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What About the Males? The *C. elegans* Sexually Dimorphic Nervous System and a CRISPR-Based Tool to Study Males in a Hermaphroditic Species

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Abstract

Sexual dimorphism is a device that supports genetic diversity while providing selective pressure against speciation. This phenomenon is at the core of sexually reproducing organisms. *Caenorhabditis elegans* provides a unique experimental system where males exist in a primarily hermaphroditic species. Early works of John Sulston, Robert Horvitz, and John White provided a complete map of the hermaphrodite nervous system, and recently the male nervous system was added. This addition completely realized the vision of *C. elegans* pioneer Sydney Brenner: a model organism with an entirely mapped nervous system. With this ‘connectome’ of information available, great strides have been made toward understanding concepts such as how a sex-shared nervous system (in hermaphrodites and males) can give rise to sex-specific functions, how neural plasticity plays a role in developing a dimorphic nervous system, and how a shared nervous system receives and processes external cues in a sexually-dimorphic manner to generate sex-specific behaviors. In *C. elegans*, the intricacies of male-mating behavior have been crucial for studying the function and circuitry of the male-specific nervous system and used as a model for studying human autosomal dominant polycystic kidney disease (ADPKD). With the emergence of CRISPR, a seemingly limitless tool for generating genomic mutations with pinpoint precision, the *C. elegans* model system will continue to be a useful instrument for pioneering research in the fields of behavior, reproductive biology, and neurogenetics.

Introduction

Sydney Brenner championed the use of *C. elegans* as a model organism to study nervous system development and function (Brenner, 1974). The genetic amenability of the *C. elegans* hermaphrodite is perfect for classic forward genetic screens, with Brenner’s first mutagenesis screen identifying nearly 100 genes, most of which were identified by movement defects. Brenner’s 1974 vision for an animal model with a completely mapped nervous system was realized - in part - by the pioneering lineage studies of John Sulston and Robert Horvitz and the circuitry diagrams of John White (Sulston et al., 1983; Sulston & Horvitz, 1977; White et al., 1986).

John Sulston also spotlighted the lesser studied sex of *C. elegans*: the male. Sulston and colleagues discovered that hermaphrodite and male *C. elegans* display striking sexual dimorphisms in development, programmed cell death, and the nervous system in hermaphrodite and male animals, with 959 and 1031 total cells in hermaphrodites and males respectively, 302 neurons in hermaphrodites and 381 in males (Sulston et al., 1983; Sulston & Horvitz, 1977; White et al., 1986). A subset of neurons are ciliated at dendritic ends: 60 in hermaphrodites and 112 in males (52 male-specific) were identified (Perkins et al., 1986; Sulston et al., 1980). Very recently, Barrios and Poole discovered four additional male-specific neurons that arise from glial cells (Molina-García et al., 2019; Sammut et al., 2015).

All these works laid the foundation for the wide body of literature that provides explanations for a large range of biological questions including those relevant to human development and disease. A key feature of this organism, one that makes it a hugely powerful model but also introduces some challenges, is that it is a hermaphroditic species with an extremely low incidence of males (0.2–0.5%). Brenner generated male stocks, a product of offspring resulting from male-mated hermaphrodites, which would result in 50% male progeny (Brenner, 1974). Jonathan Hodgkin identified high incidence of males (*him*) mutations that enable male stock maintenance without mating crosses (J. Hodgkin et al., 1979). These *him* strains are widely used and key to studying any aspect of *C. elegans* male biology.

In this paper, we focus on new advances in understanding sexually dimorphic development and behavior in the *C. elegans* male nervous system made possible by the pioneering work of John Sulston. In addition, we present a CRISPR/Cas9 method to engineer a mutation in the *him-5* gene that will facilitate study of *C. elegans* male biology in this hermaphroditic species.

***C. elegans* comes in two sexes**

C. elegans has two sexes: male and hermaphrodite. Studies of the two sexes have defined molecular mechanisms driving sexually dimorphic processes, including sex determination, differentiation, and development. However, investigations into genes influencing adult male behaviors have been limited. The *C. elegans* genome is comprised of ~20,122 protein-coding genes, of which ~6,000 have no assigned function estimated by lack of gene ontology associations (https://wormbase.org/about/wormbase_release_WS274#0--10). Hence to fully understand the organism and its genome, it is essential to study both sexes.

C. elegans sex is determined by the X to autosome ratio. Hemizygous XO animals are males. XX animals are hermaphrodites, which are morphologically female whose germ line makes sperm prior to producing oocytes. This dosage dependent sex-determination is controlled through a transcription factor, TRA-1A that has multiple target loci (Berkseth et al., 2013; J. Hodgkin, 1987a; Zarkower & Hodgkin, 1992). The self-fertilizing hermaphrodite will produce ~300 offspring, of which 99.5 – 99.8% are XX hermaphrodites and 0.2 – 0.5% are XO males due to nondisjunction (J. Hodgkin et al., 1979). The frequency of males can be increased by heat shock (J. Hodgkin, 1983), by crossing males with hermaphrodites, or by introducing mutations that increase chromosomal nondisjunction. Males crossed with hermaphrodites yield 50% XX hermaphrodites and 50% XO males with offspring counts as

high as 1,400 per mated hermaphrodite (Jeffrey R. Chasnov, 2013). Mutations that increase the frequency of nondisjunction (*him*; high incidence of males, (J. Hodgkin et al., 1979)) allow the efficient production of male progeny even if males cannot sire cross progeny. *him-5* hermaphrodites generate 35% male progeny. *him-5* males exhibit wild-type mating efficiency and behavior. The ability of hermaphrodites to self-fertilize obviates the need to propagate by mating and enables study of male related physiology/behaviors. The hermaphroditic reproduction strategy is a powerful tool to isolate mutants defective in male development, fertility, or behavior.

The neurons and mechanisms of male mating behavior

The *C. elegans* male nervous system possesses 387 neurons to the hermaphrodite's 302. The cephalic and amphid sensory organs in the head are found in both males and hermaphrodites and display sexually dimorphic features. In the male cephalic organs, the cephalic socket creates a pore through which the cephalic male CEM cilia protrude and release ciliary extracellular vesicles to the environment (Ware 1973, Ward 1975, Wang 2014, Silva 2017, Akella 2019). In the hermaphrodite cephalic organs, the four cephalic male CEM neurons that are programmed to die and the cephalic socket cell is closed to the environment. In the L4 male, the bilateral amphid socket glia undergo a male-specific division to self renew and to generate two mystery cells of the male (MCM). Hence, the male and hermaphrodite display sexual dimorphisms in sex-shared glia and in the male-specific nervous system (Molina-García et al., 2019; Sammut et al., 2015; Sulston et al., 1980; White et al., 1986). Among the sex-shared ventral cord neurons there are sex-specific motor neurons that arise from the same precursor cell lineages in males and hermaphrodites: The CA/CP motor neurons in the male and VCs in the hermaphrodite (Sulston et al., 1980; Sulston & Horvitz, 1977). With the male ventral cord CA/CP neurons and the head CEM and MCM neurons as the exceptions, the majority of the male sex-specific neurons are located in the tail.

In a landmark series of papers, Sulston characterized the lineage and development of the nongonadal structures of the *C. elegans* male (Sulston et al., 1980; Sulston & Horvitz, 1977, 1981; Sulston & White, 1980). The male tail consists of an elongated bursa, fan, and proctodeum. The fan is composed of nine pairs of bilaterally arranged rays. The proctodeum houses two spicules and the gubernaculum. Additionally, there is a hook sensillum located anterior to the cloaca and a left-right pair of postcloacal sensillae. Each ray and hook sensillum contains two ciliated sensory neurons, an A type neuron whose ciliated sensory ending is embedded within the cuticle and is predicted to be mechanosensory, and a B type neuron whose cilium is exposed to the environment (with the exception of ray 6B) and is predicted to be chemosensory. Each ray has one glial structure cell (in contrast to other sensilla that have socket and sheath glial cells). Each spicule has two ciliated sensory neurons (SPV and SPD) and one proprioceptive SPC motor neuron.

Copulation behavior is one of the more ancient social behaviors, yet remarkably little is known about the molecular basis of sexual behaviors. *C. elegans* male mating behavior offers a powerful model to study the nervous system, genes, and behavior. In *C. elegans*, the male performs most of the overt sensory and motor behaviors that occur during mating. Male mating behavior is the most complex behavior in *C. elegans*. *C. elegans* copulation

involves an innate series of behavioral substeps, raising the question of how these behaviors are genetically controlled. Though intricate, male mating behavior can be broken down into simpler, stereotypical sub-steps: response, backing, turning, location of vulva, spicule insertion, and sperm transfer (J. Hodgkin, 1983; Liu & Sternberg, 1995). In his Worm Breeders' Gazette correspondence "The Turn and the Screw" (<http://www.wormbook.org/wli/wbg7.2p22/>), Sulston et al describe the first laser ablations of *C. elegans* male-specific neuronal precursors to explore neuronal function in mating. This 1980s Worm Breeders' Gazette hardcopy version of an online BioRxiv preprint paved the way for Liu and Sternberg's seminal paper on the neuronal basis of *C. elegans* male mating behavior (J. Hodgkin, 1983; Liu & Sternberg, 1995).

Many of the male-specific neurons are associated with male-specific behaviors (Maureen M. Barr et al., 2018; Maureen M. Barr & Garcia, 2006; Liu & Sternberg, 1995). Each behavioral sub-step is regulated by a specific set of male-specific neurons, which provides great power when experimentally identifying the functional relevance of behavioral phenotypes. The shared nervous system (found in hermaphrodites and males) also contributes to male behaviors (Emmons, 2018). In this context, the shared nervous system may possess sexually dimorphic properties.

The sex-shared nervous system and male-specific remodeling:

Early pioneering studies by Sulston and White provided a fundamental understanding of how a sexually dimorphic nervous system is constructed and wired. The *C. elegans* sexes share a large portion of the nervous system (294 sex-shared neurons), however, these neurons will adopt male or hermaphrodite functionality based on their genetic sex. These sex-specific differences can range from morphological changes, altering synapse partners, and even changing their response to environmental chemical cues (Portman, 2017). The hermaphrodite-specific lineages have been extensively studied (Hobert, 2018), but the male sex has been somewhat overlooked, largely due to the challenges that arise when pursuing male-specific questions in a predominantly hermaphroditic species. Recent serial electron microscopy reconstructions and completion of the whole animal connectomes of both sexes by Emmons and Hall have provided profound new insights regarding sexual dimorphisms in the nervous system (Cook et al., 2019).

A prime example of morphological differences of sex-shared neurons and male-specific remodeling of the shared nervous system is the phasmid C PHC neuronal pair. In hermaphrodites these neurons are responsible for responses to harsh tail touch and innervate the pre-anal ganglion. In the male, these neurons extend into the tail during L4 stage, innervate male-specific motor neurons and get repurposed for male mating behavior (Serrano-Saiz et al., 2017). Male-specific remodelling can also result from past experiences. DVB neurites in males get remodeled in response to copulatory behavior. Males that are more practiced in mating demonstrate increased mating success attributed to a higher chance of spicule insertion and sperm transfer (Hart & Hobert, 2018).

The shared phasmid B PHB phasmid neurons also undergo a sex-specific rewiring program. Sexual maturation leads to synaptic pruning of connections from this neuronal pair in both

males and hermaphrodites (Oren-Suissa et al., 2016). In adults, PHB neurons connect to the AVA interneuron pair in hermaphrodites but to the AVG interneuron in males (Cook et al., 2019; Jarrell et al., 2012; White et al., 1986). As juveniles, both male and hermaphrodite PHB neurons synapse to AVA and AVG neurons, demonstrating sexually dimorphic pruning during sexual maturation (Oren-Suissa et al., 2016). This sex specific pruning has been attributed to male-specific *unc-6* netrin expression in AVG interneurons (Weinberg et al., 2018).

Nervous system developmental plasticity in the male

Two cases of the developmental plasticity in a sex-dependent manner comes from shared socket glia cells that - in only males - transdifferentiate into a male-specific neuron or that undergo a cell division to self renew and to generate a neuron. Barrios and Poole discovered that the shared amphid socket glia cells divide in the L4 male to self renew and to generate the MCM neurons that are required for sex specific learning (Sammur et al., 2015). Shortly thereafter, Barrios and Poole discovered another case of male-specific neuronal generation, but this time via transdifferentiation of glial to neuronal identity. Between the 3rd and 4th larval stages in the male tail, phasmid socket 1 (PHso1) cells transdifferentiate into ciliated phasmid D PHD neurons that control male locomotion during mating (Molina-García et al., 2019). Sulston noted that the male and hermaphrodite adult phasmid sensory organs were different (Sulston et al., 1980), but did not observe this late transdifferentiation event in his lineage studies. This brings the anatomy of the *C. elegans* male to 387 neurons (93 of which are male-specific) and 90 glia (Molina-García et al., 2019). The number of male-specific EF and DX interneurons varies between three and four depending on the worm and therefore some males have 385 neurons (Cook et al., 2019).

Sex-specific responses to neurotransmitters and external cues as a result of dimorphic circuitry

Further dimorphisms are observed between the sexes when it comes to neurotransmitter identity (Hobert & Kratsios, 2019). AIM neurons switch to a male-specific neurotransmitter identity from glutamatergic to cholinergic at the L4 larval stage, whereas hermaphrodites maintain their glutamatergic identity (Pereira et al., 2015). Environmental effects can also have an impact on sex-specific responses to chemicals. Sex-specific pruning can be disrupted by starvation of juvenile males, leading to an increase in sensitivity to chemical cues from the environment (Bayer & Hobert, 2018). The male-specific repression of the diacetyl receptor gene *odr-10* in AWA neurons prioritizes mating over feeding, demonstrating that sex-specific sensory receptor expression in a shared neuron (Ryan et al., 2014). Defecation and foraging behaviors are examples of behavioral programs that are controlled/respond through sex-shared neural circuits that are dimorphic (Gruninger et al., 2006; LeBoeuf & Rene Garcia, 2017). Neural circuits that govern sex-specific behaviors are recently reviewed by Scott Emmons (Emmons, 2018).

A class of neurons with sex-shared and sex-specific implications in behavior and physiology: The ciliary extracellular vesicle releasing neurons

Extracellular vesicles or EVs are tiny membrane bound packets that carry proteins, lipids, metabolites, and nucleic acids and are secreted from cells in all kingdoms of life. Many human cell types secrete EVs into body fluids and these EVs can potentially signal between cells and tissues. For more information about EVs, we refer the readers to reviews on EV biogenesis and function (Beer & Wehman, 2017; Cicero et al., 2015; EL Andaloussi et al., 2013; Stahl & Raposo, 2019).

Extracellular vesicle releasing neurons (EVNs) are comprised of 21 male-specific and six sex-shared ciliated neurons that possess the remarkable ability to shed and release ciliary extracellular vesicles (EVs) into the environment or extracellular space that are involved in animal-to-animal communication (Figure 1B) (Wang et al., 2014). The six sex-shared inner labial type 2 (IL2) neurons are involved in alcohol sensing, nictation behavior, and sensing blue light (Johnson et al., 2017; Lee et al., 2011; Zaslaver et al., 2015). The IL2 neurons have no known function in mating behavior. The 21 male-specific neurons are 4 cephalic male CEM neurons, 16 RnB neurons (ray 1 to 9, excluding ray 6, left and right symmetry), and one HOB hook neuron (Barrios et al., 2008; M. M. Barr et al., 2001; M. M. Barr & Sternberg, 1999). The CEM neurons respond to chemoattractants secreted by hermaphrodites for mating (J. R. Chasnov et al., 2007). The HOB neuron is required for vulva location and the ray neurons are important for response and turning behavior (Liu & Sternberg, 1995). To date, these 27 ciliated neurons are the only known neurons to release GFP-tagged ciliary EVs (or any EVs) outside the worm. *C. elegans* does produce other EVs inside the body of the animal, including EVs important for gastrulation in the embryo, cuticle formation during larval development, extrinsic repair of injured neurons, and neuronal waste expulsion in the adult (Beer & Wehman, 2017; Kolotuev et al., 2009; Liégeois et al., 2006; Melentijevic et al., 2017; Oren-Suissa et al., 2017; Wehman et al., 2011).

The cilium is a site of EV shedding and environmental release (Figure 1B). *C. elegans* cilia possess a similar basic structure to that of other ciliated organisms. At the base is the periciliary membrane compartment (akin to the ciliary pocket in mammalian cells) and transition zone. The periciliary membrane compartment acts as a trafficking hub for sorting proteins between ciliary and cell body destinations (Kaplan et al., 2012). The axoneme consists of the transition zone, middle and distal segments (Akella et al., 2019; Doroquez et al., 2014; Perkins et al., 1986; Silva et al., 2017). Environmental release of ciliary EVs is regulated by the conserved intraflagellar transport (IFT) machinery and EVN-specific ciliary resident proteins (O'Hagan et al., 2017; Silva et al., 2017; Wang et al., 2014, 2015). The kinesin-3 protein KLP-6 and myristoylated coil-coil protein CIL-7 are exclusively expressed in the 27 EVNs and regulate ciliary EV release (Maguire et al., 2015; Wang et al., 2014). Ciliary kinesin-2 and kinesin-3 KLP-6, IFT components, tubulin, and tubulin glutamylases and deglutamylases are required for ciliary EV biogenesis (O'Hagan et al., 2017; Silva et al., 2017; Wang et al., 2014). EVs may also function in neuron-glia communication through periciliary membrane compartment-released EVs at the base of CEM cilia, which get

released into the pore of the cephalic sensory organ formed by sheath and socket cells (Akella et al., 2020). The functional significance of polycystin regulation of mating behavior, interactions between the polycystins, the function of polycystins in EVs, and the function of EVs in mating behavior in general remain enigmatic and are currently being investigated.

Clinical relevance of mating behavior in *C. elegans*

Among the most important discoveries related to *C. elegans* and mating behavior, was that LOV-1 and PKD-2, orthologs of human polycystin-1 (PC1) and polycystin-2 (PC2) respectively, are involved in male mating behavior and are localized to cilia of male-specific neurons (M. M. Barr et al., 2001; M. M. Barr & Sternberg, 1999). LOV-1 and PC1 share a similar architecture as an 11 transmembrane receptor with a large extracellular domain. PKD-2 is a transient receptor protein (TRP) polycystin channel (Hanaoka et al., 2000; Nauli et al., 2003; Sharif-Naeini et al., 2009). *lov-1* and *pkd-2* are co-expressed and act in the 21 male-specific ciliated EVNs. LOV-1 and PKD-2 localize to cilia and are secreted in ciliary extracellular vesicles. In EVs isolated from mixed cultures, PKD-2 and other cargo are required to elicit male tail chasing and circling (Silva et al., 2017; Wang et al., 2014). In neurons, *lov-1* and *pkd-2* are required for sex drive, response to mate contact, and vulva location (Barrios et al., 2008; M. M. Barr et al., 2001; M. M. Barr & Sternberg, 1999). Remarkably, ciliary extracellular vesicles (EVs) carrying polycystin-2 are released upon mechanical stimulation of the sensory cilia of male and are targeted at the vulva of his mating partner. This is the first report of directional transfer of activity-evoked EVs from one animal to another (Wang et al., 2020).

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a common, life threatening disease that affects 1/400–1/1000 individuals. ADPKD is caused by mutations in PKD1 and PKD2, which encode polycystin-1 and polycystin-2 (PC1 and PC2) (Boucher & Sandford, 2004; Igarashi & Somlo, 2002). Remarkably, the function of the polycystins remains an enigma 25 years after their cloning and 17 years after their discovery on renal primary cilia. Besides cilia, PC1 and PC2 are also found in other subcellular locations including extracellular vesicles (EVs) of human urine. Urinary EVs can be used as biomarkers of renal disease (Karpman et al., 2017) including ADPKD (Hogan et al., 2015). Whether these polycystin-carrying EVs are of ciliary origin and what role EVs play in healthy and diseased kidneys remains unknown. The *C. elegans* ADPKD model is a beautiful example of the utility of model organisms in studying the underlying mechanisms of human disease. In *C. elegans*, knockouts of either *lov-1* or *pkd-2* polycystin results in a mating behavior defect but is lethal in mice (M. M. Barr et al., 2001; Boulter et al., 2001; Wu et al., 2000). In *C. elegans* and mammals the polycystins LOV-1/PC1 and PKD-2/PC2 act in the same genetic pathway and function in a sensory capacity (M. M. Barr et al., 2001; Yoder et al., 2002).

The complications that arise when studying male-specific biology in a hermaphroditic species

C. elegans is a powerful model organism that has been used by biologists for over half a century (Brenner, 1974; Fatt & Dougherty, 1963; Nigon & Dougherty, 1949). Although a

dioecious species, *C. elegans* strongly favors self-reproduction over mating and the incidence of males in a wild-type population is ~0.2 – 0.5% (J. Hodgkin, 1983). Male biology is a subject of interest for researchers, and studying genes specifically expressed in the male presents some unique challenges. The first generation of heat-shock generated ‘male stocks’ can have stress-induced epigenetic changes that persist for multiple generations (Klosin et al., 2017; Zhou et al., 2019). This dilemma is compounded when experimenting with genes that when mutated result in male mating deficiencies. When trying to make male-specific CRISPR reporters or knockouts, it seems intuitive to do this in a high incidence of males strain such as *him-5*. The *him-5* mutation increases the rate of male progeny to 35% but does not increase brood size so this comes at the cost of a decrease in hermaphrodite progeny (J. Hodgkin, 1983). This deficit of hermaphrodites is especially problematic when the CRISPR mutation efficiency is below a certain threshold. In our experience, we have not been able to successfully create a CRISPR-generated fluorescent protein fusion in *him-5* background, whereas we have no issues using N2. To further complicate the use of the *him-5* genetic background for generating CRISPR-induced mutations, *him-5* affects repair mechanism choice as a negative regulator of meiotic non-homologous end joining (NHEJ) (Macaisne et al., 2018; Mateo et al., 2016). This could severely reduce the efficiency of homology directed recombination (HDR) in *him-5* strains for CRISPR-mediated recombination. The obvious solution to this issue is to cross in the desired *Him* strain after generating CRISPR-induced mutations in the N2 background. However, this strategy has its own limitations that can be burdensome and time consuming. Examples include when genes are closely linked to the *Him* allele, when crossing mating deficient strains, or when dealing with strains containing multiple mutations that must all be homozygous after a cross. There are current methods for generating males in higher incidence that do not rely on crossing or heat shock to generate ‘male stocks.’ These strategies, such as male-producing RNAi and using transgenes to masculinize hermaphrodites, provide their own unique challenges and advantages.

RNAi has been used as a temporary means to generate males. *him-14* RNAi (also known as ‘male food’) was first described as “effective and reversible, but not efficient” in a Worm Breeder’s Gazette correspondence “RNAi feeding to produce males” (Killian and Hubbard 2001). A systematic comparison of other Him inducing RNAi targets was later performed, identifying RNAi induced knockdowns of several genes that were more efficient at generating males (Timmons et al., 2014). These *Him* inducing gene knockdowns are useful for cases where a temporary need for males is sufficient or where reversibility is required. Due to the temporary nature of this method, other approaches would be more appropriate for experiments that require permanent production of males.

Masculinization of hermaphrodites is an alternative strategy for studying the development of sex-based differences in *C. elegans* and can serve a different purpose than studying wild-type males. Specific loss of function or gain-of-function alleles can effectively masculinize hermaphrodites (J. Hodgkin, 1987b; J. A. Hodgkin & Brenner, 1977). These alleles range in phenotype from mild masculinization with no male behaviors to nearly complete masculinization with pseudo-males demonstrating mating behavior but with low fertility (J. Hodgkin, 1987b; Schedl et al., 1989). Feminization of hermaphrodites is also possible (Barton et al., 1987; J. Hodgkin, 1987b; J. A. Hodgkin & Brenner, 1977; Schedl et al.,

1989). This has been very useful at uncovering essential biological and behavioral aspects of sex differences in the *C. elegans* nervous system. Examples include using transgenics to study environmental sexual conditioning by masculinizing/feminizing the nervous system (Sakai et al., 2013) and masculinizing/feminizing single neurons or subsets of neurons to study sexual differences in sex-shared neurons (Hart & Hobert, 2018; Oren-Suissa et al., 2016). Phenotypic variation among these mutants however, necessitates studying male biology in genetically male animals to fully understand the development of the male nervous system and male-specific behaviors.

Here we propose a different approach for addressing some of the challenges associated with studying male-specific biology in a hermaphroditic species: a CRISPR/Cas-9 mutagenesis strategy, designed to effectively and reproducibly generate males in almost any genetic background.

The *him*CRISPR method

We used CRISPR/Cas9 technologies to induce the equivalent of the *him-5(e1490)* point mutation for the purposes of studying male specific physiology and/or behaviors. The *him-5(e1490)* mutant was identified in a forward genetic screen (J. Hodgkin et al., 1979) and generates a high incidence of males at ~35% as a result of a single point mutation of the splice acceptor site of intron 3 (Figure 2, Supplemental Figure 1) (Meneely et al., 2012). This method relies on the CRISPR-induced homology-directed repair (HDR) of our target locus (*him-5*) with the use of single-stranded oligonucleotides (ssODN) as the repair donor with ~70bp of homology (35bp on each side of edits) (Paix et al., 2014). Our ssODN donor is 161bp long and the homology arms of the construct are located within 100bp of the cut site. We injected a mix of Cas9 protein, crRNA, tracrRNA, ssODN, and a pRF4 (*rol-6*, dominant roller) co-injection marker to screen for transformants (Dokshin et al., 2018). In addition to the *him-5(e1490)* point mutation, the ssODN includes other silent mutations to prevent re-cutting post-recombination and to generate a restriction enzyme site for screening for successful editing. Young adult N2 worms are injected with the injection mix. F1 rollers were singled 3–4 days after injection and allowed to lay eggs. The F1 rollers were then genotyped via single-worm PCR and restriction enzyme digest to identify successful edits. F2s that were genotyped homozygous or heterozygous were singled onto multiple plates. F3s with a high incidence of males were identified as homozygous for the *him-5* CRISPR-induced mutation. Homozygotes of the gene of interest are confirmed through genotyping lysates from a pool of F3 from each *him* F2 plate, and the mutation were sequenced by Sanger sequencing of PCR products (Supplemental Figure 2). In this paper, two independent isolates *him-5(my80)* and *him-5(my81)* were phenotypically analyzed and found to recapitulate the *him-5(e1490)* phenotype.

Our *him*CRISPR approach is very efficient. With the small group of F₁ progeny selected from one round of injections, 8/11 had mutation events occur at only one of the *him-5* alleles while 1/11 had mutation events at both. In other words, ~82% of F₁ progeny genotyped for the expected mutation event (Supplemental Figure 1). This is within the range of expected efficiencies as reported previously (Dokshin et al., 2018). In some cases, NHEJ recombinants may be identified, in addition or instead of HDR recombinants, that produce

indels resulting in higher incidence of males when using this method. In this case, it is up to experimenter discretion to use this variant over the well characterized *him-5(e1490)* mutation. Our motivation for the *him*CRISPR method was to generate an efficient way to acquire high incidence of males and replicate the phenotype of the widely used *him-5(e1490)* splice-site mutation strain.

Characterizing *him-5* CRISPR strains

We analyzed brood size, male progeny count, male mating efficiency, and male mating behaviors using standard assays (J. Hodgkin, 1983; Liu & Sternberg, 1995; Meneely et al., 2012). Quantification of brood size and number of male progeny were used to characterize our CRISPR strains (Meneely et al., 2012). Brood size and incidence of males assay was performed by placing single hermaphrodites on a seeded plate, allowed to lay eggs for 24 hours, then moved to a new plate. This was done over the course of 5 days or until the hermaphrodite was sperm depleted (N=15). Our CRISPR-generated strains produce males at frequencies that are comparable to both our *him-5(e1490)* strain and the expected frequency described in the literature (Figure 3A)(Meneely et al., 2012). Brood size was also comparable to *him-5(e1490)* and *N2* (Figure 3A).

Mating efficiency data was collected according to Hodgkins (J. Hodgkin, 1983). Mating efficiency assays were conducted by placing 6 males and 6 hermaphrodites onto a mating plate for 24 hours. Males were then removed from the plate and hermaphrodites were allowed to lay eggs. After each day, cross progeny and total progeny were counted and the mated hermaphrodites were moved to a new plate. These counts were done over the course of 5 days. Three independent mating plates were analyzed for each genotype for three independent replicates (N=9, 27 total males per genotype). Test strains were mated to *dpy-17* and *unc-52* hermaphrodites and cross progeny were compared between groups. We use these two strains to distinguish between varying levels of mating defects as each strain introduces a different level of difficulty for the males. *unc-52* worms are extremely Unc (uncoordinated) and adults do not move at all except for slight head movements for feeding. This makes them much easier mating targets than *dpy-17* hermaphrodite partners. Using these two strains can give us a measure of just how mating deficient a strain is. With the mating efficiency assay, a failure to sire cross progeny may reflect a defect in mating behavior, but could just as likely be caused by defects in male tail development, sperm, or general locomotion. Both *him-5(my80)* and *him-5(my81)* strains mated at similar efficiencies to the wild-type *him-5(e1490)* when mated with either *unc-52* or *dpy-17* (Figure 3B).

Finally, mating behavior assays were performed according to Sternberg (Liu & Sternberg, 1995) and the data was used to calculate response and vulva location efficiencies. For the mating assays, day one adult virgin males (isolated at the L4 stage away from hermaphrodites) were placed on mating plates with 20 day-one *unc-31* hermaphrodites (isolated at L4 away from males). Mating was observed for 5 minutes and each mating step was observed and recorded (response, backing, turning, vulva location, spicule insertion, sperm transfer). Response and vulva location efficiencies were calculated and compared to *him-5(e1490)* as the wild-type control, and *pkd-2(sy606); him-5(e1490)* which is defective

in response and location of vulva (Lov) defective. Both *him-5(my80)* and *him-5(my81)* exhibited normal mating behavior with respect to response and location of vulva when compared to *him-5(e1490)* (Figure 3C–D). Our analysis of the *him*CRISPR demonstrates that *him-5(my80)* and *him-5(my81)* are indistinguishable from the original *him-5(e1490)* strain that is commonly accepted as a wild-type control for mating assays.

Discussion

CRISPR/Cas9-mediated mutagenesis is an effective alternative for increasing the incidence of male progeny in *C. elegans*. Our CRISPR-generated *him-5* strains are phenotypically indistinguishable from CB1490 [*him-5(e1490)*] with regard to brood size, mating efficiency, and male response and vulva location behaviors. The *him*CRISPR method can be adapted for the other commonly used *him* strain, *him-8*, by simply altering the sgRNA and homology repair construct to target that locus. We use *him-5(e1490)* for the majority of our male-related experiments, therefore, this was the subject of our investigation for validating this method. This *him*CRISPR method is a great way to circumvent experimental challenges when studying males in a hermaphroditic species by being able to generate any *Him* strain regardless of genetic background.

This tool is uniquely suited to address challenges of studying male-specific biology in a hermaphroditic species. One such challenge presents when genes are directly involved in male mating success, thus loss-of-function mutation strains that are not already in a *him-5* background will make genetic crosses complicated and time consuming at best. With this *him*CRISPR strategy, mutations that result in loss or lack of male-mating success will be less problematic since common strategies for generating high incidence of males relies on mating. Mutations that are closely linked on the same chromosome as *him-5* (or *him-8*) will no longer require reliance on recombination frequencies. Finding recombinants can be cumbersome and time consuming when the genes are closely linked (1% linkage or less). Another challenge we have faced is generating fluorescently-tagged reporters of male-specific genes using CRISPR. Given that we have had better success with generating fluorescent protein-tagged CRISPR mutations in the N2 background, we generate male stocks via heat shock to examine gene expression patterns before generating permanent male-producing lines through crossing. With this tool, we will be able to induce both the *him-5*CRISPR allele and the male-specific fluorescent-tagged protein of interest in multiplex. This can also be a tool for addressing concerns regarding phenotypic discrepancies in genetically different backgrounds, which is an issue with commonly used N2 male-stocks (Zhao et al., 2019). Heat shock alone, as a means of generating males, can be a cause for phenotypic differences through stress induced genetic changes (Klosin et al., 2017; Melnick et al., 2019). In addition to circumventing these specific challenges, it would be interesting to consider this tool as a way to study male evolution by generating males in other hermaphroditic *Caenorhabditis* and/or other nematode species (Cutter et al., 2019).

Mating behavior is the most complex behavior exhibited by *C. elegans*. As with any *C. elegans* behavioral experiment, male mating assays are influenced by many variables including environment, experimental bias, and day-to-day differences in behavioral responses (http://www.wormbook.org/chapters/www_behavior/behavior.html). For

reproducibility, controls (wild-type *him-5* and a mating defective mutant such as *pkd-2*; *him-5*) are essential. If wild-type *him-5* males do not behave normally, then assays for the day are terminated. To avoid experimental bias, the observer should be blinded to the genotype of the animals being assayed. Environmental variations include culture conditions, transfer of males to mating plates, ambient temperature, humidity, and time of day when assays are performed.

With the complexity of mating behavior in general, slight differences in how assays are performed may have profound impact on male behavioral output and its interpretation. A striking example comes from studies looking at the effect of aging on male reproductive abilities. Hart and Hobert demonstrated an increase in mating success in day 3 males over day 1 males that correlated with remodeling of the DVB neurites and repeated sensory input over time (Hart & Hobert, 2018). This study focused on spicule insertion and sperm transfer and found that day 1 males had a higher incidence of spicule prodding but lower incidence of spicule protraction whereas day 3 males demonstrated the inverse, thus were better at mating (Hart & Hobert, 2018). Mating efficiency was not measured. In contrast, others report a clear decline in mating success in day 3 or older males (Chatterjee et al., 2013; Guo et al., 2012). To assess age-associated decline in mating, Chatterjee et al. 2013 used mating efficiency, mating response, and vulva location assays in day 1 and day 5 males (only assessed mating efficiency at day 3). This study also used a ring of chemical repellent (garlic) to trap males and reduce escapees, which is not commonly used. Guo et al. 2012 measured mating potency, which is a variation on the efficiency assay. In the mating potency assay, single males are mated with temperature-sensitive *pha-1* hermaphrodites at the restrictive temperature to ensure only cross progeny can be produced. The proportion of males that can produce any cross progeny are scored. This is in contrast to the mating efficiency assay that utilizes Dpy and Unc hermaphrodites, mates multiple males per mating plate, and scores all progeny as a proportion of cross to total progeny. The ease of the mating potency assay is that one does not have to count cross progeny, rather one simply identifies whether there are any cross progeny on the plate. Although these approaches are reaching for the same outcome, differences in the assays such as hermaphrodite substrates (moving versus non moving) and the nature of the readout can influence behavioral assay interpretations. Guo et al. 2012 also used spicule insertion and sperm transfer as a measure of male-mating success and concluded that male mating was significantly reduced in day 3 males (Guo et al., 2012). Depending on specific assays and parameters, one can draw different conclusions regarding the impact of aging on male virility.

In Hodgkin 1983, an attempt to create a standardized measure of mating efficiency was proposed by calculating a tiered system of efficiencies (i.e. a 0–4 scale, 4 being perfect mating efficiency 0 being no detectable mating efficiency). Using those criteria, the negative control we use for mating assays, *pkd-2(sy606)* would be a 3 on that scale (10–30% of WT), which is considered efficient mating, even though it is vulva location defective and has a significantly lower production of cross progeny. These examples demonstrate how the complexity of behavior in a biological system is difficult to standardize, and great care must be taken when comparing results from multiple studies.

The pioneering work of John Sulston et al. led to the completion of the entire *C. elegans* connectome in both sexes and paved the way to understand how the male-specific nervous system and sex-shared nervous system to integrate organismal survival with reproductive function. New evidence of nervous system developmental plasticity in the male reveals an unexplored area of male-specific *C. elegans* physiology. The two incidences of glia to neuron transformation during sex maturation raises a fundamental question: to what extent is the *C. elegans* nervous system plastic and capable of rewiring and/or regenerating? Sex-specific responses based on dimorphic properties of sex-shared neural circuits reveals yet another layer of development that provides fundamental insights into mechanisms of sex-specific behavior and neural physiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Abbreviated wiring diagram of sexually dimorphic neurons

A) Sex-shared neurons that are sexually dimorphic after sexual maturation of the male. Includes an example of each concept described in the paper. Top worm is hermaphrodite and the bottom worm is male. Neuron cell bodies are depicted as circles, neurites as lines, and glia depicted as ovals (AMso and PMso1). Pharynx is grey. Color coded boxes represent the sexually dimorphic property of that neuron, which is also color coded. **B)** Extracellular vesicle releasing neurons (EVNs) in males (bottom) and hermaphrodites (top). Pharynx (grey) and phasmids (white circles) are shown as landmarks for head and tail, respectively. EVN cell bodies are shaded green. The structure of a CEM cilium is shown (bottom). EVs are seen inside and outside the worm (green circles).

A. *him-5* B. *him-5*

Figure 2: Genomic structure of *him-5* and mutagenesis strategy.

A) Genomic structure of *him-5* depicting the 7 exons in black. Black arrows indicate the sites of nucleotide changes introduced in the CRISPR mutagenesis specified by Roman numerals I – III (I = e1490 CAG → CAA mutation; III = silent mutation introducing HindIII restriction site; III = Silent mutations to prevent re-cutting of the sgRNA site). sgRNA target site is indicated by the bracket below exon 4. Scale bar is 100bp B) Nucleotide-level depiction of the site of mutagenesis for the *him-5* CRISPR allele. Box surrounds exon 4. Filled black arrow indicates the splice site mutation that is the causal mutation for the *him-5(e1490)* high incidence of males (AG → AA). Single-lined arrow indicates the silent point mutation that introduces a BamHI restriction enzyme site. The three double-lined arrows indicate the silent mutations introduced to disrupt the sgRNA target site to prevent re-cutting after HDR has occurred.

Figure 3: *him*CRISPR strains *him-5(my80)* and *him-5(my81)* display the same sex related characteristics as *him-5(e1490)*.

A) Total progeny of singled hermaphrodites was counted over 5 days (N=15, 3 independent replicates). *him-5(my80)* and *him-5(my81)* male percentage were not significantly different from *him-5(e1490)*. *him-5(my80)*, *him-5(my81)*, and *him-5(e1490)* were significantly different from N2 $P < 0.001$ (non-parametric one-way ANOVA, Dunn's post hoc analysis). B) Mating efficiency was assayed by comparing the ratio of cross progeny to total progeny in the F1 generation from a cross with either *dpy-17* or *unc-52* hermaphrodites. *him-5(my80)*, *him-5(my81)*, and *him-5(e1490)* exhibited statistically similar mating efficiency, whereas they all performed significantly better than *pkd-2(sy606)* ($P < 0.01$), a mating deficient strain (non-parametric one-way ANOVA, Dunn's post hoc analysis). C&D) Response and vulva location efficiency were measured in mating behavior assays. The two graphs represent

different measures of the same experiment. For *him-5(my80)*, *him-5(my81)*, and *him-5(e1490)* N=30. For *pkd-2(sys606)* N=30 for response, however only one male responded during the course of the assays, therefore for the vulva location measure, N=1. Neither response nor vulva location differed among *him-5(my80)*, *him-5(my81)*, and *him-5(e1490)* and were all significantly different from *pkd-2(sys606)* (Response P<0.001, location of vulva P<0.05, non-parametric one-way ANOVA, Dunn's post hoc analysis).