NCS-FO: Variability and Plasticity of Single Neurons in Hippocampal Learning and Memory

PROJECT DESCRIPTION

RESULTS FROM PRIOR NSF SUPPORT

1a. NSF IOS-0843712; PI: Johann "Hans" A. Hofmann; Award Amount: $515,000; Period of Support 07/15/09-07/14/14 (in no-cost extension)

1b. **Title: Molecular Basis of Social Decision Making: Towards a Mechanistic View of Mate Choice.**

1c. Summary of results: *Intellectual Merit.* Using behavioral, pharmacological, and molecular/genomic approaches, we examined the neural basis of mate choice decisions in monogamous and non-monogamous species of cichlid fish. The project had three aims: 1) Test the association of female sexual behavior with hormonal, morphological, and transcriptional changes at high temporal resolution throughout the reproductive cycle; 2) Examine the transcriptional events in candidate brain regions associated with mate choice decisions, independent of the act of mating; 3) Determine the effects of PGF2 alpha on female sexual behavior. *Broader Impact.* Three high school students from an internship program with urban Crockett High School participated in this research, and one published a paper. Eights undergraduate students participated in this research and all published papers. This research was presented in public lectures and interviews to news outlets to the US, Europe and Asia.

1d. Twenty publications produced: [1]Kidd C, Kidd MR, Hofmann HA, *Horm Behav* 165:277, 2010; [1]Brawand et al. *Nature* 513: 375-381, 2014; [2]Harris RM, Dijkstra PD, Hofmann HA, *Gen Com Endocrinol* 195:107-115, 2014; [3]Harris RM, Hofmann HA, *Adv Exp Med Biol 781*: 149-168; [4]Huffman LS, O'Connell LA., Hofmann HA, *Physiol Behav* 112:77-83, 2013; [5]Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA, *J Chem Neuroanat* 44:86, 2012; [6]Kidd MR, Dijkstra PD, Alcott C, Lavee D, Ma J, O'Connell LA, Hofmann HA, *Behav Ecol and Sociobiol* 67:1307, 2013; [7]Kidd MR, Duftner N, Koblmüller S, Sturmbauer C, Hofmann HA, *PLoS ONE* 7:e31236, 2012; [8]Kidd MR, O'Connell LA, Kidd CE, Chen CW, Fontenot MR, Williams SJ, Hofmann HA, *Gen Comp Endocrinol* 180:56, 2012; [9]Munchrath LA, Hofmann HA, *J Comp Neurol* 518:3302, 2010; [10]O'Connell LA, Ding JH, Hofmann HA, *Horm Behav*, 64:468-476; [11]O'Connell LA, Fontenot M, Hofmann HA*, J Comp Neurol* 519:75, 2011; [12]O'Connell LA, Hofmann HA, *Front Neuroendocrinol* 32:320, 2011; [13]O'Connell LA, Hofmann HA, *J Comp Neurol* 519:3599, 2011; [14]O'Connell LA, Hofmann HA, *Science* 336:1154, 2012; [15]O'Connell LA, Matthews BJ, Hofmann HA, *Horm Behav* 61:725, 2012; [16]O'Connell LA, Rigney MM, Dykstra DW, Hofmann HA, *J Neuroendocrinol* 25:644, 2013; [17]Oldfield RG, Hofmann HA, *Physiol Behav* 102:296, 2011; [18]Oldfield RG, Harris RM, Hendrickson DA, Hofmann HA, *Horm Behav* 64:44; 2013 [19]Oldfield RG, Mandrekarb K, Hendrickson DA, Chakrabarty P, Hofmann HA, *Hydrobiolgia*, in press, 2015; [20]Sessa AK, Harris RM, Hofmann HA, *Gen Comp Endocrinol* 189:59, 2013

1e. All sequence data generated from these projects are freely available at NCBI (http://www.ncbi.nlm.nih.gov/).

2a. NSF IOS-1011253; PIs: Lauren A. O'Connell, Johann "Hans" A. Hofmann; Award Amount: $15,000; Period of Support 07/15/2010-06/30/2011

2b. **Title: Dissertation Research: Social Regulation of the Estrogen Receptor Gene Regulatory Network.**

2c. Summary of results: *Intellectual Merit.* Using behavioral, pharmacological, and molecular/genomic approaches, we tested the hypothesis that estrogens modulate male aggression. We then determined how social plasticity influences gene regulatory networks by asking whether ERs regulate transcription of genes important male aggression in the preoptic

area (POA). This project provided fundamental new insights into how gene regulatory networks change with social status and subsequently influence behavior. *Broader Impact.* Five undergraduate students participated in this research and all co-authored papers. This research was presented in public lectures and interviews to news outlets to the US, Europe and Asia. The graduate student supported by this grant presented her research and related scientific activities in neuroscience outreach programs local community events, such as Austin Safe Place and UT-Explore. With this experience, the graduate student founded her own outreach program, *Little Froggers* (http://oconnell.fas.harvard.edu/little-froggers-school-program), in collaboration with Harvard University and local elementary schools.

2d. Ten publications produced: [1]Huffman LS, O'Connell Lauren A., Hofmann HA, *Physiol Behav* 112:77-83, 2013; [2]Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA, *J Chem Neuroanat* 44:86, 2012; [3]Munchrath LA, Hofmann HA, *J Comp Neurol* 518:3302, 2010; [3]O'Connell LA, Ding JH, Hofmann HA, *Horm Behav*, 64:468-476; [5]O'Connell LA, Fontenot M, Hofmann HA, *J Comp Neurol* 519:75, 2011; [6]O'Connell LA, Hofmann HA, *Front Neuroendocrinol* 32:320, 2011; [7]O'Connell LA, Hofmann HA, *J Comp Neurol* 519:3599, 2011; [8]O'Connell LA, Hofmann HA, *Science* 336:1154, 2012; [9]O'Connell LA, Matthews BJ, Hofmann HA, *Horm Behav* 61:725, 2012; [10]O'Connell LA, Rigney MM, Dykstra DW, Hofmann HA, *J Neuroendocrinol* 25:644, 2013.

2e All sequence data generated from these projects are freely available at NCBI (http://www.ncbi.nlm.nih.gov/).

3a. NSF IBN-0751311; PI: Johann "Hans" A. Hofmann; Award Amount: $160,000; Period of Support 07/15/08-07/14/12

3b. **Title: Collaborative Research: Physiological and Molecular Mechanisms Underlying Natural Variation in a Fish Escape-Circuit.**

3c. Summary of results: *Intellectual Merit.* Using behavioral, pharmacological, electrophysiological and molecular/genomic approaches, we examine the neural basis of cichlid escape behavior at the level of a single neuron, the M-cell. The project has 3 aims: 1) Test how serotonin (5-HT) affects individual and population variation in escape behavior; 2) Quantify how M-cell membrane properties are related to individual variation in escape behavior hormone levels; 3) Measure 5-HT receptor expression and genome-wide expression profiles in single M-cells (after behavioral and electrophysiological testing of the same animals). *Broader Impact.* Two high school students and two undergraduate students participated in this research and some co-authored papers. This research was presented in public lectures and interviews to news outlets to the US, Europe and Asia. The PI introduced elementary class rooms to the biology of brain and behavior and fieldwork in Africa.

3d. Four publications produced: [1]Harris RM, Hofmann HA, *Adv Exp Med Biol 781*: 149-168; [2]Neumeister H, Whitaker KW, Hofmann HA, Preuss T, *J Neurophysiol* 104:3180, 2010; [3]Whitaker KW, Neumeister H, Preuss T, Hofmann HA, *J Neurophysiol* 106:127, 2011; [4]Wong RY, Hofmann HA, *Encyclopedia of Life Sciences*, 2010; [5]Whitaker KW, Alvarez M, Preuss T, Cummings ME, Hofmann HA, *Proc R Soc B*, in review.

3e. All sequence data generated from these projects are freely available at NCBI (http://www.ncbi.nlm.nih.gov/).

CONCEPTUAL FRAMEWORK

Identifying the neuromolecular basis of individual variation is a major challenge for genotype to phenotype mapping. Diversity in neuronal structure and function is one of the most striking sources of variability in organisms with nervous systems and gives rise to complex behavior and cognition. Yet our understanding of the molecular processes underlying neuronal individuality and

plasticity is still very limited and made all the more difficult because we now know that experience changes gene expression, which itself changes neural function and thus the subsequent neural functions in future experience (Eberwine et al., 2012; Ginsberg et al., 2004; Park, Ogunnaike, Schwaber, & Vadigepalli, 2014). Progress in systems neuroscience depends on integrating data across levels of organization in order to gain a more comprehensive understanding of the plasticity and variability at the individual and cellular level. The proposed research will integrate measures of physiology and gene expression, behavioral measures of learning and memory formation, and differences across genotype to better understand variability at the individual and cellular levels (Fig. 1).

Relationship between Memory, Synaptic Plasticity, and Gene Transcription

The brain is a remarkably adaptable structure that has the capacity to convert sensory information into neuronal function. Neurons convert sensory input to electrical and chemical signals, elicit transcriptional responses, and store the information via long-term changes in neuronal circuits. Synaptic plasticity enables behavior to adapt to an ever-changing environment so that the organism achieves an evolutionary fitness benefit. The "**synaptic plasticity and memory" hypothesis** poses that memory is based on long-term changes in the function of a sparsely distributed set of synapses within neuronal circuits via specific modulation of molecular mechanisms that regulate synaptic function, and that acquisition and maintenance of different types of memory are associated with distinct region-specific changes at the level of single cells (Mayford, Siegelbaum, & Kandel, 2012; Takeuchi, Duszkiewicz, & Morris, 2014). Stimulus-

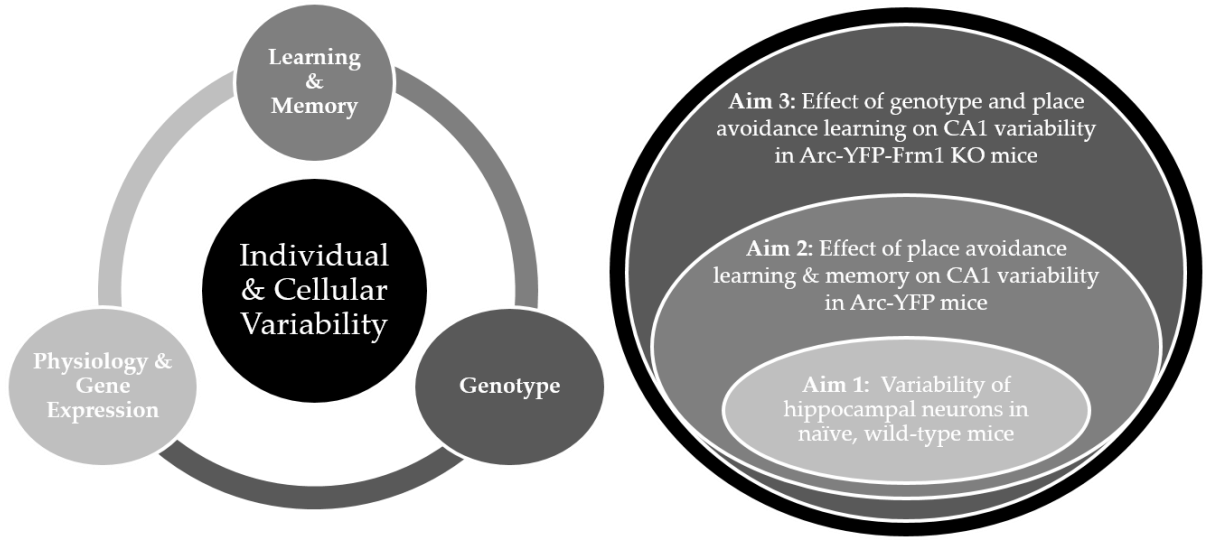

Fig 1. Conceptual Framework and Experimental Overview. A comprehensive understanding of the plasticity and variability at the individual and cellular level through integration of data across levels of organization is essential to progress in systems and behavioral neuroscience. The proposed research integrates measures of physiology, gene expression, and behavior across genotypes and in the context of learning and memory formation to better understand variability at the individual and cellular levels. Aim 1 will compare baseline variability in physiology and gene expression in single hippocampus neurons of naïve mice. Aim 2 will build on this by adding learning in an active place avoidance task. The use of Arc-YFP mice will allow us to distinguish between activated and non-activated hippocampal CA1 neurons. Aim 3 will determine the influence of genotypic differences using the Arc-YFP-Fmr1-KO mouse line. Each aim is independent, but together they provide unique insights into the mechanisms (and their interactions) that give rise to individual variation at the cellular and whole-organism level.

regulated transcription factors play a crucial role in long-term synaptic plasticity (reviewed in (Alberini, 2009)). Previous findings report activity regulated transcription of new gene expression by learning experience (Lyons & West, 2011). By analysis of transcription patterns, it has been shown that memory-related gene expression patterns can play distinct roles in memory formation (Mei et al., 2005). In addition, there is a strong association between activity-dependent gene expression and neural circuit plasticity (reviewed in (Leslie & Nedivi, 2011)).

Measuring the activity of single genes is generally insufficient to describe and understand the state of any biological system. Large-scale transcriptomic studies have given biologists the power to uncover patterns of **gene networks that are up-regulated or down-regulated in a coordinated manner** that reflects the biological phenotype (Langfelder & Horvath, 2008; Langfelder, Mischel, & Horvath, 2013). Therefore, one of the advantages of using genome-scale transcriptomics tools, such as RNA-seq to study variability and plasticity is that the activity of the entire genome can be studied at once, and the genes can be classified according to their responses to specific environmental variables. Genes that respond in the same way to a given environmental cue can be identified, and their biological and genomic characteristics studied, resulting in the analysis of higher-order biological processes linked to phenotypic variability (Aubin-Horth & Renn, 2009). Addressing higher-order processes that comprise the mechanisms of plasticity and identifying its genomic make-up may precede a thorough understanding of specific mechanisms.

Hippocampus and memory

The mammalian hippocampus is a crucial brain system for processing and storing neural representation of context, including space and time (O'Keefe & Nadel, 1978). Since the famous case of patient H.M., who reported severe deficits in new memory formation, many studies have investigated the neural mechanisms in the hippocampus that form a new memory, finding that plastic synaptic modifications are associated with novel episodic like memory formation in hippocampus in rodents, monkeys, and human (Takashima et al., 2006; Wang & Morris, 2010). Importantly, **immediate early genes (IEGs), such as Arc and c-Fos, are significantly increased in hippocampal neurons activated by context experience** (Guzowski, Setlow, Wagner, & McGaugh, 2001; L A O'Connell, Rigney, Dykstra, & Hofmann, 2013; Lauren A O'Connell, Matthews, & Hofmann, 2012). The role of the hippocampus is not limited to encoding of new information but also to retrieval of the archived information. When recalling spatial information in the Morris Water Maze task, spatial memory can be anatomically re-organized in a temporal manner, associated with IEG activation during salient memory recall (Lopez et al., 2012). During contextual fear conditioning the hippocampus is similarly characterized by memory-specific gene expression patterns (Cavallaro, D'Agata, Manickam, Dufour, & Alkon, 2002; Mei et al., 2005; Miyashita, Kubik, Lewandowski, & Guzowski, 2008).

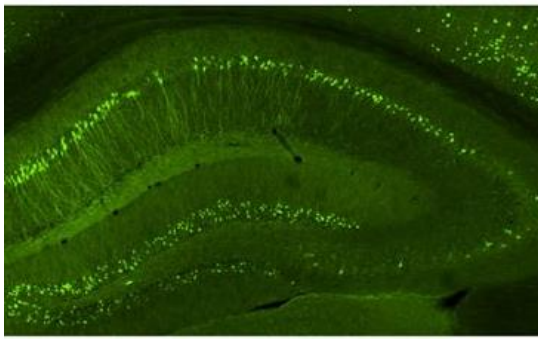

Fig. 2. Identified neurons recruited by active place avoidance training. The "Arc-YFP" mouse is a cross of ArcCreERT2 and R26R-STOP-floxed-YFP mice donated by René Hen (Denny et al., 2014). 4OH-tamoxifen injection before initial training activates Cre leading to YFP expression in neurons that transcribed Arc during training. Control mice have few YFP+ cells (not shown). We have crossed Fmr1 KO mice with this mouse to generate ArcCreERT2-R26R-STOP-floxed-YFP Fmr1 KO ("Arc-YFP-Fmr1 KO") mice for the proposed experiments. [Figure best viewed in color.]

The immediate early gene Arc can be used to understand how a hippocampal memory trace is formed and retrieved under a number of conditions. Denny et al., 2014 designed the

ArcCreERT2 transgenic mouse that allows for the comparison of cells activated during the encoding or expression of a memory versus those that were not (Denny et al., 2014). This mouse can be crossed with other mouse lines for a powerful approach to studying the effect of genotype on learning and memory (Fig 2). **Active place avoidance learning persistently changes CA1 synaptic function**, as determined by extracellular EPSP recordings from dorsal hippocampal slices (Pavlowsky & Alarcon, 2012). Place avoidance training selectively modulates the function of the CA1 synaptic circuit involving the CA3 and entorhinal cortical (EC) synapses onto CA1 neurons. These changes included increased synaptic transmission, increased likelihood of a population spike, unchanged E-S coupling, reduced potentiation and increased depression at the CA3-CA1 synapse but not at the ECIII-CA1 synapse, or in mice that received unavoidable shocks that were yoked to the trained animals. These robust changes were measured i) a day after a 1 day, 3-trials training protocol, ii) a day and iii) even 1 month after a 4-day, 3-trials/day protocol. But in the 1 month case, the changes were only in mice that expressed the place avoidance memory (Pavlowsky & Alarcon, 2012).

Translational dysregulation and cognitive impairments and in Fmr1 KO mice

Despite its successes, behavioral and neurogenomics has been criticized for its lack of hypotheses and causative insights. In a recent review, we proposed a novel avenue to overcome some of these short-comings by complementing "forward genomics" studies (i.e., from phenotype to behaviorally relevant gene modules) with a **"reverse genomics"** approach (i.e., manipulating of novel gene modules to examine effects on behavior, physiology, and the genome itself) to examine the functional causes and consequences of differential gene expression patterns (Harris & Hofmann, 2014). The fragile X mental retardation 1 gene (Fmr1) codes for a protein called fragile X mental retardation protein (FMRP). This protein is a translational repressor, is most commonly found in the brain, and is essential for normal cognitive development**. The Fmr1 knockout (KO) mouse can be used as a tool to study the effects of translation dysregulation,** because Fmr1 KO mice do not express the translational repressor FMRP. Furthermore, cognitive discriminations are impaired in humans and mice lacking the Fmr1 gene (Huber, Gallagher, Warren, & Bear, 2002; Zhong et al., 2010).

RESEARCH QUESTIONS

1. Does forming different types of memory produce distinct changes in synaptic function and molecular activity in defined regions within the hippocampal circuit?
2. Do labeled (activated during learning) and non-labeled (non-activated) neurons show distinct synaptic functional and molecular characteristics that support memory persistence?
3. Are individual differences in activity dependent transcriptional patterns correlated to behavior variability in each subject?
4. Are Fmr1 learning and neural coordination abnormalities based on altered ability of these circuits to reshape the plastic synaptic functions that reorganize neural ensemble activity to represent the updated information?

OBJECTIVES AND OVERALL RATIONALE

Progress in systems neuroscience depends on **integrating data across levels of organization** in order to gain a more comprehensive understanding of the plasticity and variability at the individual and cellular level (Fig 1). Consequently, modern research and training requires cross-disciplinary approaches that bring together concepts and methods from diverse disciplines. While imaging and molecular genetic tools have long complemented single cell electro-

physiological analyses, it has now also become possible to conduct molecular level analyses on a single neuron level (Eberwine et al., 2012).

Our integrative approach to understand neuronal variability aims to compare neuron-to-neuron variation in molecular expression in identifiable hippocampal neurons in response to learning in wild type and transgenic mice using a robust learning paradigm. The use of transgenic mice allows us to identify morphologically and/or functionally discrete populations of neurons. Unlike previous research in this area, our approach will evaluate endogenous transcriptional variability in hippocampal neurons and determine the extent to which learning alters the baseline transcriptional variability of the cells. Furthermore, we will examine how genotype affects the network of functional changes in gene expression, synaptic network and cellular electrophysiological functions in hippocampus that is caused by place learning in Fmr1 KO mice. This innovative and integrative research proposal will transform our understanding of molecular and physiological processes underlying neuronal and cognitive variability that give rise to individual differences in neural circuits.

RESEARCH APPROACH

Aim 1. Examine transcriptional variability of neurons in the hippocampal circuit.

Rationale & Significance: How variable are neurons that belong to the same cell type either within or across different functional units of the hippocampus? We will investigate key features of the "synaptic plasticity and memory" hypothesis first by examining differences between distinct but related classes of principal cells in the four major principal cell types of the dorsal hippocampus circuit: CA1, CA2, and CA3 pyramidal cells and Dentate Gyrus (DG) granule cells (Fig. 3). Our research will identify the extent to which these neurons vary in baseline physiology and gene expression of naïve mice. These studies will establish the baseline against which further studies are compared. Furthermore, they will extend preliminary work because we will then micro-aspirate the cell body of these neurons and process the material for gene expression analysis using a validated pipeline for automated RNA isolation using (Harris, Otopalik, et al., 2014) and gene expression analysis of using Nanostring® technology from single neurons (Harris, Chung, Alarcon, Hofmann, & Fenton, 2014) (Figs. 4 and 5).

Preliminary Results: Our preliminary results show that DG and CA1 neurons have distinct calcium activity patterns and molecular profiles (Fig 4). Calcium imaging of hippocampal activity in freely behaving mice show distinct patterns in calcium activity in DG and CA1 neurons (Fig. 4 left). Gene expression profiling of micro-aspirated neurons show differential gene expression of kinases, transcription factors, and metabolic genes, etc. between principal cells isolated from CA1 or DG (Fig. 4 right).

Experimental Design: The hippocampus of naïve c57b6 mice will be dissected and processed for *ex-vivo* slice physiology. **We will identify and record from principal cells in the CA1, CA2, CA3, and DG hippocampal fields**. Standard 350-µm ex

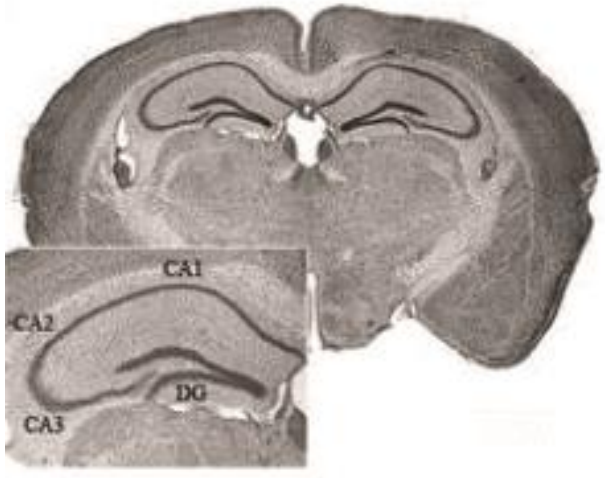

Fig. 3. The mouse hippocampus. The hippocampus is composed of multiple subfields which differ in their connectivity, function, and molecular profile. In aim 1, we will characterize single neuron variability of CA1, CA2, CA3, and DG at the single neuron level.

vivo dorsal hippocampus slices will be prepared. Recording field extracellular (Pavlowsky & Alarcon, 2012) and perforated whole-cell patch clamp (Isaac, Buchanan, Muller, & Mellor, 2009) responses to stimulation of the ECIII perforant path or CA3 Schaffer collaterals in different slices will characterize CA1 synaptic circuit function at stratum lacunosum moleculare or stratum radiatum, respectively. We will record evoked field responses to estimate input-output functions of CA1 and CA2 as an assay of network synaptic function, as well as record single cell currents under voltage clamp to determine whether the different cell types express different baseline distributions of excitatory and inhibitory and AMPA- and NMDA-mediated currents as well as their ratios to estimate excitation/inhibition balance and LTP/LTD status, which we cannot determine from the field recordings (Mercer, Trigg, & Thomson, 2007; Zhao, Choi, Obrietan, & Dudek, 2007).

Single neurons will be micro-aspirated from behaviorally naïve c57b6 mice and processed for RNA-sequencing. Briefly, RNA from single neurons will be isolated using the Promega Maxwell automated RNA isolation platform as we have done in the past (Harris, Chung, et al., 2014; Harris, Otopalik, et al., 2014). After an amplification step, RNA samples will be processed for Illumina RNA-seq (40 million reads/sample). After pre-processing and Phred-trimming reads, transcriptomes will be assembled using Trinity (Grabherr et al., 2011) and the raw contigs clustered with CD-HIT-EST (Fu, Niu, Zhu, Wu, & Li, 2012). The reads will be aligned to a species-specific reference transcriptome (Li & Durbin, 2009). We will then use the R package DESeq to identify differentially expressed genes based on read counts (Robinson, McCarthy, & Smyth, 2010).

Finally, we will use weighted gene co-expression network analysis (WGCNA; (Langfelder & Horvath, 2008)) to identify across species functional gene modules associated with behavioral and physiological measures. All analyses will be corrected for multiple hypothesis testing. We will use multivariate and clustering analyses to identify functional molecular gene co-expression modules and relate them to behavioral performance and measures of synaptic plasticity.

Potential Pitfalls and Alternative Approaches: We do not anticipate any difficulties as all behavioral and *ex vivo* physiological analyses are established in the laboratory of collaborator Fenton. Also, we have validated that sufficient high quality RNA can be isolated from micro-aspirated single neurons using RNA amplification techniques (Whitaker et al., 2011). If the RNA isolation proves difficult

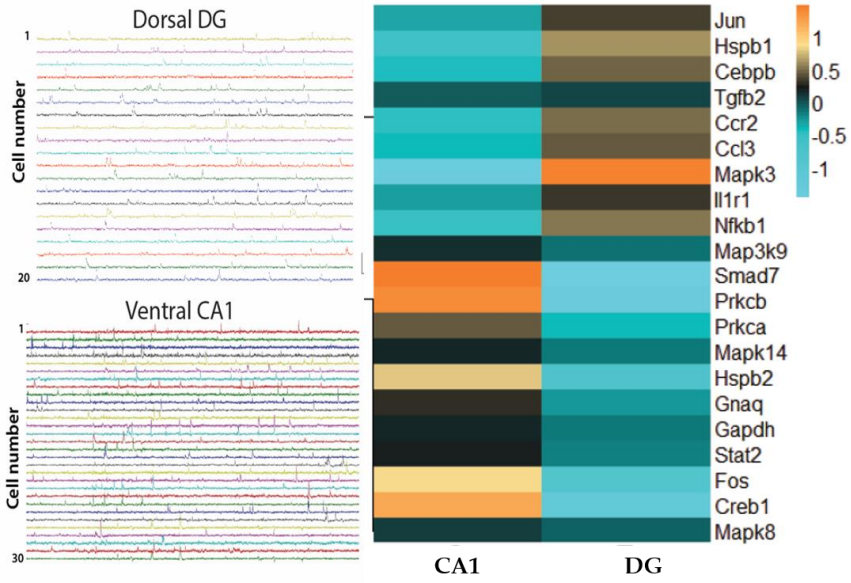

Fig 4. Single neuron cell type differences between DG and CA1. Our preliminary results show that DG and CA1 neurons have distinct calcium activity patterns and molecular profiles. **Left.** Calcium imaging of hippocampal activity in freely behaving mice show distinct patterns in calcium activity in DG and CA1 neurons. **Right**. Gene expression profiling of micro-aspirated neurons show differential gene expression of kinases, transcription factors, and metabolic genes, etc. between principal cells isolated from CA1 or DG. [Figure best viewed in color.]

we will perform the isolation analysis on small groups of neurons, which we have demonstrated is ample for the Nanostring® analysis (Fig 3D), and we do not anticipate trouble with sequencing. In the event that RNA yields are low, we will use Nanostring analysis, which will provide data on up to 800 genes.

Aim 2. Quantify transcriptional variability in active and inactive sub-populations of hippocampal principal cells after memory training.

Rationale & Significance: Aim 2 will investigate the consensus hypothesis further by examining how learning changes synaptic function and molecular expression in single cells and how these changes relate to behavioral variables. We will investigate whether the acquisition and persistence of a spatial memory alters the cellular profile of hippocampal principal neurons identified to participate in the encoding of memory (Arc-YFP+) compared to neurons identified to not participate (Arc-YFP-). We will examine only animals that received training and compare in each animal the YFP+ learning-recruited principal cells and the adjacent YFP- cells that are less likely to have been vigorously active during the training experience. According to the consensus hypothesis, particular changes in synaptic function and gene expression will be detected in the YFP^+ cells compared to the YFP^- cells, and these changes will correlate with measures of learning and memory performance.

Preliminary Results: In **a preliminary study**, mice were trained in an active place avoidance task then we identify and recorded from learning-recruited YFP+ neurons (red arrows, Fig. 5A1, 5A2) and from non-recruited YFP- neurons (black arrow, Fig. 5B1, 5B2). Then, we will micro-aspirate these neurons for molecular profiling, usual visual cues to confirm successful micro-aspiration (red arrows, C1, C2). Nanostring® gene expression assays will be used to quantify differential gene expression between YFP+ and YFP- cells (5D). The methods used were described in (Harris, Chung, et al., 2014).

In a **second preliminary study**, mice were trained an active place avoidance task, the RNA was isolated from CA3 and CA1 tissue punches for qPCR analysis of 10 candidate genes. Pair-wise correlations between gene expression values and behavioral variables were computed and statistically significant correlations are visualized as network of associations using the R package igraph (Csardi & Nepusz, 2006). The association networks for the trained and untrained mice are distinct, and largely consistent with hypothesized roles for these genes **(Fig. 6)**. Given the central role of PKMZ in memory maintenance, it is

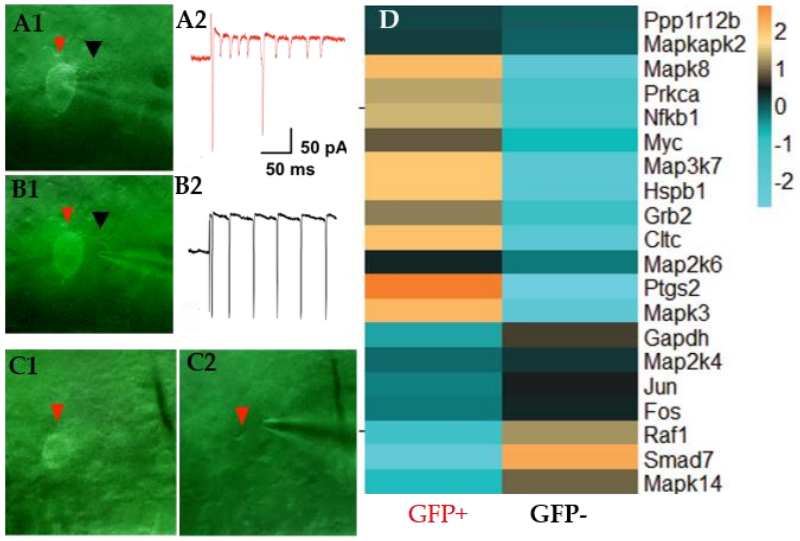

Fig 5. Comparing learning-recruited YFP^+ and non-recruited YFP^- neurons. Following training, we will identify and record from learning-recruited YFP^+ neurons (red arrows, A1, A2) and from non-recruited YFP^- neurons (black arrow, B1, B2). Then, we will micro-aspirate these neurons for molecular profiling, usual visual cues to confirm successful micro-aspiration (red arrows, C1, C2). Nanostring® gene expression assays will be used to quantify differential gene expression between YFP^+ and YFP^- cells (D). [Figure best viewed in color.]

remarkable that CA1 PKMZ gene expression is a hub that links the memory and activity variables to genes that regulate synaptic function. These preliminary data suggest that learning-induced changes at multiple levels of biological organization can be detected between regions CA1 and CA3 after spatial learning. Here, a particular innovation will be to include distinct behavioral estimates of activity, learning, and memory, with the physiological estimates of function and the estimates of molecular expression to characterize the functional networks of interactions amongst these variables, integrated across multiple levels of biological organization.

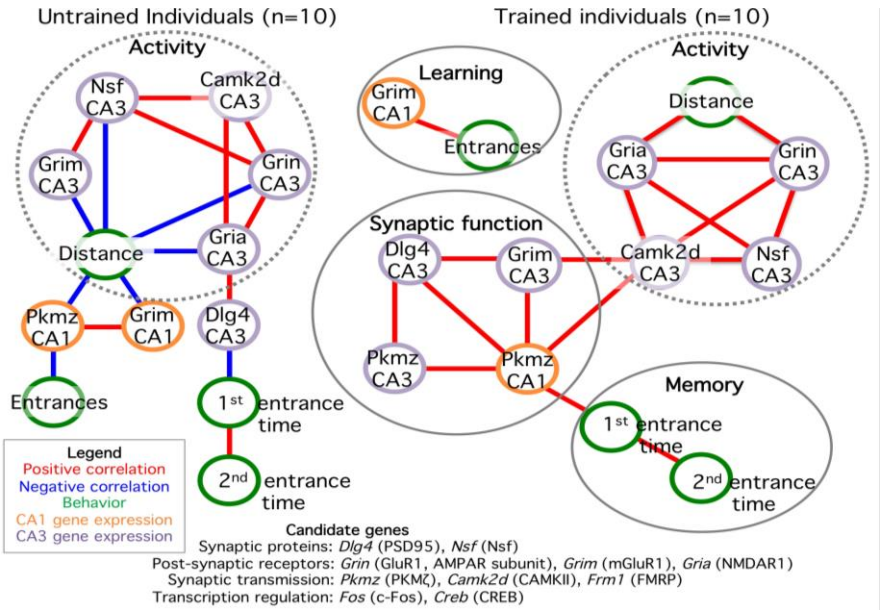

Fig 6. Distinct networks of behavior and gene expression associations in trained and untrained mice. After testing in place avoidance, trained (3 10-min trials in 1 day) or untrained (no shock) mice, RNA was isolated from CA3 and CA1 tissue punches for qPCR analysis of 10 candidate genes. Pair-wise correlations between gene expression values and behavioral variables were computed and statistically significant correlations are visualized as network of associations using the R package igraph (Csardi & Nepusz, 2006). The association networks for the trained and untrained mice are distinct, and largely consistent with hypothesized roles for these genes. Given the central role of PKMZ in memory maintenance, it is remarkable that CA1 PKMZ gene expression is a hub that links the memory and activity variables to genes that regulate synaptic function. [Figure best viewed in color.]

In a **third preliminary study**, we showed that *active place avoidance learning persistently changes CA1 synaptic function (**Fig. 7**).* Synaptic circuit function changes after place avoidance learning, measured using extracellular EPSP recordings from dorsal hippocampal slices. Place avoidance training selectively modulates the function of the CA1 synaptic circuit involving the CA3 and entorhinal cortical (EC) synapses onto CA1 neurons (Fig. 7). These changes included increased synaptic transmission, increased likelihood of a population spike, unchanged E-S coupling, reduced potentiation and increased depression at the CA3-CA1 synapse but not at the ECIII-CA1 synapse, or in mice that received unavoidable shocks that were yoked to the trained animals. These robust changes were measured i) a day after a 1 day, 3-trials training protocol, ii) a day and iii) even 1 month after a 4-day, 3-trials/day protocol. But in the 1 month case, the changes were only in mice that expressed the place avoidance memory.

Experimental Design: **Active Place Avoidance task:** Mice will be trained in the hippocampus-dependent active place avoidance task (Cimadevilla, Fenton, & Bures, 2001). Briefly, the active place avoidance task utilized a slowing rotating arena located in a rectangular room with a number of extramaze landmarks. A mouse is placed on the slowly rotating arena and allowed to explore during a 10-min pretraining session. After a 2-h rest in the home cage, the mouse is returned to the identical rotating arena for the initial learning phase. The only difference is that now whenever the mouse enters a stationary 60° zone, it receives an unpleasant foot shock. The shock zone itself is unmarked but can be located using its relationship to stationary

distal cues in the room. Mice rapidly learn to avoid the shock zone location and avoidance can be measured within the initial 5 minutes. Two more 10-min training sessions are given after 10 min rests. After a 24-h, memory retention of the conditioned avoidance is tested in the identical, initial conditions. Behavior will be quantified using digital video and automated tracking.

"Arc-YFP" mouse line: We will use activity dependent expression of YFP in an "Arc-YFP" mutant mouse to identify "learning-recruited" (YFP+) neurons (Fig. 5 A1, red arrow). This mouse is generated by crossing ArcCreERT2 mice and R26R-STOP-floxed-YFP mice (Fig. 2). 4OH-tamoxifen injection five hours before initial training in a rapidly learned active place avoidance task as well as a social learning task activates Cre leading to YFP expression in the subpopulation of neurons that transcribed Arc during training. Because Cre expression is transient, only in the presence of the drug, and once activated YFP expression is persistent, YFP is an indelible marker for the principal cells that were activated during the memory training. YFP^+ cells are enriched with the active subset, and YFP^- neurons are enriched with the inactive subset. After the memory retention test, hippocampal slices will be collected for molecular and physiological analysis as described above.

Integrating Behavioral, Physiology, and Gene Expression Analyses: We will then compare the differences in electrophysiological and molecular profiles of single learning-recruited neurons (YFP+) (Fig. 5 A2, D) with the features of YFP- neurons (Fig. 5 B2, D) that were unlikely to have been recruited by different learning episodes (Denny et al., 2014). We will collect measures of behavior (locomotor activity [path length], learning [total entrances], memory [time to first enter]), synaptic network function (synaptic transmission [paired pulse facilitation and fEPSP slope]; network excitability [population spike (PS) amplitude], [E-S coupling], [PS probability]; synaptic plasticity [100-Hz HFS potentiation] and [1-Hz LFS depression]), single cell physiology ([resting membrane potential], [input resistance], [threshold], [spike number and frequency], [AMPA/NMDA current ratio], [excitatory/inhibitory current ratio]) for the YFP+ and YFP- subpopulations. Differential network analysis of the gene expression data (Anders & Huber, 2010; Langfelder & Horvath, 2008) will be performed to separately determine if the relevant pairs of association network differ for the untrained, yoked, and trained experiences. Using the association network framework, the associations amongst all associable behavioral, physiological, and molecular measures will be characterized and compared for each genotype and for each training phase.

Potential Pitfalls and Alternative Approaches: We anticipate no difficulties as the mice are actively in use in the laboratory of collaborator Fenton. We will use appropriate controls to account for transient expression of Arc that is not indicative of learning and memory. We do not anticipate any difficulties as all behavioral and *ex vivo* physiological analyses are established in the laboratory of collaborator Fenton. Also, we have validated that sufficient high quality RNA can be isolated from micro-aspirated single neurons using RNA amplification techniques (Whitaker et al., 2011). If the RNA isolation proves difficult we will perform the isolation analysis on small groups of neurons, which we have demonstrated is ample for the Nanostring® analysis (Fig 3D), and we do not anticipate trouble with sequencing. In the event that RNA yields are low, we will use Nanostring analysis, which will provide data on up to 800 genes.

Aim 3. Determine the effect of genetically dysregulated translation on neuromolecular variability and plasticity.

Rationale and Significance: Cognitive discrimination deficits and neural discoordination are associated in Fmr1 KO mice, but we have yet to pinpoint the underlying mechanisms. Are Fmr1 learning and neural coordination abnormalities based on altered ability of these circuits to reshape the plastic synaptic functions that reorganize neural ensemble activity to represent the updated information? We will test if these abnormalities arise from altered learning- induced

changes in synaptic function, consistent with synaptic plasticity abnormalities in Fmr1 KO (Bear, Huber, & Warren, 2004). We hypothesize that place avoidance learning in Fmr1 KO mice, even when normal, results in abnormal changes in synaptic function due to dysregulated learning-driven changes in synaptic function and gene expression. The working hypothesis predicts dysregulated learning-associated changes in synaptic function due to loss of FMRP in ArcYFP-Fmr1 KO mice but similar such changes in WT and ArcYFP mice. While Fmr1 KO differences are predicted at all stages of training, the differences are predicted to be extreme after the conflict and extinction cognitive discrimination sessions. Similar association gene networks are predicted for the untrained and yoked groups, which will differ from the trained groups with FMRP. Association gene networks are expected to differ according to the absence or presence of Fmr1, and we will determine to which extent the synaptic physiology and gene expression variables are strongly correlated with the cognitive and behavioral measures.

Preliminary Results: Altered place avoidance induced changes in Fmr1 KO CA1 synaptic function (Fig. 7). Basal synaptic transmission and synaptic potentiation in Fmr1 KO mice is unchanged compared to WT mice in home caged conditions (Udagawa et al., 2013). Preliminary data indicate altered CA3 vs. CA1 synaptic function in Fmr1 KO mice after learning. WT and Fmr1 KO mice received 3 10-min place avoidance training trials during one day. Basal synaptic transmission and synaptic potentiation were tested 1 d later in ex vivo hippocampus slices. Avoidance training enhanced synaptic transmission and decreased potentiation at WT CA3-CA1 synapses. Synaptic transmission was enhanced in both trained and untrained KO mice but not further enhanced after training, possibly due to a ceiling effect. After training, the decrease in synaptic potentiation of the KO was much larger than in WT and may be associated with facilitated mGluR-mediated synaptic depression (Piskorowski & Chevaleyre, 2012).

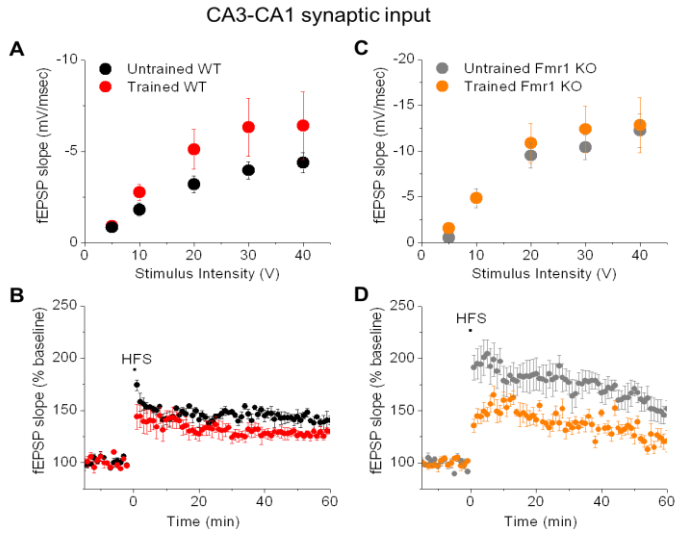

Fig. 7. Changes in CA1 synaptic circuit function in Fmr1 KO mice after place avoidance training. A,B) CA3-CA1 synaptic function in WT mice a day after place avoidance training in a 1-d/3-trial protocol (red) vs. untrained (black) mice. Trained mice show **A)** enhanced synaptic transmission and **B)** decreased synaptic potentiation to HFS compared to untrained animals. **C)** Trained (orange) and untrained (gray) KO mice show similar CA3-CA1 synaptic transmission. Note the amplitude in KO subjects is twice larger than WT. **D)** KO mice showed pronounced decrease in CA3-CA1 synaptic potentiation 1 day after training. Note the large amplitude of synaptic potentiation in untrained KO mice compared to WT. n=4 per group. [Figure best viewed in color.]

Experimental Design: We will use the Fmr1 knockout (KO) mouse model of translation dysregulation. Fmr1 KO mice do not express the Fragile X Mental Retardation Protein (FMRP) which represses translation in synaptic domains. We will use a novel "Arc-YFP-Fmr1 KO" mouse line derived from crossing the R26R-STOP-floxed-YFP mouse and the Fmr1 KO mouse and then crossing this R26R-STOP-floxed-YFP-Fmr1 KO mouse with the ArcCreERT2 mouse. This Arc-YFP-Fmr1 KO mouse will be used like in Aim 2 to understand the consequences and interaction of memory training and dysregulated molecular signaling due to translation de-repression in the absence of

FMRP. Standard 350-µm *ex vivo* dorsal hippocampus slices will be prepared after memory retention testing in the active place avoidance task (as described in Aim 2). Both WT and ArcYFP mice will serve as controls for mice expressing Fmr1. All mice will receive 4OH-tamoxifen induction of YFP expression during the target phase of training, as described. Control slices will be prepared from mice that received the same training experience with either yoked shocks or no shock. Recording field extracellular (Pavlowsky & Alarcon, 2012) and perforated whole-cell patch clamp (Isaac et al., 2009) responses to stimulation of the ECIII perforant path or CA3 Schaffer collaterals in different slices will characterize CA1 synaptic circuit function at stratum lacunosum moleculare or stratum radiatum, respectively (Fig. 7). We will measure currents under voltage clamp in the YFP+ and YFP- subpopulation of cells to determine whether individual learning-recruited and learning-quiescent cells express synaptic current changes consistent with LTP or LTD (i.e. increased or decreased AMPA/NMDA current ratios) and/or altered excitation-inhibition (excitatory/inhibitory current ratio), which we cannot determine from the field recordings. As described in preliminary studies (Fig. 4,5) we will micro-aspirate YFP+ and YFP- cells for analysis of gene expression as described in Aim 2. These experiments will yield data for the three genotypes (WT, ArcYFP, ArcYFP-Fmr1 KO). Behavioral, physiological, and gene expression data will be analyzed as described in Aim 2.

Potential Pitfalls and Alternative Approaches: We do not anticipate difficulties in collecting the data as we have worked through these complex analyses as shown by the preliminary data (Figs. 4-7). Because FMRP regulates translation and is associated with changes in the associated molecular machinery, the Fmr1 KO mice will serve as a positive control. Thus any result will be informative. Although we assume there will be a reliable pattern of molecular and synaptic physiology changes to account for the Fmr1 KO discoordination phenotype, it is also possible that loss of FMRP causes a heterogeneous set of primary and compensatory downstream changes (as suggested by (Lee et al., 2012)). Additionally, the intrinsic properties of CA1 neurons are altered in *fmr* mice, in particular the ion channel composition in the dendrites (Brager & Johnston, 2014).

We recognize that using the Arc promoter to drive expression will only identify a subclass of the neurons activated by the target experience. Using c-Fos and Arc immunohistochemistry in *ex vivo* hippocampus slices after place avoidance training, we have confirmed that the two IEGs label only weakly overlapping populations. We and others are working to engineer mice that will genetically label neurons for weeks or longer, that are either c-Fos activated or have activation of multiple IEGs, so called “Targeted Recombination in Active Populations” (TRAP) technology (Guenthner, Miyamichi, Yang, Heller, & Luo, 2013). Although beyond the scope of the current proposal, we will certainly supplement the planned studies once such mice become available. One has to start somewhere, and given the preliminary data and tools on hand, we suggest now is appropriate.

STATEMENT OF INTELLECTUAL MERIT

Identifying the neuromolecular basis of individual variation is a major challenge for genotype to phenotype mapping. Diversity in neuronal structure, function, and activity is one of the most striking sources of variability in organisms with nervous systems and gives rise to complex behavior and cognition. Yet our understanding of the molecular processes underlying neuronal individuality and plasticity is still very limited and made all the more difficult because we now know that experience changes gene expression, which itself changes neural function and thus the subsequent neural functions in future experience (Ginsberg et al. 2004, Eberwine et al. 2012, Park et al. 2014). The proposed research will investigate the influence of gene expression, physiology, learning, memory, and genotype on individual and cellular variability (Fig. 1). This innovative and integrative research proposal will transform our understanding of the molecular processes of functional variability in the hippocampus. Specifically, we will provide fundamental

new insights into how transcriptional and electrophysiological variation arises both within and between major principal cell classes of the hippocampus that are crucial for learning, memory, and cognition. The use of transgenic mice allows us to identify morphologically and/or functionally discrete populations of neurons. Unlike previous research in this area, our approach will evaluate endogenous transcriptional variability in hippocampal neurons and determine the extent to which learning alters the baseline transcriptional variability of the cells.

Using complementary approaches, PI Hofmann and collaborator Fenton have made significant progress in understanding neuron-to-neuron variation in synaptic physiology and plasticity in response to spatial learning (Fenton) and molecular variability in response to changes in behavior (Hofmann). Collaboratively, their combined expertise will shed light on individual, neural, and cognitive variability in response to two forms of hippocampal dependent learning experiences. The PI, collaborator, and their graduate students have been working together in association with the *Neural Systems and Behavior* course (co-directed by Fenton and Hofmann) at the Marine Biological Laboratory in Woods Hole (MA), conducting proof-of-principle experiments to validate the approaches used in this proposal. This research increases our ability to map genotypes to phenotypes by shedding light on the molecular and physiological mechanisms that give rise to single neuron variability. Given their positions of leadership in the neuroscience community, they have many opportunities for interdisciplinary training and outreach, and for sharing resources that will be useful to a wide range of researchers.

BROADER IMPACTS OF THE PROPOSED WORK

The proposed research and related activities have a proven track record for advancing discovery and understanding while promoting teaching, training, and learning. Here, we highlight some of the major impacts PI Hofmann and collaborator Fenton have achieved since their appointment as Course Directors of the Neural Systems & Behavior (NS&B) Course at the Marine Biological Laboratory. These activities include broadening participation and dissemination, enhancing infrastructure, improving STEM education, increased partnerships with industry, and social benefits.

Participation of women, underrepresented minorities, and persons with disabilities. The careers of women have been greatly enhanced through the previous research conducted before writing this proposal. Graduate students Ain Chung and Rayna Harris have gained considerable teaching experience as course developers and teaching assistants for NS&B, learned new techniques, presented research at multiple conferences, won travel awards, and drafted manuscripts. Additionally, at least 50% of NS&B students are females, and many come from underrepresented minorities.

Enhanced infrastructure for education and outreach. The Neural Systems & Behavior (NS&B) course, jointly directed by Fenton and Hofmann, has provided intensive training in the concepts and methodology of behavioral neurobiology and systems neuroscience to outstanding pre- and postdoctoral students since 1978. NS&B offers multiple training opportunities in modern approaches to the study of neural systems and behavior to the next generation of behavioral neuroscientists during early stages of their research careers: intensive lectures and discussion, one-on-one interaction with internationally renowned scientists, and extensive hands-on laboratory training with a variety of invertebrate and vertebrate preparations using state-of-the-art techniques and equipment. The integrative nature of this project provides unique and outstanding educational opportunities for trainees at all stages of their career. This research will be partially conducted alongside students in the Neural Systems and Behavior course at the Marine Biological Laboratories where scientific careers are transformed through learning and discovery-driven research (www.mbl.edu/nsb).

Improved STEM education and educator development. NS&B adds exceptional value to graduate and post-doctoral training. Generally, the training available for graduate students and

post-docs who are interested in understanding the neural basis of behavior is limited to the resources of their home departments and graduate programs. NS&B plays an invaluable role by providing educational training to students, post-docs, and even young faculty members in concepts and methodologies beyond what is available in any single institution. Simply bringing students to course with its 30 year history on the study of behavior and nervous system and its current community of world-renowned researchers, immediately imbues students with a sense of belonging to a larger scientific enterprise. It also provides many opportunities for cross-fertilization and collaboration that are only available through a program like NS&B. More than 30 dedicated faculty members rotate through two-week cycles with the twenty students in NS&B. This type of intense mentorship, representing so many different academic influences, is not possible except in a special summer course like NS&B. Students work hard six days a week, from 9 a.m. until past midnight for eight weeks, and form intense, life-long bonds with each other and with the faculty.

Development of innovative educational activities. In 2013, PIs and NS&B Co-Directors Hofmann and Fenton introduced single-cell molecular and genomic techniques to the NS&B curriculum to complement and extend the electrophysiological characterization of specific neuronal subclasses including identified neurons in invertebrate preparations. Working in pairs, students learned the value of integrating molecular and physiological experiments and were

Fig. 8. **Broadening the scope and impact of the Neural Systems & Behavior (NS&B) course with technological and educational innovation.** The single-neuron molecular and physiological approaches described herein have been incorporated into course laboratory exercises in 2013 (A1) and 2014 (A2) with great success. For the first time ever in 2014, all ~80 lectures associated with the course were webcast live and achieved on our website (B). We also created video tutorials for teaching complex concepts and techniques so that the expertise of NS&B instructors can be disseminated world-wide (C). [Figure best viewed in color.]

incredibly excited to successfully conduct these single-cell techniques in student-developed projects (Fig. 8A1, A2).

Broad dissemination of scientific and technological understanding. The intensive laboratory-based nature of the NS&B Course necessitates that the students must be in immersed in the total experience and therefore be in residence. This is the hallmark of all MBL courses. However, we also recognize that it is important that the large amount of information assembled for the course should be effectively disseminated to others in the field. To facilitate the dissemination of course materials we have developed course websites (http://www.mbl.edu/nsb/). These sites include the syllabus, lectures and course contact information. Starting in 2013, the faculty lectures are webcast live (Fig. 8B) and are archived and accessible to the public at: http://www.mbl.edu/nsb/about/course-videos/.

Also, with the help of a generous donation from the Grass Foundation, matched by the MBL, we have begun producing training videos that demonstrate complex dissections and surgeries as well as advanced techniques during the 2014 course. PI Hofmann has extensive experience with the design and production of didactic videos and animations from his work on transforming biology instruction at UT Austin (>200 videos and animations have been produced). The goal is twofold: To facilitate and accelerate training of NS&B course students and to build a publicly available repository of technical resources that can aid the community at large. PIs Hofmann and Fenton also created video tutorials for teaching complex concepts and techniques so that the expertise of NS&B instructors can be disseminated world-wide (Fig. 8C).

In addition, some projects started in the course are later presented at national meetings and/or provide the foundation for subsequent publications. Hofmann and Fenton and their trainees have published experiments from the course in a technical report (Harris, Otopalik, et al., 2014) and presented these findings at five scientific conferences (Fenton, Harris, & Hofmann, 2014; Harris, Chung, et al., 2014).

Increased partnerships between academia and industry. Our preliminary research has benefited greatly from the in-kind support received by Promega®. Promega's generous donation of molecular regents of supplies has allowed us to introduce an innovative molecular module into the course, which we have used to establish new protocols for single-neuron gene expression analysis. By providing state-of-the-art equipment for loan, we were able to successfully pilot the single-cell gene expression techniques described herein. We have published this work as a technical note (Harris, Otopalik, et al., 2014) with several NS&B student and course faculty as co-authors. We have also presented our use of Promega® products on an NS&B course poster at three different conferences (Fenton et al., 2014; Harris, Chung, et al., 2014). Several traditional course modules (Leech, STG, and Electric Fish) have been enhanced because of the Promega supplies and instruments made available to NS&B. The support Promega® has also facilitated collaborative research beyond the course. Specifically, it has empowered several course faculty (especially from the stomatogastric ganglion module) to begin establishing molecular techniques in their home labs. Finally, NS&B co-director and PI Hofmann arranged for a demo of the Maxwell RNA/DNA isolation robot at UT Austin, which has now resulted in a purchase of the instrument by the Genome Sequencing & Analysis core facility at UT Austin. None of these positive developments would have been possible without Promega's support! We're looking forward to the continuation of this collaboration.

More broadly, NS&B students are trained on the latest neurophysiological and imaging equipment, usually on loan from vendors with expert support. Through these collaborations with industry, students are prepared to launch their own research careers familiar with state-of-the art equipment. In addition to our long-running relationships with many vendors, we have been successful in obtaining loaned equipment from new sources as well as donations of equipment or deep discounts for purchases. For example, the course was able to purchase 10 air tables for use with the electrophysiology rigs some years ago. Each rig has a computer that is rented for use by the course and is loaded with data acquisition and analysis software that is donated.

COLLABORATION PLAN

Specific roles and complementary expertise. This collaborative effort among the The University of Texas at Austin and New York University takes advantage of the unique and complementary expertise provided by each senior researcher and provides an exceptional opportunity to integrate measures of physiology & gene expression, behavioral measures of learning & memory, and differences across genotype to better understand variability at the individual and cellular levels.

PI Hofmann's research program is aimed at elucidating the neuroendocrine, molecular, and genomic mechanisms underlying social decision-making in the context of social status/dominance and reproduction. His work has pioneered behavioral and neurogenomics in cichlids to analyze and understand the molecular and neural basis of social behavior and its evolution. His lab developed many of the functional genomics resources for cichlids with the support of the NIGMS and I was the co-initiator of the cichlid genomic consortium, which has successfully completed sequencing of five cichlid genomes with support from the NHGRI. Using comparative neuroanatomy and gene expression profiling Hofmann's research has reconstructed the evolution of the brain networks underlying social behavioral regulation (mesolimbic dopamine system; Newman's social behavior network) and shown that these systems are remarkably conserved across vertebrates, providing important neuroanatomical support for the idea that conserved molecular and neural substrates often underlie independently behavioral and physiological phenotypes. In his role as Director of the *Center for Computational Biology and Bioinformatics (CCBB)* at UT Austin, he has strong expertise in genomic and bioinformatic approaches to complex biological questions. Also, as CCBB Director he has developed a comprehensive training program for trainees at all levels in the form of workshops and short courses. **PI Hofmann will be involved** in all aspects of the proposed research, including project design, supervision of trainees, overseeing behavioral and molecular experiments and analyses, teaching, presenting findings at scientific conferences, writing manuscripts. Graduate student **Rayna Harris** will be involved in project design, supervision of trainees, RNA isolation, Illumina library preparation and sequencing, bioinformatics analyses, teaching, presenting findings at scientific conferences, writing manuscripts.

Collaborator Fenton's research aims to define the neural correlates of cognitive representations. Fenton's expertise centers on rodent behavior and in vivo single unit recordings of cell assemblies during complex behaviors. His laboratory group works to identify the neural dynamics and functional organization of neural discharge embedded within the ongoing oscillatory activity in the local field potential that defines the neural infrastructure for cognitive representations of experience. Fenton's work to identify the synaptic basis and the behavioral importance of these representations in an effort to understand neural information processing that serves learning, memory and the cognitive control of knowledge representations. **Dr. Fenton will be involved in** all aspects of the proposed research, including project design, supervision of trainees, overseeing behavioral and electrophysiological experiments and analyses, teaching presenting findings at scientific conferences, writing manuscripts. Graduate student **Ain Chung** will be involved in project design, supervision of trainees, behavioral and electrophysiological experiments and analyses teaching, presenting findings at scientific conferences, and writing manuscripts.

Using **complementary approaches**, PIs Hofmann and Fenton have made significant progress in understanding how transcriptional and electrophysiological variation in the brain influences behaviors and cognition. Their combined expertise will shed light on individual, neural, and cognitive variability and plasticity. The two PIs and their graduate students have been working together conducting proof-of-principle experiments to validate the approaches used in this study. This research increases our ability to map genotypes to phenotypes by shedding light on the molecular and physiological mechanisms that give rise to single neuron variability.

Cross-institutional and cross-discipline coordination and integration. From 2013-2017, the PIs graduate students will spend 2 summer month at the Neural Systems and Behavior Course at the Marine Biological Laboratory. At NS&B, the laboratories and office space of the PIs are adjacent, which facilitates intellectual exchange and communication (Fig. 9).

Project management across institutions and disciplines. Each summer, while at the Neural Systems & Behavior (NS&B) course, the PIs and graduate students **will communicate** daily. During the academic year, the PIs and graduate students will communicate weekly to discuss experimental design, data analysis, and all administrative responsibilities. Both PIs will share their respective research results with other PIs and key personnel. They will work together to discuss any changes in the direction of the research projects and the reprogramming of funds, if necessary. A publication policy will be established based on the relative scientific contributions of the PIs and key personnel. PI Hofmann will serve as contact PI and be responsible for submission of progress reports to NSF and all communication.

Fig. 9. **Collaboration Plan.** For two months each summer, PI Hofmann (second from left) and collaborator Fenton (far right) and graduate students Rayna Harris (far left) and Ain Chung (second from right) work side-by-side. Photo was taken just outside their adjacent laboratory and office space at the Neural Systems & Behavior course.

Regarding **intellectual property**, the PIs will grant necessary access rights to patents potentially generated within the frame of this project for the purpose of this research project to all the other PIs and key personnel on a non-exclusive royalty-free basis. Each PI shall take appropriate measures to ensure that he can grant these access rights. Right in any pre-existing intellectual property will remain the property of the party that created and/or controls it. If a PI moves to a new institution, attempts will be made to transfer the relevant portion of the grant to the new institution.

The PIs are committed to **proactively managing conflict**. We strongly believe that effective communication leads to a positive response to conflict, which leads to healthier relationships and increased productivity. During the past two years, the PIs have invested time in creating an environment that encourages individuals to interact with one other so that they have a better understanding of the colleagues whom they work. This has helped to strengthen our communication with one another and our expectations for this collaboration. If conflict develops in the future, the PIs will use proactive strategies to resolve the dispute. First, the PIs will meet and attempt to resolve the dispute. If the PIs fail to resolve the dispute, we the conflict will be presented to Dr. William Reznikoff, Director of Education at MBL (see letter of commitment) for arbitration. If the conflict still persists, it will be referred to an arbitration committee consisting of one impartial senior executive from each PI's institution and a third impartial senior executive mutually agreed upon by both PIs. No members of the arbitration committee will be directly involved in the research grant or disagreement.

Budget items supporting coordination mechanisms. As detailed in the budget and budget justification, travel is included for the graduate students and PIs to visit each other and that reciprocal institution. Travel to and from the Neural Systems & Behavior Course is support by course funds and is not included in our budget. As they did in 2014, both PIs and graduate students will attend the Society for Neuroscience Annual meeting where they will present research related to this proposal.