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EGFR signaling modulates synaptic connectivity via Gurken

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Abstract

Synaptic target selection is critical for establishing functional neuronal circuits. The mechanisms regulating target selection remain incompletely understood. We describe a role for the EGF receptor and its ligand Gurken in target selection of octopaminergic Type II neurons in the *Drosophila* neuromuscular system. Mutants in *happyhour*, a regulator of EGFR signaling, form ectopic Type II neuromuscular junctions. These ectopic innervations are due to inappropriate target selection. We demonstrate that EGFR signaling is necessary and sufficient to inhibit synaptic target selection by these octopaminergic Type II neurons, and that the EGFR ligand Gurken is the post-synaptic, muscle-derived repulsive cue. These results identify a new pathway mediating cell-type and branch-specific synaptic repulsion, a novel role for EGFR signaling in synaptic target selection, and an unexpected role for Gurken as a muscle-secreted repulsive ligand.

Keywords

EGFR; Gurken; target selection; Type II neuron; synaptic repulsion

Introduction

Neurons form synapses with appropriate partners to generate complex circuits during development. These circuits are critical for regulating behavior, homeostasis, learning and memory. Many different processes guide neurons to their target including cell migration, axon guidance, and the localization of pre- and post-synaptic components (Tessier-Lavigne & Goodman 1996). Most neurons recognize their specific targets in spite of encountering many other cells en route to their destination (Shen & Scheiffele 2010). While there are many examples of finely tuned target selection, the molecular mechanisms that govern this process remain poorly defined. A better understanding of these wiring mechanisms is crucial to appreciate circuit assembly and to begin to understand the consequences of miswiring and its implications in neurological disorders.

The *Drosophila* larval neuromuscular system is formed by identified pre- and postsynaptic cells and the wiring pattern is invariant, making it a good system to study synaptic connectivity. The primary innervations are glutamatergic, are responsible for fast synaptic transmission, and are subdivided into Type Ib and Is based on the innervation pattern and bouton morphology (J. Johansen, Halpern, et al. 1989). The second major class of neuromuscular junctions (NMJs) are termed Type II, and are octopaminergic and neuromodulatory (Monastirioti, Gorczyca, et al. 1995). Type II NMJs are present on fewer muscles of the larva and have a distinctive morphology. Neurons forming Type Is NMJs

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innervate multiple muscle targets in a given hemisegment (Landgraf, Bossing, et al. 1997) whereas Type II neurons innervate a different complement of muscle targets in the same region (Monastirioti, Gorczyca, et al. 1995). These two types of neurons encounter the same muscle field and yet make divergent target selection choices, making this an interesting system in which to study synaptic target selection.

One advantage of the *Drosophila* system is the ability to unravel mechanisms of target selection genetically (Collins & DiAntonio 2007). Many of the most striking targeting phenotypes result from gain-of-function rather than loss-of-function studies (Ghose & Van Vactor 2002). For example, overexpression of muscle-derived Fasciclin II (FasII) potently promotes the formation of ectopic synapses that are functional and stable (Davis, Schuster, et al. 1997). Loss-of-function studies may find less dramatic phenotypes because targeting decisions are complex and involve the integration of a number of positive and negative cues (Winberg, Mitchell, et al. 1998). Indeed, when their normal muscle targets are ablated, motoneurons retain the ability to synapse on alternate muscles (Cash, Chiba, et al. 1992). This suggests that the target selection of neurons is a malleable process that requires multiple simultaneous cues. In addition to molecular cues, decreases in neuronal activity via mutations in sodium channel subunits also lead to the formation and stabilization of ectopic synapses (Jarecki & H Keshishian 1995). Recent studies have highlighted the important role of electrical activity and semaphorin signaling in shaping synaptic connectivity in this system (Carrillo et al. 2010; Koon et al. 2011). Despite this progress, it is still not clear how Type II octopaminergic neurons distinguish between appropriate and inappropriate targets, and more generally, how neurons navigate to their appropriate targets to form synaptic connections.

To address this question, we undertook a genetic screen to identify mutants with defects in synaptic targeting. From this screen we identified a role for EGFR signaling in Type II neuron target selection. We find that EGFR signaling inhibits synaptic target selection in a cell-type and branch-specific manner. The inhibitory cue is provided by a muscle-derived EGFR ligand, Gurken. This is a novel function for Gurken, which was previously suggested to function exclusively in the developing oocyte (Nilson & Schüpbach 1999). This work defines EGFR and Gurken as an important receptor/ligand pair contributing to the array of signals shaping the development of neural circuits.

Methods

Fly Stocks

Flies were maintained at 25°C on standard fly food. Wild type (WT) flies were Canton S (CS) unless specified otherwise. The following flies were obtained from the Bloomington Stock Center: P{SUPor-P}*hppy*^{KG05537}, *Df(2R)Exel6069*, *Tdc2-Gal4* (Koon et al. 2011), *UAS-CD8-GFP* (T.Lee & Luo 1999), *rl^I* (Biggs, Zavitz, et al. 1994), *UAS-DCR2* (Y. S. Lee, Nakahara, et al. 2004), *G7-Gal4*, *M12-Gal4*, *UAS-EGFR* (2nd chromosome) (Carmena, Gisselbrecht, et al. 1998), *gurken*^{2B} (Neuman-Silberberg & Schüpbach 1993), *UAS-EGFR_{DN}*, and *UAS-Rhomboid* (Guichard, Biehs, et al. 1999).

UAS-hppy was a gift from Ulrike Heberlein (UCSF, CA). *UAS-Gurken RNAi* (36251 & 101701) was from the Vienna Drosophila Genome Resource Center (Vienna, Austria). *UAS-Gurken* was a gift of Bruce Edgar (Jiang & Edgar 2009).

Immunohistochemistry

Third-instar larvae were dissected in PBS and fixed in either Bouin's fixative for 5 min. or 4% paraformaldehyde for 30 min on ice. Larvae were washed with PBS containing 0.1% Triton-X-100 (PBT) and blocked in 1% NGS in PBT for 30 min, followed by overnight

incubation in primary antibodies in 1% NGS in PBT, three washes in PBT, incubation in secondary antibodies in 1% NGS in PBT for 45 min, three final washes in PBT, and equilibration in 70% glycerol in PBS. Samples were mounted in Vectashield (Vector, Burlingame, CA). The following primary antibodies were used: rabbit α -DGluRIII (Marrus et al. 2004) 1:2500 in Bouin's, mouse α -FasII (clone 1D4) 1:5 in PFA, 1:100 in Bouin's, rabbit α -Vmat (Greer, Grygoruk, et al. 2005) 1:200 in PFA, mouse α -Brp (Wagh et al. 2006) 1:200 in Bouin's, mouse α -Futsch 1:100 in Bouin's, rabbit α -Synaptotagmin1 (Mackler et al. 2002) 1:2500 in Bouin's, rat α -mCD8a (Invitrogen) 1:100 in Bouin's, rabbit α -TBH (Koon et al. 2011) 1:400 in Bouin's, rabbit anti-GFP conjugated to Alexa Fluor-488, 1:200 (Invitrogen). Goat Cy3-, Alexa-633 and FITC conjugated secondary antibodies against mouse and rabbit IgG were used at 1:1000 and were obtained from Jackson ImmunoResearch. Chicken Cy3 conjugated secondary antibody against rat IgG was used at 1:200. Antibodies obtained from the Developmental Studies Hybridoma Bank were developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences of the University of Iowa, Iowa City, IA.

Imaging and Analysis

Samples were imaged with a Nikon D-Eclipse C1 confocal microscope using 60 $\times$ oil objective. Shown images are z projections of confocal stacks acquired from serial laser scanning except for the Tdc2-GFP cell bodies which are presented as single confocal sections. Quantification was performed to determine presence or absence of FasII and Vmat positive projections on muscles 6,7,12, 13, and 4. These muscles were examined in segments A3-A5. Values were analyzed for significance using the Fisher's exact test and two-tailed P value tests. Type I boutons were counted on muscles 6 and 7 of segment A3 and analyzed with a two-sample t-test. Type Is bouton presence was examined on muscles 12, and 13 in segments A3-A5 and analyzed with Fisher's exact test and two tailed P value tests. Length of Type II synapses in segments A3-A5, was quantified using ImageJ and significance was calculated with a two-sample t-test.

Results

To identify pathways and mechanisms that govern synaptic growth and development, we performed a genetic screen of several collections of *Drosophila* mutants including p-element insertions from the BDGP project (Bellen, Levis, et al. 2004). We dissected third-instar larvae from each line and stained with a marker of the presynaptic active zone Bruchpilot (Brp) (Wagh, Rasse, et al. 2006) and the essential glutamate receptor subunit DGluRIII (Marrus & DiAntonio 2004). We identified lines with defects in synaptic development including synaptic targeting defects, active zone defects, and NMJ morphology defects. From this screen we identified an allele of *happyhour* (*hppy*), *P{SUPor-P}^{KG05537}*, which has a defect in synaptic targeting of the octopaminergic motoneurons that form Type II NMJs.

These octopaminergic motoneurons face an interesting problem: how do they choose their multiple synaptic targets and bypass other potentially suitable locations to form a synapse? The normal innervation pattern of the Type II motoneuron MNSNb/d-II and the Type Is motoneuron MNSNb/d-Is is shown in Fig 1A. Motoneuron MNSNb/d-Is forms three branches that innervate the cleft between muscles 6 and 7, muscle 13 and muscle 12 respectively. In contrast, the axon from motoneuron MNSNb/d-II extends under muscles 6 and 7 without forming a synapse, and then branches twice to form NMJs onto muscles 13 and 12. In *hppy* mutants, however, ectopic Type II NMJs are more likely to form on muscles 6 and 7 than in wild type (Fig1B, 1C). We conclude that these ectopic NMJs are Type II endings because, like wild type Type II NMJs, they 1) are long and thin with very small

synaptic boutons and 2) express the vesicular monoamine transporter (VMAT)(Greer et al. 2005), which exclusively labels octopaminergic Type II NMJs in the periphery. In the higher magnification panels, colocalization between FasII and VMAT occurs in wild type Type II NMJs as well as the ectopic Type II NMJs in *hppy* mutants.

It is possible that these ectopic Type II NMJs may represent immature or defective Type II synapses. To investigate this possibility we labeled Type II synapses in wildtype and *hppy* mutants by expressing *UAS-CD8-GFP*, a membrane targeted GFP, with *Tdc2-Gal4*, which in the periphery expresses exclusively in octopaminergic neurons (Cole, Carney, et al. 2005). We find that the ectopic Type II synapses in *hppy* exhibit expression of tyrosine beta-hydroxylase (TBH) (Fig1E). TBH is necessary to synthesize octopamine and is necessary for mature Type II NMJ function. To determine if the ectopic Type II NMJs contain machinery for synaptic release, we examined the localization of the active zone component Bruchpilot (Brp). Brp is present in the ectopic Type II NMJs in *hppy* mutants (Fig1F). In addition, the MAP1B-related protein Futsch and the synaptic vesicle protein Synaptotagmin-1 are also present in the ectopic Type II NMJs (Fig1G, H). Together, these data indicate that the ectopic Type II endings on muscles 6 and 7 in *hppy* mutants have the anatomical hallmarks (Koon et al. 2011) of mature Type II NMJs.

The establishment of Type I innervation occurs before Type II innervation and is critical to guide the Type II neurons to their appropriate locations (Halpern, Chiba, et al. 1991). To test the possibility that the ectopic Type II innervation is due to a primary defect in Type I innervation, we examined Type Ib and Is innervations on muscles 6, 7, 12, and 13. We find no difference in the number of Type I boutons on muscles 6 and 7 in the *hppy* mutant (WT= 79.1 ± 3.0 ; *hppy*= 87.5 ± 6.8 $p=0.29$) or detectable differences in their morphology. Type Is boutons are present on muscles 12 and 13 both in wild type and *hppy* mutants ($p=1$ for muscles 6/7,12 and13). Hence, Type I innervation appears to be unaffected in *hppy* mutants and so is unlikely to contribute to the formation of ectopic Type II NMJs.

To assess whether this synaptic phenotype is due to loss of *hppy*, we generated transheterozygotes for *hppy*^{KG05537} and a deficiency chromosome, *Df(2R)Exel6069*, that deletes *hppy* and adjacent genes. We find no significant difference in the presence of ectopic Type II synapses between *hppy* and *hppy/df* ($p>0.05$) (Fig1C). Hence, this phenotype is not due to a second site mutation on the *hppy*^{KG05537} chromosome. In addition, since the *hppy*^{KG05537} phenotype was not exacerbated in combination with a definitive null allele, the *hppy*^{KG05537} allele is behaving as a genetic null or a very strong hypomorph. This phenotype is not restricted to muscles 6 and 7—*hppy* mutants also have a significant increase in the likelihood of ectopic Type II innervation on muscle 4 (WT 18% $\pm$ 0.6% $n=60$; *hppy* 50% $\pm$ 0.8% $n=60$ $p=0.0005$). Type II synapses are found on muscles 12 and 13 in wild-type animals (Fig1C). Potentially, the ectopic Type II synapses on muscles 6 and 7 in *hppy* mutants are being formed at the expense of normal Type II innervation onto muscles 12 and 13. In this scenario, these Type II synapses would be significantly smaller or absent altogether. To investigate this possibility, we measured total Type II synaptic span on muscles 12 and 13 and found that Type II synapses are unaffected in *hppy* mutants (M12; WT 296 ± 25 μ m, *hppy* 301 ± 46 μ m $p=.92$, M13; WT 371 ± 45 μ m, *hppy* 308 ± 44 μ m $p=.33$ $n=10$) (Fig1D). In addition, we observed no missing type II NMJs on these muscles ($n=20$). This demonstrates that the Type II synapses at muscles 6 and 7 in *hppy* mutants are not misplaced synapses that should be located on muscles 12 and 13, but are ectopic synapses formed in addition to the normal complement of Type II synapses.

To investigate the cellular basis for these ectopic synapses, we analyzed the cell number and projection pattern of octopaminergic motoneurons. In wild-type larvae, there are clusters of three octopaminergic (Type II) neurons in the ventral nerve cord corresponding to each

segment of the animal. These neurons each have one process that bifurcates within the ventral nerve cord forming bilateral projections to each hemisegment. These projections then branch in the periphery and form Type II NMJs at appropriate locations within the hemisegment. In light of this anatomy, there are three potential explanations as to the source of the ectopic synapses: 1) *hppy* mutants have an increase in octopaminergic cell number 2) existing octopaminergic neurons form additional primary branches within the nerve cord or 3) the existing octopaminergic neuronal projections are forming ectopic branches in the periphery. To distinguish among these possibilities, we counted octopaminergic neuron cell bodies and their primary projections in wild-type and *hppy* mutant larvae. In order to count these neurons, we expressed *UAS-CD8-GFP*, a membrane targeted GFP, with *Tdc2-Gal4*. In wild-type larvae there are clusters of three octopaminergic neurons in each segment of the animal (Fig2A). In *hppy* larvae there are also three octopaminergic neurons per segment (WT-3; *hppy*-3; n=10; p=1). To assess the number of primary projections formed by these neurons, we counted the number of labeled axons innervating each hemisegment and determined that the number of projections was the same (WT-3; *hppy*-3; n=10; p=1) in *hppy* and wild-type (Fig2B). Following these GFP positive projections to their targets, we confirmed that the ectopic synapses in *hppy* mutants form from the extensions of the TDC2 positive neurons (Fig2C). These ectopic synapses in *hppy* mutants can originate from extensions of MNSNb/d-II, the Type II neuron that normally innervates muscles 12 and 13, or occasionally from MNISN-II whose projection runs in the intersegmental nerve and that normally innervates more lateral targets. Hence, the wiring defects in the *hppy* mutant are due to ectopic branching of octopaminergic projections within their muscle target field.

To test whether this phenotype is due to loss of *happyhour* in octopaminergic neurons, we expressed *UAS-hppy* with *Tdc2-Gal4* in the *happyhour* background in an effort to rescue the ectopic synapse phenotype. Expression of *happyhour* in octopaminergic neurons reduces the frequency of ectopic synapses in *hppy* mutants (Fig2D&E) ($p < .05$). While our rescue is statistically significant, it does not fully recapitulate wild-type innervation, potentially due to the strength or timing of expression from *TDC2-gal4*. Overexpression of *hppy* in a wild-type background does not show a significant change in either Type II NMJ structure or target selection (data not shown).

hppy regulates EGF receptor (EGFR) signaling that can modulate downstream activation of ERK, known in *Drosophila* as *rolled* (*rl*) (Corl, Berger, et al. 2009). We investigated whether or not this same signaling pathway is responsible for the ectopic synapse phenotype. To test whether overactivation of *rolled* downstream of *happyhour* is responsible for the ectopic Type II synapses, we made a double mutant of *happyhour* and a hypomorphic allele of *rolled* (*rl^l*) (Biggs et al. 1994). We find that homozygous *rl^l* mutants do not have any changes in Type II innervation in an otherwise wild type background (Fig3A). However, the *rl^l* mutant potently suppresses the ectopic Type II synapses in *hppy* mutants (Fig3A). This suppression is essentially complete (Fig3B) ($p < .005$).

Suppression with *rl^l* suggests that the phenotype is due to an overactivation of the EGFR pathway, however, such overactivation often leads to strong negative feedback. Such feedback ensures that strong activation of the pathway is quickly and effectively turned off (Avraham & Yarden 2011). Indeed, baseline levels of p-ERK are significantly decreased in *hppy* mutants, indicating that the pathway is turned down (Corl et al. 2009). Hence, while the findings with *hppy* and *rl* implicate EGFR signaling in the restraint of ectopic synapse formation, they are unable to distinguish the directionality of the signal. To investigate the role of EGFR directly, we manipulated the receptor in octopaminergic neurons. We expressed a dominant negative EGFR construct, *UAS-EGFR-DN* (Freeman 1996), with the *Tdc2-Gal4* driver (Fig4A). This inhibition of EGFR function in octopaminergic neurons results in a significant increase in the number of ectopic Type II NMJs on muscles 6 and 7

(Fig4B) ($p < .05$). This suggests that EGFR acts within octopaminergic neurons to inhibit inappropriate innervation. If so, then overexpression of EGFR in octopaminergic neurons may overactivate this repulsive signal and may lead to the loss of Type II innervation onto muscles 12 or 13, the normal synaptic partners of these neurons. To investigate this hypothesis, we expressed wild-type EGFR (*UAS-EGFR*) with the *Tdc2-Gal4* driver (Fig4C). This led to a significant decrease in the proportion of muscle 12 cells with Type II NMJs (Fig4D) ($p < .005$). Taken together, these loss- and gain-of-function studies demonstrate that EGFR signaling is necessary and sufficient to inhibit the capacity of octopaminergic motoneurons to form NMJs with targets.

Since EGFR signaling mediates a branch-specific repulsive signal in motoneurons, a muscle-derived ligand is likely the inhibitory cue. There are several known ligands of the EGFR, including Vein, Spitz, Keren and Gurken (Schnepf et al. 1996) (Rutledge, K. Zhang, et al. 19921) (Reich & Shilo 2002) (Neuman-Silberberg & Schüpbach 1993). To test whether any of these is the repulsive cue, we knocked down expression of each ligand via RNAi expressed under the control of a muscle specific driver, *G7-gal4* (Y. Q. Zhang, Bailey, et al. 2001). Knockdown of Vein, Spitz and Keren has no synaptic phenotype. In contrast, knockdown of Gurken recapitulates the dominant negative EGFR phenotype (Fig5A). Expression of an RNAi targeting Gurken (VDRC36251) leads to a significant increase in ectopic Type II innervations on muscles 6 and 7 (Fig5A,B) ($p < .05$). Similar results were obtained with a second Gurken RNAi (VDRC101701) ($p < .05$) that shares no common sequence with VDRC36251 as well as the homozygous *gurken*^{2B} mutant (Neuman-Silberberg & Schüpbach 1993), which has ~25% ectopic Type II NMJs at muscles 6 and 7 ($p < .05$) (Fig5A,B).

As a second test of this model, we asked whether overexpression of Gurken is sufficient to inhibit octopaminergic NMJ formation at the normal target. We expressed *UAS-Gurken* and its processing enzyme *UAS-Rhomboid* with *M12-Gal4*, which expresses in muscle 12, a normal synaptic target for MNSNb/d-II (Fig6A). Overexpression of Gurken and Rhomboid in muscle 12 is sufficient to inhibit the formation of Type II NMJs at their normal targets (Fig6B). These loss- and gain-of-function studies strongly support the model that Gurken is an inhibitory cue for octopaminergic synaptogenesis. To shape target selection, however, the model predicts that Gurken expression should be localized to muscles that do not receive octopaminergic input, such as 6 and 7. Unfortunately, we are unable to confirm this aspect of the model via *in situ* or antibody staining, as we have been unable to detect endogenous *gurken* transcript or protein in any muscles. Although it cannot speak to muscle specific expression, the modEncode RNA-seq data does demonstrate that Gurken is enriched in body wall muscle (Chintapalli, Wang, et al. 2007), supporting our genetic findings that Gurken functions outside of the developing oocyte.

Discussion

Synaptic target selection is a critical step in establishing functional neural circuits. The molecular mechanisms governing this selection have not yet been fully explored. We have taken our observation that *hppy* mutants have an increased frequency of ectopic octopaminergic Type II NMJs and identified a pathway critical for synaptic target recognition in these neurons. We find that the EGFR signaling pathway is required to prevent the development of inappropriate synaptic contacts. This inhibitory signal is mediated by muscle-derived EGFR ligand, Gurken, working through the EGF receptor in type II motoneurons. This mechanism sculpts the neuronal wiring pattern in a cell-type and branch-specific manner.

EGF as a guidance cue

There are many signaling pathways that influence the innervation pattern of *Drosophila* motor neurons. Our findings identify a novel role for EGFR signaling in mediating a repulsive guidance cue to Type II neurons. EGF has previously been demonstrated to regulate axon growth and guidance. For example, EGF positively regulates Sema-3a levels in the cornea (Ko, Morishige, et al. 2008) and interacts with NCAM-180 to promote neuritogenic activity (Povlsen, Berezin, et al. 2008). However, to our knowledge our data is the first to demonstrate that an EGF receptor and ligand provide a guidance signal.

We find ectopic Type II NMJs in *hppy* mutants, as well as when we express a dominant negative EGFR in Type II neurons or knock down its ligand Gurken in the muscle targets. This presents an apparent contradiction. Hppy is described as a negative regulator of EGFR (Corl et al. 2009), and its phenotype in this system is suppressed by a hypomorphic mutation in *rolled* (ERK), consistent with Hppy functioning as a negative regulator of EGFR signaling. Why then does loss of *hppy* have the same phenotype as inhibition of the receptor or ligand? We posit that the well-described strong negative feedback (Avraham & Yarden 2011) induced by EGFR signaling may be the explanation. We propose a model in which activation of the EGFR pathway mediates a signal that inhibits the formation or stabilization of Type II NMJs. In *hppy* mutants, however, loss of negative regulation would allow for excess activation of the EGFR that would induce a quick, strong and long-lasting negative feedback activity early in development, essentially turning off EGFR signaling in cells expressing *happyhour*. The result would be loss of the synaptic inhibitory signal mediated by EGFR at the stage when these Type II neurons are extending to their targets and the promotion of ectopic synapse formation. We appreciate that this model is speculative, and that an alternative is that *hppy* and *rolled* are regulating a pathway that is distinct from the EGFR/gurken pathway.

EGFR and synapse formation

By what mechanism does EGFR signaling affect synapse formation in Type II neurons? Presumably, there is a molecular program downstream of EGFR activation that modifies the Type II neuron such that it does not form and/or maintain an NMJ with an inappropriate target. These changes could occur at the level of the cell body or locally within individual branches. It is unlikely that cell body changes are central to the phenotype because such neuron-wide mechanisms could not easily be translated into branch-specific behavior. In contrast, local effects of EGFR signaling within neurites could explain such specificity. We do not know the cell biological mechanism mediating the branch-specific inhibition. Possibilities include alterations in the local translation or membrane insertion of synaptogenic molecules, local modulation of cytoskeletal dynamics, or failure to properly prune Type II connections.

Not only is the EGFR mediating a branch-specific effect, but it is also cell-type specific. As described above, the Type II motoneuron MNSNb/d-II and the Type I motoneuron MNSNb/d-Is travel together and presumably encounter the same cues across the hemisegment, however they generate different innervation patterns. This implies that these two types of neurons have developed cell-type specific repertoires of receptors or signaling pathways that shape their target choices.

While our data indicate a role for EGFR signaling in Type II synaptic target selection it is also likely that Type II target selection has multiple components. Our phenotypes occur at a relatively low penetrance, so it is likely that complementary and combinatorial guidance cues function with the EGFR pathway to shape target selection of Type II neurons.

A novel role for Gurken

Gurken has been studied exclusively in the developing oocyte (Schüpbach 1987) (Neuman-Silberberg & Schüpbach 1993) (González-Reyes et al. 1995) and, to our knowledge, has no known roles in other tissues. Hence, it is surprising that Gurken conveys the repulsive signal from muscle to the octopaminergic Type II neurons. In support of a function in muscle, the modEncode RNA-seq project has found that Gurken transcript is enriched 3.5 fold in larval body wall muscles (Chintapalli et al. 2007). While Gurken may be secreted from all muscles, we prefer a model in which localized expression in a muscle subset shapes the branching pattern of the innervating motoneuron. In this model, Gurken released from muscles 6 and 7, as well as other targets that should not be innervated, would locally inhibit synaptogenesis, blocking the formation of ectopic connections while allowing for the normal innervation at muscles 12 and 13. This model is consistent with our findings that knockdown of Gurken in muscle results in ectopic NMJs while localized overexpression in the normal target cell inhibits formation of appropriate NMJs. While these functional data are strong, our model must remain speculative because we have been unable to determine the localization of Gurken using currently available reagents. Future studies will investigate how this Gurken/EGFR pathway is integrated with the recently defined semaphorin- and activity-dependent mechanisms that also play an important role in shaping synaptic target selection in these neurons (Carrillo et al. 2010).

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References

- Avraham R, Yarden Y. Feedback regulation of EGFR signalling: decision making by early and delayed loops. *Nature Reviews Molecular Cell Biology*. 2011; 12(2):104–117.
- Bellen HJ, et al. The BDGP gene disruption project: single transposon insertions associated with 40% of Drosophila genes. *Genetics*. 2004; 167(2):761–781. [PubMed: 15238527]
- Biggs WH, et al. The Drosophila rolled locus encodes a MAP kinase required in the sevenless signal transduction pathway. *The EMBO Journal*. 1994; 13(7):1628–1635. [PubMed: 8157002]
- Carmena A, et al. Combinatorial signaling codes for the progressive determination of cell fates in the Drosophila embryonic mesoderm. *Genes & Development*. 1998; 12(24):3910–3922.
- Carrillo RA, et al. Presynaptic activity and CaMKII modulate retrograde semaphorin signaling and synaptic refinement. *Neuron*. 2010; 68(1):32–44. [PubMed: 20920789]
- Cash S, Chiba A, Keshishian H. Alternate neuromuscular target selection following the loss of single muscle fibers in Drosophila. 1992; 6:2051–64.
- Chintapalli V, Wang J, Dow J. Using FlyAtlas to identify better Drosophila melanogaster models of human disease. 2007; 9:715–720.
- Cole SH, et al. Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. *The Journal of Biological Chemistry*. 2005; 280(15):14948–14955. [PubMed: 15691831]
- Collins CA, DiAntonio A. Synaptic development: insights from Drosophila. *Current Opinion in Neurobiology*. 2007; 17(1):35–42. [PubMed: 17229568]
- Corl AB, et al. Happyhour, a Ste20 family kinase, implicates EGFR signaling in ethanol-induced behaviors. *Cell*. 2009; 137(5):949–960. [PubMed: 19464045]
- Davis GW, Schuster CM, Goodman CS. Genetic analysis of the mechanisms controlling target selection: target-derived Fasciclin II regulates the pattern of synapse formation. *Neuron*. 1997; 19(3):561–573. [PubMed: 9331349]

- Freeman M. Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell*. 1996; 87(4):651–660. [PubMed: 8929534]
- Ghose A, Van Vactor D. GAPs in Slit-Robo signaling. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*. 2002; 24(5):401–404.
- González-Reyes A, Elliott H, St Johnston D. Polarization of both major body axes in *Drosophila* by gurken-torpedo signalling. *Nature*. 1995; 375(6533):654–658. [PubMed: 7791898]
- Greer CL, et al. A splice variant of the *Drosophila* vesicular monoamine transporter contains a conserved trafficking domain and functions in the storage of dopamine, serotonin, and octopamine. *Journal of Neurobiology*. 2005; 64(3):239–258. [PubMed: 15849736]
- Guichard A, et al. rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during *Drosophila* wing vein development. *Development (Cambridge, England)*. 1999; 126(12):2663–2676.
- Halpern ME. Growth cone behavior underlying the development of stereotypic synaptic connections in *Drosophila* embryos. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 1991; 11(10):3227–3238. [PubMed: 1658247]
- Jarecki J, Keshishian H. Role of neural activity during synaptogenesis in *Drosophila*. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 1995; 15(12):8177–8190. [PubMed: 8613752]
- Jiang, H.; Edgar, BA. *Development*. Vol. 136. Cambridge, England: 2009. EGFR signaling regulates the proliferation of *Drosophila* adult midgut progenitors; p. 483–493.
- Johansen J, et al. Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 1989; 9(2):710–725. [PubMed: 2563766]
- Ko JA, et al. Up-regulation of semaphorin 3A in human corneal fibroblasts by epidermal growth factor released from cocultured human corneal epithelial cells. *Biochemical and Biophysical Research Communications*. 2008; 377(1):104–108. [PubMed: 18831963]
- Koon AC, et al. Autoregulatory and paracrine control of synaptic and behavioral plasticity by octopaminergic signaling. *Nature Neuroscience*. 2011; 14(2):190–199.
- Landgraf M, et al. The origin, location, and projections of the embryonic abdominal motoneurons of *Drosophila*. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 1997; 17(24):9642–9655. [PubMed: 9391019]
- Lee T, Luo L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*. 1999; 22(3):451–461. [PubMed: 10197526]
- Lee YS, et al. Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*. 2004; 117(1):69–81. [PubMed: 15066283]
- Mackler JM, et al. The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo. *Nature*. 2002; 418(6895):340–344. [PubMed: 12110842]
- Marrus SB, DiAntonio A. Preferential localization of glutamate receptors opposite sites of high presynaptic release. *Current Biology: CB*. 2004; 14(11):924–931. [PubMed: 15182665]
- Marrus SB, et al. Differential localization of glutamate receptor subunits at the *Drosophila* neuromuscular junction. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*. 2004; 24(6):1406–1415. [PubMed: 14960613]
- Monastirioti M, et al. Octopamine immunoreactivity in the fruit fly *Drosophila melanogaster*. 1995; 356(2):275–87.
- Neuman-Silberberg FS, Schüpbach T. The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell*. 1993; 75(1):165–174. [PubMed: 7691414]
- Nilson LA, Schüpbach T. EGF receptor signaling in *Drosophila* oogenesis. *Current Topics in Developmental Biology*. 1999; 4:4, 203–243.
- Povlsen GK, Berezin V, Bock E. Neural cell adhesion molecule-180-mediated homophilic binding induces epidermal growth factor receptor (EGFR) down-regulation and uncouples the inhibitory function of EGFR in neurite outgrowth. *Journal of Neurochemistry*. 2008; 104(3):624–639. [PubMed: 17995934]

- Reich A, Shilo BZ. Keren, a new ligand of the *Drosophila* epidermal growth factor receptor, undergoes two modes of cleavage. *The EMBO Journal*. 2002; 21(16):4287–4296. [PubMed: 12169631]
- Rutledge BJ, et al. The *Drosophila* spitz gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. *Genes & Development*. 1992; 6(8):1503–1517. [PubMed: 1644292]
- Schnepp B, et al. Vein is a novel component in the *Drosophila* epidermal growth factor receptor pathway with similarity to the neuregulins. *Genes & Development*. 1996; 10(18):2302–2313. [PubMed: 8824589]
- Schüpbach T. Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in *Drosophila melanogaster*. *Cell*. 1987; 49(5):699–707. [PubMed: 3107840]
- Shen K, Scheiffele P. Genetics and cell biology of building specific synaptic connectivity. *Annual Review of Neuroscience*. 2010; 33:473–507.
- Tessier-Lavigne, M.; Goodman, CS. *Science*. Vol. 274. New York, N.Y.: 1996. The molecular biology of axon guidance; p. 1123–1133.
- Wagh DA, et al. Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron*. 2006; 49(6):833–844. [PubMed: 16543132]
- Winberg ML, Mitchell KJ, Goodman CS. Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial functions of netrins, semaphorins, and IgCAMs. *Cell*. 1998; 93(4):581–591. [PubMed: 9604933]
- Zhang YQ, et al. *Drosophila* fragile X-related gene regulates the MAP1B homolog Futsch to control synaptic structure and function. *Cell*. 2001; 107(5):591–603. [PubMed: 11733059]

Figure 1. *hppy* mutants have ectopic Type II synapses

A. Schematic detailing the innervation pattern of a wild-type larval ventral muscles. Red represents Type I glutamatergic innervations. Green represents Type II octopaminergic innervations. Muscles are identified by number. L corresponds to lateral and M corresponds to medial.

B. Images of 3rd instar larval muscles 6 and 7 with neurons immunolabeled with anti-FasII (red) and anti-Vmat (green). Arrowheads indicate ectopic Type II innervations positive for Vmat. Scale bars represent 50 μ m.

C. Images of muscles 12 and 13, Arrowheads indicate normal Type II innervations that are unaffected in *hppy* mutants. Scale bars represent 50 μ m.

D. Histogram shows quantification of the presence of Type II innervation on muscles 6 and 7 (percent of muscles innervated by Type II synapses). *hppy* larvae have a significant increase in the presence of Type II innervation on muscles 6 and 7 (WT 1.8% $\pm$.4% n=54; *hppy* 16% $\pm$ 1.4% n=54; WT:*hppy* p<.05 Fisher's exact test). *hppy/df* flies also have a significant increase in the presence of Type II innervation on muscles 6 and 7 (*hppy/df* 13% $\pm$.8%; n=92; WT:*hppy/df* p<.05 Fisher's exact test). Error bars represent 95% confidence interval.

E-H. Images of wild-type (WT) Type II synapses on muscle 12 and ectopic Type II synapses in *hppy* mutants on muscles 6 and 7. Scale bars represent 1 μ m.

E. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), anti-TBH (blue) and anti-FasII (red).

- F. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), anti-TBH (blue) and anti-Brp (red).
- G. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), anti-TBH (blue) and anti-Futsch (red).
- H. Type II NMJs immunolabeled with anti-mCD8 (Tdc2-GFP)(green), and anti-Syt1 (red).

Figure 2. Ectopic synapses result from a targeting defect of the existing Type II neurons
 A. Single confocal sections of Type II neuronal cell bodies expressing *UAS-CD8-GFP* under *Tdc2-Gal4* control. *hppy* mutants do not exhibit an increased number of Type II neurons. (WT-3; *hppy*-3; n=10; p=1) Scale bars represent 5 μ m.
 B. Images of projections expressing *UAS-CD8-GFP* under *Tdc2-Gal4* control in Type II neurons. *hppy* mutants do not exhibit an increased number of projections from Type II neurons. (WT-3; *hppy*-3; n=10; p=1) Scale bar represents 1 μ m.
 C. Images of projections expressing *UAS-CD8-GFP* under *Tdc2-Gal4* control. The *Tdc2-GFP* projections form the ectopic Type II synapses in *hppy* mutants. FasII (red) labels neuronal membranes. Scale bar represents 5 μ m.
 D. Images of larval muscles 6 and 7, neurons immunolabeled with FasII (red) and Vmat (green). Ectopic Type II innervations found in *hppy* mutants are rescued by expression of *UAS-hppy* with *Tdc2-Gal4* (Type II neuron specific). Scale bar represents 10 μ m.
 E. Quantification of the presence of Type II innervation on muscles 6 and 7. Type II neuron specific rescue of *hppy* mutants significantly suppressed ectopic Type II innervations on muscles 6 and 7. *Tdc2-hppy* 7.1% $\pm$ 1.2% n=42; *hppy* 22% $\pm$.6% n=150; *hppy; Tdc2-hppy* 13% $\pm$.4% n=150 (*hppy* : *hppy;Tdc2-hppy* p < .05 Fisher's exact test). Error bars represent 95% confidence interval.

Figure 3. Overactivation of *rolled* downstream of *happyhour* is responsible for the ectopic synapses

A. Images of larval muscles 6 and 7, neurons immunolabeled with anti-FasII (red) and anti-Vmat (green). Arrowheads indicate the presence of ectopic Type II innervation.

B. Quantification of the presence of Type II innervations on muscles 6 and 7. A double mutant of *hppy* and *rolled* significantly suppresses the *hppy* phenotype (WT 3.3% ± .59% n=60; *rl¹/rl¹* 1.7% ± .4% n=59; *hppy* 18.3% ± 1.2% n=60; *hppy, rl¹/hppy, rl¹* 1.6% ± .4%) (*hppy*: *hppy, rl¹/hppy, rl¹* p < .005 Fisher's exact test). Error bars represent 95% confidence interval.

Figure 4. EGFR is necessary and sufficient to inhibit Type II innervation

A. Images of muscles 6 and 7 immunolabeled with anti-Vmat (green) and anti-FasII (red). Loss of EGFR signaling in Type II neurons results in ectopic Type II innervation on muscles 6 and 7.

B. Graph shows quantification of the presence of Type II innervation on muscles 6 and 7. Loss of EGFR signaling results in a significant increase in Type II innervation on muscles 6 and 7 (*Tdc2*/+ 3.4% ± .4% n=100; *UAS-EGFR-DN* 6.1% ± .4% n=100; *Tdc2-UAS-EGFR-DN* 14.9% ± .8% n=100) (*Tdc2*/+ : *Tdc2-EGFR-DN* p< .05; *UAS-EGFR-DN*:*Tdc2-UAS-EGFR-DN* p< .05) Fisher's exact test). Error bars represent 95% confidence interval.

C. Images of muscles 12 and 13 immunolabeled with anti-Vmat (green) and anti-FasII (red) M12 and M13 notations indicate location of muscles. Arrowhead indicates normal Type II innervation found on Muscle 12. Arrow indicates absence of Type II innervation on Muscle 12 when *UAS-EGFR* is overexpressed with *Tdc2-Gal4*.

D. Graph shows quantification of the absence of Type II innervations on Muscle 12. Overexpression of wild-type EGFR with a Type II neuron specific driver results in a significant number of muscles 12 that are not innervated by Type II synapses (*Tdc2*/+ 2% ± .6% n=50; *UAS-EGFR*/+ 0% n=50; *Tdc2-UAS-EGFR* 21% ± 1.6% n=50) (*Tdc2*/+ : *Tdc2-UAS-EGFR* p< .005; *UAS-EGFR*/+ : *Tdc2-UAS-EGFR* p= .0005 Fisher's exact test). Error bars represent 95% confidence interval.

Figure 5. Gurken ligand is necessary to mediate a repulsive signal through EGFR

A. Images of muscles 6 and 7 immunolabeled with anti-Vmat (green) and anti-FasII (red). Knockdown of Gurken in the muscle results in ectopic Type II synapses on muscles 6 and 7. *Gurken*^{2B} mutants also exhibit ectopic Type II synapses on muscles 6 and 7. Scale bars represent 10 μm.

B. Graph shows quantification of the presence of Type II innervation on muscles 6 and 7. Knockdown of Gurken in the muscle results in a significant increase in Type II innervation on muscles 6 and 7 (*UAS-Dcr2;G7gal4/+* 0% n=36; *UAS-Grk RNAi* 0% n=36; *UAS-Dcr2;G7gal4 UAS-Grk RNAi* 16% ± 2% n=36; *Gurken*^{2B} 25.9% ± 1.2% n= 77) (*UAS-Dcr2;G7gal4/+* : *UAS-Dcr2;G7gal4/ UAS-Grk RNAi* p< .05 Fisher's exact test) (*UAS-Dcr2;G7gal4/+* : *Gurken*^{2B} p<.0005 Fisher's exact test). Error bars represent 95% confidence interval.

Figure 6. Gurken ligand is sufficient to inhibit Type II innervation through EGFR

A. Images of muscles 12 and 13 immunolabeled with anti-Vmat (green) and anti-FasII (red). Expression of *UAS-Gurken* and *UAS-Rhomboïd* with the muscle 12 specific driver *M12-Gal4* results in a loss of Type II synapses on muscles 12 and 13. Scale bars represent 50 μ m. B. Graph shows quantification of the loss of Type II innervation on muscles 12 and 13. (*M12/+* 2.8% $\pm$.2% n=70; *UAS-Gurken, UAS-Rhomboïd/+* 1.4% $\pm$.2% n= 70; *M12/UAS-Gurken, UAS-Rhomboïd* 13.6% $\pm$.5% n=66) (*M12/+;M12/UAS-Gurken, UAS-Rhomboïd* p= 1; *UAS-Gurken, UAS-Rhomboïd/+; M12/UAS-Gurken, UAS-Rhomboïd* p<.05 Fisher's Exact Test). Error bars represent 95% confidence interval.