DISSERTATION

Titel der Dissertation

The developmental potential of the *C. elegans* RFX transcription factor DAF-19

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Doktorin der Naturwissenschaften (Dr. rer.nat.)

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Am I content?

No, and I hope I never will be.
The day you wake up and everything's perfect – what's left? What direction is there to go? Much better to wake every morning to matters, which worry you a bit, affairs that demand your attention and work you feel must get done. These are the things that keep me going. I love to search for solutions, to solve things. I guess you could even say that … ***Problems make me happy****.*

Renzo Rosso – Founder and Owner of Diesel

Karolinska Institutet

The experimental work presented in this thesis was carried out in the laboratory of, and supervised by, Dr. Peter Swoboda. Dr. Swoboda and his laboratory are affiliated with the Department of Biosciences and Nutrition at the Karolinska Institute and the School of Life Sciences at Södertörn University in Stockholm, Sweden.

„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“

Zusammenfassung

Die Wahrnehmung und korrekte Interpretation von Signalen aus der Umwelt ist entscheidend für das Überleben jedes Organismus. Es ist eine grosse Herausforderung die Aufnahme und Weiterleitung von sensorischer Information zu studieren, speziell in den komplexen Nervensystemen höherer Organismen. Der Rundwurm *C. elegans* besitzt ein relativ simples Nervensystem aus 302 Neuronen. 60 davon haben Cilien (haar-ähnliche, sensorische Fortsätze) und sind somit die wichtigste Aufnahmequelle externer, sensorischer Information. Die Verhaltensmuster von *C. elegans* sind zahlreich, was den Rundwurm zu einem idealen Modellorganismus macht, in dem man die Funktion von sensorischen Neuronen studieren kann.

RFX Transkriptionsfaktoren sind essentiell für die Bildung von Cilien in vielen Organismen, unter anderem in Mäusen und Menschen. Das Fehlen von DAF-19, dem einzigen RFX Protein in *C. elegans* führt zum Fehlen aller Cilien und somit zur Unfähigkeit sensorische Information aufnehmen zu können. In **Paper I** beschreiben und charakterisieren wir drei Isoformen von DAF-19. Isoform DAF-19C ist spezifisch für Neuronen mit Cilien. Der Zusatz von DAF-19C genügt alle Cilien-bedingten Phänotypen in *daf-19* Mutanten wiederherzustellen. DAF-19A/B kommen in allen nicht-ciliierten Neuronen vor und regulieren dort synaptische Funktionen. Unsere Arbeit beschreibt zum ersten Mal, dass RFX Proteine nicht nur für die Aufnahme, sondern auch für die Weiterleitung von Signalen notwendig sind.

In **Paper II** untersuchen wir mögliche zell-autonome Funktionen von DAF-19 in ciliierten Neuronen. Wir etablieren und testen ein genetisches „Reparatur“ Verfahren, welches *in vivo* die Analyse einzelner ciliierter Neuronen sowohl auf dem Zell- als auch auf dem Organismus-Niveau erlaubt. Durch Wiederherstellung der Funktion von DAF-19C nur in bestimmten, einzelnen sensorischen Neuronen (in *daf-19* genetischen Mutanten) kreieren wir Tiere mit nur einem einzelnen, isolierten, jedoch funktionsfähigen ciliierten Neuron, wohingegen jedwede anderweitige sensorische Signale von diesen Tieren nicht aufgenommen werden können. Dieses experimentelle System kann

dazu benutzt werden spezifische sensorische Fragen in Bezug auf ciliierte Neuronen oder Schritte der frühen Cilienentwicklung zu studieren.

In **Paper III** untersuchen wir die mögliche übergeordnete Rolle von DAF-19 in der Entwicklung von Cilien. Wir exprimieren DAF-19C in verschiedenen Zelltypen und versuchen so die Bildung von Cilien in nicht-ciliierten Zellen zu induzieren. Dabei entdecken wir ein wahrscheinlich regulatorisches Netzwerk, welches festlegt, in welchen Zellen Cilien gebildet werden können und in welchen nicht. Wir nehmen an, dass isoform-spezifische Suppressoren von DAF-19 das für jeweils verschiede Zelltypen spezifische Aktivierungspotential regulieren.

Summary

The detection and correct interpretation of environmental signals is crucial for the survival of every organism. Studying mechanisms of sensory perception and signal transmission is a challenging task, especially in organisms with complex neuronal networks. The nematode *C. elegans* possesses a rather simple neuronal network of 302 neurons. 60 of them have cilia (hair-like surface structures), which are the main source of external sensory input. *C. elegans* executes a large number of different behaviors and is therefore an excellent model organism in which to study sensory neuron function.

RFX transcription factors are essential for cilia formation in many organisms including mice and humans. Lack of the *C. elegans* RFX transcription factor DAF-19 leads to the complete absence of cilia and consequently of sensory input. In **Paper I** we describe and functionally characterize three different isoforms of DAF-19. We find that the short isoform DAF-19C is specifically expressed in ciliated sensory neurons and sufficient to rescue all cilia-related phenotypes of *daf-19* mutants. The long isoforms DAF-19A/B function in all non-ciliated neurons, where they are required to maintain synaptic functions. Thus, we show for the first time that an RFX protein is not only required for signal detection, but also for signal transmission.

In **Paper II** we explore cell-autonomous functions of DAF-19 in ciliated sensory neurons (CSNs). We establish and test a genetic rescue system that allows the *in vivo* analysis of isolated CSNs at both cellular and systemic levels. Using *daf-19* mutants and cell-specific rescue of DAF-19 function we generate animals with single, functional CSNs, which otherwise are completely devoid of any environmental input through cilia. This system can be used to study specific sensory issues concerning CSNs or early steps of ciliogenesis.

Finally, in **Paper III** we explore a potential master regulatory role of DAF-19. We attempt to induce ectopic cilia in *C. elegans* by expressing DAF-19C in various non-ciliated cell types and discover a likely regulatory network that governs in which cell types cilia can be made. We hypothesize that isoform-specific suppressors of DAF-19 regulate this cell-type-specific ciliogenic potential.

List of Abbreviations

Aβ	amyloid beta
AcCh	acetylcholine
AD	Alzheimer's Disease
AP	adaptor protein
bHLH	basic helix loop helix
C. elegans	*Caenorhabditis elegans*
COP	coat opsonizing protein
CSN	ciliated sensory neuron
daf	dauer formation
Daf-c	dauer formation constitutive
Daf-d	dauer formation defective
EM	electron microscopy
HD	Huntington's Disease
IFT	intraflagellar transport
L1	larval stage 1
RFX	regulatory factor binding the X-box
RNAi	RNA interference
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Saccharomyces pombe*
UV	ultraviolet
v-SNARE	vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor

List of Publications

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I. **Senti G.** and Swoboda P.: *Distinct isoforms of the RFX transcription factor DAF-19 regulate ciliogenesis and maintenance of synaptic activity.* MOLECULAR BIOLOGY OF THE CELL (2008) 19, 5517-5528.

II. **Senti G.**, Löbner J., Swoboda P.: *FRISSC -Functional rescue in ciliated sensory neurons in Caenorhabditis elegans* (Manuscript).

III. **Senti G.,** Gonzalez J.C., Ulrika Kristersson U., Löbner J., Swoboda P.: *DAF-19C – a potential master regulator of ciliogenesis in Caenorhabditis elegans?* (Manuscript).

Publications not included in this thesis:

I. Miranda-Vizuete A., Gonzalez J.C., **Gahmon G.**, Burghoorn J., Navas P., Swoboda P.: *Lifespan decrease in a Caenorhabditis elegans mutant lacking TRX-1, a thioredoxin expressed in ASJ sensory neurons*. FEBS LETTERS (2006) 580, 484-490.

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Introduction

Organ systems, such as the nervous system or the systems responsible for respiration or blood circulation, are considered the largest functional units of a living organism. Organ systems are, in turn built from interconnected organs, such as the heart, blood vessels and the lung. The correct development, integration, and functioning of organ systems and their respective components are not only essential for survival, but also for interactions with the environment and other individuals.

Our nervous system receives external input via the five external senses: vision, hearing, smell, taste, and touch. In addition, it is also required to sense the internal status of the body. Sensory information is then usually passed on to the central processing unit, the brain, which integrates all information and sends commands to executing organs, for example, the muscles.

The mammalian nervous system is large, highly complex, and inaccessible. The brain alone is built from billions of nerve cells (neurons) of various types that need to be precisely wired in order to function correctly. It is difficult to investigate circuits or even single neurons in the mammalian brain. However, neuronal building blocks are highly conserved across organisms at the cellular, subcellular, and molecular level. Therefore, studying the molecular basis of nervous system development and function is greatly facilitated through the use of simpler model organisms such as the nematode roundworm *C. elegans*.

THE MODEL SYSTEM *CAENORHABDITIS ELEGANS*

C. elegans is a free-living, bacteriovore soil nematode, also called roundworm. It was introduced as a model organism for scientific research by Sydney Brenner more than 30 years ago (Brenner, 1974). Adult worms are about one millimeter in length and color-less and can be either males or self-fertilizing hermaphrodites. A single hermaphrodite is fertile for three to four days and in this time gives rise to 300 – 350 progeny. Embryos are laid several hours after fertilization and they complete development outside the mother worm. After embryonic development, worms progress through four distinct larval stages to adulthood (**Figure 1**). Each stage ends with a period of molting, replacing the old cuticle with an underlying new one, thereby giving the growing worm sufficient space. The length of the *C. elegans* life cycle varies depending on environmental conditions, such as temperature: at 20 °C it is approximately three to four days.

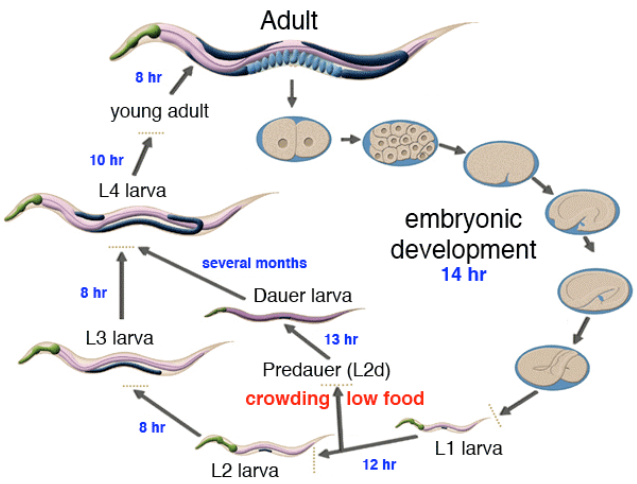

Figure 1: The C. elegans life cycle at 22 °C *(Artwork by Altun and Hall, adapted from www.wormatlas.org)*

A unique feature of *C. elegans* is its ability to overcome harsh environmental conditions by entering a special developmental stage, called the dauer larva. The decision to develop into a dauer larva is made at the early L2

stage and is triggered by a pheromone produced constitutively by every worm (Fielenbach and Antebi, 2008). A high concentration of pheromone is a result of high population density and therefore is a signal of a potential shortage of food. In addition to high levels of pheromone, food abundance and extreme temperatures can modulate the interpretation of the pheromone signal. Upon entering the dauer stage, cellular, metabolic and morphological transformations create a worm that does not feed, typically does not move and that is resistant to outside influences (e.g. extreme [both high and low] temperatures). This dauer stage is reversible. Improvement of the same conditions that trigger dauer formation (lower pheromone concentrations and temperature or increased abundance of food) can induce the worm to leave the dauer stage and resume reproductive development by progressing into the L4 larval stage.

C. elegans is now a well established model organism thanks to a large number of available techniques and tools. Transgenic animals can be generated efficiently and faster than in most other model organisms. Screens to identify gene deletions can be performed in large-scale set ups and have been recently complemented by RNAi knock-down strategies (Bargmann, 2001). The fact that RNAi has only limited effects in neurons was overcome by the generation of special sensitized backgrounds (Kennedy et al., 2004; Simmer et al., 2002; Wang et al., 2005). In addition, animals are amenable to molecular, genetic, and biochemical analyses, allowing the identification of protein interactions and the precise dissection of genetic pathways. Finally, the experimental work with C. elegans is greatly facilitated by world wide shared core facilities that provide researchers with access to information in online databases (www.wormbase.org, www.wormatlas.org), and material (for example transgenic worm strains, gene knock outs, RNAi and cDNA libraries).

C. elegans is color-less and transparent, which provides several advantages for the worm's use in biomedical research. In particular, the development of the worm from the one-cell-embryo stage to the fully-grown adult can be observed at the cellular level under the light microscope. In this way, the lineage of all 959 somatic cells in the hermaphrodite have been traced and were found to be invariant during development (Sulston et al., 1983). Individual cells in any part of the body can thus be followed and analyzed from the time they are born in mutant and wild-type animals. When using a light

microscope, this requires identification of cells based on structural features and their position within the organism. Alternatively, cells can be labeled with fluorescent markers, expressing fluorophores from cell-specific promoters. Either way, single cells or structures within the worm can thus be followed and analyzed during development.

In addition to being used to investigate developmental processes, *C. elegans* is now more frequently used as a disease model, e.g. for complex neurological disorders like Alzheimer's, Huntington's and Parkinson's Disease or Duchenne muscular dystrophy (Bessou et al., 1998; Levitan et al., 1996; Wittenburg et al., 2000). In 1998 *C. elegans* was the first multicellular organism whose genome had been completely sequenced (Caenorhabditis elegans Sequencing Consortium, 1998). Although it is a rather simple organism, a surprising 65% of human disease genes have a counterpart in the worm (Sonnhammer and Durbin, 1997). These genes can be manipulated easily in *C. elegans* and investigated in over-expression studies or ectopic expression experiments. Exceptional effort is also invested into the generation of gene deletions, providing a large, publicly available arsenal of mutants (www.shigen.nig.ac.jp/c.elegans/index.jsp; www.celeganskoconsortium.omrf.org/). Finally, behavioral studies on mutants or wild-type worms that have been exposed to pharmacological substances also provide valuable insight into disease mechanisms. These findings can subsequently be used as a basis for studies in higher animals.

OVERVIEW OVER THE *C. ELEGANS* NERVOUS SYSTEM

In contrast to vertebrate neurons, which typically develop only one axon that receives, and several branched dendrites that transmit signals, the majority of *C. elegans* neurons are mono- or bipolar. This means that every neuronal cell body sends out only one or two processes. Since neurons in the worm have synapses on (and thus receive and transmit signals from) both neurites (White, 1986), the distinction into axons and dendrites, as it is known, for example, from mice or *Drosophila*, is not entirely clear for all neurons.

The *C. elegans* nervous system is composed of neurons located in three major areas of the body: the head, the tail, and the ventral side of the body. These areas can be further subdivided into clusters (**Figure 2**). Head neurons group into anterior, dorsal, ventral, lateral, and retro-vesicular ganglions. In the tail the pre-anal, dorso-rectal and two lateral symmetric lumbar ganglions can be distinguished. A row of cell bodies, the ventral nerve cord, runs along the ventral mid line between the retro-vesicular ganglion and the pre-anal ganglion. These ganglia are connected by nerve processes sent out from the cell bodies, which form bundles running along the longitudinal axis of the animal. The majority of neurites target and meet in the circumpharyngeal nerve ring in the head, which is considered the "brain" of the worm.

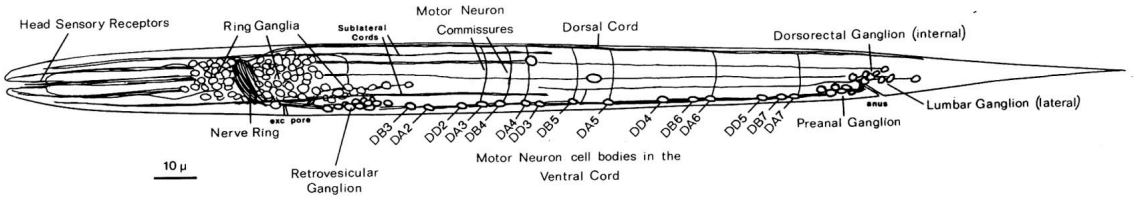

Figure 2: Schematic of the neuronal network of C. elegans. *Neurons cluster in the head and tail of the worm, their neurites run in longitudinal nerve bundles from anterior to posterior or vice versa. In addition, these nerve bundles themselves are connected via commissures. The ring ganglia in the head are: the anterior, dorsal, ventral and the lateral ganglion. (adapted from Wormatlas on www.wormbook.org)*

The neuronal network of *C. elegans* has been mapped with amazing precision based on the reconstruction of serial section electron micrographs (White, 1986). Based on the recently revised and detailed work of John White and colleagues, we know the invariant number of neurons and their positions within the body (Chen et al., 2006a; White, 1986). The hermaphrodite nervous system consists of only 302 neurons that are interconnected through around 5000 synapses. An additional 2000 synapses are established to muscles. Male worms possess additional neurons that are required for the execution of the male mating behavior. The majority of those neurons is located in the male tail, while a few can be found in the head. In addition to the location of the neuronal cell bodies, the wiring network has been described to the level of single

neuronal contact points – the synapses. Each neuron establishes contacts to several other neurons and many times a particular neuron makes several synapses to each of its partner neurons. Intriguingly, the synapse number of particular neurons is fairly consistent between individual animals. Synapses in *C. elegans* are formed *en passant*, meaning that synaptic boutons are formed along the neurite shafts of neighboring parallel nerve processes or nerve processes and muscle arms (White, 1986). Because of this detailed knowledge, the *C. elegans* nervous system is increasingly used to study the formation, positioning, and maintenance of individual synapses as well as the identification of novel molecules involved in these processes.

From environmental signals to behavior

Neurons in *C. elegans* fall into one of three functional groups: sensory neurons, interneurons, or motorneurons. In this sequence, they are responsible for the perception of environmental signals, their transmission to other neurons (higher order "brain" structures or motorneurons) and finally to muscles, where appropriate muscle contraction patterns are executed. Alternatively, the final destination of an environmental signal can trigger specific developmental programs, for example the dauer formation program.

The constant integration of multiple stimuli into a specific reaction or behavioral pattern is vital for the survival and reproduction of the worm. Sensory cues that are recognized by the worm include chemical, mechanical, olfactory and thermal stimuli, for example, light or harsh touch to the body or nose and the worm-specific dauer pheromone. The specificity of single sensory neurons for specific substances has been determined by laser ablation studies (Bargmann and Horvitz, 1991). Recently, also the detection of light by specific neurons has been demonstrated (Edwards et al., 2008; Ward et al., 2008). In addition to sensing external cues, the worm is also able to assess its internal status. Despite the relatively simple organization of its neuronal network, *C. elegans* possesses a large repertoire of complex behaviors, such as complicated patterns of movement during food search (Gray et al., 2005) and mating (Liu

and Sternberg, 1995), and different forms of associative learning (Morrison and van der Kooy, 1997; Morrison and van der Kooy, 2001; Morrison et al., 1999; Wen et al., 1997). These behaviors enable the worm to locate food sources and potential mates and to avoid harmful environments.

However, detection of environmental signals alone is not sufficient to elicit an appropriate behavior. In addition, neurons have to establish and maintain a correct wiring pattern and communication system. Only then can sensory information be propagated and translated into action. On the following pages, two morphological neuronal features essential for signal detection and transmission will be discussed in detail: (1) synapses, neuronal contact points through which signals are passed on, and (2) cilia, specialized sensory structures through which sensory neurons detect signals.

NEURONAL SUB-CELLULAR STRUCTURES: SYNAPSES

Synapse structure and function

Synapses are sites of cell-cell contact between neurons or neurons and muscles (so-called neuromuscular junctions), which serve to transmit signals. Synapses can be divided into chemical and electrical synapses. Chemical synapses transmit signals through a vesicle-mediated release of neurotransmitters (**Table 1**) or small peptides, e.g. insulin. Electrical synapses function through the direct coupling of membranes between two neurites. They are usually less abundant but seem to be associated with chemical synapses on the same neurite.

The worm offers an ideal opportunity to study all aspects of synapse formation, structure and function *in vivo* in a relatively simple organism for several reasons. First, the majority of synaptic components are highly conserved among species (**Table 2**). Also, most of the common vertebrate neurotransmitters are found in the worm (**Table 1**). These two facts allow fairly direct comparisons to be made between the worm and vertebrate species.

Finally, for many synaptic genes, mutant alleles in *C. elegans* are available and most of them are viable, allowing genetic dissection of pathways and functions.

Each synapse consists of two opposing counterparts, a pre-synaptic and a post-synaptic varicosity aligned at the synaptic cleft. The pre-synaptic terminal is characterized by the accumulation of neurotransmitter-filled synaptic vesicles around electron-dense membrane specializations (also called the pre-synaptic density or active zone; **Figure 3**). The post-synaptic site harbors various neurotransmitter receptors, ion channels, and signal transduction molecules. In chemical synapses, signals are transmitted through different neurotransmitters or neuropeptides (**Table 2**). Neurotransmitters have been assigned to single neurons, but whether individual synapses are neurotransmitter-specific is not known. Signaling molecules are packed and transported in synaptic vesicles to the active site of the synapse, where the vesicles fuse with the plasma membrane in a multi-step process that includes docking, priming, calcium sensing, membrane fusion (reviewed in (Richmond, 2005)) that leads to the release of neurotransmitter molecules into the synaptic cleft. Neurotransmitters then bind to their respective receptors at the postsynaptic terminal and thereby activate the "downstream" or receiving neuron.

Table 1: Neurotransmitter in C. elegans

Neurotransmitter	Conservation in	Action on *C. elegans* muscles[1]
Acetylcholine	Mammals	direct-excitatory
GABA (gamma-aminobutyric acid)	Mammals	weak inhibitory
Glutamate	Mammals	direct-inhibitory
Serotonin	Mammals	direct/indirect-excitatory
Dopamine	Mammals	Excitatory
Octopamine	Invertebrates only	indirect-inhibitory
Tyramine	Invertebrates only	Inhibitory

[1] This information is derived from experiments using in vitro muscle preparations to measure responses. 'direct' or 'indirect' indicates whether the effect is due to a direct action on the muscle, or indirect through a neuronal circuit.

Both vesicle proteins as well as membrane are retrieved from the presynaptic terminal by a clathrin-mediated endocytosis process. Clathrin adaptors AP2 and AP180 localize to the vesicle membrane through direct

interaction with vesicle-associated proteins (e.g. synaptobrevin or synaptotagmin) (Fukuda et al., 1995; Haucke and De Camilli, 1999; Haucke et al., 2000; Zhang et al., 1994). The role of synaptotagmin has been demonstrated by a genetic approach in *C. elegans*. In *C. elegans* synaptotagmin *snt-1* mutants synaptic transmission is reduced, and synaptobrevin *snb-1* accumulates in the membrane (Nonet et al., 1993). At the ultrastructural level, a reduction of synaptic vesicles at pre-synaptic terminals was found in these mutants (Jorgensen et al., 1995), further supporting the interpretation that *snt-1* is required for vesicle recycling. Consequently *snt-1* mutants cannot move properly and display an uncoordinated (Unc) phenotype. Upon binding to the membrane, clathrin adaptors then recruit clathrin molecules. They adapt a conformation, which facilitates curvature of the membrane during the early budding step. Completion of budding and fission finally generates vesicles that are sorted and re-filled with neurotransmitter molecules (**Figure 3**). This synaptic vesicle cycle is crucial for the maintenance of a functional synapse.

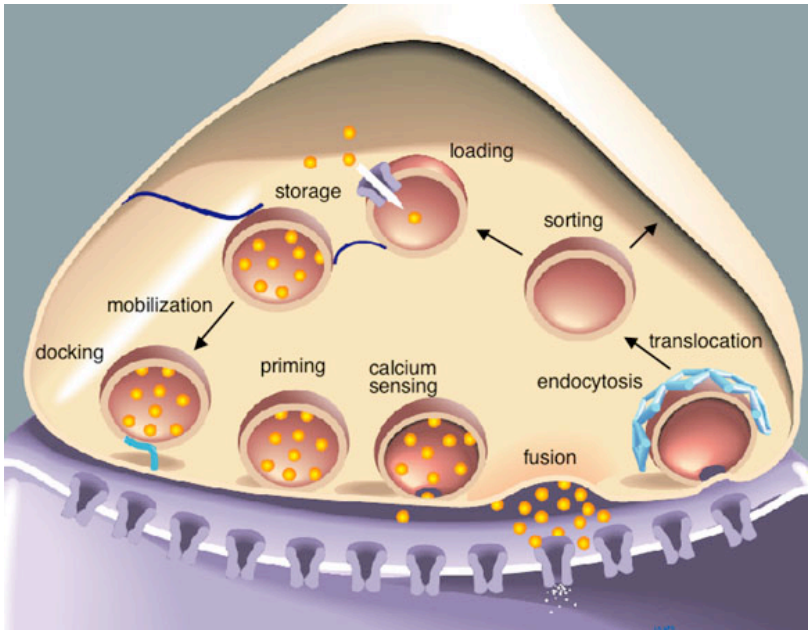

Figure 3: The synaptic vesicle cycle. *Synaptic vesicles contain transporters that load vesicles with neurotransmitter. Mobilized vesicles translocate to the terminal plasma membrane where they selectively dock close to the active zone. Docked vesicles then undergo a priming step, during which they become fusion competent. A rise in intracellular calcium binds to sensors triggering vesicle fusion and release of neurotransmitter into the synaptic cleft. Neurotransmittors can then bind and activate receptors on the post-synaptic membrane. Following full-fusion, vesicle proteins and membrane are retrieved by clathrin-mediated endocytosis. (adapted from (Richmond, 2005)).*

As the example above demonstrates, *C. elegans* has proven to be a powerful genetic model system in which to study the molecular basis of synapse structure, function, formation, and location. Several genetic screens have been conducted using behavioral or pharmacological criteria as well as fluorescent reporters to isolate synapse mutants. Subsequent modifier screens aim to uncover molecules interacting or functioning in the same pathway. Behavioral and pharmacological criteria select worms based on their aberrant movement or behavioral patterns under standard conditions (e.g. UNCoordinated, Unc mutants) or upon exposure to neuromodulatory, toxic substances such as Levamisole or Aldicarb (Lewis et al., 1980; Mahoney et al., 2006; Martin et al., 2005). Aldicarb is an acetylcholine esterase inhibitor, leading to the accumulation of acetylcholine (AcCh) in the synaptic cleft, while Levamisole, an AcCh analog, constitutively activates AcCh receptors. Both interfere with synaptic transmission and cause paralysis in wild-type worms while synapse mutants are either resistant or hypersensitive to these agents to various degrees.

Genetic screens used to isolate synapse mutants made use of synaptic vesicle clusters (that usually accumulate around synaptic terminals) marked with SNB-1::GFP in different subtypes of neurons. Abnormal accumulation of the labeled protein in mutant worms was used to identify genes involved in synapse formation. These screens identified overlapping sets of synapse genes, named *sad* (**s**ynapses of **a**mphids **d**efective), *sam* (**s**ynapse **a**bnormal **m**orphology), *syd* (**sy**napse **d**efective) and *syg* (**sy**napse **g**one) (Crump et al., 2001; Schaefer et al., 2000; Shen and Bargmann, 2003; Zhen and Jin, 1999).

In addition to classical/conventional screens, new tools to study synapses in *C. elegans* have been developed. These tools include calcium-indicators for the study of calcium dynamics in neurons and muscles (Kerr et al., 2000), styryl dyes such as FM1-43 to study vesicle cycling in sectioned worms (Kay et al., 1999), and electrophysiological methods that allow recordings at single neuromuscular junctions (Richmond et al., 1999; Richmond and Jorgensen, 1999). The development of new sample preparation protocols for transmission electron microscopy (EM) allows a more detailed analysis of morphological synaptic phenotypes (Rostaing et al., 2004). Furthermore, new immuno-EM techniques increase the resolution of studying protein localization

within the synapse (Bosher et al., 2003). These developments are essential for neurobiology research in *C. elegans* to remain competitive and to continue to contribute at the forefront to our understanding of the molecular and structural basis of synapse function.

Table 2: Selected synaptic proteins in C. elegans and their homologues in mammals

C. elegans protein	***Function***	***Mammalian homolog***
SNB-1 (Synaptobrevin, v-SNARE)	vesicle fusion	Synaptobrevin-1/VAMP-1
SNG-1 (Synaptogyrin)	Ca^{2+}-dependent exocytosis	Synaptogyrin-1
SNT-1 (Synaptotagmin)	Ca^{2+}-sensor in exocytosis, AP2-binding partner in endocytosis	Synaptotagmin-1
UNC-10 (Rim, rab interacting protein)	priming	Rim1
UNC-13 (Phorbol ester binding protein)	priming, promoting open syntaxin	Munc-13
UNC-17 (Vesicular AcCh transporter)	loading AcCh into synaptic vesicles	VAChT
UNC-18 (Syntaxin binding protein)	implicated in docking priming and fusion	Sec1 homolog
UNC-43 (CaM kinaseII)	regulation of synaptic density	CaM kinase II gamma chain
UNC-64 (Syntaxin)	vesicle fusion	Syntaxin-1A

Synapse maintenance

The process of synapse maintenance has two different aspects (1) synapse maintenance during development (establishing and strengthening of preliminary synapses) and (2) synapse maintenance during adulthood (recycling of synaptic vesicles and maintaining a constant supply of synaptic proteins).

Studying synapse development in the vertebrate central nervous system has led to a detailed understanding of the earliest events of synapse formation. A multi-step process is required, beginning with an initial contact between axons and dendrites to the induction and subsequent differentiation of synapses. The induction step of synapse formation is believed to involve several bi-directional signaling events between pre- and postsynaptic fields utilizing several classes of cell adhesion molecules (Craig et al., 2006; Waites et al., 2005). This initial phase of synapse formation is then followed by a period of

structural and functional maturation. The process of synaptic maturation leads to increased stability of the junction and a certain resistance to disassembly.

Not all synapses made during development remain present in the mature adult nervous system. Pruning, the selective removal of synapses, is an important part of neuronal circuit refinement. Development of the human brain is the most impressive example of pruning; around 40 % of all synapses made during the postnatal period are removed until adulthood (Huttenlocher and de Courten, 1987). Recently a molecular model for selective synapse removal was presented based on data from the *C. elegans* HSN neuron. Ding and co-workers demonstrated that the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex is necessary to establish the stereotypic synaptic pattern found in adults by selectively removing synapses at larval stages (Ding et al., 2007). They also identified the immunoglobulin membrane protein *syg-1*, shown earlier to direct the assembly of synapses, as a local inhibitor of SCF complex activity (Shen and Bargmann, 2003; Shen et al., 2004). Another example of synapse removal during the development of *C. elegans* involves the DD-type motor neurons. These neurons initially synapse onto ventral muscle cells. At the end of the L1 stage, these ventral synapses are removed and new ones are established with dorsal muscles (Hallam and Jin, 1998). As for synapses on HSN neurons, the synaptic pattern of DD-type motor neurons in adults is stereotypic. However, whether the same mechanism or complex is required for the establishment of this pattern is not clear.

Mature, established synapses are maintained via recycling of synaptic vesicles. This process is vital for the functionality of synapses in the adult nervous system. A large amount of data from studies in *C. elegans* on vesicle fusion, recycling, and transport is available (Jin, 2005; Richmond, 2005). However, a certain imprecision of the recycling machinery requires also a constant supply of newly formed synaptic vesicles. In the worm, UNC-104 is required for the transport of synaptic vesicles from cell bodies to synapses (Zhou et al., 2001). Upstream steps of this process, for example, how synaptic protein expression itself is regulated, and how vesicles are formed in the cell body, are not well understood, however.

Synapse positioning and patterning in C. elegans

As described above, classical views on synapse formation describe the direct pairing of synaptic partners across the synaptic cleft via adhesive molecules. However, several studies in *C. elegans* have recently demonstrated an alternative way – the use of guidepost cells. These are (non-neuronal) cells that first specify the pre-synaptic area in an adjacent neuron. In a subsequent second step, the post-synaptic partner is guided to this site. Examples for such guidepost cells are the primary vulval epithelial cells. They express *syg-2*, which recruits and interacts with the above-mentioned *syg-1*, expressed in the neuron HSNL. In such a way, the guidepost molecule SYG-2 defines pre-synaptic sites in HSNL. Glia-like sheath cells in the head of the worm also act as guideposts, coordinating the formation of synapses between AIY and RIA, two neurons in the thermosensory circuit (Colon-Ramos et al., 2007). These glia cells secrete the diffusible molecule UNC-6/Netrin, which binds to its receptor UNC-40/DCC expressed in both neurons. However, RIA and AIY respond in different ways to UNC-6. While the Netrin-DCC interaction feeds into the axon guidance pathway in RIA, it specifies the pre-synaptic site in AIY. How these different programs are activated downstream of UNC-40/DCC is not known. In another recent study by Klassen and co-workers, the role of Wnt signaling in guidepost cell-guided synapse formation was demonstrated (Klassen and Shen, 2007). Tail hypodermis cells act as guideposts by secreting LIN-44/Wnt, which binds to the LIN-17/Frizzled receptor present on the axon of the DA9 neuron. In contrast to the previous examples, this interaction does not promote, but instead inhibits, synapse formation in this area. Thus, guidepost cells can sculpt neuronal activity in two ways, supportive and/or inhibitory. The concept of guidepost is not unique to *C. elegans*, but can also be found in vertebrates (Del Rio et al., 1997; Ghosh et al., 1990). Therefore findings made in the worm may in the future also be important for other species, including humans.

NEURONAL SUB-CELLULAR STRUCTURES: CILIA

The general structure of cilia

Cilia and the structurally and functionally related flagella are ancient organelles that are found in many eukaryotic species - while *Arabidopsis thaliana*, *Saccharomyces cerevisiae* and *Dictyostelium discoideum* are some of the few species that lack these structures (Cavalier-Smith, 2002). Cilia are cellular extensions comprised of two main building blocks: a rod-like core, called the axoneme, consisting of 9 outer microtubule-doublets (and in some cases also a central pair) and a ciliary membrane that isolates the axoneme from the extra-cellular space. They are found singly or in groups and can be assigned to three major categories: motile, primary, or nodal cilia. Motile cilia are defined by their ability to move. Solitary or organized in groups, they beat in a wave-like fashion, thereby transporting extracellular fluids or small particles (e.g. mucus in the lung or cerebrospinal fluid in the brain) or generating force for cell movement (e.g. sperm cells) (Davenport and Yoder, 2005). The ability to generate force is achieved by the presence of a central microtubule pair in the axoneme, resulting in a so-called 9+2 pattern. Primary cilia are solitary organelles that display a 9+0 pattern and are consequently non-motile (Davis et al., 2006). Their function on most cells remains to be elucidated, however. In the vertebrate kidney and also in *C. elegans* (as will be discussed later) they serve sensory purposes. The third category, nodal cilia, is found on the node of gastrulation-stage mammalian embryos. Their axonemes display a 9+0 pattern, nevertheless they can move - although in a propeller like fashion. This movement is vital to generate a morphogenetic gradient that sets up the left-right asymmetry in mammals.

Despite the growing interest of researchers in ciliogenesis in recent years, we still have only a rudimentary understanding of cilium formation. Cilium formation begins with the formation of a basal body, which is derived from the centriole, a structure that organizes and arranges the microtubular spindle during cell division. In non-dividing cells, one centriole is positioned close to the membrane and it serves as a microtubule-organizing center for the

ciliary axoneme. Cilia in all organisms are constructed through a conserved process called **I**ntra **F**lagellar **T**ransport (IFT) (Rosenbaum and Witman, 2002). IFT is characterized by the bipolar movement of particles along the axoneme, more precisely between the outer microtubule doublets and the membrane. This movement is facilitated through two classes of motor proteins: kinesins are required for the transport from the proximal to the distal end of the cilium and dyneins move particles from the distal to the proximal end. One can distinguish two different groups of IFT components: complex A particles and complex B particles. Pioneering biochemical work in Chlamydomonas purified and identified a large number of complex A and B components (Luck et al., 1977). The absence of a single component results in drastic shortening of cilia, indicating that IFT components are also essential for cilium formation. This is not surprising. Since cilia do not harbor ribosomes, they are highly dependent on IFT as a supply-mechanism for structural and functional components (Mukhopadhyay et al., 2007; Ou et al., 2007; Pan et al., 2006).

Table 3: Prominent examples of human cilia-related diseases
Polycystic kidney disease (PKD)
Bardet Biedl Syndrome (BBS)
Kartagener Syndrome or Primary Ciliary Dyskinesia (PCD)
Jeune asphyxiating thoracic dystrophy (JATD)
Meckel Syndrome (MKS)
Alström Syndrome (AS)

Cilia and flagella are found on nearly every mammalian cell type. For a comprehensive list of all cell types that have been reported to have primary or other types of cilia, visit the following web sites: http://members.global2000.net/browser/cilialist.html or http://www.bowserlab.org/primarycilia/ciliumpage2.html. Structurally impaired cilia have in recent years been associated with severe diseases and syndromes in humans, indicated that their diverse functions are vital for most organs (**Table 3**). Molecular investigations to determine the underlying causes for these diseases even in mouse models are not always possible or, at least, are very time consuming. Therefore simple animals like *C. elegans* are the models of choice to study cilia defects *in vivo*.

Table 4: Amphid ciliated sensory neurons and their specificity

Neuron	Cilium	Sensory specificity	Function	Dye filling
ASE	single rod	Na^+, Cl^-, K^+, cAMP, biotin, lysine	Water-soluble chemotaxis	no
ASG	single rod	Na^+, Cl^-, cAMP, biotin, lysine	Dauer formation, Lifespan, Chemotaxis	no
ASH	single rod	Cd^{2+}, Cu^{2+}, 1-octanol	Nociception, Social feeding	FITC, DiI, DiO
ASI	single rod	Na^+, Cl^-, K^+, cAMP, biotin, lysine	Dauer formation, Chemotaxis	FITC, DiI, DiO
ASJ	single rod	dauer pheromone	Dauer formation/recovery, Chemotaxis, Lifespan	FITC, DiI, DiO
ASK	single rod	lysine	Avoidance, Chemotaxis, Lifespan	FITC, DiI, DiO
ADF	double rod	Na^+, Cl^-, cAMP, biotin	Dauer formation, Chemotaxis	FITC
ADL	double rod	Cd^{2+}, Cu^{2+}, 1-octanol	Avoidance, Social feeding	FITC, DiI, DiO
AWA	wing-like	diacetyl, pyrazine, trimethylthiazole	Volatile chemotaxis, Lifespan	no
AWB	wing-like	2-nonanone, 1-octanol	Volatile avoidance	DiI, DiO
AWC	wing-like	benzaldehyde, butanone, isoamylalcohol, …	Volatile chemotaxis, Lifespan	no
AFD	microvillae	temperature	Thermosensation	no

Cilia in C. elegans

In contrast to the widespread distribution of cilia in mammals, in *C. elegans* cilia are restricted to 60 neurons located mainly in the head and the tail of the animal. These 60 neurons define a subset of sensory neurons in *C. elegans*, the ciliated sensory neurons (CSNs). Their precise positions within the body as well as their functions are well described. Most CSNs in the head are part of two lateral-symmetric sensilla, specialized sense organs called the amphids (**Figure 4**). Those in the tail are part of the phasmids. Amphids as well as phasmids consist of CSNs and two support cells (one socket and one sheath cell) that form a channel and connect the sensillum to the hypodermis. Cell ablation studies have identified numerous volatile and soluble chemical compounds that can be sensed by *C. elegans* and have assigned those compounds to the sensory function of single neurons (**Table 4**). Typically,

several neurons are able to detect one particular substance. One neuron is a primary sensor while the others have minor roles in detection.

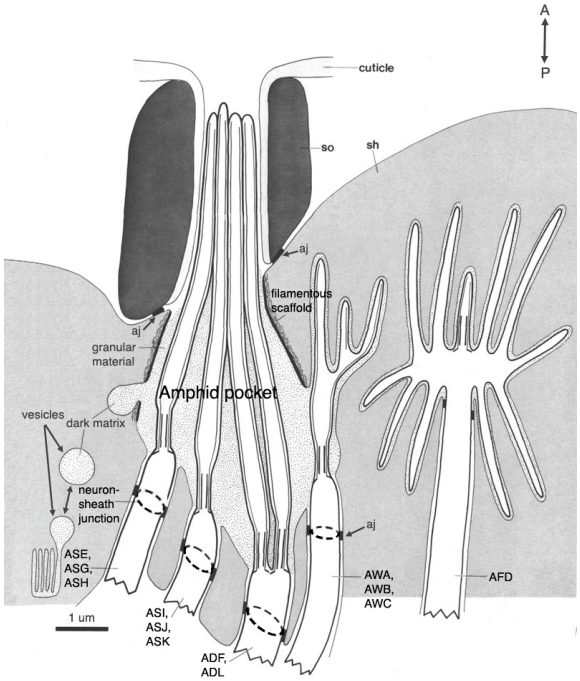

Figure 4: Schematic of amphid cilia. *The amphids, a pair of lateral sensilla in the head, are the principal chemosensory organs of nematodes. Each amphid is comprised of the ciliated dendrites of 12 sensory neurons. These cilia can have different shapes (single or double rod-like, wing-like or with numerous microvillae) and locations (projecting through a pore with direct contact to the environment, or remaining within the amphid pocket). (adapted from (Perkins et al., 1986))*

Several screens performed in *C. elegans* have generated a large number of mutants that have structurally or functionally impaired cilia (Bargmann et al., 1993; Culotti and Russell, 1978; Efimenko et al., 2005; Emery et al., 1996b; Malone and Thomas, 1994; Ou et al., 2007; Perkins et al., 1986; Starich et al., 1995). These mutants were categorized and named based on their phenotypes: *daf* (**da**uer **f**ormation affected), *che* (**che**motaxis response defective), *osm* (**osm**otic avoidance defective), *odr* (**od**orant **r**esponse defective) or *dyf* (**dy**e-**f**illing defective). Dye-filling mutants are characterized by the inability of CSNs to take up lipophilic dyes such as DiI, DiO or FITC. This uptake is facilitated in wild-type worms by cilia. After an incubation time of about one hour the entire CSN is saturated and visible under the UV

microscope. Mutants with structurally defective cilia do not incorporate dye in their CSNs and are therefore dye-filling defective.

RFX TRANSCRIPTION FACTORS

RFX proteins belong to the winged-helix family of transcription factors. They are defined by a 76 amino acid DNA-binding domain and are present in many eukaryotes. *S. cerevisiae*, *S. pombe*, and *C. elegans* each contain one RFX gene, the *Drosophila* genome encodes two, and seven have been identified in mice and humans (Aftab et al., 2008). Certain individual RFX proteins have been implicated in the regulation of single genes (Iwama et al., 1999; Wolfe et al., 2006). Other RFX proteins regulate similar processes in several species, such as the cell cycle, brain development, and cilium formation. The RFX transcription factors in *S. pombe*, *S. cerevisiae* and *Drosophila* dRFX2 are implicated in the regulation of cell cycle check points (Huang et al., 1998; Otsuki et al., 2004; Wu and McLeod, 1995). Neuron-specific functions have been described for mammalian RFX1 and RFX4. RFX1 was recently identified as a regulator of the EAAT3 neuronal glutamate transporter, which suggests a possible role in the regulation of neurotransmitters (Ma et al., 2006). Several isoforms of RFX4 have been isolated, one transcript of which, RFX4_v3, is implicated in early brain development (Zhang et al., 2006). Mammalian RFX3 functions mainly in cilia. During embryogenesis, it is responsible for nodal cilium development, the specification of left-right asymmetry, and the differentiation of ciliated ependymal cells in the brain (Baas et al., 2006; Bonnafe et al., 2004). Similarly, Drosophila RFX, dRFX, is expressed in the peripheral nervous system, where it is essential for proper signaling in ciliated type I sensory organs (Dubruille et al., 2002). The regulation of cilium formation is so far the only process regulated by RFX proteins that is conserved across species.

RFX proteins are characterized through two functional domains, a highly conserved DNA binding domain (DBD) and a dimerization domain (DIM). The DBD recognizes and binds to a short DNA sequence motif, the x-box, present in the promoter region of direct target genes. A consensus sequence for mammalian RFX proteins and the single *C. elegans* RFX protein

have been defined based on *in vitro* binding studies and *in vivo* gene expression studies, respectively (Efimenko et al., 2005; Emery et al., 1996b).

THE ROLE OF DAF-19 DURING CILIOGENESIS

The *C. elegans* genome encodes a single RFX transcription factor, DAF-19. It was the first transcription factor shown to be essential for ciliogenesis. DAF-19 was also the first member of the RFX transcription factor family identified as part of cilium development (Swoboda et al., 2000). Subsequently a conserved role for RFX proteins in ciliogenesis was also discovered in other organisms (Baas et al., 2006; Bonnafe et al., 2004; Dubruille et al., 2002). The gene *daf-19* is expressed in all CSNs (Swoboda et al., 2000). These neurons enable the worm to sense cues from the environment through the cilium. The cilia of those sensory neurons are therefore the major source of input for environmental signals for the worm. Mutations in *daf-19* result in animals completely devoid of any ciliated structure. Such worms are therefore unable to respond to environmental signals like food, dauer pheromone, or nose touch (Perkins et al., 1986). *daf-19* mutant worms display a highly penetrant dauer formation phenotype, with over 90 % of worms activating the dauer formation program. Nevertheless, in the laboratory *daf-19* mutants are viable and thus a suitable model to study ciliogenesis. Several labs have identified a large number of direct DAF-19 target genes based on the presence of the x-box promoter sequence motif, the binding site for DAF-19. The expression of these genes is dependent on DAF-19 and the cis-acting x-box; these genes are specifically expressed in CSNs, and they are frequently directly involved in cilia structure and function (Blacque et al., 2005; Chen et al., 2006b; Efimenko et al., 2005).

THE CONCEPT OF ECTOPIC EXPRESSION

Necessity and Sufficiency – two related genetic concepts

Studying the function of a particular gene can be attempted in several ways. A loss-of-function (LOF) mutation of the gene of interest can be targeted by homologous recombination in yeast, flies, or mice. Random mutagenesis in flies or worms can also result in the deletion of the gene product. The resulting phenotype reveals the process or program for which the gene is required. In other words, a null or LOF mutation of a gene will determine its necessity. Another approach to studying gene function is the over-expression of the gene of interest in a wild type organism (gain-of-function, GOF). This is particularly useful if deletion of the gene does not result in a phenotype, e.g. because of functional redundancy of several genes. GOF experiments can be carried out in the cells that the gene is usually expressed or in other, unrelated, cell types. Gene expression in unrelated cell types can lead to surprising results that can indicate the sufficiency of a gene to induce a certain function or developmental program. The genetic concepts of necessity and sufficiency are especially important with respect to developmental events. Gene products that can induce an entire developmental program have been identified and are called "master regulators" or "master switch genes." These genes can lead to reprogramming of cell fates and transformation of cell types.

Master regulators

eyeless – ectopic eye formation in Drosophila melanogaster

eyeless, the *Drosophila* homolog of the paired transcription factor Pax6, was one of the first genes found to induce a developmental program and therefore was labeled a "master regulatory gene". LOF mutations in *eyeless* generate, as the name suggests, flies without eyes. Conversely, ectopic activation of *eyeless* induces the formation of this complex organ consisting of

many different cell types (Halder et al., 1995). The first intriguing pictures of compound eyes growing on antennae and legs of the fly were followed by the identification of other genes required for eye development. Many of them are also able to induce ectopic eye development. To date, PAX6, EYA (Eyes absent), SO (sine occulis) and DAC (Dachshund) form the key members of the retinal determination gene network, which directs eye development in *Drosophila*. These genes, even though they can be placed into a hierarchical structure, regulate each other through feedback loops, and some of them even interact physically. In addition, TOY (Twin of eyeless) was found to act upstream of *eyeless.* OPTIX and EYG (Eyes gone) function independently from the other members in proper eye development. Thus, *eyeless*, despite its necessity and apparent sufficiency for eye development in *Drosophila*, is not the sole determining factor for this process. Instead, it is embedded in a complex determination cascade consisting of several master switch genes.

MyoD – induction of a muscle specific developmental program

In tissue culture experiments MyoD (*myoblast determination 1*; (Davis et al., 1987)) was found to transform a variety of cell types (e.g. nerve cells, fat cells, fibroblasts, and liver cells) into muscle-like cells that express muscle-specific genes like desmin, myosin heavy chain, muscle specific receptors, and membrane molecules (Weintraub et al., 1989). These experiments showed that MyoD is sufficient to induce a muscle-specific developmental program. Surprisingly, in MyoD knock out mice muscles still form, indicating that MyoD is not required (necessary) for this developmental program (Wang et al., 1996). This was due to the redundancy of MyoD and Myf, a MyoD-like gene that also has the potential to induce muscle-specific gene expression. Muscles develop normally without either MyoD or Myf. However, simultaneous deletion of both genes leads to the absence of muscle development (Rudnicki et al., 1993). These results indicate that MyoD and Myf are master regulatory genes that are sufficient for skeletal muscle development, but individually they are not necessary. In addition to MyoD and Myf, several other genes important for muscle development were identified as belonging to the myogenic bHLH

protein family: myogenin, Myf5, and Mrf4, all of which have a key role in muscle cell specification and differentiation (Buckingham et al., 2003; Molkentin and Olson, 1996; Perry et al., 2001; Pownall et al., 2002; Puri and Sartorelli, 2000).

What defines a master regulator?

Approaching to explain the term "master regulator" from an unbiased point of view, one would describe it as a protein that is the one and only inducer of the start of a (developmental) expression cascade. Furthermore, this master regulator should ideally be both necessary and sufficient to trigger a certain developmental process, activating this cascade wherever and whenever expressed. However, looking at the examples of master regulators identified, two facts become apparent: (1) there are not many master regulators reported and (2) the situation is usually more complex than outlined above. Two examples of master regulatory genes were presented in the previous section - their range of action is, however, very different. MyoD induces the identity of a specific cell type and is sufficient for this process. Eyeless on the other hand, induces several different cell types that are ultimately arranged into a functional organ. In contrast to MyoD, Eyeless is both necessary and sufficient for inducing a particular developmental program. However, in both cases these genes were first identified as THE master regulator. Subsequent studies then discovered redundant proteins with identical functions, epistatic genes in the same pathway, or parallel pathways with similar activation potential.

Thus, one can state that a real master regulator actually does not exist. Genes act in networks and important pathways are often backed up by (other) genes, which can exert the same function. In this sense, the term 'key regulator' would be more appropriate.

Thesis Aim

Past and present efforts in the Swoboda lab and several other labs focus mainly on the identification and characterization of genes required for cilia formation (Blacque et al., 2005; Chen et al., 2006b; Efimenko et al., 2005). Such genes can be direct or indirect target genes of the RFX transcription factor DAF-19, depending on their method of identification. The role of DAF-19 in ciliated sensory neurons and during ciliogenesis is therefore being extensively studied.

However, preliminary results suggested that DAF-19 may also be expressed in cells other than ciliated sensory neurons (Swoboda et al., 2000). The function of the transcription factor in these cells remained largely unexamined. The work presented in this thesis aims to investigate novel functions of DAF-19. It also explores the transcription factors' potential to induce cilia during development in different cell types. In particular this work tries to answer the following questions:

- Does DAF-19 have a function in other neurons or cell types in *C. elegans*?
- Can DAF-19 be used to generate a novel tool to study all aspects of sensory neuron function from sensory input to behavior *in vivo*?
- Is DAF-19 a master regulator of ciliogenesis and thus sufficient to induce cilia formation in different cell types?

Results and Discussion

This section presents and discusses all findings that are part of this thesis, based on the specific aims presented in the section “Thesis Aims”. Paper I is included as published in the journal, while Paper II and III are written in manuscript form. Thus Paper I follows its own page numbering.

Paper I

DISTINCT ISOFORMS OF THE RFX TRANSCRIPTION FACTOR DAF-19 REGULATE CILIOGENESIS AND MAINTENANCE OF SYNAPTIC ACTIVITY

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Distinct Isoforms of the RFX Transcription Factor DAF-19 Regulate Ciliogenesis and Maintenance of Synaptic Activity

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Neurons form elaborate subcellular structures such as dendrites, axons, cilia, and synapses to receive signals from their environment and to transmit them to the respective target cells. In the worm *Caenorhabditis elegans*, lack of the RFX transcription factor DAF-19 leads to the absence of cilia normally found on 60 sensory neurons. We now describe and functionally characterize three different isoforms of DAF-19. The short isoform DAF-19C is specifically expressed in ciliated sensory neurons and sufficient to rescue all cilia-related phenotypes of *daf-19* mutants. In contrast, the long isoforms DAF-19A/B function in basically all nonciliated neurons. We discovered behavioral and cellular phenotypes in *daf-19* mutants that depend on the isoforms *daf-19a/b*. These novel synaptic maintenance phenotypes are reminiscent of synaptic decline seen in many human neurodegenerative disorders. The *C. elegans daf-19* mutant worms can thus serve as a molecular model for the mechanisms of functional neuronal decline.

INTRODUCTION

RFX proteins belong to the winged-helix family of transcription factors. They are defined by a 76-amino acid DNA-binding domain and are present in many eukaryotes. The genomes of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans* each harbor one RFX gene, *Drosophila* contains two, and five have been identified in mice and humans. Individual RFX proteins regulate related processes in several species, such as the cell cycle (Wu and McLeod, 1995; Huang *et al*., 1998; Otsuki *et al*., 2004), brain development and neuronal functions (Ma *et al*., 2006; Zhang *et al*., 2006), and ciliogenesis. Cilia develop as specialized subcellular structures with sensory or motile functions that project off many different cell types. Their structure and function have been investigated in mammals, *Drosophila*, and *C. elegans*. Initially, the characterization of the single *C. elegans* RFX transcription factor, DAF-19, had established for the first time a connection between RFX transcription factors and cilia development (Swoboda *et al*., 2000) and provided a basis for subsequent studies in other species. *Drosophila* dRFX is expressed in the peripheral nervous system, where it is essential for the proper function of ciliated type I sensory organs (Dubruille *et al*., 2002; Laurencon *et al*., 2007). Mammalian RFX3 is responsible for nodal cilia development, the specification of left-right asymmetry and the differentiation of ciliated ependymal cells in the brain (Bonnafe *et al*., 2004; Baas *et al*., 2006). Thus, the role of RFX transcriptions factors in ciliogenesis is conserved across species. In *C. elegans*, the gene *daf-19* is expressed in ciliated sensory neurons mostly located in the head and tail of the worm (Swoboda *et al*., 2000). These neurons are the major source of input for environmental signals for the worm. *daf-19* mutant worms are completely devoid of ciliated structures and are consequently unable to respond to environmental signals such as food, dauer pheromone, or nose touch (Perkins *et al*., 1986). Nevertheless, in the laboratory *daf-19* mutants are viable and thus a suitable model to study ciliogenesis. We and others have identified a large number of direct DAF-19 target genes based on the presence of the x-box promoter sequence motif, the binding site for DAF-19. Their expression in ciliated sensory neurons was dependent on both *daf-19* and the promoter x-box, and many of them are required for cilia structure and function (Blacque *et al*., 2005; Efimenko *et al*., 2005; Chen *et al*., 2006).

In the present study, we show that DAF-19 not only regulates the formation of cilia in sensory neurons but also is required for the maintenance of synaptic functions in the remainder of the nervous system. The hermaphrodite *C. elegans* nervous system consists of 302 neurons (60 of which are ciliated) that are connected via ~7000 chemical synapses and 700 gap junctions (White *et al*., 1986). Chemical synapses are established either between neurons or between neurons and muscle cells, at the so-called neuromuscular junctions. Each synapse consists of three major areas: 1) the synaptic vesicle pool, made up of vesicles at various stages of the recycling process or ready for neurotransmitter release; 2) the presynaptic terminal, where synaptic vesicles fuse in a multistep process and release neurotransmitters into the synaptic cleft; and 3) the postsynaptic target area in the receiving neuron, the receptive field, in which neurotransmitter receptors cluster. The isolation of a large number of *C. elegans* synapse mutants has provided us with detailed knowledge about the function of the synapse, especially the life cycle of synaptic vesicles. Recent work addressed the hierarchical assembly of the presynaptic terminal, providing detailed insight into the interdependence of assembly steps at a molecular level (Dai *et al*., 2006; Patel *et al*., 2006).

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However, how the expression of individual synaptic components is regulated after their initial establishment and how their constant supplies are maintained, remains largely unknown.

Here, we present a detailed analysis of three different *daf-19* transcripts. We show that the short isoform *daf-19c* is expressed in all ciliated sensory neurons and is sufficient to rescue ciliogenesis phenotypes of *daf-19* mutants. The two long isoforms *daf-19a/b* are expressed in basically all nonciliated neurons. We describe novel behavioral and cellular phenotypes of *daf-19*. In particular, we demonstrate that DAF-19A/B are necessary to maintain expression levels of several synaptic proteins, which assigns DAF-19 a function in neurotransmission. Surprisingly, this reduced synaptic protein expression is rather mild at larval stages but declines progressively as adult *daf-19* mutants age. Therefore, our study for the first time establishes a member of the RFX transcription factor family as a regulator of synaptic maintenance. Intriguingly, the synaptic defects in *daf-19* mutants display strong parallels to the synaptic decline observed in human neurodegenerative disorders, suggesting that similar mechanisms may be affected.

MATERIALS AND METHODS

Strains and Culture Methods

Culture of *C. elegans* strains was carried out as described previously (Brenner, 1974). The strains and transgenes used in this work are summarized in Supplemental Table 4. All strains were grown at 20°C. At this temperature, *daf-19* mutants display a highly penetrant Daf-c phenotype. However, ~10% of the population does not activate the dauer formation program and can be used for experiments (Swoboda *et al*., 2000). Worms were picked singly at larval stage 4 (L4) before behavioral and paralysis assays that required a small number of worms (<50 animals/assay). Antibody stainings of mixed stage populations were performed on large batches of *daf-19* worms. For all experiments that required large populations of staged worms (Western blot, quantitative polymerase chain reaction [PCR], and antibody stainings) or that involved the analysis of nonrescuing transgenes (transcriptional *gfp* fusions of x-box candidate genes, intron-*gfp* fusions, translational *gfp* fusions of synaptic genes), we used the *daf-12* (*sa204*) background. The *daf-12* mutation suppresses the Daf-c phenotype of *daf-19* and prevents dauer formation.

Injection Constructs, Germ Line Transformation, and Green Fluorescent Protein (GFP) Expression Analyses

pGG20 and pGG21 contain the last 250 base pairs of *daf-19* intron 3 and *daf-19* intron 4 fused to *gfp*, respectively. The *daf-19* rescue and deletion constructs pTJ803, pGG14, and pGG18 (see Figure 2) were derived from pTJ786 (*daf-19* genomic plus 2.9-kb promoter). pGG67 is a genomic/cDNA fusion rescue construct specific for *daf-19a* (see Figure 4). Transcriptional *gfp* fusions of *daf-19* were injected at 100 ng/μl and *daf-19* rescue constructs were injected at 10 ng/μl. Synaptic markers and promoter *gfp* fusions were injected at 50–70 ng/μl. Adult hermaphrodites were transformed using standard techniques (Mello *et al*., 1991).

Behavioral Assays

Paralysis assays were performed on nematode growth medium agar plates containing 500 μM aldicarb or 100 μM levamisole. In addition, the resistance of *daf-19* mutants to levamisole was confirmed at concentrations up to 1 mM (data not shown). At least 25–30 1-d-old adult worms were examined for each strain. Worms were classified as paralyzed when they did not move upon prodding with a pick three times in a row.

For dwelling/roaming assays, 1-d-old adult worms were transferred singly to fresh plates with a bacterial lawn of standardized size. After 1 h, worms were removed, each plate was put on a transparency with a grid (5 × 5 mm), and the number of squares that were filled with worm tracks was counted (Figure 4A). Each assay was repeated at least twice, with two independent lines for each transgene. More than 30 worms were examined in paralysis and dwelling/roaming assays.

DiI Staining, Microscopy, and Fluorescence Imaging

Fluorescent dye-filling assays with DiI were performed as described previously (Starich *et al*., 1995). For live imaging of GFP expression, worms were anesthetized in 0.1% sodium azide in M9 buffer and immobilized on a 2% agar pad. Differential interference contrast and fluorescence pictures were taken on an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). We also used the microscope together with the OpenLab software (Improvision, Coventry, United Kingdom) for the analysis of expression levels of synaptic proteins (antibody stainings). Pictures of the comarker UNC-10 (unchanged between *wild type* and *daf-19*) and the synaptic protein under investigation were taken at fixed exposure times (optimized for the UNC-10 staining intensity). The intensity of the signal for the synaptic protein under these conditions was classified as "strong" when the picture was overexposed and as "weak" when the picture was underexposed (cf. Figures 6 and 7 and Supplemental Table 2).

Northern Blot Analysis and RNase Protection Assay

Embryos were isolated from gravid wild-type adults grown on egg medium by hypochlorite treatment. Embryonic total RNA was extracted using TRIzol (Invitrogen, Paisley, United Kingdom). For Northern blot, radioactive probes were prepared using the Prime-It random labeling kit (Stratagene, La Jolla, CA) and purified over ProbeQant G50 columns (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). RNase protection assay: Radioactive probes were prepared according to the manufacturer's instructions (MAXIscript kit; Ambion, Austin, TX), and hybridization to 20 μg total RNA was carried out according to the instructions in the manual for the RPA III kit (Ambion).

Quantitative Real-Time PCR

We used TRIzol and the RNeasy kit (QIAGEN, Dorking, Surrey, United Kingdom) to extract total RNA from staged 2-d-old adult worms. All samples were checked for RNA integrity (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA) and subjected to DNase digestion and single-strand cDNA synthesis (iScript; Bio-Rad, Hemel Hempstead, United Kingdom). Expression levels of selected genes were analyzed in an Applied Biosystems 7300 thermocycler (Applied Biosystems, Foster City, CA) by using actin as a reference gene. Each reaction was run in triplicates on two independent biological samples for each strain. All primers had a melting temperature of 58–60°C and produced a single amplicon. Data were analyzed using the Fast SDS software 1.3.1 (Applied Biosystems).

Antibody Production

cDNA fragments corresponding to DAF-19 amino acids 2–212 (for AbDAF19N) and 340–513 (for AbDAF19C), respectively, were expressed in BL21 (DE3) bacterial cells. Immunization of rabbits was carried out at Gramsch Laboratories (Schwabhausen, Germany). On Western blots, AbDAF19N detected a specific band of 120 kDa, by using *wild-type* protein extracts, corresponding to DAF-19A/B. This band was absent from protein extracts from *daf-19* mutant worms (Supplemental Figure 1A). AbDAF19C was not suitable for Western blot analysis. On worm whole-mount stainings, both antibodies detected a signal in neuronal nuclei at all stages (Supplemental Figure 1, B and D). Aside from that, DAF-19 was also detectable in hypodermal cells at larval stages (data not shown).

Western Blot Analysis

Worms were staged by hypochlorite treatment of gravid adults. Western blots were incubated with AbDAF19N (1:250), anti-tubulin (YOL 1/34; 1:100), anti-UNC-17 (1:200), anti-SNB-1 (SN1; 1:200), horseradish peroxidase (HRP) anti-rat (1:10,000), and HRP anti-mouse (1:5000).

Antibody Staining

Staining with antibodies against UNC-29 and UNC-49 required permeabilization through freeze-fracture (Gally and Bessereau, 2003). For all other antibodies, whole-mount fixation and permeabilization were carried out as described previously (Finney *et al*., 1988). Worms were incubated with a 1:400 dilution of affinity-purified anti-DAF-19 antibodies. Other antibodies used were anti-OSM-5 (1:200), anti-SNB-1 (Ab1092; 1:2000), anti-SNB-1 (SN1; 1:200), anti-SNT-1 (R558; 1:100), anti-UNC-10 (RIM; 1:200), anti-UNC-13 (1:800), anti-UNC-17 (1:1000), anti-UNC-18 (G247; 1:100), anti-UNC-29 (1:200), anti-UNC-31 (1:200), anti-UNC-49 (1:800), anti-UNC-64 (Ab940; 1:5000), Alexa488 and Alexa546 (1:250: Jackson ImmunoResearch Laboratories, West Grove, PA), and Cy5 (1:1000; Rockland Immunochemicals, Gilbertsville, PA). The SN1 and RIM antibodies were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). For all antibodies, we investigated the entire nervous system. However, for reasons of equal comparisons, we mainly focused on the head region/nerve ring. Confocal pictures of antibody stainings were taken on a TCS SP microscope (Leica, Wetzlar, Germany).

DNA Sequence Motif Searches

DNA sequences of *C. elegans* and *Caenorhabditis briggsae* synapse genes (3-kb promoter, the entire coding region and 1-kb downstream of the stop codon) were scanned for possible matches to an x-box consensus sequence RYYNYY(N)$_{1-3}$RRNRRY with VectorNTI (Invitrogen). Candidate motifs were analyzed for 1) motifs that are conserved between both species and occur in several genes, or 2) motifs that occur in several *C. elegans* genes, in case the

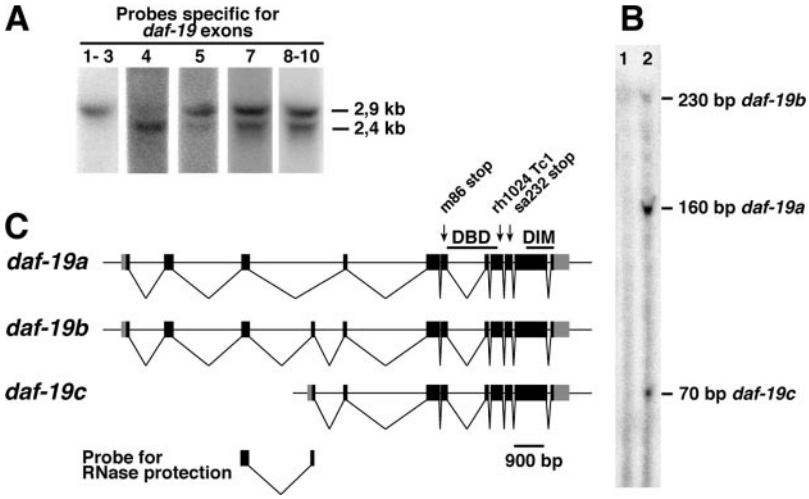

Figure 1. Identification of a third, novel *daf-19* transcript, *daf-19c*. (A) Northern blot analysis of total wild-type RNA. Probes against specific *daf-19* exons (depicted above each lane) detect a novel *daf-19* transcript of 2.4 kb. The two previously described transcripts *daf-19a/b* (Swoboda *et al*., 2000) differ only by ~70 nt and run as one band of 2.9 kb. (B) RNase protection assay using an exons 3–4 probe visualizes all three *daf-19* transcripts, which differ in the composition of exons 3 and 4 (lane 1, unspecific tRNA; lane 2, total RNA from wild-type worms). (C) Genomic organization of the three *daf-19* transcripts. The arrows indicate the three *daf-19* mutant alleles investigated. The RNA probe used for the RNase protection assay is indicated below. DBD, DNA-binding domain; DIM, dimerization domain.

candidate motif lacked conservation in other nematodes species. Candidate motifs conserved between *C. elegans* and *C. briggsae* were not found.

To identify conserved motifs unrelated to the x-box, we searched 1.5 kb upstream of the ATG of the same genes. We scanned for motifs of 5–10, 8–14, and 10–16 nucleotides length using MEME (http://meme.sdsc.edu).

RESULTS

Evidence for a New daf-19 Transcript

To study the subcellular localization and developmental dynamics of DAF-19, we generated antibodies against N- and C-terminal epitopes AbDAF19N and AbDAF19C, respectively. In wild-type worms, these two antibodies detected different DAF-19 expression patterns (see below), which suggested the existence of different DAF-19 isoforms. To determine the corresponding transcripts, we performed Northern blot experiments (Figure 1A). A probe specific for exons 1-3 hybridized to a single 2.9-kb band. This band corresponds to the two known transcripts, the long isoforms *daf-19a/b*, which differ only by the small, alternatively spliced exon 4 (Swoboda *et al*., 2000). In addition to the 2.9-kb band, probes specific for the remaining exons also detected a 2.4-kb transcript, which we termed short isoform *daf-19c*. To visualize all three isoforms *daf-19a/b/c* in one experiment, we conducted an RNase protection assay. A cDNA probe against exons 3–4 protected fragments of three different sizes, corresponding to the transcripts *daf-19a* (containing only exon 3), *daf-19b* (containing exons 3 and 4), and *daf-19c* (containing only exon 4) (Figure 1, B and C). Using 5′-rapid amplification of cDNA ends, we amplified a fragment that includes *daf-19c* exon 4 fused to the SL1 splice leader (data not shown). This is consistent with a trans-splice at the beginning of exon 4 followed by an ATG at position +9, in-frame with the remainder of the *daf-19* transcript. In summary, these results show that in addition to the known long isoforms *daf-19a/b*, a third short isoform *daf-19c* exists that comprises SL1-spliced exons 4–12.

DAF-19C Is Specifically Expressed in Ciliated Sensory Neurons and Regulates Ciliogenesis

A full-length genomic translational *gfp* fusion was shown to be sufficient to rescue the major cilia-related phenotypes of *daf-19*, dye-filling defective (Dyf) and dauer formation constitutive (Daf-c) (Swoboda *et al*., 2000). The identification of *daf-19c* raised the question about the functional significance of each transcript. To test for isoform-specific functions, we generated genomic deletion constructs and introduced them into a *daf-19* mutant background (Figure 2A). Fragments of *daf-19* lacking the promoter and the region up to intron 3 were still able to express DAF-19, as determined by staining with AbDAF19C (Supplemental Figure 2F). The expression was restricted to a small set of neurons in the head and the tail, a pattern reminiscent of ciliated sensory neurons. Consistent with the expression pattern, these constructs were sufficient to activate the expression of *osm-5* and *bbs-7*, two well characterized, direct *daf-19* target genes that are expressed in ciliated sensory neurons and function in cilia formation (Figure 2B and Supplemental Figure 2, A′ and A″) (Haycraft *et al*., 2001; Blacque *et al*., 2004). As expected, these *daf-19* deletion constructs also rescued the Dyf and Daf-c phenotypes of *daf-19* mutants (Figure 2, A and C; data not shown). By contrast, DNA constructs starting downstream of exon 4 failed to rescue (Figure 2, A, D, and E). We also expressed either *daf-19a* or *daf-19c* cDNAs from the *gpa-13* promoter in a *daf-19* mutant background. *gpa-13* drives expression in five ciliated sensory neurons: ADF, ASH, AWC, PHA, and PHB (Jansen *et al*., 1999), out of which ASH (in the head) and PHA and PHB (in the tail) can be stained with the fluorescent dye DiI (Hedgecock *et al*., 1985). We found that *gpa-13(p)::daf-19c*, but not *gpa-13(p)::daf-19a* was sufficient to rescue cilia formation in sensory neurons as visualized by fluorescent dye DiI filling (Figure 2, F–I).

That a 5′-deleted genomic *daf-19* fragment was able to express *daf-19c* and rescue ciliogenesis suggests that an internal promoter drives its expression. We generated *gfp* fusions to intronic sequences flanking exon 4 to investigate their expression patterns. A 250-base pair fragment upstream of exon 4 (Figure 2, J and K) and intron 4 (Figure 2, L and M) were sufficient to drive *gfp* expression in ciliated sensory neurons from the mid-embryonic stage to hatching and from the mid-embryo to adult stage, respectively. Intron 5 was unable to drive *gfp* expression (data not shown). We conclude that introns 3 and 4 contain promoter elements that are sufficient to initiate and maintain the expression of *daf-19c*. In summary, the novel isoform DAF-19C is specifically expressed in ciliated sensory neurons from its own promoter within the gene. *daf-19c*, in contrast to *daf-19a*, is sufficient to rescue the major, cilia-related phenotypes of *daf-19* mutants.

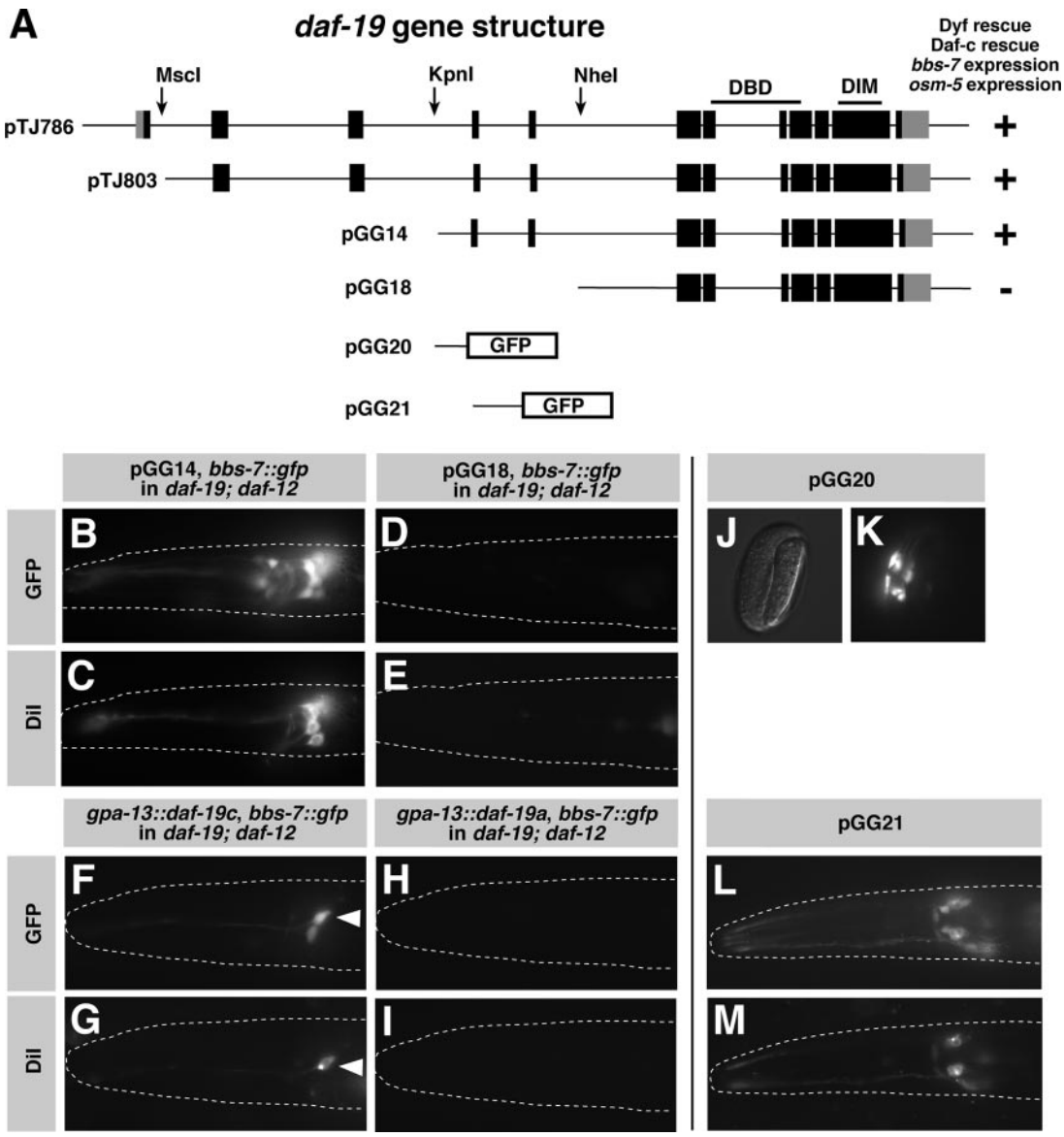

Figure 2. *daf-19c* is transcribed from an internal promoter and regulates cilia formation. (A) Genomic organization of *daf-19* and different deletion constructs. (B–E) *daf-19* worms expressing pGG14 are rescued for the expression of the direct DAF-19 target cilia gene *bbs-7::gfp* and DiI fluorescent dye filling. *daf-19* worms expressing pGG18 are not rescued. (F and G) *daf-19* worms expressing *daf-19c* from the *gpa-13* promoter are rescued for the expression of *bbs-7::gfp* and DiI fluorescent dye filling. The arrowhead depicts ASH; the other neuron in F is AWC. (H and I) Expression of *daf-19a* from the same promoter does not rescue. (J–M) Intron 3 (pGG20) and intron 4 (pGG21) contain regulatory elements driving *gfp* expression in ciliated sensory neurons in the embryo (J and K) and at all developmental stages (L), respectively. *gfp* expressing neurons were identified as ciliated sensory neurons by DiI fluorescent dye filling (M).

DAF-19A/B Are Expressed in Nonciliated Neurons

To elucidate the functions of DAF-19A/B, we analyzed their expression patterns in detail, by using antibodies against the N- and C-terminal regions of DAF-19, AbDAF19N, and AbDAF19C, respectively. Although the N-terminal antibody AbDAF19N recognizes epitopes unique to the long isoforms DAF-19A/B, the C-terminal antibody AbDAF19C recognizes the same epitopes common to isoforms DAF-19A/B/C (Figure 3A). To prove that AbDAF19N specifically detects DAF-19A/B and not C, we compared both antibodies on transgenic rescue lines expressing only DAF-19A or DAF-19C, respectively. As expected, we could detect DAF-19A with the N- and C-terminal antibodies, but DAF-19C only with the C-terminal antibody (Supplemental Figure 2, A–F). Stainings of wild-type worms with both antibodies detected DAF-19 in the majority of neuronal nuclei in the head and tail ganglia and in the ventral nerve cord. This signal was absent in all *daf-19* mutant alleles tested (*m86*, *m334*, *m407*, *rh1024*, *sa190*, and *sa232* affect all three isoforms equally; Swoboda *et al.*, 2000), proving the specificity of both antibodies (Supplemental Figure 1, B–E). Although the AbDAF19N and AbDAF19C staining patterns overlapped in large parts, they were not identical. Posterior to the nerve ring, where the cell bodies of the amphid ciliated sensory neurons are located, we observed a group of cells, which stained only with AbDAF19C, but not with AbDAF19N (Supplemental Figure 1, B and D).

Our analysis revealed that DAF-19A/B are expressed in a larger number of neurons than DAF-19C, which is restricted

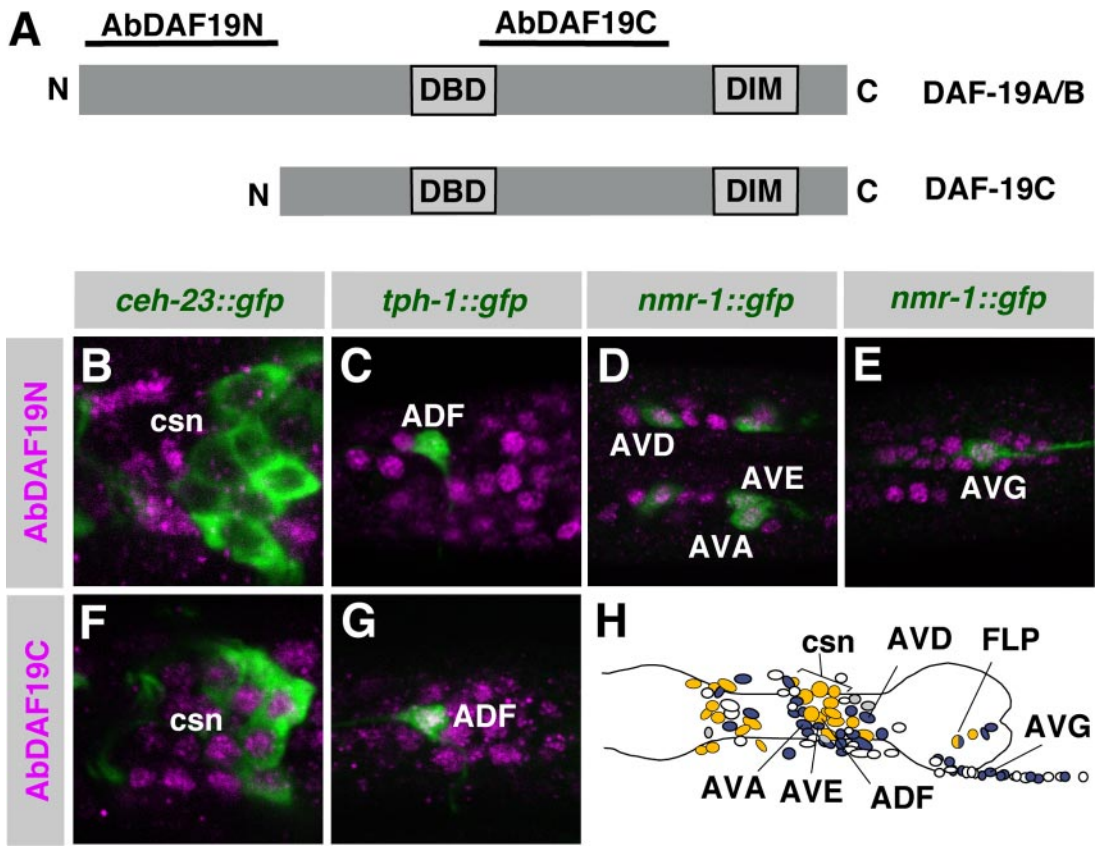

Figure 3. An antibody specific for DAF-19A/B detects DAF-19 in all nonciliated neurons. (A) DAF-19 epitopes recognized by two different antibodies. Antibody AbDAF19N is specific for the long isoforms DAF-19A/B, whereas antibody AbDAF19C recognizes all three isoforms DAF-19A/B/C. (B–G) *gfp* reporter lines stained with antibodies against DAF-19. AbDAF19N detects DAF-19A/B only in nonciliated neurons (D and E) and not in ciliated sensory neurons (csn) (B and C). Ciliated sensory neurons express only DAF-19C. Because ciliated sensory neurons do not express DAF-19A/B, DAF-19C can be visualized by AbDAF19C (F and G). *ceh-23::gfp* marks ciliated sensory neurons, *tph-1::gfp* marks the serotonergic neuron ADF (ciliated), and *nmr-1::gfp* marks interneurons (nonciliated); see Supplemental Table 1 for details. (H) Schematic summary of all neurons investigated in the head region (blue, AbDAF19N; yellow, AbDAF19C; white, not determined neurons; and gray, no DAF-19 expression detected).

to ciliated sensory neurons. From a rough cell count of all neurons that stained with AbDAF19N in the adult hermaphrodite, we estimate that DAF-19A/B are expressed in ~200–240 neurons (data not shown). To understand where DAF-19A/B may exert their functions, we determined their expression patterns in detail. We stained *gfp* reporter lines, which mark subgroups of neurons, with anti-GFP and with AbDAF19N antibodies to determine whether they label the same neurons. Using nine different markers, we tested nearly half of all 302 neurons in the adult hermaphrodite, corresponding to ~60 different classes of neurons (Figure 3H and Supplemental Table 1). We found that DAF-19A/B were expressed only in nonciliated neurons and not in ciliated sensory neurons (Figure 3). For example, *ceh-23::gfp* is expressed in many ciliated sensory neurons and the nonciliated neurons AIY and CAN. DAF-19A/B were detected in AIY and CAN but not in ciliated sensory neurons (Figure 3B; data not shown). Similarly, the nonciliated neurons marked with *nmr-1::gfp* stained with AbDAF-19N and therefore express DAF-19A/B (Figure 3, D and E). Thus, DAF-19C is specific for ciliated sensory neurons, and DAF-19A/B are specific for nonciliated neurons. In total, we found in 86 of 92 tested nonciliated neurons expression of DAF-19A/B, representing many different neuronal classes. In summary, DAF-19A/B are expressed in 200–240 nonciliated neurons and DAF-19C is expressed in 60 ciliated sensory neurons, which adds up to a basically pan-neuronal expression pattern of DAF-19 in the *C. elegans* hermaphrodite.

Dwelling/Roaming Behavior Depends on Multiple daf-19 Isoforms

Mutations in genes with broad neuronal expression often lead to the impaired movement of worms (*UN*Coordinated phenotype). *daf-19* mutants move in a wild-type like manner and show no obvious Unc phenotype. We also tested *daf-19* mutants in body bend assays to determine their movement speed, and we found that they can move as fast as *wild type* (data not shown). More specific aspects of *C. elegans* behavior (mating, feeding, egg laying, or patterns of movement) are usually dependent on or influenced by sensory abilities of the worm and thus depend on *daf-19c*. We did not identify a specific behavior that exclusively required nonciliated neurons or DAF-19A/B (data not shown). However, when performing body bend assays, we observed in *daf-19* mutants severe defects in their dwelling/roaming behavior, which was dependent on all three DAF-19 isoforms. When put on a fresh plate seeded with bacteria, a single wild-type worm covers the entire bacterial lawn with tracks within a short time (dwelling/roaming) (Figure 4, A and B). In contrast, *daf-19* mutants (we tested *m86*, *rh1024*, and *sa232*) move only for a short time and then start feeding locally (Figure 4D; data not shown). A similar behavior is observed in many

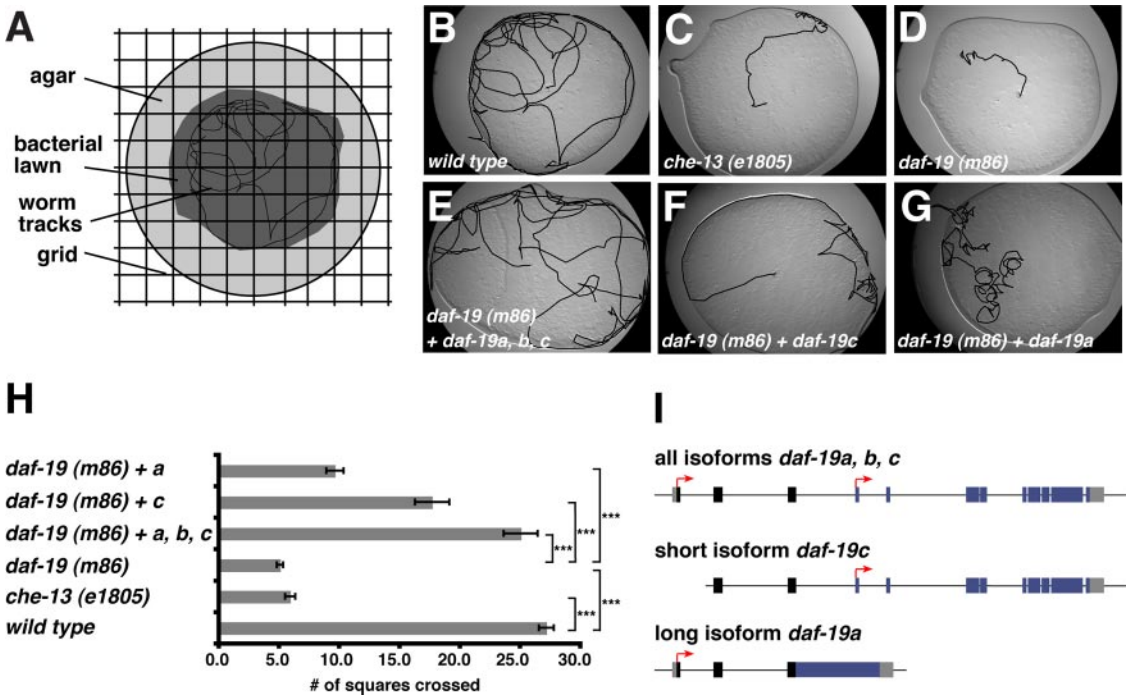

Figure 4. DAF-19A and DAF-19C are required for complete rescue of the dwelling/roaming phenotype of *daf-19* mutants. (A) Schematic visualizing the method for analyzing the dwelling/roaming assay. (B–G) Representative pictures of worm tracks on a bacterial lawn after 1 h (dwelling/roaming). Black lines visualize the worm tracks. (H) Quantification of the dwelling/roaming phenotype. Error bars show SEM values; ***$p < 0.001$, as detected by two-sample *t* test. (I) Genomic organization of the isoform-specific rescue constructs. Arrows mark the beginning of the three isoforms, respectively.

cilia mutants, in which genes that are expressed exclusively in ciliated sensory neurons are mutated (e.g., *che-13*; Haycraft *et al*., 2003) and *che-11* (Bell *et al*., 2006; Figure 4C; data not shown). To test the functions of the different DAF-19 isoforms in the dwelling/roaming behavior, we generated isoform-specific rescue constructs (Figure 4I). The dwelling/roaming phenotype of *daf-19* mutants could partially be rescued by *daf-19a* or *daf-19c* (Figure 4, F and G). Complete rescue occurs only when all isoforms were present via a full-length genomic *daf-19* construct (Figure 4, E and H). From these behavioral experiments, we conclude that the dwelling/roaming phenotype of *daf-19* mutants is not merely caused by the lack of cilia, because the function of both the long and the short *daf-19* isoforms are required.

daf-19 Mutants Are Resistant to Aldicarb and Levamisole

DAF-19C regulates cilia formation in ciliated sensory neurons. Do DAF-19A/B regulate an analogous, common function in nonciliated neurons? Neurons must establish synaptic connections to multiple partners to guarantee the correct wiring and function of the neuronal network. To test for connectivity, we visualized the nervous system with the pan-neuronal marker *unc-104::gfp* and other markers. *daf-19* mutants develop a grossly normal neuronal network that includes all the required neurons and processes (data not shown). To examine the efficiency of synaptic transmission, we exposed *wild type* and *daf-19* mutants to the pharmacological substances aldicarb and levamisole. Aldicarb, an acetylcholine esterase inhibitor, leads to the accumulation of acetylcholine in the synaptic cleft and the paralysis of wild-type animals. *daf-19* mutants (*m86*, *rh1024*, and *sa232*) showed moderate, but statistically significant, resistance to aldicarb compared with wild-type worms (Figure 5A; data not shown). This resistance could be the result of a presynaptic defect (synthesis or release of acetylcholine [ACh]), or a postsynaptic defect (response to ACh). To determine

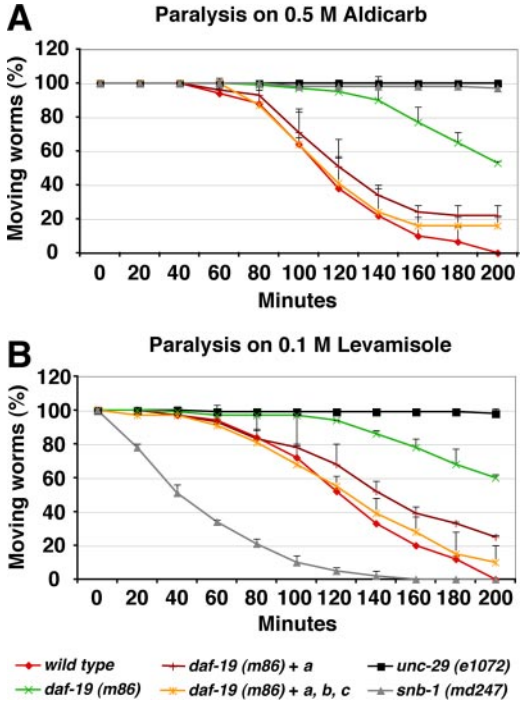

Figure 5. Paralysis assays on aldicarb and levamisole. *daf-19* mutants show resistance to aldicarb (A) and levamisole (B) in paralysis assays. *daf-19a* rescues to a similar extent as a genomic full-length fragment. Error bars show SEM values.

Table 1. Comparison of general neuronal and synaptic markers for their expression in wild-type and *daf-19* mutant adult worms

Protein	Molecular function or similarity	Detection method	*daf-19 (m86)* vs. *wild type*
General neuronal proteins			
JNK-1	Serine/threonine kinase	gfp reporter	No change
UNC-104	Kinesin-like protein	gfp reporter	No change
Pre- and postsynaptic proteins			
SYD-1	PDZ and rhoGAP domain protein	gfp reporter	No change
UNC-10	Rim1 homologue	Antibody	No change
UNC-13	Neurotransmitter release regulator	Antibody	No change
UNC-18	Sec1 homologue	Antibody	No change
UNC-31	PH-domain protein	Antibody	No change
GLR-1	Glutamate receptor	gfp reporter	No change
UNC-29	Acetylcholine receptor	Antibody	No change
UNC-43	CaM kinase II	gfp reporter	No change
UNC-49	GABA receptor	Antibody	No change
UNC-64	***Syntaxin***	***Antibody***	***Reduced***
Synaptic vesicle proteins			
IDA-1	***Tyr phosphatase-like receptor***	***gfp reporter***	***Reduced***
SNB-1	***Synaptobrevin, v-SNARE***	***Antibody, gfp reporter***	***Reduced***
SNG-1	Synaptogyrin	gfp reporter	No change
SNT-1	***Synaptotagmin***	***Antibody***	***Reduced***
UNC-17	***Vesicular acetylcholine transporter***	***Antibody***	***Reduced***

All GFP reporters are translational fusions to *gfp*.

whether *daf-19* mutants had any postsynaptic deficiencies, we tested *daf-19* mutants on levamisole, an acetylcholine receptor agonist, which activates cholinergic receptors independent of presynaptic input. Strikingly, *daf-19* mutants (*m86*, *rh1024*, and *sa232*) were also resistant to levamisole compared with *wild type* (Figure 5B; data not shown). To exclude that these phenotypes are caused by the lack of cilia, we performed paralysis assays on the cilia mutants *che-11*, *che-13*, and *osm-5*. None of them showed the same phenotype as *daf-19* mutants; rather, they behaved similar to *wild type* (Supplemental Figure 3A; data not shown). Furthermore, the resistance of *daf-19* to both aldicarb and levamisole was rescued by a genomic *daf-19* fragment or by *daf-19a* alone (Figure 5). This directly demonstrates that the long isoform DAF-19A is required to regulate synaptic transmission. Finally, because levamisole is thought to mainly act on postsynaptic acetylcholine receptors at neuromuscular junctions, we tested the function of DAF-19A in body wall muscles. Ectopic expression of *daf-19a* in muscle tissue did not alter the resistance of *daf-19* mutants to levamisole (Supplemental Figure 3B). Together, our experiments uncover a hitherto undescribed neuronal function of *daf-19a* in synaptic signal transmission.

Diminished Expression of Synaptic Vesicle Proteins in daf-19 Mutants

To elucidate the reason for the reduced synaptic transmission efficiency in *daf-19* mutants, we investigated the expression and localization of several types of neuronal proteins that may explain the aldicarb and levamisole phenotypes of *daf-19* mutants (Table 1). The expression of general neuronal proteins (JNK-1 and UNC-104) and pre- and postsynaptic proteins (SYD-1, UNC-10, UNC-13, UNC-18, UNC-31, GLR-1, UNC-29, UNC-43, and UNC-49) did not differ between *wild type* and *daf-19* mutants. These results suggest that the overall abundance of synapses and synaptic proteins is not affected in *daf-19* mutants.

However, we also found proteins whose abundance was reduced in *daf-19* mutants. Of all pre- and postsynaptic proteins tested only one component of the presynaptic terminal, UNC-64/syntaxin, was reduced in *daf-19* mutants compared with *wild type* (Figure 6B and Supplemental Table 2). UNC-64 is a plasma membrane receptor for intracellular vesicles and part of the core synaptic vesicle fusion machinery, involved in the release of neurotransmitters. Among the synaptic vesicle markers investigated, all with the exception of SNG-1/synaptogyrin were reduced in *daf-19* mutants: IDA-1 (tyrosine phosphatase-like receptor that interacts with UNC-31 and UNC-64), UNC-17 (acetylcholine transporter), SNB-1 (synaptobrevin, v-SNARE/vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor), and SNT-1 (calcium-dependent phospholipid-binding protein) (Figure 6, A, C–H; Table 1; and Supplemental Table 2). To ensure that these observations were not a result of staining artifacts, we analyzed in each individual animal in parallel the expression of UNC-10, which remained unchanged between *wild type* and *daf-19*. Thus, our analysis discovered a so far undescribed *daf-19* phenotype, the reduced expression of selective synaptic components. Interestingly, the analysis of mixed stage populations revealed that this reduction was prominent, particularly at adult stages. To analyze this observation in detail, we performed the same analysis on staged worms at different times during adulthood. Intriguingly, the difference of SNB-1 and UNC-64 levels between *wild type* and *daf-19* became stronger as they progressed through adulthood (Figure 7, A and B). Corroborating evidence for the gradual reduction of synaptic proteins in the absence of DAF-19 was obtained by Western blot analysis. Protein extract from *daf-19* (−) worms contained less SNB-1 and UNC-17 compared with *daf-19* (+)

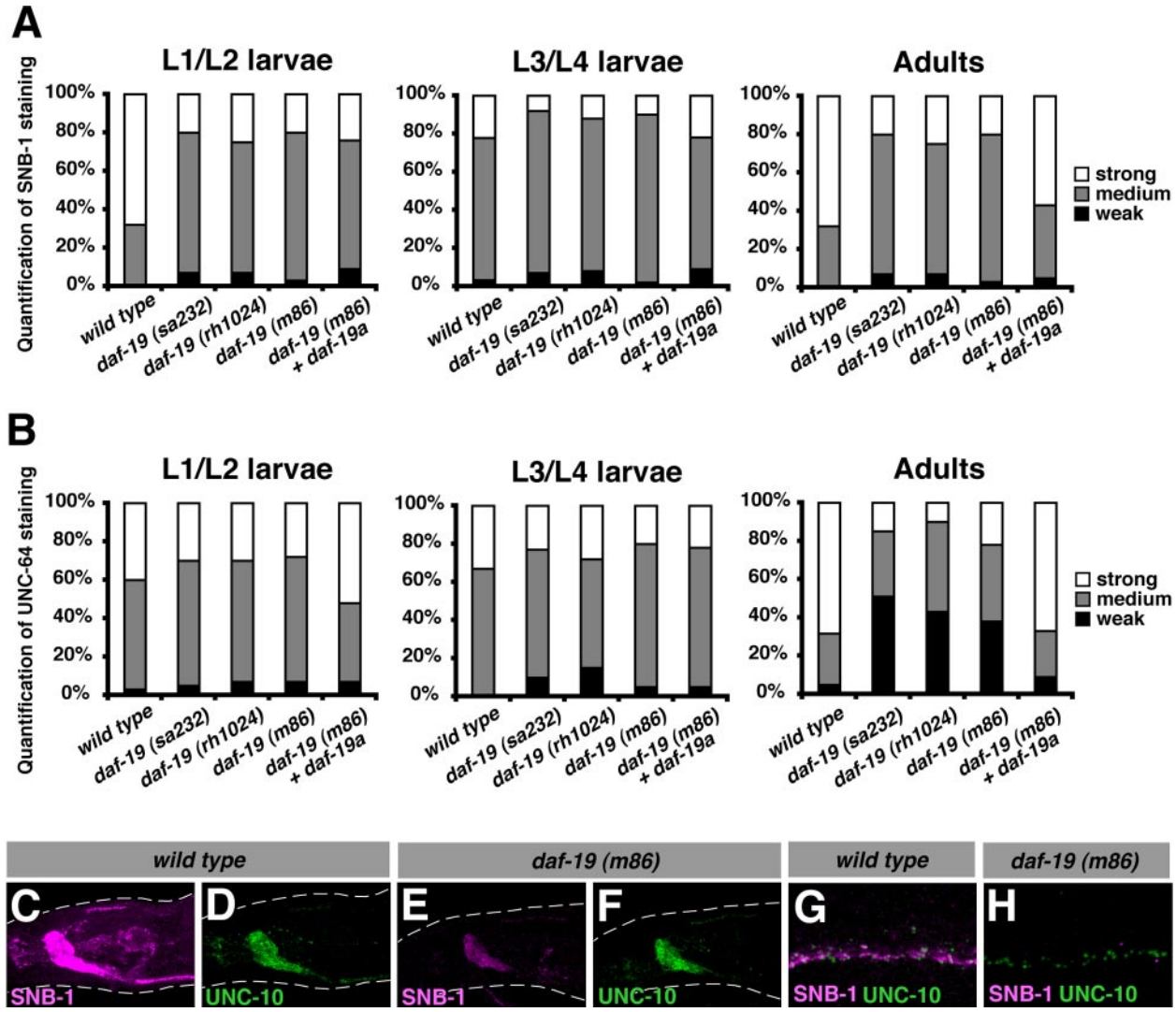

Figure 6. Mutations in *daf-19* result in the down-regulation of the synaptic vesicle proteins SNB-1 and UNC-64 (cf. Table 1). (A and B) Quantification of SNB-1 (A) and UNC-64 (B) antibody stainings in mixed stage populations of *wild type*, *daf-19*, and rescued worms (see Supplemental Table 2). The unchanged UNC-10 staining was used as a reference. At least 40 animals were scored for each genotype and stage. (C–H) Confocal micrographs of adult worms stained with antibodies against SNB-1 (C, E, G, and H) and UNC-10 (D and F–H). C–F show the nerve ring in the head; G and H show a magnification of the ventral nerve cord. Genotypes are indicated above the panels. Representative pictures show SNB-1 staining classified as strong in *wild type* and weak in *daf-19*, both with regard to the unchanged UNC-10 signal.

worms and this difference increased dramatically with the age of the worms (Figure 7C). We were interested in whether this stage-related observation correlated with behavioral phenotypes, and we compared L4 larvae and adults in dwelling/roaming and aldicarb assays. In both experiments, *daf-19* mutant adult worms showed stronger phenotypes than L4 larvae (data not shown). Reduced neuronal expression of SNB-1 and UNC-64 could be rescued by a full-length genomic *daf-19* rescue fragment that expresses all three isoforms as well as by isoform-specific rescue for *daf-19a* alone (Figure 6, A and B; data not shown). These results support those obtained in the paralysis assays (Figure 5), in which DAF-19A could rescue the resistance of *daf-19* mutants to aldicarb and levamisole. Furthermore, the rescue by DAF-19A alone indicates that the reduction of SNB-1 and UNC-64 in nonciliated neurons is not merely a result of the lack of cilia and consequently the lack of environmental stimuli. To support this notion, we investigated SNB-1 and UNC-64 expression in the cilia mutants *che-11* and *che-13* and we found that protein levels were similar to *wild type* (Supplemental Table 2). Therefore, we conclude that the reduced SNB-1 and UNC-64 levels seen in *daf-19* mutant adults are not caused by the lack of cilia or lack of sensory input but are a consequence of the absence of DAF-19A/B.

In summary, these experiments show that *daf-19* mutants have reduced levels of several synaptic proteins (e.g., SNB-1 and UNC-64). *snb-1* and *unc-64* mutants are resistant to aldicarb and levamisole, suggesting that their gradual loss in *daf-19* mutants directly causes changes in synaptic transmission. Interestingly, the reduced expression of synaptic proteins in *daf-19* mutants affects mostly components of the synaptic vesicle pool and is increasingly evident at adult stages, whereas larval stages are not or only mildly affected. Thus, the synaptic defects seen in *daf-19* mutants are likely not caused by early developmental deficiencies. We speculate that they are the consequence of a problem arising during the maintenance of synaptic protein expression in the aging adult.

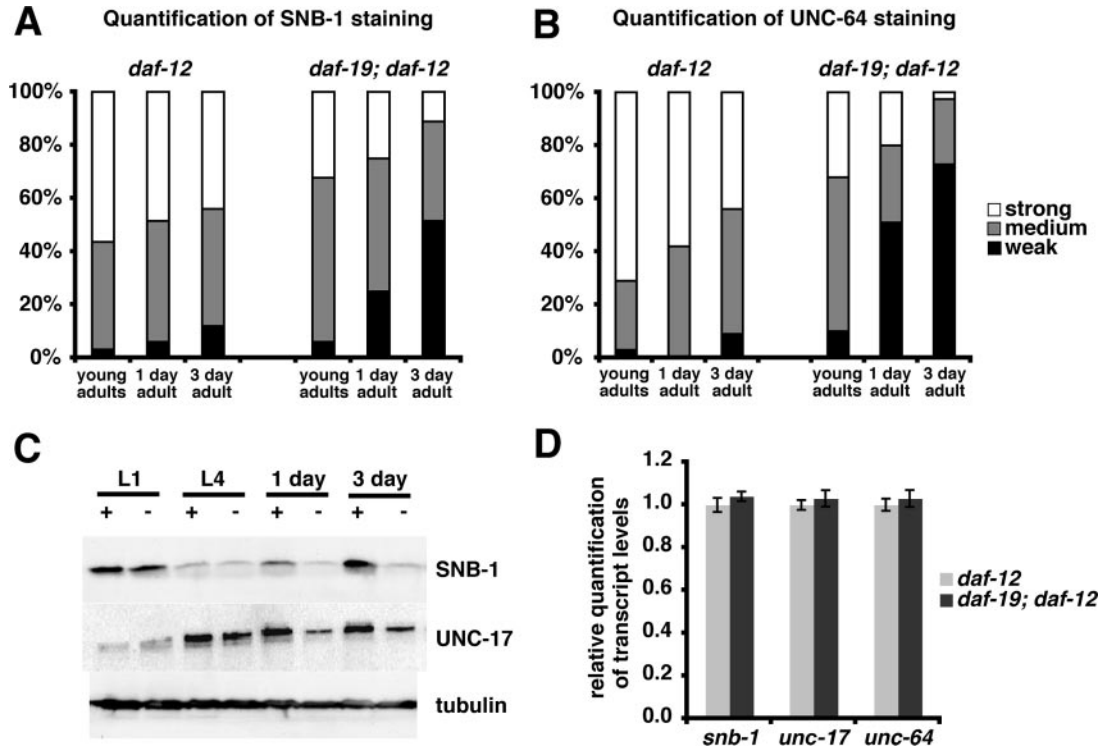

Figure 7. Mutations in *daf-19* result in the down-regulation of the synaptic vesicle proteins SNB-1, UNC-17, and UNC-64 but not of their transcripts. (A and B) Quantification of SNB-1 (A) and UNC-64 (B) antibody staining in staged adult *daf-12* and *daf-19; daf-12* worms. The unchanged UNC-10 staining was used as a reference. At least 40 animals were scored for each genotype and stage. (C) Western blots comparing SNB-1 and UNC-17 levels in protein extracts of staged *daf-12* (+) and *daf-19; daf-12* (−) worms. Within each developmental stage, proteins were isolated from an equal number of worms (note: this number varies for the different stages); 1 day and 3 day denote adults grown for 1 and 3 d after reaching L4, respectively. (D) Quantification of transcript levels of synaptic vesicle genes in *daf-12* and *daf-19; daf-12* adults by quantitative real-time PCR.

DAF-19A/B Regulate Synaptic Protein Expression Indirectly

DAF-19C regulates target cilia genes directly through a conserved promoter motif, the x-box. Are synaptic genes regulated by DAF-19A/B in a similar manner? Because all DAF-19 isoforms contain the same DNA binding domain (Figures 1C and 3A), we reasoned that they could bind to overall very similar DNA sequence motifs and that direct target genes for DAF-19A/B are included in published lists of predicted x-box genes (Blacque *et al*., 2005; Efimenko *et al*., 2005; Chen *et al*., 2006). We filtered those lists for all genes with functions at synapses or in vesicle formation/transport (Supplemental Table 3). *ida-1*, *snb-1*, *snt-1*, *unc-17*, and *unc-64* were not among them. In addition, we searched those five genes for degenerated, x-box-like or other conserved sequence motifs. None of these searches revealed any common motifs (data not shown), suggesting that they do not harbor a binding site for DAF-19A/B. To search for other possible direct DAF-19A/B targets, we checked the expression of multiple candidates from the above-mentioned lists for their dependence on the transcription factor. None of them was affected in *daf-19* mutants (Supplemental Table 3). To finally test whether *snb-1*, *unc-17*, and *unc-64* are directly or indirectly regulated at the transcript level, we compared their expression levels by quantitative real-time PCR. We did not detect any difference between *wild type* and *daf-19* mutants in transcript levels of these three genes (Figure 7D). Thus, we conclude that DAF-19A/B do not regulate synaptic genes at the transcriptional level. We speculate that DAF-19A/B maintain synaptic protein expression in nonciliated neurons via an indirect mechanism, yet to be discovered (Figure 8).

DISCUSSION

Different DAF-19 Isoforms Have Distinct Functions in Subsets of Neurons

C. elegans DAF-19 was shown to regulate the expression of genes required for the structure and function of cilia (Swo-

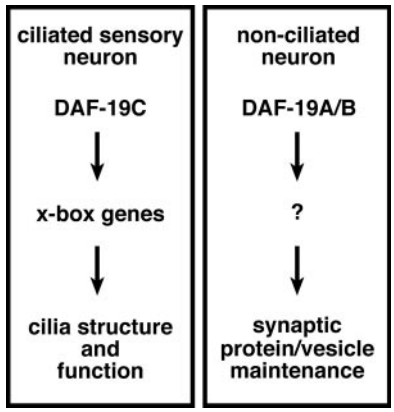

Figure 8. DAF-19A/B and DAF-19C execute distinct functions in synapses and cilia, respectively.

boda *et al*., 2000). Here, we identified a novel short transcript *daf-19c* that lacks exons 1-3. This short isoform DAF-19C is specifically expressed in ciliated sensory neurons from an internal promoter, and it is sufficient to rescue all cilia-related phenotypes of *daf-19* mutants (Dyf, Daf-c, expression of cilia-specific, direct target genes). In contrast, the long isoforms DAF-19A/B are expressed from a different promoter in almost all nonciliated neurons, resulting in a basically pan-neuronal expression pattern of DAF-19. This expression of multiple isoforms via the so-called two-promoter system is common to many genes in *C. elegans* and crucial for the execution of their isoform-specific functions (Choi and Newman, 2006). We discovered that *daf-19* mutants are resistant to the pharmacological substances aldicarb and levamisole, both of which modulate cholinergic synaptic transmission and lead to paralysis. The reason for this resistance was found in strongly reduced levels of synaptic vesicle proteins that were observed in adult but not juvenile animals. In addition, the lack of DAF-19 results in impaired dwelling/roaming behavior of the worm. These phenotypes can be rescued by the long isoform DAF-19A and therefore implicate a novel role of DAF-19 in the maintenance of synaptic neurotransmission.

How Do the Different DAF-19 Isoforms Activate Different Groups of Target Genes?

A large number of direct target genes has been identified for the cilia-specific short isoform DAF-19C. All those genes have in common that they 1) are expressed and function in ciliated sensory neurons and 2) contain an x-box promoter motif. Direct target genes of DAF-19A/B in nonciliated neurons currently remain unidentified. Furthermore, DAF-19A is not sufficient to replace DAF-19C in ciliated sensory neurons, indicating that these isoforms activate different target genes. Therefore, what determines the respective functions of the different isoforms?

First, the x-box DNA sequence motifs bound by DAF-19A/B could vary slightly but significantly from the motifs bound by DAF-19C. In *C. elegans*, the consensus in cilia-specific x-box genes contains a defined spacer of two central nucleotides (Efimenko *et al*., 2005), whereas the consensus sequence for hRFX has a variable spacer of zero to three nucleotides (Emery *et al*., 1996; Gajiwala *et al*., 2000). It is possible that the larger DAF-19A/B also could bind a consensus sequence with no or three spacer nucleotides, like hRFX proteins do. Alternatively, DAF-19A/B could act on x-box motifs in positions different from hitherto proven x-box motifs (i.e., >250 base pairs upstream of the ATG or within introns).

In another scenario, DAF-19–interacting proteins could decide which genes can be transcribed. DAF-19A/B contain an N-terminal part encoded by exons 1-3 lacking in DAF-19C. This N-terminal extension might serve as a site for protein interactions through which isoform-specific binding partners regulate the affinity to synaptic x-box genes instead of cilia x-box genes. Interestingly, RFX genes in all eukaryotes encode proteins of a size similar to the long isoforms DAF-19A/B, having a long N-terminal part upstream of the DNA binding domain. In addition, for some RFX genes, such as *daf-19*, alternative splicing of different isoforms has been demonstrated (e.g., Zhang *et al*., 2006). However, the protein part encoded by *daf-19* exons 1-3 is not highly conserved at the amino acid level across species. Conservation between RFX proteins of different organisms could thus exist at a structural level. We assume that the N-terminal part of the protein, despite the lack of any assigned conserved domains, is important for the specific function of DAF-19A/B and other RFX proteins. It will thus be essential to characterize the function of the protein domains encoded by exons 1-3.

DAF-19A/B Are Required for Pre- and Postsynaptic Functions in Neurons

We discovered novel *daf-19* mutant phenotypes that are caused by the lack of DAF-19A/B and suggest pre- and postsynaptic maintenance defects in neurotransmission. In agreement with these defects, we found that the abundance of several synaptic proteins, especially SNB-1, UNC-17, and UNC-64, was gradually reduced during adulthood. Three characteristics set the synaptic defects of *daf-19* apart from all other synapse mutants identified so far: 1) Intriguingly, the decline of synaptic protein levels was most prominently seen in adult worms, whereas larval stages were hardly affected. 2) In neurons both pre- and postsynaptic functions are affected. 3) Because DAF-19A/B are expressed in neurons but not in muscles, it is likely that muscular postsynaptic terminals are intact. The absence of DAF-19 in muscular tissue indicates that the protein does not have a function in muscle cells. This explains why ectopic expression of *daf-19* in body wall muscles does not rescue the levamisole-induced paralysis phenotype of *daf-19* mutants. The facts listed above also help explain why *daf-19* mutants do not have a severe Unc phenotype and are only moderately resistant to paralyzing substances such as aldicarb and levamisole as opposed to the complete resistance seen, for example, in the Unc mutants *unc-29*, *unc-64*, or *snb-1* (Nonet *et al*., 1998; Saifee *et al*., 1998).

Although our paralysis experiments using levamisole revealed deficiencies at postsynaptic terminals in *daf-19* mutants, we currently do not know their cause. All postsynaptic proteins checked were unchanged in *daf-19* mutants. It is unlikely that the presynaptic effects found induce an indirect postsynaptic defect (resistance to levamisole) through a feedback mechanism. In that case, *daf-19* mutants should on levamisole phenocopy other presynaptic mutants, such as *snb-1*. We therefore hypothesize that in addition to the presynaptic proteins we describe, so far unidentified postsynaptic molecules are also affected by the lack of DAF-19.

Maintaining Synaptic Protein Expression: A Novel Role for DAF-19A/B

Several screens have been performed that used SNB-1::GFP as synaptic vesicle marker (Zhen and Jin, 1999; Schaefer *et al*., 2000; Zhen *et al*., 2000; Crump *et al*., 2001; Shen and Bargmann, 2003). Others investigated genes with predicted roles in synaptic functions (Sieburth *et al*., 2005), synaptic vesicle recycling and transport (Koushika *et al*., 2004; Dittman and Kaplan, 2006). These screens uncovered genes required for the localization of SNB-1::GFP at the synapse but not for the maintenance of SNB-1 function. Therefore, *daf-19* is the first *C. elegans* mutant that shows a strong reduction of several synaptic proteins, especially during the later phases of adulthood. This suggests that DAF-19A/B are required for the maintenance of synaptic components rather than for their expression during development.

We identified several synaptic vesicle proteins that are reduced upon loss of *daf-19*. Two possible scenarios could explain these findings: 1) DAF-19A/B have an influence on synaptic vesicle biogenesis/recycling, or 2) DAF-19A/B regulate a neuronal gene or process that is required for synaptic vesicle protein expression or maintenance. If a general reduction of synaptic vesicles was taking place, one would expect all vesicle proteins to be reduced to similar extents. Although we formally cannot rule out this possibility, the

various degrees of reduction between different vesicle proteins (strong reduction of SNB-1 and UNC-64, mild reduction of UNC-17, and no reduction of SNG-1) argue against a general vesicle problem and indicate that these proteins are regulated differentially. Work from mammalian systems supports the notion of individual regulation of synapse components (Shimohama *et al*., 1998). Furthermore, the increase of synaptic proteins during neuronal development is not due to the increase of the transcriptional rate, but it is regulated at the level of protein stability (Daly and Ziff, 1997). Because most synaptic proteins are highly conserved, it is very likely that also in *C. elegans* the expression, maintenance, or both of synaptic proteins is individually regulated. We hypothesize that if DAF-19A/B regulate synaptic protein expression, they execute this function indirectly at a posttranscriptional level, because transcript abundance of the corresponding genes in *daf-19* mutants were similar to *wild type* (Figure 8).

Cilia development is an essential process regulated by RFX transcription factors across species. Is it similar with regard to the functional maintenance of synapses? Although brain defects have been reported for Rfx3- and Rfx4_v3-deficient mice (Baas *et al*., 2006; Zhang *et al*., 2006), embryonic lethality precluded the analysis of late brain defects. Our analysis of *daf-19* mutants suggests that the specific investigation of synapse-related functions of RFX transcription factors in other organisms is relevant to synaptic maintenance.

The C. elegans daf-19 Mutant: A New Disease Model for Functional Synaptic Decline?

Deregulation of synaptic proteins has been described for several neurological diseases, such as Huntington's disease (Morton *et al*., 2001) or Alzheimer's disease (Sze *et al*., 2000; Reddy *et al*., 2005). Research concerning neurodegeneration nowadays increasingly focuses on the loss of synaptic proteins, which is thought to trigger synaptic loss (Selkoe, 2002). The phenotypes seen in *daf-19* mutants show parallels to the loss of synaptic proteins described for neurodegenerative diseases. RFX transcription factors as well as the majority of synaptic proteins in *C. elegans* are highly conserved, which suggests that synaptic protein stability in different organisms may be similarly regulated. Therefore, *C. elegans* and the *daf-19* mutant in particular may in the future prove to be a useful model system to experimentally dissect the mechanisms that maintain synaptic function.

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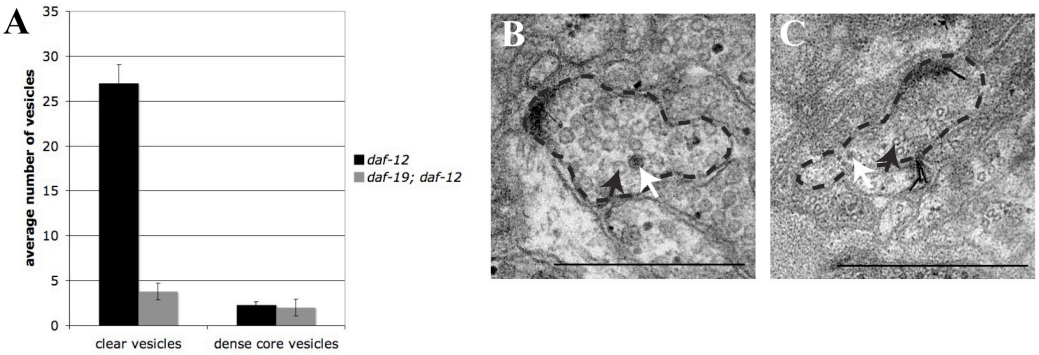
PAPER I – APPENDIX

We found that levels of synaptic vesicle proteins were reduced in *daf-19* mutants. This reduction could be caused by defects during protein expression. Alternatively, the number of synaptic vesicles themselves could be affected. To investigate these possibilities further, we performed EM experiments on *daf-19* mutants.



Two-day old adults were used to determine the number of synaptic vesicles in the pre-synaptic terminals of motor neurons in the ventral nerve cord. (A) Synapses of *daf-19; daf-12* mutants contain considerably less clear synaptic vesicles as compared to *daf-12* mutants. The number of dense core vesicles is similar between both genotypes. (B) Synaptic profile of a *daf-12* mutant. (C) Synaptic profile of a *daf-19; daf-12* mutant. The error bars in the graph show s.e.m. The dashed lines in B and C outline the border of a single synapse; black arrows mark clear vesicles and white arrows mark dense core vesicles. The scale bar marks 2 µm. These preliminary data suggest that the loss of DAF-19 causes a decrease in synaptic vesicles.

Sample preparation: Animals were transferred into ice-cold fixative containing 1% formaldehyde/2% glutaraldehyde. Heads and tails of the worms were removed with a syringe to ensure proper penetration of the fixative into the worm and conservation of the tissue. Fixed specimens were once more

transferred into fresh ice-cold fixative and stored over night at 4°C. All subsequent steps of sample preparation and sectioning were performed according to standard procedures by the staff of Kjell Hultenby at the local EM facility (Karolinska Hospital, Huddinge, Sweden).

Data collection and analysis: Sections (50 nm thick) of animals were collected and profiles containing a pre-synaptic density were chosen for analysis. For *daf-12* mutants (control) three profiles from three worms (coming from two independent sample preparations) were analyzed. For *daf-19; daf-12* mutants three profiles from three worms (coming from one sample preparation) were analyzed. Table 1 shows in detail the numbers for each genotype that also summarized in panel A.

Table 1: Vesicle number in single synapse profiles (three per genotype)

Genotype	Clear vesicles	Dense core vesicles
daf-12	30/26/24	3/2/2
daf-12; daf-19	8/7/4	1/6/2

Paper II

FUNCTIONAL RESCUE IN SINGLE SENSORY CILIA (FRISSC) IN *C. ELEGANS*

Functional rescue in single sensory cilia (FRISSC)

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Running Title: Single cilia rescue in *C. elegans*

Keywords: DAF-19, RFX transcription factor, ciliated sensory neuron (CSN), FRISSC, drop assay, quadrant assay

ABSTRACT

The detection and correct interpretation of environmental signals is crucial for the survival of every organism. Studying mechanisms of sensory perception is a challenging task, especially in organisms with complex neuronal networks. The nematode *C. elegans* possesses a rather simple neuronal network of 302 neurons, including 60 ciliated sensory neurons (CSNs), which are the main source of external sensory input. In spite of this simplicity, *C. elegans* executes a large number of different behaviors and is therefore an excellent model organism in which to study sensory neuron function. We have generated a genetic rescue system – FRISSC (**F**unctional **R**escue **I**n **S**ingle **S**ensory **Ci**lia) – that for the first time allows the *in vivo* analysis of isolated CSNs at both a cellular and systemic level. FRISSC makes use of the RFX transcription factor DAF-19, a key regulator of a large number of cilia genes and, therefore, of cilia formation. Mutations in *daf-19* result in the complete absence of all sensory cilia and therefore of external sensory input. We use *daf-19* mutants and cell-specific rescue of DAF-19 function in only selected neurons. This generates animals with single, fully functional CSNs. Otherwise and elsewhere these animals are completely devoid of any environmental input through cilia. We show that the rescue of cilia formation in single sensory neurons is sufficient to restore their function, and is cell-autonomous and cell-specific. The high penetrance of rescue makes FRISSC an excellent tool to study behaviors triggered by sensory input in single animals as well as in populations of worms. FRISSC can easily be adapted to any CSN. It can be used to study DAF-19 related developmental aspects, specific sensory issues concerning CSNs or early steps of ciliogenesis.

INTRODUCTION

The detection of environmental cues is essential for the survival of every organism. These cues trigger and modulate essential behaviors like the search for food, avoidance of harmful conditions, or reproduction. Sensory perception in humans and animals relies on several different modalities including vision, touch, hearing, taste, and smell. Input via several or all of these senses is received simultaneously through sensory neurons. They transmit their signals to the brain, where all the information is integrated and processed, resulting in behavioral responses. As a consequence, studying sensory behavior in higher organisms is a very challenging task.

The nematode *C. elegans* responds to most sensory input except for hearing. It responds to a wide variety of environmental signals and executes a large number of different behaviors. *C. elegans* possesses a simple, well-described, and invariantly wired neuronal network of only 302 neurons (White, 1986). It is therefore an excellent model organism in which to study the underlying neuronal logic that determines behavior. To sense environmental and internal cues, *C. elegans* possesses a rather small number of sensory neurons. Some of them are located throughout the body, where they are responsible for sensing body touch (Bounoutas and Chalfie, 2007). The majority is located in the head and tail of the worm and has access to the environment. Best described and most studied is a subgroup of ciliated sensory neurons (CSNs) organized in two symmetric lateral sense organs – the amphids in the head and the phasmids in the tail (Perkins et al., 1986). All amphid and phasmid neurons are bipolar neurons that extend two processes from the cell body, one dendrite and one axon. At their tips, some amphid CSN dendrites project a small process, a cilium, with access to environmental cues, through a pore in the cuticle of the worm. Cilia function as compartments to localize receptors and downstream signaling molecules that are necessary to receive environmental input. Each CSN is unique in its expression of receptors; these determine its sensory spectrum and trigger distinct behaviors in the worm (Figure 1A).

Several techniques have been developed to study the sensory function of individual CSNs. One strategy is based on the elimination of individual

CSNs with a focused laser beam (Bargmann and Avery, 1995; Bargmann and Horvitz, 1991). In this technique, a single CSN or combinations of CSNs are deleted in individual animals, which are later tested for their behavioral response to distinct sensory cues. Genetic ablation of single CSNs can also be achieved by expressing cytotoxic genes (e.g. *ced-4* or *mec-4(d)*) from a cell-specific promoter. Targeted cell ablation can also be induced by cell-specific expression of caspases, cysteine proteases that are key regulators of induced cell death (Chelur and Chalfie, 2007; Harbinder et al., 1997; Shaham and Horvitz, 1996). However, all cell ablation methods reach limitations when complex or redundant sensory functions are studied. Such redundancies are only uncovered if, by chance, the right combination of sensory neurons is eliminated. Furthermore, these techniques only allow a certain cue to be matched to a specific behavior. Analyzing sensory perception inside the CSN under investigation itself is not possible (Figure 1B).

Another recent development used to investigate sensory neuron function is optical imaging using fluorescent reporters. These molecules, e.g. cameleon, are used to measure neuronal activity when single neurons are exposed to sensory cues (Figure 1C; (Kerr et al., 2000)). In contrast to ablation methods, neuronal activity imaging is used to study sensory functions of single neurons, both in wild type as well as in mutant animals. However, the experimental imaging procedure requires immobilization of the animal. Thus, direct correlation between sensory input and behavioral output is not possible in most cases.

In summary, the methods described above are used very successfully to determine the requirement of single CSNs for the detection of a specific sensory cue. However, they do not allow the examination of sufficiency of single CSNs isolated from all other sensory input. Neither do any of the available methods cover all aspects of a sensory cue/perception/neuron/behavior system. This issue could be solved by an *in vivo* system that relies on the presence and function of only a single or a small number of selected ciliated neurons in an otherwise cilia-free background. In one possible approach, a heat shock system that has been adapted for cell- and stage-specific expression of transgenes was tested in CSNs (Bacaj and Shaham, 2007). This tool was successfully used to extend short and partially defective cilia structures to their

full length in a small number of neurons. However, it remains unclear how the acute exposure to heat shock temperatures affects the stability of cilia extension, cilia functionality, as well as behavioral outputs.

In our current work we use the *C. elegans daf-19* mutant as a tool with which sensory input through a single, fully functional cilium can be assayed in the background of a transgenic animal that is otherwise fully defective with regard to sensory input through cilia (Figure 1D). *daf-19* encodes an RFX transcription factor that in CSNs directly controls the expression of a large number of structural and functional cilia genes (Efimenko et al., 2005). In *daf-19* mutants no cilia are formed and the animals are completely sensory defective. However, even though sensory cilia are entirely absent, the remainder of the neurons (cell body, dendrite, axon) is intact and properly localized (Swoboda et al., 2000). We recently identified the relevant *daf-19* transcript, *daf-19c*, which controls the development of structurally and functionally intact cilia (Senti and Swoboda, 2008). This transcript now enables us – for the first time – to specifically and fully rescue functional cilia development. We have developed a genetic rescue system, FRISSC (**F**unctional **R**escue **I**n **S**ingle **S**ensory **C**ilia), in which we restore single cilia by expressing *daf-19c* from specific neuron-specific promoters in a *daf-19* mutant background. We show that restored single cilia are structurally as well as functionally complete and are sufficient to trigger correct behavioral responses. Thus, FRISSC allows one to assay *in vivo* the function of a single CSN, isolated from any other sensory input through cilia. Sensory perception through this CSN can be analyzed at a cellular level, as can its contribution to behavior at a systemic level. The heritable nature of the system allows the analysis of a large number of animals, making it also optimal to investigate even subtle phenotypes or redundant functions. FRISSC can also be adapted to dissect the signal reception and transduction machinery localized to cilia in *C. elegans*. By inducing heterologous expression of neuronal proteins from other organisms the relevance of this tool can be extended beyond the worm.

MATERIALS AND METHODS

Strains and culture methods

Growth and culture of *C. elegans* strains were carried out as described (Brenner, 1974). All strains were grown at 20 °C unless stated otherwise. Strains used for this study were: CB3323 [*che-13* (*e1805*)], JT204 [*daf-12* (*sa204*)], JT5010 (wild-type N2 Bristol), JT6924 [*daf-19* (*m86*); *daf-12* (*sa204*)], MT3665 [*osm-9* (*n1601*)]. The extra-chromosomal array transgenes used in this work are summarized in Supplementary Table 1. All of them were injected into JT6924.

Injection constructs, germ line transformation and GFP expression analyses

Transcriptional fusions to fluorescent reporters were injected at 20 to 60 ng/µl and *daf-19c* rescue constructs were injected at 50 ng/µl. Promoters of the following genes were selected to drive *daf-19c* expression: *gcy-5* (encoding a guanylyl cyclase), *gpa-13* (encoding a G protein alpha), *dat-1* (encoding a dopamine transporter), *gpa-11* (encoding a G protein alpha), *trx-1* (encoding a thioredoxin). Adult hermaphrodites were transformed using standard techniques (Mello et al., 1991). Transgenic extra-chromosomal arrays were analyzed in a *daf-19*; *daf-12* mutant background (JT6924). The *daf-12* mutation completely suppresses the Daf-c phenotype of *daf-19* and prevents dauer formation.

Behavioral assays

To assess avoidance behavior, drop assays were performed as described (Hilliard et al., 2002). For each assay 30 worms were picked as L4 larvae and examined 24 hours later (one-day-old adults). The animals were tested for their avoidance behavior to 3-5 consecutive drops of 0.1 % SDS or 1 M glycerol. Each assay was repeated at least twice and data were pooled for statistical analysis. The avoidance index (a.i.) was determined as the number of correct responses to a drop of repellent (backward movement) divided by the total number of drops applied. Staged young adults (obtained by growing up the progeny of bleached gravid adults) were used for chemotaxis assays. Attraction to 10 mM NaCl was assessed in quadrant assays, as previously described (Jansen et al., 2002; Wicks et al., 2000). Each strain was tested in at least three

independent assays. A chemotaxis index (c.i.) was calculated as (A-C)/(A+C), where A is the number of worms in quadrants with NaCl and C the number of worms in quadrants without the attractant.

DiI staining, microscopy and fluorescence imaging

Fluorescent dye filling assays with DiI or FITC were performed as previously described (Starich et al., 1995). For the investigation of GFP expression and dye filling, worms were anesthetized in 0.1 % sodium azide in M9 buffer and immobilized on a 2.0 % agar pad. Analyses were performed on a Zeiss Axioplan 2 microscope with OpenLab software. The data presented in Tables 1-3 are based on the analysis of at least 30 transgenic adult animals per line. Frequencies of reporter gene expression, rescue of dye filling, and cilia formation were confirmed in larvae and found to be very similar to adults (data not shown). Confocal micrographs of anesthetized worms were taken on an LSM 510 META laser-scanning microscope (Zeiss) equipped with an Argon 2/488 nm and a HeNe 543 nm laser.

Antibody staining

Staining with antibodies against DAF-19C, OSM-5, and GFP was carried out as described (Senti and Swoboda, 2008). The secondary antibodies Alexa488 and Alexa546 (Jackson Immunoresearch) were used 1:250, Cy3 was used 1:1000. Incubations with primary antibodies were performed overnight at 4 °C and incubations with secondary antibodies were for three hours at room temperature.

RESULTS

Rescue of cilia on selected, single ciliated sensory neurons (CSNs)

DAF-19 is required in all CSNs to activate the cilia formation program. *daf-19* mutants fail to activate direct target cilia genes and completely lack all ciliated structures. Expression of *daf-19c*, the CSN-specific isoform of *daf-19*, in a *daf-19* mutant background rescues cilia formation and all cilia-related phenotypes, thereby restoring all sensory functions (Figure 2B; (Senti and Swoboda, 2008)). In this work we asked whether we could use a modified rescue strategy to generate worms with cilia on only a few selected or even single CSNs in a worm that otherwise completely lacks sensory, environmental input through cilia. In such an experimental setup, we would be able to investigate the cell-autonomy of cilia formation and of sensory behavior at the level of single CSNs.

To assess the rescue potential of DAF-19C in a single CSN, we initially chose the *gcy-5* promoter to express *daf-19c* and consequently generate only a single cilium in a *daf-19* mutant background. *gcy-5* encodes a guanylyl cyclase and its promoter drives expression exclusively in a single CSN, the neuron ASER (Figure 2A). To facilitate the identification of ASER in transgenic worms, we co-transformed the reporter gene *gcy-5::gfp* together with the rescue construct. When stained with an antibody against DAF-19C, we detected only one nucleus expressing DAF-19C in these rescue lines. This nucleus always resided in the *gfp*-marked ASER neuron (data not shown). DAF-19C expression in ASER was observed from embryonic to adult stages - expression dynamics that followed the expression pattern of the *gcy-5::gfp* reporter. By contrast, *daf-19c* expressed from its own promoter restored direct target cilia gene expression as well as cilia formation in all CSNs in the head and tail of the worm (Figure 2B and data not shown). To demonstrate that DAF-19C expressed from a heterologous promoter was functional, we investigated the expression of reporter constructs (promoter fusions to *gfp* or *DsRed*) of established direct DAF-19C target cilia genes (x-box genes) (Blacque et al., 2004; Haycraft et al., 2003; Haycraft et al., 2001). Expression of DAF-19C in the single ASER neuron activated *bbs-7::gfp*, *che-13::DsRed* and OSM-5 expression, tested by antibody staining, in the neuron (Figure 2, C vs. D, and E;

and data not shown). These results demonstrate that *daf-19c* under the control of a heterologous promoter is able to induce cilia-specific gene expression in selected, single neurons.

Several features can be used to verify that a particular cilium or CSN is structurally and functionally complete. One method is visual inspection of cilia morphology. We investigated visually the rescue of cilia structures in the *gcy-5* rescued lines and found that ASER neurons formed cilia at the tip of their dendrites. By contrast, cilia were never found on ASER neurons in *daf-19* mutants (Figure 2, F vs. G). Some CSNs are characterized by their direct contact to the environment. Consequently, when wild-type worms are exposed to fluorescent lipophilic dyes (DiI, DiO, FITC, etc.), 12 out of 60 CSNs take up the dye through their cilia and fluorescent labeling occurs throughout the cell body and both neurites. Structurally impaired, short, or missing cilia lead to a phenotype described as dye filling defective, Dyf (Perkins et al., 1986; Starich et al., 1995). To confirm the structural integrity of rescued single cilia by dye filling, we generated another rescue line, using the *gpa-13* promoter. This promoter drives *daf-19c* expression in the following CSNs: ADF, ASH, AWC in the head and PHA/B in the tail (Figure 3 A, B). Again we confirmed the expected expression pattern of DAF-19C and the activation of its direct target cilia genes *bbs-7::gfp*, *che-13::DsRed* (Figure 3, C to E; and data not shown). *gpa-13* activates expression in two neuron pairs, ASH and ADF, which can be labeled with the lipophilic dyes DiI and FITC, respectively. *daf-19* mutants are completely dye filling defective. However, when *daf-19c* is expressed in those mutants from the *gpa-13* promoter, ASH neurons expressing DAF-19C regain their ability to fill with DiI and ADF neurons fill with FITC (Figure 3E). These results show that expression of DAF-19C in selected CSNs can restore not only cilia gene expression, but also structurally complete cilia.

Quantitative evaluation of the rescue system

Since we can restore cilia structures on single CSNs in *daf-19* mutants, we asked if we could establish a tool that allows the functional investigation of any single CSN. One pre-requisite would be that the rescue works efficiently from a large variety of CSN-specific promoters. Thus, we quantified the rescue

efficiency of DAF-19C expressed from promoters that are active in different sets of CSNs (Table 1).

We ensured that all promoters tested, *gcy-5*, *gpa-13*, *dat-1*, *gpa-11*, and *trx-1*, are active at late embryonic stages, the time period when cilia develop in *C. elegans*. The onset of promoter activity was monitored by transcriptional *gfp* fusions. Consistent with the *gfp* expression profile of the promoters, all were able to induce DAF-19C expression from the late embryonic to adult stages as confirmed by immunofluorescent staining with antibodies against DAF-19C (data not shown). We detected DAF-19C expression exclusively in those neurons where the promoters were expected to be active.

All transgenes were studied in the worm as extra-chromosomal arrays. As a consequence of not being integrated into the genome, extra-chromosomal arrays are occasionally lost in certain cells during mitosis. Thus, to mark transgenic, DAF-19C expressing, and therefore potentially rescued neurons we used cell-specific promoter::*gfp* fusions. To then quantify the expression of DAF-19C in those neurons we co-expressed a transcriptional *DsRed* fusion of the direct DAF-19C target cilia gene *che-13*. Each transgenic, *gfp* positive neuron was then scored for the induction of *che-13::DsRed*. We found that in all transgenic rescue lines the induction of cilia gene expression in transgenic neurons was above 80 %, that is, exceptionally high (Table 2). Transgenic lines expressing *daf-19c* from the *gpa-13* promoter did not contain a *gfp* marker for neurons harboring the extra-chromosomal expression array. Thus, we were not able to analyze specifically only transgenic neurons. Instead we assumed that all neurons were transgenic. In such a scenario, the efficiency of DAF-19C to activate direct target cilia genes in those CSNs where *gpa-13* was active was above 65 %. However, since extra-chromosomal arrays are never transmitted to all cells, the number of actual transgenic neurons is unknown. The fraction of *gpa-13* expressing neurons, therefore, is necessarily a conservative (under-) estimate.

Next we asked how the efficiency to induce (direct DAF-19C target) cilia genes corresponded to the structural formation of complete cilia. We examined single transgenic neurons marked with *bbs-7::gfp* by visual inspection for the presence of cilia at the tip of their dendrites (Table 3). Typically more than half, in some cases up to 80 % of all transgenic, *gfp*

positive neurons in the various rescue lines formed cilia compared to the complete absence of cilia in *daf-19* mutants (Table 3). When applicable, dye filling assays were performed to demonstrate that the structural rescue of cilia was indeed complete. As expected, rescued cilia also facilitated fluorescent dye filling with lipophilic dyes in most neurons (Table 3). We did not observe dye filling in rescued ASJ neurons, which is likely due to misshaped or short cilia. Also the phasmid CSNs PHA/B did not fill with DiI. In summary, using five different promoters to control *daf-19c* expression we examined 11 different classes of CSNs: ADE, ADF, ADL, ASE, ASH, ASJ, AWC, CEP, PDE, PHA, and PHB. In 11/11 CSNs (100 %) we were able to induce expression of direct DAF-19 target cilia genes and in 7/7 examined CSN types (100 %) the morphological rescue of cilia. Testing the structural integrity of those cilia by dye filling was successful in 3/6 (50 %) cases.

Finally, to ensure that the rescue of functional cilia was specific for the rescued CSN, we analyzed all lines for non-specific induction of cilia reporter genes *bbs-7::gfp* and *che-13::DsRed*. We did not find activation of cilia genes in non-transgenic neurons (data not shown). In addition, we did not find neurons that were rescued for their Dyf phenotype, except for those sensory neurons in which we intentionally expressed DAF-19C (data not shown). In summary, we conclude that the expression of *daf-19c* from CSN-specific promoters in *daf-19* mutants restores cilia-specific gene expression and cilia structure only in those CSNs in which the promoter is active. This rescue is specific for the rescued CSN and cell-autonomous as it does not lead to rescue in other, neighboring neurons. Thus, this rescue system, which we call FRISSC (**F**unctional **R**escue **I**n **S**ingle **S**ensory **Ci**lia), can easily be adapted to any CSN.

Applying the daf-19c rescue system to test the autonomous function of single CSNs

The highly efficient rescue of cilia structures on selected neurons prompted us to test whether sensory functions of these cilia were restored to a similar extent. In such a case our rescue system would be suitable to investigate behaviors mediated by single neurons in a population of transgenic worms. As proof of principle, we used two different assays that investigate sensory

behaviors either at the level of single worms or at the level of a population of worms.

First, we used the *gpa-13* rescued lines to investigate the sensory function of rescued ASH neurons in a drop test. This test was established to investigate the aversive behavior of the worm to water-soluble repellents (Hilliard et al., 2002). Aversion to these substances is mediated mainly by ASH and to a minor extent also by ASK or ADL, depending on the nature of the repellent. We performed drop assays using 0.1 % SDS and 1 M glycerol (Figure 4, A and B). In these assays, *daf-19; daf-12* mutants and the control strains *che-13* and *osm-9*, which are defective in the detection of repellents, had very low aversion indices (a.i.) – less than 0.1 for SDS and 0.2 for glycerol – compared to an a.i. of 0.96 ($p < 0.001$) and 0.95 ($p < 0.001$) in *wild type* or *daf-12* mutants, respectively. As expected, expression of *daf-19c* from the *gpa-13* promoter in ASH substantially improved the avoidance behavior. We obtained an a.i. of 0.5 ($p < 0.001$) and 0.6 ($p < 0.001$) for the two rescue lines tested (Figure 4A). Intriguingly, this 50 – 60 % increase in the avoidance behavior matched the 50 – 60 % morphological rescue of ASH cilia in the two rescue lines (compare Figure 4A and Table 3). These results suggest that the rescue of cilia structure and sensory behavior is tightly correlated. We repeated the drop assay and followed it with a dye filling assay for each individual worm analyzed. Indeed, we saw a strong correlation between the rescue of cilia structure and aversion. All worms in which cilia formation on ASH neurons was rescued and dye filling restored responded correctly to the aversive stimulus, resulting in an a.i. higher than 0.9 ($p < 0.001$) for the rescue lines (Figure 4B). As a negative control, we included the *gcy-5* rescue lines in our assays; these lines show functional rescue only in ASER neurons (Figure 4A). These CSNs are required for detection of the attractant NaCl, but not for repelling compounds. As expected, rescue of cilium structure on ASER did not restore aversion, resulting in an a.i. below 0.1.

To specifically test the rescue of sensory functions in ASER, we performed quadrant assays that investigate the attraction to NaCl (Figure 4C). The attraction to salt is mediated by several CSNs, among others the bilateral neuron pairs ASEL/R and ADFL/R. Wild-type worms, *daf-12* mutants, as well as *osm-9* mutants, which are defective in the detection of repellents, but not of

NaCl, scored chemotaxis indices (c.i.) of 0.8 and higher. Mutations in *daf-19* lead to a very low c.i. of 0.09 ($p < 0.001$). As expected, based on their sensory capacity, worms with rescued cilia on either ASER (in *gcy-5* rescue lines) or ADF (in *gpa-13* rescue lines) were able to detect NaCl correctly. Their c.i. increased to 0.32 and 0.47 ($p < 0.05$) in *gcy-5* rescue lines and 0.45 ($p < 0.1$) in *gpa-13* rescue lines, respectively. The partial rescue observed in all lines most likely uncovers the redundant functions of several CSNs in the attraction to NaCl sensory behavior. In addition to transgenic, rescued animals ("trans") we also analyzed non-transgenic ("non-trans"), mutant worms of each strain in the very same experiments. As expected for this internal control, non-transgenic worms were not rescued for their ability to detect NaCl. In summary our results show that the expression of *daf-19c* in single CSNs is highly penetrant and sufficient to functionally restore the sensory capacity of a single CSN and its associated behaviors.

DISCUSSION

FRISSC – a new tool to analyze CSN-specific sensory function

Formation of cilia is dependent on DAF-19C, a recently discovered novel isoform of the RFX transcription factor DAF-19 (Senti and Swoboda, 2008). While all other cilia structure mutants show short or misshaped cilia, *daf-19* mutant animals are completely devoid of cilia structures. Therefore, *daf-19* mutants are guaranteed to be free from all sensory input through cilia. FRISSC uses this mutant to generate single functional CSN on demand by expressing *daf-19c* from CSN-specific promoters. Thus, FRISSC experimentally introduces a simple morphological change from ”without cilium” to “with cilium” on an otherwise structurally intact neuron. This results in the transformation of the sensory status of the animal from “no input” to “input”.

FRISSC is not only “clean” with regard to the sensory ability before and after the rescue, it is also cell-autonomous. We tested FRISSC with regard to cilia gene expression, cilia morphology, and cilia sensory function. We observed rescue of cilia morphology and sensory behavior only in those neurons where we intentionally expressed DAF-19C, but never in other CSNs. This strictly neuron-specific cilia rescue by DAF-19C is crucial for the analysis and correct interpretation of single CSN functions and their respective contributions to neuronal networks and sensory behaviors. Furthermore, we found that the rescue potential of the different promoter::*daf-19* transgenes is highly efficient and penetrant. Thus, FRISSC fulfills all requirements as a tool to study the autonomous function of single CSNs without sensory input from other CSNs.

Noteworthy features of FRISSC

Three protein isoforms are expressed from the gene *daf-19*: DAF-19A, B, and C. DAF-19A/B, which are not expressed in CSNs, are required for the maintenance of synaptic vesicle proteins at later stages of the *C. elegans* life cycle (Senti and Swoboda, 2008). All *daf-19* mutant alleles currently available affect all three isoforms equally. This may be a concern when using FRISSC to conduct behavioral studies in *daf-19* mutants. However, we have shown that the

synaptic defects in *daf-19* mutants affect adult worms only as they age (Senti and Swoboda, 2008). The defects do not affect larvae or young adults that are typically used for behavior assays. Thus, late synaptic defects due to the loss of daf-19A/B are not problematic, as is apparent from the behavioral assays we performed in this study.

Furthermore, we performed our analyses in a *daf-19; daf-12* double mutant background to suppress the Daf-c (**Da**uer **f**ormation **c**onstitutive) phenotype of *daf-19*. In all behavioral tests we performed, *daf-12* single mutants did not exhibit any sensory defects. Therefore, we conclude that mutations in *daf-12* do not interfere with our behavioral analyses. However, in some instances a pure *daf-19* mutant background might be advantageous, e.g. for dauer-related CSN functions. In that case, the genetic background can be easily changed.

Expanding the versatility of FRISSC

Several methods are available to study sensory functions and related behaviors of CSNs. Some of them are based on the elimination of the neuron in question (cell ablation techniques using a laser beam or expression of cytotoxic molecules). Other techniques focus on isolated investigation in an environment where all other CSNs are also functional (neuronal activity imaging). Each of these methods has specific advantages; choosing one method over another is typically based on experimental circumstances.

A unique feature of FRISSC is the isolation under which a particular sensory event can be studied. All currently available cilia structure mutants have cilia remnants of various lengths, categorized as likely non-functional (Haycraft et al., 2003; Haycraft et al., 2001; Perkins et al., 1986). However, when studying these mutants, residual function, especially for sensing volatile chemicals (cues), cannot be excluded. *daf-19* mutants are currently unique, as they are completely devoid of any cilia structures and therefore lack any sensory input via cilia.

Another strong advantage of FRISSC is its heritability. Transgenic worms can be generated easily and the rescuing transgenes are then maintained as extra-chromosomal arrays (Mello et al., 1991). In contrast, laser ablation experiments yield only a limited number of manipulated animals and require

specific technical equipment and skills (Bargmann and Avery, 1995; Bargmann and Horvitz, 1991). We have shown that FRISSC is sufficiently quantitative as well as efficient for analyzing a particular behavior even in a population of transgenic worms. Thus, the constant supply of a large number of transgenic animals allows for the examination of even subtle phenotypes that otherwise might be missed. Though possibly advantageous, it is therefore not necessary to integrate into the genome extra-chromosomal arrays prior to behavioral analyses. In contrast, as demonstrated in drop and quadrant assays, non-transgenic or mosaic animals can serve as important internal negative controls.

We developed FRISSC with the aim of studying the functions of single CSNs. Although the list of promoters specific for single CSNs is long, some CSNs share specific promoters with at least one other or more types of CSNs. In that case, the use of transgenes as extra-chromosomal arrays, which are occasionally lost during mitosis, can be exploited. Since extra-chromosomal arrays give rise to a heterogeneous population that does not express the transgene in every target cell, mosaic analysis can be applied. Alternatively, our rescue system can be combined with a tool that superimposes second level expression-control (both spatial and temporal), e.g. the FLP recombinase system (Davis et al., 2008).

Future applications

FRISSC allows the creation and *in vivo* analysis of single functional CSNs independent of any other sensory input through cilia on other neurons. We envision three major areas of applications for this tool. First, FRISSC will facilitate the investigation of single cilia and CSN function from the cellular level to isolated sensory circuits and finally to isolated behaviors. It will be the tool of choice when sensory redundancies need to be understood. The investigation of the role of selected interneurons, which typically receive input from multiple CSNs, in a particular sensory behavior will be possible with FRISSC. Second, single isolated cilia can also be used in a gain-of-function or sufficiency approach, by expressing and analyzing individual molecular components of the signal reception and transduction machinery in different types of CSNs. The function and effect of these proteins can subsequently evaluated from cellular to organismal level *in vivo* in transgenic animals.

Finally, FRISSC combined with other methods creates a powerful tool to study questions concerning the development of functional cilia as such. Adding time-control to the spatial expression of DAF-19, e.g. by adapting the *hsf-1* model (Bacaj and Shaham, 2007), or the FLP-recombinase system (Davis et al., 2008), allows the precise initiation of ciliogenesis. Thus, one could investigate the potential of DAF-19 induced ciliogenesis at different developmental stages, or could focus on the early steps of cilia assembly. The latter application will be made considerably easier, as not all cilia develop at the same time or in the same manner (Sulston and Horvitz, 1977; Sulston et al., 1983). Being able to selectively focus on only one cilium will help to solve time-related aspects of ciliogenesis, including when and how sensory cilia attain their signal reception and transduction capacities. Overall, FRISSC will allow to answer questions regarding sensory neuron development and sensory mechanisms from a new angle. It will therefore provide a valuable addition to the toolbox for sensory behavior analysis.

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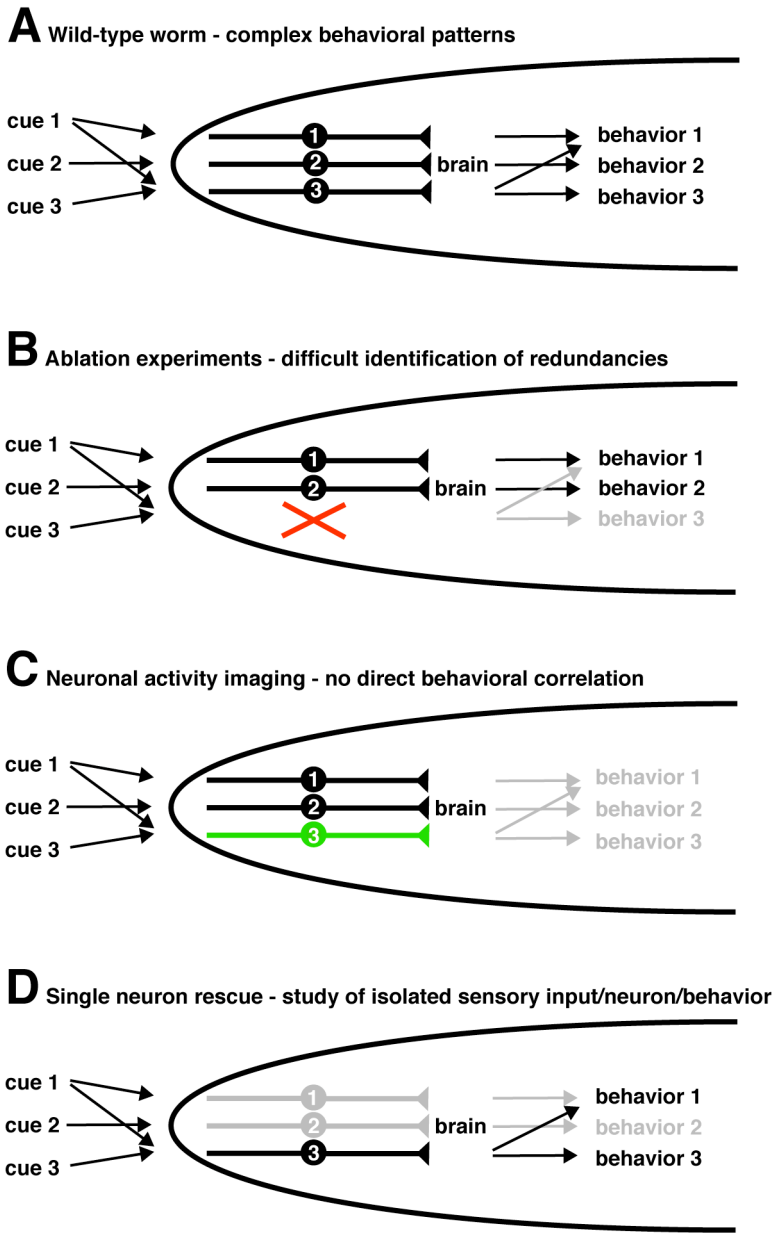
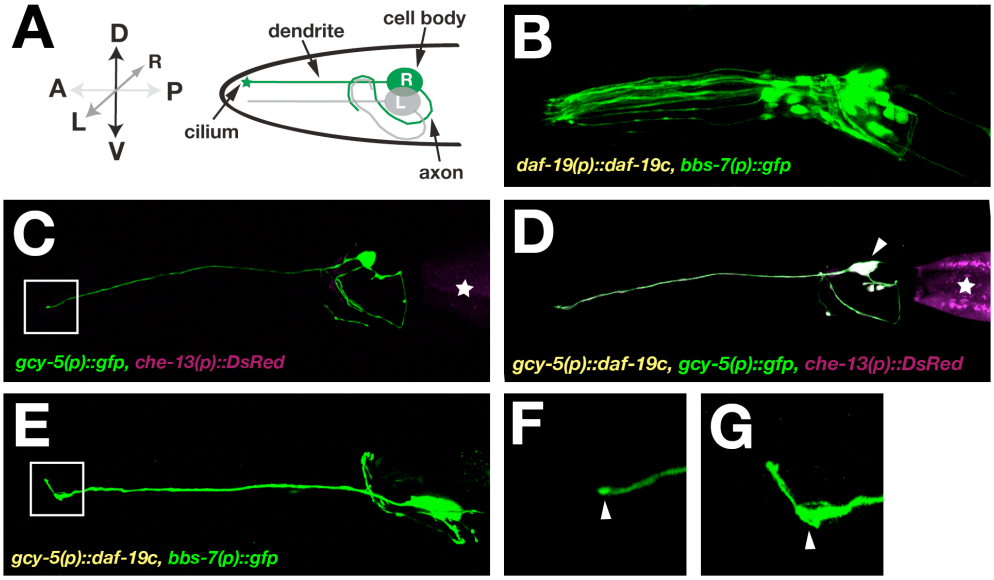
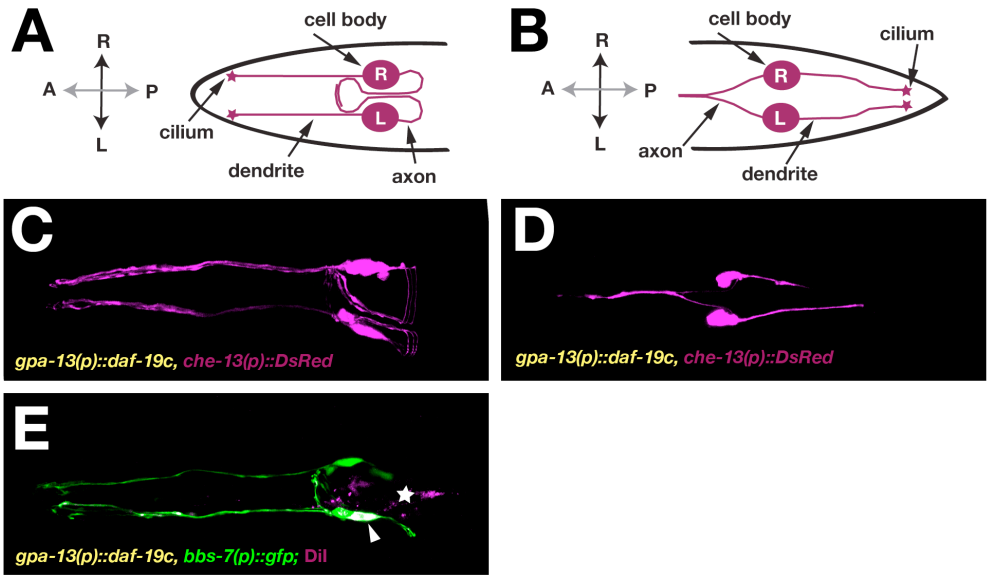
Senti et al. Figure 1



Senti et al. Figure 2



Senti et al. Figure 3



Senti et al. Figure 4

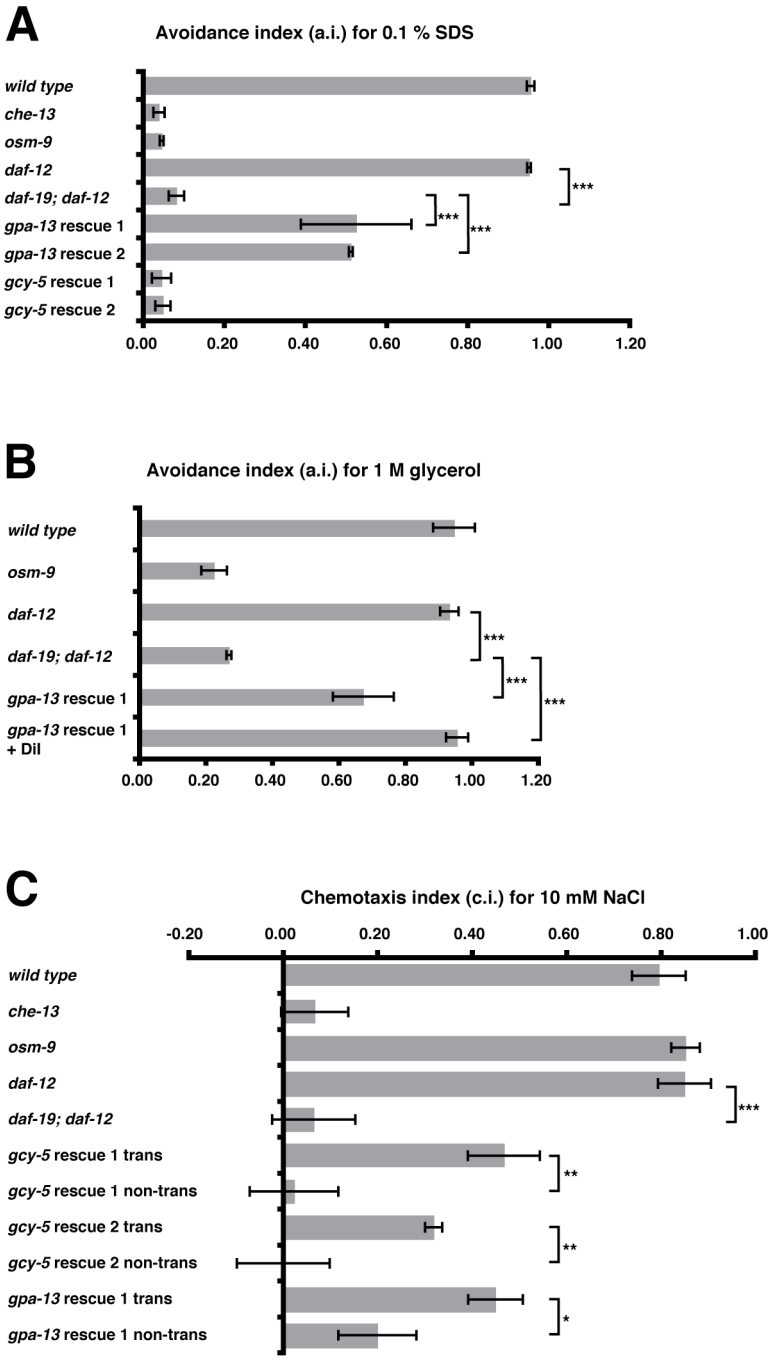


Table 1. Rescue of cilia formation and sensory function by expressing *daf-19c* from different ciliated sensory neuron-specific promoters.

			rescue of			
promoter	**start of expression**	**site of expression**	***che-13::DsRed* / *bbs-7::gfp***	**cilia length**	**dye filling**	**sensory behavior**
gcy-5	embryo	ASER	yes / yes	yes	n.a.	yes
gpa-13	embryo	ADF, ASH, AWC, PHA/B	yes / yes	yes	yes[2]	yes
dat-1	embryo (PDE in L1)	ADE, CEPs, PDE	yes / yes	yes	n.a.	n.d.
gpa-11	embryo	ADL, ASH	yes / yes	yes	yes	n.d.
trx-1	embryo	ASJ	yes / yes	yes[1]	no	n.d.

n.a. ….. not applicable; n.d. ….. not determined; [1] cilia appeared shorter than wild-type cilia; [2] analyzed in ADF and ASH

Table 2. Rescue efficiency of cilia gene expression in single neurons that express *daf-19c* from ciliated sensory neuron-specific promoters. Transgenic neurons were marked with *gfp* and scored for the activation of the direct DAF-19C target *che-13*::*DsRed*.

promoter	cell type	transgenic line	transgenic neurons expressing *che-13::DsRed* (%)
gcy-5	ASER	line 1	100
		line 2	100
*gpa-13**	ADF L/R	line 1	> 68
		line 2	> 73
	ASH L/R	line 1	> 72
		line 2	> 80
	AWC L/R	line 1	> 72
		line 2	> 78
	PHA/B L/R	line 1	> 65
		line 2	> 78
dat-1	ADE L/R	line 1	100
		line 2	100
	PDE L/R	line 1	100
		line 2	83
	CEPs	line 1	98
		line 2	98
gpa-11	ADL L/R	line 1	98
		line 2	93
	ASH L/R	line 1	100
		line 2	100
trx-1	ASJ L/R	line 1	87
		line 2	95

* The *gpa-13* lines do not contain a *gfp* marker for transgenic neurons. Therefore, the % calculations are based on the assumption that all neurons carry the extra-chromosomal array transgene. Since extra-chromosomal arrays can be lost during mitosis, these numbers are therefore an underestimate.

Table 3. Rescue efficiency of cilia structures in single neurons that express *daf-19c* from ciliated sensory neuron-specific promoters. Transgenic neurons were marked with *bbs-7::gfp*.

promoter	cell type	transgenic line	transgenic neurons with rescued cilium (%)	rescue confirmed by dye filling (d) or visibility of cilium (c)
gcy-5	ASER	line 1	71	c
		line 2	82	c
gpa-13	ADF L/R	line 1	yes	d
		line 2	yes	d
	ASH L/R	line 1	67	d
		line 2	54	d
	AWC L/R	line 1	yes	c
		line 2	yes	c
	PHA/B L/R	line 1	0	c
		line 2	0	c
dat-1	ADE L/R	line 1	n.d.	n.d.
		line 2	n.d.	n.d.
	PDE L/R	line 1	n.d.	n.d.
		line 2	n.d.	n.d.
	CEPs	line 1	59	c
		line 2	56	c
gpa-11	ADL L/R	line 1	35	d
		line 2	57	d
	ASH L/R	line 1	48	d
		line 2	54	d
trx-1	ASJ L/R	line 1	15	c
		line 2	33	c

n.d. … not determined; yes … representative spot-checks were done

Supplementary Table 1. Strains and extra-chromosomal arrays analyzed in JT6924 [*daf-19* (*m86*); *daf-12* (*sa204*)].

strain	transgene	presence of DAF-19	direct DAF-19 target gene	visualization of neuron of interest	transgenesis marker
gcy-5 promoter constructs					
OE3761	ofEx559		*che-13::DsRed*	*gcy-5::gfp*	*elt-2::mCherry*
OE3762	ofEx560		*che-13::DsRed*	*gcy-5::gfp*	*elt-2::mCherry*
OE3791	ofEx590	*gcy-5::daf-19c*	*che-13::DsRed*	*gcy-5::gfp*	*elt-2::mCherry*
OE3792	ofEx591	*gcy-5::daf-19c*	*che-13::DsRed*	*gcy-5::gfp*	*elt-2::mCherry*
OE3798	ofEx597	*gcy-5::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*
OE3799	ofEx598	*gcy-5::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*
gpa-13 promoter constructs					
OE3773	ofEx570	*gpa-13::daf-19c*	*che-13::DsRed*		*unc-122::gfp*
OE3774	ofEx571	*gpa-13::daf-19c*	*che-13::DsRed*		*unc-122::gfp*
OE3200	ofEx160	*gpa-13::daf-19c*	*bbs-7::gfp*		*unc-122::gfp*
OE3203	ofEx166	*gpa-13::daf-19c*	*bbs-7::gfp*		*unc-122::gfp*
OE3789	ofEx588	*gpa-13::daf-19c*			*unc-122::gfp*
OE3790	ofEx589	*gpa-13::daf-19c*			*unc-122::gfp*
OE3847	ofEx620	*gpa-13::daf-19c*		*gcy-5::gfp*	*elt-2::mCherry*
OE3848	ofEx621	*gpa-13::daf-19c*		*gcy-5::gfp*	*elt-2::mCherry*
OE3849	ofEx622	*gpa-13::daf-19c*		*gcy-5::gfp*	*elt-2::mCherry*

dat-1 promoter constructs

OE3518	ofEx367	*dat-1::daf-19c*	*che-13::DsRed*	*dat-1::YC2.3*	*elt-2::mCherry*
OE3519	ofEx368	*dat-1::daf-19c*	*che-13::DsRed*	*dat-1::YC2.3*	*elt-2::mCherry*
OE3793	ofEx592	*dat-1::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*
OE3794	ofEx593	*dat-1::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*

gpa-11 promoter constructs

OE3765	ofEx563		*che-13::DsRed*	*gpa-11::gfp*	*elt-2::mCherry*
OE3766	ofEx564		*che-13::DsRed*	*gpa-11::gfp*	*elt-2::mCherry*
OE3796	ofEx595	*gpa-11::daf-19c*	*che-13::DsRed*	*gpa-11::gfp*	*elt-2::mCherry*
OE3797	ofEx596	*gpa-11::daf-19c*	*che-13::DsRed*	*gpa-11::gfp*	*elt-2::mCherry*
OE3828	ofEx601	*gpa-11::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*
OE3830	ofEx603	*gpa-11::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*

trx-1 promoter constructs

OE3769	ofEx567		*che-13::DsRed*	*trx-1::gfp*	*elt-2::mCherry*
OE3770	ofEx568		*che-13::DsRed*	*trx-1::gfp*	*elt-2::mCherry*
OE3777	ofEx574	*trx-1::daf-19c*	*che-13::DsRed*	*trx-1::gfp*	*elt-2::mCherry*
OE3778	ofEx576	*trx-1::daf-19c*	*che-13::DsRed*	*trx-1::gfp*	*elt-2::mCherry*
OE3795	ofEx594	*trx-1::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*
OE3523	ofEx372	*trx-1::daf-19c*	*bbs-7::gfp*		*elt-2::mCherry*

Paper III

DAF-19C, A POTENTIAL MASTER REGULATOR OF CILIOGENESIS IN *CAENORHABDITIS ELEGANS*?

DAF-19C – a potential master regulator of ciliogenesis in *Caenorhabditis elegans*?

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Running Title: Ectopic expression of DAF-19C in *C. elegans*

Keywords: cilia, DAF-19, RFX, transcription factor, x-box

ABSTRACT

Master regulators are developmental switch molecules that govern the formation of an organ or differentiation of a specific cell type. Their most important feature is the ability to transform different types of tissue or cells into a particular differentiated state. A few master regulators that act during invertebrate and vertebrate development have so far been described. *eyeless*, which induces the formation of the *Drosophila* compound eye, and MyoD, which induces differentiation of muscle tissue in mammals, are two such examples. In this study we investigated whether the RFX transcription factor DAF-19 can act as a master regulator of cilia formation in *C. elegans*. Cilia are the main sensory structures in the worm, found on 60 ciliated sensory neurons (CSNs) in the head and tail. The isoform specific for CSNs, DAF-19C, induces cilia formation in these sensory neurons by activating a large number of cilia genes that confer structure and function to the sensory organ. In this work we attempted to induce ectopic cilia in *C. elegans* by expressing DAF-19C in various non-ciliated cell types. We find that ectopic expression of DAF-19C can induce target genes in muscle and hypodermis cells, but not in non-ciliated neurons. Surprisingly, DAF-19A, which specifically functions in synapse maintenance in non-ciliated neurons, can also activate cilia genes when expressed ectopically in muscle or hypodermis cells. We hypothesize that isoform-specific suppressors regulate the cell-type-specific activation potential in of different DAF-19 isoforms. Whether the activation of cilia genes in muscles and hypodermis leads to the formation of cilia remains to be shown by more detailed experiments.

MATERIAL AND METHODS

Strains and culture methods

Growth and culture of *C. elegans* strains were carried out as described (Brenner, 1974). All strains were grown at 20 °C unless noted otherwise. Strains used for this study were: JT6924 [*daf-19* (*m86*); *daf-12* (*sa204*)], JT8651 [*daf-19* (*m86*)/mnC1; *lin-15* (*n765* ts)]. The extra-chromosomal arrays used in this work are summarized in Table 3.

Injection constructs, germ line transformation, and GFP expression analyses

Transcriptional fusions to fluorescent reporters were injected at 20 to 60 ng/µl and *daf-19c* ectopic expression constructs were injected at 50 ng/µl. Adult hermaphrodites were transformed using standard techniques (Mello et al., 1991). Transgenic arrays were analyzed in a *daf-19;daf-12* mutant background, except for some *lin-44* and *mec-7 lines*, which were analyzed in the strain JT8651. For each transgene typically three independent lines were generated, of which two were analyzed in detail (see Table 3). For each line more than 30 transgenic animals of all different larval stages as well as adults were looked at.

Microscopy and fluorescence imaging

For the investigation of GFP and DsRed expression, worms were anesthetized in 0.1% sodium azide in M9 buffer, and immobilized on a 2% agar pad. The analysis was performed on a Zeiss Axioplan 2 microscope connected to a computer running OpenLab software.

Antibody staining

Staining with antibodies against DAF-19 and GFP were carried out as described (Senti and Swoboda, 2008). The secondary antibodies Alexa488 and Alexa546 (Jackson Immunoresearch) were used 1:250, Cy3 was used 1:1000. Incubations with primary antibodies were performed overnight at 4°C and incubations with secondary antibodies for 3 hours at room temperature.

INTRODUCTION

Cilia are hair-like cellular extensions that are structurally conserved in most eukaryotic organisms. In mammals they occur on many different cell types where they fulfill vital functions. Motile cilia are required for the movement of extracellular fluids (clearing the lungs of mucus, generation of the nodal flow in early embryogenesis, etc.) while non-motile cilia typically have sensory functions. Loss or malformations of cilia are associated with severe diseases or syndromes and can in some cases even be fatal in humans (Bisgrove and Yost, 2006; Eley et al., 2005; Ibanez-Tallon et al., 2003; Pazour, 2004; Satir, 2008; Yoder, 2007). RFX transcription factors have been associated to cilia formation in many different organisms (Bonnafe et al., 2004; Dubruille et al., 2002; Swoboda et al., 2000). They recognize and bind a short DNA sequence motif, the x-box, in the promoter of their target genes, which leads to the activation of transcription (Emery et al., 1996a).

In *C. elegans*, cilia are restricted to 60 ciliated sensory neurons (CSNs), mostly located in the head and tail of the animal. All of them are non-motile cilia and have sensory functions (Inglis et al., 2007; Perkins et al., 1986). Loss of these cilia does not affect the viability of the animal. While several RFX genes are found in, for example, mice and flies, there is only one such family member, DAF-19, encoded in the genome of *C. elegans*. Several isoforms of this RFX transcription factor have been identified (Senti and Swoboda, 2008; Swoboda et al., 2000). The short isoform, DAF-19C, is a key regulator of ciliogenesis in the worm. It activates a large number of cilia genes including structural and functional components (Efimenko et al., 2005; Senti and Swoboda, 2008). Mutations in the gene *daf-19* deplete worms of all ciliated structures, abolishing all cilia-mediated sensory perception (Perkins et al., 1986). DAF-19C re-expression in CSNs of *daf-19* null mutant animals is sufficient to rescue all cilia-related phenotypes such as cilia structure, sensory function, and behavior (Senti and Swoboda, 2008).

Since DAF-19C has such a central function in cilia formation, we wondered if it functions as a master regulator of this process. We hypothesized that ectopic expression of DAF-19C in originally non-ciliated cells could induce target gene expression or eventually lead to the assembly of cilia.

RESULTS AND DISCUSSION

To test this hypothesis, we conducted a pilot study whereby we expressed *daf-19c* in non-ciliated cells. We generated several constructs that drive *daf-19c* expression from cell-specific promoters. DAF-19C may not be able to activate cilia gene expression in all different cell types. Therefore we chose promoters active in non-ciliated neurons as well as non-neuronal tissue (**Table 1**). We expected that a pre-set neuronal environment in non-ciliated neurons would be more advantageous for ectopic cilia formation than non-neuronal environments. Promoters directing expression in non-ciliated neuronal cell types were *mec-7* (in touch sensory neurons) and *unc-30* (in D-type motor neurons). For non-neuronal cell types we chose *myo-2* (in the pharynx muscle), *unc-54* (in body wall muscles), and *lin-44* (in tail hypodermis cells) (**Table 3**). Details for those five promoters and genes are available at www.wormbase.org. Each promoter was cloned upstream of the *daf-19c* cDNA. To monitor the specificity of DAF-19C function, we generated similar constructs with the long isoform DAF-19A (**Table 3**). In wild-type worms DAF-19A is expressed in non-ciliated neurons where it is required for the maintenance of synaptic function. Importantly, it is not able to replace the function of DAF-19C in CSNs (Senti and Swoboda, 2008).

All constructs were injected together with marker DNA to generate transgenic over-expression strains. Mammalian RFX transcription factors can form heterodimers via their dimerization domain (Emery et al., 1996a; Emery et al., 1996b). To avoid any dimerization and possible interference of the different DAF-19 isoforms in neurons, we used a daf-19 null mutant strain for all *daf-19* isoforms. Ectopic expression of DAF-19C in all lines was visualized by antibody staining (**Figure 2**). To monitor the activity of the transcription factor, we co-injected fluorescent reporters of direct DAF-19C cilia target genes (*bbs-7::gfp*, *bbs-2::gfp* and *che-13::DsRed;* **Table 3**).

myo-2*:* Expression of DAF-19A in the pharynx muscle (*myo-2* promoter) induced severe malformations, most likely through structural changes in the muscle cells (**Figure 1**). These malformations caused the impairment of feeding and consequently death at various larval stages. It was not possible to

propagate and analyze these lines. We therefore eliminated both *myo-2* constructs from our study.

mec-7* and *unc-30: Expression of DAF-19C from the *mec-7* and *unc-30* promoters did not activate direct DAF-19 target genes *bbs-7::gfp* and *che-13::DsRed* in non-ciliated neurons (**Table 1** and **2**). For the analysis of all D-type motorneurons in the ventral nerve cord (*unc-30* promoter) and the touch sensory neurons in the head, body and tail of the worm (*mec-7* promoter) were looked at. Importantly, however, *unc-30* is also expressed in one ciliated sensory neuron, ASG (Jin et al., 1994). Thus, we expected *unc-30*-driven expression of DAF-19C to induce target gene expression and cilia formation in ASG. Unlike the case in non-ciliated D-type motorneurons, we detected clear reporter gene expression in ASG (**Table 1**). In addition, the formation of a cilium on its dendrite was also fully restored (data not shown). From this internal control we conclude that the DAF-19C expression system itself was functional, but that target genes were activated only in ciliated but not in non-ciliated neurons. We conclude that DAF-19C is not able to induce cilia formation when ectopically expressed in non-ciliated neurons. As expected DAF-19A expression from the same promoters did not activate cilia gene expression in both ciliated and non-ciliated neurons, either (**Table 1**).

***unc-54 and lin-44*:** In contrast to the lack of apparent DAF-19C function in non-ciliated neurons, DAF-19C was able to activate target genes in body wall muscle cells when expressed from the *unc-54* promoter and in hypodermal cells when expressed using the *lin-44* promoter (**Table 1**). In body wall muscles, apart from the induction of cilia genes, we detected no further effect on cell structure or identity. Animals that activated cilia genes in their body wall muscles were viable and healthy and did not appear different from their non-transgenic siblings. When using the *lin-44* promoter, we saw activation of *bbs-7::gfp* and *bbs-2::gfp* in hypodermal cells of the tail and occasionally observed severe deformations of the tail (**Figure 2**).

Intriguingly, we saw more substantial effects when DAF-19C was expressed in non-neuronal tissue than in non-ciliated neurons. To test if this activation of cilia genes was specific for DAF-19C, we investigated the effect of DAF-19A isoform expression in body wall muscle and tail hypodermis cells. Surprisingly, DAF-19A expression from the *unc-54* promoter was also able to

trigger DAF-19C cilia target gene expression in body wall muscles. Furthermore, ectopic expression of DAF-19A from the *lin-44* promoter in the tail hypodermis generated similar and even more frequent tail deformation phenotypes than did similar expression of DAF-19C (**Table 1** and **Figure 1**). We saw in these lines activation of *bbs-7::gfp* and observed in more than 50% of transgenic animals severe deformations of the tail, including complete loss of the tail. We also observed constipated, sick L1 larvae with severe tail deformations (data not shown). This is likely du to the fact that expression of the different DAF-19 isoforms in the posterior most hypodermis cells interferes with their proper differentiation. Consequently the anus, which is built by these hypodermis cells, is structurally deformed or missing and the defecation program cannot be executed properly. Those larvae did not develop any further, frequently disintegrated at the posterior end, and consequently died.

In summary, we can state that neither DAF-19C nor DAF-19A are able to induce cilia gene expression in non-ciliated neurons. In CSNs DAF-19C but not DAF-19A can activate cilia target genes. Surprisingly, both isoforms, DAF-19A and DAF-19C, were able to induce cilia gene expression in non-neuronal cell types (**Table 2**).

Although DAF-19C is essential for cilia formation, we did not find that it is sufficient to induce ectopic cilia gene expression in any given cell type. This suggests that the activity of DAF-19 is strongly dependent on cellular context. Furthermore, we suspect that the potential to ectopically activate cilia genes is regulated differentially for the two isoforms, DAF-19A and C. Interestingly none of the isoforms can activate cilia genes in non-ciliated neurons. Only isoform C in CSNs and both isoforms A and C can activate cilia genes in non-neuronal cells (**Table 2**). How is this context-dependent activity of DAF-19A and C regulated? Based on our results we hypothesize that ciliogenesis in non-ciliated neurons is actively suppressed. This scenario is especially attractive since isoforms DAF-19A/B are expressed in all non-ciliated neurons, where they are required for the maintenance of synaptic function (Senti and Swoboda, 2008). All DAF-19 isoforms contain the same DNA binding domain and therefore should recognize the same x-box DNA sequence motif leading to the activation of cilia genes. Thus, a suppressor of

DAF-19 (A and C) in non-ciliated neurons could prevent the transcription factor from activating cilia genes.

This suppressor theory assumes that all DAF-19 isoforms have the potential to activate cilia genes. However, only DAF-19C, not isoform A, is able to rescue cilia formation in CSNs in *daf-19* mutants. This was shown by expression of either isoform in ASG in our current work, as well as in our previous work on the molecular characterization of the three DAF-19 isoforms (Senti and Swoboda, 2008). We thus further speculate that suppression of DAF-19A also takes place in CSNs. However, in this cell type, suppression would target only DAF-19A without affecting the transcriptional activity of DAF-19C. Alternatively, DAF-19C may require another protein X to fully induce the ciliogenic program in CSNs. This factor should only be present in CSNs. It needs to be pointed though out that although many screens for cilia mutants yielded a large number of mutants, none of them show the same profound cilia phenotype as *daf-19*. We therefore hypothesize that a protein X that acts together with or upstream of DAF-19 would have to have multiple, maybe even essential, functions. Mutations in gene X would be lethal and therefore would not be found in a screen for cilia mutants. Finally, in non-neuronal cell types, suppressors or regulators of DAF-19 are not required since the transcription factor is originally not expressed in these cells (Senti and Swoboda, 2008). Consequently ectopic expression of either DAF-19 isoform is able to activate cilia target genes in these cell types.

We detected substantial activation of cilia target genes in muscle and hypodermis cells upon ectopic expression of DAF-19A and C. Was this activation of the cilia transcriptional cascade followed by the assembly of ectopic cilia? It is possible that cilia or rudimentary ciliary structures were formed. However with the analysis we performed so far, we were not able to detect them. Experiments using fluorescently tagged proteins that localize to the basal body, transition zone, or the cilium itself could visualize ectopic cilia on muscles or hypodermis cells. Alternatively, immunofluorescent staining against acetylated tubulin could answer this question.

How are cilia formation and the activity of RFX proteins regulated in other species? Recently, a number of studies have identified genes that function upstream of RFX transcription factors in mice, zebrafish, and *Xenopus* (Beckers

et al., 2007; Stubbs et al., 2008; Yu et al., 2008). In the zebrafish pronephros the Jagged 2/Notch signaling pathway suppresses *rfx2*, thereby regulating the number of multi-ciliated versus transporting epithelial cells (Liu et al., 2007). Mouse Foxj1 (also known as HFH-4) has previously been associated with cilia formation. Beckers and colleagues found that the homeobox gene *noto* is an essential regulator of node morphogenesis and ciliogenesis in the posterior notochord and acts upstream of *foxj1* and *rfx3* (Beckers et al., 2007). Recent studies in zebrafish now demonstrate that *foxj1* is a target of the Hedgehog signaling pathway, transcriptionally activating genes essential for motile cilia formation. Intriguingly, these studies also demonstrate a master regulatory switch function of Foxj1 in cilia formation. Ectopic expression in non-ciliated tissue results in the assembly of motile cilia. Molecularly, Foxj1 can be placed upstream of Rfx2. However, whereas embryos deficient for Foxj1 display a lack of motile cilia, Rfx2 function is associated with primary (non-motile) cilia. The precise mode of interaction between Foxj1 and Rfx2 in zebrafish remains, therefore, unclear. However, *C. elegans* does not develop motile cilia, neither does its genome encode a clear Foxj1 homolog. Proteins that function together with, or have regulatory functions upstream of DAF-19C, remain, therefore, to be identified. Their discovery may be essential to induce ectopic sensory cilia in *C. elegans*.

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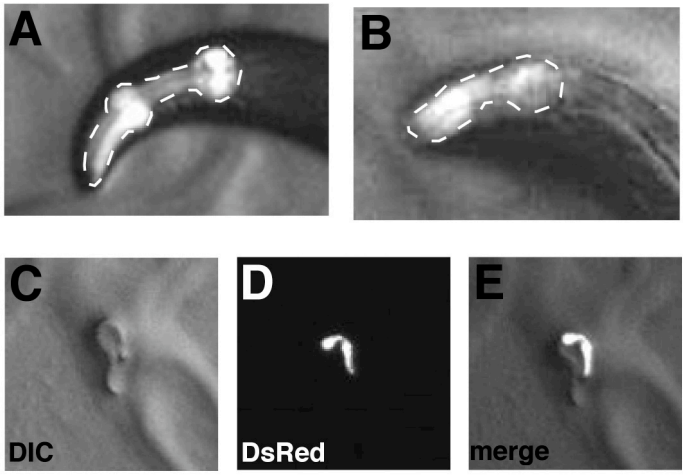

Figure 1: Ectopic expression of DAF-19A in pharynx muscle causes the deformation of the pharynx. (A, B) Heads of (A) wild-type and (B) transgenic (F1 generation) adult worms. (C-E) Transgenic L1 larvae (F2 generation). In all animals the pharynx is visualized by DsRed being expressed from the *myo-2* promoter. Ectopic expression causes a severe deformation of the pharynx in all transgenic worms and larval lethality in the F2 generation.

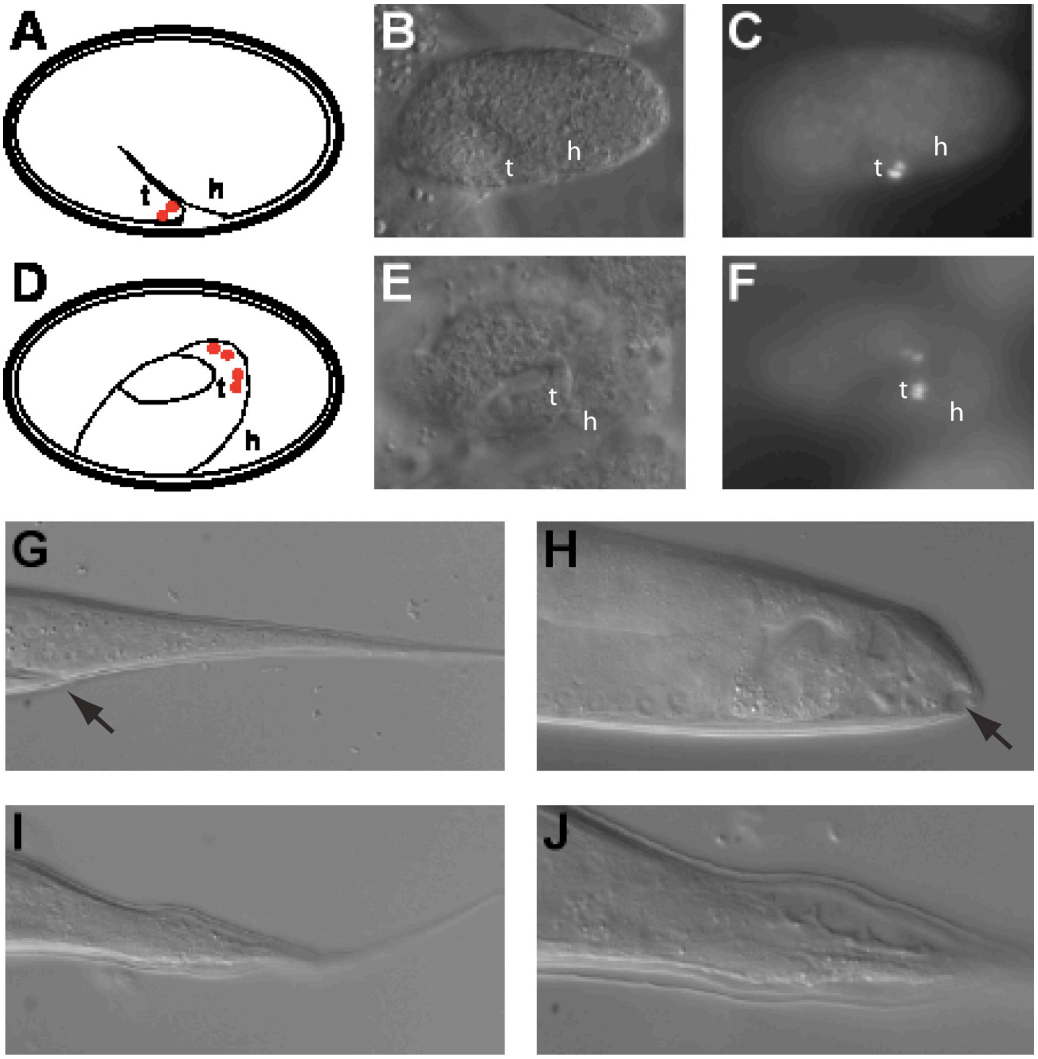

Figure 2: Ectopic expression of DAF-19A in the tail hypodermis. (A-C) Comma stage embryo and (D-F) 3-fold stage embryo expressing DAF-19A in the tail. (A, D) Cartoons: h marks the head, t the tail region, and red dots represent nuclear DAF-19A (B, E) DIC image and (C, F) fluorescent image where DAF-19A is visualized by a DAF-19 specific antibody. (G) Tail of a wild-type worm. (H-J) Tails are missing (H) or deformed (I, J) in worms ectopically expressing DAF-19A in the tail hypodermis. J is a magnification of I. Arrows in G and H mark the location of the anus.

Table 1. Ectopic expression of the DAF-19 isoforms C or A from different promoters. Induction of cilia gene expression, of cilia, and of phenotypes caused by ectopic expression results are marked in red. Fields marked in blue describe results where no effects were observed.

DAF-19 Isoform	Promoter	Start of Expression	Site of Expression	Induction of		
				che-13::DsRed* / *bbs-7::gfp	**Cilia**	**Abnormalities**
DAF-19C	*mec-7*	embryo	touch neurons in the body (ALM, AVM, PLM, PVM)	no / no	n.d.	n.d.
DAF-19A				no / no	n.d.	n.d.
DAF-19C	*unc-30*	embryo	D-type motorneurons, ASG	yes (ASG) / n.d.	yes (ASG)	n.d.
DAF-19A				n.d. / no	n.d.	n.d.
DAF-19C	*lin-44*	embryo	tail hypodermis (hyp 8-11)	n.d. / yes	n.d.	tail morphology
DAF-19A				n.d. / yes	n.d.	tail morphology
DAF-19C	*unc-54*	embryo	body wall muscle	yes / yes	n.d.	n.d.
DAF-19A				n.d. / yes	n.d.	n.d.

n.d. ….. not done

Table 2. Potential of DAF-19A and C to activate cilia gene expression in different cell types

	Promoter	DAF-19 isoform	Target gene activation	Rescue/formation of cilia
Ciliated sensory neurons	*gpa-13#* *unc-30**	C	**YES**	**YES**
		A	NO	NO
Non-ciliated neurons	*mec-7* *unc-30***	C	NO	NO
		A	NO	NO
Non-neuronal cells	*unc-54* *lin-44*	C	**YES**	**N.D.**
		A	**YES**	**N.D.**

 # … shown in (Senti and Swoboda, 2008); * … in ASG; ** … in motorneurons, N.D. … not done

Table 3. Strains and extra-chromosomal arrays analyzed in JT6924 [*daf-12* (*sa204*); *daf-19* (*m86*)] and JT8651 [*daf-19* (*m86*)/mnC1; *lin-15* (*n765* ts)]

strain	transgene	presence of DAF-19	direct DAF-19 target gene	cellular marker	transgenesis marker
mec-7 promoter constructs					
OE3514	ofEx363	*mec-7::daf-19c*	*bbs-7::gfp, che-13::DsRed*		*myo-2::DsRed*
OE3515	ofEx364	*mec-7::daf-19c*	*bbs-7::gfp, che-13::DsRed*		*myo-2::DsRed*
OE3647	ofEx495	*mec-7::daf-19c*	*che-13::DsRed*	*mec-4::gfp (zdIs5)*	*elt-2::mCherry, lin-15*
OE3648	ofEx496	*mec-7::daf-19c*	*che-13::DsRed*	*mec-4::gfp (zdIs5)*	*elt-2::mCherry, lin-15*
OE3189	ofEx153	*mec-7::daf-19c*	*che-13::DsRed*	*mec-4::gfp (zdIs5)*	*lin-15*
OE3190	ofEx154	*mec-7::daf-19c*	*che-13::DsRed*	*mec-4::gfp (zdIs5)*	*lin-15*
OE3044	ofEx32	*mec-7::daf-19a*	*bbs-7::gfp*		*lin-15*
OE3045	ofEx33	*mec-7::daf-19a*	*bbs-7::gfp*		*lin-15*
OE3046	ofEx34	*mec-7::daf-19a*	*bbs-7::gfp*		*lin-15*
OE3191	ofEx155	*mec-7::daf-19a*	*bbs-7::gfp*		*lin-15*
OE3187	ofEx151	*mec-7::daf-19a*	*che-13::DsRed*	*mec-4::gfp (zdIs5)*	*lin-15*
OE3188	ofEx152	*mec-7::daf-19a*	*che-13::DsRed*	*mec-4::gfp (zdIs5)*	*lin-15*
unc-30 promoter constructs					
OE3771	ofEx569			*unc-30::gfp*	*elt-2::mCherry*
OE3779	ofEx577		*che-13::DsRed*	*unc-30::gfp*	*elt-2::mCherry*
OE3780	ofEx578		*che-13::DsRed*	*unc-30::gfp*	*elt-2::mCherry*
OE3772	ofEx581	*unc-30::daf-19c*		*unc-30::gfp*	
OE3781	ofEx579	*unc-30::daf-19c*	*che-13::DsRed*	*unc-30::gfp*	*elt-2::mCherry*
OE3782	ofEx580	*unc-30::daf-19c*	*che-13::DsRed*	*unc-30::gfp*	*elt-2::mCherry*

lin-44 promoter constructs

OE3512	ofEx361	*lin-44::daf-19c*	*bbs-2::gfp*	*myo-2::DsRed*
OE3513	ofEx362	*lin-44::daf-19c*	*bbs-2::gfp*	*myo-2::DsRed*
OE3186	ofEx149	*lin-44::daf-19c*	*bbs-7::gfp*	*lin-15*
OE3185	ofEx150	*lin-44::daf-19a*	*bbs-7::gfp*	*lin-15*
OE3184	ofEx148	*lin-44::daf-19a*	*bbs-7::gfp*	*lin-15*

unc-54 promoter constructs

OE3775	ofEx572	*unc-54::daf-19c*	*bbs-7::gfp*	*elt-2::mCherry*
OE3776	ofEx573	*unc-54::daf-19c*	*bbs-7::gfp*	*elt-2::mCherry*
OE3786	ofEx585	*unc-54::daf-19a*	*bbs-7::gfp*	*elt-2::mCherry*
OE3787	ofEx586	*unc-54::daf-19a*	*bbs-7::gfp*	*elt-2::mCherry*
OE3788	ofEx587	*unc-54::daf-19a*	*bbs-7::gfp*	*elt-2::mCherry*

gpa-13 promoter constructs

OE3773	ofEx570	*gpa-13::daf-19c*	*che-13::DsRed*	*unc-122::gfp*
OE3774	ofEx571	*gpa-13::daf-19c*	*che-13::DsRed*	*unc-122::gfp*
OE3200	ofEx160	*gpa-13::daf-19c*	*bbs-7::gfp*	*unc-122::gfp*
OE3203	ofEx166	*gpa-13::daf-19c*	*bbs-7::gfp*	*unc-122::gfp*
OE3217	ofEx171	*gpa-13::daf-19a*	*bbs-7::gfp*	*unc-122::gfp*
OE3218	ofEx172	*gpa-13::daf-19a*	*bbs-7::gfp*	*unc-122::gfp*

Conclusions

From these studies, the following conclusions can be drawn based on the specific aims of the thesis:

- Several isoforms are expressed from the *daf-19* gene. DAF-19C is specific for ciliated sensory neurons (CSNs) and activates genes essential for cilia structure and function. DAF-19A/B are required for synaptic functions in non-ciliated neurons. Loss of DAF-19A/B results in reduced levels of synaptic vesicle proteins, a condition that becomes increasingly prominent in aging adult worms. Thus DAF-19A/B are not essential for synapse development, but are essential for synapse maintenance in the mature nervous system.
- FRISSC is a novel tool to study cilia function in *C. elegans* that uses animals with only a single functional cilium. In contrast to all other tools available to study sensory cilia, FRISSC allows investigation of all aspects of cilia function *in vivo*: the cilium itself, the sensory neuron, the neuronal circuit it participates in and the evoked behavioral response. Thus FRISSC animals represent a unique experimental model to study the effect of isolated sensory input at the cellular and organism level.
- The different isoforms of DAF-19 (A/B and C) have distinct potentials to activate cilia genes in cell types. This potential is strongly dependent on the cellular context in which they are expressed and may be due to a repressor system that differentially regulates DAF-19A/B, and C. If DAF-19 expression in non-neuronal cells can lead to the assembly of cilia needs further and more detailed experiments.

Concluding Remarks and Future Perspectives

With the novel findings presented in this thesis, new questions arise and follow-up projects can be designed. This chapter is dedicated to suggesting and outlining the most interesting and immediate lines of investigation that could be followed.

THE EFFECTS OF DAF-19 ACTIVITY ON LEARNING AND MEMORY

We analyzed the functions of the long DAF-19 isoforms A/B (Paper I). They are expressed in the entire nervous system (except for CSNs) and loss of DAF-19A/B impairs synaptic functions. However, in *daf-19* mutants we never observed severe behavioral phenotypes, for example movement defects that one would expect from this expression pattern. This could be explained by the fact that (a) the reduced levels of synaptic proteins occur rather late at adult stages and/or (b) that levels are only reduced, but not eliminated. Either way, these mild defects might require the search for more subtle phenotypes.

By employing classic learning and conditioning experiments, it has been shown that *C. elegans* is capable of learning (Rankin et al., 1990). In addition, a variety of behavioral assays are now standard that allow testing the function of groups of neurons or even single neurons in *C. elegans.* Associative learning has been demonstrated in experiments in which chemicals, to which *C.*

elegans is not attracted, are paired with food, followed by testing attraction to the chemicals alone (Wen et al., 1997). More recent studies of *C. elegans* learning and memory have shown that acquisition of knowledge can be differentiated from recall of learned information (Atkinson-Leadbeater et al., 2004). Interestingly, even aging has an influence on learning and memory *C. elegans* (Murakami, 2007).

Synaptic activity is not only required for the transmission of signals. During nervous system development, increased synaptic activity is required to stabilize synapses. In the mature nervous system on the other hand, high levels of synaptic activity are implicated in positive feedback loops that ultimately lead to the process of learning and memory. Thus it would be interesting to investigate whether the loss of DAF-19A/B causes learning defects in *daf-19* mutants. Employing assays for learning and memory on *daf-19* mutant worms of various ages could give an insight into this matter.

EVOLUTIONARY ASPECTS ON THE DEVELOPMENT OF THE DIFFERENT DAF-19 ISOFORMS

Cilia in all organisms rely on an intraflagellar transport (IFT) mechanism for their assembly and maintenance. In recent years two major theories on how cilia and IFT developed in these organisms were established. The symbiontic scenario proposed by Margulis and co-workers suggests that the merge of a Spirochete with the host cell is the origin of the cilium (Bermudes and Margulis, 1987; Margulis et al., 2006). However, several facts argue that this might not be the case (summarized in (Satir et al., 2007)).

Autogenous scenarios on the other hand support that cilia were founded by the duplication and subsequent divergence of pre-existing components of the eukaryotic cell (Cavalier-Smith, 2002). According to this theory, IFT evolved as a specialized form of coated vesicle transport from the protocoatomer complex, a prototypic membrane-curving module present in a pre-karyotic cell. Consequently IFT shares common ancestry with other protocoatomer derivates like COPI and II, clathrin coats, and the nuclear pore complex. Today this relationship is reflected by the similarity of sequence and

structures of IFT, COPI, and clathrin-vesicle components, as most of these proteins have several N-terminal WD40 repeats and C-terminal TRP motifs. Interestingly, in *C. elegans* the transport of membrane receptors to the base of the cilium is dependent on the AP-1 clathrin adaptor complex (Bae et al., 2006; Dwyer et al., 2001). In another study, Vieira and co-workers demonstrated a link between vesicle trafficking in the Golgi apparatus and cilium formation in vertebrates (Vieira et al., 2006). Therefore not only cilia function, but also cilia formation seems to be dependent on clathrin-mediated processes. It should be noted that although the theory of the autogenous evolution of cilia is well supported, it does not account for the origin of the centriole, the precursor of the basal body that serves as the base and template of the cilium. Satir and co-workers suggest that a self-assembly RNA enveloped virus formed the basis of the cilia origin – a theory that complements the autogenous evolution of cilia theory developed by Cavalier-Smith and his colleagues (Satir et al., 2007).

In light of the theories presented above, it is possible that the different isoforms of DAF-19 co-evolved with the formation and generation of the IFT transport system and/or other vesicular transport systems. In Paper I we demonstrate that DAF-19C is essential for the expression of cilia genes, including IFT components. Strikingly we found a connection between DAF-19A/B and synaptic vesicle proteins. The recycling of synaptic vesicles proteins is highly dependent on clathrin-coats (as outlined in the section *Synapse structure and function*). Thus, it is believed that IFT and COPI-clathrin like vesicle coats diverged from an ancestral vesicular transport mechanism (Cavalier-Smith, 2002). It is possible that this separation was induced, or maybe even preceded by, the differential expression of the proteins specific for each process. We speculate that in the ancestral ciliated prokaryote, one single RFX transcription factor activated all necessary vesicle proteins. As IFT evolved from the ancient vesicular process, gene modifications gave rise to two different RFX transcription factor isoforms necessary to specifically regulate two different processes: one that activates synaptic vesicle genes (DAF-19A/B in *C. elegans*), and one that activates genes involved in the cilia transport system (DAF-19C in *C. elegans*). A careful analysis and comparison of all RFX transcription factors and their different isoforms in ciliated and non-ciliated organisms could shed more light on these speculations.

THE ROLE OF DAF-19 IN SYNAPTIC MAINTENANCE

We have successfully demonstrated that DAF-19 regulates also a second process in addition to cilium formation, the maintenance of synaptic vesicle proteins (Paper I). Based on our current knowledge, we conclude that DAF-19 very likely regulates the maintenance of individual synaptic vesicle proteins or synaptic vesicles (biogenesis or recycling) themselves. Despite the detailed description of the progressive loss of synaptic components and the behavioral consequences in *daf-19* mutants, we are still missing the direct molecular link between DAF-19 and the maintenance of synaptic function. The maintenance of the mature *C. elegans* nervous system has so far not been studied in detail. Therefore, genes or mechanisms required for this process are not known. Clearly, a set of direct and indirect target genes of DAF-19A/B would immensely increase our understanding of the processes required for the continued function of a fully developed neuronal network. An unbiased approach for the isolation of direct and indirect target genes of DAF-19A/B could include gene expression profiling using microarray technology or tiling arrays. DAF-19A/B are indirectly required for the maintenance of synaptic vesicle protein levels (Paper I). Based on this knowledge, we would expect to isolate genes encoding transmembrane or membrane-associated proteins, genes involved in vesicular processes like vesicle formation or recovery, endo- and exocytosis, but also mRNA decay or protein degradation, which - as recently demonstrated - can also impact synaptic functions (Ding et al., 2007; Wang et al., 2006).

Our analysis of the synaptic defects in *daf-19* mutants revealed a rather unique phenotype. While no defects are seen in *daf-19* larvae, synaptic protein levels decrease in adults as they age. Reports about the loss of synaptic maintenance during aging have so far not been made in *C. elegans* or in other model organisms. However, we performed transmission electron microscopy to investigate whether the loss of synaptic vesicle proteins was caused by a reduced number of synaptic vesicles. Indeed, we have now preliminary results that the number of synaptic vesicles in synapses of *daf-19* mutants is strongly reduced compared to *wild type* (see Appendix to Paper I). A similar observation was made in *C. elegans* clathrin mutants (Erik Jorgensen, personal

communication; (Sato et al., 2009). Interestingly, *Drosophila* clathrin heavy chain (*chc*) mutants display a dramatic defect in neurotransmission maintenance during intense synaptic activity. A condition caused by a defect in synaptic vesicle recycling (Kasprowicz et al., 2008). Very recently Gu and co-workers presented their findings on *apm-2* (adaptor protein medium subunit 2), an AP2 subunit that functions in clathrin-mediated endocytosis. Intriguingly, *apm-2* mutants show only mild synaptic defects, which suggests that the gene is not essential, but that it facilitates synaptic vesicle recycling (Gu et al., 2008). Although not completely identical, these defects remind us of those seen in *daf-19* mutants and thus strengthen the hypothesis that DAF-19A/B target genes could participate in synaptic vesicle endocytosis. Based on these observations, it is tempting to speculate that DAF-19 may regulate one or more components of the clathrin-mediated endocytic process. It is a particularly attractive theory in the light of the previously described models of cilia evolution. A focused approach that directly investigates genes required for synaptic vesicle recycling in a *daf-19* mutant background – for example by quantitative real-time PCR – could complement the unbiased attempt to identify DAF-19 target genes.

THE DAF-19 MUTANT – A NEW MODEL FOR NEURODEGENERATIVE DISEASES?

A large number of vertebrate and invertebrate disease models for Alzheimer's disease (AD), Huntington's disease (HD) and other neurodegenerative diseases are nowadays available. They mainly focus on the few genes/proteins that were shown to induce pathologies in humans, for example the Huntingtin protein or the β-amyloid peptide (Aβ) and the microtubule-associated protein tau, which are the primary components of senile plaques and intracellular neurofibrillary tangles, respectively. Vertebrate and invertebrate disease models analyze the function of these proteins by overexpressing the corresponding human genes in neuronal or muscle cells and are to large extents able to reproduce certain aspects of human pathologies (Driscoll and Gerstbrein, 2003; Link, 2005; Link, 2006; McGowan et al., 2006). However, the cellular and molecular mechanisms underlying the toxicity of

these proteins, e.g. Aβ, are still debated. Although senile plaques and neurofibrillary tangles count as hallmarks of AD pathology, they can – however, less frequently – also be found in humans who did not show any signs of dementia. On the other hand, senile plaques and neurofibrillary tangles have never been detected in unmodified (wild-type) rodents, resulting in 'natural' AD models. Therefore, modified transgenic animals are currently the system of choice to investigate neurodegenerative diseases such as AD. Finally, it is important to note, that most cases of AD are sporadic and do not involve modifications of known disease genes. This suggests that many other disease genes causing neurodegeneration remain to be discovered.

The cause of AD is far from being defined. Clearly, senile plaques and neurofibrillary tangles are late stage symptoms associated with AD, but the ultimate sequence of events that leads to those neuronal changes is still unknown. In recent years, increasing attention is being paid to the role of synapses in neurodegenerative diseases. Deregulation of SNAREs (soluble NSF attachment receptors) and other synaptic proteins has been described for a number of neurological diseases, such as HD (Morton et al., 2001), schizophrenia (Halim et al., 2003) or AD (Reddy et al., 2005; Sze et al., 2000). In AD, synaptic loss is the earliest and strongest correlating feature to and therefore likely a major reason for cognitive decline (Masliah et al., 1989; Terry et al., 1991). Increasing focus is now put on the loss of synaptic proteins, thought to trigger synaptic loss (Selkoe, 2002). AD is associated with a reduction of different synaptic proteins to various degrees (Reddy et al., 2005; Shimohama et al., 1997; Sze et al., 2000), where levels of synaptobrevin are usually affected more severely compared to other synaptic proteins. The molecular phenotypes seen in *daf-19* mutants are very similar, suggesting that *daf-19* may regulate a mechanism that – when defective – can lead to synaptic decline.

Although this is still highly speculative, a connection between RFX proteins and synaptic decline may even exist in humans. Recently, a novel locus for late-onset AD has been identified (Wijsman et al., 2004), that maps to a locus that includes hRFX2, one of the closest human *daf-19* homologs. In another study, hRFX2 was found to be a candidate for regulating the expression of amyloid beta peptides, which form the plaques that are characteristic of late

AD stages (Lahiri et al., 2005). Since both RFX transcription factors and the majority of synaptic proteins in *C. elegans* are highly conserved, parallels in the regulation of synaptic protein expression in different organisms, including humans, are very likely. Therefore, the *C. elegans daf-19* mutant may prove to be a useful model organism to dissect experimentally the mechanisms that maintain synaptic function. It may ultimately also help us to better understand the process of neurodegeneration that is associated with human diseases.

FRISSC – A NOVEL TOOL TO STUDY CILIATED NEURON FUNCTION

Because of its simple nervous system and invariant number of CSNs, *C. elegans* is an exceptionally well-suited model organism in which to study sensory neuron function. Initially, the assignment of sensory specificity was investigated by laser ablation of single neurons or combinations thereof (Bargmann and Horvitz, 1991). The molecular dissection of sensory neuron pathways can also be achieved via mutant analysis (if such mutants are available). Recently, a number of techniques were added to the toolbox for sensory neuron analysis, significantly extending our experimental possibilities: heat-shock inducible expression systems, laser ablation of individual neurons, control of expression in time and space via the FLP recombinase system and optical imaging methods (Bacaj and Shaham, 2007; Bargmann and Horvitz, 1991; Davis et al., 2008; Kerr et al., 2000). Each of these methods offers unique advantages and possibilities for investigating specific aspects of CSN function. However, none of them allows the study of a single CSN isolated from any other sensory input. This might be desirable in the case of redundant functions, shared downstream circuits, or integration of several sensory modalities into one behavior. Furthermore, the above listed methods have shortcomings in that they do not allow analysis of all aspects of sensory perception (detection of the cue, physiological aspects in the neuron, resulting behavior in the worm). In Paper II we present FRISSC (**F**unctional **R**escue **I**n **S**ingle **S**ensory **C**ilia), a novel *in vivo* tool for the analysis of CSN function that makes all of this possible. By rescuing cilia formation in a *daf-19* mutant background in specific CSNs, we generate worms with only a single structurally and functionally intact

cilium. We demonstrate that the full cascade from signal detection to behavioral analysis can be carried out with this system. Thus, FRISSC is a valuable addition to the tools for studying CSN function. In Paper II we also suggest novel experimental paths that FRISSC enables one to explore and we suggest ways to combine it with other experimental approaches to further improve its versatility (Bacaj and Shaham, 2007; Davis et al., 2008).

THE DEVELOPMENTAL ROLE AND POTENTIAL OF THE RFX TRANSCRIPTION FACTOR DAF-19

In Paper III we conducted a pilot study with the aim to investigate whether DAF-19 functions as a master regulator of ciliogenesis. We expected that ectopic expression of DAF-19C, which activates cilia genes in CSNs, would lead to cilia formation in originally non-ciliated cell types (neurons, hypodermal cells and muscle cells). Surprisingly, both DAF-19 isoforms A and C can activate cilia genes. However, the potential to do so is dependent on the cellular context. In CSNs it is only DAF-19C that is active, while in non-ciliated neurons neither of the isoforms can activate cilia genes. Surprisingly, both isoforms can activate cilia genes in non-neuronal cells. We hypothesize that one or more isoform-specific repressors could regulate the ability of DAF-19A or C to induce cilia genes. Such repressors could be identified in screens for mutants that activate cilia genes in non-ciliated neurons.

Alternatively, although in a less straightforward scenario, non-ciliated neurons could lack a co-regulator of ciliogenesis. Recent studies on motile cilia in zebrafish demonstrated that Foxj1a acts upstream of Rfx2 (the DAF-19 homolog in zebrafish) and is required for the expression of components specific for motile cilia (Stubbs et al., 2008; Yu et al., 2008). *C. elegans* does not have motile cilia, neither does its genome encode a clear Foxj1 homolog that shows overlapping expression with DAF-19. Nevertheless, in analogy, co-activators for cilia gene expression could be needed in addition to DAF-19 for a successful induction of non-motile cilia in originally non-ciliated cell types. However, the search for such regulators might turn out to be difficult. Several screens for cilia mutants in *C. elegans* failed to uncover any key regulators other than DAF-19

(Bargmann et al., 1993; Culotti and Russell, 1978; Malone and Thomas, 1994; Perkins et al., 1986; Starich et al., 1995). Consequently, the *daf-19* mutant is, to date, the only one mutant that does not develop cilia at all; all other cilia mutants have at least short cilia remnants. A co-regulator that induces cilia in concert with DAF-19, if it exists, might have additional phenotypes or even be lethal when mutated, and thus would not appear in a genetic screen for cilia defects.

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Publications

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