to the important questions of how olfactory systems evolved and allow variation to the odors within a species.

AIM 3. FOSTER A WORLD-CLASS UNDERGRADUATE NEUROSCIENCE PROGRAM

UNDERGRADUATE NEUROSCIENCE MAJOR

With aid from the first year of funding on this project, Dominican University established an undergraduate neuroscience program in 2008. This program has thrived over the past two years. In the 2009-2010 academic year, we had our first graduate from the program. For this academic year, 6 students are on track to graduate. To provide some context, the psychology department, which is typically the 1st or 2nd biggest major on campus, graduates about 30 students per year.

Consistent with the strong growth in student interest, we have founded a neuroscience social club and a local chapter of Nu Rho Psi, the national honors society for undergraduate neuroscience majors. These social organizations have begun sponsoring programming (movie screenings, journal clubs, etc.) that will help ensure a strong base of recruits for future years.

UNDERGRADUATE RESEARCH

A key to a strong neuroscience major is participation in research. Therefore, we funded 14 students with modest stipends to participate in research across the different labs participating in the neuroscience program at Dominican (see outcomes section, below). This included 6 students from under-represented minorities (42%). Together, these students accomplished almost all of the research reported in the first two aims, plus considerably more that went beyond the first two research aims.

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 (Tim Lazicki), 1st **and** 3rd place in a regional poster competition (Tim Lazicki and Benora McBride), 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).

These research experiences are having a large impact on our students. 9 of the 14 students funded are approaching graduation or have graduated. While most of them are still waiting to hear about their post-graduate applications, 3 of these 9 have already been accepted into high quality post-graduate programs (Tim Lazicki – Neuroscience Program at University of New Mexico; Benora McBride – Pharmacy School at the College of Notre Dame, Nadine Nitsusanta – Dental School at Tufts University).

EXTRAMURAL FUNDING

When the first year of earmark funding began in 2008, Dominican University had very little active research. With the help of these funds, we now have 5 active undergraduate research labs in the natural sciences. Moreover, the quality of the work funded has yielded the first competitive NIH grant ever awarded to the university, an R-15 jointly awarded to the PI and Irina Calin-Jageman (see outcomes section, below).

SUMMER PROGRAM TO ENHANCE STEM PARTICIPATION

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.

The SURE program was initiated in the summer of 2008 with the first year of funding on this project. It continued in 2009, and this year's funding allowed continuation in 2010. SURE 2010 was held May 23 - 28. We welcomed twelve community college students from six different community colleges: Harold Washington, Truman, Triton, Wilbur Wright, Kennedy King and Malcolm X.

SURE is a five-day boot camp introduction to life and laboratory instruction at a four-year school. CC students who participate in the event spend the five days in the Dominican University dormitory. A curriculum has been designed that bolsters quantitative research skills, enhances comfort in performing basic laboratory techniques, and fosters a cohesive social group where students encourage each other as they transition from Community College to Baccalaureate Granting Institution. It is important to note that Dominican University is situated close to the city of Chicago, close to public transportation lines, and within 20 miles of several city and suburban community colleges.

The boot camp experience involves 1) preparation for the project through reading and discussing relevant scientific articles, 2) introduction to basic laboratory techniques, 3) hands on laboratory work, and 4) collection, interpretation, and presentation of data. Student participants are typically divided into teams of three or four as they perform their experiment. The groups then are charged to give a report of their laboratory activities from the day before to their peers and instructors the next day. Each of the five days focuses on a different scientific discipline. SURE has been successful in helping CC students who are interested in STEM related careers gain confidence in their ability to be successful academically at a four-year college. Former SURE participants have enrolled in a 4-year college of their choice and are actively interested in assisting other students like themselves who are preparing for a STEM related career.

Seven Dominican faculty members from chemistry, biology and neuroscience disciplines and one Triton College faculty participated in the program in 2010. Speakers were invited from Elmhurst College (Venkatesh Gopal) and Wilbur Wright Community College (Joseph Oyugi).

The response from the students was very positive for the most part. Selected responses to the question "Overall what do you feel you gained from participating in SURE?"

- "overall i gained alot through this experience. i learned how to analyze data and make conclusions. it opened my mind to pursuing more scientific research opportunities, because its so interesting and i would love to do research as much as i can and learn more about it."
- "I feel that I have a better understanding of what is available to me in terms of education and careers. I was able to ask many questions on a variety of topics and felt that all of the professors were not only educating me on their topics, but were helping me plan my future as well"
- "I gained a greater understanding of what pathways are available to me in science, and I established valuable connections with other professors that can offer networking and advice in the future. I've also been able to evaluate my interests in a much broader context to help decide where I really feel comfortable, and where I should investigate a career more. Thank you for offering this program! The skills that were taught are important, but these can be learned in school. Instead, I think the program's strengths lie in networking with professors in your interest areas, and the variety of subjects offered. These strengths give students a greater pool of niches to explore, and a greater support system to get them to where they want to go."

OUTCOMES

COMPETITIVE FUNDING

- Mechanisms in the Expression and Decay of Long-Term Habituation
  NIH Grant #1R15MH090998-01, co-PI
  $249,000 – direct costs
  Funding period: 7/15/2010-7/14/2010

STUDENT RESEARCH ASSISTANTS FUNDED

Rose Beausoliel	Behavioral Neuroscience Lab		
Kristine Bonnick	Neurobiology Lab	M	Applications for Med School Pending
Kevin Buchik	Behavioral Neuroscience Lab		
Tim Lazicki	Alexian Health Care		Accepted to Grad Program in Neuroscience
Karolina Matel	Behavioral Neuroscience Lab		Applications for Med School Pending
Benora McBride	Behavioral Neuroscience Lab	M	Accepted to Pharmacy School
Nadine Nitsusanta	Neurobiology Lab	M	Accepted Dental School
Ike Ofaku	Alexian Health Care	M	
Patrycja Oleksiak	Neurobiology Lab		Applying for jobs as research tech
John Pontikis	Behavioral Genetics Lab		

Karl Rickert	Neurochemsitry Lab		
Raquel Robles	Neurobiology Lab	M	Accepted to Medical School
Laura Salazar	Behavioral Neuroscience Lab	M	Apps to Clinical Psych Pending
Natalie Thompson	Behavioral Genetics Lab		

M = Minority Student

Where no plans are noted, the student is not yet a senior

UNDERGRADUATE PROFESSIONAL PRESENTATIONS

- McBride B*, Salazar *L, Bonnick K*, Matel 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 and *Faculty for Undergraduate Neuroscience Meeting*, Chicago, IL.
- McBride B*, Bonnick K*, Matel K*, Oleksiak P*, **Calin-Jageman RJ** & Calin-Jageman IE (2009). Behavioral physiological, and genetic analysis of habituation of the Aplysia tail-elicited siphon withdrawal reflex. *Molecular and Cellular Cognition Meeting,* Chicago, IL.
- Bonnick K*, McBride B*, Salazar L*, Kroes R, Moskal J, **Calin-Jageman RJ** & Calin-Jageman IE (2010). Behavioral, physiological, and genetic mechanisms of habituation in *Aplysia californica*. *Association for Psychological Science,* Boston, MA.
- Bonnick K*, McBride B*, Salazar L*, Kroes R, Moskal J, **Calin-Jageman RJ** & Calin-Jageman IE (2010). Behavioral, physiological, and genetic analysis of habituation of the Aplysia tail-elicited siphon withdrawal reflex. *Society for Neuroscience Abstracts*, 35, 409.7 and *Faculty for Undergraduate Neuroscience Meeting*, San Diego, CA.

* Denotes undergraduate researcher

UNDERGRADUATE HONORS AND AWARDS

- McBride B & Calin-Jageman RJ (2009). SOMAS grant, HHMI and Davidson College.
- Bonnick K (2009). 3rd Place, Undergraduate Poster Competition, Chicago Chapter of the Society for Neuroscience
- Lazicki (2009). 1st Place, Undergraduate Poster Competition, Chicago Chapter of the Society for Neuroscience
- Lazicki T (2009). Nu Rho Psi national travel award for the Society for Neuroscience Annual Meeting

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