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

PhD thesis outline

Bánk Gábor Fenyves, MD

Molecular Medicine Doctoral School

Semmelweis University

Supervisors: Péter Csermely, member of the Hung. Acad. of Sci.
Csaba Sőti, MD, DSc

Official reviewers: Christos Chinopoulos, MD, PhD
Csaba Cserép, MD, PhD

Head of the Complex Examination Committee:
János Réthelyi, MD, PhD

Members of the Complex Examination Committee:
Judit Bak, PhD
Balázs Hangya, MD, PhD

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1 Introduction

Despite the pursuit of understanding the brain since ancient times, today we still face a growing number of fundamental questions. To address the challenges, neuroscience utilizes a wide range of disciplines from anatomy to molecular biology to systems theory. Neuronal functioning can be studied in multiple species, offering a variety of methods with different resolutions and scopes. The most interesting of all, the human brain is yet too complex for our current knowledge to fully comprehend but several primitive species exist serving as models for *in vitro*, *in silico,* and *in vivo* research.

Ultimately, the functioning of a nervous system can be described as synchronized activation and inhibition of neuronal cells and cell-groups, varying in time, resulting in some type of behavior. Activation and inhibition are due to ion channel-dependent ion-flux and subsequent membrane potential changes resulting in intracellular molecular changes. These delicate processes are tightly regulated on multiple levels.

Behind all functionality, the connectivity of neurons and neuronal areas, hence the structural organization of the nervous system is a fundamental determinant. Thus, studying the physical wiring of a nervous system (also called the connectome) is often useful. Methods of graph theory, network theory and other mathematical models are often used to aid the understanding of large, complex systems. The brain is a complex system that can be described as a network of neurons (nodes) and synapses (edges). A complete wiring of a nervous system is currently available only for the nematode *Caenorhabditis elegans* (*C. elegans*) but other species are in the focus of research as well. Importantly, the network theory approach of nervous systems allows the better understanding of brain

diseases and pathologies, serving potentially diagnostic or predictive purposes.

The nematode *C. elegans* proved to be an ideal model organism in neuroscience for multiple reasons: for example, it has a reconstructed neuronal wiring, a fully sequenced genome, a short life-span and a transparent body. About 40% of its genes have human orthologs. The hermaphrodite worms have 302 uniquely identified neurons which are connected by ~3,000 chemical connections and ~600 gap junctions. Interneuronal communication is similar to that observed in mammals.

Despite the simple organization, *C. elegans* is capable of surprisingly complex behavior. It can distinguish multiple forms of environmental stimuli and respond to them adaptively by changing its motor patterns and sustain the newly learnt behavior - manifestations of learning and memory. The molecular processes underlying the different memory phases (i.e. short- vs. long-term memory) are initiated in parallel, utilizing partially independent genetic resources for transcription, translation and post-transcriptional modifications. A comprehensive list and understanding of the genes required for memory activation (learning), maintenance, and removal (forgetting) is yet to be achieved.

On a structural level, cytoskeletal actin-remodeling is an example of memory-related synaptic plasticity. Pathways which regulate cytoskeletal changes are potentially linked to memory. Members of the RNA-binding protein family Musashi (*msi-1* in *C. elegans*, *msi1/msi2* in human) are such proteins due to their suspected role in the regulation of actin-branching.

Electron microscopy can provide detailed structural information about the type and direction of a synapse, but it's unable to tell the neurotransmitter used or the polarity of the connection (i.e. whether being excitatory or inhibitory) which is a crucial

functional information. This gap can possibly be filled by analyzing neuron specific gene expression data which are increasingly available for *C. elegans* and have, for instance, resulted in a comprehensive knowledge about neuronal neurotransmitter usage. However, no brain-scale synaptic polarity data is currently available.

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* I investigated, experimentally, the genetic and molecular underpinnings of aversive olfactory long-term associative memory, on genome-wide and single gene-level as well. I specifically studied the neuron-specific role of Musashi (MSI-1, *msi-1*) in long-term memory and forgetting, and related subcellular synaptic dynamics. Additionally, I aimed to modulate long-term memory with pharmacophores using a novel treatment methodology.

As all behavior, learning and remembering is a result of complex neurobiological activity, carried out by a physically constraining synaptic infrastructure. Therefore, I 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. I aimed to predict the synaptic polarities of the ionotropic chemical synapse connectome by utilizing gene expression data in a novel conceptual framework.

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 this section. Similarly to the thesis, methods already published with my authorship are briefly described or excluded from this thesis, following the guidance of the Doctoral School.

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)*. 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% dimethyl-sulfoxide 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.

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 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, 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.

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. To identify memory-related genes we performed genome-scale microarray experiments on neuronally enriched RNA at multiple time points before and after memory induction. We defined a core set of 538 upregulated and 174 downregulated long-term memory-associated genes. Fifty percent of the upregulated genes were found generally activated in LTAM, many of those being CREB-dependent (Figure 1).

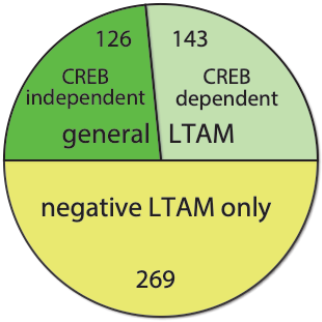

Figure 1. Distribution of differentially expressed (upregulated) genes. Amongst the 538 genes identified as upregulated after aversive olfactory conditioning, 50% was also overexpressed in a positive training paradigm previously published. Of these genes, 126 and 143 were CREB-independently and -dependently regulated, respectively.

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

While CREB activity is associated with cognitive function and memory, there is yet no approved drug that would improve memory *via* CREB-modulation. We tested the effect of two pharmacological compounds with CREB-modulatory effect on the memory phenotype of *C. elegans,* by using a novel test protocol. We observed a prolonged aversive olfactory memory phenotype after the application of Naphthol AS-E, while observed no effect of the compound 666-15i.

4.3 MSI-1 regulates long-term memory/forgetting via translational regulation of cytoskeletal plasticity

The role of cytoskeletal remodeling and actin-reconfiguration in long-term memory-associated synaptic plasticity has been shown previously. Musashi, a neuronally expressed RNA-binding protein was found to interact with members of the actin-branching complex Arp2/3, marking it as a potential regulator of long-term memory.

In our work Musashi (*C. elegans msi-1*) 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. The wild phenotype can be rescued by aspecific and even AVA interneuron-specific re-expression of *msi-1*. Thus, *msi-1* possibly decreases memory length by expression in the AVA interneuron. We showed that MSI-1 interacted with and downregulated the expression of three subunits (*arx-1, arx-2, arx-3*) of the Arp2/3 protein complex which regulates cytoskeletal actin branching. We also showed the subcellular localization of ARX-2 and demonstrated

its colocalization with filamental actin and GLR-1 (a non-NMDA-type glutamate receptor) in the AVA interneuron (Figure 2).

This suggests that MSI-1 is involved in the regulation of forgetting by interfering with the structural remodeling of glutamatergic synapses.

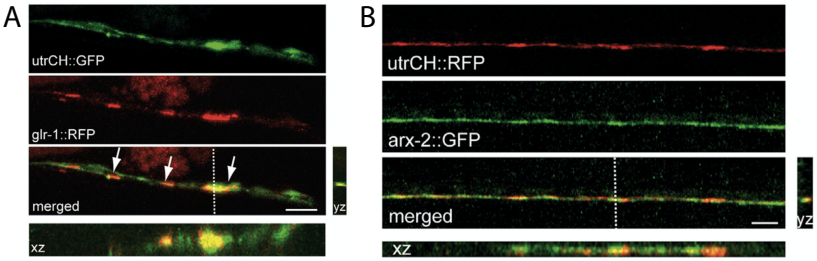

Figure 2. Confocal imaging of fluorescently labeled actin, ARX-2 and GLR-1 receptor in the AVA interneuron. A Distribution of F-actin along the ventral nerve cord was detected with GFP-fused utrophin CH-domain (green, upper panel), together with RFP-fused GLR-1 (red, middle panel). **B** Distribution 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.

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 (i.e. whether being excitatory or inhibitory) of otherwise well-described synapses. In case of ionotropic chemical neurotransmission presynaptic release of neurotransmitters activate postsynaptic ligand-binding receptor

ion channels which then allow in- and outflux of ions, resulting in neuron excitation or inhibition. We proposed that not only the neurotransmitter type but also the postsynaptic receptor gene expression is important to approach the excitation/inhibition phenomenon.

To 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. We sorted the ionotropic 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). 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. 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. We predicted that 9,034 of the synapses are excitatory and 2,580 are inhibitory, while 3,431 synapses have complex function (Figure 3).

We found that with this prediction method the ratio of excitatory and inhibitory synapses is close to 4:1. In contrast to other prediction methods when either only the neurotransmitter expression or only the receptor gene expression 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.

Notably, in subsets of connections which connect neurons of different modalities of sensory neurons, motor neurons, interneurons and polymodal neurons, the E:I ratios varied between 1:10 (motor » sensory) and 14:1 (inter » motor). Importantly, we observed a dominant excitation excess in the feedforward direction (sensory » inter » motor) and inhibition excess in the feedback direction (motor » inter » sensory).

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

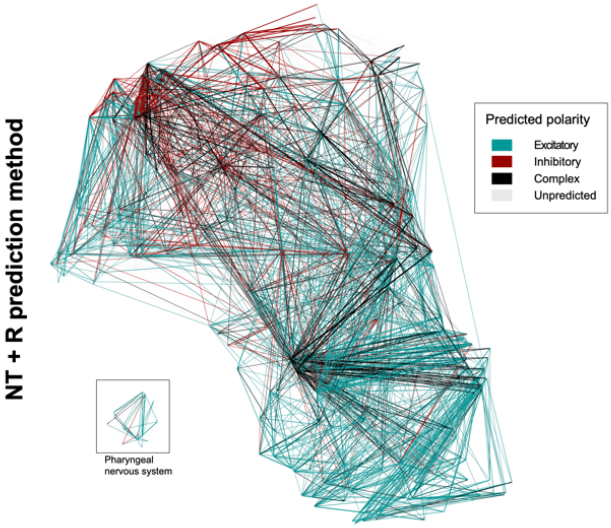

Figure 3. Network representation of the *C. elegans* chemical synapse connectome. Network representation using the EntOpt layout plugin in Cytoscape. Edges represent excitatory (blue), inhibitory (red), or complex (black) chemical connections predicted by combining presynaptic neurotransmitter and postsynaptic receptor gene expression data.

5 Conclusion

In the work summarized in this doctoral thesis, we contributed to a better understanding of the nervous system of *C. elegans* by using experimental, network science, and computational methods, 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. 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 (*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 interneuron. Since Musashi 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 that. We predicted polarities for 73% of all ionotropic chemical synapses and showed that the excitatory-inhibitory sign-balance is 4:1, similar to what had been described in many stable systems. We argue that polarity should be predicted by assessing not only presynaptic but postsynaptic neuronal properties as well. Our findings suggest a forward excitatory and backward inhibitory excess that is in line with the expected behavior of a functionally compartmentalized signal processing system.

6 Publications

6.1 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

6.2 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