KU LEUVEN

FACULTY OF SCIENCE

Discovering biological functions of *Caenorhabditis elegans* two-pore domain potassium ion channels in egg-laying behaviour

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Host lab information page

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Abbreviations

C. elegans	*Caenorhabditis elegans*
CeNGEN	*C. elegans N*euronal Gene Expression Network
CGC	*Caenorhabditis* Genetics Centre
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DMP	defecation motor program
E. coli	*Escherichia coli*
FLP	FMRFamide-like peptide
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
HSN	hermaphrodite-specific neurons
IP_3	inositol triphosphate
IDT	Integrated DNA Technologies
kb	kilobase
MARK	MAP/microtubule affinity regulating kinase
NGM	nematode growth medium
NLP	neuropeptide-like peptide
ns	non-significant
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
TAE	tris-acetate EDTA buffer
TALK	TWIK-related alkaline-sensitive potassium channel
TASK	TWIK-related acid-sensitive potassium channel
TEVC	two-electrode voltage clamp
THIK	tandem pore domain halothane-inhibited potassium channel
TPM	transcripts per million
TREK	TWIK-related potassium channel
TRESK	TWIK-related spinal cord potassium channel
TWK/K2P	two-pore domain potassium channel
um	uterine muscles
UV	ultraviolet
VC	ventral cord neurons
vm	vulval muscles

Samenvatting

Kaliumkanalen kunnen de exciteerbaarheid van de cel reguleren en daardoor verschillende fundamentele activiteiten in verschillende organismen zoals voortbeweging en het leggen van eieren beïnvloeden. K2P kanalen vormen een familie van kaliumkanalen die kaliumionen zowel naar binnen als naar buiten kunnen rectificeren. Tot op heden zijn er 47 genen gekend die coderen voor K2P kanalen in *Caenorhabditis elegans*. Op basis van gegevens uit single cell RNA-sequencing is gebleken dat meerdere K2P kanalen tot expressie komen in neuronen die verband houden met het leggen van eieren in *C. elegans*. De functie van deze kanalen bij het leggen van eieren is echter niet duidelijk. In dit project hebben we de rol bestudeerd van drie K2P kanalen bij het leggen van eieren in *C. elegans*, gecodeerd door de genen *twk-9*, *twk-26* en *twk-46*. Om de biologische functies van K2P kanalen in het eierlegcircuit van *C. elegans* te onthullen, hebben we eerst terugkruisingen uitgevoerd met mutanten die defecten vertoonden in het *twk-9* gen en we hebben een volledige knock-out mutant van het *twk-26* gen gegenereerd door middel van CRISPR genoombewerking. Daarna werden er eierleg- en eierretentie experimenten uitgevoerd op de mutanten *twk-9(ok1611)*, *twk-26(ibt22*) en *twk-46(ibt17)*. We hebben ontdekt dat er wat betreft eierretentie geen effect van de mutaties was op de eierproductie *in vivo* in vergelijking met het wild type. Echter, het eierleg experiment in afwezigheid van voedsel toonde aan dat de mutanten *twk-26(ibt22)* en *twk-46(ibt17*) meer eieren legden dan het wild type. Deze resultaten suggereren dat *twk-26* en *twk-46* het leggen van eieren remmen wanneer er geen voedsel aanwezig is. Om verder te verduidelijken hoe *twk-26* en *twk-46* het eierleggedrag bij *C. elegans* remmen, kunnen er in de toekomst meer experimenten worden uitgevoerd, zoals rescue experimenten, het genereren van gain-of-function mutanten en het gebruik van dubbele of driedubbele mutanten.

Summary

Potassium channels can regulate the cell excitability, therefore modulating various fundamental activities in different organisms such as locomotion and egg-laying. Two-pore domain potassium ion (K2P) channels are one potassium channel family that can rectify potassium ions inwardly and outwardly. To date, 47 genes are known to encode K2P channels in *Caenorhabditis elegans*. Based on single cell RNA sequencing data, it has been found that multiple K2P channels are expressed in egg-laying related neurons of *C. elegans*. However, the function of these channels in egg-laying behaviour is not clear. In this project, we studied the putative role of three K2P channels in *C. elegans* egg-laying, encoded by the *twk-9*, t*wk-26* and *twk-46* genes. To reveal biological functions of K2P channels in the *C. elegans* egg-laying circuit, we firstly outcrossed mutants defective in the *twk-9* gene and generated a full knock-out mutant of the *twk-26* gene by CRISPR genome editing. Afterwards, egg-laying and egg retention assays were conducted on *twk-9(ok1611)*, *twk-26(ibt22)* and *twk-46(ibt17)* mutants. We found that in terms of egg retention, there was no effect of the mutations on egg production *in vivo* compared to the wild type. However, the egg-laying assays in the absence of food showed that *twk-26(ibt22)* and *twk-46(ibt17)* mutants laid more eggs than the wild type. These results suggest that *twk-26* and *twk-46* inhibit egg-laying when food is absent. To further elucidate how *twk-26* and *twk-46* inhibit egg-laying behaviour in *C. elegans*, more experiments, such as rescue experiments, generating gain-of-function mutants and using double or triple mutants, can be implemented in the future.

Introduction

1. Potassium channels

Potassium channels, which are important for maintaining the resting membrane potential, are characterized by one or more pores that allow the passage of K^+ ions. Inside a cell the K^+ concentration is much higher than outside and the opposite is true for the Na^+ concentration. This difference in ion concentration results in transport of these ions across the membrane, regulating cell excitability. The permeability of K^+ is higher than that of other ions because of the presence of potassium leak channels in the membrane, which contributes to generating resting potentials (Kew & Davies, 2010). Hodgkin and Katz discovered in 1949 that the resting membrane potential becomes less negative with increasing K^+ concentrations outside the cell. More specifically, the resting membrane potential changes with the logarithm of the K^+ concentration, which is calculated using the Goldman-Hodgkin-Katz equation (Purves, 2001).

There are several types of potassium channels that differ in structure and function. Ion selectivity is conserved in all potassium channels and relies on the presence of the signature sequence TXXTXGYG in the pore selectivity filter (Heginbotham *et al.*, 1994). Potassium channels contain transmembrane helices, the number of which varies among the different types of potassium channels (Kuang *et al.*, 2015). Potassium channels can be classified into three main classes based on the number of pore regions and transmembrane domains: inwardly rectifying, voltage-gated, and two-pore domain potassium channels (Salkoff *et al.*, 2005). Two-pore domain potassium channels are being studied in drug discovery research, but are less intensively studied than other potassium channels. Because of their insensitivity to classical K^+ channel blockers and their independence from voltage and time, they have been neglected, although they are present in many tissues and cells (Feliciangeli *et al.*, 2015). These channels are sometimes also called K2P or TWK (two-P domain K^+) channels.

1.1 Two-pore domain potassium channels

1.1.1 The structure of two-pore domain potassium channels

All two-pore domain potassium channels share a common basic structure. They consist of two pore domains (P1-P2) and four transmembrane domains (TM1-TM4) per subunit. Because they have two pore domains, they can function as dimers rather than tetramers like the other potassium channels. TM1 and TM2 are connected by an extracellular loop that contains a pore domain (P1) and two α-helices (C1 and C2). TM3 and TM4 are linked via another extracellular loop containing P2 (Royal, 2018). The N-terminus of K2P channels is very short compared to the C-terminus, and both termini project to the intracellular region (Rajan *et al.*, 2002). K2P channels generate mostly instantaneous and non-inactivating currents over the entire range

of membrane potentials; they are leaky channels, as predicted by the Goldman-Hodgkin-Katz equation (Feliciangeli *et al.*, 2015).

1.1.2 The classification of two-pore domain potassium channels in mammals

Mammals have a family of 15 two-pore domain potassium channels that can be divided into six subfamilies based on their functions and structures (Lotshaw, 2007) (Figure 1).

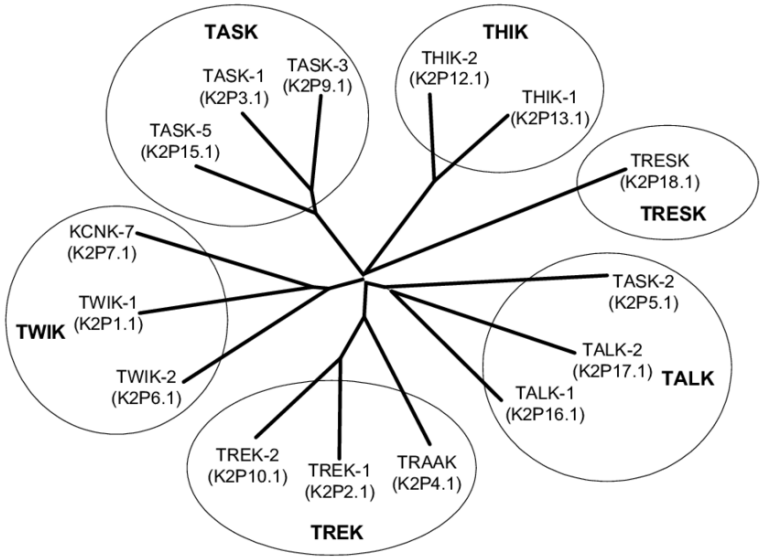


Figure 1: Classification of mammalian K2P channels. This represents a schematic phylogenetic tree of mammalian K2P channels, which contains TWIK, TREK, TASK, TALK, THIK and TRESK channels. (Veale, 2000)

One class of mammalian K2P channels is the TWIK family, which comprises TWIK-1, the first mammalian K2P channel discovered. TWIK-1 produces very low current levels, for which there are two possible hypotheses. First, SUMOylation, in which TWIK-1 channels are conjugated to a small ubiquitin-like polypeptide, is thought to silence TWIK-1 channels on the cell surface (Christensen *et al.*, 2016). This conjugation can be prevented by a mutation, namely the exchange of lysine for glutamic acid, which results in TWIK-1 producing stronger currents (Feliciangeli *et al.*, 2010). For the second hypothesis a recent study suggested that TWIK-1 is endocytosed from the plasma membrane and accumulates in recycling endosomes (Christensen *et al.*, 2016). This endocytosis can also be prevented by mutation of a di-isoleucine motif in the C-terminus, which maintains TWIK-1 in the plasma membrane (Feliciangeli *et al.*, 2010). Protein kinase C and activation of G_i-coupled serotoninergic and adrenergic receptors increase TWIK-1 currents, whereas acidic pH, Ba^{2+}, and some drugs such as quinine and quinidine decrease TWIK-1 currents. TWIK-1 is expressed in the heart, especially in the atria of humans and zebrafish (Christensen *et al.*, 2016).

Members of the TREK (TWIK-related K^+ channel) family are TREK-1, TREK-2, and TRAAK (Honoré, 2007). They are mechanosensitive, and their activity can be regulated by membrane stretch, membrane depolarization, heat, and unsaturated fatty acids (Royal, 2018). These three channels can cluster to form a heterodimeric channel. In TREK-1, strong outward rectification was observed, which can be characterized by an external Mg^{2+} blockade at negative membrane potentials and an intrinsic voltage-dependent mechanism. The expression of TREK channels in the nervous system is high; in humans, TREK-1 is strongly expressed in the amygdala, basal ganglia, cortex, and hippocampus (Honoré, 2007).

Members of the TASK (TWIK-related acid-sensitive K^+ channel) family are TASK-1, TASK-3, and TASK-5. These channels can be inhibited by extracellular acidification (Duprat *et al.*, 1997). G protein-coupled receptors can also inhibit these channels in two ways. One way is: after the activation of G_q, phospholipase C can be activated resulting in the depletion of PIP_2 in the plasma membrane, which reduces the activity of the channel. The other way involves a direct interaction between the channel and $G_{\alpha q,}$ which inhibits channel activity (Chemin *et al.*, 2003). In addition, halothane, a type of anaesthetics, has been found to be able to activate TASK-1 and TASK-3 (Karschin *et al.*, 2001). Surprisingly, alkalization can also activate TASK-1 (Shvetsova *et al.*, 2022). When examining the rat brain, they found that TASK-3 is mainly expressed in cholinergic, serotonergic, and noradrenergic neurons. In contrast, TASK-5 is expressed in the olfactory bulb and TASK-1 in the cardiac atria and ventricles (Karschin *et al.*, 2001).

The TALK (TWIK-related alkaline-sensitive K^+ channel) and THIK (tandem pore domain halothane-inhibited) channels have diverse physiological properties. TALK channels are activated by alkaline pH and are sensitive to Ba^{2+}, quinine, quinidine, chloroform, halothane, and isoflurane. Members of this family are TALK-1 and TALK-2, with TALK-1 expressed exclusively in the pancreas and TALK-2 expressed mainly in the pancreas, but also to a lesser extent in the liver, placenta, heart, and lungs of humans (Girard *et al.*, 2001). THIK channels can be inhibited by the volatile anaesthetic halothane and activated by arachidonic acid. There are two known members of this family, THIK-1 and THIK-2, THIK-1 was found to be expressed in the rat brain, but only at low levels. THIK-2 has also been found in the rat brain, specifically in the granule cell layer of the cerebellum, olfactory bulb, cortex, and hypothalamus (Rajan *et al.*, 2001).

The last family of K2P channels discovered is the TRESK (TWIK-related spinal cord K^+ channel) family. TRESK channels have been found in humans in the spinal cord, as well as in ganglia, cerebrum, cerebellum, brainstem, spleen, thymus, etc. It has low sequence similarity to other mammalian K2P channels, with a longer intracellular loop of more than 120 amino acids between TM2 and TM3 and a shorter C-terminal segment downstream of TM4. It is

suspected that there is more than one TRESK gene because of the large differences between human and mouse orthologs with respect to the intracellular N-terminus, the sequence between TM1 and the signature sequence, and the proximal segment of the intracellular loop (Enyedi & Czirják, 2015). Recently, the mechanism of activating and inhibiting TRESK channels has been revealed by Enyedi & Czirják, (2015) (Figure 2). They found that TRESK channels can be activated by the stimulation of the G_q-coupled receptor. In detail, TRESK channels are phosphorylated at serine residues 252 and 262, 264 and 267 by PKA and MARK kinase, respectively, and are thereby inhibited. Phosphorylation of serine 252 promotes the binding of the 14-3-3 protein to the channel, resulting in the inhibition of calcineurin, a serine/threonine phosphatase, bound to the PQIIIS site of the TRESK channel. An increase in intracellular Ca^{2+} concentration activates calcineurin, allowing it to bind to the LQLP site. Activated calcineurin dephosphorylates serine, detaches the 14-3-3 protein, and increases TRESK channel activity. The rephosphorylation of serine residues by PKA and MARK kinase returns the channel to its initial state (Enyedi & Czirják, 2015). Similar to TREK channels, TRESK channels can also be regulated by membrane stretch, which may be due to a conformational change in the membrane structure (Callejo *et al.*, 2013).

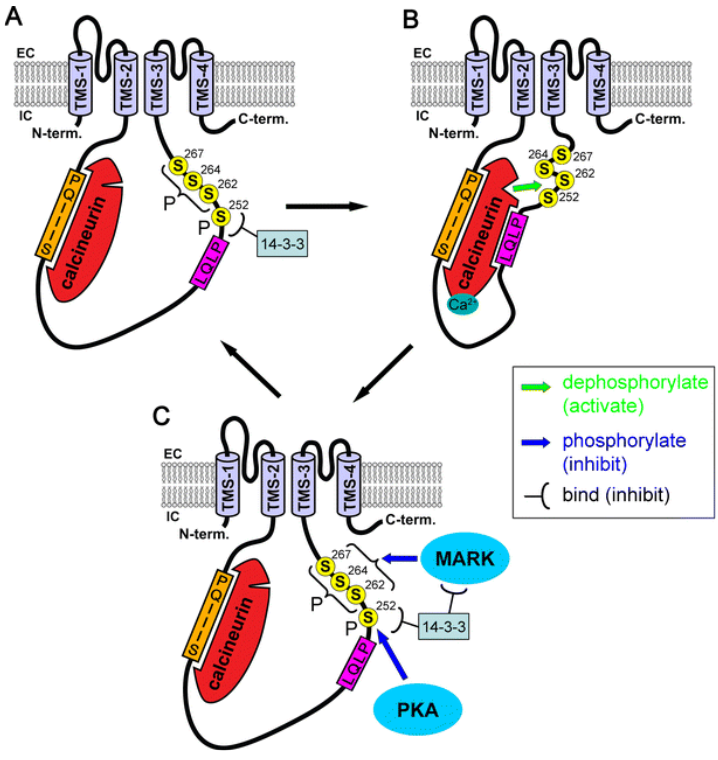


Figure 2: The activation and inhibition of the human TRESK channel regulated by calcineurin and Ca^{2+}. PKA and MARK kinase phosphorylate the serine residues of the TRESK channel, which inactivates the channel. The 14-3-3 protein binds to the channel, which inhibits calcineurin that is bound to the PQIIIS site of the channel. Calcineurin can be activated by an increase in Ca^{2+} concentration, this allows calcineurin to bind to another site of the channel, the LQLP site. Activated calcineurin dephosphorylates the serine residues, the 14-3-3 protein detaches and the channel activity increases (Enyedi & Czirják, 2015)

Members of the TWIK, TREK, TASK, and TALK families respond differently to pH via various pH-sensing mechanisms. The TASK and TALK channels are inhibited by acidic extracellular pH. In TASK-1 and TASK-3, a histidine residue in the P1 domain senses pH changes, whereas in TALK channels histidine in the extracellular loop between P2 and TM4 interacts with the selectivity filter (Rajan *et al*., 2000; Royal, 2018). The TALK channel can be constitutively opened over a wide pH range when this basic residue is replaced by an uncharged amino acid, such as alanine (Royal, 2018). TREK-1 and TREK-2 are activated by acidic internal pH, and the sensor that leads to this activation is located at the C-terminus. However, external acidity can inhibit TREK-1 while activating TREK-2, this strange scenario is caused by the interaction of a histidine residue in the loop between TM1 and P1 with residues in the loop between P2 and TM4. These residues are negatively charged in TREK-1 and positively charged in TREK-2, implying electrostatic attraction/repulsion, the protonated side chain of the histidine sensor causes the pore to open/close. Interestingly, the ion selectivity of TWIK-1 changes as a function of pH. At neutral pH, it is strictly selective for K^+ ions; however, at acidic pH, it becomes permeable to Na^+ ions (Feliciangeli *et al*., 2015).

1.1.3 *Caenorhabditis elegans* as a model organism

Although much has been learned about K2P channel physiology, many questions remain about their *in vivo* roles in the nervous system. One way to address such questions is by using model organisms with simpler nervous systems and powerful genetics. In particular, the nematode *C. elegans* has been proven useful to answer many of these research questions. Sydney Brenner chose this organism because it is small, easy to grow in the lab, and has a rapid adult-to-adult life cycle of approximately 3.5 days. *C. elegans* has two natural sexes: hermaphrodites and males. Hermaphrodites are self-fertilizing females that can produce their own sperm. Males are formed by meiotic nondisjunction only 0.1% of the time (Brenner, 1973).

1.1.4 The functions of two-pore domain potassium channels in *Caenorhabditis elegans*

Compared to mammals, *C. elegans* is known to have many more K2P channels, with at least 47 genes known to encode K2P channels to date (Soussia *et al*., 2019). This seems to be a surprising number of potassium channel-encoding genes, especially considering that *C. elegans* has a compact nervous system of only 302 neurons (White *et al*., 1986). K2P channels have been found to be present not only in neurons but also in other cell types, such as muscle cells (Salkoff *et al*., 2005).

TWK-18 is one of the K2P channels in *C. elegans* that has been extensively studied. Using transcriptional reporters, TWK-18 channels were found to be expressed only in body wall muscles (Kunkel *et al*., 2000). Little is known about activators of K2P channels in *C. elegans*, but Kunkel *et al*. (2000) recently found that TWK-18 channels can be activated by temperature. *twk-18(cn110)* is a mutant allele with a substitution in the second and fourth TM domains,

which shows uncoordinated movements when the temperature increases, and becomes paralyzed when the temperature increases further. The potassium currents of TWK-18 increase when the temperature increases, resulting in the inhibition of the excitation of body wall muscles required for locomotion. Another mutant, *twk-18(e1913),* exhibits uncoordinated movement at all temperatures. It is not yet known why *twk-18(cn110)* shows a temperature-dependent behavioural phenotype and *twk-18(e1913)* does not (Kunkel *et al*., 2000).

TWK-40 is another K2P channel in *C. elegans* that has been studied in detail for its roles in defecation and locomotion. First, TWK-40 was found to regulate the rhythmic defecation motor program (DMP) in *C. elegans* (Yue *et al*., 2022). DMP begins with contraction of the posterior body wall muscles, followed by contraction of the anterior body wall muscles and finally contraction of the enteric muscles, resulting in expulsion of intestinal contents (Riddle *et al.,* 1997a). One of the important neurons that innervate DMP muscles are DVB neurons. Secretion of the neuropeptide NLP-40 activates the GPCR AEX-2, which is located in DVB neurons. This activates an ion channel in the enteric muscles, which regulates their contraction (Figure 3). TWK-40 regulates the resting membrane potential of DVB neurons by providing an outward K^+ current (Yue *et al*., 2022). Yue *et al*., 2022 found that *twk-40(gf)* mutants show a decrease in expulsion frequency and *twk-40(lf)* mutants show an increase in expulsion frequency.

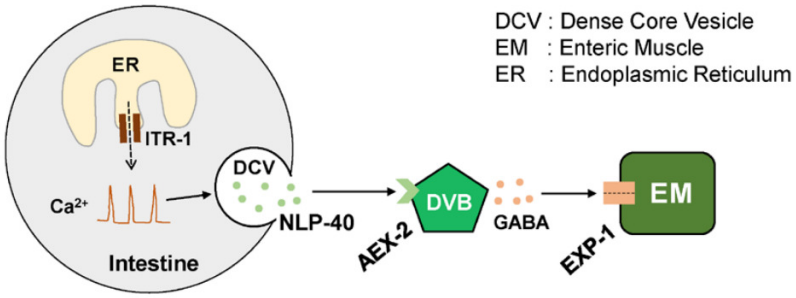


Figure 3: Mechanism of expulsion in *C. elegans*. The IP_3 receptor ITR-1 drives Ca^{2+} oscillations in the intestine, which leads to secretion of the neuropeptide NLP-40. This activates the GPCR AEX-2 in the DVB neurons. DVB neurons release GABA, which leads to the activation of an ion channel EXP-1 in the enteric muscles. Thus, contraction of the DMP muscles is controlled. (Yue *et al*., 2022)

Meng *et al*. (2022) investigated an important role of *twk-40* in *C. elegans* locomotion. They used a reporter transgene, a red fluorescent protein expressed under control of the *twk-40* promoter, to identify cells expressing *twk-40*. The *twk-40* gene is highly expressed in AVA, AVB, and DVA neurons, and its expression in AVA is significantly higher. AVA interneurons regulate backward movement and AVB interneurons control forward movement. TWK-40 is important in regulating the resting membrane potential and membrane excitability of AVA interneurons, similar to DVB motor neurons. In this study, they also used *twk-40(lf)* and *twk-*

40(gf) mutants to study the effects on locomotion. In the *twk-40(lf)* mutants, overall motor activity increased, backward movement was more frequent, reversal movements were prolonged, curvature increased, and exaggerated body bends occurred. In the *twk-40(gf)* mutants, there was a decrease in total motor activity, a drastic decrease in forward movement, and no reversal. To summarize, TWK-40 has a negative effect on expulsion because of defects in the DMP. In addition, TWK-40 also affects locomotion, leading to a decrease in forward movements and no reversal movements (Meng *et al.*, 2022).

Apart from locomotion and defecation, K2P channels may also function in the egg-laying circuit. Based on single-neuron RNA sequencing data (Taylor *et al.*, 2021), it has been found that multiple K2Ps have high expression levels in HSN and VC neurons governing egg-laying behaviour in *C. elegans* (Table 1). However, the question regarding whether and how these K2P channels function is not yet fully understood.

Table 1: Overview of the expression levels of different K2P genes expressed in VC4-5 and HSN neurons. The expression levels of the genes in VC4-5 and HSN are expressed in transcripts per million (TPM).

gene	VC4_5 (TPM)	HSN (TPM)
twk-2	0	9.83
twk-46	165.05	76.86
twk-26	1137.13	93.68
twk-17	46.84	0
twk-39	4.36	0
twk-23	171.98	0
twk-9	37.97	0
egl-23	704.33	0
unc-58	153.70	38.17

2. Egg-laying behaviour in *Caenorhabditis elegans*

2.1 Anatomy of the reproductive system of *C. elegans*

2.1.1 Components of the reproductive system

The reproductive system of *C. elegans* consists of the uterus, the vulva, their associated muscles, and the neurons that innervate these muscles. The uterus is a central chamber that links the two arms of the gonad, and this central chamber is linked to the vulva. Eggs travel from the spermatheca to the uterus after fertilisation, in the uterus the eggs further develop until they are eventually expelled via the vulva (Riddle *et al.*, 1997b). Egg-laying is promoted by sixteen sex muscles: eight vulval muscles, four vm1s and four vm2s, attached to the lips of the vulva, and eight uterine muscles, four um1s and four um2s, encircling the uterus. Only vm2 muscles receive direct synaptic input from egg-laying neurons, vm1 muscles are connected to

these vm2 muscles via gap junctions. Eight neurons are important in the egg-laying process: six VC neurons and two HSN neurons (Lint & Hall , 2009).

2.1.2 Egg-laying circuit

Two types of neurons are important in the egg-laying process of *C. elegans,* HSN and VC neurons. HSN neurons direct their synaptic input to the vm2 vulval muscles, leading to their contraction and eventually egg expulsion (Schafer, 2006). HSN neurons release serotonin and NLP-3, which work together to induce the onset of the active egg-laying phase (Brewer *et al*., 2019). VC4 and VC5 neurons release acetylcholine, which can function in three ways; VC4 and VC5 can be activated to release acetylcholine, which excites vulval muscles. VC4 and VC5 can also release acetylcholine thereby inhibiting the release of serotonin by HSN, which inhibits egg-laying. VC neurons can also activate body wall muscles, leading to a decrease in velocity during egg-laying (Kopchock *et al*., 2021). All sex muscles are needed for eggs to be expelled, since only vm2 muscles receive direct synaptic input, vm1 muscles and uterine muscles form gap junctions with vm2 muscles so they can all together contract for egg-laying to take place (Schafer, 2006).

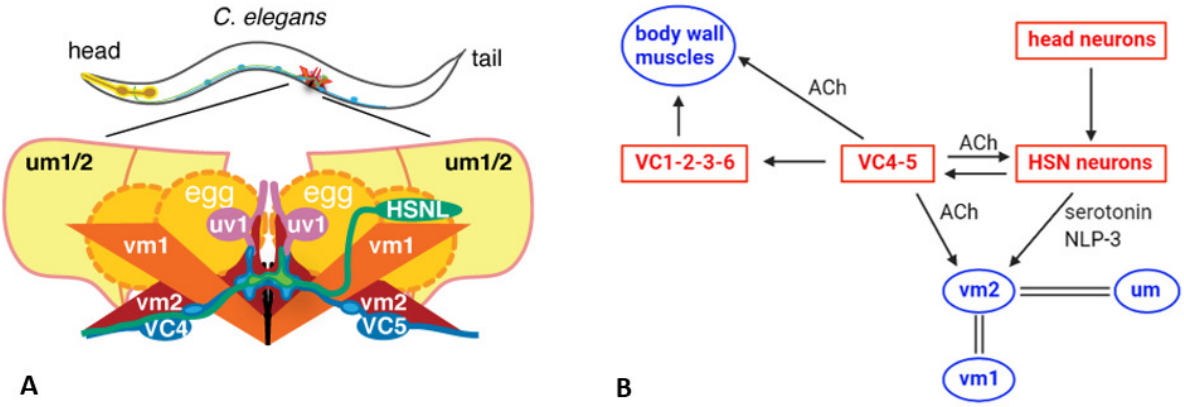


Figure 4: Schematic overview of the egg-laying circuit in *C. elegans*. (A) Positioning of the sex muscles and neurons important for egg-laying. The uterine muscles, um1 and um2, are located around the uterus and vulval muscles, vm1 and vm2, are located anterior and posterior to the vulva. HSN neurons can be found posterior to the vulva and the vulva-proximal VC neurons, VC4 and VC5, are positioned anterior and posterior to the vulva (Collins *et al*., 2016). **(B) Scheme of interactions between egg-laying neurons and muscles.** HSN neurons receive synaptic input from head neurons and direct synaptic input to VC neurons and vm2 vulval muscles. HSN neurons release the neuromodulators, serotonin and NLP-3. VC4 and VC5 release acetylcholine and thereby activate body wall muscles and vm2 muscles or inhibit serotonin release by HSN neurons. Vm2 muscles are linked to vm1 muscles and uterine muscles via gap junctions.

2.1.3 HSN neurons

HSN neurons are hermaphrodite-specific neurons that play an important role in egg-laying in *C. elegans,* and are situated immediately posterior to the vulva. During the development of *C. elegans* HSN neurons migrate from the tail of the embryo to the midbody (Sulston *et al*., 1983).

In the second and third larval stages, HSN neurons begin axonal outgrowth, and during the fourth larval stage, they form axonal branches near the developing vulva (Figure 5) (Gian *et al*., 1993). They eventually form neuromuscular junctions with the vm2 vulval muscles, this way they can innervate these muscles. HSN neurons also direct synaptic output to VC motor neurons, which are another set of neurons important in egg-laying processes. HSN neurons receive synaptic inputs from head neurons. HSN neurons are the only egg-laying neurons that are necessary for normal egg-laying; when they are defective or absent the worms are egg-laying defective and the uterus is bloated with eggs (Hall *et al*., 2013a).

Figure 5: Localisation of HSN neurons in *C. elegans*. HSN neurons are positioned just posterior to the vulva. The axons of HSN neurons join the ventral nerve cord and extend into the ventral nerve ring. When passing the vulva HSN neurons provide synaptic input to the vulval muscles and the VC neurons (Hall *et al*., 2013a).

HSN neurons are only present in hermaphrodites, in males they undergo programmed cell death. The *egl-1* gene is found to play an important role in this apoptotic process. TRA-1 is a transcription factor, found downstream of the *egl-1* gene, that determines the fate of HSN neurons. TRA-1 is active in hermaphrodites and binds to its binding site and thereby inhibits the activity of a HSN-specific transcriptional activator of *egl-1*, repressing *egl-1* transcription and promoting HSN survival. In males TRA-1 is inactive and unable to bind to its binding site, the HSN activator activates the transcription of *egl-1*, resulting in HSN death (Nehme & Conradt, 2008).

2.1.4 VC neurons

In addition to HSN neurons, VC neurons located in the ventral nerve cord also play an important role in egg-laying. VC neurons arise during the first larval stage, begin axonal outgrowth during the third larval stage, and form axonal branches during the fourth larval stage (Hall *et al*., 2013b). There are two types of VC neurons: vulva-proximal VC4 and VC5 neurons, and vulva-distal VC1, VC2, VC3, and VC6 neurons (Figure 6). VC4 and VC5 are located immediately anterior and posterior to the vulval opening, and they extend short neurites that direct their synaptic output to the vulval muscles and other VC neurons. The four other VC neurons are located further away from the vulval opening, and they extend longer neurites into the ventral nerve cord that direct their synaptic output to body muscles and to other motor neurons (Schafer, 2006).

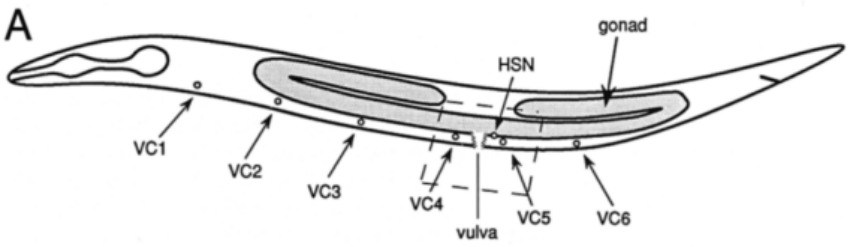


Figure 6: Schematic overview of the positioning of the vulva, the HSN neurons and the VC neurons in *C. elegans*. VC4 and VC5 are vulva-proximal neurons located anterior and posterior, respectively, to the vulva. VC1, VC2, VC3 and VC6 are vulva-distal neurons located further from the vulva. HSN neurons are positioned posterior to the vulva. (Li *et al*., 1990)

2.2 Temporal pattern of egg-laying in *C. elegans*

In *C. elegans* egg-laying follows a specific temporal pattern. This pattern consists of short bursts lasting 1-2 minutes (t1), separated by longer resting periods of 20 minutes (t2). The worms are either in an active phase, in which eggs are laid regularly, or in an inactive phase, in which no eggs are laid (Figure 7) (Schafer, 2005).

Figure 7: Temporal pattern of egg laying in *C. elegans*. Each red line corresponds to an egg laying event. t1 is the time between two egg-laying events within a cluster, t2 is the time between the last egg-laying event of one cluster and the first egg-laying event of the next cluster, and t2 corresponds to the time that an inactive phase lasts.

2.3 Environmental factors affecting *C. elegans* egg-laying behaviours

Many environmental factors can affect the egg-laying processes of *C. elegans*. For instance, vibration and hypertonic salt solutions inhibit the egg-laying rate (Schafer, 2005). Inhibition of egg-laying due to vibration requires the mechanosensory neurons, ALM and PLM (Sawin, 1996). When *C. elegans* is placed in a hypertonic salt solution, such as M9, it loses water via osmosis and egg-laying is inhibited (Wheeler & Thomas, 2006).

Temperature is also an important environmental factor that has an effect on egg-laying behaviour. *C. elegans* reproduction is very sensitive to temperature changes, as the brood size decreases with increasing temperatures. The ideal temperature is 20°C, in this condition an individual hermaphrodite lays approximately 300 eggs. When the temperature rises (28-32°C) only a few eggs are laid after 24 hours and no eggs are laid after more than 24 hours (Aprison & Ruvinsky, 2014).

Besides several factors that inhibit egg-laying, there are also factors that increase the egg-laying rate, such as the presence of abundant food. When responding to food, ciliated chemosensory neurons AWC and ASK are necessary for the modulation of normal egg-laying. AWC neurons are needed for chemotaxis towards lysine and ASK neurons are needed for the response towards volatile attractants including isoamyl alcohol. The combination of isoamyl alcohol and the presence of abundant food leads to an increase of the egg-laying rate (Sawin, 1996). FLP-1-encoded peptides, released by AIA and AIY interneurons, signal the presence or absence of food to the egg-laying circuit. In the absence of food, low levels of FLP-1 are released leading to long inactive egg-laying phases and slow egg-laying. In the presence of abundant food, there is an increase in FLP-1 release and egg-laying is stimulated (Waggoner *et al*., 1998).

2.4 The role of neurotransmitters in egg-laying behaviour

In addition to environmental factors, egg-laying behaviour in *C. elegans* is regulated by neurotransmitters released from HSN and VC neurons (Waggoner *et al*., 1998). In most animals, neurotransmitters are released into the synaptic cleft, where they bind to receptors. This typically involves the opening of voltage-gated sodium channels, enabling the influx of Na^+ ions into the post-synaptic cell and generating an action potential (Purves *et al*., 2004). However, in the neurons of *C. elegans*, voltage-gated sodium channels are absent. As a result, it is believed that depolarization in *C. elegans* neurons primarily relies on the activation of voltage-gated calcium channels (Williams *et al*., 2018).

2.4.1 Serotonin

One important neurotransmitter for egg-laying in *C. elegans* is serotonin. Its biosynthesis requires two genes: *tph-1*, which encodes tryptophan hydroxylase, and *cat-4*, a gene required for the activity of amino acid decarboxylase (Hardaker *et al*., 2001). HSN neurons release serotonin and thereby promote egg-laying by inducing the onset of the active egg-laying phase. Serotonin is released by HSN neurons into the synaptic cleft and is then taken up by serotonin receptors on the vulval muscles. To date, four serotonin receptors have been discovered in *C. elegans*: three G protein-coupled receptors, SER-1, SER-4 and SER-7, and one chloride channel, MOD-1 (Carre-Pierrat *et al*., 2006). When HSNs are ablated, the frequency of egg-laying events decreases; however, the egg-laying rate within the clusters remains unaltered. Exogenous serotonin can cause HSN-ablated animals to be continuously in the active egg-laying phase. VC4 and VC5 are weakly serotonergic; therefore, they can also stimulate egg-laying by releasing serotonin (Waggoner *et al*., 1998).

2.4.2 Acetylcholine

Another important neurotransmitter for egg-laying in *C. elegans* is acetylcholine. It is synthesized by choline acetyltransferase and activates postsynaptic cells by binding acetylcholine receptors, this way it stimulates egg-laying events within the active phase

(Waggoner *et al.*, 1998). Acetylcholine has been shown to have both excitatory and inhibitory effects on egg-laying. It is released by VC neurons and can inhibit egg-laying by inhibiting the release of serotonin by HSN neurons. Ca^{2+} transients are observed in VC neurons during the active phase of egg-laying, but only one-third approximately coincides with vulval muscle contractions that result in egg release. The remaining Ca^{2+} transients coincide with weak vulval muscle contractions and partial opening of the vulva, these are called vulval muscle "twitches". These vulval muscle "twitches" can be a part of a negative feedback loop by activating VC neurons, which then release acetylcholine, but this acts on G protein-coupled receptors, such as GAR-2, on HSN neurons. Thus, egg-laying is inhibited by acetylcholine (Figure 8). The vulval muscle "twitches" can also be a part of a positive feedback loop. Activation of VC neurons causes them to release the neurotransmitter acetylcholine, which excites the vulval muscles, resulting in ubiquitous and coordinated contractions (Kopchock *et al.*, 2021).

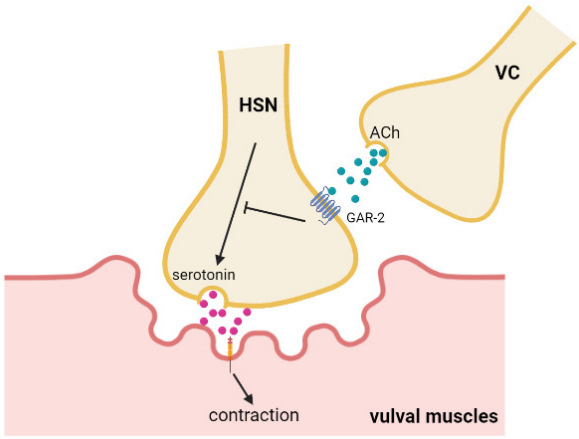


Figure 8: The release of acetylcholine by VC neurons inhibits the release of serotonin by HSN neurons. Acetylcholine binds to G-protein coupled receptors, such as GAR-2, on HSN neurons, inhibiting egg-laying by inhibiting the release of serotonin by HSN neurons. This figure was generated using Biorender.com.

2.5 The role of neuropeptides in egg-laying behaviour

Neuropeptides can function as neurotransmitters, but are larger polypeptide molecules that are encoded by precursor genes rather than enzymatically synthesized. Several neuropeptides are known to play a role in egg-laying behaviour in *C. elegans*, a few of them are shortly explained here.

HSN neurons release both the neuropeptide NLP-3 and serotonin to regulate egg-laying behaviours. Serotonin or NLP-3 alone can partially stimulate egg-laying. However, when both signals are lacking, there is no significant stimulation of egg-laying by HSN neurons. Calcium recordings in vm1 and vm2 indicate that serotonin or NLP-3 released by HSN neurons promotes Ca^{2+} activity specifically in vm2, while vm1's activity remains unaffected. A successful egg-laying event requires contractions from both vm1 and vm2, therefore it is

demonstrated that NLP-3 and serotonin can directly activate vm2 and indirectly vm1 (Brewer *et al*., 2019).

FLP-1 is a FMRF amide-related neuropeptide that plays a role in egg-laying behaviour in *C. elegans.* It promotes the transition from the inactive to the active egg-laying phase and slows the egg-laying rate in the absence of food (Nelson *et al*., 1998). The function of FLP-1 in egg-laying behaviour in the presence or absence of food has previously been described. FLP-1 functions together with the neurotransmitter serotonin to stimulate the initiation of the active phase. FLP-1 is not expressed in HSN neurons, but is expressed in cells in the head that lie in close proximity to the HSN dendrite in the nerve ring; thus, HSN neurons may mediate the effects of FLP-1 (Nelson *et al*., 1998).

2.6 The role of ion channels in egg-laying behaviour

In addition to genes affecting neurotransmitters and neuropeptides, various ion channel genes have been shown to affect egg-laying. Ion channels are used in excitable cells, such as neurons and muscles, to convert chemical or mechanical signals into electrical signals (Chalfie, 2009). Egg-laying neurons are required to pass along electrical synapses and to innervate the muscles, resulting in egg-laying. Most of the ion channels known to be important in the egg-laying behaviour of *C. elegans* are potassium channels. Potassium channels that are important in egg-laying behaviour include EGL-2, EGL-23, EGL-36, SUP-9 and UNC-103 (Schafer, 2006). EGL-36 for example is a voltage-gated potassium channel that controls the excitability of egg-laying muscles (Elkes *et al*., 1997; Salkoff *et al*., 2005). UNC-103 is a potassium channel expressed in vulval muscles and HSN neurons and is involved in muscle contraction (D. Reiner *et al*., 2006). Besides potassium channels, there are other ion channels that play a role in egg-laying behaviour. EGL-19 is a calcium channel expressed in egg-laying muscles, *egl-19(gf*) mutants show a hyperactive phenotype, whereas *egl-19(lf)* mutants show a decrease in Ca^{2+} channel activity and accumulation of unlaid eggs (Collins & Koelle, 2013; Jospin *et al*., 2002). Chloride channels can also play a role in egg-laying, such as MOD-1, which is a serotonin-gated chloride channel expressed in HSN neurons, VC neurons or vulval muscles (Schafer, 2006).

Research outline

The main purpose of this research is to elucidate the role of two-pore domain potassium channels in egg-laying behaviour of *C. elegans*. In this project three different K2P channels, TWK-9, TWK-26 and TWK-46, have been identified as potential candidates based on their expression in egg-laying neurons, HSN and VC4-5, as indicated by CeNGEN data. When a K2P channel is inactivated, it results in reduced outward K^+ current, leading to an increased intracellular K^+ concentration and a more positive resting membrane potential. The specific effects of channel inactivation on egg-laying behaviour depend on the channel's characteristics, particularly the extent of K^+ current conducted through it. Firstly, the corresponding mutant strains are outcrossed or generated using the CRISPR-Cas9 technique. Given that the proper functioning of K2P channels may influence various aspects of the reproductive process, such as egg production rates, the number of eggs retained within the uterus, and the timing and frequency of egg-laying, different egg-laying related assays are conducted to investigate the functions of these three K2P channels in egg-laying behaviour. These assays encompass the measurement of egg-laying events in the presence and absence of food. Food availabilty is an external factor known to influence egg-laying. Studying the effects of K2P channels under both conditions provides insights into how these channels respond to food stimuli. Additionally, the research also involves quantifying the number of unlaid eggs in young adults. This measurement serves as an additional parameter to assess the impact of K2P channels on *C. elegans* reproduction.

Materials and methods

1. *C. elegans* maintenance

For regular *C. elegans* maintenance, 5 adult worms from one genotype were transferred to one 55mm plate seeded with 100µL OP50 every Monday and Thursday. In this way, worms always have enough food and can therefore not starve. All maintenance plates were incubated at 20°C.

1.1 Male generation

In a typical *C. elegans* population only 0.1-0.2% are males, therefore males needed to be generated using a heat shock treatment. 8 L4 hermaphrodites were transferred on seeded 90mm NGM plates, the plates were placed in a 30°C incubator for 4-6 hours. After that the plates were kept overnight at 15°C and the next morning the plates were placed at 20°C. After 3 to 4 days, the F1 progenies should contain 2-5% males. To keep males in culture 8-20 males were placed together with 2-4 L4 hermaphrodites on a seeded 55mm NGM plate.

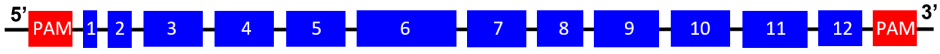
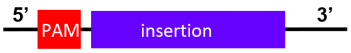
1.2 *C. elegans* strains

All the *C. elegans* strains used in this study are listed in Table 2 and the genetic mutations are represented in Table 3.

Table 2: list of different *C. elegans* strains

genotype	description	outcrossed
wild type	/	/
twk-9 (ok1611)	1.8 kb deletion	2x
twk-26(ibt22)	full knock-out	0x
twk-46(ibt17)	full knock-out	1x

Table 3: representation of the genetic mutations.

genotype	sequence
twk-9	
twk-9(ok1611)	
twk-26	
twk-26(ibt22)	
twk-46	
twk-46(ibt17)	

1.3 Preparation NGM plates

C. elegans worms were maintained on Nematode Growth Medium (NGM) plates. 1L NGM contains 3.0g NaCl (Sigma-Aldrich), 17g Bacto™ Agar (BD Biosciences) and 2,5g Bacto™ Peptone (Gibco™), which are dissolved in 1L AD water. To avoid contamination, the NGM was autoclaved. After autoclaving, the mixture was placed in a 60°C oven for cooling. The following steps were performed in a laminar flow. 1mL of 1M $MgSO_4$ solution, 1mL of 1M $CaCl_2$ solution, 1mL of 5mg/mL cholesterol in ethanol solution, and 25mL of 1M P buffer were added to the NGM mixture. Using a pipette boy and a serological pipette (Greiner CELLSTAR®), 12mL NGM was added to each 55mm diameter for regular maintenance. While 5mL NGM was added to each baby plate with a diameter of 35mm for the behavioural assays. After pouring, the plates remained in the laminar flow to solidify and were turned upside down in a box to be stored in a 4°C room.

1.4 Inoculation of OP50

In the lab *C. elegans* feeds on OP50, which is an *E. coli* strain. The night before seeding, a single colony of OP50 was picked and inoculated in an autoclaved glass vial containing 10mL 2XTY medium in a laminar flow. The glass vial was put overnight in a 37°C shaking incubator, and the next morning, the vial was taken out from the incubator and put in a 4°C room.

1.5 Seeding plates

NGM plates were seeded with the prepared OP50 using a stepper pipette (Eppendorf Multipette® plus) and a stepper pipette tip (Eppendorf). The medium plates were seeded with 100µL. The bacteria were spread evenly on the plates using a glass spreader sterilized in ethanol and flame. For the behavioural assays, baby plates were seeded with a droplet of 5µL, this was not spread. The seeded plates were left open in the laminar flow to dry; when dried, they were stored upside down in a box at room temperature.

2. *twk-9(ok1611)* verification and outcrossing

Strain RB1414 with genotype *twk-9(ok1611)* was ordered from CGC, with a deletion in *twk-9*. Genotyping was initially performed to check if the received strain had this deletion. To avoid affects from background mutations, two rounds of outcrossing were performed.

2.1 Primer design

For genotyping, the design of specific primers is required. Because the mutation was a deletion, two primer pairs were required. An external primer pair, forward and reverse primer, and an internal primer pair, forward or reverse primer and poison primer. If there was a deletion the poison primer could not bind.

The application used for primer design was SnapGene. A number of conditions must be considered when designing primers. First, the primers should be 19-20 bp in length. The

sequence should contain 40-60% GC content with no repeats. The primer sequences are shown in Table 4. The annealing temperature was calculated by subtracting 5°C from the melting temperature. Before performing the actual PCR, a gradient PCR was performed to find the optimal annealing temperature, which is 55°C.

Table 4: primer sequences for genotyping *twk-9(ok1611)*

forward primer	5' gagaatgtagtccgcagagc 3'
reverse primer	5' gccaaaaaatacggtacttg 3'
internal primer	5' acatgctatctcgtggccg 3'

2.2 Worm lysis

The first step was to break the cuticle of the worms to release the genomic DNA. This was performed by adding one or more worms to a mixture of 5µL proteinase K (1mg/mL, Tritirachium album, Sigma-Aldrich) and 100µL PCR template buffer. In the case of genotyping a single worm, one worm was added per 5µL mixture, and in the case of genotyping a population, ten worms were added to 10µL mixture. The vials were placed for at least 15min at -80°C to ensure that the cuticles were broken. Subsequently, the worm lysis program was initiated using a thermocycler (Biometra) (Table 5).

Table 5: Worm lysis program

Release of genomic DNA	60°C	60 min
Inactivation Proteinase K	95°C	15 min
Hold	10°C	

2.3 Genotyping PCR

Amplification of the *twk-9(ok1611)* allele was performed using REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich). An overview of the volumes of this reaction mix is given in Table 6. To each sample vial 2µL of the genomic DNA from the worm lysis step was added. The PCR program was initiated using a thermocycler (Biometra) (Table 7).

Table 6: Overview of the volumes of the reaction mix for PCR 2 using REDTaq ReadyMix

component	**volume**
Readymix	6.25µL
Forward primer	0.5µL
Reverse/poison primer	0.5µL
Milli Q water	5.25µL
total	12.5µL

Table 7: PCR program using REDTaq ReadyMix

Initial denaturation	95°C	2min
Denaturation	95°C	1min
Hybridisation	55°C	2min
Extension	72°C	2min (1min/kb)
➔ 30 cycles		
Final extension	72°C	5min
Hold	10°C	

2.4 Gel electrophoresis

The final PCR products were analysed using agarose gel electrophoresis. A large gel was prepared by adding 2g of agarose (Sigma-Aldrich) to 200mL Tris-acetate EDTA buffer (TAE), which was heated in a microwave until the solution was clear and boiled. 10µL Gelred (10000x in Dimethylsulfoxide (DMSO), Biotum) was added to this mixture, which intercalates between nucleic acids and fluoresces strongly when exposed to UV light. Thus, the position of the DNA fragments in the gel could be observed. The solution was then poured into an electrophoresis chamber (Bio-Rad PowerPac™ Basic). When the gel solidified, the combs were taken out and the gel was wrapped in plastic foil and stored in the fridge until use.

The gel was placed in an electrophoresis chamber filled with TAE buffer. The gel was loaded by pipetting 5µL of each sample into gel wells. In the first and last wells, 5µL of a 1 kb plus ladder (Invitrogen), of which the length is known, was added as a reference. For genotyping, a large gel with two combs was used. In the upper row, the forward/reverse primer pair was added to the mix, and the forward/poison primer was added to the lower row. Electrophoresis was started at 130V for 40min. The negatively charged DNA fragments travelled to the positive pole through the gel, and DNA fragments of different sizes migrated at different rates.

After 40min, the gel was observed using the Proxima 2500-T gel imaging system (Isogen Life Sciences). The gel was placed inside this apparatus and excited with UV light; thus, the positioning of the DNA fragments could be observed.

2.5 Outcrossing of *twk-9(ok1611)*

After checking the genotype of *twk-9(ok1611)*, the worms were outcrossed twice. This was performed by crossing *twk-9(ok1611)* with the wild type to remove the background (Figure 9). Homozygous *twk-9(ok1611)* worms from the second outcross were used in the behavioural assays.

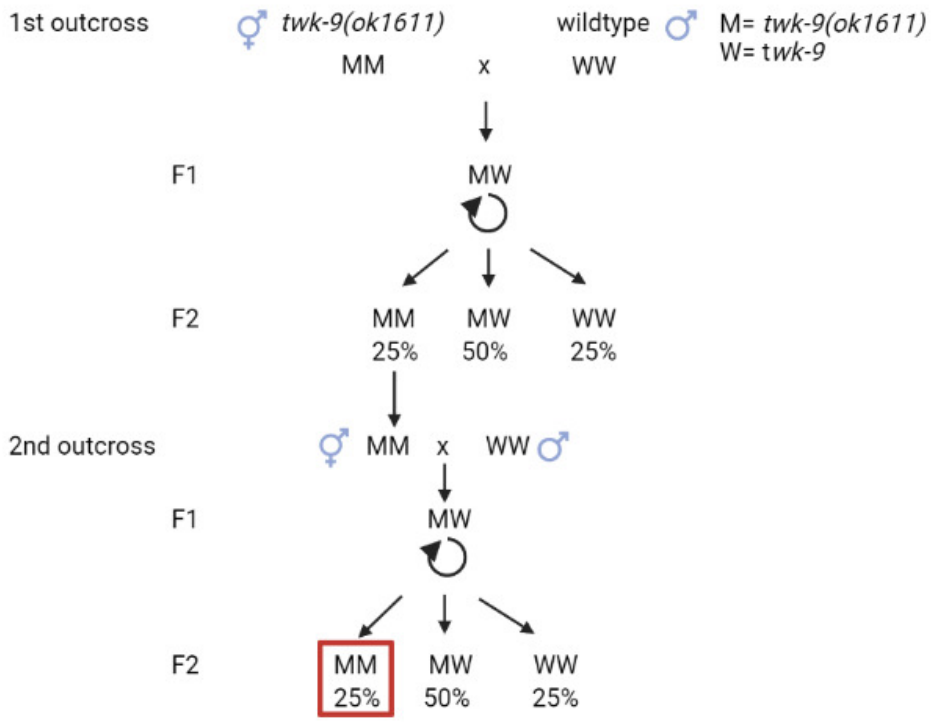


Figure 9: Crossing scheme for outcrossing *twk-9(ok1611)* with wild type. To create a crossing plate 2 L4 hermaphrodite *twk-9(ok1611*) worms were co-cultured with 10 male wild-type worms. After 3-4 days, the progenies (F1), which are expected to be heterozygous, were singled out. When the F1 worms laid eggs, they were genotyped using single worm PCR and gel electrophoresis to confirm heterozygosity. 25% of the progenies of F1 (F2) should be homozygous *twk-9(ok1611)*, this was again confirmed using single worm PCR and gel electrophoresis. Homozygous *twk-9(ok1611)* worms were used to perform a second outcross by crossing them again with male wild-type worms. The same procedure was performed as for the first outcross. The scheme was generated using Biorender.com.

3. Generating full knock-out of *twk-26* using CRISPR-Cas9

3.1 crRNA design

Two double-stranded breaks located along *twk-26* were required to create a deletion in this gene, for which two crRNAs needed to be designed. A PAM sequence was chosen close to the location of the double-stranded breaks, which is required for Cas9 to bind and cut. The upstream and the downstream crRNAs were selected next to or around the PAM sequence. The program CRISPOR (Tefor Infrastructure) was used for off-target analysis. When this program gave a good score for both crRNA sequences, they were ordered from Integrated DNA Technologies (IDT). Using this method, two crRNAs have been determined as below (Table 8).

3.2 Repair template design

Second, the repair templates must be designed within the crRNA region. There is a 5' homology arm of 35 bp located at the 3' end of the upstream crRNA, and a 3' homology arm of 35 bp located at the 5' end of the downstream crRNA. The repair template had a total of 70bp and was ordered from Sigma (Table 8).

Table 8: crRNA and repair template sequences for the generation of a full knock in *twk-26* using CRISPR-Cas9

crRNA1	5' attgcctcccgtttctacag 3'
crRNA2	5' atgggacactgacaccatgt 3'
repair template	5' cacttttccgttcaacgcgggttcggagacaaaagttcagtttgatctacgaagatctacaaaatcgcgg 3'

3.3 Preparation CRISPR-Cas9 injection mix

In addition to the gene of interest mix, a *dpy-10* mix was prepared. *dpy-10* serves as a selectable marker to identify successful gene modifications, in this case a successful knock-out of the *twk-26* gene. The *dpy-10* mix was prepared by mixing the volumes of Cas9, tracrRNA and crRNA, shown in Table 9. This mixture was incubated at 37°C for 10min. Subsequently, the volumes of the repair template and Milli Q water were added. The same was done for the gene of interest mix. Thereafter, both mixes were pipetted together and spun at maximum speed for 2min. The final mixture was kept on ice.

Table 9: Overview of the volumes and concentrations of the *dpy-10* and gene of interest mixes

	***dpy-10* mix**		**Gene of interest mix**	
	volume	**concentration**	**volume**	**concentration**
Cas9	0.25µL	5µg/µL	0.25µL	5µg/µL
tracrRNA	0.26µL	3.77µg/µL	0.26µL	3.77µg/µL
crRNA	0.24µL	2.33µg/µL	2x0.20µL	1.40µg/µL
Repair template	2.20µL	0.50µg/µL	1.10µL	1.00µg/µL
Milli Q	2.05µL	/	2.99µL	/

3.4 Preparation of injection pads

A 2% agarose solution was placed in a microwave to become liquid. From this solution a droplet was pipetted onto a glass coverslip and another glass coverslip was placed on top of it. After the agarose was solidified, one coverslip was removed by gently sliding. The slides were placed overnight in a 37°C incubator.

3.5 CRISPR-Cas9 microinjection

To perform injections young adults are the best stage, so the day before the injections L4 worms were picked. The microinjections were performed by trained lab technicians. The

worms were injected in the cytoplasm of the syncytium of the gonads. After the injection, the worms were transferred onto newly seeded plates and placed in a 20°C incubator.

3.6 Identification of homozygous *twk-26* knock-out worms

A co-CRISPR marker, *dpy-10*, which has a visual phenotype, was used. Heterozygous *dpy-10* worms have a roller phenotype and homozygous *dpy-10* worms have a dumpy phenotype. Three days after the injection two plates with the highest number of F1 worms with the roller phenotype were picked. Around 24 worms were picked from these plates and transferred onto separate plates. When the F1 worms laid eggs, they were genotyped by using PCR and gel electrophoresis. Worm lysis was done using the same program as for *twk-9(ok1611),* shown in Table 5. However, for *twk-26(ibt22)* OneTaq Quick-Load® 2x Master Mix with Standard buffer (NEB) was used instead of REDTaq ReadyMix. To each sample vial 2 µL of the genomic DNA resulting from the worm lysis was added. The PCR program was initiated using a thermocycler (Biometra) Table 11.

Table 10: : Overview of the volumes of the reaction mix for genotyping PCR using OneTaq Quick-Load® 2x Master Mix with Standard buffer

component	**volume**
OneTaq Quick-Load® 2X Master Mix with Standard buffer	6.25µL
Forward primer	0.25µL
Reverse/poison primer	0.25µL
Milli Q water	5.75µL
total	12.5µL

Table 11: PCR program using OneTaq Quick-Load® 2x Master Mix with Standard buffer

Initial denaturation	94°C	30 sec
Denaturation Hybridisation Extension ➔ 30 cycles	94°C 55°C 68°C	30 sec 1 min 5 min
Final extension	68°C	5 min
Hold	10°C	

Worms that were homozygous for the *twk-26* deletion, which was checked using gel electrophoresis, were sent for sequencing and further used in the behavioural assays. In Figure *10* an overview of the screening procedure is depicted.

Table 12: primer sequences for genotyping *twk-26(ibt22)*

forward primer	5' agcacaattccataaacggt 3'
reverse primer	5' cccgtaggtttttcagtagt 3'
internal primer	5' gacagggtatactttgatag 3'

3.7 Sequencing

The Wizard® SV Gel and PCR Clean-Up System (Promega) kit was used to purify the PCR products. Firstly, 18µL of Membrane Binding Solution was added to 18µL of PCR product. The SV Minicolumn was inserted into the Collection Tube. The prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1min. Subsequently, this was centrifuged at the maximum speed for 1min. 700µL of Membrane Wash Solution was added and this was again centrifuged at maximum speed for 1min. The flow-through was discarded, and the Minicolumn was reinserted into the Collection Tube. Thereafter, 500µL of Membrane Wash Solution was added and this was centrifuged for 5min at the maximum speed. The Collection Tube was emptied, and the column assembly was centrifuged for 1min. Eventually, the Minicolumn was transferred to a clean 1,5mL microcentrifuge tube, and 50µL of Nuclease-Free Water was added. This was incubated at room temperature for 1min and then centrifuged at the maximum speed for 1min. The Minicolumn was discarded and the DNA was stored at 4°C. The concentration was then measured using a nanophotometer. Finally, 4µL of forward primer was added to 10µL of the sample, which was sent for sequencing.

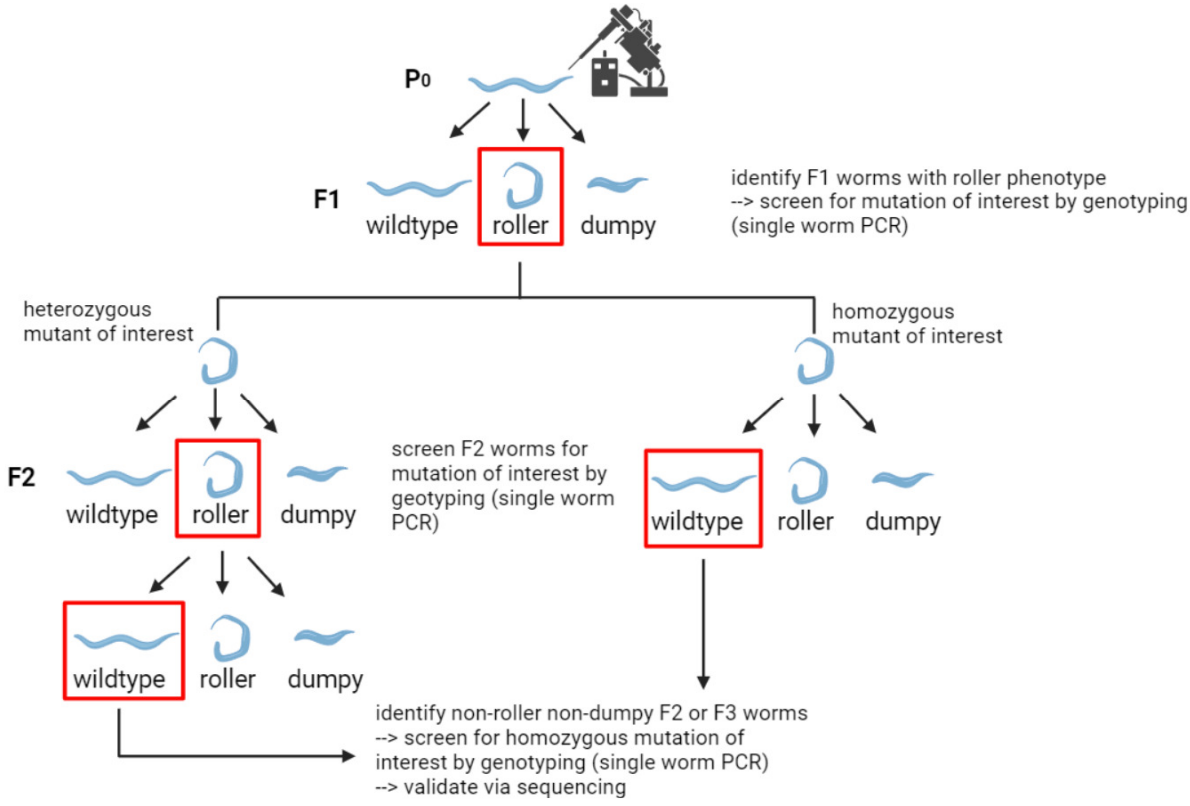


Figure 10: Scheme for the screening procedure of injected *C. elegans* worms using CRISPR-Cas9 genome editing.

4. Phenotyping: behavioural assays

4.1 Egg-laying assays on/off food

Egg-laying events were compared between worms on plates with bacteria and worms on plates without bacteria. The day before the assay L4-stage worms were picked in the afternoon. On the morning of the assay, these worms were young adults and were singled out on plates with/without food. Exactly two hours after isolating the worms, they were burned and the number of eggs was counted. These experiments were performed with the wild type, *twk-9(ok1611), twk-46(ibt17)* and *twk-26(ibt22)* mutants.

4.2 Egg retention assays

In egg retention assays, the number of eggs inside the uterus was counted. The day before the assay, L4-stage worms were picked; thus, the worms were adults on the assay day. NGM plates without bacteria were used. A droplet of bleaching solution was added to the NGM plate, and a worm was placed inside the bleaching solution. After a few minutes, the cuticle was broken and the eggs were exposed. The number of eggs was counted and compared among the wild type, *twk-9(ok1611)*, *twk-46(ibt17),* and *twk-26(ibt22)* mutants.

Results

1. *twk-9(ok1611)* outcrossing

Prior to the egg-laying assays, the *twk-9(ok1611)* mutant was outcrossed twice with the wild type. After the second outcross the progenies from a heterozygous worm were evaluated by PCR and gel electrophoresis and the results are shown in Figure 11. The worms in lane 7, 8, 13, 16 and 26 are homozygous individuals. Compared to N2 bands, for the PCR reaction with primers flanking the full version of *twk-9*, these worms only have one short band at the position at around 780bp. For the PCR reaction with poison primers, there is no band showing in those worms, indicating that they are homozygous for the partial deletion allele of *twk-9* after outcrossing. After two outcrosses, one individual worm was picked and maintained for the following behavioural assays.

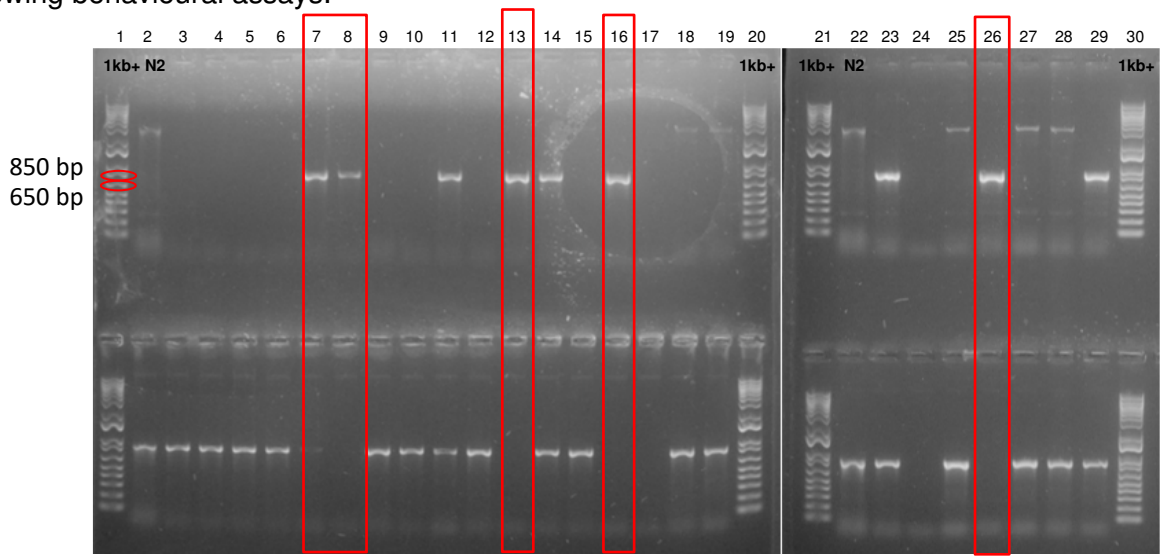


Figure 11: Gel electrophoresis result of *twk-9(ok1611)* strain after two rounds of outcrossing. Lane 1, 20, 21 and 30 contains the 1kb plus ladder and lane 2 and 22 contain the wild type control. Lane 3-19 and lane 23-29 contain the progenies from a worm that was previously confirmed to be heterozygous. On the left side of the figure sizes of two bands of the 1kb plus ladder are indicated, which helps annotating the size of the bands in the gel.

2. *twk-26* full knock out by CRISPR-Cas9 technique

CRISPR-Cas9 was used to create a knock-out strain for the *twk-26* gene. PCR and gel electrophoresis were done to identify the worms with the homozygous mutant phenotype, the results are shown in Figure 12. The worms in lane 8, 9 and 15 are homozygous individuals. Compared to N2 bands, for the PCR reaction with primers flanking the full version of *twk-26*, these worms only have one short band at the position around 500 bp. For the PCR reaction with poison primers, there is no band showing in those worms, indicating that they are homozygous for the partial deletion allele of *twk-26*. After this identification, one individual homozygous worm was picked and maintained for the following behavioural assays.

Sequencing of *twk-26(ibt22)* confirmed that the targeted gene sequence was removed from the genome of the knock-out mutant. The sequences of wild type *twk-26* and the mutant allele *twk-26(ibt22)* are attached in the appendices.

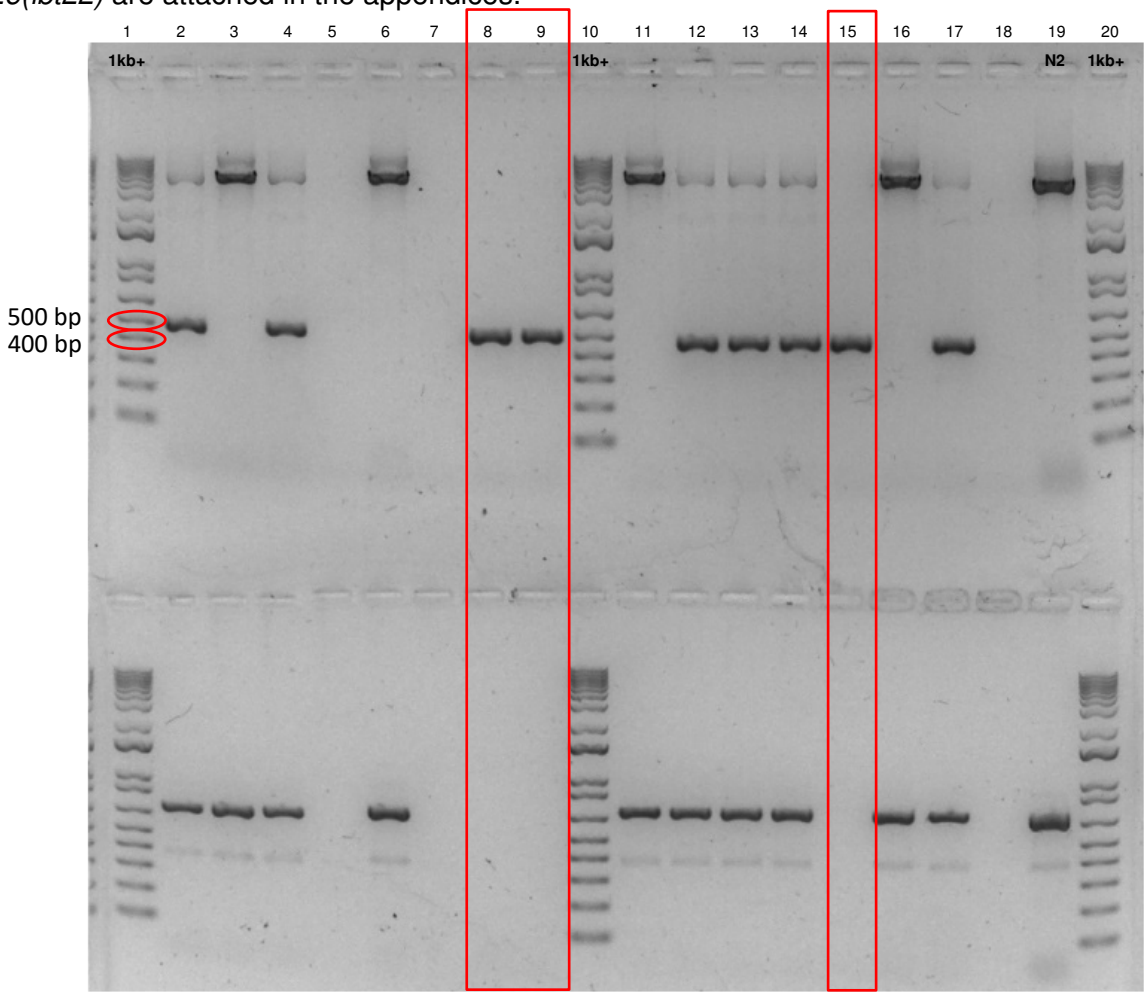


Figure 12: Gel electrophoresis result of *twk-26* after CRISPR-Cas9 knock-out. Lane 1, 10 and 20 contain the 1kb plus ladder and lane 19 contains the wild type control. Lane 2-9 and lane 11-18 contain progenies from the worms that were injected with the CRISPR-Cas9 injection mix. On the left side of the figure sizes of two bands of the 1kb plus ladder are indicated, which helps annotating the size of the bands in the gel.

3. Egg-laying behaviour on/off food

In the egg-laying assays the number of eggs laid after exactly 2 hours was compared between the wild type, *twk-9(ok1611), twk-46(ibt17)* and *twk-26(ibt22)* mutants in two different conditions: on and off food. The results of these assays are shown in Figure 13, every dot represents the number of eggs laid by one worm. Around 50 worms per strain were tested on 4 independent assay days. To see if there were significant differences between the mutants and the wild type a statistical test, Mann-Whitney test, was performed. The Mann-Whitney test assumes a null hypothesis, where there is no significant difference between the number of eggs laid by the mutant and the wild type. Based on the resulting p-values a decision can be made to either reject or keep this null hypothesis. When the p-value is above 0.05, the null hypothesis can be kept. In the egg-laying assays on food, no significant differences were

observed between the mutants and the wild type. However, for the egg-laying assays off food, significant differences were observed between *twk-46(ibt17)* and the wild type and between *twk-26(ibt22)* and the wild type. No significant differences were observed between *twk-9(ok1611)* and the wild type.

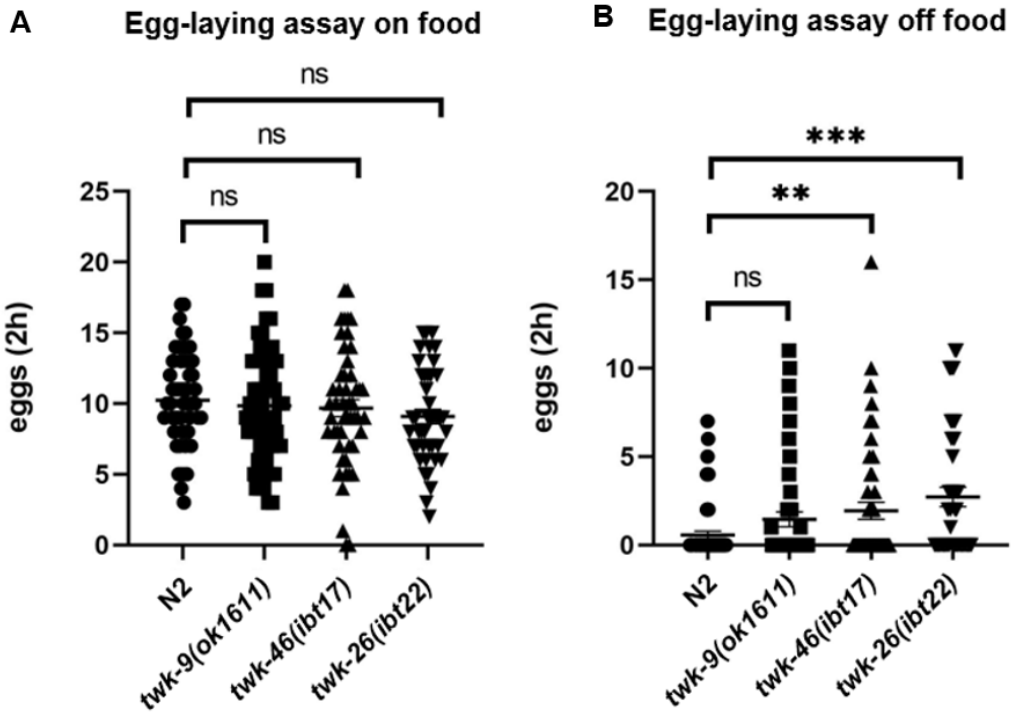


Figure 13: Egg-laying assay on and off food. This figure shows the number of eggs that every worm laid in two hours. A comparison is made between the wild type, *twk-9(ok1611)*, *twk-46(ibt17)* and *twk-26(ibt22)* mutants using the Mann-Whitney test. **A.** For the egg-laying assays in the presence of food, four trials were conducted, three trials with 10 worms and one trial with 20 worms. All three comparisons are considered non-significant (ns) (p-value>0.05). **B.** For the egg-laying assays in the absence of food, four trials were conducted with an average of 13 worms per trial. The comparison of *twk-9(ok1611)* with the wild type is considered non-significant (p-value>0.05). The comparisons of *twk-46(ibt17)* and *twk-26(ibt22)* with the wild type are significantly different (p-value<0.05).

4. Egg retention assay

In the egg retention assays using the bleaching method the worms were placed in a bleaching solution, which exposes the eggs that were inside the uterus, and the number of eggs was counted. The number of eggs inside the uterus was compared between the wild type, *twk-9(ok1611), twk-46(ibt17)* and *twk-26(ibt22)* mutants. The results of these assays are shown in Figure 14, every dot represents the number of eggs inside the uterus of one worm. Around 35 worms per strain were tested on 4 independent assay days. To see if there were significant differences between the mutants and the wild type a statistical test, Mann-Whitney test, was performed. No significant differences were observed between the mutants and the wild type.

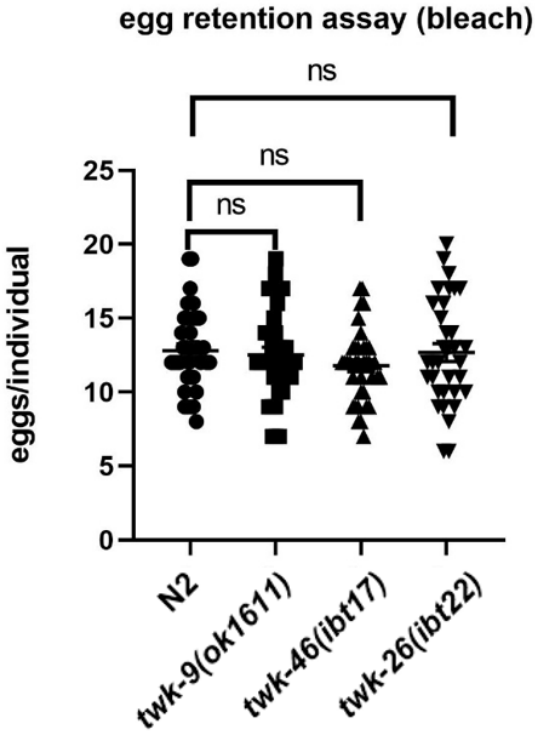


Figure 14: Egg retention assay using the bleaching method. This figure shows the number of eggs inside the uterus of the worm. A comparison is made between the wild type, *twk-9(ok1611)*, *twk-46(ibt17)* and *twk-26(ibt22)* mutants using the Mann-Whitney test. Four trials were conducted with an average of 9 worms per trial. All three comparisons are considered non-significant (ns) (p-value>0.05).

Discussion

In this study we investigated the role of three two-pore domain potassium channels in egg-laying behaviour of *C. elegans*. Three genes encoding K2P channels were chosen, *twk*-9, *twk-26* and *twk-46*, based on their expression level in the egg-laying neurons HSN and VC.

A strain with a deletion in *twk-9*, *twk-9(ok1611)*, was successfully outcrossed twice. A full knock-out was generated of *twk-26, twk-26(ibt22)*, and a strain with a full knock-out of *twk-46, twk46(ibt17)*, was already present in the lab stock. Following that, two different assays were conducted to characterize the roles of those K2P channels in egg-laying behaviour in *C. elegans*. The egg retention experiments showed that *twk-9*, *twk-26* and *twk-46* are not involved in egg production *in vivo*. However, *twk-26* and *twk-46* mutants had a slightly, but significantly higher number of eggs laid off food, compared to the wild-type strain. These results suggest that *twk-26* and *twk-46* could inhibit egg-laying off food in *C. elegans.*

1. Knocking out *twk-26* and *twk-46* results in subtle phenotypes in egg-laying behaviour in the absence of food

In the absence of food, *twk-26(ibt22)* and *twk-46(ibt17)* mutants display a subtle increase in the number of eggs laid compared to the wild type. We expected to see more eggs, because the inactivation of a K2P channel causes depolarization in the neuronal membrane, which leads to less inhibition of egg-laying. This result suggests that the *twk-26* and *twk-46* genes may play a role in modulating the excitability of HSN and/or VC neurons, thereby influencing egg-laying behaviours.

A previous study by Trent *et al*. (1983) demonstrated that a gain-of-function mutation in *egl-23,* a K2P channel, led to egg-laying defects. This mutation disrupts the normal regulation of the K2P channel, resulting in impaired egg-laying behaviour (Trent *et al*., 1983). In our study, we found that loss-of-function mutations in K2P channels increase egg-laying. These combined findings suggest that K2P channels serve to inhibit egg-laying behaviour, and their dysregulation, whether through gain-of-function mutations or loss-of-function mutations, affects the normal coordination of egg-laying in *C. elegans*.

Additionally, Branicky *et al*. (2014) demonstrated that a loss-of-function mutation in *clh-3*, a gene encoding a voltage-gated chloride channel, resulted in increased HSN activity and subsequently increased egg-laying. Despite differences in charge and direction of ion movement compared to K2P channels, a similar trend of slightly increased egg-laying was observed. Another study conducted by Emtage *et al*. (2012) found an inwardly rectifying potassium channel IRK-1, whose null mutant showed a minor increase in egg-laying in the

presence of food. Surprisingly, a cooperative effect has been observed in a double mutant of *egl-6* and *irk-1*. The mechanism underlying this cooperative effect is the inhibition of HSN's excitability by EGL-6, a GPCR, in a way that is dependent on IRK-1 channels. This research not only illustrated the role of *irk-1*, but also linked its function with a GPCR pathway, putting forward the interesting model that K2P channels might play a role in egg-laying behaviours by interacting with GPCR pathways. However, this hypothesis needs further experiments to verify in the context of the K2P channels studied in this project. The results of the studies involving *clh-3* and *irk-1* suggest that a loss-of-function mutation in an ion channel may elicit relatively subtle effects (Emtage *et al*., 2012). In our case, subtle effects are primarily attributed to the presence of multiple K2P channels that collectively contribute to the establishment of the resting membrane potential. The different K2P channels expressed in egg-laying neurons are represented in Table 1. Consequently, the knock-out of a single channel causes only a limited impact.

2. Previous studies on K2P channels result in subtle phenotypes in other behaviours

So far, no work has been done to systemically study the potential functions of K2P channels in *C. elegans* egg-laying behaviour. In our study, very subtle phenotypes were observed in the *twk-26* and *twk-46* single null mutants. This result is not surprising as in the other studies on *C. elegans* K2P channels, most loss-of-function alleles of K2P channels do not have strong phenotypes as gain-of-function alleles do. For example, extensive research has been conducted to unravel roles of multiple K2P channels in locomotion circuits of *C. elegans*. Zhou *et al*. (2022) found that loss-of-function mutations in several K2P channels, such as *twk-2* and *twk-17*, only cause defective body curvature and/or locomotion, while gain-of-function mutations in K2P channels, such as *twk-40* and *twk-2*, cause paralysis. In a study of Reiner *et al*. (1995) several potassium channel mutants were examined that affect muscle activation in *C. elegans*. They observed that loss-of-function mutations would not significantly affect muscle function, while gain-of-function mutations would cause defective or hyperactive muscle activation (Reiner *et al*., 1995). In addition, Soussia *et al*. (2019) found that simply substituting the sixth residue in the second transmembrane region (TM2.6) of K2P channels with asparagine can cause hyperactive K2P channels. This finding provides a potential approach to study the function of K2P channels by making gain-of-function alleles. In our case, that subtle phenotypes (for *twk-26* and *twk-46*) or no phenotypes (for *twk-9*) were found in egg-laying behavioural assays might be attributed to high possibility of redundancy in egg-laying neurons. CenGEN is a database that provides information about the cell-specific gene expression patterns in *C. elegans* (Taylor *et al*., 2021). According to CeNGEN data, there are

9 K2P channels expressed in HSN and VC neurons. To further elucidate each K2Ps function in the egg-laying circuit, more experiments are necessary in the future.

3. Future perspectives

3.1 Rescue experiments

Our findings indicate the involvement of TWK-26 and TWK-46 in egg-laying behaviour. However, further verification of this involvement requires additional rescue experiments. A rescue experiment aims to determine whether the abnormal behaviour caused by the mutation can be restored by reintroducing the wild-type gene. To conduct this experiment, a genetic construct can be generated using a vector containing a promoter specific to *twk-26* or *twk-46*, the respective *twk-26* or *twk-46* DNA and a reporter such as GFP. This construct can be injected into the gonads of mutant worms. As the construct is taken up as an extrachromosomal array, it is not passed on to all progenies. Worms that have successfully taken up the construct can be identified by their green fluorescence, facilitated by the presence of the reporter gene (Calahorro *et al*., 2012). Subsequently, behavioural assays can be performed to compare the behaviour of the mutant worms with that of the rescue worms. It is possible to restrict rescue experiments to specific cell types by using cell-specific promoters. This way, one can gain insights into the gene's role and its contribution to the phenotype within those cell types (Sweeney, 2014). In our study, it would be particularly intriguing to employ HSN- or VC-specific promoters to gain deeper understanding of the gene's functions in these specific egg-laying neurons.

3.2 Finding stronger phenotypes

As mentioned before, one possible reason why *twk-26*, *twk-46* and *twk-9* single mutants do not show a strong phenotype could be redundancy. Multiple K2P channels can be expressed in the same neuron. In cases where one K2P channel loses its function due to a deletion, (an)other K2P channel(s) expressed within the same neuron can potentially compensate the effect of this mutation. Redundancy makes it difficult to decipher the individual contributions of each channel to neuronal activity. To reduce the possibility of compensation, double or triple mutants can be generated, where two or three K2P channels expressed in the same neuron are knocked out. This way, it is possible to study the effects displayed by single, double and triple mutants.

Alternatively, CRISPR-Cas9 can be used to create gain-of-function alleles. After introducing mutations at certain locations, one can then screen for a phenotype with pronounced effect. Soussia *et al*. (2019) created gain-of-function mutations in K2P channels using CRISPR/Cas9 gene editing. By substituting hydrophobic residues for hydrophilic residues in TM2.6 the channel activity can be increased. These gain-of-function mutations are known to result in strong phenotypes. Since there are multiple K2P channels expressed within a neuron,

knocking out a single channel might have minimal impact on the total potassium current. The introduction of a gain-of-function mutation in a K2P channel can cause the channel to be continuously open, increasing the total potassium current. As a result, this sustained opening is likely to induce hyperpolarization of the neuron, thereby inhibiting its activity (Soussia *et al*., 2019).

Another fact that should be noted is that *twk-26* and *twk-46* are expressed in both HSN and VC neurons. Previous studies suggested that VC4 and VC5 neurons can inhibit HSN's activities by releasing acetylcholine, which acts on the muscarinic acetylcholine receptor GAR-2 present on the HSN neurons (Bany *et al*., 2003; Kopchock *et al*., 2021). Activation of this receptor by acetylcholine inhibits serotonin release from HSN neurons, thereby suppressing egg-laying (Bany *et al*., 2003). On the other hand, VC neurons can stimulate vulval muscles by releasing acetylcholine. Acetylcholine binds to its receptor on vulval muscles, which depolarizes the muscle cell membrane (Waggoner *et al*., 1998). Given that the VC neurons exert both excitatory and inhibitory effects on egg-laying behaviour, it is reasonable to see subtle phenotypes in *twk-26* and *twk-46* null mutants due to the possible counteracting effects between HSN and VC neurons. Therefore, to rule out the possible counteracting effect, generating cell-specific knock-out of *twk-26* and *twk-46* can give us more information of how they function in HSN and VC neurons respectively. The other way to find a stronger phenotype could be working on K2P channels exclusively expressed in HSN neurons. A potential candidate for investigation is *twk-2*, as it is exclusively expressed in HSN and not in VC4-5, as indicated in Table 1.

3.3 Examination of egg-laying neurons by calcium imaging

In our study, our primary focus was to investigate these K2P channels' function by doing egg-laying related behavioural assays. However, an additional and intriguing approach is to investigate their roles at neuronal level. Calcium imaging serves as a valuable tool for tracking neuronal activity over time. By comparing the calcium dynamics of wild-type neurons with those of neurons with specific K2P channel knock-outs, we can gain a deeper understanding of the impact of the genetic modifications on neuronal activity.

The previous work conducted by Ravi *et al*. (2018) encompassed calcium imaging of individual HSN neurons. To record intracellular changes in calcium levels, they employed a Ca^{2+}-sensitive reporter called GCaMP5. Simultaneously, they used a Ca^{2+}-insensitive fluorescent protein, mCherry, to track the positioning of the HSN neurons. This approach allowed them to correlate HSN neuronal activity with egg-laying events and changes in locomotion. They observed a HSN Ca^{2+} transient, coinciding with slowing of locomotion and ending with egg release. They were able to record a series of closely spaced HSN Ca^{2+} transients, coinciding with egg-laying events (Ravi *et al*., 2018).

In another study, a different research group employed calcium imaging to investigate the relationship between circuits governing reproduction and defecation. By comparing HSN Ca^{2+} transients with defecation events, they made a significant discovery. Specifically, they found that the presence of one or more HSN Ca^{2+} transient(s) correlated with notably longer intervals between defecation events. This finding indicates that signals from the HSN neurons might inhibit the defecation motor rhythm (Brewer *et al*., 2019).

Activation of K2P channels results in hyperpolarization of neuronal membranes, leading to a reduction in intracellular Ca^{2+} levels. Conversely, the knock-out of K2P channels is expected to cause an increase in intracellular Ca^{2+} levels. Consequently, in our mutants, we anticipate observing increased Ca^{2+} transients within HSN neurons. Although mainly associated with egg-laying behaviour, recent studies have revealed that HSN neurons also play a role in locomotion and defecation. To regulate these behaviours, HSN neurons communicate with other neurons (Brewer *et al*., 2019; Ravi *et al*., 2018). Given the findings by Brewer *et al*. (2019), which indicate that HSN neurons inhibit defecation, the increased HSN activity in our mutants suggests an increased inhibition of defecation. Furthermore, as our mutant strains, *twk-26(ibt22)* and *twk-46(ibt17)*, exhibit a higher egg-laying rate compared to the wild type, it is reasonable to expect a greater reduction in locomotion since Ravi *et al*. (2018) observed decreased locomotion during egg-laying.

3.4 Examination of the K2P channels by TEVC

Up to now, only minor differences have been observed between the mutants and the wild type. Delving deeper into cellular dynamics and measuring cell activity may yield interesting findings. One promising method for such investigations is the utilisation of two-electrode voltage clamp (TEVC) to study ion channel currents expressed in *Xenopus laevis* oocytes. In this approach, the gene encoding a K2P channel, such as *twk-9*, *twk-46* or *twk-26,* is cloned in an expression vector, which is subsequently introduced into *Xenopus laevis* oocytes. The process entails the insertion of two electrodes in the oocyte, namely a voltage-sensing electrode and a current-sensing electrode. The voltage-sensing electrode facilitates the application of diverse voltages to the oocyte, thereby enabling the activation and characterization of the K2P channel's currents. Simultaneously, the current-sensing electrode measures and records the currents that traverse the K2P channels (Wang *et al*., 2017). The resulting current-voltage relationship obtained through this technique provides valuable insights into the conductance and voltage sensitivity of the channel. Consequently, this experimental framework offers a means to examine the manner in which K2P channels contribute to the regulation of cellular excitability (Cucu *et al*., 2005).

3.5 Examination of the developmental stage of the eggs inside the uterus

In addition to the typical egg-laying assays we did in this project, another comparison between mutants and wild type can be made by looking at the developmental stage of the eggs inside the uterus. Eggs in wild-type worms are laid at the 30-cell gastrulation stage, approximately 5 hours after fertilization (Bucher and Seydoux, 1994). During our project an experiment was started where the developmental stage of the eggs was observed under a microscope. In this experiment, no apparent differences were observed between the mutants and the wild type in terms of the developmental stage of the eggs. However, further data is required before any definitive conclusions can be drawn.

In a study conducted by Lee *et al*. (2011), the percentage of early stage eggs laid by both wild type and mutant worms was determined. The mutant strain, *str-33(ykp001)*, exhibited a hyperactive egg-laying phenotype. The researchers placed staged adult worms on a NGM agar plate and allowed them to lay eggs for one hour before killing them. Each egg was examined under a microscope, with eggs containing eight or fewer cells categorized as “early stage”. The findings revealed that the wild type worms laid multicellular eggs, while the mutant worms laid eggs at the four-cell stage (Lee *et al*., 2011).

Similarly, (Koelle & Horvitz, 1996) also investigated the developmental stage of newly deposited eggs. They isolated L4 stage worms and gave them 39 hours to develop at 20°C. Subsequently, 20 adult worms were transferred to fresh plates and the developmental stage of the freshly laid eggs was determined by microscopic examination. The eggs were classified into three categories: one to eight-cell stage, nine-cell stage to comma stage and post-comma stage he “comma stage” denotes a stage where numerous cell divisions have already taken place, leading to an increased total cell count (Tserevelakis *et al*., 2010).

Furthermore, an alternative technique known as third harmonic generation (THG) microscopy can be employed to obtain high-resolution images of *C. elegans* eggs. By analysing the recorded THG signals effectively, it becomes possible to determine the overall structure of each cell. The cell nucleus, having a homogenous structure, does not emit a THG signal. Conversely, non-linear THG signals are generated by discontinuities in the region surrounding the cell nucleus, originating from the cytoplasm, various organelles and lipid deposits. Eggs in the comma stage can be identified by an increased number of individual cells and the initiation of embryo elongation. On the other hand, eggs in the 3-fold stage exhibit fully elongated embryos folded inside the egg, which eventually hatch into L1 worms (Tserevelakis *et al*., 2010).

4. Limitations

One limitation of our experiment is that we relied solely on RNA-seq data and did not experimentally verify the expression pattern of the K2P channels. The CeNGEN (*C. elegans* Neuronal Gene Expression Map and Network) database utilizes single-cell RNA sequencing to provide valuable insights into the expression patterns of various neuron classes in the entire nervous system of *C. elegans* (Taylor *et al.*, 2021). As indicated in Table 1 *twk-9*, *twk-26* and *twk-46* exhibit high expression in the egg-laying neurons HSN and VC4-5. The CeNGEN data offers four threshold values (1-4) that present different trade-offs between the risks of false positives and false negatives (Taylor *et al.*, 2021). Among these thresholds, threshold 1 is the least stringent and yields the highest number of expressed genes per neuron type, albeit a higher risk of false positives. On the other hand, threshold 4 is the strictest and results in the fewest genes being identified for each neuron type, leading to fewer false positives but potentially more false negatives. To strike a balance between false positives and false negatives, we chose threshold 2, which was estimated to have a true positive detection rate of 0.81 and a false discovery rate of 0.14 (Hammarlund *et al.*, n.d.).

Although threshold 2 gives a good balance between false positives and false negatives, it is unlikely to achieve a threshold that completely eliminates all errors. Therefore, there is still some uncertainty in the analysis results, so we need to interpret CeNGEN data with caution. Thus, there remains a possibility that the K2P channels TWK-9, TWK-26, and TWK-46 are either not expressed or only expressed to a limited extent in the HSN and/or VC4-5 neurons. Consequently, it is possible that only minor differences in egg-laying behaviour are observed between the mutants and the wild type. Thus, it would be advisable to experimentally verify the expression pattern. Considering that *C. elegans* is a transparent organism, *in vivo* visualization of expression can be accomplished by employing a fluorescent protein, such as GFP. A constructed vector containing a promoter sequence along with the *twk-9*, *twk-46*, or *twk-26* open reading frame and a fluorescent reporter sequence can be introduced into *C. elegans* hermaphrodites through techniques like microinjection or microparticle bombardment (Evans, 2006). This approach would enable the visualization of the expression patterns of *twk-9*, *twk-46*, and *twk-26* using a fluorescent microscope.

Conclusion

Our research has contributed to the understanding of the functions of two-pore domain potassium channels in egg-laying behaviour of *C. elegans*. To investigate this, we conducted egg-laying assays and egg retention assays using three mutant strains, *twk-9(ok1611)*, *twk-26(ibt22)* and *twk-46(ibt17)*. Interestingly, we did not observe any significant differences in the number of eggs retained in the uterus between the wild type and mutants.The egg-laying in the presence of food did not differ between the wild type and the mutants. However, the egg-laying in the absence of food showed a subtle increase in egg-laying for *twk-26(ibt22)* and *twk-46(ibt17)*. Based on these findings we can conclude that *twk-26* and *twk-46* inhibit egg-laying in the absence of food.

Although this research provides some insights into the role of K2P channels in egg-laying neurons, further experiments are necessary to draw definitive conclusions. To verify the involvement of these K2P channels in egg-laying behaviour additional rescue experiments are required. Future investigations could explore stronger phenotypes by utilizing double/triple mutants, generating gain-of-function mutants or creating cell-specific knock-outs. Additionally, a comparative analysis between the wild type and the mutants focusing on the developmental stage of the eggs could be performed. Alternatively, future studies may shift their focus to the neurons themselves or specifically investigate the K2P channels using advanced techniques such as calcium imaging and two-electrode voltage clamp.

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Addendum

1. Risk analysis

1.1 General

The lab in which the experiments were done has biosafety level 1. Only non-pathogenic organisms were used: *C. elegans* and *E. coli* OP50. While working in the lab a number of precautions must be taken into account. During experimental work a lab coat and gloves need to be worn. The lab coat needs to be closed, can never leave the lab environment and can never enter rooms in which food and/or drinks are stored. Food, drinks and smoking are prohibited in the lab. When leaving the lab hands are washed with soap and disinfectant. When working sterile the laminar flow is used. Before and after use, the laminar flow, materials and gloves are disinfected with 70% alcohol.

Waste needs to be sorted into the right waste containers. Liquids need to be poured in the designated jerry cans. Solid biohazardous waste needs to go into the cardboard box with the yellow plastic bag inside. Sharp materials, such as cover glasses, needles… need to go into the designated small containers. Reusable materials are sterilized in the autoclave. Trained people in the lab do this, they use heat-resistant gloves.

1.2 Products

1.2.1 GelRed

GelRed is used to stain DNA in gel electrophoresis. The risks are still not fully clear, so it needs to be handled with care. GelRed is only used in the gel electrophoresis room, all materials used here cannot leave this area. In this room a specific lab coat with a red collar and gloves are worn. All GelRed contaminated materials need to be discarded in the biohazard waste box. Physical contact needs to be avoided at all times.

1.2.2 $CaCl_2$

$CaCl_2$ can be irritating for the eyes therefore eye and face protection needs to be worn when using this product. In case of contact, rinsing with water for several minutes is needed.

1.2.3 EtOH

Ethanol is a highly flammable product that can cause eye irritation. Therefore, it needs to be stored in a closed container far away from heat.

2. Solutions and buffers

2.1 1M $MgSO_4$

To prepare 200mL of a 1M $MgSO_4$ solution 49.3g $MgSO_4$ (246.5g/mol, Supelco®) was added to a 500mL Duran bottle. Milli Q water was added up to 200mL, this was swirled gently to homogenise and the bottle was autoclaved. Under the laminar flow aliquots of 50mL were made in Falcon tubes.

2.2 1M $CaCl_2$

To prepare 200mL of a 1M $CaCl_2$ solution 147g $CaCl_2$ (147.01g/mol, Sigma-Aldrich) was added to a 500mL Duran bottle. Milli Q water was added up to 200mL, this was swirled gently to homogenise and the bottle was autoclaved. Under the laminar flow aliquots of 50mL were made in Falcon tubes.

2.3 5mg/mL Cholesterol

To prepare 200mL of a 5mg/mL cholesterol solution 1g Cholesterol (386.65g/mol, Sigma-Aldrich) was added to a 500mL Duran bottle. 100% EtOH absolute (VWR Prolabo Chemicals) was added up to 200mL, this was swirled gently to homogenise and the bottle was autoclaved. Under the laminar flow aliquots of 50mL were made in Falcon tubes.

2.4 1M P buffer

To prepare 200mL of 1M P buffer 21.77g KH_2PO_4 (136.09g/mol, Sigma-Aldrich) and 6.97g K_2HPO_4 (174.18g/mol, VWR Prolabo Chemicals) were added to a 500mL Duran bottle. Milli Q water was added up to 200mL, this was swirled gently to homogenise and the bottle was autoclaved.

2.5 2xTY medium broth

To prepare 200mL of 2xTY medium broth 3.2g Bactotryptone (Gibco), 2g Yeast extract (Sigma-Aldrich) and 1g NaCl (58.44g/mol, Sigma-Aldrich) were added to a 500mL Duran bottle. Milli Q water was added up to 200mL, this was swirled gently to homogenise and the bottle was autoclaved.

2.6 50x TAE buffer

To prepare 1L of 50x TAE buffer 242g Trizma base (121.14g/mol, Sigma-Aldrich), 18.61g Disodium EDTA (372.24 g/mol, Sigma-Aldrich) and 700mL AD water to a plastic bottle. A stirrer was added until everything was dissolved. 57.1 mL acetic acid (60.05g/mol, Sigma-Aldrich) was added and this was swirled gently to homogenise. AD was added to the bottle up until 1L.

2.7 PCR template buffer

To prepare 100 mL of PCR template buffer, the following quantities are mixed together:

- 1Ml of 1% Gelatin

- 5mL of KCl
- 0.25mL of 1M $MgCl_2$
- 1mL of 1M Tris in HCl (pH 8.3)
- 4.5mL of 10% NP-40
- 4.5mL of 10% Tween 20

2.8 S basal

To prepare 200mL of S basal 1.17g NaCl, 1.2 KH_2PO_4 and 0.2g K_2HPO_4 were added to a 500mL Duran bottle. Milli Q water was added up to 200mL, this was swirled gently to homogenise and the bottle was autoclaved.

3. CRISPR-Cas9 knock-out mutants

3.1 *twk-46* wild type and mutant

PAM

Exon

SSODN

- **wild type *twk-46***

tatcctccaagaaaattttcccttctctccgggaccaatttgtaagcggagagcacattgcaaatcgcccacgccaacccaaaaag
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- mutant *twk-46*

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3.2 *twk-26* wild type and mutant

PAM

Exon

SSODN

insertion

- **wild type *twk-26***

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- mutant *twk-26*

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