

NALCN Channels Are Not Major targets of $G\alpha_o$ or $G\alpha_q$ Modulation in the *C. elegans* Egg-Laying Behavior Circuit

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Abstract

Sodium leak channels (NALCN) are regulators of cell membrane potential. Previous studies in mammalian neurons and *C. elegans* have shown that $G\alpha_q$ and $G\alpha_o$ signaling antagonistically modulates NALCN activity to regulate neuron excitability and neurotransmitter release for behavior. Here, we test whether NALCNs mediate the effects of $G\alpha_q$ and/or $G\alpha_o$ signaling in the *C. elegans* egg-laying circuit. We find that while gain-of-function NALCN mutants exhibit hyperactive egg-laying behavior, NALCNs are not required for the effects of $G\alpha_q$ or $G\alpha_o$ signaling for egg laying. These results show that NALCNs are not major effectors of G-protein signaling for *C. elegans* egg-laying behavior.

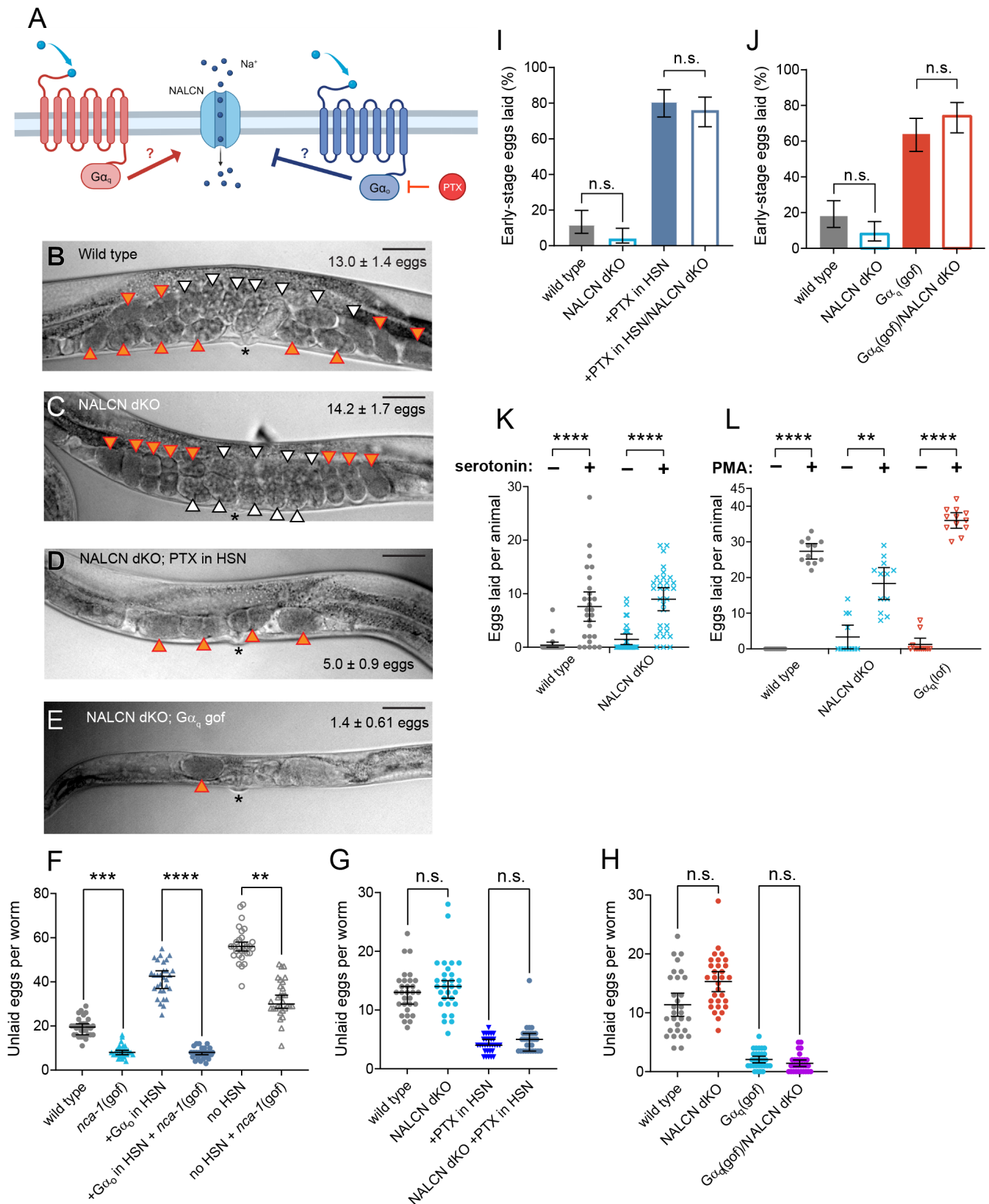


Figure 1. NALCN channels are not required for G α_q , G α_o , serotonin, or DAG regulation of *C. elegans* egg-laying behavior.:

(A) Cartoon of G-protein signaling pathways through hypothesized NALCN modulation. $G\alpha_q$ and $G\alpha_o$ are activated by G-protein-coupled receptors (red or blue, respectively) and signal through either known or unknown effectors, respectively, to promote or inhibit cell excitability through ion channels, one of which may be NALCN. Pertussis Toxin (PTX) inhibits $G\alpha_o$ signaling.

(B-E) Micrographs of egg accumulation in wild-type (B) and mutant strains [ZM2960](#) (*nca-1(gk9)* IV; *nca-2(gk5)* III) (C), [MIA524](#) (*nca-1(gk9)* IV; *nca-2(gk5)* III; *vsIs50* [*tph-1::PTX*, [LIN-15](#)] *lite-1(ce314)* *lin-15(n765ts)* X) (D), and [EG4036](#) (*egl-30(tg26)* I*; *nca-1(gk9)* IV; *nca-2(gk5)* III) (E). Scale bars indicate 100 μm . *: denotes a gain-of-function allele of the $G\alpha_q$ ortholog, *egl-30*.

(F-H) Scatterplots of egg accumulation in wild-type and indicated mutant or transgenic animals with altered NALCN function, G-protein signaling, and/or HSN development. Error bars represent 95% confidence intervals for the mean. n.s. indicates $P > 0.05$; ** indicates $P = 0.0036$; *** indicates $P = 0.0005$; **** indicates $P < 0.0001$ (Kruskall-Wallis test with Dunn's correction for multiple comparisons; $n = 30$ per genotype). (F) Animals expressing the *nca-1(e625)* gain-of-function mutant along with either a gain-of-function $G\alpha_o$ (Q205L) mutant transgenically expressed in the HSNs or a whole-animal *egl-1(n986dm)* mutation that drives HSN programmed cell death (e.g. no HSNs). (G) Egg accumulation in *nca-1;nca-2* double mutants (NALCN dKO) alone, in animals expressing Pertussis Toxin in the HSNs, or (H) *egl-30(tg26)* $G\alpha_q$ gain-of-function mutants.

(I-J) Bar graphs indicate percent of embryos laid at early stages of development (8 cells per embryo or less). Error bars indicate 95% confidence intervals for the mean proportion. n.s. indicates $P > 0.05$ (Fisher's exact test; $n = 100$ embryos per genotype).

(K) Scatterplots indicating the number of eggs laid per worm in two hours in M9 buffer alone or M9 plus 18.5 mM serotonin. Error bars indicate 95% confidence intervals for the mean. **** indicates $P < 0.0001$ (Kruskall-Wallis test with Dunn's correction for multiple comparisons; $n \geq 26$ animals per condition).

(L) Scatterplots indicating the number of eggs laid per worm in two hours in M9 buffer alone or M9 plus 10 μM PMA. Error bars indicate 95% confidence intervals for the mean. ** indicates $P = 0.0071$, and **** indicates $P < 0.0001$ (Kruskall-Wallis test with Dunn's correction for multiple comparisons; $n = 12$ animals for each indicated group).

Description

Signaling through alpha subunits of heterotrimeric G-proteins such as $G\alpha_q$ and $G\alpha_o$ regulates cell electrical excitability and neurotransmitter release, yet which downstream ion channel effectors mediate their signaling *in vivo* is not well understood. *C. elegans* is an ideal system for the study of these pathways as its simple nervous system is fully mapped by electron microscopy, uses the same signaling molecules as in humans, and is rich in GPCRs homologous to those targeted therapeutically.

The egg-laying circuit of *C. elegans* consists, in part, of a pair of serotonergic hermaphrodite-specific neurons (HSNs), which innervate two pairs of vulval muscle cells that contract for egg release (Schafer, 2006). $G\alpha_q$ and $G\alpha_o$ signal in both the HSNs and vulval muscles to promote and inhibit egg-laying behavior, respectively (Collins et al., 2016; Tanis et al., 2008), and electrophysiological and Ca^{2+} imaging experiments show that $G\alpha_o$ signaling serves to stabilize the resting membrane potential of the HSNs, reducing their electrical excitability and Ca^{2+} transient activity in the vulval muscles (Dhakal et al., 2022; Ravi et al., 2021; Shyn et al., 2003).

Several studies in both mammals and worms have identified NALCN Na^+ leak channels as a potential effector of both $G\alpha_q$ and $G\alpha_o$ signaling ((Lu et al., 2009; Lutas et al., 2016; Philippart & Khaliq, 2018; Swayne et al., 2009; Topalidou et al., 2017a; Topalidou et al., 2017b); Fig. 1A). In worms, double knockout mutants of NALCN genes *unc-77* (also known and referred to herein as *nca-1*) and *nca-2* suppress the loopy locomotor waveform of either activated $G\alpha_q$ *egl-30(tg26)* mutants, animals expressing activated Rho, or loss-of-function $G\alpha_o$ *goa-1* mutants (Topalidou et al., 2017a). Additionally, genetic experiments with several genes involved in the $G\alpha_o$ and $G\alpha_q$ -Rho pathways showed that dopamine signaling through $G\alpha_o$ -coupled [DOP-3](#) receptors negatively modulates *NCA-1* and *NCA-2* channels (Topalidou et al., 2017b). Whether NALCN channels play a similar role in other *C. elegans* behaviors including egg laying is not clear. *nca-1* and *nca-2* are expressed in cells of the egg-laying circuit (Taylor et al., 2021), and gain-of-function *nca-1* mutations lead to increased HSN Ca^{2+} activity (Yeh et al., 2008) and hyperactive egg-laying behavior (Yeh et al., 2008) (Fig. 1A), consistent with a role for NALCN channels in promoting egg laying.

To determine whether NALCN channels regulate egg laying downstream of G protein signaling, we used a genetic epistasis approach. Animals expressing the GTPase-deficient $G\alpha_o$ mutant (Q205L) in the HSN neurons (Tanis et al., 2008) have reduced HSN Ca^{2+} activity and delayed egg laying (Ravi et al., 2021). Double mutants expressing both $G\alpha_o$ (Q205L) and *nca-1(gf)* have strongly hyperactive egg-laying behavior that is not statistically different from *nca-1(gf)* mutant animals alone (Fig. 1F), consistent with NCA-1 channels acting downstream of $G\alpha_o$ in egg laying. Depolarization in the egg-laying muscles can overcome defects in HSN function, including loss of HSNs altogether (Bastiani et al., 2003). To test if the hyperactive egg laying of *nca-1(gf)* mutants represented HSN-specific suppression of $G\alpha_o$ (Q205L) or a bypass, we created *nca-1(gf)* mutant animals lacking HSNs using the [egl-1\(n986dm\)](#) mutation that causes premature death of the HSN neurons (Conradt & Horvitz, 1998). Unlike the results with $G\alpha_o$ (Q205L), *nca-1(gf)* only partially suppressed the egg-laying defects caused by loss of HSNs, suggesting NCA-1 NALCN channels promote egg-laying behavior both in and outside of the HSNs.

To clarify the role of these channels more directly, we analyzed egg-laying behavior in *nca-1*, [nca-2](#) double knockout (dKO) mutants completely lacking NALCN channels. NALCN dKO animals retained a similar number of eggs in their uterus compared to wild-type animals (compare Fig. 1B to 1C; Fig. 1G). We next tested how loss of NALCN channels affected egg-laying behavior in animals expressing Pertussis Toxin (PTX) in HSN to inactivate $G\alpha_o$ (Tanis et al., 2008) or in [egl-30\(tg26\)](#) gain-of-function mutants with a whole-animal increase in $G\alpha_q$ signaling (Doi & Iwasaki, 2002). We measured steady-state egg accumulation in each mutant and found that complete loss of NALCN function failed to suppress the hyperactive egg-laying behavior of either mutant (Fig. 1D-E, G-H). Since mutants like [egl-30\(tg26\)](#) grow slowly and have reduced brood sizes (Bastiani et al., 2003; Williams et al., 2007), we complemented these experiments with assays of the developmental stage of freshly-laid eggs (Chase & Koelle, 2004), a measure less dependent on brood size and animal growth rates (Fig. 1I-J). We found that loss of NALCN channels did not suppress the laying of early stage eggs by the [egl-30\(tg26\)](#) gain-of-function mutant. Together, these results show that NALCN channels are not required for the increased egg laying in mutants with too much excitatory $G\alpha_q$ or lacking inhibitory $G\alpha_o$ signaling.

Egg laying in NALCN dKO mutants was similarly responsive to exogenous serotonin and phorbol esters. Worms placed in hypertonic M9 buffer are inhibited for egg laying, and this is restored in M9 buffer containing serotonin. NALCN dKO mutants laid eggs in response to exogenous serotonin at a rate comparable to that of the wild type (Fig. 1K). NALCN dKO mutants also showed a robust egg-laying response to exogenous phorbol-12-myristate-13-acetate (PMA), an analog of diacylglycerol (DAG) – one of two second messengers produced by the $G\alpha_q$ signaling pathway (Fig. 1L). Taken together, these experiments indicate that while elevated NALCN channel activity can promote egg-laying behavior, NALCN channels are not major effector targets of either $G\alpha_q$ or $G\alpha_o$ signaling for egg laying.

Methods

Strains

All *C. elegans* strains, derived from the [N2](#) wild-type strain, were cultured on a lawn of *E. coli* strain [OP50](#) on nematode growth media (NGM) agar plates as previously described (Brenner, 1974). The following strains were provided by the Caenorhabditis Genetics Center: [N2](#) (wild type), [VC12 nca-1\(gk9\) IV](#) (Consortium, 2012), [VC9 nca-2\(gk5\) III](#) (Consortium, 2012), [ZM2960 nca-1\(gk9\) IV](#); [nca-2\(gk5\) III](#) (Humphrey et al., 2007), [DR1089 nca-1\(e625\) IV](#) (Yeh et al., 2008), [MT1434 egl-30\(n686\) I](#) (Trent et al., 1983), and [CG21 egl-30\(tg26\) I](#); [him-5\(e1490\) V](#) (Garcia et al., 2001). [LX1832 lite-1\(ce314\) X lin-15\(n765ts\) X](#) was a generous gift from Dr. Michael Koelle and has been previously described (Collins et al., 2016). [EG4036 egl-30\(tg26\) I](#); [nca-1\(gk9\) IV](#); [nca-2\(gk5\) III](#) was a generous gift from Dr. Erik Jorgensen and has been previously described (Topalidou et al., 2017a).

Strain [MIA27 egl-1\(n986dm\) V](#) in which the HSNs undergo programmed cell death was generated as previously described (Garcia & Collins, 2019). Strains [MIA244 vsIs49 V](#); [lite-1\(ce314\) X lin-15\(n765ts\) X](#) expressing activated [GOA-1\(Q205L\)](#) in the HSNs from the [tph-1](#) promoter and [MIA218 vsIs50 X](#); [lite-1\(ce314\) X](#); [lin-15\(n765ts\) X](#) expressing Pertussis Toxin (PTX) in the HSNs from the [tph-1](#) promoter have been described previously (Ravi et al., 2021). The following strains were newly generated in this study:

[MIA469: vsIs49 V](#); [nca-1\(e625\) IV](#); [lite-1\(ce314\) lin-15\(n765ts\) X](#)

[MIA496: nca-1\(gk9\) IV](#); [lite-1\(ce314\) lin-15\(n765ts\) X](#)

[MIA497: nca-2\(gk5\) III](#); [lite-1\(ce314\) lin-15\(n765ts\) X](#)

[MIA506: nca-1\(gk9\) IV](#); [vsIs50 lite-1\(ce314\) lin-15\(n765ts\) X](#)

[MIA507: nca-2\(gk5\) III](#); [vsIs50 lite-1\(ce314\) lin-15\(n765ts\) X](#)

[MIA508](#): [egl-1\(n986dm\)](#) V; [nca-1\(e625\)](#) IV

[MIA524](#): [nca-1\(gk9\)](#) IV; [nca-2\(gk5\)](#) III; [vsIs50](#) [lite-1\(ce314\)](#) [lin-15\(n765ts\)](#) X

[MIA525](#): [nca-1\(gk9\)](#) IV; [nca-2\(gk5\)](#) III; [lite-1\(ce314\)](#) [lin-15\(n765ts\)](#) X

Behavior Assays

Egg retention in the uterus and early developmental stages of laid eggs were measured as previously described (Chase & Koelle, 2004). Both assays were conducted with adult animals staged approximately 30 h after the late L4 stage.

Pharmacological Assays

Egg laying in response to either exogenous serotonin or PMA was measured as previously described (Banerjee et al., 2017; Dhakal et al., 2022; Kopchock et al., 2021). Briefly, adult animals 30 h after the late L4 stage were placed in individual wells containing 100 μ L of either M9 buffer (a hypertonic solution that normally inhibits egg laying) or either 18.5 mM serotonin (creatinine sulfate monohydrate salt, Sigma-Aldrich # H7752) or 10 μ M PMA (Phorbol-12-myristate-13-acetate, Calbiochem # 524400) in a 96-well microtiter dish. The number of eggs and/or L1 larvae released in each well were counted after 2 h.

Statistical Procedures

Mean numbers of eggs retained in the uterus or of eggs laid in pharmacological assays were compared between genotypes via Kruskal-Wallis test with Dunn's correction for multiple comparisons. Mean proportions of early-stage eggs laid were compared by Fisher's exact test. All P values were calculated in GraphPad Prism version 9.2.0.

Microscopy

Animals were immobilized at 30 hours post-L4 with M9 buffer as previously described (Moresco, 2005) and images of eggs in the uterus taken using a Zeiss Axio Observer Z1 microscope.

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