




# Serotonin signals through postsynaptic $G\alpha_q$ , Trio RhoGEF, and diacylglycerol to promote *Caenorhabditis elegans* egg-laying circuit activity and behavior

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## Abstract

Activated  $G\alpha_q$  signals through phospholipase-C $\beta$  and Trio, a Rho GTPase exchange factor (RhoGEF), but how these distinct effector pathways promote cellular responses to neurotransmitters like serotonin remains poorly understood. We used the egg-laying behavior circuit of *Caenorhabditis elegans* to determine whether phospholipase-C $\beta$  and Trio mediate serotonin and  $G\alpha_q$  signaling through independent or related biochemical pathways. Our genetic rescue experiments suggest that phospholipase-C $\beta$  functions in neurons while Trio Rho GTPase exchange factor functions in both neurons and the postsynaptic vulval muscles. While  $G\alpha_q$ , phospholipase-C $\beta$ , and Trio Rho GTPase exchange factor mutants fail to lay eggs in response to serotonin, optogenetic stimulation of the serotonin-releasing HSN neurons restores egg laying only in phospholipase-C $\beta$  mutants. Phospholipase-C $\beta$  mutants showed vulval muscle  $Ca^{2+}$  transients while strong  $G\alpha_q$  and Trio Rho GTPase exchange factor mutants had little or no vulval muscle  $Ca^{2+}$  activity. Treatment with phorbol 12-myristate 13-acetate that mimics 1,2-diacylglycerol, a product of PIP<sub>2</sub> hydrolysis, rescued egg-laying circuit activity and behavior defects of  $G\alpha_q$  signaling mutants, suggesting both phospholipase-C and Rho signaling promote synaptic transmission and egg laying via modulation of 1,2-diacylglycerol levels. 1,2-Diacylglycerol activates effectors including UNC-13; however, we find that phorbol esters, but not serotonin, stimulate egg laying in *unc-13* and phospholipase-C $\beta$  mutants. These results support a model where serotonin signaling through  $G\alpha_q$ , phospholipase-C $\beta$ , and UNC-13 promotes neurotransmitter release, and that serotonin also signals through  $G\alpha_q$ , Trio Rho GTPase exchange factor, and an unidentified, phorbol 12-myristate 13-acetate-responsive effector to promote postsynaptic muscle excitability. Thus, the same neuromodulator serotonin can signal in distinct cells and effector pathways to coordinate activation of a motor behavior circuit.

**Keywords:** serotonin; *Caenorhabditis elegans*; neurotransmission; G protein; synapse; circuit activity; optogenetics; calcium imaging; Trio RhoGEF; DAG

## Introduction

Neurons communicate in circuits via synaptic transmission to initiate, sustain, and terminate behaviors. During neurotransmission, both synaptic vesicles and dense-core vesicles fuse with the presynaptic membrane, releasing neurotransmitters and neuropeptides that activate postsynaptic ion channels and G-protein-coupled receptors (GPCRs) (Betke et al. 2012; Geppetti et al. 2015). While much has been learned about neurotransmitter signaling pathways through ionotropic receptors, the diversity of GPCRs and their signaling pathways has complicated our understanding of how their signaling exerts changes on cell excitability and behavior. The G protein,  $G\alpha_q$ , is one of the major G proteins expressed in all excitable cells (Simon et al. 1991; Wilkie et al. 1992; Offermanns 2001). Activated  $G\alpha_q$  signals through PIP<sub>2</sub>-specific phospholipase-C $\beta$  (PLC $\beta$ ) to generate the second messengers, inositol 1,4,5 trisphosphate (IP<sub>3</sub>) and 1,2-diacylglycerol (DAG). IP<sub>3</sub> activates the IP<sub>3</sub> receptor to release  $Ca^{2+}$  from intracellular stores and activate downstream kinases, lipases, and ion channels

(Huang 1989; Berridge et al. 2000; Mujica and Gonzalez 2011; Li et al. 2014). The membrane lipid DAG has been shown to recruit and activate numerous effector proteins including UNC-13 and protein kinase C (PKC) (Maruyama and Brenner 1991; Rozengurt et al. 1997; Brose and Rosenmund 2002; Ananthanarayanan et al. 2003; Silinsky and Searl 2003; Thore et al. 2005; Lou et al. 2008), but whether these or other identified DAG targets function to transduce all forms of  $G\alpha_q$  signaling in vivo remains an open question.

Genetic studies in the nematode worm *Caenorhabditis elegans* have shown that  $G\alpha_q$  signaling through both PLC $\beta$  and Trio promotes neurotransmitter and neuropeptide transmission. In *C. elegans*, *unc-73* gene encodes at least 8 isoforms of Trio, which has both Rac and Rho GTPase exchange factor (GEF) DH/PH domains (Steven et al. 2005; Williams et al. 2007). *unc-73* mutations that specifically affect the second Rho activating DH/PH GEF domain of Trio Rho GTPase exchange factor (RhoGEF) disrupt locomotion, feeding, and egg-laying behaviors (Williams et al. 2007), without causing the axon pathfinding and neurodevelopmental defects

Received: March 09, 2022. Accepted: April 26, 2022

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observed in animals bearing *unc-73* Trio RacGEF mutations that affect the first, Rac activating DH/PH GEF domain (Steven *et al.* 1998). In worms,  $G\alpha_q$  knockouts are lethal while PLC $\beta$  or Trio RhoGEF single knockouts show defects in neurotransmission that disrupt locomotion, feeding, egg-laying and other behaviors, resembling  $G\alpha_q$  loss-of-function mutants (Brundage *et al.* 1996; Hajdu-Cronin *et al.* 1999; Lackner *et al.* 1999; Bastiani *et al.* 2003). Worms bearing mutations that disrupt both PLC $\beta$  and Trio RhoGEF phenocopy the larval arrest phenotype of  $G\alpha_q$  null mutants, consistent with these two effectors relaying most or all of the relevant  $G\alpha_q$  signaling (Williams *et al.* 2007). Genetic and biochemical studies showed that  $G\alpha_q$  binding to and activation of the Trio RhoGEF domain to promote Rho signaling is conserved in mammals (Chhatriwala *et al.* 2007; Rojas *et al.* 2007); however, it remains unclear how PLC $\beta$  and Rho signaling promotes neurotransmitter and neuropeptide release *in vivo*. The larval lethality of  $G\alpha_q$  null mutants can be rescued by the DAG-mimetic phorbol ester, PMA (Reynolds *et al.* 2005), which can also rescue the egg-laying defects of PLC $\beta$ , Trio RhoGEF double mutants (Williams *et al.* 2007). These results suggest  $G\alpha_q$  signaling through both PLC $\beta$  and Trio may ultimately converge to regulate DAG levels and the activation of downstream effectors. Both PLC $\beta$  and Trio RhoGEF promote acetylcholine (ACh) release from motor neurons that control locomotion (Lackner *et al.* 1999; Miller *et al.* 1999; Williams *et al.* 2007), although mutations in Trio RhoGEF cause behavior defects more aligned with a function in dense core vesicle release (Hu *et al.* 2011).  $G\alpha_q$ , Trio, and PLC $\beta$  are expressed in the nervous system and in muscles (Steven *et al.* 1998, 2005; Lackner *et al.* 1999; Bastiani *et al.* 2003; Taylor *et al.* 2021). While re-expression of PLC $\beta$  in motor neurons (Lackner *et al.* 1999) or Trio in all neurons (Williams *et al.* 2007) rescues the locomotion behavior defects of their mutants, prior work has shown that  $G\alpha_q$  has additional functions to promote egg laying in muscles (Bastiani *et al.* 2003) where Trio RhoGEF is also expressed (Steven *et al.* 2005). Loss of PLC $\beta$  fails to suppress the hyperactive egg-laying phenotypes of  $G\alpha_q$  gain-of-function mutants, consistent with  $G\alpha_q$  signaling through other effectors like Trio RhoGEF to regulate egg laying (Bastiani *et al.* 2003). Indeed, *unc-73* RhoGEF mutations strongly suppress the hyperactive egg-laying behavior phenotypes of  $G\alpha_q$  mutants unable to inhibit  $G\alpha_q$  signaling (Williams *et al.* 2007).

Using genetics, optogenetics, pharmacology, and  $Ca^{2+}$  imaging techniques, we have investigated how  $G\alpha_q$  and its two effector pathways regulate egg-laying circuit activity and behavior. We find that PLC $\beta$  functions in the neurons while Trio RhoGEF signals in both neurons and muscles to promote egg laying. Loss of each of these effectors imparts specific defects in egg-laying behavior that indicate these proteins function in distinct cells to promote egg-laying circuit activity and behavior. Many of these defects can be rescued in part by treatment with phorbol esters that mimic DAG production. Thus, despite  $G\alpha_q$  signaling through independent PLC $\beta$  and Trio RhoGEF pathways, these effectors may ultimately converge to increase DAG levels which promote egg-laying behavior.

## Materials and methods

### Strains

*Caenorhabditis elegans* worms were maintained at 20°C on Nematode Growth Medium (NGM) agar plates with *Escherichia coli* OP50 as a source of food as described previously (Brenner 1974). All behavior assays and fluorescence imaging experiments were performed with age-matched adult hermaphrodites aged 24–36 h

after the late L4 stage. Strains used in this study are listed in Table 1.

## Molecular biology and transgenes

### Vulval muscle GCaMP5 strains

Vulval muscle  $Ca^{2+}$  activity was recorded using GCaMP5G (Akerboom *et al.* 2013), which was expressed along with mCherry from the *unc-103e* promoter (Collins and Koelle 2013), as previously described (Collins *et al.* 2016; Ravi, Garcia, *et al.* 2018). The wild-type reporter strain, LX1918 *vsIs164 [unc-103e::GCaMP5::unc-54 3'UTR + unc-103e::mCherry::unc-54 3'UTR + lin-15(+)] lite-1(ce314) lin-15(n765ts) X* was described previously (Collins *et al.* 2016). LX1918 males were crossed separately into DA823 *egl-30(ad805) I*, MT1434 *egl-30(n686) I*, JT47 *egl-8(sa47) V*, MT1083 *egl-8(n488) V*, KG1278 *unc-73(ce362) I*, LX1226 *eat-16(tm761) I*, CG21 *egl-30(tg26) I*, *him-5(e1490) V*, or KP1097 *dgk-1(nu62) X* hermaphrodites to generate MIA140 *egl-30(ad805) I*; *vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA139 *egl-30(n686) I*; *vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA109 *egl-8(sa47) V*; *vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA288 *egl-8(n488) V*; *vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA141 *unc-73(ce362) I*; *vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA287 *eat-16(tm761) I*; *vsIs164 lite-1(ce314) lin-15(n765ts) X*, MIA286 *egl-30(tg26) I*; *vsIs164 lite-1(ce314) lin-15(n765ts) X*, and MIA296 *dgk-1(nu62) vsIs164 lite-1(ce314) lin-15(n765ts) X*, respectively. The corresponding gene mutation was confirmed by phenotype, genotype, or both. The presence of *vsIs164* was confirmed observing the mCherry marker, and *lite-1(ce314) X* was confirmed with PCR genotyping. Oligo sequences used for genotyping the corresponding mutations are shown in Table 2.

### Trio RhoGEF-E transgenes

#### Pan-neuronal expression

The *rab-3* promoter was used to drive the expression of GFP alone or with Trio-RhoGEF-E. Briefly, plasmids KG#68 (*rab-3p::GFP*; 15 ng/ $\mu$ L) alone or with KG#281 (*rab-3p::unc-73e*; 50 ng/ $\mu$ L) (Williams *et al.* 2007) were injected into KG1278 *unc-73(ce362) I*. For behavior experiments, 5 independent GFP-expressing transgenic lines were used, from which a single transgenic line from each was kept: MIA374 *unc-73(ce362) I*; *keyEx66* (expressing GFP alone) and MIA375 *unc-73(ce362) I*; *keyEx67* (expressing GFP and Trio RhoGEF-E). Plasmids KG#281(*rab-3p::unc-73e*) and KG#68(*rab-3p::GFP*) were kind gifts from Dr. Kenneth Miller.

#### Pan-muscle expression

Plasmid pKMC33 (*rgs-1p::mCherry*) was digested with NheI/KpnI and ligated with similarly digested pPD96.52 (Fire lab *C. elegans* Vector Kit 1999; 1608: L2534, Addgene) to generate pKMC166 (*myo-3p::mCherry*). Plasmid KG#281 (*rab-3p::unc-73e*) was digested with NheI and KpnI, and the insert was ligated into similarly digested pKMC166 to generate pPD3 (*myo-3p::unc-73e*). pKMC166 (15 ng/ $\mu$ L) alone or with pPD3 (50 ng/ $\mu$ L) was injected into KG1278 *unc-73(ce362) I* mutants. Five independent mCherry-expressing transgenic lines were used for behavior experiments from which a single transgenic line from each was kept: MIA376 *unc-73(ce362) I*; *keyEx68* (expressing mCherry alone) and MIA377 *unc-73(ce362) I*; *keyEx69* (expressing mCherry + Trio RhoGEF-E).

#### Neuron and muscle co-expression

Plasmids KG#68 (15 ng/ $\mu$ L; pan-neuronal GFP) or pKMC166 (15 ng/ $\mu$ L; pan-muscle mCherry) alone or with KG#281 (50 ng/ $\mu$ L; pan-neuronal *unc-73e*) and pPD3 (50 ng/ $\mu$ L; pan-muscle *unc-73e*) were injected into KG1278 *unc-73(ce362) I*, generating 5 independent

**Table 1.** *C. elegans* strains used in this study.

Strains	Genotype	Feature	Source
N2	Wild type	Bristol wild-type strain	Brenner (1974)
MT1434	<i>egl-30(n686)</i> I	$G\alpha_q$ loss-of-function mutant, egg-laying defective	Trent et al. (1983)
DA823	<i>egl-30(ad805)</i> I	$G\alpha_q$ strong loss-of-function mutant, egg-laying defective	Brundage et al. (1996) and Mendel et al. (1995)
KG1278	<i>unc-73(ce362)</i> I	Trio RhoGEF loss-of-function mutant, egg-laying defective	Williams et al. (2007)
KG1397	<i>unc-73(ev802)</i> I	Trio RhoGEF deletion mutant, egg-laying defective	Williams et al. (2007)
JT47	<i>egl-8(sa47)</i> V	PLC $\beta$ null, egg-laying defective	Thomas (1990)
MT1083	<i>egl-8(n488)</i> V	PLC $\beta$ null, egg-laying defective	Trent et al. (1983)
CB6614	<i>egl-8(e2917)</i> V	PLC $\beta$ null, egg-laying defective	Yook and Hodgkin (2007)
RM1221	<i>egl-8(md1971)</i> V	PLC $\beta$ null, egg-laying defective	Miller et al. (1999)
JIP2081	<i>egl-23(bln360[n601])</i> IV; <i>lin-15AB(n765)</i> <i>vsIs164 lite-1(ce314)</i> X	Egg-laying defective	Thomas (1990) and this study
MT1212	<i>egl-19(n582)</i> IV	Egg-laying defective	Trent et al. (1983)
MT1444	<i>egl-2(n693)</i> V	Egg-laying defective	Trent et al. (1983)
LX1226	<i>eat-16(tm761)</i> I	$G\alpha_q$ RGS null, hyperactive egg laying	Porter and Koelle (2010)
CG21	<i>egl-30(tg26)</i> I; <i>him-5(e1490)</i> V	$G\alpha_q$ gain-of-function mutant, hyperactive egg laying	Garcia et al. (2001)
LX1832	<i>lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Used for transgenic line creation	Gürel et al. (2012)
LX1286	<i>egl-8(sa47)</i> I; <i>lin-15(n765ts)</i> X	PLC $\beta$ null mutant, egg-laying defective; multi-vulva	This study
LX1674	<i>egl-8(sa47)</i> I; <i>lin-15(n765ts)</i> X; <i>vsEx679</i>	PLC $\beta$ null, egg-laying defective; non-Muv, expresses GFP in neurons from <i>rgs-1</i> promoter	This study
LX1675	<i>egl-8(sa47)</i> I; <i>lin-15(n765ts)</i> X; <i>vsEx680</i>	PLC $\beta$ null, egg laying defective; non-Muv, expresses PLC $\beta$ and GFP in neurons from <i>rgs-1</i> promoter	This study
MIA26	<i>egl-1(n986dm)</i> V	Lacks HSNs	Ravi, Garcia, et al. (2018)
LX1918	<i>vsIs164 lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles	Li et al. (2013)
MIA109	<i>egl-8(sa47)</i> V; <i>vsIs164 lite-1(ce314)</i> , <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-8(sa47)</i> mutant	This study
MIA139	<i>egl-30(n686)</i> I; <i>vsIs164 lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-30(n686)</i>	This study
MIA140	<i>egl-30(ad805)</i> I; <i>vsIs164 lite-1(ce314)</i> , <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-30(ad805)</i>	This study
MIA141	<i>unc-73(ce362)</i> I; <i>vsIs164</i> , <i>lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>unc-73(ce362)</i>	This study
MIA286	<i>egl-30(tg26)</i> I; <i>vsIs164</i> , <i>lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-30(tg26)</i>	This study
MIA287	<i>eat-16(tm761)</i> I; <i>vsIs164 lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>eat-16(tm761)</i>	This study
MIA288	<i>egl-8(n488)</i> V; <i>vsIs164 lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>egl-8(n488)</i>	This study
MIA296	<i>dgk-1(nu62)</i> <i>lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses GCaMP5 and mCherry in the vulval muscles of <i>dgk-1(nu62)</i>	This study
MIA372	<i>unc-73(ce362)</i> I; <i>keyEx64</i>	Expresses GFP in the neurons and mCherry in the muscles of <i>unc-73(ce362)</i>	This study
MIA373	<i>unc-73(ce362)</i> I; <i>keyEx65</i>	Expresses GFP and Trio RhoGEF-E cDNA in the neurons, and mCherry and Trio RhoGEF-E cDNA in the muscles of <i>unc-73(ce362)</i>	This study
MIA374	<i>unc-73(ce362)</i> I; <i>keyEx66</i>	Expresses GFP in neurons	This study
MIA375	<i>unc-73(ce362)</i> I; <i>keyEx67</i>	Expresses GFP and Trio RhoGEF-E cDNA in neurons	This study
MIA376	<i>unc-73(ce362)</i> I; <i>keyEx68</i>	Expresses mCherry in muscles	This study
MIA377	<i>unc-73(ce362)</i> I; <i>keyEx69</i>	Expresses mCherry and Trio RhoGEF-E cDNA in muscles	This study
LX1836	<i>wzIs30</i> IV; <i>lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses Channelrhodopsin-2 (ChR2) in HSNs from the <i>egl-6</i> promoter	Collins et al. (2016)
MIA229	<i>keyIs48</i> ; <i>lite-1(ce314)</i> <i>lin-15(n765ts)</i> X	Expresses ChR2 in vulval muscles from the <i>ceh-24</i> promoter	Kopchock et al. (2021)
MIA300	<i>egl-30(ad805)</i> I; <i>wzIs30</i> IV; <i>lite-1(ce314)</i> X	Expresses ChR2 in HSN of <i>egl-30(ad805)</i> mutant	This study
MIA301	<i>egl-30(ad805)</i> I; <i>keyIs48</i> ; <i>lite-1(ce314)</i> X	Expresses ChR2 in vulval muscles of <i>egl-30(ad805)</i> mutant	This study
MIA304	<i>wzIs30</i> IV; <i>egl-8(n488)</i> V; <i>lite-1(ce314)</i> X	Expresses ChR2 in HSNs of <i>egl-8(n488)</i> mutant	This study

(continued)

**Table 1.** (continued)

Strains	Genotype	Feature	Source
MIA305	<i>egl-8(n488) V</i> ; <i>keyIs48</i> ; <i>lite-1(ce314) X</i>	Expresses ChR2 in vulval muscles of <i>egl-8(n488)</i> mutant	This study
MIA308	<i>wzIs30 IV</i> ; <i>egl-8(sa47) V</i> ; <i>lite-1(ce314) X</i>	Express ChR2 in HSNs of <i>egl-8(sa47)</i> mutant	This study
MIA309	<i>egl-8(sa47) V</i> ; <i>keyIs48</i> ; <i>lite-1(ce314) X</i>	Expresses ChR2 in vulval muscles of <i>egl-8(sa47)</i> mutant	This study
MIA247	<i>unc-73(ce362) I</i> ; <i>wzIs30 IV</i> ; <i>lite-1(ce314) X</i>	Expresses ChR2 in HSN of <i>unc-73(ce362)</i> mutant	This study
MIA248	<i>unc-73(ce362) I</i> ; <i>keyIs48</i> ; <i>lite-1(ce314) X</i>	Expresses ChR2 in vulval muscles of <i>unc-73(ce362)</i> mutant	This study
LX1615	<i>vsSi3[Punc-103e::unc-103(e1597dm)-GFP::unc-103, cb-unc-119(+)] II</i>	Expresses gain-of-function A331T UNC-103 ERG channel in the vulval muscles from the <i>unc-103e</i> promoter	This study
MT7929	<i>unc-13(e51) I</i>	UNC-13 loss-of-function	Brenner (1974)
EG9631	<i>unc-13(s69) I</i>	UNC-13 null	Rose and Baillie (1980)
IK105	<i>pkc-1(nj1) V</i>	nPKC $\epsilon$ null	Okochi et al. (2005)
IK130	<i>pkc-1(nj3) V</i>	nPKC $\epsilon$ null	Okochi et al. (2005)
RB781	<i>pkc-1(ok563) V</i>	nPKC $\epsilon$ null	C. elegans Deletion Mutant Consortium (2012)
VC127	<i>pkc-2(ok328) X</i>	cPKC $\alpha/\beta$ null	C. elegans Deletion Mutant Consortium (2012)
MJ500	<i>tpa-1(k501) IV</i>	nPKC $\delta/\theta$ null	Tabuse et al. (1989)
MJ563	<i>tpa-1(k530) IV</i>	nPKC $\delta/\theta$ null	Tabuse et al. (1989)

**Table 2.** Oligonucleotide sequences used in this study.

Oligo name	Sequence (5' > 3')	Use
<i>lite-1(ce314)</i> -fwd	ACGGGAGACGAAGAGCTAAAT AGG	Genotyping of <i>lite-1(ce314)</i>
<i>lite-1(ce314)</i> -rev	CTAAGTTGCCGGTTGCCTTAG AAC	Genotyping of <i>lite-1(ce314)</i>
<i>egl-30(n686)</i> -F	GCCAAACCGAGCAGGACATTCTGCG	Genotyping of <i>egl-30(n686)</i>
<i>egl-30(n686)</i> -R	CGGGAAAGTAGTCAGCGAGATGCG	Genotyping of <i>egl-30(n686)</i>
<i>egl-30(ad805)</i> -F	GCCAGGGCTGTCCCATTACGG	Genotyping of <i>egl-30(ad805)</i>
<i>egl-30(ad805)</i> -R	TCGGAAAGCGCCACCAGGAAC	Genotyping of <i>egl-30(ad805)</i>
<i>egl-8</i> -cDNA-fwd	CTTGGCTAGCGTAGAAAAAATGGCAAAGGAG-TTCCAGTTC	For amplification of <i>egl-8</i> coding sequences
<i>egl-8</i> -cDNA-rev	CGCCCATGGTTATCAAACGACAGAAGTCGGT-TGAGC	For amplification of <i>egl-8</i> coding sequences
<i>egl-8</i> -Cterm-NotI-fwd	GTGGGTACTCCACTGGGGTGCGGCCGCTGG-AGGTCTTCGACACCGGT	Quick change mutagenesis of <i>egl-8</i> cDNA to insert in-frame NotI enzyme for GFP insertion
<i>egl-8</i> -Cterm-NotI-rev	ACCGGTGTCGAAGGACCTCCAGCGGCCGCAC-CC CCAGTGGAGTACCCAC	Quick-change mutagenesis of <i>egl-8</i> cDNA to insert in-frame NotI enzyme for GFP insertion
NotI-GFP-FWD	GGTGGCCGCTGGAAGTAAAGGAGAAGAAC-TTTC	For amplification of GFP with flanking, in-frame NotI enzyme sites for insertion into <i>egl-8</i> cDNA
NotI-GFP-REV	TCCAGCGGCCGCTCCTTTGTATAGTTCATCC-ATGCC	For amplification of GFP with flanking, in-frame NotI enzyme sites for insertion into <i>egl-8</i> cDNA
RE-GFP-FWD-new	GCGTCTAGAACCGGTGCTAGCGTAGAAAAA-TGGTCAGTAAAGGAGAAGAACTTTTC	For amplification of sequences encoding GFP for cloning into pGP3 <i>rgs-1</i> promoter containing plasmid
RE-GFP-REV	TACGAATTCGGTACCTCAGATTTATTTGTAT-AGTTCATCCATG	For amplification of sequences encoding GFP for cloning into pGP3 <i>rgs-1</i> promoter containing plasmid
<i>unc-103(gf)</i> -QC-fwd	CTC GGT TCT TTG ATG TAC ACC TCT GTG TTC GGT AAT G	For QuickChange mutagenesis of <i>unc-103</i> coding sequences to insert the A331T mutation present in the <i>e1597gf</i> mutant.
<i>unc-103(gf)</i> -QC-rev	CAT TAC CGA ACA CAG AGG TGT ACA TCA AAG AAC CGA G	For QuickChange mutagenesis of <i>unc-103</i> coding sequences to insert the A331T mutation present in the <i>e1597gf</i> mutant.

mCherry(+), GFP(+) transgenic lines for behavior experiments from which a single transgenic line from each was kept: MIA372 *unc-73(ce362)*; *keyEx64* expressing mCherry (muscles) and GFP (neurons) only and MIA373 *unc-73(ce362)*; *keyEx65* expressing GFP (neurons), mCherry (muscles) and TrioRhoGEF-E (both neurons and muscles).

### PLC $\beta$ transgenes

To generate a control plasmid expressing GFP in neurons, GFP coding sequences were amplified from pJM60 (Moresco and Koelle

2004) using oligonucleotides RE-GFP-FWD/-REV, digested with NheI/KpnI, and ligated into similarly digested pGP3 bearing the *rgs-1* promoter (Dong et al. 2000), generating pKMC78. An *egl-8* cDNA was used to generate and express a functional GFP fusion protein in neurons. Briefly, oligonucleotides *egl-8*-cDNA-fwd/-rev were used to amplified *egl-8* coding sequences from a plasmid bearing an *egl-8* cDNA provided by Dr. Kenneth Miller (pKP309). This amplicon was digested with NheI/NcoI and ligated into a similarly digested pPD49.26 plasmid, generating pKMC193. Quickchange mutagenesis with oligonucleotides *egl-8*-Cterm-NotI-fwd/-rev was

used to insert an in-frame NotI site near the 3' end of the *egl-8* cDNA in a divergent region of the coding sequence, generating plasmid pKMC194. Coding sequences for *egl-8* bearing this NotI site were then moved to pKMC78 by digestion of pKMC194 with NheI/NcoI followed by ligation into a similarly digested pKMC78, generating pKMC195. Oligonucleotides NotI-GFP-FWD/-REV were used to amplified GFP coding sequences from pKMC78, digested with NotI, and ligated into a similarly digested pKMC195, generating pKMC196. A strain bearing the *egl-8(sa47)* mutation was generously provided by Dr. Joshua Kaplan and backcrossed 4 times to N2 wild-type animals to generate LX1225 *egl-8(sa47)* V. MT8189 *lin-15(n765ts)* males were mated to LX1225 to generate LX1287 *egl-8(sa47)* V; *lin-15(n765ts)* X hermaphrodites that were kept at 15°C prior to injection. Plasmids expressing GFP alone (pKMC78; 5 ng/μL) or *egl-8* CDNA fused to GFP (pKMC196; 5 ng/μL) from the *rgs-1* promoter were injected along with pL15EK (50 ng/μL) into LX1287 hermaphrodites. For behavior experiments, 5 independent GFP-expressing lines were used from which a single transgenic line (*vsEx679* [GFP] and *vsEx680* [EGL-8::GFP], respectively) was kept. We noted that transgenic expression of GFP alone did cause a modest, but significant reduction of egg accumulation compared to LX1225 *egl-8(sa47)* V mutant animals. This effect appeared to be specific for egg accumulation, as these same *egl-8(sa47)* GFP-only expressing transgenic lines showed similar resistance to 1 mM aldicarb as LX1225 *egl-8(sa47)* V ( $0 \pm 2.1\%$  vs.  $9 \pm 5.9\%$  of animals paralyzed at 4 h, respectively). In contrast,  $48 \pm 7.9\%$  of transgenic *egl-8(sa47)* animals expressing EGL-8::GFP were paralyzed at 4 h, not significantly different to wild-type N2 animals ( $51 \pm 9.6\%$  of animals paralyzed at 4 h).

### Vulval muscle Channelrhodopsin-2 strains

N2 males were crossed into MIA229 *keyIs48* [*ceh-24p::Chr2::unc-54* 3'UTR + *lin-15(+)*], *lite-1(ce314)*, *lin-15(n765ts)* X (Kopchock et al. 2021) to produce F1 heterozygous males, which then were crossed separately into MIA211 *unc-73(ce362)* I; *lite-1(ce314)* *lin-15(n765ts)* X, MIA299 *egl-30(ad805)* I; *lite-1(ce314)* *lin-15(n765ts)* X, MIA303 *egl-8(n488)* V; *lite-1(ce314)* *lin-15(n765ts)* X, or MIA307 *egl-8(sa47)* V; *lite-1(ce314)* *lin-15(n765ts)* X hermaphrodites to generate vulval muscle-specific Channelrhodopsin-2 (Chr2) expressing transgenic lines MIA248 *unc-73(ce362)* I; *keyIs48*; *lite-1(ce314)* X, MIA301 *egl-30(ad805)* I; *keyIs48*; *lite-1(ce314)* X, MIA305 *egl-8(n488)* V; *keyIs48*; *lite-1(ce314)* X, and MIA309 *egl-8(sa47)* V; *keyIs48*; *lite-1(ce314)* X, respectively. The presence of *lite-1(ce362)* was confirmed by genotyping as above, and the presence of the Chr2 transgene was confirmed by rescue of the *lin-15(n765ts)* multi-vulva (Muv) phenotype.

### HSN Channelrhodopsin-2 strains

Chr2 was expressed in the HSNs from the *egl-6* promoter via an integrated *wzIs30* transgene (Emtage et al. 2012). This transgene was crossed into  $G\alpha_q$  signaling mutants as follows. N2 males were crossed into LX1836 *wzIs30* IV; *lite-1(ce314)* *lin-15(n765ts)* X to generate heterozygous F1 males, which were then crossed separately into MIA211 *unc-73(ce362)* I; *lite-1(ce314)* *lin-15(n765ts)* X, MIA299 *egl-30(ad805)* I; *lite-1(ce314)* *lin-15(n765ts)* X, MIA303 *egl-8(n488)* V; *lite-1(ce314)* *lin-15(n765ts)* X, or MIA307 *egl-8(sa47)* V; *lite-1(ce314)* *lin-15(n765ts)* X hermaphrodites to generate MIA247 *unc-73(ce362)* I; *wzIs30* IV; *lite-1(ce314)* X, MIA300 *egl-30(ad805)* I; *wzIs30* IV; *lite-1(ce314)* X, MIA304 *wzIs30* IV; *egl-8(n488)* V; *lite-1(ce314)* X, and MIA308 *wzIs30* IV; *egl-8(sa47)* V; *lite-1(ce314)* X, respectively. The presence of *lite-1(ce362)* was confirmed by PCR

genotyping, and the *wzIs30* transgene was confirmed by rescue of the *lin-15(n765ts)* Muv phenotype.

### Other strains

A single-copy MosSCI knock-in strain expressing *unc-103* bearing the *e1597dm* gain-of-function mutation was constructed as described (Collins and Koelle 2013). Briefly, plasmid pKMC179 bearing *unc-103* coding sequences behind the *unc-103e* promoter/enhancer was mutagenized by QuickChange using primers *unc-103(gf)-QC-fwd* and *unc-103(gf)-QC-rev* to generate pKMC183. Digestion with BsrGI and Sanger sequencing confirmed that the mutagenesis was successful. *unc-103(e1597gf)* coding sequences were then PCR amplified from pKMC183 using Phusion polymerase (NEB), digested with NheI/MluI enzymes, and then ligated into pKMC176, a *ttTi5606* site MosSCI donor plasmid, generating pKMC185. pKMC185 was then injected at 50 ng/μL along with plasmids expressing Mos1 transposase and mCherry co-injection markers into EG4322 *ttTi5605* II; *unc-119(ed9)* III, as described (Frokjaer-Jensen et al. 2008), generating LX1565 *vsSi3[Punc-103e::unc-103e(e1597dm)-GFP, cb-unc-119(+)]* II; *unc-119(ed3)* III, which had a strong Egl phenotype but markedly reduced Unc phenotype compared to the reference CB1597 *unc-103(e1597dm)* strain. LX1565 was then outcrossed to N2 4 times to generate LX1615 *vsSi3[Punc-103e::unc-103e(e1597dm)-GFP, cb-unc-119(+)]* II.

### Behavior assays

Quantification of egg accumulation was performed as described (Chase and Koelle 2004). Staged adults were obtained by picking late L4 animals and culturing them 24–30 h at 20°C. Each animal was placed in 7 μL of 20% hypochlorite (bleach) solution and eggs were counted after animals had dissolved. Numbers of eggs and any internally hatched L1 animals were combined.

### Pharmacological assays

Egg laying in response to exogenous serotonin was performed as described (Banerjee et al. 2017; Kopchock et al. 2021). Individual staged adult animals were placed in 100 μL of either M9 buffer alone, or M9 containing 18.5 mM serotonin (creatinine sulfate monohydrate salt, Sigma-Aldrich # H7752) or M9 containing 10 μM PMA (Phorbol-12-myristate-13-acetate, Calbiochem # 524400) in a 96-well microtiter dish. After 1 h, the number of released eggs and L1 larvae in each well were counted. Since egg-laying defective animals sometimes release 1 or 2 eggs/L1 larvae when they are first picked into the well in response to mechanical stimulation, animals were only recorded as responding if they laid 3 or more progeny. For  $Ca^{2+}$  imaging, NGM plates containing either PMA or ethanol solvent were prepared as described (Reynolds et al. 2005). Age-matched adult worms from each genotype were placed on separate PMA or control NGM plates at room temperature for 1.5 h. An agar chunk was then placed between 2 glass coverslips for  $Ca^{2+}$  activity recording as described (Ravi, Nassar, et al. 2018). The unused plates were kept at 4°C for future use.

### Optogenetic assay

All-trans retinal (ATR) (Sigma Aldrich, R2500) was resuspended in ethanol (100%) to make 100 mM solution and added to a warmed culture of OP50 bacteria grown in B Broth media to a final concentration of 0.4 mM. Individual NGM agar plates were seeded with 200 μL of freshly prepared +ATR food and were grown in the dark for ~24 h prior to use. In all photo-stimulation experiments, a set of control animals were grown in the absence of ATR. Animals were imaged at 4× magnification on a Leica M165FC

stereomicroscope and illuminated with  $3.3 \text{ mW/cm}^2$  of  $\sim 470 \pm 20 \text{ nm}$  blue light produced using a EL6000 metal halide light source and a GFP excitation/emission filter set. The 30 s on/off sequence was programmed and controlled using a Doric Optogenetics TTL Pulse Generator (OTPG-4, Version 3.3) triggering a SHB1 series shutter controller (ThorLabs; 170712-1).

## Microscopy

### Ratiometric $\text{Ca}^{2+}$ imaging

Vulval muscle  $\text{Ca}^{2+}$  activity was performed in freely behaving adult animals at 24–30 h past the late L4 larval stage, as described previously (Collins et al. 2016; Collins and Koelle 2013; Ravi, Garcia, et al. 2018). Worms co-expressing GCaMP5G and mCherry under the *unc-103e* promoter transgene *vsIs164* were mounted beneath the chunk of agar over the glass coverslip, and reporter fluorescence was recorded through an  $20\times$  Apochromatic objective (0.8 NA) mounted on an inverted Zeiss Axio Observer.Z1. A Colibri.2 LED illumination system was used to excite GCaMP5 at 470 nm and mCherry at 590 nm for 10 ms every 50 ms. GFP and mCherry fluorescence emission channels were separated using a Hamamatsu W-VIEW Gemini image splitter and recorded simultaneously for 10 min with an ORCA-Flash 4.0 V2 sCMOS camera at 256/256-pixel resolution ( $4 \times 4$  binning) at 16-bit depth. A motorized stage was manually controlled using a joystick to maintain the freely behaving animal in the field of view. For experiments without treatment of PMA or vehicle control, animals were recorded until each entered into an egg-laying active state. The recording was then cropped to a 10-min (12,000 frame) 2 channel image sequence and centered on the first egg-laying event observed, for subsequent ratiometric analysis. The egg-laying active state was operationally defined as starting 1 min before the first egg-laying event and ending 1 min after the last egg-laying event observed in the 10-min recording. For egg-laying defective mutants like *egl-30(ad805)* and *unc-73(ce362)* that lay essentially no eggs, the 10-min extraction was not centered on any specific behavioral feature. For drug and vehicle control assays, the 10-min recording period started immediately, whether or not animals laid eggs or were seen to enter into an active state. Image sequences were exported to Volocity software (Quorum Technologies Inc.) for segmentation and ratiometric analysis.  $\text{Ca}^{2+}$  transient peaks from ratio traces were detected using a custom MATLAB script, as described (Ravi, Nassar, et al. 2018).

### Experimental design and statistical analysis

Sample sizes for behavioral assays followed previous studies (Chase and Koelle 2004; Collins et al. 2016). Statistical analysis was performed using Prism v.8 or v.9 (GraphPad).  $\text{Ca}^{2+}$  transient peak amplitudes, widths, and inter-transient intervals were pooled from multiple animals (typically  $\geq 10$  animals per genotype). All statistical tests were corrected for multiple comparisons (Bonferroni for 1-way ANOVA or Fisher's exact tests; Dunn's correction for Kruskal–Wallis tests). Each figure legend indicates individual P-values with  $P < 0.05$  being considered significant.

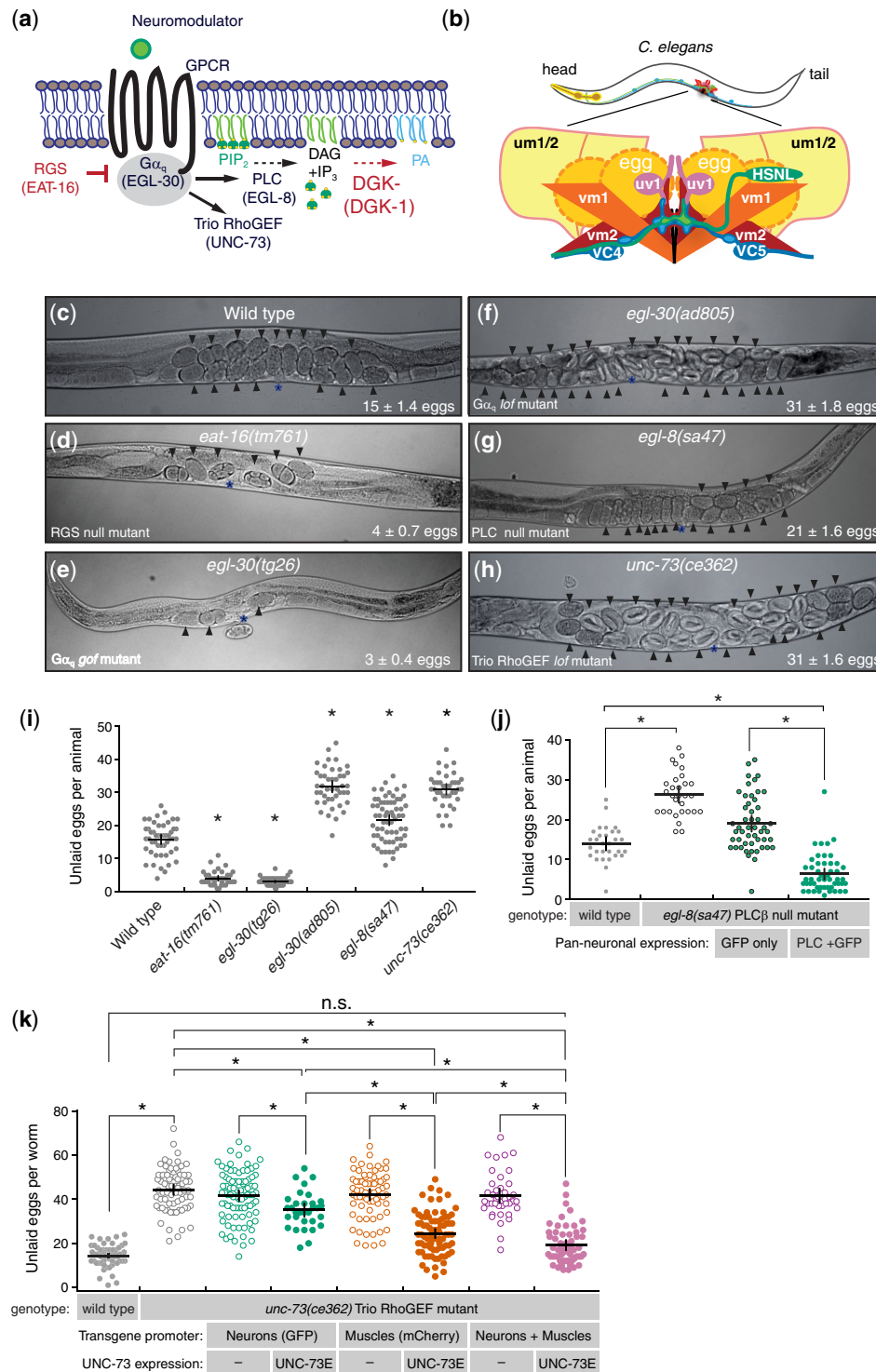
## Results

### Trio RhoGEF acts in both neurons and muscles to drive egg-laying behavior

Prior studies have shown that  $G\alpha_q$  signaling (Fig. 1a) through PLC $\beta$  and Trio RhoGEF promotes neurotransmitter release and locomotion (Brundage et al. 1996; Miller et al. 1999; Williams et al. 2007). How  $G\alpha_q$  signaling regulates other *C. elegans* behavior circuits is less well established. To address this uncertainty, we

chose to examine the neural circuit driving egg-laying behavior. Egg-laying behavior in *C. elegans* is regulated by a small motor circuit with defined neurons and muscle connectivity (White et al. 1986; Cook et al. 2019). The HSNs are serotonergic command motor neurons (Fig. 1b) that initiate the egg-laying active state and promote the excitability of the *vm1* and *vm2* egg-laying vulval muscles (Waggoner et al. 1998; Emtage et al. 2012; Collins et al. 2016). Innervating ventral cord motor neurons release acetylcholine to regulate muscle contraction (Waggoner et al. 2000; Kim et al. 2001; Bany et al. 2003; Kopchock et al. 2021). We first examined the steady-state accumulation of eggs in the uterus as a proxy for changes in egg-laying circuit activity and behavior. As previously shown, animals bearing mutations in the EAT-16 RGS protein, which inhibits  $G\alpha_q$  signaling (Hajdu-Cronin et al. 1999), or gain-of-function mutations in  $G\alpha_q$  itself, (Doi and Iwasaki 2002; Bastiani et al. 2003) showed a significant increase in egg laying resulting in a significant reduction in egg accumulation compared to the  $\sim 15 \pm 1.4$  embryos retained in wild-type animals (Fig. 1, c–e and i). Conversely, animals bearing mutations which reduce  $G\alpha_q$  signaling showed the opposite phenotype. Animals bearing an early nonsense mutation predicted to be a PLC $\beta$  null mutant, *egl-8(sa47)* (Lackner et al. 1999; Williams et al. 2007), accumulated an average of 21 eggs, showing a significant increase in egg retention (Fig. 1g). Animals bearing a missense mutation in the RhoGEF domain of Trio, *unc-73(ce362)* (Williams et al. 2007), showed an even stronger egg-laying behavior defect, accumulating more than 30 eggs in the uterus, closely resembling animals bearing loss-of-function mutations in  $G\alpha_q$  itself (Fig. 1, f–i). Together, these results confirm that  $G\alpha_q$  and its effectors PLC $\beta$  and Trio RhoGEF are required for egg-laying behavior in *C. elegans* and that loss of the Trio RhoGEF branch causes a stronger behavior impairment compared to loss of PLC $\beta$ .

Previous work has shown that  $G\alpha_q$ , Trio, and PLC $\beta$  are expressed in neurons and muscles of the egg-laying circuit (Brundage et al. 1996; Steven et al. 1998; Lackner et al. 1999; Miller et al. 1999; Bastiani et al. 2003; Taylor et al. 2021). To understand where  $G\alpha_q$  and its effectors function to regulate egg laying, we used tissue-specific promoters to express cDNAs encoding PLC $\beta$  or Trio RhoGEF in either all neurons, in the body wall and egg-laying vulval muscles, or in both neurons and muscles. We found that transgenic expression of PLC $\beta$  from the pan-neuronal *rgs-1* promoter (Dong et al. 2000) in PLC $\beta$  null mutants was sufficient to rescue their defects in egg laying (Fig. 1j) and acetylcholine (ACh) release as measured by restoration of sensitivity to aldicarb, a cholinesterase inhibitor (see Materials and Methods). This suggests PLC $\beta$  functions in neurons to regulate egg laying. Previous work has indicated the presence of 8 transcript variants of Trio (A, B, C1, C2, D1, D2, E, and F), which are differentially expressed in *C. elegans* (Steven et al. 1998; Steven et al. 2005). Transgenic expression of Trio RhoGEF-E in neurons is sufficient to rescue the locomotion defects of Trio RhoGEF mutants (Williams et al. 2007). To explore whether Trio RhoGEF acts similarly in neurons to promote egg laying, we used the *rab-3* pan-neuronal promoter (Williams et al. 2007) to express Trio RhoGEF-E and measured egg accumulation in these animals. We observed a modest, but significant reduction in the number of eggs retained in Trio RhoGEF mutants ( $\sim 36$  eggs) compared to control Trio RhoGEF mutant animals ( $\sim 42$  eggs; Fig. 1k). Transgenic expression of Trio RhoGEF-E in the egg-laying vulval muscles from a muscle-specific promoter showed a greater rescue of egg accumulation ( $\sim 25$  eggs), and this rescue of egg laying was improved to nearly wild-type levels when Trio RhoGEF-E was expressed in both neurons and muscles ( $\sim 19$  eggs; Fig. 1k). Although these rescue lines



**Fig. 1.** Trio RhoGEF acts in both neurons and muscles to regulate egg-laying behavior. a) Schematics of excitatory and inhibitory  $G\alpha_q$  signaling pathway. *C. elegans* gene names are beneath the protein they encode. b) Cartoon of the *C. elegans* egg-laying circuit from a lateral view. Only the left side of the bilaterally symmetric circuit is shown. HSNL, Hermaphrodite Specific Neuron (left); VC4 and VC5 Ventral C neurons; vm1 and vm2 vulval muscles, um1 and um2 uterine muscles; uv1 uterine-vulval neuroendocrine cells. c–h) Bright field images of worms of the indicated genotypes; arrowheads indicate accumulated eggs. Mean number of accumulated eggs  $\pm$  95% confidence intervals is also indicated. Position of the vulva is shown with an asterisk (\*). i) Scatterplot of egg accumulation in wild-type, *eat-16(tm761)*, *egl-30(tg26)*, *egl-30(ad805)*, *egl-8(sa47)*, and *unc-73(ce362)* mutant animals. Line indicates mean eggs  $\pm$  95% confidence intervals. Asterisks indicate  $P \leq 0.0001$  [1-way ANOVA with Bonferroni's correction]; wild type ( $n = 49$ ); *eat-16(tm761)* ( $n = 36$ ); *egl-30(tg26)* ( $n = 47$ ); *egl-30(ad805)* ( $n = 44$ ); *egl-8(sa47)* ( $n = 65$ ); *unc-73(ce362)* ( $n = 38$ ). j) Transgenic rescue of *egl-8* PLC $\beta$  egg-laying defects. Scatterplot of egg accumulation in transgenic animals expressing GFP only or EGL-8/PLC $\beta$  fused to GFP expressed from the *rgs-1* promoter in *egl-8(sa47)* mutants ( $n = 50$ ) compared to wild-type ( $n = 30$ ) and *egl-8(sa47)* mutant animals ( $n = 30$ ). Bar indicates mean eggs  $\pm$  95% confidence intervals. Asterisks indicate  $P \leq 0.0001$  (1-way ANOVA with Bonferroni's correction). k) Transgenic rescue of *unc-73* Trio RhoGEF egg-laying defects. Scatterplot of egg accumulation in wild-type ( $n = 60$ ), *unc-73(ce362)* mutants ( $n = 72$ ), and transgenic animals expressing a fluorescent protein with or without Trio/UNC-73E in neurons from the *rab-3* promoter ( $n \geq 32$ ) or in muscles from the *myo-3* promoter ( $n \geq 69$ ), or in both neurons and muscles ( $n \geq 41$ ) in *unc-73(ce362)* mutants. Horizontal line indicates mean accumulated eggs  $\pm$  95% confidence intervals. Asterisks indicate  $P \leq 0.0145$ ; n.s., not significant ( $P > 0.05$ ; 1-way ANOVA with Bonferroni's correction for multiple comparisons).

are extrachromosomal arrays and likely expressed at different levels and with different amounts of mosaicism, these results support a general interpretation that *unc-73* functions in both neurons and muscles to promote egg laying. However, because previous work showed that expression of *unc-73* in neurons can rescue locomotion defects (Williams et al. 2007) but not egg laying (Fig. 1k and data not shown), these results indicate that *unc-73* has additional functions in muscle that cannot be bypassed or rescued by expression just in neurons. Together, these results suggest that  $G\alpha_q$  effectors PLC $\beta$  and Trio RhoGEF function in neurons to regulate egg-laying behavior. Our results also suggest Trio RhoGEF also functions in the postsynaptic vulval muscles for proper regulation of egg laying, a finding consistent with previously results regarding  $G\alpha_q$  (Bastiani et al. 2003).

### Serotonin signals through $G\alpha_q$ , Trio, and PLC $\beta$ to promote egg laying

Previous studies have shown that serotonin released from the HSN signals through G-protein-coupled serotonin receptors expressed on the vulval muscles (Bastiani et al. 2003; Dempsey et al. 2005; Xiao et al. 2006; Tanis et al. 2008; Fernandez et al. 2020). The vulval muscles are also innervated by cholinergic ventral cord motor neurons (White et al. 1986; Cook et al. 2019) whose release of ACh is regulated by serotonin and G protein signaling (Nurish et al. 1999). To test how serotonin promotes egg laying via  $G\alpha_q$ , we measured the egg-laying response to serotonin in  $G\alpha_q$  signaling mutants. Serotonin promotes egg laying in hypertonic M9 buffer, a condition that normally inhibits egg laying in both wild-type and HSN-deficient *egl-1(n986dm)* mutants, which developmentally lack the HSNs (Fig. 2b). Consistent with previous results (Trent et al. 1983; Brundage et al. 1996; Bastiani et al. 2003), >62% of wild-type animals and 57% of HSN-deficient *egl-1(n986dm)* mutant animals laid eggs in response to serotonin compared to only 13% of  $G\alpha_q$  mutant animals (Fig. 2b). Serotonin response was similarly and significantly reduced to 23% and 3% in PLC $\beta$  and Trio RhoGEF mutant animals, respectively (Fig. 2b). Our results are consistent with the previous data reporting that  $G\alpha_q$  loss-of-function mutants and the PLC $\beta$  deletion mutant, *egl-8(n488)* do not lay eggs in response to exogenous serotonin (Trent et al. 1983; Bastiani et al. 2003).

To confirm whether PLC $\beta$  is required for egg laying in response to serotonin, we tested other PLC $\beta$  mutants including *sa47* and *md1971*, both of which carry nonsense mutations predicted to terminate the protein prematurely, and *e2917* in which the coding sequence is disrupted by a Mos1 transposon (Lackner et al. 1999; Miller et al. 1999; Yook and Hodgkin 2007). All PLC $\beta$  mutants tested failed to lay eggs in response to exogenous serotonin after 60 min (Fig. 2b), but only *egl-8(n488)* animals remained resistant to serotonin after 90 min (Table 3), with the other PLC $\beta$  mutant animals beginning to lay eggs by 90 min, consistent with previous observations (Bastiani et al. 2003). Taken together, these results indicate that  $G\alpha_q$ , PLC $\beta$ , and Trio act at least in part outside of HSNs to promote egg laying in response to serotonin. To determine where in the animal the Trio RhoGEF deficiency caused serotonin insensitivity, we measured egg laying in Trio RhoGEF mutant animals re-expressing Trio RhoGEF-E in either neurons, muscles, or both. Transgenic expression of Trio RhoGEF-E in neurons failed to rescue egg laying in response to serotonin (Fig. 2c), but expression of Trio RhoGEF-E in muscles, or in both neurons and muscles, restored egg laying of *unc-73(ce362)* mutant animals (Fig. 2c), suggesting that Trio RhoGEF mediates serotonin signaling by acting in the vulval muscles. Together, these results indicate that  $G\alpha_q$ , PLC $\beta$ , and Trio RhoGEF function at least in part

outside of the HSNs to drive egg laying in response to serotonin with Trio RhoGEF likely functioning in the muscles.

### Optogenetic stimulation of the HSNs and vulval muscles suggests cellular specificity of $G\alpha_q$ effectors for egg-laying

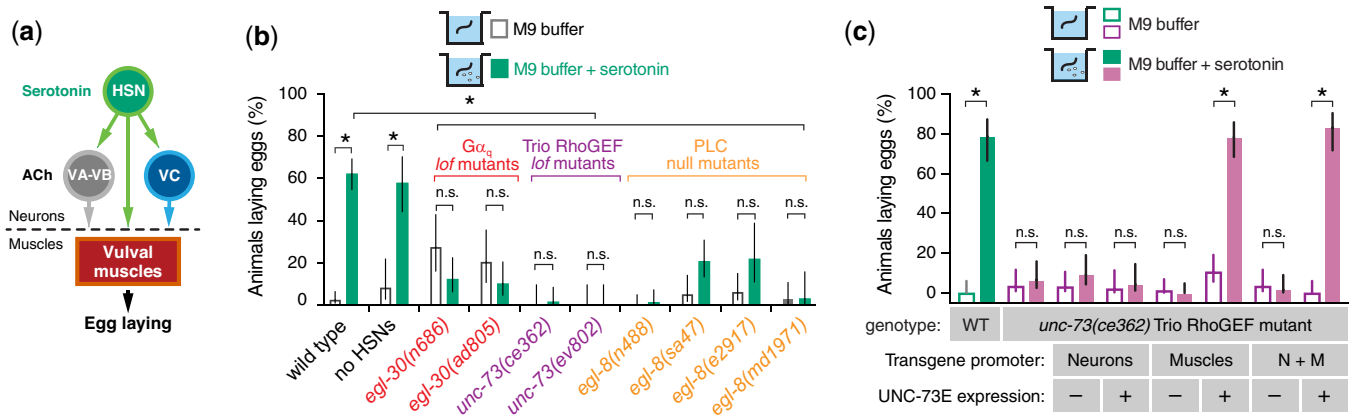
Optogenetic stimulation of ChR2 expressed in either the HSNs (Emtage et al. 2012) or vulval muscles (Kopchok et al. 2021) can drive egg laying. To test whether and how  $G\alpha_q$  and its effectors mediate this response, we expressed ChR2 in HSNs in  $G\alpha_q$  and effector mutants and measured egg laying during 30s of exposure to blue light. Blue light stimulation of the HSNs drove an average release of ~3 eggs in wild-type animals, which was reduced to essentially zero in  $G\alpha_q$  [*egl-30(ad805)*] and Trio RhoGEF [*unc-73(ce362)*] mutants (Fig. 3a), consistent to our previous results showing Trio RhoGEF acts in part downstream of the HSNs in the postsynaptic vulval muscles. In contrast, optogenetic stimulation of the HSNs in PLC $\beta$  null mutants [*egl-8(sa47)* or *egl-8(n488)*] drove robust egg release, releasing an average of ~4 and ~6 embryos, respectively, in 30s (Fig. 3a). To test whether the failure of egg laying in  $G\alpha_q$  and Trio RhoGEF mutants was a consequence of muscle developmental defects, rather than excitability deficits, we expressed and stimulated ChR2 in the vulval muscles. Blue light exposure drove the release of ~4 eggs in 30s in wild-type animals. Both PLC $\beta$  [*egl-8(sa47)* and *egl-8(n488)*] and Trio RhoGEF mutants [*unc-73(ce362)*] laid a similar number of eggs as wild-type control animals after blue light stimulation (Fig. 3b). The *egl-30(ad805)*  $G\alpha_q$  mutant laid slightly fewer eggs on average (~3) but this was not significant. Thus, the failure of  $G\alpha_q$  and Trio mutants to lay eggs in response to exogenous serotonin or optogenetic stimulation of the HSNs does not arise from some intrinsic defect in vulval muscle contractility, but rather a specific deficiency in muscle excitability. These results are also consistent with prior findings showing rescue of egg-laying behavior defects of  $G\alpha_q$ , PLC $\beta$ , and Trio RhoGEF mutants by exogenous phorbol esters (Lackner et al. 1999; Williams et al. 2007).

Collectively, we interpret the previous serotonin experiments and these optogenetic results as showing that  $G\alpha_q$  signals through PLC $\beta$  in HSN and/or in other neurons to promote release of neurotransmitters that signal through vulval muscle receptors coupled to  $G\alpha_q$  and Trio RhoGEF. That HSN optogenetic stimulation, but not exogenous serotonin, stimulates egg laying in PLC $\beta$  mutants suggests HSN releases other factors such as NLP-3 neuropeptides (Brewer et al. 2019), which signal to promote egg laying in parallel to serotonin and PLC $\beta$ . Our data do not support a model where PLC $\beta$  acts only in HSNs as *egl-1(n986dm)* animals lacking HSNs still lay eggs in response to serotonin while *egl-8* PLC $\beta$  null mutants do not (Fig. 2b). Together, these results support the conclusion from our rescue experiments (Fig. 1, j and k) that PLC $\beta$  and Trio RhoGEF function in distinct cells and through unique mechanisms to promote egg-laying circuit activity and behavior.

### $G\alpha_q$ and Trio RhoGEF are required for vulval muscle $Ca^{2+}$ activity

Egg laying is a 2-state behavior where ~20-min inactive states are punctuated by ~2-minute active states with high levels of rhythmic  $Ca^{2+}$  transient activity in the egg-laying circuit driving release of 3-5 eggs (Waggoner et al. 1998; Zhang et al. 2008; Zhang et al. 2010; Collins et al. 2016). Loss of  $G\alpha_q$  signaling in *egl-30(n686)* animals causes a significant reduction in spontaneous and serotonin-induced vulval muscle  $Ca^{2+}$  transients in immobilized animals (Shyn et al. 2003). We therefore tested whether these  $G\alpha_q$ -dependent  $Ca^{2+}$  activity defects were similarly seen in freely





**Fig. 2.** Serotonin signals through  $G\alpha_q$ , Trio, and PLC $\beta$  to promote egg laying. a) A working model of serotonin and acetylcholine (ACh) signaling in the egg-laying circuit. b) Bar plots showing the percentage of animals laying eggs in M9 buffer alone (open boxes) or M9 + 18.5 mM serotonin (filled boxes) after 1 hr. Bar indicates mean percent  $\pm$  95% confidence intervals. Asterisks indicate  $P < 0.0007$ ; n.s., not significant ( $P > 0.05$ , Fisher's exact test with Bonferroni's correction for multiple comparisons;  $n > 30$  animals for each genotype and condition). c) Bar plot showing percent of animals laying eggs in M9 buffer or M9 + 18.5 mM serotonin in wild-type or Trio RhoGEF mutant animals expressing nothing or Trio/UNC-73E in neurons, muscles, or both. Bar indicates mean percent  $\pm$  95% confidence intervals. Asterisks indicate  $P < 0.0007$ ; n.s., not significant ( $P > 0.05$ , Fisher's exact test with Bonferroni's correction for multiple comparisons;  $n > 30$  animals).

**Table 3.** Serotonin-induced egg laying in *egl-8* PLC $\beta$  mutants.

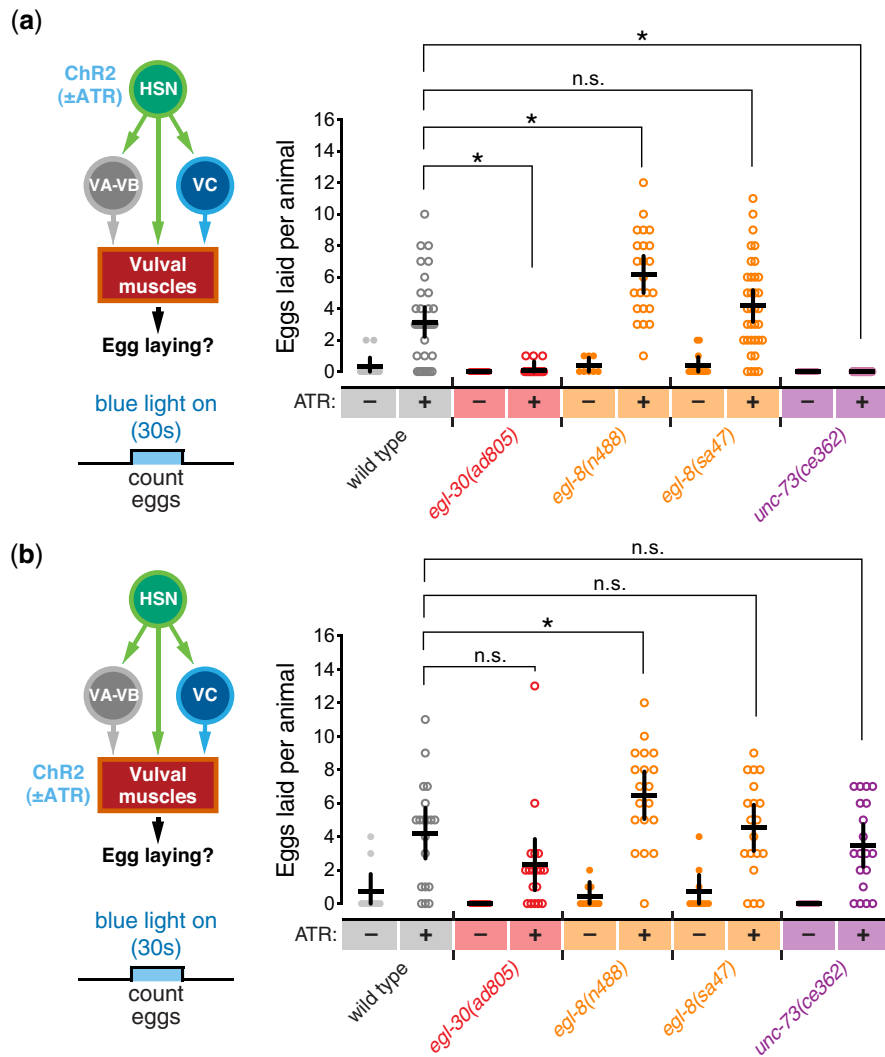
Genotype	N	60 min		90 min		P-value (paired t test)
		Average eggs laid in 18.5 mM serotonin	Std. deviation	Average eggs laid in 18.5 mM serotonin	Std. deviation	
wild type (N2)	64	1.8	1.7	5.8	4.5	<0.0001
<i>egl-8(e2917)</i>	32	0.2	0.6	1.4	2.5	0.0091
<i>egl-8(n488)</i>	32	0.0	0.2	0.1	0.3	0.0831
<i>egl-8(md1971)</i>	32	0.3	0.8	1.3	2.1	0.0075
<i>egl-8(sa47)</i>	32	0.8	1.4	2.5	2.5	0.0002

behaving animals on solid media and whether they were shared in PLC $\beta$  and Trio RhoGEF mutants. We expressed the genetically encoded  $Ca^{2+}$  reporter, GCaMP5, along with mCherry in the vulval muscles of mutant animals with either too much or too little  $G\alpha_q$  signaling and performed ratiometric imaging as they entered and left the egg-laying active state.

As shown in Fig. 4A, the normal 2-state pattern of rhythmic vulval muscle  $Ca^{2+}$  activity (and egg laying) is lost in animals bearing strong loss-of-function  $G\alpha_q$  or Trio RhoGEF mutations. To further quantify these activity defects, we compared vulval muscle  $Ca^{2+}$  transient amplitudes and frequencies in wild-type and  $G\alpha_q$  signaling mutant animals. In *egl-30(ad805)*  $G\alpha_q$  and *unc-73(ce362)* Trio mutants, which laid no eggs during the recording period, we failed to see the large amplitude egg-laying  $Ca^{2+}$  transients ( $1.4 \pm 0.1 \Delta R/R$ ) typically observed in wild-type animals where both the vm1 and vm2 muscles contract or even the smaller amplitude rhythmic "twitch"  $Ca^{2+}$  transients ( $0.4 \pm 0.03 \Delta R/R$ ) that are localized primarily to the vm1 muscles (Fig. 4, a and b). As a result, the wild-type frequency of  $3.5 \pm 1.0 (\Delta R/R) Ca^{2+}$  transients per min was significantly reduced to essentially zero in *egl-30(ad805)*  $G\alpha_q$  and *unc-73(ce362)* Trio mutants (Fig. 4c). In contrast, we did not observe a significant reduction in the amplitude or frequency of vulval muscle  $Ca^{2+}$  transients in *egl-30(n686)*  $G\alpha_q$  weak loss-of-function mutants or *egl-8(sa47)* and *egl-8(n488)* PLC $\beta$  null mutants compared to wild-type control animals (Fig. 4, a–c). Such animals entered infrequent active states but their rhythmic vulval muscle twitching and egg-laying  $Ca^{2+}$  transients were grossly intact. In fact, inspection of  $Ca^{2+}$  traces suggested an apparent increase in vulval muscle

$Ca^{2+}$  transient activity in *egl-30(n686)*  $G\alpha_q$  and PLC $\beta$  mutants (Fig. 4a) including a significant increase in the amplitude and frequency of twitch  $Ca^{2+}$  transients in *egl-8(n488)* PLC $\beta$  mutant animals (Fig. 4, b and c). This elevated vulval muscle  $Ca^{2+}$  activity was reminiscent of that observed in *egl-1(n986dm)* animals lacking the HSNs (Collins et al. 2016) as these animals still enter and leave infrequent egg-laying active states, possibly driven by the stretch-dependent feedback of egg accumulation in the uterus (Ravi, Garcia, et al. 2018) which does not appear to act by modulating HSN activity (Ravi et al. 2021). These data further indicate that the egg-laying defects of PLC $\beta$  mutants are not caused by a loss of vulval muscle  $Ca^{2+}$  activity.

Increased  $G\alpha_q$  signaling enhances vulval muscle activity. *egl-30(tg26)* or *eat-16(tm761)* mutant animals with elevated  $G\alpha_q$  signaling showed even stronger egg-laying  $Ca^{2+}$  transients with average amplitude  $> 2 \Delta R/R$ , a significant difference (Fig. 4, a and b).  $Ca^{2+}$  transients were also significantly more frequent in *egl-30(tg26)*  $G\alpha_q$  gain-of-function mutants ( $\sim 7$  transients per min) and in *eat-16(tm761)*  $G\alpha_q$  RGS protein loss-of-function mutants ( $\sim 5$  transients per min) (Fig. 4c). DAG Kinase- $\theta$  (DGK- $\theta$ ) is thought to antagonize DAG signaling by catalyzing its conversion to phosphatidic acid (Fig. 1a). *dgk-1(nu62)* mutants lacking DGK- $\theta$ /DGK-1 have increased neurotransmitter release and egg laying (Miller et al. 1996; Nurrish et al. 1999; Jose and Koelle 2005), likely through elevation of DAG levels and activation of effectors downstream of  $G\alpha_q$ . Somewhat surprisingly, *dgk-1(nu62)* mutant animals did not show a significant increase in vulval muscle  $Ca^{2+}$  transient amplitude or frequency (Fig. 4, a–c). Like PLC $\beta$ , DGK-1 is expressed in



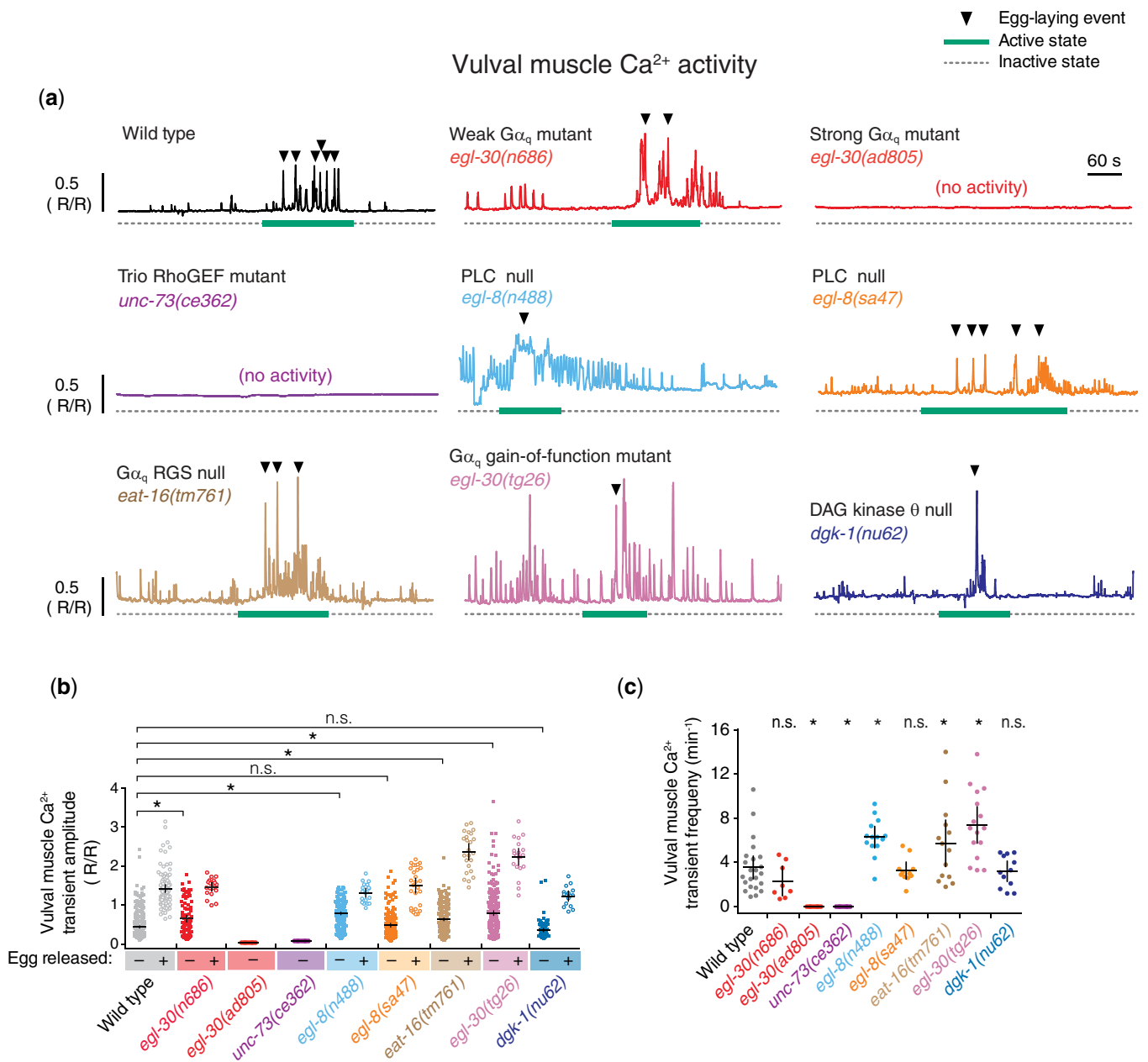
**Fig. 3.** Optogenetic stimulation of the HSNs or vulval muscles reveals distinct cellular specificity of  $G\alpha_q$  effectors for egg laying. a) On left, cartoon of the egg-laying circuit and experiment showing blue light activation of HSN for 30 s. On right, scatterplot showing eggs laid per worm in the presence (+) or absence (-) of ATR cofactor during the blue light activation of ChR2 expressed in HSNs of wild-type, *egl-30(ad805)*  $G\alpha_q$  strong loss-of-function mutants, *egl-8(n488)* and *egl-8(sa47)* PLC $\beta$  mutants, and *unc-73(ce362)* Trio mutant animals. Line indicates mean eggs laid  $\pm$ 95% confidence intervals. Asterisks indicate  $P < 0.0001$ ; n.s., not significant ( $P > 0.05$ , 1-way ANOVA with Bonferroni's correction for multiple comparisons;  $n > 10$ ). b) On the left, cartoon of the egg-laying circuit and experiment showing blue light activation of vulval muscles for 30 s (left). On the right, scatter plots of eggs laid per worm in presence (+) or absence (-) of ATR during blue light activation of ChR2 expressed in the vulval muscles of wild type, *egl-30(ad805)*  $G\alpha_q$  strong loss-of-function mutants, *egl-8(n488)* and *egl-8(sa47)* PLC $\beta$  mutants, and *unc-73(ce362)* Trio mutant animals. Line indicates mean eggs laid  $\pm$ 95% confidence intervals. Asterisk indicates  $P \leq 0.0255$ ; n.s., not significant,  $P > 0.05$  (1-way ANOVA with Bonferroni's correction for multiple comparisons;  $n > 10$ ).

neurons (Nurrish et al. 1999), suggesting alterations in IP $_3$  and/or DAG levels in neurons may affect the frequency of egg-laying active states without altering the overall pattern or strength of vulval muscle Ca $^{2+}$  activity within those active states. Indeed, *goa-1(n1134)* mutants that reduce inhibitory  $G\alpha_o$  signaling have hyperactive egg-laying behavior defects that strongly resemble *dgk-1(nu62)* mutants without a significant increase in vulval muscle Ca $^{2+}$  activity (Ravi et al. 2021). Together, these results indicate  $G\alpha_q$  and Trio RhoGEF, but not PLC $\beta$ , are required for vulval muscle activity that drives twitching and egg-laying Ca $^{2+}$  transients during egg-laying active states.

### DAG mimetics restore muscle activity and egg laying to $G\alpha_q$ signaling mutants

How does serotonin signaling through  $G\alpha_q$  promote vulval muscle activity? Previous results have shown that DAG mimetic phorbol

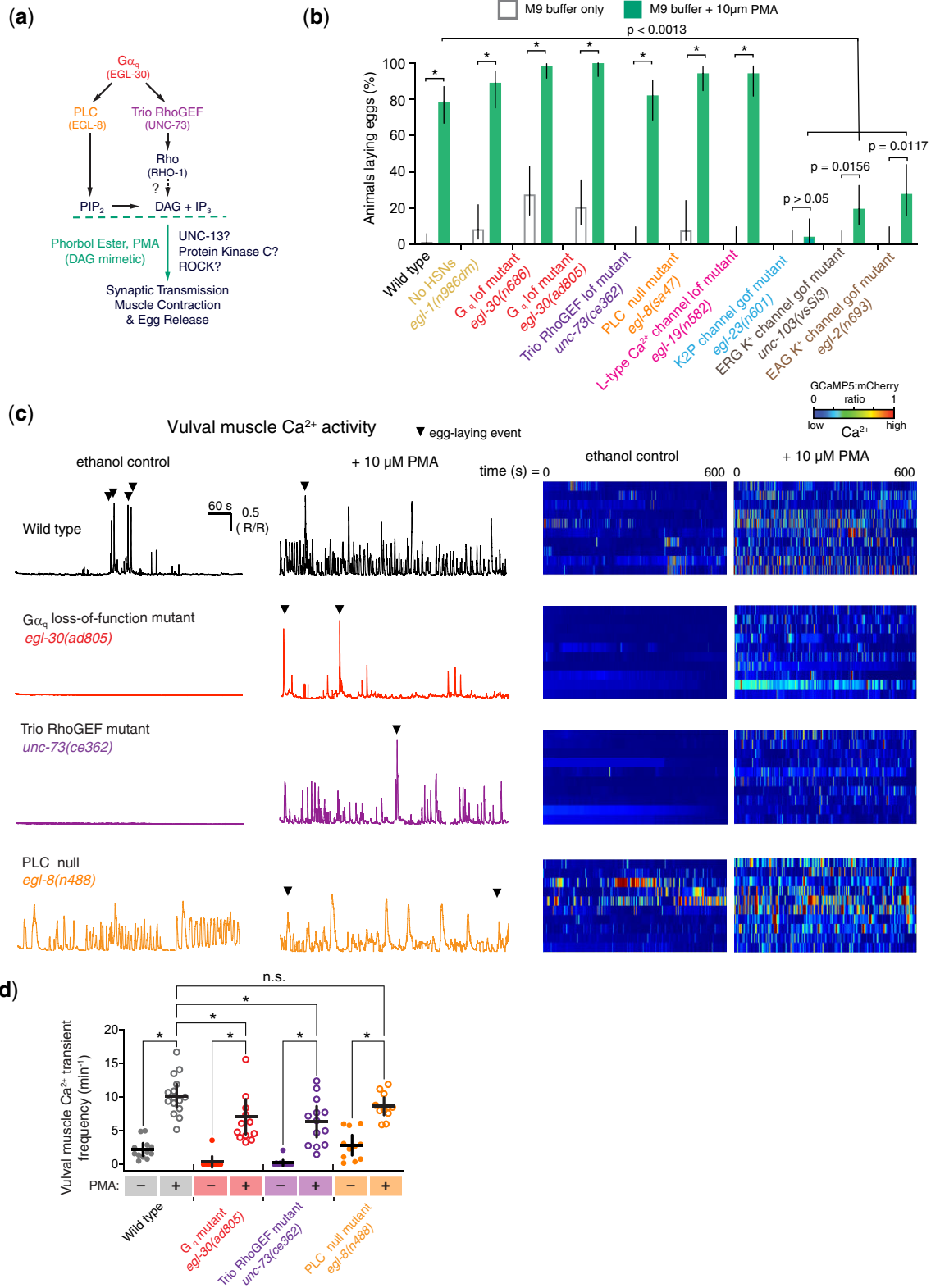
esters restore locomotion and animal viability to  $G\alpha_q$  null mutants and restore egg laying in PLC $\beta$ , Trio RhoGEF double mutants (Williams et al. 2007). However, the effects of phorbol esters on egg laying in single mutants were not clear, raising questions as to whether PMA rescued egg laying downstream of PLC $\beta$ , Trio RhoGEF, or both (Fig. 5a). We measured the egg-laying responses of wild-type animals and  $G\alpha_q$  signaling mutants to Phorbol 12-myristate 13-acetate (PMA). As shown in Fig. 5b, 10  $\mu$ M PMA treatment strongly stimulated egg laying in wild-type animals and all mutants with reduced  $G\alpha_q$  signaling ( $\geq 80\%$  animals laying eggs). Like serotonin (Fig. 2b), PMA also rescued egg laying in *egl-1(n986dm)* mutant animals lacking the HSNs (Fig. 5b), but only PMA rescued egg laying in  $G\alpha_q$  and effector signaling mutants. These results are consistent with PMA acting downstream of both serotonin release from the HSNs and its subsequent signaling through  $G\alpha_q$ -coupled receptors. To test whether



**Fig. 4.**  $\text{G}\alpha_q$  and Trio signaling promotes vulval muscle activity. a) Representative GCaMP5::mCherry ( $\Delta\text{R}/\text{R}$ ) ratio traces showing vulval muscle  $\text{Ca}^{2+}$  activity in freely behaving wild-type, *egl-30(n686)*  $\text{G}\alpha_q$  weak loss-of-function mutant, *egl-30(ad805)*  $\text{G}\alpha_q$  strong loss-of-function mutant, *unc-73(ce362)* Trio strong loss-of-function mutant, *egl-8(n488)* PLC $\beta$  null mutant, *egl-8(sa47)* PLC $\beta$  null mutant, *eat-16(tm761)*  $\text{G}\alpha_q$  RGS protein null mutant, *egl-30(tg26)* strong  $\text{G}\alpha_q$  gain-of-function mutant, and *dgk-1(nu62)* DAG Kinase null mutant animals during active (solid bar) and inactive (dotted line) egg-laying behavior states. Arrowheads indicate egg-laying events. Vertical and horizontal scale bars show GCaMP5/mCherry fluorescence ratio ( $\Delta\text{R}/\text{R}$ ) and time, respectively. b) Scatterplots of  $\text{Ca}^{2+}$  transient peak amplitudes for the indicated genotypes during twitch (closed square) and egg-laying transients (open circles). Asterisks indicate  $P < 0.0001$ , n.s. indicates not significant ( $P > 0.05$ , Kruskal–Wallis test with Dunn’s correction for multiple comparisons). c) Scatterplots of  $\text{Ca}^{2+}$  transient frequency for indicated genotypes. Line indicates mean eggs laid  $\pm 95\%$  confidence intervals; asterisks indicate  $P \leq 0.0340$ ; n.s. indicates not significant ( $P > 0.05$ , 1-way ANOVA with Bonferroni’s correction for multiple comparisons;  $n > 10$  animals recorded per genotype).

PMA and DAG act upstream to modulate vulval muscle electrical excitability, we also tested the PMA response in animals carrying mutations in voltage-gated channels that reduce or block egg laying. Loss of L-type  $\text{Ca}^{2+}$  channel activity in *egl-19(n582)* hypomorphic mutants impairs egg laying downstream of serotonin (Trent et al. 1983; Waggoner et al. 1998), but we find *egl-19(n582)* mutant animals still lay eggs in response to  $10\ \mu\text{M}$  PMA (Fig. 5b). The n582 mutation alters but does not eliminate  $\text{Ca}^{2+}$  channel function (Jospin et al. 2002; Gao and Zhen 2011), possibly explaining how

PMA could still rescue egg-laying behavior. Indeed, *tpa-1(k530)* mutants originally identified by their resistance to phorbol esters like PMA show synthetic egg-laying defects when combined with *egl-19(n582)* (Waggoner et al. 1998). We next tested gain-of-function  $\text{K}^+$  channel mutants that block egg laying. Animals expressing A383V gain-of-function EGL-23 K2P channels (Trent et al. 1983; Ben Soussia et al. 2019), A331T gain-of-function UNC-103 ERG  $\text{K}^+$  channels (Reiner et al. 1999; Reiner et al. 2006; Collins and Koelle 2013), or A478V gain-of-function EGL-2 EAG channels



**Fig. 5.** The DAG mimetic PMA rescues egg-laying circuit activity and behavior defects of  $G\alpha_q$  signaling mutants. a) Diagrams showing working model of  $G\alpha_q$  and DAG signaling pathway during egg-laying behavior. b) Bar plots showing the percentage of animals showing egg laying in M9 buffer (open bars) or M9 buffer +10  $\mu$ M PMA (filled bars). Error bars indicate 95% confidence intervals for the proportion; asterisks indicate  $P < 0.0013$  (Fisher's exact test with Bonferroni's correction for multiple comparisons;  $n \geq 30$  animals per genotype and condition). c) Left, representative GCaMP5::mCherry ( $\Delta R/R$ ) ratio traces showing vulval muscle  $Ca^{2+}$  activity in wild-type or the indicated  $G\alpha_q$  signaling mutant animals in the absence or presence of 10  $\mu$ M PMA. Arrowheads indicate egg-laying events. Vertical and horizontal scale bars show GCaMP5/mCherry fluorescence ratio ( $\Delta R/R$ ) and time, respectively. Right, heat map showing intensity modulated color spectrum of GCaMP5::mCherry ( $\Delta R/R$ ) ratio of vulval muscle  $Ca^{2+}$  activity ranging from low to high  $Ca^{2+}$ . Rows indicate ratio changes in each of 10 animals. d) Scatterplots of  $Ca^{2+}$  transient frequency in the absence (-) and presence (+) of 10  $\mu$ M PMA for the indicated genotypes. Lines indicate mean eggs laid  $\pm$ 95% confidence intervals; asterisk indicates  $P \leq 0.0275$ ; n.s., not significant ( $P > 0.05$ , 1-way ANOVA with Bonferroni's correction for multiple comparisons;  $n \geq 10$  animals per genotype and condition).

(Weinshenker et al. 1999) showed reduced egg laying in response to PMA (Fig. 5b). PMA-induced egg laying was completely blocked in *egl-23* K2P gain-of-function mutants, and the PMA response was significantly reduced in both *unc-103* ERG and *egl-2* EAG mutant animals compared to wild type (Fig. 5b). Although these results do not rule out that phorbol esters like PMA may be stimulating egg laying in a manner independent of its DAG mimetic effects, our data support a model where  $G\alpha_q$  and Trio RhoGEF signal upstream of DAG to promote vulval muscle excitability and/or contractility.

We next imaged how PMA affected vulval muscle  $Ca^{2+}$  activity. We performed 10-min GCaMP5  $Ca^{2+}$  recordings of wild-type or  $G\alpha_q$  signaling mutants after 2 h of exposure to 10  $\mu$ M PMA (Supplementary Movies 1–3). Quantitation of  $Ca^{2+}$  transients showed that PMA significantly increased vulval muscle  $Ca^{2+}$  activity in wild-type animals to  $10 \pm 1.6$  transients per minute from an average  $2 \pm 0.9$  transients per min in vehicle-treated, wild-type animals (Fig. 5, c and d and Supplementary Movie 1). PMA also restored both rhythmic twitch and egg-laying  $Ca^{2+}$  transients to strong  $G\alpha_q$  and Trio RhoGEF signaling mutants, with  $Ca^{2+}$  transients frequencies increasing from essentially  $0 \pm 0.8$  transients per minute in vehicle-treated controls to  $7 \pm 2$  transients per minute after PMA treatment, almost but not quite to the level of PMA-treated wild-type control animals (Fig. 5, c and d and Supplementary Movies 2 and 3). Together, these studies show that DAG-mimetic phorbol esters restore muscle excitability and contractility defects of  $G\alpha_q$  and Trio RhoGEF mutants, suggesting that DAG production could be a major and necessary consequence of both  $G\alpha_q$  and Trio RhoGEF signaling.

### Phorbol esters promote egg laying independent of UNC-13 or single protein kinase C isoforms

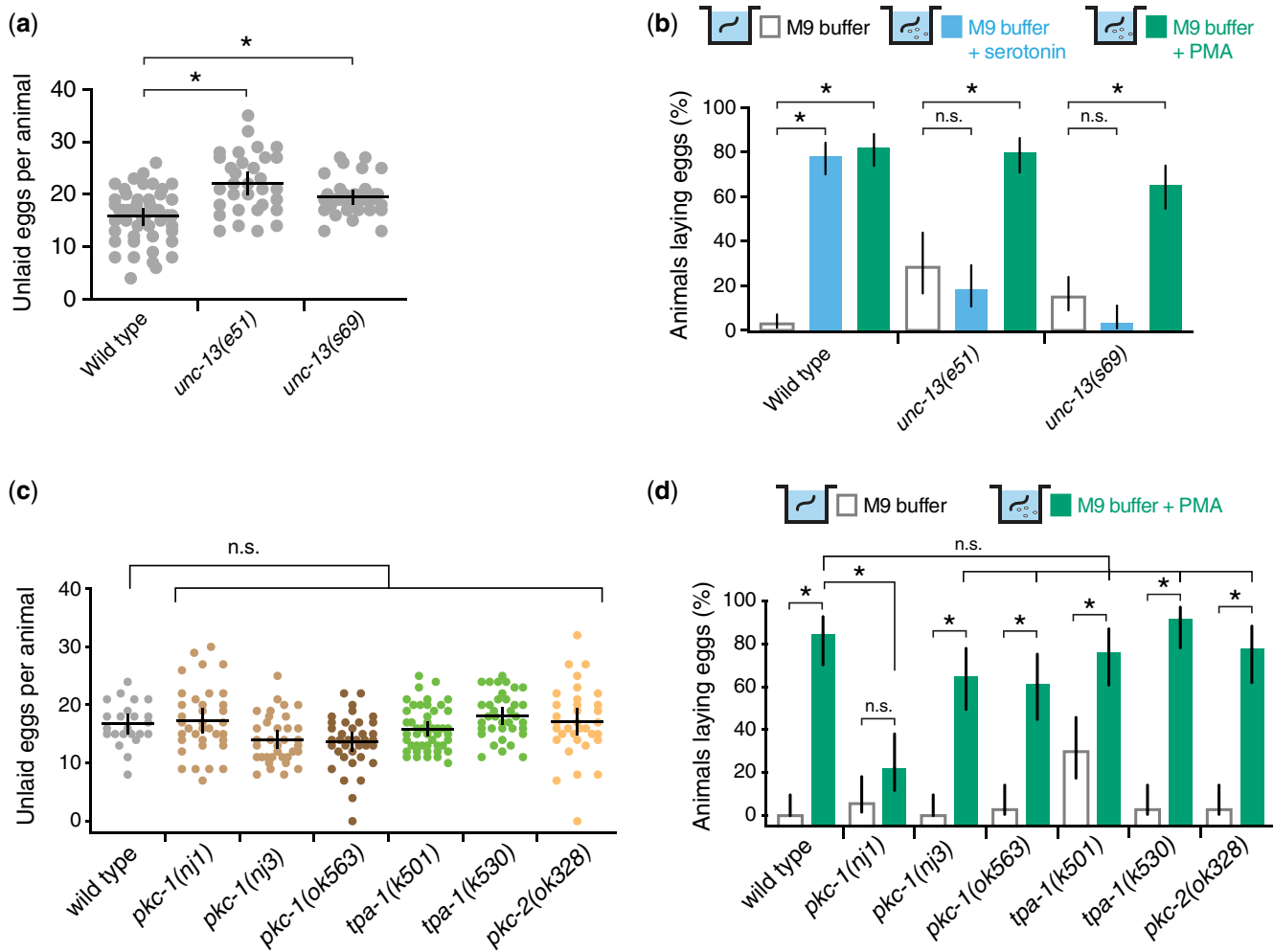
How do phorbol esters like PMA rescue vulval muscle  $Ca^{2+}$  activity and egg laying? Previous results have shown that DAG and PMA bind to C1 domain-containing proteins such as mUNC-13/UNC-13 and PKC to regulate their activity (Konig et al. 1985; Huang 1989; Betz et al. 1998; Newton 2001; Silinsky and Searl 2003). To test if DAG regulates egg-laying behavior through activation of UNC-13 or PKC, we tested whether mutants lacking these proteins still have a robust serotonin and/or PMA egg-laying response (Fig. 6). Mutants that eliminate axonal and synaptic UNC-13 show reduced egg laying, accumulating an average of 22 eggs compared to 15 seen in wild-type animals (Fig. 6a), like PLC $\beta$  mutants but significantly fewer than the >30 eggs that accumulate in  $G\alpha_q$  and Trio RhoGEF mutants (Fig. 1, c–i). *unc-13* mutants also resemble PLC $\beta$  mutants in their egg-laying response to serotonin and PMA. Egg laying in *unc-13* mutants was stimulated by PMA but was resistant to serotonin (Fig. 6b). While the serotonin resistance we observed for *unc-13* mutants after 1 h differs from that seen by Bastiani et al. (2003) at 90 min, we saw similar differences for the PLC $\beta$  mutants (Table 3). These results show that serotonin promotes egg laying through a PLC $\beta$  and UNC-13-dependent pathway that may be distinct from the PMA-stimulated pathway.

To determine whether  $G\alpha_q$  and Trio RhoGEF signaling through the PMA-responsive pathway is mediated by PKC, we analyzed egg accumulation in animals bearing predicted null mutations in different PKC isoforms (Tabuse et al. 1989; Tabuse 2002; Okochi et al. 2005; Hyde et al. 2011; Edwards et al. 2012). The *C. elegans* genome encodes 4 PKCs isoforms PKC-1, PKC-2, PKC-3, and TPA-1. PKC-1 has previously been shown to promote neuropeptide transmission (Sieburth et al. 2007). While neuropeptides signal to promote egg laying (Avery et al. 1993; Kass et al. 2001; Jacob and

Kaplan 2003; Brewer et al. 2019), PKC-1 (nPKC- $\epsilon$ ) null mutants showed a grossly normal egg accumulation of 14~17 eggs (Fig. 6c). Animals bearing predicted null mutants of novel and conventional PKCs such as nPKC $\delta/\theta$  (TPA-1) and cPKC $\alpha/\beta$  (PKC-2) orthologs also show no significant differences in egg accumulation (Fig. 6c), suggesting that, unlike loss of  $G\alpha_q$  or Trio RhoGEF signaling, disruption of individual PKC signaling pathways does not strongly affect egg-laying behavior. We next tested whether PKC mediates the egg-laying response to PMA. All the PKC single mutant animals laid eggs in response to PMA except for *pkc-1(nj1)* (Fig. 6c). The *nj1* allele is predicted to be a missense mutation that may lead to the expression of a mutant PKC protein with altered function (Okochi et al. 2005). Indeed, previous experiments using this mutant have shown that *pkc-1(nj1)* mutant animals have stronger behavior defects (Okochi et al. 2005; Ventimiglia and Bargmann 2017), suggesting that the mutant protein may be expressed and interfere cell signaling, possibly by interfering with the function of other co-expressed PKC isoforms like TPA-1. Together, these results support a model where  $G\alpha_q$  and Trio RhoGEF signaling in the vulval muscles drives elevation of DAG, which activates targets like PKCs and/or other effectors to promote cell electrical excitability for egg laying.

### Discussion

In this study, we explored the cellular and molecular specificity of  $G\alpha_q$  effector signaling as it regulates egg-laying circuit activity and behavior using molecular genetics, optogenetics, pharmacology, and  $Ca^{2+}$  imaging techniques. We found that  $G\alpha_q$  effectors PLC $\beta$  and Trio RhoGEF differentially act in neurons and muscles to promote synaptic transmission and egg-laying behavior, supporting a working model where  $G\alpha_q$  signals through Trio RhoGEF in both neurons and muscles while PLC $\beta$  functions outside of HSN to promote egg laying (Fig. 7). Although  $G\alpha_q$ , PLC $\beta$ , and Trio RhoGEF mutants fail to lay eggs in response to serotonin, optogenetic stimulation of HSNs fully rescued egg laying in PLC $\beta$  but not  $G\alpha_q$  or Trio RhoGEF mutants. Recent work has shown that the HSNs release NLP-3 neuropeptides which can promote egg laying even in *tph-1* mutants lacking serotonin (Brewer et al. 2019), possibly explaining why optogenetic activation of HSNs rescues egg laying to PLC $\beta$  mutants even when exogenous serotonin cannot. The HSNs are also predicted to release ACh (Pereira et al. 2015), and nAChR receptors are expressed on the vulval muscles and can stimulate egg laying (Waggoner et al. 2000; Kim et al. 2001). Because serotonin drives egg laying in animals lacking HSNs but not in animals lacking PLC $\beta$  and that expression of PLC $\beta$  is sufficient to rescue normal egg laying, we propose working model (Fig. 7) where  $G\alpha_q$  and PLC $\beta$  act in neurons other than HSNs to promote release of neurotransmitters like ACh onto the vulval muscles to stimulate egg laying. The cholinergic VA, VB, and VC neurons also innervate the vulval muscles alongside the HSNs and express PLC $\beta$  (White et al. 1986; Cook et al. 2019; Taylor et al. 2021). Consistent with this model, we have recently shown that blocking VC synaptic transmission reduces egg laying in response to serotonin (Kopchok et al. 2021). Optogenetic stimulation of the VCs (Kopchok et al. 2021) or VA/VB neurons (Kopchok 2021) stimulates vulval muscle  $Ca^{2+}$  activity, but the resulting  $Ca^{2+}$  activity is insufficient to drive the strong egg-laying contractions. Because we find that mutations that increase  $G\alpha_q$  signaling have stronger vulval muscle  $Ca^{2+}$  transients, we propose that serotonin and NLP-3 released from HSN potentiate ACh release from other motor neurons and promote the electrical excitability and/or contractility of the vulval muscles, converting rhythmic twitch

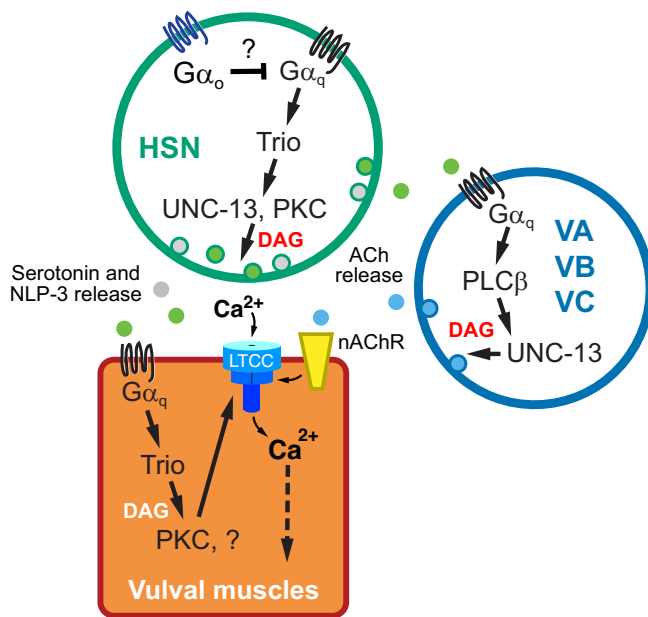


**Fig. 6.** DAG promotes egg laying independent of UNC-13 or PKC. a) Scatterplot of egg accumulation in wild-type, *unc-13(e51)* loss-of-function mutant, and *unc-13(s69)* null mutant animals. Lines indicate mean eggs laid  $\pm$ 95% confidence intervals. Asterisk indicates  $P \leq 0.0021$  (1-way ANOVA with Bonferroni's correction for multiple comparisons;  $n \geq 36$  per genotype). b) Bar plots showing the percentage of wild-type, *unc-13(e51)*, or *unc-13(s69)* mutant animals laying eggs in M9 buffer, 18.5 mM serotonin, or 10  $\mu$ M PMA. Asterisks indicate  $P < 0.0006$ ; n.s., not significant ( $P > 0.05$ , Fisher's exact test with Bonferroni's correction for multiple comparisons;  $n \geq 36$  animals per genotype and condition). c) Scatterplot of egg accumulation in wild type ( $n = 24$ ) and the indicated PKC mutant animals ( $n \geq 35$  per genotype). Line indicates mean eggs accumulated  $\pm$ 95% confidence intervals. n.s., not significant ( $P > 0.05$ , 1-way ANOVA with Bonferroni's correction for multiple comparisons). d) Bar plots showing the percentage of wild-type and PKC mutant animals showing egg laying in M9 buffer or 10  $\mu$ M PMA. Bar indicates mean eggs  $\pm$ 95% confidence intervals for the proportion. Asterisks indicate  $P < 0.0007$ ; n.s., not significant ( $P > 0.05$ , Fisher's exact test with Bonferroni correction for multiple comparisons;  $n \geq 35$  animals per genotype and condition).

$Ca^{2+}$  transients into stronger egg-laying transients. Previous studies have shown that Trio RhoGEF acts in neurons to regulate locomotion behavior (Steven et al. 2005; Williams et al. 2007; Hu et al. 2011). Our studies show that transgenic Trio RhoGEF expression in either neurons or muscles alone is insufficient to restore wild-type level of egg-laying behavior, but expression in both is sufficient. These results mirror previously published results for  $G\alpha_q$  (Bastiani et al. 2003), further supporting a model where PLC $\beta$  and Trio RhoGEF functions during locomotion and egg-laying behaviors are distinct.

How does  $G\alpha_q$ , PLC $\beta$ , and Trio-RhoGEF signaling promote egg-laying behavior? Earlier studies have suggested that Rho ortholog RHO-1 in *C. elegans* regulates synaptic activity in a mechanism that involves the G12 family protein GPA-12 (Lutz et al. 2005; Hiley et al. 2006). Activated RHO-1 also directly binds to and inhibits the DGK-1 diacylglycerol kinase expressed in neurons that signals to reduce DAG available to bind effectors (Hiley et al. 2006; McMullan et al. 2006). Our data are consistent with the previous results reporting that  $G\alpha_q$  signaling regulates postsynaptic vulval

muscle activity mainly through  $G\alpha_q$ -Trio pathway as  $G\alpha_q$  and Trio mutants show a similarly strong reduction in vulval muscle  $Ca^{2+}$  activity. Because muscle activity defects in  $G\alpha_q$  and Trio mutants can be restored by the DAG-mimetic PMA, we suggest that insufficient levels of DAG are responsible for the circuit activity and behavior defects of  $G\alpha_q$  and Trio RhoGEF mutants. In the absence of PLC $\beta$ , how would parallel  $G\alpha_q$  signaling through Trio RhoGEF and RHO-1 generate DAG? Besides PLC $\beta$  (EGL-8), *C. elegans* expresses 4 other PLC orthologs: PLC $\epsilon$  (PLC-1), PLC-2, PLC $\gamma$  (PLC-3), and PLC $\delta$  (PLC-4) (Vázquez-Manrique et al. 2008). In vitro studies with cultured mammalian cells show that small G proteins like Rho can bind to and activate PLC $\epsilon$  (Wing et al. 2003; Seifert et al. 2008). Genetic and molecular expression evidence in *C. elegans* suggests a model where PLC $\epsilon$  is activated downstream of  $G\alpha_q$  and Rho to promote cell activity (Kunitomo et al. 2013; Yu et al. 2013; Taylor et al. 2021), but whether Trio activation of Rho ultimately acts through these PLCs to produce DAG is not clear. One approach to test if these other PLCs mediate Rho signaling would be to perform genetic epistasis experiments. However, loss



**Fig. 7.** Working model of  $G\alpha_q$  signaling in the egg-laying circuit. See text for details.

of Rho-1 causes lethality (Jantsch-Plunger et al. 2000; McMullan and Nurrish 2011) and loss of PLC $\epsilon$  cause sterility defects (Yin et al. 2004), limiting our ability to measure differences in egg laying. Alternatively,  $G\alpha_q$  signaling through the Rho-1 branch may be independent of PLCs and DAG production where exogenous PMA is instead activating factors downstream of a parallel PLC $\beta$  pathway. While our rescue data are consistent with PLC $\beta$  acting in neurons and Trio RhoGEF acting in muscles, we cannot rule out a PLC $\beta$  function for DAG or IP $_3$  production in the vulval muscles. Imaging or biochemical approaches documenting  $G\alpha_q$ -dependent changes in PIP $_2$  (Stauffer et al. 1998) and/or DAG (Tewson et al. 2012; Ohno et al. 2017) in vivo, along with cell-specific rescue and knockout experiments (LeBoeuf et al. 2020), should resolve whether the Rho-1 branch acts through PLCs and/or inhibits DAG lipases to promote DAG levels.

Do phorbol esters like PMA stimulate *C. elegans* egg laying by acting as DAG mimetics? Previous studies have shown that phorbol esters promote both synaptic vesicle and dense core vesicle release from neurons and neurosecretory cells (Silinsky and Searl 2003). DAG and phorbol esters activate many effectors including mUNC-13 in the brain and PKC in nearly all cells (Huang 1989; Betz et al. 1998). In *C. elegans*, exogenous treatment with phorbol esters causes growth inhibition, uncoordinated movement, and lethality, which can be suppressed by loss-of-function mutations in a single gene, *tpa-1*, which encodes nPKC $\delta/\theta$  (Tabuse and Miwa 1983; Tabuse et al. 1989). Acute PMA treatment promotes hypersensitivity to the paralytic effects of aldicarb (Sieburth et al. 2007). Double mutants of both PKC-1 and UNC-13 (H17K) show increased resistance to phorbol esters compared to either mutant alone suggesting that PMA acts in part through these effectors to regulate ACh release (Silinsky and Searl 2003; Sieburth et al. 2007). Our data show that animals lacking UNC-13 still lay eggs in response to PMA. Mutant animals with defects in single PKC isoform encoding genes were similarly responsive to PMA, with the notable exception of *pkc-1(nj1)* mutant animals. *pkc-1(nj1)* results in a missense mutation and shows a significantly reduced PMA response compared to 2 other putative null mutants. Such allele-specific differences among *pkc-1* alleles have been observed

previously in experiments studying PKC function in nose touch response, octanol and high osmolarity avoidance (Hyde et al. 2011), regulation of AWC<sup>ON</sup> glutamate release (Ventimiglia and Bargmann 2017), and in regulation of PKC by DAG or Ca<sup>2+</sup> (Okochi et al. 2005). The *nj1* allele may impart a dominant-negative effect, affecting the recruitment or function of other PKC isoforms. For example, TPA-1 has been shown to function redundantly with PKC-1 (also known as TTX-4), a nPKC- $\epsilon$  ortholog (Okochi et al. 2005). Studies have shown that activated  $G\alpha_q$  and accumulation of DAG recruit TPA-1 to compensate for the loss of PKC-1 (Hiroki and Iino 2022). Egg-laying defects of *egl-19(n582)* L-type Ca<sup>2+</sup> channel mutants are enhanced when combined with *tpa-1(k530)* PKC null mutants (Waggoner et al. 1998), suggesting that TPA-1 may mediate some of the DAG and/or PMA response for egg laying. Future work testing compound mutants disrupting UNC-13 and different PKC isoforms should reveal whether PMA acts as a DAG mimetic in neurons and muscle cells to promote egg laying.

Besides the compensatory effect of various PKCs and UNC-13, this study does not rule out other effectors as potential targets of DAG and/or PMA. In vitro studies show ROCK (Rho-associated coiled-coil kinase) activation in PMA-induced apoptosis and macrophage differentiation (Chang et al. 2006; Yang et al. 2017). ROCK has a predicted C1 domain that mediates protein interaction with DAG and might bind and be similarly activated by PMA (Xiao et al. 2009). In *C. elegans*, RHO-1 signals through LET-502/ROCK to phosphorylate nonmuscle myosin light chain (Shimizu et al. 2018).  $G\alpha_q$  also promotes neurotransmitter release via additional kinase targets including SEK-1 Mitogen-Activated Protein Kinase in the p38 MAPK pathway and KSR-1 in the ERK MAPK pathway (Hoyt et al. 2017; Coleman et al. 2018). KSR-1 is particularly interesting in that its N-terminus shares sequence similarity with C1 domains that might mediate regulation by DAG. Loss of KSR-1 and other ERK MAPK components also suppress the loopy locomotion defects caused by gain-of-function mutations in Rho-1 (Coleman et al. 2018). Taken together, our work is consistent with a model where additional DAG-sensitive effectors act downstream of  $G\alpha_q$ , Trio, and Rho to promote muscle excitability and/or contractility for egg laying.

Phorbol esters and locally produced DAG may promote egg laying via activation of distinct effectors. Apart from the activation of C1 domain containing effectors, emerging evidence indicates that phospholipase C-dependent production of DAG directly modulates the gating of ion channels for membrane excitability. For example, DAG activates several ion channels including canonical transient receptor potential cation channels (Hofmann et al. 1999) while also inhibiting other ion channels including 2-pore domain TASK potassium channels via an unknown mechanism (Wilke et al. 2014). PIP $_2$  has also been shown to modulate some ion channels like KCNQ directly (Suh et al. 2006), although DAG and PMA modulate *C. elegans* KCNQ channels likely via the intermediate activation of protein kinases including PKC (Wei et al. 2005). Thus,  $G\alpha_q$  modulation of PIP $_2$  and DAG levels could directly or indirectly affect several postsynaptic ion channels to shape electrical excitability. DAG is also precursor in the production of several signaling lipids, including the endocannabinoid 2-arachidonoylglycerol (2-AG), which has been shown to signal from dendrites in a retrograde manner through neuronal  $G\alpha_o$ -coupled endocannabinoid receptors to inhibit neurotransmitter release (Hashimoto et al. 2005; Wettschreck et al. 2006; Tanimura et al. 2010; Hashimoto et al. 2013; Soltesz et al. 2015). In *C. elegans*, 2-AG activates the NPR-19 endocannabinoid receptor ortholog that couples to  $G\alpha_o$  to modulate serotonin transmission, pharyngeal, feeding, and locomotory

behaviors (Pastuhov *et al.* 2016; Oakes *et al.* 2017; Oakes *et al.* 2019). We have recently shown that feedback of egg accumulation alters vulval muscle  $\text{Ca}^{2+}$  activity, which subsequently signals to regulate bursts of  $\text{Ca}^{2+}$  transients in the HSNs that accompany the onset of the egg-laying active state (Ravi, Garcia, *et al.* 2018; Ravi *et al.* 2021). These results support a model where stretch-dependent feedback of egg accumulation stimulates postsynaptic vulval muscle  $\text{Ca}^{2+}$  signaling. This  $\text{Ca}^{2+}$  could then activate PLCs to generate DAG and 2-AG, which signal to modulate HSN activity, serotonin release, and egg laying. The genetic and experimental accessibility of the *C. elegans* egg-laying circuit should allow us to determine if conserved G proteins like  $\text{G}\alpha_q$  act generally to drive neural circuit activity via changes in DAG, subsequent activation of effectors, and retrograde messengers like 2-AG.

## Data availability

All the data, reagents, and strains used in this study are available from the corresponding author upon request.

Supplemental material is available at GENETICS online.

## Acknowledgments

We thank Drs. Kenneth Miller, Joshua Kaplan, and Thomas Boulin for sharing plasmids and strains. We thank Drs. Julia Dallman, Laura Bianchi, and Athula Wikramanayake along with members of the Collins lab for helpful discussions and feedback on the manuscript.

## Funding

This work was funded by grants from the National Institutes of Health (R01-NS086932) and National Science Foundation (IOS-1844657) to KMC. PD is supported by an American Heart Association predoctoral fellowship Award (20PRE35210233). Some of the strains used in this study were provided by the *C. elegans* Genetics Center, which is funded by the National Institutes of Health Office of Research Infrastructure Programs (P40-OD010440).

## Conflicts of interest

The authors declare no conflicts of interest.

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