Electrical Synapses are Drivers of Neural Plasticity Through Passage of Small Molecules

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Abstract

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In order to respond to changing environments and fluctuations in internal states, animals adjust their behavior through diverse neuromodulatory mechanisms. In this study we show that electrical synapses between the ASH primary quinine-detecting sensory neurons and the neighboring ASK neurons are required for modulating the aversive response to the bitter tastant quinine in *C. elegans*. Mutant worms that lack the electrical synapse proteins INX-18 and INX-19 become hypersensitive to dilute quinine. Cell-specific rescue experiments indicate that *inx-18* operates in ASK while *inx-19* is required in both ASK and ASH for proper quinine sensitivity. Imaging analyses find that INX-19 in ASK and ASH localizes to the same regions in the nerve ring, suggesting that both sides of ASK-ASH electrical synapses contain INX-19. While *inx-18* and *inx-19* mutant animals have a similar behavioral phenotype, several lines of evidence suggest the proteins encoded by these genes play different roles in modulating the aversive quinine response. First, INX-18 and INX-19 localize

to different regions of the nerve ring, indicating that they are not present in the same synapses. Second, removing *inx-18* disrupts the distribution of INX-19, while removing *inx-19* does not alter INX-18 localization. Finally, by using a fluorescent cGMP reporter, we find that INX-18 and INX-19 have distinct roles in establishing cGMP levels in ASK and ASH. Together, these results demonstrate that electrical synapses containing INX-18 and INX-19 facilitate modulation of ASH nociceptive signaling. Our findings support the idea that a network of electrical synapses mediates cGMP exchange between neurons, enabling modulation of sensory responses and behavior.

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Introduction

1. Neuroplasticity

In order to navigate an ever-changing environment, organisms must adapt their behavior to best fit the present state. They do this by interrogating their memories of the past and using these, combined with current environmental and internal information, to predict the behaviors that will produce the most favorable outcome. This alteration of behavior happens at the level of the neural circuit where neural plasticity enables organisms to learn and adapt. Several broad categories of plasticity are known to alter neural activity, and this review will cover synaptic plasticity and neuromodulation. Synaptic plasticity is the process by which individual synapses are altered in strength. Neuromodulation affects large numbers of neurons and can affect diverse populations of neurons at once. Structural plasticity occurs when the architecture of a neural circuit is physically remodeled to add or remove connections. Together, these processes alter neural activity in

response to internal or external environmental changes, enabling the organism to change its behavior to suit its present needs.

Research was not always interested in the ways that circuits change and adapt over time; it was not until the 1970s and 1980s that synaptic plasticity and neuromodulation respectively became topics of study[1]. Despite its limited tenure as a research interest, much progress has been made in understanding the molecular mechanisms behind neural plasticity. Today we still don't fully understand how the changing patterns of activity of neural circuits contribute to behavioral adaptation and learning. However, we are beginning to understand how the different components of neural plasticity interact to create meaningful patterns of activity within the physical bounds of the connectome.

I will discuss what is known about the types and mechanisms and consequences of synaptic plasticity and neuromodulation. I will then explore how use of common molecules and regulatory overlap allow synaptic plasticity and neuromodulation to interact.

2. Synaptic Plasticity

Synaptic plasticity is a term that refers to several different types of modifications that occur at synapses, but in the most basic sense synaptic plasticity refers to the increase or decrease in efficacy of a particular synapse in response to prior activity. It has long been known that synapses can be potentiated. In the late 1960s Bliss and Lømo[2] first observed long term potentiation (LTP) when they saw spike potentiation following stimulation of the perforant path in the rabbit

hippocampus. Since then, the field of synaptic plasticity has greatly expanded to include a number of types of plasticity with a variety of mechanistic bases. Synaptic plasticity can last milliseconds to minutes, as in synaptic enhancement and depression, or minutes to hours or even longer, as in LTP and long-term depression (LTD)[3]. The mechanisms of synaptic plasticity vary, and can be presynaptic or postsynaptic.

2.1 Presynaptic Plasticity

2.1.1 Paired-Pulse Facilitation

When two stimuli reach the presynaptic terminal within quick succession, the second may provoke a larger response, a process known as paired-pulse facilitation (PPF). Facilitation of this type lasts for a few hundreds of milliseconds and is thought to be largely presynaptic in origin as facilitation is associated with an increase in the number of vesicles released and a greater spontaneous release probability without a corresponding increase in the postsynaptic response to a single vesicle fusion[4]. It was originally hypothesized that this short term enhancement was due to residual calcium ions in the presynaptic terminal leading to facilitation of synaptic vesicle release[5]. However, the amount of residual calcium from the first action potential would need to be considerable in order to achieve the enhancement of the second response[6], so another mechanism is likely. It is possible that the presynaptic terminal contains a high-affinity calcium binding protein with slow kinetics, such that even small amounts of residual calcium are registered and that signal is amplified over time through the actions of this protein[7]. Another

possibility is that a calcium binding protein such as calbindin in the presynaptic terminal acts as a buffer, reducing the response to the first stimulus[8]. The influx of calcium from the second action potential would then have a stronger effect as the calcium buffer is already saturated so greater amounts of calcium would be available to stimulate vesicle release. Lastly, calcium binding proteins such as calmodulin are known to impact the function of voltage gated calcium channels in the presynaptic bouton, so prior stimuli can cause facilitation by making future influx of calcium more likely[9]. This last model is explained in more detail below in the section on voltage gated calcium channel modulation.

2.1.2 Synaptic Depression

Paired stimuli don't always result in facilitation. At some synapses, the second of two action potentials will be lesser in magnitude, a property known as depression. The simplest explanation for this phenomenon is that the presynaptic bouton depletes the readily releasable pool of vesicles so fewer can be released upon the second stimulation. If this is model is true, the magnitude of depression will depend on the initial release probability and the size of the readily releasable pool of vesicles, a property that appears to be true in many cases[3]. Depression following tetanic stimulation may be more pronounced and longer lasting than paired-pulse depression, an observation that fits the depletion model as the actively recycling pool of vesicles may be depleted in addition to the readily releasable pool, meaning that the synapse must replenish from the slowly mobilized non-recyling reserve pool. While the depletion model is sufficient to explain presynaptic depression in many cases, particularly after rapid stimulation, another key factor at

play is the likelihood of release as dictated by calcium entry. This mechanism is discussed in more detail below.

2.1.3 Posttetanic Potentiation and Augmentation

Posttetanic potentiation (PTP) and augmentation occur after trains of stimuli and have longer time constants than facilitation or depression do. While PTP typically lasts longer than augmentation, they are thought to be mechanistically similar[10]. Long trains of action potentials cause substantial calcium build-up in the presynaptic terminal that mirrors the PTP time course as it returns to baseline, suggesting that residual calcium may be the mechanism for PTP and augmentation. There is evidence that mitochondrial uptake of calcium contributes to increase of residual calcium following tetanic stimulation, as blocking mitochondrial uptake of calcium blocks both the slow efflux of calcium from the presynaptic terminal and PTP[11,12]. In addition, calcium efflux after tetanic stimulation is likely slowed by the inefficiency of the Na^+/Ca^{2+} exchange pump. Following long trains of stimuli and the initial quick reduction in calcium levels due to the Na^+/Ca^{2+} pump, there is a build-up of sodium ions, leading the exchange pump to work poorly or even in reverse[12]. This inefficiency would contribute to a slow drop off of calcium during augmentation or PTP. However, at some synapses presynaptic calcium levels return to baseline much faster than PTP, suggesting that there must be alternative biochemical mechanisms. As in facilitation and depression, these mechanisms likely center on the presynaptic voltage gated calcium channels.

2.1.4 Regulation of Calcium Channels in Facilitation, Depression, and Potentiation

Given that calcium levels play a main role in determining the probability of vesicle release from the presynaptic terminal, altering the opening, closing, or inactivation kinetics of presynaptic voltage gated calcium channels will alter the activity of the synapse. If calcium channels are easier to open (ie, open threshold voltage is decreased), this will likely contribute to facilitation, augmentation, or PTP. Conversely, decreasing the voltage threshold or increasing inactivation will cause decreased presynaptic calcium, contributing to synaptic depression.

Numerous mechanisms can cause alterations to voltage gated calcium channels. In particular, the calcium binding protein calmodulin appears to play a role in some cases[13], in addition to related proteins calcium binding protein 1 (CaBP-1) and visin-like protein 2 (VILIP-2). All three proteins are able to directly bind to and regulate the activity of voltage gated calcium channels present in presynaptic terminals[10]. Calmodulin can impact either facilitation or inactivation of voltage gated calcium channels, depending on what (calcium-dependent) conformation it is in and therefore which part of the calcium channel it binds to[14]. The current model suggests that the binding of calcium to the rapid high-affinity binding site on the C-terminus of calmodulin contributes to facilitation, while the slower low-affinity site on the N-terminus contributes to depression. CaBP-1 inhibits voltage gated calcium channel activity by binding to the same inhibitory site that calmodulin binds to, though curiously this regulatory action of CaBP-1 does not appear to require calcium[15]. VILIP-2 also binds to voltage gated calcium channels but causes facilitation, perhaps by competing with calmodulin for its inhibitory binding site, thus blocking its inactivation effects[16].

The variation of effects of presynaptic calcium sensing proteins helps explain how different synapses may respond differently to the same repetitive stimulation. Local variation in gene expression might bias a particular synapse towards synaptic depression instead of facilitation, even if the synapse contains the same type of calcium channels and releases the same neurotransmitter. In addition, the multiple competing regulatory mechanisms help contribute to homeostatic plasticity, that is, changes in neuronal activity that stabilize the circuit.

2.1.5 Autoreceptor-Associated Negative Feedback

After the release of synaptic vesicles, binding of vesicle contents to autoreceptors present on the presynaptic cell membrane can decrease synaptic activity[17]. Autoreceptors are typically metabotropic and may respond to neurotransmitters such as glutamate or dopamine or to other vesicle contents such as ATP (extracellular ATP is broken down into adenosine which binds to the autoreceptors)[17]. In these cases, the inhibition appears similar to what is seen with voltage-gated calcium channel blockers or reduction of external calcium, suggesting that the mechanism of action is through decreasing calcium influx[18]. Dopamine, acting through presynaptic D2 receptors, is also able to activate G-protein activated inwardly rectifying potassium channels (GIRKs) which causes potassium efflux and thus hyperpolarization, further decreasing the potential of synaptic release[19]. Though it appears that presynaptic autoreceptors do function in synaptic depression, their impact may not be great; metabotropic glutamate receptors at the calyx of Held appear to only contribute 10% to overall depression[20].

2.1.6 Retrograde Signaling

In addition to receiving signals through autoreceptors, presynaptic terminals respond to signals from their postsynaptic partners. Retrograde signaling molecules come in many types, including gases, proteins, and lipid-derived signals, and may be released or membrane bound[21]. Retrograde signals can either function in negative feedback, much like autoreception, or they can upregulate presynaptic activity in association with postsynaptic LTP.

The most well understood gaseous retrograde transmitter is nitric oxide (NO), which is manufactured by the glutamatergic NMDA receptor-bound nitric oxide synthase in response to NMDA receptor activity[22]. After diffusing across the synaptic cleft, NO binds to soluble guanylyl cyclases (GCYs) that produce cyclic guanosine monophosphate (cGMP), which in turn bind to and activate various cGMP-dependent protein kinases, as well as regulating cyclic-nucleotide gated ion channels[23]. While it has been suggested that NO increases LTP as disruption of the neuronal NO pathway inhibits LTP[24], it has difficult to determine what is happening on a molecular level due to the multitude of downstream targets of NO.

Proteins such as conventional neurotransmitters or trophic factors can be released postsynaptically and may have presynaptic actions. Just as in autoreception, postsynaptic GABA may bind to $GABA_B$ receptors, causing synaptic depression likely through voltage gated calcium channels[25]. Growth facters like brain derived neurotropic factor (BDNF) can be released upon postsynaptic activity. BDNF binds to TrkB receptors and potentiates presynaptic signaling[26,27]. Just like

presynapic neurotransmitter release, retrograde release of protein signals typically requires depolarization and calcium influx. In addition, membrane bound proteins such NCAM appear to stabilize LTP formation, though LTP maintenance is unaffected[28].

Lipid based signals are also key players in retrograde signaling[22]. Endocannabinoids are the best studied of this group and cannabinoid 1 (CB1) receptors are found presynaptically throughout the central nervous system. In glutamatergic synapses, depolarization, calcium influx, and metabatropic glutamate receptors contribute to the activation of phospholipase C β which converts the lipid PIP_2 to DAG which is further altered to become the endocannabinoid 2-AG[29]. 2-AG is then released and binds to CB1 receptors, which, in an unknown mechanism likely involving voltage gated calcium channels, decrease the probability of vesicle release.

2.2 Postsynaptic Mechanisms

In addition to presynaptic changes, neural plasticity is achieved through changes in protein function and expression in the postsynaptic bouton. These changes have been the best characterized at glutamatergic synapses in the mammalian hippocampus, and naturally differ in other cases. Nonetheless, the mechanistic themes are likely to carry through to other synapses.

2.2.1 Long Term Potentiation (LTP)

Long term potentiation refers to the process by which frequent stimulation of a synapse leads to potentiation that lasts for minutes to hours or longer. This form of plasticity is seen at glutamatergic synapses that have been stimulated at

high frequency[30]. These synapses contain two different types of glutamate receptors: AMPA (α-amino-3-hydroxy-5-methyl-isoxazaleproprionic acid) receptors and NMDA (N-methyl-D-aspartate) receptors. While both receptors are permeable to cations, the NMDA receptor is controlled by postynaptic depolarization in addition to glutamate binding[30,31]. In its inactive state, the pore is blocked by a magnesium ion that is only released after AMPA receptors have allowed for sufficient ion influx. Thus, NMDA receptors act as coincidence detectors for multiple instances synaptic activity[32]. Once active, NMDA receptors allow for calcium influx, which can then activate numerous downstream pathways that ultimately lead to greater synaptic strength. Downstream of calcium influx, LTP is associated with greater trafficking of AMPA receptors to the postsynaptic membrane[33], but can also be achieved by postranslational modifications of glutamate receptors or associated proteins within the postsynaptic density[31], or changes to the synaptic cytoskeleton that result in larger and stronger synapses[34].

2.2.1.1 Ca^{2+}/Calmodulin Dependent Kinase II (CaMKII)

CaMKII is highly abundant in the brain and is particularly enriched at the postsynaptic density, where it is a key player in LTP. CaMKII is found as an auto-inhibited hexamer until activated by Ca^{2+}/calmodulin after calcium influx through NMDA receptors. Once a CaMKII subunit is bound by Ca^{2+}/calmodulin, that subunit is freed from auto-inhibition and is able to auto-phosphorlylate neighboring subunits that have also been freed[35]. With enough calcium entry, this has a cascading effect that results in persistently active CaMKII that continually phosphorylates itself faster than phosphatases can dephosphorylate. Once CaMKII is

activated, it translocates to the postsynaptic density and binds to NMDA receptors. This is critical to localize CaMKII to the postsynaptic density where it is able to directly act on synaptic proteins to increase synaptic strength[30,35].

CaMKII can directly phosphorylate AMPA receptors, leading to increased ion conductance even at intermediate levels of glutamate binding[36,37]. This results in greater postsynaptic depolarization upon glutamate binding, and thus stronger synaptic connection. In addition, CaMKII activity appears to increase exocytosis of AMPA receptor containing vesicles to the postsynaptic membrane, and enhance the ability of the postsynaptic density to capture AMPA receptors[37]. It is still unclear how CaMKII achieves these effects, although phosphorylation of other synaptic proteins such as stargazin appears to play a critical role in targeting AMPA receptors to the synapse through binding with PSD-95[38]. This increases strength of active synapses and allows silent synapses (synaptic structures that lack AMPA receptors) to respond to glutamate release, both hallmarks of LTP. Knock-down of CaMKII or mutation of its autophosphorylation site causes loss of both LTP and of behavioral memory[39,40]. Critically, perfusion of CaMKII itself appears to be able to achieve many of these effects without electrical stimulation or NMDA receptor activation, suggesting that it is sufficient for LTP induction[41].

While the majority of CaMKII is inactivated a minute or two after stimulation ceases, LTP persists. In addition, compounds that block the catalytic abilities of CaMKII block induction but not maintenance of LTP[37]. This would suggest that despite the autophosphorylation abilities of CaMKII that allow it to be persistently

active, it might not be "the" memory molecule. However, there is evidence of CaMKII maintaining binding with NMDA receptors for at least 60 minutes following LTP induction, and disruption of this binding reverses LTP, so it is possible that CaMKII has a structural function in maintenance of LTP[37,42].

2.2.1.2 MAPK/ERK pathway

The mitogen-activated kinase MAPK (also known as extracellular-signal regulated kinase ERK) pathway has been implicated in many aspects of cell proliferation and differentiation, but also appears to play a role in the late stages of LTP[31]. There are several ways to activate ERK, but I will focus on calcium given its importance in synaptic plasticity. Calcium influx via NMDA receptors or voltage gated calcium channels causes the G-protein Ras to bind GTP. Ras-GTP triggers the protein kinase Raf. Raf then phosphorylates and activates the MAPK/ERK kinase MEK, which in turn activates ERK[43]. Active ERK is able to trigger diverse signaling cascades that target transcription factors, cytoskeletal elements, and other kinases, causing a broad spectrum of synaptic changes that are associated with synapse growth, LTP, and learning[43–45].

Active ERK is associated with increased glutamate response due to an increase in AMPA receptors in the postsynaptic membrane. This particular mechanism of LTP induction depends on CaMKII, which appears to be able to activate Ras[46]. In addition, ERK and its downstream kinase targets may be able to directly regulate cytoskeletal elements at the synapse, causing an increase in synaptic size and new synapses. Importantly for late stage LTP, ERK and its target

kinases can act in the nucleus, where they are thought to regulate protein synthesis by phosphorylation of transcription factors such as CREB[47].

2.2.1.3 CREB

While short term plasticity requires local changes to protein function typically mediated by phosphorylation, for long term plasticity, protein synthesis is required[48]. For this to occur, multiple genes need to be regulated in a coordinated fashion. A candidate for this coordination is CREB (cAMP response element binding protein). cAMP responsive elements (CREs) can be found in the regulatory regions of many genes, and it is a protein on which many LTP-associated pathways converge[49]. Indeed, increases in phosphorylated CREB and activity of CRE-driven reporter genes can be seen after LTP or some types of learning paradigms[50,51]. CREs can be found in regulatory regions of many diverse gene families that are activated by different stimuli and in different tissues, and it is still not clear exactly what changes in gene expression are required for lasting LTP[49]. It is additionally unclear how global changes in gene transcription can have local effects at specific synapses, but this is likely achieved through some sort of synaptic tagging mechanism that marks highly active synapses as sites of new protein integration[52].

As with many paths that lead to LTP, CREB can be activated in response to influx of calcium through NMDA receptors or voltage gated calcium channels. Calcium stimulates some types of adenylyl cyclases, which manufacture cAMP. cAMP in turn activates protein kinase A (PKA), which directly phosphorylates CREB, enabling it to drive gene expression in the nucleus[53]. Animals with defects in this

pathway have learning defects, and in slice culture, stimulation of PKA can mimic the effects of LTP[54–56]. Despite being the canonical pathway for CREB activation, PKA is not the only activator. As mentioned above, ERK and other kinases downstream of it are able to phosphorylate CREB. The ERK pathway can be triggered by neurotransmitters or neurotropic factors binding to tyrosine kinase receptors[57]. In addition, calcium influx can trigger CaMKIV, which also phosphorylates CREB[53]. The convergence of these and other pathways on CREB indicate that CREB is likely an integration point for many different types of stimulation that influence synaptic strength.

2.2.2 Long Term Depression (LTD)

In contrast to LTP, LTD refers to the depression of a synapse that lasts for minutes to hours or longer. This type of plasticity is typically seen with low-frequency stimulation[58]. Despite being the functional opposite of LTP, hippocampal LTD shares some molecular commonalities. Surprisingly, NMDA receptor activation and subsequent calcium rise seems to be necessary for both, though the magnitude and duration of calcium signaling may be the crucial difference with LTD resulting from shorter, smaller calcium increases. If this is the case, the simplest explanation for how a rise in calcium could be responsible for both LTP and LTD is the differential affinity of calcium sensing proteins in the synapse[30]. CaMKII, which is of utmost importance to LTP induction, has a low affinity for calcium and is thus only able to become active after stimuli that produce high levels of calcium. Calcinuerin, a phosphotase linked to LTD, has a high affinity for calcium[59]. This means that low levels of calcium would result in much greater phosphotase than kinase activity. In

the reverse of LTP, dephosphorylation of AMPA receptors and associated postsynaptic density proteins would decrease synapse activity, leading to lowered conductivity and LTD. Again in the reverse of LTP, dephosphorylation of AMPA receptor is not only associated with decreased channel conductivity, but also internalization of receptors[60]. There is some evidence that the proteasome is necessary for LTD, and that ubiquitination of postsynaptic proteins plays a role in the active deconstruction of the synapse through LTD[61,62]. As maintaining and modifying synaptic strength through a balance of LTP, LTD, and other forms of plasticity is extremely important, there are likely to be multiple regulatory pathways that influence synaptic depression. Unfortunately, while there are a great deal of manipulations that modify the expression of LTD, a basic molecular model is still not agreed upon.

3. Neuromodulation

Classical neurotransmitters largely bind to membrane bound ionotropic receptors that are directly responsible for EPSCs and IPSCs in the postsynaptic neuron. As I've discussed above, activity can alter the way each synapse behaves primarily by changing the activity and number of these receptors. Another class of neurotransmitters known as neuromodulators is able to modify the behavior of whole cells or circuits at once by changing the intrinsic firing properties of neurons. Neuromodulation may make a cell more or less likely to have an EPSC in response to stimulation, or change its intrinsic firing rhythm[1]. A classic example of the power of neuromodulation comes from *Aplysia*. This marine mollusc is able to regulate its gill withdrawal reflex by habituating to soft touches and sensitizing to strong noxious

stimuli[63]. This happens in a serotonin dependent manner, where release of serotonin by interneurons affects signaling at the sensory-motor synapse. This leads to phosphorylation of potassium channels, inhibiting the potassium current and lengthening action potentials[64,65]. This change in signaling is dependent on neurons outside of the gill-withdrawal circuit and relies on the overall state of the animal rather than the activity of a single circuit or synapse.

In the mammalian brain, neuromodulators are produced by small numbers of cells which project to many diverse brain regions[66]. In general neuromodulators are thought to be responsible for regulating mood, arousal, and attention, and neuromodulators heavily alter synaptic plasticity.

3.1 Signaling Mechanisms in Neuromodulation

Unlike the actions of classical neurotransmitters, neuromodulators typically act through metabotropic receptors. The activity of these receptors depends on the type of associated G protein, and can influence diverse signaling pathways. This slower form of neurotransmission does not directly cause EPSCs or IPSCs, but instead can change the properties of ion channels in the membrane. This may cause the resting membrane potential to depolarize or hyperpolarize, affecting the cell's ability to respond to stimuli. In addition to direct effects on cells' intrinsic firing properties, neuromodulators can affect gene expression, modifying the long-term changes of synaptic plasticity.

Major neuromodulators include serotonin, dopamine, and noradrenaline, though neuropeptides and acetylcholine can also act as neuromodulators. Many

neuromodulators can act like classical neurotransmitters by binding to ionotropic receptors, and some classic neurotransmitters can also behave like neuromodulators by acting through metabotropic receptors[67,68]. The difference in action is then not due to only the neurotransmitter itself, but the receptor to which it binds. Accordingly, G-protein coupled receptor types show incredible diversity and act through many different pathways, allowing neuromodulation to affect many brain areas at once in very different ways.

3.1.1 The cAMP Signaling Pathway

A primary target of neuromodulators is adenylyl cyclase, which manufactures cAMP. cAMP activates protein kinase A (PKA) which has many targets including CREB, NMDA receptors, AMPA receptors, and other ion channels. In addition, cAMP can directly influence the opening of cyclic-nucleotide gated ion channels[69]. By influencing the relative membrane permeability to various ions, activation of this pathway affects overall excitability, and can affect how the cell responds to LTP- or LTD-inducing stimuli. For example, activation of the serotonin receptor 5-HT_4 is coupled to a rise in cAMP that results in increased excitability by inhibiting calcium sensitive potassium channels. Without potassium leakage after EPSCs, afterhyperpolarization is decreased and neurons are more likely to have further EPSCs[70]. Binding of neuromodulators does not always increase excitability. Serotonin, dopamine, and noradrenaline each have the capacity to both stimulate and inhibit the cAMP signaling pathway depending on which G protein is associated with their receptors[66].

3.1.2 The Phospholipase C Pathway

The other primary target of G protein signaling is the PLC pathway. In this pathway the G protein stimulates phospholipase C (PLC). Activated PLC cleaves the lipid PIP2 into IP3 and DAG[71]. DAG activates protein kinase C (PKC), while IP3 binds to calcium channels on the membrane or the endoplasmic reticulum, thus increasing intracellular calcium. This both depolarizes the cell and also contributes to the activation of numerous cell signaling pathways[67]. Activation of PKC by serotonin binding to 5-HT_2 receptors can inhibit opening of voltage gated sodium channels, leading to increased excitation in a similar manner as blocking potassium channels[72].

3.2 Interactions Between Neuromodulation and Synaptic Plasticity

Given the overlap in signaling molecules present in synaptic plasticity and neuromodulation, it is no surprise that neuromodulators interact with synaptic plasticity. Indeed, because they have cell-wide influences, neuromodulators may be responsible for the sort of homeostatic plasticity that maintains healthy activity levels in cells undergoing a lot of LTP[1,32]. Synaptic scaling allows a neuron to sustain synapses with a variety of weights without overloading metabolic capacity, thus a synapse that has been strengthened through LTP might be weakened, but continue to be stronger than its neighbors as they are similarly weakened. This type of plasticity is achieved by affecting synaptic strength on a whole cell basis.

Neuromodulators such as dopamine can enhance LTP by increasing AMPA receptor glutamatergic transmission, either by direct phosphorylation of AMPA

receptors or by increasing AMPA receptor trafficking[73,74]. Dopamine in particularly appears to play a significant role in both the likelihood of LTP/LTD establishment and also the maintenance of synaptic plasticity. It does this through a variety of mechanisms, including PKA signaling, CREB dependent protein synthesis, and the MAPK pathway[75]. These impacts are all likely due to influence on AMPA receptor expression, trafficking, and trapping within the postsynaptic site. Thus, neuromodulation has impacts beyond modifying the intrinsic excitability of neurons and is also able to directly modify a neuron's ability to maintain synaptic plasticity.

Consistent with the apparent role of synaptic plasticity in learning, neuromodulators have profound effects on memory acquisition and maintenance. If neuromodulators truly are the basis of emotion, arousal, and attention, it makes sense that they would have this effect as mental states impact learning. Numerous pharmacological studies bear out this hypothesis. Serotonin receptor antagonists have been shown to facilitate certain memory tasks, while agonists have the opposite effect[76,77]. Dopamine antagonists are able to impair persistence of spatial memory in rats[78]. Given the variety of targets of neuromodulatory signaling it comes as no surprise that while manipulation of neuromodulatory systems clearly impacts memory, the effects vary depending on the type of learning measured and the specific manipulation, and the exact mechanisms remain a subject of study.

4. Electrical Synapses

In contrast to chemical synapses wherein electrical signals from the presynaptic neuron must transform into a chemical neurotransmitter message that can influence

postsynaptic receptors, electrical synapses are a site of direct communication between cells. Signals present in the cytoplasm of one cell are able to diffuse to the cytoplasm of the second without changing form. While the nature of chemical synapses has been more studied, these days the dynamic contributions of electrical synapses are also being recognized[79].

A word on terminology: Functionally, gap junctions and electrical synapses are thought of as synonymous, but I will use the term electrical synapse to distinguish those coupling electrically active cells (ie, between neurons or the neuromuscular junction) from those present between other cell types such as glia or epithelial cells. Connexin- and innexin-containing gap junctions are found in most tissue types, where they allow for passage of cell signals just as in the nervous system.

4.1 Structure of electrical synapses

4.1.1 Channel structure

Electrical synapses are made up of channels that bridge the extracellular space between two neurons (fig 1). These channels consist of two hemichannels that come from each cell. While vertebrate and invertebrate electrical synapses appear

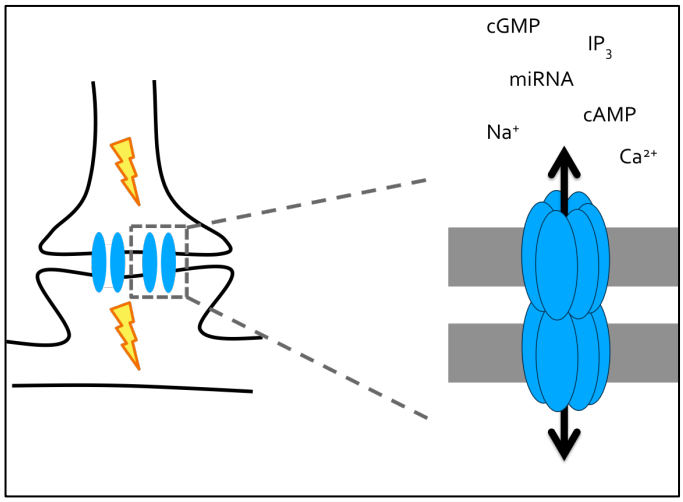

Fig 1 An electrical synapse. The electrical synapse is made of two hemichannels with a central pore that can transmit electrical information and signaling molecules between the cytoplasm of two cells.

structurally similar, they are actually formed of protein subunits from two evolutionarily unrelated protein families with structural but not sequence homology: connexins in vertebrates and innexins in invertebrates[80,81]. These subunits organize into hexameric hemichannels[82,83] (although there is evidence now that the one innexin with a solved protein structure makes octameric hemichannels, suggesting that perhaps one major difference between connexin- and innexin-based channels is their oligomerization[84,85]).

Channels can have different configurations of connexins or innexins to support different functions. Hemichannels may be homomeric (made of one type of connexin or innexin) or heteromeric (made of more than one type)(fig2)[86]. There are many different connexins and innexins (ex: the human genome has 21 connexin genes, Drosophila has 8 innexin genes, and *C. elegans* has 25)[87–89] and they are differentially expressed in different tissues and neuronal populations (and most cell types seem to express multiple connexins or innexins), implying that there is a great diversity in electrical synapses[90]. In addition, innexin genes in particular are known

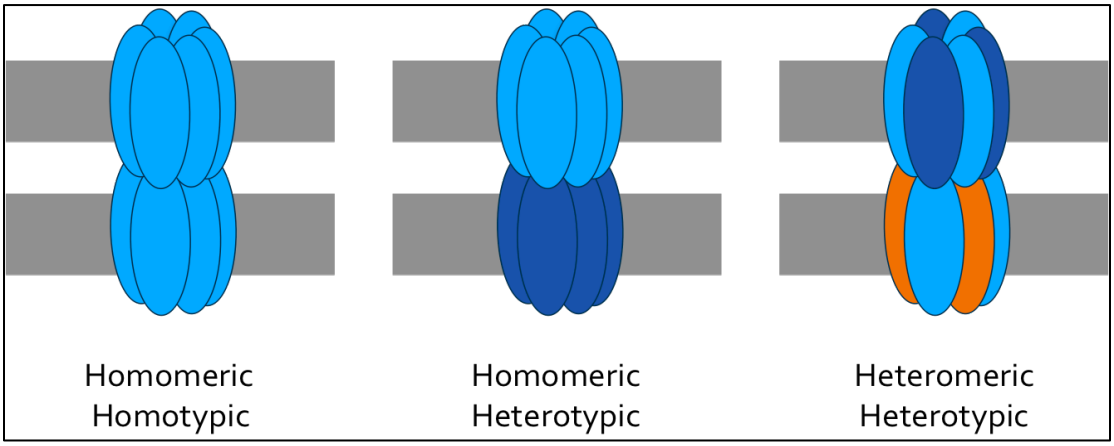

Fig 2. Organization of electrical synapses. Electrical synapses may be made up of the same type of connexin/innexin or different connexins/innexins.

to produce multiple splice variants, leading to an even greater number of combinations (in fact, different splice variants of a single gene can form heterotypic channels)[87,91].

While connexins and innexins do not share sequence homology and appear to have arisen separately evolutionarily, structurally they are very similar and are both found at electron micrographs of gap junction plaques (as well as other functional similarities discussed in section 4.2), suggesting that despite their differences, they are functional analogues[92]. The major difference between connexin- and innexin-based channels appears to be size: innexin-based channels are larger. The gap between apposing cells is wider in invertebrates, and the diameter of individual channels seems to be bigger owing to the larger central pore, possibly caused by octameric rather than hexameric hemichannel organization[93].

4.1.2 Protein Structure

Connexins and innexins share a remarkably similar protein structure despite having no sequence homology. Both connexins and innexins are proteins that have four alpha-helical transmembrane domains with two extracellular loops, one intracellular loop, and intracellular N- and C-termini[80,86] (Fig 3). Crucially, the extracellular loops contain cysteines in conserved

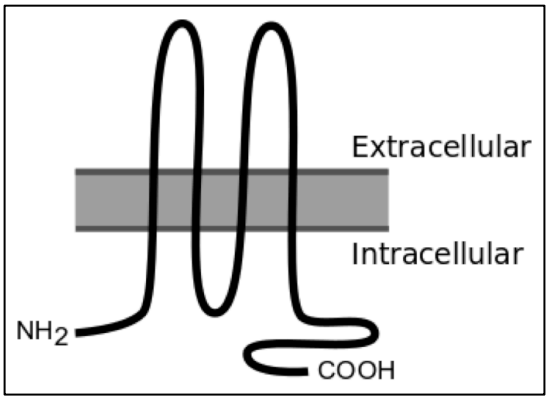

Fig 3 Structure of electrical synapse proteins Connexins and innexins are both 4 transmembrane domain having proteins with intracellular N- and C-termini and two extracellular loops.

locations (3 each in connexins, 2 each in innexins), which play a key role in the docking of hemichannels together across the intracellular gap[85,94,95], lending to the evidence that innexins and connexins form functionally analogous structures.

Both the N- and C-termini are important for the successful functioning of channels. The N-terminus is not required for subunit oligomerization, but appears to play a crucial role in gating the channels, and has been observed forming a plug- or funnel-like structure in the pore of both vertebrate and invertebrate channels[85,96–98]. The C-terminus is the most variable region between the connexins, and varies widely in both sequence and in length. It is the site of many protein modifications including phosphorylation, acetylation, and ubiquitination, and contains numerous sites of protein interaction including with the cytoskeleton and with regulators of cell growth and migration[99]. Most of the work illuminating the structural properties of the N- and C-termini has been done on relatively few connexins and one innexin, so it remains to be seen if these properties hold up more generally.

4.2 Function of electrical synapses

Electrical synapses are important units of signaling between cells of many tissues, though I will focus on their role in neuronal tissues. Electrical synapses are the neuronal version of what is known in other tissues as gap junctions, but have similar functions and properties. The channels in these electrical synapses do far more than their name would suggest. In addition to allowing passage of electrical signals and ions from one cell to another[100–102], electrical synapses can pass a wide

variety of signaling molecules such as calcium[103,104], cAMP[105–108], cGMP[106,109], IP_3[104,110], ATP and ADP[111–113], metabolites such as glucose[114,115], and even small RNAs[116,117]. Given this variety, electrical synapses can play roles in a number of different cellular processes, and their importance in both developing and mature tissue is likely still underestimated.

4.2.1 Electrical Synapse Permeability

Despite their fairly large pore size, electrical synapses can be highly selective in terms of what they allow to pass. Like many ion channels, the selectivity filter appears to be in the pore of the channel. The filtering ability comes from the pore size and subunit composition[106,118]. A good deal of work has gone into determining what molecules are able to pass connexin channels, but unfortunately a similar effort has not yet been completed for innexins, so for the vast majority of innexin channels we do not know what they are capable of passing beyond electrical potential. From studies probing permeability with neutral compounds of different sizes, we know that size of the pore is one factor in determining the permeability of channels and that innexin-based channels have a larger pore size[119–121], but that is not the whole story. Presence of charged amino acids lining the pore opening also contribute to the types of molecules that pass through channels[118,122]. There are clearly other factors playing a role in determining permeability however. Some cation-preferring channels nonetheless easily pass larger negatively charged molecules and break the patterns they otherwise match with regard to size and charge[123]. Additionally, single missense mutations that result in replacing one amino acid with a similar one within the pore can affect permeability despite not

changing charge or hydrophobicity[124]. It may be that the subunit composition creates low-affinity binding sites to certain molecules and thus preferential passage is gained, but this remains to be seen.

While initially assumed to be essentially always-open channels that allow for free-flowing traffic of signals, in fact electrical synapses can be highly dynamic. Electrical synapse opening is regulated by a number of stimuli including physiological state (ie, membrane voltage, pH, and calcium levels[125–129]) and by protein-protein interactions such as binding to cytoskeletal proteins[130–132]. Their conductance can be further regulated by the speed at which individual channels are introduced to and removed from membranes[133–135], and by post-translational modifications (generally on the C-terminal tail) such as phosphorylation and acetylation[99]. Phosphorylation of connexin channels generally results in decreased junctional conductance, though it can also affect the trafficking of immature channels[136,137]. Acetylation of connexin channels promotes removal from the cell membrane and internalization[138,139]. Interestingly, some of the phosphorylation sites are regulated by the same pathways that are known to regulate chemical synapse activity, namely the cAMP/PKA pathway[140,141], the Ca^{2+}/CaMKII pathway[142], and NO/cGMP/PKG pathway[143]. Thus, it is wrong to think of electrical synapses as static structures; their activity is being constantly tuned as the needs of the cells change.

Electrical synapses can be either bidirectional (passing signals equally between both neurons) or rectified (passing signals only one direction) [79,91,144]. Rectification

of electrical synapses can occur in several different ways. When channels are heterotypic, and the apposing hemichannels are formed of different connexins or innexins (see Fig 2), the difference in hemichannels properties can lead to rectification[90,91,145–147]. This may occur because the amino acids lining each pore opening are differently charged, such that charged ions or molecules have an easier time traveling one direction[90,146]. Additionally, each hemichannel may have different open probabilities, leading to signals to preferentially enter on the more open side[148]. Another major cause of rectified electrical synapses is the physiological state of each cell (ex: intrinsic membrane potential, cell size, etc)[79,149]. A cell that is naturally in a more depolarized state will be more likely to depolarize its neighbor, resulting in an electrical synapse that is functionally rectified even if it is made up of homotypic channels. Lastly, electrical synapses may be rectified as a result of differential regulation even if they are homotypic. As intracellular environments vary, the relative levels of modifications can differ on either side of the synapse. For example, one side of the channel may undergo conformational changes due to phosphorylation or voltage gating, decreasing the probability of signaling molecule entry on that side[90,148]. Rectification is one way in which electrical synapses are significantly more complex that a simple pore and can have dynamic and important roles within neural circuits.

Given the number of different connexins and innexins expressed in the animal kingdom, the potential for functional diversity is vast. This is diversity is multiplied in invertebrates as unlike connexins, innexin genes generally have introns and therefor the potential for splice variants[93]. Because the channel's rectification and

permeability depends on subunit composition and modification in both cells, a single cell that expresses a single connexin or innexin could nonetheless have electrical synapses with different properties. In order to begin to resolve this complexity, more study is needed into the permeabilities of different electrical synapses (especially heterotypic or heteromeric channels), particularly innexin-based, as there is a dearth of information regarding their properties. Additionally, the field would benefit from studies into which connexins and innexins are able to form heterotypic or heteromeric channels and which only form homomeric/homotypic channels.

4.2.2 Roles of electrical synapses in the nervous system

While connexins and innexins function in numerous tissue types (and much of the data on permeability comes from non-neuronal tissues and in-vitro approaches), I will focus on the roles of electrical synapses in the nervous system.

Electrical synapses are abundant in the mature nervous system, found in all major brain regions in both vertebrates and invertebrates. Roughly half of mammalian connexins are expressed in the brain, though most are found in glia rather than in neurons[81]. Connexin 36 (Cx36) is the primary vertebrate neuronal connexin, and is commonly expressed by interneurons[81,150]. Innexins are broadly expressed in invertebrate nervous systems; in *C. elegans* in particular, electrical synapses are extremely common. 80% of worm innexin genes are expressed in neurons, and many express multiple innexins simultaneously (including a few that express up to a dozen innexins)[89]. Despite their abundance, it remains largely

mysterious what roles electrical synapses are playing in the adult brain. While they are capable of passing small molecules, much of the research on electrical synapses in the nervous system has focused on their ability to pass electrical signals. These studies have illuminated several distinct functions of electrical synapses: they allow for synchronized activity of neural populations, impact network organization, and allow for signal amplification and coincidence detection.

4.2.2.1 Network Synchronization

Neural oscillations are patterns in neural activity where neurons fire together in a repetitive manner. In most cases, it is unclear what purpose these oscillations serve, but they are considered to be indicators of neural connectedness and they can be found in numerous cell types. In the vertebrate nervous system Cx36 is particularly important in the propagation of oscillatory behavior, although not in the initial generation of these rhythmic spikes[151,152]. In Cx36 knock out (KO) mice, gamma oscillations are reduced in power in the mouse hippocampus during running behavior, suggesting these neurons are less coordinated, though this does not seem to have a behavioral consequence[153]. A similar pattern is found in the neocortex, where Cx36 contributes to synchronicity in interneurons, particularly in larger populations of cells[154], in the inferior olive where it drives coordination between pairs of cells[152], and in the retina where Cx36-based coupling between AII amacrine cells and ON cone bipolar cells is required for oscillatory waves involved in of light signals[155]. Similarly, innexins are highly present in rhythm generating networks, where they increase synchronicity between populations of neurons such as the stromatogastric ganglion neurons of crustaceans[156]. Interestingly, in the rat

somatosensory cortex, synchronicity between pairs of interneurons is conferred through the presence of both electrical synapses and GABAergic chemical synapses. In pairs of cells that lack electrical synapses there is no synchronicity in firing, while in pairs lacking chemical synapses, the synchronicity is delayed[151]. For these cases the behavioral consequence is unknown, and surprisingly Cx36 KO mice show no gross behavioral defects (though some visual and memory defects will be discussed below), which may mean other channels are able to compensate.

4.2.2.2 Network Organization

Another function for electrical synapses in the vertebrate nervous system is to actively shape the networks in which they work. This is very evident in the retina, where electrical synapses are found in all cell types[157]. Horizontal cells are extensively electrically coupled with their neighbors, which increases the size of their receptive fields[158,159]. This coupling is reduced during the day due to the presence of dopamine which, via cAMP and protein kinase A (PKA), causes phosphorylation of electrical synapses and reduces the channel open probability[159]. This affectively narrows their receptive fields, likely contributing to higher acuity vision in bright light. In the hippocampus of Cx36 KO mice, the spatial selectivity of place cells is reduced, leading to each cell having larger regions of interest that are not stable over time[160]. These cells have slower theta oscillations, suggesting that they are not correctly integrating various excitable inputs, and this results in impaired short-term spatial memory. In both of these cases, electrical synapses shape the areas of interest for downstream neurons and at least in the retina allow for this area to be dynamic.

4.2.2.3 Amplification of Signals

Lastly, electrical synapses function to reduce noise and amplify signals in neural circuits. In vertebrates this has been most extensively studied in the retina, where electrical synapses between cones allow for correlated visual stimuli to be amplified, improving the signal-to-noise ratio by around 80%[161]. Rods and cones also share electrical synapses, and like those of horizontal cells, these are modulated by dopamine via PKA[162]. During the day, increased dopamine levels mean electrical synapses are weak and that cone horizontal cells are receiving input primarily from cones. At night, the rod-cone electrical synapses are strengthened, meaning that cones carry visual information from rods to their horizontal cells, thus increasing the signal. This property increases dim light vision. This function to increase signal and decrease noise comes at least partly because electrical synapses are considered low-pass filters, meaning they preferentially transmit lower frequency signals and block higher frequency signals. This is because of the innate electrical properties of cells and the time it takes membrane capacitance to charge[125].

Much like the vertebrate examples above, electrical synapses allow for amplification of signals through a variety of mechanisms. Principally, electrical synapses underlie coincidence detection in circuits with "hub and spoke" architecture. Hub and spoke refers to circuits where multiple presynaptic "spoke" cells connect to a central "hub" neuron with electrical synapses, and is a common motif within the *C. elegans* nervous system. When two spoke cells depolarize close in time, together they are more cause a larger depolarization in the hub neuron. This same architecture can also work in the opposite manner whereby a hyperpolarized

neuron can act as a shunt to minimize the impact that a single depolarizing event can have. In this manner, multiple types of input can converge upon a single integration hub such that the behavioral output is the sum of multiple types of information simultaneously.

Additionally, electrical synapses can act as amplifiers of chemical synapses. In this case, chemical and electrical synapse components are found at the same sites known as mixed electrical synapses. When a chemical synaptic event causes depolarization within the post synaptic cell, this depolarization is able to diffuse through electrical synapses into the presynaptic terminal (and others nearby in lateral excitation), causing greater pre-synaptic depolarization, more calcium influx, and increased release of synaptic vesicles, leading to a larger postsynaptic event[163]. This ultimately leads to a faster response to stimuli, and is thus common in circuits that govern escape behavior in non-mammalian animals (while electrical synapses hypothetically provide faster signal transmission than chemical synapses, the higher body temperature of mammals speeds up chemical synaptic transmission and eliminates the speed benefit of electrical synapses). Mutations in the *Drosophila* innexin Shaking-B result in a failure of flight muscle activation and delay in jump muscles in response to a manual stimulus[164]. This type of amplification at mixed chemical and electrical synapses has also been observed in vertebrates, particularly in teleost Mauthner cells where heterotypic electrical synapses mediate signal transmission from auditory afferents[147,165].

Clearly connexin- and innexin- based channels play many different roles in the nervous system, but more study is needed, particularly on their potential to pass small signaling molecules between neurons as that is an understudied possibility.

4.2.2.4 Electrical Synapse Functions in Glia and Developing Neurons

Beyond expression in mature neurons, there are gap junctions between some types of glia, particularly astrocytes (and far fewer between glia and neurons) that make up a glial syncytium. This network have been shown to be necessary for propagation of small molecules and metabolites[166,167]. In particular, gap junctions between astrocytes may be crucial in transporting molecules such as glucose from blood vessels to neurons to feed the energetic needs of neurons[168]. Fulfilling another homeostatic purpose, gap junctions between astrocytes and oligodendrocytes have been hypothesized to buffer the extracellular K^+ remaining after action potentials. Astrocytes would take up this K^+ through ion channels or gap junction hemichannels, and then through the glial gap junction network, the K^+ is redistributed such that it does not disrupt the ion balance[167,169]. In fact, extracellular K^+ has been shown to increase junctional coupling between glial cells, meaning this intercellular diffusion would be easier[170]. In myelinated neurons, the maintenance of homeostasis may be managed by reflexive gap junctions: those that are between the wrapped layers of the same Schwann cell. These allow for radial diffusion of ions and small molecules up to one million times faster than if the same molecules diffused outward in a circumferential manner. Mutations in Cx32, which is the main connexin found at these gap junctions can cause peripheral demyelination neuropathies[171]. In the fly retina, gap junctions between glia are crucial in trafficking

metabolites of neurotransmitters from the synaptic cleft back to the neuronal cell body where they are re-used[172]. Knock-down of Inx-2 in the retinal glia results in delayed or deficient responses to visual stimuli. Glial gap junction networks are not just passively supporting neuronal needs however. They also play a role in propagating waves of calcium within glia (either through direct passage of calcium or through passage of the small molecule IP_3)[173,174], which can result in release of gliotransmitters that regulate the activity both synapses and blood vessels[175,176]. The gap junction network allows glial cells to support and actively modify neuronal signaling.

Electrical synapses are also known to function during neural development, where they define populations of cells destined to become functional regions by propagating both electrical and small molecule signals[177–179]. For example, in the developing mouse retina, electrical synapses composed of Cx36 and Cx45 specifically link retinal ganglion cells to their close neighbors, allowing them to fire together, and in knock-outs, these cells' firing patterns are actually more similar to other more distant cells[180]. Without local synchronization, the retinal axons that project to the lateral geniculate nucleus of the thalamus do not project to the correct region, suggesting that electrical synapses are necessary for defining cellular maps in the retina. The formation of functional units of electrically coupled neurons has been hypothesized to lay down the blueprint for future chemical synapse in mature circuits, as transient electrical coupling during development is common in both vertebrates and invertebrates, and in at least some cases required for correct chemical synapse formation[181–185]. Beyond merely connecting populations of cells

together, electrical synapses can also impact cell fate in another manner: by passage of signaling molecules between neurons. In *C. elegans*, innexins underlie a left/right asymmetry decision, whereby the cell fate of the AWC pair of sensory neurons is determined by signaling through an inx-19 electrical synapse network[186,187]. In this case, stochastic differences in cytosolic calcium levels in previously identical immature neurons are propagated through a network of electrically coupled neurons (the exact signal may be calcium itself, membrane potential, or something else yet to be determined) such that the cell with higher calcium levels becomes AWCoff and the cell with lower calcium levels becomes AWCon[188]. Additionally, connexin channels are used in migrating neurons not for their ability to pass signals, but as cell-adhesion molecules[189]. A similar function has not been confirmed for invertebrate neurons, though there is no obvious reason they would not serve as adhesive molecules as well. Electrical synapses in the developing nervous system play many roles, defining both the fate of neurons as well as the pattern of their mature synapses.

4.2.3 Potential role in neuroplasticity

Neurons can affect the activity of those around them through lateral excitation wherein electrical potential diffuses through electrical synapses, as mentioned above in section 4.2.2.3. In this way, the membrane potential of a neuron can change the excitability of connected cells. Potential from depolarized cells can diffusion through electrical synapses to depolarize neighbors, thus making them more likely to depolarize themselves. On the contrary, hyperpolarized cells can draw positive charge from their neighbors, making them less likely to depolarize in the future.

These kinds of electrical impacts would directly affect processes like LTP and LTD, increasing or decreasing the threshold required. Given that electrical activity can enhance junctional conductivity, it seems likely that passage of electrical signals would work in a positive feedback loop as well, such that the more depolarization that occurred, the more easily that depolarization would be able to travel to nearby cells.

As a prominent player in both baseline neural signaling and in neural plasticity, Ca^{2+} passage through electrical synapses is likely to have effects on neural plasticity. Increasing presynaptic calcium levels of neighboring neurons could impact them similar to how paired-pulse facilitation works (see section 2.1.1 for details). This would mean that even in the absence of their own firing events, these neurons would be primed for faster and higher action in the next event. Postsynaptically, calcium diffusion could have a number of effects on LTP and LTD, either facilitating or decreasing the strength thereof. Calcium ions can activate the CaMKII pathway (see section 2.2.1.1), the MAPK/ERK pathway (see section 2.2.1.2), as well as CREB via CaMKIV (see section 2.2.1.3), all of which ultimately strengthen chemical synapses. Conversely however, low levels of postsynaptic Ca^{2+}, can contribute to LTD by activating to high binding affinity phosphatases (see section 2.2.2). Thus altering postsynaptic calcium levels could tip the balance towards LTP or LTD.

Another way that electrical synapses could contribute to neuroplasticity is through the passage of small molecules. Molecules such as cAMP, cGMP, and IP3 have documented roles in neural plasticity through their effects on kinases (which

can promote the activity of neurotransmitters receptors or modulate membrane potential by changing the type and number of membrane channels present) and small molecule gated ion channels that directly impact membrane potential. (Details of small molecule affects on chemical synapses are described in depth in sections above. For information on signaling pathways in neural plasticity involving cAMP, see sections 2.2.1.3 and 3.1.1; cGMP, see section 2.1.6; IP_3, see section 3.1.2). In this manner, neurons undergoing small molecule mediated plasticity could prime their electrically-coupled neighbors for similar changes by providing them with the same small molecules. While passage of such small molecules has been well documented in non-neuronal tissues, in glial cells, and in developing neurons, there has been very little study on the potential for neuronal electrical synapses to do so. This is likely because of the unique role that electrical synapses play in neurons, as well as the difficulty in separating the potentially simultaneous transmission of both electrical and small molecule signals.

One region where electrical synapses are known to impact neural plasticity is in the hippocampal CA1 region. To date there is one study that shows Cx36 KO results in elimination of LTP generation following high frequency stimulation. This is correlated with increases in the ratio of GluN2A/GluN2B NMDA receptor subunits[190]. This higher 2A/2B ratio is associated with the need for stronger stimulation in order to induce LTP and a wider ranger of stimulation resulting in LTD[191–193], so lack of these electrical synapses seems to change the plasticity threshold. The mechanism whereby electrical synapses mediate this ratio change is unknown. As described in section 4.2.2.2, Cx36 KO results in reduced spatial

memory but this study examine the impact on oscillatory patterns rather than LTP, so it remains unclear what, if any, impact electrical synapses have on neural plasticity in cells beyond the CA1 region.

5. C. elegans neuroplasticity

5.1 Signaling in C. elegans sensory neurons

Sensation is extremely important to *C. elegans* as it uses chemical and physical cues to find food and mates as well as avoid environmental danger such as pathogens. A large portion of the nervous system (upwards of 52 neurons out of 302 in hermaphrodites, and nearly double that in males) are sensory neurons[194,195]. Sensory neurons generally come in pairs given three-letter names (ie, AWC, ASI, ALM), and in the case of chemosensory neurons they make contact with the outside world with their ciliated endings through pores in the cuticle. Pairs of sensory neurons generally express multiple different sensory receptors[194]. In several cases, this leads sensory neurons (such as ASH) to be highly polymodal and able to respond to such diverse cues as touch, hyperosmolarity, and volatile odors[196]. While mechanosensation receptors are thought to be ionotropic, causing direct depolarization of the cell upon activation[197], most known chemsensory receptors are GPCRs. Within chemosensory neurons, these receptors lead to two primary mechanisms of signal transduction that result in depolarization: the cGMP/TAX-2/TAX-4 pathway and the TRPV pathway. In the first, G protein signaling can activate guanylyl cyclases or phosphodiesterases that produce or hydrolyze cGMP. In the presence of cGMP, cation-permeable channels made of TAX-2 and TAX-4

subunits open[198,199]. This pathway is similar to vertebrate phototransduction and is thought to be the primary sensory transduction pathway in ASE, AWC, AWB, ASI, ASG, ASJ, and ASK, though not all players and their functionalities have been confirmed in all cells. The alternative pathway downstream of G-protein signaling is slightly less clear, but it involves a cation TRPV channel made of OCR-2 and OSM-9 subunits and is thought to function in ADF, ADL, ASH, and AWA[200,201]. These channels are thought to be gated by lipids, particularly polyunsaturated fatty acids (PUFAs), as mutations within the PUFA synthesis pathway disrupt chemosensation in the neurons that utilize the TRPV channel[202]. Both types of signaling ultimately result in changes to calcium levels within the sensory cell.

5.1.1 Plasticity in C. elegans sensory neurons

C. elegans chemosensation is constantly modulated by the worm's physiological state and environmental signals, allowing it to fine-tune responses to sensory input. In addition, the activity of sensory neurons is modified by habituation (diminished responses after repeated stimulation) and by associative learning (pairing of sensory input with unconditioned stimuli such as presence of food or noxious bacteria). Food is a potent modulator of chemosensory signaling. The *C. elegans* behavior pattern changes between the on-food state (largely made up of periods of dwelling occasionally interspersed with short bouts of crawling) and the off-food state (initially made up of rapid series of reversals in a local search mode and then followed by long distance travel)[203]. Additionally, while on food, worms are more sensitive to noxious stimuli, whereas worms that have been starved are more sensitive to odors and are better able to learn from odors in their environment[204].

This behavioral plasticity is driven by monamine signaling, particularly dopamine and serotonin[205,206]. The particular receptors and their downstream signaling targets are not known in most cases, though these details have been worked out in mechanosensory neurons and are discussed in more detail below.

When looking at the general patterns of sensory neuroplasticity in *C. elegans*, one sees some parallels with the types of changes that are seen in vertebrate systems. Unfortunately, the molecular details are significantly less well studied than in vertebrates, but what we do know suggests common mechanisms throughout the animal kingdom as glutamate receptors, CaMKII, and CREB all play important roles. As in vertebrates, *C. elegans* AMPA-type glutamate receptors are critical in adaptation and learning. Mutants lacking the receptor subunit GLR-1 are deficient in olfactory learning[207]. In touch circuitry, release of glutamate from sensory neurons is required form memory formation and training results in changes in the sizes of AMPAR subunit GLR-1 and GLR-2 puncta in the ventral nerve cord[208,209]. This change is mediated in part by the scaffolding molecule MAGI-1 that regulates AMPA receptor localization in multiple forms of sensory plasticity[210]. GLR-1 localization can also be regulated by the *C. elegans* CaMKII ortholog UNC-43, though the mechanism by which it does so is poorly understood[211]. CaMKII activity in *C. elegans* neurons has not been extensively studied, but it does appear to regulate presynaptic strength at the neuromuscular junction[212] and is required for learning in response to aversion training with pathogenic bacteria[213].

Despite having only 302 neurons and a limited set of behaviors, *C. elegans* has the ability to form long term memories that last for days. As in vertebrates, this type of memory requires CREB and protein synthesis. Worms lacking CREB are unable to form long-term memories, while those with an overexpression of CREB in the nervous system are able to increase the duration of memory maintenance[214]. In some cases, CREB acts within sensory neurons themselves to maintain memory, as in the thermosensory neuron AFD, while in others[121], CREB functions in interneurons where it may affect the strength of multiple sensory inputs simultaneously[215,216]. The genomic targets of CREB in the *C. elegans* nervous system are only starting to be worked out, but they include many genes involved in neurotransmitter release, synaptogenesis, synaptic scaffolding, calcium signaling, ion channels, and numerous kinases that can regulate such processes[215]. CREB, CaMKII, and AMPA receptors are all important players in vertebrate neuroplasticity as well, indicating the basics of neuroplasticity are similar across species.

While there is much still to learn about the various ways the *C. elegans* nervous system engages in neuroplasticity, there are a few cases where the mechanism is well studied: olfactory plasticity in the AWC neurons; gustatory plasticity in the ASE neurons, and touch habituation in the mechanosensory neurons. I will explain what is known in these cases in the sections below. Additionally, and of particular interest for this thesis, much work has been done on the signaling and plasticity within the nociceptive ASH neurons and that will be covered in section 5.2.

5.1.1.1 Olfactory plasticity in the AWC neurons

AWC neurons are two of the primary sensors of neutral-to-attractive odors such as benzaldehyde, isoamyl alcohol, and butanone, and respond to odors with a decrease in calcium levels[217,218]. Despite sensing multiple different odorants, AWC neurons can selectively alter their responses to individual odorants, as prolonged exposure to one odorant results in adaptation to that specific odorant[219]. *C. elegans* adapts to odors in the environment by decreasing responses after extended exposure, and can also learn to associate particular odors with food or lack thereof, either strengthening or weakening chemotaxis. There are several different mechanism that function in AWC plasticity. The sensory receptors themselves can be directly affected, as prolonged odor exposure leads to GPCR internalization upon binding of ARR-1 (beta arrestin), which decreases sensitivity to that odor[220]. Two other proteins are key in short-term adaptation in AWC, though their exact roles are not determined: OSM-9, a subunit of the TRPV receptor which is thought to be lipid-gated and functions in primary sensory transduction in other cells[200,219,221]; and ADP-1, an as yet uncloned gene[219]. Having multiple different pathways function in AWC to modulate signaling allows the neuron to be able to selectively modify responses to odors.

One modulatory pathway within AWC that has a more complete mechanism is the EGL-4 pathway. EGL-4 is a cGMP dependent protein kinase, and EGL-4 mutants are deficient in both short and long term adaptation. In short-term adaptation, EGL-4 is predicted to phosphorylate both the TAX-2 subunit of the cGMP gated cation channel[222], and CNG-3[223] (a putative alternative subunit of the same channel) directly impacting sensory signaling. It may do so at particular populations that are

linked to odorant receptors, as it is otherwise unclear how this can be odorant specific since all odorant signaling converges upon the TAX-2/TAX-4 channel. Prolonged exposure to an odorant produces long lasting adaptation, and this requires a nuclear localization signal and cGMP binding sites on EGL-4[224]. It is thought that decreasing cGMP levels due to G-protein signaling within the sensory cilia of AWC during prolonged odor exposure are what results in this nuclear translocation, as it does not depend on calcium changes[225,226]. It is unclear exactly what the nuclear targets of EGL-4, but it is known to upregulate the GPCR STR-2, and may result in upregulation of PUFA signaling, thus activating TRPV channels and decreasing responsiveness to odors[224,226]. EGL-4 nuclear localization and thus odor adaptation are further modulated by the presence of food via the activity of other neurons. In the absence of food, aversive olfactory conditioning requires insulin signaling from the AIA neurons through the AGE-1 PI3-kinase in AWC. This contributes to EGL-4 nuclear localization[227]. Thus multiple different signals converge upon EGL-4 within AWC, allowing it to regulate odorant signaling in several different ways.

5.1.1.2 Gustatory plasticity in the ASE neurons

The ASE neurons respond to soluble tastants such as salts, biotin, and lysine[228]. They do so in a partly asymmetrical manner: the left neuron (ASEL) responds to increases in concentration of Na^+, Li^+, K^+, and Mg^{2+}, and the amino acid methionine while the right (ASER) responds more strongly to decreases in concentration of K^+, Cl^-, I^-, and Br^-[229,230]. This asymmetry exists at the receptor level and translates into opposite influences on behavior, with ASEL promoting lengthy forward locomotion

and ASER promoting turns[231]. Like AWC, ASE signaling can be plastic, and is especially responsive to the presence of food. In the absence of food, AIA interneurons secrete INS-1 (the *C. elegans* homologue of insulin), which acts on the PI3-kinase pathway within ASER via the insulin-like receptor DAF-2. The PI3K AGE-1 phosphorylates PIP2 to PIP3, which then leads to activation of phosphoinosotide dependent kinases and protein kinase B[232]. Ultimately, in the absence of food prolonged exposure to NaCl results in increased calcium signals within ASER in response to downshifts in concentration of NaCl and a decreased synaptic output to interneurons[233]. Worms starved in the presence of NaCl have decreased attraction toward it as a result.

5.1.1.3 Touch habituation in mechanosensory neurons

The anterior (ALM) and posterior (PLM) mechanosensory neurons of *C. elegans* respond to touch. While tapping on their culture plate initially results in >90% of animals reversing, repeated stimulation results in habituation (<50% of animals reverse after 15 taps). Touch habituation memory comes in three different time frames: short-term (immediate), intermediate-term (lasting ~12hr), and long term (lasting >24hr), and each is controlled by a different mechanism. Short-term habituation results from auto-phosphorylation of potassium channels, which causes inhibition. These channels likely function to repolarize cells after depolarization, so closure leads to sustained depolarization and a decreased ability of calcium channels to re-activate in response to future stimuli[234]. Intermediate-term habituation requires release of a putatively inhibitory neuropeptide FLP-20 from mechanosensory neurons, though the downstream targets are unknown[235]. Long-

term touch habituation training results in changes in AMPA receptor subunit (GLR-1 and GLR-2) levels which rely on the scaffolding molecule MAGI-1[208,209]. This requires glutamate signaling from the mechanosensory neurons, where EAT-4, a homologue of the vesicular glutamate transporter VGLUT1, is involved[236,237]. This process also requires CREB, though the targets are not understood[238]. The existence of multiple different pathways to modulate mechanosensitivity indicates that *C. elegans* is able to fine-tune its behavior depending on the type stimulus.

Touch habituation is modulated by the presence of food: on food, worms habituate slowly (taking 15+ taps to go from >90% responding to <50% responding), while off food they habituate rapidly (reaching a <50% response rate within about 5 taps)[239]. This process is mediated by dopaminergic signaling as animals deficient in dopamine synthesis or lacking the dopamine receptor DOP-1 behave as if they are off food even when on food during touch habituation training, and this can be rescued by exogenous dopamine[239,240]. In the presence of food, dopamine levels increase[241], and within the mechanosensory neurons this is sensed by DOP-1 receptors[240]. DOP-1 is a GPCR, and the Gq subunit EGL-30 as well as other members of the canonical PLCβ pathway are required downstream of DOP-1 for modulation of tap habituation[239]. They likely do so through generation of both DAG (which typically acts by activating protein kinase C) and IP3 (which can activate calcium channels in the ER) as members of both of these downstream pathways are important for tap habituation[239,242]. This shows that like chemoreception, mechanosensory plasticity is sensitive to the environment and that it is modulated by the same types of pathways as vertebrates utilize.

5.2 Nociception and plasticity: ASH

5.2.1 Nociceptive signaling

ASH is the primary nociceptive neuron in *C. elegans* and responds to a number of different stimuli including hyperosmolarity, heavy metals, nose touch, noxious odors, and bitter molecules such as quinine[196]. When the worm encounters these stimuli, ASH responds by increasing calcium levels. ASH then signals to the command interneurons that control forward and backwards locomotion and this causes the animal to reverse away from the stimulus[243]. Calcium transients within ASH require G-protein signaling and the TRPV channel composed of OSM-9 and OCR-2, thought to be activated by polyunsaturated fatty acids. While the TRPV channel is generally required within ASH for responses to multiple stimuli, the upstream GPCR signaling is thought to be distinct for different stimuli as ASH expresses multiple different G proteins[244] and knocking them out individually reduces ASH responses to specific stimuli rather than reducing nociception generally[245]. For example, knock-out of the Gγ subunit GPC-1 selectively reduces calcium signals in response to quinine, but not glycerol, SDS, or Cu^{2+} (though GPC-1 mutants fail to adapt normally to prolonged or repeated exposure to Cu^{2+})[196], while the Gα subunit ODR-3 is primarily required for response to hyperosmolarity and nose touch but plays a minor role in quinine sensation[245,246]. In addition to G proteins, multiple other molecules are critical for responding to specific noxious stimuli sensed by ASK[243,247–249]. The diversity of signaling mechanisms converging upon a common calcium channel allows for modulation to occur both on the individual stimulus level and for avoidance as a whole.

5.2.2 Nociceptive plasticity

Like other sensory neurons, ASH responses are plastic. After repeated exposure to aversive stimuli, ASH calcium responses are diminished and the worm is less likely to reverse. This habituation is stimulus specific as other stimuli evoke normal levels of aversion, so it likely occurs at the level of G-protein signaling, either affecting the GPCRs themselves or the effects of G-proteins[196,248]. For most stimuli, the exact players in down-regulating responses are unknown, but in quinine sensation it is known that cGMP desensitizes ASH neurons in a similar manner as in AWC (see section 5.1.1.1). Within ASH, cGMP binds to and activates the protein kinase-G EGL-4, which in turn phosphorylates and activates the G-protein regulators RGS-2 and RGS-3[250]. These in turn down regulate G-protein signaling downstream of the quinine receptor, though the players involved are unknown. Decreased G-protein signaling leads to smaller calcium transients and thus less quinine avoidance. In cases of low cGMP (ie, knock-out of the guanylyl cyclases ODR-1, GCY-27, GCY-33, and GCY-34 that manufacture cGMP) animals are hypersensitive to quinine, while in cases of high cGMP (ie, over-expression of guanylyl cyclases within ASH), quinine responses are dampened[250]. Interestingly, there are no known guanylyl cyclases natively expressed within ASH, suggesting that such regulation comes from an external source. Indeed, expression of the guanylyl cyclase GCY-27 within ASK is sufficient to rescue quinine hypersensitivity in GCY-27 knock-outs[250] and expression of ODR-1 within AWC is sufficient to rescue quinine hypersensitivity in ODR-1 knock-outs[251]. It is unclear why cGMP must come from external sources rather than within ASH itself, though as cGMP levels also impact responses to other,

non-ASH-sensed stimuli, cGMP levels within a network of sensory neurons may serve to simultaneously regulate multiple processes.

In addition to being able to specifically modulate responses to individual noxious stimuli, ASH is sensitive to environmental signals such as food, which regulate its sensitivity more broadly through monoamines. In the presence of food, *C. elegans* becomes more sensitive to noxious stimuli such as Cu^{2+}, hyperosmolarity, and bitter tastants[205]. This occurs via strengthened calcium responses mediated by dopamine signaling through the DOP-4 receptors, though it is unclear how DOP-4 signaling ultimately affects calcium levels. The impact of serotonin (levels of which are also increased in the presence of food) on ASH signaling is complex. While serotonin increases sensitivity to many, if not all, noxious stimuli, there are conflicting reports regarding whether it does so by increasing or decreasing calcium levels, and in fact may do both through different actions mediated by the SER-5 receptor. Calcium transients in response to nose touch are potentiated through an unknown mechanism in the presence of serotonin[196]. In response to 1-octanol however, sensitization occurs in a complex concentration-dependent manner both up- and downstream of calcium signaling. In the presence of food, calcium responses to dilute 1-octanol are increased by serotonin through a mechanism involving the Gα protein GPA-11[252,253]. In response to high concentrations of 1-octanol however, additional neurons (ADL and AWB) are recruited and serotonin strengthens the synapses between the sensory neurons and command interneurons[252]. Confusingly in some cases serotonin even seems to decrease calcium signaling within ASH. In this case, the data suggests that in response to serotonin signaling via the SER-2

receptor, calcium may act not as a depolarizing ion, but as an intracellular messenger that inhibits L-type voltage gated calcium channels (EGL-19) that normally inhibit ASH responses by activating the K+ channel SLO-1[254]. Thus, calcium levels within ASH may not always be a reliable indicator of depolarization, but the presence of food nonetheless sensitizes ASH via monoamine signaling.

ASH has an extremely diverse range of plasticity mechanisms among sensory neurons. There are a few reasons this may be the case: 1) the potential diversity of plasticity mechanisms within other cells has not been fully explored. This reason can not be ruled out before more research is done into modulatory mechanisms in other cells. 2) ASH is especially polymodal. *C. elegans* sensory neurons generally respond to multiple different stimuli, though in many cells all the stimuli are in only one or two classes (ie, volatile odorants or soluble tastants). Additionally, ASH is the primary nociceptor; sensation of attractive cues is distributed over a number of neurons[194]. With such diversity in stimuli type and importance, ASH has a uniquely wide variety of signals that each must be carefully modulated. This seems likely to be one reason for the diversity of modulatory mechanisms. 3) Nociception is extremely important for an organism's survival, so fine-tuning responses to the wide variety of negative stimuli an animal encounters in its environment is crucial for survival. The existence of so many modulatory pathways allows *C. elegans* to both adjust its sensitivity to individual stimuli and regulate its overall sensitivity. If aversive stimuli are indicative of dangerous environments, why would an organism ever want to decrease its sensitivity to such cues? The answer to this question can be illustrated with the example of reciprocal inhibition between ASH and ASI. ASI

responds to nutritional cues such as soluble tastants and bacterial metabolites and regulates the sensation of satiety[228,255]. When presented with two opposing cues, *C. elegans* must make a choice which to prioritize, and this choice is influenced by physiological state. While the presence of food increases ASH responses, in starved worms ASH responses to aversive cues are weakened and ASI responses to nutritious cues are potentiated[256]. This is mediated in part by a mutual inhibition between the two cells involving the neurons ADF and RIC[257]. This allows for the organism to balance opposing cues. Well-fed worms can afford to ignore signals of food in order to avoid potentially harmful environments. Starved worms however, must risk being exposed to noxious chemicals or pathogenic bacteria in order to eat, so any hint of food suppresses avoidance. Regulating this balance in signaling is also likely to be one of the reasons ASH is so heavily modulated as its many different sensory pathways need to be regulated by a lot of environmental and physiological information. This thesis will introduce yet another modulatory mechanism to the existing framework.

6. Manuscript

INX-18 and INX-19 play distinct roles in electrical synapses that modulate aversive behavior in Caenorhabditis elegans

Short Title: Electrical synapses in behavior modulation

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Abstract

In order to respond to changing environments and fluctuations in internal states, animals adjust their behavior through diverse neuromodulatory mechanisms. In this study we show that electrical synapses between the ASH primary quinine-detecting sensory neurons and the neighboring ASK neurons are required for modulating the aversive response to the bitter tastant quinine in *C. elegans*. Mutant worms that lack the electrical synapse proteins INX-18 and INX-19 become hypersensitive to dilute quinine. Cell-specific rescue experiments indicate that *inx-18* operates in ASK while *inx-19* is required in both ASK and ASH for proper quinine sensitivity. Imaging analyses find that INX-19 in ASK and ASH localizes to the same regions in the nerve ring, suggesting that both sides of ASK-ASH electrical synapses contain INX-19. While *inx-18* and *inx-19* mutant animals have a similar behavioral phenotype, several lines of evidence suggest the proteins encoded by these genes play different roles in modulating the aversive quinine response. First, INX-18 and INX-19 localize to different regions of the nerve ring, indicating that they are not present in the same synapses. Second, removing *inx-18* disrupts the distribution of INX-19, while removing *inx-19* does not alter INX-18 localization. Finally, by using a fluorescent cGMP reporter, we find that INX-18 and INX-19 have distinct roles in establishing cGMP levels in ASK and ASH. Together, these results demonstrate that electrical synapses containing INX-18 and INX-19 facilitate modulation of ASH nociceptive signaling. Our findings support the idea that a network of electrical synapses mediates cGMP exchange between neurons, enabling modulation of sensory responses and behavior.

Author Summary

Animals are constantly adjusting their behavior to respond to changes in the environment or to their internal state. This behavior modulation is achieved by altering the activity of neurons and circuits through a variety of neuroplasticity mechanisms. Chemical synapses are known to impact neuroplasticity in several different ways, but the diversity of mechanisms by which electrical synapses contribute is still being investigated. Electrical synapses are specialized sites of connection between neurons where ions and small signaling molecules can pass directly from one cell to the next. By passing small molecules through electrical synapses, neurons may be able to modify the activity of their neighbors. In this study we identify two genes that contribute to electrical synapses between two sensory neurons in *C. elegans*. We show that these electrical synapses are crucial for proper modulation of sensory responses, as without them animals are overly responsive to an aversive stimulus. In addition to pinpointing their sites of action, we present evidence that they may be contributing to neuromodulation by facilitating passage of the small molecule cGMP between neurons. Our work provides evidence for a role of electrical synapses in regulating animal behavior.

Introduction

A defining feature of animal behavior is its plasticity. Animals adapt their behavior in order to respond to environmental challenges and physiological changes. Such behavioral plasticity is essential for animal survival and is achieved by changing the activity of neurons and circuits in a variety of ways. One way is through neuromodulation, whereby diffusible signals such as neuropeptides, dopamine, and serotonin are used to tune brain activity in broad regions[1,63,258]. By contrast, neuronal activity can be altered locally by changing the strength of individual synapses[17,259]. In order to understand dynamic brain function, it is crucial to uncover mechanisms that drive neuroplasticity at various levels.

Electrical synapses (also known as gap junctions) are composed of membrane channels that join the cytoplasm of two cells[260]. They are found throughout vertebrate and invertebrate nervous systems[81,87,89,260] where they pass both electrical and chemical signals between connected cells[86]. Electrical synapses have been primarily studied for their ability to synchronize electrical activity between pairs or groups of neurons[100–102], but can also pass small molecules such as calcium[103,104], cAMP[105–108], cGMP[106,109], IP_3[104,110], and even small miRNA[116,117]. Interestingly, while electrical synapses share similar function and protein topology in vertebrates and invertebrates[93], genes encoding electrical synapse components are evolutionarily unrelated[86,260]. As a result, electrical synapses in vertebrates are composed of connexins, while those in invertebrates are composed of innexins (INXs). The separate evolution of electrical synapses suggests the functional

necessity of these channels, although their role in neural plasticity and brain function is not fully understood.

Recently, it was discovered that innexin networks play a crucial role in cGMP-dependent sensory modulation in *Caenorhabditis elegans*[251]. Krzyzanowski and colleagues found that cGMP functions within the sensory neuron ASH to dampen nociceptive sensitivity but is produced in neighboring neurons[250]. They further showed that cGMP-mediated dampening of ASH nociceptive sensitivity requires an innexin-based network[251]. These findings uncover a new strategy of network regulation that may contribute to the modulation of neural activity. ASH is the primary nociceptive neuron pair in *C. elegans* and responds with increased calcium levels to diverse aversive stimuli including hyperosmolarity, nose touch, heavy metals such as copper, volatile repellents such as octanol and alkaloids such as quinine[194,196,243,245,261–263]. ASH controls movement away from noxious stimuli through synapses on the forward and backward command interneurons.[264,265] Nociception in ASH is extensively modulated, and reactivity to aversive stimuli such as quinine is regulated by the presence of food and the satiety state of the worm[205,251,252,266–268]. Notably, ASH forms electrical synapses with multiple other sensory neurons and a few interneurons[269,270], suggesting electrical synapses may be crucial in modulating its activity.

We investigated the impact of electrical synapses between ASH and its neighbor ASK on behavioral sensitivity to the bitter tastant quinine. ASK forms multiple electrical synapses with ASH[270] and expresses several innexins[89,186,271], making it a

candidate for directly modifying ASH activity. Results of this study show that the electrical synapse proteins INX-18 and INX-19 function within ASK and ASH to allow for modulation of the quinine avoidance response. Through imaging, we found that INX-18 and INX-19 localize to known sites of electrical synapses. Our data further suggest that INX-19 plays a principle role in diffusion of cGMP from ASK to ASH. Our study identifies a direct connection between two sensory neurons that modulates neuronal activity and thus regulates behavior in *C. elegans*.

Results

Innexin-18 and innexin-19 are required for modulation of the quinine response

A recent study suggests that a network of electrical synapses is involved in modulation of the quinine response[251], however the exact composition of those electrical synapses has not been determined. ASH is a multimodal nociceptive neuron that responds to quinine and forms direct electrical synaptic connections with the sensory neuron ASK[269,270], which is also involved in quinine sensation[245]. To explore whether the electrical synapses between ASK and ASH play a role in modulating quinine sensitivity, we investigated the innexins INX-18 and INX-19 that are expressed in these two sensory neurons[89,186,271]. While INX-4 is also expressed in ASH, we did not include it in our analyses as it has already been explored in a previous study[251].

To determine whether INX-18 and/or INX-19 play a role in modulating the behavioral response to quinine, we assayed *inx-18(ok2454), inx-19(ky634)* and *inx-19(tm1896)* mutant animals (figure 1A-B) for quinine sensitivity. We placed drops

of quinine solution in front of freely crawling worms and recorded their responses as "responding" if they reverse or "non-responding" if they continue forward[245,247]. We found that these mutant animals were hypersensitive to 1 mM quinine in the quinine drop test (figure 1C). As a negative control, we examined the response of mutant animals to M13 buffer. Both *inx-18(ok2454)* and *inx-19(tm1896)* animals responded to M13 buffer at similar levels to wild-type (N2) animals, *inx-19(ky634)* animals, however, were slightly more responsive than wild-type animals (figure S1A). This may be because this strain has mildly increased spontaneous reversal rates (see below). As a positive control, we tested the response of mutant animals to a high concentration of quinine (10 mM) that that is strongly aversive to wild-type animals. We found that that all strains respond similarly to presentation of 10 mM quinine (figure S1B). Together, these data show that *inx-18(ok2454), inx-19(ky634)* and *inx-19(tm1896)*mutant animals have increased quinine avoidance, suggesting that ASH activity is increased in the absence of these electrical synapse components.

The inx-19(tm1896) allele alters quinine responses without affecting locomotion

Two different *inx-19* alleles (*tm1896* and *ky634*) have been identified and implicated in sensory neuron function[186]. While mutant animals with either allele show increased response to 1 mM quinine (figure 1C), these two alleles have different impacts on locomotion. First, *inx-19(ky634)* mutant animals exhibited more reversals in response to M13 (figure S1A). Second, during locomotion, *inx-19(ky634)* animals spontaneously reversed more frequently in the absence of stimuli (figure S2A). Third, the average crawling velocity of *inx-19(ky634)* mutant animals was lower than that of wild-type animals (figure S2B). These data suggest

that *inx-19(ky634)* animals have altered movement in addition to changes in quinine response. At a molecular level, *inx-19(ky634)* is a G→A single nucleotide polymorphism causing an E70K substitution within the first extracellular loop of INX-19, while *inx-19(tm1896)* is a 546 basepair deletion that removes the majority of the first intracellular loop and a portion of the second transmembrane domain of INX-19 (figure 1A). Because the function of innexins requires their transmembrane domains, *tm1896* is likely to be a strong loss-of-function or null allele. By contrast, a substitution within the extracellular docking domain may have a more complicated effect on protein function. For this reason, *inx-19(tm1896)* animals were utilized for the remainder of the experiments.

Inx-19 is required in both ASK and ASH for modulation of the quinine response

Inx-19 is expressed in multiple tissues such as neurons and muscles. Even within the nervous system, *inx-19* is expressed in ASH as well as a number of other neurons, including ASK, which has been implicated in quinine sensation and its regulation[186,245,271]. To determine the site of action of INX-19, we performed a series of rescue experiments with *inx-19* cDNA fused to fluorphores in the *inx-19(tm1896)* background. We found that, under the control of the native *inx-19* promoter[186], expression of *inx-19* cDNA fully rescued quinine hypersensitivity in response to 1 mM quinine (figure 2A). This demonstrates that *inx-19* cDNA is functional and the *inx-19* mutation is responsible for the quinine hypersensitivity phenotype. Interestingly, these worms also showed reduced response to 10 mM quinine, suggesting that INX-19 overexpression could cause over-correction of the quinine sensitivity defects (figure S3A).

We then expressed GFP or mCherry-tagged *inx-19* cDNA under the control of cell-selective promoters to determine in which neurons INX-19 acts to regulate quinine sensitivity. We found that expression of *inx-19* cDNA in either ASK or ASH (using *Psra-9*[272] and *Posm-10*[248,273], respectively) did not significantly restore the quinine response to 1 mM quinine in *inx-19(tm1896)* animals. In contrast, simultaneous expression of *inx-19* in both ASK and ASH brought 1 mM quinine response rates back to wild-type levels (figure 2A). As controls, we tested the response of these animals to M13 buffer and 10 mM quinine and found no change in sensitivity (figure S3A, B). These data indicate that INX-19 is required in both ASK and ASH for appropriate modulation of quinine sensitivity.

Inx-18 is required in ASK for modulation of the quinine response

Inx-18 is expressed in a subset of neurons including ASK[89,271]. However, unlike *inx-19, inx-18* is not expressed in ASH, indicating that its site of action resides outside of ASH. To determine whether the altered quinine response rate of *inx-18* mutant animals is due to the lack of INX-18 function, we performed rescue experiments using *inx-18*. *Inx-18* does not have an obvious promoter, as several genes lie directly upstream of its genomic position. However, the second intron has been successfully used to drive its expression[274]. To test whether the *inx-18(ok2454)* mutation is responsible for the quinine hypersensitivity phenotype, we cloned *inx-18* gDNA, which included the intronic regions. Expression of *inx-18* gDNA was sufficient to restore responses to 1 mM quinine in *inx-18(ok2454)* mutant animals to wild-type levels, indicating that loss of *inx-18* is the reason for quinine hypersensitivity (figure 2B). Next, we found that the site of action of *inx-18* is in ASK,

as expression of *inx-18* cDNA fused to GFP using the *Psra-9* promoter rescued the quinine hypersensitivity phenotype (figure 2B). As controls, we tested the response of these animals to M13 buffer and 10 mM quinine and found no change in sensitivity (figure S3C, D) These results show that *inx-18* and *inx-19* have distinct, but partially overlapping, sites of action. Combined, our data indicate that INX-19 must be present in both ASK and ASH, while INX-18 in ASK alone is sufficient to modulate the quinine response.

ASK INX-19 and ASH INX-19 localize to the same regions in neighboring axons.

The *C. elegans* wiring diagram suggests that the ASK and ASH neurons form electrical synapses with one another in the nerve ring[269,270], which raises the possibility that INX-18 and INX-19 are components of these electrical synapses. As our behavioral results show that *inx-19* functions in both ASK and ASH, we examined the subcellular localization of INX-19 in these two neurons using fluorescence microscopy. We drove expression of GFP-tagged INX-19 in ASK and mCherry-tagged INX-19 in ASH. These fluorophore-tagged INX-19 constructs are functional as they can restore quinine responses in *inx-19(tm1896)* mutant animals (figure 2A). If INX-19 is a component of electrical synapses between ASK and ASH, we reasoned that INX-19 expressed in ASK would localize to the same regions of the nerve ring as INX-19 expressed in ASH. Our imaging data show that INX-19 forms punctate structures along the axons in the nerve ring when expressed in both cells. As expected, most ASK INX-19 and ASH INX-19 is localized to overlapping puncta, despite the fact that these innexin proteins are in two distinct neurons (figure 3A-D). Quantification of these images show that INX-19 expressed in ASK and ASH

produces puncta that colocalize 67% of the time (figure 3H). These data indicate that INX-19 is present on both sides of the ASK-ASH electrical synapses.

INX-18 rarely colocalizes with INX-19

Our behavioral results indicate that INX-18 functions within ASK to modulate the behavioral response to quinine. To investigate where INX-18 resides in ASK, and whether it is functioning in the same synapses as INX-19, we expressed GFP-tagged INX-18 and asked whether it colocalizes with INX-19 (figure 3E-G). We found that, like INX-19, GFP-tagged INX-18 forms puncta along the axons (figure 3F). However, INX-18 showed low levels of colocalization with mCherry-tagged INX-19 expressed in ASH (~4% colocalization, figure 3H), demonstrating that the vast majority of INX-18 is not in the same synapses as INX-19 in adult animals.

INX-19 localization in ASK requires both inx-18 and inx-19

To determine the relationship between INX-18 and INX-19 localization, we investigated whether the expression patterns of INX-18 and INX-19 are influenced by one another. We expressed fluorescently-tagged *inx-18* and *inx-19* cDNA in ASK and ASH individually and examined their expression patterns in mutant backgrounds. We found that the number of INX-19 puncta in the ASK axon was significantly reduced in *inx-18* mutant animals (figure 4A). In addition, localization of INX-19 within ASK requires INX-19 in other neurons, as the number of ASK INX-19 puncta was diminished in *inx-19(tm1896)* mutant animals (figure 4A). In no cases were the puncta fully eliminated, indicating that only some electrical synapses are affected in each case. We did not observe significant differences in the number of

INX-19 puncta in ASH in *inx-18(ok2454)* or *inx-19(tm1896)* animals, although the downward trend (figure 4B) suggests that INX-19 localization in ASH may need both *inx-18* and *inx-19*. In contrast, INX-18 localization does not appear to require INX-19, as the number of INX-18 puncta in the nerve ring remained unchanged in *inx-19(tm1896)* mutant animals (figure 4C). This indicates that the localization of INX-18 is independent of INX-19. Taken together, these data suggest that *inx-18* plays a role in INX-19 electrical synapse assembly and/or maintenance. Perhaps INX-18 is transiently present in the ASK-ASH synapses during development, but by adulthood INX-18 has been removed from these synapses. Indeed, a number of studies have shown that innexin expression can be developmentally contolled[89,186,271].

Inx-18 and inx-19 have largely overlapping functions

To investigate the functional relationship between *inx-18* and *inx-19*, we assessed the behavioral responses of *inx-18; inx-19* double mutant animals. If these two genes act in parallel to regulate quinine sensitivity, the phenotype of the double mutant should be stronger than that of the single mutants. If, however, *inx-18* and *inx-19* are acting together in the same pathway, we would expect animals with mutations in both genes to have a phenotype of similar strength to the single mutant animals. The *inx-19(tm1896); inx-18(ok254)* double mutants responded at somewhat higher rates than both the *inx-18(ok2454)* and *inx-19(tm1896)* single mutants (figure 4D), but this difference was statistically insignificant. This suggests that the two genes function largely in the same pathway to modulate the quinine response. Together with the visualization data, these findings suggest that while

INX-18 is localized to different electrical synapses than INX-19, its primary function is to set up or maintain INX-19 localization.

Three different possibilities for the function of the ASK-ASH electrical synapses in quinine regulation

In order to determine how *inx-18* and *inx-19* affect ASH activity, we considered three potential mechanisms: First, *inx-18* and *inx-19* mutations may alter the cell fate of ASK or ASH, leading to changes in the quinine sensing circuit. Second, the ASK-ASH electrical synapses could function to shunt calcium, depressing ASH activity by allowing calcium ions to flow out to ASK. In this case, we expect that removal of ASK-ASH electrical synapses would result in increased Ca^{2+} signals in ASH and decreased Ca^{2+} levels in ASK. Finally, the ASK-ASH electrical synapses could pass cGMP from ASK to ASH, thus down-regulating the quinine response in ASH. Indeed, it was previously demonstrated that expressing the guanylyl cyclase GCY-27 in ASK rescued the quinine hypersensitivity in *gcy-27(ok3653)* mutant animals[250], suggesting an important role of cGMP in ASK in modulating quinine responses. We tested these three possibilities by examining cell fate markers, the calcium indicator GCaMP6s, and the fluorescent cGMP reporter FlincG3 in ASK and ASH.

ASK and ASH cell fate and morphology are unchanged in inx-19 and inx-18 mutant animals

Electrical synapse channels are known to regulate cell fate decisions during development[275,276], in particular, *inx-19* has been shown to regulate neural differentiation in *C. elegans*[186]. Thus, it is possible that *inx-19* or *inx-18* also impacts

ASK and/or ASH cell fate or morphology. To test this possibility, we expressed mCherry in ASK (using the *sra-9* promoter) and mTagBFP2 in ASH (using the *osm-10* promoter, which also expresses weakly in ASI). We found that the cell fate of ASK and ASH remained the same in the *inx-18(ok2454)* and *inx-19(tm1896)* mutant animals, as the number of neurons that expressed these fluorescent markers and their positions were unaltered (figure 5). Furthermore, we showed that the morphology of ASK and ASH were identical between wild-type and the mutant animals. Specifically, both ASK and ASH have cell bodies near the terminal bulb of the pharynx, while dendrites extend to the nose tip and axons project into the nerve ring. Additionally, the cell bodies, dendrites, and axons remained clearly visible In wild-type, *inx-19(tm1896)* and *inx-18(ok2454)* mutant animals (figure 5B). Together, these data indicate that there is no gross morphological or cell fate changes to either ASK or ASH upon removal of INX-18 and INX-19.

ASK calcium responses remain unchanged upon removal of ASK-ASH electrical synapses

We examined the possibility that the ASK-ASH electrical synapses function to shunt calcium, thus decreasing behavioral responses to quinine. Previous studies have shown that the ASH neurons respond strongly to quinine with an increase in intracellular calcium[196]. While ASK is known to be a minor player in the quinine response[245], the calcium response of ASK neurons to quinine is unknown. In ASK, attractive stimuli typically result in a decrease in calcium levels, while the aversive stimulus SDS results in a calcium increase[277]. Thus, it is possible that the aversive stimulus quinine also directly triggers a calcium increase in ASK. Alternatively, ASK

may receive calcium ions from the primary quinine-sensing neuron ASH via the ASK-ASH electrical synapses. If the ASK-ASH electrical synapses pass calcium from ASH to ASK, this shunting effect would decrease ASH calcium levels in response to quinine as some of the calcium ions in ASH would flow to ASK in wild-type worms. In contrast, in animals lacking the ASK-ASH electrical synapses, we would expect increased calcium levels in ASH as the flow to ASK would be blocked. If ASK receives calcium from ASH, we would expect any quinine-induced calcium signal in ASK to decrease in mutant animals lacking the ASK-ASH electrical synapses.

We expressed GCaMP6s in ASK and ASH to visualize calcium dynamics in those cells in response to quinine presentation. Because both ASK and ASH are involved in blue-light avoidance behavior[278], the GCaMP6s experiments were carried out in a *lite-1(ce314)* background to eliminate blue-light induced changes of GCaMP6s fluorescence in ASK and ASH. Our results showed that CGaMP6 fluorescence in ASK and ASH increased after switching from buffer to quinine, indicating increased Ca^{2+} levels in response to quinine (figure 6A-B, blue traces). However, Ca^{2+} signals in ASH were much more robust than those in ASK, consistent with the role of ASH as the primary quinine-sensing neuron[245].

To examine the impact of electrical synapses on Ca^{2+} dynamics, we monitored ASK and ASH GCaMP6s fluorescence in mutant *inx-18(ok2454)* and *inx-19(tm1896)* animals. We found that the increase in ASK GCaMP6s fluorescence remained the same between wild-type and mutant worms (figure 6B, 6D, 6F), suggesting that the ASK-ASH electrical synapses are not a main conduit for the ASK Ca^{2+} signal. When

we imaged GCaMP6s fluorescence in ASH, we found the increase in ASH GCaMP6s fluorescence were enhanced in *inx-18 (ok2454)* and *inx-19(tm1896)* animals (figure 6A, 6C, 6E). These results are consistent with the behavioral quinine hypersensitivity observed in these mutant worms. Together, these data show that ASK Ca^{2+} signals do not rely on the ASK-ASH electrical synapses, indicating that Ca^{2+} shunting to ASK is not the primary mechanism of quinine response regulation.

cGMP levels in ASK and ASH are influenced by ASK-ASH electrical synapses

cGMP is required within ASH for down regulation of the quinine response[250]. However, ASH is not known to express any guanylyl cyclases, which produce cGMP. Recently, two studies suggested that guanylyl cyclase expression in other neurons plays a key role in modulating the quinine response[250,251]. These findings prompted us to examine whether ASH acquires cGMP through the ASK-ASH electrical synapses. Indeed, ASK expresses the guanylyl cyclases ODR-1 and GCY-27[279], both of which are known to modify the quinine response[250,251]. If ASK supplies ASH with cGMP through the ASK-ASH electrical synapses, we would expect to observe diminished levels of cGMP in ASH with a compensatory increase within ASK in *inx-18(ok2454)* and *inx-19(tm1896)* mutant animals.

To visualize levels of cGMP within ASK and ASH, we utilized the *C. elegans* codon-optimized version of FlincG3, which contains the cGMP binding domains of protein kinase G1α fused to cpEGFP[280,281]. Binding of cGMP increases FlincG3 fluorescence. We co-expressed FlincG3 and the red fluorescent protein mScarlet under control of the same promoters in ASK and ASH in the *lite-1(ce314)* background (figure 7A).

After crossing the transgenes into *inx-18(ok2454)* and *inx-19(tm1896)*, we imaged FlincG3 fluorescence in ASK and ASH. FlincG3 fluorescence was compared to mScarlet fluorescence to account for variations in expression levels. We found that ASH FlincG3 fluorescence was decreased in both *inx-18(ok2454)* and *inx-19(tm1896)* mutant animals (figure 7B), suggesting a reduction of the basal cGMP levels in ASH. These data are consistent with the behavioral hyper-responsiveness of *inx-18* and *inx-19* mutant worms to dilute quinine, as decreased cGMP levels could lead to increased ASH calcium levels in response to quinine[250,251]. In ASK, FlincG3 fluorescence was increased in *inx-19(tm1896)* mutant animals but was unchanged in *inx-18(ok2454)* animals (figure 7C), suggesting that INX-19-based electrical synapses are primarily responsible for supplying ASH with cGMP from ASK. Together, our data suggest that INX-18 and INX-19 are major components of the ASK-ASH electrical synapses that modulate behavioral sensitivity to quinine, and that they do so by affecting transport of cGMP into ASH.

Discussion

We showed that electrical synapses between the *C. elegans* sensory neurons ASK and ASH play an active role in modifying nociceptive behavior via the passage of cGMP between cells. We found that the innexins INX-18 and INX-19 are required within ASK and ASH for proper modulation of the quinine response, as mutant animals lacking these innexins are hyperresponsive to quinine. These innexins form electrical synapses between ASK and ASH, in which INX-19 is a major component, though INX-18 is important for correct localization of INX-19 synapses in ASK. Our study supports a model in which ASK-ASH electrical synapses facilitate the passage

of cGMP from ASK to ASH. Within ASH, cGMP downregulates calcium signals in response to quinine stimulation, likely by binding to and activating the cGMP-dependent protein kinase EGL-4[250], ultimately leading to a reduction neural activity and thus aversive behavior (figure 8).

Electrical synapses can be made of different combinations of innexin subunits. Homotypic channels contain hemichannels that are composed of the same innexins, while heterotypic channels are made up of hemichannels that are composed of different innexins. The channel composition determines permeability, as heterotypic channels are thought to produce rectified electrical synapses: those that preferentially pass ions and small molecules in one direction rather than equally in both[90,91,163]. Our data suggest that INX-19 is a major component of the ASK-ASH electrical synapses. One possibility is that INX-19 forms homotypic channels. However, some INX-19 synapses do contain INX-18, suggesting that at least some are heterotypic. Though the number of electrical synapses containing both INX-18 and INX-19 is quite small, it is possible that levels of INX-18 within such synapses are generally low, making their visualization difficult. INX-18 could also make electrical synapses with other innexins in ASH. Nonetheless, our results suggest that the main function of INX-18 is carried out through its regulation of INX-19, as the *inx-18* and *inx-19* mutants do not show additive responses to quinine.

The structural makeup of the ASK-ASH electrical synapses has functional implications for ASH modulation. The composition of electrical synapses is key in determining their permeability, and heterotypic composition is a major cause of

rectification[90,91,145,147]. If the ASK-ASH electrical synapses are heterotypic (*i.e.*, consist of both INX-18 and INX-19 hemichannels) and rectified, this could explain why ASK cGMP levels, but not calcium levels, are affected by *inx-18* and *inx-19* mutations. Rectified channels bias the direction of movement of ions and molecules, making it more likely for signals to travel in one direction. If small molecule signals could easily pass from ASK to ASH but not in the reverse direction, cGMP may be more likely to travel from ASK to ASH than Ca^{2+} would be from ASH to ASK. This mechanism could explain why our data suggest movement of cGMP but not Ca^{2+}. Additionally, the permeability of electrical synapses is dependent on the subunits that make up the channels[106,118]. While the permeability of most innexin-based channels is unknown, it is possible that the ASK-ASH electrical synapses are more permeable to cGMP than Ca^{2+}, particularly given the timescales upon which each operate. Electrical synapses have long been considered low-pass filters, preferentially passing signals that change over longer time periods as opposed to quick oscillations[125,282]. Regardless of the molecular reason, the selectivity of electrical synapses to either particular molecules or directions means that they can be sophisticated players within neural circuits. Changes in innexin composition during development or in mature circuits could dramatically impact how the neurons are regulated through the electrical synaptic network.

Electrical synapses are not static structures; they are regulated developmentally as well as in mature circuits[125,127,129,133,271]. Our data suggest that innexins can impact the localization of other innexins even if they are not a permanent part of the same synapses. INX-18 plays a crucial role in the localization of INX-19. Thus, its

main impact on modulating the quinine response may be in supporting the function of INX-19. While INX-18 is required for proper localization of INX-19, an *inx-18* mutation does not eliminate INX-19 synapses completely. This may explain why the *inx-18(ok2454)* mutation does not have an impact on cGMP levels in ASK, as some signaling could still occur through the remaining INX-19-based electrical synapses even in the absence of INX-18.

ASH activity is modulated by cGMP, and yet ASH is not known to express any guanylyl cyclases, which produce cGMP[279,283,284]. This suggests that other neurons may regulate its activity. Such modulation occurs in the context of a larger sensory neuron network that simultaneously assesses many different sensory inputs, any of which could be affecting baseline levels of cGMP within sensory neurons. Thus, by being sensitive to changes in cGMP levels, ASH is able to receive modulatory information from many neurons simultaneously. ASH receives cGMP from its immediate neighbor ASK as well as other neurons[251], suggesting that cGMP levels within ASH (and thus nociceptive sensitivity) are under the control of a number of external signals. If this is the case, cGMP could be a general signal of the state of the worm, integrating multiple signals to indicate whether it is in a favorable or unfavorable circumstance[285–289]. Our data support the notion that electrical synapses regulate function in a sensory neuron network by modulating the passage of small molecules into neurons such as ASH. In this way, multiple sensory inputs such as availability of food or sexual partners, presence of pathogens or other environmental conditions could alter various different behaviors at once.

Figures

Figure 1: Mutations in inx-19 and inx-18 result in hypersensitivity to quinine.

A,B) Diagram of *inx-19* and *inx-18* alleles used. Innexin genes code for proteins that consist of 4 transmembrane helices with intracellular N and C tails. *Inx-19(ky634)* is a SNP resulting in an E>K substitution within the first extracellular loop, while *inx-19(tm1896)* is an in-frame deletion of 546bp that removes most of the intracellular loop and a portion of the third transmembrane domain. *Inx-18(ok2454)* is a ~1800bp deletion that removes the second-fourth transmembrane domains and a portion of the C-terminus. **C)** Quinine Drop Test with 1 mM quinine. *Inx-19(ky634)*, *inx-19(tm1896)*, and *inx-18(ok2454)* mutant animals are hypersensitive to 1 mM quinine, responding a greater percentage of the time. N2 (wild-type)=18%, n=510; *inx-19(ky634)*=65%, n=120, $p<0.0001$; *inx-19(tm1896)*=44%, n=390, $p<0.0001$; *inx-18(ok2454)*=44%, n=350, $p<0.0001$. All groups were compared with a Chi-square test ($p<0.0001$, $\alpha=0.05$), and post-hoc Fisher's Exact tests with Bonferroni's correction ($\alpha=0.0167$) were computed to compare each group to the control.

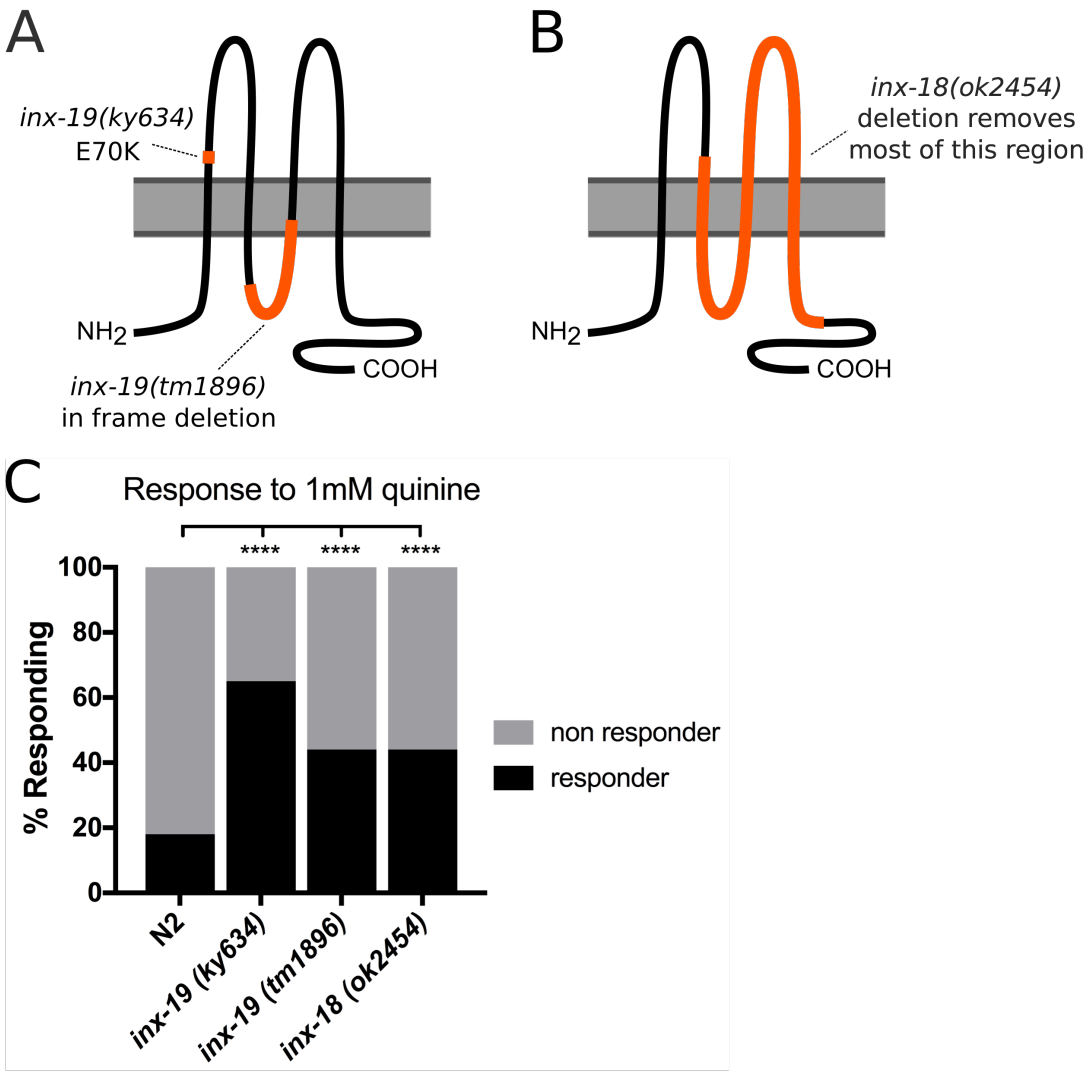

A
inx-19(ky634)
E70K
NH2
COOH
inx-19(tm1896)
in frame deletion
B
inx-18(ok2454)
deletion removes
most of this region
NH2
COOH
C
Response to 1mM quinine
****
****
****
100
80
60
40
20
0
% Responding
non responder
responder
N2
inx-19 (ky634)
inx-19 (tm1896)
inx-18 (ok2454)

Figure 2: Expression of inx-19 and inx-18 in ASK and ASH restores wild-type quinine sensitivity.

A) Expression of *inx-19* isoform A cDNA under the native promoter in *inx-19(tm1896)* animals rescued quinine sensitivity to N2 (wild-type) levels. Expression in ASK (*Psra-9*, which expresses solely in ASK[272]) or ASH (*Posm-10*, which also expresses in the tail neurons PHA and PHB as well as weakly in ASI[248,273]) alone did not significantly rescue the behavior, while simultaneous expression did. N2=15%, n=220; *inx-19(tm1896)*=46%, n=210; *inx-19;Pinx-19::inx-19cDNA*=18%, n=100, p=0.62 vs N2, p<0.0001 vs *inx-19*; *inx-19;Psra-9::inx-19cDNA*=32%, n=100, p=0.0009 vs N2, p=0.02 vs *inx-19*; *inx-19;Posm-10::inx-19cDNA*=37%, n=110, p<0.0001 vs N2, p=0.13 vs *inx-19*; *inx-19;Psra-9::inx-19cDNA; Posm-10::inx-19cDNA* =22%, n=110, p=0.16 vs N2, p<0.0001 vs *inx-19*. All groups were compared with a Chi-square test (p<0.0001, α=0.05), and post-hoc Fisher's Exact tests with Bonferroni's correction (α=0.006) were computed to compare each group to N2 and *inx-19(tm1896)*. All rescues were performed with C-terminal mCherry- or GFP-tagged INX-19 and expression was verified visually before behavioral experiments. **B)** Expression of *inx-18* gDNA in *inx-18(ok2454)* animals rescued the quinine hypersensitivity phenotype, as did expression of *inx-18* cDNA in ASK (*Psra-9*). N2=13%, n=120; *inx-18(ok2454)*=48%, n=120; *inx-18;inx-18gDNA*=12%, n=100, p=0.84 vs N2, p<0.0001 vs *inx-18*; *inx-18;Psra-9::inx-18cDNA*=14%, n=120, p>0.99 vs N2, p<0.0001 vs *inx-18*. All groups were compared with a Chi-square test (p<0.0001, α=0.05), and post-hoc Fisher's Exact tests with Bonferroni's correction (α=0.013) were computed to compare each group to N2 and *inx-18(ok2454)*. All rescues except for gDNA were

performed with C-terminal GFP-tagged INX-18 and expression was verified visually before behavioral experiments.

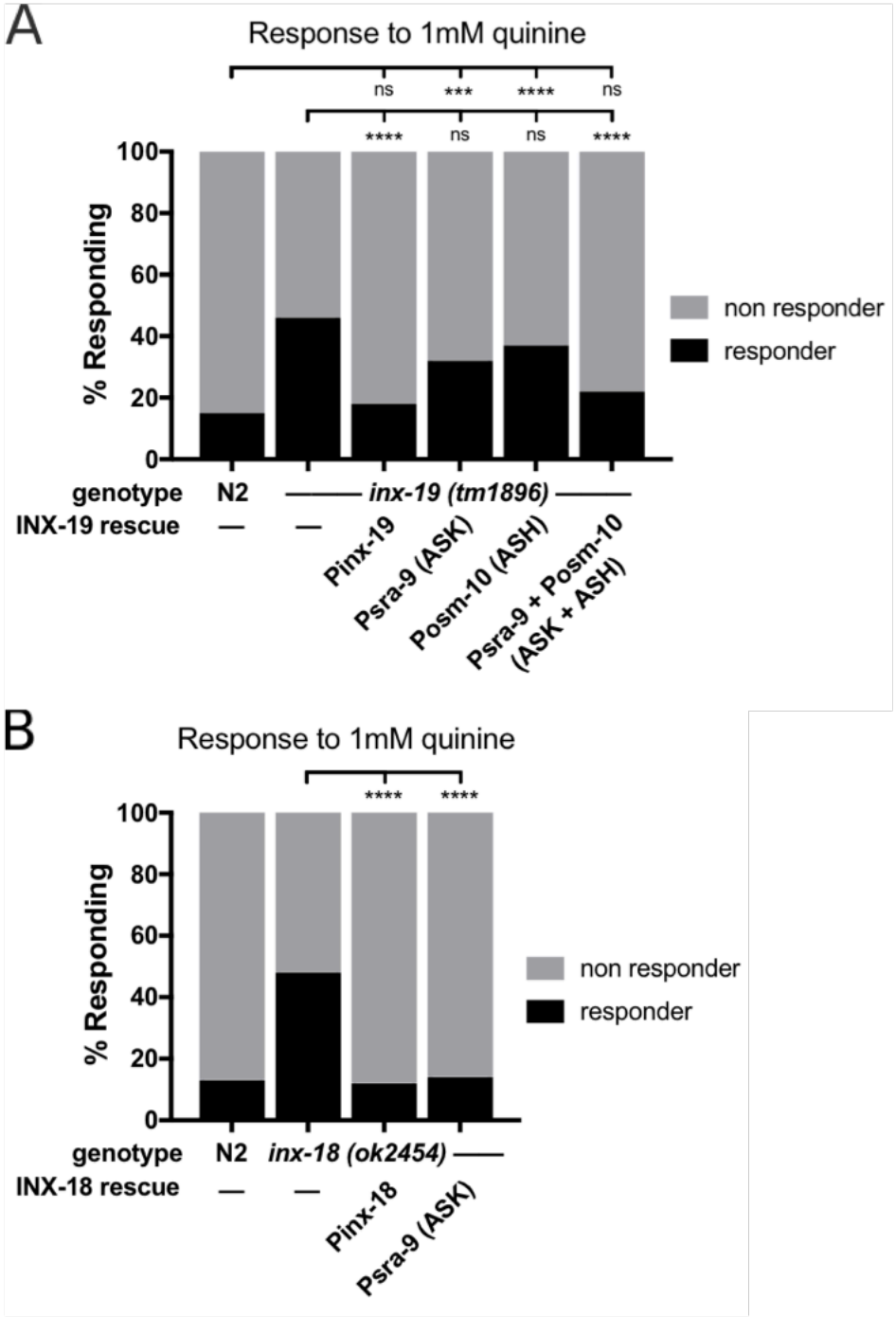
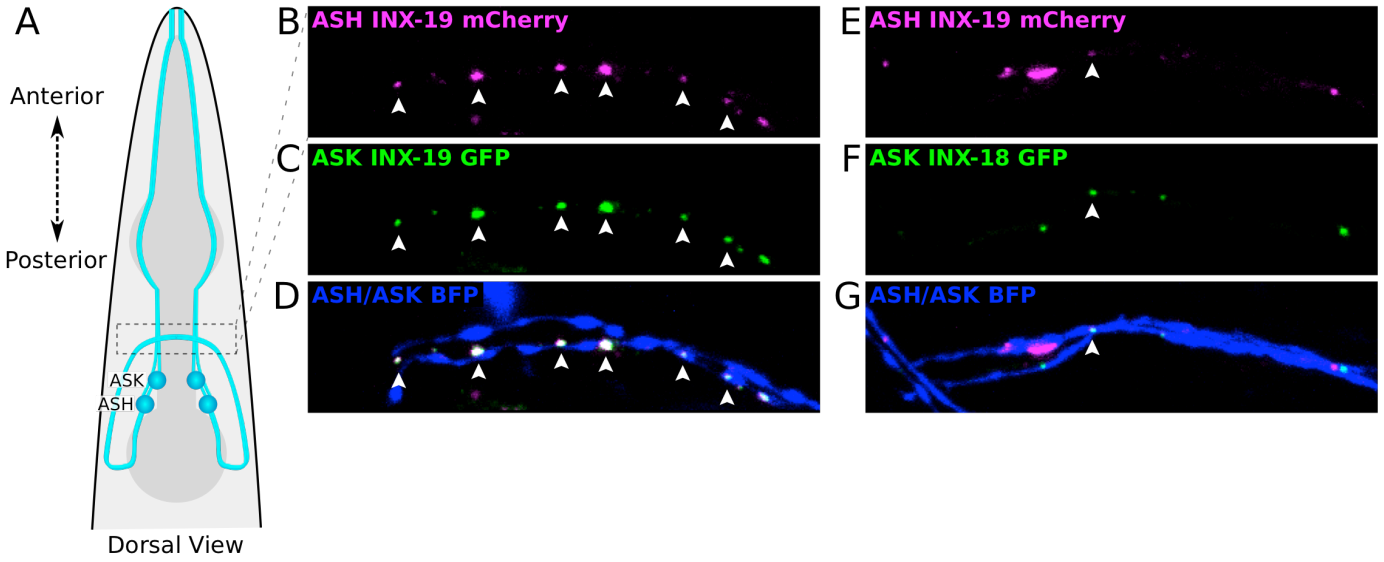

A
Response to 1mM quinine
ns
***
****
ns
****
ns
ns
****
100
80
60
40
20
0
% Responding
non responder
responder
genotype
N2
inx-19 (tm1896)
INX-19 rescue
—
—
Pinx-19
Psra-9 (ASK)
Posm-10 (ASH)
Psra-9 + Posm-10 (ASK + ASH)
B
Response to 1mM quinine
****
****
100
80
60
40
20
0
% Responding
non responder
responder
genotype
N2
inx-18 (ok2454)
INX-18 rescue
—
—
Pinx-18
Psra-9 (ASK)

Figure 3: INX-19 and INX-18 colocalize in the nerve ring when expressed in ASK and ASH

A) Diagram of the *C. elegans* head in a dorsal view. Dashed box indicates the location of imaging of ASK and ASH axons in the nerve ring. **B-D)** INX-19 expressed in both ASK (where it is tagged with GFP) **(B)** and ASH (where it is tagged with mCherry) **(C)** forms multiple puncta that colocalize along the ASK-ASH axons. Points of colocalization are indicated with white arrowheads. ASK and ASH are additionally expressing cytosolic mTagBFP2, seen in the axons that traverse the image, highlighted in **D**. **E-G)** INX-19 tagged with mCherry expressed in ASH **(E)** colocalizes in the nerve ring with GFP-tagged INX-18 expressed in ASK **(F)**. A white arrowhead indicates a point of colocalization. Cytosolic BFP fills the ASK-ASH axons, highlighted in **G**. **H)** Quantification of colocalization. In worms expressing INX-19 in ASK and ASH, 67% of nerve ring puncta colocalize (n=144 puncta in 14 animals). In worms expressing INX-18 in ASK and INX-19 in ASH, ~4% of nerve ring puncta colocalize (n=81 puncta in 10 animals). Each dot represents an individual worm, and error bars are ±SEM.


A
Anterior
Posterior
ASK
ASH
Dorsal View
B
ASH INX-19 mCherry
C
ASK INX-19 GFP
D
ASH/ASK BFP
E
ASH INX-19 mCherry
F
ASK INX-18 GFP
G
ASH/ASK BFP

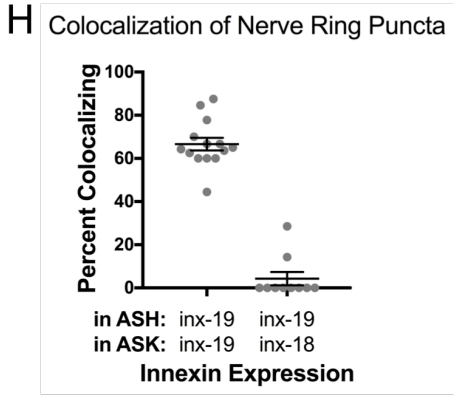

H
Colocalization of Nerve Ring Puncta
Percent Colocalizing
100
80
60
40
20
0
in ASH: inx-19 inx-19
in ASK: inx-19 inx-18
Innexin Expression

Figure 4: inx-18 and inx-19 play distinct roles in ASK-ASH electrical synapse localization and function

A) *inx*-19 cDNA was expressed using *Psra-9* and fluorescent puncta in the nerve ring were counted in N2 (wild-type), *inx-18(ok2454)* and *inx-19(tm1896)* backgrounds. Each dot represents an individual worm and error bars are ±SEM. Ordinary one-way ANOVA between three groups showed significant differences ($F[2,12]=5.763$, $p=0.02$, $\alpha=0.05$). Dunnett's multiple comparison test showed that INX-19 ASK puncta were decreased in *inx-18(ok2454)* (n=5, p=0.01) and in *inx-19(tm1896)* (n=5, p=0.05) in comparison to N2 (n=5). **B)** *inx*-19 cDNA was expressed using *Psrd-10* and puncta in the nerve ring were counted in N2, *inx-18(ok2454)* and *inx-19(tm1896)* backgrounds. Each dot represents an individual worm and error bars are ±SEM. Ordinary one-way ANOVA between three groups showed no significant differences ($F[2,14]=0.814$, $p=0.46$, $\alpha=0.05$). **C)** *inx*-18 cDNA was expressed using *Psra-9* and puncta in the nerve ring were counted in N2, *inx-18(ok2454)* and *inx-19(tm1896)* backgrounds. Each dot represents an individual worm and error bars are ±SEM. Ordinary one-way ANOVA between three groups showed no significant differences ($F[2,13]=1.637$, $p=0.23$, $\alpha=0.05$). **D)** *Inx-18(ok2454);inx-19(tm1896)* double mutant animals were assayed for sensitivity to 1 mM quinine using the quinine drop test. Double mutants responded at higher rates than either *inx-18* or *inx-19* single mutants. N2=18%, n=510; *inx-19(tm1896)*=44%, n=390; *inx-18(ok2454)*=44%, n=350; *inx-19;inx-18*=53%, n=180, p=0.05 vs *inx-19*, p=0.05 vs *inx-18*. All groups were compared with a Chi-square test ($p<0.0001$,

α=0.05), and post-hoc Fisher's Exact tests with Bonferroni's correction (α=0.025) were computed to compare the double mutant to single mutant animals.

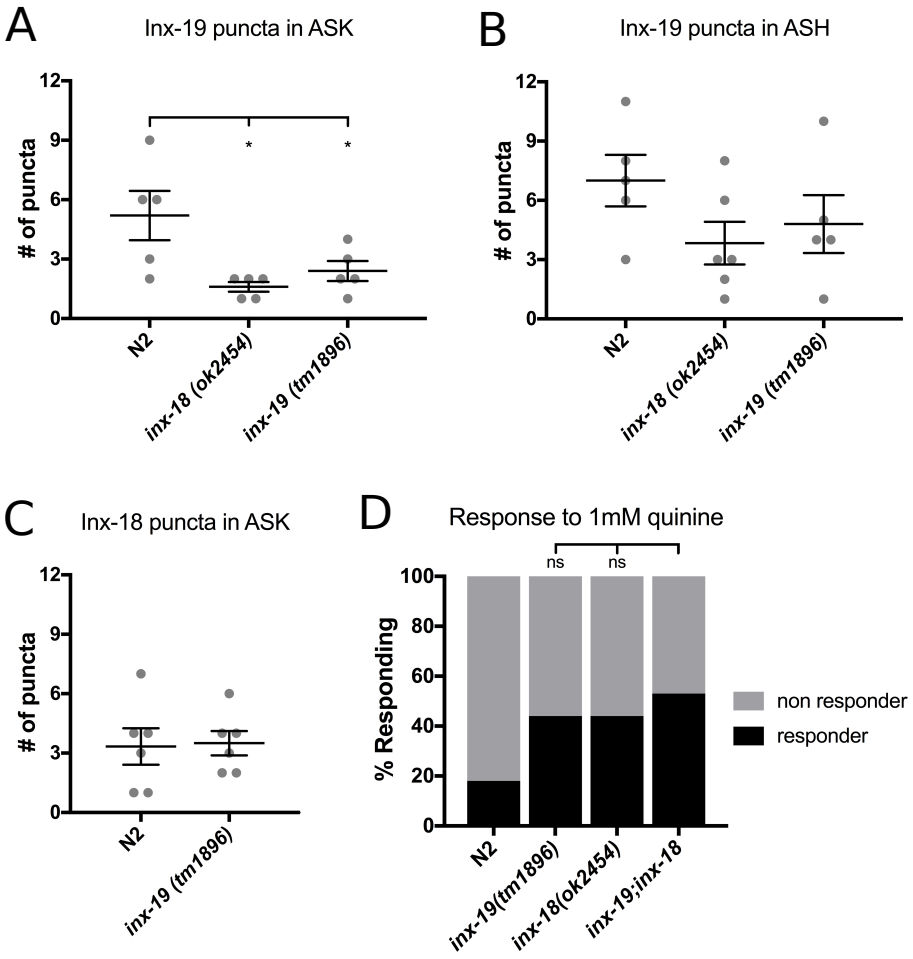

A
Inx-19 puncta in ASK
# of puncta
12
9
6
3
0
*
*
N2
inx-18 (ok2454)
inx-19 (tm1896)
B
Inx-19 puncta in ASH
# of puncta
12
9
6
3
0
N2
inx-18 (ok2454)
inx-19 (tm1896)
C
Inx-18 puncta in ASK
# of puncta
12
9
6
3
0
N2
inx-19 (tm1896)
D
Response to 1mM quinine
ns
ns
% Responding
100
80
60
40
20
0
non responder
responder
N2
inx-19(tm1896)
inx-18(ok2454)
inx-19;inx-18

Figure 5: ASK and ASH architecture is unaltered in inx-18 and inx-19 mutant animals

A) Diagram of neural architecture of ASK, ASH, and ASI in the *C. elegans* head. The dendrites reach out to the nose while the axons extend from the cell body into the nerve ring around the isthmus of the pharynx. **B-D)** Representative confocal images of the worm head with *Psra-9::mCherry* (ASK) and *Posm-10::bfp* (ASH and weakly in ASI) show cell bodies, dendrites extending to the nose, and axons projecting into the nerve ring. Images on the left include maximum intensity projections of the mCherry and BFP images superimposed upon a brightfield image to show location of cells; images on the right are maximum intensity projections of the mCherry and BFP channels without the brightfield image to show details of the cell architecture. Comparison between N2 (wild-type), *inx-19(tm1896)*, and *inx-18(ok2454)* (15-20 animals per genotype were imaged) show no major differences in cell architecture.

A
Dorsal
Ventral
ASH
ASI
ASK
B
N2
C
inx-19
(tm1896)
D
inx-18
(ok2454)
ASK ASH +ASI

Figure 6: ASK Ca^{2+} responses to quinine presentation are unaltered in inx-18 and inx-19 mutant animals while ASH Ca^{2+} responses are heightened in both

A) GCaMP6s fluorescence intensity in ASH in response to 10 mM quinine. Cells were imaged for 30s with presentation of quinine at 10s. The *lite-1(ce314)* mutation was included to eliminate blue-light induced calcium responses in ASK and ASH. All genotypes showed an increase in ASH GCaMP6s fluorescence in response to quinine presentation, though for *lite-1;inx-19(tm1896)* and *lite-1;inx-18(ok2454)* animals the response is larger and faster than that of *lite-1(ce314).* Averaged GCaMP6s traces are shown and error bars are ±SEM. n=48 animals for all genotypes tested. **B)** GCaMP6s fluorescence intensity in ASK in response to 10 mM quinine. ASK showed small increases of GCaMP6s signals and there were no significant differences between genotypes. Averaged GCaMP traces are shown and error bars are ±SEM. n=24, n=21 and n=22 animals imaged for *lite-1(ce314)*, *lite-1;inx-19* and *lite-1;inx-18*, respectively. **C, D)** Heatmaps showing individual traces from all worms analyzed. Data points in the heatmaps represent GCaMP6s signals normalized to the averaged fluorescence intensity of the first 3 seconds of imaging. **E)** Quantification of ASH fluorescence change at four seconds after quinine stimulation. One-way ANOVA between three groups showed significant differences ($F[2,141]=3.89$, $p=0.02$, $\alpha=0.05$), and Dunnett's multiple comparison test showed that mean ASH GCaMP6s fluorescence change in *lite-1(ce314)* animals (n=48) differed from both *lite-1;inx-19* (n=48, p=0.02) and *lite-1;inx-18* (n=48, p=0.05) animals. **F)** Quantification of ASK fluorescence change four seconds after quinine stimulation. One-way ANOVA between three groups showed no significant differences in ASK GCaMP6s

fluorescence ($F[2,64]=0.202$, $p=0.817$, $\alpha=0.05$) between *lite-1(ce314)* (n=24), *lite-1;inx-19* (n=21) and *lite-1;inx-18* animals (n=22).

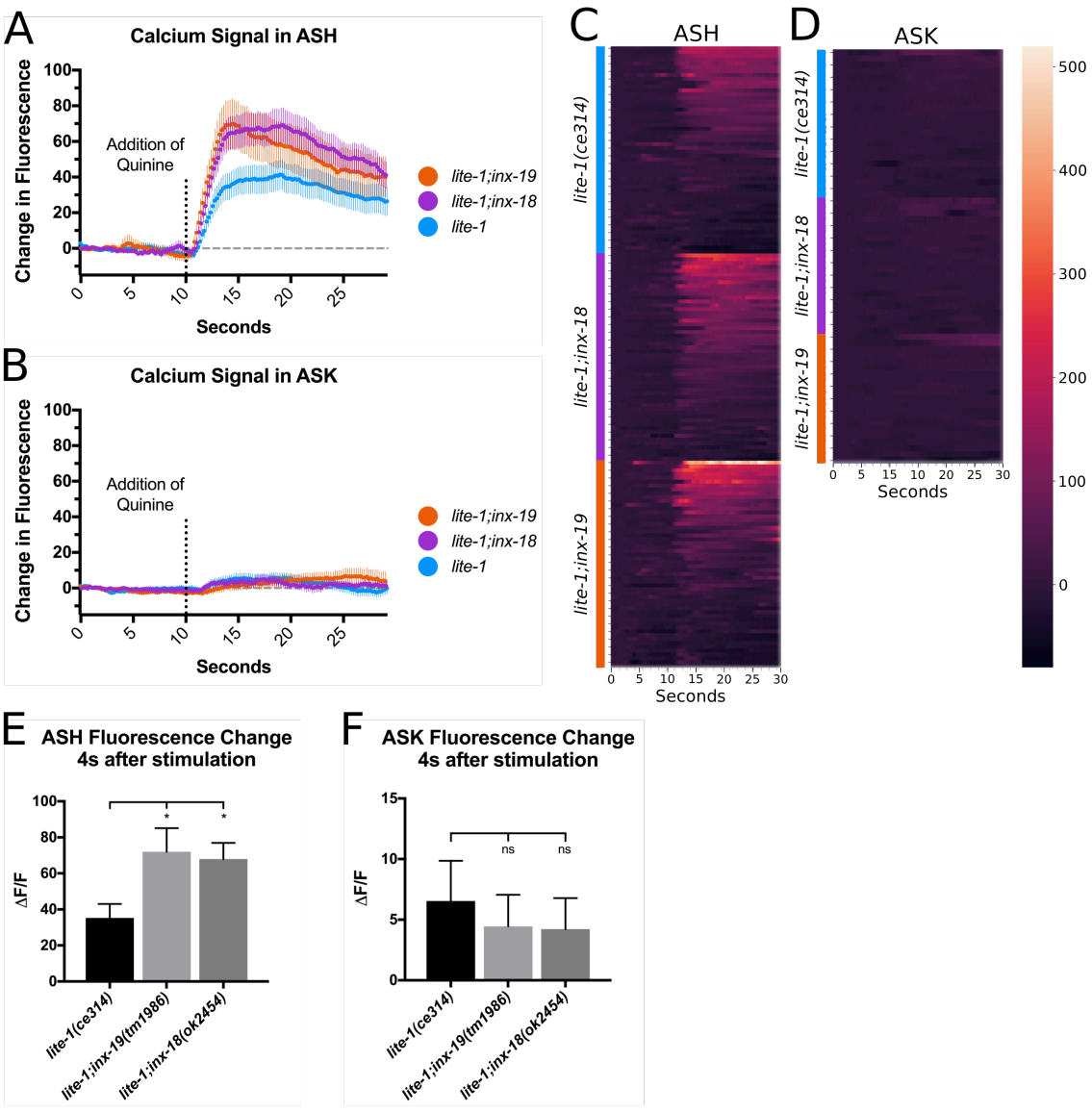

A
Calcium Signal in ASH
Change in Fluorescence
Addition of Quinine
Seconds
lite-1;inx-19
lite-1;inx-18
lite-1
B
Calcium Signal in ASK
C
ASH
D
ASK
lite-1(ce314)
lite-1;inx-18
lite-1;inx-19
E
ASH Fluorescence Change 4s after stimulation
ΔF/F
F
ASK Fluorescence Change 4s after stimulation
ns

Figure 7: Mutations in inx-18 and inx-19 disrupt endogenous cGMP levels in ASK and ASH

A) Diagram of FlincG3 .The cGMP binding domains of PKG 1α (blue) are followed by circularly permuted EGFP (green) and a short PKG 1α tail (blue). WingG2 increases in brightness in response to cGMP. **B)** Example of FlincG3 and mScarlet expression within ASH. Ellipses were drawn around the cell body to measure fluorescence intensity. **C)** cGMP levels within the ASH cell body. The ratio between mean fluorescence intensity of FlincG3 and mScarlet signals was determined for each genotype. Decreases in ASH FlincG3 fluorescence were found in *inx-18(ok2454)* and *inx-19(tm1896)* mutant animals when compared to wild-type worms. Each data point was obtained from a single cell; error bars are ±SEM. One-way ANOVA between three groups showed significant differences ($F[2,68]=3.643$, $p=0.03$, $\alpha=0.05$), and Dunnett's multiple comparison test showed that mean fluorescence intensity in *lite-1(ce314)* (n=24) cells differed from both *lite-1;inx-18* cells (n=24, $p=0.05$) and *lite-1;inx-19* cells (n=23, $p=0.04$). **D)** cGMP levels within the ASK cell body. ASK FlincG3 fluorescence was not altered in *inx-18(ok2454)* mutant animals, and increased in *inx-19(tm1896)* mutant animals when compared to wild-type animals. Each data point was obtained from a single cell; error bars are ±SEM. One-way ANOVA between three groups showed significant differences ($F[2,72]=8.115$, $p=0.0007$, $\alpha=0.05$), and Dunnett's multiple comparison test showed that mean fluorescence intensity in *lite-1(ce314)* cells (n=26) did not differ from *lite-1;inx-18* cells (n=25, $p=0.87$) but was increased in *lite-1;inx-19* cells (n=24, $p=0.0008$).

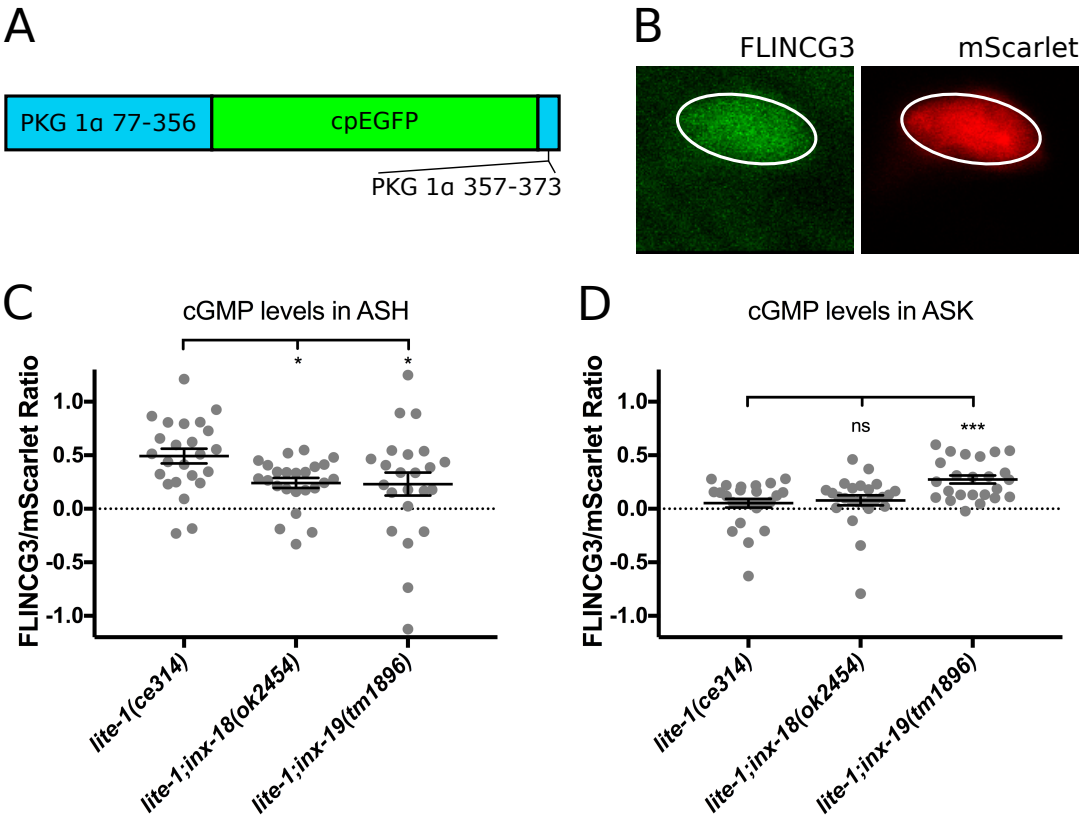

A
PKG 1α 77-356
cpEGFP
PKG 1α 357-373
B
FLINCG3
mScarlet
C
cGMP levels in ASH
FLINCG3/mScarlet Ratio
1.0
0.5
0.0
-0.5
-1.0
*
*
lite-1(ce314)
lite-1;inx-18(ok2454)
lite-1;inx-19(tm1896)
D
cGMP levels in ASK
FLINCG3/mScarlet Ratio
1.0
0.5
0.0
-0.5
-1.0
ns
***
lite-1(ce314)
lite-1;inx-18(ok2454)
lite-1;inx-19(tm1896)

Figure 8: Model of ASK-ASH electrical synapse facilitation of ASH modulation

Our study supports a model in which ASK-ASH electrical synapses facilitate the passage of cGMP from ASK to ASH. Within ASH, cGMP downregulates calcium signals in response to quinine stimulation, leading to a reduction in aversive behavior. INX-19 (orange) is shown on both sides of the ASK-ASH electrical synapses while INX-18 (purple) is shown joining with an unknown innexin and contributing to INX-19-based synapse localization.

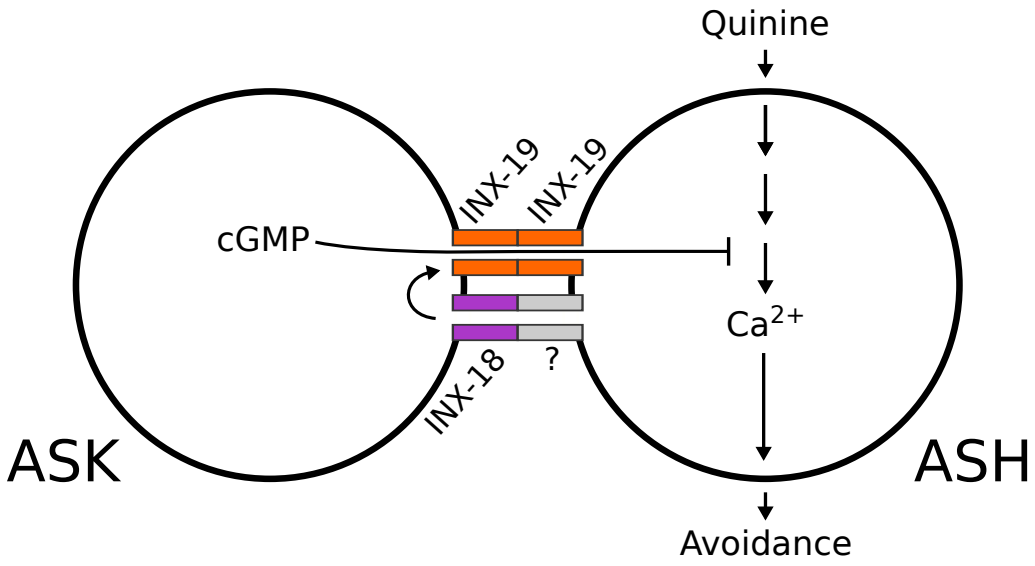


Figure S1: inx-18 and inx-19 mutant animals respond normally to control solutions

A) *inx-19(tm1896)* and *inx-18(ok2454)* mutant animals respond at N2 (wild-type) levels when presented with M13 buffer, while *inx-19(ky634)* animals respond slightly more than wild-type animals. N2=13%, n=330; *inx-19(ky634)*=23%, n=120, p=0.012 ; *inx-19(tm1896)*=19%, n=210, p=0.07; *inx-18(ok2454)*=16%, n=160, p=0.33. All groups were compared with a Chi-square test (p=0.05, α=0.05), and post-hoc Fisher's Exact tests with Bonferroni's correction (α=0.017) were computed to compare each group to the control. **B)** *inx-19(ky634)*, *inx-19(tm1896)*, and *inx-18(ok2454)* mutant animals respond at wild-type levels when presented with 10 mM quinine. N2=93%, n=330; *inx-19(ky634)*=97%, n=120, p=0.18; *inx-19(tm1896)*=97%, n=210, p=0.03; *inx-18(ok2454)*=98%, n=120, p=0.02. All groups were compared with a Chi-square test (p=0.02, α=0.05), and post-hoc Fisher's Exact tests with Bonferroni's correction (α=0.017) were computed to compare each group to the control.

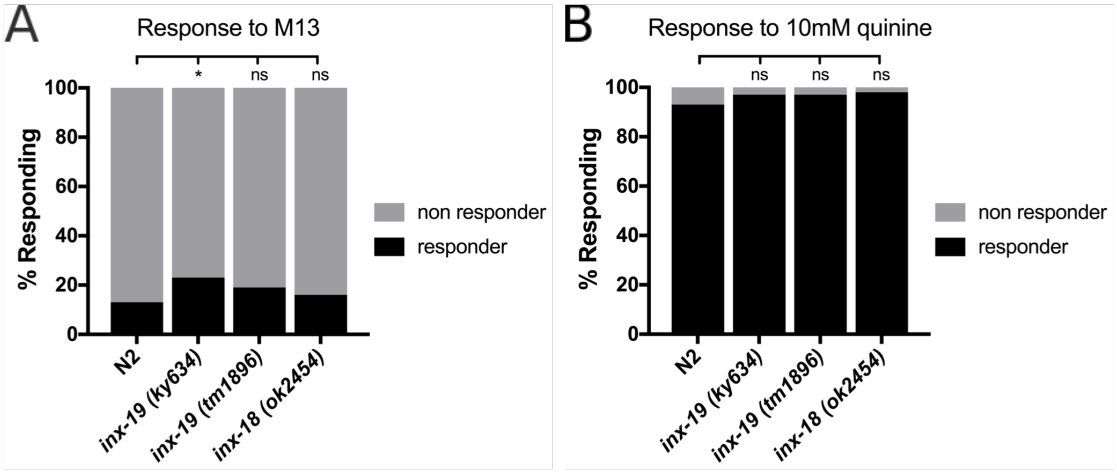


Figure S2: inx-19(ky634) mutant animals have movement defects

A) *Inx-19(ky634)* mutant animals reverse more frequently than N2 (wild-type) animals. Number of reversals were counted from a one-minute video. One-way ANOVA between three groups showed significant differences ($F[2,99]=6.943$, $p=0.0015$, $\alpha=0.05$), and Dunnett's multiple comparison test showed that N2 (n=34) differed from *inx-19(ky634)* (n=33, p=0.0006) but not *inx-19(tm1896)*(n=35, p=0.097). **B)** *inx-19(ky634)* mutant animals have lower average movement velocity than N2 animals. One-way ANOVA between three groups showed significant differences ($F[2,99]=6.089$, $p=0.003$, $\alpha=0.05$), and Dunnett's multiple comparison test showed that N2 (n=34) differed from *inx-19(ky634)* (n=33, p=0.021) but not *inx-19(tm1896)*(n=35, p=0.677). Each data point represents a single worm and error bars are ±SEM.

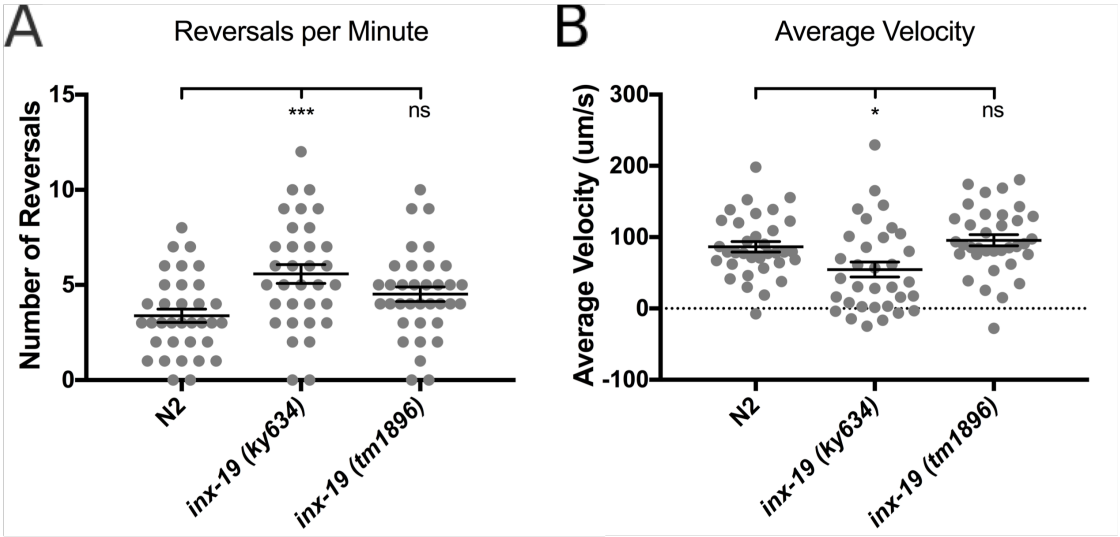
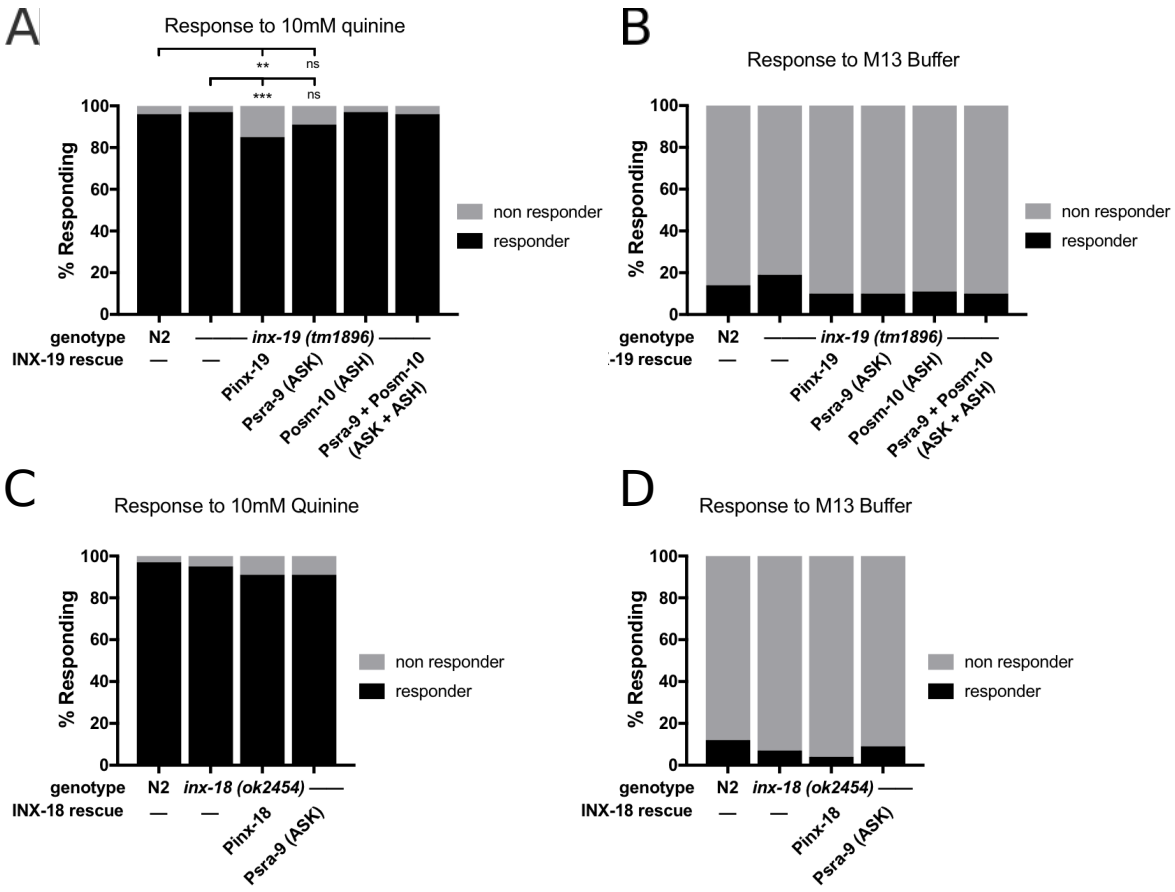


Figure S3: Responses of worms carrying rescue transgenes to negative and positive control solutions

A) *Inx-19(tm1896)* animals carrying rescue transgenes behaved like N2 (wild-type) animals when presented with M13 buffer. N2=14%, n=220; *inx-19(tm1896)*=19%, n=210; *inx-19;Pinx-19::inx-19cDNA*=10%, n=100; *inx-19;Psra-9::inx-19cDNA*=10%, n=100; *inx-19;Posm-10::inx-19cDNA*=11%, n=110; *inx-19;Psra-9::inx-19cDNA; Posm-10::inx-19cDNA* =10%, n=110. All groups were compared with a Chi-square test ($p=0.12$, $\alpha=0.05$) **B)** *Inx-18(ok2454)* animals carrying rescue transgenes behaved like N2 animals when presented with M13 buffer. N2=12%, n=120; *inx-18(ok2454)*=7%, n=120; *inx-18;inx-18gDNA*=4%, n=100; *inx-18;Psra-9::inx-18cDNA*=9%, n=120. All groups were compared with a Chi-square test ($p=0.16$, $\alpha=0.05$). **C)** *Inx-19(tm1896)* animal carrying neuron-specific transgenes behaved like N2 animals when presented with 10 mM quinine, but expression of *inx-19* cDNA using the native promoter reduced the responses to 10 mM quinine below wild-type levels. N2=96%, n=220; *inx-19(tm1896)*=97%, n=210; *inx-19;Pinx-19::inx-19cDNA*=85%, n=100, $p=0.002$ vs N2, $p=0.0004$ vs *inx-19*; *inx-19;Psra-9::inx-19cDNA*=91%, n=100, $p=0.10$ vs N2, $p=0.04$ vs *inx-19*; *inx-19;Posm-10::inx-19cDNA*=97%, n=110, $p=0.76$ vs N2, $p>0.99$ vs *inx-19*; *inx-19;Psra-9::inx-19cDNA; Posm-10::inx-19cDNA* =96%, n=110, $p>0.99$ vs N2, $p=0.74$ vs *inx-19*. All groups were compared with a Chi-square test ($p<0.0002$, $\alpha=0.05$), and post-hoc Fisher's Exact tests with Bonferroni's correction ($\alpha=0.006$) were computed to compare each group to N2 and *inx-19(tm1896)*. **D)** When expressing *inx-18* cDNA under the native promoter or in ASK, *inx-18(ok2454)* animals behaved like wild-type when presented with 10 mM quinine. N2=97%, n=120; *inx-18(ok2454)*=95%, n=120; *inx-18;inx-*

18gDNA=91%, n=100; *inx-18;Psra-9::inx-18cDNA*=91%, n=120. All groups were compared with a Chi-square test (p=0.21, α=0.05)



Materials and Methods

C. elegans Culture

Strains were maintained at room temperature (20-21°C) on NGM agar plates seeded with OP50 *E. coli* bacteria. The N2 strain (Bristol, England) was used as wild type. The following mutant strains were used in this study: CX6161 *inx-19 (ky634) I,* FX01896 *inx-19 (tm1896) I*, RB1896 *inx-18 (ok2454) IV,* BJH2183 *inx-18 (ok2454) IV;inx-19(tm1896) I,* BJH2259 *lite-1 (ce314) X,* BJH2304 *lite-1(ce314);inx-19(tm1896), and* BJH2303 *lite-1(ce314);inx-18(ok2454).*

Transgenes

Transgenic strains for rescue experiments were generated by microinjection[290] of various innexin-containing plasmids (30-40 ng/µl) together with co-injection markers. The co-injection markers were *Punc-122::gfp* (BJP-I15, 20-40 ng/µl) and *Punc-122::mcherry* (BJP-I14, 30-40 ng/µl). Cytoplasmic fluorophores (mCherry, mTagBFP2, and mScarlet) were injected at 30-40ng/µl. For GCaMP imaging experiments, plasmids (BJP-L136, *Psrbc-66::GCaMP6s::SL2::mCherry::let-858utr* or BJP-L137, *Posm-10::GCaMP6s::SL2::mCherry::let-858utr*) were injected at 70 ng/µl into the light-insensitive *lite-1(ce314)* worms. To quantify cGMP levels, FlincG3 plasmids (pFG270, *Psrbc-66::FlincG3::unc-54utr* or pFG250, *Psrd-10::FlincG3::unc-54utr*) were injected at 20 ng/µl into *lite-1(ce314)* worms.

Behavioral Assays

Well-fed day 1 adults were used for all analyses. To ensure uniformity of worm age and feeding status, L4 animals were picked onto fresh plates the afternoon

before behavior tests. Behavior assays were performed on at least 5 separate days in parallel with controls.

Quinine Drop Test

The quinine drop test was performed as described previously[243,245,247]. Quinine HCl (Sigma-Aldrich Q1125) was dissolved in M13 Buffer pH 7.4 (30 mM Tris-HCl pH 7.0, 100 mM NaCl, 10 mM KCl) to 10 mM. Aliquots were frozen at -20°C. Aliquots were defrosted on the day of the experiment and allowed to reach room temperature before use. Solutions were loaded into glass needles via mouth pipetting through long silicone tubing. Needles were formed from 1.5 mm filamented glass capillaries (World Precision Instruments, Inc.) with a Sutter micropipette puller and the tips opened by breaking with fine forceps. 10cm NGM plates were brought to room temperature on the bench overnight and then left open at room temperature to dry for 2.5-4 hours before being used (plates are appropriately dry when worms leave tracks on the agar that do not immediately disappear). For each assay, 15 worms were placed on a plate and allowed to acclimate for 30 min. Small drops (approximately 1 body length in diameter) of M13, 1 mM quinine, or 10 mM quinine were then delivered via glass needle approximately 1 body length in front of worms. When worms encountered the drop, they were scored as avoiding the drop if they initiated a reversal within 4 s and reversed at least half a body length backwards. To avoid desensitization, worms were not tested with a new solution within 2min of initial drop presentation. The experimenter was blind to the strain when scoring reversals.

Movement Assays

5 worms at a time were placed on 10 cm NGM plates and allowed to acclimate for 1 minute. Video capturing was then carried out using an imaging set up from MBF Bioscience. Freely crawling worms were monitored for 60 seconds at 5 frames per second. Moving velocity at each frame was calculated by the WormLab 4.1 from MBF Bioscience after confirming correct assignment of head location throughout the video. Reversals were denoted with negative values. Comparison of number of reversals/min and mean velocity was calculated using an ordinary one-way ANOVA using Dunnett's correction for multiple comparisons between all groups. The alpha value was set at 0.05.

Confocal Microscopy for Imaging Synapse and Cell Architecture

Young adults were paralyzed using 30 mg/ml 2,3-butanedione monoxime (BDM) dissolved in M9. Worms were imaged using an Olympus FV1000 confocal system with a 60x oil objective (NA 1.4). Z-stacks of fluorescent images (0.40 μm step-size for synapses, or 1.20 μm step-size for cell architecture) were taken at the region of interest. Maximum intensity projections of images were obtained using Fiji. For colocalization analysis, the number of puncta within the nerve ring in each channel was counted, and scored as colocalizing (containing signal from both channels) or non-colocalizing (containing signal from a single channel). Percentage colocalization was calculated by determining the ratio between the number of colocalizing puncta and the total number of puncta in each maximum intensity projection.

Calcium Imaging

GCaMP6s[291] was used for all calcium imaging. *Lite-1(ce314)* worms were injected with either *Psra9::GCaMP6s::SL2::mCherry::let-858utr* (ASK) or *Posm-10::GCaMP6s::SL2::mCherry::let-858utr* (ASH) along with the co-injection marker *Punc-122:mCherry*. Transgenic lines were crossed with mutant animals to generate *lite-1(ce314);inx-19(tm1896)* and *lite-1(ce314);inx-18(ok2454)*, which the identical extrachromosomal arrays for imaging. Worms were imaged using a microfluidic olfactory chip[292]. M13 buffer was used to load worms into the chip, and their nose tips were washed with M13 buffer for 30 seconds before each recording. At the start of the recording, animals were exposed to M13 buffer for 10 s before 10 mM Quinine dissolved in M13 was washed in to the chip. The images were captured at 5 frames per second with an exposure time of 100ms on a Leica DMI3000B inverted microscope with a 63x Oil objective and a QImaging OptiMOS camera. The region of interest was defined as a square-shaped area surrounding the desired cell body. Background-subtracted fluorescence intensity values were collected from every sample's ROI and stored into MATLAB formatted files. Change in fluorescence intensity (ΔF/F%) was calculated by dividing each value by the average intensity of the first 3 seconds of imaging.

cGMP Imaging

FlincG3[280,281] was used for cGMP imaging. *Lite-1(ce314)* worms were injected with either *Psrbc-66::FlincG3::unc-54utr* and *Psrbc-66::mScarlet::unc-54utr* (ASK) or *Psrd-10::FlincG3::unc-54utr* and *Psrd-10::mScarlet::unc-54utr* (ASH) along with the co-injection marker *Punc-122:mCherry*. Transgenic lines were crossed with mutant

animals to generate *lite-1(ce314);inx-19(tm1896)* and *lite-1(ce314);inx-18(ok2454),* which carry the identical extrachromosomal arrays for imaging. L4 worms were picked onto fresh OP50-seeded NGM plates 6 hours before imaging to ensure synchronization of age and feeding status. Young adults were paralyzed with 30 mg/ml BDM dissolved in M9. Immobilized worms were imaged using an Olympus FV1000 confocal microscope with a 60x Water objective. Kalman filtering was used to reduce noise. Z-stacks (1.28 μm step-size) were taken through the cell body. Maximum intensity projections were obtained using Fiji[293]. Two elliptical ROIs were drawn in the mScarlet channel: one surrounding the cell body and one capturing background fluorescence from a region near the cell body that did not contain an axon or dendrite. Mean pixel intensity in both the FlincG3 and mScarlet channels was calculated using Fiji and background intensity was subtracted from cell body intensity. The ratio between FlincG3 and mScarlet mean intensity was calculated to control for expression variation.

DNA constructs

Name	**Construct**	**Construction Notes**
BJP-L109	*Pinx-19::inx-19a::gfp::unc-54utr*	*Pinx-19* (5556bp) is from Dr. Cornelia Bargmann and primers were: GATAAGCGCGGATGCTCCT and TGACAGTGCTCTCAGAGGGA. *Inx-19a* cDNA is from Dr. Cornelia Bargmann and primers were: ATGTGGCGGACACCAGCATC and AAGAAACGATTTCGTCTGTCCAGGA.
BJP-I15	*Punc-122::gfp::unc-54utr*	
BJP-L99	*Psra-9::inx-19a::mCherry::gdp-2utr*	*Psra-9* is 3012bp and primers were: GCATGCTATATTCCACCAAA and GAAATCTTGAAACTGAAAAATACA
BJP-L112	*Psra-9::inx-19a::gfp::unc-54utr*	

BJP-L125	*Psra-6::inx-19a::mCherry::gdp-2utr*	*Psra-6* is 2018bp and primers were TTCCAGTGCTCTGAAAATCTTG and GGCAAAATCTGAAATAATAAATATT
BJP-L114	*Posm-10::inx-19a::gfp::unc-54utr*	*Posm-10* (900bp) is from Dr. Josh Kaplan and primers were: CTTGACACCGACTGGCAC and GCGTTCGACACCTTGTAAGAT
BJP-L120	*Psrd-10::inx-19a::gfp::unc-54utr*	*Psrd-10* (1841bp) is from Dr. Denise Ferkey and primers were: AGCCACGGCTAGCTACAG and GTTGAATTTGGTCTGTGAGCT
	inx-18 gDNA PCR	*Inx18* gDNA (7646bp) used the primers: ACAGTCGAGTCGTCGTCGTCG and TAATTTTGAAACAAAAATCGGAAAG AA
BJP-L46	*Psra-9::inx-18::gfp::unc-54utr*	*Inx-18* cDNA (1308bp) is from Dr. Zhao-Wen Wang and primers were: ATGGTCGGTGGATTCCG and AACATAATGTGTCCGTGTCGGA
BJP-L115	*Psrbc-66::mTagBFP2::unc-54utr*	*Psrbc-66* is 2055bp and used the primers: CAACGATGAAATATTGATCGTACAAA and TTCTGAGACACCTGACTTTCTGTC
BJP-L116	*Posm-10::mTagBFP2::unc-54utr*	
BJP-L143	*Psrbc-66::mScarlet::unc-54utr*	
BJP-L142	*Psrd-10::mScarlet::unc-54utr*	
BJP-L139	*Psra-9::mCherry::unc-54utr*	
BJP-L136	*Psrbc-66::GCaMP6s::SL2::mCherry::let-858utr*	
BJP-L137	*Posm-10::GCaMP6s::SL2::mCherry::let-858utr*	
pFG270	*Psrbc-66::FlincG3::unc-54utr*	Received from Dr. Denise Ferkey
pFG250	*Psrd-10::FlincG3::unc-54utr*	Received from Dr. Denise Ferkey

Statistical Analyses

Statistical analyses for all experiments except calcium imaging were carried out as described in the legends for each figure using GraphPad Prism Statistical analysis of the calcium imaging experiments was carried out using a custom written MATLAB program and GraphPad Prism.

Acknowledgments

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Authors' Contributions

L.V., I.R., and J.B. conceived of experiments. L.V. designed and performed experiments and analyzed data. B.U. performed and analyzed GCaMP6s experiments. S.W., D.M.F., and N.D.L. provided unpublished reagents and assisted with experimental design. L.V. wrote the manuscript with I.R. and J.B.

7. Addenda

What follows are the results of several experiments that provide interesting results regarding the impact of electrical synapses and cGMP on behavior, but that were not explored deeply and thus not included in the manuscript. Their relation to the story I have laid out is considered in the discussion (section 8)

7.1 Results:

Innexin-19 and guanylyl cyclase-27 are required for lysine chemotaxis.

As the ASK/ASH electrical synapse and cGMP are necessary for proper quinine modulation, we hypothesized they might impact other chemosensory behaviors in

these cells as well. If cGMP is diffusing from ASK, leading to measurably lower levels of ASK cGMP, it stands to reason that ASK function may also be impacted. ASK is involved in sensation of and chemotaxis towards lysine, a weakly attractive soluble tastant[228,277]. If cGMP levels within ASK are important for lysine chemotaxis, we would expect that lowering cGMP levels by disruption of the ASK/ASH electrical synapse or knocking out a guanylyl cyclase would impact this behavior. We tested attractance to lysine in N2, *inx-19(tm1896)* and *gcy-27(ok3653)* strains by using a chemotaxis assay (Add. Fig 1A) and calculating the attractance index(AI). As expected, lysine is weakly attractive to N2 worms (AI=0.32). Chemotaxis is significantly disrupted in both *inx-19(tm1896)* (AI=0.15) and *gcy-27(ok3653)* (AI=0.015) strains (Add. Fig 1B). This suggests that the ASK/ASH electrical synapse may impact activity of ASK as well as ASH, and that proper cGMP levels are required within ASK for lysine chemotaxis.

Mutations in innexin-19 and guanylyl cyclase-27 increase burrowing activity

While in the lab, *C. elegans* spends its time crawling on the surface of agar plates seeded with *E. coli*, but this is not a native behavior for the worm. In its natural habitat, *C. elegans* is found burrowing through rotting material or the soil in search of food. Worms on the soil surface risk desiccation and UV damage, so natural selection resulted in a species that prefers the cool, dark, low-oxygen environment below the surface[294]. Through laboratory domestication *C. elegans* has acquired mutations that give it better fitness in laboratory conditions including tolerance for high oxygen[294–296], but it will still burrow if there are breaches in the agar surface. We noticed that several strains we were using seemed to have a natural tendency to

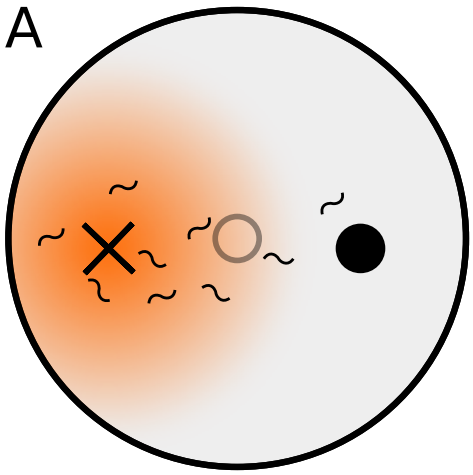
burrow below the agar more frequently than N2 animals and adapted a burrowing assay to test this behavior (Add. fig 2A)[297]. Worms were injected into the center of an agar-filled plastic pipette, sealed in, and then evaluated for how far they traveled from the start site in 2 hours. As expected given there was no attractive stimulus driving travel[298], N2 generally did not burrow far from where they started (mean=17.66mm traveled), but both *inx-19(tm1896)* (mean=32.94mm) and *gcy-27(ok3653)* (mean=34.09mm) burrowed farther, frequently making it to the ends of the tubes (60mm in either direction) by the end of the experiment (Add. Fig 2B).

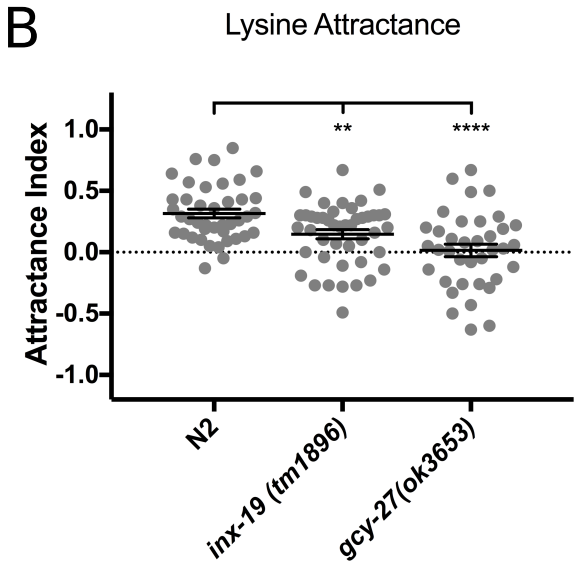
Multiple possibilities could explain this result including changing the tactile or oxygen preferences of *C. elegans*, changes to the off-food local search/sustained travel switch, or changes to their movement. While we did not test either of the former possibilities, all strains had the same average movement velocity (Add. Fig 2C), indicating there were not major changes to their crawling behavior. On the other hand, *gcy-27(ok3653)* worms show fewer spontaneous reversals per minute than N2 (Add. Fig 2D). While this could explain the longer travel distance as these animals may be less likely to turn around once they have left the start site, we tested reversal frequency on the 2-dimensional plane of the agar plate surface rather than in a 3-dimensional matrix like the one in the burrowing assay. Worms change their crawling behavior in response to tactile influence[299], and the kinematics of burrowing are distinct from either swimming or crawling[298], so it would be unsurprising if reversal frequency were modified by being within the agar rather than on top of it and a more direct measure of crawling behavior within agar tubes is required.

7.2 Figures:

Addendum Figure 1: Mutations in innexin-19 and the guanylyl cyclase-27 disrupt lysine chemotaxis

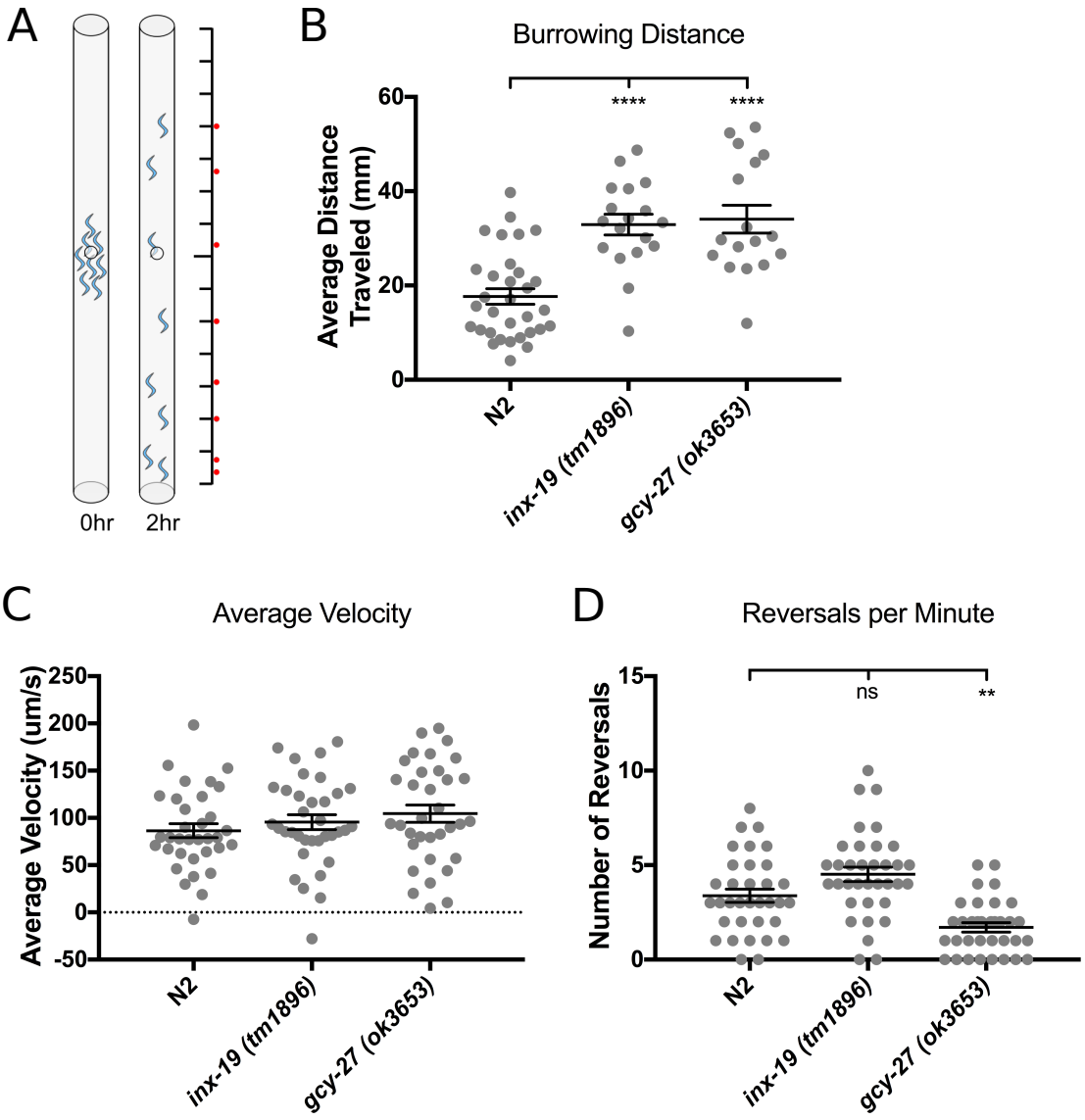
A) Diagram of a lysine chemotaxis assay. *C. elegans* were washed and placed at the start location (grey circle) on an agar plate with the choice to move towards high lysine concentration (black X) or to move away from lysine (black circle). After 1 hour, the number of worms at each spot was counted and attractance index (AI) was calculated. An AI of 1 indicates 100% attractance to a lysine, 0 indicates the *C. elegans* is neutral towards the lysine, while an AI of -1 indicates complete avoidance. **B)** Attractance indices towards lysine of N2, *inx-19(tm1896)*, and *gcy-27(ok3653)*. N2 (AI=0.32) has a greater preference toward lysine than either *inx-19(tm1896)* (AI=0.15) or *gcy-27(ok3653)* (AI=0.015). One-way ANOVA between three groups showed significant differences ($F[2,120]=13.0$, $p<0.0001$, $\alpha=0.05$), and Dunnett's multiple comparison test showed that N2 (n=41) differed from both *inx-19(tm1896)*(n=45, p=0.0065) and *gcy-27 (ok3653)*(n=37, p<0.0001). Each data point represents the results from one plate and error bars are SEM.


A


B
Lysine Attractance
Attractance Index
1.0
0.5
0.0
-0.5
-1.0
**
****
N2
inx-19 (tm1896)
gcy-27(ok3653)

Addendum Figure 2: Mutations in innexin-19 and the guanylyl cyclase gcy-27 lead to greater burrowing activity

A) Diagram of burrowing assay. Worms were deposited into the center of agar-filled plastic tubes and were left to burrow for 2 hours. After 2 hours, their distance from the start site was measured. **B)** Average distance traveled by N2, *inx-19(tm1896)*, and *gcy-27(ok3653)*. N2 worms did not generally move very far from their start site, while both *inx-19(tm1896)* and *gcy-27(ok3653)* burrowed significantly farther. One-way ANOVA between three groups showed significant differences ($F[2,64]=20.48$, $p<0.0001$, $\alpha=0.05$), and Dunnett's multiple comparison test showed that N2 (n=32) differed from both *inx-19(tm1896)*(n=18, $p<0.0001$) and *gcy-27 (ok3653)*(n=17, $p<0.0001$). Each data point represents the results from one burrowing assay (~50 worms) and error bars are SEM. **C)** *gcy-27(ok3653)* and *inx-19(tm1896)* mutants do not differ in their average movement velocity compared to N2 animals. One-way ANOVA between three groups showed no significant differences ($F[2,100]=1.223$, $p=0.299$, $\alpha=0.05$). Each data point represents a single worm and error bars are SEM. **D)** *gcy-27(ok3653)* mutants reverse less frequently than N2 animals. Number of reversals were counted from a one minute video. One-way ANOVA between three groups showed significant differences ($F[2,100]=18.01$, $p<0.0001$, $\alpha=0.05$), and Dunnet's multiple comparison test showed that N2 (n=34) differed from *gcy-27(ok3653)* (n=33, $p=0.0012$) but not *inx-19(tm1896)*(n=35, $p=0.0972$). Each data point represents a single worm and error bars are SEM.


A
0hr
2hr
B
Burrowing Distance
Average Distance Traveled (mm)
60
40
20
0
****
****
N2
inx-19 (tm1896)
gcy-27 (ok3653)
C
Average Velocity
Average Velocity (um/s)
250
200
150
100
50
0
-50
N2
inx-19 (tm1896)
gcy-27 (ok3653)
D
Reversals per Minute
Number of Reversals
15
10
5
0
ns
**
N2
inx-19 (tm1896)
gcy-27 (ok3653)

7.3 Methods

Lysine chemotaxis assay

Chemotaxis assays were carried out as described in Shingai et al 2005[300]. Chemotaxis plates were 9cm petri dishes containing 2% agarose dissolved in the following solution (pH 6.0): 1 mM CaCl2, 1 mM MgSO4, 5 mM potassium phosphate. Start, test, and control spots were drawn onto the plate, with the start spot being at the center and test/control spots being 1.5cm in opposite directions (see Add. Fig 1A for diagram). Roughly 24hr before experiments commenced, 10µL of 3M L-Lysine was dotted onto the test spot, and then again 4hr before. This creates a gradient of lysine. Plates were kept at room temperature for the 24hr before experiments. At the start of experiments, 1µL of 0.1M sodium azide was pipetted onto the control and test spots in order to anaesthetize the animals once they reached either spot. Worms were washed off culture plates into microfuge tubes using low-salt buffer (1mM CaCl2, 1mM MgSO4, 5mM KPO, pH 6.0), let settle, and then washed two more times to remove residual bacteria before pipetting onto the start site. Excess liquid was removed with a Kimwipe. After 1hr, anaesthesized worm at either the test or control spot were counted and the attractance index (AI) was calculated as follows: $\frac{\#\ worms\ at\ test\ substance - \#\ worms\ at\ control}{total\ \#\ worms}$

Burrowing assay

Burrowing tubes were constructed by adapting the method described by the Vidal-Gadea lab[297]. Plastic Pasteur pipettes were filled with 2% burrowing agar: 2% agarose in 5mM KH_2PO_4 [pH 6.0], 1mM $CaCl_2$, 1mM $MgSO_4$. These were let cool and

cut using a razor blade heated in the flame of a Bunsen burner to score a ring in the plastic before snapping into 12cm lengths. An opening was created in the center of the tube by heating the end of paperclip and melting a hole that extended partway into the agar. Excessive melted plastic was removed using forceps to clear the opening. Both ends and the opening were sealed with Parafilm and tubes were placed in 4C until use. Tubes were brought to room temperature before commencing experiments. 50 worms were picked without bacteria into a small droplet of M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 litre. Sterilize by autoclaving), concentrated, and then sucked into a glass capillary using a (Drummond Scientific Wiretrol 10uL micropipet). The end of the capillary tube was gently inserted into the agar at the center of the burrowing tubes and worms were expelled into the agar. Residual M9 was carefully removed using a Kimwipe and the opening was sealed with Parafilm. Tubes were placed vertically at room temperature for 2 hours. Using a dissecting scope, worm location was marked on the outside of each tube at the end of the experiment and distance from start location was measured in mm. Some worms would borrow to the Parafilmed ends of the tubes so Parafilm seals were removed and checked for worms, which were counted as being 60mm from the start.

Movement Assay

See methods in manuscript.

8. Discussion

The data presented indicate that electrical synapses are important regulators of neural activity and animal behavior, and that they can do so via passage of small molecules. This provides another mechanism for neural plasticity. Being restricted by the limits of diffusion to impacting activity of nearby cells, electrical synapse driven plasticity operates on a level between that of synaptic plasticity and neuromodulation. Together, these three different plasticity mechanisms are able to fine-tune activity by responding to different types of cues. Synaptic plasticity occurs when specific synapses undergo changes in activity, and thus responds to and directly targets individual nodes in a network. These small changes alter how a network behaves, making a particular path more or less likely to fire. Neuromodulation uses small numbers of cells to modulate entire brain regions in response to general physiological change. This type of plasticity can target multiple networks simultaneously, allowing for large-scale changes in multiple behaviors. In contrast, electrical synapse mediated plasticity functions between directly connected cells. This means that the neurons that participate in this type of plasticity are likely modulating and being modulated by functionally related cells. Electrical connectivity is already known to facilitate lateral excitation where electrical potential diffuses between cells, making local groups more or less likely to fire. As these junctions are also passing small molecules such as Ca^{2+}, cAMP, and cGMP, it makes sense that these signals could achieve similar ends. If one neuron had elevated cAMP levels leading to changes in ion channel function, some cAMP could diffuse into neighboring cells and potentiate similar changes. Conversely a cell

with lowered cAMP levels compared to its neighbors could act as a cAMP sink and inhibiting plasticity. In this way, one cell or group of cells could affect the strength of changes within a local network. As the small molecules that can pass through electrical synapses are the same messengers often used in synaptic plasticity and neuromodulation, it seems likely that these two mechanisms are actively regulated by this type of diffusion.

By having multiple mechanisms modified by electrical synapse mediated plasticity, the nervous system may provide a buffer against noise and ensure that local networks are more synchronized in activity levels. This would function similarly to what is described for electrical synchronization in sections 4.2.2.1 and 4.2.2.3. Briefly, if we consider that small molecule concentration in the cytoplasm can rise and fall in waves much like electrical potential but on a longer time scale, low-pass filters like electrical synapses would preferentially pass these types of changes among neighboring cells. If one cell were behaving abnormally in response to neuromodulatory signals and producing excessive levels of particular small molecules, these diffusing to neighboring cells would dampen the impact of any neuron unable to regulate its signaling appropriately. It would be interesting to test this by challenging a cell with excessive amounts of these small molecules such as cAMP or cGMP and testing the network robustness with or without electrical synapses. This could be done by overexpressing enzymes such as guanylyl cyclases or adenylate cyclases or using inducible systems such as BeCyclOp that generates cGMP in response to blue light stimulation[301]. Measuring neural activity after this

perturbation with and without electrical synapses would enable us to see whether their presence affects network stability in aberrant contexts.

In *C. elegans*, electrical synapses modulate multiple behaviors. In addition their necessity for suppressing the response to quinine (discussed in the manuscript above), innexins promote lysine chemotaxis and inhibit burrowing behavior (see addenda above). These findings suggest that the mechanism we described above is not a unique case to just quinine or even just nociception, but rather a more general mechanism within the nervous system. Of course, with just the data presented, we cannot be certain that the lysine and burrowing phenotypes are due to electrical synapse disruption: as explained within the manuscript, innexin genes may play multiple roles including changing cell fate or membrane potential. These may be the cause of the changes to lysine chemotaxis and burrowing behavior as we did not interrogate the mechanisms, but given mutations in both inx-19 and gcy-27 cause similar phenotypes, it is tempting to imagine they function similarly to quinine modulation. This could be tested using similar methods as described in the above manuscript, although narrowing the exact synapses involved may provide a challenge as lysine chemotaxis is distributed among 4 pairs of neurons (ASE, ASG, ASI, and ASK)[228], and numerous neurons likely contribute to the complex burrowing behavior.

One area in electrical synapse biology that has been ill-explored is the impact of channel rectification on neural activity. While the existence of rectified channels has long been known, their impact is less well understood (for review see section 4.2.1).

Rectification has largely been considered to give directionality to electrical signals, but it may also lead to directional permeability of small molecule diffusion. If some portion of the ASK/ASH electrical synapses are heterotypic with INX-18 and INX-19 being present, this likely indicates some amount of rectification. It would be interesting to test whether this is the case and what impact it has on the modulation of ASH and ASK. Heterotypy, permeability, and rectification of channels could be tested in vitro using *Xenopus* oocytes[302], and then the composition of the ASK/ASH electrical synapses could be manipulated by expressing different combinations of innexins in each cell. Testing behavior, calcium, and cGMP signaling in these manipulated systems would allow us to discover whether rectification plays a role in this synapse. Perhaps experiments like this would demonstrate that modulation is normally bi-directional with signals from ASK down regulating quinine sensation and signals from ASH regulating ASK functions. Knowing more about the permeability of the ASK/ASH electrical synapses would also allow us to better understand the nature of ASH modulation.

The fact that ASH activity is modulated by cGMP and yet it is not known to manufacture its own cGMP seems at first to be counterintuitive, particularly given that neurons that habituate often do so by internal signaling or because of the intrinsic properties of their membrane channels. However, when one thinks about this sort of modulation in the context of the larger sensory neuron network, responding to (and modulating the impact of) multiple different stimuli at once, this makes sense. To be a fully functional part of the sensory network, ASH must be regulated by stimuli that are not its own, including things that are known to be

influenced by cGMP such as satiety[285–287]. High levels of cGMP in sensory neurons may be a signal of satiety given that gain-of-function (gof) mutations in the cGMP dependent protein kinase G EGL-4 result in worms that behave as if they are sated (quiescence, low levels of dauer formation) even when they are actually starved[286]. In this case, the gof EGL-4 enzyme acts as if cGMP levels are high. In contrast, loss of function EGL-4 mutants act as if they are starved: decreased quiescence and increased dauer formation[286,288,289]. Additionally, loss of function mutants of the cGMP gated channel subunit TAX-2 (this functions as if cGMP levels were low), show reduced quiescence and have a tendency to leave the bacterial lawn as if they need to search for food[287]. These data suggest that high levels of cGMP within the sensory neurons is associated with satiety and lead to quiescence, a lethargic sleep-like state wherein the worm is less sensitive to noxious stimuli[287]. As sensing satiety requires multiple cues from multiple cells and affects multiple different behaviors[255,287,303,304], it makes sense for satiety signaling to be distributed both in origin (ie, many cells contribute to cGMP levels) and in reception (ie, many cells are able to respond to cGMP). Now that reliable a reliable, easy to use cGMP sensor has been developed for *C. elegans*,[280] imaging cGMP levels in multiple neurons that participate in this sort of signaling is possible.

Often when we study neural activity and/or modulation, we focus on individual neurons without seeing the entire network. Partly this is out of necessity, as the complexity of multiple cells making numerous different types of connections presents too many variables to manipulate easily in a lab, especially the added complication of neuromodulation, a network that cannot be seen in a connectome

map. Thankfully, we are getting there with sophisticated computer models that can account for changing synapse strength or ablating neurons[305]. If signaling molecule levels and permeability through electrical synapses were accounted for in such models, we could start to appreciate the impact such signaling has on the activity of networks. This would get us closer to the elusive promise that connectomics makes: that if we understand the patterns of connection between cells, we will be able to decode how meaning arises from neural activity. As this thesis demonstrates, just having the map is not enough. To better model the ever-changing neural environment, we must take into account the impact electrical synapses as facilitators of local network plasticity.

9. References

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