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Single-Neuron Gene Expression Analysis Using the Maxwell® 16 LEV System in the Neural Systems and Behavior Course

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24 **ABSTRACT**

25 Gene expression analysis from single cells has become increasingly prominent across
26 biological disciplines; thus, it is important to train students in these approaches. Here, we
27 present an experimental and analysis pipeline that we developed for the Neural Systems
28 & Behavior (NS&B) course at Marine Biological Laboratory. Our approach used the
29 Maxwell® 16 LEV simplyRNA Tissue Kit and GoTaq® 2-Step RT-qPCR System for gene
30 expression analysis from single neurons of the crustacean stomatogastric ganglion, a
31 model system to study the generation of rhythmic motor patterns. We used double-
32 stranded RNA to knockdown expression of a putative neuromodulator-activated sodium
33 channel. We then examined the electrophysiological responses to known
34 neuromodulators and confirmed that the response was reduced. Finally, we measured
35 how mRNA levels of several ion channel genes changed in response. Our results provide
36 new insights into the neural mechanisms underlying the generation and modulation of
37 rhythmic motor patterns.

38 INTRODUCTION

39 The Neural Systems & Behavior (NS&B) course at Marine Biological Laboratory (MBL)
40 has provided intensive training in the concepts and methodology of behavioral neurobiology
41 and systems neuroscience to doctoral and postdoctoral students since 1978. NS&B offers
42 multiple training opportunities in modern approaches to the study of neural systems and
43 behavior for the next generation of behavioral neuroscientists during early stages of their
44 research careers. This approach includes intensive lectures and discussion, one-on-one
45 interaction with scientists and extensive hands-on laboratory training with a variety of
46 invertebrate and vertebrate preparations using state-of-the-art techniques and equipment.
47 Building on this success, we aimed to enhance and expand the course by incorporating single-
48 cell molecular and genomic techniques to complement and extend the electrophysiological
49 characterization of specific neuronal subclasses, including identification of neurons in
50 invertebrate preparations.

51 The stomatogastric nervous system of the Jonah crab, *Cancer borealis*, is a model
52 system to study neural mechanisms underlying the generation of rhythmic motor patterns and
53 their modulation (1). The crab stomatogastric ganglion (STG) contains just 26 neurons, and the
54 electrical properties, functional connectivity and neuromodulatory regulation of this neural
55 network have been well characterized. Thus, the STG is well suited for analysis of gene
56 expression at the single-neuron level (2).

57 Of particular interest is the examination of genes encoding ion channels that regulate
58 sodium and potassium conductances, especially channels regulated by neuromodulatory
59 substances. We investigated whether a qPCR approach could be implemented in the NS&B
60 course to quantify gene expression levels of ion channels that are important for regulating
61 electrophysiology and behavior in this system

62

63 METHODS

64 Adult Jonah crabs, *Cancer borealis*, were obtained from Yankee Lobster (Boston, MA)
65 and maintained in tanks with running/ chilled seawater until used. Crabs were anesthetized, and
66 the complete stomatogastric nervous system was dissected out of the animal and pinned out in
67 a dish containing chilled (12–13°C) physiological saline solution. The ganglia were then
68 desheathed, and the lateral pyloric (LP) and pyloric dilator (PD) neurons were identified based
69 on morphology and electrical activity. One of the two PD and the LP neurons were injected with
70 double-stranded RNA (dsRNA), targeting the sodium leak channel nonselective (*NaLCN*). After

71 24 or 48 hours, electrical activity of individual neurons and the entire network in response to
72 bath-applied neuromodulators was examined before treating with collagenase and harvesting
73 single PD and LP neurons using fine forceps under a dissecting scope. The PD and LP neurons
74 were then individually placed in 200µl of homogenization buffer with 1-thioglycerol, frozen in
75 liquid nitrogen and stored at -80°C until further processing (2–3 days later).

76 RNA was isolated using the Maxwell® 16 LEV simplyRNA Tissue Kit (Cat.# AS1280)
77 according to the manufacturer's instructions and subsequently eluted with 30µl of nuclease-free
78 water. A 9.5µl aliquot was reverse transcribed in a 20µl reaction using the GoTaq® 2-Step RT-
79 qPCR System (Cat.# A6010). Briefly, RNA, random hexamers and oligo(dT) were denatured at
80 70°C for 5 minutes, then reduced to 4°C. The tube was placed on ice, GoScript™ Reverse
81 Transcriptase added and cDNA was synthesized on the Veriti® Thermal Cycler (Applied
82 Biosystems) with the following settings: 25°C, 5 minutes; 42°C, 60 minutes; 70°C, 15 minutes.
83 The cDNA was diluted 1:5 in nuclease-free water, and 6µl was amplified in a 20µl real- time
84 PCR using the GoTaq® qPCR Master Mix and 0.25µM (final concentration) of each forward
85 and reverse primer with the StepOnePlus™ Real-Time PCR System (Applied Biosystems).
86 qPCR was performed using primers to amplify 18S rRNA and three candidate ion channel
87 genes: *shal*, *para* and *NaLCN*. The sequences of primers were as follows: 18S forward,
88 AGGTTATGCGCCTACAATGG; 18S reverse, GCTGCCTTCCTTAGATGTGG; *shal* forward,
89 CTACATCGGTCTTGGCATCA; *shal* reverse, AGATCCTGAACACGCGAAAC; *para* forward,
90 TCGGTATGGTGTGAAGGAT; *para* reverse, CAGTGTTCCGATACCCTTGG; *NaLCN*
91 forward, ATGCTGACTGTGGGTGTGTC; *NaLCN* reverse, GACAGTGCCAAAGAGGATG.

92 Primer efficiencies were determined by amplifying a series of twofold dilutions of *C.*
93 *borealis* whole ganglia cDNA covering four orders of magnitude of template (100–0.01ng of
94 cDNA per reaction). The primer-specific amplification efficiency E (the amplification factor per
95 PCR cycle) was derived from the slope of the regression using formula $E=2^{-(1/\text{slope})}$. The
96 results were plotted as the cycle of quantification (Cq) vs. $\log_2[\text{cDNA}]$ (Figure 1). For all
97 samples, the Cq values generated from the Applied BioSystems StepOnePlus™ software were
98 converted to counts using $\text{count} = E(\text{Cq}_1 - \text{Cq})$, where Cq₁ is the Cq of a single target molecule
99 (set to 37 for all genes) using the R package MCMC.qpcr (3). Then a Markov Chain Monte
100 Carlo (MCMC) algorithm was used to sample from the joint posterior distribution over all model
101 parameters, thereby estimating the effects of all factors (cell type and dsRNA treatment) on the
102 expression of every gene (3). The estimated stability of 18S rRNA was directly incorporated into
103 the model as a control gene. To examine Pearson correlations in expression level between

104 gene, counts were log transformed expression graphed using R (4).

105

106 RESULTS

107 Primer efficiencies (E) were all within the acceptable range (1.9–2.1; Figure 1). Sufficient
108 RNA quantities were isolated from all samples for quantification of the four genes. A statistical
109 analysis of changes in expression due to the factors of cell type (LP or PD) and treatment
110 (dsRNA *NaLCN* treatment versus control) revealed that *NaLCN* expression is significantly
111 decreased with dsRNA treatment independent of cell type ($p = 0.048$; Figure 2), but not for
112 *para* ($p = 0.992$), *shal* ($p = 0.784$) or 18S RNA ($p = 0.316$). Across samples, *shal* and *para*
113 expression levels were significantly correlated ($r^2 = 0.93$, $p = 1.39 \times 10^{-5}$; Figure 3).
114 Electrophysiological responses to two neuromodulators suspected of activating NaLCN
115 channels (Crustacean cardioactive peptide [CCAP] and proctolin) were significantly reduced as
116 expected if *NaLCN* expression was inhibited by dsRNA (data not shown).

117

118 DISCUSSION AND CONCLUSIONS

119 Our results show that high-quality RNA can be obtained from single neurons in a course
120 setting. Importantly, the amplification reactions produced valid and biologically meaningful data.
121 *NaLCN* dsRNA treatment was sufficient at reducing levels of *NaLCN* RNA in the cells after 24
122 or 48 hours. The high degree of variance in expression between samples as seen in Figure 2
123 may be due to compensatory actions of the STG network through gap junctions and other
124 molecular events. While there was no significant effect of dsRNA treatment on *para* or *shal*
125 expression, we found that the expression of these two genes was highly correlated, indicating
126 that the expression of these two ion channel-encoding genes may be tightly coregulated. We
127 also showed that RNA interference in conjunction with electrophysiological recordings and
128 manipulations provide an effective means for studying central pattern generation. Our results
129 serve as a proof of concept that single-cell molecular approaches can be incorporated into the
130 NS&B curriculum for generating insight into the neuromolecular mechanisms regulating
131 rhythmic motor patterns in the crab STG.

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140 trademark of Promega Corporation. StepOnePlus is a trademark of Applied Biosystems. Verti is
141 a registered trademark of Life Technologies, Inc.

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143 AUTHOR CONTRIBUTIONS

144 All authors conceived and designed the experiment. RMH, AGO, and CS conducted the
145 experiments, RMH and HAH analyzed the data and wrote the paper. All authors approved the
146 final version of the manuscript.

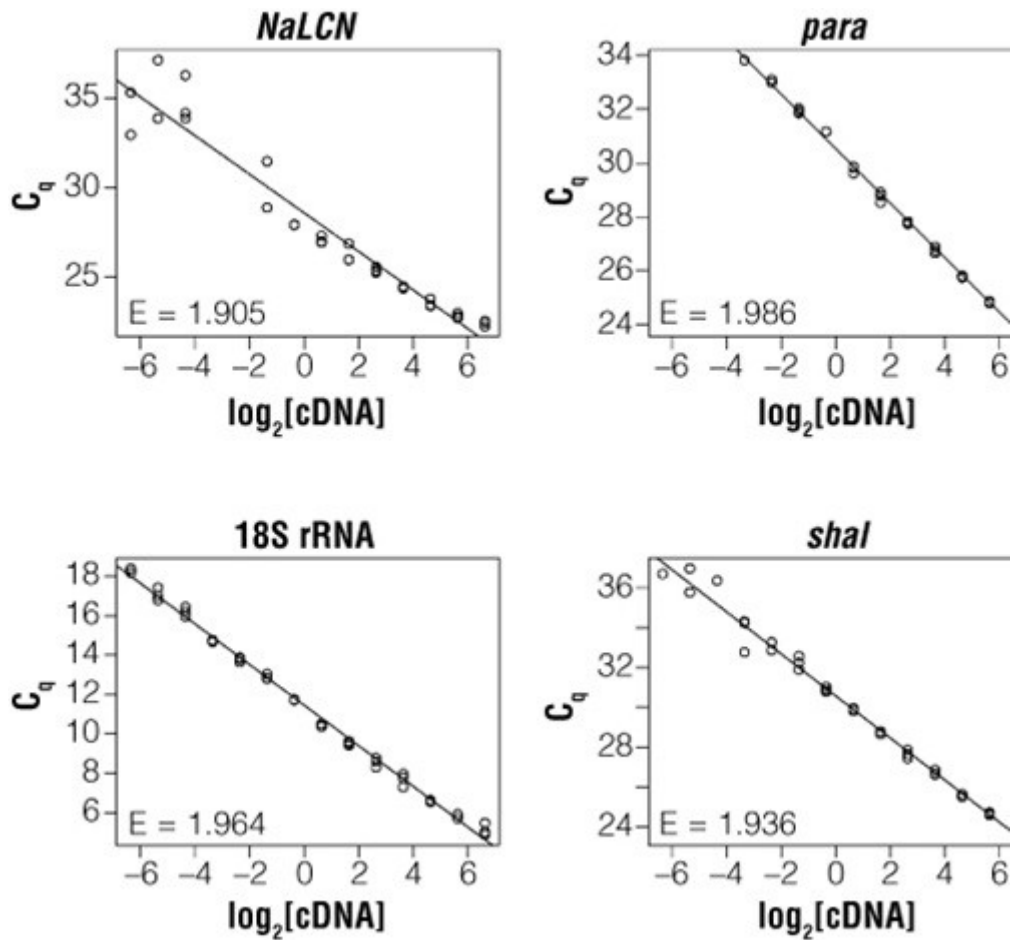
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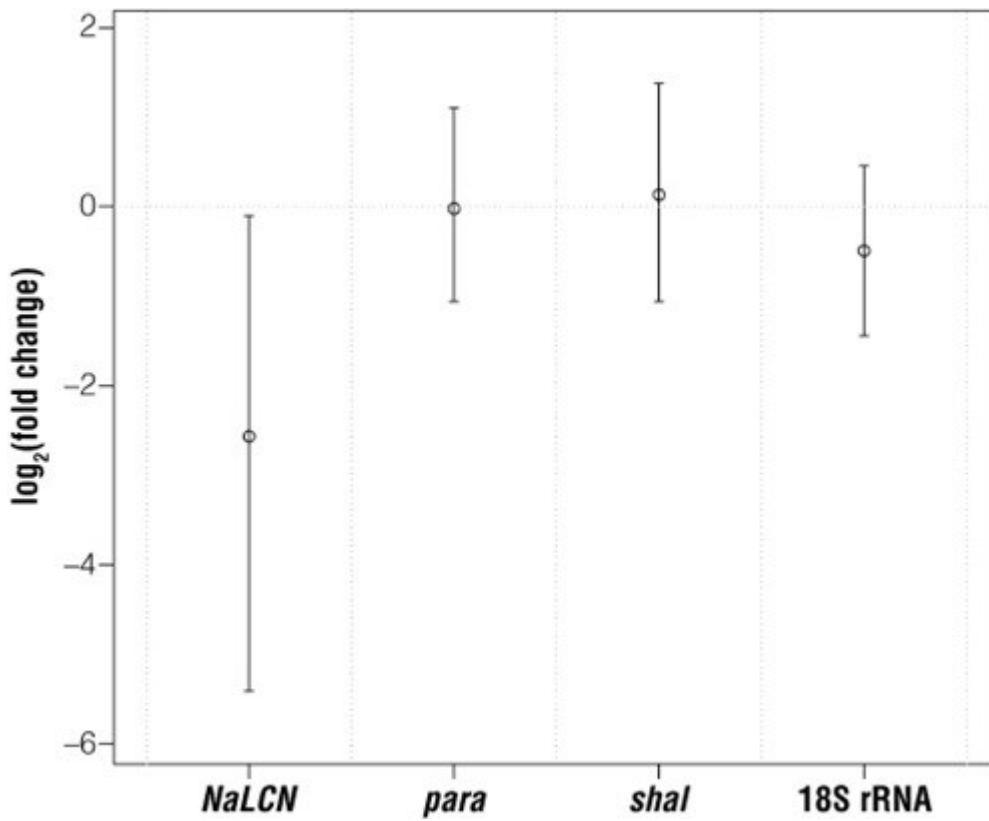
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159 **FIGURES**



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161 **Figure 1. Primer efficiencies.** Primer efficiencies (E, the amplification factor per PCR
162 cycle) were determined by amplifying a series of twofold dilutions of *Cancer borealis*
163 cDNA covering four orders of magnitude of template amount (100–0.01ng cDNA per
164 reaction). The results were plotted as the cycle of quantification (C_q) vs. $\log_2[\text{cDNA}]$. E
165 was derived from the slope of the regression using formula $E=2^{-(1/\text{slope})}$. *NaLCN*:
166 sodium leak channel nonselective; *para*: voltage-gated sodium channel; *shal*: potassium
167 channel; 18S RNA: 18S ribosomal RNA subunit.

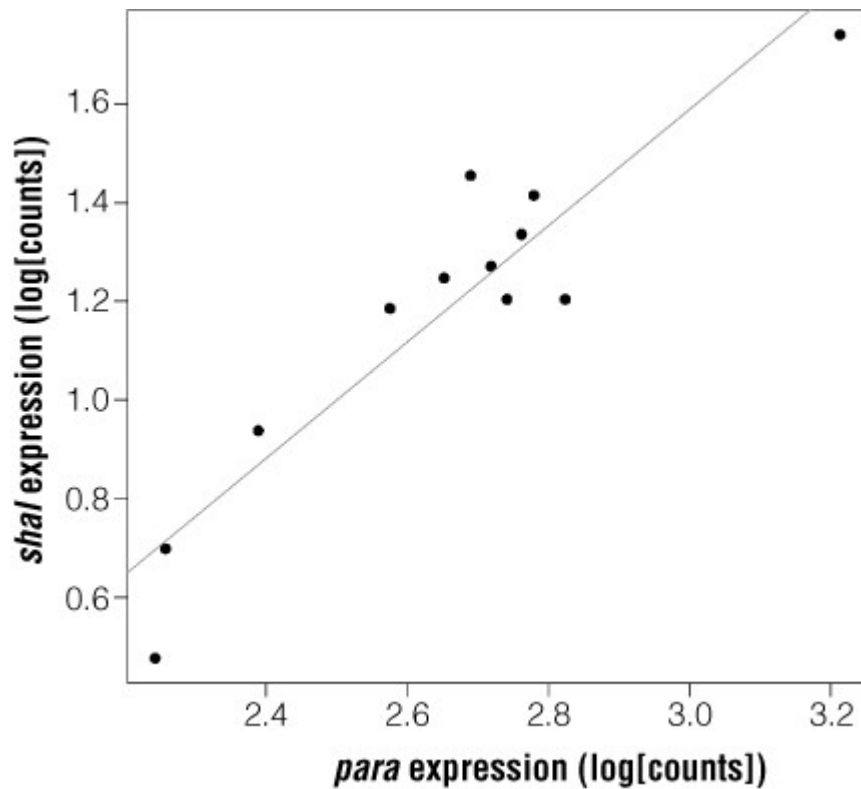
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171 **Figure 2. NaLCN dsRNA treatment reduces NaLCN expression.** The effect of *NaLCN*
172 dsRNA treatment on expression is plotted for each gene as log₂(fold change), with
173 positive and negative values indicating increased or decreased expression, respectively.
174 The treatment occurred over 24 or 48 hours with both times included in the plotted data.
175 The points are posterior means, the whiskers denote 95% confidence intervals.

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179 **Figure 3. *para* and *shal* expression levels are correlated.** A Pearson correlation
180 of gene expression (log[counts]) for all samples analyzed shows that *para* and *shal*
181 expression levels are significantly correlated ($r^2 = 0.93$, $p = 1.39 \times 10^{-5}$). *para*:
182 voltage-gated sodium channel; *shal*: potassium channel.
183