Serotonin differentially modulates Ca++ transients and depolarization in a *C. elegans* nociceptor

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ABSTRACT

Monoamines and neuropeptides modulate neuronal excitability and synaptic strengths, shaping circuit activity to optimize behavioral output. In *C. elegans*, a pair of bipolar polymodal nociceptors, the ASHs, sense 1-octanol to initiate escape responses. In the present study, 1-octanol stimulated large increases in ASH Ca++, mediated by L-type voltage-gated Ca++ channels (VGCCs) in the cell soma and L- plus P/Q-type VGCCs in the axon, which were further amplified by Ca++ released from intracellular stores. Importantly, 1-octanol-dependent aversive responses were not inhibited by reducing ASH L-VGCC activity genetically or pharmacologically. Serotonin (5-HT), an enhancer of 1-octanol avoidance, potentiated 1-octanol-dependent ASH depolarization measured electrophysiologically, but surprisingly, decreased the ASH somal Ca++ transients. These results suggest that ASH somal Ca++ transient amplitudes may not always be predictive of neuronal depolarization and synaptic output. Therefore, although increases in steady state Ca++ can reliably indicate when neurons become active, quantitative relationships between Ca++ transient amplitudes and neuronal activity may not be as straightforward as previously anticipated.
INTRODUCTION

The potential outputs of a neural circuit are specified by the chemical and electrical synapses that interconnect the neurons. However, the actual output is largely determined by the concentrations of neuromodulatory monoamines and neuropeptides present at a given moment, which can drastically reconfigure circuits by modulating intrinsic cell excitability and synaptic strengths (Briggman and Kristan 2008; Komuniecki et al. 2014; Marder and Bucher 2007). Therefore to understand animal behavior in terms of individual neurons, we need both an anatomical map of their neuronal connectivity and an understanding of the prevalent neuromodulatory states and their functional consequences (Bargmann 2012; Bargmann and Marder 2013). To achieve this goal, it is necessary to quantitatively record neuronal activity states during circuit operation, compare activity patterns under different modulatory conditions, and relate the observed activity patterns to overall behavior. The complexity of this task grows exponentially as nervous system size increases. Not surprisingly, the most detailed insights thus far have come from studies of small discrete circuits with well-defined outputs such as the crustacean somatogastric ganglion. These circuits can orchestrate multiple distinct patterns of muscle contraction depending on which monoamines and neuropeptides are present, to select behaviorally-appropriate rhythmic outputs (Blitz and Nusbaum 2011; Marder and Bucher 2007).

Similarly, neuromodulators can reconfigure more complex mammalian circuits to modulate sensory function and behavior (Gleason 2012; Hermans et al. 2011; Pena-Ortega 2012), suggesting neuromodulatory strategies are highly conserved.

An important current goal in neuroscience is to extend these studies to the whole animal, to explain complex behaviors in terms of neuronal activity patterns and understand
how monoamines and neuropeptides alter behavioral states. The *C. elegans* hermaphrodite provides a useful model system, with only 302 neurons that are interconnected by about 7000 chemical and about 1000 electrical synapses, fully mapped in a wiring diagram based on serial-section electron microscopy (White et al. 1986). Importantly, *C. elegans* is transparent, so nervous system-wide neuronal activity patterns can be recorded from immobilized (glued to a substrate or held in microfluidics devices), or freely moving worms using Ca++ imaging techniques. These approaches have been very fruitful in elucidating the circuitry that controls sensory function and locomotion, and have begun to unravel the mechanisms by which neuromodulators control behavior (Chalasani et al. 2007; Ezcurra et al. 2011; Hilliard et al. 2005; Macosko et al. 2009; Piggott et al. 2011; Suzuki et al. 2008). Ca++ imaging has the advantage of being completely non-invasive, and may be used to monitor many neurons simultaneously, and in *C. elegans*, perhaps even the entire nervous system. (Chen et al. 2011; Grienberger and Konnerth 2012; Schrodel et al. 2013; Seelig and Jayaraman 2013). However, Ca++ influx is an indirect measure of neuronal activity, since the Ca++ enters the cytoplasm through VGCCs that become activated upon depolarization. Ca++ influxes may underestimate depolarization where VGCCs are relatively scarce or become downregulated through intracellular signaling cascades (Budde et al. 2002). Conversely Ca++ influxes may overestimate depolarization if the VGCCs are coupled to inositol tris-phosphate receptors (IP$_3$Rs) or ryanodine receptors (RYRs), which can amplify Ca++ signals by Ca++-induced Ca++ release (CICR) from internal stores (Busch et al. 2012; Goto and Mikoshiba 2011; Kato et al. 2012; Simpson et al. 1995). Importantly, *C. elegans* neurons are isopotential and do not fire action potentials (Goodman et al. 1998), so membrane depolarization can spread the length of the neuron independently of voltage-
gated channels. For these reasons, care must be taken to properly interpret changes in Ca++
dynamics caused by monoamines and neuropeptides because they can potentially reflect
direct modulation of the VGCCs or their coupling to IP$_3$Rs or RYRs rather than altered
neuronal excitation *per se.*

We are studying neuromodulation in an aversive olfactory circuit mediated by the *C. elegans*
nociceptive ASH sensory neurons, focusing particularly on the functional
significance of ASH Ca++ dynamics. The ciliated endings of the ASHs sense the noxious
odorant 1-octanol through the amphid opening at the tip of the nose, and stimulate
backwards locomotion escape behavior. This response is modulated at multiple levels in
the sensorimotor circuitry by 5-HT, octopamine (OA), tyramine (TA), dopamine (DA), and
several neuropeptides (Chao et al. 2004; Ezak and Ferkey 2010; Hapiak et al. 2013; Mills et
al. 2012; Wragg et al. 2007). Worms off food reverse around 10s after exposure to 30% 1-
octanol, but reverse in around 5s when pre-incubated on 5-HT containing plates (Chao et
al. 2004; Harris et al. 2009). 5-HT acts through three distinct receptors to potentiate
aversive behavior, including SER-5 acting in the ASHs (Harris et al. 2011; Harris et al. 2010;
Harris et al. 2009). 1-octanol induces large Ca++ transients in the soma of the ASHs (Mills et
al. 2012). Here, we dissected the Ca++ signal genetically and pharmacologically, and
compared it to behavioral responses and ASH depolarization measured directly. The
magnitude of the ASH somal Ca++ transient did not predict the depolarization strength or
synaptic output as inferred from the initiation of aversive responses, and in fact, ASH somal
Ca++ transients varied inversely with depolarization strength during 5-HT modulation.
These results highlight the potential for non-linearity in the relationship between Ca++
signal amplitude and neuronal depolarization strength.
RESULTS

Distinct ASH Ca++ pools mediate ASH-driven aversive responses

The two ASH nociceptive neurons are necessary and sufficient for aversive responses to dilute 1-octanol; i.e. laser ablation of the ASHs or the ASH-selective RNAi knockdown of the EAT-4 vesicular glutamate transporter, essential for glutamatergic signaling, abolish the aversive response (Chao et al. 2004; Harris et al. 2010). Transgenic animals expressing the Ca++ reporter GCaMP3 under the control of the ASH-selective sra-6 promoter (P_{sra-6}:GCaMP3) display large increases in ASH Ca++ when exposed to buffer saturated with 1-octanol (2.4 µM) (Mills et al. 2012), making them a useful strain to study the modulation of 1-octanol-dependent aversive behavior. To begin probing the significance of these increases in ASH Ca++, we identified the underlying Ca++ channels using genetic and pharmacological approaches. 1-octanol-dependent increases in ASH Ca++ were observed in the soma, dendrite, and axon (Fig. 1A). Somal signals were strongly reduced in the presence of nemadipine-A, a specific inhibitor of the C. elegans EGL-19 L-type VGCC (Kwok et al. 2006), and in egl-19 loss-of-function mutants, but unaffected in mutants lacking the UNC-2 P/Q-type VGCC, indicating that L-type VGCCs predominate in the soma (Fig 1A, B; dendritic signals behaved the same as somal signals). Axonal signals were much less sensitive to nemadipine-A, significantly reduced by loss of UNC-2, and nearly abolished by nemadipine-A plus unc-2 mutation, suggesting both channels are important in axons (Fig. 1A, C).

Ca++ entering the cytoplasm from outside the cell can stimulate Ca++ release from internal stores through the inositol tris-phosphate receptor (IP_3R, encoded by itr-1) and...
the ryanodine receptor (RYR, encoded by unc-68) (Baker et al. 2013). This Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) appears to operate in both the soma and axon of ASHs, dependent on IP\textsubscript{3}Rs and RYRs in the soma, but only IP\textsubscript{3}Rs in the axon (Fig. 2). For example, loss of either channel reduced 1-octanol-dependent increases in ASH peak Ca\textsuperscript{2+} fluorescence by roughly half in the soma (Fig. 2A, B), suggesting that much of the observed Ca\textsuperscript{2+} signal was not the direct result of voltage-gated processes. Loss of IP\textsubscript{3}Rs also altered the kinetics of the 1-octanol-induced Ca\textsuperscript{2+} signal, increasing the rise time (Fig. 2A, E) and changing the decay time course. In wild-type animals, the Ca\textsuperscript{2+} signal peaked and began to desensitize within 10s, while 1-octanol was still present, then continued decreasing at the same rate after 1-octanol withdrawal, indicating the ASH somal Ca\textsuperscript{2+} pool was no longer detectably responsive to the presence or absence of stimulus. In contrast, in IP\textsubscript{3}R mutants, the Ca\textsuperscript{2+} signal desensitized more slowly, and responded to 1-octanol withdrawal with an accelerated return to baseline (Fig. 2A, F). Together, these results suggest IP\textsubscript{3}Rs first amplify the somal Ca\textsuperscript{2+} signal initiated by depolarization/EGL-19 VGCC activation, then release additional Ca\textsuperscript{2+} driven by positive feedback and eventually desensitize due to high cytoplasmic Ca\textsuperscript{2+} levels (Keizer et al. 1995), resulting in a persistent somal Ca\textsuperscript{2+} pool that eventually decays independent of stimulus withdrawal. Similar trends are observed in axons (comparing IP\textsubscript{3}R mutants to wild type), with decreased amplitudes (Fig 2C, D) increased rise times (Fig. 2G), and decreased desensitization rates (Fig. 2H). In contrast, loss of RYRs had no effect on Ca\textsuperscript{2+} kinetics in either the somas or axons (Fig. 2A, C, E-H). In summary, 1-octanol depolarizes ASH neurons at the amphid (Roayaie et al. 1998), activating L-type VGCCs in the soma and a combination of L- and P/Q-type VGCCs in the axon, and stimulates significant Ca\textsuperscript{2+} release from internal stores through IP\textsubscript{3}Rs and RYRs;
amplification of Ca++ signals in ASHs by IP₃Rs adds a layer of kinetic complexity that obscures the relationship between ASH stimulation and optically-measurable Ca++ signals. Surprisingly, the ASH RNAi knockdown of EGL-19 L-type VGCC had no effect on ASH-mediated aversive responses to 30% 1-octanol off food, with wild type and transgenic animals initiating backward locomotion in about 10 s after 1-octanol exposure, even though ASH EGL-19 RNAi dramatically reduced 1-octanol dependent ASH somal Ca²⁺ transients (Fig. 3). These data strongly suggest that large increases in ASH somal Ca²⁺ are not required for ASH-mediated aversive responses to 1-octanol off food.

5-HT decreases 1-octanol-dependent ASH somal Ca²⁺ transients, but increases ASH depolarization

Food and 5-HT significantly decrease the time taken to initiate backward locomotion in response to dilute 1-octanol, i.e. they increase aversive responses, through a complex extrasynaptic modulatory circuit involving three distinct 5-HT receptors, including the Gαᵣ-coupled SER-5 in the ASHs (Harris et al. 2009). Therefore, we examined the role of 5-HT on 1-octanol-dependent Ca²⁺ dynamics. To confirm that ASH expression of Psra-6::GCaMP3 did not perturb 1-octanol avoidance, we showed 5-HT still potentiated 1-octanol aversive responses off food (from about 10 s to about 5 s) in the Psra-6::GCaMP3 transgenics (Fig. 4A); these responses did not differ significantly from wild-type (Fig. 4F, P>0.05). Surprisingly, although 5-HT increased aversive responses, it dramatically inhibited 1-octanol-dependent increases in ASH somal Ca²⁺ (Fig. 4B, C). As predicted (Harris et al. 2009), both SER-5 and EGL-30 Gαᵣ were required for the 5-HT inhibition of the 1-octanol-dependent somal Ca²⁺ signal (Fig. 4C). Interestingly, axonal Ca²⁺ signals were not
significantly reduced by 5-HT treatment (Fig. 4D), reinforcing the earlier observation that the Ca** pools in the ASH soma and axon are independent. To test whether this 5-HT effect was general or 1-octanol-specific, we compared responses to 1 mM dihydrocaffeic acid (DHCA), another ASH-sensed soluble ligand (Aoki et al. 2011; Kato et al. 2014), and found a similar 5-HT-dependent decrease in ASH somal Ca** (Fig. 4E). This result suggests that the 5-HT sensitization of ASH-mediated aversive responses occurs, at least in part, through decreased, not increased ASH somal Ca** transients. In animals with the EGL-19 L-VGCC knocked down in the ASHs using RNAi, basal aversive responses were wild type, but 5-HT failed to potentiate, and even slightly inhibited, aversive responses (Fig. 4F). To eliminate the possibility that the ASI neurons were the relevant site of EGL-19 L-VGCC knockdown (i.e. Psra-6 is active in ASHs and ASIs), we performed a control using the srb-6 promoter (active in ASHs, ADLs, PHAs and PHBs, but not ASIs), and observed the same result. The abolition of 5-HT modulation by EGL-19 L-VGCC knockdown reinforces a role for ASH somal Ca** transients in modulation. That the decrease in ASH Ca** transients in ASH-selective EGL-19 RNAi knockdown animals was not sufficient to potentiate aversive responses in the absence of 5-HT (Fig. 3B, 4F) suggests that other aspects of 5-HT signaling are also required for the more rapid initiation of aversive responses.

To better understand the relationship between the 1-octanol-dependent ASH somal Ca** signal and ASH depolarization, we turned to electrophysiology. It was necessary to modify standard C. elegans neuronal patch clamping protocols by adding a second flow pipette to deflect the stream of 1-octanol-saturated external solution away from the exposed neuron during recordings, since long chain alcohols can directly partition into cell membranes and affect ion channel function (Fig. 5A, Materials and Methods). Using this
system, we measured robust 1-octanol-evoked depolarizations that developed slowly (relative to touch responses (Geffeney et al. 2011)) and returned to baseline once 1-octanol was removed (Fig. 5B). These depolarizations, measured at the cell soma, are likely to reflect membrane potential changes throughout the ASH neuron, due to the presumed isopotentiality of *C. elegans* neurons (Goodman et al. 1998). Surprisingly, pre-exposure of the animals to 5-HT (using the same protocol as in behavioral and Ca**⁺** imaging experiments) led to a significant potentiation of 5-HT-evoked depolarization amplitudes (Fig. 5B, C), even though 5-HT appeared to decrease 1-octanol-dependent increases in somal Ca**⁺** (Fig. 4B, C). To ensure that dissection and exposure of ASHs to the bath solution did not disrupt 5-HT signaling, we also measured Ca**⁺** transients in dissected ASHs that were not further processed for patch-clamp recording. As observed in intact animals, 5-HT pre-treatment also decreased 1-octanol-evoked Ca**⁺** signals in the somas of these dissected neurons (Fig. 5D). Together, these data demonstrate that 5-HT pre-exposure reduces the 1-octanol-dependent increase in ASH somal Ca**⁺**, but increases ASH depolarization; i.e. the somal Ca**⁺** transient varies inversely with depolarization. Therefore, although increases in steady state Ca**⁺** can reliably indicate when neurons become active, the quantitative relationship between steady state Ca**⁺** and neuronal activity may not be as straightforward as previously anticipated.

DISCUSSION

The two ASH sensory neurons are necessary and sufficient for aversive responses to dilute 1-octanol. In the present study, we have recorded 1-octanol-dependent ASH somal and axonal calcium transients, using the genetically-encoded Ca**⁺** indicator GCaMP3, and ASH
depolarization by direct electrophysiological measurement, and shown that 1) the amplitude of the ASH somal Ca\(^{++}\) transient does not accurately predict the strength of aversive response off food, as ASH-selective RNAi knockdown of EGL-19 dramatically decreases the 1-octanol-dependent ASH somal Ca\(^{++}\) signal, but has no effect on aversive responses (Fig. 3); and 2) 5-HT pretreatment potentiates aversive responses and increases 1-octanol-dependent ASH depolarization, but dramatically depresses 1-octanol-dependent Ca\(^{++}\) transients in the ASH soma (Fig. 4C, Fig. 5). These data show that the magnitude of the ASH somal Ca\(^{++}\) transient does not necessarily predict the depolarization strength or synaptic output, and suggest that receptor potentials generated at the amphid may be sufficient to activate VGCCs and synaptic vesicle release in the axons. Consistent with this interpretation, input resistances of C. elegans neurons are very high (Goodman et al. 1998), so for a neuron the size of ASH, depolarization can spread passively from distal dendrite to axon with little loss of strength; however, this does not exclude the possibility that small rises in Ca\(^{++}\) are required to initiate the behavior. In contrast, ASH somal Ca\(^{++}\) clearly plays a role in 5-HT modulation, since ASH-selective EGL-19 RNAi knockdown prevents the 5-HT potentiation of aversive behavior (Fig. 3). However, while the depression of ASH Ca\(^{++}\) influx is probably necessary, it is not sufficient for 5-HT modulation, as the ASH RNAi knockdown of EGL-19 does not stimulate aversive responses in and of itself (Fig. 3). These observations raise two questions: 1) how does decreasing 1-octanol-dependent ASH somal Ca\(^{++}\) signal increase ASH depolarization and 2) what additional aspects of serotonergic signaling are required for the 5-HT sensitization of aversive responses? A Ca\(^{++}\)-dependent negative feedback loop controlling ASH excitability would provide a parsimonious explanation for how reduced Ca\(^{++}\) influx could lead to increased
depolarization amplitude (Fig. 6). 1-octanol, activating an as-yet-unidentified sensory transduction cascade, causes depolarization of the ASH plasma membrane (based on other ASH stimuli, this cascade may involve the ODR-3 G protein, and polyunsaturated fatty acid (PUFA) signaling to activate the cationic OSM-9/OCR-2 TRP channels (Bargmann 2006; Colbert et al. 1997; Kahn-Kirby et al. 2004; Roayaie et al. 1998)). The depolarization spreads passively with little attenuation through the soma to the axon, where it activates VGCCs in the soma (EGL-19 L-type VGCCs), and the axon (EGL-19 plus UNC-2 P/Q-type VGCCs). Ca++ influx then stimulates Ca++ release from internal stores through IP₃Rs and RYRs, thus amplifying and shaping the kinetics of the Ca++ transient (Fig. 2). This Ca++ pool could then activate a number of different hyperpolarizing conductances, including the SLO-1 Ca++-activated K⁺ channel, or the anoctamin or bestrophin Cl⁻ channels (Francois et al. 2012; Hogan 2007; Jeon et al. 2013; Liu et al. 2013), to potentially brake the 1-octanol-induced depolarization. Direct inhibition of the Ca++ influx by 5-HT through SER-5 and Gα₉ could disinhibit ASHs by reducing negative feedback, and in fact, Gα₉-dependent pathways for inhibition of neuronal L-type VGCCs have been described in mammals (Olson et al. 2005).

Besides reducing ASH somal Ca++ transients and potentiating ASH depolarization, 5-HT activates at least two other pathways relevant to potentiation of 1-octanol aversive behavior. First, Gα₉ signaling (i.e. downstream of SER-5) regulates synaptic vesicle fusion at C. elegans neuromuscular junctions (Miller et al. 1999; Perez-Mansilla and Nurrish 2009), and potentially also in ASHs (Esposito et al. 2010). Second, 5-HT potentiation of 1-octanol avoidance requires neuropeptide release from ASHs, specifically NLP-3-encoded neuropeptides (Harris et al. 2010). Maximal 5-HT potentiation of aversive responses may
require all of these pathways simultaneously, whereas the ASH-selective EGL-19 RNAi knockdown may only activate one pathway (i.e. increased ASH excitability) while failing to activate (or even inhibiting) others. Finally, the potential role of intrinsic homeostatic adaptation in the ASHs to maintain normal electrical excitability must be considered. In many types of neurons, loss of one inhibitory conductance is compensated for by upregulation of another (Bergquist et al. 2010; Misonou 2010; Nerbonne et al. 2008; Turrigiano 2011). For example, the $I_{\text{A}}$ K$^+$ conductance is upregulated after loss of $I_{\text{KGa}}$ Ca$^{++}$-activated K$^+$ conductance following blockade of L-type VGCCs with Cd$^{++}$ in the cardiac ganglion of the crab *Cancer borealis* (Ransdell et al. 2012). By analogy, the replacement of the SLO-1 Ca$^{++}$-activated K$^+$ conductance by a Ca$^{++}$-insensitive K$^+$ conductance (such as the $I_{\text{A}}$ K$^+$ channel KVS-1) in response to ASH-selective EGL-19 RNAi knockdown could explain both the failure to observe fast (5s) basal 1-octanol responses, and the loss of 5-HT potentiation in the Ca$^{++}$-dependent negative feedback model described above.

The relationship between 5-HT signaling, Ca$^{++}$ influx and electrical excitability in ASH nociceptors bears a striking similarity to inflammation-induced changes in mammalian somatic nociceptors associated with chronic pain. At sites of injury, the ‘inflammatory soup’ of signaling molecules, including 5-HT, initiates signaling cascades that chronically hypersensitize nociceptors (Basbaum et al. 2009; Reichling et al. 2013). In several rodent models of nerve injury and inflammation, hyperalgesia is observed coincident with nociceptor hyperexcitability, reduced Ca$^{++}$ influxes through N- and L-type VGCCs, and a loss of Ca$^{++}$-activated K$^+$ conductances in dorsal root ganglion neurons (Hendrich et al. 2012; Hogan 2007; McCallum et al. 2011; Zhang et al. 2012). However to definitively show that these events are causally related, it will be necessary to demonstrate
that inflammatory signaling leads to increased excitability by decreasing Ca++ influx and reducing Ca++-activated K+ currents, all within a single cell, which is difficult in rodents because each dorsal root ganglion contains thousands of sensory neuron cell bodies representing multiple nociceptive and non-nociceptive classes (Hogan 2010), and absolute cell identities cannot be assigned between animals. The *C. elegans* system importantly complements the mammalian model, because its neuroanatomy is stereotyped and relatively simple. With only two ASH neurons (left and right), we can be certain that correlated changes in multiple parameters are taking place within the same cells. Future studies of this conserved nociceptive signaling pathway in *C. elegans* should provide important insights into the initiation and progression of chronic pain in humans.

Our results add to a growing body of observations highlighting the diversity of roles for somal Ca++ in the ASH nociceptors. Several studies support the conventional view, that Ca++ transient amplitudes are predictive of the strength of ASH output. First, six out of six ASH-sensed noxious stimuli produced ASH somal Ca++ transients, while non-noxious stimuli did not (Hilliard et al. 2005). In this study, the Ca++ signals were EGL-19 dependent, and Ca++ transients elicited by exposure to Cu++ ions (an ASH-sensed chemorepellent) were diminished by 5-HT treatment (Hilliard et al. 2005). Second, dopamine positively modulates Cu++-dependent avoidance and ASH Ca++ transients in parallel, through the DOP-4 receptor (Ezcurra et al. 2011). Third, ASH-mediated avoidance of high pH shows strong correlation between stimulus intensity, ASH Ca++ signal peak amplitudes, and induction of avoidance behavior (Sassa et al. 2013), and fourth, *egl-4* mutation affects ASH Ca++ signal intensity and avoidance behavior in parallel for the ASH-sensed repellent quinine (Krzyzanowski et al. 2013). Other studies, however, reveal alternative roles for ASH somal
Ca++. For example, loss of RGS-3 proteins that dampen G-protein signaling abolish aversive responses to 100% 1-octanol, but sensitivity is restored when Ca++ buffering proteins are expressed in ASHs, implying that excessive ASH Ca++ levels inhibit activation (Ferkey et al. 2007). Additionally, loss of function egl-19 mutation does not affect aversive responses to high osmotic strength glycerol despite reducing ASH Ca++ transient peak amplitude, but does result in a failure to adapt (i.e. downregulate) during repeated stimulation (Kato et al. 2014). Similarly, a point mutation in the OSM-9 TRPV channel subunit reduces somal ASH Ca++ transients below the level of detectability by GCaMP3, but does not prevent osmotic avoidance (Lindy et al. 2014). Interestingly, these animals also showed defects in adaptation and modulation of osmotic avoidance by monoamines (Lindy et al. 2014). These studies support our conclusion that amplitudes of the somal Ca++ transients in ASHs are not always predictive of response strength, and confirm our observation that ASH somal Ca++ transients are involved in response plasticity.

These findings have important implications for neural circuit analysis. Circuits in both vertebrates and invertebrates switch among several different ‘output states’, dependent on the intrinsic excitability of the neurons and the synaptic strengths of their connections, subject to modulation by neuropeptides and monoamines (Bargmann 2012; Bargmann and Marder 2013; Briggman and Kristan 2008; Gleason 2012; Komuniecki et al. 2014; Pena-Ortega 2012). Therefore quantitative measures of neuronal excitation are crucial for understanding how neuromodulators select one circuit output pattern over another. In vertebrates, brain-wide Ca++ imaging will continue to be a powerful tool (Chen et al. 2013), and even though vertebrate neurons use all-or-none action potentials, Ca++ transient amplitudes still provide important information about action potential frequency.
and sub-threshold excitation events (Chen et al. 2013; Glickfeld et al. 2013; Sasaki et al. 2008; Vogelstein et al. 2009). Accounting for modulation of Ca++ entry dynamics by neuromodulatory signaling cascades will be important for the correct interpretation of these data sets. In *C. elegans*, appreciating the relationship between depolarization and Ca++ influx may be even more important, because neurons signal through graded potentials, whose amplitudes probably encode analog information about excitation strength. Ca++ signals are usually equated with membrane potential changes but, as we show here, somal Ca++ signals can become uncoupled from, or even vary inversely with depolarization amplitude and behavioral output. Consistent with our observations, an independent study showed Ca++ signals can vary inversely with synaptic vesicle release (measured optically using a synaptobrevin-pHluorin fusion reporter in *C. elegans* salt sensing neurons (Oda et al. 2011)). So while Ca++ influxes can reliably indicate when neurons become active, they are potentially error prone for reporting the degree of activation, a critical parameter for comparing circuit configurations under different modulatory conditions. This potential non-linearity in the input-output relationships in Ca++ imaging approaches may be a significant source of error, and direct measurement of depolarization amplitude and/or neurotransmitter release should be performed in parallel to confirm Ca++ imaging results where possible.
MATERIALS AND METHODS

Strains and constructs. Strains were maintained on NGM agar plates seeded with OP50 bacteria according to standard protocols. The following strains were used: N2, CX10979 kyEx2865[Pssa-6::GCaMP3], FY907 grls17[Pssa-6::GCaMP3], FY867 ser-5(tm2654) I; kyEx2865[Pssa-6::GCaMP3], FY882 itr-1(sa73) IV; grls17[Pssa-6::GCaMP3], FY933 egl-19(n582) IV; grls17[Pssa-6::GCaMP3], FY934 egl-30(n686sd) I; grls17[Pssa-6::GCaMP3], FY935 unc-68(e540) V; grls17[Pssa-6::GCaMP3]. Neuron-specific RNAi transgenes were created as previously described (Esposito et al. 2007), and co-injected with Punc-122::RFP at a concentration of 50ng/µl. Animals were analyzed within four generations of original injection. The primers used for Pssa-6::egl-19RNAi transgene construction were:

- sra-6F: 5’-CTTTTCATCTCGACCAGACGGTG-3’;
- sra-6F*: 5’-CAATGTCCACTGATGTACCTTTCTATC-3’;
- egl-19 Tf: 5’-GTTCGTGTGATGCGTCTCGTG-3’;
- egl-19 Tf*: 5’-GCGTCTCGTGAAGCTGCTTTC-3’
- egl-19 Tr: 5’-CTTGCCAGGCTTCTCCAGTTG-3’
- egl-19 Tr*: 5’-CAGGCTTCTCCAGTTGCTGATC-3’
- Psra-6::egl-19 P: 5’-
- Psra-6::egl-19 P: 5’-

Calcium imaging. Calcium imaging experiments were performed essentially as previously described (Mills et al. 2012). Animals were glued to 1.5 mm round coverslips-coated with Sylgard (Dow Corning, Midland, MI), immersed in electrophysiology external solution (see
below) using WormGlu cyanoacrylate glue (GluStitch, Delta, Canada). Coverslips were placed in a laminar flow chamber (Warner RC26G, Warner Instruments, Hamden CT) and perfused continuously with fresh external solution. Saturated 1-octanol solution (~2.37μM in electrophysiological external) or 1 mM DHCA was delivered under gravity feed through solenoid valves using a Perfusion Pencil (AutoMate Scientific, Berkeley, CA) or homemade equivalent. All odorant solutions also contained the fluorescent tracer sulforhodamine 101 (SR101, 1 μM), which stains animals on contact. After exposure, animals were visually examined for staining to confirm successful application. No response was observed in ASHs exposed to 1 μM SR101 alone; in the ASI neurons (which are not required for 1-octanol sensitivity, but express the Psra-6::GCaMP3 reporter transgene); and osm-9 mutants (lacking the TRP channel required downstream of ODR-3 in the ASH chemosensory signaling pathway (Colbert et al. 1997)), demonstrating specificity in the ASH 1-octanol responses (not shown). To control for the possibility that high Ca++ levels produced GCaMP3 saturation in these experiments that could obscure the kinetics of the off responses, we applied 150 mM K+-containing external solution to dissected ASHs expressing GCaMP3 to artificially depolarize them, obtaining ΔF/Fo values comparable to the 1-octanol-evoked responses. These transients activated and deactivated very quickly, relative to 1-octanol responses (not shown), suggesting that kinetic difference between 1-octanol responses of wild-type and itr-1 mutants were not artifacts of GCaMP3 saturation by relatively high ASH Ca++ levels in the wild type. Recordings were performed on an Axioskop 2 FS Plus upright compound microscope (40X Achroplan water immersion objective, GFP filter set #38), fitted with an Orca ER CCD camera (Hamamatsu, Skokie, IL) and an automated shutter (Uniblitz, Vincent Associates, Rochester, NY). Minimal
illumination intensity was used to minimize GCaMP3 photobleaching, and we did not observe differential photobleaching rates between different genotypes and treatment groups. 5-HT exposure was performed by incubating animals on NGM agar plates containing 4 mM 5-HT for 30 min before recording. Nemadipine-A exposure was performed by incubating animals on NGM agar plates containing 5µM nemadipine-A spread across the top of the plate for 30 min, and recordings were performed with nemadipine-A in the bath perfusion. 5-HT and nemadipine-A plates were prepared fresh each day. Animals had typically been removed from food for 10-20 minutes prior to recording, so were considered to be in the ‘off food’ state. Fluorescent images were acquired using MetaVue 7.6.5 (MDS Analytical Technologies, Sunnyvale, CA), and analyzed with Jmalyze software (Rex Kerr). Exposure times were 50ms with 4x binning. For quantifying soma fluorescence changes, square regions of interest (ROIs) were drawn centered on the starting position of the soma, corresponding to approximately 100x soma area. For axons, roughly equal size ROIs were chosen, excluding the soma and dendrite. We routinely compared baseline fluorescence values between mutant or drug treated worms and corresponding controls, and observed no significant differences.

**Electrophysiology.** For patch-clamp analyses, animals were glued and placed in the recording chamber as described above. ASH cell bodies (identified by GCaMP3 expression) were exposed for whole-cell recordings by slitting the cuticle with a glass patch pipette (TW150-3, World Precision Instruments, Sarasota, FL) that had been melted and drawn to a fine point on a Narishige MF-83 microforge (Narishige, Setagaya-ku, Tokyo, Japan), and mounted on a micromanipulator (Sutter MP285, Sutter Instruments, Novato, CA). Whole-cell recordings were performed using pressure-polished patch pipettes (15-30MΩ
resistance (Johnson et al. 2008)) Internal solution contained 15 mM KCl, 115 mM K gluconate, 10 mM HEPES, 5 mM MgCl₂, 0.25 mM CaCl₂, 5 mM EGTA, 20 mM sucrose, 5 mM MgATP, 0.25 mM NaGTP; pH 7.20, 315 mOsm. External solution contained 150 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 15 mM HEPES; pH 7.30, 327-333 mOsm.

1-octanol solution was delivered using the Perfusion Pencil as described above, mounted on a Warner SF77B Perfusion Fast Step device (step size 100µm; Warner Instruments) to permit precise computer control of pipette position. A second patch pipette (20-30 µm opening, driven by a syringe pump; KD Scientific, Holliston, MA) was placed in the bath to provide a shielding stream of external solution that could be used to precisely steer the odorant stream. (See Figure 5). The shielding stream diverted the 1-octanol solution away from the animal’s nose prior to and after application, and prevented 1-octanol from contacting the exposed ASH neuron during application, thus ensuring precise timing, and preventing direct contact of the odorant solution with the exposed cell body. After termination, the animal was inspected and any sample with visible SR101 staining of the exposed neuron due to faulty laminar flow or pipette positioning error was discarded.

Signals were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale CA) in current clamp mode (0 pA injected current; 10 kHz sampling, 2 kHz filtering), digitized with a Digidata 1440A digitizer, and analyzed using pCLAMP 10 software (Molecular Devices). 5-HT treatment was performed as described above.

**Behavioral assays.** Behavioral responses to 1-octanol were assayed as previously described (Chao et al. 2004; Harris et al. 2009). L4 animals were picked the night before assaying to fresh plates seeded with OP50. 5-HT-containing NGM plates were prepared 2 hours prior to assays by adding 4 mM 5-HT creatinine sulfate monohydrate to molten solid
media (~50°C) before pouring. A glass capillary or paintbrush hair was dipped in 30% 1-
-octanol (dissolved in 100% ethanol, v/v) and placed in front of a forward-moving animal.
For experiments in the absence of 5-HT, animals were transferred to an intermediate
(food-free) plate for one minute, and then transferred to assay plates and tested after 10
minutes. In contrast, for assays in the presence of 4mM 5-HT, worms were transferred to
an intermediate plate for one minute, but transferred to the 5-HT-containing plate (food-
free), and assayed after 30 minutes.

All reagents were obtained from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich
(St. Louis, MO). Statistical analysis was performed using unpaired, two-tailed Student’s t-
tests unless otherwise stated, using Prism software with data presented as mean ± SEM.

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**Figure 1. Ca++ channels mediating 1-octanol-evoked Ca++ influx into the ASH soma and axon**

A) 1-octanol stimulates Ca++ transients in the soma and axon of ASHs; NemA preferentially inhibited somal Ca++ signals. B) Loss or block of the L-type (EGL-19), but not the P/Q-type (UNC-2) Ca++ channels strongly decreased the somal Ca++ signal. C) Axonal Ca++ signals required both L-type and P/Q-type channels. * P<0.05 compared to wild type/untreated; †P<0.05 compared to unc-2 untreated. Numbers within/above bars indicate n.
Figure 2. Ca++ release from intracellular stores amplifies and shapes Ca++ dynamics in ASH neurons

A, C) Time courses of 1-octanol-evoked Ca++ transients in ASH somata (A) and axons (C) in wild type and IP3R mutants (left) or RYR mutants (right; *itr-1* and *unc-68* respectively). 1-octanol applied between 0 and 20s (grey box). B, D) Peak amplitudes of somal (B) and axonal (D) Ca++ transients. E-H) Kinetic analysis of somal (E, F) and axonal (G, H) Ca++ transients: (E, G) signal rise times, (F, H) decay slopes. Black bars in (F, H) indicate desensitization in the continued presence of 1-octanol, white bars indicate decay rate after 1-octanol removal (rates measured at the intervals denoted by the black and white boxes in panels A and C, respectively; n=13, 12, and 7 for wild type, *itr-1* and *unc-68*, respectively, in (A, F); n=6, 8, and 6 for wild type, *itr-1* and *unc-68*, respectively, in (C, H). *P<0.05 compared to corresponding wild type measurement; †P<0.05 compared to *itr-1* desensitization rate. Numbers within bars indicate n.
Figure 3. Aversive responses not affected by reduction of Ca\(^{++}\) influx through EGL-19 L-type VGCC

A) ASH-selective RNAi knockdown of the EGL-19 L-type channel (using the sra-6 promoter) did not alter 1-octanol response times \((P>0.05)\), even though Ca\(^{++}\) signals in the ASH soma in response to 1-octanol exposure \((B)\) were strongly reduced \(*P<0.05\) compared to wild type\).
Figure 4. 5-HT modulation of ASH chemosensory responses

A) Aversive behavioral responses to 1-octanol in Psra-6::GCaMP3 worms; basal responses and 5-HT stimulation were normal, indicating that GCaMP3 expression in ASHs does not interfere with ASH function or 5-HT modulation. B) 5-HT treatment reduced Ca++ signals in ASHs stimulated by 1-octanol (saturated aqueous solution); n=7 and 13 for untreated and +5-HT, respectively, dashed lines are SEM, grey box indicates 1-octanol exposure. C) 5-HT modulation of ASH Ca++ transients required the 5-HT receptor SER-5 and the Goq subunit EGL-30 (wt is wild type). D) Axonal Ca++ signals were not significantly reduced by 5-HT. E) ASH responses to 1mM DHCA were also reduced by 5-HT treatment. F) ASH-selective RNAi knockdown of EGL-19 prevented 5-HT potentiation of 1-octanol aversive responses; instead, 5-HT slightly inhibited them. Two different ASH-selective promoters were used, sra-6 and srb-6, as indicated in each bar (*P<0.05, **P<0.0001 compared to untreated, number in bar on graphs indicates n).
Figure 5. 5-HT potentiates ASH depolarization in response to 1-octanol

A) Diagram of recording setup. Left panel: arrangement of pipettes before exposure, as whole-cell recording is being established. At lower right, the recording pipette and protruding neuronal cell bodies. At upper left, flow pipette providing 1-octanol solution and a fluorescent dye to monitor flow (shaded plume), at lower left, flow pipette providing a stream of buffer to shield exposed neuron. Exposure is initiated by moving 1-octanol pipette to preset position closer to nose (middle panel), and terminated by returning 1-octanol pipette to original position (right panel). B) Representative traces of ASH depolarization in response to 1-octanol exposure in control (left) or 5-HT treated worms (right). C) 5-HT effect on ASH 1-octanol-induced depolarization (*P<0.0005). D) 1-octanol responses of ASH neurons dissected for electrophysiology, but analyzed by Ca++ imaging, showing that 5-HT signaling to reduce Ca++ signals remained intact through the dissection process (*P<0.05).
Figure 6. Model of a hypothetical Ca++-driven negative feedback loop controlling ASH excitability

The activation of the ASH sensory neuron by exposure to 1-octanol involves an as-yet-unidentified amphidial signal transduction cascade, resulting in a membrane depolarization ("ΔV"), which spreads down the ASH dendrite to the soma and axon, probably passively, given the apparent isopotentiality (Goodman et al. 1998) of *C. elegans* neurons (Goodman et al. 1998). The amphidial ASH signal transduction cascade activated by other stimuli includes the ODR-3 G protein, and polyunsaturated fatty acid (PUFA) signaling to activate the cationic OSM-9/OCR-2 TRP channels (Bargmann 2006; Colbert et al. 1997; Kahn-Kirby et al. 2004; Roayaie et al. 1998)), and these components are also probably involved in activation by 1-octanol. The stimulus-dependent ASH depolarization activates L-type VGCCs (EGL-19) in dendritic, somal, and axonal membranes, and P/Q-type VGCCs (UNC-2) at synaptic vesicle (SV) release sites in the axon, leading to glutamate release. Ca++ influx through EGL-19 leads to a rise in cytoplasmic Ca++ that is further amplified by the Ca++-dependent release of Ca++ from endogenous stores through both the RYR (UNC-68) and IP3R (ITR-1). This cytoplasmic Ca++ pool can potentially drive neuronal repolarization by activating Ca++-dependent inhibitory ion channels (Ca++-activated K+ channels, anoctamins, or bestrophins; dashed line). Importantly, ASH 5-HT signaling through the SER-5 receptor has the potential to regulate ASH excitability at many levels, including the modulation of ASH Ca++ dynamics.
A

Time to reverse (s)

wt 18 19
ASH::EGL-19 18 19
RNAi

B

ΔF/Φo (%)

wt 15 7
ASH::EGL-19 15 7
RNAi

*
1-octanol $\Delta V$ sensory ending

$\Delta V$ cell soma, dendrite, axon

$\Delta V$ axonal SV release sites

$\Delta V$ UNC-2 NT release

5-HT $\rightarrow$ Ca$^{++}$ L-VGCC EGL-19

CICR (IP$_3$R/RyR)