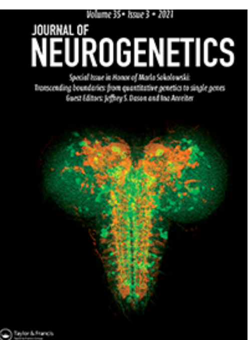

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ORIGINAL RESEARCH ARTICLE

A cGMP-dependent protein kinase, encoded by the *Drosophila foraging* gene, regulates neurotransmission through changes in synaptic structure and function

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ABSTRACT

A cGMP-dependent protein kinase (PKG) encoded by the *Drosophila foraging* (*for*) gene regulates both synaptic structure (nerve terminal growth) and function (neurotransmission) through independent mechanisms at the *Drosophila* larval neuromuscular junction (nmj). Glial *for* is known to restrict nerve terminal growth, whereas presynaptic *for* inhibits synaptic vesicle (SV) exocytosis during low frequency stimulation. Presynaptic *for* also facilitates SV endocytosis during high frequency stimulation. *for*'s effects on neurotransmission can occur independent of any changes in nerve terminal growth. However, it remains unclear if *for*'s effects on neurotransmission affect nerve terminal growth. Furthermore, it's possible that *for*'s effects on synaptic structure contribute to changes in neurotransmission. In the present study, we examined these questions using RNA interference to selectively knockdown *for* in presynaptic neurons or glia at the *Drosophila* larval nmj. Consistent with our previous findings, presynaptic knockdown of *for* impaired SV endocytosis, whereas knockdown of glial *for* had no effect on SV endocytosis. Surprisingly, we found that knockdown of either presynaptic or glial *for* increased neurotransmitter release in response to low frequency stimulation. Knockdown of presynaptic *for* did not affect nerve terminal growth, demonstrating that *for*'s effects on neurotransmission does not alter nerve terminal growth. In contrast, knockdown of glial *for* enhanced nerve terminal growth. This enhanced nerve terminal growth was likely the cause of the enhanced neurotransmitter release seen following knockdown of glial *for*. Overall, we show that *for* can affect neurotransmitter release by regulating both synaptic structure and function.

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Glia; exocytosis; endocytosis; synaptic transmission; presynaptic; neurotransmitter release; nerve terminal growth; axon

Introduction

Synaptic strength is influenced by changes in both synaptic structure (nerve terminal growth) and function (neurotransmission). The *Drosophila* larval neuromuscular junction (nmj) is a well-established model system for studying synaptic structure and function (reviewed in Harris & Littleton, 2015). Effects on these processes have been shown to be interrelated in some cases (Budnik, Zhong, & Wu, 1990; Davis & Goodman, 1998; Sigrist, Thiel, Reiff, & Schuster, 2002; Stewart, Schuster, Goodman, & Atwood, 1996; Zhong, Budnik, & Wu, 1992; Zhong & Wu, 2004), but not others (Dason *et al.*, 2009; Romero-Pozuelo, Dason, Atwood, & Ferrús, 2007; Romero-Pozuelo *et al.*, 2014). While most studies using this model have focused on presynaptic and postsynaptic regulation of synaptic structure and function, the importance of glia in these processes is becoming increasingly apparent (Brink, Gilbert, Xie, Petley-Ragan, & Auld, 2012; Dason, Allen, Vasquez, & Sokolowski, 2019; Fuentes-Medel *et al.*, 2009; Keller *et al.*, 2011; Kerr *et al.*, 2014).

The *Drosophila foraging* (*for*) gene encodes a cGMP-dependent protein kinase (PKG) (Osborne *et al.*, 1997) that affects numerous behaviours (see Anreiter & Sokolowski, 2019 for a detailed review). *for*'s influence on these behaviours likely arise from *for*'s effects at synapses. We recently found that *for* is expressed in both presynaptic neurons and glia at the *Drosophila* larval nmj and affects both synaptic structure and function (Dason *et al.*, 2019). Specifically, we found that for^0 null mutants have increased nerve terminal growth (Dason *et al.*, 2019). Tissue specific rescues demonstrated that this increased nerve terminal growth was due to the absence of *for* in glia (Dason *et al.*, 2019). Furthermore, for^0 null mutants have increased neurotransmission in response to low frequency stimulation due to increased presynaptic Ca^{2+} entry and decreased neurotransmission in response to high frequency stimulation due to reduced synaptic vesicle (SV) recycling (Dason *et al.*, 2019). Tissue specific rescues showed that both effects were due to presynaptic *for* (Dason *et al.*, 2019).

Fluorescein-assisted light inactivation of *for* revealed that *for*'s effects on neurotransmission can occur independent of its effects on nerve terminal growth (Dason *et al.*, 2019). However, it remains possible that *for*'s effects on nerve terminal growth can also affect neurotransmission and *for*'s effects on neurotransmission may contribute to changes in

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nerve terminal growth. To address these possibilities, we used RNA interference (RNAi) to selectively knock down all *for* transcripts in neurons or glia at the *Drosophila* larval nmj and examined whether *for*'s effects on synaptic structure and function were interrelated.

Material and methods

Fly stocks

Fly stocks were grown on molasses based fly medium in uncrowded conditions at 25 °C (Anreiter, Vasquez, Allen, & Sokolowski, 2016). Mid third instar larvae were used for all experiments. All transgenic lines were backcrossed into a wild-type *sitter* background (Allen, Anreiter, Neville, & Sokolowski, 2017). The GAL4/UAS system was used for neuron or glial specific expression of transgenes (Brand & Perrimon, 1993). A *UAS-forRNAi* line targeting all *for* transcripts and *UAS-Dcr* were used to knock down *for* in selected tissues (Dason *et al.*, 2020). *n-syb-GAL4* (Verstreken *et al.*, 2009) was used to drive expression of *UAS-forRNAi, UAS-Dcr* in neurons and *Repo-GAL4* (Sepp, Schulte, & Auld, 2001) was used to drive expression of *UAS-forRNAi, UAS-Dcr* in glia.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Cantarutti, Burgess, Brill, & Dason, 2018). Larvae were dissected, fixed and then incubated overnight at 4 °C with primary antibodies diluted in blocking solution. FITC-conjugated anti-horseradish peroxidase (HRP) antibody (1:800 dilution; Jackson ImmunoResearch) was used to visualize neurons and the mouse monoclonal bruchpilot (brp) antibody (1:100 dilution; Iowa Hybridoma Bank; Wagh *et al.*, 2006) was used to visualize active zones. Larval fillet preparations were mounted in Permafluor (Immunon, Pittsburgh, PA) on a glass slide with a cover slip. Preparations were viewed under a TCS SP5 confocal laser-scanning microscope (Leica, Heidelberg, Germany) with a 63× oil-immersion objective (1.4 NA).

Electrophysiology

Intracellular recordings were performed in HL6 saline (Macleod, Hegstrom-Wojtowicz, Charlton, & Atwood, 2002) supplemented with 0.5 mM $CaCl_2$ as previously described (Dason, Smith, Marin, & Charlton, 2014). Sharp glass electrode filled with 3 M KCl (~40MΩ) were used to measure spontaneously occurring miniature excitatory junction potentials (mEJPs) and stimulus-evoked excitatory junction potentials (EJPs) from the ventral longitudinal muscle fiber 6 (abdominal segment 3) of dissected larvae. A suction electrode was used to stimulate cut segmental nerves at 0.05 Hz. Electrical signals were recorded using the MacLab/4S data acquisition system (ADInstruments).

FM1-43 imaging

FM1-43 experiments were performed as previously described (Dason, Smith, Marin, & Charlton, 2010). The following high K^+ saline: 25 mM NaCl, 90 mM KCl, 10 mM $NaHCO_3$, 5 mM HEPES, 30 mM sucrose, 5 mM trehalose, 10 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.2 (Verstreken, Ohyama, & Bellen, 2008) was to induce high K^+ depolarization for FM1-43 loading and unloading. Presynaptic boutons were loaded with FM1-43 (Invitrogen) by high K^+ depolarization for 2 min and subsequently washed for 5 min in Ca^{2+} free HL6. 75 µM Advasep-7 was included for the first 1 min of the wash to reduce background fluorescence from extracellular FM1-43 (Kay *et al.*, 1999). A Leica TCS SP5 confocal laser-scanning microscope with a 63X water dipping objective (0.9 NA) was used to take an image of FM1-43 uptake and then high K^+ depolarization for 2 min was used to induce exocytosis. A second image was then taken to document FM1-43 unloading. The released fraction was calculated using the following formula: (fluorescence of load – fluorescence of unload)/fluorescence of load.

Statistical analysis

SigmaPlot (version 11.0; Systat Software) was used for statistical analysis. One-way ANOVA tests (with a Holm–Sidak *post hoc* test) were used for comparing datasets. Error bars in all figures represent ± standard error of the mean (sem).

Results

Glial for negatively regulates nerve terminal growth and evoked neurotransmission

We previously found that *for* expression in glia rescued the increased nerve terminal growth seen in the for^0 null mutant (Dason *et al.*, 2019). We hypothesized that knockdown of glial *for* would phenocopy the increased nerve terminal growth seen in the for^0 null mutant. We used RNAi to knockdown *for* in glia at the *Drosophila* larval nmj and then stained nmjs with HRP (a neuronal membrane marker) and brp (an active zone marker). We counted the number of synaptic boutons on segment 3 of muscle fibers 6 and 7 in experimental and control genotypes. Two types of axons innervate these muscle fibers and result in 1 b and 1 s boutons, which differ in their morphological and physiological properties (Atwood, Govind, & Wu, 1993; Kurdyak *et al.*, 1994). As predicted, we found that glial knockdown of *for* increased the number of 1 b and 1 s boutons compared to controls (Figure 1(A,B)). The number of active zones per bouton was not significantly different between genotypes (Figure 1(C,D)). We next examined the role of glial *for* in neurotransmitter release by recording the compound EJP generated by tonic-like type 1 b and phasic-like type 1 s boutons from segment 3 of muscle fiber 6 in third-instar larvae in response to low frequency stimulation (0.05 Hz). Surprisingly, we found glial knockdown of *for* significantly increased the amplitude of EJPs compared to controls (Figure 1(E,F)). There were no significant differences in the

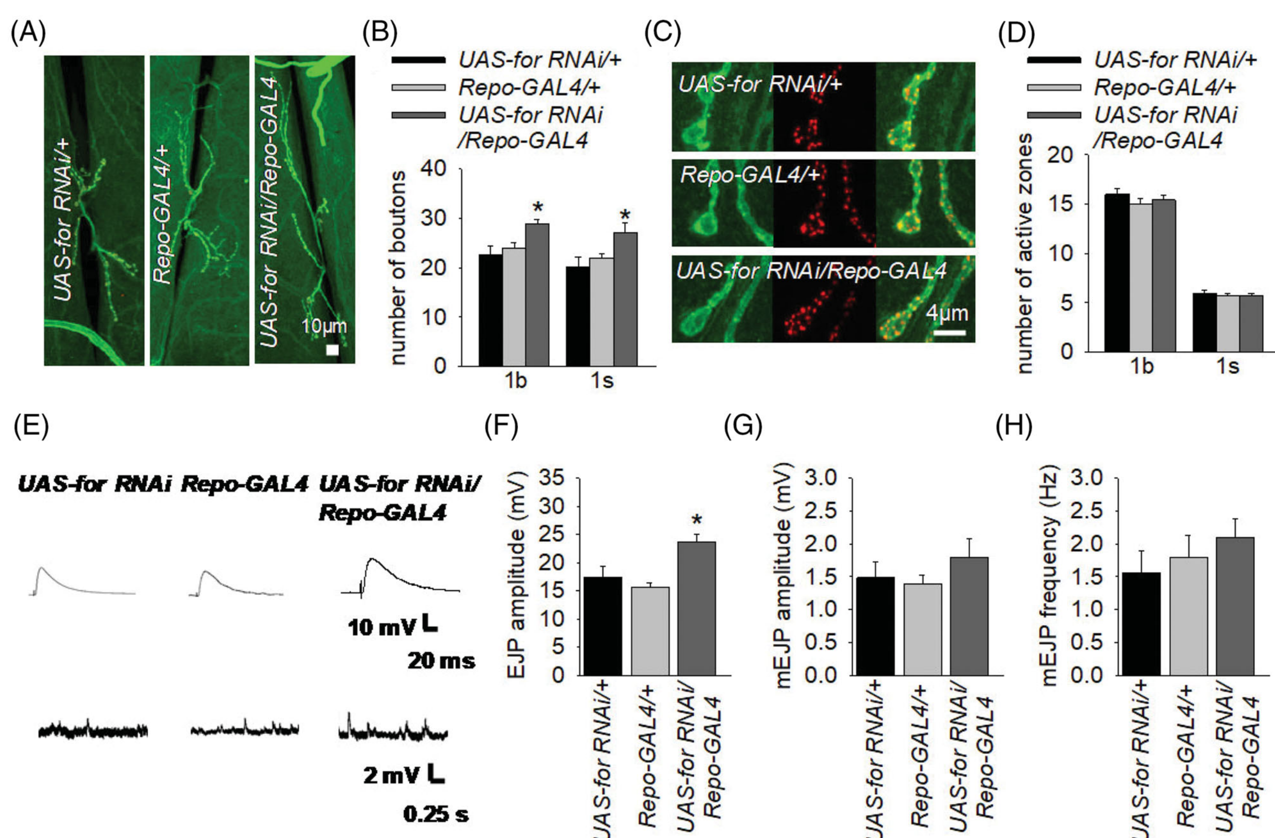


Figure 1. Glial *for* negatively regulates neurotransmitter release and nerve terminal growth. A, Fixed larval nmjs stained with FITC-conjugated anti-HRP antibody. B, Glial specific knockdown of *for* (+;+;*UAS-for RNAi, UAS-dcr/Repo-GAL4*) significantly increased the number of 1 b ($F(2,26) = 7.162$, $p < .05$; $n = 7$–12) and 1 s boutons ($F(2,26) = 5.166$, $p < .05$; $n = 7$–12) in comparison to controls (+;+;*UAS-for RNAi, UAS-dcr/+* and +;+;*Repo-GAL4/+*). C, Representative images of 1 b and 1 s boutons stained with FITC-conjugated anti-HRP antibody and anti-BRP. D, The number of active zones per 1 b ($F(2,34) = 0.725$, $p > .05$; $n = 10$–14) or 1 s bouton ($F(2,35) = 0.159$, $p > .05$; $n = 11$–14) was not significantly different between experimental and control genotypes. E, Representative traces of EJPs and mEJPs. Preparations were maintained in HL6 (0.5 mM Ca^{2+}) saline. F, The amplitude of evoked EJPs were significantly enhanced when glial *for* was knocked down in comparison to control genotypes ($F(2,28) = 9.427$, $p < .05$; $n = 8$–13). G,H, There were no significant differences in mEJP amplitude ($F(2,24) = 0.821$, $p > .05$; $n = 7$–11) or frequency ($F(2,24) = 0.713$, $p > .05$; $n = 7$–11) between genotypes. Error bars represent ± sem.

amplitude (Figure 1(E,G)) or frequency (Figure 1(E,H)) of mEJPs between genotypes. Thus, glial *for* negatively regulates both nerve terminal growth and evoked neurotransmission.

Presynaptic for negatively regulates neurotransmission

Presynaptic *for* negatively regulates evoked neurotransmission in response to low frequency stimulation (Dason *et al.*, 2019). To determine if this increased neurotransmission alters nerve terminal growth, we used RNAi to knock down *for* in presynaptic neurons at the *Drosophila* larval nmj and assessed whether presynaptic *for* regulated nerve terminal growth. Presynaptic knockdown of *for* had no effect on the number of 1 b and 1 s boutons or the number of active zones per bouton compared to controls (Figure 2(A–D)). We next examined the role of presynaptic *for* in neurotransmitter release by recording EJPs in response to low frequency stimulation (0.05 Hz). As expected, we found that presynaptic knockdown of *for* significantly increased the amplitude of EJPs compared to controls (Figure 2(E,F)). There were no significant differences in the amplitude (Figure 2(E,G)) or frequency (Figure 2(E,H)) of spontaneously occurring mEJPs between genotypes. Thus, presynaptic *for* negatively regulates evoked neurotransmission and this increased neurotransmission does not affect nerve terminal growth.

Presynaptic for is required for SV endocytosis

We previously found that expression of presynaptic *for*, but not glial *for*, could rescue the impaired SV endocytosis seen in the for^0 null mutant (Dason *et al.*, 2019). To determine if reduced levels of presynaptic or glial *for* phenocopies the SV endocytosis impairment of the for^0 null, we used the lipophilic dye FM1-43 (Betz & Bewick, 1992) to monitor SV cycling. We assessed SV cycling in control and experimental genotypes by measuring FM1-43 uptake by stimulating preparations with high K^+ saline for 2 min in the presence of FM1-43 (Figure 3(A)). FM1-43 uptake was significantly reduced when presynaptic *for* was knocked down in comparison to controls (Figure 3(A,B)). No effect was observed when glial *for* was knocked down (Figure 3(B)). We next measured FM1-43 unloading by applying high K^+ saline for 2 min to determine if the impairment seen when presynaptic *for* was knocked down was due to a defect in SV exocytosis. We found that a similar fraction was released by all genotypes (Figure 3(A,C)) demonstrating that an impairment in SV exocytosis was not the cause of the reduced FM1-43

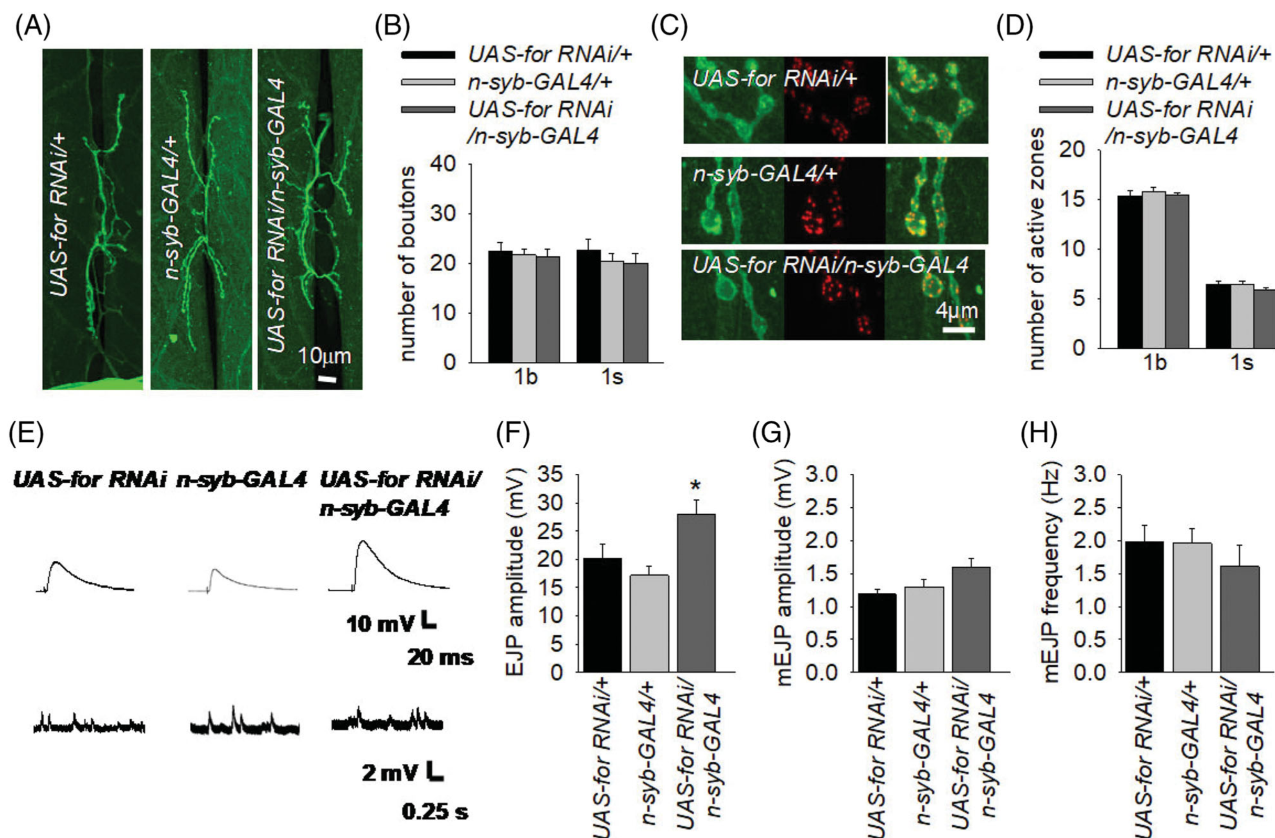


Figure 2. Presynaptic *for* negatively regulates neurotransmitter release but has no effect on nerve terminal growth. A, Fixed larval nmjs stained with FITC-conjugated anti-HRP antibody. B, Neuronal specific knockdown of *for* (+;+;*UAS-for RNAi, UAS-dcr/n-syb-GAL4*) had no effect on the number of 1 b ($F(2,21) = 0.166$, $p > .05$; $n = 7–9$) and 1 s boutons ($F(2,21) = 0.488$, $p > .05$; $n = 7–9$) in comparison to controls (+;+;*UAS-for RNAi, UAS-dcr/*+ and +;+;*n-syb-GAL4/*+). C, Representative images of 1 b and 1 s boutons stained with FITC-conjugated anti-HRP antibody and anti-BRP. D, The number of active zones per 1 b ($F(2,30) = 0.313$, $p > .05$; $n = 10–13$) or 1 s bouton ($F(2,27) = 0.954$, $p > .05$; $n = 9–12$) was not significantly different between experimental and control genotypes. E, Representative traces of EJPs and mEJPs. Preparations were maintained in HL6 (0.5 mM Ca^{2+}) saline. F, The amplitude of evoked EJPs were significantly enhanced when presynaptic *for* was knocked down in comparison to control genotypes ($F(2,25) = 7.117$, $p < .05$; $n = 7–11$). G,H, There were no significant differences in mEJP amplitude ($F(2,22) = 3.015$, $p > .05$; $n = 6–11$) or frequency ($F(2,22) = 0.577$, $p > .05$; $n = 6–11$) between genotypes. Error bars represent ± sem.

uptake seen in larvae with presynaptic *for* knocked down. Thus, presynaptic *for* is required for SV endocytosis.

Discussion

Our study characterized the effects of presynaptic or glial knockdown of *for* on synaptic structure and function. In agreement with our previous findings using a *for⁰* null mutant, we found presynaptic *for* negatively regulates neurotransmitter release in response to low frequency stimulation and is required for SV endocytosis, whereas glial *for* functions to restrain nerve terminal growth. Expanding on our previous work, we found that the enhanced neurotransmission resulting from presynaptic knockdown of *for* did not alter nerve terminal growth. Furthermore, we found that glial *for* can affect neurotransmission through its effects on nerve terminal growth.

for and axonal growth

Data from our study (Figure 1) and several others demonstrates that *for* functions to restrain axonal growth (Dason *et al.*, 2019; Peng *et al.*, 2016; Renger, Yao, Sokolowski, & Wu, 1999; Song *et al.*, 2019). Higher levels of *for* in allelic *for* variants have fewer ectopic nerve entry points in muscles at the *Drosophila* larval nmj (Renger *et al.*, 1999). *Drosophila* *for⁰* null mutant larvae have increased nerve terminal growth and this increased nerve terminal growth is rescued by expression of *for* in glia (Dason *et al.*, 2019). Consistent with these findings, we show that selectively knocking down *for* in glia enhanced nerve terminal growth (Figure 1(A,B)). *for*'s effects on nerve terminal growth are not a consequence of increased neurotransmission, as presynaptic knockdown of *for* increases neurotransmission without affecting nerve terminal growth (Figure 2). Genetic experiments in *Drosophila* suggest that *for* regulates nerve terminal growth by modulating TGF-β/BMP retrograde signaling (Dason *et al.*, 2019). Specifically, *for* may affect the secretion of the glial TGF-β ligand, Mav, which regulates nerve terminal growth through muscle derived Glass bottom boat (Gbb), the *Drosophila* TGF-β/BMP homologue (Fuentes-Medel *et al.*, 2012). Consistent with these *Drosophila* studies, a role for glial PKG in synaptogenesis was demonstrated in *Xenopus laevis* tadpole (Sild, Van Horn, Schohl, Jia, & Ruthazer, 2016). *for* is also required for restraining axonal growth in *Drosophila* embryos. *for²⁰⁻²⁹* null mutants display axonal overgrowth, incorrect axon pathfinding and incorrect target recognition (Peng *et al.*, 2016). *for* physically interacts with the transcription factor *longitudinal lacking* (*lola*) (Peng *et al.*, 2016),

which is required for repelling longitudinal axons away from the midline (Crowner, Madden, Goeke, & Giniger, 2002). *for* regulates axon guidance by antagonizing the effects of Lola in *Drosophila* embryos (Peng *et al.*, 2016).

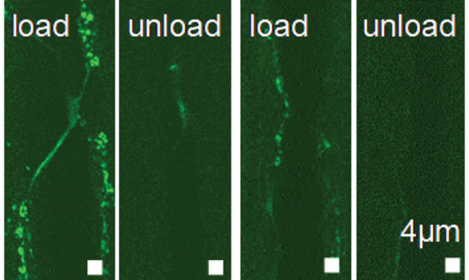
Drosophila for was also recently shown to restrict axon regeneration. Specifically, *Drosophila for* inhibits axon regeneration of larval sensory neurons (Song *et al.*, 2019). During axon regeneration, a mechanosensitive ion channel called Piezo is activated and induces local Ca^{2+} transients at the growth cone, leading to activation of nitric oxide synthase and *for*, which restrict axon regrowth (Song *et al.*, 2019). Overexpression of *for* impedes axon regeneration demonstrating that *for* functions as a brake for axon regeneration (Song *et al.*, 2019). These findings are consistent with the reported roles of *for* negatively regulating axonal growth (Dason *et al.*, 2019; Peng *et al.*, 2016; this study). Collectively, these studies suggest that *for* negatively regulates axonal growth and that *for* could be a potential target for increasing axon regeneration and modulating nervous system repair.

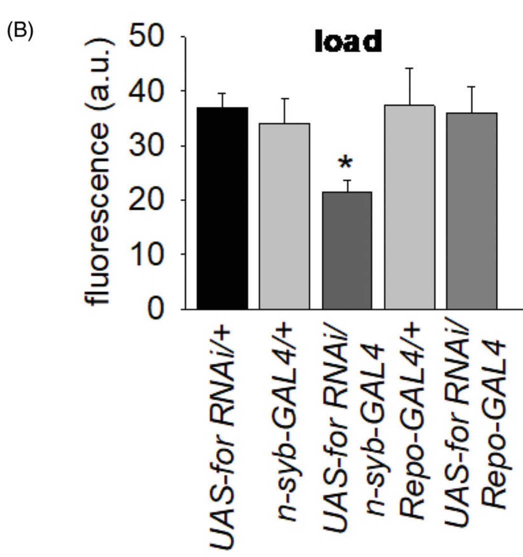
Presynaptic for and neurotransmission

We found that knockdown of *for* in presynaptic neurons increased evoked neurotransmission in response to low frequency stimulation (Figure 2(E,F)). These findings are in agreement with our previous work that demonstrated that for^0 null mutants have increased evoked neurotransmission and presynaptic Ca^{2+} entry in response to low frequency stimulation and that these effects could be rescued by expressing *for* in presynaptic neurons (Dason *et al.*, 2019). Consistent with a role of *for* in negatively regulating evoked neurotransmission, an earlier study found that overexpression of *for* reduced evoked neurotransmission (Renger *et al.*, 1999). Cultured *Drosophila* neurons of allelic variants of *for* with reduced PKG activity were previously shown to have decreased voltage-dependent K^+ currents (Renger *et al.*, 1999). A similar reduction of K^+ currents in for^0 null mutants or larvae with *for* knock downed in presynaptic neurons could increase action potential duration, leading to increased presynaptic Ca^{2+} entry and increased evoked neurotransmission.

Knockdown of *for* in presynaptic neurons did not affect spontaneous neurotransmission (Figure 2(E,G,H)). Similarly, we previously found that spontaneous neurotransmission was not altered in for^0 null mutants (Dason *et al.*, 2019). Our data suggests that *for* has a selective effect on evoked neurotransmission. These findings are consistent with growing evidence that evoked and spontaneous neurotransmission have distinct molecular mechanisms (Kavalali, 2015; Melom, Akbergenova, Gavornik, & Littleton, 2013; Pang *et al.*, 2011; Ramirez, Khvotchev, Trauterman, & Kavalali, 2012).

We previously used fluorescein-assisted light inactivation to acutely inactivate *for* and found that *for*'s effects on neurotransmission can occur independent of any changes in nerve terminal growth (Dason *et al.*, 2019). In agreement with this, knockdown of presynaptic *for* had no effect on nerve terminal growth (Figure 1(A,B)), but did increase





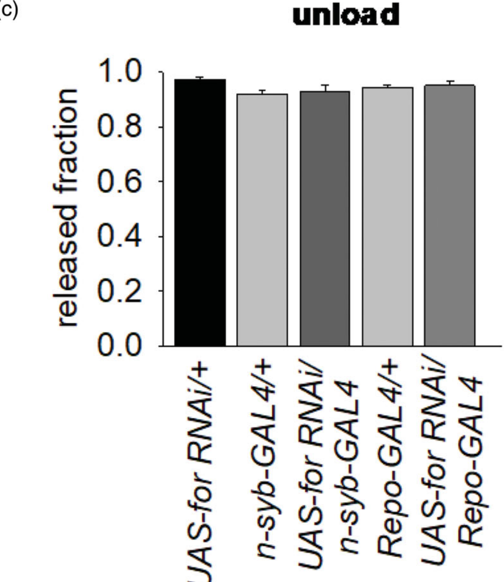


Figure 3. Presynaptic *for* regulates SV endocytosis. A, Representative images of presynaptic boutons loaded with FM1–43 during high K^+ stimulation for 2 min. High K^+ saline was then reapplied for 2 min and fluorescence was measured again (unload). B, Neuronal specific knockdown of *for* (+;+;*UAS-for RNAi, UAS-dcr/n-syb-GAL4*) significantly reduced the amount of FM1-43 uptake compared to controls (+;+;*UAS-for RNAi, UAS-dcr/*+ and +;+;*n-syb-GAL4/*+) ($F(2,17) = 10.170$, $p < .05$; $n = 4$–10), demonstrating impaired SV recycling. Glial specific knockdown (+;+;*UAS-for RNAi, UAS-dcr/Repo-GAL4*) had no effect in comparison to controls (+;+;*UAS-for RNAi, UAS-dcr/*+ and +;+;*Repo-GAL4/*+) ($F(2,15) = 0.0197$, $p > .05$; $n = 4$–8). C, A similar fraction of FM1-43 was released in controls and RNAi lines, demonstrating that recycled SVs could undergo exocytosis (presynaptic knockdown: ($F(2,17) = 3.301$, $p > .05$; $n = 4$–10 and glial knockdown: ($F(2,15) = 1.702$, $p > .05$; $n = 4$–8). Fluorescence (F) was reported with background F subtracted. Scale bars represent 4 μm. Error bars represent ± sem.

evoked neurotransmission (Figure 1(E,F)). Thus, presynaptic *for* directly regulates evoked neurotransmission.

Presynaptic for and SV endocytosis

We found that knockdown of presynaptic *for* impaired SV endocytosis (Figure 3). We previously used fluorescein-assisted light inactivation of FOR and a temperature-sensitive dynamin mutant, *shibire^{ts1}*, to demonstrate that FOR is necessary for endocytosis of SVs that have undergone exocytosis using a functional FOR protein (Dason *et al.*, 2019). These data show that FOR's effects on SV endocytosis are not simply a consequence of altered SV exocytosis. These findings are consistent with several studies that propose that PKG plays a key role in balancing SV exocytosis and endocytosis (Collado-Alsina, Ramírez-Franco, Sánchez-Prieto, & Torres, 2014; Dason *et al.*, 2019; Eguchi, Nakanishi, Takagi, Taoufiq, & Takahashi, 2012; Petrov, Giniatullin, Sitdikova, & Zefirov, 2008; Taoufiq, Eguchi, & Takahashi, 2013). During periods of sustained synaptic transmission, SV recycling is upregulated through a retrograde pathway that involves the release of nitric oxide (NO) from the postsynaptic cell and a subsequent increase in presynaptic cGMP and PIP_2 in cultured hippocampal neurons and synapses of the rat Calyx of Held (Eguchi *et al.*, 2012; Micheva, Buchanan, Holz, & Smith, 2003). Similarly, application of NO donors at the *Drosophila* nmj induces cGMP immunoreactivity in presynaptic boutons (Wildemann & Bicker, 1999) and both presynaptic cGMP (Shakiryanova & Levitan, 2008) and PIP_2 levels (Dason *et al.*, 2014) were found to be elevated during high frequency stimulation. PKG is activated by cGMP and thought to upregulate PIP_2 levels through a Rho-kinase (Eguchi *et al.*, 2012; Taoufiq *et al.*, 2013). PIP_2 recruits AP-2 and clathrin to sites of endocytosis (Micheva *et al.*, 2003). Thus, a reduction in PIP_2 is likely the cause of the impaired SV endocytosis observed following the inhibition of PKG in mammalian studies or the absence of *for* in *Drosophila* studies.

Glial for's effects on synaptic structure and function

Growing evidence demonstrates a role for glia in regulating both synaptic structure and function. Specifically, glia are important mediators of processes such as neurotransmission (Panatier *et al.*, 2011), synapse formation (Mauch *et al.*, 2001) and synaptic plasticity (Henneberger, Papouin, Oliet, & Rusakov, 2010). We previously found that expressing *for* in glia in a *for^0* null mutant background rescued the enhanced nerve terminal growth seen in the *for^0* null mutant but did not rescue the enhanced neurotransmission seen in the *for^0* null mutant (Dason *et al.*, 2019). This clearly demonstrates that glial *for* is not having a direct effect on neurotransmission. In the present study, we found that knocking down glial *for* enhanced both nerve terminal growth and neurotransmission (Figure 1). This strongly suggests that while presynaptic *for* has direct effects on neurotransmission, glial *for* regulates neurotransmission through its effects on nerve terminal growth.

The for gene and behaviours

The *for* gene is required for survival (Anreiter *et al.*, 2021) and known to affect many behaviours, such as foraging (Allen *et al.*, 2017; Anreiter, Kramer, & Sokolowski, 2017; Anreiter & Sokolowski, 2018), learning and memory (Kaun, Hendel, Gerber, & Sokolowski, 2007; Mery, Belay, So, Sokolowski, & Kawecki, 2007), stress responses (Caplan, Milton, & Dawson-Scully, 2013; Dawson-Scully, Armstrong, Kent, Robertson, & Sokolowski, 2007; Krill & Dawson-Scully, 2016), sleep (Donlea *et al.*, 2012) and nociception (Dason *et al.*, 2020). Neuronal and glial *for* likely contribute in regulating many of these responses and behaviours. *for* is expressed in both neurons and glia in the larval CNS (Allen, Anreiter, Vesterberg, Douglas, & Sokolowski, 2018; Dason *et al.*, 2020). Interestingly, *for* appears to be expressed primarily in glia in the adult CNS (Allen & Sokolowski, 2021). However, some expression of *for* in neurons in the adult CNS has also been reported (Eddison, Belay, Sokolowski, & Heberlein, 2012). In recent years, the importance of glia in flies and mammals have been shown in behaviours such as learning and memory (Adamsky *et al.*, 2018; Frankland & Josselyn, 2020; Kol *et al.*, 2020), sleep (Artiushin & Sehgal, 2020) and pain (Salter & Beggs, 2014). Future behavioural studies on FOR/PKG should examine the potential contributions of both neuronal and glial FOR/PKG. Our data demonstrates that *for* has pleiotropic effects at synapses, with presynaptic *for* directly regulating synaptic function, while glial *for* regulates synaptic function through effects on nerve terminal growth at the larval nmj.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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