Species and individual differences in serotonin receptor expression in identified neurons are correlated with a motor behavior.

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Abstract (253 words)

G protein-coupled receptors (GPCRs) activated by biogenic amines such as serotonin (5-HT) can modulate neuronal signaling and membrane properties. Expression differences in GPCRs could underlie species or individual differences in behaviors. This study examined correlations between behavior and GPCR gene expression in single identified neurons. In the nudibranch *Tritonia diomedea* (Mollusca; Gastropoda; Nudipleura), 5-HT plays an essential role in the generation of dorsal-ventral (DV) escape swimming by enhancing the strength of synapses made by neuron C2, which is a member of the swim central pattern generator (CPG). Neurons homologous to C2 are present across Nudipleura. Previous research found that in the nudibranch, *Hermissenda crassicornis*, which does not swim, C2 synapses are not modulated by 5-HT. The pleurobranchomorph, *Pleurobranchaea californica*, has variable swimming, which correlates with the extent of modulation of C2 synaptic strength. We tested whether the expression of particular 5-HT receptor genes correlated with species and individual variation in swimming. Using single C2 neuron transcriptome sequencing and absolute quantitative PCR (qPCR), we found that 5-HT2a and 5-HT7 receptor expression was high in C2 neurons isolated from *Tritonia* and individual *Pleurobranchaea* that swam, but not

Hermissenda or non-swimming *Pleurobranchaea*. In contrast, C2 neurons isolated from *Hermissenda* exhibited higher 5-HT4 and 5-HT6 expression, neither of which was expressed in *Tritonia* C2 neurons. The results suggest that differences in the expression of particular GPCRs in single neurons could account for differences in behavior both across and within species. This finding has implications for our understanding of the evolution of species-typical behaviors.

Introduction

It has been proposed that differences in behavior could arise through differences in neuromodulatory signaling[1-5]. Serotonin (5-HT) alters behavior by modulating neuronal and synaptic properties[4,6-11]. Many 5-HT receptor subtypes have been identified[12]. If the same neuron varied its expression of particular 5-HT receptor subtypes, it could cause 5-HT to act differently on that neuron. If such variation in 5-HT receptor expression occurred across species, it might underlie production of species-specific behavior. Furthermore, differences in receptor expression across individuals within a species could also account for individual variability in behavior.

We addressed this question by examining 5-HT receptor expression in single neurons of Nudipleura (Mollusca; Gastropoda). These sea slugs have large neurons that can be individually identified from animal to animal within a species as is common with invertebrates[13,14]. Furthermore, homologous neurons can be identified across species[15]. Moreover, the roles of these neurons in simple behaviors have been determined.

The nudibranch *Tritonia diomedea* (synonymous with *Tritonia tetraquetra*, Pallas 1888) swims by producing alternating dorsal and ventral (DV) body flexions. The central pattern generator (CPG) underlying the swim motor pattern contains the

identified neurons C2 and DSI[16,17]. C2 is identified in each species by its white soma in the cerebral ganglion[18,19], which is immunoreactive to the neuropeptides FMRFamide and Small Cardioactive Peptide (SCP), and its contralaterally-projecting axon[20]. DSIs are identified in each species by 5-HT immunoreactivity and their position in the brain[21-23]. These neurons can be identified in the nudibranch, *Hermissenda crassicornis* even though *Hermissenda* lacks this swimming behavior[24].

The more distantly related *Pleurobranchaea californica* also has C2 and DSI neurons known as A1 and As1-3 respectively, which are identified by the same criteria as in *Tritonia* and *Hermissenda*[20,25,26]. Like *Tritonia*, *Pleurobranchaea* swims with DV body flexions. Furthermore, as in *Tritonia*, these neurons are members of the *Pleurobranchaea* DV swim CPG[22,26]. In this paper, we will refer to the neurons using the *Tritonia* nomenclature.

Serotonergic neuromodulation plays a central role in the production of the swim motor pattern in *Tritonia*. Application of 5-HT to the isolated *Tritonia* brain is sufficient to evoke a swim motor pattern and injection of the animal with 5-HT evokes a swim[27]. Stimulation of the serotonergic DSI or 5-HT application increases neurotransmitter release from C2 through an increase in spike-evoked Ca^{2+} levels in C2 terminals and affects C2 spiking [28,29]. The 5-HT receptor antagonist methysergide blocks the neuromodulatory actions of DSI on C2 synapses[30]. Methysergide also blocks production of the swim motor pattern in the isolated brain and prevents swimming when injected into the intact animal[27].

Unlike in *Tritonia*, neither DSI nor 5-HT modulates C2 synaptic strength in *Hermissenda*. Furthermore, application of 5-HT to the isolated *Hermissenda* brain fails to evoke a swim motor pattern, and injection of *Hermissenda* with 5-HT does not cause

the animal to swim[31]. This correlation between swimming behavior and serotonergic modulation of C2 neurons could be caused by species differences in expression of particular 5-HT receptors in C2.

In *Pleurobranchaea*, DSI stimulation and 5-HT application both enhance the strength of C2 synapses. Additionally, 5-HT induces swimming following injection in live *Pleurobranchaea* and methysergide blocks swimming[31]. Although *Pleurobranchaea* and *Tritonia* appear to have evolved DV swimming behaviors independently, the neural mechanisms underlying these convergent behaviors both involve serotonergic neuromodulation of homologous neurons.

Unlike in *Tritonia*, swimming in *Pleurobranchaea* is not consistent; the same animal varies in its propensity to swim when tested over the course of several days[25,26,31]. Similarly, some isolated brain preparations do not exhibit bursting activity typical of a swim motor pattern. It was shown that the extent of serotonergic enhancement of C2 synaptic strength correlated with the number of burst cycles in the motor pattern[31]. Therefore, there is a relationship between C2 modulation by 5-HT and individual variability in swimming behavior.

Here, we identified the 5-HT receptor subtypes expressed by C2 homologues. Seven receptor subtypes, from five families of 5-HT receptors, were previously identified in whole brain tissue of *Tritonia*, *Hermissenda*, and *Aplysia californica*[32,33] (Figure 1). We performed Illumina RNA sequencing (RNA-seq) on *Pleurobranchaea* brains to identify 5-HT receptor subtypes in that species. Using single-neuron RNA sequencing (snRNA-seq) and single-cell quantitative PCR (qPCR), we examined the expression of 5-HT receptor genes in C2 neurons from *Tritonia*, *Hermissenda*, and *Pleurobranchaea*. We

found that differences in 5-HT receptor mRNA expression by C2 homologues correlates with species and individual differences in swimming behavior.

Methods

Animals

Specimens of *Tritonia diomedea* were collected by Living Elements LLC (Vancouver, B.C.), *Hermissenda crassicornis* and *Pleurobranchaea californica* specimens were collected by Monterey Abalone Co. (Monterey, CA). All animals were housed at 10°C in recirculating artificial seawater (ASW, Instant Ocean). Individual *Tritonia* were anaesthetized before dissection using cold temperature and the other species were anaesthetized using 0.33 M magnesium chloride.

Whole-brain RNA extraction, cDNA production, and transcriptome sequencing

For whole-brain RNA extraction, brains were dissected and cleaned of connective tissue, flash-frozen using liquid nitrogen, then stored at -80°C. RNA extraction was performed using the RNeasy Plus Universal Mini Kit (Qiagen). RNA extracts were quantified using Nanodrop (Thermo Fisher). RNA was reverse transcribed to cDNA using Superscript IV (Thermo Fisher) following manufacturer's instructions.

For the *Pleurobranchaea* whole brain transcriptome, additional whole-brain RNA was extracted as described above. RNA was treated and sequenced on an Illumina Hi-Seq2500 as described previously for *Tritonia*[34] and *Hermissenda*[32], and similarly assembled using the *de novo* assembler Trinity (citation). Metrics for the *Pleurobranchaea* CNS shotgun transcriptome assembly are provided in supplementary table x.

5-HT receptor plasmid cloning

Species-specific primers were designed using transcriptome-derived 5-HT receptor sequences from *Tritonia*[34], *Hermissenda*[32], and *Pleurobranchaea* (NCBI SRA TBA) transcriptomes. The primers (Eurofins) are listed in Supplemental Table 1. PCR was performed to amplify putative 5-HT receptor genes from whole-brain cDNA from each species using *Taq* polymerase (Thermo Fisher). PCR products were gel purified using the Qiaquick Gel Extraction Kit (Qiagen). Resulting DNA was ligated using T4 DNA ligase and inserted into JM109 competent cells (Promega). Cloned plasmids were extracted using GenElute Plasmid Mini-prep Kit (Sigma Aldrich). Plasmids were sequenced on a 3730 DNA Analyzer (Thermo Fisher), and sequences were aligned against transcriptome sequences using MUSCLE[35] to verify gene identity.

Phylogenetic analysis of 5-HT receptors

A phylogenetic tree was created using methods described previously for *Tritonia* and *Hermissenda* receptors[32]. Previously unpublished *Pleurobranchaea californica* 5-HT receptor sequences were determined from a whole brain transcriptome, and sequences were confirmed by plasmid cloning. MUSCLE alignment followed by maximum likelihood tree construction with bootstrapping was done using MEGA6 software[36].

Synthetic RNA production for quantitative PCR standards

Plasmid DNA sequences for each species-specific 5-HT receptor subtype were linearized with T7- or Sp6-oriented enzyme digests (New England Biosciences) and gel-

verified. Digested plasmids were purified using phenol-chloroform extraction and alcohol precipitation, and quantified using a Nanodrop 2000C (Thermo Fisher). Synthetic RNA was produced using either T7 or Sp6 MegaScript Kit (Thermo Fisher) and purified. RNA was quantified using Nanodrop. The copy number for each 5-HT receptor synthetic RNA sample was calculated using the gene-specific molecular weight and RNA concentration[37]. RNA standards were then serially diluted and individually reverse transcribed to cDNA using SuperScript IV (Thermo Fisher).

Single-neuron isolation

Following initial dissection, the brain, which consists of the fused cerebral, pleural, and pedal ganglia, was placed in either artificial sea water (Instant Ocean) or normal saline (NS) (420 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 11 mM d-glucose, and 10 mM HEPES, pH 7.5). The connective sheath was removed using fine scissors and forceps. C2 was visually identified as the white neuron in the anterolateral cerebral ganglion[18-20].

Intracellular recordings were made using 10-30 MΩ glass microelectrodes connected to an IX2-700 Intracellular Amplifier (Dagan). Extracellular recordings and stimulation of body wall nerves were made by placing them in polyethylene tubing connected to a Model 1700 differential amplifier (A-M Systems). Signals were digitized using the Micro1401 and Spike2 software (Cambridge Electronic Design Limited).

To identify C2, neurons were recorded intracellularly and a swim motor pattern was evoked by stimulating a body wall nerve with 20–35 V at 5 Hz for 3 s. In *Pleurobranchaea*, C2 neurons were isolated from bursting and non-bursting preparations; individual preparations were defined as “swimming” if two or more bursts

could be evoked, and "non-swimming" if one or no bursts were elicited (Figure 2). Contralateral C2 neurons are electrically coupled to each other in all three species[19,20,25,26,38,39]. Electrical coupling to the contralateral white cell was tested in some *Hermissenda* and non-swimming *Pleurobranchaea* preparations when participation in the swim motor pattern could not be used to help identify C2.

After identification of C2, the brain was bathed in 0.2% Protease IX for five min and washed with ASW or NS. For neurons that were to be sequenced, two additional washes of filtered ASW were performed. Individual C2 neurons were removed with forceps and a suction pipette.

After removal from the brain, single C2 neurons were placed in individual 1.5 mL tubes containing distilled water and RNaseOut (Thermo Fisher), and visually inspected under a light microscope to verify that cells were present, intact, and were the only cells in the tube. C2 neurons were discarded if they appeared damaged at any point during isolation, or did not meet species-specific physiological hallmarks of C2 neurons[20]. Neurons were not isolated if the central ganglia appeared damaged. C2 neurons that appeared to have other neurons attached to them following initial isolation were also discarded.

Single-cell transcriptome assembly and gene identification

For *Tritonia* and *Hermissenda*, five individual C2 cell samples were prepared. One swimming and one non-swimming *Pleurobranchaea* C2 cell sample was prepared. Single cell cDNA was synthesized using the Clontech SmartSeq v4 Ultra-Low Input RNA Kit (Takara). Libraries were prepared and indexed using the Nextera XT DNA Library Preparation Kit and 96-Sample Index Kit (Illumina). Qubit (Thermo Fisher) and

Bioanalyzer (Agilent) analyses were used to judge quantity and quality of cDNA outputs during both protocols. cDNA samples were sequenced on a Hi-Seq2500 (Illumina) with a read depth of 10 million reads per sample. Sequencing reads as fastq files were analyzed using FastQC (Andrews 2010) via BaseSpace analysis (Illumina). For *Tritonia* and *Hermissenda*, the single sample raw reads were concatenated before assembly. All files were then assembled with Trinity[40]. BLAST+ (version 2.4.0)[41] was used to identify genes with queries from whole-brain transcriptomes of the same species. NCBI tBLASTx was used to confirm phylogenetic identity of each identified gene. MUSCLE[35] was used to align individual sequences. The resulting transcriptomes are published as NCBI SRA TBA.

Single-Neuron cDNA synthesis for quantitative PCR

Neurons used for qPCR were isolated as described above and were transcribed to cDNA using Superscript IV (Thermo Fisher). Following isolation, the cells were added to a mixture of distilled water and RNaseOut and frozen at -80°C. A master mix was created following the Superscript IV manufacturer's protocol, and aliquots were added directly to the RNA. The RNA was annealed and cooled on ice. The annealed RNA was then divided by volume equally into two tubes. One tube received reverse transcriptase enzyme plus additional Superscript IV reagents (+RT). The other tube lacked reverse transcriptase (-RT) and received a volume of distilled water in place of the enzyme volume. The RNA samples were then reverse-transcribed following manufacturer's instructions.

mRNA quantification using single-neuron and whole brain absolute qPCR

Absolute qPCR was performed for individual genes from each species. qPCR-specific primers were designed against each gene orthologue (Supplemental Table 2). For

5-HT2a in *Tritonia* and *Hermissenda*, two sets of primers were used to measure mRNA expression at multiple locations along the 5-HT2a mRNA. Other gene orthologues were measured with one primer pair. RNA standards were prepared as described above, and were run alongside cell cDNA, -RT samples, and without-template control samples on every qPCR trial. C2 samples were run either as single cells, or pooled cells from multiple animals; raw copy numbers were divided by starting cell amount. Whole brain tissue was run with 150 ng RNA calculated per one triplicate sample. All C2 and whole brain samples, standards, and controls were run in triplicate using Perfecta SYBR Green Supermix with ROX or Low ROX (Quanta Bio) on an Applied Biosystems 7500 or StepOne Plus qPCR machine.

Absolute mRNA copy number values were calculated using the standard curve generated during each trial. If amplification occurred in the –RT samples, it was subtracted from the +RT samples. Trials were omitted if the standard curve efficiency was calculated to be outside the range of 85 to 110%. A melt curve was run on every trial, and samples were omitted if a double peak was detected, or if samples showed multiple bands in an agarose gel (Sigma Aldrich) following the qPCR trial. mRNA copy numbers were analyzed using SigmaPlot (version 10, Systat Software Inc.).

Results

Small cardioactive peptide precursor was expressed in C2 neurons from each species

C2 neurons are immunoreactive for SCP across nudipleura species[20]. Therefore, the SCP precursor gene was used as a positive marker of C2 identity in this study. The gene for SCP was expressed in C2 neuron transcriptomes from all three species, including swimming and non-swimming *Pleurobranchaea* (Table 1). Of note, there are two SCP

precursor gene alternative splice isoforms present in molluscan brains, a short and long isoform (cite Tritonia TSA paper). In C2 neuron transcriptomes, only the short isoform was present. Single-neuron qPCR confirmed that SCP precursor expression was present in all three species (Figure 3).

A subset of 5-HT receptors were expressed in Tritonia *C2 neurons*

Although it is known that the *Tritonia* C2 neuron responds to 5-HT, the identity of the receptors expressed by this cell were previously unknown. We measured 5-HT receptor subtype gene expression from single *Tritonia* C2 neurons by performing snRNA-seq. Five of the seven receptor subtypes previously identified from whole brain transcriptomes were expressed in the *Tritonia* C2 neuron concatenated transcriptome. These were receptors 5-HT1a, 1b, 2a, 2b, and 7 (Table 2). 5-HT 4 and 5-HT6 were not present in the transcriptome. Thus, only a subset of receptor subtypes were present in *Tritonia* C2 neurons according to snRNA-seq.

qPCR measurements validated the transcriptome results. 5-HT2a and 5-HT7 receptor subtypes both had median expression well above 200 copies per cell (Figure 4). Within these two data sets, variability in copy number across samples was high, with 5-HT2a showing a range between 152 and 655 copies, and 5-HT7 ranging between 209 and 611 copies. 5-HT4 and 5-HT6 were not detected in *Tritonia* C2 neurons, consistent with the RNA-seq results.

The remaining receptors were variably expressed at low levels. 5-HT1a was identified in the transcriptome. However, qPCR was only able to detect this particular transcript in one out of the five *Tritonia* C2 neurons. 5-HT1b was also found in the transcriptome, and consistently expressed at fewer than 200 copies per cell according to

qPCR. 5-HT2b was detected in the single-cell transcriptome, but only three out of six *Tritonia* samples expressed 5-HT2b according to qPCR. All seven of the 5-HT receptor subtypes were measured in whole brain tissue using qPCR, however, indicating that the qPCR primers were able to amplify the gene of interest when it was present (Figure 5).

In Hermissenda, *C2 expressed a different subset of 5-HT receptors compared with* Tritonia

Hermissenda C2 5-HT receptor expression was compared to that of *Tritonia* using both snRNA-seq and qPCR. The C2 transcriptome in *Hermissenda* contained 5-HT1a, 1b, 4 and 6 receptors (Table 2). These results were again validated and quantified using single neuron qPCR (Figure 4). 5-HT2a and 5-HT7, which were highly expressed in *Tritonia* were not found in the *Hermissenda* C2 transcriptome or using single neuron qPCR. One *Hermissenda* qPCR sample, however, showed low expression of 5-HT7. In constrast, 5-HT4 and 5-HT6, which were not expressed in *Tritonia* C2 neurons, were found to be expressed at greater than 200 copies per cell in *Hermissenda* using single neuron qPCR. Thus, there was a clear species difference in the expression of 5-HT receptors in homologous neurons that differ in their responses to 5-HT.

Single-neuron qPCR showed that the other receptors were expressed at low levels as in *Tritonia*. 5-HT1a and 5-HT1b expression in *Hermissenda* C2 samples were below 200 copies per cell. The 5-HT2b sequence was found in the *Hermissenda* C2 transcriptome, but as in *Tritonia,* it was measured in three of eight qPCR samples. The similarities in the low-level expression of 5-HT1a, 5-HT1b, and 5-HT2b in both *Tritonia* and *Hermissenda* suggests that these receptors would not be responsible for the species-differences in the effect of 5-HT on C2.

5-HT receptor subtypes were identified in Pleurobranchaea *whole-brain tissue*

Before testing which 5-HT receptor subtypes were expressed in *Pleurobranchaea* C2 neurons, we generated a *Pleurobranchaea* whole brain transcriptome (suppl. table x as suggested in methods?), and mined it for 5HT receptor sequences using BLAST. All seven molluscan 5-HT receptor subtype orthologues were identified (Supplemental Table 3). To confirm their sequence identities, we created consensus sequences of each receptor protein coding sequence via cloning and sequencing of complementary DNA generated from whole-brain tissue. The *Pleurobranchaea* 5-HT receptor subtype consensus protein sequences phylogenetically clustered with other subtypes from each 5-HT receptor family (Figure 1).

Receptor expression differed in swimming and non-swimming Pleurobranchaea

Since *Pleurobranchaea* does not reliably swim, we partitioned samples from this species based on the motor pattern produced at the time that the C2 sample was taken. If the motor pattern consisted of more than a single burst, it was considered a "swimmer" (Figure 2c). On the other hand, if stimulation of the body wall nerve produced one or fewer bursts, the individual was categorized as a "non-swimmer" (Figure 2d).

5-HT2a and 5-HT7 gene sequences were found in the transcriptome of C2s isolated from *Pleurobranchaea* that swam. This was validated by qPCR. Only one sample from the non-swimming *Pleurobranchaea* group showed low expression of 5-HT2a according to qPCR, calculated at 45 mRNA copies (Figure 6). There was a large degree of variability in the amount of 5-HT2a expressed in individual C2 samples from swimming individuals, from 100 to 1276 copies. All but one sample expressed 5-HT7 genes according to qPCR

(Figure 6). Variability in qPCR-measured expression was highest for this gene, with a range of 0 to 2446.

In the C2 transcriptome from non-swimming *Pleurobranchaea* neither 5-HT2a nor 5-HT7 sequences were present. This was validated with qPCR, except that one of the C2 neurons from the seven non-swimming swimming animals exhibited 5-HT7 gene expression (Fig. 4). Thus, *Pleurobranchaea* that exhibited a swim motor pattern on the day of testing resembled *Tritonia* in the expression of 5-HT2a and 5-HT7 receptor genes, whereas non-swimmers resembled *Hermissenda* in the absence of this expression.

We did not perform qPCR on the remaining receptor types, but relied on the transcriptomes. The swimming *Pleurobranchaea* C2 transcriptome contained sequences for 5-HT1b expression but none of the other receptors whereas the C2 transcriptome of non-swimmers did not contain any 5-HT receptor gene sequences. This suggests that on the days that *Pleurobranchaea* does not swim when tested, C2 did not express any 5-HT receptor genes, but it expressed 5-HT1b, 5-HT2a, and 5-HT7 receptor genes on days when it did swim.

Additional G-protein coupled receptors were measured using single-cell transcriptomes

The absence of any 5-HT receptor sequences from the C2 transcriptome of non-swimming *Pleurobranchaea* was not due to the inability to detect GPCR sequences. Several other types of GPCR were found in both swimmers and non-swimmers. These GPCRs were also identified in the C2 transcriptomes from *Tritonia* and *Hermissenda*. (Table 3). However, the presence or absence of these other GPCRs did not correlate with swimming. A greater number of GPCR types were found in the two nudibranchs,

compared with the *Pleurobranchaea* data sets. This is not due to an absence of these genes in the brain transcriptome, but a lack of expression in C2.

Molluscs express three identified dopamine receptor subtypes, known as D1, D2, and DInv[33,42]. D2 expression was found in C2 in all three species, including both the swimming and non-swimming *Pleurobranchaea* C2 transcriptomes (Table 4). Overall, the presence or absence of GPCR sequences in the C2 transcriptomes of the two nudibranchs, *Tritonia* and *Hermissenda*, was more similar to each other than to the more distantly related *Pleurobranchaea*, however, there were still many species differences.

Discussion

Our results provide evidence that differences in expression of particular 5-HT receptor genes in an identified neuron correlate with the presence of a specific type of swimming behavior. Using a combination of single-neuron transcriptome sequencing and absolute qPCR, gene expression for 5-HT receptors was measured in C2 neurons from three species of nudipleura sea slugs. The nudibranchs, *Tritonia* and *Hermissenda* differ in swimming behavior and in the expression of particular 5-HT receptor subtypes. *Pleurobranchaea* exhibits variations in swimming behavior and coordinate variations in the expression of 5-HT receptor genes; C2 neurons extracted from swimming individuals shared a profile of 5-HT receptor subtype expression with *Tritonia*, whereas non-swimming *Pleurobranchaea* did not express any 5-HT receptor genes in this neuron. Overall, these results indicate a role for neuromodulatory gene expression in species-specific behaviors, as well as in individual variability in behaviors.

C2 synaptic modulation may be controlled via specific 5-HT receptors

In *Tritonia* C2 neurons, two subtypes were consistently measured across samples and showed expression above 200 copies per cell with single-neuron qPCR: 5-HT2a and 5-HT7. C2 neurons isolated from swimming *Pleurobranchaea* expressed 5-HT2a and 5-HT7 subtypes according to both the C2 transcriptome and qPCR. In contrast, the *Hermissenda* C2 neurons did not express 5-HT2a or 5-HT7 receptor genes. Thus, the expression of these two receptors correlated with the presence of the swimming behavior and the modulation of C2 synaptic strength by serotonin.

The expression of 5-HT2a and 5-HT7 in C2 in *Tritonia* and swimming *Pleurobranchaea*, but not *Hermissenda*, indicates that one or both of these subtypes likely contributes to species-specific modulation and swimming. On the other hand, *Hermissenda* C2 neurons expressed 5-HT4 and 6, which were not found in *Tritonia* or *Pleurobranchaea* C2 neurons. The functions of these 5-HT receptors in *Hermissenda* C2 neurons are currently unknown. It is unclear if the presence of 5-HT4 and 5-HT6 actively prevents modulation of C2 synaptic strength or if they are present for other purposes unrelated to swimming.

Methysergide, a mammalian 5-HT1/2 family antagonist, blocks swimming in both *Tritonia* and *Pleurobranchaea*[27,31]. Additionally, it reduces DSI-mediated modulation of C2 when bath applied to isolated *Tritonia* brain preparations[30]. It is possible that methysergide acts on 5-HT2a receptors in *Tritonia* and *Pleurobranchaea* C2 neurons, consistent with its actions on 5-HT2 family receptors in mammals. However, methysergide binding selectivity may differ between receptor families in different animal phyla, as has been reported previously with respect to receptor pharmacology[43,44].

Tritonia C2 neurons exhibit an increase is spike-evoked calcium in synaptic terminals following DSI stimulation or puffs of serotonin[29,45]. This calcium increase was

blocked by methysergide, indicating that it could be mediated by 5-HT2 family receptors, which have conserved G-protein coupling to the Phospholipase C/IP3/DAG pathway. Consistent with this, peptidergic neurons in *Aplysia* increase membrane expression of pre-synaptic voltage-gated Ca^{2+} channels in response to activation of this GPCR pathway and the downstream effector kinase, PKC (citation). Thus the synaptic modulation may be mediated by the 5-HT2A receptors expressed by C2.

Other serotonin receptors may mediate additional responses.

DSI stimulation and serotonin application cause three additional effects on C2 beside synaptic enhancement: a decrease in spike frequency adaptation that is associated with a decrease in C2 spike after-hyperpolarization, a fast depolarizing potential, and a slow depolarization[30,46]. The slow depolarization is blocked by methysergide, suggesting that it may also be mediated by 5-HT2a receptors. The fast potential is blocked by the antagonist gramine, which does not block the synaptic enhancement or the slow potential, suggesting that it is mediated by a different 5-HT receptor. The "fast" potential is still fairly slow by synaptic standards with a decay time constant of 0.28 seconds, compared to a decay time constant of 4.4 seconds for the slow potential, indicating that both could be mediated by a GPCR.

C2 reliably expressed 5-Ht1b receptor genes in both *Tritonia* and *Hermissenda,* and this sequence was present in the transcriptome of swimming *Pleurobranchaea*. However, this gene was expressed at fewer than 200 copies per cell and thus was not as prevalent as the other 5-HT receptor genes. Because it was expressed in both *Tritonia* and *Hermissenda*, the 5-HT1b receptor may mediate an action of serotonin on C2 other than enhancement of synaptic release or synaptic potentials, none of which were seen in

Hermissenda. However, the effect of serotonin on spike frequency adaptation was not tested in *Hermissenda* or *Pleurobranchaea*, leaving open the possibility that it is mediated b 5-HT1b receptors.

The expression of multiple neuromodulatory receptors is a feature that has been observed in several single-neuron studies. In the anaspid mollusc *Aplysia californica*, pleural ganglion sensory neurons express at least three 5-HT receptor subtypes, identified with single cell PCR[33]. In crustaceans, single identified neurons have been studied with respect to their neuromodulatory receptor expression. The pyloric dilator (PD) pacemaker cells, for example, have been shown to express multiple 5-HT receptor subtypes, an acetylcholine receptor[47-49], and dopamine receptors[50], as determined by a combination of pharmacology, immunohistochemistry, and single-cell RT-PCR.

Previous pharmacological research has found receptor expression differences between homologous neurons from different species. The LP neurons of crabs, for example, respond to 5-HT[47], proctolin[51], and CCAP[52], whereas their homologues in lobsters respond to DA through D1Rs [53], mGLUR agonists[54], and orcokinin[55]. Species differences in the identity of neurotransmitter receptors expressed by homologous neurons might be expected, given the fact that homologous neurons can have species-specific functions[56].

Other genes did not show a correlation with behavior

Although the expression profile of 5-HT receptors in C2 was species specific and variable across individuals with different behaviors, the SCP precursor gene was expressed in all C2 neurons, regardless of species and behavior. SCP immunoreactivity was previously identified as a defining characteristic of C2 neurons and an indication of

their homology across species[20]. The presence of SCP mRNA in C2 neurons from each species confirmed the identity of the cell, and showed that there are gene markers found across homologous neurons, regardless of species. It is likely that there are many more gene expression similarities shared by these homologous cells.

Other GPCRs did not show a correlation with behavior, although their expression did vary across species. Several GPCRs were identified in C2 neurons from each species, indicating that C2 may have several neuromodulatory inputs. The transcriptomes from each species showed that many receptors were species-specific in the nature of their expression in C2. There may be other, as yet unidentified, species-specific behaviors mediated by C2 neurons. In the case of *Pleurobranchaea*, however, it is worth noting that the sample size for the transcriptome sequencing was lower than that of the other two species (see methods). Therefore, it is possible that *Pleurobranchaea* C2 neurons express more GPCRs than those captured by sequencing in this study.

Parallel evolution of neuromodulation

Most nudipleura species do not exhibit DV swimming. Furthermore, based on their phylogenetic tree, it appears that *Tritonia* and *Pleurobranchaea* do not share a most recent common ancestor that exhibited this behavior and thus likely evolved DV swimming independently[6]. Both species also gained serotonergic modulation of C2 neurons independently[31]. The expression of orthologous 5-HT2a and 5-HT7 receptor genes in C2 neurons from swimming *Pleurobranchaea* and *Tritonia* suggests that the neuromodulatory responses in these two species is a result of parallel evolution, where homologous genes came to be used independently for the same function[57]. It was previously proposed that serotonergic modulation transformed C2 and other neurons

from a latent circuit into the functional swim motor pattern CPG circuit observed in *Tritonia* and *Pleurobranchaea*[31,58]. In fact, it may be that the presence of 5-HT2a and/or 5-HT7 is the "switch" in this evolutionary transformation.

A similar neuromodulatory "switch" affecting behavior is found in prairie and montane voles. The neuromodulatory vasopressin receptor V1a is expressed by neurons in the ventral pallidum of monogamous prairie voles, but not in the closely related, non-monogamous montane voles[59-62]. The non-monogamous voles exhibit behavior characteristic of monogamy when the V1a receptor is exogenously expressed in their ventral pallidum[63]. In this example, the presence or absence of the V1a receptor is the underlying mechanism that facilitates the behavioral switch.

Although the mechanism by which the 5-HT receptor subtypes affect behaviors involving C2 is currently unknown, the correlation between their expression and swimming behaviors shown here presents a tantalizing possibility that manipulating 5-HT2a or 5-HT7 expression in non-swimmer C2 neurons might cause DV swimming to occur, while knocking down their expression in DV-swimmers may reduce or eliminate the swim motor pattern.

C2 showed within-species variability in amount of 5-HT receptor mRNA expressed according to qPCR

There was variability in the level of expression of some receptor subtypes within each species, according to single-neuron qPCR. 5-HT7 was expressed in one of six *Hermissenda* C2 samples, and in one non-swimming *Pleurobranchaea* sample. 5-HT1a was expressed in one *Tritonia* C2 neuron sample, while it was below the level of qPCR detection in the other samples tested for that gene. Similarly, 5-HT2b was expressed in

only a few of the *Tritonia* and *Hermissenda* C2 neurons tested. In the remaining qPCR trials, there was variability in the amounts of each subtype expressed between samples from within a species.

This variability could be due to natural fluctuations in the amount of mRNA for a given gene, which occurs randomly in many cell types[64,65]. In all eukaryotic cells, mRNA molecules are transcribed in the nucleus, translated to proteins by ribosomes, and then degraded as part of normal cell cycling. It is therefore to be expected that mRNA amounts would wax and wane over time, causing variability in qPCR measurements. It is possible that 5-HT receptor mRNA fluctuates randomly in this way.

In other systems, 5-HT receptor mRNA expression variability correlates with specific behaviors. In the neural circuit controlling song production in birds, microRNAs alter seasonal expression of 5-HT and other receptors[66]. Estrogen produced during reproductive phases causes a reduction in 5-HT receptor mRNA in mussel gonads[67]. In non-human primates and rats, 5-HT receptors and other 5-HT-related genes vary with stress in several brain regions[68-71]. 5-HT receptor mRNA was found to vary with circadian rhythm and hibernation periods in rodents[72,73]. The fluctuation of 5-HT receptor mRNA in C2 neurons may have as yet undiscovered functional consequences. It is also worth noting that the animals used in this study were wild-caught adults. They may have exhibited varying mRNA expression because of their individual experiences in the wild before ending up in the laboratory.

Non-swimming Pleurobranchaea *did not express any 5-HT receptors in C2.*

DV swimming varies daily in *Pleurobranchaea*, as measured using both *in vivo* behavioral assays and *in vitro* fictive swimming[25,26,31]. This variability was found

previously to correlate with serotonergic enhancement of C2 synaptic strength[31]. Here, we found that for the most part, C2 in non-swimming *Pleurobranchaea* did not express any 5-HT receptor genes. Although the presence of mRNA in a cell does not necessarily indicate that the corresponding protein is present[74], the correlation of 5-HT receptor mRNA expression and swim motor pattern bursts by C2 in *Pleurobranchaea* points to a genetic mechanism that may at least partially facilitate the mechanism by which C2 and other neural components become functional as the swim motor pattern circuit[31].

Although there was variability between almost all of the individual qPCR samples for each gene and species combination, *Pleurobranchaea* C2 samples were the most variable by qPCR for 5-HT2a, 5-HT7, and SCP. Perhaps some species-specific aspect of mRNA cycling resulted in the increased variability observed in this species. If this were the case, then over time there could be periods where few 5-HT receptor proteins were expressed at C2 synapses in *Pleurobranchaea*, which could cause temporary loss of DV swimming and serotonergic modulation. This predicted fluctuation in 5-HT receptor protein could be an explanation for the previously observed individual variability in this species related to C2 modulation and swimming[31].

Conclusion

Measuring 5-HT receptor gene expression in single C2 neurons uncovered a correlation between neuromodulatory gene expression and swimming behavior. Neuromodulation has been hypothesized to be a means of functionally repurposing a tissue or cell to facilitate the evolution of species-specific behaviors[1-4]. The identification of 5-HT2a and 5-HT7 mRNA in C2 neurons, by two methods, from two DV swimming species can help in our understanding of how neuromodulators can facilitate adaptation

of existing network structures, and is an example of a potential neural mechanism underlying the evolvability of behaviors.

Table 1: SCP Identified in C2 Transcriptome Assemblies.

Table 2: 5-HT Receptors Identified in C2 Transcriptome Assemblies.

Table 3: Other GPCRs Identified in C2 Transcriptome Assemblies.

Table 4: Dopamine Receptors Identified in C2 Transcriptome Assemblies.

Figure 1: Phylogenetic Tree of 5-HT and Dopamine Receptor Subtypes. Maximum likelihood phylogeny showing seven 5-HT receptor subtypes spanning five families, with dopamine D1 and D2 receptors included because they fall within the same ancestral grouping. Bootstrap values represent percentage of predicted replicates at each node. Receptor subunits were aligned using conserved transmembrane domain regions.

Figure 2: Swim Motor Patterns in Three Nudipleura Species. Intracellular recordings of C2 neurons in response to body wall nerve stimulation (black bar) from *Tritonia* (a), *Hermissenda* (b), and swimming (c) and non-swimming (d) *Pleurobranchaea*. Only *Tritonia* and swimming *Pleurobranchaea* show action potential bursts, forming the swim motor pattern.

Figure 3: Small Cardioactive Peptide (SCP) Gene Expression in C2 Neurons. SCP gene expression was measured in C2 neurons from *Tritonia* (light gray), *Hermissenda* (dark gray), and *Pleurobranchaea* (white), using absolute qPCR. Individual samples are represented as open circles on each graph. The boxes represent the range from 25% to 75% of sample expression levels. The median is represented by a black line

in each box. When the standard error of the mean was beyond the 25% to 75% range, it is represented as grey error bars.

Figure 4: 5-HT Receptor Expression in C2 from *Tritonia* and *Hermissenda*. 5-HT receptor expression was measured using absolute qPCR to determine mRNA copy number in individual C2 neurons samples from each species. Individual samples are shown as open circles overlaid on whisker plots for *Tritonia* (light gray) and *Hermissenda* (dark gray). The boxes represent the range from 25% to 75% of sample expression levels. The median is represented by a black line in each box. When the standard error of the mean was beyond the 25% to 75% range, it is represented as grey error bars.

Figure 5: Whole-brain Expression of 5-HT Receptors. qPCR measured 5-HT receptor subtype expression from whole brain tissue in *Tritonia* (a), *Hermissenda* (b), and *Pleurobranchaea* (c).

Figure 6: 5-HT2a and 5-HT7 Receptor Expression in C2 from *Pleurobranchaea* with Variable Swimming Behaviors. 5-HT2a and 5-HT7 receptor expression was measured in *Pleurobranchaea* C2 neurons, which were categorized as “Swimmers” (gray) and “Non-swimmers” (white). Individual samples are represented as circles on each graph. The boxes represent the range from 25% to 75% of sample expression levels. The median is represented by a black line in each box. When the standard error of the mean was beyond the 25% to 75% range, it is represented as grey error bars.

Supplemental Table 1: List of primers used for initial amplification and plasmid cloning.

Supplemental Table 2: List of primers used for absolute qPCR.

Supplemental Table 2: C2 transcriptome contig identification numbers.

Supplemental Table 3: *Pleurobranchaea* whole brain transcriptome contig identification numbers.

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Figure 1

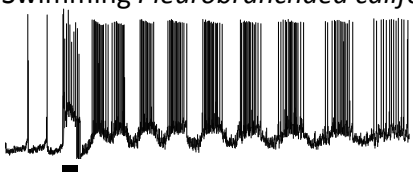
5-HT2a Aply
5-HT2a Pleu
5-HT2a Trit
5-HT2a Herm
5-HT2a Dros
5-HT2b Dros
5-HT2b Herm
5-HT2b Trit
5-HT2b Aply
5-HT2b Pleu
5-HT2 Rat
D2 Herm
D2 Pleu
D2 Aply
D2 Rat
D2 Dros
D2 Trit
5-HT7 Herm
5-HT7 Trit
5-HT7 Aply
5-HT7 Pleu
5-HT7 Dros
5-HT7 Rat
5-HT1 Rat
5-HT1a Dros
5-HT1b Dros
5-HT1a Herm
5-HT1a Trit
5-HT1a Pleu
5-HT1a Aply
5-HT1b Herm
5-HT1b Trit
5-HT1b Aply
5-HT1b pleu
5-HT4 Herm
5-HT4 Trit
5-HT4 Pleu
5-HT4 Aply
5-HT4 Rat
5-HT6 Herm
5-HT6 Trit
5-HT6 Pleu
5-HT6 Aply
5-HT6 Rat
D1 Rat
D1 Dros
D1 Aply
D1 Pleu
D1 Herm
D1 Trit

Figure 2

A. *Tritonia diomedea*

B. *Hermissenda crassicornis*

C. Swimming *Pleurobranchaea californica*



D. Non-swimming *Pleurobranchaea californica*

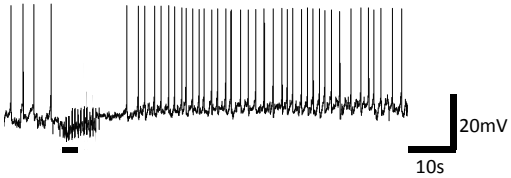


Figure 3

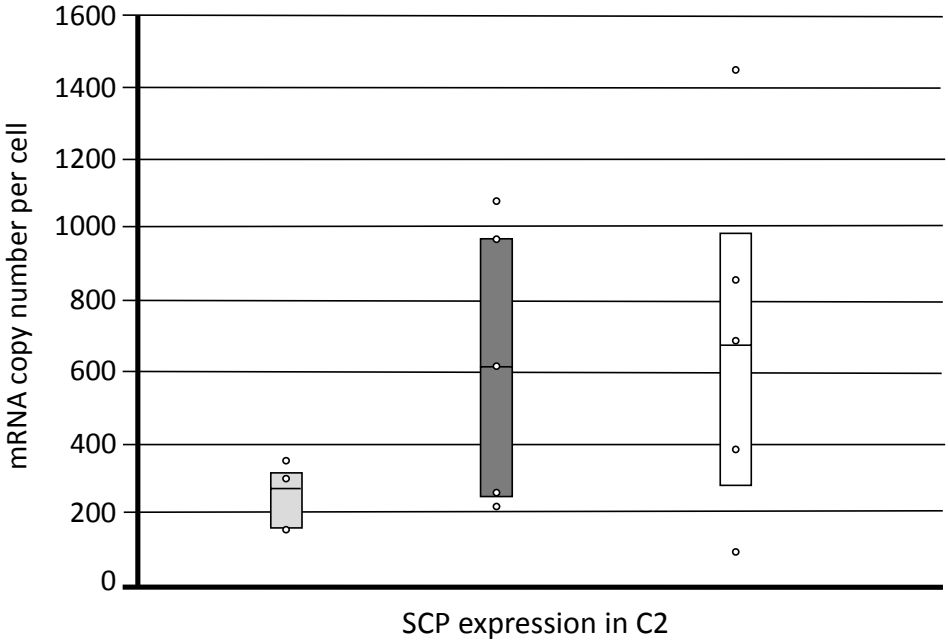

1600
1400
1200
1000
800
600
400
200
0
mRNA copy number per cell
SCP expression in C2

Figure 4

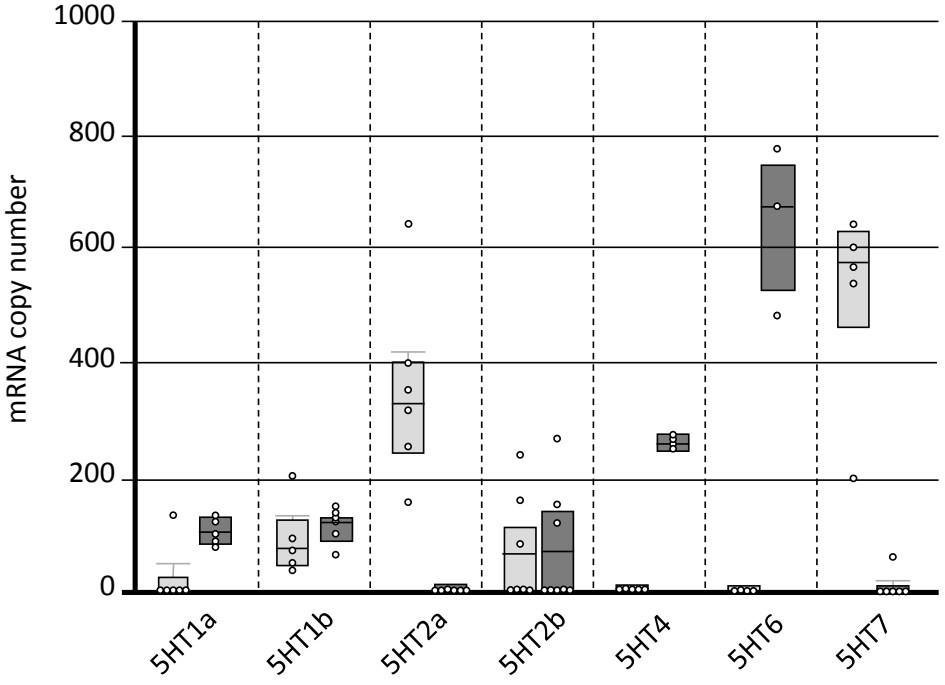
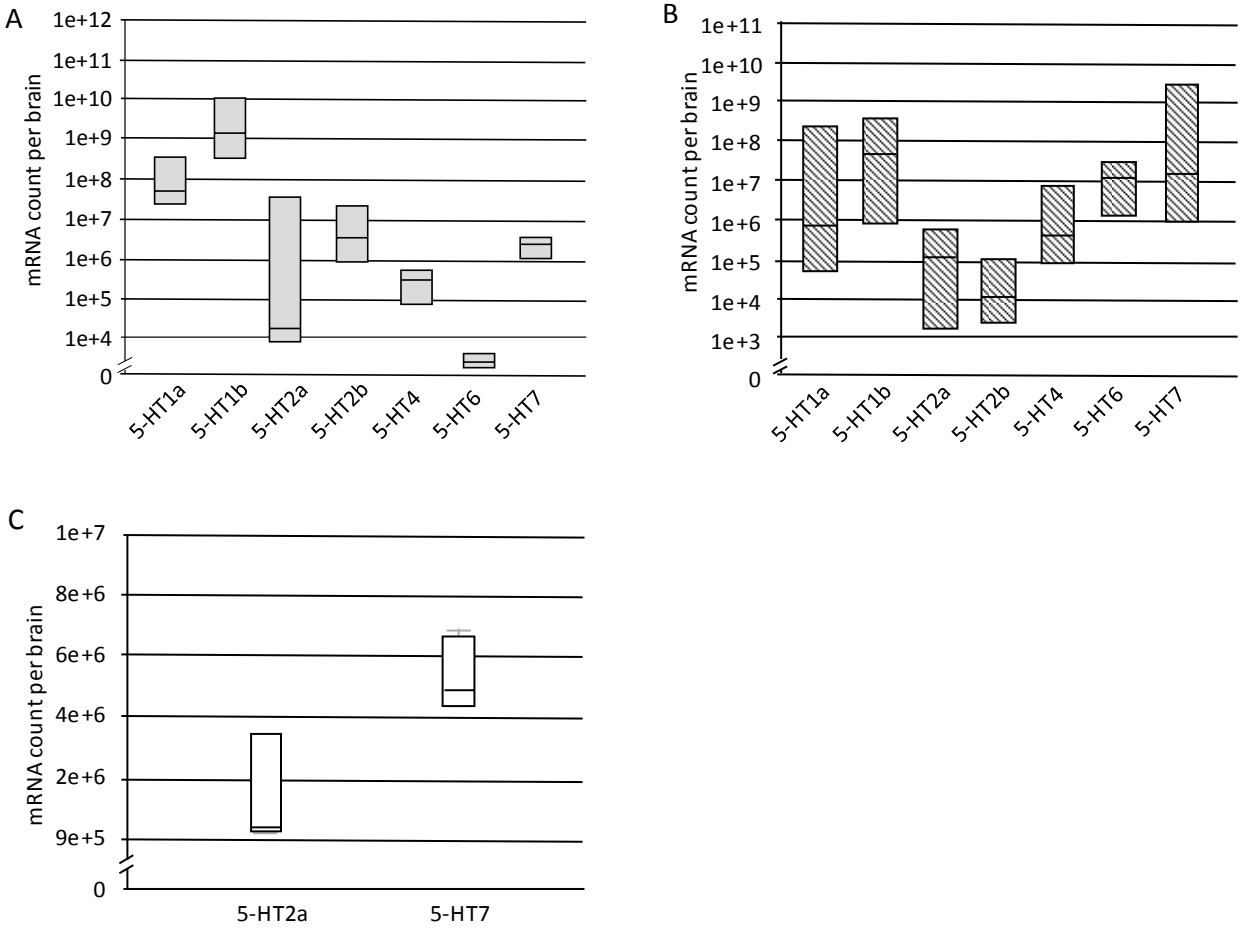

1000
800
600
400
200
0
mRNA copy number
5HT1a
5HT1b
5HT2a
5HT2b
5HT4
5HT6
5HT7

Figure 5


A
1e+12
1e+11
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
0
mRNA count per brain
5-HT1a
5-HT1b
5-HT2a
5-HT2b
5-HT4
5-HT6
5-HT7
B
1e+11
1e+10
1e+9
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
0
mRNA count per brain
5-HT1a
5-HT1b
5-HT2a
5-HT2b
5-HT4
5-HT6
5-HT7
C
1e+7
8e+6
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4e+6
2e+6
9e+5
0
mRNA count per brain
5-HT2a
5-HT7

Figure 6

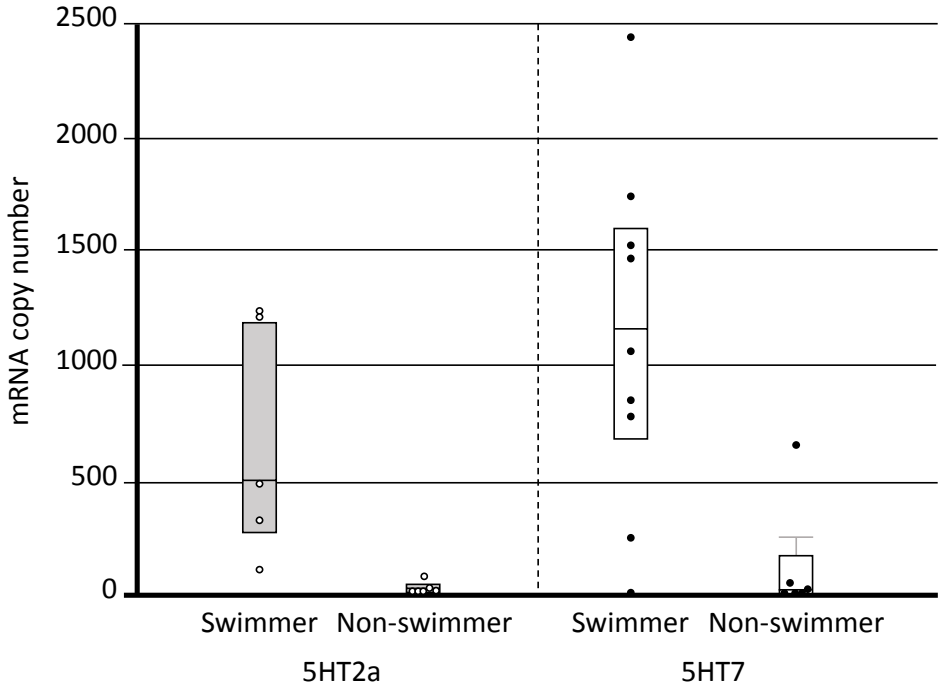
2500
2000
1500
1000
500
0
mRNA copy number
Swimmer
Non-swimmer
Swimmer
Non-swimmer
5HT2a
5HT7

Table 2

Gene	***Tritonia***	***Hermissenda***
5-HT1a	TRINITY_DN44848_c3_g1_i3	TRINITY_DN63691_c1_g1_i1
5-HT1b	TRINITY_DN48323_c0_g1_i1	TRINITY_DN103990_c0_g1_i1
5-HT2a	TRINITY_DN61866_c0_g1_i1	not identified
5-HT2b	TRINITY_DN24124_c0_g1_i1	TRINITY_DN122702_c0_g1_i1
5-HT4	not identified	TRINITY_DN86843_c0_g1_i1
5-HT6	not identified	TRINITY_DN57740_c0_g1_i1
5-HT7	TRINITY_DN1861_c0_g1_i1	not identified
SCP	TRINITY_DN42320_c1_g1_i2	TRINITY_DN79728_c0_g1_i1

Pleurobranchaea **(swim)**	***Pleruobranchaea*** **(non-swim)**
not identified	not identified
TRINITY_DN12981_c0_g1_i1	not identified
TRINITY_DN7433_c0_g2_i1	not identified
not identified	not identified
not identified	not identified
not identified	not identified
TRINITY_DN6728_c0_g1_i1	not identified
TRINITY_DN3137_c0_g1_i1	TRINITY_DN4239_c0_g1_i1

Table 3

	Tritonia C2	*Hermissenda* C2
Octopamine Receptor 1	TRINITY_DN282_c0_g3_i1	TRINITY_DN80161_c8_g1_i2
Octopamine Receptor 2	TRINITY_DN46207_c10_g1_i1	TRINITY_DN61974_c0_g1_i1
Adrenergic Receptor	TRINITY_DN28513_c0_g1_i1	TRINITY_DN68537_c0_g2_i1
mAChR	TRINITY_DN44275_c2_g1_i1	TRINITY_DN81430_c5_g1_i1
Histamine H1 Receptor	TRINITY_DN39405_c0_g1_i1	TRINITY_DN81430_c5_g1_i1
Neuropeptide Y receptor	TRINITY_DN46118_c9_g2_i3	TRINITY_DN73680_c1_g3_i2
Prolactin releasing receptor	TRINITY_DN45282_c6_g3_i1	TRINITY_DN77362_c1_g2_i1
Somatostatin Receptor	TRINITY_DN19522_c0_g1_i1	TRINITY_DN82721_c1_g1_i4
Orexin Receptor 1	not identified	TRINITY_DN76532_c0_g1_i1
Orexin Receptor 2	not identified	TRINITY_DN45196_c1_g1_i1
GPCR83	not identified	TRINITY_DN61803_c0_g1_i1
GPCR161	TRINITY_DN42626_c1_g1_i2	not identified
Cardioacceleratory Peptide Receptor	not identified	TRINITY_DN79207_c0_g1_i1
Neuromedin U Receptor	TRINITY_DN69884_c0_g1_i1	TRINITY_DN73225_c0_g2_i1

***Pleurobranchaea* (S) C2**	***Pleurobranchaea* (NS) C2**
not identified	not identified
TRINITY_DN32740_c0_g1_i1	not identified
not identified	TRINITY_DN16230_c0_g1_i1
not identified	not identified
not identified	not identified
not identified	not identified
not identified	not identified
not identified	not identified
TRINITY_DN2724_c1_g1_i1	TRINITY_DN2748_c0_g1_i1
not identified	not identified
not identified	not identified
TRINITY_DN36378_c0_g1_i1	not identified
not identified	not identified
not identified	not identified