Mechanisms Mediating Synchronous Membrane Potential Oscillations in Projection Neurons in the Adult *Drosophila* Brain

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Adam Truong studied the role of gap junctions in mediating synchronous membrane potential oscillations in projection neurons in *Drosophila* pupae. He then designed experiments using the more developed and complex neural connections of adult *Drosophila* brains and discovered a novel connection between projection neurons. In 2012, Adam received a Master's Degree in Global Medicine from the University of Southern California, and is currently studying to receive his degree in medicine from the University of California, Irvine School of Medicine. He hopes to use both degrees to unite his twin passions of international public health and allopathic medicine.

Abstract

Chemical synaptic transmission mediates information transfer between neurons in the insect antennal lobe and plays an important role in processing olfactory information. In contrast, while electrical synapses (gap junctions) play a clear role in neuronal communication in the mammalian olfactory bulb, relatively little is known about their role in the antennal lobe. A recent study reported that electrical synapses mediated by *innexin8* encoded gap junction proteins form specifically between projection neurons (PNs) that innervate the same but not different antennal lobe glomeruli in the intact adult fly brain. The current study showed correlated membrane potential oscillations between randomly selected PNs. This led to the hypothesis that gap junctions are important for synchronizing activity between PN pairs that innervate both the same and different glomeruli. Recordings were made from pairs of projection neurons and the dendrites for each projection neuron were located. This demonstrated that PN pairs innervating different glomeruli showed correlated electrical activity even with chemical synaptic transmission blocked. Correlated activity also was reduced in PN pairs in *innexin8* mutants compared to wildtype. These data show that *innexin8* encoded gap junction proteins are important in synchronizing activity between PNs that innervate different and the same antennal lobe glomeruli.

Faculty Mentor

Adam joined the lab in the summer following his freshman year and did research every quarter until he graduated. Early on he enthusiastically applied himself to developing the fine micro-dissection skills required to remove the brain from the head of a tiny fruit fly. Adam then undertook the heroic task of recording simultaneously from two neurons in a well-defined neural circuit that processes olfactory information in the fly brain. The data in his excellent paper demonstrate coordinated activity between two different neuronal subtypes, revealing a novel pathway of communication between neurons in this circuit. Adam took full advantage of his time in the lab, learning do science and to think like a scientist.

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Key Terms

- *Drosophila*
- Gap Junction
- Heterotypic
- *innexin8*
- Projection Neuron

Introduction

Generation of behavioral responses to olfactory signals in higher organisms requires rapid and specific communication between neurons in the central olfactory circuits. *Drosophila* has served as an excellent model system for studying information processing in the olfactory system as the circuits involved are relatively simple and well defined. An odor, when introduced to fly antennae, stimulates specific olfactory receptor neurons that send their axons into one of approximately 50 segregated areas called glomeruli in the antennal lobe. Each glomerulus is specific to a unique odor type. Two major neuronal cell types in the antennal lobe process incoming sensory information. The first are unipolar cholinergic projection neurons (PNs) with an initial segment that bifurcates in the antennal lobe. One branch forms a dendritic spread contained within a single glomerulus where it receives information from the primary olfactory receptor neurons. The other branch forms an axonal projection that carries information to higher processing centers (Jefferis and Hummel, 2006). Pairs of projection neurons either share the same glomerulus or are confined to different glomeruli, homotypic and heterotypic projection neuron pairs, respectively (Figure 1). The second major neuronal type in the *Drosophila* antennal lobe is the local neuron, the majority of which are inhibitory GABAergic interneurons. While projection neuron dendrites are contained within single glomeruli, local neurons have axonal and dendritic processes that extend broadly throughout the antennal lobe where they form connections with PNs, olfactory receptor neurons, and other local neurons across glomeruli (Chou et al., 2010).

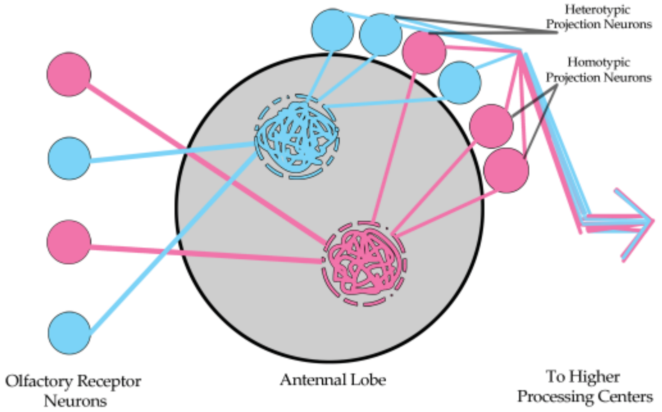

Figure 1

Drosophila olfactory system map. The olfactory receptor neurons receive input and send their axons to specific antennal lobe glomeruli. Here they synapse onto projection neuron dendrites and information is sent to higher processing centers.

In the vertebrate olfactory system, both electrical and chemical synapses are important in information processing. For example, in mammals, Connexin 36 was found to be responsible in producing correlated oscillations between mitral cells (the vertebrate equivalent of the unipolar *Drosophila* PN) (Christie et al., 2005). Recent data suggest that electrical communication, mediated by gap junctions, is also important in processing olfactory information in insect olfactory circuits. Physical connections with other PNs in dissociated antennae lobe cultures have been shown to exhibit strongly correlated calcium transients. RNAi mediated knockdown of a specific gap junction gene, *innexin7*, showed a significant reduction in correlated activity in PN pairs in culture. These studies demonstrate that PNs form Innexin7 containing gap junctions that coordinate intracellular calcium levels between PNs in culture. Another recent study has also demonstrated that paired recordings from homotypic PNs that innervate the same antennal lobe glomerulus in the brain of the intact adult fly exhibit highly synchronized membrane potential oscillations in the absence of odor stimulation (Kazama and Wilson 2009). In addition, they have shown homotypic PN pairs are electrically coupled, and coupling is disrupted by the *ShB* mutation in the gap junction gene *innexin8* (Yaksi and Wilson, 2010). This suggests that *innexin8* encoded gap junction proteins are important in synchronizing activity in homotypic PNs in the adult brain.

Wilson's group also reported that heterotypic PNs innervating different glomeruli did not exhibit coordinated spontaneous activity or evidence of electrical coupling when examined in the intact fly (Kazama and Wilson, 2009). In contrast to recordings in an intact living fly, my preliminary data from isolated brains revealed coordinated activity in several randomly selected PNs. This led to the hypothesis that PN-PN communication is much more complex than initially thought and that heterotypic PN interaction is more prevalent in the absence of coordinated sensory input. I tested this by simultaneous paired whole cell recordings of spontaneous activity in heterotypic PN pairs brains isolated from wildtype, *innexin7* knockdown, and *innexin8* mutant PNs to explore the presence and mechanism of heterotypic neuronal communication.

Materials and Methods

Fly Stocks

Three *Drosophila* lines were used:

1. Wildtype (wildtype): CantonS stock (W1) and the uncrossed transgenic line, 22948, (Vienna Drosophila

RNAi center). Data from the two lines were similar so results were pooled.

2. *Innexin7* knockdown (*inx7* KD): The 22948 line was crossed to a PN specific GAL4 driver line, GH146-Gal4, resulting in RNAi-knocked down expression of *inx7* specifically in PNs.

3. *Innexin8* mutant (*inx8* mutant): a point mutation in *inx8* (*ShB*) (Godenschwege lab)

Identification of PN Pairs in Isolated Brains

To determine if projection neurons display correlated activity in the absence of coordinated sensory input, pairs of antennal lobe PNs were recorded in isolated adult fly brains. By surgically removing the brain from the fly during preparation the central antennal lobe circuits remain intact, but there are no afferent inputs since the antennal nerves have been severed. PN cell bodies are located in three clusters surrounding each antennal lobe. The dorsolateral cluster was targeted for recording because it has been found to have the highest density of PNs. Recording electrodes were visually guided under DIC optics to the dorsal cluster PN cell bodies to be recorded from.

In order to identify PN pairs that were heterotypic, electrodes were filled with Lucifer yellow dye or biocytin. Whole cell recordings were maintained for an average of 40–60 minutes to allow sufficient time for dye to diffuse throughout the cell. Post-staining confocal analysis revealed that all randomly selected PN pairs in wildtype brains were heterotypic (n=6) with their dendrites occupying distinct glomeruli (Figure 2).

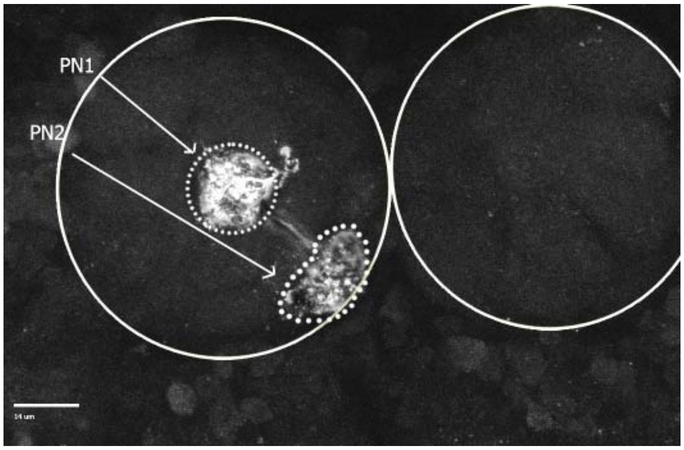

Figure 2

Heterotypic wildtype PN pair (PN1-PN2) identified by dendritic branching in distinct glomeruli in antennal lobe. Solid circles outline bilaterally symmetric antennal lobes. Dotted regions outline filled glomeruli from PN1 and PN2. Patched dorsolateral cell bodies cannot be seen.

Staining and Imaging

To label individual cells, pipettes were filled with internal recording solution containing either biocytin (0.4%) or Lucifer yellow (0.4%). After recording, electrodes were removed (often times removing the cell body too) and the brains were fixed in cold 4% Paraformaldehyde in PBS for 1 hour. To image the glomeruli, brains were stained the following day with a primary antibody directed against the synaptically localized protein Bruchpilot (NC-82, 1:1000, 1 hour) followed by staining with a fluorescently tagged secondary antibody (Alexafluor 546, 1:1000, 1 hour). PNs filled with biocytin were visualized by staining with fluorescently tagged streptavadin against biocytin (Alexafluor 488 biocytin, 1:1000, 1 hour). PNs filled with Lucifer yellow were visualized directly using a BP 535-590 filter. Images were taken with a Zeiss META multi photon confocal microscope and analyzed using Volocity imaging software. 1024x1024 resolution confocal Z-series were imaged by single pass scan method and 1μm slices.

Whole Brain Electrophysiology

Electrophysiology was conducted as previously described (Gu and O'Dowd, 2006; Gu et al., 2009). Briefly, brains were dissected from adult flies (1–3 days after eclosion) in recording solution. Papain (20 U/ml) and cysteine (1mM) were added for 3–10 minutes to soften the brain and connective tissue during extraction. The brain was placed on a glass-bottom chamber held down with a platinum frame and imaged with an Axioskop 2FS Zeiss microscope under DIC optics. Data were acquired using an Axopatch 200B amplifier and Clampex 9.0. Electrodes were pulled from glass pipettes to a resistance ranging from 9–11 MΩ. All recordings were started in saline recording solution and synaptic transmitter drugs were perfused in after 10 minutes. Neurotransmitter drugs used included picrotoxin (PTX, 1 μM) and curare (10 μM) to block GABAergic and nicotinic acetylcholine receptors, respectively.

Data analysis was done using Clampfit 9.2. Ten-second intervals were selected one minute before drug perfusion and five minutes after to represent activity in saline and synaptic neurotransmitter blockers. Cross correlation analysis was done using Clampfit's cross-correlation analysis function. Data were processed with 1,000 lags producing ±1 second of time shift. The degree of correlation was quantitatively assessed using a cross correlation function analysis. A cross correlation function describes the correlation of an event occurring in both cells. If one cell depolarizes/hyperpolarizes as the other does across each sample interval, the correlation reaches a maximum value of 1.0. Peak CC values were used in quantitative analysis of real-time electrical coordination and the cross correlation graph was used in qualitative analysis of oscillation regularity.

Results

Paired Current Clamp Recordings from Heterotypic PN Pairs in Wildtype

Simultaneous current clamp recordings from heterotypic wildtype PN pairs revealed regular low frequency membrane potential oscillations in both cells (Figure 3A). The cross correlation function at lag time 0 for PN1-PN2 in saline shows a peak value of 0.72 at lag time 0 (Figure 3B). The high correlation coefficient, designated at the value at lag time zero, indicates a high degree of correlation in real time. To determine if this was dependent on chemical synaptic transmission, picrotoxin (PTX) and curare were applied to the bath to block GABA and nicotine acetylcholine receptors, respectively. There was no decrease in the correlation coefficient after pharmacologically blocking synaptic transmission (Figure 3C, D). For all six wildtype heterotypic PN pairs the average correlation coefficient was high, and was not significantly reduced when PTX and curare were perfused into the bath (Figure 3E). These data demonstrate that, in contrast to recordings *in vivo*, heterotypic wildtype PN pairs in isolated brains exhibit highly correlated activity. This is independent of excitatory cholinergic and inhibitory GABAergic chemical synaptic transmission, suggesting coordination is mediated by gap junctions.

Paired Current Clamp Recordings from Heterotypic PN Pairs after Inx7 Knockdown

Previous experiments in my lab suggested that coordinated activity between PN pairs examined in the isolated brain may be mediated by *inx7* encoded gap junction proteins. To test this hypothesis paired heterotypic PN recordings were made in the *inx7* knocked-down brains, in which *inx7* expression was reduced by >90%. Similar to wildtype strain physiology, *innexin7* RNAi knockdowns showed robust coordinated oscillations in saline (Figure 4A). Quantitative analysis indicated a high degree of coupling (Figure 4B). In the presence of PTX and curare, the cells remained coupled (Figure 4C, D). Quantitative analysis confirms no significant difference between wildtype and *inx7* RNAi knockdown correlation coefficients in saline (Figure 4E) or in PTX, curare (Figure 4F). These data suggest that gap junctions encoded by *innexin7* in projection neurons are not necessary for synchronous membrane potential oscillations in heterotypic cells.

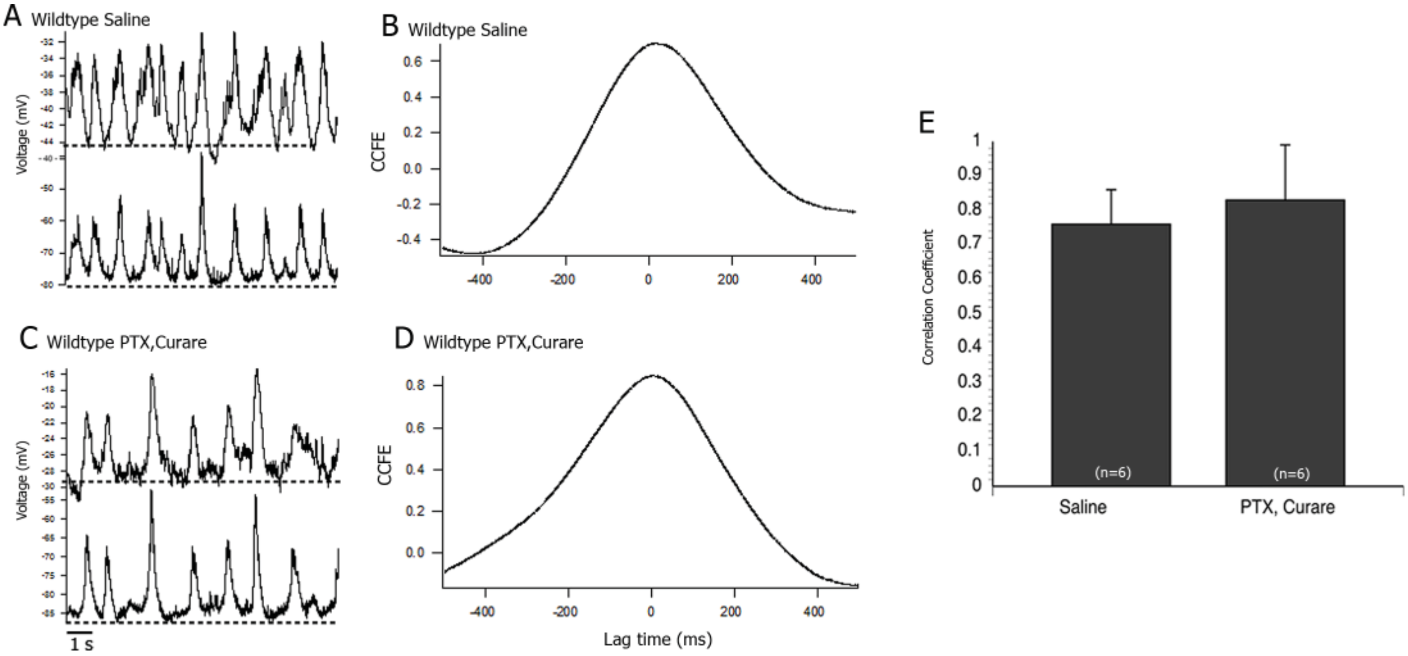

Figure 3

Wildtype heterotypic PN-PN current clamp recordings. (A) Simultaneous recording showing spontaneous membrane potential oscillations in two PNs in standard saline occurring at the same time over a 10 second period. (B) Cross correlation graph of recordings in A with a value of 0.72 at lag time=0. (C) Same PN-PN recording after perfusing in PTX and Curare. (D) Cross correlation graph of PNs in PTX, Curare. Correlation coefficient at lag time 0=0.88. (E) Quantitative analysis shows no significant difference in mean correlation coefficient in wildtype PN pairs between saline and neurotransmitter blockers (t-test, P=0.11, n=6).

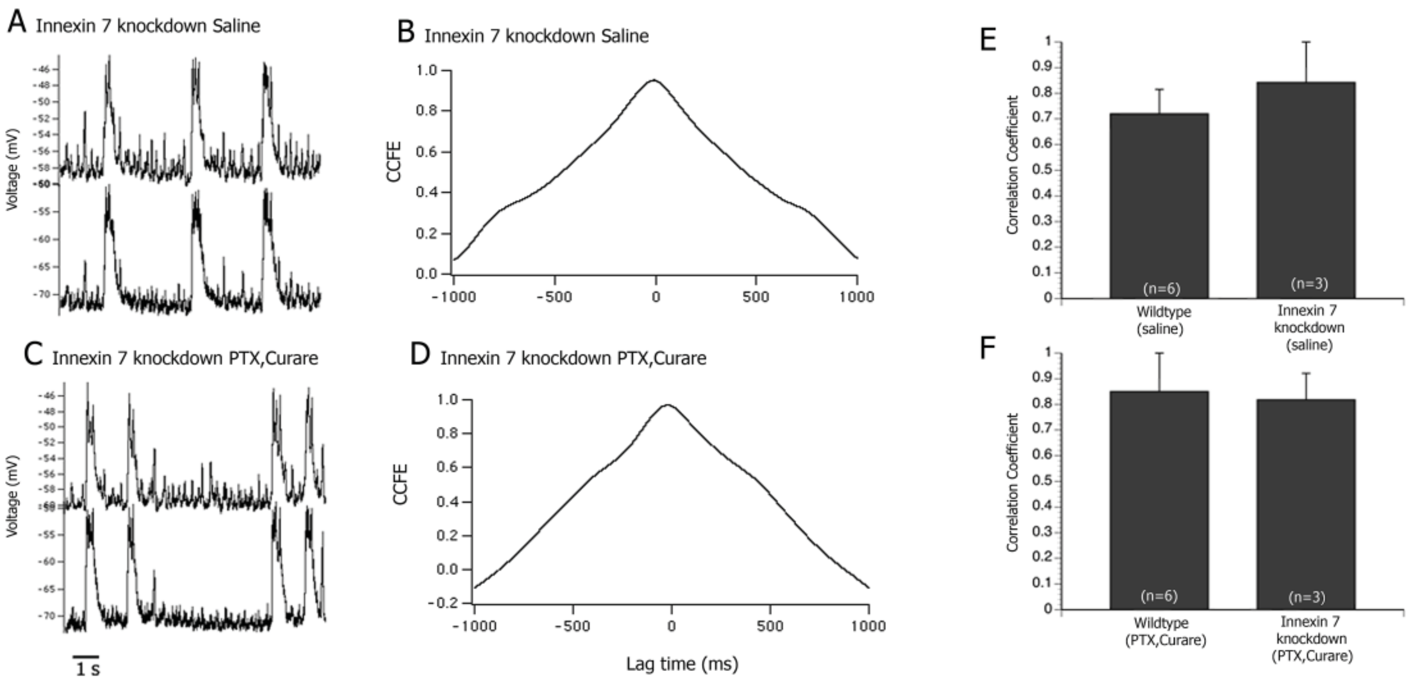

Figure 4

Inx7 KD paired PN-PN current clamp recordings. (A) Simultaneous recordings from two PNs in saline. (B) Cross correlation graph of pair in A showing a value of 0.92 at lag=0. (C) Recordings from same pair in A after perfusion of PTX and Curare into the chamber. (D) Cross correlation graph for records shown in C shows a coupling efficiency=0.96. (E) Mean correlation coefficients of wildtype and *innexin7* RNAi knockout heterotypic PNs (n=6, 3, respectively) in saline. (F) Mean correlation coefficients in PTX, curare showing no significant difference (t-test, P=0.37).

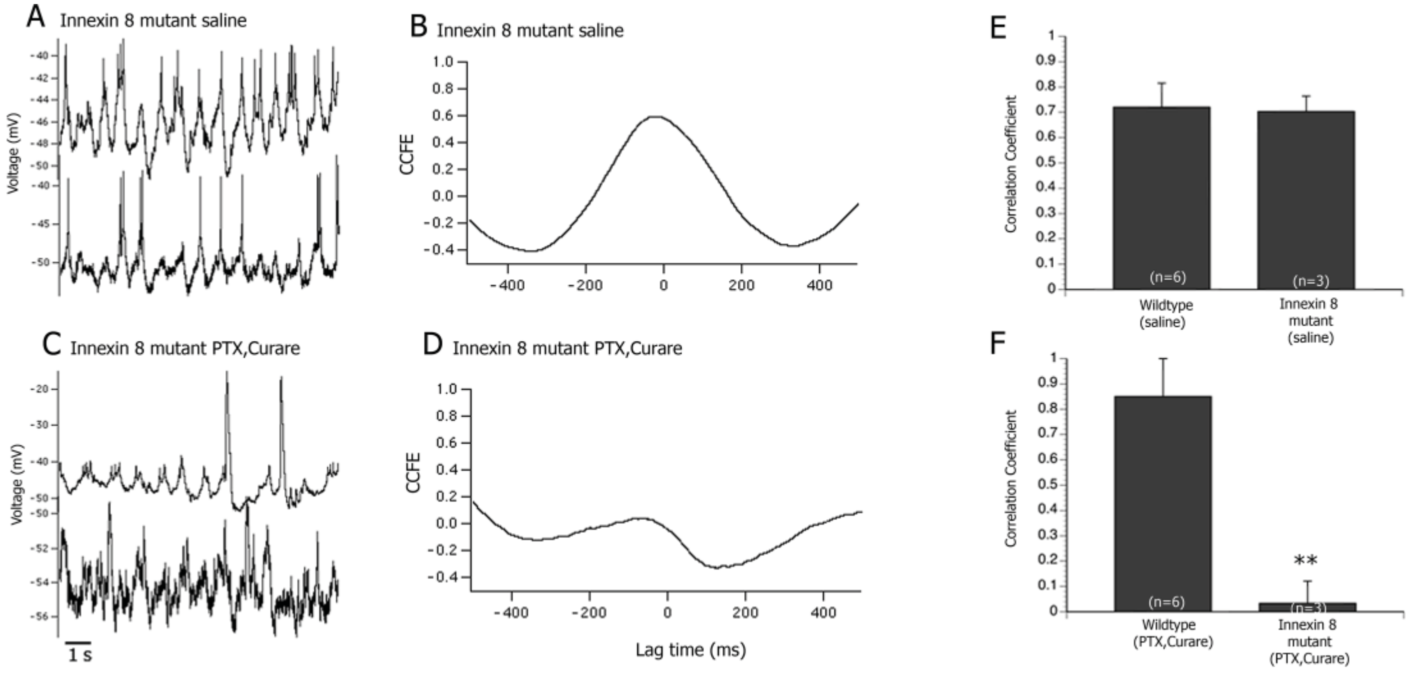

Figure 5

Innexin8 mutant paired PN-PN current clamp recordings. (A) Simultaneous PN-PN recording from *inx8* mutant in saline. (B) Cross correlation graph of pair in A showing a value of 0.60 at lag time=0 (n=3). (C) Same PN-PN recording in PTX and Curare (D) Cross correlation graph of PNs in PTX,Curare. Coupling coefficient at lag time 0=0.02. (E) Quantitative analysis shows no significant difference of correlation coefficient between wildtype and *innexin8* mutant in saline. (F) A significant difference is seen between the wildtype and *innexin8* mutant the presence of PTX, curare (t-test, **P<0.01), (n=3).

Paired Current Clamp Recordings from Heterotypic PN Pairs in Innexin 8 Mutant

Together with *innexin7*, *innexin8* encoded gap junction proteins are also found in the *Drosophila* olfactory system and genetic elimination of these gap junctions in a mutant (*inx8* mutant) has been shown to disrupt electrical coupling between homotypic projection neurons when recorded *in vivo*. I used *inx8* mutants and tested for coordinated PNs using paired electrophysiology in brains removed and isolated. In normal saline, heterotypic PNs showed coupled membrane potential oscillations and quantitative analysis showed a correlation coefficient of 0.60, similar to wildtype and *inx7* knockdowns (Figure 5A, B, E). However, addition of synaptic transmitter blockers caused a dramatic reduction in the degree of correlation between the PNs. The membrane potential oscillations persisted; however, they were no longer synchronized (Figure 5C). Quantitative analysis of this pair revealed a major decrease in the correlation coefficient (Figure 5D). In the presence of synaptic transmitter blockers, the mean correlation coefficient in *inx8* mutants was significantly reduced compared to wildtype (Figure 5F). These data indicate that *inx8* encoded gap junction proteins are involved in mediating correlated membrane potential oscillations in heterotypic PN pairs.

Paired Stimulus

To determine if there was direct electrical coupling between PNs, I injected one cell with three voltage steps (10, -40, and +40 mV) and evaluated the response in the other cell. Depolarization induced in the stimulated PN did not cause a measurable depolarization in the second PN (n=3). This suggests there is no direct electrical linkage between these two cells.

Discussion

A previous study reported coordinated activity mediated by *inx8* encoded gap junctions between PNs innervating the same glomerulus but not those innervating different glomeruli in brains of intact adult *Drosophila* (Kazama and Wilson, 2009; Yaksi and Wilson, 2010). By removing the brain from the fly and isolating it from all afferent olfactory inputs I was able to use a very different preparation to conduct a similar experiment. When olfactory input was eliminated, heterotypic PN pairs that innervate different glomeruli exhibited coordinated membrane potential oscillations in saline. Synchrony was reduced in *inx8* mutants only after classic chemical synaptic transmission was blocked. This suggests that both synaptic transmission and *innexin8* encoded gap junction proteins are important for coordination of heterotypic PN coupling. However, coupling can be maintained by chemical synaptic transmission when the *innexin8* gene is mutated. The question still remains whether these *innexin8* encoded gap junctions play a small active role in the background of dominant synaptic transmission, are designed to regulate spontaneous activity in the antennal lobe circuit only when coordinated sensory input is removed, or both. The unexpected complexity of signal regulation in *Drosophila* displays the possibility of hidden communication pathways or regulation, changing how we perceive sensory information flow both in the fly and the mammal.

Previous studies in my lab have shown that *inx7* is a gap junction gene that regulates coordination of spontaneous changes in intracellular calcium between PNs in dissociated culture. However, when *inx7* expression was knocked down in PNs, this did not decrease the strong correlation of activity between PNs. This indicated that *innexin7* expression is not necessary for generation of coordinated membrane oscillations between heterotypic PNs in the isolated brain. This could be explained by *inx7* encoded gap junction proteins mediating coordinated changes in calcium level, but the amount and/or timing of the changes do not influence membrane potential oscillations. It is also possible that PNs in culture, made from brains of late stage pupae, express *inx7* at much higher levels than they do in the adult. This may be important in coordinating coupling that is important for development but is not necessary in the mature brain. Future studies to address this question could evaluate calcium levels and membrane potential oscillations in PN pairs in pupal brains isolated from both wildtype and *inx7* knockdowns.

My data indicate that all heterotypic PN pairs exhibit coordinated oscillations, but I did not find direct evidence of electrical connections. These coordinated oscillations could be due to up-regulation of gap junction activity in PNs in the isolated brain resulting in an expansive antennal lobe PN network. In this case, injecting current into one cell of a large network would have little effect on any other cell. A mechanism that could underlie such an effect could involve gating of gap junctions (Delmar et al., 2000). Removal of coordinated sensory input could alter levels of phosporylation, which could increase gap junction function and result in formation of an extensive PN network. Future experiments that introduce phosphatases or kinases into the cell through the pipette could be used to determine if coordinated oscillatory activity is regulated by levels of phosphorylation.

A second possible explanation for coordinated oscillations in the absence of direct electrical coupling between two PNs is that GABAergic local interneurons could coordinate activity across glomeruli (Lagier et al., 2004). These interneurons span glomeruli and they may be important in coordinating activity of multiple PNs. This could be tested by examining the effect of removing these cells by laser ablation or genetic manipulations.

Acknowledgements

To everyone in the O'Dowd and Smith lab, thank you. Thanks especially to Dr. Diane O'Dowd. Without her tireless effort and concern for my progress, both as a person and as a scientist, none of this would be possible.

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