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CHARACTERIZATION OF MEMORY, FORGETTING AND SYNAPTIC POLARITIES IN THE *CAENORHABDITIS ELEGANS* NERVOUS SYSTEM

PhD thesis

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Abbreviations, Definitions

ANOVA	Analysis of variance
AP-1	Activating protein 1
C/EBP	CCAAT enhancer binding protein
cDNA	Complementary deoxyribonucleic acid
CREB	cAMP response element binding protein
DA	Diacetyl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEG	Electroencephalography
Egr	Early growth response protein
fMRI	Functional Magnetic Resonance Imaging
GluA2 AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor glutamate A2 subunit
HEN-1	Small RNA 2'-O-methyltransferase
JNK-1	Stress-activated protein kinase jnk-1
LTAM	Long-term associative memory
MACO-1	Macoilin
NMDA	N-methyl-D-aspartic acid
RIP-Chip	Ribonucleoprotein immunoprecipitation–microarray
RNA	Ribonucleic acid
SCD-2	ALK tyrosine kinase receptor homolog scd-2
TIR-1	NAD(+) hydrolase tir-1
UTR	Untranslated region

1 Introduction

1.1 Experimental and network theory approaches in neuroscience

There has always been an interest in the human mind ever since early history. In ancient Egypt cognition was attributed to the heart, so the brain was removed during the preparation of dead. However, Greek scientists like Erasistratus, Hippocrates or Herophilus, and later Galenus from the Roman Empire, emphasized and investigated the role of the brain as an organ of higher mental functions, who were followed by Vesalius and Willis in the middle age (Gross, 1987).

In the pursuit of a better understanding of the nervous system milestone innovations like light microscopy, tissue staining (e.g. silver impregnation) and later electron microscopy, molecular methods, and functional magnetic resonance imaging all contributed grossly to successful experimentation. Hungarian scientists like István Apáthy, János Szentágothai, followed by contemporary masters like Szilveszter E. Vizi, or Brain Prize-awarded Péter Somogyi, Tamás Freund and György Buzsáki have always been at the frontline of neurosciences.

Recently, the spectrum of neurosciences has broadened in two directions. On one side, the focus is increasing on smaller and smaller compounds of the brain – enlarging single neurons, investigating individual receptors and molecular interactions. In the other direction, brain is increasingly approached with methods of systems theory – recognizing its complexity. Currently, the term neuroscience covers and incorporates a wide range of disciplines such as anatomy, biology, bioinformatics, genetics, histology, molecular biology, neuropsychology, and network and systems theory.

The extent to which neuronal functioning can be studied differs between species. The human brain, most interesting amongst all, can be studied *in vivo* with fMRI or EEG which methods have a clear limitation in their resolution. On the contrary, model species can be used to dissect their brains in high resolution, *in vivo* or *in vitro*. (Bliss & Gardner-Medwin, 1973; Kandel et al., 2014; Menzel, 1990; Menzel & Muller, 1996; Sandi & Rose, 1994; Stock & Zhang, 2013). However, the trade-off is evident: the nervous systems of primitive species can be better resolved on a neuronal level, but their behavioral palette is inevitably simpler and far from human. Complex behavior of

developed species is attributed to significantly more complex nervous system, studying of which requires ethically and technologically demanding resources.

Ultimately, the functioning of a nervous system can be described as synchronized activation and inhibition of neuronal cells and cell-groups. Activation and inhibition are due to ion channel-dependent ion-flux and membrane potential dynamics leading to intracellular molecular changes. These dynamics have been measured for a long time *in vitro*, and recently *in vivo* techniques are also increasingly available by applying genetically coded fluorophores and automated imaging (Nguyen et al., 2016). In parallel, behavior analysis also became increasingly automated thus less prone to observation bias (Albrecht & Bargmann, 2011; Brown & Schafer, 2015; Hong et al., 2015; Hu et al., 2015; Itskov et al., 2014; Ohayon et al., 2013; Wakabayashi et al., 2004). Despite all the advancements, a comprehensive understanding of the neuronal functioning behind behavior is still lacking.

The fundament of all functionality is the connectivity of neurons and neuronal areas, i.e. the structural organization of the nervous system. Thus, studying the physical wiring of a nervous system (also called the connectome) is often useful. Methods of graph theory and network theory are often used to target the understanding of large, complex systems with mathematical models (Bullmore & Sporns, 2009; Fornito et al., 2016; Tononi et al., 1994). A graph is a collection of nodes which are connected by edges. A real-world system can be modeled as a network if it can be represented as a set of nodes and edges. A nervous system is evidently such a system comprising of neurons and interconnecting synapses. This connectivity network can be studied on multiple scales which approaches are collectively referred to as connectomics (Bullmore & Sporns, 2009; Zeng, 2018). Macroscale connectomics focuses on large brain areas and the fibre tracts connecting them, utilizing techniques such as diffusion tensor imaging or diffusion spectrum imaging. These modalities are applicable on large organs like the mammalian brain. Mesoscale approach deals with cell-groups and their connections, often focusing on the synchronicity of activity between different groups of cells. (Oh et al., 2014; Zeng, 2018). Microscale connectomics being the highest-resolution, targets neurons and their synaptic connections. On this level the number and localization of individual synapses can be investigated with confocal or electron microscopy. Complete reconstruction of a nervous system is currently available only for the nematode *Caenorhabditis elegans* (Varshney et al., 2011; White et al., 1986), but other species are in the focus as well (Kuan et al., 2020;

Scheffer et al., 2020; Zeng, 2018). For mammals, smaller brain regions have been described for mice and cats on microscale level, but the human brain is still far from a full reconstruction (Oh et al., 2014; Reimann et al., 2019). These efforts are significantly supported by novel techniques like super-resolution microscopy, *in vivo* neuronal tracing or computer-assisted automated analytics.

Importantly, the network theory approach of nervous systems allows the better understanding of brain diseases and pathologies, serving diagnostic or predictive purposes. For example, fMRI-based functional reconstructions of human brain networks show different patterns in patients with Alzheimer's disease, epilepsy or schizophrenia (Ponten et al., 2007; Supekar et al., 2008; Yu et al., 2011). Moreover, network theory tools can be used in the differential diagnosis, and the follow-up of progression and therapeutic effect of dementias (de Waal et al., 2014; Raj et al., 2012; J. Zhou et al., 2010).

From an information theory aspect, nervous systems are networks which particularly function as communication systems. Network topological features such as small-worldness, modularity, and rich club organization are energetically costly but evolutionarily advantageous properties serving the ultimate purpose of a neural network: to efficiently communicate. These features are universally found in brain networks of many species, from rodents to the human connectome (Bassett & Bullmore, 2017; Harriger et al., 2012; Meunier et al., 2010), and contribute to effectively collect, integrate, and propagate information (Bullmore & Sporns, 2009; Rubinov et al., 2015). Missing a clear understanding of how information is routed and communicated through the brain, many models have been proposed to characterize neural communication such as optimal routing (Bullmore & Sporns, 2009; Rubinov & Sporns, 2010), diffusion models (Abdelnour et al., 2014), spreading dynamics (Mišić et al., 2015), or navigation (Seguin et al., 2018).

1.2 The nervous system of *Caenorhabditis elegans*

1.2.1 Introducing *C. elegans*

The introduction and establishment of *Caenorhabditis elegans* (*C. elegans*) as an experimental model species for genetic and molecular studies is largely acknowledged to Sydney Brenner (Brenner, 1974). This simple animal proved to be an ideal model organism for multiple reasons:

- maintenance of populations is cheap,
- population-level testing is possible due to small (1mm) size and short (12-18 days) life cycle,
- hermaphrodites can provide stable genetic (including transgenic) lines,
- it is the first animal of a fully sequenced genome (The *C. elegans* Sequencing Consortium et al., 1998),
- its nervous system is small and fully reconstructed (Cook et al., 2019; Varshney et al., 2011; White et al., 1986),
- the transparency of the animal allows the observation of internal /cell-level processes *in vivo.*

The *C. elegans* genome consists of approximately 20,000 genes in 97 million base pairs which is 300-fold less than the number of base pairs in the human genome. Still, about 80% of the proteins shows some degree of homology with human proteins. The majority (99.9%) of *C. elegans* populations are hermaphrodites which are capable of self-reproduction of isogenic progenies. The hermaphrodite *C. elegans* is built up of 959 cells, of which 302 are neurons. According to their function, neurons can be classified as sensory, inter- and motor neurons, though some neurons have mixed functions. The first detailed wiring reconstruction of the nervous system was published in 1986 (White et al., 1986) describing approximately 3,000 chemical connections and 600 gap junctions. The localization and the connectivity of neurons was considered largely conserved between individuals, allowing unique labeling of neurons. Interneuronal communication is carried out *via* synaptic (both through chemical synapses and gap junctions) and extrasynaptic transmission. Most of the neurotransmitters and neuromodulators expressed by *C. elegans* (e.g. acetylcholine, glutamate, dopamine, serotonin, GABA) are identical to mammals' (Chase & Koelle, 2007). The animal also expresses a wide range of neurotransmitter receptors and ion channels (Altun, 2011).

1.2.2 Adaptive behaviors: learning, memory, and forgetting

Despite its simple organization, *C. elegans* is capable of surprisingly complex behavior. It can distinguish multiple forms of environmental stimuli (e.g. oxygen, salt, temperature, odor, and touch) and respond to them adaptively by changing its motor patterns. Its main motor drive is search for food and avoidance of noxious stimuli. The worm can persistently change its reaction to the environment by learning and inducing short- and long-term memory as well (Ardiel & Rankin, 2010; Sasakura & Mori, 2013). The intensity and frequency of stimuli determines the length of memory induced. Short term

memory lasts for minutes-hours which can consolidate to long-term memory lasting for days, comparable to the short life-span of animals. Notably, worms also exhibit imprinting, i.e. life-long memories formed under specific developmental or environmental circumstances.

Induction of (short-term) memory is regulated on the post-translational level, through mechanisms like phosphorylation/dephosphorylation or receptor trafficking. Conversion to long-term memory (also called consolidation) is realized by structural changes requiring new proteins, mediated by transcription and translation as well (Kandel et al., 2014). For example, structural actin cytoskeleton remodeling is a manifestation of memory-related synaptic plasticity (Okamoto et al., 2004). In terms of timing, the molecular processes underlying the different memory phases occur in a parallel fashion rather than sequentially, utilizing independent genetic resources (McGaugh, 2000).

Assessment of learning in *C. elegans* is possible through multiple assays. For example, in aversive olfactory conditioning worms are exposed to a chemoattractant (e.g. diacetyl) while being starved. This leads to an association between diacetyl and starvation, thus worms will avoid diacetyl instead of being attracted to it. This change of behavior is not permanent (lasts only for a couple of hours), but repeated associations can increase the length of the new behavior up to days.

An important feature of adaptive behavior is the capability to erase old and unused information, i.e. to forget. Historically, two psychological models of forgetting have been proposed (decay and interference), both assuming that forgetting is a (molecularly) passive loss of memories (Jonides et al., 2008; Wixted, 2004). However, recent studies suggest forgetting is actively regulated by multiple molecular mechanisms (Berry et al., 2012; Davis & Zhong, 2017) in many species including *C. elegans*, such as the endocytosis of GluA2 AMPA receptors (Hardt et al., 2014; Migues et al., 2016; Sachser et al., 2017), the dopamin/Rac/Cofilin-activated cytoskeleton reorganization (Berry et al., 2012, 2015; Cervantes-Sandoval et al., 2016), or the TIR-1/JNK-1 and MACO-1 and SCD-2/HEN-1 pathways (Inoue et al., 2013; Kitazono et al., 2017). Notably, mechanisms of forgetting seem to be separated from that of learning and memory on the cellular level as well, as neurons independent from the formation and maintenance of memory can act as regulators of forgetting (Inoue et al., 2013; Shuai et al., 2015).

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1.2.3 Genetic background of long-term memory

The formation and maintenance of long-term memory requires *de novo* protein synthesis, of which many proteins have been identified and studied extensively (Gal-Ben-Ari et al., 2012; Rosenberg et al., 2014). Recently, gene expression regulatory mechanisms of memory have been studied in many species including *C. elegans* on a genome-scale as well (Borovok et al., 2016; Centeno et al., 2016; Jiang et al., 2011; Lakhina et al., 2015). Results suggest that multiple genes are activated or inhibited in parallel during the memory phases, mediated by multiple transcription factors (e.g. CREB, C/EBP, Egr, AP-1, and Rel) (Alberini, 2009). See also Table 1 for a list of learning and/or memory-associated genes.

Table 1. Overview of genes regulating learning and memory in *C. elegans*. Deletion of the gene doesn't change (✓) or impairs (✕) the function or the effect is unknown (?).

protein	gene	learning	STM	LTM	forgetting	Source
CREB	*crh-1*	✓	✓	✕	?	(Kauffman et al., 2010; Lau et al., 2013)
TIR-1/ JNK-1		✓	✓	✓	✕	(Inoue et al., 2013)
adducin	*add-1*	✕	✕	✕	?	(Vukojevic et al., 2012)
rasGAP1	*gap1*	✕	✓	✓	✓	(Gyurkó et al., 2015)
rasGAP2	*gap2*	✓	✕	?	?	
rasGAP3	*gap3*	✓	✕	?	?	
AMPAR	*glr-1*	✓	✕	✕	?	(Lau et al., 2013)
MAGI	*magi-1*	✕	✕	?	?	(Stetak et al., 2009)
NMDAR	*nmr-1*	✕	✕	✕	?	(Lau et al., 2013)

Amongst all genes, perhaps the cAMP response element binding protein (CREB) is one of the most studied. CREB is a fundamental and evolutionarily conserved regulator of long-term memory as has been shown in invertebrates and vertebrates as well (Alberini, 2009; Kandel, 2012). Recently, 757 genes have been identified in *C. elegans* that are

upregulated CREB-dependently in a positive olfactory conditioning assay (Lakhina et al., 2015). Interestingly, different learning and memory forms require CREB in distinct neurons: mechanosensory habituation, thermotaxis memory and positive olfactory associative memory necessitates CREB in the AVD, AFD, and AIM neurons, respectively (Lakhina et al., 2015; Nishida et al., 2011; Timbers & Rankin, 2011). Yet, several questions remain regarding memory associated gene expression changes like the temporal dynamics or the extent of modality-specificity.

Previous studies found that cytoskeletal changes and more specifically, actin remodeling play a role in the formation and regulation of long-term memory (Lamprecht, 2014; Vukojevic et al., 2012). Thus, pathways which act on cytoskeletal changes are potentially interesting. Musashi is an RNA-binding protein family (coded by *msi1/msi2*, *musashi*, and *msi-1* in human, *Drosophila*, and *C. elegans*, respectively) (Sakakibara et al., 2002). In nematodes, *msi-1* is present during embryogenesis and in the adult nervous system as well (Yoda et al., 2000). Musashi represses protein translation by binding to the 3'UTR region of target mRNA molecules (Ohyama et al., 2012). In a RIP-ChIP assay, 64 mRNAs were found to interact with Musashi, one of them being involved in cytoskeletal actin branching regulation (de Sousa Abreu et al., 2009). The potential interaction of Musashi with actin remodeling makes it a candidate protein of memory regulation.

1.3 Network approach of the *C. elegans* nervous system

As any nervous system, the one of *C. elegans* can be depicted as a network of neurons and interconnecting synapses. The *C. elegans* neuronal network is outstanding from any other connectomes by being the most comprehensive. The first connectome reconstruction published more than thirty years ago (White et al., 1986) was revised and updated several times as new electron microscopy images were generated. These neuronal networks are publicly available and are subjects of extensive analyses (Cook et al., 2019; Jarrell et al., 2012; Varshney et al., 2011). The connectome reconstructions are partially overlapping and to some extent different in the number of neurons and connections covered (Table 2, also Bentley, 2017).

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Table 2. Differences between connectome reconstructions of C. elegans

	WormWiring.org	Varshney *et al.*	Cook *et al.*
Number of interneuronal chemical connections	3,638	2,575	3,242
Number of interneuronal chemical synapses	20,589	6,394	10,203
Number of neurons (non-isolated; including the pharyngeal nervous system)	297	-	279
Number of neurons (non-isolated; without the pharyngeal nervous system)	278	279	259

In the following, some network properties of the *C. elegans* neuronal connectome are briefly described.

1.3.1 Scale-free, small-world and rich club properties

Real world networks are often scale-free, meaning that their degree distribution follows a power law. This results in a network where many low-degree nodes are connected by only few high-degree nodes (also called hubs). Scale-free networks are quite resilient to errors and permit cost-effective distribution of information. The *C. elegans* connectome is not completely scale-free (Varshney et al., 2011), but consists a *rich club* of high-degree and highly interconnected nodes (Towlson et al., 2013). Its distribution of synaptic weights also follows a power law (Sohn et al., 2011). Another property of communication networks is small-worldness which is defined by high clustering coefficient and low average path length. The *C. elegans* connectome is a small-world network (Varshney et al., 2011).

1.3.2 Other network properties: reciprocity, modularization

Reciprocity is the measure of bi-directional connectivity of neurons A reciprocal connection can participate in important functional operations such as signal enhancement, integration, and feedback. *C. elegans* presents more bidirectional connections than expected (Reigl et al., 2004). The proportion of reciprocal connections is between 17-27% (Varshney et al., 2011; White et al., 1986) which is important in coordinating

movement and mating behavior (Correa et al., 2012; Roberts et al., 2016; White et al., 1986).

Modularization is the tendency of a network to form large subgraphs (modules) where intra-modular connectivity is more dense than extra-modular connectivity. Modules are important as they can provide the structural basis for parallel and independent execution of tasks. In nervous systems modules can often be identified as anatomically defined, functionally specific areas as have been shown in multiple species (Bassett & Bullmore, 2006; Hilgetag et al., 2000; Pan & Sinha, 2009; C. Zhou et al., 2006). Similar studies in *C. elegans* have revealed several different (anatomy-based, function-based or multi-aspect) modularization features of the connectome (Pan et al., 2010; Pavlovic et al., 2014; Sohn et al., 2011).

1.4 Excitation and inhibition in the *C. elegans* neuronal network

Electron microscopy can provide detailed structural information about the type and direction of a synapse, but it is unable to tell the neurotransmitter used or the polarity of the connection (i.e. whether being excitatory or inhibitory) which are a crucial functional information. This gap can be filled by analyzing neuron specific gene expression data which are increasingly available for *C. elegans* and have resulted in a comprehensive knowledge about neuronal neurotransmitter usage (Hammarlund et al., 2018; Loer & Rand, 2016; Taylor et al., 2019). Experimental confirmation of synaptic signs *in vivo* is difficult and resource-demanding (Goodman et al., 2012; Warrington et al., 2019), but *in silico* predictions have been made for subcircuits of the connectome (Rakowski et al., 2013; Rakowski & Karbowski, 2017; Wicks et al., 1996). However, yet no brain-level synaptic polarity information exists for *C. elegans*.

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2 Objectives

The goal of my doctoral studies was to better understand the nervous system and behavior of the nematode *C. elegans* by using experimental and network science tools. Out of the many adaptive behavior forms of *C. elegans* we investigated, experimentally, the genetic and molecular underpinnings of aversive olfactory long-term associative memory, on genome-wide and single gene-level as well. We specifically studied the neuron-specific role of Musashi (MSI-1, *msi-1*) in long-term memory and forgetting, and related subcellular synaptic dynamics. Additionally, we aimed to modulate long-term memory with pharmacophores using a novel treatment methodology.

As all behaviors, learning and remembering are results of complex neurobiological activity, carried out by a physically constraining synaptic infrastructure. Therefore, we aimed to analyze the available connectivity map (connectome) of the *C. elegans* nervous system, from a systems perspective. My goal was to combine structural and genomic data for a better understanding of neuronal function. We aimed to predict the synaptic polarities in the ionotropic chemical synapse connectome by utilizing gene expression data in a novel conceptual framework. Since the premotor interneuron AVA has a structurally central position in the network and is also crucial in many behaviors, we aimed to further characterize its functional connectivity.

Altogether, the objective of this dissertation was to contribute to a better understanding of the *C. elegans* nervous system, a bridge towards more complex, ultimately the human, nervous systems.

3 Methods

Methods not described in associated co-authored or first-authored publications are detailed in the following section. Methods already published with my authorship are briefly described or excluded from this thesis, following the guidance of the Doctoral School.

3.1 Experimental methods

For the pharmacologic experiments the following strains were acquired from the *C. elegans* Genetic Center (Minneapolis, USA): wild type Bristol strain N2 variant; *crh-1(tz2)*; *msi-1(os1)*.

Assessment of chemotaxis and aversive olfactory associative learning and memory was carried out as described previously (Stetak et al., 2009), summarized briefly in Figure 1.

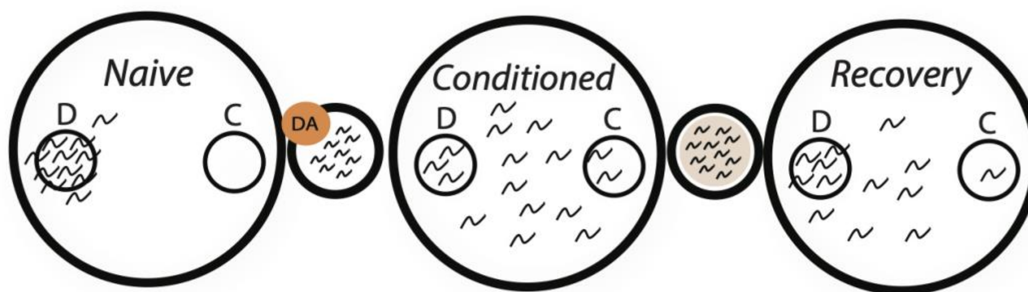

Figure 1. Aversive olfactory conditioning and chemotaxis. Diacetyl is a natural attractant for worms. To assess chemotaxis towards diacetyl (DA), worms are put on the middle of a chemotaxis test plate and are freely crawling until immobilized either in the diacetyl-containing (D) or the non-containing control (C) area. Chemotaxis index (CI) is calculated to quantify positive chemotaxis ([worms in D – worms in C] / all worms on the plate). Untreated (*Naive)* worms show a native attraction towards diacetyl. During long-term aversive olfactory training (i.e. conditioning), worms are exposed to diacetyl in the absence of food. Conditioned worms develop aversion towards diacetyl (*Conditioned*), which learnt behavior diminishes gradually over time (*Delayed*).

A novel conditioning protocol was developed to apply pharmacological treatment during aversive conditioning. Instead of using traditional CTX-plates, worms were conditioned in 15 ml 5% w/v mannose solution (isotonic for worms) mixed with 0.02% v/v diacetyl. Treatment solutions contained 1% DMSO to prevent precipitation of compounds.

Pharmacons were applied in 10-500 µM concentrations, dissolved in CTX or DMSO solutions. Assay-ready worms were washed from NGM feeding plates to 15-ml tubes and re-washed with CTX solution two times to remove residual OP50 bacteria. Worms were

treated in a 15-ml tube before (for 1 hour), during (for 2 x 1 hours), and after (for 0.5 hour) conditioning.

3.2 Sign prediction methods

For synaptic polarity predictions, the chemical connections subset of the WormWiring hermaphrodite connectome reconstruction (http://wormwiring.org) – consisting of 3,638 connections (20,589 synapses) and 297 neurons – was used. Neurotransmitter and receptor gene expression data were obtained from previous publications (Altun, 2011; Gendrel et al., 2016; Hobert et al., 2016; Loer & Rand, 2016; Pereira et al., 2015; Serrano-Saiz et al., 2017; Taylor et al., 2019) and from Wormbase (http://wormbase.org) and manually curated. Only genes encoding ionotropic receptor subunits for the three major synaptic neurotransmitters (glutamate, acetylcholine, GABA) were evaluated and scored as binary information (i.e. expressed, non-expressed). Polarities of synapses were predicted based on presynaptic neurotransmitter and postsynaptic receptor gene expression data (Figure 2), using nested logical and conditional formulas. Synapses were predicted as *excitatory* or *inhibitory* if only cation channel or only anion channel receptor genes matched the presynaptic neurotransmitter, respectively; *complex* if both types of receptor genes matched; and *unpredicted* if no receptor gene matched. Exact formulas are available on the website http://EleganSign.linkgroup.hu.

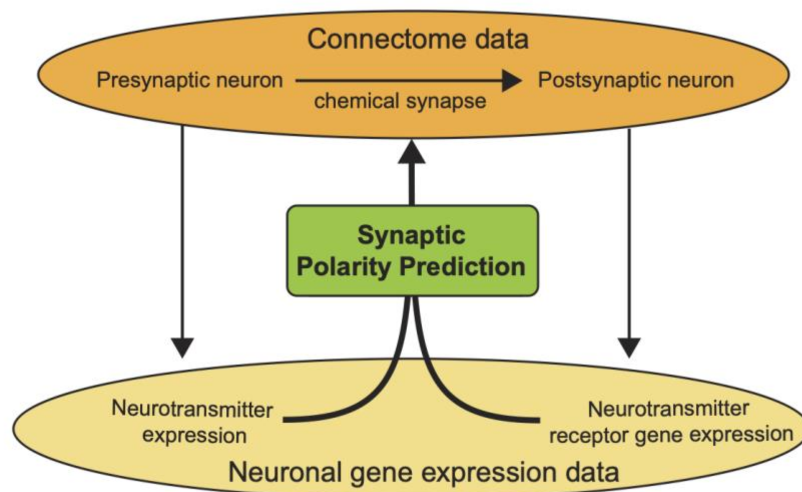

Figure 2. Prediction method of synaptic polarities of the *C. elegans* connectome. Connectome and gene expression data from various databases were manually curated. Polarities of chemical synapses were predicted based on the neurotransmitter expression of presynaptic neurons and ionotropic receptor gene expression of postsynaptic neurons. (Fenyves et al., 2020)

4 Results

4.1 Gene activation patterns during long-term memory-induction

Transcription and translation are required for the formation and consolidation of long-term memory. One of the key transcription factors in long-term memory is CREB, which regulates the expression of many genes in multiple species (Barco et al., 2003; Josselyn et al., 2002; Scott et al., 2002). The role of CREB has also been shown in *C. elegans* in associative and non-associative memory (Kauffman et al., 2010; Lau et al., 2013; Timbers & Rankin, 2011) and was found to be a significant transcriptional regulator of numerous memory genes (Lakhina et al., 2015).

To establish an aversive olfactory LTAM activated gene-set (both CREB-dependent and -independent) and to define the temporal patterns of gene expression during aversive olfactory LTAM we performed genome-scale microarray experiments at multiple time points (before and after conditioning). To obtain tissue-specificity we used the pan-neuronal promoter driven PAB-1::FLAG system (Von Stetina et al., 2007) to enrich neuronal RNA. For the first experiment, we collected samples at 8 time points after conditioning, and also after adaptation and starvation (Figure 3A). Contrasting to naive worms, gene expression analysis revealed 3,563 differentially expressed transcripts after conditioning, of which 639 were also differentially expressed after starvation or adaptation (Figure 3B).

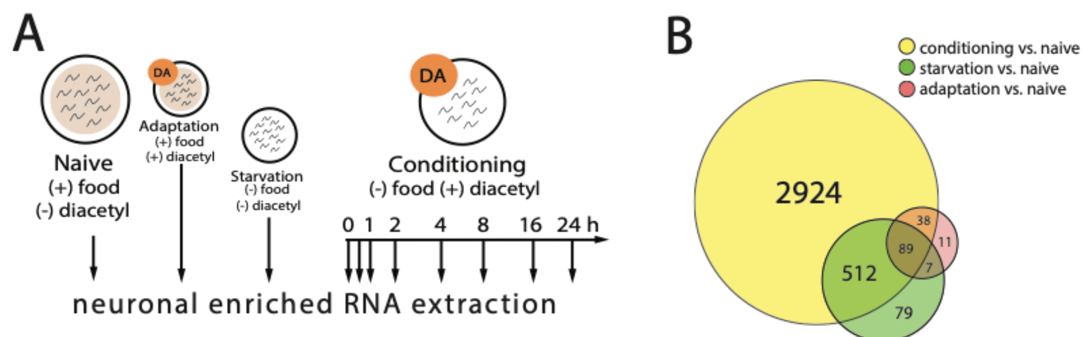

Figure 3. Sample collection and expression profiling. A For the first analysis, neuronally enriched mRNA was collected from worms before conditioning (*naive*), and after adaptation, starvation, and multiple time points after conditioning (marked by arrows in the figure). **B** Differential gene expression was measured between each conditioning time point and adaptation or starvation vs. the naive state, resulting in 3563 probe sets expressed differentially after conditioning (yellow circle). Amongst these probe sets, 639 were also differentially expressed in naive vs. adaptation or naive vs. starvation (overlapping red and green circles, respectively). (Freytag et al., 2017)

Using hierarchical clustering, these transcripts were grouped into six clusters. Three of the clusters corresponded to upregulated genes differing in the temporal kinetics of their expression patterns, suggesting three waves of gene activation (Figure 4).

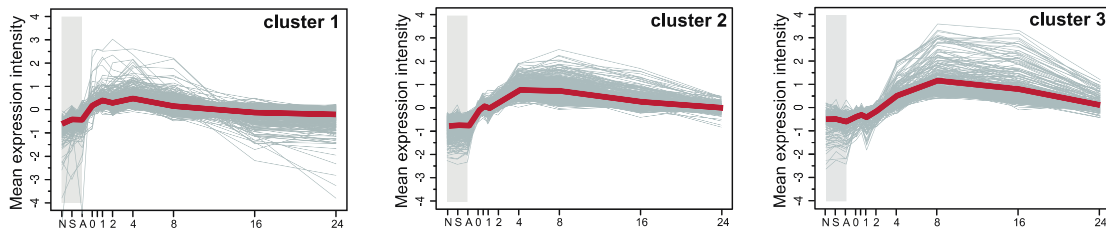

Figure 4. Three clusters of gene activation. Hierarchical clustering of differentially expressed probe sets revealed three upregulation clusters (clusters 1, 2, and 3). Naive (N), starvation-only (S), adaptation-only (A), and conditioning time points (0, 1, 2, 4, 8, 16, 24) are represented in the X axis. N, S, and A states are in grey boxes. (Freytag et

In a second experiment, to further differentiate LTAM-specific genes from starvation- and adaptation-related genes, we collected RNA 4 and 8 hours after conditioning, starvation, and adaptation (Figure 5A). Contrasting 4h+8h conditioning to naive, 4h+8h starvation and 4h+8h adaptation, we identified a differentially expressed gene set of 1,039 probes (706 upregulated and 333 downregulated), suggesting that these probe sets represent memory-specific transcripts (Figure 5B).

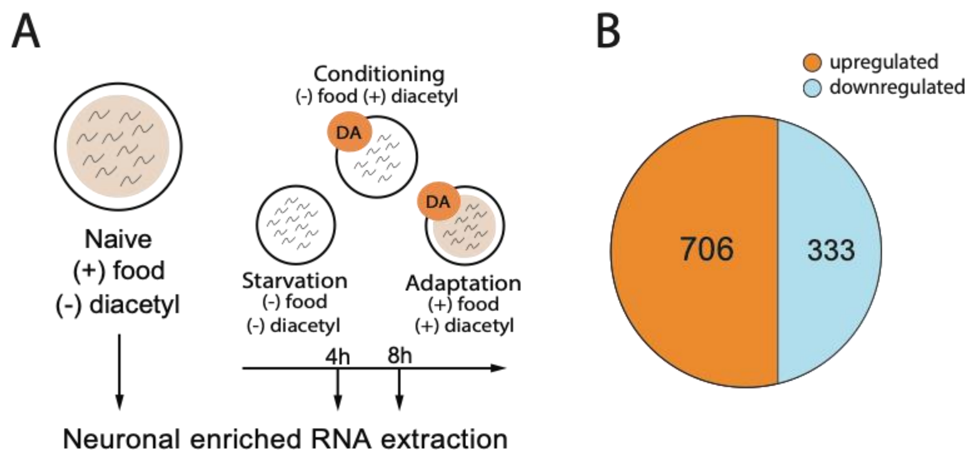

Figure 5. LTAM-specific expression profiling. A For the second analysis, neuronally enriched mRNA was collected before conditioning and two time points (4h and 8h) after starvation, adaptation, or conditioning. The presence or absence of food or diacetyl on the plate before chemotaxis was tested is marked by (+) and (-). **B** Contrasting probe sets of naive, adaptation (4h+8h), and starvation (4h+8h) states to the combined 4h+8h after conditioning state revealed 1039 differentially expressed conditioning-specific probes, of which 706 and 333 was upregulated and downregulated, respectively, during long-term memory. (Freytag et al., 2017)

By compiling results from both experiments, we defined a core set of 582 upregulated and 185 downregulated transcripts, corresponding to 538 and 174 genes, respectively. We compared the gene set of 538 with a previous paper describing positive olfactory LTAM genes (Lakhina et al., 2015), and found that exactly 50% of the upregulated genes overlap between the two datasets (Figure 6). These genes possibly serve a stimulus-independent role in long term memory. Integrating findings from that study (Lakhina et al., 2015), we concluded that more than half of the stimulus-independent general LTAM genes were classified as CREB-dependent.

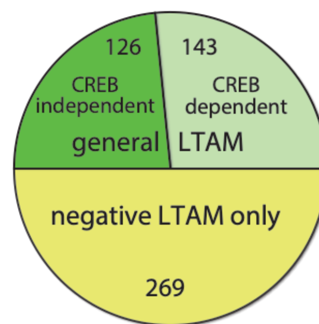

Figure 6. Distribution of differentially expressed (upregulated) genes. Amongst the 538 genes identified as overexpressed after aversive olfactory conditioning, 50% was also overexpressed in a positive training paradigm described (Lakhina et al., 2015). Of these genes, 126 and 143 were CREB-independently and -dependently regulated, respectively. (Freytag et al., 2017)

4.2 The effect of potential CREB-inhibitors on long-term memory

It has been shown in *Drosophila* and mice that pharmacological or transgenic modulation of CREB activity can have a positive effect on cognitive function and memory (Kudo et al., 2005; Scott et al., 2002). However, yet there is no approved drug that would improve human memory *via* CREB-modulation. Also, pharmacological CREB-modulation in *C. elegans* has not been carried out before. This motivated us to pharmacologically manipulate long-term memory *via* CREB in *C. elegans*. We first confirmed the phenotype of the loss-of-function strain *crh-1(tz2)* in diacetyl-associated aversive long-term memory. We found that memory measured at 16 and 24 hours after conditioning is significantly impaired, but learning is not affected (Figure 7).

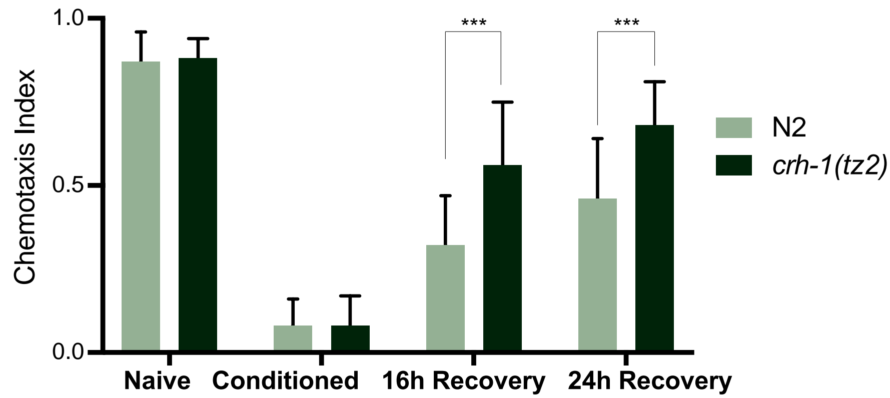

Figure 7. Chemotaxis and memory phenotype of *crh-1* strain. The CREB loss-of-function strain *crh-1(tz2)* shows intact chemotaxis towards diacetyl (naive) and learning (*conditioned*) and has a significantly worse long-term memory measured 16 and 24 hours after conditioning (*16hR and 24hR*). N=4 independent experiments in triplicates. *** p<0.001. 2-way ANOVA, Bonferroni-correction.

Next, we developed a novel experimental procedure which allowed an extended time-window for pharmacological treatment, even during conditioning. We established a protocol which was based on liquid conditioning of worms (Methods). This protocol was first validated for efficacy: we found that the effect of aversive solution-based conditioning with 0.02% diacetyl is non-toxic and is non-inferior in terms of memory-induction to the widely used plate-based conditioning method (Figure 8).

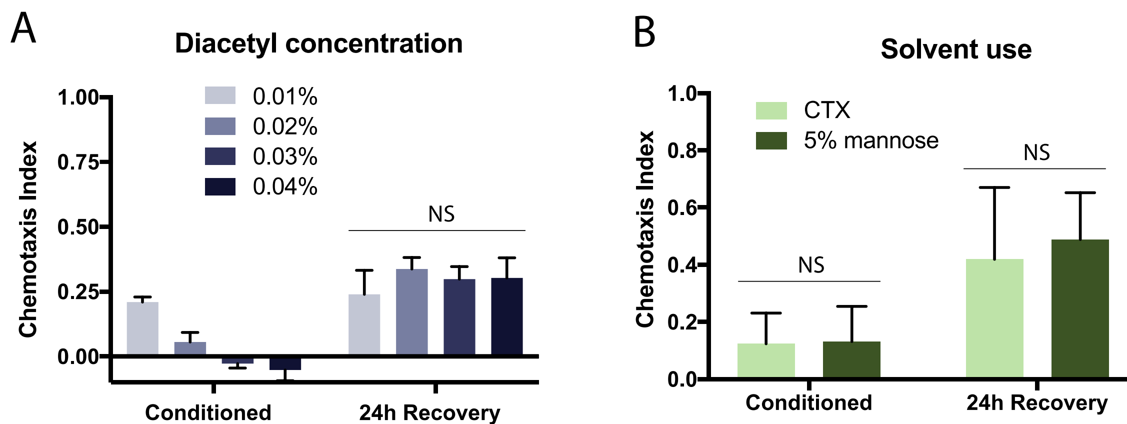

Figure 8. Efficacy of liquid conditioning. Worms were conditioned in 15 ml Falcon tubes in the presence of diacetyl solved in CTX or mannose solution in different concentrations. **A** Different concentrations of diacetyl were tested for efficacy in memory-induction. **B** Dissolving diacetyl in 5% mannose or in CTX to a final concentration of 0.02% did not alter the efficacy of conditioning nor long-term memory measured at 24h. N=4 independent experiments in triplicates. NS: p>0.05, 2-way ANOVA, Bonferroni-correction.

Then, we tested the effect of two compounds (previously shown to possess CREB-inhibitory effect) on aversive olfactory long-term memory in wild type worms (Li et al., 2016; Xie et al., 2015). First, we applied Naphthol AS-E, a CREB-CBP interaction inhibitor acting at the KID-KIX domain, in two concentrations. Application of Naphthol AS-E (for 3h 20 minutes in total) did not affect chemotaxis or learning, but surprisingly, it improved long-term memory measured both 8 and 16 hours after conditioning (Figure 9).

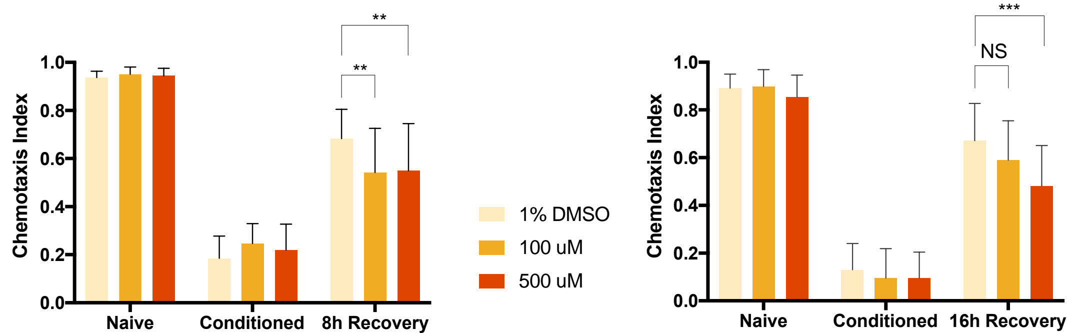

Figure 9. Chemotaxis of trained worms, treated with Naphthol AS-E. N2 worms were liquid conditioned for 2 x 60 min, with 30 min rest in between. Worms were treated 1 hour before conditioning, during conditioning, and 20 min after conditioning (total of 3h 20'). Chemotaxis index was measured before (naive), directly after (conditioned), and 8 and 16 hours after conditioning (8h and 16h recovery, respectively). Naive worms show normal chemotaxis with or without treatment, and neither learning is affected by the treatment. N=4 independent experiments, in triplicates. *** $p < 0.001$, ** $p<0.01$, NS: $p>0.05$. 2-way ANOVA, Bonferroni-correction.

Next, we applied a recently discovered selective inhibitor of CREB-mediated transcription, 666-15i, in different concentrations. We found that none of the applied concentrations affected either chemotaxis or learning or, unexpectedly, long-term memory (Figure 10).

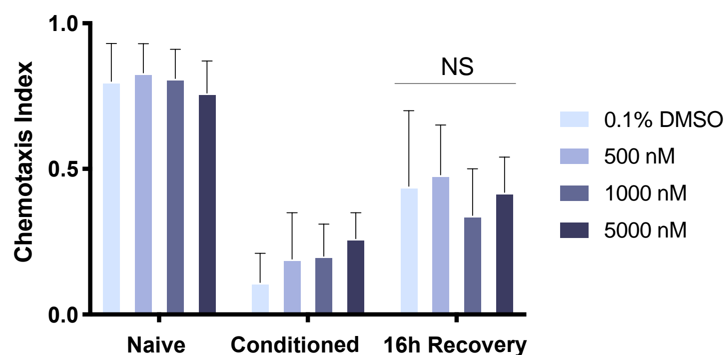

Figure 10. Chemotaxis and memory phenotype after treatment with compound 666-15i. Chemotaxis of 666-15i-treated and control N2 worms towards diacetyl was measured before (Naive), immediately after (Conditioned) and 16 hours after (16h Recovery) conditioning. N=4 independent experiments in triplicates. NS: $p>0.05$. Two-way ANOVA, Bonferroni correction.

In conclusion, our findings suggest an effect of Naphthol AS-E on long-term memory in *C. elegans*.

4.3 MSI-1 regulates long-term memory/forgetting *via* translational regulation of ARX-2 at the GLR-1 synaptic densities of the AVA interneuron

The role of cytoskeletal remodeling in synaptic plasticity and actin-reconfiguration during long-term memory has been shown previously (Okamoto et al., 2004). Musashi, a neuronally expressed RNA-binding protein was found to interact with members of the actin-branching complex Arp2/3 (de Sousa Abreu et al., 2009), marking it as a potential regulator of long-term memory.

In our work Musashi (MSI-1, encoded by *msi-1* in *C. elegans*) was investigated for its role in aversive olfactory learning and memory. We found that the *msi-1(os1)* partial deletion mutant strain has intact learning but significantly improved memory (Figure 11). The wild phenotype can be rescued by unspecific re-expression of *msi-1* (Figure 11A). Tissue-specific rescue experiments showed that re-expression of *msi-1* cDNA in the AVA interneuron group in the loss-of-function *msi-1(os1)* strain is sufficient to achieve wild-type memory (Figure 11B) Thus, *msi-1* possibly decreases memory length by expression in the AVA interneuron.

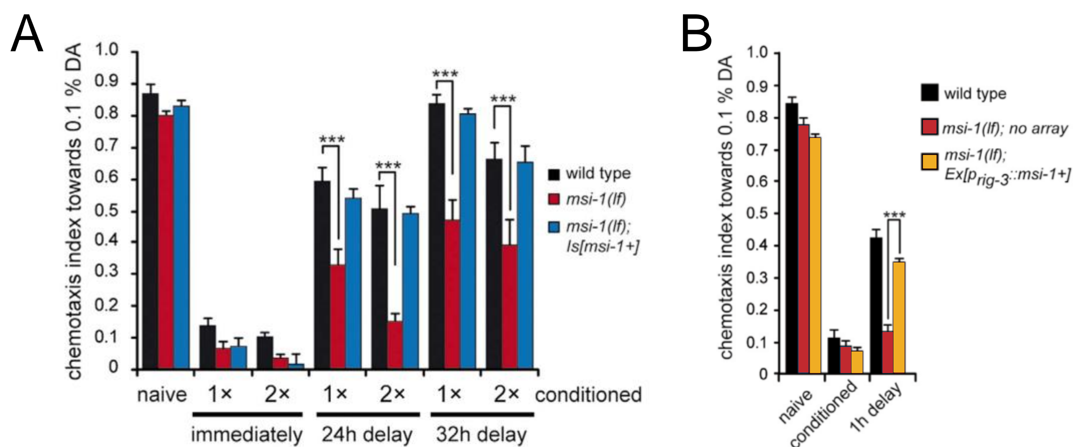

Figure 11. Learning and memory phenotype of MSI-1 loss-of-function and rescue strains. A Aversive long-term-memory was tested following single (1x) and repeated (2x) conditioning at 0h (*immediately*), 24h (*24h delay*), and 32h (*32h delay*) after training. Wild-type (*black*), loss-of-function (*red)* and *msi-1* rescue (*blue*) strains were tested. **B** Aversive memory was tested immediately (*conditioned*) and at 1h (*1h delay*) after conditioning in wild-type (*black*), loss-of-function (*red*) and AVA interneuron-specific rescue strain (*yellow*). All experiments were done in triplicates and repeated at least three times (N>3). Bars represent mean ± SEM. $**p < 0.01$, $***p < 0.001$. (Hadziselimovic et at., 2014)

Next, we investigated whether MSI-1 modulates the expression of actin branching proteins as a possible mechanism of the memory-effect. In a co-immunoprecipitation assay we found that MSI-1 interacted with three subunits (*arx-1, arx-2, arx-3*) of the Arp2/3 actin branching complex (Figure 12A). In a subsequent fusion-protein fluorescent intensity experiment, we showed that the expression of the same three subunits is repressed upon memory induction (Figure 12B). This suggests that MSI-1 targets and downregulates the expression of three actin-branching proteins.

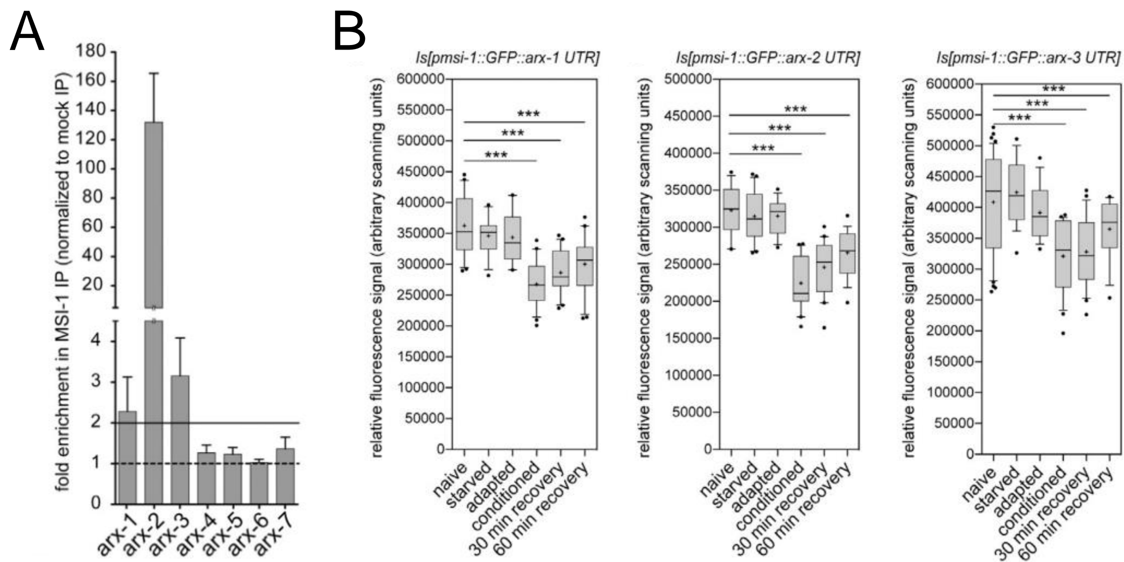

Figure 12. MSI-1 interaction with ARX proteins. A Co-immunoprecipitation assay shows a minimum 2x enrichment for ARX-1, ARX-2 and ARX-3. **B** The fluorescent intensities of GFP-tagged ARX-1, ARX-2, and ARX-3 proteins are decreased after memory induction. *** $p < 0.001$. (Hadziselimovic et at., 2014)

We approached the subcellular localization of one of the target subunits, *arx-2,* using confocal microscopy. Since both the non-NMDA type glutamate receptor GLR-1 (*glr-1*) and the filamental actin (F-actin, marked by utrCH) are closely associated with memory (Lamprecht, 2014; Morrison & van der Kooy, 2001; Rose et al., 2003), we investigated the co-localization of these proteins with ARX-2. We generated stable transgenic lines fluorescently labeling the above proteins with either green (GFP) or red (RFP) fluorescent proteins.

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We found that along the axon of the AVA interneuron, all three proteins co-localize (Figure 13). This suggests that MSI-1 is involved in the regulation of forgetting by interfering with the structural remodeling of glutamatergic synapses.

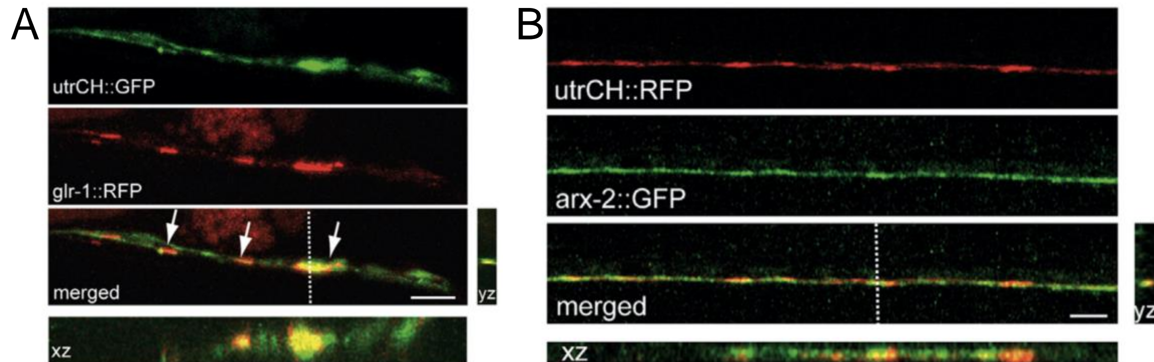

Figure 13. Confocal imaging of fluorescently labeled actin, ARX-2 and GLR-1 receptor. A Distribution of F-actin along the ventral nerve cord was detected with GFP-fused actin-marker utrophin CH-domain (green, upper panel), together with RFP-fused GLR-1 (red, middle panel). **B** Distribution of F-actin (red, upper panel) and ARX-2 (green, middle panel) along the ventral nerve cord. Overlapped images show that GLR-1 dense synapses (marked by arrows) co-express F-actin and ARX-2 (yellow, merged panels). The position of yz-projection is marked with dotted line. Scale bar represents 1 mm. (Hadziselimovic et at., 2014)

4.4 Polarity prediction in the *C. elegans* ionotropic chemical synapse network

A major flaw of any available structural connectome is the lack of polarity information of otherwise (e.g. type- and direction-wise) well-described synapses. While electrical synapses are generally considered bidirectional, chemical synapses are unidirectional where the presynaptic neuron either excites or inhibits the postsynaptic neuron. This momentary effect is a result of the interplay of a number of factors e.g. the type neurotransmitter(s) and receptor(s) expressed by the pre- and postsynaptic neurons, respectively, the biophysical properties of the neurons (membrane potential, intracellular/extracellular ion gradients), or cellular signal transduction protein assembly. A specific type of transmission is *via* ionotropic chemical synapses when the presynaptic release of neurotransmitters activates postsynaptic ligand-binding receptor ion channels which then allow in- and outflux of ions.

Many models assume that out of the three main neurotransmitters, glutamate and acetylcholine are excitatory, while GABA is an inhibitory neurotransmitter, thus the expression of one or the other neurotransmitter defines the modality of all the outgoing connections of a neuron – as proposed by the Dale's principle (Dale, 1935). However, in *C. elegans* certain non-conventional receptor ion channels exist (e.g. ACh-mediated anion or GABA-mediated cation channels) which necessitates a prediction approach that considers neurotransmitter and receptor expression as well. This approach would allow synapse-level instead of neuron-level polarity prediction.

To address this issue and predict polarities of ionotropic chemical synapses, we first created a custom neuronal gene expression map by manually curating datasets available on Wormbase and in other publications (Methods). We sorted the previously identified 62 ionotropic postsynaptic receptor genes into six functional classes based on their suggested neurotransmitter ligand (glutamate, acetylcholine or GABA) and putative ion channel type (cationic or anionic, i.e. excitatory or inhibitory), as shown in Table 3. The distribution of neurons according to their neurotransmitter and receptor gene expression patterns is showed in Figure 14.

Table 3. Ionotropic receptor genes. The C. elegans genome contains 62 ionotropic postsynaptic receptor genes for glutamate, acetylcholine, and GABA. In this table genes are grouped according to their neurotransmitter ligand and whether forming cationic (+) or anionic (-) ion channels. In C. elegans "unconventional signaling", namely, glutamate-mediated inhibition, cholinergic inhibition and GABA-ergic excitation, is facilitated by 6, 6, and 2 receptor genes, respectively. In the gene expression database used in this work, expression in at least one neuron was found in the case of 42 genes (marked bold), while for 20 genes no neuronal expression was found. (Fenyves et al., 2020)

	Glutamate	**Acetylcholine**		**GABA**
cation channel receptor gene	***glr-1*** ***glr-2*** ***glr-3*** ***glr-4*** ***glr-5*** ***glr-6*** ***glr-7*** ***glr-8*** ***nmr-1*** ***nmr-2***	*acr-1* ***acr-2*** ***acr-3*** *acr-4* ***acr-5*** ***acr-6*** *acr-7* *acr-8* *acr-9* *acr-10* ***acr-11*** ***acr-12*** *acr-13* ***acr-14*** ***acr-15***	***acr-16*** *acr-17* ***acr-18*** *acr-19* ***acr-20*** *acr-21* ***acr-23*** *acr-25* ***deg-3*** ***des-2*** *eat-2* ***lev-8*** ***unc-29*** *unc-38* ***unc-63***	***exp-1*** ***lgc-35***
anion channel receptor gene	*glc-1* *glc-2* ***glc-3*** *glc-4* ***avr-14*** ***avr-15***	***acc-1*** ***acc-2*** *acc-3* ***lgc-47*** *lgc-48* *lgc-49*		***gab-1*** ***ggr-1*** ***ggr-2*** ***ggr-3*** ***lgc-36*** ***lgc-37*** ***lgc-38*** *unc-49*

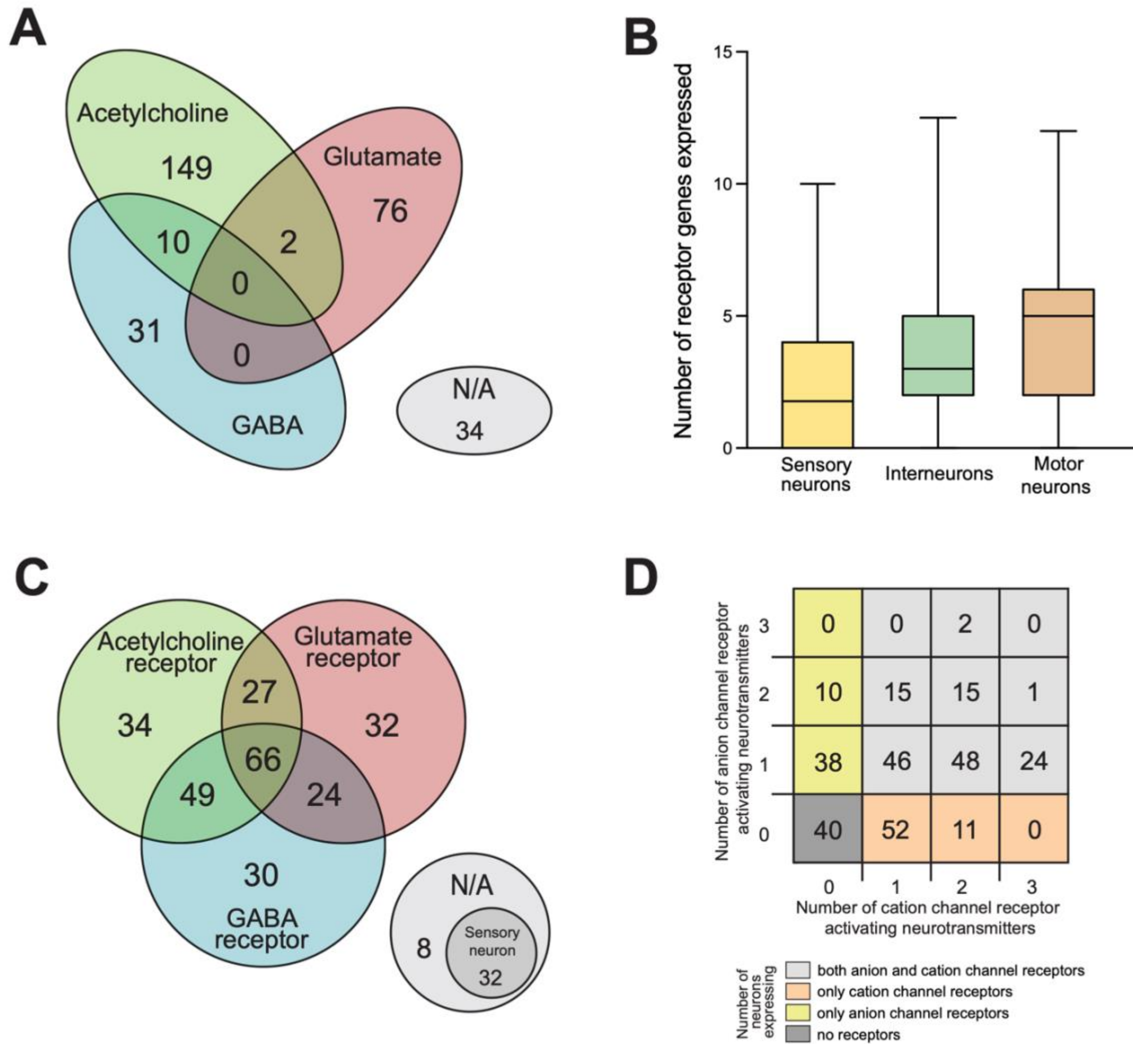

Figure 14. Neurotransmitter and receptor expression patterns of *C. elegans* neurons. Expression data of the three major synaptic neurotransmitters and their receptors of *C. elegans* were collected from multiple datasets and were manually curated (see Methods). **A** Distribution of neurons according to their neurotransmitter expression: glutamate (red), acetylcholine (green), GABA (blue) or none (grey). **B** Number of receptor genes expressed by neurons, grouped by neuron modality. **C** Distribution of neurons based on their neurotransmitter receptor gene expression (colors are the same as in panel **A**). **D** Distribution of neurons according to the number of neurotransmitters for which anion and/or cation channel receptor genes are expressed. (Fenyves et al., 2020)

Next, we constructed a tool that predicts polarities based on the neurotransmitter expression of the presynaptic neuron and ionotropic receptor gene expression of the postsynaptic neuron (Methods). We labeled synapses as *excitatory* or *inhibitory* when the neurotransmitter-matched postsynaptic receptor genes were only cation or anion channel

related, respectively; *complex* if the receptor genes were both cation and anion channel related.

We successfully predicted polarities for 73% of the 20,589 chemical synapses in 3638 connections. Only 27% of synapses couldn't be predicted, due to insufficient or non-matching data (Figure 15B). We predicted that 9,034 of the synapses are excitatory and 2,580 are inhibitory, while 3,431 synapses have complex function.

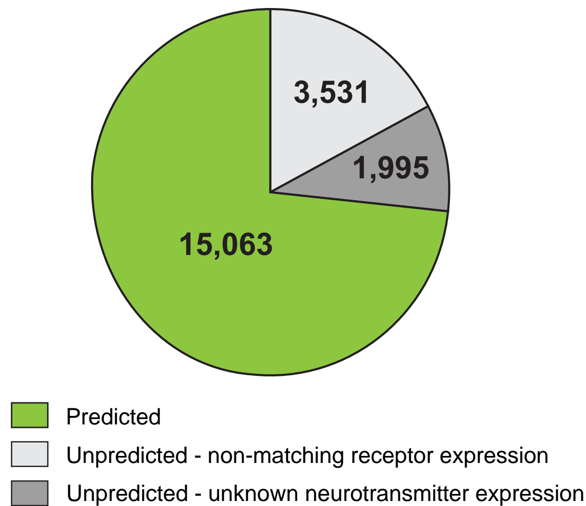

Figure 15. Prediction of synaptic polarities of the *C. elegans* connectome. Distribution of predicted and unpredicted synapses. We were able to predict the polarity of 73% of chemical synapses (green). The polarities of the rest of synapses were unpredicted due to unknown neurotransmitter expression of the presynaptic neurons (dark grey) or non-matching receptor gene expression of the postsynaptic neurons (light grey). (Fenyves et al., 2020)

We found that with this prediction method the ratio of excitatory and inhibitory synapses is close to 4:1 (Figure 17; *NT+R method*). In contrast to other prediction methods when either only the neurotransmitter expression (*NT only* method) or only the receptor gene expression (*R only* method) was taken into account, only the combined neurotransmitter and receptor expression-based prediction method yielded an excitatory:inhibitory (E:I) ratio that is close to what has been found stable in other networks (Discussion). The major source of difference between the results of different methods is that 30% of cholinergic and 5% of glutamatergic synapses were predicted inhibitory with the *NT+R method*, a significant fraction otherwise predicted excitatory with the *NT only* method (Figure 17B).

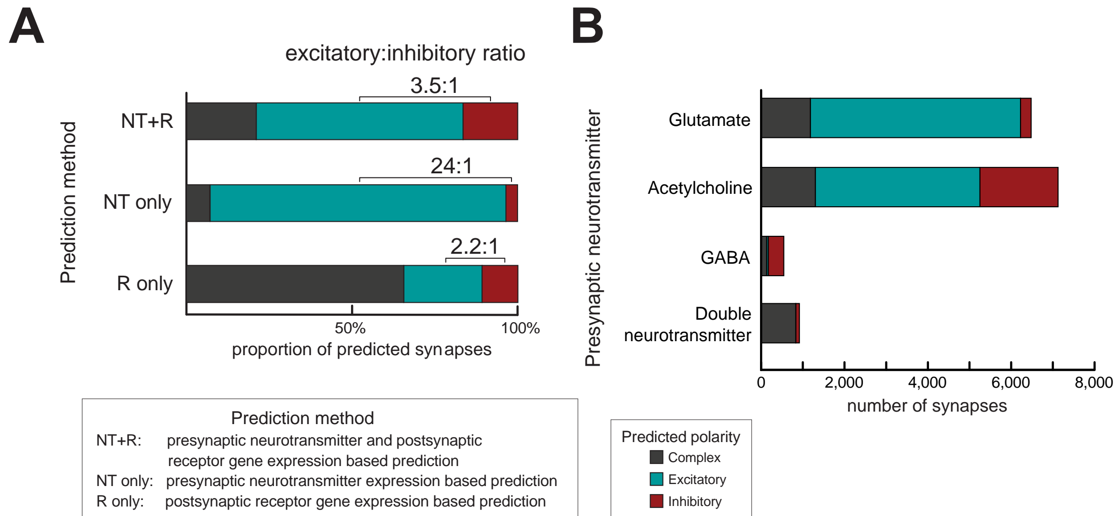

Figure 17. Predicted synaptic polarities. A Distributions of predicted polarities, using the method developed in this paper (*NT+R*) and two alternative methods as comparison (*NT-only* and *R-only*). Polarities were predicted by considering the neurotransmitter expression of the presynaptic neuron and/or the receptor gene expression of the postsynaptic neuron (see Methods). **B** Distributions of predicted synaptic polarities (using the *NT+R* method) grouped by the presynaptic neurotransmitter. Unpredicted synapses are not shown. (Fenyves et al., 2020)

Notably, in subsets of connections which connect neurons of different modalities (i.e. sensory neurons, motor neurons, interneurons and polymodal neurons) the E:I ratios varied between 1:10 (motor » sensory) and 14:1 (inter » motor), as shown in Figure 16. Importantly, we observed a dominant excitatory excess in the feedforward direction (sensory » inter » motor) and inhibitory excess in the feedback direction (motor » inter » sensory), which had been discussed in the literature previously (Dalenoort & de Vries, 2004; Martikainen et al., 2005).

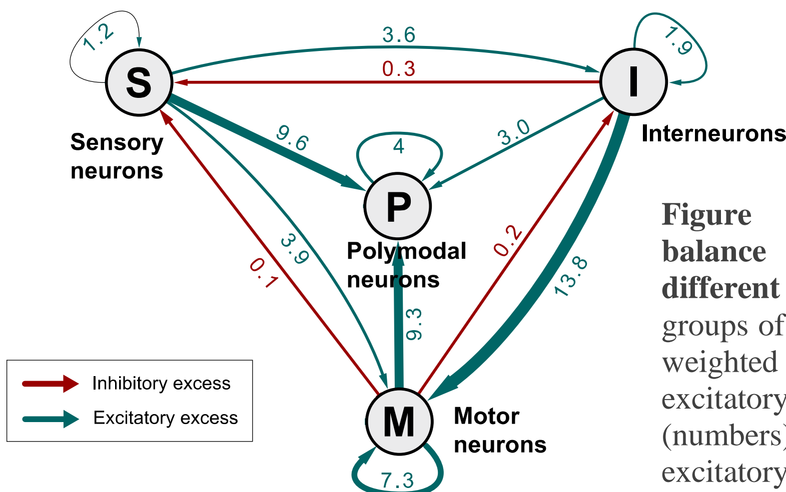

Figure 16. Excitatory:inhibitory balance between neuron groups of different modalities. Nodes represent groups of neurons by modality. Edges are weighted according to the excitatory:inhibitory (E:I) ratios (numbers). Green and red colors represent excitatory (E:I > 1) and inhibitory (E:I < 1) excess in sign-balances, respectively. (Fenyves et al., 2020)

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4.4.1 Polarity-balance of AVA interneuron group connections

As has been shown previously, the AVA interneuron group has a central role in many forms behavior, e.g. in locomotion or memory. Thus, adding polarity information to its connectivity can further characterize its nature of involvement in these behaviors. AVA interneurons express two types of neurotransmitters (both glutamate and GABA) and 4 classes of ion-channel receptor genes (6 glutamate-driven cation, 4 and 1 acetylcholine-driven cation and anion, respectively, and 2 GABA-driven anion). This allows both incoming and outgoing connections of AVA to be potentially excitatory or inhibitory, depending on the synaptic partners.

Using the polarity prediction tool described above we labeled that the extensive majority of connections going out from AVA as complex (potentially both excitatory and inhibitory function), and the incoming connections as almost exclusively half-half excitatory or complex (Figure 18).

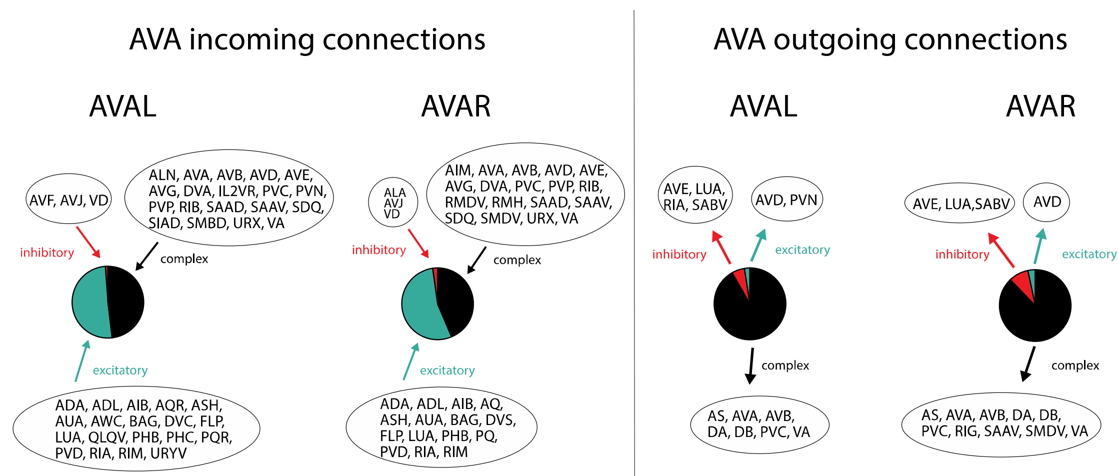

Figure 18. Distribution of polarities of the AVA interneurons' synapses. Neuron groups connecting to the AVA interneuron are grouped according to being presynaptic or postsynaptic to AVAL and AVAR neurons (incoming and outgoing connections, respectively) and grouped by their polarity (excitatory (blue), inhibitory (red), complex (black). Pie slices are proportional to synapse numbers. Unpredicted synapses are not accounted for.

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4.4.2 Polarity-balance in the AVA-centered locomotor subnetwork

The complex setup of polarities was also highlighted in the visualization of the locomotor subnetwork in which AVA has a central role driving backward motion (Figure 19). According to our model the only neurons AVA inhibited were the AVE, while they excited only the AVD neurons of the subnetwork. All other outgoing connections were predicted complex. Some motor neurons can inhibit the AVAs, but most others act on it in a complex manner.

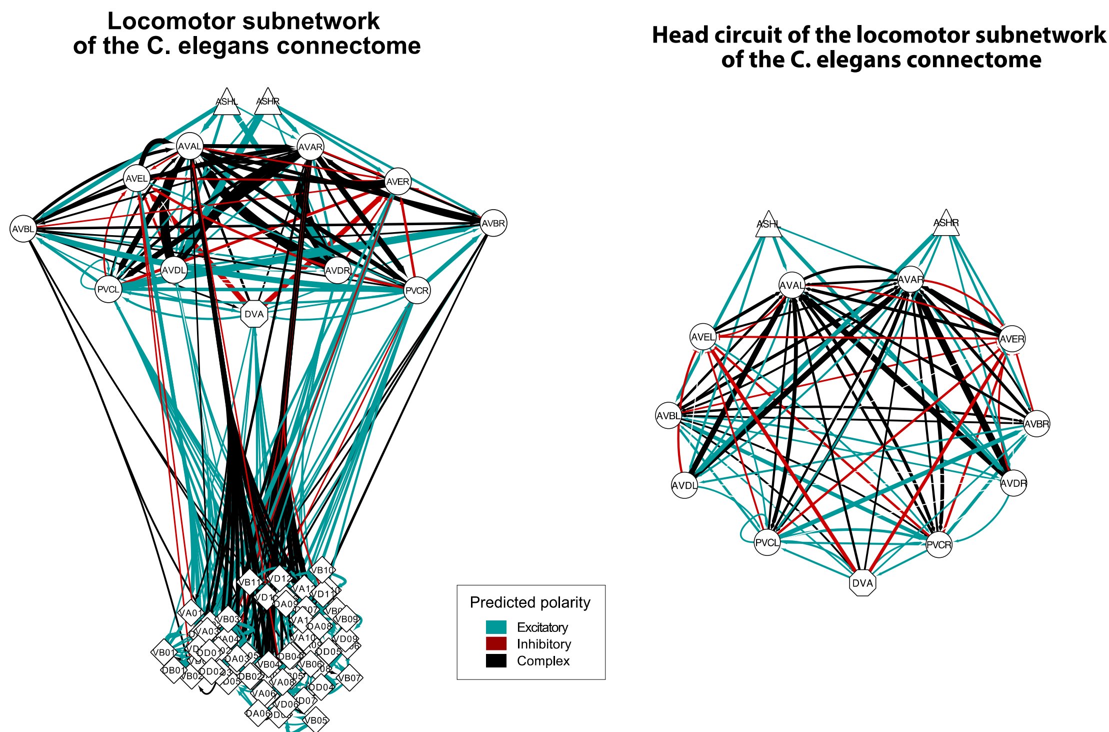

Figure 19. Network representation of the locomotion subnetwork. Edges represent excitatory (blue), inhibitory (red), or complex (black) chemical connections. Edges are weighted according to synapse number. Shape of vertices (△,○,◇) represents the modality (sensory, inter, motor, respectively) of neurons. (Fenyves et al., 2020)

Network representation using the EntOpt layout plugin nicely captured some anatomical locations of the worm (Figure 20A) and revealed that the majority of inhibitory connections in the connectome are localized to the ventral ganglion (Figure 20B). Prediction of polarities based on neurotransmitters only (*NT only method*) resulted in a large excitatory excess (Figure 20C) compared to our method, apparent in the head neurons and premotor interneurons.

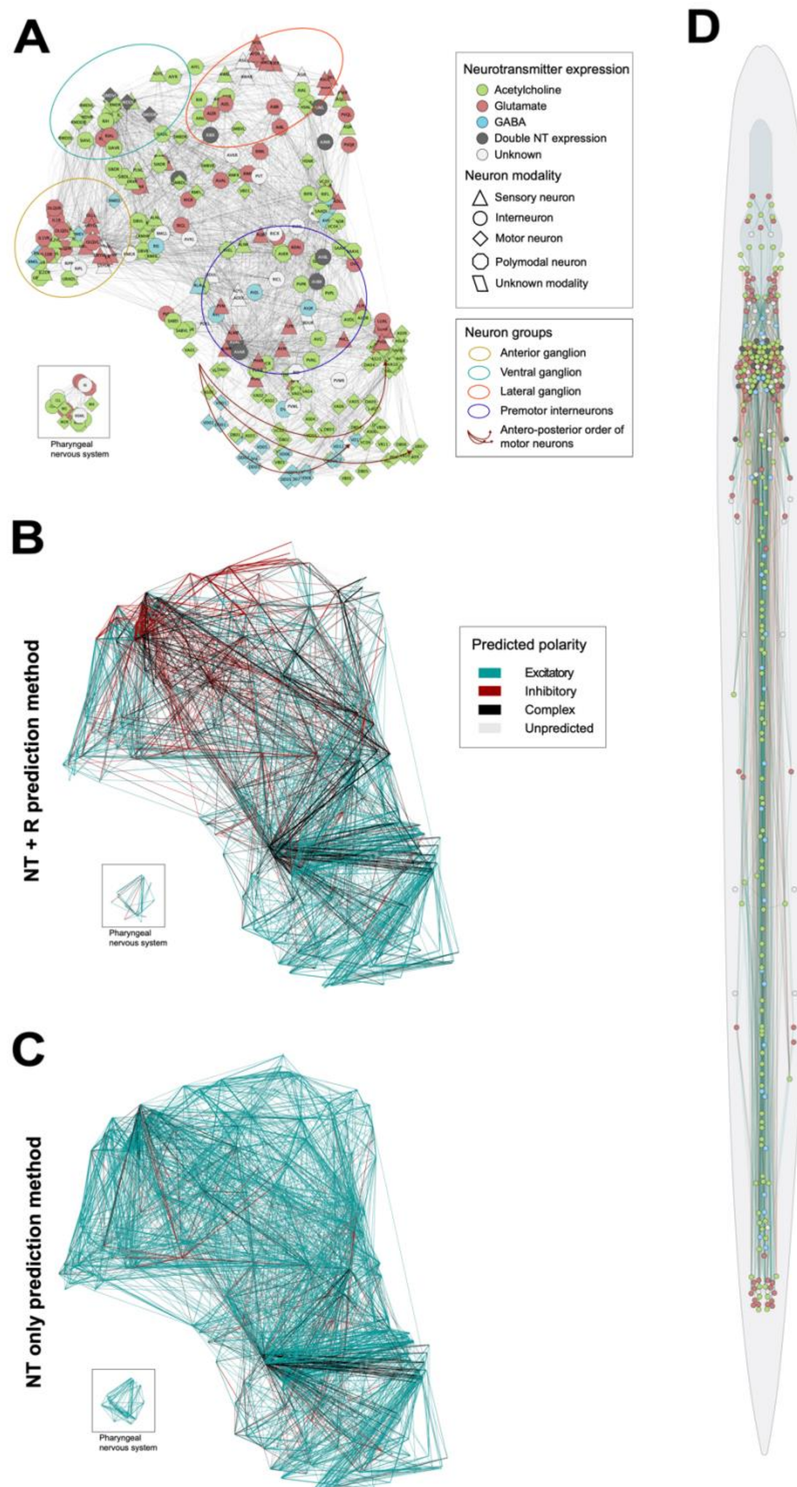

Figure 20. Network representations of the *C. elegans* chemical synapse network. A-C Network representations using the EntOpt layout plugin in Cytoscape (Ágg et al., 2019). **A** Color and shape of vertices represent neurotransmitter expression and modality of neurons, respectively (see inset for definitions)**. B** Edges represent excitatory (blue), inhibitory (red), or complex (black) chemical connections predicted by the NT+R method (see Methods), weighted according to synapse numbers. **C** Colors of edges (see panel B) represent the polarities of chemical synapses predicted by the NT-only method. **D** Layout of vertices represents the anatomic position of neurons. Node and edge colors are as in panels **A** and **B,** respectively**.** (Fenyves et al., 2020)

4.5 Data availability

The sign prediction tool is available at http://EleganSign.linkgroup.hu. Scripts are available in GitHub [https://github.com/bank-fenyves/CeConn-SignPrediction].

5 Discussion

Understanding how the brain works is an extremely challenging historic aim of mankind. We are far from fully comprehending the probably most important of all – the human nervous system – but we can study in detail less complex systems showing evolutionarily conserved features. Such a simple organism is the nematode *C. elegans* which provides an ideal framework for neuroscientific research by its deeply investigated and well-known neuroanatomic and genomic properties, and behavior.

This doctoral thesis summarizes our research in studying the nervous system of *C. elegans* to better understand its functioning in different behaviors, by applying both experimental, network science, and computational methods.

Similar to more advanced organisms, *C. elegans* shows remarkably complex behaviors like permanent adaptation to environment by learning and memory. Molecular mechanisms of long-term memory have been investigated for a long time, and recently the genetic requirements of this process have been explored on a transcriptomic level (Lakhina et al., 2015). Long-term memory is (at least partially) a CREB-regulated process in *C. elegans*, similarly to other species, and requires the up- and down-regulation of several genes. Our findings suggest that multiple temporal waves of gene expression take place during long-term memory formation, which is in line with similar studies in other species (Bozler et al., 2017; Lefer et al., 2012).

Since many nematode genes have human orthologs, identifying a memory-associated gene in *C. elegans* has potential implications in humans. For example, the human *ADD1* gene was significantly associated with episodic memory performance while the *C. elegans* ortholog *add*-1 was also found to regulate short- and long-term memory (Vukojevic et al., 2012). We found that the *C. elegans* ortholog of the *musashi* gene (*msi-1)* is necessary for intact memory removal, thus its human ortholog (*MSI1*) is a candidate gene in memory-related disorders (Mastrandreas et al., 2019). During long-term memory, MSI-1 regulates the translation of actin-branching proteins which accumulate at the ionotropic glutamatergic postsynaptic sites of the AVA interneurons, suggesting a complex molecular process in forgetting involving cytoskeletal changes at distinct neuronal locations. Our findings on an active regulatory mechanism of forgetting are in line with the proposed role of other mechanisms in memory decay, suggesting that

multiple molecular processes are responsible for active memory loss (Davis & Zhong, 2017; Inoue et al., 2013; Shuai et al., 2010).

Pharmacological modulation of worm behavior can potentially lead to the discovery of novel drugs (Artal-Sanz et al., 2006; O'Reilly et al., 2014). The transcription factor CREB is a fundamental and evolutionarily conserved regulator of long-term memory thus serves as an ideal candidate for drug testing. In our work we applied two compounds as possible modulators of CREB activity. While one compound was found ineffective even in high concentrations, application of Naphthol AS-E unexpectedly resulted in prolonged long-term memory: our *in vivo* results are in contrast with previous *in vitro* studies which have classified Naphthol AS-E as a CREB-activity inhibitor thus postulating a memory-shortening effect (Xiao et al., 2010; Xie et al., 2015). The possible mechanism of a memory-prolonging effect is currently unclear. Regarding our results, there are several limitations of the pharmacological treatment method to be discussed: a) the chemical properties of a compound determine how resistant the cuticle is against it, which is unknown; b) ingestion *via* the pharynx possibly leads to enzymatic digestion of the compound which is hard to track; c) the correlation of the applied and the final effective concentrations of the drug might not be linear. For the above reasons, our results of pharmacological testing require further experimental validation with other techniques.

Direct evidence to determine the polarity of a synapse can only be given by delicate experimental methods. Technical difficulties yet prevent us from obtaining brain-scale *in vivo* polarity information, so alternatively *in silico* methods are often used. We approached the question from a gene expression perspective, which allowed us to predict polarities for ~70% of the ionotropic chemical synapses. Overall, the observed 4:1 excitatory:inhibitory ratio is completely in line with previous *in vivo* and *in vitro* results of neuronal and other real-world networks (Gulyás et al., 1999; Leskovec et al., 2010; Liu, 2004; Markram et al., 2015). This ratio can only be predicted if not only the presynaptic neurotransmitter expression but also the postsynaptic receptor gene expression is taken into consideration.

A surprisingly high proportion of synapses were predicted as "complex" (i.e. both excitatory and inhibitory) that can be explained in at least two (non-exclusive) ways. Firstly, neurotransmitter receptors are not homogenously spread across the plasma membrane, but their subcellular distribution is regulated. This allows distinct

compartments of a neuron to act independently, even to the same neurotransmitter, as have been shown in *C. elegans* as well (Arey et al., 2019; Chalasani et al., 2010; Choi et al., 2015; Kuramochi & Doi, 2019; Nusser, 2012; Tao et al., 2019; Zou et al., 2018). Secondly, dynamic changes in gene expression allow neurons to change their neurotransmitter or receptor gene expression patterns (Hammond-Weinberger et al., 2020; Spitzer, 2017). Once temporally specific and subcellular-level expression data become available, synaptic polarities can be further specified.

The AVA premotor interneuron group plays a central role in controlling behavior, e.g. in learning, memory, and simple mechanosensory reflexes (Chalfie et al., 1985; Stetak et al., 2009). According to our polarity predictions, this neuron group has a very low number of stable inhibitory connections (both incoming and outgoing) but has a high number of complex connections, in light of the observed brain-wide polarity distributions. Thus, our findings further support the special role of the AVA interneuron by its potential to (simultaneously and/or sequentially) integrate and distribute excitatory and inhibitory connections.

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6 Conclusion

In the work summarized in this doctoral thesis, we contributed to a better understanding of the nervous system of *C. elegans*, in several ways.

1. We explored previously unknown molecular mechanisms behind learning, memory and forgetting. We identified 143 CREB-dependent genes which are overexpressed during associative long-term memory regardless of the training paradigm. We showed that long-term memory-induced gene expression activation happens in three temporal waves. Long-term memory can possibly be modulated by pharmacological treatment targeting CREB-activity, a finding which needs to be confirmed in subsequent experiments.
2. We discovered that the RNA-binding protein Musashi (*C. elegans* MSI-1, encoded by *msi-1)* actively regulates the loss of previously learnt behavior (i.e. regulates forgetting) by modulating actin-branching at the glutamatergic synapses, especially in the AVA interneurons. Since MSI-1 has human orthologs, this mechanism can be a candidate target for subsequent drug discoveries.
3. We built a custom neuronal neurotransmitter and receptor gene expression database and developed an algorithm that predicts the polarities of chemical synapses based on presynaptic neurotransmitter and postsynaptic receptor expression. We predicted polarities for 73% of all ionotropic chemical synapses and showed that the excitatory-inhibitory sign-balance is close to 4:1. This ratio is similar to what have been described in many stable systems. We argue that polarity should be predicted by assessing not only presynaptic, but postsynaptic neuronal properties as well.

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7 Summary

The nematode *C. elegans* is a simple organism that shows a wide range of behaviors and allows detailed analysis of molecular and genetic features underlying complex behavioral phenotypes. Moreover, its well-described nervous system provides an excellent framework for studying various network properties on the local and global scale as well.

In my doctoral research work I pursued to reveal molecular, genomic, and network-level features of nematode behavior and brain structure, applying *in vivo* and *in silico* techniques.

We contributed to the better understanding of the genetic and molecular underpinnings of long-term memory on the genomics and molecular level. We identified a core gene set that is upregulated during long-term memory. This set partially consists of CREB-dependent and stimulus-independent genes. By applying a novel pharmacological assay, we demonstrated a potential LTAM-modulatory effect of pharmacological CREB-manipulation.

We identified a translational regulatory mechanism which is responsible for the active removal of long-term memory. The RNA-binding protein MSI-1 represses the translation of actin-branching proteins which localize at the glutamate receptor-containing postsynapstic sites of the AVA interneuron and contributes to physiological forgetting. This is the first demonstration of an active molecular mechanism underlying the removal of associative memories in *C. elegans.*

We created an algorithm that predicts polarities of ionotropic chemical synapses in the *C. elegans* neuronal network using gene expression data. We predicted a well-balanced distribution of ionotropic chemical synapse polarities in the *C. elegans* connectome, since the 4:1 excitatory:inhibitory ratio observed is similar to what is widely found in other balanced real-world networks. Our findings suggest a feedforward excitatory and feedback inhibitory excess that is in line with the expected behavior of a functionally compartmentalized signal processing system.

8 References

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9 Publications

9.1 Publications directly related to this thesis

Freytag V, Probst S, Hadziselimovic N, Boglari C, Hauser Y, Peter F, Fenyves BG, Milnik, A., Demougin, P., Vukojevic, V., de Quervain, D. J.-F., Papassotiropoulos, A., Stetak, A. Genome-wide temporal expression profiling in *Caenorhabditis elegans* identifies a core gene set related to long-term memory. *J Neurosci*. 2017;37: 6661–6672. doi:10.1523/JNEUROSCI.3298-16.2017

Hadziselimovic N, Vukojevic V, Peter F, Milnik A, Fastenrath M, Fenyves BG, Hieber, P., Demougin, P., Vogler, C., de Quervain, D. J.-F., Papassotiropoulos, A., Stetak, A. Forgetting is regulated via Musashi-mediated translational control of the Arp2/3 complex. *Cell*. 2014;156: 1153–1166. doi:10.1016/j.cell.2014.01.054

Fenyves BG, Szilágyi GS, Vassy Z, Sőti C, Csermely P. Synaptic polarity and sign-balance prediction using gene expression data in the *Caenorhabditis elegans* chemical synapse neuronal connectome network. *PLoS Comput. Biol.* 2020;16: e1007974. doi:10.1371/journal.pcbi.1007974.

9.2 Publications not directly related to this thesis

Fenyves BG, Arnold A, Gharat VG, Haab C, Tishinov K, Peter F, de Quervain D, Papassotiropoulos A, Stetak A. Dual role of an *mps-2/KCNE*-dependent pathway in long-term memory and age-dependent cognitive decline. *Curr. Biol.* 2020;31: 1-13. doi:10.1016/j.cub.2020.10.069

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