



Gene regulatory mechanisms underlying the emergence of novel neuronal functions

Doctoral Thesis by Andrea Millán Trejo

Thesis supervisor: Dr. Nuria Flames Bonilla

Doctoral Programme in Neurosciences

January 2025



CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS



UNIVERSITAT
DE VALÈNCIA



INSTITUTO DE
BIOMEDICINA DE
VALENCIA CSIC

Nuria Flames Bonilla, Doctora en Neurociencias, Investigador Científico y Directora de la Unidad de Neurobiología del Desarrollo del Instituto de Biomedicina de Valencia del CSIC

CERTIFICA:

Que la presente memoria titulada 'Gene regulatory mechanisms underlying the emergence of novel neuronal functions ', corresponde al trabajo realizado bajo su dirección por Doña Andrea Millán Trejo, para su presentación como Tesis Doctoral en el Programa de Doctorado en Neurociencias de la Universitat de València.

Firman el presente certificado en Valencia, a 10 de enero de 2025:

Firmado por FLAMES BONILLA
NURIA - DNI ***3311** fecha
15/01/2025 mediante un
certificado expedido por AC
Sector Público

ENRIQUE|
LANUZA|
NAVARRO

Firmado digitalmente
por ENRIQUE|LANUZA|
NAVARRO
Fecha: 2025.01.16
09:09:00 +01'00'

Fdo. Directora de tesis

Dra. Nuria Flames Bonilla

Fdo. Tutor académico

Dr. Enrique Lanuza Navarro

This Thesis has been made possible thanks to a pre-doctoral fellowship from the Subprograma Estatal de Formación del Programa Estatal de Promoción del Talento y su Empleabilidad en I+D+i, en el marco del Plan Estatal de Investigación Científica y Técnica y de Innovación 2017-2020

The investigation has been funded by the following investigation projects: 'Programas de regulación transcripcional asociados a enfermedades genéticas' Grant SAF2017-84790-R.

Agradecimientos

En primer lugar, me gustaría dar las gracias a Nuria Flames por darme la oportunidad de trabajar en su laboratorio, mostrarme las bondades de trabajar con *C. elegans*, y enseñarme que hay que ponerle ilusión a nuestro trabajo incluso cuando todo se pone cuesta arriba. También me gustaría darle las gracias a Arantza Barrios por dejarme disfrutar de un huequito de su laboratorio, acogerme como una más del grupo y darme la oportunidad de conocer Londres.

Por supuesto quiero darle las gracias a todas las personas que han pasado por el Flames' lab y que de una forma u otra han aportado su granito de arena para construir no solo esta tesis, si no la persona (más vieja, pero mas madura) en la que me he convertido. Empezando con lo que para mí es el antiguo lab, con Miren que fue mi primera mentora a la que le debo prácticamente casi todo lo que sé A Rebeca, Isa, Isa Jr., Noemí y Erick, con quienes he compartido alegrías y penas. Gracias, chicos, por haberme acompañado en tantos momentos y por permitirme desahogarme con vosotros. Luego llegó mi queridísima Pilar, sin la que hoy en día estaría completamente desquiciada, es mi terapia quincenal, que seguimos manteniendo religiosamente (y que nos dure mucho). También quiero agradecerle a Andrés su paciencia conmigo como su profe, y que siempre tenga una palabra amable para mí (ya me contratarás cuando seas PI). Después llegaron el yin y el yang de Anna y Adrián: Anna, mi terremoto preferido, que prácticamente lo sabe todo de mí y que siempre dice las cosas como son; y Adri, que aunque sigue perteneciendo al lab, cada vez se le ve menos por aquí... aunque siempre está para un sarao y para una charla sobre la precariedad del predoc. Tampoco me quiero olvidar de Carla y Estela, que hicieron de mi estancia en Londres una aventura mucho más cómoda y amable, gracias chicas por abrirme vuestra casa.

A los que quedamos en el nuevo laboratorio, gracias. A Toni, por la serenidad y paciencia que transmites; a Rafa, por ser el showman que necesitamos, capaz de diseñar el CRISPR más fantástico del siglo y, al mismo tiempo, montar una performance. Y, por último, a mis chicas del lab, Yaiza y Laura, con quienes comparto el día a día más pesado, pero con un toque de humor que lo hace todo mucho más fácil. Gracias a tod@s por hacer que ir al laboratorio fuera una alegría y hacerme sentir en casa.

Además, quiero dar las gracias a quienes me han aguantado y apoyado fuera del ámbito laboral. A mis amigos: Mari, Buri, María, Lulu, Javi y Alfonso, por ser mi familia en Valencia y hacer de los primeros de años de tesis los más divertidos a pesar del Covid. Y a mi familia de verdad, a vosotras Mamá y Elena, por escucharme cada día y por hacer que os sienta siempre cerquita, aunque estéis lejos. Os quiero mucho. Y a ti Papá, gracias por haber creído siempre en mí. Seguro que estarías orgulloso.

Por último, a ti Mario, por haber estado a mi lado todos estos años acompañándome en cada paso, y escuchándome relatar hasta la infinity, por lo vivido y por lo que nos queda, gracias.

A todos, gracias por haber sido parte de este viaje. Esta tesis no solo es un trabajo, sino un reflejo de todas las personas que han estado a mi lado.

Contents

1.	INTRODUCTION	13
1.1.	Complexity of nervous system development and evolution.	14
1.2.	Generation of neuronal diversity in development	18
1.2.1.	Cell potency	18
1.2.2.	Neuronal cell fate specification and differentiation	19
1.3.	Gene regulatory networks controlling gene expression programs	21
1.3.1.	<i>cis</i> -regulatory modules	22
1.3.2.	<i>trans</i> -regulatory elements	26
1.3.3.	Transcription factor binding through the genome.	28
1.3.4.	Combinatorial action of transcription factors	33
1.3.5.	Chromatin accessibility and epigenetics.	35
1.4.	Plasticity and evolution of cell-types	40
1.4.1.	Basic concepts in the evolution of novelty	40
1.4.2.	Evolution of cell type diversity and the emergence of new cell types.	42
1.5.	<i>C. elegans</i> as a model organism	48
1.5.1.	<i>C. elegans</i> nervous system	50
1.5.2.	Neurogenesis in <i>C. elegans</i>	53
1.5.3.	Epigenetics and repressive chromatin in <i>C. elegans</i>	57
1.5.4.	The serotonergic system of <i>C. elegans</i>	61
1.5.5.	<i>C. elegans</i> egg-laying behaviour	66
1.5.6.	Evolutionary studies in other <i>Caenorhabditis</i> nematodes	70
1.6.	Previous unpublished results related to this PhD project	75
1.6.1.	Serotonergic diversity in the <i>Caenorhabditis</i> genus	75

1.6.2.	VC4 and VC5 neurons in <i>C. angaria</i> are serotonergic reuptakers.....	77
1.6.3.	<i>C. elegans cat-1</i> expression in VC4 and VC5 is dependent on the pro-neural factor HLH-3/Ascl1 and the homeobox UNC-4/UNCX.....	77
1.6.4.	The serotonergic fate of VC4 and VC5 is actively repressed in <i>C. elegans</i> by cell-autonomously mono and di-methylation of H3K9.	79
1.6.5.	VC4 and VC5 serotonergic phenotype in H3K9me deficient <i>C. elegans</i> depends on serotonin reuptake	81
1.6.6.	VC4 and VC5 serotonergic phenotype in H3K9me deficient <i>C. elegans</i> depends on <i>hlh-3</i> and <i>unc-4</i>	82
2.	OBJECTIVES.....	84
3.	MATERIAL AND METHODS	86
3.1.	<i>Caenorhabditis</i> strains and culture.....	87
3.1.1.	Strains and standard growth	87
3.1.2.	Semi-liquid media.....	87
3.1.3.	Population synchronization without bleaching	88
3.1.4.	Population synchronization with bleaching	88
3.2.	Immunohistochemistry	89
3.2.1.	Serotonin immunohistochemistry tube fixation.....	89
3.2.2.	VMAT/ChAT immunostaining freeze-cracking	90
3.2.3.	Immunostaining after semi-liquid culture.....	91
3.3.	Cloning and reporter strain generation.....	92
3.3.1.	Cloning into pPD95.75.....	92
3.3.2.	Site directed mutagenesis.....	93
3.3.3.	Generation of reporter transgenic lines in <i>C. elegans</i> ..	94
3.3.4.	Strain crossing.....	95
3.3.5.	Genotyping.....	96

3.3.6.	<i>C. angaria</i> transgenesis	98
3.4.	Heat-shock induction	99
3.5.	Microscope scoring	100
3.5.1.	Fluorescence reporter scoring.....	100
3.5.2.	Immunostaining scorings.....	101
3.5.3.	Fluorescence intensity analysis.....	101
3.6.	Egg-laying behavioural assays	102
3.6.2.	Egg-laying overnight	102
3.6.3.	Egg-laying upon 5-HT stimuli.....	102
3.6.4.	Egg-laying video recordings and analysis.....	102
3.7.	Curling tail in males	103
3.8.	Alignment	104
3.9.	<i>C. elegans</i> ChIP-seq profiles.....	104
3.10.	Statistics.....	105
3.11.	Materials.....	106
4.	RESULTS	109
4.1.	Gene regulatory logic of <i>C. angaria</i> and <i>C. elegans</i> VC4 and VC5 neuron serotonergic phenotype.	111
4.1.1.	Study of <i>mod-5</i> regulatory regions suggests changes in <i>cis</i> -regulatory regions are responsible for the <i>C. angaria</i> VC4 and VC5 neurons serotonergic phenotype.	111
4.1.2.	UNC-4/UNCX homeodomain directly activates the expression of <i>C. angaria mod-5/SERT</i> enhancer in <i>C. elegans</i> VC4 and VC5 neurons.....	121
4.1.3.	<i>C. angaria mod-5/SERT</i> enhancer is sufficient to induce VC4 and VC5 serotonergic phenotype in <i>C. elegans</i>	124
4.1.4.	Low levels of homeodomain transcription factor <i>unc-4/UNCX</i> restricts VC4 and VC5 serotonergic phenotype in <i>C. elegans</i>	127

4.1.5. <i>C. elegans mod-5</i> locus shows high levels of H3K9me marks and HPL-2/HP1 binding	131
4.1.6. H3K9me signaling and UNC-4/UNCX overexpression seem to act in parallel	132
4.2. Behavioural consequences of the VC4 and VC5 neuron serotonergic phenotype in <i>C. angaria</i>	137
4.2.1. Comparative study of the number of eggs laid in <i>C. elegans</i> and <i>C. angaria</i>	137
4.2.2. Egg-laying dynamics study in <i>C. elegans</i> and <i>C. angaria</i>	140
4.2.3. Egg laying induction in high serotonin concentration media.	145
4.2.4. VC4 and VC5 from <i>C. elegans mod-5(vlc47 syb7715)</i> strain can reuptake serotonin from the environment.....	149
4.3. Characterization of the plasticity in VC4 and VC5 serotonergic phenotype in <i>C. elegans</i>	153
4.3.1. Study of VC4 and VC5 serotonergic phenotype in <i>C. elegans</i> wild isolates	153
4.3.2. Egg-laying behaviour comparison between N2 and CB4856 Hawaiian wild isolate.	154
4.3.3. Mating as trigger for the VC4 and VC5 serotonergic phenotype in <i>C. elegans</i>	157
4.3.4. Egg-laying behaviour in cross progeny	159
4.3.5. Growing in a high 5-HT concentration environment induces the serotonergic phenotype in <i>C. elegans</i> VC4 and VC5 neurons.....	162
4.3.6. <i>C. elegans</i> VC4 and VC5 are able to reuptake 5-HT from the environment.....	165
4.3.7. VC4 and VC5 plastic response to external 5-HT is not present in <i>C. briggsae</i> and <i>C. tropicalis</i>	165

4.3.8.	VC4 and VC5 serotonergic phenotype when growing in high 5-HT environments is dependent on UNC-4 and MOD-5 expression.....	167
4.3.9.	VC4 and VC5 can reuptake external 5-HT in a <i>mod-5</i> independent manner.....	171
4.3.10.	<i>C. elegans</i> VC4 and VC5 plastic serotonergic phenotype could protect worms from egg-laying due to exogenous 5-HT.....	173
4.4.	Analysis of serotonergic phenotype of CPs and CAs male specific neurons in <i>C. elegans</i> and <i>C. angaria</i>.....	177
4.4.2.	<i>C. angaria</i> serotonergic CA neurons re-uptake 5-HT in a <i>mod-5</i> dependent manner.....	178
4.4.3.	Study of <i>mod-5</i> regulatory regions activity in <i>C. angaria</i> and <i>C. elegans</i> transgenic animals.	178
4.4.4.	Role of CAs serotonergic phenotype in spiral mating in <i>C. angaria</i>	181
5.	DISCUSSION.....	185
5.1.	Gene regulatory changes underlying the emergence of novel neuronal functions.....	186
5.1.1.	<i>Angaria</i> group of species VC4 and VC5 serotonergic phenotype was achieved through co-option of <i>mod-5</i> intron1 enhancer in the VC4 and VC5 GRN.....	186
5.1.2.	UNC-4 VC4 and VC5 neuron sub-type selector TF directly activates <i>mod-5</i> intron 1 enhancer.	187
5.2.	<i>C. elegans</i> displays a variable VC4 and VC5 serotonergic phenotype.	189
5.2.1.	Epigenetic repression and restricted UNC-4 levels prevent VC4 and VC5 serotonergic phenotype in <i>C. elegans</i> N2.	189
5.2.2.	<i>C. elegans</i> VC4 and VC5 variability in wild isolates serotonergic phenotype.....	191

5.2.3. <i>C. elegans</i> serotonergic VC4 and VC5 in N2 strain is plastic to environmental grow conditions	192
5.2.4. VC4 and VC5 serotonergic plasticity to high 5-HT environments is not a general feature of non- <i>Angaria Caenorhabditis</i> species.....	195
5.3. Behavioural relevance of serotonergic phenotype of VC4 and VC5	196
5.3.1. Quantification of egg-laying deposition in <i>C. angaria</i> and <i>C. elegans</i> strains under laboratory conditions does not reveal a role for VC4 and VC5 serotonergic phenotype.....	197
5.3.2. Study of egg-laying behaviour dynamics in <i>C. angaria</i> and <i>C. elegans</i> strains under laboratory conditions also failed to reveal a role for VC4 and VC5 serotonergic phenotype.....	198
5.3.3. Study of egg-laying behaviour dynamics in <i>C. elegans</i> wild isolates and self and mating egg laying reveals interesting differences among strains.....	198
5.3.4. Protective role for the serotonergic phenotype in VC4 and VC5 neurons.....	199
5.4. A novel example of emergence of novelty: CA male specific neurons are serotonergic in <i>C. angaria</i>.....	201
5.5. Relevance of the project beyond nematode's biology	203
5.5.1. Developmental plasticity and evolution.....	203
5.5.2. Human SERT transient expression and psychiatric disorders.....	204
6. CONCLUSIONS	207
BIBLIOGRAPHY	211
RESUMEN.....	239
ANNEX.....	267

Index of figures and tables

Introduction

Figure 1. 1. Waddington's Classical Epigenetic Landscape for development .	18
Figure 1. 2. Neuronal specification and differentiation.	20
Figure 1. 3. Representations of gene regulatory networks	22
Figure 1. 4. Schematic representation of key components in the transcriptional regulation of gene expression.....	25
Figure 1. 5. Position Weight Matrix.	26
Figure 1. 6. Transcription is a complex and highly regulated process.	27
Figure 1. 7. Overview of chromatin accessibility and TF binding profiling techniques.....	30
Figure 1. 8. Primary remodellers of chromatin accessibility.....	36
Figure 1. 9. Phenotype norm of reaction	40
Figure 1. 10. Mechanisms of cell type evolution.	46
Figure 1. 11. Anatomical features of adult <i>Caenorhabditis elegans</i>	50
Figure 1. 12. Nervous system of adult <i>Caenorhabditis elegans</i>	52
Figure 1. 13. The terminal selector model for transcriptional regulation of neuron-type effector genes	56
Figure 1. 14. Constitutive heterochromatin in <i>C. elegans</i>	60
Figure 1. 15. Serotonergic system in <i>C. elegans</i>	62
Figure 1. 16. Serotonin biosynthesis	64
Figure 1. 17. Egg-laying behavior in <i>C. elegans</i>	69
Figure 1.18. 5-HT staining in <i>Caenorhabditis</i> genus.....	76
Figure 1.19. Previous results.....	80

Material and Methods

Figure 3.1. Schematic of the pPD95.75 plasmid	94
Table 3. 1. . Primers used for <i>C. virilis mod-5</i> intron cloning	93
Table 3. 2. Primers used for directed site mutagenesis	93
Table 3. 3. Oligos to genotype mutants and reporters	97
Table 3. 4. Materials.....	106

Results

Figure 4. 1. Models for the evolutionary mechanisms generating gene expression diversity between orthologous cell types.....	112
Figure 4. 2. Previous analysis of <i>C. elegans</i> and <i>C. angaria</i> regulatory regions of 5-HT pathway genes through transgenesis in <i>C. elegans</i>	113
Figure 4. 3. Analysis of <i>C. elegans</i> and <i>C. angaria</i> regulatory regions of 5-HT pathway genes through transgenesis in <i>C. angaria</i>	116
Figure 4. 4. VMAT staining in <i>C. angaria</i>	117
Figure 4. 5. Analysis of <i>mod-5</i> regulatory sequences from other <i>Caenorhabditis</i> species through transgenesis in <i>C. elegans</i>	118
Figure 4. 6. <i>C. angaria</i> VC4 and VC5 maintain a cholinergic fate.	120
Figure 4. 7. UNC-4 directly activates <i>C. angaria mod-5</i> regulatory sequence	123
Figure 4. 8. First intron of <i>C. angaria</i> is sufficient to allow 5-HT staining in <i>C. elegans</i>	126
Figure 4. 9. UNC-4 induction is sufficient to drive a 5-HT phenotype in VC4 and VC5. A.....	130
Figure 4. 10. H3K9 methylation and HPL-2 binding profile in <i>C. elegans</i> serotonergic pathway genes and <i>unc-4</i> loci.....	133
Figure 4. 11. Quantification UNC-4::GFP fluorescence reporter intensity in <i>C. elegans</i> VC4 and VC5 neurons.....	135
Figure 4. 12. Number of eggs laid by different species of the <i>Caenorhabditis</i> genus.	138
Figure 4. 13. Egg laying dynamics in <i>C. elegans</i> and <i>C. angaria</i>	142
Figure 4. 14. Egg laying behavior upon acute 5-HT exposure	147
Figure 4. 15. VC4 and VC5 can reuptake serotonin from the outside.....	151
Figure 4. 16. Variability in serotonergic staining in VC4 and VC5 neurons in different <i>C. elegans</i> wild isolates and experimental replicates	155
Figure 4. 17. Egg laying dynamics in <i>C. elegans</i> N2 and <i>C. elegans</i> CB4856 WT	157
Figure 4. 18. Analysis of VC4 and VC5 5-HT staining induction by mating..	159
Figure 4. 19. Egg laying dynamics in <i>C. elegans</i> N2 self-crossed and <i>C. elegans</i> N2 crossed with males	161
Figure 4. 20. <i>C. elegans</i> cultured in 5-HT 10mM show an increase in the percentage of VC4 and VC5 neurons stain with 5-HT	163
Figure 4. 21. <i>C. briggsae</i> and <i>C. tropicalis</i> do not show serotonin staining in their VC4 and VC5 after culture in 5-HT 10mM	166

Figure 4. 22. 5-HT media induction of VC4 and VC5 neuron serotonergic phenotype is dependent on <i>unc-4</i> and <i>mod-5</i> function.....	169
Figure 4. 23. VC4 and VC5 neurons can reuptake exogenous 5-HT in a <i>mod-5</i> independent manner.....	172
Figure 4. 24. Animals grown in high environmental 5-HT conditions are protected from egg-laying induction due to exposure to acute 5-HT.	175
Figure 4. 25. Scheme of the lineage of the CP and CA in males.....	177
Figure 4. 26. Analysis of <i>C. angaria</i> and <i>C. elegans</i> serotonergic identity in CA and CP neurons.....	180
Figure 4. 27. Analysis of male tail curling in <i>C. elegans</i> and <i>C. angaria</i>	183

1. INTRODUCTION

1.1. Complexity of nervous system development and evolution.

The nervous system is the most complex tissue, with an estimated 86.1 ± 8.1 billion neurons in the human brain (Azevedo et al., 2009; Herculano-Houzel, 2009) accompanied by a greater number of synapses. Only in the human neocortex, there are, on average, 6,900 synapses per neuron and an estimated number of 164×10^{12} synapses (Tang et al., 2001). However, these numbers only scratch the surface of the nervous system's complexity, which is further enriched by its extraordinary neuron diversity, comprising over an estimated number of 10,000 different neuronal types (Muotri & Gage, 2006). Moreover, this complexity, together with the diverse structural networks in which the neurons are embedded, serves as the fundamental substrate for a multitude of essential biological functions, from primal instincts such as appetite and respiration to higher-order cognitive processes such as learning and memory. Finally, nervous systems have an astonishing ability to adapt to external stimuli, a process known as neuronal plasticity.

The initial descriptions of nerve cells are credited to Ehrenberg and Purkinje who almost simultaneously began to describe certain large cells in the mammalian cerebellum in the mid-1830s. In the early 1870s, Camillo Golgi developed the silver-chromate technique for staining the nervous system. Then, in the late 1880s Santiago Ramón y Cajal, often regarded as the "father of modern Neuroscience," proposed the "neuron doctrine," which identified neurons as the functional units of the nervous system. Sherrington subsequently provided a functional explanation for Cajal's structural postulates, combining anatomical and physiological concepts into a unified context and introducing the idea of synapse (López-Muñoz et al., 2006). Nonetheless, it was not until the following century that Dale and Loewi

discovered the communication between nerve cells through neurotransmitter release (Valenstein, 2002).

More recently, neuroscience research has been (and still is) advancing rapidly thanks to new technologies such as deep sequencing and single-cell transcriptomics, facilitating a deeper dive into molecular neurobiology and gene expression analysis. This has revealed the vast molecular diversity that defines the variety of neuron types found in different animal nervous systems (Darmanis et al., 2015; Zeng & Sanes, 2017; Johansen et al., 2023; Siletti et al., 2023).

To start uncovering the mechanisms underlying the impressive complexity of the nervous systems we need to begin by understanding their developmental principles: from the generation of neurons to the acquisition of specific functions and their integration into circuits that drive specific behaviours. Despite significant progress in elucidating the basic principles of nervous system development, many questions remain unanswered regarding not only its development but also the origin or origins of nervous systems and the evolutionary mechanisms driving the generation of increasingly complex nervous systems with greater neuronal diversity acquiring novel neuronal functionalities.

This thesis aims to contribute to a better knowledge for some of these fundamental questions by exploring how changes in regulatory networks contribute to the emergence of new neuronal identities. To achieve this aim, we take advantage of the simple model organism *C. elegans* and study its serotonergic system, then considering the highly conserved developmental cell lineage in all *Caenorhabditis* genus species we investigate their differential serotonergic phenotypes, seeking to elucidate the changes that have led the

appearance of new phenotypes. Furthermore, we examine the behavioural variations that may arise from this diversity.

To provide the reader with some background, in the introduction, we will first explore the principles of neuronal specification, outlining how cells commit to a neuronal lineage and differentiate **(Section 1.2)**. Then we will investigate gene regulatory networks (GRNs), highlighting their architecture, the interplay between *cis*- and *trans*-regulatory elements, the combinatorial action of transcription factors, and the roles of chromatin accessibility and epigenetic modifications in gene expression **(Section 1.3)**. Next, we are going to examine the concept of plasticity and the evolution of cell types, emphasizing phenotypic plasticity and evolutionary mechanisms that contribute to cellular diversity and adaptability **(Section 1.4)**. Moreover, we will also discuss the utility of *C. elegans* as a model organism, focusing on its nervous system structure, neurogenesis, terminal selector genes, and the role of epigenetics in defining serotonergic identity and regulating egg-laying behaviour **(Section 1.5)**. Finally, I will summarize unpublished findings from Dr. Carlos Mora, which reveal insights into the serotonergic phenotype of VC4 and VC5 neurons in the *Angaria* species group and its epigenetic regulation in *C. elegans* **(Section 1.6)**, which then will lead us to the objectives of this project.

1.2. Generation of neuronal diversity in development

1.2.1. Cell potency

During embryonic development, cells restrict their potential to generate different cell types, a concept known as potency, as they commit to a specific cell lineage (reviewed in (Singh et al., 2016)). This concept was illustrated by Waddington through his landscape metaphor, in which a ball rounds through a valley with various paths, unable to turn back once a direction is chosen (Waddington, 1957)(**Fig 1.1**).

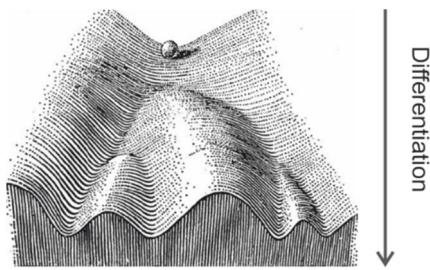


Figure 1. 1. Waddington's Classical Epigenetic Landscape for development.

This metaphor represents the decision-making process of a cell (represented by the ball) during development. Different decision points will lead the cell to its final fate. Adapted from (Waddington, 1957).

Potency begins with totipotency, defined as ‘the ability of one cell to give rise to a fully functional organism’. Under this definition, only the zygote and the blastomeres of the 2-cell stage embryo are considered totipotent cells (Riveiro & Brickman, 2020). Subsequently, before the gastrulation stage, all cells become pluripotent, capable of giving rise to any of the three germ layers generated during gastrulation: ectoderm, mesoderm, and endoderm. Following this, tissue-specific stem cells emerge. These undifferentiated cells possess self-renewal capabilities and are classified as multipotent when they can give rise to multiple cell types within a tissue (e.g., blood) or unipotent when they give rise to only one cell type (e.g., sperm-forming stem cells of the testis) (Slack, 2021).

Stem cells then give rise to tissue-specific progenitors which proliferate in large but finite numbers. These progenitors further differentiate into immature unipotent cells specific to the tissue they belong, which eventually mature into terminally differentiated cells of a given type (Bhartiya, 2015).

1.2.2. Neuronal cell fate specification and differentiation

This transition from the loss of potency to the increase in specificity also applies to neurons: the progenitor cell must decide between a neuronal or not neuronal fate, then it must comprise to a specific neuronal lineage and finally, it must mature, and terminally differentiate to a specific neuronal class or subclass (Hobert, 2005).

This first “decision” of committing to a neural or not neural fate is more a transition marked by external cues which are spatial and temporal regulated gradients of signalling molecules known as morphogens that induce specific gene regulatory networks (GRNs) mediated by the expression of a particular pattern of transcription factors (TFs) that commit progenitors towards neuronal fate, generating neuroblast that will eventually generate specific neuron types. These GRNs activate proneuronal genes that encode mainly basic-helix-loop-helix (bHLH) TFs that initiate Notch-mediated lateral inhibition in neighboring cells (Cabrera, 1990). This process is known as neuronal specification.

Once the cells exit the cell cycle, and they have committed to a neuron lineage, they become postmitotic neuronal precursors that begin to migrate to their final position. However, they are still immature and in order to acquire their highly specialized characteristics, they should go through a maturation step, in which axonal dendritic morphogenesis, synaptogenesis and elimination of redundant synapses allow them to assemble into functional circuits, a process

known as terminal differentiation (**Fig 1.2 A**). Simultaneously, during terminal differentiation, neurons begin to express a combination of differentially expressed effector genes, which are the ones that provide each neuron type the unique properties and functions of a mature neuron (Hobert, 2016).

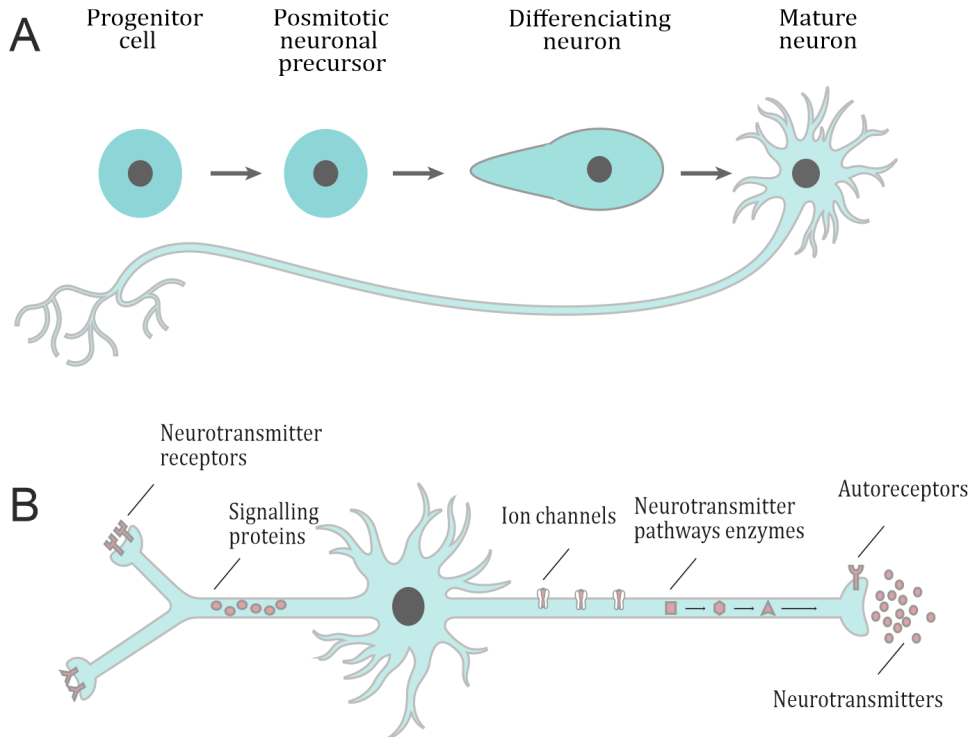


Figure 1. 2. Neuronal specification and differentiation. **A.** Stages of neuronal development. First, progenitor cells acquire a neuronal fate (commitment or specification). Next, they exit the cell cycle to become postmitotic neuronal precursors. Then identity is revealed during neuronal differentiation through coordinate expression of batteries of neuron-specific genes. Neurons remain immature until they start the differentiation process that includes cell migration, extension of projections, synapse formation and expression of their specific effector genes. **B.** Terminal features of a mature neuron. Set of terminal features that allow a neuron to perform its particular function. In addition to these neuron-type specific effector genes other effector genes are shared among many or all neurons in the organisms, such as basic structural synaptic or sensory cilia component.

1.3. Gene regulatory networks controlling gene expression programs

As mentioned before, developmental events are regulated by GRNs that regulate temporal and spatial expression of thousands of target genes, including additional TFs. These GRNs are not only in charge of the commitment of a cell to a certain lineage but also take part in their terminal differentiation. Then commitment and differentiation could be described as a sequence of partially overlapping regulatory states, defined by the activity of specific transcription factors that interact with the genome to regulate transitions between regulatory steps (Davidson, 2006b).

The architecture of an organism GRN is modular and coordinated, meaning that each network node serves a different function contributing to the overall regulatory state (defined as the set of gene expression regulators present in a cell at a given developmental time and under specific environmental conditions), meaning that a network node interprets spatial signals (inputs to the network), and then activate another set of regulatory gene to coordinate the expression of specific genes to respond properly to that conditional regulatory state (output) (Davidson, 2006a; Davidson, et al., 2002) **(Fig 1.3)**.

An important architectural feature of GRNs in neuronal terminal differentiation is that the same battery of TFs typically co-regulates mostly all the effector genes of a given cell type (Davidson, 2006a; Hobert, 2008). Given the numerous cell types found in higher organisms, understanding how GRNs can activate different combinations of effector genes in each cell could shed light on how cell diversity is achieved.

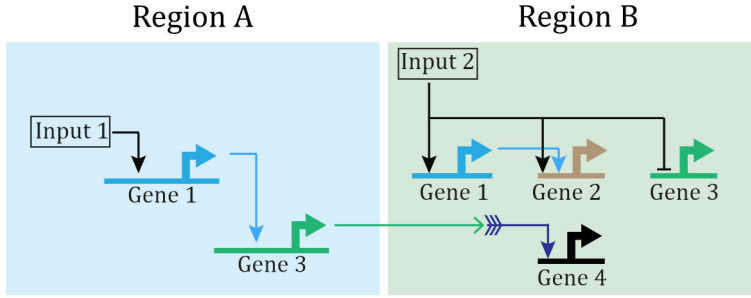


Figure 1. 3. Representations of gene regulatory networks. An example of a simple gene regulatory network submodule occurring in two different regions of the body. Inputs could be any cue coming from other subnetworks, such as morphogens. Thick horizontal bars represent *cis*-regulatory modules, arrows emerging from them represent transcription, while thin arrows are protein products of TFs regulating target gene expression. (Adapted from Davidson, 2006)

GRNs have diverse classes of functional regulatory elements, some of them defined as the battery of active transcription factors, co-regulators, or regulatory non-coding transcripts in each cell which are known as *trans*-regulatory modules. Then these trans elements are recruited to specific *cis*-regulatory modules (CRMs) that control activation or repression of specific target genes, and thus mediate most developmental information regulating gene expression in time and space (Davidson, 2006a; Dsilva & Galande, 2024).

1.3.1. *cis*-regulatory modules

CRMs or *cis*-regulatory elements (CREs) consist of clusters of small sequence motifs of about 6-12 bp called transcription factor binding sites (TFBS) because they are recognized by specific transcription factors through their DNA-binding domains (DBD) to activate or repress transcription of the gene regulated by that CRM (Dsilva & Galande, 2024; Kim & Wysocka, 2023). Thus, the activity of a specific CRMs is not rigid, in each cell at each particular time is determined by the collective of TFs bound to it.

CRMs are classified according to their position relative to the genes they control, transcription start site-proximal promoter or distal element, or their effect, being separated into 1) enhancers that upregulate transcription, which are the most studied CRMs and were originally defined as short sequences that independently of its distance or orientation respect to the target gene, drive transcription of the promoter, 2) silencers that downregulate transcription, they have been less studied 3) insulators to separate regulatory domains, mediated by CCCTC-binding factor (CTCF), that prevent enhancers and silencers from affecting close genes, and specify the spatial organization of chromatin into Topologically Associated Domains (TAD) (Kim & Wysocka, 2023; Panigrahi & O'Malley, 2021) (**Fig 1.4**). However, this classification is dependent on the context because, as their activity depends on specific complement of TF-binding, a CRE that acts as an enhancer could act as a silencer in another cell type, as has been described in *Drosophila* (Gisselbrecht et al., 2020).

Enhancers consist of compact clusters of transcription factor binding sites (TFBS) located in intergenic and intronic regions, and even, but not usually, in coding exons (Birnbaum et al., 2014) and they can be as far from the promoter they regulate as a million bases pair away (Lettice et al., 2003; Sanyal et al., 2012). The role of enhancers in activating gene expression involves the recruitment of transcription factors, which in turn serve as adaptors to diverse cofactors (COFs), with no capacity of direct DNA binding, that could be co-activators or co-repressors, to their binding sites. If they are distal to the promoter, enhancers establish physical connections with the core promoter (50–100 bp DNA segment around the TSS (Transcription Start Site)) of their target genes, generating chromatin loops (Pennacchio et al., 2013; Ptashne, 1986; Roeder, 1996), and they also interact with the transcription machinery (**Fig 1.4**). The rate at which transcription occurs is determined by the combined

activity of the COFs attached to both the enhancer and the core promoter (Kim & Wysocka, 2023; Panigrahi & O'Malley, 2021; Ptashne & Gann, 1997). Nonetheless, the criteria that separate enhancers and promoters are not yet clarified, given that some studies demonstrate that several promoters show enhancer activity (Diao et al., 2017; Engreitz et al., 2016; Rajagopal et al., 2016) while there are some enhancers with the ability to drive transcription initiation by themselves (Andersson et al., 2014; De Santa et al., 2010).

There is a technique called 'promoter-bashing' that enables the identification of the regions that contain the biological information needed to drive transcriptional activity, to do that, selected sub-regions of the genome (either putative promoters or enhancers) are fused to a reporter gene (most commonly fluorescent proteins). These constructs are used to generate transgenic animals and then expression is analysed. The smallest regions that produce expression in specific cell types or tissues are known as the 'minimal CRMs (Andersson & Sandelin, 2020).

However, some questions typically discussed in enhancer biology, are still not completely unanswered (Heinz et al., 2015; Levo & Segal, 2014; Reiter et al., 2017; Rogers & Bulyk, 2018). One of them is what are the specific characteristics that differentiate enhancers from the rest of genomic regions and allow them to be selectively bound by TFs, the second one is what determines promoter-enhancer selectivity taking into account the long distances that sometimes separate these two elements, and it is related to the 3D structure of the chromatin, and the last question regarding enhancers is how do they, with the action of TFs and COFs, actually promote transcription.

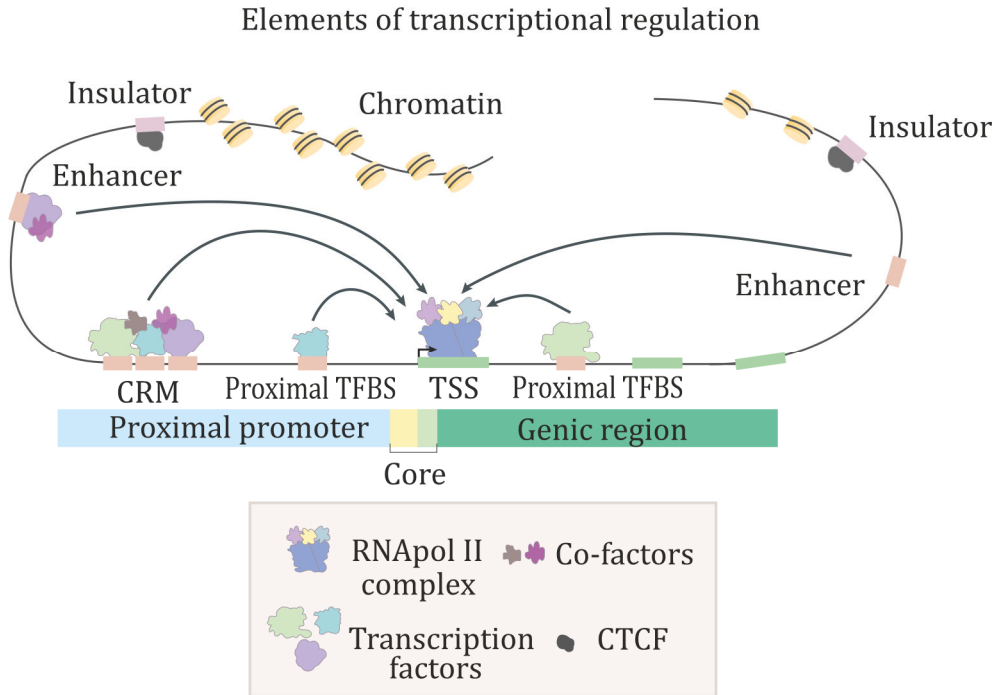


Figure 1. 4. Schematic representation of key components in the transcriptional regulation of gene expression. DNA is wrapped around histones, forming nucleosomes. Proximal and distal *cis*-regulatory elements, such as promoters or enhancers, serve as scaffolds in which *trans*-acting elements like transcription factors or cofactors bind to activate transcription by triggering RNAPol II activity. Transcription factors are bound to specific motifs in regions that typically lack nucleosomes. Enhancers interact with nearby promoters to control the expression of specific genes, moreover, they can activate different promoters, independently of their orientation, within a given transcriptional domain. Three-dimensional structural elements also influence transcription outcomes with insulators marking the chromatin boundaries of the regions accessible for the transcriptional machinery. CTCF is one of the proteins that establishes the limits of these domains, by binding to insulator elements. Modified from (Lenhard et al., 2012). *C. elegans* does not code for CTCF but similar actions to maintain TADs have been proposed for condensing (Das et al., 2024)

1.3.2. *trans*-regulatory elements

As mentioned above, transcription is regulated by the coordinated action of *cis* and *trans*-acting elements. Inside this last group, the most studied elements have been the TFs that have an important role in the initiation of the transcription and elongation of the nascent mRNA. TFs contain evolutionary conserved DNA binding domains that recognize and bind to short, degenerated 6-12 bp DNA sequences called TFBS, a single TF can recognize a diverse variety of similar TFBS. The set of TFBS the TF binds conform a ‘motif’. These short sequence motifs are usually represented as a sequence logo or position weight matrix (PWM) that represents the degeneracy in TF binding specificity (Berg, 1988; Inukai et al., 2017; Stormo & Zhao, 2010). In these graphs, letters represent the nucleotides and their size indicates the probability of occurrence (Gao et al., 2017; Schneider & Stephens, 1990) (**Fig 1.5 A**).

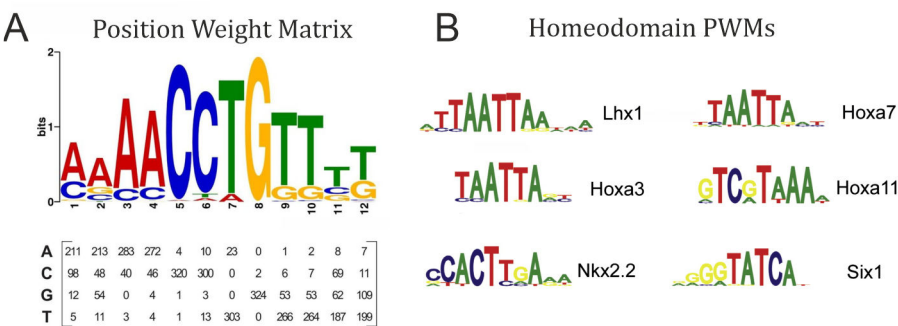


Figure 1. 5. Position Weight Matrix. A. Graphical representation of a PWM, where letters represent nucleotides, and their sizes indicate the probabilities of occurrence. The matrix used to generate the graph displays the frequency of each base at each position. Adapted from (Leiz et al., 2021). **B.** PWM analysis of homeodomains reveals that most homeodomain (HD) transcription factor families are characterized by a TAAT core binding motif (M. F. Berger et al., 2008).

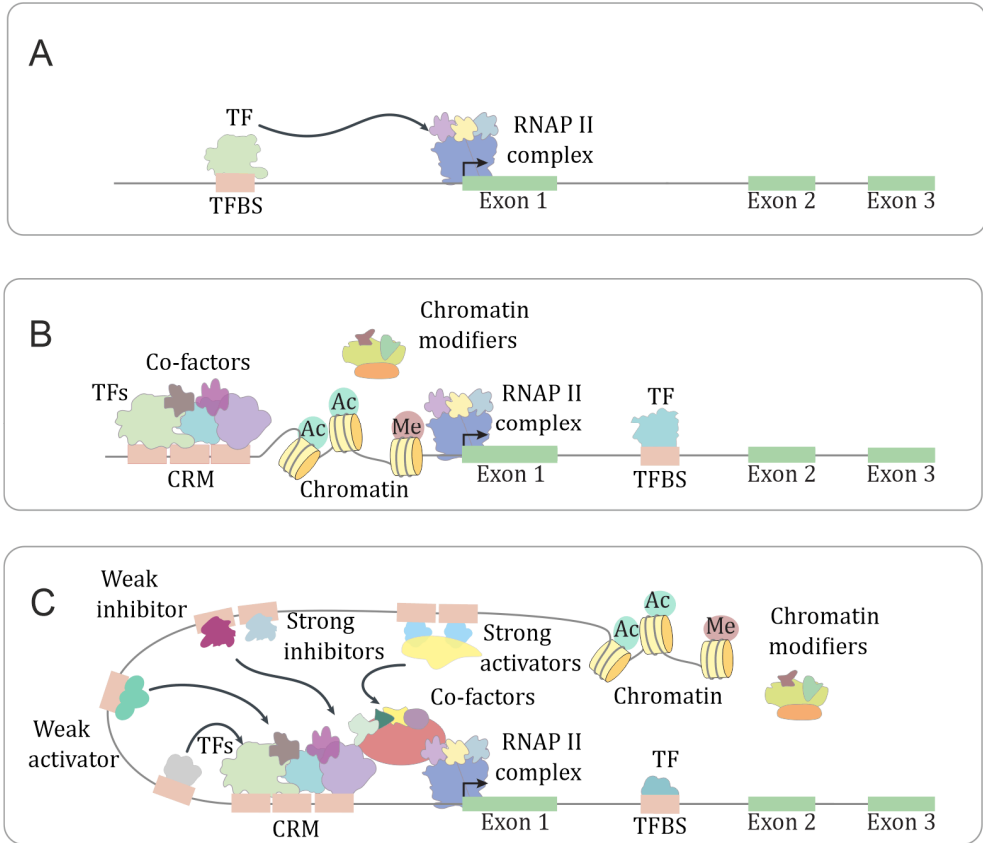


Figure 1. 6. Transcription is a complex and highly regulated process. Increasingly complex models for the regulation of gene transcription are represented. A. Simplification of the transcription in which a single transcription factor (TF) triggers the activation of the RNAP II complex. **B.** Generalized landscape with the main characters involved in the regulation of gene expression. Regulatory regions can be localized upstream and downstream the TSS. Many TFs are needed to enable transcription, binding and activity of co-factors is also necessary. Accessibility provided by the chromatin modifiers and additional TFs is also a key component in gene transcription. **C.** Scheme of the high complexity found in the regulation of transcription, involving *cis*- and *trans*-acting elements that mediate the process in long and close distances. DNA three-dimensional structure also allows the interaction between distal regions. Ac: acetylation, Me: methylation. Adapted from (Nam & Reineke, 2017).

Thus, DBDs have features that give TF the ability to recognize and bind to their specific motifs. DBDs present deep conservation of a diverse set of sequence specificities which have enabled the classification of TFs in different families. (Wingender et al., 2013). In principle, TFs from the same family recognize similar TFBSs, for example, most homeodomain (HD) TF families are defined by a TAAT core binding motif (M. F. Berger et al., 2008)- See **Fig 1.5 B** for examples of HD PWMs. Nonetheless, transcription is a complex process, that must coordinate the action of many different players with their regulatory routines (**Fig 1.6**). In the most simplified scenario, one TF binds to the promoter of a target gene, recruits the transcription machinery, and enables the expression of that gene (**Fig 1.6 A**).

However, although actions of specific individual TFs on enhancers are often studied, this model is not realistic, as TFs act in a coordinated way to modify the chromatin around the target gene promoter before the transcription machinery can bind and begin the RNA synthesis (**Fig 1.6 B**). However, this model is still a simplified version because the expression of a single gene involves the coordination of many proteins including the exit of gene-negative regulators, and the entrance of positive regulators that can bind close to the TSS of the target gene or many kilobases away due to chromatin looping achieved by other proteins such as cohesins (**Fig 1.6 C**) and all of these elements suffer their own regulatory processes (Nam & Reineke, 2017).

1.3.3. Transcription factor binding through the genome.

Given that the binding of TFs to their TFBS is the initial step to allow the expression of a target gene, many efforts have been made to find TFBS along the genome. However, DNA-binding *in vivo* is not predictable through TF-binding

motifs, because considering the small and degenerate nature of TFBS, most TFs bind only a small fraction of their motif matches in the genome (J. Wang et al., 2012). Nonetheless, in the last years, many high-throughput methods have been developed to characterize TFBSs and active enhancers through the genome both *in vitro* and *in vivo* (Levo & Segal, 2014).

Generally, techniques to measure *in vivo* occupancy of sites to deduce the binding preferences of TFs have been done with chromatin immunoprecipitation followed by microarray (ChIP-chip) (Harbison et al., 2004; Venters et al., 2011) or followed by high-throughput sequencing (ChIP-seq) (Arvey et al., 2012). ChIP phase in both technologies uses antibodies that recognize specific TFs, cell samples are prepared to crosslink TFs to their DNA bound regions, DNA is then fragmented, and the specific antibodies are used to pull down the TF bound to DNA. Then TFBS sequences were initially identified by microarray and now by deep sequencing. There are variations of this technology such as ChIP-exo (Rhee & Pugh, 2011) that uses exonuclease trimming following immunoprecipitation to increase the resolution of binding events being mapped, ORGANIC (occupied regions of genomes from affinity-purified naturally isolated chromatin) in which the ChIP step is replaced by a micronuclease digestion (MNase-seq) (Kasinathan et al., 2014), and more recently CUT&RUN (Cleavage Under Target and Release Using Nuclease) (Skene & Henikoff, 2017) and CUT&TAG (Cleavage Under Target and Tagmentation) (Kaya-Okur et al., 2019) which are modified variations of ChIP, that use antibodies fused to MNases or Tagmentases, that enable the decrease of sample size and have reduced the signal to background ratio improving the data quality and reliability.

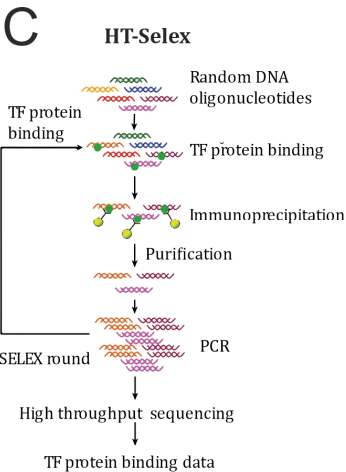
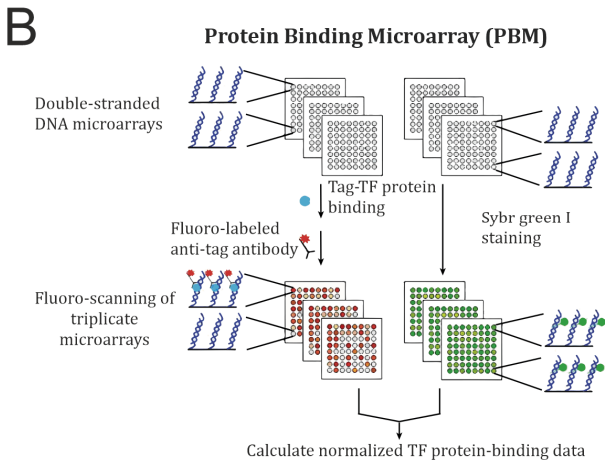
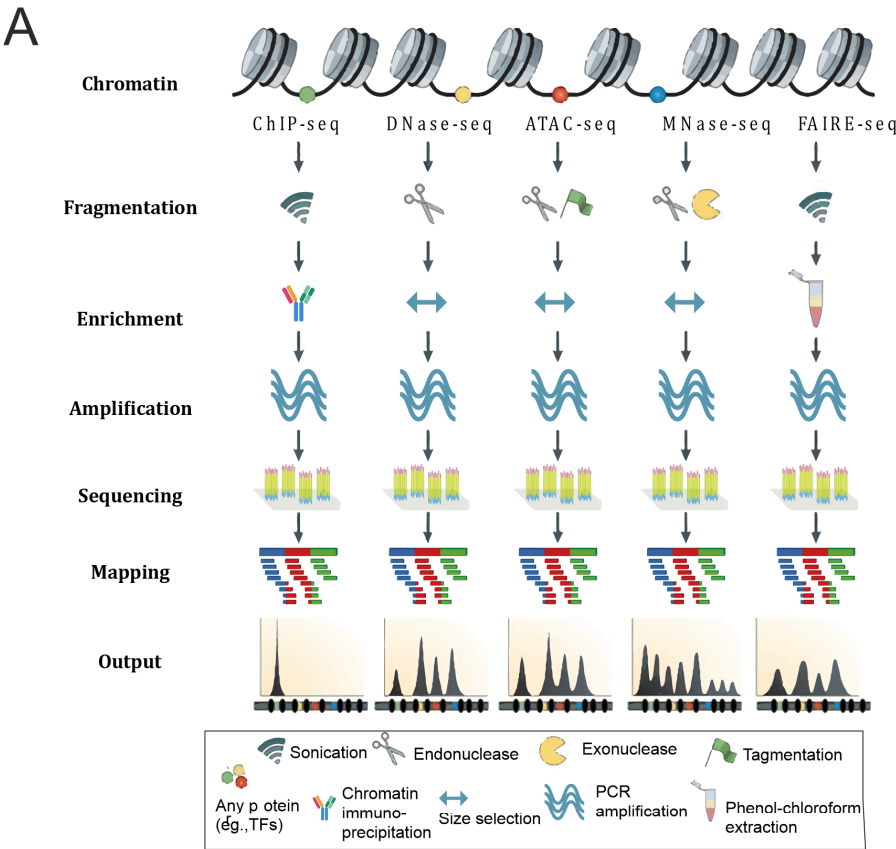


Figure 1. 7. Overview of chromatin accessibility and TF binding profiling techniques. **A.** In vivo techniques. ChIP-seq analysis reveals genomic regions of TF binding using specific antibodies that target a certain TF to pull it down together with its bound DNA that is then sequenced. DNase-seq and ATAC-seq reveal open-chromatin regions. DNase-seq is based in light digestion of the chromatin with DNase I endonuclease, while ATAC-seq uses an engineered Tn5 transposase to cleave DNA and add tags to it. MNase-seq provides the location of nucleosomes with high precision. FAIRE-seq uses cross-linking with formaldehyde which is more efficient in nucleosome-bound DNA than in nucleosome-depleted regions. Then the non-cross-linked DNA is sequenced. Adapted from (Meyer & Liu, 2014). **B.** In vitro techniques, in a protein binding microarray (PBM), high-density dsDNA microarray is prepared and a purified protein is added to let it bound to the DNA. The protein is tagged with a fluorophore to measure the interactions. **C.** The in vitro technique, HT-SELEX, uses a library of random dsDNA to bind a specific TF. Then bound molecules are isolated by immunoprecipitation, amplified and used for another binding round. After several rounds, the final DNA molecules bound by the TF are sequenced (Wang et al., 2011)

Simultaneously many technologies have been developed for the identification of open chromatin regions such as DNase I hypersensitive site sequencing (DNase-seq) (Hesselberth et al., 2009; Neph et al., 2012) the formaldehyde-assisted isolation of regulatory elements sequencing (FAIRE-seq) (Simon et al., 2012) or the most popular one, the assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Buenrostro et al., 2013)(**Fig 1.7 A**).

Another approach used to identify TF binding motifs has been *in vitro* occupancy assays, such as protein binding microarrays (PBMs) (M. F. Berger & Bulyk, 2009) in which the DNA-binding domain of a target protein is expressed, purified, and then added to microwell plates with known sequences of double strand DNA (dsDNA), and then identified using fluorescent antibodies to quantify the protein bound (**Fig 1.7 B**) or high-throughput systematic evolution of ligands by exponential enrichment (HT-SELEX) in which dsDNA mixture is incubated with DBDs and then after washing out non-specific dsDNA, the selected ones are extracted from the proteins, amplified and the process is

repeated several times, finally dsDNA population is sequenced to deduce TFs binding specificities (Y. Zhao et al., 2009) (**Fig 1.7 C**), among other technologies.

All this knowledge has been (and still is) collected in many TFBD databases such as TRANSFAC (Matys et al., 2006), CIS-BP (Weirauch et al., 2014), ChIPBASE (Yang et al., 2013), HOCOMOCO (Kulakovskiy et al., 2016), JASPAR (Rauluseviciute et al., 2024) and others. All these databases are used to predict potential binding events both through the genome or focused on putative regulatory sequences of interest and all of them are free and accessible.

Despite all these titanic efforts in understanding and predicting TF binding to the genome, the prediction capability is very low. This is in part due to the fact, that they are all based on studying TFs individually, and as explained above, the genome of a cell in a particular time is characterized by the complex nature of its layers of regulation that are the result of its past events in development and the combinatorial mode of action of TFs.

More complex approaches have also been undertaken. For example, performing functional characterization of enhancer activity using massively parallel reporter assay (MPRA) of many genomic sequences (Kheradpour et al., 2013; Mogno et al., 2013; M. A. White et al., 2013; Whitfield et al., 2012), where the transcriptional activity of hundreds of thousands of different sequences (synthetic CRMs) are measured in parallel *in vivo*. Using this method, it was described that only a small fraction of the predicted TFBSs in eukaryotic genomes are indeed occupied by TFs and the occupancy has highly variable across cell types and contexts (Segal et al., 2008; Spitz & Furlong, 2012). Moreover, only a subset of bound TFBSs relate to active enhancers meaning their ability to drive transcription in reporter assays (Fisher et al., 2012;

Kwasnieski et al., 2014). Thus, binding is not directly related to activity, what difficult even more our ability to predict functional sites and TFBSs that are bound by TFs from the ones unoccupied. More recently, all these techniques and knowledge are being combined with the use of artificial intelligence tools (or deep learning) to increase our understanding of the function of the non-coding genome (Taskiran et al., 2024).

Understanding the ‘transcriptional code’ involves not only WHERE in the genome TFs can bind, but also WHEN they do so in the context of a particular cell. This timing is influenced by factors such as chromatin accessibility, histone modification, or interaction with other TFs and COFs, all of which are regulatory components that affect TFs activity.

1.3.4. Combinatorial action of transcription factors

As expected due to the combinatorial activity of TFs, enhancers usually contain clusters of TFBS for the same TF (homotypic clusters) or different TF (heterotypic clusters) (Reiter et al., 2017). The observation that batteries of TFs tend to bind the same set of CRMs in the genome evidence that TFs may collaborate to bind CRMs, moreover, disrupting the TFBS of a certain TF or eliminating one can disturb other TF from binding to its site leading to the loss of gene expression (Heinz et al., 2010; Junion et al., 2012; Siersbæk et al., 2014; Yan et al., 2013). This combinatorial activity allows gene expression in a cell-type and time-specific manner through the precise combinatorial pattern of TFs that results in a vast range of accurate enhancer activities. One example of this cooperativity can be seen in *D. melanogaster* where pMAD (phosphorylated form of MAD TF) allows the acquisition of a terminal fate in cells in the dorsal mesoderm when combined with Tinman, and in the wing imaginal disc when combined with Scalloped (Guss et al., 2013; Xu et al., 1998). And this is only one

example of the several studies that have demonstrated the combinatorial effect of TFs in many organisms such as *Drosophila*, *C. elegans*, or mammals (Brocal-Ruiz et al., 2023; He et al., 2011; Lloret-Fernández et al., 2018; Stefflova et al., 2013). Therefore, understanding how enhancers allow cell diversity involves considering TFs activity in a combinatorial way.

TF cooperativity can be achieved through diverse mechanisms; from direct TF-TF interactions before DNA binding to collaboration mediated through DNA, both, with additional stability to protein-protein contacts provided by DNA or even without any direct TF-TF interaction (Reiter et al., 2017; Vandel et al., 2019). Moreover, interactions between TFs or between a TF and a cofactor (protein-protein interactions; PPIs) can result in directly or indirectly modified DNA binding preferences (Slattery et al., 2011).

Combinatorial binding can also occur in sequential rather than simultaneous binding of TFs, as it happens with pioneer TFs which are specialized TFs with specific properties that allow them to bind to nucleosomal DNA within closed chromatin and facilitate the binding of other TFs. Here, cooperativity arises from the fact that neither type of TFs can activate transcription without the other.

Ultimately, another possibility is that TF cooperativity takes place after DNA binding. Individual TFs have different functions and can interact with different elements of the core transcriptional machinery and recruit co-factors. Indeed, in a study where 5000 synthetic regulatory sequences were tested by MPRA in the murine hepatocellular carcinoma cell line (HemG2), they found that sequences with heterotypic clusters of TFBSs conferred stronger activation than homotopic clusters in transcriptional reporter assays (R. P. Smith et al.,

2013). This can be explained through the many TF cofactors recruited with enzymatic activity including Mediator complex, chromatin-remodelling complexes, and histone-modifying complexes. Then, the necessity of different enzymatic activities recruited by specific TFs explains why different combinations of TFs result in higher reporter activity than multiple TFBSs for the same TF.

1.3.5. Chromatin accessibility and epigenetics.

Finally, another layer of complexity in the regulation of gene expression which is not directly related to the DNA sequence but to its accessibility is the epigenome of a cell. In the nucleus of the cells, the chromatin is composed of nucleosomes which consist of an octamer of histone proteins (H2A, H2B, H3, and H4) encircled by ~147 bp of DNA. Chromatin is classified as euchromatin, found in an 'open' and hyper-accessible state, which is normally related to active chromatin, intermediate states or 'permissive' chromatin, and heterochromatin, corresponding to more inaccessible or repressed state known as 'close' (Annunziato, 2008; Klemm et al., 2019).

Chromatin accessibility plays an important role in modulating gene expression throughout development, differentiation, and in response to environmental stimuli (S. L. Berger, 2007). The different 'open' to 'close' states are governed by multiple factors, including post-translational modifications (PTM) of histones, chromatin remodelling complexes, DNA methylation or histone variants (**Fig 1.8**). For instance, DNA methylation, especially in promoter regions have been found to act as a repressive signal that unable the transcription factor binding, being Cytosine methylation at the C5 position (5-methylcytosine, 5mC) the predominant DNA modification in mammals, in the context of this PhD thesis, it is important to mention that *C. elegans* lacks DNA

methylation (Dan & Chen, 2023; Z. D. Smith & Meissner, 2013) (**Fig 1.8 B**); during chromatin remodelling, multiproteic complexes (SWI/SNF, ISWI, INO80 or CHD) use the energy of ATP hydrolysis to mobilize and restructure nucleosomes (Becker & Workman, 2013) (**Fig 1.8 A**); also histone variants such as H2A.Z or H3.3 are able to modify the nucleosome structure or chromatin dynamics, restricting or enabling the access to certain regions (Martire & Banaszynski, 2020).

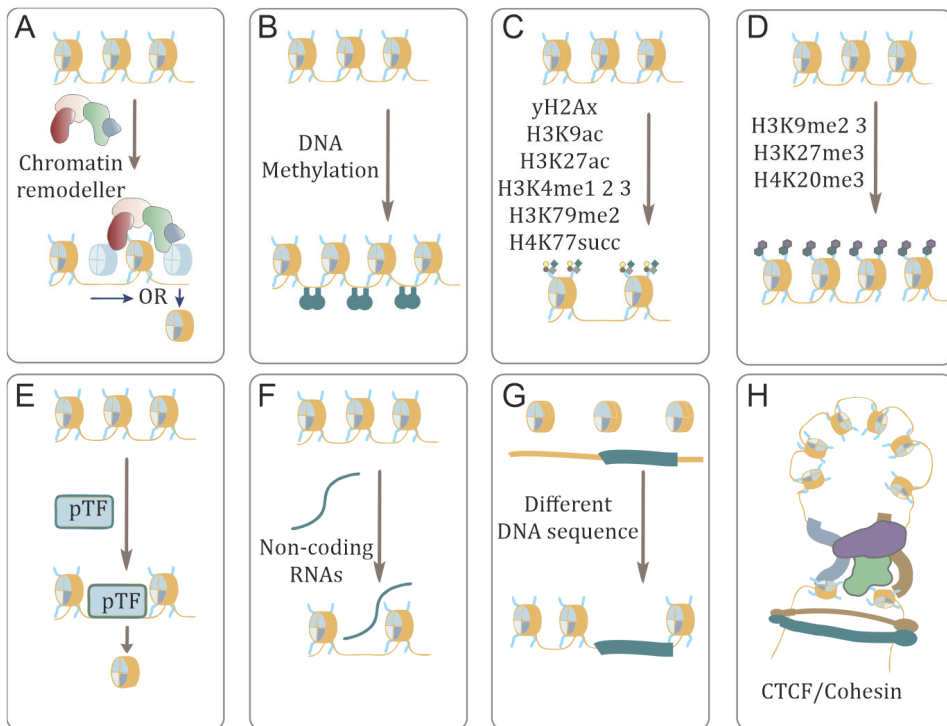


Figure 1. 8. Primary remodellers of chromatin accessibility. Chromatin accessibility is determined by different regulators such as: chromatin remodellers (A), DNA methylation (B), histone post-translational modifications that induce transcription (C) or repress it (D), pioneer transcription factors, that open the chromatin (E), non-coding RNAs (F), DNA sequence itself (G), and chromatin 3D structure that allows or impedes the entrance of TFs and the transcriptional machinery (H). Adapted from (Y. Chen et al., 2024).

Nonetheless, the most studied mechanism are the PTMs in which covalent modifications of histones including methylation, acetylation, phosphorylation or ubiquitination, among others, affect the structure of the chromatin, making it more or less accessible to transcription machinery (**Fig 1.8 C, D**). These PTMs, also known as epigenetic marks, have been suggested to serve as an epigenetic code and their landscape is controlled by an interconnected set of ‘writers’, ‘readers’ or ‘erasers’ proteins that catalyze the formation, recognize or erase particular PTMs. They could be found either in the terminal tail of histone on the core domains principally in lysine residues but also in other residues (Millán-Zambrano et al., 2022).

The most studied modifications have been methylation and acetylation. Histone acetylation generally correlates with less-compact chromatin structure and transcriptional active regions, and it is enriched in promoters and enhancers (Verdin & Ott, 2015). However, in the case of methylation, the relation to transcription activity depends on which residue it is found, and it is also found in three different states, mono, di, or trimethylation which increases the complexity. For instance, monomethylated H3K4 (H3K4me1) is known to be found in ‘poised’ enhancers, which are not strictly active, but they are prepared to a potential activation, and in these regions is also found the repressive mark H3K27me3 (Bonn et al., 2012; Heintzman et al., 2007; Rada-Iglesias et al., 2012; Shlyueva et al., 2014). In the case of active enhancers both H3K4me1 and acetylation marks in H3K27 (H3K27ac) can be found (Creyghton et al., 2010; Heintzman et al., 2009). However, the trimethylated H3K4 (H3K4me3) mark has been observed to be highly enriched around the TSSs of transcriptionally active genes, together with H3K27ac (Bonn et al., 2012). On the other hand, H3K9me3 is an epigenetic mark of constitutive heterochromatin. Nevertheless, the roles of repression for H3K9 methylation has been recently expanded, as it

has also been described to have important role in silencing effector gene expression from undesired cellular lineages (Padeken et al., 2022).

The regulation of these methylation marks in lysine residues is controlled by a huge set of lysine MTases (KMTs), lysine demethylases (KDMs) and effector proteins that recognize specific methyllysine residues. (Reviewed at (Dan & Chen, 2023).

More recently, other novel PTMs have been described, such as serotonylation and dopaminylation of glutamine 5 in histone 3 (H3Q5) specifically in the brain (Farrelly et al., 2019; Lepack et al., 2020). The dopaminylation of histone is associated with aberrant expression of reward-related behaviour and synaptic plasticity-related neuronal genes (Lepack et al., 2020). While in serotonylation, researchers demonstrated that transglutaminase 2 can serotonylate H3K4me3 marked nucleosomes ending up with H3K4me3Q5ser nucleosomes that are enriched in euchromatin and correlate with permissive gene expression (Farrelly et al., 2019).

As for the case of histone methylations and demethylations, specialized protein complexes deposit the marks in particular regions of the genome. These complexes are generally ubiquitously expressed. The current model states that TFs have an important and instructive role determining where and when epigenetic marks are deposited, however, we are still in the early days for the understanding of the interplay between TFs and epigenetic modifiers.

As mentioned above, several attempts have been made to find the transcriptionally active regions on the DNA and the TFBSs such as ATAC-seq, DNase-seq, ChIP-seq (and its variants) or epigenetic profiling, among others (Inukai et al., 2017). In order to annotate enhancers based on chromatin accessibility and histone tail marks, one remarkable project has been the ENCODE Consortium (Encyclopedia of DNA Elements) (ENCODE Project

Consortium, 2012) which integrates data from a variety of assays that include information of DNA binding, accessibility and methylation, but also data about 3D chromatin structure, gene expression or gene variants.

The study of how gene regulatory networks, in all their layers of complexity, work will help us understand how gene expression is both established, maintained, and changed through the life of organisms. Changes in the DNA sequences, epigenetic mechanisms, and chromatin remodelling facilitate phenotypic diversity, enabling populations to explore a range of adaptive responses. Thus, a deep understanding of gene regulatory networks will also teach us how organisms can rapidly adapt to changing conditions in developmental time (intrageneration) and also in a slower path, how they evolve across generations to give rise to novel cell types, structures and functions.

1.4. Plasticity and evolution of cell-types

1.4.1. Basic concepts in the evolution of novelty

Organisms have the ability to express different phenotypes in different environments. This ability is what is known as phenotypic plasticity. This characteristic intrinsic to organisms is specific for a given genotype in a given environment and is theoretically termed as the ‘norm of reaction’. This includes all the potential outcomes a genotype has in response to different environments. If the phenotype is constant is termed canalized. When a genotype generates markedly different phenotypes in presence of different environments, if the phenotypes either are differentially discrete it is considered a polyphonic trait while if phenotypes reveal in a continuum are considered plastic (**Fig 1.9**) (Pfennig, 2021; Sarkar, 2004).

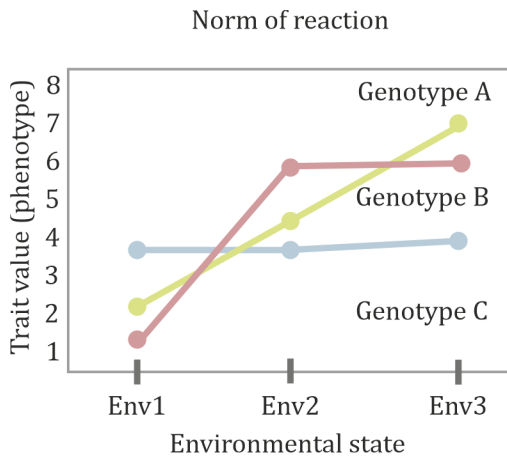


Figure 1. 9. Phenotype norm of reaction. For each genotype (A, B and C), a specific phenotype is expressed in a specific environmental state. The points are joined to form a response curve (reaction norm). The reaction of norm for a given trait may vary continuously (genotype A, plasticity), consist of a few discrete alternatives (genotype B, polyphenism) or be canalized (no variation in the phenotype observed). Adapted from (Pfennig, 2021).

The classical theory of evolution has been mutation-led evolution (MLE), in which genetic mutations introduce heritable variations that are then the subject of natural selection. However, currently, the idea of plasticity (also) -led evolution (PLE) has been taking relevance, and both ideas should be taken into account when studying evolution (Ng & Kinjo, 2024; Pfennig, 2021).

However, the idea that plasticity is involved in evolution is not recent, in fact, Waddington already explored that theory in 1952 with the “genetic assimilation” process. *‘This mechanism would provide a means by which an “acquired character” in the conventional sense could be “assimilated” by the genotype, and eventually appear comparatively independent of any specific environmental influence’* (Waddington, 1953), this potential evolutionary mechanism was demonstrated to be possible under laboratory conditions, using heat-shock over *D. melanogaster* that induced phenotypic changes, that then became fixed in the population, illustrating how environmental stress shift reaction of norm revealing additional developmental phenotypes (or new traits) that may become genetically assimilated over time (Waddington, 1953). More recently, many studies involving different organisms, have demonstrated the genetic assimilation mechanism, such as laboratory induced fixation of a seasonal wings colour phenotype (normally plastic) in the buckeye butterfly (van der Burg et al., 2020), or the environmentally induced matricidal hatching (triggered by maternal nutrient deprivation) in *C. elegans* that in a particular wild isolate has evolved into a genetically encoded trait that becomes constitutive (Vigne et al., 2021). Nevertheless, the role of genetic assimilation in evolution of novelty is still controversial. Moreover, molecular mechanisms underlying this process are not known.

Indeed, plasticity has not only been proposed to be important in inducing evolution but, in some cases could prevent it. Selection could favor plastic individuals just because plasticity will give them the ability to adapt to changing environments (‘Baldwin effect’) (Baldwin, 1896). This could then avoid the “need” of further genetic mutations and evolution or could allowing populations under strong environmental changes to live long enough to let new adaptations to evolve in a classic mutation first model, what is known as the ‘buying time’ theory (Diamond & Martin, 2021).

Finally, another direct way in which plasticity could facilitate evolution is by the inheritance of epigenetic factors (Bonduriansky & Day, 2009). This last idea is still controversial, even when there are many ways in which this information could be transferred to the offspring such as DNA methylation, by specific enzymes that copy the methyl tag from a cell to its daughter during DNA replication (Y. Wang et al., 2017); histone modifications that can be inherited (Audergon et al., 2015); or small RNA sequences present in sperm that carry epigenetic information (Q. Chen, Yan, Cao, et al., 2016; Q. Chen, Yan, & Duan, 2016; Pfennig, 2021).

1.4.2. Evolution of cell type diversity and the emergence of new cell types.

Cell types serve as fundamental building blocks of multicellular organisms, they are highly diversified in animals allowing for specialized functions. This diversity is a product of evolutionary adaptation. However, cell type evolution contrasts with the fact that all cells in an organism share the same genome. It has been proposed that novel cell types emerge as the result of cells reaching stable ‘transcriptional regulatory states’, meaning that the cell has acquired a ‘type’ defined by the effector genes it expresses (Arendt et al., 2016; Callier, 2020). The first step towards understanding cell type evolution is the classification of cell types present in the organisms across the tree of life. Classically, cells have been classified according to their morphology or function, however, the development of single-cell transcriptomics such as single-cell RNAseq has allowed for the fast and high throughput classification of different cell types in many different organisms, not only model systems. For instance, in *Spongilla lacustris*, a species of sponge, 18 different cell types were found, including one with hybrid immune and neuronal properties. This example

could be the starting point for acquiring complex nervous systems in animals (Musser et al., 2021).

A conceptual framework emphasizing the importance of understanding the molecular mechanisms controlling cell identity when studying evolutionary relationships between cell types was proposed by Arendt et al. (2016). In this article it was introduced the 'serial sister cell type' concept that distinguishes evolutionary history from developmental lineage. This means that while cells may share a common developmental origin, they may not be closely related evolutionarily. For instance, in *Caenorhabditis elegans*, neurons and muscle cells can originate from the same developmental lineage, while cells with similar functions may arise from entirely different lineages (Sulston et al., 1983). Similarly, in vertebrates, horizontal cells and cone photoreceptors in the retina share developmental precursors but have highly divergent structures and functions (Emerson et al., 2013). The idea of serial sister cell types proposes that during early animal evolution, repeated divisions of the body into distinct regions led to the duplication and diversification of cell types within those regions. This resulted in a series of topographically separate sister cell types arising from different regions and produced by different stem cells, even though they are evolutionarily related. This concept helps explain why developmental and evolutionary lineages can differ (Arendt et al., 2016).

When it comes to identifying homologous cell types between distant species (those sharing a common ancestral cell type), the task is challenging using only morphology or function. This is because these features could be similar due to convergent evolution, rather than shared ancestry. Therefore, comparing molecular fingerprints offers a more reliable method to identify homologous cell types across species. The molecular fingerprint of a cell comprises the unique combination of active genes that give rise to the cell type's specific

identity together with the transcription factors, acting as terminal selectors, that define the effector genes that provide that identity (Arendt et al., 2016, 2019). Certainly, in many cases, homologous cell types share similar developmental origins, however, when considering cell types separated by long evolutionary distances, this is not applicable, thus is why it is important to differentiate developmental lineages from evolutionary lineages.

Beyond the establishment of cell-type classification or the homology of cell types between species, one important unanswered question is the mechanisms that underlie the emergence of new cell types, that is the diversification of stable transcriptomes. Several models of cell type evolution and diversification have been proposed: One of the ways is functional segregation, in which one sister cell type loses a function that is maintained in the other, in analogy to gene duplications (**Fig 1.10 A**). One classical example of this mechanism is the segregation of functions in the vertebrate retina, where some cells have maintained light sensitivity (cones and rods) while others have retained the ability to integrate and transmit signals from photoreceptors (bipolar, amacrine and horizontal cells). While the common ancestor of these cells is proposed to be a cell with bipolar morphology, dendrites with a photosensitive cilium and an axon that established direct synaptic contact with the ganglion cells. This cell would have divided in two sister cell types, one that retained the photoreceptor and lost the axonal connection to ganglion cells, with the lack of expression of *Chx10* and *Vsx* (rods and cones), and one that retains the interneuron function but loses the light-sensitive cilium and maintained the expression of that genes (bipolar cells) (Arendt, 2008; Arendt et al., 2009).

Another mechanism of cell type evolution is the divergence of functions between cell types (**Fig 1.10 B**), in this case, there is no division of functions, but sister cells just evolve in different directions. That would be the case of the

cones and rods, each cell type evolved to detect different wavelengths. This was achieved in a process of gene duplication and gene subfunctionalization, meaning that after gene duplication the divergent evolution between two distinct cell types is stabilized by the loss of regulatory elements that drive their expression in both cell types (Force et al., 1999). For instance, the evolution of olfactory receptor sister cell types has been driven by the expansion of the olfactory G-protein-coupled receptor (GPCR) family and the emergence of regulatory mechanisms that ensure the expression of a unique GPCR per neuron type (Arendt, 2008).

The acquisition of completely novel functions through the recruitment of additional GRN modules also allows for the emergence of novel cell types (**Fig 1.10 C**). For example, new cell-types could appear by activating pre-existing batteries of differentiation genes that were present only in other cell-types (co-option)(Arendt, 2008). A typical example of the acquisition of new functions in a cell is observable in the cnidarian nematocyte, a highly specialized cell type that evolved novel functions through the acquisition of unique genes, many of which have no orthologues in other species. This emergence probably involved extreme modification of a pre-existing cell type, possibly a sensory neuron with exocrine capabilities, as suggested by the similarity of the cnidarian nematocyte to a sensory cilium (Hwang et al., 2007; Miljkovic-Licina et al., 2004).

Finally, a new cell type could emerge due to the fixation of a transient state, a process termed genetic assimilation as discussed above (**Fig 1.10 D**). At first, this induced state can be reversible, however, the induced gene expression program can evolve to be constitutive. Then, cell type diversification will occur when alternative states of an ancestral cell type become fixed. This process is exemplified in the origin of a novel cell type due to the co-option of an ancestral stress response as a constitutive trait (Love & Wagner, 2022). For instance, the

emergence of the decidual stromal cell (DSC), a novel cell type of eutherians mammals has been proposed to evolved from the stress response of an ancestral cell (Erkenbrack et al., 2018).

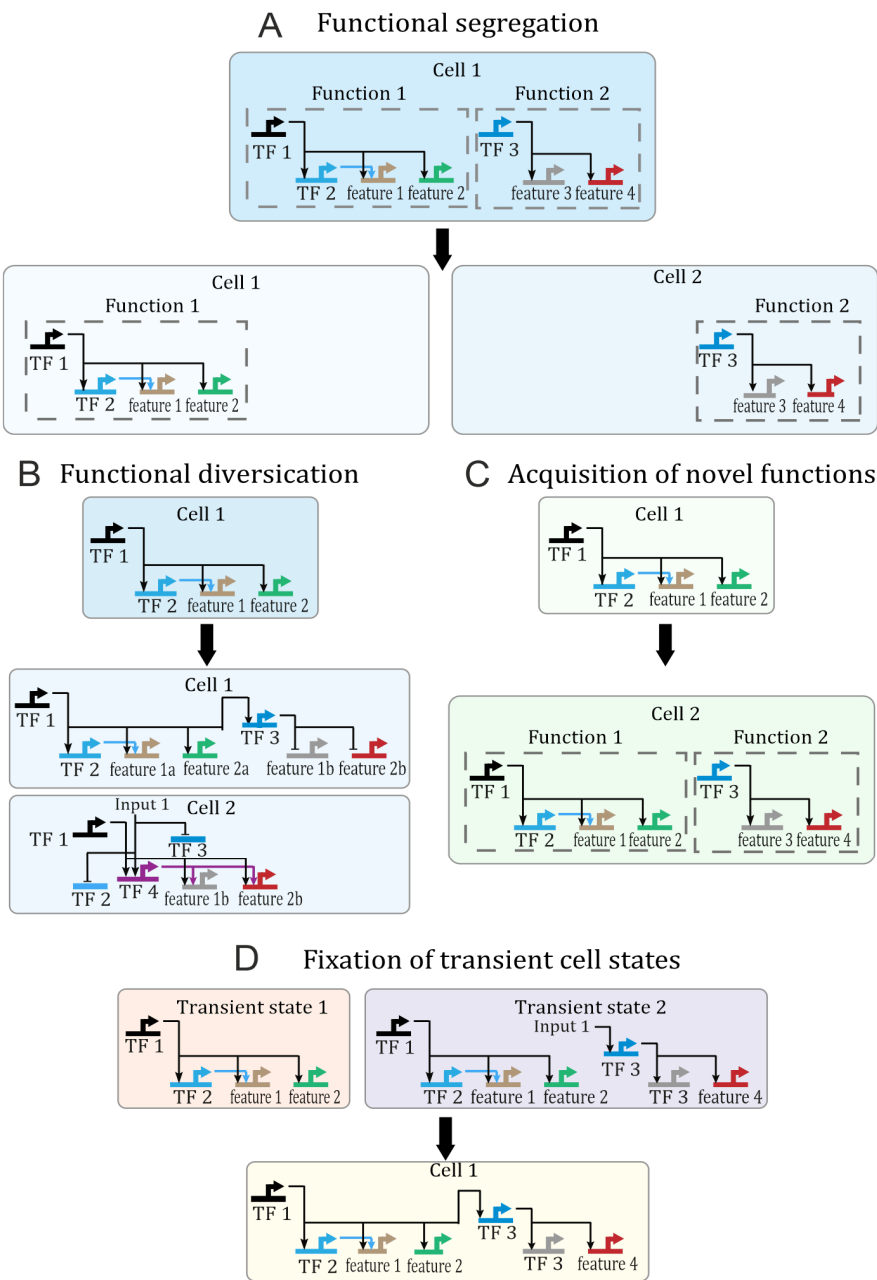


Figure 1. 10. Mechanisms of cell type evolution. **A.** Functional segregation. Each of the two sisters' cell types loses some functions. **B.** Functional diversification. Takes place after gene duplication and subfunctionalization. **C.** Acquisition of novel functions. Emergence of novel genes contributes to the expression of new batteries of genes. **D.** Fixation of transient states. A cell state triggered by facultative stimuli becomes fixed independently of the trigger.

In my PhD project, we aim to get a better understanding of the relationship and complex interplay between development, plasticity and evolution, with a major focus in understanding the regulatory mechanisms driving cell type development and evolution. These are very fundamental questions. To be able to perform in depth regulatory studies and also be able to identify homologous cell types among species we take advantage of the simplicity of the model organism *C. elegans*, and its well-characterized developmental lineage and functionality of the nervous system. We combine the use of *C. elegans* with other *Caenorhabditis* species to contribute to uncovering the fundamental mechanisms driving evolutionary innovation.

1.5. *C. elegans* as a model organism

Caenorhabditis elegans is a free-living transparent nematode (round worm), of the *Rhabditae* family. It is 1mm of length and in the wild it can be found in soil or rotten fruits, where it feeds on bacteria. Sydney Brenner defined this little organism as '*an experimental organism which was suitable for genetical study and in which one could determine the complete structure of the nervous system*' (Brenner, 1974). With these words Brenner highlighted the great applicability that *C. elegans* could give to the neurobiology field, however, the usage of the worm has exponentially increased and expanded to many other research fields such as genetics, cancer, toxicity, aging or evolution, among others. In fact, 4 Nobel prizes, including this year's Nobel prize in Physiology or Medicine, have been awarded for studies performed in *C. elegans* (in 2002, 2006, 2008 and 2024). But what makes these animals such a good model?

One important characteristic is that *C. elegans* is easy and cheap to grow in laboratory, they are normally cultured in agar plates with a layer of *Escherichia coli* on which they feed. They are also androdioecious, which means it is primarily self-fertilising (hermaphrodite), making it easy to maintain isogenic populations. Nonetheless, males appear with a probability of 0.1-0.2%, or higher probabilities when exposed to stressful conditions such as heat, due to meiotic non-disjunction of the X chromosome (hermaphrodites are XX and males X0), which allows crossing between males and hermaphrodites. Moreover, each individual can lay around 300 eggs, and they have a rapid life cycle of 3 days at 20°C, making it easy to increase populations in a very short time (Corsi et al., 2015).

C. elegans was the first multicellular organism to be entirely sequenced since its genome is pretty compact with 100Mb organized in 5 autosomes and one X chromosome (*C. elegans* Sequencing Consortium, 1998), and there are 28590

CDSs (Coding DNA Sequences) from which the 82.4% have been fully confirmed (WormBase release WS294, Nov. 2024). Importantly, approximately 83% of *C. elegans*' protein sequences have a homolog in humans, while 70% of human genes have an ortholog in *C. elegans* (Lai et al., 2000). Moreover, *C. elegans* develops through an invariable number of somatic cells, and the lineage of each cell is known from the embryo to the adult organism (Sulston & Horvitz, 1977), and give rise to different tissues such as cuticle, gut, pharynx, muscle, reproductive system and nervous system (**Fig 1.11**).

Another important feature that makes *C. elegans* a good model system is its availability to genetic studies. Many already generated mutants are available upon request to the scientific community through consortia like the CGC (*Caenorhabditis* Genetics Center). Germline transformation is easily achieved by microinjection of DNA into the gonad, yielding transgenic lines in a few days (Mello et al., 1991) for example expressing fluorescent proteins under the control of specific promoters that thanks to the transparency of the worm allows *in vivo* visualization of specific neuron types (Chalfie et al., 1994). Moreover, the introduction of CRISPR-Cas9 technology to *C. elegans* has further improved the specificity in the generation of mutant and transgenic worms and also in generating endogenous reporters for specific genes (Dickinson et al., 2013; Friedland et al., 2013; Shen et al., 2014). Thanks to these advantages, forward and reverse genetics have been used to identify key genes involved in developmental and cellular processes. More recently, the use of scRNAseq methodologies has provided the array of cellular transcriptomes in *C. elegans* in the second and fourth larval stage as well as for all progenitor cells present during its full embryonic development (Cao et al., 2017; Packer et al., 2019; Taylor et al., 2021).

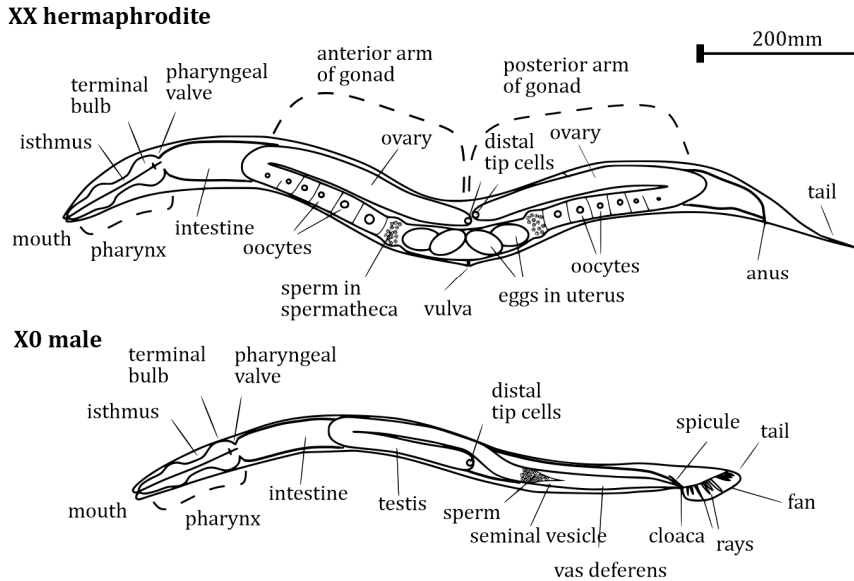


Figure 1. 11. Anatomical features of adult *Caenorhabditis elegans*. *C. elegans* adult hermaphrodite (top) and male (bottom), side view. Hermaphrodites and males differ in overall body size and structures such as the somatic gonad and tail, also other tissues and organs are sexually dimorphic, notably the nervous system and musculature. Adapted from (Zarkower, 2006).

1.5.1. *C. elegans* nervous system

As mentioned above, *C. elegans* has an invariable cell lineage that gives rise to 959 somatic cells of which 302 are neurons in the case of the hermaphrodites while in males 387 are neurons in a total of 1031 somatic (Molina-García et al., 2020; Sammut et al., 2015; Sulston & Horvitz, 1977; J. G. White et al., 1986) (**Fig 1.12**). Neurons are grouped in several ganglia in the head, called the nerve ring, the ventral nerve cord along the worm's body, and in the animal's tail (J. G. White et al., 1986). In the hermaphrodite, 282 neurons constitute the somatic nervous system located between the hypodermis and the body wall muscle, while the 20 remaining neurons, conform the pharyngeal or enteric system and are embedded in the pharyngeal muscle, and both systems remain separated

and only communicate through RIP interneurons (Altun & Hall, 2011; White et al., 1986). In males, the nervous system is additionally composed by 85 other neurons, most of them involved in mating behaviour and located in the posterior body, in the region of the tail, that has a major role in mating behaviour (Altun & Hall, 2011; Emmons & Lipton, 2003; Sulston et al., 1980)(Fig 1.12).

C. elegans neurons can be classified according to morphology, anatomical position, and synaptic connections. According to anatomical and morphological features, there are 118 classes of neurons. In many cases, the class is defined by a unique bilateral pair of neurons (70) or even a unilateral neuron (26) (Hobert et al., 2016). In terms of functionality, neurons can be classified as interneurons, motoneurons, sensory neurons or polymodal neurons (if they have more than one of the mentioned functions) they release neurotransmitters similar to vertebrates such as acetylcholine, glutamate, GABA, serotonin, dopamine, octopamine and tyramine (Altun & Hall, 2011; Hobert et al., 2016). In addition, recent analysis using CRISPR/Cas9-engineered reporter strains have revealed neurons that may only release neuropeptides and also found evidence that suggests the existence of novel monoaminergic transmitters (C. Wang et al., 2024).

Neuronal connectome of *C. elegans* was the first one to be completely mapped by electronic microscopy reconstruction, finding about 5000 chemical synapses, 2000 neuromuscular junctions and 600 gap junctions (J. G. White et al., 1986). More recently, both the hermaphrodite and the male connectome have been studied, corroborating the around 5000 chemical synapses in hermaphrodite but expanding the number of gap junctions to 1447. Males showed 5,315 chemical synapses and 1,755 gap junction edges (Cook et al., 2019). More recent studies have reported the neuropeptide connectome of *C.*

elegans brain showing a complex and decentralized network that operates differently from traditional wired brain circuits (Ripoll-Sánchez et al., 2023).

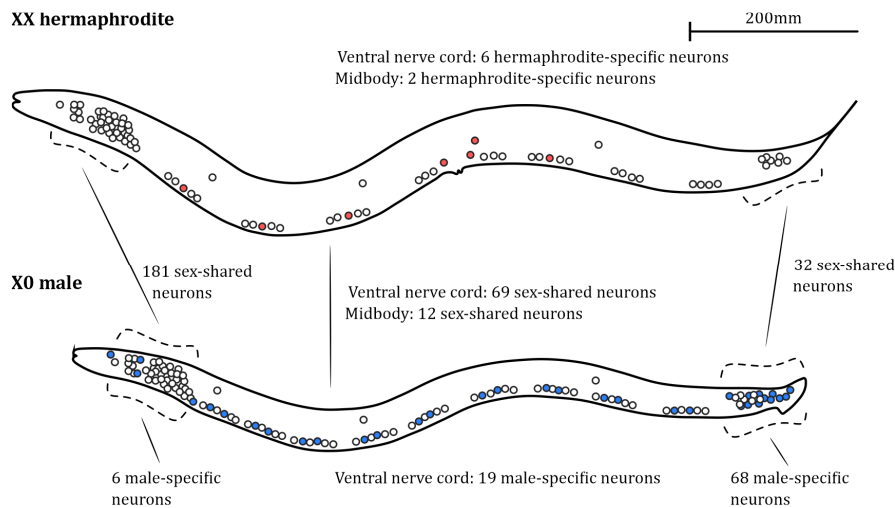


Figure 1. 12. Nervous system of adult *Caenorhabditis elegans*. *C. elegans* adult hermaphrodite (top) and male (bottom) share 294 neurons, represented here by colourless circles. Adult hermaphrodites contain a total of 302 neurons; red circles indicate 8 hermaphrodite-specific neurons. Adult males possess 387 neurons; blue circles show 93 male-specific neurons. Adapted from (Serrano-Saiz et al., 2017)

Thus, even this "simple" model organism reveals a high level of complexity in the organization of its nervous system. This complexity is responsible for the variety of behaviours linked to its locomotion, sensory processing, decision-making mechanisms, memory, etc. which has allowed researchers to study the relation between neural networks and behaviour, taking advantage of a 'simple' organism.

1.5.2. Neurogenesis in *C. elegans*

As explained above, (section 1.1.2) in the generation of the nervous system during embryonic development, a key decision will be choosing between a neuronal or not neuronal fate. Unlike other organisms, *C. elegans* neurons are not clonally derived which means that many lineages produce both neuronal and non-neuronal cells some of which are even in the last division, and also many neurons are derived from terminal divisions in which the sister cell undergoes apoptosis (Poole et al., 2024; Sulston et al., 1983; Sulston & Horvitz, 1977).

Since the origin of many tissues in *C. elegans*, including neurons, is nonclonal, the concept of layers that define the different tissues found in other organisms does not apply to this model. However, some TF families that have a role in specifying neuronal progenitors in vertebrates are shared with *C. elegans* [reviewed in (Poole et al., 2024)]. For instance, HOX cluster TFs, a specific subfamily of homeodomain TFs, are known to have an important role in the anterior-posterior body patterning and segmentation across metazoans, and also are important during early steps of neuronal specification (Bello et al., 2003; Gouti & Gavalas, 2008; Maurange & Gould, 2005). These roles have also been found in the homologs of these HOX cluster genes in *C. elegans* (*ceh-13*, *lin-39*, *mab-5*, *egl-5*, *nob-1* and *php-3*) (J. J. Smith & Kratsios, 2024). Also TFs from the bHLH (basic helix-loop-helix), known as proneural factors (Bertrand et al., 2002), have been found to have a role in the specification of neuroblasts such as *lin-32* involved in the postembryonic development of the sensory rays in the male (C. Zhao & Emmons, 1995) or *cnd-1*, that regulates the specification of some neuroblast that give rise to some motor neurons in the ventral cord (Hallam et al., 2000). It is important to mention that not only induction but repression also has an important role in the specification of neuroblast, for

instance, *lin-26* is indispensable to avoid the transformation of ventral epidermal cells in neurons (Horvitz et al., 1983).

Once a cell has committed to a neuronal fate, it will make specific lineage choices. These choices are also regulated by TFs. For instance, UNC-86 acts in neuroblast lineages to promote their correct specification, its absence produces various lineage-specific transformation (Hobert, 2010).

Finally, the postmitotic neuron will start terminally differentiating expressing the battery of effector genes that provides its own identity and functions. In *C. elegans* the set of transcription factors that directly control the expression of the specific effector genes are termed ‘terminal selectors’ (Hobert, 2008). Neurons that come from the same lineage do not generally share many features, in fact, they are quite diverse in the synaptic connections, neurotransmitter usage, receptor expression or components of gap junctions, in general in their expression profiles (Poole et al., 2024). Indeed, it has been described that every one of the 118 neuron classes expresses a unique combination of neuropeptides (Ripoll-Sánchez et al., 2023). Thus, in the last terminal division, even related (sister) neurons end up expressing very different combinations of TFs that directly control the expression of neuron-type specific transcriptomes. How this regulatory landscape is conformed in such a differential way between sister cells is not completely understood, however, *wnt* signalling has been implicated in segregation of fates in dividing events, as has been described in other animals including mammals (Poole 2024).

Terminal selector (TS) role in regulating maturation of neurons has been deeply studied in *C. elegans*, and it is thought that every neuron class expresses a unique combination of TSs that define the transcriptome and subsequently the functions of each class (**Fig 1.13**). For instance the HSN motorneuron needs the

combinatorial action of at least 6 TFs that act collectively to ensure the expression of the effector genes of this neuron (Lloret-Fernández et al., 2018).

Most effector genes are shared (independently) between neurons, meaning what makes a neuron type unique is not the expression of a given gene but the unique combination of effector gene expression (unique transcriptome). These specific expression patterns are regulated by specific gene regulatory networks in which particular *cis*-regulatory modules (CRMs) integrate the inputs of different sets of terminal selectors (**Fig 1.13 C**). For example, *tph-1* expression in different serotonergic neurons is achieved by non-overlapping enhancers that regulate its neuron type expression (Lloret-Fernández et al., 2018). For other effector genes, the same enhancer is controlled by different TFs (belonging to the same family) expressed in different neurons, for example, all glutamatergic neurons express *eat-4* VGLUT, which is controlled by different HD TFs in each neuron class, but often acting through the same TFBS (Serrano-Saiz et al., 2013).

Another characteristic of terminal selectors is that their expression is necessary throughout the life of the neuron to maintain the expression of the effector genes and thus the identity. This is normally regulated through a feedback loop of autoactivation (Etchberger et al., 2007; Leyva-Díaz & Hobert, 2019) (**Fig 1.13 A**). Among all different TF families, Homeodomain TFs (HD) seem enriched for terminal selectors functions in *C. elegans* (~70%) and they are reused for the specification of different neurons in combination with other TFs. In fact, there are 5 TFs recurrently found acting as TS in 70 different classes, 4 of them being HD (UNC-86, CEH-14, UNC-42, CEH-34)(Reilly et al., 2022).

Although terminal selectors are indispensable for the terminal specification of neurons, there are some genes shared by many or all neurons that are regulated independently of the specific collective of terminal selectors in each neuron.

This kind of shared features are for example panneuronal effector genes or genes involved in regulating the sensory cilia. Panneuronal gene expression has been recently found to be regulated by the CUT subfamily of HD (Leyva-Díaz & Hobert, 2022) that act redundantly in a dosage-dependent manner. In the case of genes that code for structural cilia components, they are under the control of the Forkhead TF FKH-8 and the RFX TF DAF-19 (Brocal-Ruiz et al., 2023; Swoboda et al., 2000) that physically interact to allow the expression of the cilia coding genes (**Fig 1.13 B**).

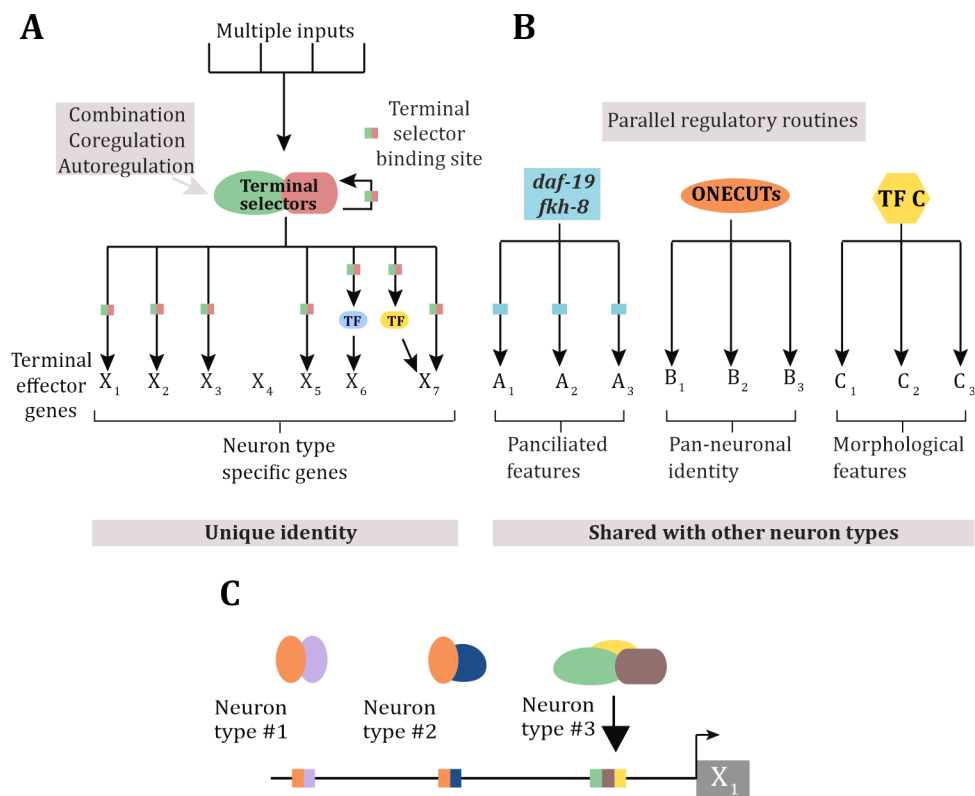


Figure 1. 13. The terminal selector model for transcriptional regulation of neuron-type effector genes. **A.** Specific terminal selector (TS) combinations directly regulate batteries of effector genes in each neuronal type. TSs recognize specific motifs (green/pink rectangle) and induce transcription of specific target genes. Their expression is maintained through autoregulation. Xn represents the effector genes. Sometimes TS activates additional TFs that control other sub-routines (blue and yellow circles). **B.** Parallel regulatory routines to TS have also been described such as pan-neuronal features (regulated via ONECUT TFs) or panciliated features (regulated via DAF-19 and FKH-8 TFs). These effector features are controlled by transcription factors that do not assign unique identities. **C.** Modular organization of the regulatory sequence of effector gene X1 allows for its regulation by different combinations of terminal selectors. Adapted from (Hobert, 2016).

1.5.3. Epigenetics and repressive chromatin in *C. elegans*

As discussed previously in section 1.2., the specific targets of TFs result from the combinatorial actions of TFs found in a cell at a particular time and also the specific regulatory context (epigenetic landscape) found in the cell. Naturally, neurons and neuronal development are not exception. TF actions are influenced by the chromatin landscape, which is acquired during the development of the neuron, and it can determine the accessibility of the TF to certain binding sites. One example of the importance of the chromatin landscape can be observed in the silencing of the HD UNC-4 through the methylation of the histone 3 in the methyl 9 (H3K9). UNC-4 is an HD TF important for VC4 and VC5 neuron terminal differentiation. VC neurons are classified into VC1 to VC6 along the ventral nerve cord, they all share many features in their transcriptome but VC4 and VC5 receive signals from the developing vulva that differentiate them from the rest of VCs. One of those differentiating factors is UNC-4. However, UNC-4 is initially expressed in all VCs and is actively epigenetically repressed in the VC1-3 and VC6 through the H3K9 methylation mechanisms (Zheng et al., 2013). This example points out the importance not only of activation mechanisms but also active repression for the correct neuron fate acquisition.

Part of this PhD thesis has studied in more depth the role of H3K9me in neuron specification and evolution. Thus, I will explain in more detail epigenetic repression and its known role in *C. elegans* development. As in other organisms, *C. elegans* has both euchromatin and heterochromatin. In the case of heterochromatin, we can differentiate between permanently silenced constitutive heterochromatin, found generally in the distal arms of the chromosomes, enriched in H3K9me_{2/3} and its ligand HP1 (Heterochromatin Protein 1)(Eissenberg & Elgin, 2014); and facultative heterochromatin containing potentially active genes, these regions are enriched in H3K27me₃, which is deposited by PRC2 (Polycomb Repressive Complex 2)(Ahringer & Gasser, 2018; Wiles & Selker, 2017).

In central regions, where active genes are found, H3K27me₃ marks are found alone, while in the distal arms of chromosomes, enriched in repetitive elements, H3K27me₃ colocalize with H3K9me₃ (T. Liu et al., 2011; Meister et al., 2010). Central and distal arms also show a different distribution within the nucleus. Indeed LMN-1 (lamin protein), and lamin-associated factors such as LEM-2 and EMR-1 are enriched in distal arms, which indicates its association with the nuclear envelope. Indeed LEM-2 associated domains are enriched in H3K9-methylated histones, and this mark is important for its positioning since a nuclear chromodomain protein CEC-4 recognizes it (Ahringer & Gasser, 2018; Gonzalez-Sandoval et al., 2015; Towbin et al., 2012).

The principal Histone methyl transferases (HMTs) that target H3K9me are MET-2 (SETDB1 homolog) and SET-25; H3K9 mono and di-methylation is deposited by MET-2, while trimethylation of H3K9 is deposited by SET-25. There have been other genes postulated to encode HMTs that target H3K9, however, double knockout of *set-25* and *met-2* is sufficient to eliminate all detectable H3K9me analysed with mass spectrometry (Towbin et al., 2012).

H3K9 methylation is a sequential process in which MET-2 initiates the process by adding the first two methyl groups, this first step takes place partially in the cytoplasm, before nucleosome assembly. Then, SET-25, which preferentially localizes to perinuclear foci, acts on the already modified H3K9me1/2 to add the third methyl group. This process is essential for the formation of stable binding to the nuclear lamina (Towbin et al., 2012). SET-25 is also able to compensate for MET-2 lost by depositing the two first methyl groups. Both HTMs have affinity for their own products which make them accumulate in H3K9me rich nuclear foci (Ahringer & Gasser, 2018; Towbin et al., 2012).

Specific histone modifications can create binding sites for proteins called 'readers' that recognize a specific modified amino acid. For H3K9me, the prototype reader is HP1a from *Drosophila* (Clark & Elgin, 1992). In *C. elegans*, five proteins have been shown to recognize H3K9me, four containing chromodomains, HPL-1, HPL-2, CEC-3 and CEC-4, and the other one, LIN-61, with four MTB motifs. HPL-1 and HPL-2 are the orthologous of HP1, but they have different functions (Ahringer & Gasser, 2018). HPL-2 is preferentially found in distal arms of autosomes, enriched in repetitive genomic elements, and shows a strong correlation with repressive histone marks, and it have been associated with gene repression, while HPL-1 has been found in both arms and central regions of autosomes, suggesting a role in regulating gene expression, and it has been particularly related to the regulation of genes associated with male tail development. However, none of them participates in the anchoring of chromatin to the nuclear periphery, role that CEC-4 regulates as mentioned above (De La Cruz-Ruiz et al., 2023). LIN-61 can recognize H3K9me2 and me3 (Harrison et al., 2007; Koester-Eiserfunke & Fischle, 2011). It forms a complex with HPL-2 and LIN-13 (multi-zinc finger) involved in vulva development (Coustham et al., 2006). Mutations in CEC-3, HPL-2 or LIN-13 and also MET-2 eliminate the repression of the transcription factor *unc-4*, which is only

expressed in VC4 and VC5 in wildtype backgrounds and becomes expressed in all VCs with mutant backgrounds of these genes (**Fig 1.14**) (Zheng et al., 2013).

Histone methyl marks can be removed by demethylases, they can be classified in two different classes, one contains amine oxidases, such as SPR-5/LSD1, that eliminates mono and di-methylation marks, and the class that contain the Jumonji C (JmjC) domain that can eliminate the three states of methylation, but only two proteins of this group are involved in H3K9 demethylation JMJD-2/JMJD2a and JMJD-1.2/CeKDM7a (Ahringer & Gasser, 2018). *Spr-5*, *jmjd-2* and *jmjd-1.2* are required to alleviate the *unc-4* repression mark in VC4 and VC5 to allow for its expression (Zheng et al., 2013).

Importantly, although H3K9me has been traditionally associated to heterochromatin, a more dynamic role in cell type specific repression of undesired lineage-related effector genes has been recently revealed (Methot et al., 2021; Padeken et al., 2022).

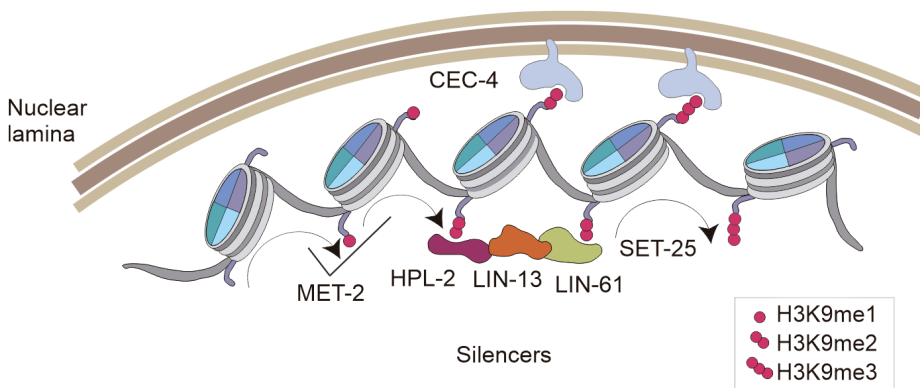


Figure 1. 14. Constitutive heterochromatin in *C. elegans*. MET-2 is responsible for H3K9me1/2 establishment, whereas SET-25 is mostly responsible for H3K9me3. HPL-2, LIN-13 and LIN-61 form a complex to bind to H3K9me1/2/3, and mediate gene repression. H3K9me2/3 is anchored to the nuclear lamina by the direct binding of CEC-4. Modified from (Gonzalez-Sandoval et al., 2015)

1.5.4. The serotonergic system of *C. elegans*

Among the 118 different neuron types present in hermaphrodite *C. elegans* five neuronal types share the use of 5-HT as neurotransmitter. This PhD thesis has a major focus in the study of the serotonergic system. Thus, in this section, I will explain it in more depth.

Serotonin (5-HT) is an ancestral signalling molecule present in all kingdoms of life, even before the emergence of animals or nervous systems, accordingly 5-HT can be synthesized through different biosynthesis pathways in fungi, plants or animals (Hay-Schmidt, 2000). In *C. elegans* the serotonergic system is defined by five neuronal subtypes, four in the head region, the bilateral NSM (neurosecretory motorneuron) ADF (amphid sensory neuron), and AIM (anterior interneuron) the unilateral RIH (ring interneuron) and one, the HSN motoneuron (hermaphrodite-specific neuron) located in the vulva region (Chase & Koelle, 2007; Desai et al., 1988; Duerr et al., 1999; McIntire et al., 1992; Sze et al., 2000). All of them are reliably immunoreactive to 5-HT antibodies.

Additionally, the hermaphrodite-specific VC4 and VC5 cholinergic motoneurons have been reported to have dim and variable staining for 5-HT (Duerr et al., 1999, 2001), but this phenotype has not been further studied due to low penetrance and low reliability (**Fig 1.15 A**). On the other hand, in males there are also some male-specific serotonergic neurons, the six CPs in the ventral nerve cord that strongly stain for 5-HT and also express the 5-HT pathway genes for its synthesis and three neurons located in the rays of the tail, R1, R3 and R9 that also strongly stain for 5-HT and are likely synthesizers, demonstrate by the expression of 5-HT synthesis gene. Also PGA neurons stain for 5-HT but they reuptake serotonin rather than synthesize it since it only expresses *mod-5* and *cat-1* genes (Loer & Kenyon, 1993; Serrano-Saiz et al., 2017; Sze et al., 2000; C. Wang et al., 2024) In addition, PVW neurons in males

also stain for 5-HT , however, these neurons do not express the genes for the synthesis of 5-HT nor the reuptaker *mod-5*, so authors proposed an alternative synthesis or reuptake mechanisms should be happening .

Finally, CEMs neurons (2 ventral and 2 dorsal) also male-specific, located in the head of the worm have been described to also weakly stain for serotonin antibody, but they do not express *bas-1* or *cat-1* , indicating that they may be producing 5-HTP rather than 5-HT (Serrano-Saiz et al., 2017; C. Wang et al., 2024) (Fig 1.15 A).

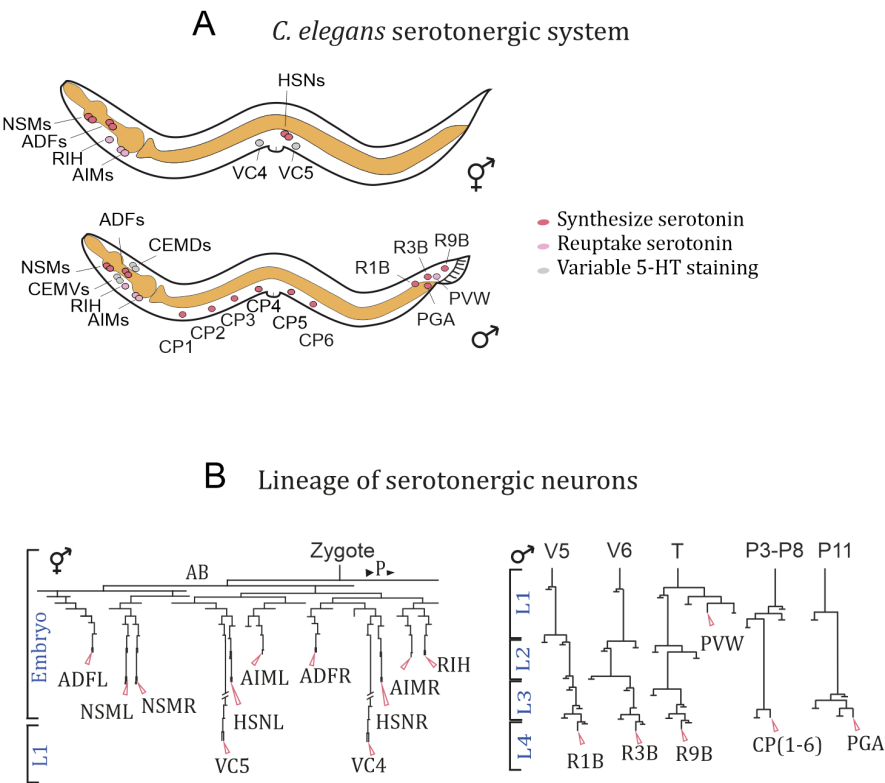


Figure 1. 15. Serotonergic system in *C. elegans*. **A.** *C. elegans* hermaphrodite (top) and male (bottom) serotonergic neurons both reuptakers and synthesizers. **B.** *C. elegans* hermaphrodite lineage of serotonergic neurons (left). All of them are born embryonically, except for VC4 and VC5 which are born in the first larval stage (L1). Male-specific serotonergic neurons lineage (right), all of them but PVW are born after hatching.

Serotonin immunostaining of neurons can be achieved in two alternative ways, either by 5-HT biosynthesis (the case of NSM, ADF, HSN, CPs and ray neurons for example) or through the external reuptake of available 5-HT provided by synthesizer neurons (AIM and RIH neurons for example). The 5-HT biosynthesis pathway in animals is a highly conserved pathway that occurs in two steps, in the first place, L-tryptophan amino acid is hydroxylated to be transformed in 5-hydroxytryptophan (5-HTP), this process is catalysed by tryptophan hydroxylase (TPH-1 in *C. elegans* and TPH1 and TPH2 in human), but it requires the cofactor tetrahydrobiopterin (BH4) that requires the enzyme GTP cyclohydrolase I (CGH1 in human or CAT-4 in *C. elegans*) to be synthesized. Then 5-HTP is decarboxylated by the aromatic amino acid decarboxylase (AADC in human and BAS-1 in *C. elegans*) to be transformed into 5-hydroxytryptamine, or serotonin (Chase & Koelle, 2007) (**Fig 1.16 A**). Recently, an alternative pathway to TPH-1 function has been found, the enzyme PAH-1, primarily involved in phenylalanine metabolism, has been described to be able to produce 5-HTP, by tryptophan hydroxylation, which must be converted to serotonin by the enzyme BAS-1 as in the classical pathway, giving BAS-1 the role of essential enzyme for serotonin synthesis. Although PAH-1 is involved in the synthesis of 5-HT as an alternative to TPH-1, it might play a more significant role in producing specific serotonin-modified metabolites, while TPH-1 is more prevalent in the synthesis of free serotonin (Yu et al., 2023).

However, not all serotonin immunoreactive neurons express this set of enzymes for serotonin biosynthesis. In the case of AIM and RIH interneurons, they are not able to synthesize serotonin but they rather re-uptake it from the extrasynaptic space, because both of them express the serotonin transporter, MOD-5 (SERT in human) (**Fig 1.15 A, 1.16 B**).

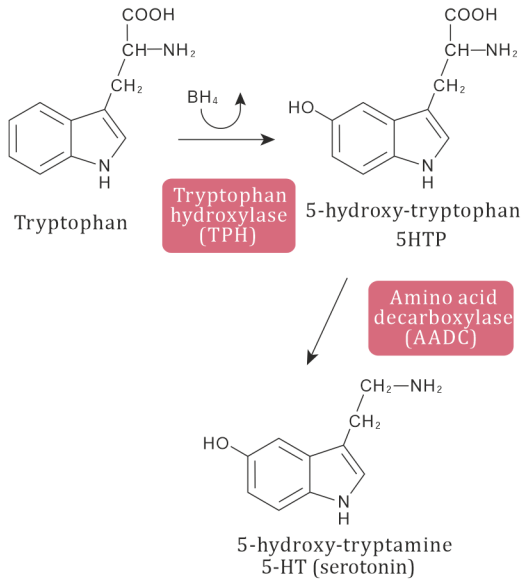
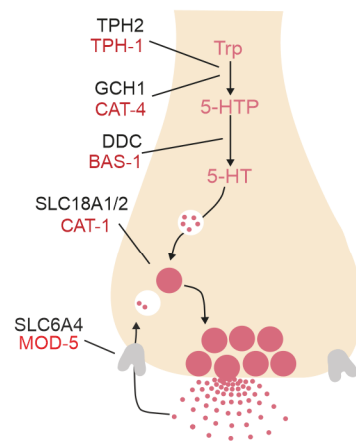
A Serotonin biosynthesis pathway**B Serotonergic neuron**

Figure 1. 16. Serotonin biosynthesis pathway **A.** Serotonin biosynthesis pathway in neurons is conserved in animals from nematodes to mammals. **B.** Serotonin pathway genes in *C. elegans*. Serotonin synthesizers neurons express TPH-1/TPH2, CAT-4/GCH1, BAS-1/DDC, essential for 5-HT synthesis, and both neurons that synthesize and the ones that reuptake express CAT-1/SLC18A1/2, the vesicular monoamine transporter, and MOD-5/SLC6A4, a serotonergic reuptaker. Human protein names are represented in black and worm names in red. Adapted from (Flames & Hobert, 2011)

These neurons lose its staining when treating the worms with Fluoxetine, a SERT inhibitor, (Kullyev et al., 2010). In the hermaphrodite NSM and ADF express MOD-5, but not HSN. In males, CEM (Cephalic male sensory neuron in the head) and PGA (Preanal Ganglion Cell) neurons are also thought to reuptake serotonin (Serrano-Saiz et al., 2017), however recently, TPH-1 (involved in synthesis) reporters have been identified in CEM, and also in R4B and R7B that also express BAS-1 (C. Wang et al., 2024).

Finally, all the neurons that are able to use 5-HT as a neurotransmitter, either by synthesis or by reuptake, also express the monoamine vesicular transporter CAT-1 (VMAT in human). CAT-1 is also expressed in the rest of monoaminergic neurons such as dopaminergic, tyraminerbic and octopaminergic neurons. In the hermaphrodite, interestingly, CAT-1 antibody show staining in VC4 and VC5 neurons, although they are cholinergic neurons (Duerr et al., 2001). More recently TPH-1 expression has also been reported in these two neurons, thus it is unclear if these neuron pair is or not serotonergic (because they do not normally show staining for 5-HT) and if so, if they are synthesizers or reuptakers (**Fig 1.15, 1.16**) (C. Wang et al., 2024).

Functions of the different serotonergic neurons in *C. elegans* are quite diverse and so are the transcription factors in charge of their terminal differentiation (terminal selectors). The NSM neurons located in the anterior pharyngeal bulb are known to have an important role in sensing the presence of food (Albertson & Thomson, 1976) and modulating locomotion, pharyngeal pumping and egg-laying behaviour (Rhoades et al., 2019; Sawin et al., 2000). Known terminal selectors for the NSM are CEH-34, TTX-3 and UNC-86 (Vidal et al., 2022; F. Zhang et al., 2014). ADF neurons in the lateral head ganglia are involved in food detection, immunity, dauer entry, fat mobilization, and chemosensation (Bargmann & Horvitz, 1991; Chang et al., 2006; Noble et al., 2013; Pocock & Hobert, 2010; Xie et al., 2013). In our lab we have found LAG-1 to be a terminal selector in this neuron (Maicas et al., 2021). The HSNs are motoneurons, that are both serotonergic and cholinergic, are the main neurons involved in egg-laying process together with VC4 and VC5 neurons (explained below). A collective of 6 transcription factors (SEM-4, AST-1, EGL-46, EGL-18, HLH-3, and UNC-86) are essential for the terminal differentiation of these neurons (Desai et al., 1988; Lloret-Fernández et al., 2018). AIM and RIH neurons are thought to modulate the activity of the behavioural circuits since they regulate the

serotonin available in the extrasynaptic media (Jafari et al., 2011). UNC-86 is again terminal selector of both types of neurons (Serrano-Saiz et al., 2013; Sze et al., 2002), while CEH-14 is known to be also a terminal selector for AIM (Pereira et al., 2015). In males, AIMS are important for the mating-searching behaviour (Barrios et al., 2012).

There are also different serotonergic receptors expressed in many other post-synaptic neurons or tissues in the body of the worm. To date, six receptors of serotonin have been identified: SER-4, MOD-1, and LGC-50, classified as driver receptors that induce locomotion and SER-1, SER-5, and SER-7 that serve as modulator receptors that regulate these effects (Chase & Koelle, 2007; Dag et al., 2023; Hamdan et al., 1999; Hobson et al., 2006; Olde & McCombie, 1997; Ranganathan et al., 2001). Analysis of mutants for these receptors has found serotonin as modulator of mechanisms like locomotion, pharyngeal pumping, egg-laying or feeding.

1.5.5. *C. elegans* egg-laying behaviour

Egg-laying is one of the simplest locomotory events a nematode can achieve (Schafer, 2005). Since *C. elegans* is a hermaphrodite organism, fertilized eggs tend to accumulate in its uterus, that start to be laid at Young Adult (YA) stage. The reference strain N2, normally has around 15 fertilized eggs in the uterus. The egg-laying process has been described to be a rhythmic behaviour transitioning with active and inactive phases. Active phases last approximately ~2 min, during which the probability of laying an egg is higher, and they are separated by a mean of 20 min of inactive phases (Waggoner et al., 1998) (**Fig 1.17 A**). However, historically, egg-laying behaviour has been studied in N2, and not in other *C. elegans* wild strains, and this is important because recent studies have found for example that the number of eggs retained is variable

within strains, finding even internal hatching (Mignerot et al., 2024; Vigne et al., 2021).

The circuit that controls egg-laying is formed by two vulva muscles, vm1 and vm2, the uv1 neurosecretory cells together with two classes of motoneurons, the HSNs, and VC1-6 (especially VC4 and VC5). In this context, HSN and VCs make direct neuromuscular synapses with vm2 muscle, which is connected to vm1 through gap junctions. The HSN, VC4, and VC5 neurons also form direct synapses with one another (Schafer, 2005). Ablation of the vm2 vulval muscles showed they were essential for egg-laying, their contraction correlates with egg-laying events (although not all calcium transients end up in egg-laying events) (Collins et al., 2016; M. Zhang et al., 2008). HSN ablation produced a reduction in the egg-laying rate, although it did not abolish the release of eggs (Desai & Horvitz, 1989). However, the role of VCs specifically VC4 and VC5, is more ambiguous, they have been related to both activation and inhibition of the circuit, on the one hand, they have been found to release acetylcholine to activate nicotinic receptors in vm2 muscles, facilitating egg release (Collins et al., 2016), however, on the other hand, they have been also linked to an inhibitory effect on the HSNs, reducing their activity (M. Zhang et al., 2008). Both HSN and VC4 and VC5 also express and release neuropeptides. Finally, uv1s cells release tyramine and neuropeptides to inhibit egg-laying (Banerjee et al., 2017; Collins et al., 2016)(**Fig 1.17 B**).

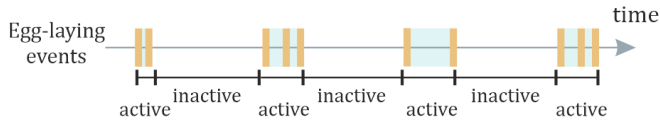
The circuit exhibits a rhythmic activity started by HSN serotonergic release that initiates the active state. These neurons show long calcium transients that are linked to egg-laying events. In contrast VCs exhibit shorter calcium transients that peak just before the egg is released, however, their activity is not enough to allow the egg-laying event. VCs activity is more related to the modulation of

locomotion during egg-laying events, as the two processes seem to be tightly synchronized (**Fig 1.17 B**) (Collins et al., 2016).

The main neurotransmitters controlling egg-laying are serotonin and acetylcholine and both are thought to function as co-transmitters at egg-laying neuromuscular synapses. Acetylcholine receptors are found in HSN, vm1/2 and uv1 leading to both activation or repression of egg-laying. Although stimulation of nicotinic receptors with levamisole showed an increase in egg-laying, the stimulation of both nicotinic and muscarinic receptors led to an inhibitory effect (Bany et al., 2003). Indeed, overexpression of ACh receptor *gar-1*, showed an accumulation of unlaidd eggs that could suggest an inhibitory effect acting in vm1 and vm2, authors also proposed that ACh activation role could happen through inhibiting uv1 cells that in turn will not inhibit HSNs (Fernandez et al., 2020). (**Fig 1.17 B**).

Serotonin seems to have a prevalent role in the circuit since exposure to exogenous serotonin induces an increase in the number of eggs laid even in the absence of HSN or VCs (Shyn et al., 2003). 5-HT receptors such as *ser-1* and *ser-7*, essential for egg-laying response in the presence of serotonin, are expressed in vm2 and act as activators (Fernandez et al., 2020; Hapiak et al., 2009). *ser-7* is also expressed in VCs which could explain the activation of these neurons through 5-HT release from the HSN, and finally, this receptor is also found in HSN, suggesting an autoactivation. Surprisingly serotonin receptors were also found in cells that do not directly receive HSN synapsis, such as vm1 and um1/2 muscles cells, meaning that HSN serotonin may travel extrasynaptically to get to these other cells and induce egg-laying (Fernández et al., 2020).

A *C. elegans* egg-laying dynamics



B *C. elegans* egg-laying circuit

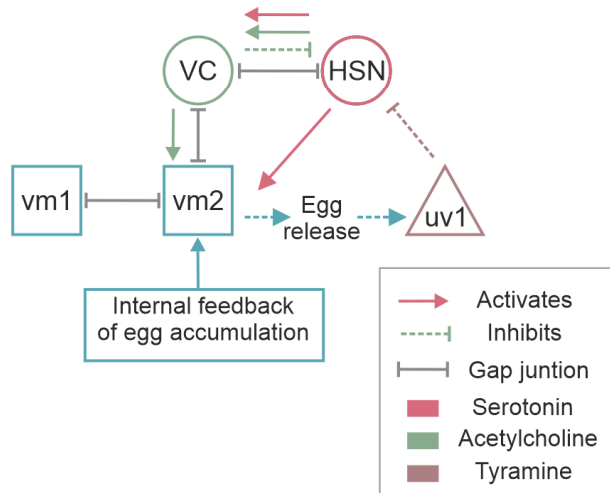


Figure 1. 17. Egg-laying behaviour in *C. elegans*. **A.** *C. elegans* egg-laying behaviour transitions between active and inactive states. Egg-laying events are represented by orange lines, located inside active states represented by blue squares. **B.** Egg-laying circuit. HSN and VC motoneurons, together with UV1 neurosecretory cells use different neurotransmitters and neuropeptides to activate or inhibit egg laying behaviour through the activation of vulval muscles (vm2/vm1) or the autoregulation of the mentioned neurons. Modified from (Collins et al., 2016).

Despite the predominant activator role of 5-HT, it also has an inhibitor role, through SER-4 receptor, which mutation leads to fewer eggs retained, and mutants were hyperreactive to egg-laying upon serotonin exposure (Hobson et al., 2006). However, its expression have been only observed in vm2 and more weakly in vm1 (Hobson et al., 2006).

In summary, although the egg-laying circuit was initially considered one of the simplest motor behaviours, it is now clear that its function and tight regulation is far from simple. It is a highly modulated circuit that is regulated by internal signals of different nature; such as the number of eggs in the uterus that have been proposed to induce egg laying through mechanical activation of vulval muscles (Medrano & Collins, 2023) or nutritional state; and external cues, like presence of food or different stressors, such as hypoxic conditions, osmotic stress or presence of pathogens (Mignerot et al., 2024; Schafer, 2005). In addition, there is high interconnectivity and feedback among the circuit components with combined short-range synaptic (ACh) transmission and diffusible extrasynaptic modulation (5-HT and neuropeptides).

1.5.6. Evolutionary studies in other *Caenorhabditis* nematodes

Although *C. elegans* has become the most used nematode as a model organism, there are other *Caenorhabditis* species that have been the subject of study. To date, over 70 different species have been discovered (Barrière & Félix, 2014; Dayi et al., 2021; Kiontke et al., 2011; Sloat et al., 2022; Stevens et al., 2019). Importantly all species in the genus seem to share a deeply conserved embryonic cell lineage, generating the same complement of cells roughly in the same locations (Memar et al., 2019; Z. Zhao et al., 2008). However, comparative studies have reveal both similarities and differences in cells sharing location since variations in lineage specification and cell fate determination have been observed in related species in developmental mechanisms (Haag et al., 2018). These species also show a variety of life-styles and habitats that have induced genomic, morphological, and behavioural adaptations (Kiontke & Sudhaus, 2006). These characteristics, combined with the easiness of maintaining them in the laboratory and the deep comprehension of *C. elegans* species, make the

Caenorhabditis genus a useful tool to explore how genetic, developmental, and ecological factors interact over evolutionary timescales.

Caenorhabditis species have been classified as generalist or specialist species, based on the degree of ecological specialization (Futuyma & Moreno, 1988). The generalist group is composed by *C. briggsae*, the most common species across the globe, *C. elegans* and *C. remanei*, found in temperate regions and *C. tropicalis* and *C. brenneri* found in tropical areas, all of which are often found in human-associated niches. (Kiontke et al., 2011). Specialist species display strong specificity in their ecological niches, for instance, *C. bovis* was isolated from the ears of cattle at 37°C (Kreis, 1964; Stevens et al., 2020), or *C. inopinata* is found in fresh figs and its dispersal between fruits seems to depend on pollinating wasps (Kanzaki et al., 2018; Woodruff & Phillips, 2018).

Many different evolutionary studies have been performed involving this genus. For example, there is extensive work on the evolution of mating systems within the *Caenorhabditis* genus, since three species have independently derived to androdioecy (*C. elegans*, *C. briggsae* and *C. tropicalis*) (Thomas et al., 2012). Molecular genetic studies have found that only the mutation of *fog-2* in *C. elegans* is sufficient to transform self-fertile hermaphrodites into spermless females (Schedl & Kimble, 1988); while lowering the expression of *tra-2* and *swm-1* allow the transformation of *C. remanei* females, in a self-fertilizing worms (Baldi et al., 2009). Other studies have focused on how the development of different tissues have changed through evolution, such as the specification of the vulva. In *C. elegans* inappropriate expression of LIN-3/EGF due to mutations in redundant synMuv (Synthetic Multivulva) genes give rise to a multivulva phenotype (Cui et al., 2006). Surprisingly, in *C. briggsae* also ectopic expression of *lin-3* led to multivulva phenotype, but the genes involved in the repression of it, which are noncanonical histone HTZ-1/H2A.z and three nematode-specific

proteins, exhibit distinct functions and genetic interactions compared to their orthologs in *C. elegans* (Chamberlin et al., 2020).

Recently, development of bioinformatic tools that allows to analyse big data together with better sequencing techniques, has enable to perform comparative genomics between different species, for example Stevens et al. (2019) analysed the genome of 10 species and found for example that *C. sulstoni* has the smallest genome (65.1 Mb) known in the genus, or that *C. parvicauda* has the fewest predicted genes (~16400) and *C. waitukubuli* has the higher (~30000)(Stevens et al., 2019). Moreover, we can get information about non-coding elements in the genome of *Caenorhabditis* species that can be related to the regulation of expression of genes, as in *C. inopinata* in which changes in conserved non-coding elements have been associated with body morphology and behaviour (Tamagawa et al., 2024). Also, analysis of Single-Cell RNAseq data in *C. elegans* has allowed us to get a better understanding of the transcriptome of certain cell types but also in different developmental stages (Cao et al., 2017; Roux et al., 2023; Taylor et al., 2021). This approach is now being expanded to other organisms such as *C. briggsae* (Large et al., 2024). These datasets will be extremely useful for comparative molecular studies and the study of GRN evolution.

Another important resource is the use of diverse wild isolates of *C. elegans*, which also shows great genetic diversity. The *Caenorhabditis* Natural Diversity resource (CaeNDR) (Crombie et al., 2024) has become a very useful tool for the acquisition of information both genomic and ecological of the different isolates for studies exploring natural variation and evolutionary processes (Cook et al., 2019; Crombie et al., 2024). The expansion of *C. elegans* in diverse geographic and ecological environments (all continents) offers an enormous reservoir of natural genetic variation associated with different traits (Crombie et al., 2019,

2024). For instance, sensitivity to external zinc was found in a *C. elegans* strain isolated in Hawaii (CB4856), while the laboratory-derived N2 was resistant, and it was identified a naturally deletion in the gene *sqst-5* associated with resistance to high exogenous Zinc (Evans et al., 2020). There is also pheromone response variation linked to *srg-37* gene (Lee et al., 2019). More recently, a study related to intra-uterine egg retention in different wild isolate revealed a huge variability with examples of even internal hatching. Such phenotypes have different evolutionary origins, suggesting that strong egg retention could be a plastic adaptative response and confer a competitive advantage in limited-food or harsh environments (Mignerot et al., 2024). Evolutionary studies comparing wild isolates and N2 have also been important to reveal multiple mutations in N2 derived laboratory strain. For instance, fixation of alleles for the genes *npr-1*, *glb-5*, and *nath-10* altered traits that could affect experimental interpretation (Sterken et al., 2015).

Finally, non-*Caenorhabditis* nematode species have also been studied in different contexts and to a different extend. For example, *P. pacificus* (Sommer, 2006) has been established as a satellite model species to study the evolution of vulval developmental mechanisms in comparison to *C. elegans* over large evolutionary distances which has revealed significant variations in the induction mechanisms and competence of vulval precursor cells (VPCs) between these two species (Zauner & Sommer, 2007). Another recently described genus *Auanema*, closely related to *Caenorhabditis*, has found to exhibit a unique reproductive polyphenism where the mode of reproduction (female vs. hermaphrodite) is determined by developmental conditions (Kanzaki et al., 2017). Also, different parasitic nematodes with biomedical or economic impact have also been studied, such as the *Brugia malayi* species that causes human filariasis, a debilitating disease affecting millions of people, and hosts *Wolbachia* bacteria as obligate symbionts. It has been found that treating

these nematodes with antibiotics kills the *Wolbachia*, disrupting the reproductive cycle of the nematode and becoming a promising strategy for controlling filariasis (Slatko et al., 2010).

1.6. Previous unpublished results related to this PhD project

This project builds on the work presented by Dr. Carlos Mora in his thesis. Here, I summarize previous results he obtained. Additionally, some of the experiments I performed were initiated by Dr. Mora; in order to provide better continuity of the story I decided to include those experiments in the result section, where the contribution from Dr. Mora is in all cases, clearly stated.

1.6.1. Serotonergic diversity in the *Caenorhabditis* genus

When this project was initiated by Dr. Mora, the serotonergic system in the *Caenorhabditis* genus had not been fully characterized. He performed an intense characterization of the serotonergic phenotype in this genus (**Fig 1.18**), and he could observe that the serotonergic staining in the head remained highly conserved among species, even though RIH was not stained in *C. angaria* consistent with previous studies (Rivard et al., 2010). Other species also showed variable staining in RIH, ADF or AIM (this variability could be technical, due to differences in cuticle strength). HSN neurons, located near the vulva, were also consistently stained for serotonin, except for *C. monodelphis*. However, the most robust difference observed was the strong staining of two somas located in the vulva region present only in the *Angaria* group of species composed of five species, *C. angaria*, *C. castelli* (sp. 12), *C. quiockensis* (sp. 38), *C. sp. 8* and *C. sp. 24*. The somas are located in a region where in *C. elegans* only two neurons are present VC4 and VC5 cholinergic motor neurons, what made the identification of these two neurons a very easy task. In addition, this phenotype had already been described, for *C. angaria* species (Loer & Rivard, 2007). Thus, Dr. Mora concluded the most likely explanation is that in the ancestral species of *Angaria* group acquired the serotonergic phenotype. To have a more comprehensive view of 5-HT immuno staining in the genus, in my thesis work I have completed staining for additional species such as *C. remanei*,

C. tropicalis, *C. doughertyi*, or *C. inopinata*, and repeated some species such as *C. kamaaiina*, *C. angaria*, *C. briggsae* or *C. elegans* with comparable results to the ones shown previously (Fig 1.18) (Annex 4.1)

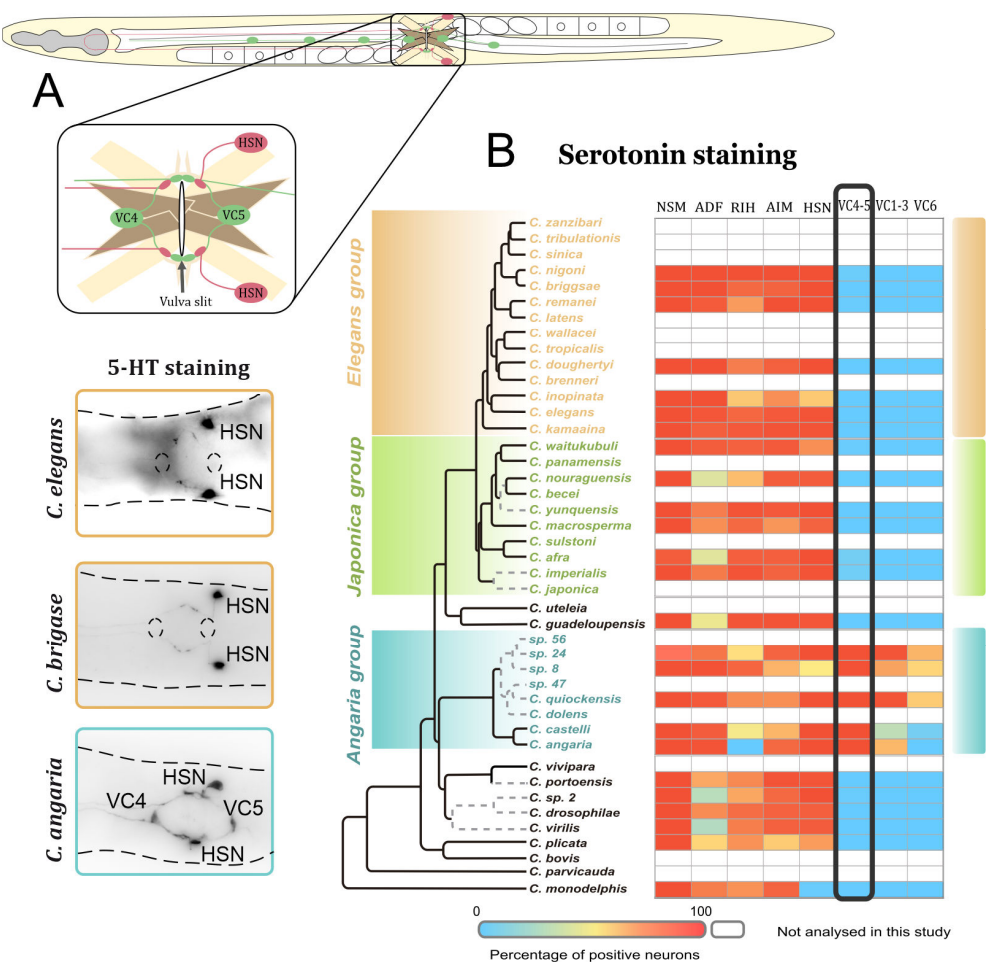


Figure 1.18. 5-HT staining in *Caenorhabditis* genus. **A.** Relative position of VC4, VC5 (green circles) and HSNs (pink circles) in the body of the worm. The yellow and brown structures represent the vulval muscles. **B.** Analysis of the serotonergic system in different species of *Caenorhabditis* genus. *Angaria* group of species show a defining serotonergic phenotype in VC4 and VC5 neurons —representative images of *C. elegans*, *C. briggsae* and *C. angaria* 5-HT staining are shown (left).

1.6.2. VC4 and VC5 neurons in *C. angaria* are serotonergic reuptakers

As described in the introduction, there are two types of serotonergic neurons in the nematode *C. elegans*: those that synthesize 5-HT, expressing the enzymes required for serotonin biosynthesis (*tph-1*, *cat-4*, and *bas-1*), such as NSM, ADF, and HSN neurons, and those that reuptake 5-HT from the environment (expressing *mod-5/SERT* and *cat-1/VMAT*), which are RIH and AIMs neurons (Jafari et al., 2011; Kullyev et al., 2010).

Intriguingly, VC4 and VC5 neurons in *C. elegans* expressed the monoamine vesicular transporter *cat-1/VMAT*, although the function of this aminergic gene in otherwise cholinergic neurons is so far unknown. VC4 and VC5 neurons are located near the HSN serotonin producer neuron, thus our working hypothesis for 5-HT staining of VC4 and VC5 neuron in *Angaria* species, was that they could have become reuptakers. To test this hypothesis, we collaborated with Dr. Arantza Barrios in UCL and Dr. Carla Lloret-Fernández, another past member of the lab, performed laser ablations of the HSN neurons in *C. angaria*. They found that the elimination of these two neurons was enough to abolish the serotonergic phenotype found in VC4 and VC5 in *C. angaria*. This result supported the hypothesis of VC4 and VC5 neurons in *C. angaria* being 5-HT reuptakers (**Fig 1.19 A**).

1.6.3. *C. elegans cat-1* expression in VC4 and VC5 is dependent on the pro-neural factor HLH-3/Ascl1 and the homeobox UNC-4/UNCX.

In *C. elegans*, VC4 and VC5 cholinergic motoneurons are born post embryonically at the end of L1 stage, but they remain undifferentiated until the end of L3, when they start to extend their processes (**Fig 1.15 B**) (Sulston et al., 1980). At the end of L4 stage they complete their maturation and acquire

terminal morphology and start to express effector genes necessary for their function. These two particular neurons are located anterior-posterior to the vulva and close to the serotonergic HSNs. Generation of VCs from the P3.a to P8.a lineages and achievement of their terminal fate is dependent on LIN-39 and LIN-11, while VC4 and VC5 is dependent on EGF signalling from vulva and UNC-4 TF (Freyd et al., 1990a; B. B. Wang et al., 1993; Zheng et al., 2013).

As already stated, *C. elegans* VC4 and VC5 cholinergic neurons have been reported to exhibit unreliable 5-HT staining and constitutively express *cat-1*, which is involved in monoaminergic metabolism since codes for monoamine vesicular transporter. Dr. Mora also characterised how the expression of *cat-1* is regulated in *C. elegans*.

hlh-3 is a bHLH proneural factor that has already been described as necessary for VC4 and VC5 differentiation (Perez & Alfonso, 2020), thus Carlos determined that *hlh-3* was also essential for *cat-1* expression in VC4 and VC5 in *C. elegans*. In addition, *unc-4* a homeodomain transcription factor is also required in VC4 and VC5 neurons for proper expression of terminal effector genes (Lickteig et al., 2001), and Dr. Mora determined *unc-4* is also necessary for the expression of *cat-1* in *C. elegans* VC4 and VC5 (**Fig 1.19 B**). These results are consistent with the observation described by Perez and Alfonso, 2020 in which *hlh-3* is required for the correct expression of *unc-4* in VC4 and VC5 neurons.

1.6.4. The serotonergic fate of VC4 and VC5 is actively repressed in *C. elegans* by cell-autonomously mono and di-methylation of H3K9.

As part of Dr. Mora's project, he tested many environmental conditions to try to identify cues that could trigger a strong serotonergic phenotype in *C. elegans* VC4 and VC5 neurons (Mora-Martínez, 2019), however, none of the stimuli was successful in triggering such transition. Given the important role of epigenetic factors in controlling cellular transitions (Klosin et al., 2017), we reasoned epigenetic factors could be regulating this 5-HT phenotype.

Through a candidate approach, Dr. Mora found that the serotonergic phenotype of *C. elegans* VC4 and VC5 neurons is actively repressed by mono and di-methylation of H3K9 and repressor recruitment, a classic mark for heterochromatin more recently involved in lineage refinement (Destain et al., 2024). This conclusion was driven by the analysis of predicted null alleles for histone methyltransferases *met-2* and *set-25* and the ortholog of mouse H3K9me readers, Heterochromatin Protein 1 (HP1), *hpl-2*, and a Malignant Brain Tumor (MBT) protein *lin-61*. Serotonin staining in these strains showed a significant increase in the percentage of strong VC4 and VC5 signal (**Fig 1.19 C, H**). Dr. Mora also demonstrated that *hpl-2*, *lin-61*, and *met-2* act cell-autonomously in VC4 and VC5 neurons to restrain serotonergic phenotype in VC4 and VC5 in *C. elegans* (Mora-Martínez, 2019), moreover, he showed that H3K9 methylation during L4 (stage in which VC4 and VC5 mature) is critical for the repression of the serotonergic phenotype (**Fig 1.19 D, H**).

Thus, we could conclude that in *C. elegans* VC4 and VC5 serotonergic phenotype is actively repressed by H3K9 epigenetic modifications.

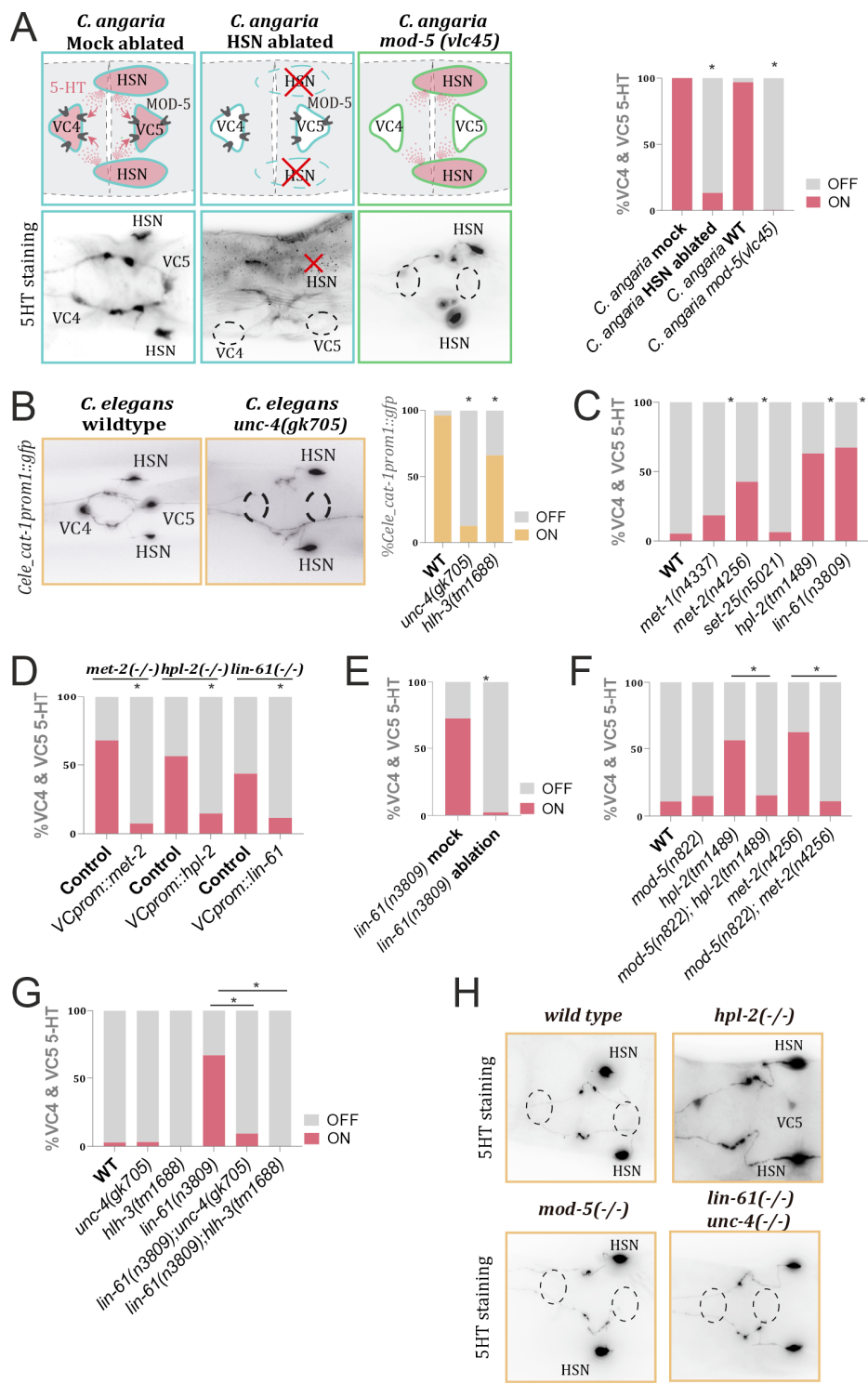


Figure 1.19. Previous results. **A.** *C. angaria* serotonergic phenotype is dependent on *mod-5* reuptake of the serotonin released by the HSN. Images and quantification of the serotonergic phenotype of VC4 and VC5 in *C. angaria* WT, with HSN ablation and *mod-5(vlc45)* mutant **B.** In *C. elegans*, UNC-4 HD is required for the expression of *cat-1* in VC4 and VC5 neurons. Images and quantification of *Cele_cat-1prom1::gfp* expression in *C. elegans* WT and *unc-4(gk705)* mutant **C.** *C. elegans* serotonergic phenotype in VC4 and VC5 is restricted by H3K9me2 and me3. Quantification of serotonergic phenotype of *C. elegans* WT, *met-1(n4337)*, *met-2(n4256)*, *set-25(n5021)*, *hpl-2(tm1489)* and *lin-61(n3809)*. **D.** H3K9 methylation effect in restricting 5-HT fate in VC4 and VC5 is cell autonomous. Quantification of *met-2(n4256)*, *hpl-2(tm1489)* and *lin-61(n3809)* mutants and VCs cell autonomous rescues. **E.** VC4 and VC5 H3K9me mutant serotonergic phenotype is dependent on HSN serotonergic phenotype **F.** VC4 and VC5 H3K9me mutant serotonergic phenotype is dependent on *mod-5* **G.** *unc-4* and *hlh-3* are required for the VC4 and VC5 H3K9me mutant serotonergic phenotype. **H.** Images of VC4 and VC5 phenotype in *C. elegans* WT, *hpl-2(tm1489)*, *mod-5(n822)* and double *lin-61(n3809);unc-4(gk705)* mutant background

1.6.5. VC4 and VC5 serotonergic phenotype in H3K9me deficient *C. elegans* depends on serotonin reuptake.

Dr. Mora also explored the nature of the VC4 and VC5 serotonergic phenotype in H3K9me pathway mutants. First, HSN laser ablation experiments in *C. elegans* *lin-61* mutants show similar results as *C. angaria* ablation, supporting a similar 5-HT reuptaker phenotype for VC4 and VC5 in these mutants (**Fig 1.19 E, H**). The only serotonin reuptaker gene known in *C. elegans* genome is *mod-5*, thus Dr. Mora analysed *met-2(4256); mod-5(n822)* and *hpl-2(tm1489); mod-5(n822)* double mutants showing a significant reduction in VC4 and VC5 serotonin staining compared to *met-2(4256)* or *hpl-2(tm1489)* single mutants, respectively. Moreover, expression of *mod-5* cDNA specifically in VC4 and VC5 is enough to become serotonin immunoreactive (**Fig 1.19 F, H**).

Altogether, these results suggest that in *C. elegans* N2 strain epigenetic repression in VC4 and VC5 leads to repression of *mod-5* locus, preventing the serotonergic phenotype under laboratory conditions.

1.6.6. VC4 and VC5 serotonergic phenotype in H3K9me deficient *C. elegans* depends on *hlh-3* and *unc-4*.

Finally, Dr. Mora also determined that, in addition to their role in VC4 and VC5 correct specification and *cat-1* expression, *hlh-3* and *unc-4* are also required for 5-HT staining in H3K9me deficient mutants. This was demonstrated through serotonin staining analysis in *lin-61* mutants crossed to *hlh-3* and *unc-4* mutants, that show loss of the serotonergic phenotype in VC4 and VC5 neurons. This phenotype could be due to *unc-4* and *hlh-3* effect on *mod-5* expression or alternatively their action could be only on *cat-1* as these factors are required for *cat-1* expression in VC4 and VC5 (**Fig 1.19 G**).

In summary, when I started my PhD project we knew 1) VC4 and VC5 neurons in *Angaria* group of species have acquired the ability to stain for 5-HT, through reuptake from HSN, likely due to constitutive *mod-5* 5-HT transporter expression in these neurons; 2) In *C. elegans* VC4 and VC5 have a partial 5HT phenotype (*cat-1* expression), which is controlled by *hlh-3* proneural TF and *unc-4* homeodomain TF, 3) Epigenetic modifications in *C. elegans* prevent the full serotonergic phenotype in VC4 and VC5 likely through direct or indirect repression of *mod-5* locus.

2. OBJECTIVES

In this thesis, we aim to study the molecular evolutionary changes that took place in the *Angaria* group of species concerning the serotonergic phenotypes of VC4 and VC5 neurons and what could be the functional behavioural relevance of the acquired trait. Regarding *C. elegans* repression of VC4 and VC5 serotonergic phenotype, I wanted to explore if environmental signals release that repression which would demonstrate that the trait is indeed plastic. Finally, I also wanted to explore the molecular mechanisms for the evolution of other neuronal phenotypes specifically focusing on CA male neurons.

Specifically, my PhD thesis is subdivided my PhD project in four main objectives:

- 1) Molecular characterization of GRN changes that underlie *Angaria* VC4 and VC5 serotonergic phenotype.
- 2) Egg laying behavioural analysis of different *C. angaria* and *C. elegans* strains to understand the physiological role of the acquired trait in *Angaria* species.
- 3) Study of the possible plastic nature of VC4 and VC5 serotonergic phenotype in *C. elegans*: identification of environmental triggers and functional relevance.
- 4) Description of the emergence of another novel trait in *Angaria* species: CA male specific neuron serotonergic phenotype.

3. MATERIAL AND METHODS

3.1. *Caenorhabditis* strains and culture.

3.1.1. Strains and standard growth

The *C. elegans* wildtype strain used was N2 (Bristol strain). N2 and all mutant strains were obtained from the *Caenorhabditis* Genetics Center (CGC), except for *mod-5* null allele *knu383*, which was bought from Knudra Transgenics, and wild isolate strains that were requested to CaeNRD (*Caenorhabditis* Natural Diversity Resource). *C. angaria mod-5(vlc45)* was generated in our laboratory and *mod-5(mod-5(vlc47, syb7715))* was designed in our laboratory and generated by SunnyBiotech. All non-*C. elegans* species were also obtained from the CGC except for *C. sp. 24* and *C. sp. 39*, which were kindly provided by Karin Kiontke at New York University.

A list of all strains used is included in **Annex 3.1**.

For standard growth conditions, all species were cultured in Nematode Growth Medium (NGM, see **Materials**), in agar plates (60 x 15 mm, no vents, Corning #BP50-01) seeded with *E. coli* OP50. Before immunohistochemistry or other experiments, all strains were kept at 20°C unless indicated otherwise.

3.1.2. Semi-liquid media

The semi-liquid media, known as Nematode Gelrite (now Gelzan) Growth Medium (NGG) was prepared as previously described (Gilarte et al., 2015; Muschiol & Traunspurger, 2007). It is analogous to the standard Nematode Growth Medium (NGM)(Brenner, 1974) but bacto agar is replaced by 1.5 g of gellan gum per liter medium (Gelzan™ CM; PhytoTech, #G3251). Briefly, freshly prepared NGG is autoclaved, and during the cooling period, CaCl₂, MgSO₄, KH₂PO₄, and cholesterol were added aseptically. Then 5mL of NGG was directly poured into agar plates for control conditions or was previously supplemented

with 10mM of Serotonin creatinine sulfate monohydrate (Sigma-Aldrich, #H7752) for NGG-5HT growth media. Immediately after pouring, *E.coli* OP50 lawn scraped from two seeded NGM plates was mixed well with NGG to provide food in the media. The resulting media has a semi-liquid consistency, where worms are embedded within media in the 3 dimensions and can transition from swimming to locomotion without sinking.

3.1.3. Population synchronization without bleaching

This protocol was adapted from Emerson and Lee, 2022. Briefly, 12 gravid hermaphrodites were placed in 55 mm NGM plates seeded with OP50 and incubated overnight (12-20h) at 20°C. The mothers were removed the following day and plates were incubated at 20°C. Embryos were grown until day 1-2 of adulthood (3 days) when they start laying eggs. Then plates were washed 3 times with M9 1X (see **Materials**) to remove all adults. After that 1mL of M9-0.5%Tween was added to each plate, and they were scratched to recover as many eggs as possible and transferred to 1.5 mL Eppendorfs. The eggs were washed with M9-T until the supernatant is clear and then another 3 washes were performed with M9 1 X. Finally, eggs were seeded in new plates and incubated until the desired stage.

3.1.4. Population synchronization with bleaching

Well-fed, gravid worms were collected from 4 different NGM plates with M9 1X buffer and transferred to 1.5 mL Eppendorf tubes. Tubes were centrifuged at 2,000 rpm for 1 minute at 4°C and worms were washed 3 times with M9 1X buffer to remove bacteria. Then egg-preparation solution (see **Materials**) was added at a 1:1 volume corresponding to the worm pellet. Then worms were vortexed for 2-4 minutes depending on the strain and checking for bleaching

disintegration of adult bodies under the scope. Then tubes were filled with ice-cold M9 1X to dilute the bleaching action, and tubes were centrifuged at 3,500 rpm for 2 minutes at 4°C and the supernatant was discarded. Washes were repeated 4 times. Finally, tubes were filled with 1 mL of M9 1X buffer at room temperature and incubated for 12 to 18 hours on a rocking nutator at 20°C before plating the hatched larvae on fresh NGM plates.

3.2. Immunohistochemistry

3.2.1. Serotonin immunohistochemistry tube fixation

The protocol was adapted from (McIntire et al., 1992). Mixed worm populations were used unless indicated. Worms were collected with M9 1X (**table 3.4**) in 1.5 mL DNA LoBind tubes (Eppendorf, #0030108051), washed with M9 1X 3 times or until the supernatant was clear, for each wash worms were centrifuged for 1 min at 950 rpm, and then a last wash with dd (double destiled) water was performed before fixation in 1 mL 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 12-14 h at 4°C while shaking in a nutator. After fixation, worms were washed 3 times with wash solution, TrPBS (0.5% Triton X-100 in PBS) and incubated for 12-14 h with a solution containing 5% β -mercaptoethanol, 1% Triton X-100 and 0.1 M Tris (Tris stock 1M and pH 7.5), at 37°C while shaking on a nutator.

The next day, worms were rinsed 3 times in TrPBS and once in collagenase buffer. Afterward, worms were treated with collagenase type IV (Sigma, #C5138) for 1 h 20 min (1mg of collagenase in 1mL of collagenase buffer), at 37°C with shaking at 650-700 rpm followed by 30min at 37°C without shaking. After collagenase treatment, 3 washes were performed with TrPBS.

Worms were incubated in blocking solution (TrPBS, 1%BSA) (Sigma-Aldrich, #05470) for 1 hour at room temperature, without shaking. After blocking

solution removal, rabbit anti-5-HT antibody (Sigma, #S5545) diluted 1:5000 in 1 mL PGT (**table 3.4**) was added. Worms were incubated in primary antibody for at least 24 h at 4°C without shaking.

The last day worms were rinsed five times, tubes were inverted but no shaking was applied (20 min per wash) in TrPBS with 0.1% BSA and incubated with the secondary antibody (Alexa 555-conjugated donkey anti-rabbit (Molecular Probes, #A31572) diluted 1:1000 in the same medium as primary antibody, for 3 h at room temperature in darkness. Finally, worms were washed five more times (20 min each) in TrPBS with 0.1% BSA and mounted with FluorSave (Merck Millipore, #34578920ML) or ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen, # P36966) on standard microscope slides (Rogo Sampaic #11854782) and covered with cover glasses (Corning, #CLS284525). One day after, mounted slides were sealed with common nail polish to avoid dryness. Samples were visualized within a few days under a Zeiss Axioplan 2 epifluorescence microscope.

3.2.2. VMAT/ChAT immunostaining freeze-cracking

This protocol was performed using the freeze-cracking protocol provided by Marie Gendrel (adapted from (Duerr et al., 1999)). Worms were collected with M9 1X in a 1.5 mL Eppendorf then washed 3 times or until supernatant was clear and then a last wash with dd water before resuspending the pellet in 80 µl of 0.025 % glutaraldehyde in PBS 1X. Then worms were placed in a SuperFrost slide, covered with another slide perpendicularly, slightly pressed, and then put in dry ice for at least 20 min.

With a quick flick of the wrist, the slide sandwich cracked. Next, slides were incubated in ice-cold methanol for 2 min and then transferred and incubated for another 4 minutes in ice-cold acetone. Then slides were placed in 50 mL

falcon tubes with 40 mL of PBS 1X and worms were rinsed off the slides with a Pasteur pipette.

Then worms were pelleted by spinning for 2 min at 1500 rpm and transferred to a 1.5 mL Eppendorf after pipetting off the supernatant. Worms were incubated in blocking solution (TrPBS, 1%BSA) for 1 hour at room temperature. After blocking solution removal, goat anti-VMAT and mouse anti-UNC-17 antibodies (kindly provided by Janet Duerr) were diluted 1:1000 in PGT and added. Worms were incubated in primary antibody for 24 h at 4°C without shaking.

From this point, secondary antibody incubation and slide preparation were similar to 5-HT immunostaining protocols. Secondary antibodies used were Alexa 488 for UNC-17, Alexa 555 for VMAT.

3.2.3. Immunostaining after semi-liquid culture

Before fixing, the semi-liquid media with worms from a plate was transferred to 50 mL falcons and approximately 20 mL of M9 1X was added to the falcon and mixed by agitation, the media was filtered with 20 µm filters (pluriStrainer, #43-50020-50). After all the media had passed through, the filter was put upside down in another 50 mL falcon, and clean M9 1X buffer was added to the filter to recover the worms. The falcon with the worms is filled with M9 1X - 0.05%Tween till 30 mL and put in a tube revolver (ThermoFisher #88881001) at 20 rpm for 20-30 minutes. Next worms are let to settle down and supernatant is removed and fresh M9 1X - 0.05% Tween is added. Washes are repeated at least 4 times. Finally, worms were transferred to a 1.5 mL LoBind tube, and the immunostaining was performed as indicated above.

3.3. Cloning and reporter strain generation

3.3.1. Cloning into pPD95.75

To build reporter constructs, genomic DNA of the corresponding species was obtained using QIAGEN DNeasy (QIAGEN #69504) and resuspended in nuclease-free water (Sigma, #W4502). Regions of interest were PCR amplified and introduced by standard cloning into the multi-cloning site (MCS) of pPD95.75 plasmid which also contains the GFP coding sequence, the muscle myosin unc-54 3' UTR, and seems to have a basal promoter in the synthetic intron ahead of the gfp (Addgene Plasmid #1494) (**Fig. 3.1**).

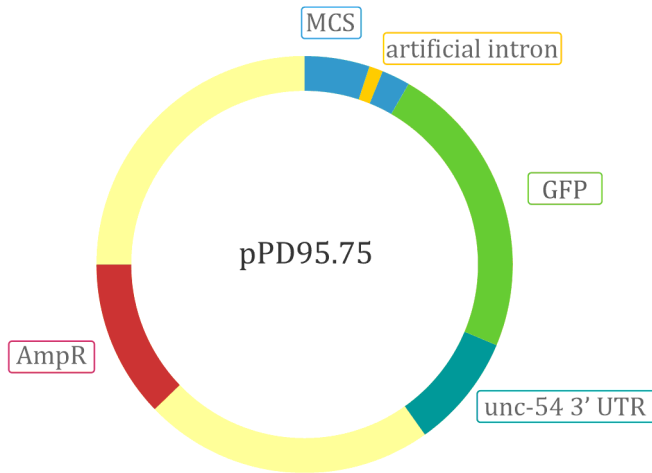


Figure 3. 1. Schematic of the pPD95.75 plasmid. MCS: multiple cloning site, where *cis*-regulatory modules (CRM) are cloned.

To obtain the *Cvir_mod-5prom1* insert, after several attempts we failed to amplify the region of interest, thus we ordered it by gene synthesis [Biomatik Corporation (Ontario, CA)]. Primers used in **table 3.1**

Once the construct was cloned, plasmid DNA was purified using QIAprep Spin Miniprep Kit (QIAGEN #27106) and resuspended in miliQ water (Sigma,

#W4502). The resulting plasmid DNA was sent to Macrogen to sequence for verification.

Table 3. 1. Primers used for *C. virilis mod-5* intron cloning

Target	Oligo name	Direction	Sequence
<i>C. virilis mod-5 intron1</i>	oc699	Fwd	ATGTTACGGTGGCATTTCGGTAC
	oc800	Rev	GCATACTGTTAGCACCGCTCGAC

3.3.2. Site directed mutagenesis.

To test for the functionality of TFBS, we designed specific mutations of predicted TAAT consensus homeodomain binding sites found in the minimal promoter region of *C. angaria mod-5* (*Cang_mod-5prom9*). Primers used for the site-directed mutagenesis are indicated in **Table 3.2**.

In the case of *C. ang_mod-5prom9X3HD*, we asked for a GBlock to IDT company, containing the 3 mutated sequences and then it was also cloned by standard cloning into the pPD.95.75.

Table 3. 2. Primers used for directed site mutagenesis

Template promoter	Resulting promoter	Oligo name	Direction	Sequence
<i>Cang_mod5prom9</i>	<i>Cang_mod5prom9#HD1</i>	oAM63	Fwd	GAAATAAGCTTGCATGCC
				TGCAGGAcTAGGCCCGAT
				TTTAGGC
		oAM64	Rev	CCTATAAGAGCCTAAAAT
				CGGGCCTAgTCCTGCAGGC ATGC

Cang_mod5p rom9	<i>Cang_mod5 prom9#HD</i> 2	oAM65	Fwd	GAGCCCAAAATTTTAGGA TTTTTCCAGccTATTTTGTG GGATC
		oAM66	Rev	GCACGTTTTTGGACTTTT TGATCCCAAAAATAggCTG GAAAAATCC
	3	oAM67	Fwd	CCGCAAAACCGCAGCCTG AGATAAccTACCCTCACAT TTTTTC
		oAM68	Rev	GATGAATGAAAAATGTGA GGGTAggTTATCTCAGGCT GCGG

3.3.3. Generation of reporter transgenic lines in *C. elegans*

Reporter transgenic lines were generated by simple array intragonadal microinjection. The injection mix was composed of 50 ng/ μ L of the construct of interest and 100 ng/ μ L of pFR4 plasmid that carries *rol-6(su1006)* gain of function allele as a co-injection marker (Mello et al., 1991). *rol-6(su1006)* is a dominant negative mutation of a collagen gene that confers worms a helically right-twisted body phenotype known as “roller” that allows for the easy identification of the transgenic animals under the dissecting scope (Kramer et al., 1990). Additionally, for some experiments, a plasmid carrying a *ttx-3* minimal promoter, only expressed in AIYs, (pNF95) upstream of DsRed fluorescent protein was added at 20 ng/ μ L, allowing the identification of worms carrying the extrachromosomal arrays under the fluorescent microscope.

Before the microinjection, 2% agarose pads were prepared (Mello & Fire, 1995), and to avoid needle blocking DNA mixes were centrifuged at full speed for 5 minutes. Then 1 μ L of the supernatant was aspirated with a microloader

(Eppendorf, #930001007) and loaded into a 0.5 μm diameter Femtotip II needle (Eppendorf, #930000043). The needle was then adjusted to a micromanipulator (Narishigue, MN-151).

Young adult hermaphrodites were placed in halocarbon oil 700 (Sigma, #H-8898) to eliminate the bacteria from around the body and then they were placed over the agarose pad for immobilization with some oil remaining to prevent dehydration.

The microinjection process was performed using a FemtoJet (Eppendorf, #5274) coupled to an inverted microscope (Zeiss, Axio Vert.A1). Worms were microinjected into the syncytial arms of both gonads, where DNA mixes were released. Then, hermaphrodites were placed in individual fresh NGM plates and incubated at 20°C. Typically, at least 10 animals were injected per mix.

Three days after the microinjection, plates were inspected to look for the presence of the co-marker *rol-6(su1006)* in the F1 progeny. All the “rollers” found were placed in individual NGM plates for a maximum of 150 animals. Three days later, 3 or 4 transgenic lines were selected and maintained by the mosaic presence of the *rol-6(su1006)* phenotype in the F2 progeny.

3.3.4. Strain crossing

Strains were crossed to study the influence of certain genetic backgrounds over the expression of several fluorescent reporters and/or to obtain combinations of gene reporters or double mutant backgrounds.

For crossings, 3-4 L4 hermaphrodites and 12 or more males were put together in NGM plates seeded with small bacterial lawns placed in the centre of the plate to increase encounter probability. Plates were incubated for approximately 3 days at 20°C. Then 10 L4 hermaphrodites from the F1 were singled into new

NGM agar plates (once F1 males have been found on the plate to ensure mating has taken place) and incubated again at 20°C for 3 days. F2 progeny was then properly segregated to obtain all possible genetic backgrounds. Finally, F2 worms were genotyped to identify desired genotypes.

3.3.5. Genotyping

To ensure the presence of the desired genetic background, newly generated mutant strains were genotyped. First, to extract the DNA template to perform PCRs (Polymerase Chain Reaction), individual F2 worms were incubated in 20 μ L of lysis solution with 2% Proteinase K (Roche Life Science, #3115879001) for 90 min at 65°C and 30 min at 95°C, for Proteinase K inactivation.

Then NZYTaQ II 2X Green Master Mix (NZYtech, #MB345) was used to perform PCRs, following manufacturer instructions. For each PCR annealing temperature was set at 5°C under the lower primer T_m .

Appropriate primers were designed (**Table 3.3**), accounting for the specific nature of the mutation to perform standard PCRs using 3 μ L of F2 lysates as DNA templates. Deletion alleles were genotyped using two pairs of primers, one pair inside the deletion that only appears when the wildtype allele is present, and another pair outside the deletion that allows us to differentiate between heterozygous and mutant alleles according to the size of the amplicon, the presence of the amplified sequences was checked through agarose gel electrophoresis. For point mutations, only one pair of primers was used, and then the amplified region containing the region of the mutation was Sanger sequenced (Macrogen). Finally, for crosses with fluorescent reporters, the offspring was selected under a fluorescence dissecting scope (Zeiss, 1FAxioZoom V16) when possible, or when the fluorescence was not intense enough a microscope was used to select the offspring. Moreover, when

genotyping by eye was not clear enough, the presence of the reporter tag was also checked by PCR amplifying the sequence of the fluorophore.

Table 3. 3. Primers to genotype mutants and reporters

Target gene	Desing	Oligo name	Oligo type	Sequence
<i>hpl-2(1489)</i>	deletion	oc190	FWD, external to deletion	TGCGAATTGGTTTCT CTCTCTG
		oc191	REV, external to deletion	TCAGAGCTCCTAACCT ACCT
		oc213	FWD, internal to deletion	AAATTCCCTCTCAAAC TCGTCC
		oc214	REV, internal to deletion	AGCAAATCAACAAAG CGAGC
<i>unc-4(gk705)</i>	deletion	oc119	FWD, external to deletion	CAAAATACGTGCAAA GTATTTC
		oc120	REV, external to deletion	CCATGGGACGTTGAA ATCAC
<i>mod-5(n802)</i>	substitution	oc408	FWD	CAGACGACTGTGGAC CCTTCC
		oc409	REV, mut. allele	AAGTTCCGTGGGCGT CATGT
		oc410	REV, wt allele	AAGTTCCGTGGGCGT CATGA
		oc420	REV, sequencing	GAAAAGACGCCAACC ATTTTCG

mod- 5(<i>knu383</i>)	deletion	oC194	FWD, internal to deletion	GGCTTACGCTCACGCT TAGGC
		oC201	REV, external to deletion	GACGGAGTGGTCAAT GGTGTCCC
		oC560f	FWD, external to deletion	GCGGCCGACATCTCAC GGGTTC
		oC560r	REV, internal to deletion	CTCCAGAAATAATTC GAGTTTCG
bas- 1(<i>ad446</i>)	deletion	oAM96	FWD external to deletion	CAATTAGGCCGCAAA CCCTT
		oAM97	REV external to deletion	CTTCCCACACAGAAAC GTAGC
		oAM98	FWD internal to deletion	CAATACTGATCTCCAC AAGGCG
		oAM99	REV internal to deletion	AACCCTCTTCTACACA GACTGG
mNG	Tag	oMM113	FWD	CAAGGAACCGGAAAC
		3		CCAAAC
		oMM113	REV	CCGGATTGAAAGTAC
		4		AGATTCTCCT

3.3.6. *C. angaria* transgenesis

Transgenesis in non-model species was challenging, to increase the likelihood of obtaining transgenic animals, complex arrays were used. Genomic DNA of the corresponding species was digested overnight with SpeI restriction enzyme at 37°C and purified using QIAquick (QIAGEN #28104). Then the microinjection mix was prepared containing 60 ng/μL of digested genomic DNA, 2.5 ng/μL

pCFJ90 (*Pmyo-2::mCherry*, Addgene #19328) as a co-injection marker, and finally 10 ng/ μ L of the construct of interest. Microinjection was performed as in *C. elegans*.

We were able to obtain transgenic lines or enough F1 animals for scoring. However, transmission was poor. This, together with the gonochoristic nature of *Angaria* species, which requires crossing animals carrying the construct, made it impossible to maintain the lines or freeze them.

3.4. Heat-shock induction

For overexpression of *unc-4* in the whole worm, a plasmid containing heat-shock promoter followed by *unc-4* cDNA pNF855 (already designed by Dr. Carlos Mora Martinez) was microinjected in worms together with *rol-6(su1006)* as coinjection marker.

First, this construct was microinjected into the endogenous reporter of *mod-5*, NFB1719 *mod-5(vlc47)*, in *C. elegans*. Since we did not detected NeonGreen expression after a heat-shock of 20 minutes at 34°C, we next co-injected in wild type worms a DNA mix containing the heat-shock promoter followed by *unc-4* cDNA (pNF855) together with *Cel_mod5prom3::gfp* construct and the *rol-6(su1006)* as co-injection marker. All additional *hs::unc-4* microinjections in different genetic backgrounds were co-injected with the *mod-5prom3::gfp* construct. YA or L4 worms from all the lines were then heat-shocked for 30 minutes at 37°C and 3 hours later GFP fluorescence was analysed, or worms were fixed and stained for 5HT. Cases in which the time between heat-shock and fixation was longer are specified in the text.

3.5. Microscope scoring

Scoring and images were performed using a Zeiss Axioplan 2 epifluorescence microscope using the 63X objective. Percentages of GFP expression in the cell were calculated as the total number of GFP-positive cells over the total number of scored cells.

For *unc-4* endogenous expression analysis, photos were taken with a confocal TCS-SP8 Leica microscope due to the low intensity of the signal.

3.5.1. Fluorescence reporter scoring

Reporter scoring was performed using YA females, hermaphrodites, or males both in *C. elegans* and *C. angaria*. Worms were maintained at 20°C except for the strains containing *hpl-2(tm1489)* which is temperature sensitive and were kept at 25°C. In the case of extrachromosomal reporter arrays, at least two independent lines were scored per construct. In all cases, at least 30 worms were scored when possible. For the scorings, worms were mounted in 4% agarose pads and placed over standard microscope slides using a drop of 0.5 M sodium azide (Sigma, #26628-22-8) to immobilize worms. Worms were let a few minutes in the NaN₃ drop to allow them to turn upwards, making the vulva and VC easily observable, putting the slide in a wet chamber, after which the slides were covered with standard cover slides (Corning, #CLS284518).

For the analysis of the intensity of the signal in the endogenous *unc-4* reporter strain, Z-stack photos of the vulva region were taken maintaining the same exposure time for both genotypes or conditions. Images were acquired with confocal TCS-SP8 Leica microscope.

3.5.2. Immunostaining scorings

For immunostaining preparation analyses, scorings were performed in a categorical manner: ON when staining was visible, OFF when not detected and FAINT if VC soma was barely visible. At least 40 YA worms were scored whenever it was possible.

3.5.3. Fluorescence intensity analysis

In the case of worms grown in environmental 5HT conditions, or mated worms, intensity analysis was performed to acquire a quantitative phenotype.

For intensity analysis, exposure time was maintained identical for all conditions for the same experiment and Z-stack photos were taken with an AxioCam MRm camera (Zeiss, #426509-9901-000) coupled to a computer running the Zen System 2011 (Zeiss). Fiji (ImageJ 1.5i) (Schindelin et al., 2012) software was used to open and assess all the measurements needed to perform the calculation.

Fluorescence intensity was measured by applying CTCF (Corrected Total Cell Fluorescence). A measurement of the neuron of interest was taken together with three different measurements of background readings around the cell studied to generate the mean background value.

$$\text{CTCF} = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background readings})$$

When the neuron was not visible, an area similar to the neurons visible was measured and three measurements around that area were taken, this explains why some CTCF values are below 0.

3.6. Egg-laying behavioural assays

3.6.2. Egg-laying overnight

In order to study the total number of eggs laid by one worm 24, 48 or 72 h after YA, L4 hermaphrodites were placed in a fresh NGM plate, or a cross was put in gonochoristic species, and in both cases, worms were incubated at 20°C overnight. Then each female or hermaphrodite was singled in a fresh NGM plate and incubated at 20°C for 24 h, next day worms were put in new NGM plates and incubated another 24 h (will be 48 h after YA), and this process was again repeated for a third time. In all the cases, eggs or L1 larvae of each plate were scored.

3.6.3. Egg-laying upon 5-HT stimuli

5-HT effects on egg-laying were performed as previously described (Y.-C. Chen et al., 2020; Hart, 2006), with slight modifications. Individual worms were distributed in 96-well plates with 50 µL of S basal or S basal with 5 or 10 mM of serotonin creatinine sulfate monohydrate (Sigma, #H7752). Serotonin solution was always freshly prepared, and the mix was vortexed until dissolved. Then worms were incubated for 2 hours at 20°C, then the number of eggs was scored for each well. The experiment was repeated three times with 12 worms per experiment, except for the assays of exposure to 5 mM 5HT in *C. elegans* wildtype, *C. elegans lin-11prom::mod-5* transgenic animals, *C. angaria* wildtype and *C. angaria mod-5(vlc45)* that was performed only once.

3.6.4. Egg-laying video recordings and analysis

To better understand the dynamics of the egg-laying behaviour, *C. elegans* L4 or *C. angaria* crosses were put in new NGM plates overnight. The day after, YA females or hermaphrodites were put alone NGM plates (100 x 20mm) seeded

with 300 μ L of OP50 the day before. Then 6-hour movies of single worms were recorded with Motif video recording system (Loopbio, Austria). Videos were taken at 5 fps with an exposure of 29000 μ s and using the hq-fast codec record. Then, videos were visually inspected, registering the video time when an egg was laid.

Downstream statistical analysis was performed using R and Rstudio, using custom scripts that are available at Git Hub (Ahlmann-Eltze & Patil, 2021; Auguie & Antonov, 2017; Grolemond & Wickham, 2011; R. Core Team, 2013). For each worm (w), the 6-hour video was fragmented into smaller recordings ($r, r = 1, 2, 3, \dots, N$) and compressed to have a maximum duration of 12 minutes. We manually annotate the duration of the last recording (t_n , in seconds) and fix the total duration to 6 hours (21600 seconds), being able to define the compression motif of each video with the following equation:

$$Compression_w = \frac{21600}{N_w \cdot 12 \cdot 60 + t_n}$$

Then, the custom function get Analysis() was used to decompress the time and obtain the statistics. Summarizing, the function takes each egg annotation file and discards the worms that laid less than 10 eggs in 6 hours, then converts each egg laying moment to seconds and applies the decompression correction ($Compression_w$). The egg laying intervals are calculated by the difference between consecutive egg laying events.

3.7. Curling tail in males

A previously described method was used to study the effect of 5-HT in the behaviour of tail curling (Loer & Kenyon, 1993), YA male worms were exposed to 5mM 5-HT in M9 1X buffer, or the buffer alone for 15 seconds. Worms with

the tail curled for 5 seconds were scored as “curled.” As in the egg-laying assay, 5-HT solution was always freshly prepared.

3.8. Alignment

To perform the alignments of *cat-1* and *unc-17* coding sequences in the Fig 4.4 and Fig 4.6 respectively, we blast the conceptual translation of *C. elegans* of each gene, and then we perform a BLAST search against the database ‘*Caenorhabditis_angaria*.PRJNA51225.WBPS16.protein.fa’ in the *Caenorhabditis* Genome Project webpage (<http://Caenorhabditis.org/>).

To align the coding sequences of *cat-1* and *unc-17* shown in Figures 4.4 and 4.6, we first obtained the conceptual translations of the *C. elegans* sequences for each gene from the WormBase webpage (<https://wormbase.org/#012-34-5>) (Davis et al., 2022). These translations were then subjected to a BLAST search against the *Caenorhabditis angaria* protein database (*Caenorhabditis_angaria*.PRJNA51225.WBPS16.protein.fa) available on the *Caenorhabditis* Genome Project webpage (<http://Caenorhabditis.org/>).

3.9. *C. elegans* ChIP-seq profiles

ChIP-seq profiles were generated by Antonio Jorda, a postdoctoral researcher in our laboratory. All ChIP-seq datasets from L3 larvae were obtained from the GPL9309 GEO platform. The series numbers are: GSM1195399 (H3K9me1 Input Rep 1), GSM1195401 (H3K9me1 IP Rep 1), GSM1206324 (H3K9me2 Input Rep 1), GSM1206326 (H3K9me2 IP Rep 1), GSM1206288 (H3K9me3 Input Rep 1), GSM1206290 (H3K9me3 IP Rep 1), GSM1206406 (HPL2 Input Rep 1), and GSM1206408 (HPL2 IP Rep 1). Wiggle tracks were loaded onto IGV and inspected against the *C. elegans* (ce11) reference genome annotation from

IGV. Background signal was subtracted from each IP track by elevating the baseline to the corresponding Input's local maximum.

3.10. Statistics

Reporter expression data from transgenic lines in *C. elegans* and *C. angaria*, and mutant strains or 5-HT immunostainings were categorically classified as “on” or “off,” and the significance of the difference to the wildtype was performed using the two-tailed Fisher exact test; “faint” phenotype was included as “on” for the statistics.

For intensity level of the *unc-4(syb1658[unc-4::GFP]* fluorescent reporter, 5-HT immunostaining in which intensity was analysed and egg-laying experiments, normality was studied using D'Agostino & Pearson test, then distributions that followed the normality were analysed with an unpaired t-test, unless the variance between groups were not the same in which case Welch test was used. Distributions that did not follow normality were analysed using Mann–Whitney nonparametric test.

3.11. Materials

This section includes the composition of buffers not detailed above.

Table 3. 4. Materials

Component	Concentration	Component	Concentration
NGM agar		Egg preparation solution	
Agar	16 g/L	NaClO	50% V/V
NaCl	0.05 M	KOH 5 M	20% V/V
Peptone	2.5 g/L	ddH ₂ O	30% V/V
MgSO ₄	1 M	PBS buffer 10X	
Cholesterol	1 mL/L of 5 mg/mL solution in ethanol	NaCl	1.37 M
CaCl ₂	1 M	KCl	27 mM
KPO ₄	1 M, pH 6.0	Na ₂ HPO ₄	100 mM
Nystatin	2.5 mL/L	KH ₂ PO ₄	18 mM
Streptomycin	0.15 g/L	Collagenase Buffer	
Worm lysis solution		Triton X-100	1%
Proteinase K	2% V/V	Tris pH 7.5	0.1M
GoTaq® Colorless Reaction Buffers for PCR	20% V/V	CaCl ₂	1mM
Nuclease-free water	78% V/V	TrPBS	
M9 buffer		PBS	1X
Na ₂ HPO ₄ ·12H ₂ O	80 mM	Triton X-100	0.5%
KH ₂ PO ₄	22 mM	S basal	
NaCl	8.5 mM	NaCl	100mM
NH ₄ Cl	0.2 M	KH ₂ PO ₄	44mM
PGT		K ₂ HPO ₄	5.7mM
PBS	1X	Cholesterol	1 mL/L of 5 mg/mL solution in ethanol
Triton X-100	0.25%		
Gelatin	0.1%		

4. RESULTS

This thesis explores the evolution of serotonergic fate in VC4 and VC5 motoneurons of *C. elegans* and *C. angaria*, which exhibit differential serotonergic phenotypes. We explore the genetic and molecular mechanisms underlying these differences, examine the behavioural implications of this evolutionary change, and explore the plasticity of the serotonergic phenotype in *C. elegans*. The findings are presented in four main sections, in **section 4.1** we investigate the gene regulatory logic responsible for the distinct serotonergic fates of VC4 and VC5 neurons in *C. elegans* and *C. angaria*. In **section 4.2** we explore the behavioural consequences of the serotonergic phenotype in *C. angaria* VC4 and VC5 neurons focusing on egg-laying behaviour and investigating how the presence of serotonin in these neurons influences this process. Then in **section 4.3** we turn the focus to *C. elegans*, examining the plasticity of the serotonergic phenotype in VC4 and VC5 neurons identifying the environmental triggers that activate this plasticity and investigating the regulatory mechanisms involved. Finally, in **section 4.4** we extend the investigation to male-specific neurons, CPs and CAs, which are homologous to VCs. This section aims to determine if these neurons share similar gene regulatory logic and explores the potential role of serotonergic CAs in *C. angaria* mating behaviour.

4.1. Gene regulatory logic of *C. angaria* and *C. elegans* VC4 and VC5 neuron serotonergic phenotype.

4.1.1. Study of *mod-5* regulatory regions suggests changes in *cis*-regulatory regions are responsible for the *C. angaria* VC4 and VC5 neurons serotonergic phenotype.

Previous results strongly suggested that gained expression of the serotonin reuptaker *mod-5* in VC4 and VC5 neurons in *Angaria* species could be a key event explaining the emergence of the serotonergic reuptaker phenotype. As already mentioned in the introduction, the 5-HT reuptaker phenotype requires the expression of two genes, the vesicular monoamine transporter *cat-1/vmat* and the 5-HT reuptaker *mod-5/ser* (**Fig 1.15 B**). Thus, we aimed to explore the expression of these two genes in both species to understand better the evolution of this new trait in *Angaria* species.

A priori, this event could be explained either by gain of expression of specific TFs in *Angaria* VC4 and VC5 neurons (or the loss of TF repressors) that activate *mod-5* locus (TRANS model) or by the acquisition of a new *cis*-regulatory module (CRM, a.k.a enhancer) in the *mod-5* locus in *Angaria* genome recognized by TFs already present in ancestral species (CIS model) (**Fig 4.1**). These possible scenarios make specific predictions for the outcome of heterologous gene reporter experiments. If trans-environment changes are mainly responsible for the phenotype, *C. elegans* regulatory region reporters (*Cele_mod-5/ser*) would be activated in *C. angaria* VC4 and VC5 neurons while *C. angaria* reporter (*Cang_mod-5/ser*) would be inactive in the context of *C. elegans* neurons. Conversely, if *cis*-regulatory changes are the main drivers of the phenotype, the *Cang_mod-5/ser* reporter would be active in *C. elegans* VC4 and VC5 neurons and *Cele_mod-5/ser* would not be active in *C. angaria*.

Models for the acquisition of the new trait

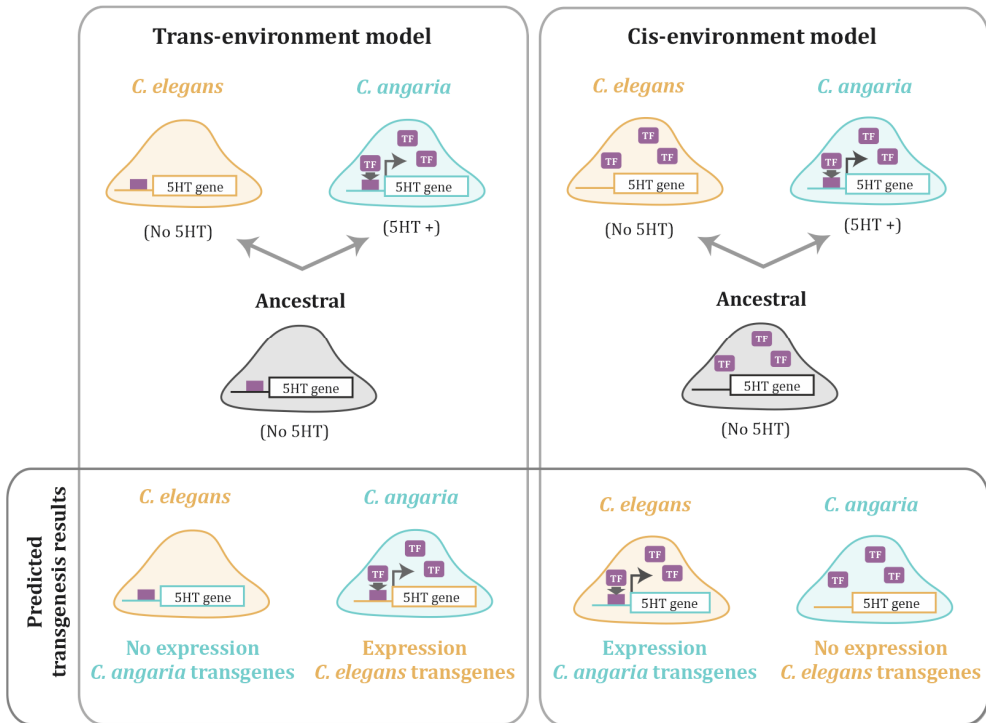


Figure 4.1. Models for the evolutionary mechanisms generating gene expression diversity between orthologous cell types. In the trans-environmental model (left), transcription factors that activate ancient enhancers present in serotonin pathway genes gain expression in *C. angaria* VC4 and VC5 neurons. If this was the case, *C. angaria* fluorescence reporter constructs from 5-HT pathway genes should not be expressed in the context of *C. elegans* VC4 and VC5 neurons. In the *cis*-environmental model (right), evolution of the trait is explained by the emergence of new enhancers in 5-HT pathway genes that are recognized by transcription factors ancestrally expressed in VC4 and VC5 neurons. If this is the case, reporters of *C. angaria* 5-HT pathway genes should be expressed in *C. elegans* VC4 and VC5 neurons.

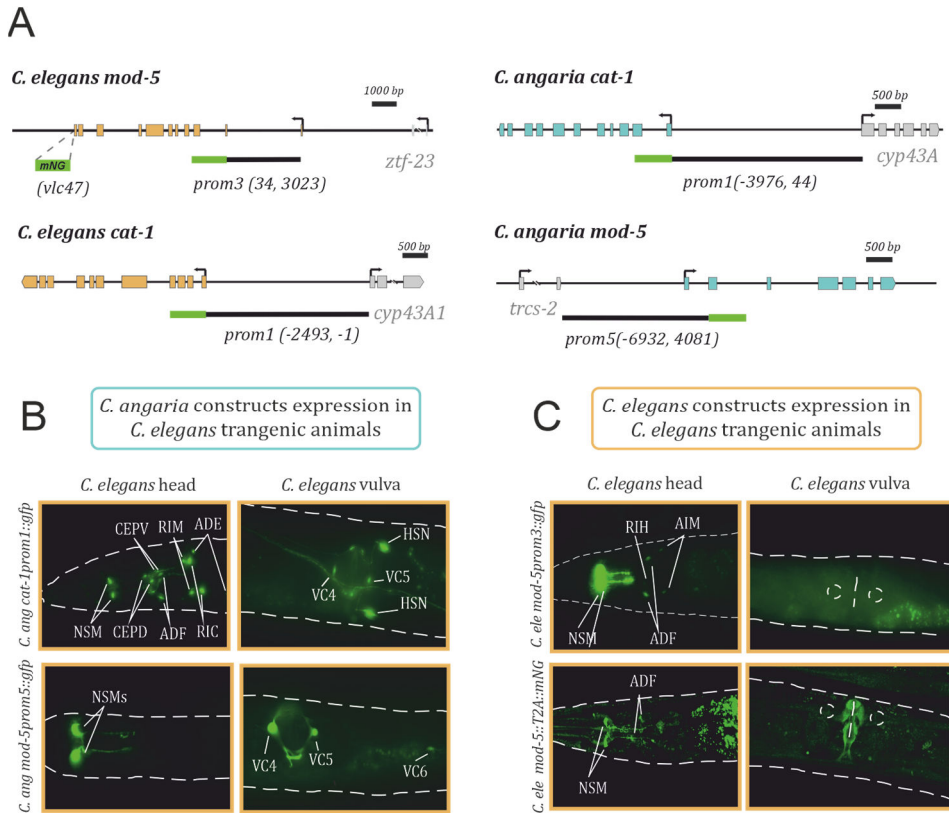


Figure 4. 2. Previous analysis of *C. elegans* and *C. angaria* regulatory regions of 5-HT pathway genes through transgenesis in *C. elegans*. **A.** Scheme of *C. elegans* and *C. angaria* used reporters. For *C. elegans* (**left**) there is a representation of both a transgenic and endogenous reporter of *mod-5* and transgenic reporter of *cat-1*. Exons are represented in orange; exons of adjacent genes appear in gray. Regulatory region comprised in the transgenic reporters are represented by a thick black line with a green rectangle as GFP. Endogenous reporter is a CRISPR tagged T2A::Neongreen allele (*vlc47*) at the end of *mod-5* coding sequence. For *C. angaria* reporters (**right**) blue squares represent *cat-1* and *mod-5* exons, while in gray are the exons of adjacent genes. **B.** Expression of *C. angaria* constructs in *C. elegans* transgenic animals. *C. angaria cat-1prom1* and *C. angaria mod-5prom5* are both expressed in *C. elegans* VC4 and VC5 neurons. **C.** Expression of *C. elegans mod-5* constructs in *C. elegans*. *mod-5* reporters both transgenic and endogenous are not expressed in VC4 and VC5 neurons in *C. elegans*. This data was generated by Dr. Carlos Mora.

To distinguish between these two models Dr. Mora performed homologous and heterologous reporter assays and started analysing the activity of different regulatory regions of serotonergic genes from *C. elegans* and *C. angaria* genome in *C. elegans* and *C. angaria* transgenic animals (Mora-Martínez, 2019). The most relevant findings from this study were: 1) *C. angaria cat-1* CRM (*Cang_cat-1prom1*) is activated in *C. elegans* VC4 and VC5 neurons; 2) *C. angaria mod-5* CRM (*Cang_mod-5prom5*) is also activated in *C. elegans* VC4 and VC5 neurons 3) none of the tested *C. elegans mod-5* CRM, or the *mod-5::T2A::mNeonGreen* CRISPR strain are expressed in *C. elegans* VC4 and VC5 (**Fig 4.2**).

Next, I performed reciprocal experiments, analysing *C. elegans* and *C. angaria* constructs in *C. angaria* transgenic animals. Despite the difficulties that bring the use of non-model organisms and the challenge of setting up transgenesis in *C. angaria*, I was able to obtain *C. angaria* lines for these constructs, *Cele_cat1prom1::gfp*, *Cele_mod-5prom3::gfp* and *Cang_cat-1prom1::gfp*, however, transmission was very low. This fact, together with the challenges of maintaining an extrachromosomal array in a gonochoristic species limited the number of worms I could score before the lines were lost (**See Annex 4.1**). Alternatively, I injected and scored F1 animals instead of established transgenic lines to increase the number of worms when necessary. *Cele_cat-1prom1::gfp* construct (**Fig 4.2 A**) injected in *C. angaria* drove *gfp* expression in all the monoamine synthesizer neurons (ADF, NSM, CEPV, CEPD, ADE, PDE, HSN, RIC and RIM) except for AIM and RIH which is consistent with the lack of serotonin staining in *C. angaria* in these neuron type. In the region of the vulva, I could only detect *gfp* expression in the HSN but not VC4 or VC5 (**Fig 4.3 A, B**) meaning that *C. elegans cat-1* enhancer is not active in *C. angaria* VC4 and VC5 environment, maybe due to genetic drift mechanisms. Similar loss of expression of heterologous reporters was found for *C. angaria cat-4* and *bas-1* reporter in *C. elegans* ADF and HSN, even when they are serotonin synthesizers, as already

described by Carlos Mora (Mora-Martínez, 2019). *Cele_mod-5prom3::gfp* is active in NSM, ADF, and AIM, but not in RIH nor in VC4 or VC5 neurons (**Fig 4.3 A, B**)(**Annex 4.1**). Altogether, these results suggest *C. elegans mod-5* regulatory regions are not active either in VC4 or VC5 neurons of *C. elegans* or *C. angaria*.

To better understand the behaviour of *C. angaria* constructs in their endogenous cellular environments Dr. Mora also injected the same reporters in *C. angaria*. Surprisingly, *Cang_cat-1* reporter did not produce detectable *gfp* expression in VC4 and VC5 neurons, in contrast to its expression in *C. elegans*. Since Dr. Mora obtained very few numbers of worms carrying the *Cang_cat-1prom1* reporter, I injected that construct again, generating one line and I saw results similar to the previous ones (**Annex 4.1**), *Cang_cat-1prom1* was expressed in NSM, ADF, CEPV, CEPD, RIC, RIM, ADE, HSN and PDE, but not in VC4 or VC5 neurons (**Fig 4.3 C,D**). These unexpected results suggest CRM driving *cat-1* vesicular monoamine expression in VC4 and VC5 in *C. angaria* might light outside the tested region. Alternatively, to the use of reporter assays, to confirm *cat-1* expression in VC4 and VC5 neurons of *C. angaria* I performed immunostaining using an antibody for *C. elegans cat-1* (Duerr et al., 1999), *C. angaria cat-1* coding regions shares 76% identity to *C. elegans cat-1* (**Fig 4.4 A**) what suggests the antibody can be used in both species. I performed an immunostaining against VMAT, and I localized the transporter in the head zone, around the nerve ring, and also in the ventral nerve cord, however, the identification of individual cells in the head was not possible. In the vulva region, we found staining in two somas located in the VC4 and VC5 positions, but we failed to identify the HSN, it is worth noting that the staining was not easy to perform and animals were damaged which could explain the lack of staining in these neurons. (**Fig 4.4 B**).

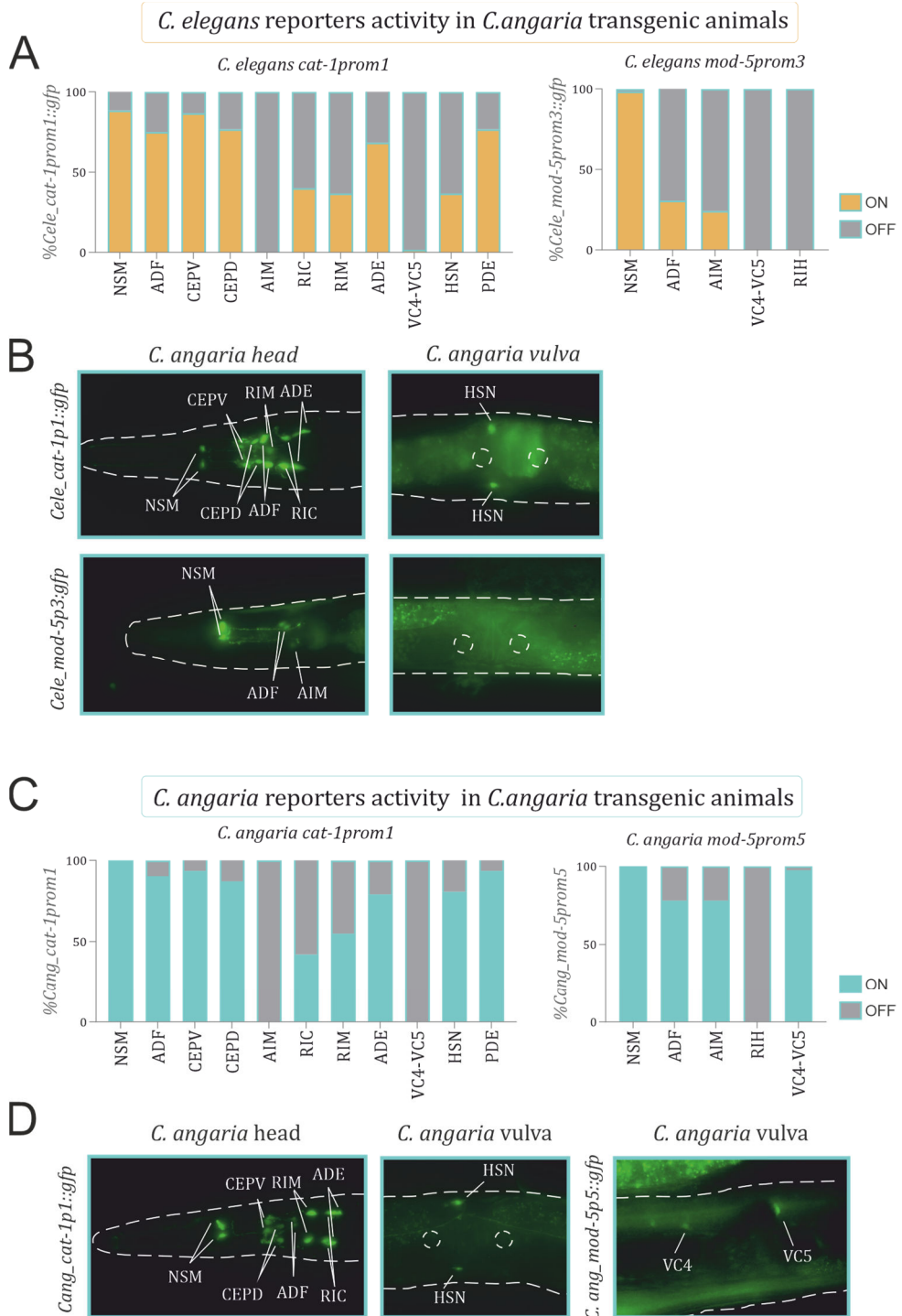
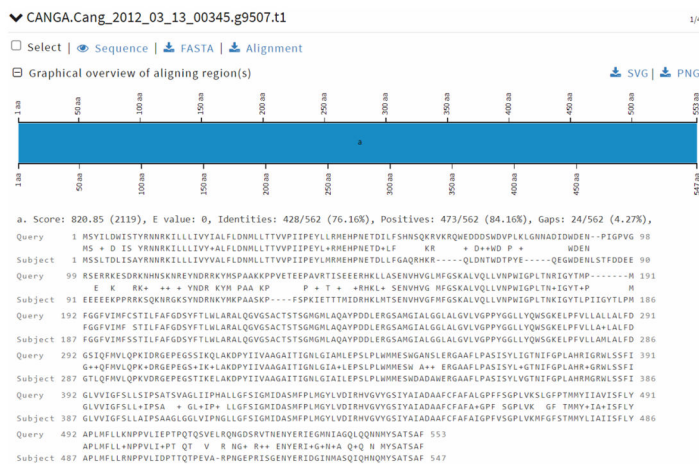


Figure 4. 3. Analysis of *C. elegans* and *C. angaria* regulatory regions of 5-HT pathway genes through transgenesis in *C. angaria*. **A.** Quantification of *C. elegans* *gfp* reporter expression for *cat-1* (n=30 worms) and *mod-5* (n=31 worms) constructs in *C. angaria*. **B.** Representative images for *C. elegans* *cat-1* and *mod-5* fluorescence reporters' expression in *C. angaria*. *C. elegans* 5-HT reporters are inactive in *C. angaria* VC4 and VC5 neurons. **C.** Quantification of *C. angaria* *gfp* reporter expression for *cat-1* (n=27 worms) and *mod-5* (n=28 worms) constructs in *C. angaria*. **D.** Representative images for *C. angaria* *cat-1* and *mod-5* fluorescence reporters' expression in *C. angaria*. *Cang_mod-5prom9* reporter but not *Cang_cat-1prom1* reporter is active in *C. angaria* VC4 and VC5 neurons

A



B

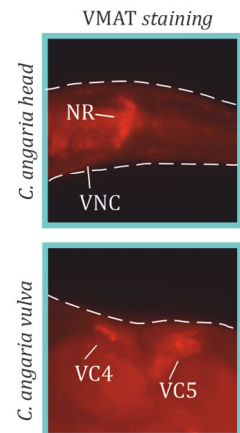


Figure 4. 4. VMAT staining in *C. angaria*. **A.** Alignment of *C. elegans* *cat-1* coding sequence to *C. angaria* *cat-1* shows 76% of identities. **B.** Representative images of VMAT staining in the head and the vulva of *C. angaria* show expression in the nerve ring and in VC4 and VC5 neurons.

Finally, *Cang_mod-5prom5*, injected in *C. angaria* also by Dr. Mora, was seen in NSM, ADF, and AIM. This is different from the absence of GFP protein in ADF and AIM neurons for this construct in *C. elegans*, indicating an additional example of genetic drift. Moreover, strong GFP expression was detected in VC4 and VC5 neurons, similar to those observed when injected in *C. elegans*. *Cang_mod-5prom5* expression in VC4 and VC5 neurons of both species is in

great contrast to *Cele_mod-5* reporter which did not drive *gfp* expression in VC4 and VC5 neurons in either species. This difference indicates that *cis*-regulatory changes in *C. angaria mod-5* locus is the most likely mechanisms driving the emergence of serotonergic reuptaker phenotype in *C. angaria* VC4 and VC5 neurons.

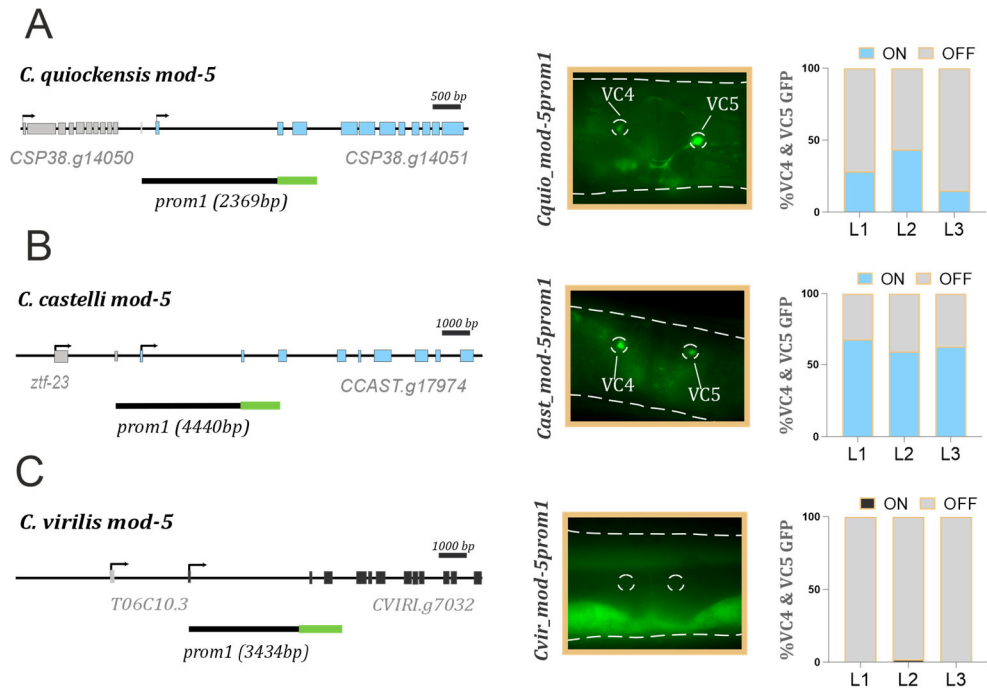


Figure 4. 5. Analysis of *mod-5* regulatory sequences from other *Caenorhabditis* species through transgenesis in *C. elegans*. *mod-5* CRMs from Angaria species but not from other *Caenorhabditis* species drive *gfp* expression in *C. elegans* VC4 and VC5 neurons. The left schemes represent *C. quiockensis*, *C. castelli* and *C. virilis mod-5* locus, blue squares represent exons of *mod-5* in Angaria species and dark grey squares represent exons of non-Angaria species, grey squares exons of adjacent genes. Regulatory region comprised in the transgenic reporters are represented by a thick black line and a green square as GFP. A. Images and quantification of three different lines of *Cquo_mod-5prom1* reporter that showed expression in VC4 and VC5 neurons in *C. elegans* (n=30,31,30 worms). B. Images and quantification of three different lines of *Ccast_mod-5prom1* showed expression in VC4 and VC5 neurons in *C. elegans* (n=30,32,30 worms). C. Images and quantification of three different lines for *Cvir_mod-5prom1* reporter which is not expressed in VC4 and VC5 neurons in *C. elegans* (n=50,52,54 worms), but shows expression in NSM neurons.

Dr. Mora's analysis of several species of the *Angaria* group indicated that all of them share VC4 and VC5 staining, suggesting that VC4 and VC5 neurons acquired the ability to contain 5-HT in the common ancestor of the *Angaria* group (synapomorphy). Then, if the key molecular mechanism for this synapomorphic trait is the emergence of a new enhancer in *mod-5* locus active in these neuron types, we hypothesized that we should be able to isolate such enhancers in additional *Angaria* species.

As transgenesis in non-model species is challenging, Dr. Mora performed *C. elegans* reporter strains carrying the regulatory regions of *mod-5* from two additional species in the *Angaria* group, *C. castelli* (*Ccast_mod-5prom1*) and *C. quiockensis* (*Cquio_mod-5prom1*) finding *gfp* expression in VC4 and VC5 neurons with both constructs, further supporting the *cis*-environment model (**Fig 4.5 A and B**). Next, we hypothesized that, if the key regulatory event took place in the ancestral species of the *Angaria* group, then *Caenorhabditis* species outside of the *Angaria* group but phylogenetically close to them should not contain a *mod-5* enhancer active in VC4 and VC5 neurons. To test this hypothesis, I focused on *C. virilis* species, from which the genome was available (for the phylogenetic distribution) (**Fig 1.17**). *C. virilis*, *Cvir_mod-5prom1* reporter does not drive GFP expression in VC4 and VC5 neurons, however, this same region is active in NSM neurons similar to *C. elegans* homologous reporter (**Fig 4.5 C**) (**Annex 4.2**).

Finally, we studied the cholinergic phenotype of *C. angaria* VC4 and VC5 neurons. In *C. elegans*, VC4 and VC5 neurons are cholinergic, thus we wanted to assess if in *Angaria* species these neuron types swapped the cholinergic fate to a serotonergic fate or if, alternatively, both functionalities coexisted in these neurons. To test for the cholinergic fate, I performed a UNC-17/VChAt immunostaining (Duerr et al., 2001). UNC-17 is the acetylcholine

transmembrane transporter specifically expressed in cholinergic neurons (Hobert, 2013), *C. angaria* UNC-17 shares 84% identity with *C. elegans*, suggesting that the antibody should work in both species (**Fig 4.6 A**). I could detect strong UNC-17 immunostaining in VC4 and VC5 neurons in *C. angaria* which allowed me to conclude that these neurons are both serotonergic and cholinergic (**Fig 4.6 B**). Similar serotonergic and cholinergic neurotransmission is observed in *C. elegans* HSN (Duerr et al., 2001).

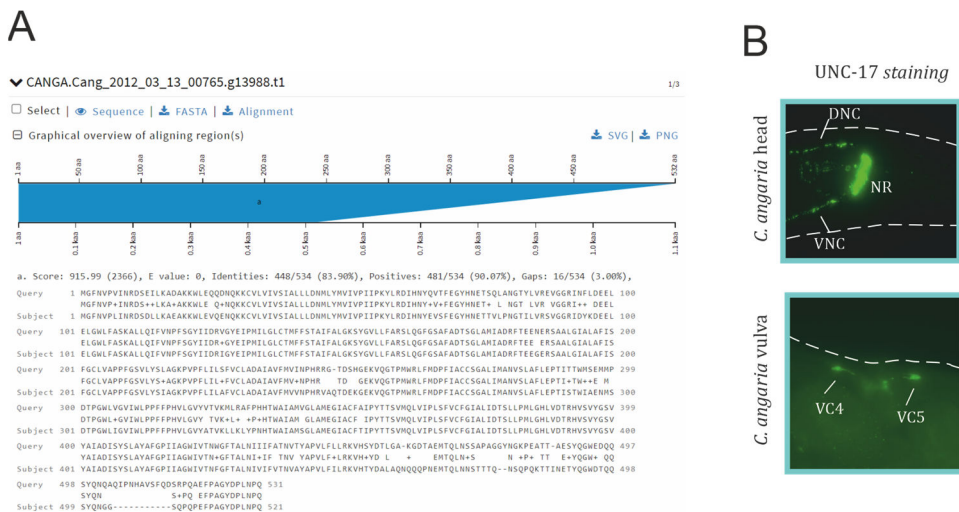


Figure 4. 6. *C. angaria* VC4 and VC5 maintain a cholinergic fate. **A.** Alignment of *C. elegans* *unc-17* coding sequence to *C. angaria* *cat-1* shows 83% of identities. **B.** Representative images of UNC-17/CHAT immunostaining in *C. angaria* show that VC4 and VC5 cholinergic fate is also present in this species.

Altogether, these results indicated that *C. angaria* VC4 and VC5 neurons as well as additional species in the *Angaria* group acquired the serotonergic phenotype through changes in the *mod-5* *cis*-regulatory regions located in the first intron of this locus, this new enhancer allows its VC4 and VC5 neuron activation not only in *C. angaria* but also in the context of *C. elegans*, what shows that the TRANS environment (TFs) necessary for its activation is shared by both species.

Accordingly, next I aimed to identify what TF is involved in the activation of *Cang_mod-5prom5* construct.

4.1.2. UNC-4/UNCX homeodomain directly activates the expression of *C. angaria mod-5/SERT* enhancer in *C. elegans* VC4 and VC5 neurons

Several TFs are known to play a role in VC4 and VC5 neuron differentiation (Perez & Alfonso, 2020; Zheng et al., 2013). Among them UNC-4 homeodomain TF seemed a good candidate for playing a role in the activation of *Cang_mod-5prom5*, as we had already determined that *C. elegans unc-4* is necessary for *cat-1* expression in *C. elegans* VC4 and VC5 neurons (Mora-Martínez, 2019). Indeed, Dr. Mora found that *Cang_mod-5prom5* expression in VC4 and VC5 neurons in *unc-4* mutants is severely affected (**Fig 4.7 A**). Next, I wanted to explore whether *unc-4* activity on *C. angaria mod-5* enhancer was direct or indirect. *UNC-4/UNCX* is a homeodomain that recognizes a TAAT core binding site motif (HD sites) (**Fig 4.7 B**). Then, we looked for this motif in the minimal promoter region of *C. angaria mod-5* active in VC4 and VC5 neurons (*Cang_mod-5prom9*, a region of almost 500pb) and we found three different putative HD sites (**Fig 4.7 C**).

Then, we designed a reporter construct mutating all three predicted binding motifs *Cang_mod-5prom9X3HD* (**Fig 4.7 D**). Since transgenesis in *C. angaria* is challenging, at first, we checked reporter construct expression in *C. elegans*. I found strong GFP expression defects for two different lines of the 3XHD mutated construct (**Fig 4.7 E**) (**Annex 4.3**).

Next, I studied the functionality of *UNC-4* binding sites in their endogenous *C. angaria* context, I injected *C. angaria* animals with the construct carrying the mutations at all three predicted HD sites. Although I was unable to obtain lines,

I was able to score some F1 females carrying the mutated construct, and none of them expressed GFP in VC4 and VC5 neurons in contrast to the WT construct in which I observed about 70% of VC4 and VC5 expressing GFP. **(Fig 4.7 F) (Annex 4.3).**

Then to explore which of the three predicted HD sites could be functional, or if there was more than one that could be active, I mutated each site separately and generated *C. elegans* transgenic lines with each individual construct **(Fig 4.7 G)**. I found significant defects when mutating any of the three sites, however, a single point mutation in the third HD (HD3) had the strongest effect. These results indicate that even though all sites seem to be functional, the third one seems to be the most important **(Fig 4.7 G)**. Importantly, this third site contains an extended TAATT motif, which best matches the UNCX HD motif **(Fig 4.7 B)(scorings Annex 4.3).**

In summary, these results suggest a direct action of *UNC-4/UNCX* on the activation of *mod-5* locus in *C. angaria* in VC4 and VC5 neurons.

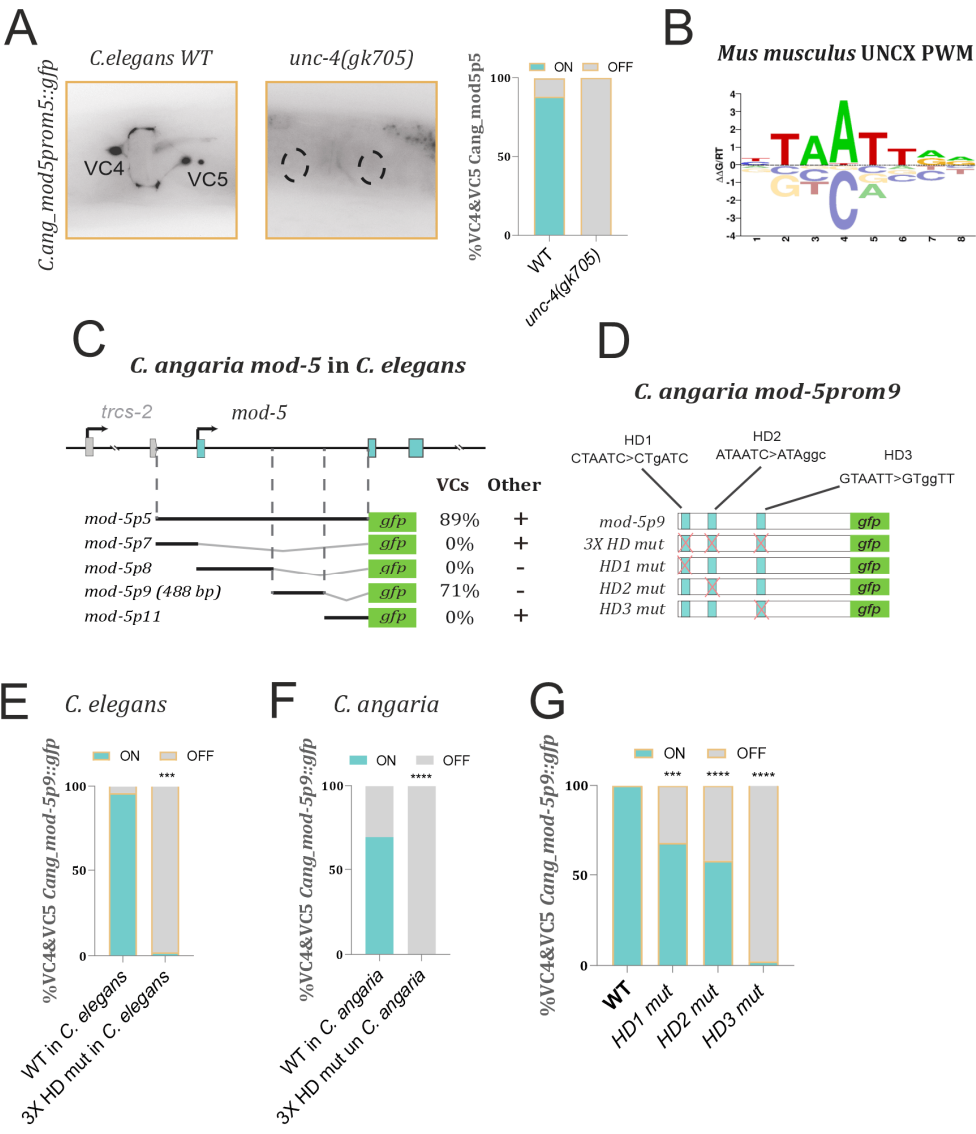


Figure 4. 7. UNC-4 directly activates *C. angaria mod-5* regulatory sequence. A. *unc-4* is essential for *C. ang_mod-5prom5* expression. Images and quantification of the loss of *Cang_mod-5prom5* expression in *C. elegans unc-4* mutants. For each genotype, the mean percentage of expression of two different lines is represented (n=63, 79 worms). B. Position Weight Matrix (PWM) generated from protein binding microarray experiments for the mouse homeodomain TF UNCX, homolog of UNC-4, (Source PMID: 18585359). C. Promoter bashing constructs of *C. angaria mod-5*. *C. ang_mod-5prom9* is the minimal promoter region driving *gfp* expression in VC4 and VC5 neurons (Mora, 2019). D. *C. angaria mod-5prom9* contains three putative homeodomain binding sites. Individual and combined HD point mutations were tested for their functionality in *C. elegans* and *C. angaria* transgenic animals. E. Quantification of combined HD point mutations in *C. elegans* reporter lines, point mutation in the three HD binding sites shows defects in *gfp* expression in VC4 and VC5 neurons. Mean of three different lines is represented (n=101, 99 worms). F. Quantification of combined HD point mutations in *C. angaria* reporter line, point mutation of the three HD binding sites affects *gfp* expression in VC4 and VC5 neurons (n=28, 35 worms) G. Quantification of individual HD point mutations in two independent *C. elegans* reporter lines. Mutation in the third putative HD binding site shows higher defects. The mean of two different lines is represented (n=64, 61, 65, 66 worms). Fisher exact test was used for statistical analyses.

4.1.3. *C. angaria mod-5*/SERT enhancer is sufficient to induce VC4 and VC5 serotonergic phenotype in *C. elegans*.

Our results suggest changes in the *cis*-environment of *mod-5* locus are responsible for the difference in the serotonergic phenotype of VC4 and VC5 neurons between *C. elegans* and *C. angaria* species. In *Angaria* species, including *C. angaria*, CRM driving *mod-5* activation in VC4 and VC5 neurons is located in the first intron of the gene. As shown above, out of its genomic context, in the form of an extrachromosomal array, *Cang_mod-5* intron 1 (*prom5*) is sufficient to drive GFP expression in VC4 and VC5 in *C. elegans* (**Fig 4.2 B**). Next, we tested if, replacement of this *C. angaria* CRM in *C. elegans mod-5* endogenous locus would be sufficient to drive *mod-5* expression and thus the serotonergic phenotype. We substituted the first intron of *C. elegans mod-5* with its homologous sequence in *C. angaria*. To do that, Dr. Rafael Alís, a postdoctoral researcher in our laboratory, designed the CRISPR construct and we ordered

the strain to be built from Sunny biotech company in the strain in which *mod-5* locus is endogenously tagged with *mNeonGreen* [*mod-5::T2A::mNeonGreen (vlc47)*] generating the final strain *mod-5(vlc47 syb7715)* (**Fig 4.8 A**).

I was not able to see *mNeonGreen* expression in VC4 or VC5 neurons in *C. elegans mod-5(vlc47 syb7715)*. However, when I performed 5-HT staining in these worms, they showed strong and highly penetrant staining in VC4 and VC5 neurons, very similar to the staining observed in *C. angaria*. The staining for 5-HT in the other neurons was unaffected as expected (**Fig 4.8 B and C**)(**Annex 4.4**).

As we had determined that UNC-4 was directly activating the *C. angaria mod-5* reporter constructs, I wanted to explore the role of UNC-4 in providing the serotonergic phenotype observed in *C. elegans mod-5(vlc47 syb7715)* animals. I crossed this strain to *unc-4(gk705)* mutant and observed a complete loss of the serotonergic phenotype in VC4 and VC5 (**Fig 4.8 D**).

Thus, *C. angaria mod-5* enhancer located in the first intron of *mod-5* is sufficient to provide the serotonergic phenotype to *C. elegans* when placed in its genomic context, providing further support to the importance of this *cis*-regulatory change in the emergence of the serotonergic phenotype of VC4 and VC5 neurons. Failure to observe *mNeonGreen* expression in this strain might be due to low levels of expression below the detection threshold. Similar undetectable expression of CRISPR strains has been reported for genes expressed at low levels such as some TFs.

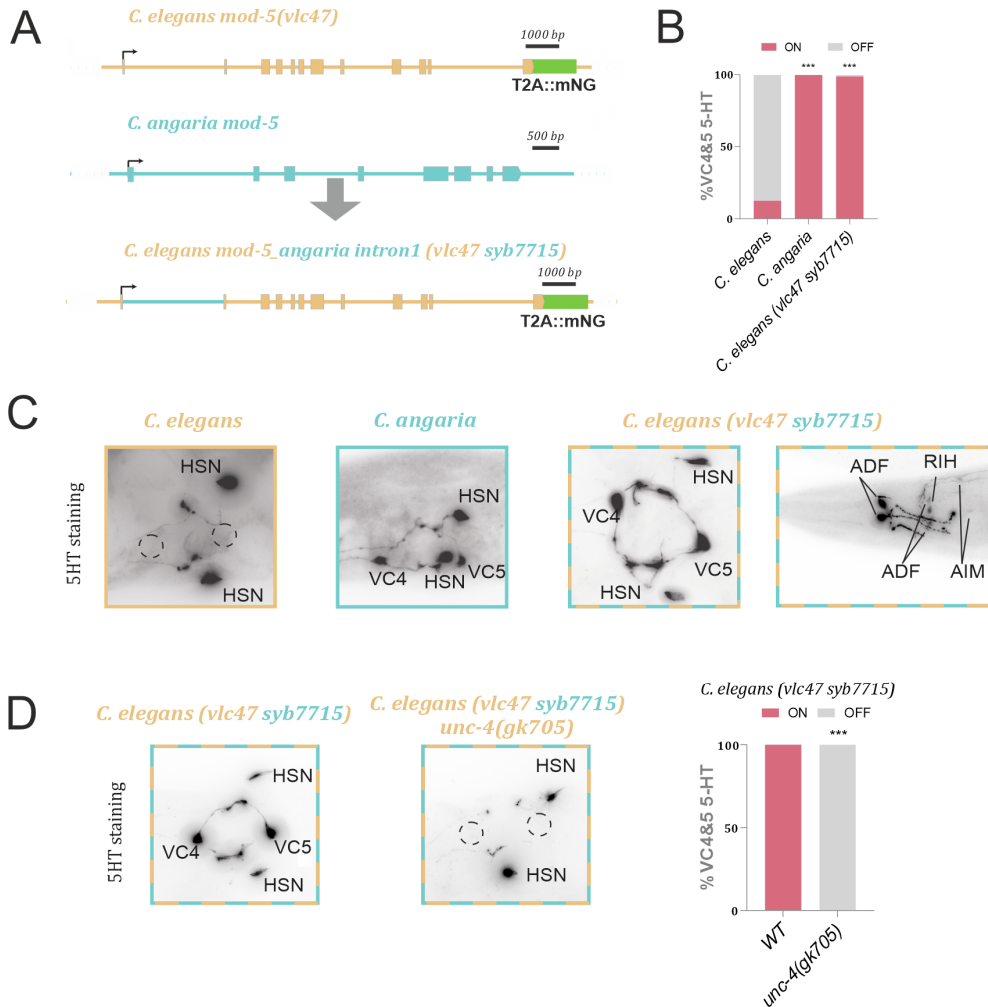


Figure 4. 8. First intron of *C. angaria* is sufficient to allow 5-HT staining in *C. elegans*. A. Scheme of *mod-5/sert* locus in *C. elegans* and *C. angaria*. *C. elegans mod-5(vlc47)* corresponds to the CRISPR tagged T2A::Neongreen allele, while *C. elegans mod-5(syb7715)* substitutes *C. elegans* intron1 sequence by *C. angaria* intron1. B. Quantification of 5-HT staining shows intron 1 swap is sufficient to drive strong 5-HT phenotype in *C. elegans* VC4 and VC5 neurons (n=40,40,40 worms). C. Representative images of 5-HT staining in the different genetic backgrounds (B). *C. elegans mod-5(vlc47, syb7715)* show expected staining of NSM, ADF, RIH and AIM in the head. D. Images and quantification show that UNC-4 is necessary for VC4 and VC5 neuron serotonergic phenotype in *C. elegans (vlc45 syb7715)* strain (n=42, 40 worms).

4.1.4. Low levels of homeodomain transcription factor *unc-4*/UNCX restricts VC4 and VC5 serotonergic phenotype in *C. elegans*.

Previous work on the origin of enhancer activities using homologous sequences from different *Drosophila* species determined that what initially was reported as a new enhancer in a species, that showed robust activation in particular tissues, when checking the activity of the homologous sequences of other species that were initially reported as inactive, often, low residual activity was detected, suggesting suboptimal enhancers can more easily evolve into robustly active regulatory regions (Rebeiz et al., 2011). Dr. Mora previous results showed that *mod-5* expression in VC4 and VC5 neurons is actively repressed by the action of the H3K9me pathway. Indeed, mutants for H3K9me show strong VC4 and VC5 neuron 5-HT staining, which is dependent on UNC-4 (Mora-Martínez, 2019), suggesting enhancer activity in the *C. elegans mod-5* locus mediated by UNC-4 is normally blocked by active H3K9me repression. However, extrachromosomal reporter arrays comparing *Cang_mod-5prom5* and *Cele_mod-5prom3* (both including the first intron of *mod-5* in each species) show strong activity for the *C. angaria* construct and no detectable activity of the *C. elegans* homolog sequence, suggesting either H3K9me repression also takes place in the extrachromosomal array, other factors are differentially expressed in H3K9me mutants, or *C. elegans* intron 1 regulatory region is suboptimal for its activation by UNC-4. Thus, to try to reveal any possible residual activity in *C. elegans* sequence we designed an experiment to overexpress *unc-4*. To get high levels of *unc-4* I used a construct for *unc-4* cDNA overexpression under a heat shock promoter (which induces *unc-4* not only in VC neurons but in the whole worm). *unc-4* overexpression was found to be sufficient to provide VC4 and VC5 *gfp* expression from the multicopy extrachromosomal reporter *Cele_mod-5prom3::gfp* (**Fig 4.9 A**), but not detectable NeonGreen was observed from the endogenous reporter *mod-*

5::T2A::mNG. (**Fig 4.9 B**) (**Annex 4.5**). However, 5-HT staining in these animals shows that *unc-4* cDNA overexpression is sufficient to significantly increase the percentage of animals showing detectable 5-HT staining in VC4 and VC5 neurons (**Fig 4.9 D and F**) and that this staining requires an intact *mod-5* locus (**Fig 4.9 H**) (**Annex 4.6**), demonstrating that even though we fail to detect NeonGreen expression from the endogenous reporter *mod-5::T2A::mNG*, *UNC-4* overexpression is sufficient to drive some residual activity of the locus. We also checked that the increase in serotonin staining upon *UNC-4* expression was due to the rise in *unc-4* levels and not just because of the heat shock, by scoring worms with and without the array (**Fig 4.9 E**) (**Annex 4.6**).

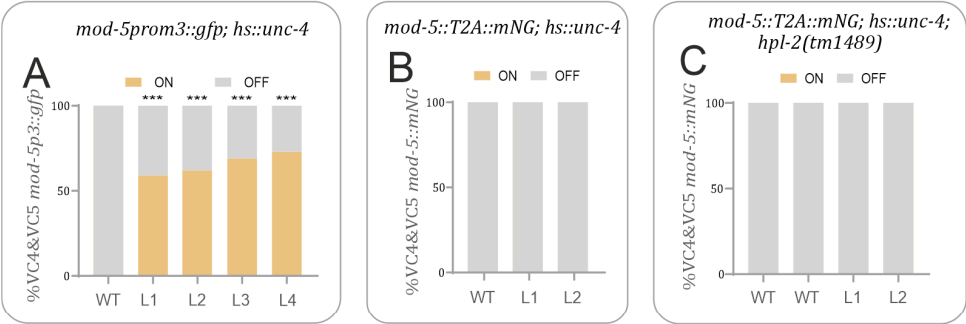
These results suggest *C. elegans* intron1 has a suboptimal capacity to act as enhancer which can be revealed with increased levels of *UNC-4*. In addition, as shown previously by Carlos Mora, this low activity of the enhancer is further silenced by active repression via H3K9me activity. Since H3K9me mutants showed a serotonergic phenotype in VC4 and VC5 neurons but, endogenous *mod-5* expression was also undetectable, I combined both *unc-4* overexpression in a *hpl-2* mutant, however, we could still not detect *Neongreen* expression from *mod-5* locus in this genetic background (**Fig 4.9 C**) (**Annex 4.5**).

All heatshock experiments were analysed for either GFP or 5-HT staining 3.5 h after the heat shock induction using mixed populations of non-synchronized adult worms. Next, I performed longitudinal studies to check if longer periods could allow us to see a higher percentage of VC4 and VC5 serotonergic neurons. I found that worms fixed 10h after heat shock showed a higher percentage of 5-HT staining, interestingly worms fixed 18h after induction had lost is increased in 5-HT staining in VC4 and VC5 compared to controls (**Fig 4.9 G**) (**Annex 4.6**). These results indicate that there is a progressive accumulation of 5-HT in VC4 and VC5 neurons upon *UNC-4* induction that seems to reach a peak after 10h

and importantly, the reduction in worms staining for 5-HT at 18h suggests 5-HT uptaken in VC4 and VC5 neurons is either released or degraded.

Finally, previous results from Dr. Mora had determined that L4 stage (period of VC4 and VC5 differentiation) is critical for the H3K9me repression of the serotonergic phenotype (Mora-Martínez, 2019). Using temperature-sensitive alleles Dr. Mora found H3K9 activity is required during this larval stage but not before or after. Thus, I also tried to determine if this larval stage was also more receptive to the effects of UNC-4 induction. I performed the heat shock both at L4 and at Young Adult stages and checked the expression of GFP in the strain carrying *mod-5prom3::gfp*. I found that *unc-4* overexpression in L4 drove a higher percentage of *gfp*-positive VC4 and VC5 neurons (85%) compared to worms exposed to *unc-4* overexpression at YA stage (53%) (**Fig 4.9 I**), meaning that L4 stage as expected is critical for the activation of *mod-5* (**Annex 4.7**). I did not perform 5-HT staining on these animals due to the significant loss of worms during the tube-fixing protocol and the requirement to select animals at the desired stage containing the extrachromosomal array.

mod-5 expression



5HT staining

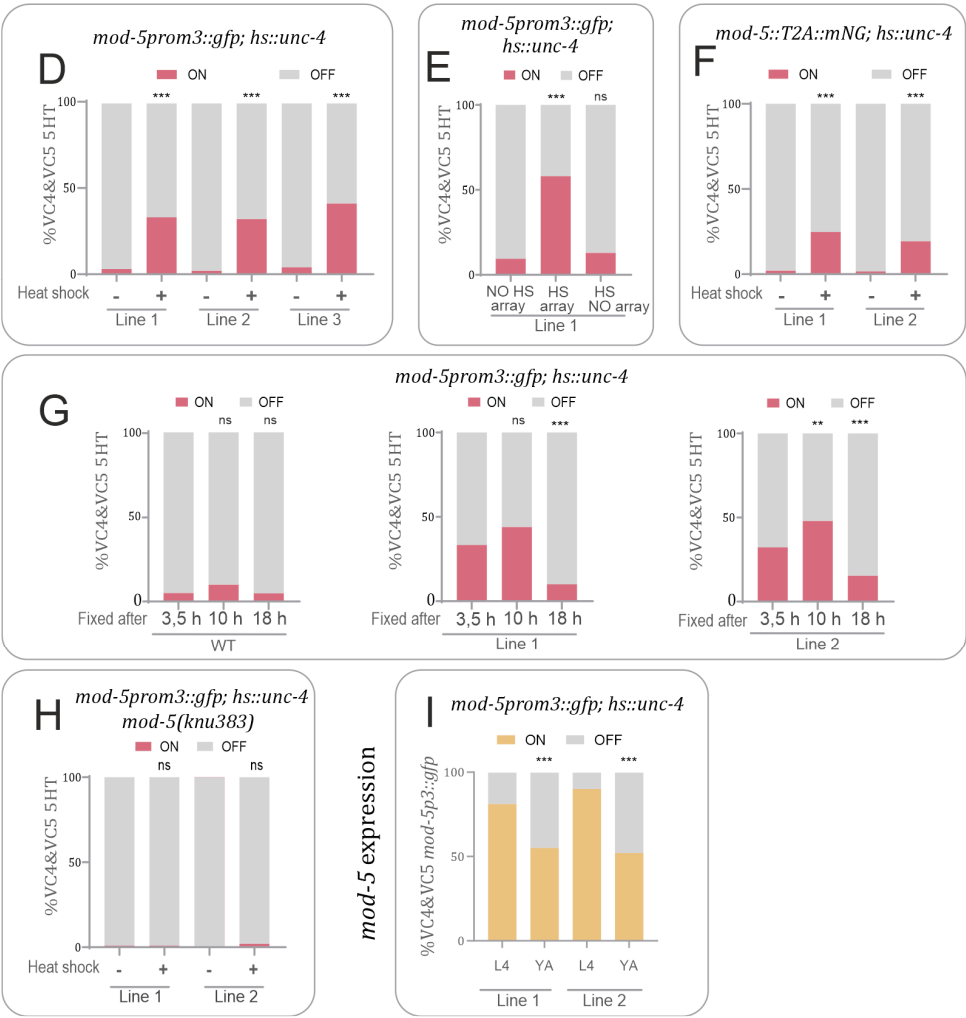


Figure 4. 9. UNC-4 induction is sufficient to drive a 5-HT phenotype in VC4 and VC5. **A.** *C. ele_mod-5prom3* expression is increased in 4 different lines when UNC-4 is overexpressed (n=30, 29,17,19,33 worms). **B.** *C. elegans mod-5* endogenous expression is not visible in two different lines with UNC-4 induction (n=30, 29, 17 worms). **C.** *C. elegans mod-5* endogenous expression is not visible in two different lines in *hpl-2(tm1489)* mutant background with UNC-4 induction (n=30 worms). **D.** Serotonin staining in three different lines containing *unc-4* cDNA under a heat-shock promoter together with *Cele_mod-5prom3* reporter. UNC-4 induction by heat shock is enough to increase 5-HT in VC4 and VC5. Experiments were performed at least twice; the mean is represented for each line (n>90 worms). **E.** Serotonin staining is increased due to UNC-4 induction and not due to the heat-shock alone. The first column represents the population with the array that has not been heat-shocked, the second population with the array and with UNC.4 induction, and the third population without the array that had been heat-shocked (n=31,31,31 worms). **F.** Serotonin staining in two different lines containing *unc-4* cDNA under a heat-shock promoter together with the endogenous reporter of *mod-5*. UNC-4 induction increases the percentage of serotonergic VC4 and VC5 neurons (n>80 worms). **G.** Time between heat-shock and fixation alters the percentage of VC4-5 stained. The first graph represents a strain with no *unc-4* array, the second and the third are the strains used in (D) Fixation was performed 3.5, 10 and 18h later (n>40 worms). **H.** Serotonin staining in VC4-5 due to UNC-4 induction is lost in *mod-5(knu383)* mutant background (n>45 worms). Fisher exact test was used for statistical analysis. **I.** UNC-4 induction in L4 shows a higher percentage of VC4 and VC5 neurons expressing *Cele_mod-5prom3* (n>25 worms).

4.1.5. *C. elegans mod-5* locus shows high levels of H3K9me marks and HPL-2/HP1 binding

Dr. Mora previous analyses of H3K9me mutants suggest that H3K9me activity prevents UNC-4 from directly activating *C. elegans mod-5* locus, however, alternative models (such as repression of UNC-4) can be envisioned, and we had no evidence H3K9me action is direct on *mod-5* locus. Thus, we explored H3K9me marks and recruitment of HPL-2/HP1 silencer in *C. elegans* genome using whole worm available datasets from modENCODE. In addition to *mod-5* locus, we also analysed methylation marks in *cat-1* which is constitutively expressed in VC4 and VC5 neurons, other genes coding for the 5-HT pathway

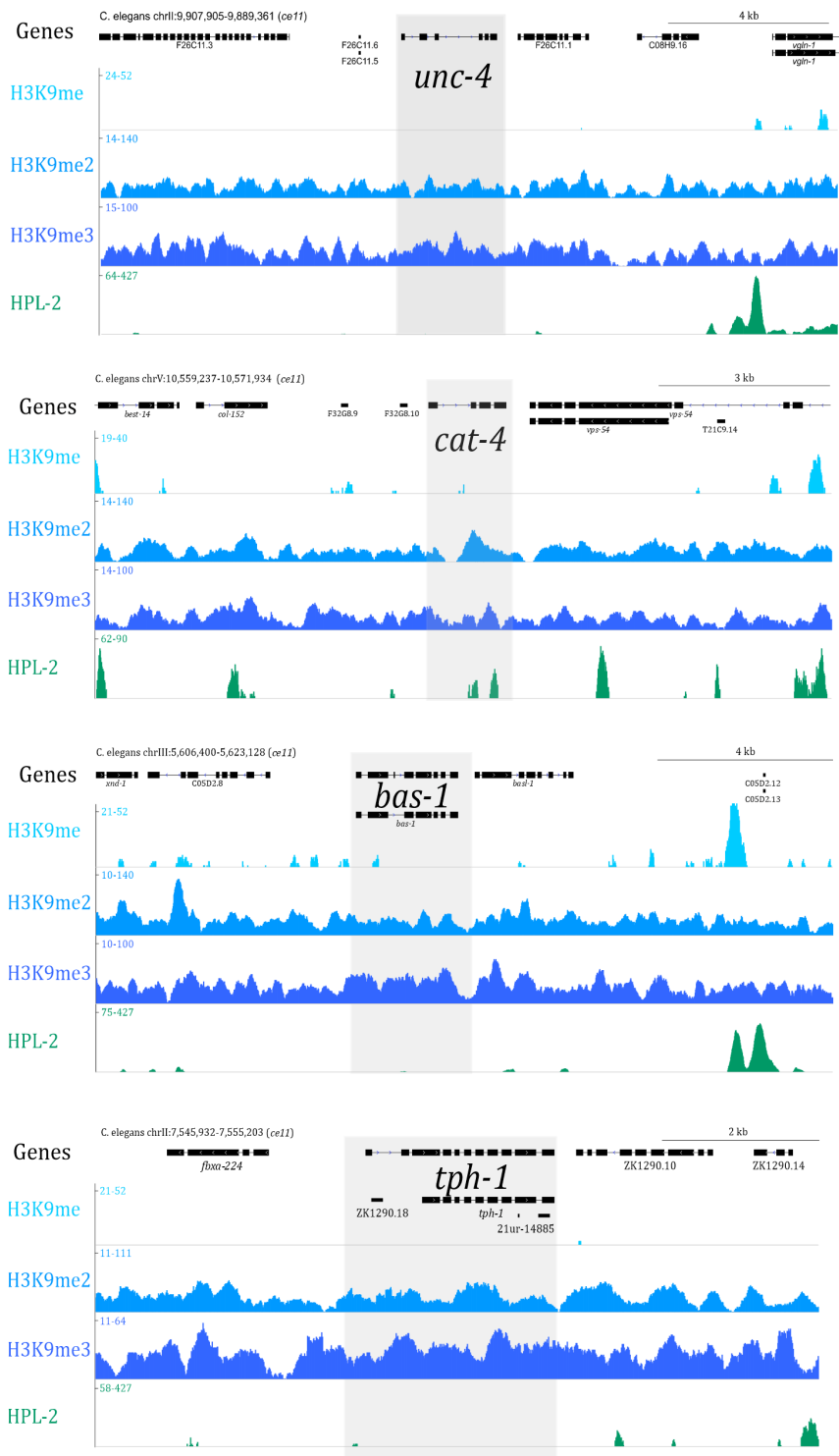
synthesis (*tph-1*, *bas-1* and *cat-4*), which we do not have any evidence of expression in VC4 and VC5 neurons and *unc-4* transcription factor.

We observed high levels of di and tri methylation (H3K9me2 and me3) in all the serotonergic pathway genes and also (but not as high) in the *unc-4* locus. However, in the case of *cat-1* and *mod-5*, we also observed another layer of repression not found in the other loci, namely high levels of monomethylation (H3K9me1 peaks) that coincide with HPL-2 binding (**Fig 4.10**). *cat-4* locus also shows some H3K9me1 marks and HPL-2 recruitment, but minor compared to both *cat-1* and *mod-5* loci.

It is important to point out these data sets are from the whole worm and not specifically from VC4 and VC5 epigenetic status. Nevertheless, we can conclude that *mod-5* and also *cat-1* loci but not *unc-4* seem to have direct H3K9me repression marks and HPL-2 recruitment. Future experiments, using isolated VC4 and VC5 neurons should be performed to conclude this epigenetic repression is specifically present in this neuron type.

4.1.6. H3K9me signalling and UNC-4/UNCX overexpression seem to act in parallel

My results show that *unc-4* overexpression is sufficient to provide a serotonergic phenotype in VC4 and VC5 neurons. In addition, Dr. Mora results showed that H3K9me mutants' induction of the VC4 and VC5 serotonergic phenotype requires *unc-4* activity. Then, next I tried to explore if the phenotype of epigenetic mutants could be mediated through increasing *unc-4* expression levels.



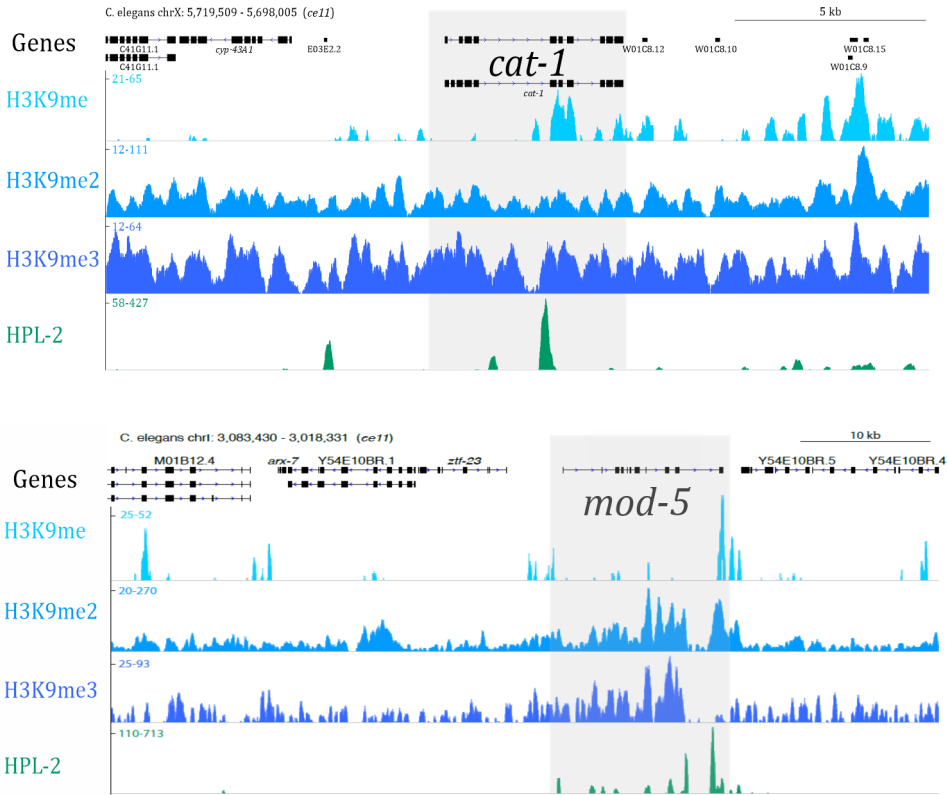


Figure 4. 10. H3K9 methylation and HPL-2 binding profile in *C. elegans* serotonergic pathway genes and *unc-4* loci. Mono-, di- and tri- methylation levels and HPL-2 binding are represented. *cat-1* and *mod-5*, show high levels of mono- di- and tri- methylation (H3K9me1 peaks) that coincide with HPL-2 binding.

For this I analysed *unc-4* endogenous expression levels (using a CRISPR tagged allele) between wildtype worms and *hpl-2* mutants, all grown at 25°C (*hpl-2* mutation is temperature sensitive). However, contrary to our initial hypothesis, I found *unc-4* expression levels to be significantly reduced in this mutant background (**Fig 4.11**) indicating that in the serotonergic phenotype in VC4 and VC5 observed in H3K9me mutants *unc-4* overexpression is not needed (**Annex 4.8**). Importantly, in agreement with my observation recent results show that

H3K9me repression of undesired effector genes in muscle and hypodermal lineages do not affect expression of key cell fate selector TFs (Methot et al., 2021).

Altogether, we found that 1) in wildtype *C. elegans* animals, both modest levels of *unc-4* and epigenetic silencing of *mod-5* locus repress the serotonergic phenotype in VC4 and VC5 2) overexpression of *unc-4* is sufficient to activate at low levels *C. elegans mod-5* intron 1 enhancer, both in the exogenous and endogenous locus, even in the context of wildtype epigenetic factors 3) *mod-5* locus, but not *unc-4* show strong marks of H3K9me2, suggesting it is directly epigenetically repressed 4) induced serotonergic phenotype in H3K9me mutants requires *unc-4* but does not require increased levels of UNC-4.

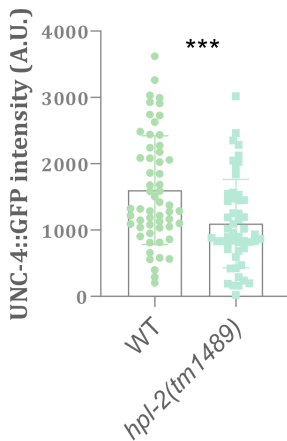


Figure 4.11. Quantification UNC-4::GFP fluorescence reporter intensity in *C. elegans* VC4 and VC5 neurons. UNC-4::GFP expression levels are significantly reduced in *hpl-2(tm1489)* mutants (n=57,52 neurons). T-test was applied for statistical analysis.

In conclusion, results from this first section allow me to conclude 1) *Angaria* species serotonergic phenotype in VC4 and VC5 is mediated by changes in the *mod-5 cis-regulatory* regions, 2) *unc-4* directly activates *C. angaria mod-5* locus, 3) *C. angaria mod-5* enhancer is sufficient to induce VC4 and VC5 neuron serotonergic phenotype in *C. elegans*, 4) *unc-4* overexpression can induce *C.*

elegans serotonergic phenotype in VC4 and VC5 neurons even in the context of wild type H3K9me repression, 5) *mod-5* has high levels of H3K9me1 and me2 and HPL-2 recruitment, 6) serotonergic phenotype depression in *C. elegans* VC4 and VC5 neurons of H3K9me pathway mutants or of *unc-4* induction are independent from one another. Next, to complement these molecular studies I explored the possible behavioural consequences of VC4 and VC5 serotonergic phenotype in *Angaria* species.

4.2. Behavioural consequences of the VC4 and VC5 neuron serotonergic phenotype in *C. angaria*

Once we identified the molecular changes responsible for the induction of VC4 and VC5 neuron serotonergic phenotype in *C. angaria*, we aimed to understand the functional implications of this evolutionary change. Specifically, we investigated the effects of VC4 and VC5 neuron serotonergic phenotype on egg-laying behaviour.

4.2.1. Comparative study of the number of eggs laid in *C. elegans* and *C. angaria*

As explained in the introduction, VC4 and VC5 together with the HSN, are the main neurons controlling egg-laying behaviour, and 5-HT is released by HSN, together with other acetylcholine and other neuropeptides, to activate vulval muscles. VC4 and VC5 neuron synapses lie in close proximity to HSN synapses, thus we hypothesized that VC4 and VC5 could reuptake 5-HT released from HSN to control the egg-laying process differently from *C. elegans*, for example by restricting the action of 5-HT and thus reducing the contractions of the vulva. Then, a simple hypothesis could be that having *C. angaria* VC4 and VC5 serotonergic neurons will lead to a decrease in the number of laid eggs or a change in egg-laying dynamics.

To test this hypothesis as a first approximation I studied the total number of eggs laid each day in 3 consecutive days (24h, 48h and 72h period). *C. elegans* and *C. angaria* are two different species, separated by millions of years of independent evolution, thus differences between species could be attributed to many different factors and not just the VC4 and VC5 differential phenotype. Thus, in order to drive useful conclusion, we took advantage of strains we had

already generated in the project that compare the same species but with the only difference of the VC4 and VC5 serotonergic phenotype.

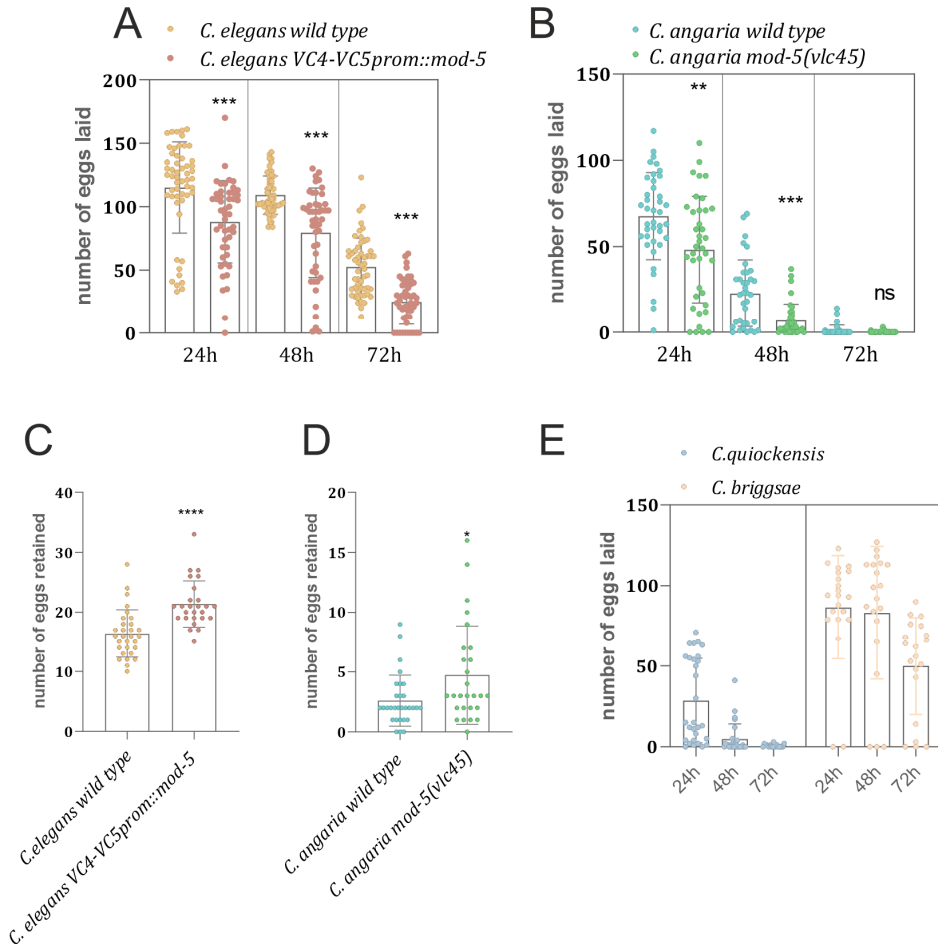


Figure 4. 12. Number of eggs laid by different species of the *Caenorhabditis* genus.

A. *C. elegans* wildtype animals lay more eggs than *C. elegans* expressing *mod-5* cDNA in its VC4 and VC5 scored in 24h periods: at 24h, 24-48h and 48-72h after young adult (n=54, 54). **B.** *C. angaria* wildtype lay more eggs than *C. angaria* *mod-5(vlc45)* scored in 24h periods: at 24h, 24-48h and 48-72h after young adult (n=39, 39). **C.** *C. elegans* animals with *mod-5* expression in VC4 and VC5 neurons retains more eggs than *C. elegans* wildtype (n=30,26). **D.** *C. angaria* *mod-5(vlc45)* retains more eggs than *C. angaria* wildtype (n=30,27). **E.** *C. briggsae*, specie with no serotonergic VC4 and VC5 neurons, lay similar number of eggs to *C. elegans* and more than *C. angaria* and *C. quiockensis* which have serotonergic VC4 and VC5 (n=29,32). T-test was applied when distributions were normal, if not, Mann-Whitney test was used for statistical purposes.

Therefore, we compared wild type N2 *C. elegans* animals with a *C. elegans* transgenic strain that carries a construct to express *mod-5* cDNA in its VCs (emulating what we term an “angarised” scenario). In the case of *C. angaria*, we compared *C. angaria* control animals to a *C. angaria mod-5* mutant strain (generated by Dr. Mora) that we consider a proxy for an “eleanised” worm. As *C. angaria* needs to be mated to generate offspring I crossed females from both the wildtype and the mutant strain to WT males to avoid introducing variability through *mod-5* mutant males.

I scored the number of laid eggs in three consecutive days (24, 48 and 72h after young adult stage). As expected, *C. elegans* wild type animals show a decrease in the number of eggs laid 3 days after reaching young adult stage due to sperm depletion (Angeles-Albores et al., 2017). In the case of *C. elegans* transgenic animals with expression of *mod-5* in the VC neurons there is also a decrease in the number of eggs laid in the third day (72h) (**Fig 4.12 A**). Importantly, there is a significant decrease in the number of eggs laid in *C. elegans* transgenic animals at all stages compared to controls, what supports our hypothesis that *mod-5* expression in VC4 and VC5 neurons could uptake 5-HT from HSN and prevent its action.

C. angaria egg-laying behaviour has not been studied in the past. We found that the number of eggs laid by *C. angaria* is smaller than *C. elegans* at all time periods analysed. Similar to *C. elegans*, *C. angaria* animals also show a reduction in the number of eggs laid 72h post mating. Indeed, this decrease is already apparent 48h post mating (**Fig. 4.12B**). When comparing wild type *C. angaria* with *C. angaria mod-5* mutants I found significant defects in egg laying in all stages (**Fig 4.12 B**). These results were contradictory to our initial hypothesis, moreover, as both the gain and loss of VC4 and VC5 serotonergic phenotype in

both species lead to the same phenotype the results did not help us identify the possible role of *mod-5* expression in VC4 and VC5 neurons.

I performed similar studies with two additional species, *C. quiockensis*, with VC4 and VC5 serotonergic phenotype, which belongs to the *Angaria* group, and *C. briggsae* which does not show VC4 and VC5 neuron 5-HT staining. *C. quiockensis* laid very few eggs, most of them in the first 24h after mating while *C. briggsae* released many more eggs, alike *C. elegans*, with a drop in egg laying 72 h after reaching young adult stage (**Fig 4.12 E**)(**Annex 4.9**).

We thought that maybe our experimental set up was not adequate to identify complementary VC4 and VC5 serotonergic phenotypes because *C. angaria* lays fewer eggs than *C. elegans* (**Annex 4.9**). Thus, alternatively, I quantified the number of eggs inside worms 24 hours after young adult stage instead of eggs laid. In this case we found that both *C. elegans* transgenic animals expressing *mod-5* in VC4 and VC5 neurons as well as *C. angaria mod-5* mutants both retain more eggs inside the mother compared to controls (**Fig 4.12 C and D** and **Annex 4.12**). Thus, this alternative quantification method also not reveal any differential behaviour that could be attributed to the VC4 and VC5 serotonergic phenotype.

4.2.2. Egg-laying dynamics study in *C. elegans* and *C. angaria*

We reasoned that maybe our initial approach of quantifying laid eggs in 24h periods did not have enough resolution to detect small changes in egg laying dynamics. Thus, we turned into a more precise analysis through single worm recordings of 6h to quantify individual egg laying events. All recordings were performed 12 to 24 hours after mating, to cover the periods of highest rates of egg laying. To study this process, we took as reference previous models,

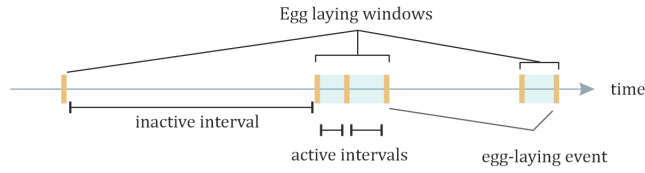
explained in the introduction, in which this behaviour was modeled as a rhythmic two-state behaviour with active phases of egg-laying with variable egg-laying events (in 1-2 min periods) and inactive phases of no egg laying of variable lengths (Waggoner et al., 1998).

Recordings were performed in collaboration with Dr. Arantza Barrios, at University College London, who recorded single worms for 6 hours. Then, videos were manually analysed, and I registered the time when every egg was laid. Adrian Tarazona, another PhD student in our lab, performed statistical analysis to model egg-laying behaviour in the four different genetic conditions: *C. elegans*, *C. elegans mod-5 VC4-VC5* expression, *C. angaria*, and *C. angaria mod-5* mutant.

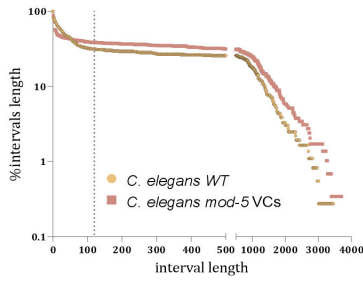
To specifically investigate the dynamics of the egg-laying behaviour, we defined some important concepts similar to previous work (Waggoner et al., 1998). Every egg laid is termed as an egg-laying event, the time between two egg-laying events is an interval. If the time between two egg-laying events is longer than 120 seconds, the interval is considered an inactive interval. Alternatively, all egg-laying events separated by less than 120s will belong to the same egg-laying active window and the time between 2 egg-laying events inside an egg-laying window will be known as an active interval (**Fig 4.13 A**).

We set 120 seconds as the threshold to delimitate the active state as previously described (Waggoner et al., 1998) (however we also performed similar analyses with a threshold of 1 minute obtaining similar results). For each genotype and condition, at least six different animals were recorded (**Annex 4.11**).

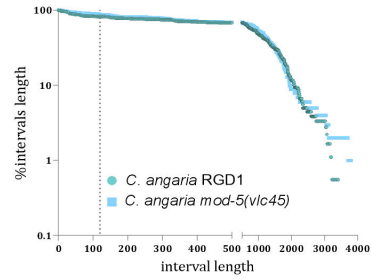
Egg laying dynamics



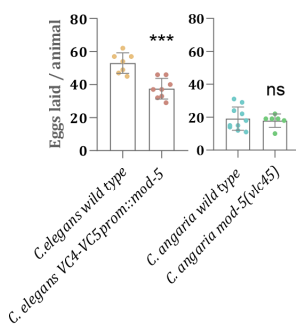
C. elegans interval length distribution



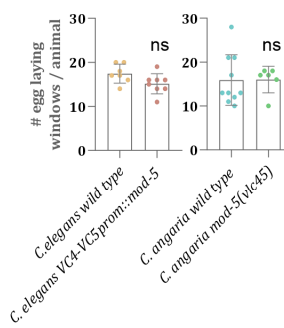
C. angaria interval length distribution



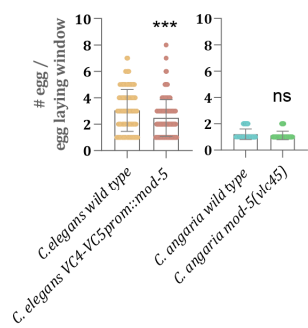
Number of eggs



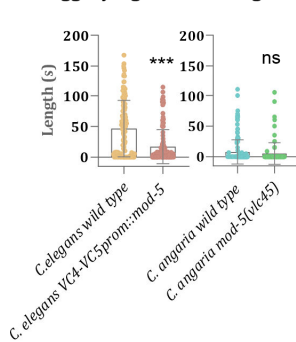
D Egg-laying windows



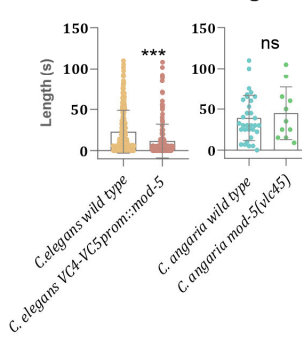
E Eggs per egg laying window



Egg laying window length



G Active interval length



H Inactive interval length

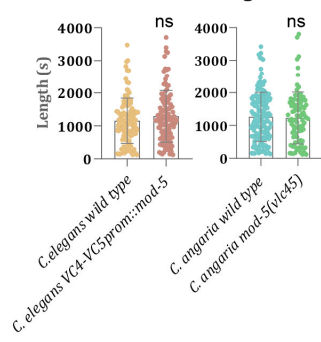


Figure 4. 13. Egg laying dynamics in *C. elegans* and *C. angaria*. **A.** Schematic representation of egg laying behaviour and terms used in the analysis. Animals were recorded moving freely in OP50 plates for 6h and egg-laying events (shown as orange vertical lines) were manually annotated. *C. elegans* egg-laying behaviour transitions from active periods of egg-laying (shown in blue) to inactive periods with no egg laying. Transition into an inactive interval is assigned when the time between two egg-laying events is longer than two minutes (Waggoner et al., 1998). An egg-laying window is defined by the time comprised between the first and the last egg-laying event with intervals shorter than two minutes (active intervals). **B.** Interval length distribution in *C. elegans* wild type (n=7) compared to transgenic animals expressing *mod-5* cDNA in VC neurons (n=8) (**left graph**) or *C. angaria* wild type (n=10) compared to *C. angaria mod-5(vlc45)* mutant (n=6) (**right graph**). Both *C. elegans* strains show a bi-phasic behaviour, while *C. angaria* strains do not. Dotted lines mark 120s intervals, dividing active from inactive intervals. **C.** Quantification of total number of laid eggs. *C. elegans* transgenic animals expressing *mod-5* cDNA in VC neurons lay significantly fewer eggs than *C. elegans* wild type. **D.** There are no significant differences in the number of egg-laying windows among groups. **E.** Quantification of the number of eggs laid per egg-laying window shows a significantly smaller number of eggs laid by *C. elegans* transgenic animals expressing *mod-5* cDNA in VC neurons compared to wild type. **F.** Egg laying window length quantification, showing significantly shorter windows in *C. elegans* transgenic animals compared to wild type. **G.** Active interval length is also significantly shorter in *C. elegans* transgenic animals compared to wild type. **H.** No differences in the length of inactive phases were found between genotypes of the same species. T-test was applied when distributions were normal, if not, Mann-Whitney test was used for statistical purposes.

In the first place, to have a general view of how worms lay eggs, we represented the accumulative frequency of intervals with respect to the length of those intervals (**Fig 4.13 B**). The dotted line in the graph (at 120s) divides active from inactive intervals. Our data on *C. elegans* N2 wildtype strain reproduces previous reports on egg-laying dynamics (Waggoner et al., 1998). Next, we aimed to compare the different genetic backgrounds. In general, we found that *C. elegans* both wildtype and the strain expressing *mod-5* in VC4 and VC5 neurons have a bi-phasic behaviour that is represented by a initial steep slope seen for the high frequency of short active intervals. In contrast, *C. angaria*, both wildtype and mutant strains, do not show the initial slope in the graph, showing a lack of bi-phasic behaviour between active and inactive states. Indeed, there

are very few active intervals in *C. angaria* species, suggesting they do not transition from active to inactive periods of egg laying. This conclusion was possible thanks to the use of egg laying recordings which reveals the importance of correct analytical set ups to drive insightful conclusions.

Despite the interesting finding of the different egg-laying dynamics between *C. elegans* and *C. angaria* species, the interval frequency distributions did not reveal major differences between *C. elegans* and *C. elegans* animals expressing *mod-5* in VC4 and VC5 neurons nor between *C. angaria* and *C. angaria mod-5* mutant animals. Thus, we compared different specific features among genotypes. Similar to our previous analysis, we found that in this 6h period, *C. elegans* WT laid significantly more eggs than *C. elegans* expressing *mod-5* in their VCs. However, in the case of *C. angaria*, in these smaller time frames analysed at the initial stages of egg laying, there was no difference in the number of eggs laid between *C. angaria* WT and *C. angaria mod-5* mutants (**Fig 4.13 C**). Comparing the number of egg-laying windows, we could not find differences between *C. elegans* strains, nor between *C. angarias*' (**Fig 4.13 D**). However, we found that wildtype worms in *C. elegans* laid more eggs per active windows than *C. elegans* with *mod-5* in VCs, but no differences were found in *C. angaria* strains (**Fig 4.13 E**). Finally, we also studied the length of egg-laying windows, active and inactive intervals. In the case of *C. angaria* wildtype and the *mod-5* mutant, we could not find differences in the length of any of these periods. However, for the *C. elegans* genotypes, we found longer active intervals and egg-laying windows in the wild type than in the *C. elegans* expressing *mod-5* in VCs (**Fig 4.13 F, G and H**).

Thus, in conclusion, our results in *C. elegans* are consistent with the idea of VC4 and VC5 neuron reuptake having a role in preventing prolonged 5-HT signaling from HSN: we found that worms with VCs expressing *mod-5*, tend to have

shorter active intervals and shorter egg-laying windows that result in fewer eggs laid per active window (even when the number of egg-laying windows is similar). (**Annex 4.11**).

However, when studying *C. angaria* egg-laying dynamics, we could see that *C. angaria* egg-laying events were grouped in the form of active windows as we could see in *C. elegans*, in contrast, they have individual egg-laying events more randomly distributed (**Fig 4.13 B**). However, when comparing *C. angaria* with *C. angaria mod-5* mutant we could not detect any significant difference between these strains.

Thus, despite major efforts studying egg laying dynamics at high resolution in different *C. elegans* and *C. angaria* strains we could not identify any behavioural difference on egg laying that could be attributed to the VC4 and VC5 serotonergic phenotype.

4.2.3. Egg laying induction in high serotonin concentration media.

Our failure to assign functional relevance to the VC4 and VC5 serotonergic phenotype made us reflect on our experimental procedures. Environmental change and adaptation are important driving forces for evolution; however, our behavioural studies had been performed in standard laboratory conditions that relate very little to the real changing environment that nematodes inhabit. Indeed, several external conditions can modify egg laying such as starvation (reviewed in Schafer, 2005), in addition, exogenous serotonin itself is able to induce egg laying (Horvitz et al., 1982), although the mechanisms mediating this effect are not completely characterized.

In his thesis, Dr. Mora analysed the response to high exogenous 5-HT in several *Caenorhabditis* species, belonging to and not to the *Angaria* group. He found that exposure to exogenous serotonin induced an increase in the number of eggs laid in *C. elegans* N2 (as already described Horvitz et al., 1982) and other species without VC4 and VC5 serotonergic phenotype, however, for tested species in the *Angaria* group, with serotonergic VC4 and VC5 neurons, this induction was avoided (Mora-Martínez, 2019).

This made us wonder if VC4 and VC5 serotonergic phenotype could be related to this different behaviour. Importantly, although 5-HT is mostly known as a classical neurotransmitter in animal nervous systems, it is a very ancient molecule that is also synthesized and used as a hormone by some plants and or a signal molecule by microorganisms (Reviewed at (Gonçalves et al., 2022)). Nematodes live in complex environments of rotten fruits and vegetables with a high diversity of fungi and bacteria. Indeed, some *C. angaria* isolates have been found in fruits with high levels of 5-HT such as tomatoes, bananas, or pineapples. Thus, it is conceivable that these nematodes could potentially encounter environments with high levels of exogenous serotonin. How could then this exogenous 5-HT affect egg-laying behaviour and what is the role of VC4 and VC5 serotonergic phenotype in protecting worms from that effect?

In the first place, I wanted to repeat Dr. Carlos Mora experiment of exogenous 5-HT exposure but including the genotypes of *C. angaria* (wildtype and *mod-5* mutant) and *C. elegans* (wildtype and transgenic animals with *mod-5* expression in VC4 and VC5 neurons) and try to evaluate the importance of *mod-5* in VC4 and VC5 neurons in this experimental setup.

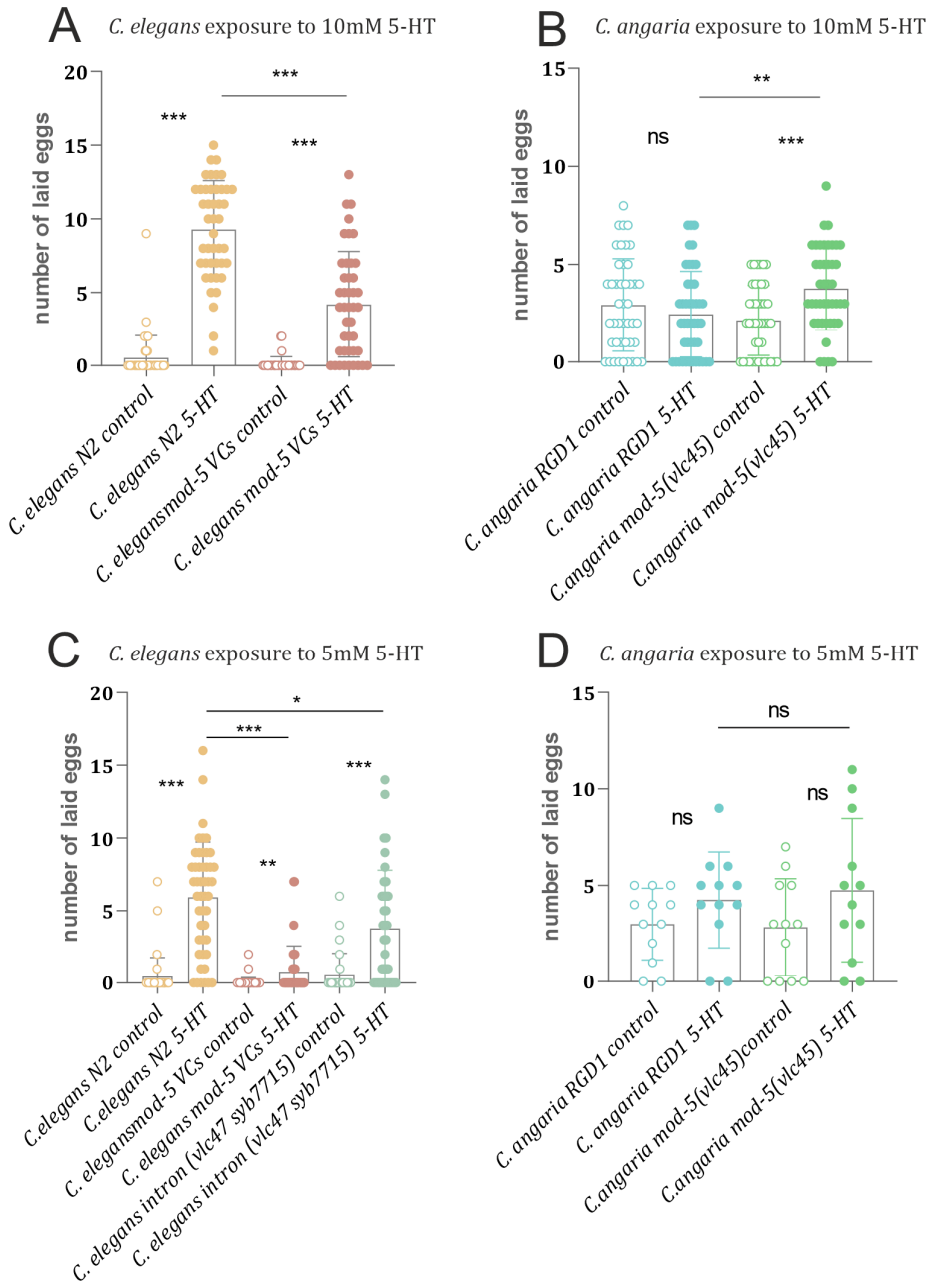


Figure 4.14. Egg laying behaviour upon acute 5-HT exposure. **A.** 10 mM 5-HT exposure induces egg laying in both *C. elegans* genotypes. Importantly, when *C. elegans mod-5* cDNA is expressed in VC4 and VC5 animals show significantly fewer laid eggs when exposed to 10mM 5-HT compared to wildtype (n=43). **B.** *C. angaria* wildtype egg-laying is not affected by exogenous 5-HT but *C. angaria mod-5(vlc45)* mutants lay significantly more eggs when exposed to 5-HT (n=44). **C.** 5mM 5-HT exposure induces egg-laying in the three genotypes of *C. elegans*. There are significant differences between *C. elegans* wildtype exposed to 5-HT 5mM and both *C. elegans* expressing *mod-5* in VC4 and VC5 neurons and *C. elegans (vlc45 syb7715)* (n=47,47,48,40,28,36). **D.** 5-HT 5mM exposure does not induce egg laying in *C. angaria* wildtype nor *C. angaria (vlc45)* mutants (n=12). T-test was applied when distributions were normal, if not, Mann-Whitney test was used for statistical purposes.

Worms were placed in S complete media, as control, or media supplemented with 5-HT 10mM for 2 hours, next, I scored the number of laid eggs. In the case of *C. elegans* as expected we observed a significant increase in the number of laid eggs when exposed to exogenous 5-HT compared to control conditions. Importantly, when examining exposure to 5-HT in *C. elegans* worms with *mod-5* expressed in VCs, despite that we also found egg-laying induction compared to control media, we could observe that the behaviour of laying eggs due to external serotonin was significantly different to *C. elegans* N2 wildtype. Thus, we found that worms that express *mod-5* in their VC4 and VC5 neurons were less responsive to the external serotonin than the wildtype worms (**Fig 4.14 A**)(Annex 4.12).

Next, when studying *C. angaria* response to external 5-HT, I could observe that worms do not respond to external serotonin and lay a similar number of eggs when exposed or not to 5-HT. In contrast, in the case of *C. angaria mod-5* mutants, I observed an increase in the number of eggs laid when exposed to exogenous serotonin compared to control conditions. These results demonstrate that, in *C. angaria*, *mod-5* is important in controlling the egg-laying response to external serotonin (**Fig 4.14 B**) (Annex 4.12).

I also repeated the same experiments at lower 5-HT concentration (5mM) and this time I also included the *C. elegans* strain carrying the first intron of *C. angaria mod-5*, *C. elegans mod-5(vlc47 syb7715)*, which shows a serotonergic phenotype in their VC4 and VC5 neurons alike *C. angaria*. Using this 5-HT concentration I could observe behaviour differences between *C. elegans* N2 and *C. elegans* worms with *mod-5* expressed in VCs.

Indeed, lack of response to external 5-HT in *mod-5* transgenic animals was more pronounced at this concentration. In the case of the *C. elegans* strain carrying *C. angaria* intron1, I found a significant response in the 5-HT induction of egg laying, however, the response was significantly lower than in N2 *C. elegans* control strain (**Fig 4.14 C**). In the case of the *C. angaria* strains, we could not find differences between both genotypes even comparing only the worms exposed to 5-HT, yet it is important to mention that the number of worms in both *C. angaria* strains was lower than in the 10mM serotonin concentration condition, thus additional experiments at this concentration should be added to be able to conclude the effect in *C. angaria* (**Fig 4.14 D**) (**Annex 4.12**).

4.2.4. VC4 and VC5 from *C. elegans mod-5(vlc47 syb7715)* strain can reuptake serotonin from the environment.

Experiments obtained in the previous section were very exciting and at the same time puzzling. For the first time we could identify a behavioural phenotype that we could directly attribute to the presence of VC4 and VC5 serotonergic phenotype in both *Caenorhabidits* species. However, this phenotype was unconventional, as it did not involve internal egg-laying circuits but exposure to external environmental signals. Then we thought of a possible explanation for a hypothetical purpose of this phenotype in the wild. Since the vulva structure is exposed to the external media, and so are vulval muscles and

VC4 and VC5 neurons termini projections (**Fig 4.15 A**) and one of the most important neurotransmitters used by the HSN to activate vulva muscles contraction is the 5-HT (Desai et al., 1988), we hypothesized that maybe the ability of VC4 and VC5 neurons to reuptake serotonin could be related to the elimination of the external serotonin from the proximity of HSN 5-HT synapses to avoid responses to external 5-HT that could be creating noise to the HSN endogenous communication with the vulva muscles. This scenario implies that VC4 and VC5 neurons expressing *mod-5* are able to re-uptake 5-HT not only from the HSN but also from the environment. To probe this in *C. angaria* was challenging because it is difficult to generate new mutants, thus alternatively we used the *C. elegans* strain carrying the *C. angaria* intron 1 of *mod-5* (*vlc47 syb7715*) that we know its VC4 and VC5 neurons are able to reuptake 5-HT. First, to eliminate all the endogenously synthesized 5-HT I crossed this strain to a *bas-1(ad446)* mutant, since this enzyme is necessary in all the possible pathways in the worm that lead to 5-HT synthesis (Yu et al., 2023). As expected, 5-HT staining in *mod-5 (vlc47 syb7715); bas-1(ad446)* double mutant show absence of 5-HT in the whole body, including VC4 and VC5 neurons. After that, I grew the worms in soft NGG media at both control and high serotonin concentrations (10mM) (see methods for more information about this media) and then I performed serotonin staining. As expected, I found that in control conditions there was no 5-HT staining, however, these worms grown in 10mM 5-HT media show 5-HT staining in VC4 and VC5 neurons, meaning that these two neurons are capable of reuptaking serotonin from the environment. We

also observed some staining in the NSM neurons in the head, but not in other neurons (**Fig 4.15 B**) (**Annex 4.13**).

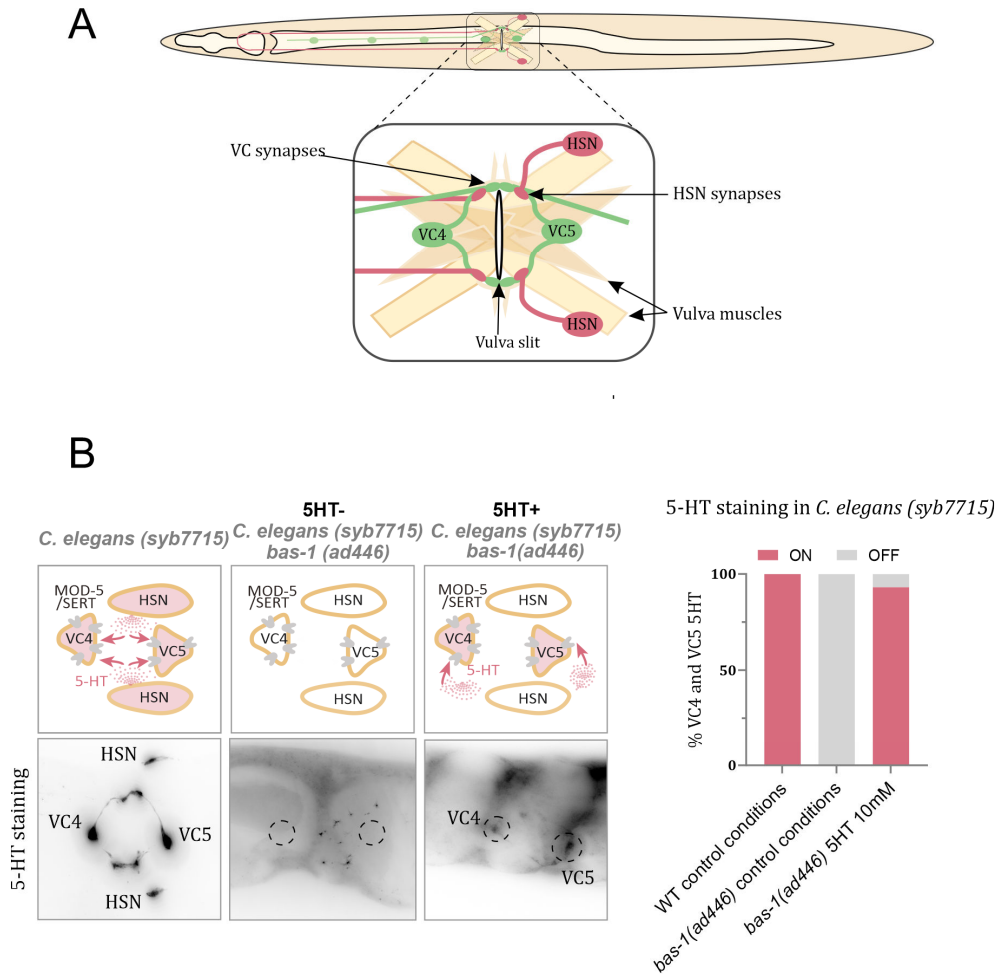


Figure 4.15. VC4 and VC5 can reuptake serotonin from the outside. **A.** Representation of the position of VC4 and VC5 neurons and their projections in the body of the worm showing the proximity of their termini projections to the vulva slit and to the HSN neuromuscular synapses. **B.** Schematic depiction (top left), representative micrographs (bottom left) and quantification (right) of VC4 and VC5 neuron serotonin staining in *C. elegans mod-5(vlc47, syb7715)* animals, *C. elegans (vlc47, syb7715); bas-1(ad446)* animals that lack serotonin biosynthesis and *C. elegans mod-5(vlc47,syb7715); bas-1(ad446)* animals exposed to high levels of external 5-HT. Quantification shows VC4 and VC5 neurons can uptake 5-HT from external sources. (n=40; 50; 51).

In summary, this result's section allows me to conclude that 1) species with fully serotonergic VC4 and VC5 tend to lay fewer eggs than worms with variable or no 5-HT in these neurons, however, as both tested species without serotonergic VC4 and VC5 are hermaphroditic (*C. elegans* and *C. brigssae*) in contrast to gonochoristic *Angaria* group, the correlation would be related to this fact, thus future experiments with additional gonochoristic species outside the *Angaria* group would be necessary to drive strong conclusions in this regard 2) *C. angaria* egg-laying dynamics do not follow the bimodal pattern found in *C. elegans* 3) VC4 and VC5 serotonergic fate in *C. angaria* have a role in controlling the egg-laying behaviour in environments with high 5-HT concentration 4) Genetically induced serotonergic VC4 and VC5 in *C. elegans* protects from the egg-laying induction produced by exogenous serotonin 5) VC4 and VC5 neurons expressing *mod-5* can reuptake serotonin of the external media.

Considering, as already mentioned, that *C. angaria* has been sometimes isolated from high serotonergic environments, this result could explain the fixed serotonergic condition found in their VC4 and VC5 neurons.

Given that we found that *Angaria* species show a constitutive VC4 and VC5 serotonin staining and that *C. elegans* N2 strain actively represses through epigenetic mechanisms the VC4 and VC5 serotonergic phenotype, but sometimes we find weak 5-HT staining in some animals, in the next section we will explore if VC4 and VC5 5-HT repressed phenotype in *C. elegans* could indeed be a plastic phenotype released under specific stimuli and also if genetic variability among different wild isolates that could potentially show diversity on the basal levels of repression in VC4 and VC5 5-HT staining.

4.3. Characterization of the plasticity in VC4 and VC5 serotonergic phenotype in *C. elegans*

4.3.1. Study of VC4 and VC5 serotonergic phenotype in *C. elegans* wild isolates

Since I started this project, I have been detecting some 5-HT staining in VC4 and VC5 when performing staining assays on the N2 strain in *C. elegans*, this staining is generally found in a small percentage of the population and is quite variable between assays. *C. elegans* N2, the strain used in the laboratory, has been grown at standard laboratory conditions for more than a decade before it was frozen, thus it is a derived strain with several examples of laboratory adaptation (Sterken et al., 2015). This situation made us think that maybe the VC4 and VC5 5-HT status of N2 strain was not exactly reflecting the behaviour in the wild. Recent efforts from the *Caenorhabditis* Natural Diversity Resource (CaeNDR) have isolated, sequenced and characterized the genetic variability of thousands of *C. elegans* wild isolates.

CaeNDR has built a collection of strains that contain a wide representation of the wild isolate genetic variability. Thus, we ordered this collection and added a few more isolates to build a collection of eighteen different strains isolated from all around the world (**Fig 4.16 A**). I performed three independent sets of 5-HT staining assays with these strains. The first staining was performed in a mixed population of stages, although I only scored HSN-positive worms (meaning there are already adults, they could be younger or older). We found a surprising variability, from strains with hardly any 5-HT staining in their VC4 and VC5 neurons (QX1211, JU1581 and MY23 showed levels similar or lower than N2), to others with percentages of 80% of animals with 5-HT staining (E.g JU775 and JU782) (**Fig 4.16 B**). To try to avoid the possible effect of age in the variable serotonergic phenotype of VC4 and VC5, I performed two replicates of the staining in synchronized populations of young adults. However,

synchronization did not decrease variability in 5-HT staining, in addition, we also found that the variability is not only high among different isolates but also between replicates using the same isolate. Indeed, in the two N2 replicates with synchronized worms, we found an unexpected high percentage of animals with VC4 and VC5 staining, not observed previously (**Fig 4.16 C**)(**Annex 4.14**).

These results point out the huge variability that we could find between different strains of *C. elegans* or even in different replicates. We hypothesized that a particular environmental trigger (e.g particular humidity, temperature, plate contaminant, etc) might have been responsible for the increase in staining observed in N2. Further experiments need to be performed to evaluate more systematically the source of variability and to distinguish genetic from the environmental components of such variability. Nevertheless, these preliminary results showed that this particular serotonergic phenotype certainly is a plastic trait in *C. elegans*.

4.3.2. Egg-laying behaviour comparison between N2 and CB4856 Hawaiian wild isolate.

Given that we saw some *C. elegans* strains with important percentages of VC4 and VC5 stained for 5-HT and the previous evidence suggesting N2 adaptation to laboratory conditions, we decided to compare egg-laying dynamic between N2 and one of the most genetically diverse from N2 and well-studied wild isolates CB4856 (Hawaiian strain). We performed 6 hours movies and analysed them similar to analysis in **Fig 4.13**. In first place, we found that CB4856 slope of short intervals was less pronounced than in N2, and more similar to N2 transgenic animals expressing *mod-5* in VC4 and VC5 (Compare **Fig 4.17A** to **Fig 4.13B**).

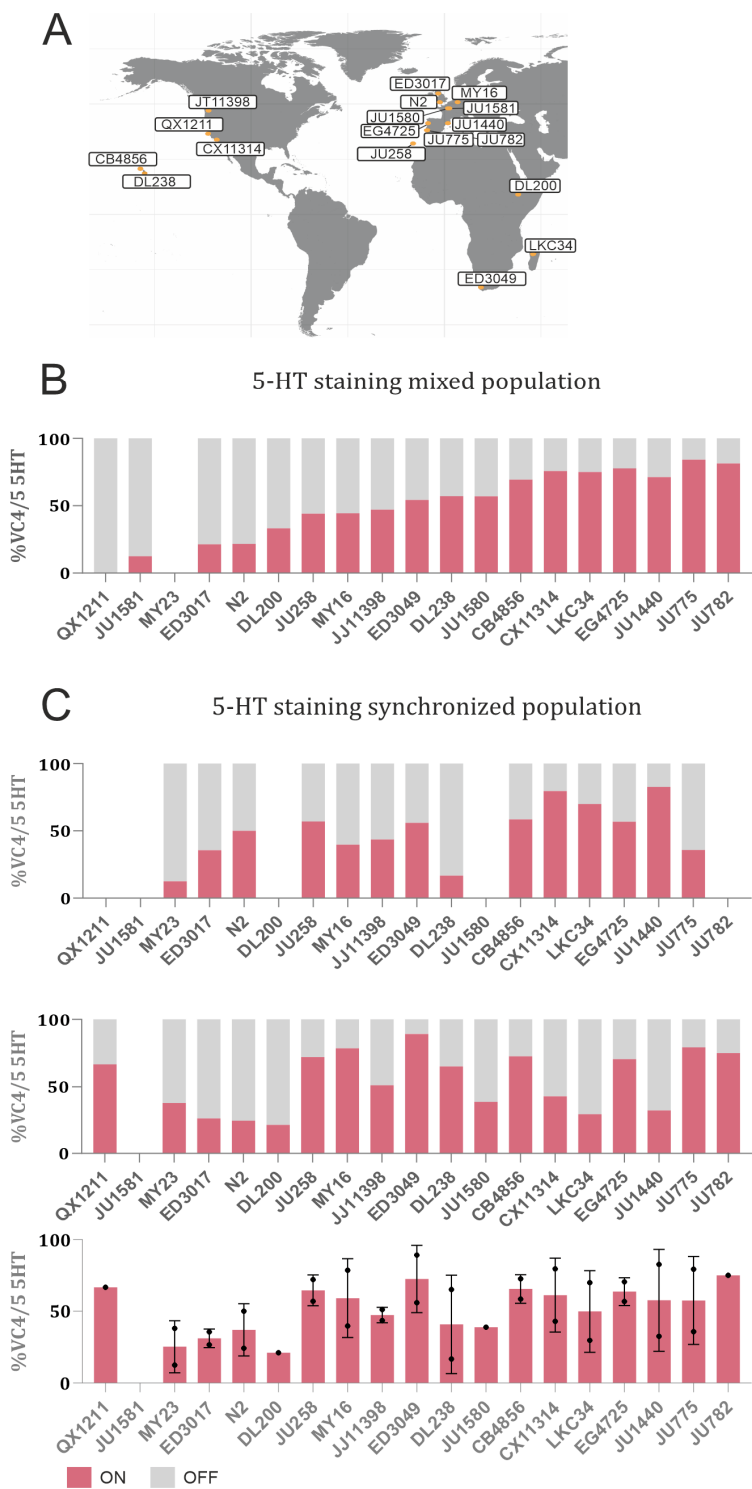


Figure 4. 16. Variability in serotonergic staining in VC4 and VC5 neurons in different *C. elegans* wild isolates and experimental replicates. **A.** Map representing the origin of the different wild isolates used. **B.** Quantification of the percentage of VC4 and VC5 neurons that stain for 5-HT in mixed populations of different *C. elegans* wild isolates and N2 **C.** Quantification of the percentage of VC4 and VC5 neurons that stain for serotonin in synchronized populations of *C. elegans* isolates in two independent assays. ($n > 30$ in all assays per genetic background). Last graph represent the mean of the two synchronized assays.

This is due to a drastic reduction in the frequency of very short active intervals in the wild isolate compared to N2. We could observe a reduced number of eggs laid in CB4856 worms compared to N2 (**Fig 4.17 B**) even less than N2 with *mod-5* expression in VCs (compare to **Fig 4.13C**). We also found that N2 had shorter inactive intervals than CB4856, and N2 had longer egg-laying windows with a higher number of eggs laid per window and a higher number of active windows than CB4856 (**Fig 4.17 C, D, E, F, and G**)(**Annex 4.11**). In general, CB4856 egg laying behaviour is more similar to N2 transgenic animals expressing *mod-5* in VC4 and VC5 than to N2 wild type, with some notable examples, namely, CB4856 has less egg laying windows than transgenic *mod-5*.

Transition from inactive to active state has been described to depend on HSN 5-HT release, thus this phenotype could be explained by lower HSN 5-HT content in the hawaiian strain. Accordingly, this leads to longer inactive intervals in CB4856 which are not observed in transgenic *mod-5* animals. Finally, active interval lengths are shorter in the transgenic animals than in CB4856, the duration of these intervals might be directly related to the ability of clear 5-HT from the synapse, that could be stronger in transgenic animals expressing *mod-5* under a heterologous promoter than in the wild isolate strain.

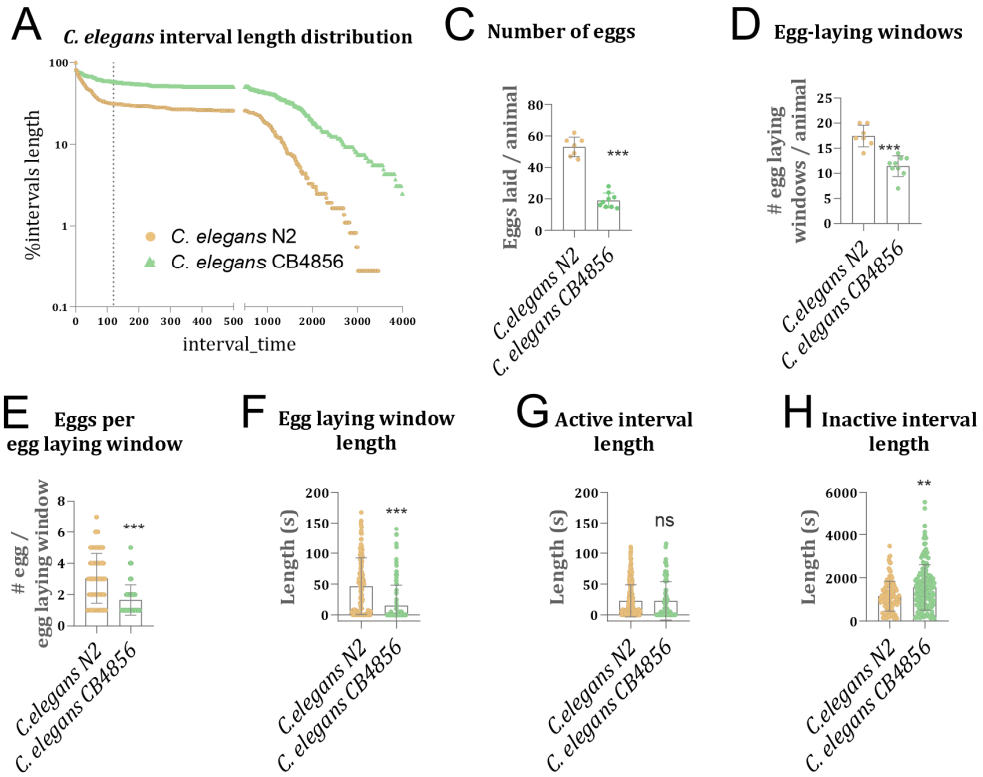


Figure 4. 17. Egg laying dynamics in *C. elegans* N2 and *C. elegans* CB4856 WT. A. Egg-laying dynamics show that CB4856 isolate seem to have a less steep slope in the active intervals compared to N2, which could mean that these animals lack the bi-phasic behaviour (n=7,8). **B.** Quantification of total number of laid eggs. CB4856 strain also significantly fewer eggs than N2. **C.** Number of egg laying windows in N2 is significantly higher than in CB4856. **D.** Quantification of the number of eggs laid per egg-laying window shows significantly fewer laid eggs by CB4856 than N2. **F.** Egg laying window length quantification, show significantly shorter windows in CB4856 compared to N2.

4.3.3. Mating as trigger for the VC4 and VC5 serotonergic phenotype in *C. elegans*.

After finding the vast variability in the serotonergic phenotype upon diverse *C. elegans* isotypes and replicates and then a clear plasticity in this phenotype, we searched for the possible trigger that could activate the serotonergic phenotype

in *C. elegans* VC4 and VC5 neurons. Stress is one of the main triggers of plasticity, accordingly, Dr. Mora in his thesis tried some classical stressors in N2, such as starvation, high temperature, hypoxia or pathogenic infection, without finding any significant induction of the VC4 and VC5 serotonergic phenotype (Mora-Martínez, 2019).

Alternatively, I decided to test another environmental trigger. VC4 and VC5 neurons have been reported to respond to mechanosensory stimuli (Medrano & Collins, 2023), on the other hand, it is well known that a different array of stressful conditions results in an increase production of males in the population, thus I reasoned that the mechanosensory stimuli of mating could be a possible trigger of the VC4 and VC5 5-HT phenotype. To test this hypothesis, I performed a serotonergic staining in N2 wild type compared to *C. elegans him-5(e1490)* in which the number of males is increased, and we supposed that should mean an increase also in the number of females exposed to mating. I performed 5-HT staining and measured the intensity in the VC4 and VC5 neurons. Although I did not find significant differences between both genotypes, there is a tendency to higher intensities in the *him-5(e1490)* strain **(Fig 4.18) (Annex 4.15)**. We hypothesize that maybe those females showing greater 5-HT staining could be the ones exposed to mating males, nevertheless, with this first approach we cannot determine if this is the case and considering the great variability we found in the previous section, we cannot conclude mating induces an increase in VC4 and VC5 phenotype. In the future we will perform experiments using *plg-1(e2001)* males, that leave a plug after mating, we will then pick those females to perform 5-HT staining and more accurately determine if mating induces the serotonergic fate.

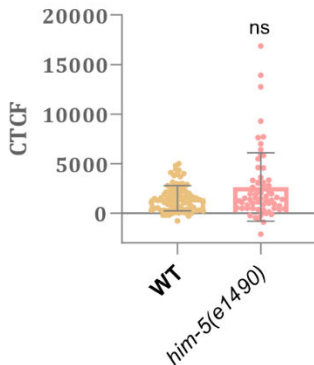


Figure 4. 18. Analysis of VC4 and VC5 5-HT staining induction by mating. Quantification of VC4 and VC5 5-HT staining intensity between a mixed population of wildtype worms and *him-5(e1490)*, there is a tendency of higher intensity in *him-5* animals that is not significant ($n > 60$ neurons). Mann-Whitney test was used for statistical purposes. **G.** Active interval length is significantly shorter in N2 mated compared to self-fertilized

Nevertheless, performing 5-HT staining in a few individuals might be challenging, so in light of these preliminary results we decided to study the egg-laying behaviour of mated hermaphrodites compared to unmated hermaphrodites to determine if mating induces changes in the behaviour that could be related to VC serotonergic phenotype.

4.3.4. Egg-laying behaviour in cross progeny

As a first approximation to determining if egg laying behaviour is modified by mating we performed the scoring of number of eggs in 24h windows after 24, 48, and 72h with *C. elegans* N2 hermaphrodites self-fertilized compared to hermaphrodites crossed to males. We found *C. elegans* not mated laid more eggs in the first 24h after young adult, however, when mated, they tended to release more eggs on the second day, and 72h after young adult stage, these worms still laid an important number of eggs compared to not mated worms (**Fig 4.19 A**)(**Annex 4.9**). The availability of exogenous sperm could explain this effect.

As we have learnt from our previous analysis with *C. elegans* and *C. angaria*, quantification of number of laid eggs is only a rough approximation that does

not allow to identify more subtle changes in the dynamics of egg laying behaviour.

Thus, I moved on to study 6h egg laying recordings of *C. elegans* N2 worms crossed with males. In this case, I found interesting differences, in first place N2 mated animals show similar bimodal dynamics similar to N2, with a high frequency of short intervals.

However, animals from cross progeny tend to lay fewer eggs in the 6 hours of video recording than the self-crossed worms (Fig 4.19 C). We also saw less egg laying windows that were shorter in length and longer inactive intervals. However, we observed that active interval length was shorter, thus resulting in similar number of eggs laid per active window compared to controls (Fig 4.19 F, G and H). Thus I conclude that mating induces a reduction in the number of egg laying windows and increase in inactive interval length, which could be due to a decrease in 5-HT released from HSN, it also shows a decrease in active window and active interval length what could be related to VC4 and VC5 serotonergic induction, however the number of eggs per active window is not significantly different from unmated animals, probably because the reduction in active interval length compensates for the reduction in the active windows length. In summary, mated animals display less processes of active egg laying separated longer intervals of time but lay the same number of eggs in each location. These results could suggest that mated animals will disperse more the population than self-fertilized animals, however, further experiments, with a different experimental set up should be designed to test this hypothesis.

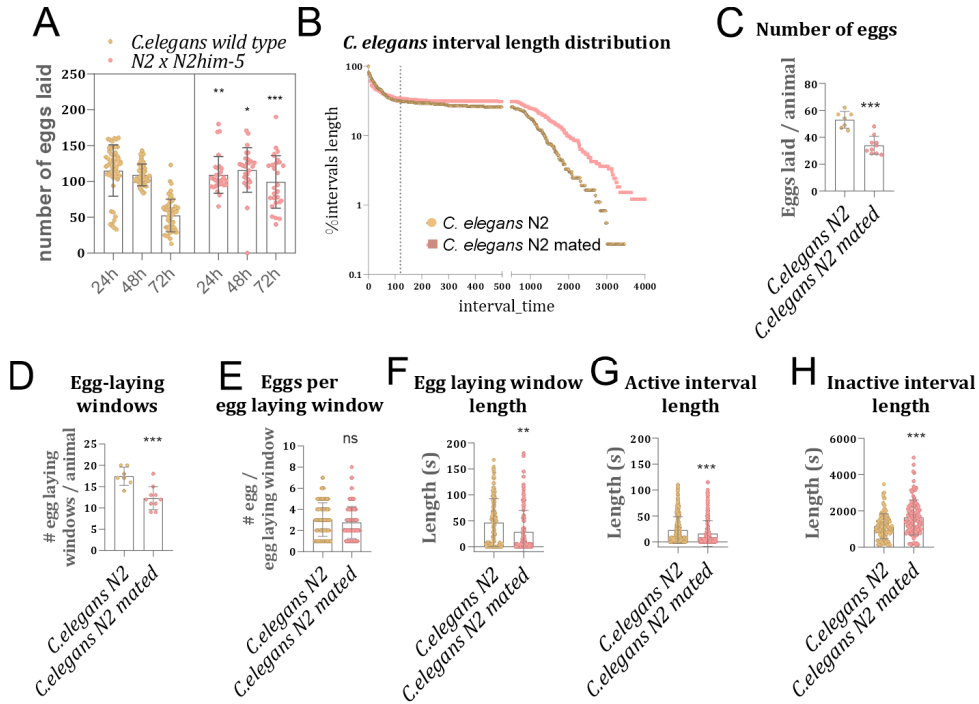


Figure 4. 19. Egg laying dynamics in *C. elegans* N2 self-crossed and *C. elegans* N2 crossed with males. **A.** *C. elegans* N2 hermaphrodites crossed with wildtype males lay fewer eggs at 24h and 24-48h after YA than self-crossed ones, however, hermaphrodites mated lay significantly more eggs than the wildtype in the 48-72 h period, likely due to male deposited sperm (n=54,40). **B.** Egg-laying dynamics show a very similar behaviour between N2 self-fertilized and crossed with males (n=7,9). **C.** Quantification of total number of laid eggs show that N2 mated release fewer eggs compared to N2 self-crossed. **D.** Number of egg laying windows in N2 self-crossed is significantly higher than in N2 crossed with males. **E.** Quantification of the number of eggs laid per egg-laying window shows significantly fewer laid eggs by N2 mated than N2 self-crossed. **F.** Egg laying window length quantification, show significantly shorter windows in N2 crossed compared to N2 self-crossed. **G.** Active interval length is significantly shorter in N2 mated compared to self-fertilized. **H.** Longer inactive phases were found in N2 crossed and in CB4856 compared to self-fertilized N2. T-test was applied when distributions were normal, if not, Mann-Whitney test was used for statistical purposes.

4.3.5. Growing in a high 5-HT concentration environment induces the serotonergic phenotype in *C. elegans* VC4 and VC5 neurons.

Even though mating itself seems to have a role in egg-laying behaviour and might induce some changes in VC4 and VC5 5-HT staining, our results were not very conclusive. Thus, I decided to keep exploring other possible triggers of this phenotype. Considering that my results had shown that the serotonergic VC4 and VC5 phenotype was very important in *C. angaria* to control the egg-laying behaviour in environments with high 5-HT concentration, we thought that this kind of environments would constitute the trigger of the phenotype. Thus, I start growing *C. elegans* N2 animals living in high 5-HT concentration semi-solid media.

Since we did not know how many generations could be needed to acquire the phenotype (if indeed was acquired), we decided to grow the worms in control or serotonergic-supplemented media (10mM) for 6 generations. Previous reports on epigenetic inheritance have described very different number of generations where the trait is maintained, from 3-4 to transgenerational small RNA silencing (Houri-Zeevi et al., 2020), 5 generations to study the inheritance of temperature-induced expression or even 10-15 generations to establish a behaviour (Toker et al., 2022). Thus, as starting point I decided to try for 6 generations but performing a 5-HT staining in the third and the sixth generation **(Fig 4.20 A)(Annex 4.16)**.

To our surprise, we found 87% of VC4 and VC5 stained for serotonin already in the third generation, and that percentage increased to 97% in the sixth generation **(Fig 4.20 B)**. However, we were obtaining a high percentage (48 and 51%) of serotonergic VC4 and VC5 also in control conditions as well, that were not like previous experiments (similar to our results with the wild isolates strains). As already described, this could be due to some uncontrolled

environmental factor or alternatively by some change in the N2 strain. To try to come back to the habitual serotonergic percentages in the control animals, we decided to unfreeze N2 again and repeat the same experiment, but only for one generation. **(Annex 4.16).**

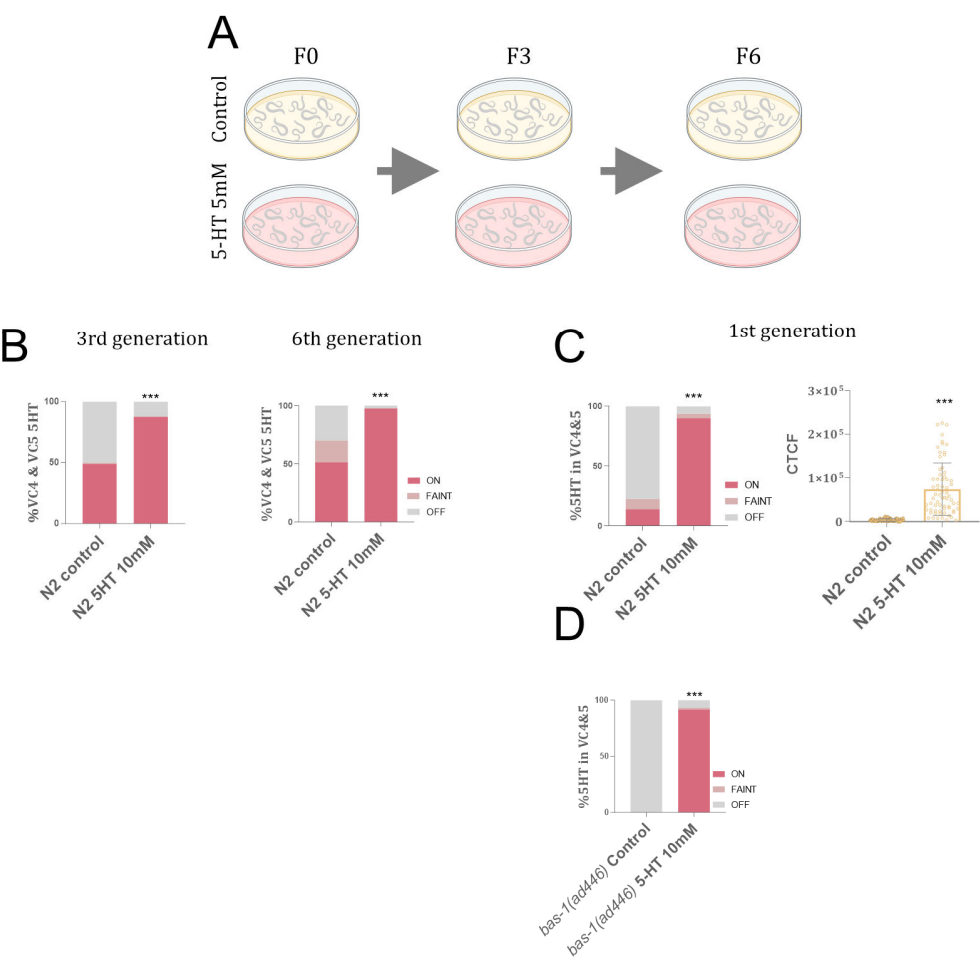


Figure 4.20. *C. elegans* cultured in 5-HT 10mM show an increase in the percentage of VC4 and VC5 neurons stain with 5-HT. **A.** Scheme of the cultures of worms with 10mM 5-HT or without 5-HT. **B.** Worms grown for 3 or 6 generations in media supplemented with 10mM of 5-HT show a significant increase in the percentage of VC4 and VC5 neurons stained for 5-HT compared to worms grown in control conditions (n=40 worms). **C.** Worms grown for 1 generation in 10mM of 5-HT show a higher percentage of neurons with signal (n>100 worms) **(left)** and higher 5-HT intensity quantification (n>50 worms) **(right)** compared to worms grown in control conditions. **D.** VC4 and VC5 neurons can reuptake 5-HT from the outside, shown by the quantification of *bas-1(ad446)* animals grow in 5-HT media that show staining in VC4 and VC5 neurons while there is no 5-HT in *bas-1* mutants grown in control conditions. (n=100 worms). Fisher exact test was used in all statistics but in the intensity quantification in which Mann-Whitney test was used.

Interestingly, we found that animals living only for one generation in 5-HT 10mM acquired a robust serotonergic phenotype in these two neurons. Besides the habitual ON/OFF scoring, we also wanted to fully probe this phenotype by performing an intensity analysis which again supports our point that VC4 and VC5 in *C. elegans*, only need 1 generation living in a serotonergic environment to become serotonergic **(Fig 4.20 C). (Annex 4.16 and 4.17)**. Importantly, we find that after thawing N2 strain 5-HT ON levels in control animals came back to our previous results with very low % of animals showing VC4 and VC5 staining in control conditions.

In summary, results in this section give further support to the hypothesis that VC4 and VC5 5-HT fate is plastic and is subordinated to the specific environment in which worms grow, being high levels of external 5-HT a strong inducer of this fate induction.

4.3.6. *C. elegans* VC4 and VC5 are able to reuptake 5-HT from the environment.

As mentioned above, we demonstrate that VC4 and VC5 in the *C. elegans* strain carrying *C. angaria* first *mod-5* intron (*vlc47 syb7715*), is capable of reuptaking external serotonin when no internal biosynthesis is possible. This phenomenon is seen in a synthetic strain that combines both *C. elegans* and *C. angaria* genome. In light of our results that show that when N2 *C. elegans* is exposed to high levels of external 5-HT their VC4 and VC5 neurons show robust 5-HT staining, we repeated that same experiment by growing *bas-1* mutant animals (that lack the ability to synthesize 5-HT) in both control and 5-HT supplemented media. We found that VC4 and VC5 in *C. elegans* is able to reuptake serotonin from the outside in a non-serotonin synthesizer background (**Fig 4.20 D**). It is important to mention that I could also observe some dim staining in NSM (58%), RIH (17%) and AIM (43%), neurons with previously reported *mod-5* expression (**Annex 4.16**).

4.3.7. VC4 and VC5 plastic response to external 5-HT is not present in *C. briggsae* and *C. tropicalis*

In Evolutionary Biology, the concept of Developmental plasticity is very important as the gain and subsequent loss of plasticity are proposed to have a role in the emergence of phenotypic novelty, a concept known as genetic assimilation. My results demonstrate that, under laboratory conditions, VC4 and VC5 are constitutively serotonergic in the *Angaria* group of species, and constitutively non-serotonergic in *C. elegans*, this fate is revealed to be plastic when *C. elegans* is exposed to external 5-HT as an environmental trigger. These results support the importance of genetic assimilation but do not demonstrate it as we cannot assess if the ancestry of *Angaria* species had a plastic phenotype that then evolved into a fixed one. Thus, next I wanted to further explore if this

plasticity could be also found in other *Caenorhabditis* species, as a wide distribution of this plasticity trait would provide strong evidence for genetic assimilation in *Angaria* species.

To check that, I used two additional species of the elegans group, *C. briggsae* and *C. tropicalis* and grew them for 1 generation both at control and high 5-HT media.

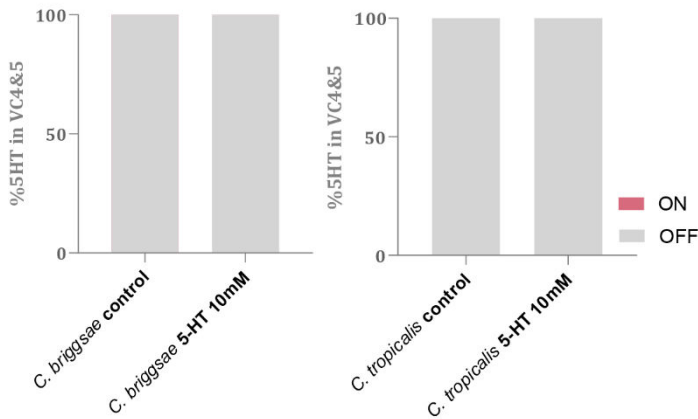


Figure 4.21. *C. briggsae* and *C. tropicalis* do not show serotonin staining in their VC4 and VC5 after culture in 5-HT 10mM. In contrast to *C. elegans*, no staining was observed in VC4 and VC5 neurons in both species when grown in 5-HT (n=40 worms)

I found that none of them showed serotonin staining in their VC4 and VC5 neurons after growing in an environment with exogenous 5-HT. This experiment was performed three times with same results (**Fig 4.21**)(Annex 4.18).

Thus, I conclude that plasticity, or at least plasticity to this specific environmental trigger does not seem to be widely distributed in the *Caenorhabditis* genus. We cannot assure that this plasticity could not be found in other species or that plasticity of these neurons in *C. briggsae* and *C. tropicalis*

could be revealed under different growing conditions. Future work will be required to determine if genetic assimilation occurred in the *Angaria* group of species or if alternatively, plastic and constitutive ON VC4 and VC5 phenotypes evolved independently from an ancestral epigenetically repressed state.

4.3.8. VC4 and VC5 serotonergic phenotype when growing in high 5-HT environments is dependent on UNC-4 and MOD-5 expression.

Given that we had demonstrated that UNC-4, overexpression in VC4 and VC5 is enough to acquire a serotonergic phenotype in *C. elegans* in these neurons and that this phenotype in *C. elegans* bearing *C. angaria* intron 1 does also require UNC-4, we wanted to explore if *unc-4* was also required for the serotonergic phenotype found in VC4 and VC5 when *C. elegans* is exposed to external 5-HT.

To check that, I used *unc-4(gk705)* strain to grow with and without serotonin in the media for one generation to check its necessity for the phenotype. We found that in the absence of *unc-4* we were able to see some staining in VC4 and VC5, but the percentage of worms showing staining was severely decreased compared to what we observed in wildtype background (**Fig 4.22 A**). To obtain a quantitative measure to complement my qualitative ON/OFF scoring, I measured the intensity of the fluorophore. I found the difference between *unc-4* and the wildtype was very dramatic (**Fig 4.22 B**), meaning that the homeodomain transcription factor is required for the acquisition of the plastic serotonergic phenotype in VC4 and VC5 neurons in *C. elegans* (**Fig 4.22 B and D**)(Annex 4.19 and 4.20).

Once I established the necessity of *unc-4* for the serotonergic phenotype in VC4 and VC5, the logical next step was to further demonstrate that *C. elegans* is reuptaking 5-HT through *mod-5*. Then I took two different mutant alleles *n822*,

a substitution that generates an early STOP codon, and *knu383*, described to be a null mutation that lacks all *mod-5* locus (**Fig 4.22 H**).

I performed the same experiment growing both strains in control and serotonin-supplemented media for one generation. When *mod-5* mutant worms were grown in 5-HT they showed a significant decrease both in the percentage of worms stained and in the intensity of 5-HT staining (**Fig 4.22 A and B**). However, to my surprise, there was still some staining in both alleles. I could observe for the *n822* allele that around 36% of VC4 and VC5 neurons stained, but what was more surprising was the null allele *knu383*, because I found 88% of serotonergic VC4 and VC5 when worms were grown in high 5-HT, although this staining was much weaker than in wildtype worms (**Fig 4.22 A, B**).

To facilitate comparison between control conditions and 5-HT I plotted each genotype individually (Fig 4.22 E and F), which allows to conclude that, in each of the mutants, for both *unc-4* and *mod-5* there is still a significant response to 5-HT although to a much lower level than in wild type animals (Annex 4.19 and 4.20). Further experiments should be aim to study compensatory effects in these mutant backgrounds.

Thus, I concluded that both *unc-4* and *mod-5* are required to mediate VC4 and VC5 response to environmental 5-HT.

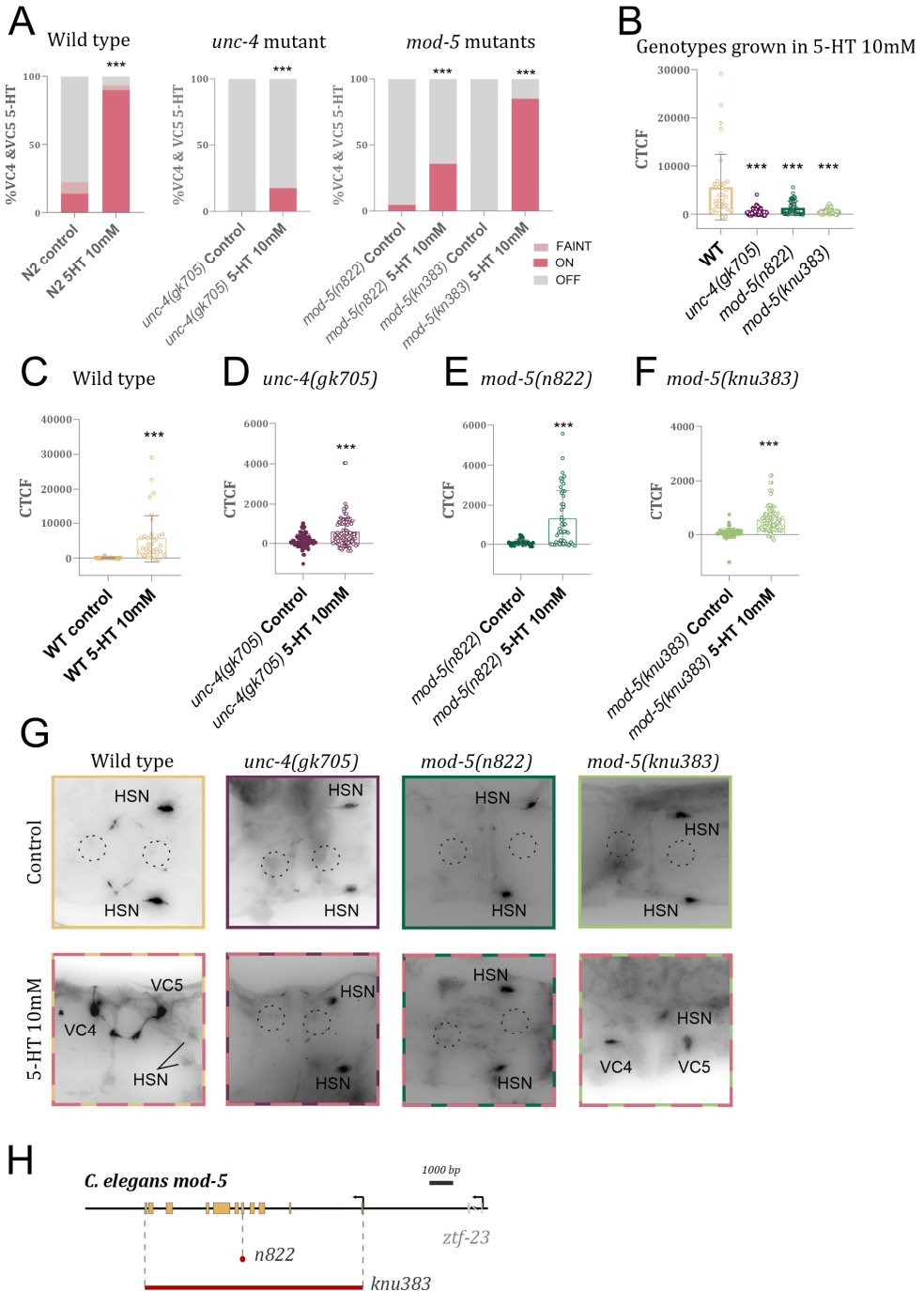


Figure 4.22.5-HT media induction of VC4 and VC5 neuron serotonergic phenotype is dependent on *unc-4* and *mod-5* function. **A.** Quantification of the percentage of serotonergic VC4 and VC5 neurons in *unc-4(gk705)*, *mod-5(n822)* and *mod-5(knu383)* mutant worms grown with or without 10mM 5-HT. In the three mutant backgrounds there is an increase of 5-HT in VC4 and VC5 staining but is significantly less penetrant than in wild type worms (n=40 worms). **B.** 5-HT intensity quantification shows significant differences in *unc-4(gk705)*, *mod-5(n822)* and *mod-5(knu383)* mutants compared to wildtype worms cultured in a 5-HT media (n=30 worms). **C, D, E, F.** Comparison in 5-HT intensity between worms cultured in control condition compared to animals grown in 10mM 5-HT in WT, *unc-4(gk705)*, *mod-5(n822)* and *mod-5(knu383)* respectively [same experiment as in (B)]. In all cases there is a significant increase in the intensity of the signal when animals are grown in 5-HT. **G.** Images of the vulva in WT, *unc-4(gk705)*, *mod-5(n822)* and *mod-5(knu383)* grown with (bottom) or without(top) 5-HT. Fisher exact test was used in (A), Kustal-Wallis nonparametric test was applied in (B) with Dunn's correction for multiple comparisons, Mann-Whitney test was used in (C,D,E,F). **H.** Scheme of *C. elegans mod-5* mutant alleles used. The locus of *mod-5* is represented, *knu383* represents the null allele in which the whole locus is eliminated. The allele *n822* is a substitution allele that codes for a stop codon.

4.3.9. VC4 and VC5 can reuptake external 5-HT in a *mod-5* independent manner.

Since we found residual staining in *C. elegans* VC4 and VC5 neurons when exposed to external 5-HT in *mod-5* mutant backgrounds, one possibility could be that the serotonin “might enter the cells by reuptake from vulval muscle neuromuscular junctions” as commented before by Schafer (2005) or through the synapses VC4 and VC5 neurons share with vulva muscles and HSN neurons (J. G. White et al., 1986). Then to explore if the serotonin observed in VC4 and VC5 in *mod-5* mutants exposed to external 5-HT could come from the HSN or not, I crossed *mod-5(knu383)* null mutant to *bas-1(ad446)* mutant. That way, we eliminate any possibility of serotonergic biosynthesis inside the worms. Then I grew *bas-1(ad446); mod-4(knu3839)* strain in control and 10mM 5-HT and after one generation I fixed the worms and performed 5-HT staining.

I found that, although 5-HT staining intensity was significantly decreased in *bas-1(ad446); mod-4(knu3839)* double mutant compared to *bas-1(ad446)* mutant (**Fig 4.23 A, B**), this effect was quite small. I found for both the single *bas-1* mutant and the double mutant, that the intensity of the signal was significantly stronger in the worms grown in 5-HT than in the control conditions (**Fig 4.23 A**). However, when specifically comparing the signal in *bas-1(ad446)* and *bas-1(ad446); mod-5(knu383)*, when exposed to 5-HT, we found a small but significant decrease in the staining signal in the absence of *mod-5(knu383)* (**Fig 4.23 B**). In conclusion, putting together results from the previous section and this one I see that on the one hand *mod-5* is in part responsible for the VC4 and VC5 serotonergic phenotype but on the other hand, it seems *mod-5* induction is mainly responsible for reuptake of endogenous 5-HT and that there must be another complementary mechanism by which external serotonin is being uptaken by VC4 and VC5 from the outside (**Annex 4.20**). Future experiments, such as VC4 and VC5 transcriptional profiling from

control and 5-HT grown animals could help identify this alternative mechanism.

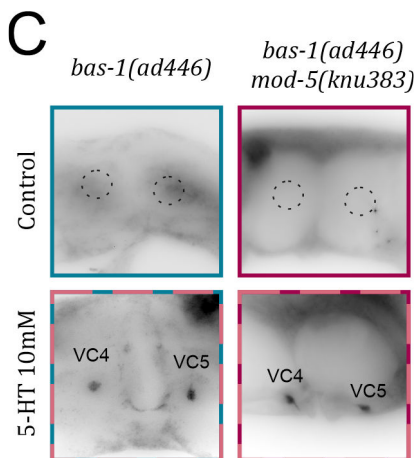
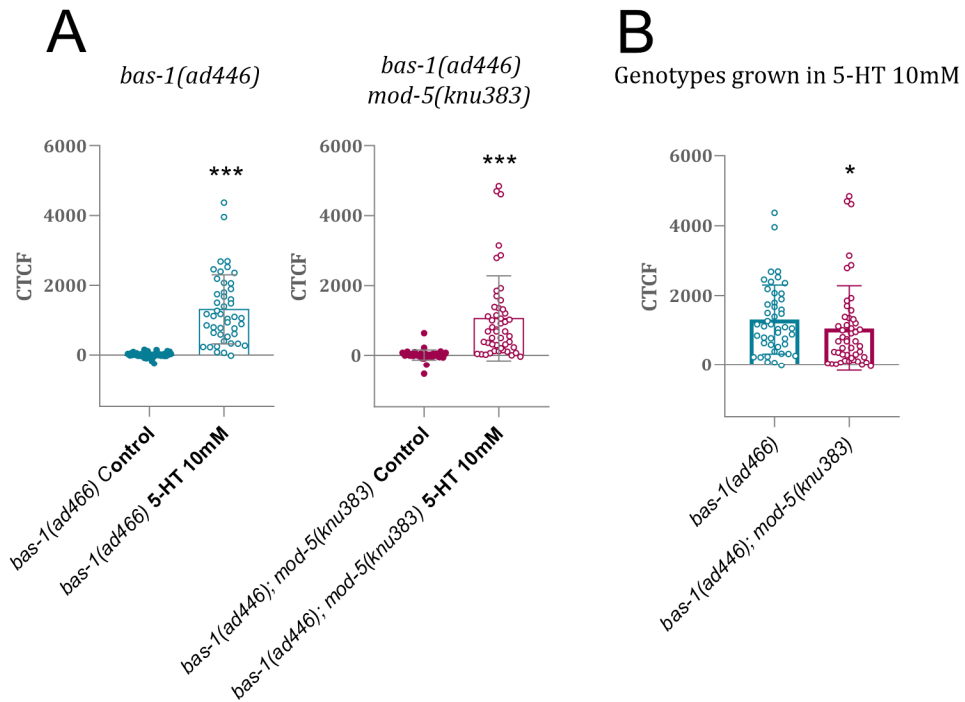


Figure 4.23. VC4 and VC5 neurons can reuptake exogenous 5-HT in a *mod-5* independent manner. **A.** 5-HT intensity quantification of *bas-1(ad446)* mutants and *bas-1(ad446); mod-5(knu383)* double mutant shows an increase in the intensity in animals grown in 5-HT compared to those grown in control conditions. (n=30 worms) **B.** Comparison between both genotypes of worms grow in 5-HT (results from A) show a small but significant decrease in VC4 and VC5 neuron 5-HT intensity in *mod-5(knu383)* animals **C.** Images *bas-1(ad446)* mutant and *bas-1(ad446); mod-5(knu383)* cultures with and without 5-HT. Mann-Whitney test was used for statistics.

4.3.10. *C. elegans* VC4 and VC5 plastic serotonergic phenotype could protect worms from egg-laying due to exogenous 5-HT

After the discovery of the plasticity of the serotonergic phenotype in VC4 and VC5 neurons in *C. elegans*, and identifying one trigger of that response, we focused on the behavioural importance of the phenotype.

Since we had found that *C. angaria* serotonergic VC4 and VC5 seem to be buffering the possible noise produced by external serotonin and then worms were capable of avoiding the egg-laying induction produced by this 5-HT, we explored if the acquisition of serotonergic VC4 and VC5 in *C. elegans* due to the characteristics of the environment could also generate a protective response against exogenous 5-HT.

To perform this experiment, I grew wildtype animals in control and serotonergic supplemented media, and after 1 generation, I took young adult worms and put them in Scomplete or 5mM serotonin (for acute 5-HT treatment) and scored the eggs laid after 2h. As already shown, worms grown in control conditions and exposed to 5-HT for 2h have an increase in the number of eggs laid. However, worms grown in a serotonergic environment show a significant decrease in the eggs laid compared to control growth conditions. This result highlights the importance of the plastic phenotype, the worms grown in serotonergic environments were now protected from acute exposure to 5-HT **(Fig 4.24 A)(Annex 4.21)**.

Finally, as I have determined the requirement of *unc-4* and at least partially of *mod-5* for the serotonergic plastic phenotype, I aimed to further support this idea by studying their requirement in the behavioural responses. Thus, I repeated the experiment using *unc-4(gk705)* and *mod-5(knu383)*, growing the worms for one generation in the two different conditions (with and without 5-

HT) and then assessing their response to acute 5-HT exposing them to 5-HT or serotonin 5mM for 2h.

For *unc-4* mutants, I found that worms grown in serotonin showed a slight difference with the ones grown in control conditions when exposed to acute 5-HT stimuli, meaning that growing in 5-HT media made them less responsive to the exogenous 5-HT (**Fig 4.24 B**), similar to what we found in the wildtype strain. But when comparing wildtype animals and *unc-4* mutants grown in 5-HT and their response to the acute 5-HT stimuli, I found a significant difference between them (**Fig 4.24 D**). This means that *unc-4* is required not only for the serotonergic phenotype but also to mediate proper behavioural response.

Regarding *mod-5* mutants, the behavioural phenotypes were much stronger than for *unc-4* mutants. Indeed, I found *mod-5* mutants show no difference in their response to acute serotonin treatment between worms grown in 5-HT compared to worms cultured in control media (**Fig 4.24 C**). This lack of external 5-HT buffering capacity is further evident when directly comparing *mod-5* mutants and wildtype worms grown in 5-HT when exposed to 5-HT (**Fig 4.24 D**). These results indicate that *mod-5* is essential for the protective response to exogenous 5-HT (**data Annex 4.24**).

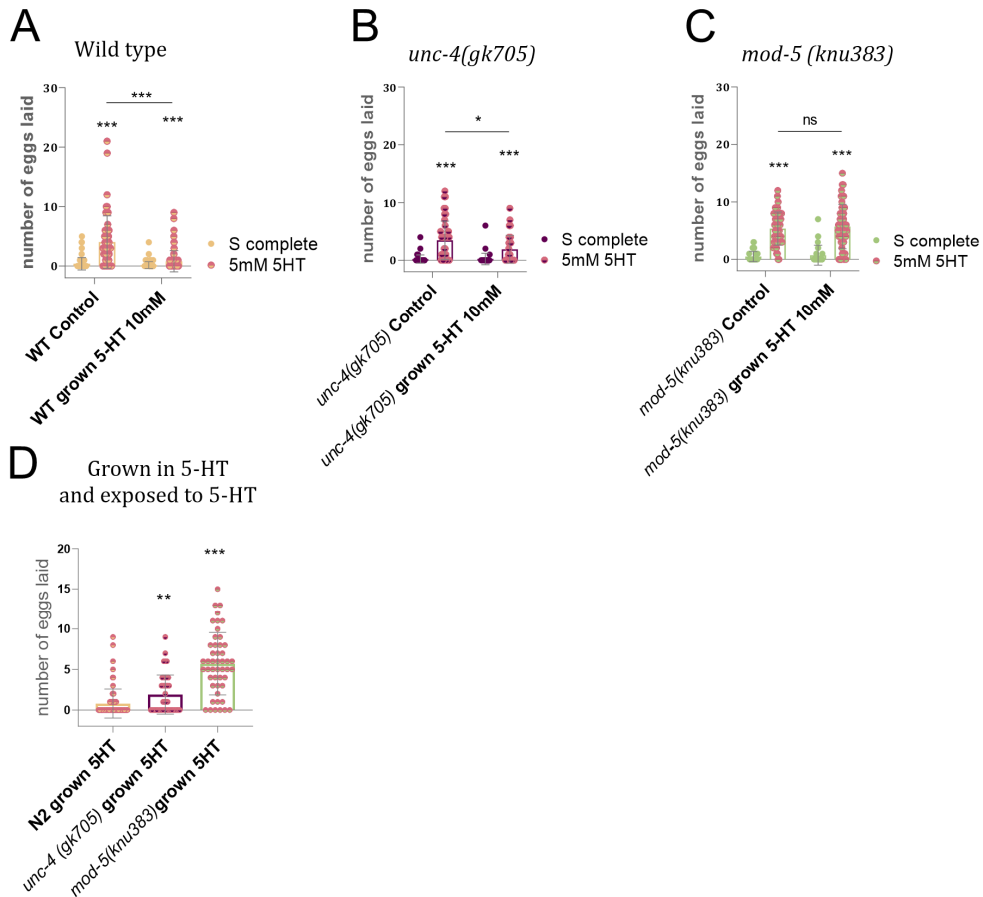


Figure 4. 24. Animals grown in high environmental 5-HT conditions are protected from egg-laying induction due to exposure to acute 5-HT. **A.** N2 worms cultured for one generation in 5-HT show significantly fewer eggs laid when exposed to acute 5-HT compared to control conditions, even though they are still affected by exogenous 5-HT ($n > 70$). **B.** *unc-4(gk795)* mutants grown with and without 5-HT for one generation show an increase in the number of eggs laid when exposed to exogenous 5-HT, there are also a slight difference between animals grown with and without 5-HT when exposed to 5-HT ($n > 45$), suggesting *unc-4* is not required for preventing egg laying induction by acute 5-HT. **C.** There are not significant differences between worms grown with and without 5-HT for one generation when exposed to acute 5-HT in *mod-5(knu383)* ($n > 45$), suggesting *mod-5* is required for preventing egg laying induction by acute 5-HT. **D** Comparison between *unc-4(gk705)* and *mod-5(knu383)* and WT grown in 5-HT for one generation and exposed to 5-HT acute stimuli (same experiment A,B,C) shows a defect in the protection from external serotonin in animals lacking *unc-4* and *mod-5* compared to wild type animals. T-test was applied when distributions were normal, if not, Mann-Whitney test was used for statistical purposes.

Altogether, results from section 3, allow me to conclude that 1) serotonergic phenotype in VC4 and VC5 neurons is a plastic trait in *C. elegans* 2) VC4 and VC5 neurons become serotonergic when worms are cultured for one generation in high 5-HT concentration environments 3) VC4 and VC5 in *C. elegans* are capable of reuptaking 5-HT from the outside 3) the serotonergic phenotype in VC4 and VC5 neurons triggered by the exposure to exogenous 5-HT is not observed in two additional analysed species in *Caenorhabditis* genus 4) serotonergic phenotype in VC4 and VC5 neurons, when cultured with exogenous 5-HT, is dependent on *unc-4* and partially dependent on *mod-5* 5) exposure to 5-HT in culture make worms to acquire a protective behaviour from the egg-laying induction produced by acute exposure to 5-HT, this response is also dependent on *unc-4* and *mod-5*.

Finally, for the last chapter of my thesis, after the profound investigation of VC4 and VC5 neurons in hermaphrodites in *C. elegans* and females in *C. angaria*, we turned to the male CPs and CAs neurons which are the homologous cells to the VCs in the lineage of males. In the next section, I will summarize the results obtained from studying males in this new project that is just starting.

4.4. Analysis of serotonergic phenotype of CPs and CAs male specific neurons in *C. elegans* and *C. angaria*.

VC neurons are only present in females or hermaphrodites. In males, the neuroblast giving rise to VCs goes through an extra division cycle to generate two sister neuron types CA and CPs (**Fig 4.25**). CAs and CPs are both motoneurons, CPs 1-6 are serotonergic synthesizer neurons in *C. elegans*, while CAs are cholinergic, and only from 1 to 4 have been described to show unreliable serotonergic staining, in a similar manner to VC4 and VC5 unreliable staining (Loer & Kenyon, 1993). In *C. angaria* males, previous published results and results from our lab found that both CPs and CAs stain for serotonin (Loer & Kenyon, 1993; Mora-Martínez, 2019). The CAs serotonergic phenotype present in *C. angaria* but not in *C. elegans* is homologous to VC4 and VC5 in hermaphrodites. Thus, we decided to explore if the regulation of CAs serotonergic phenotype could be the same as in the VCs.

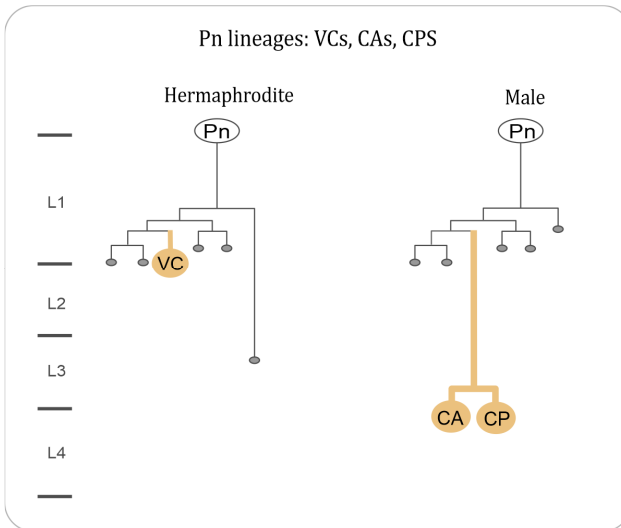


Figure 4.25. Scheme of the lineage of the CP and CA in males. CA and CPs arise from the same lineage as in VCs in the hermaphrodites. VCs are born at the end of L1 but in males divide once again at the end of L3.

4.4.2. *C. angaria* serotonergic CA neurons re-uptake 5-HT in a *mod-5* dependent manner.

First, I analysed if CA serotonergic phenotype in *C. angaria* does also require *mod-5* reuptake. I performed a serotonin staining in *C. angaria* wildtype worms and in *C. angaria mod-5(vlc45)* mutants and scored the males' CPs and CAs. I found a significant reduction in the number of CAs stained in the mutants (**Fig 4.26 A**)(**Annex 4.22**), meaning that similar to VCs in females, CAs in *C. angaria* seemed to have acquired a serotonergic reuptaker phenotype rather than being able to synthesize 5-HT. Interestingly, there is some residual 5-HT staining present in *C. angaria* CA neurons that displays an anteroposterior gradient. This observation suggests: 1) there are mechanisms of 5-HT reuptake independent of *mod-5* activity (as described for *C. elegans* in section 4.3.9) and 2) those compensatory mechanisms seem to respond to A-P gradient, thus suggesting the possible involvement of HOX genes.

4.4.3. Study of *mod-5* regulatory regions activity in *C. angaria* and *C. elegans* transgenic animals.

We also took advantage of the microinjections made in *C. angaria* to study the activity of *C. elegans cat-1* and *mod-5* reporter constructs and *C. angaria cat-1* reporter construct. We found all reporters, both from *C. angaria* and *C. elegans* genomes to be active in CP serotonergic neurons. However, none of the reporters were active in CA neurons, with only very few CA showing some expression for *C. elegans mod-5prom3::gfp* reporter (**Fig 4.26 B**)(**Annex 4.23**). Analysis of *C. angaria mod-5* regulatory regions needs to be performed in the future to identify if, *C. angaria mod-5* minimal CRM that is active in VC4 and VC5 neurons (*Cang_mod-5prom5*) is also active in CA neurons. Also, the lack of *C. angaria cat-1* reporter expression in CA neurons is homologous to the lack of

expression detected for VC4 and VC5 neurons suggesting the enhancer active in these neurons lies outside the regions studied in this reporter.

We also analysed *mod-5* expression from *C. elegans* and from *C. angaria* genome in *C. elegans* CA and CP neurons. The first thing we did was to check the expression of the endogenous *mod-5* reporter (*vlc47*) in CPs and CAs in *C. elegans*. We observed that only CP neurons expressed the reporter, while it was impossible to see Neongreen signal in CA neurons (**Fig 4.26 C**). Next, we studied if *Cang_mod-5prom5* reporter activity (which is active in VC4 and VC5 neurons both in *C. angaria* and *C. elegans*) in *C. elegans* males. I found both CPs and CAs neurons activate this reporter, although expression in CPs was more prevalent than in CAs (**Fig 4.26 C**)(**Annex 4.24**). We also observed some A-P effect for reporter activation as *Cang_mod-5prom5* reporter was not active in the most posterior CA neuron (CA6).

After that, the next logic step was to look for 5-HT staining in CAs in the *C. elegans* strain carrying the first intron of *C. angaria mod-5*, as we had detected staining in VC4 and VC5 in *C. elegans* just by substituting that intron. I detected 100% of staining in CPs (as expected as these neurons are 5-HT synthesizers) and some significant staining in CAs (**Fig 4.26 D**)(**Annex 4.22**). Similar to *Cang_mod-5prom5* reporter, 5-HT staining also shows A-P with no staining for 5-HT in the most posterior CA neurons (CA5 and CA6). However, is important to mention that the number of worms scored was very low (n=20) and the staining should be repeated.

In summary, these results, although preliminary, are compatible with a *cis*-regulatory model for the evolution of the CA 5-HT reuptaker trait in *C. angaria*, homologous to VC4 and VC5 neurons in the hermaphrodite.

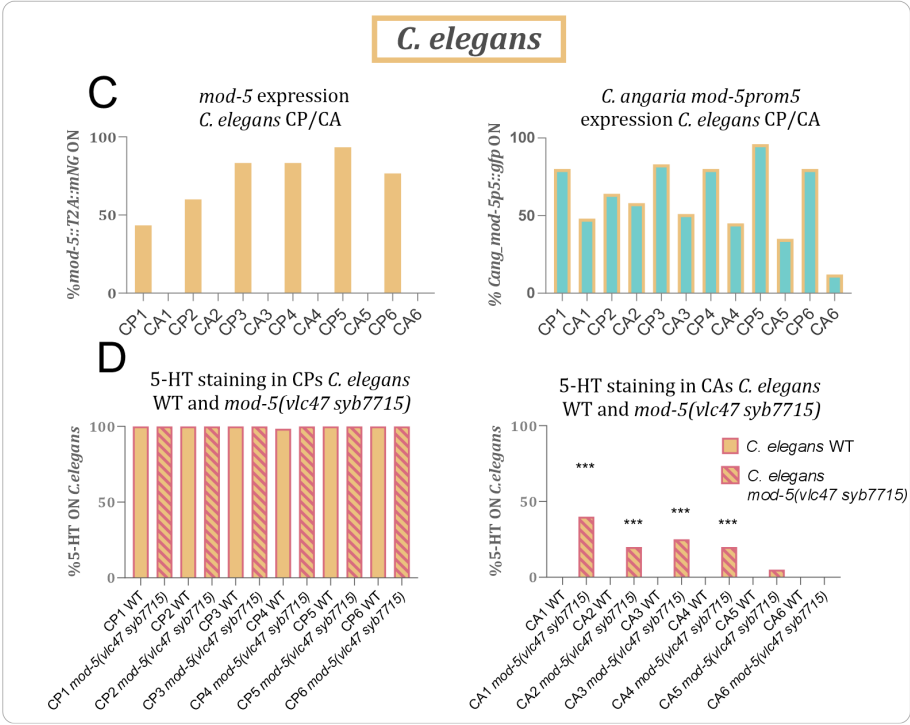
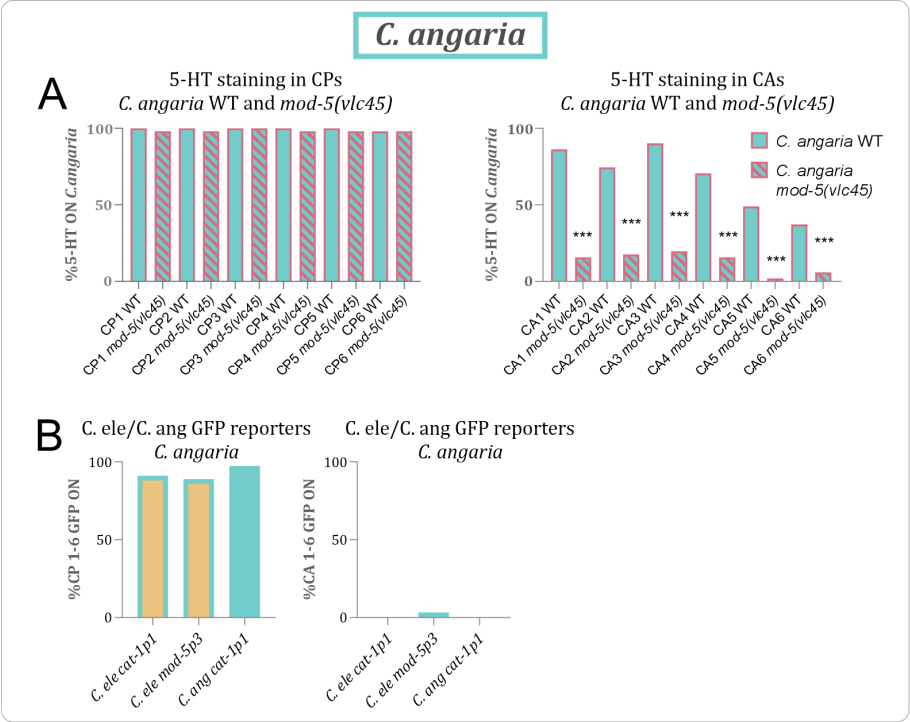


Figure 4. 26. Analysis of *C. angaria* and *C. elegans* serotonergic identity in CA and CP neurons. **A.** *C. angaria mod-5(vlc45)* mutants CA neurons show significant defects in their serotonergic fate while CP neurons are unaffected (n=50 worms). **B.** *C. angaria* CP neurons but not CA neurons show expression of *Cele_cat-1prom1*, *Cele_mod-5prom3* and *Cang_cat-1prom1* (n=32,24,31 worms). **C.** *C. elegans* CP neurons, but not CA neurons express *C. elegans mod-5(vlc47)* reporter. *Cang_mod-5prom5* is expressed in both CP and CA neurons in *C. elegans* (n=30,31 worms). Fisher exact test was used for statistical analysis **D.** CP neurons show serotonergic phenotype in both *C. elegans* WT and *C. elegans mod-5(vlc47, syb7715)*, CAs show some 5-HT staining in *C. elegans mod-5(vlc47, syb7715)* but not in *C. elegans* WT (n=40,20 worms)

4.4.4. Role of CAs serotonergic phenotype in spiral mating in *C. angaria*.

For our studies on VC4 and VC5 neuron evolution, we combined both molecular approaches to identify the mechanisms leading to the emergence of the new trait together with behavioural analysis that could help us better understand the evolutionary meaning of the acquired trait. Thus, I have also started performing behavioural analysis that could help us better understand if CA 5-HT staining in *C. angaria* specie has any functional relevance. As with any aspect of nematode biology, *C. elegans* male mating behaviour is much more studied than *C. angaria*'s. Loer and Kenyon (1993) described the role of CPs in the mating behaviour in *C. elegans*. They demonstrated that CPs or CPs and CAs' precursor ablation was related to a poorly mating behaviour, specifically the turning behaviour around the hermaphrodite in the vulva searching (spiral movement). They also described the importance of 5-HT for tightly curling of the tail and posterior body ventral-inward, which they described to be similar to the tail curling exhibited by males during mating behaviour.

Later on, a specific type of mating was described, the spiral mating in which the male is coiled around the female, that was only detectable in the *Angaria* group of species (Kiontke et al., 2011). Although the neuronal circuit involved in spiral mating is completely unknown, it is striking that this group of species also

shows the serotonergic CAs which are localized in the ventral cord, and as mentioned before, CPs which are just next to CAs are known to be required for the correct turning around the hermaphrodites in *C. elegans*, which involves the correct contraction of body wall muscles.

More recently, Susoy and Samuel (2023) reported that in *C. elegans* males, contrary to previous description of only displaying parallel mating, spiral mating can also be observed when worms are placed in semi-liquid media (Susoy & Samuel, 2023). Moreover, spiral mating in *C. elegans* seems to be a plastic behaviour that is learnt in young adults exposed to such soft media. This plasticity once more was reminiscent of the scenario found for VC4 and VC5 in hermaphrodites exposed to high levels of 5-HT.

Thus, we started exploring the possible role of serotonergic CAs (together with CPs) in the spiral mating behaviour in *C. angaria* and possibly in *C. elegans* learnt behaviour. As the first experimental approach we studied tail curling both under basal laboratory conditions and in presence of acute 5-HT. To investigate the need of serotonergic CAs in *C. angaria* we used the wildtype animals compared to *mod-5(vlc45)* mutant (with no serotonergic CAs) and in *C. elegans* we explore the behaviour in the wildtype N2 strain and the strain with the first intron of *C. angaria mod-5, syb7715*, since we observed some 5-HT staining in their CAs.

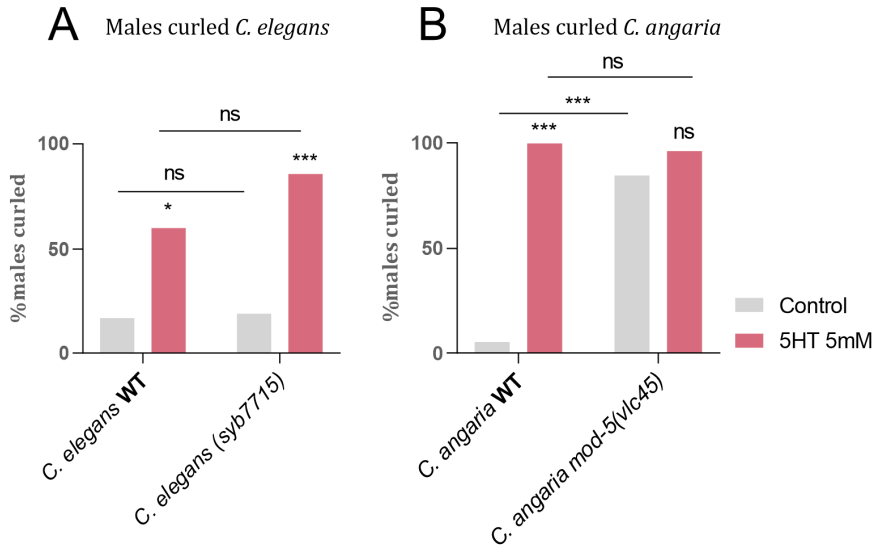


Figure 4. 27. Analysis of male tail curling in *C. elegans* and *C. angaria*. **A.** 5mM of 5-HT exposure increases the number of male tails curled in both *C. elegans* WT and *C. elegans* *mod-5(vlc47, syb7715)* (n=12,15,16,14), not significant differences were found between genotypes. **B.** Serotonin exposure (5mM) significantly increases the number of male tails curled in *C. angaria* WT, but not in *C. angaria* *mod-5(vlc45)* since males showed curled tails even in the absence of exogenous 5-HT (n=19,22,26,26). Fisher exact test was used for statistical analysis

We found in the case of *C. elegans*, that both the wildtype and the strain with the *C. angaria* intron show a low percentage of tail curling under normal conditions but this curling effect is increased in both phenotypes when exposed to exogenous 5-HT, with no significant differences between the wildtype and the *C. elegans* (syb7715) (**Fig 4.27 A**)(**Annex 4.25**). Considering we observed only partial CA 5-HT staining in *C. elegans* (syb7715), this might explain the lack of differences in behaviour between both genotypes. Future experiments using *C. elegans* transgenic animals expressing *mod-5* under a CA-specific and strong promoter (similar to our *VC prom:mod-5* strain) will be required to assess possible roles of *mod-5* expression in CA neurons.

In the case of *C. angaria*, similar to *C. elegans* we find a low percentage of male tail curling in wild type under basal conditions. However, we find that *mod-5(vlc45)* mutants show a significant percentage of males curled, which could mean an overactivation of body wall muscles from 5-HT serotonin released from CPs in the absence of *mod-5* reuptake. In agreement with this hypothesis, exogenous 5-HT induces curling in *C. angaria* but does not further increase curling in *mod-5(vlc45)* mutants. **(Fig 4.27 B) (Annex 4.25)**. It is important to mention that the number of males scored goes from 12 to 26 worms, so we need to increase the sample size of these experiments.

In conclusion, from this last part of my thesis we can conclude that 1) CAs neurons in *C. angaria* have acquired a 5-HT reuptaker phenotype in a *mod-5* dependent manner, 2) *Cang_mod-5prom5* is expressed in *C. elegans* CAs in support of a *cis*-regulatory mechanisms also taking place in the evolutionary acquisition of this trait 3) the first intron of the *C. angaria mod-5* locus is not enough to recapitulate all the serotonergic phenotype in *C. elegans* CAs 4) *C. angaria mod-5(vlc45)* mutants male show a higher percentage of curled male tail in control conditions that could mean an overactivation of body wall muscles.

5. DISCUSSION

5.1. Gene regulatory changes underlying the emergence of novel neuronal functions

5.1.1. *Angaria* group of species VC4 and VC5 serotonergic phenotype was achieved through co-option of *mod-5* intron1 enhancer in the VC4 and VC5 GRN.

Building on previous work of Mora-Martínez (2019), my findings confirm that the regulatory region of *C. angaria mod-5* active in VC4 and VC5 neurons (as well as in the 5-HT NSM neurons) is located in its first intron (**Fig 4.7 C**) (Mora-Martínez, 2019). This region is not only active in these neurons in *C. angaria* transgenic lines but also is sufficient to drive reporter expression in the *C. elegans* VC4 and VC5 (**Fig 4.2**). This *cis*-regulatory activity for *mod-5* intron1 found in *C. angaria*, was conserved across other *Angaria* group of species including, *C. castelli* and *C. quiocensis* (**Fig 4.5**), supporting the hypothesis of gene regulatory network changes as drivers of evolutionary divergence (Dsilva & Galande, 2024). Interestingly, *C. angaria* VC4 and VC5 still retained their cholinergic fate as in *C. elegans* (**Fig 4.6**), suggesting that VC4 and VC5 in the *Angaria* group of species likely acquired novel functions rather than undergoing a functional substitution. This aligns with Arendt's (2008) model of evolutionary innovation by 'acquisition of novel functions', where changes in *cis*-regulatory modules permit ancestral transcription factors to drive new gene expression patterns (Arendt, 2008).

Our results demonstrated that substituting the first intron of *C. elegans mod-5* with its homologous sequence from *C. angaria* is sufficient to induce a serotonergic phenotype in the endogenous context of *C. elegans* VC4 and VC5 neurons (**Fig 4.8**). For *C. elegans*, and other species of *Caenorhabditis* long first introns have been described to be conserved and to have an important role in regulating gene expression (Fuxman Bass et al., 2014), evolutionarily, it is possible that regulatory regions contained in introns, and thus inside the gene

locus are more likely to be conserved along evolutionary distances, even if there are translocation events of big genomic rearrangements that might affect intergenic regions or even chromosomal location.

We have never been able to detect endogenous *mod-5* expression in VC4 and VC5 using a CRISPR-engineered endogenous NeonGreen reporter (**Fig 4.2 C**), probably due to low levels of *mod-5* expression, however, we have demonstrated several times the necessity and sufficiency of the *mod-5* serotonin reuptaker for acquiring the phenotype (**Fig 1.19 F, Fig 4.9 H, Fig 4.22**), strongly supporting that the acquisition of *mod-5* expression was the key molecular event driving the phenotype in *Angaria* species. In addition, the expression we detected from *C. angaria_mod-5 intron1* reporter in *C. elegans* VC4 and VC5 neurons indicates that transcription factors necessary for activating this enhancer are expressed in *C. elegans* and probably are ancestral to the split of *Angaria* and *Elegans* groups of species. In the future, it would be interesting to try to generate and analyse additional transgenic animals with *C. angaria_mod-5 intron1* reporter in other species such as *C. monodelphis*, *C. parvicauda*, *C. bovis* or *C. plicata* that diverged earlier from *Angaria* and *Elegans* group to assess how ancestral the VC4 and VC5 "trans" environment is.

5.1.2. UNC-4 VC4 and VC5 neuron sub-type selector TF directly activates *mod-5* intron 1 enhancer.

VC are motoneurons located along the antero-posterior axis of the ventral nerve cord. All VCs (from 1 to 6) share the expression of many effector genes, however, VC4 and VC5 neurons, located near the vulva are further diversified inducing the expression of additional effector genes, such as *cat-1* or *mir-124*. Transcriptionally, all VCs depend on the action of HLH-3 proneural factor and LIN-11 HD TF (Freyd et al., 1990b; Hobert et al., 1998; Perez & Alfonso, 2020).

In addition, LIN-11 together with EGF signaling from the vulval cells induces the expression of UNC-4 specifically in VC4 and VC5 neurons (Zheng et al., 2013).

Mora-Martínez (2019) previously showed that *cat-1* expression in *C. elegans* VC4 and VC5 depends on both HLH-3 proneural factor and UNC-4 HD TF and are also essential for *C. angaria mod-5* reporter expression in *C. elegans* (**Fig 1.19 B, Fig 4.7 A**). HLH-3 is known to regulate at least in part UNC-4 expression (Perez & Alfonso, 2020). Moreover, the idea of the requirement of UNC-4 to activate *C. angaria mod-5* regulatory region was further supported when I found that the serotonergic phenotype induced by the first intron swap between *C. angaria* and *C. elegans* was abolished in a *C. elegans unc-4* mutant background (**Fig 4.8 D**).

UNC-4 has been shown to act both as a repressor or activator depending on the cellular context (Winnier et al., 1999). Thus, building on this, I wanted to assess if UNC-4 was directly activating *mod-5* expression or if alternatively, the effect was indirect through the activation of additional TFs activators of *mod-5* or repressing expression of a direct *mod-5* repressor. My analysis of the minimal *mod-5* CRM active in VC4 and VC5 neurons identified 3 putative UNC-4 binding sites, it determined that all of them seem to be functional (in agreement with the current model of CRM function with the presence of several homotypic TFBS for specific TFs) (Reiter et al., 2017). However, the effect of the mutations was not the same for all three, the most critical site corresponds precisely to the one matching the UNCX homeodomain extended motif (TAATT), reinforcing the idea of a direct action of UNC-4 in *mod-5* transcriptional activation, in analogy to the terminal selector model. However, as UNC-4 is only expressed in a specific subset of VC neurons (VC4 and VC5 neurons) and the "pan-VC" effector

genes do not seem to be affected in UNC-4 neurons, we propose that UNC-4 acts as a sub-type selector.

Our conclusions regarding a conserved role of *C. angaria* UNC-4 in *C. angaria mod-5* expression are based on heterologous reporter analysis (*C.ang_mod-5* reporters) in *C. elegans unc-4* mutants, nevertheless, it would be advisable in the future to generate *C. angaria unc-4* mutants to directly check the VC4 and VC5 5-HT phenotype in this mutant background.

5.2. *C. elegans* displays a variable VC4 and VC5 serotonergic phenotype.

5.2.1. Epigenetic repression and restricted UNC-4 levels prevent VC4 and VC5 serotonergic phenotype in *C. elegans* N2.

In *C. elegans*, the serotonergic phenotype in VC4 and VC5 neurons is constrained by epigenetic modifications, specifically H3K9 mono- and di-methylation (Mora-Martínez, 2019). We found that in Chip-seq data, *mod-5* locus shows high H3K9 methylation marks as well as recruitment HPL-2, which acts as silencers (**Fig 4.10**). These marks are also observable in the locus of *cat-1*. Although these data set are obtained from whole L4 worms, they support a direct role of H3K9me in repression of *mod-5* expression in VC4 and VC5. Nevertheless, H3K9me has been also described to repress *unc-4* expression in non-vulval VC neurons (Zheng et al., 2013), thus indirectly helping to provide VC4 and VC5 its subtype features. As we have determined that in VC4 and VC5 UNC-4 controls *C. elegans cat-1* expression and *C. angaria mod-5* reporter expression (Mora-Martínez, 2019), we also wanted to assess if increasing *unc-4* levels in *C. elegans* could be sufficient to induce a serotonergic phenotype in *C. elegans* VC4 and VC5. Indeed, this proved to be true (**Fig 4.9 D, E, F**). Thus, our results suggest that even in a context of wild type H3K9me repression, high levels of the

inductor TF (UNC-4) is able to overcome that repression. Although we were not able to detect transcription from *C. elegans mod-5* endogenous locus using our NeonGreen CRISPR tagged allele, we determined that UNC-4 overexpression induction of 5HT phenotype requires *mod-5*, supporting the model of UNC-4 activating *mod-5* locus (**Fig 4.9 H**).

Considering UNC-4 induction can overcome H3K9me, we wondered if H3K9me mutants show an increase in expression of UNC-4, which could explain the phenotype. However, we found indeed a small but significant downregulation of UNC-4 expression in H3K9me mutants (**Fig 4.11**). *C. elegans mod-5* first intron also contains predicted UNCX motifs, we suggest that either these regions are not as efficient recruiting UNC-4 and maybe other TFs as *C. angaria*'s ones or H3K9me of the locus takes place to a lesser extend in *C. angaria mod-5* intron1 sequence. Future experiments should be aimed to profile H3K9me marks and repressor recruitment in VC4 and VC5 of N2 *C. elegans* compared to *C. elegans* bearing *mod-5 C. angaria intron1*.

Altogether, our findings support a model in which *C. elegans mod-5* locus maintains some latent enhancer activity in VC4 and VC5 neurons. This activity is suppressed under laboratory conditions by two parallel mechanisms, restricted UNC-4 levels and epigenetic H3K9 methylation of *mod-5* locus. Recent reports on the role of H3K9me silencing undesired lineage effector gene expression are in agreement with our model, as they also observed ectopic expression of effector genes in H3K9me mutants but not induction of TFs controlling effector gene expression (Padeken et al., 2022).

5.2.2. *C. elegans* VC4 and VC5 variability in wild isolates serotonergic phenotype

The active repression of VC4 and VC5 serotonergic phenotype in *C. elegans* N2 strain made us wonder if this trait might show genetic variability among wild isolates. Analyzing the serotonergic phenotype of VC4 and VC5 neurons across different isolates of *C. elegans* revealed a wide range of serotonergic phenotypes, ranging from 0% to nearly 90%. This finding highlights the interindividual plasticity of this phenotype in *C. elegans* and contrasts with the low penetrant phenotype typically observed in the standard N2 strain (Duerr et al., 1999, 2001 and our own observations). The restricted serotonergic phenotype in N2 could be attributed to the highly controlled laboratory environment under which this strain has been cultured for decades, which might have favored repression of VC4 and VC5 5-HT phenotype that leads to earlier egg laying deposition, faster development of the embryo outside the mother and thus an advantage in population competition (Mignerot et al., 2024). This could be yet another example of the many mutations fixed in N2 such as *npr-1*, *glb-5* and *nath-10* (Sterken et al., 2015).

The diverse array of penetrance in the VC4 and VC5 5-HT phenotypes found in wild isolates gives us an extraordinary opportunity to keep exploring the genetic basis of this variability. Indeed, recombinant inbred lines (RILs) derived from *Caenorhabditis elegans* wild-type N2 and CB4856 (which shows higher 5-HT VC4 and VC5 penetrance) have been generated and are being used for mapping genes underlying complex traits (Doroszuk et al., 2009). Thus, we could use this approach in the future to identify the additional genetic components of 5-HT VC4 and VC5 phenotype and its variability. However, in my experiments using wild isolates I notice an abnormally high percentage of N2 5-HT VC4 and VC5 staining, which we do not currently know how to explain, it is possible that some environmental confounding factor was also present (such

as different humidity, temperature, chemical or biological contaminant, etc.) that could be responsible for the shift in phenotype. Thus, before moving on to use N2 and CB4856 RIL we should be able to decrease the variability in our staining under laboratory conditions to distinguish genetic from environmental triggers.

5.2.3. *C. elegans* serotonergic VC4 and VC5 in N2 strain is plastic to environmental grow conditions

In addition to genetic factors that could modulate *C. elegans* VC4 and VC5 serotonergic phenotype, considering the variability that we saw even for the same genetic background, we also wanted to explore environmental conditions as possible triggers for a serotonergic phenotype in VC4 and VC5 in *C. elegans* N2. Previous work from Dr. Mora fail to detect strong induction using different conditions, such as heat stress, pathogenic bacteria or hypoxia (Mora-Martínez, 2019). As VC4 and VC5 are located in the vulva and have been reported to respond to mechanosensation (Medrano & Collins, 2023) we tested mating as a trigger. We found a modest increase in serotonin staining intensity in these neurons when animals came from a *him-5(e1490)* plate compared to a wild-type one, although the trend was not statistically significant. While this could point that mating might enhance the ability of VC4 and VC5 to reuptake 5-HT the inability to distinguish mated from unmated hermaphrodites in *him-5(e1490)* animals may have diluted the results, future experiments using mated verified hermaphrodites will be required to drive stronger conclusions.

Alternatively, we turned into a different environmental stimulus, as we had determined that *C. angaria* is able to buffer the effect of high levels of exogenous 5-HT in contrast to egg laying induction in *C. elegans* we wondered if, exogenous 5-HT itself could be the trigger of *C. elegans* VC4 and VC5 developmental

plasticity. Indeed, I found that exposure to elevated 5-HT levels for just one generation resulted in nearly 100% of VC4 and VC5 neurons acquiring a strong serotonergic phenotype. We found this phenotype to be dependent on UNC-4 and MOD-5, supporting the hypothesis that these neurons actively reuptake serotonin. We also demonstrate that the serotonin reuptake was not only the one released by the HSN, but these neurons were able to reuptake the external serotonin. Once found that VC4 and VC5 serotonergic phenotype is plastic in *C. elegans*, considering the proximity of the VC4 and VC5 to the vulva slit, their role in regulating the circuit of the egg-laying process (Collins et al., 2016), and the importance of 5-HT as the main activator of the circuit, it makes sense that the VC4 and VC5 reuptake the exogenous serotonin to control the behaviour by the reuptake of the excess of 5-HT to avoid the constant contraction of the vulva muscles.

The identification of a robust environmental trigger for VC4 and VC5 phenotype is an important step, however, we currently do not know how the external 5-HT signal is sensed and how is transduced into the generation of a robust response of *mod-5* expression and 5-HT reuptake. Future experiments should be aimed to address this important question. We envision at least two possibilities: 1) 5-HT could be acting through 5-HT receptors. Among different receptors, SER-7, is the only known serotonergic receptor present in VC4 and VC5 (Fernandez et al., 2020). Thus, we would use available *ser-7* mutants and grow them in high 5-HT environments and see if they show defects in VC4 and VC5 plasticity. Alternatively, 5-HT signal could be detected by vulval cells expressing 5-HT receptors and then transduce the stimuli to VC4 and VC5 neurons. Thus, other 5-HT receptor mutants could also be tested. 2) An alternative to 5-HT acting through 5-HT receptors, we could also envision a direct role of 5-HT itself in VC4 and VC5 gene expression. Serotonylation, as mentioned in the introduction, has been recently found to be a class of post-

translational modification of histones, that correlates with permissive gene expression. The modification occurs in glutamine residues on histone H3 (H3Q5ser), via transglutaminases and it has been found also to be dependent on SERT activity (Farrelly et al., 2019; Chan et al., 2024). We hypothesize that high levels of environmental 5-HT could result in H3Q5ser in VC4 and VC5 neurons, precluding H3K9me deposition or blocking its repressive effect. We are currently using immunostaining with antibodies specific to H3Q5ser to study if there is serotonylation in *C. elegans* under these circumstances.

Finally, one interesting result that is worth noting is that VC4 and VC5 were able to reuptake 5-HT when grown in a high 5-HT environment, even in a double *bas-1(ad446)*, *mod-5(knu383)* mutant background (**Fig 4.23**), where neurons cannot synthesize or reuptake serotonin through the only known SERT coding gene in *C. elegans*. In addition, previously in our laboratory we have observed some residual staining in VC4 and VC5 doubles mutants *hpl-2(tm1489)*; *mod-5(n822)*, in *hpl-2(tm1489)*; *mod-5(knu383)* and also in *met-2(n4256)*; *mod-5(n822)* (Mora-Martínez, 2019). These results suggest alternative mechanisms available in these neurons to reuptake the serotonin. This observation is very important given the key role of 5-HT and SERT in mental disorders as well as the wide use of SERT inhibitors (SRIs) in psychiatric treatments (Pourhamzeh et al., 2022). This particular set up, growing *bas-1(ad446)*, *mod-5(knu383)* mutants in high 5-HT could be used to perform a forward genetic screen to identify other genetic components responsible for 5-HT reuptake in addition to *mod-5*, which could be of biomedical importance. Indeed *mod-5* independent actions of SRIs have been reported in *C. elegans* (Ranganathan et al., 2001) but are not currently understood.

5.2.4. VC4 and VC5 serotonergic plasticity to high 5-HT environments is not a general feature of non-*Angaria Caenorhabditis* species.

Our results show that *Angaria* species have a constitutive VC4 and VC5 5-HT phenotype and *C. elegans* a constitutively repressed phenotype that can be released upon specific stimuli, constituting then a plastic trait. The next question then to address was, is the plasticity generally distributed in the *Caenorhabditis* genus? If that would be the case then it would suggest that plasticity constitutes the ancestral state that was then fixed (genetic assimilation) in *Angaria* species. ‘Genetic assimilation’ refers to the evolutionary process by which a trait that is produced specifically in response to some environmental stimulus, becomes stably expressed independently of the evoking environment and thus genetically fixed (Waddington, 1953; Braendle & Flatt, 2006). Thus, to see how is plasticity distributed in the genus I performed similar experiments with two additional species, *C. briggsae* and *C. tropicalis* which both failed to exhibit serotonin reuptake in these neurons even when grown for one generation under serotonergic environments (**Fig 4.21**). Thus, the serotonergic phenotype of VC4 and VC5 neurons across *Caenorhabditis* species seems to exhibit a remarkable diversity, reflecting distinct evolutionary trajectories probably influenced by different environmental pressures.

Thus, *C. angaria* has a constitutive VC4 and VC5 serotonergic phenotype and *C. elegans* a repressed and plastic one. We have determined in *C. briggsae* and *C. tropicalis* this trait is not plastic, at least to this stimulus, but it is still possible that other triggers might drive the serotonergic VC4 and VC5 phenotype in these species. Thus, an important remaining question is to determine if *C. briggsae* and *C. tropicalis* do show active epigenetic repression for VC4 and VC5

serotonergic phenotype. There are available *C. briggsae* *met-2* mutants that we are currently checking for 5-HT staining.

Importantly, outside the genus *Caenorhabditis*, the constitutive serotonergic phenotype in VC4 and VC5 neurons has independently evolved in other nematode clades (Loer & Rivard, 2007), suggesting that similar environmental pressures repeatedly favour this adaptation and that mechanistically, this shift from no 5-HT to 5-HT phenotype is relatively easy to happen. The recurrence of this phenotype points to a convergent evolution in shaping this particular trait of neuronal diversity. Future experiments using non-*Caenorhabditis* species will be necessary to test if, similar to *Angaria* group of species, *cis*-regulatory changes are the main drivers for the VC4 and VC5 constitutive 5-HT phenotype in those species and if the enhancer is also located in the first intron of the gene.

Thus, according to our results, we hypothesize that an ancestral *Caenorhabditis* species likely exhibited either a constitutively repressed or a plastic serotonergic phenotype in VC4 and VC5 neurons. Then this trait evolved into a constitutive one in *Angaria* species and maybe to a plastic one in *C. elegans*.

5.3. Behavioural relevance of serotonergic phenotype of VC4 and VC5

In addition to our molecular characterization of the mechanisms underlying *C. angaria* constitutive and *C. elegans* plastic VC4 and VC5 5-HT phenotype, we wanted to complement our studies genetic with behavioural approximations that could help us better understand the purpose of this trait (if any). This aimed turned out to be challenging, due to the great lack of knowledge that we currently have on the ecology of the different *Caenorhabditis* species (Schulenburg & Félix, 2017). However, we finally found a hypothetical

environmental function for *C. angaria* VC4 and VC5 phenotype that was key to later allow us to identify the trigger of VC4 and VC5 phenotypic plasticity in N2.

5.3.1. Quantification of egg-laying deposition in *C. angaria* and *C. elegans* strains under laboratory conditions does not reveal a role for VC4 and VC5 serotonergic phenotype.

Considering the known role of VC4 and VC5 together with the HSNs on the egg-laying behaviour (Schafer, 2005), in first place we studied the total number of eggs released by the different species. Species without a fixed serotonergic phenotype in these neurons (*C. elegans* and *C. briggsae*) tend to release more eggs compared to species with a fixed serotonergic phenotype (*C. angaria* and *C. quiockensis*) (**Fig 4.12**). In *C. angaria* and *C. elegans*, this difference can partly be attributed to the total number of containing eggs, which is significantly lower in *C. angaria*. To explore the role of MOD-5 on this behaviour, we used *C. elegans* strain that expresses *mod-5* in VCs, which would mimic *C. angaria* context. We found indeed a reduction in the number of eggs laid and an increase in the number of unreleased eggs. In this context, *mod-5* expression in VCs would be reuptaking the serotonin released by the HSN and modulating the vulva contractions and in turn egg-laying. However, in a *C. angaria mod-5(vlc45)* mutant, the expected increase in egg-laying was not observed; instead, there was also a reduction in egg laying, what might indicate VC4 and VC5 5-HT could be released in this species to participate in egg laying induction (**Fig 4.12**). Alternatively, as *mod-5* mutation in *C. angaria* is constitutive, it is likely affecting other neurons expressing the gene, potentially altering additional behaviours that could indirectly influence egg-laying (Ranganathan et al., 2001).

5.3.2. Study of egg-laying behaviour dynamics in *C. angaria* and *C. elegans* strains under laboratory conditions also failed to reveal a role for VC4 and VC5 serotonergic phenotype.

Then, to more precisely study egg-laying behaviour we recorded animals for 6 h and then manually annotated egg-laying events as previously done (Hardaker et al., 2001; Waggoner et al., 1998). We looked for differences in the dynamics of egg-laying behaviour. The most interesting difference observed is the overall dynamic. In *C. elegans* N2 strain, the egg-laying process has been described as a two-state behaviour with active and inactive phases. This biphasic process is maintained in all the strains derived from the N2 (*mod-5* expression in VC4 and VC5 or N2), but, in the case of *C. angaria*, worms do not follow a biphasic process with active and inactive periods, eggs are laid as individual events without really showing active egg laying windows. However, when trying to assess the relevance of *mod-5* in egg-laying dynamics we found no differences between *C. angaria* wild-type and the *mod-5(vlc45)* mutant. In *C. elegans* N2 dynamics, wildtype worms laid more eggs, had longer active intervals and egg laying windows in which they also laid more eggs than the animals with *mod-5* expression in their VCs (**Fig 4.13**). These findings also support the idea that at least in *C. elegans*, the expression of *mod-5* in VCs restricts to some extent egg laying.

5.3.3. Study of egg-laying behaviour dynamics in *C. elegans* wild isolates and self and mating egg laying reveals interesting differences among strains.

We also performed experiments on the dynamics of the egg-laying behaviour with animals mated and also with a wild isolated of *C. elegans* that usually had higher percentages of serotonergic VC4 and VC5, the strain CB4856. The effect of mating was similar to the one observed with the *C. elegans mod-5* VCs, with fewer number of eggs, fewer and smaller egg-laying windows and longer

inactive periods, showing a clear effect of the mating in the egg-laying dynamics (**Fig 4.19**). We think this is a case of behavioural plasticity not described before, and it will be interesting to further study its molecular mechanisms in the future. Male sperm is known to contain many components different to hermaphrodite sperm (Ebbing et al., 2018), thus it would be interesting to test if male sperm is required for this plastic behaviour.

In the case of the CB4856 strain, the dynamics on the egg-laying process are very different than the ones in the N2 strain, with fewer eggs, fewer and shorter egg-laying windows, and fewer eggs laid per egg-laying window, in general, a much more controlled behaviour (**Fig 4.17**). Indeed, it seems CB4856 follows a dynamic more similar to *C. angaria*'s suggesting that the N2 laboratory derivation could also impact how N2 lays its eggs, with a high number of short interval events of egg laying.

5.3.4. Protective role for the serotonergic phenotype in VC4 and VC5 neurons.

Preliminary results of Mora-Martinez (2019) showed that the species of the *Angaria* group, with a constitutive serotonergic phenotype in their VC4 and VC5 neurons, were normally more protected from egg-laying induction produced by external 5-HT compared to the rest of tested *Caenorhabditis* species (Mora-Martínez, 2019). However, as discussed above, differences found among different species can be due to multiple factors, thus I aimed to assess if this correlation was indeed due to *mod-5* activity in VC4 and VC5 neurons. Initially, we were able to recapitulate the results previously generated with both 10 and 5mM concentrations while *C. angaria* remained unaffected. Then, we compared *C. elegans* with *C. elegans mod-5* overexpression in VC4 and VC5 neurons and *C. angaria* with *C. angaria mod-5* mutants to see if the 5-HT phenotype was responsible for the differential response to 5-HT. When exposed to 5-HT, although *C. elegans* animals with *mod-5* expression in their VCs showed an

increase in the number of eggs laid compared to control conditions, this response was diminished compared to *C. elegans* WT exposed to 5-HT (**Fig 4.14**), suggesting that the expression of *mod-5* in VCs, protects *C. elegans* worms from external serotonin, probably thanks to their capacity of reuptaking external serotonin. We also performed a similar experiment with the *C. elegans* strain containing the first intron of *C. angaria mod-5*, (*vlc47 syb7715*), and we saw a similar effect, however the protection seems to be more diluted in the *mod-5(vlc47 syb7715)* strain. In *C. angaria*, on the other hand, the effect was concentration-dependent, we observed an increased in the number of eggs laid in the *C. angaria mod-5(vlc45)* mutant when exposed to 10mM 5-HT, suggesting a loss of the protection offered by the serotonergic phenotype of VCs against external serotonin, but this effect was diluted when the concentration was reduced to 5mM, although there is an obvious tendency, that may become significant with an increase in the population (**Fig 4.14**).

In summary, under this specific experimental paradigm, we were able for the first time to assign a behavioural difference due to the VC4 and VC5 serotonergic phenotype. Our findings suggest a protective role of the serotonergic phenotype of VC4 and VC5 in modulating egg-laying behaviour under high-serotonin conditions. This phenotype is constitutive in *Angaria* group of species like *C. angaria*, which confers resistance to exogenous serotonin. Then these results allowed us to hypothesize that the environmental trigger for *C. elegans* could be indeed external 5-HT, as we determined. In *C. elegans*, the plasticity of this phenotype allows for environmental adaptability, suggesting an evolutionary advantage in changing or difficult environments.

The question then is, what could be the changing environmental conditions that *C. elegans* is exposed to and that could have selected the plasticity of VC4 and VC5 phenotype? Here we can just speculate with our knowledge on *C. elegans*

ecology. *C. elegans* is frequently found in slug's intestines where it can survive intestinal transit (Petersen et al., 2015). This behaviour has been suggested to be a dispersal mechanism for *C. elegans*. Intestine is the major source of 5HT in the body, including snails and slugs (Hernádi et al., 1998; N. Liu et al., 2021; Rao & Gershon, 2018). Considering that phenotypic plasticity is generally favored in changing environments where predictive cues are present, it is tempting to speculate *C. elegans* VC4 and VC5 serotonergic plasticity might be key to induce egg retention when present in intestinal environments, which in turn will favour offspring survival, but not in external habitats that will favour population dispersal and colonization of new food resources. This finding aligns well with recent studies proposing that strong egg retention is a plastic adaptive response, offering competitive advantages in resource-limited or harsh conditions (Mignerot et al., 2024).

5.4. A novel example of emergence of novelty: CA male specific neurons are serotonergic in *C. angaria*.

CA and CP neurons are male-specific neurons in *C. elegans* that arise from the same lineage as VC neurons found in hermaphrodites, however, in the males this neuroblast divides once more generating CA and CP sister neurons. CPs 1-6 stain for serotonin both in *C. angaria* and *C. elegans*, being probably synthesizers, at least in *C. elegans* where they express the genes necessary for synthesizing serotonin (C. Wang et al., 2024). In the case of CAs, they are cholinergic in *C. elegans* but in *C. angaria* they show a serotonergic phenotype, mostly due to 5-HT reuptake through *mod-5*, demonstrated by the reduction on the 5-HT staining in *C. angaria mod-5(vlc45)* mutant (**Fig 4.26**). *Cele_cat-1prom1* and *Cele_mod-5prom3* reporters were not expressed in CAs nor *Cang_cat-1prom1*, although all were expressed in CPs in *C. angaria*. This could

suggest that any of these constructs contain the active enhancers for their expression in CAs in *C. angaria*. If similar *cis*-regulatory mechanisms operate for *C. angaria* CA serotonergic phenotype than for VC4 and VC5 neurons, we would expect *C. angaria_mod-5_intron1* reporter is active both in *C. angaria* and *C. elegans* CA neurons. However, when expressing *Cang_mod-5prom5* in *C. elegans*, we were able to observe expression in both CPs and CAs, but CAs expression was around 50% or below. Moreover, the introduction of the first intron of *C. angaria mod-5* in *C. elegans* strain (*vcl47 syb7715*) which we saw was sufficient to drive a serotonergic phenotype in VC4 and VC5 neurons, had a milder phenotype for CA 5-HT phenotype. Although we detected some staining in CAs, the effect was not as dramatic as in the hermaphrodites. These two results point to the idea that either the main enhancer regulating the expression of *mod-5* could be located outside the first intron of *C. angaria mod-5* or that additional trans-environment changes are responsible for *C. angaria* CA phenotype.

For a better understanding of the molecular mechanisms underlying CA serotonergic phenotype in *C. angaria* future experiments should be done: 1) comparative studies of *C. angaria mod-5* enhancers in *C. angaria* and *C. elegans* transgenic animals, 2) study the serotonergic phenotype in CAs in H3K9me mutants (as in VC4 and VC5) 3) Assess if the partial CA 5HT phenotype in *C. elegans* strain (*vcl47 syb7715*) is dependent on UNC-4 activity.

Finally, considering the role of CPs and CAs in the locomotory and mating behaviour, and especially in the spiral mating behaviour that is only constitutively shown by *Angaria* group of species (Kiontke et al., 2011), but a plastic behaviour in *C. elegans* (Susoy & Samuel, 2023) we studied the tail curling in the presence of 5-HT. In *C. angaria mod-5(vlc45)* it was surprising how males tend to curl even in the absence of external 5-HT, pointing to an

important role of CAs that are able to reuptake 5-HT for the correct locomotory behaviour of the male. Nonetheless, the population used for this experiment is too small to be able to make conclusions, future experiments will elucidate these questions.

5.5. Relevance of the project beyond nematode's biology

Since the establishment of *C. elegans* as model system by Sidney Brenner, key discoveries have been made thanks to the study of this organism, including but not limited to findings related to apoptosis, non-coding RNAs, longevity, etc. Resulting in four Nobel awards to discoveries produced using this system.

We hope results from my PhD will have a significant impact, not only in the field of *C. elegans* and nematode biology but in the field of Nervous system evolution and Biomedicine.

5.5.1. Developmental plasticity and evolution.

The role of phenotypic plasticity in evolution is an important controversy in the field. There is a general consensus about the importance of plasticity for adapting to changing environments, however, its role driving evolutionary change is still debated. For many years in the ‘Synthesis Era’ of evolutionary studies, the focus was put on the role of genes in evolution, but forgetting about phenotypes and their development (Pfennig, 2021). However, natural selection works on phenotypes not genes. On this project we provide relevant results that support an important role for plasticity, or at least on release of lineage repression to drive evolution of novelty. We find changes in regulatory networks were able to lead to new neuronal identities and behavioural

variations. Similarly, our characterization of specific GRN changes agrees with proposed models for nervous system evolution that have hypothesize that changes in regulatory regions are key to drive novel gene expression (Arendt, 2008; Arendt et al., 2019).

5.5.2. Human SERT transient expression and psychiatric disorders.

In the human brain, serotonin (5HT) is the most widely distributed neurotransmitter controlling essential functions, including sensory processing, cognitive control, internal states, autonomic responses and motor activity (Gaspar et al., 2003; Lesch & Waider, 2012). As in *C. elegans*, mammalian serotonergic neuron types can be classified as *bona fide* 5HT synthesizing neurons or 5HT reuptakers, which express the 5HT reuptaker gene (*Sert*) to upload 5HT secreted from nearby synthesizer neurons (Gaspar et al., 2003). 5HT synthesizer neurons are born early in embryonic development, are restricted to the Raphe region and perdure in adulthood. On the contrary, 5HT reuptaker neurons are present in diverse brain regions, including cortex, thalamus and retina but this phenotype is embryonically restricted due to transient SERT expression. This ephemeral serotonergic feature has been involved in axon guidance, synapse formation or cell survival (Gaspar et al., 2003). In addition, the use of SERT inhibitors during pregnancy and *sert* genetic variants have been linked to pathological conditions such as depression, anxiety, autism or pain perception (Murphy & Lesch, 2008; Murphy & Moya, 2011) and as key component for environmental interactions to disease (vulnerability) (Hankin et al., 2011). However, mechanisms controlling temporally restricted 5HT reuptaker phenotype are currently not well understood (Gaspar et al., 2003). Indeed, mouse retinal ganglion cells, that transiently express SERT in development, reactivate its expression after optic

nerve injury, leading to increased vulnerability of this neuron type (Kingston et al., 2021). Importantly, H3K9me pathway has also been genetically linked to these SERT-linked disorders (Peña et al., 2014; Walker et al., 2015). In light of our results, we speculate that plastic H3K9me epigenetic silencing of *sert* locus might be phylogenetically conserved in mammals, could potentially be environmentally modulated and have biomedical relevance in 5-HT related psychiatric disorders.

6. CONCLUSIONS

- 1.1. *C. angaria* species acquired a constitutive serotonergic phenotype in VC4 and VC5 neurons through changes in the *cis*-regulatory regions of the *mod-5/Sert* locus.
 - 1.2. The *C. angaria mod-5/Sert* enhancer is sufficient to induce a VC4 and VC5 serotonergic phenotype in *C. elegans* when placed in its endogenous context and it is dependent on UNC-4 homeodomain TF activity.
 - 1.3. Overexpression of *unc-4* is sufficient to activate, at low levels, the *C. elegans mod-5* intron 1 enhancer and induce the serotonergic phenotype in VC4 and VC5 neurons.
 - 1.4. The *mod-5* locus has high levels of H3K9me1 and me2 and HPL2 repressor recruitment in *C. elegans*.
 - 1.5. VC4 and VC5 serotonergic phenotype induced in H3K9me mutants requires *unc-4* activity but H3K9me mutants do not increase UNC-4 levels.
-
- 2.1. Species with fully serotonergic VC4 and VC5 neurons tend to lay fewer eggs than worms with variable or no 5-HT staining in these neurons.
 - 2.2. *C. angaria* egg-laying dynamics do not follow the bimodal pattern found in *C. elegans*.
 - 2.3. The VC4 and VC5 serotonergic fate in *C. angaria* buffers egg-laying induction effects in environments with high 5-HT concentration.
 - 2.4. Genetically induced serotonergic VC4 and VC5 neurons in *C. elegans* protect from egg-laying induction produced by exogenous 5-HT in a way similar to *Angaria* species. This response is also dependent on *mod-5/Sert* expression and UNC-4 transcription factor activity.

- 3.1. VC4 and VC5 neurons in *C. elegans* are capable of reuptaking 5-HT from the environment when expressing *mod-5/Sert*.
 - 3.2. In *C. elegans*, the serotonergic phenotype in VC4 and VC5 neurons is a plastic trait. *C. elegans* growth in environments high in serotonin triggers the serotonergic phenotype that is dependent on *unc-4* transcription factor and partially dependent on *mod-5/Sert*.
 - 3.3. Exposure to 5-HT in culture causes *C. elegans* to acquire a protective behaviour from the egg-laying induction produced by acute exposure to 5-HT. This response is similar to *Angaria* species responses and is also dependent on *mod-5/Sert* and *unc-4* transcription factor.
 - 3.4. Serotonergic plasticity of VC4 and VC5 neurons is not observed in two additional analysed species in the *Caenorhabditis* genus, *C. briggsae* and *C. tropicalis*.
-
- 4.1. CA neurons in *C. angaria* seem to be 5-HT reuptakers and they reuptake serotonin in a *mod-5*-dependent manner.
 - 4.2. *C. angaria mod-5* reporter is expressed in *C. elegans* CAs, supporting a *cis*-regulatory mechanism also responsible for the evolutionary acquisition of this male specific trait.
 - 4.3. The first intron of the *C. angaria mod-5* locus is not sufficient to recapitulate the serotonergic phenotype in *C. elegans* male CAs suggesting the enhancer for CA expression could be located outside intron 1 or *trans*-factor could also be different between *C. elegans* and *C. angaria* CA neurons.
 - 4.4. *C. angaria mod-5(vlc45)* mutant males show a higher percentage of male tail curling in control conditions, which could mean an overactivation of body wall muscles.

BIBLIOGRAPHY

- Ahlmann-Eltze, C., & Patil, I. (2021). *ggsignif: R Package for Displaying Significance Brackets for "ggplot2."* OSF. <https://doi.org/10.31234/osf.io/7awm6>
- Ahringer, J., & Gasser, S. M. (2018). Repressive Chromatin in *Caenorhabditis elegans*: Establishment, Composition, and Function. *Genetics*, 208(2), 491–511. <https://doi.org/10.1534/genetics.117.300386>
- Albertson, D. G., & Thomson, J. N. (1976). The pharynx of *Caenorhabditis elegans*. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 275(938), 299–325. <https://doi.org/10.1098/rstb.1976.0085>
- Altun, Z. F., & Hall, D. H. (2011). Nervous system, general description. *Wormbook*. <https://doi.org/10.3908/wormatlas.1.18>
- Andersson, R., Gebhard, C., Miguel-Escalada, I., Hoof, I., Bornholdt, J., Boyd, M., Chen, Y., Zhao, X., Schmidl, C., Suzuki, T., Ntini, E., Arner, E., Valen, E., Li, K., Schwarzfischer, L., Glatz, D., Raithel, J., Lilje, B., Rapin, N., ... Sandelin, A. (2014). An atlas of active enhancers across human cell types and tissues. *Nature*, 507(7493), 455–461. <https://doi.org/10.1038/nature12787>
- Angeles-Albores, D., Leighton, D. H. W., Tsou, T., Khaw, T. H., Antoshechkin, I., & Sternberg, P. W. (2017). The *Caenorhabditis elegans* Female-Like State: Decoupling the Transcriptomic Effects of Aging and Sperm Status. *G3: Genes/Genomes/Genetics*, 7(9), 2969–2977. <https://doi.org/10.1534/g3.117.300080>
- Annunziato, A. (2008). DNA packaging: Nucleosomes and chromatin. *Nature Education*, 1(1), 26.
- Arendt, D. (2008). The evolution of cell types in animals: Emerging principles from molecular studies. *Nature Reviews Genetics*, 9(11), 868–882.
- Arendt, D., Bertucci, P. Y., Achim, K., & Musser, J. M. (2019). Evolution of neuronal types and families. *Current Opinion in Neurobiology*, 56, 144–152. <https://doi.org/10.1016/j.conb.2019.01.022>
- Arendt, D., Hausen, H., & Purschke, G. (2009). The ‘division of labour’ model of eye evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1531), 2809. <https://doi.org/10.1098/rstb.2009.0104>
- Arendt, D., Musser, J. M., Baker, C. V. H., Bergman, A., Cepko, C., Erwin, D. H., Pavlicev, M., Schlosser, G., Widder, S., Laubichler, M. D., & Wagner, G. P. (2016). The origin and evolution of cell types. *Nature Reviews Genetics*, 17(12), 744–757. <https://doi.org/10.1038/nrg.2016.127>
- Arvey, A., Agius, P., Noble, W. S., & Leslie, C. (2012). Sequence and chromatin determinants of cell-type-specific transcription factor binding. *Genome Research*, 22(9), 1723–1734.
- Audergon, P. N. C. B., Catania, S., Kagansky, A., Tong, P., Shukla, M., Pidoux, A. L., & Allshire, R. C. (2015). Restricted epigenetic inheritance of H3K9 methylation. *Science*, 348(6230), 132–135. <https://doi.org/10.1126/science.1260638>
- Auguie, B., & Antonov, A. (2017). gridExtra: Miscellaneous functions for “grid” graphics. *R Package Version*, 2(1).
- Azevedo, F. A. C., Carvalho, L. R. B., Grinberg, L. T., Farfel, J. M., Ferretti, R. E. L., Leite, R. E. P., Jacob Filho, W., Lent, R., & Herculano-Houzel, S. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-

- up primate brain. *The Journal of Comparative Neurology*, 513(5), 532–541. <https://doi.org/10.1002/cne.21974>
- Baldi, C., Cho, S., & Ellis, R. E. (2009). Mutations in two independent pathways are sufficient to create hermaphroditic nematodes. *Science*, 326(5955), 1002–1005.
- Baldwin, J. M. (1896). A New Factor in Evolution. *The American Naturalist*, 30(354), 441–451.
- Banerjee, N., Bhattacharya, R., Gorczyca, M., Collins, K. M., & Francis, M. M. (2017). Local neuropeptide signaling modulates serotonergic transmission to shape the temporal organization of *C. elegans* egg-laying behavior. *PLoS Genetics*, 13(4), e1006697. <https://doi.org/10.1371/journal.pgen.1006697>
- Bargmann, C. I., & Horvitz, H. R. (1991). Chemosensory neurons with overlapping functions direct chemotaxis to multiple chemicals in *C. elegans*. *Neuron*, 7(5), 729–742. [https://doi.org/10.1016/0896-6273\(91\)90276-6](https://doi.org/10.1016/0896-6273(91)90276-6)
- Barrière, A., & Félix, M.-A. (2014). Isolation of *C. elegans* and related nematodes. *WormBook: The Online Review of C. Elegans Biology*, 1–19. <https://doi.org/10.1895/wormbook.1.115.2>
- Barrios, A., Ghosh, R., Fang, C., Emmons, S. W., & Barr, M. M. (2012). PDF-1 neuropeptide signaling modulates a neural circuit for mate-searching behavior in *C. elegans*. *Nature Neuroscience*, 15(12), 1675–1682. <https://doi.org/10.1038/nn.3253>
- Becker, P. B., & Workman, J. L. (2013). Nucleosome Remodeling and Epigenetics. *Cold Spring Harbor Perspectives in Biology*, 5(9), a017905. <https://doi.org/10.1101/cshperspect.a017905>
- Bello, B. C., Hirth, F., & Gould, A. P. (2003). A Pulse of the *Drosophila* Hox Protein Abdominal-A Schedules the End of Neural Proliferation via Neuroblast Apoptosis. *Neuron*, 37(2), 209–219. [https://doi.org/10.1016/S0896-6273\(02\)01181-9](https://doi.org/10.1016/S0896-6273(02)01181-9)
- Berg, O. G. (1988). Selection of DNA binding sites by regulatory proteins. Functional specificity and pseudosite competition. *Journal of Biomolecular Structure & Dynamics*, 6(2), 275–297. <https://doi.org/10.1080/07391102.1988.10507713>
- Berger, M. F., Badis, G., Gehrke, A. R., Talukder, S., Philippakis, A. A., Peña-Castillo, L., Alleyne, T. M., Mnaimneh, S., Botvinnik, O. B., Chan, E. T., Khalid, F., Zhang, W., Newburger, D., Jaeger, S. A., Morris, Q. D., Bulyk, M. L., & Hughes, T. R. (2008). Variation in Homeodomain DNA Binding Revealed by High-Resolution Analysis of Sequence Preferences. *Cell*, 133(7), 1266–1276. <https://doi.org/10.1016/j.cell.2008.05.024>
- Berger, M. F., & Bulyk, M. L. (2009). Universal protein-binding microarrays for the comprehensive characterization of the DNA-binding specificities of transcription factors. *Nature Protocols*, 4(3), 393–411.
- Berger, S. L. (2007). The complex language of chromatin regulation during transcription. *Nature*, 447(7143), 407–412. <https://doi.org/10.1038/nature05915>
- Bertrand, N., Castro, D. S., & Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nature Reviews. Neuroscience*, 3(7), 517–530. <https://doi.org/10.1038/nrn874>

- Bhartiya, D. (2015). Stem cells, progenitors & regenerative medicine: A retrospection. *The Indian Journal of Medical Research*, 141(2), 154–161.
- Birnbaum, R. Y., Patwardhan, R. P., Kim, M. J., Findlay, G. M., Martin, B., Zhao, J., Bell, R. J. A., Smith, R. P., Ku, A. A., Shendure, J., & Ahituv, N. (2014). Systematic Dissection of Coding Exons at Single Nucleotide Resolution Supports an Additional Role in Cell-Specific Transcriptional Regulation. *PLOS Genetics*, 10(10), e1004592. <https://doi.org/10.1371/journal.pgen.1004592>
- Bonduriansky, R., & Day, T. (2009). Nongenetic Inheritance and Its Evolutionary Implications. *Annual Review of Ecology, Evolution, and Systematics*, 40(Volume 40, 2009), 103–125. <https://doi.org/10.1146/annurev.ecolsys.39.110707.173441>
- Bonn, S., Zinzen, R. P., Girardot, C., Gustafson, E. H., Perez-Gonzalez, A., Delhomme, N., Ghavi-Helm, Y., Wilczyński, B., Riddell, A., & Furlong, E. E. M. (2012). Tissue-specific analysis of chromatin state identifies temporal signatures of enhancer activity during embryonic development. *Nature Genetics*, 44(2), 148–156. <https://doi.org/10.1038/ng.1064>
- Braendle, C., & Flatt, T. (2006). A role for genetic accommodation in evolution? *BioEssays*, 28(9), 868–873. <https://doi.org/10.1002/bies.20456>
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77(1), 71–94. <https://doi.org/10.1093/genetics/77.1.71>
- Brocal-Ruiz, R., Esteve-Serrano, A., Mora-Martínez, C., Franco-Rivadeneira, M. L., Swoboda, P., Tena, J. J., Vilar, M., & Flames, N. (2023). Forkhead transcription factor FKH-8 cooperates with RFX in the direct regulation of sensory cilia in *Caenorhabditis elegans*. *eLife*, 12, e89702. <https://doi.org/10.7554/eLife.89702>
- Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y., & Greenleaf, W. J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nature Methods*, 10(12), 1213–1218.
- Cabrera, C. V. (1990). Lateral inhibition and cell fate during neurogenesis in *Drosophila*: The interactions between scute, Notch and Delta. *Development*, 109(3), 733–742. <https://doi.org/10.1242/dev.109.3.733>
- Callier, V. (2020). Understanding the evolution of cell types to explain the roots of animal diversity. *Proceedings of the National Academy of Sciences*, 117(11), 5547–5549. <https://doi.org/10.1073/pnas.2002403117>
- Cao, J., Packer, J. S., Ramani, V., Cusanovich, D. A., Huynh, C., Daza, R., Qiu, X., Lee, C., Furlan, S. N., Steemers, F. J., Adey, A., Waterston, R. H., Trapnell, C., & Shendure, J. (2017). Comprehensive single-cell transcriptional profiling of a multicellular organism. *Science (New York, N.Y.)*, 357(6352), 661–667. <https://doi.org/10.1126/science.aam8940>
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science (New York, N.Y.)*, 263(5148), 802–805. <https://doi.org/10.1126/science.8303295>
- Chamberlin, H. M., Jain, I. M., Corchado-Sonera, M., Kelley, L. H., Sharanya, D., Jama, A., Pabla, R., Dawes, A. T., & Gupta, B. P. (2020). Evolution of Transcriptional

- Repressors Impacts *Caenorhabditis* Vulval Development. *Molecular Biology and Evolution*, 37(5), 1350–1361. <https://doi.org/10.1093/molbev/msaa009>
- Chan, J. C., Alenina, N., Cunningham, A. M., Ramakrishnan, A., Shen, L., Bader, M., & Maze, I. (2024). Serotonin Transporter-dependent Histone Serotonylation in Placenta Contributes to the Neurodevelopmental Transcriptome. *Journal of Molecular Biology*, 436(7), 168454. <https://doi.org/10.1016/j.jmb.2024.168454>
- Chang, A. J., Chronis, N., Karow, D. S., Marletta, M. A., & Bargmann, C. I. (2006). A Distributed Chemosensory Circuit for Oxygen Preference in *C. elegans*. *PLOS Biology*, 4(9), e274. <https://doi.org/10.1371/journal.pbio.0040274>
- Chase, D. L., & Koelle, M. R. (2007). Biogenic amine neurotransmitters in *C. elegans*. *WormBook: The Online Review of C. Elegans Biology*, 1–15. <https://doi.org/10.1895/wormbook.1.132.1>
- Chen, Q., Yan, M., Cao, Z., Li, X., Zhang, Y., Shi, J., Feng, G., Peng, H., Zhang, X., Zhang, Y., Qian, J., Duan, E., Zhai, Q., & Zhou, Q. (2016). Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science*, 351(6271), 397–400. <https://doi.org/10.1126/science.aad7977>
- Chen, Q., Yan, W., & Duan, E. (2016). Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. *Nature Reviews Genetics*, 17(12), 733–743. <https://doi.org/10.1038/nrg.2016.106>
- Chen, Y., Liang, R., Li, Y., Jiang, L., Ma, D., Luo, Q., & Song, G. (2024). Chromatin accessibility: Biological functions, molecular mechanisms and therapeutic application. *Signal Transduction and Targeted Therapy*, 9(1), 1–39. <https://doi.org/10.1038/s41392-024-02030-9>
- Chen, Y.-C., Seyedsayamdost, M. R., & Ringstad, N. (2020). A microbial metabolite synergizes with endogenous serotonin to trigger *C. elegans* reproductive behavior. *Proceedings of the National Academy of Sciences*, 117(48), 30589–30598. <https://doi.org/10.1073/pnas.2017918117>
- Clark, R. F., & Elgin, S. C. R. (1992). Heterochromatin protein 1, a known suppressor of position-effect variegation, is highly conserved in *Drosophila*. *Nucleic Acids Research*, 20(22), 6067–6074. <https://doi.org/10.1093/nar/20.22.6067>
- Collins, K. M., Bode, A., Fernandez, R. W., Tanis, J. E., Brewer, J. C., Creamer, M. S., & Koelle, M. R. (2016). Activity of the *C. elegans* egg-laying behavior circuit is controlled by competing activation and feedback inhibition. *eLife*, 5, e21126. <https://doi.org/10.7554/eLife.21126>
- Cook, S. J., Jarrell, T. A., Brittin, C. A., Wang, Y., Bloniarz, A. E., Yakovlev, M. A., Nguyen, K. C. Q., Tang, L. T.-H., Bayer, E. A., Duerr, J. S., Bülow, H. E., Hobert, O., Hall, D. H., & Emmons, S. W. (2019). Whole-animal connectomes of both *Caenorhabditis elegans* sexes. *Nature*, 571(7763), 63–71. <https://doi.org/10.1038/s41586-019-1352-7>
- Corsi, A. K., Wightman, B., & Chalfie, M. (2015). A Transparent Window into Biology: A Primer on *Caenorhabditis elegans*. *Genetics*, 200(2), 387–407. <https://doi.org/10.1534/genetics.115.176099>
- Coustham, V., Bedet, C., Monier, K., Schott, S., Karali, M., & Palladino, F. (2006). The *C. elegans* HP1 homologue HPL-2 and the LIN-13 zinc finger protein form a

- complex implicated in vulval development. *Developmental Biology*, 297(2), 308–322. <https://doi.org/10.1016/j.ydbio.2006.04.474>
- Creyghton, M. P., Cheng, A. W., Welstead, G. G., Kooistra, T., Carey, B. W., Steine, E. J., Hanna, J., Lodato, M. A., Frampton, G. M., Sharp, P. A., & others. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proceedings of the National Academy of Sciences*, 107(50), 21931–21936.
- Crombie, T. A., McKeown, R., Moya, N. D., Evans, K. S., Widmayer, S. J., LaGrassa, V., Roman, N., Tursunova, O., Zhang, G., Gibson, S. B., Buchanan, C. M., Roberto, N. M., Vieira, R., Tanny, R. E., & Andersen, E. C. (2024). CaeNDR, the Caenorhabditis Natural Diversity Resource. *Nucleic Acids Research*, 52(D1), D850–D858. <https://doi.org/10.1093/nar/gkad887>
- Crombie, T. A., Zdraljevic, S., Cook, D. E., Tanny, R. E., Brady, S. C., Wang, Y., Evans, K. S., Hahnel, S., Lee, D., Rodriguez, B. C., Zhang, G., van der Zwagg, J., Kiontke, K., & Andersen, E. C. (2019). Deep sampling of Hawaiian *Caenorhabditis elegans* reveals high genetic diversity and admixture with global populations. *eLife*, 8, e50465. <https://doi.org/10.7554/eLife.50465>
- Cui, M., Kim, E. B., & Han, M. (2006). Diverse Chromatin Remodeling Genes Antagonize the Rb-Involved SynMuv Pathways in *C. elegans*. *PLOS Genetics*, 2(5), e74. <https://doi.org/10.1371/journal.pgen.0020074>
- Dag, U., Nwabudike, I., Kang, D., Gomes, M. A., Kim, J., Atanas, A. A., Bueno, E., Estrem, C., Pugliese, S., Wang, Z., Towilson, E., & Flavell, S. W. (2023). Dissecting the functional organization of the *C. elegans* serotonergic system at whole-brain scale. *Cell*, 186(12), 2574–2592.e20. <https://doi.org/10.1016/j.cell.2023.04.023>
- Dan, J., & Chen, T. (2023). Chapter 3—Writers, erasers, and readers of DNA and histone methylation marks. In S. G. Gray (Ed.), *Epigenetic Cancer Therapy (Second Edition)* (pp. 39–63). Academic Press. <https://doi.org/10.1016/B978-0-323-91367-6.00012-X>
- Darmanis, S., Sloan, S. A., Zhang, Y., Enge, M., Caneda, C., Shuer, L. M., Hayden Gephart, M. G., Barres, B. A., & Quake, S. R. (2015). A survey of human brain transcriptome diversity at the single cell level. *Proceedings of the National Academy of Sciences of the United States of America*, 112(23), 7285–7290. <https://doi.org/10.1073/pnas.1507125112>
- Das, M., Semple, J. I., Haemmerli, A., Volodkina, V., Scotton, J., Gitchev, T., Annan, A., Campos, J., Statzer, C., Dakhovnik, A., Ewald, C. Y., Mozziconacci, J., & Meister, P. (2024). Condensin I folds the *Caenorhabditis elegans* genome. *Nature Genetics*, 56(8), 1737–1749. <https://doi.org/10.1038/s41588-024-01832-5>
- Davidson, E. H. (2006a). CHAPTER 1—The “Regulatory Genome” for Animal Development. In E. H. Davidson (Ed.), *The Regulatory Genome* (pp. 1–29). Academic Press. <https://doi.org/10.1016/B978-012088563-3.50019-5>
- Davidson, E. H. (2006b). *The Regulatory Genome: Gene Regulatory Networks In Development And Evolution*. Elsevier.
- Davidson, E. H., Rast, J. P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.-H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C. T., Livi, C. B., Lee, P. Y., Revilla, R., Rust, A. G., Pan, Z. jun, Schilstra, M. J., Clarke, P. J. C., ... Bolouri, H.

- (2002). A genomic regulatory network for development. *Science (New York, N.Y.)*, 295(5560), 1669–1678. <https://doi.org/10.1126/science.1069883>
- Davis, P., Zarowiecki, M., Arnaboldi, V., Becerra, A., Cain, S., Chan, J., Chen, W. J., Cho, J., da Veiga Beltrame, E., Diamantakis, S., Gao, S., Grigoriadis, D., Grove, C. A., Harris, T. W., Kishore, R., Le, T., Lee, R. Y. N., Luybaert, M., Müller, H.-M., ... Sternberg, P. W. (2022). WormBase in 2022-data, processes, and tools for analyzing *Caenorhabditis elegans*. *Genetics*, 220(4), iyac003. <https://doi.org/10.1093/genetics/iyac003>
- Dayi, M., Kanzaki, N., Sun, S., Ide, T., Tanaka, R., Masuya, H., Okabe, K., Kajimura, H., & Kikuchi, T. (2021). Additional description and genome analyses of *Caenorhabditis auriculariae* representing the basal lineage of genus *Caenorhabditis*. *Scientific Reports*, 11(1), 6720. <https://doi.org/10.1038/s41598-021-85967-z>
- De La Cruz-Ruiz, P., Rodríguez-Palero, M. J., Askjaer, P., & Artal-Sanz, M. (2023). Tissue-specific chromatin-binding patterns of *Caenorhabditis elegans* heterochromatin proteins HPL-1 and HPL-2 reveal differential roles in the regulation of gene expression. *GENETICS*, 224(3), iyad081. <https://doi.org/10.1093/genetics/iyad081>
- De Santa, F., Barozzi, I., Mietton, F., Ghisletti, S., Polletti, S., Tusi, B. K., Muller, H., Ragoussis, J., Wei, C.-L., & Natoli, G. (2010). A Large Fraction of Extragenic RNA Pol II Transcription Sites Overlap Enhancers. *PLOS Biology*, 8(5), e1000384. <https://doi.org/10.1371/journal.pbio.1000384>
- Desai, C., Garriga, G., McIntire, S. L., & Horvitz, H. R. (1988). A genetic pathway for the development of the *Caenorhabditis elegans* HSN motor neurons. *Nature*, 336(6200), 638–646. <https://doi.org/10.1038/336638a0>
- Desai, C., & Horvitz, H. R. (1989). *Caenorhabditis elegans* mutants defective in the functioning of the motor neurons responsible for egg laying. *Genetics*, 121(4), 703–721. <https://doi.org/10.1093/genetics/121.4.703>
- Destain, H., Prahlad, M., & Kratsios, P. (2024). Maintenance of neuronal identity in *C. elegans* and beyond: Lessons from transcription and chromatin factors. *Seminars in Cell & Developmental Biology*, 154, 35–47. <https://doi.org/10.1016/j.semcdb.2023.07.001>
- Diamond, S. E., & Martin, R. A. (2021). Buying Time: Plasticity and Population Persistence. In *Phenotypic Plasticity & Evolution*. CRC Press.
- Diao, Y., Fang, R., Li, B., Meng, Z., Yu, J., Qiu, Y., Lin, K. C., Huang, H., Liu, T., Marina, R. J., Jung, I., Shen, Y., Guan, K.-L., & Ren, B. (2017). A tiling-deletion-based genetic screen for cis-regulatory element identification in mammalian cells. *Nature Methods*, 14(6), 629–635. <https://doi.org/10.1038/nmeth.4264>
- Dickinson, D. J., Ward, J. D., Reiner, D. J., & Goldstein, B. (2013). Engineering the *Caenorhabditis elegans* genome using Cas9-triggered homologous recombination. *Nature Methods*, 10(10), 1028–1034. <https://doi.org/10.1038/nmeth.2641>
- Doroszuk, A., Snoek, L. B., Fradin, E., Riksen, J., & Kammenga, J. (2009). A genome-wide library of CB4856/N2 introgression lines of *Caenorhabditis elegans*. *Nucleic Acids Research*, 37(16), e110. <https://doi.org/10.1093/nar/gkp528>

- Dsilva, G. J., & Galande, S. (2024). From sequence to consequence: Deciphering the complex cis-regulatory landscape. *Journal of Biosciences*, 49(2), 46. <https://doi.org/10.1007/s12038-024-00431-0>
- Duerr, J. S., Frisby, D. L., Gaskin, J., Duke, A., Asermely, K., Huddleston, D., Eiden, L. E., & Rand, J. B. (1999). The *cat-1* Gene of *Caenorhabditis elegans* Encodes a Vesicular Monoamine Transporter Required for Specific Monoamine-Dependent Behaviors. *The Journal of Neuroscience*, 19(1), 72–84. <https://doi.org/10.1523/JNEUROSCI.19-01-00072.1999>
- Duerr, J. S., Gaskin, J., & Rand, J. B. (2001). Identified neurons in *C. elegans* coexpress vesicular transporters for acetylcholine and monoamines. *American Journal of Physiology-Cell Physiology*, 280(6), C1616–C1622. <https://doi.org/10.1152/ajpcell.2001.280.6.C1616>
- Ebbing, A., Vértesy, Á., Betist, M. C., Spanjaard, B., Junker, J. P., Berezikov, E., Oudenaarden, A. van, & Korswagen, H. C. (2018). Spatial Transcriptomics of *C. elegans* Males and Hermaphrodites Identifies Sex-Specific Differences in Gene Expression Patterns. *Developmental Cell*, 47(6), 801–813.e6. <https://doi.org/10.1016/j.devcel.2018.10.016>
- Eisenberg, J. C., & Elgin, S. C. R. (2014). HP1a: A structural chromosomal protein regulating transcription. *Trends in Genetics*, 30(3), 103–110. <https://doi.org/10.1016/j.tig.2014.01.002>
- Emmons, S. W., & Lipton, J. (2003). Genetic basis of male sexual behavior. *Journal of Neurobiology*, 54(1), 93–110. <https://doi.org/10.1002/neu.10163>
- ENCODE Project Consortium. (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature*, 489(7414), 57–74. <https://doi.org/10.1038/nature11247>
- Engreitz, J. M., Haines, J. E., Perez, E. M., Munson, G., Chen, J., Kane, M., McDonel, P. E., Guttman, M., & Lander, E. S. (2016). Local regulation of gene expression by lncRNA promoters, transcription and splicing. *Nature*, 539(7629), 452–455. <https://doi.org/10.1038/nature20149>
- Erkenbrack, E. M., Maziarz, J. D., Griffith, O. W., Liang, C., Chavan, A. R., & Wagner, G. P. (2018). *The mammalian decidual cell evolved from a cellular stress response*. *Evolutionary Biology*. <https://doi.org/10.1101/246397>
- Etchberger, J. F., Lorch, A., Sleumer, M. C., Zapf, R., Jones, S. J., Marra, M. A., Holt, R. A., Moerman, D. G., & Hobert, O. (2007). The molecular signature and cis-regulatory architecture of a *C. elegans* gustatory neuron. *Genes & Development*, 21(13), 1653–1674. <https://doi.org/10.1101/gad.1560107>
- Evans, K. S., Zdraljevic, S., Stevens, L., Collins, K., Tanny, R. E., & Andersen, E. C. (2020). Natural variation in the sequestosome-related gene, *sqst-5*, underlies zinc homeostasis in *Caenorhabditis elegans*. *PLOS Genetics*, 16(11), e1008986. <https://doi.org/10.1371/journal.pgen.1008986>
- Farrelly, L. A., Thompson, R. E., Zhao, S., Lepack, A. E., Lyu, Y., Bhanu, N. V., Zhang, B., Loh, Y.-H. E., Ramakrishnan, A., Vadodaria, K. C., Heard, K. J., Erikson, G., Nakadai, T., Bastle, R. M., Lukasak, B. J., Zebroski, H., Alenina, N., Bader, M., Berton, O., ... Maze, I. (2019). Histone serotonylation is a permissive modification that enhances TFIID binding to H3K4me3. *Nature*, 567(7749), 535–539. <https://doi.org/10.1038/s41586-019-1024-7>

- Fernandez, R. W., Wei, K., Wang, E. Y., Mikalauskaite, D., Olson, A., Pepper, J., Christie, N., Kim, S., Weissenborn, S., Sarov, M., & Koelle, M. R. (2020). Cellular Expression and Functional Roles of All 26 Neurotransmitter GPCRs in the *C. elegans* Egg-Laying Circuit. *The Journal of Neuroscience*, 40(39), 7475–7488. <https://doi.org/10.1523/JNEUROSCI.1357-20.2020>
- Fisher, W. W., Li, J. J., Hammonds, A. S., Brown, J. B., Pfeiffer, B. D., Weiszmann, R., MacArthur, S., Thomas, S., Stamatoyannopoulos, J. A., Eisen, M. B., & others. (2012). DNA regions bound at low occupancy by transcription factors do not drive patterned reporter gene expression in *Drosophila*. *Proceedings of the National Academy of Sciences*, 109(52), 21330–21335.
- Flames, N., & Hobert, O. (2011). Transcriptional Control of the Terminal Fate of Monoaminergic Neurons. *Annual Review of Neuroscience*, 34(Volume 34, 2011), 153–184. <https://doi.org/10.1146/annurev-neuro-061010-113824>
- Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y., & Postlethwait, J. (1999). Preservation of Duplicate Genes by Complementary, Degenerative Mutations. *Genetics*, 151(4), 1531–1545. <https://doi.org/10.1093/genetics/151.4.1531>
- Freyd, G., Kim, S. K., & Horvitz, H. R. (1990a). Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-II*. *Nature*, 344(6269), 876–879. <https://doi.org/10.1038/344876a0>
- Freyd, G., Kim, S. K., & Horvitz, H. R. (1990b). Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-II*. *Nature*, 344(6269), 876–879. <https://doi.org/10.1038/344876a0>
- Friedland, A. E., Tzur, Y. B., Esvelt, K. M., Colaiácovo, M. P., Church, G. M., & Calarco, J. A. (2013). Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system. *Nature Methods*, 10(8), 741–743. <https://doi.org/10.1038/nmeth.2532>
- Futuyma, D. J., & Moreno, G. (1988). The Evolution of Ecological Specialization. *Annual Review of Ecology and Systematics*, 19, 207–233.
- Fuxman Bass, J. I., Tamburino, A. M., Mori, A., Beittel, N., Weirauch, M. T., Reece-Hoyes, J. S., & Walhout, A. J. M. (2014). Transcription factor binding to *Caenorhabditis elegans* first introns reveals lack of redundancy with gene promoters. *Nucleic Acids Research*, 42(1), 153–162. <https://doi.org/10.1093/nar/gkt858>
- Gao, Z., Liu, L., & Ruan, J. (2017). Logo2PWM: A tool to convert sequence logo to position weight matrix. *BMC Genomics*, 18(6), 709. <https://doi.org/10.1186/s12864-017-4023-9>
- Gaspar, P., Cases, O., & Maroteaux, L. (2003). The developmental role of serotonin: News from mouse molecular genetics. *Nature Reviews Neuroscience*, 4(12), 1002–1012. <https://doi.org/10.1038/nrn1256>
- Gilarte, P., Kreuzinger-Janik, B., Majdi, N., & Traunspurger, W. (2015). Life-History Traits of the Model Organism *Pristionchus pacificus* Recorded Using the Hanging Drop Method: Comparison with *Caenorhabditis elegans*. *PLOS ONE*, 10(8), e0134105. <https://doi.org/10.1371/journal.pone.0134105>
- Gisselbrecht, S. S., Palagi, A., Kurland, J. V., Rogers, J. M., Ozadam, H., Zhan, Y., Dekker, J., & Bulyk, M. L. (2020). Transcriptional Silencers in *Drosophila* Serve a Dual Role as Transcriptional Enhancers in Alternate Cellular Contexts. *Molecular Cell*, 77(2), 324–337.e8. <https://doi.org/10.1016/j.molcel.2019.10.004>

- Gonçalves, S., Nunes-Costa, D., Cardoso, S. M., Empadinhas, N., & Marugg, J. D. (2022). Enzyme Promiscuity in Serotonin Biosynthesis, From Bacteria to Plants and Humans. *Frontiers in Microbiology*, 13, 873555. <https://doi.org/10.3389/fmicb.2022.873555>
- Gonzalez-Sandoval, A., Towbin, B. D., Kalck, V., Cabianca, D. S., Gaidatzis, D., Hauer, M. H., Geng, L., Wang, L., Yang, T., Wang, X., Zhao, K., & Gasser, S. M. (2015). Perinuclear Anchoring of H3K9-Methylated Chromatin Stabilizes Induced Cell Fate in *C. elegans* Embryos. *Cell*, 163(6), 1333–1347. <https://doi.org/10.1016/j.cell.2015.10.066>
- Gouti, M., & Gavalas, A. (2008). Hoxb1 Controls Cell Fate Specification and Proliferative Capacity of Neural Stem and Progenitor Cells. *Stem Cells*, 26(8), 1985–1997. <https://doi.org/10.1634/stemcells.2008-0182>
- Grolemund, G., & Wickham, H. (2011). Dates and Times Made Easy with lubridate. *Journal of Statistical Software*, 40, 1–25. <https://doi.org/10.18637/jss.v040.i03>
- Guss, K. A., Benson, M., Gubitosi, N., Brondell, K., Broadie, K., & Skeath, J. B. (2013). Expression and function of scalloped during *Drosophila* development. *Developmental Dynamics*, 242(7), 874–885. <https://doi.org/10.1002/dvdy.23942>
- Haag, E. S., Fitch, D. H. A., & Delattre, M. (2018). From “the Worm” to “the Worms” and Back Again: The Evolutionary Developmental Biology of Nematodes. *Genetics*, 210(2), 397–433. <https://doi.org/10.1534/genetics.118.300243>
- Hallam, S., Singer, E., Waring, D., & Jin, Y. (2000). The *C. elegans* NeuroD homolog *cnd-1* functions in multiple aspects of motor neuron fate specification. *Development (Cambridge, England)*, 127(19), 4239–4252. <https://doi.org/10.1242/dev.127.19.4239>
- Hamdan, F. F., Ungrin, M. D., Abramovitz, M., & Ribeiro, P. (1999). Characterization of a Novel Serotonin Receptor from *Caenorhabditis elegans*. *Journal of Neurochemistry*, 72(4), 1372–1383. <https://doi.org/10.1046/j.1471-4159.1999.721372.x>
- Hankin, B. L., Nederhof, E., Oppenheimer, C. W., Jenness, J., Young, J. F., Abela, J. R. Z., Smolen, A., Ormel, J., & Oldehinkel, A. J. (2011). Differential susceptibility in youth: Evidence that 5-HTTLPR x positive parenting is associated with positive affect “for better and worse.” *Translational Psychiatry*, 1(10), e44. <https://doi.org/10.1038/tp.2011.44>
- Hapiak, V. M., Hobson, R. J., Hughes, L., Smith, K., Harris, G., Condon, C., Komuniecki, P., & Komuniecki, R. W. (2009). Dual Excitatory and Inhibitory Serotonergic Inputs Modulate Egg Laying in *Caenorhabditis elegans*. *Genetics*, 181(1), 153–163. <https://doi.org/10.1534/genetics.108.096891>
- Harbison, C. T., Gordon, D. B., Lee, T. I., Rinaldi, N. J., Macisaac, K. D., Danford, T. W., Hannett, N. M., Tagne, J.-B., Reynolds, D. B., Yoo, J., & others. (2004). Transcriptional regulatory code of a eukaryotic genome. *Nature*, 431(7004), 99–104.
- Hardaker, L. A., Singer, E., Kerr, R., Zhou, G., & Schafer, W. R. (2001). Serotonin modulates locomotory behavior and coordinates egg-laying and movement in *Caenorhabditis elegans*. *Journal of Neurobiology*, 49(4), 303–313. <https://doi.org/10.1002/neu.10014>

- Harrison, M. M., Lu, X., & Horvitz, H. R. (2007). LIN-61, One of Two Caenorhabditis elegans Malignant-Brain-Tumor-Repeat-Containing Proteins, Acts With the DRM and NuRD-Like Protein Complexes in Vulval Development but Not in Certain Other Biological Processes. *Genetics*, 176(1), 255–271. <https://doi.org/10.1534/genetics.106.069633>
- Hart, A. C. (2006). Behavior. WormBook, ed. The C. elegans research community. *WormBook*.
- Hay-Schmidt, A. (2000). The evolution of the serotonergic nervous system. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 267(1448), 1071–1079. <https://doi.org/10.1098/rspb.2000.1111>
- He, Q., Bardet, A. F., Patton, B., Purvis, J., Johnston, J., Paulson, A., Gogol, M., Stark, A., & Zeitlinger, J. (2011). High conservation of transcription factor binding and evidence for combinatorial regulation across six Drosophila species. *Nature Genetics*, 43(5), 414–420. <https://doi.org/10.1038/ng.808>
- Heintzman, N. D., Hon, G. C., Hawkins, R. D., Kheradpour, P., Stark, A., Harp, L. F., Ye, Z., Lee, L. K., Stuart, R. K., Ching, C. W., Ching, K. A., Antosiewicz-Bourget, J. E., Liu, H., Zhang, X., Green, R. D., Lobanenkov, V. V., Stewart, R., Thomson, J. A., Crawford, G. E., ... Ren, B. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature*, 459(7243), 108–112. <https://doi.org/10.1038/nature07829>
- Heintzman, N. D., Stuart, R. K., Hon, G., Fu, Y., Ching, C. W., Hawkins, R. D., Barrera, L. O., Van Calcar, S., Qu, C., Ching, K. A., Wang, W., Weng, Z., Green, R. D., Crawford, G. E., & Ren, B. (2007). Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nature Genetics*, 39(3), 311–318. <https://doi.org/10.1038/ng1966>
- Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., Cheng, J. X., Murre, C., Singh, H., & Glass, C. K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Molecular Cell*, 38(4), 576–589.
- Herculano-Houzel, S. (2009). The human brain in numbers: A linearly scaled-up primate brain. *Frontiers in Human Neuroscience*, 3, 31. <https://doi.org/10.3389/neuro.09.031.2009>
- Hernádi, L., Erdélyi, L., Hiripi, L., & Elekes, K. (1998). The organization of serotonin-, dopamine-, and FMRFamide-containing neuronal elements and their possible role in the regulation of spontaneous contraction of the gastrointestinal tract in the snail Helix pomatia. *Journal of Neurocytology*, 27(10), 761–775. <https://doi.org/10.1023/a:1006955018882>
- Hesselberth, J. R., Chen, X., Zhang, Z., Sabo, P. J., Sandstrom, R., Reynolds, A. P., Thurman, R. E., Neph, S., Kuehn, M. S., Noble, W. S., & others. (2009). Global mapping of protein-DNA interactions in vivo by digital genomic footprinting. *Nature Methods*, 6(4), 283–289.
- Hobert, O. (2005). Specification of the nervous system. *WormBook: The Online Review of C. Elegans Biology*, 1–19. <https://doi.org/10.1895/wormbook.1.12.1>
- Hobert, O. (2008). Regulatory logic of neuronal diversity: Terminal selector genes and selector motifs. *Proceedings of the National Academy of Sciences of the United*

- States of America*, 105(51), 20067–20071.
<https://doi.org/10.1073/pnas.0806070105>
- Hobert, O. (2010). Neurogenesis in the nematode *Caenorhabditis elegans*. *WormBook: The Online Review of C. Elegans Biology*, 1–24.
<https://doi.org/10.1895/wormbook.1.12.2>
- Hobert, O. (2013). The neuronal genome of *Caenorhabditis elegans*. *WormBook: The Online Review of C. Elegans Biology*, 1–106.
<https://doi.org/10.1895/wormbook.1.161.1>
- Hobert, O. (2016). Terminal Selectors of Neuronal Identity. In *Current Topics in Developmental Biology* (Vol. 116, pp. 455–475). Elsevier.
<https://doi.org/10.1016/bs.ctdb.2015.12.007>
- Hobert, O., D'Alberti, T., Liu, Y., & Ruvkun, G. (1998). Control of Neural Development and Function in a Thermoregulatory Network by the LIM Homeobox Gene *lin-11*. *Journal of Neuroscience*, 18(6), 2084–2096.
<https://doi.org/10.1523/JNEUROSCI.18-06-02084.1998>
- Hobert, O., Glenwinkel, L., & White, J. (2016). Revisiting Neuronal Cell Type Classification in *Caenorhabditis elegans*. *Current Biology: CB*, 26(22), R1197–R1203. <https://doi.org/10.1016/j.cub.2016.10.027>
- Hobson, R. J., Hapiak, V. M., Xiao, H., Buehrer, K. L., Komuniecki, P. R., & Komuniecki, R. W. (2006). SER-7, a *Caenorhabditis elegans* 5-HT7-like Receptor, Is Essential for the 5-HT Stimulation of Pharyngeal Pumping and Egg Laying. *Genetics*, 172(1), 159–169. <https://doi.org/10.1534/genetics.105.044495>
- Horvitz, H. R., Chalfie, M., Trent, C., Sulston, J. E., & Evans, P. D. (1982). Serotonin and octopamine in the nematode *Caenorhabditis elegans*. *Science*, 216(4549), 1012–1014.
- Horvitz, H. R., Sternberg, P. W., Greenwald, I. S., Fixsen, W., & Ellis, H. M. (1983). Mutations that affect neural cell lineages and cell fates during the development of the nematode *Caenorhabditis elegans*. *Cold Spring Harbor Symposia on Quantitative Biology*, 48 Pt 2, 453–463.
<https://doi.org/10.1101/sqb.1983.048.01.050>
- Houri-Zeevi, L., Korem Kohanim, Y., Antonova, O., & Rechavi, O. (2020). Three Rules Explain Transgenerational Small RNA Inheritance in *C. elegans*. *Cell*, 182(5), 1186–1197.e12. <https://doi.org/10.1016/j.cell.2020.07.022>
- Hwang, J. S., Ohyanagi, H., Hayakawa, S., Osato, N., Nishimiya-Fujisawa, C., Ikeo, K., David, C. N., Fujisawa, T., & Gojobori, T. (2007). The evolutionary emergence of cell type-specific genes inferred from the gene expression analysis of *Hydra*. *Proceedings of the National Academy of Sciences*, 104(37), 14735–14740.
<https://doi.org/10.1073/pnas.0703331104>
- Inukai, S., Kock, K. H., & Bulyk, M. L. (2017). Transcription factor-DNA binding: Beyond binding site motifs. *Current Opinion in Genetics & Development*, 43, 110–119.
<https://doi.org/10.1016/j.gde.2017.02.007>
- Jafari, G., Xie, Y., Kullyev, A., Liang, B., & Sze, J. Y. (2011). Regulation of extrasynaptic 5-HT by serotonin reuptake transporter function in 5-HT-absorbing neurons underscores adaptation behavior in *Caenorhabditis elegans*. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 31(24), 8948–8957. <https://doi.org/10.1523/JNEUROSCI.1692-11.2011>

- Johansen, N., Somasundaram, S., Travaglini, K. J., Yanny, A. M., Shumyatcher, M., Casper, T., Cobbs, C., Dee, N., Ellenbogen, R., Ferreira, M., Goldy, J., Guzman, J., Gwinn, R., Hirschstein, D., Jorstad, N. L., Keene, C. D., Ko, A., Levi, B. P., Ojemann, J. G., ... Miller, J. A. (2023). Interindividual variation in human cortical cell type abundance and expression. *Science (New York, N.Y.)*, 382(6667), eadf2359. <https://doi.org/10.1126/science.adf2359>
- Junion, G., Spivakov, M., Girardot, C., Braun, M., Gustafson, E. H., Birney, E., & Furlong, E. E. M. (2012). A Transcription Factor Collective Defines Cardiac Cell Fate and Reflects Lineage History. *Cell*, 148(3), 473–486. <https://doi.org/10.1016/j.cell.2012.01.030>
- Kanzaki, N., Kiontke, K., Tanaka, R., Hirooka, Y., Schwarz, A., Müller-Reichert, T., Chaudhuri, J., & Pires-daSilva, A. (2017). Description of two three-gendered nematode species in the new genus *Auanema* (Rhabditina) that are models for reproductive mode evolution. *Scientific Reports*, 7(1), 11135. <https://doi.org/10.1038/s41598-017-09871-1>
- Kanzaki, N., Tsai, I. J., Tanaka, R., Hunt, V. L., Liu, D., Tsuyama, K., Maeda, Y., Namai, S., Kumagai, R., Tracey, A., Holroyd, N., Doyle, S. R., Woodruff, G. C., Murase, K., Kitazume, H., Chai, C., Akagi, A., Panda, O., Ke, H.-M., ... Kikuchi, T. (2018). Biology and genome of a newly discovered sibling species of *Caenorhabditis elegans*. *Nature Communications*, 9(1), 3216. <https://doi.org/10.1038/s41467-018-05712-5>
- Kasinathan, S., Orsi, G. A., Zentner, G. E., Ahmad, K., & Henikoff, S. (2014). High-resolution mapping of transcription factor binding sites on native chromatin. *Nature Methods*, 11(2), 203–209.
- Kaya-Okur, H. S., Wu, S. J., Codomo, C. A., Pledger, E. S., Bryson, T. D., Henikoff, J. G., Ahmad, K., & Henikoff, S. (2019). CUT&Tag for efficient epigenomic profiling of small samples and single cells. *Nature Communications*, 10(1), 1930.
- Kheradpour, P., Ernst, J., Melnikov, A., Rogov, P., Wang, L., Zhang, X., Alston, J., Mikkelsen, T. S., & Kellis, M. (2013). Systematic dissection of regulatory motifs in 2000 predicted human enhancers using a massively parallel reporter assay. *Genome Research*, 23(5), 800–811. <https://doi.org/10.1101/gr.144899.112>
- Kim, S., & Wysocka, J. (2023). Deciphering the multi-scale, quantitative cis-regulatory code. *Molecular Cell*, 83(3), 373–392. <https://doi.org/10.1016/j.molcel.2022.12.032>
- Kingston, R., Amin, D., Misra, S., Gross, J. M., & Kuwajima, T. (2021). Serotonin transporter-mediated molecular axis regulates regional retinal ganglion cell vulnerability and axon regeneration after nerve injury. *PLoS Genetics*, 17(11), e1009885. <https://doi.org/10.1371/journal.pgen.1009885>
- Kiontke, K. C., Félix, M.-A., Ailion, M., Rockman, M. V., Braendle, C., Pénigault, J.-B., & Fitch, D. H. (2011). A phylogeny and molecular barcodes for *Caenorhabditis*, with numerous new species from rotting fruits. *BMC Evolutionary Biology*, 11(1), 339. <https://doi.org/10.1186/1471-2148-11-339>
- Kiontke, Karin. C., & Sudhaus, W. (2006). Ecology of *Caenorhabditis* species. *WormBook: The Online Review of C. Elegans Biology*, 1–14. <https://doi.org/10.1895/wormbook.1.37.1>

- Klemm, S. L., Shipony, Z., & Greenleaf, W. J. (2019). Chromatin accessibility and the regulatory epigenome. *Nature Reviews Genetics*, 20(4), 207–220. <https://doi.org/10.1038/s41576-018-0089-8>
- Klosin, A., Casas, E., Hidalgo-Carcedo, C., Vavouri, T., & Lehner, B. (2017). Transgenerational transmission of environmental information in *C. elegans*. *Science (New York, N.Y.)*, 356(6335), 320–323. <https://doi.org/10.1126/science.aah6412>
- Koester-Eiserfunke, N., & Fischle, W. (2011). H3K9me2/3 binding of the MBT domain protein LIN-61 is essential for *Caenorhabditis elegans* vulva development. *PLoS Genetics*, 7(3), e1002017. <https://doi.org/10.1371/journal.pgen.1002017>
- Kramer, J. M., French, R. P., Park, E. C., & Johnson, J. J. (1990). The *Caenorhabditis elegans* rol-6 gene, which interacts with the sqt-1 collagen gene to determine organismal morphology, encodes a collagen. *Molecular and Cellular Biology*, 10(5), 2081–2089. <https://doi.org/10.1128/mcb.10.5.2081-2089.1990>
- Kreis, H. (1964). *A nematode from the external auditory ineatus of zebu cattle in East Africa (Tanganyika): Rhabditis bovis, n, sp.*
- Kulakovskiy, I. V., Vorontsov, I. E., Yevshin, I. S., Soboleva, A. V., Kasianov, A. S., Ashoor, H., Ba-Alawi, W., Bajic, V. B., Medvedeva, Y. A., Kolpakov, F. A., & others. (2016). HOCOMOCO: expansion and enhancement of the collection of transcription factor binding sites models. *Nucleic Acids Research*, 44(D1), D116–D125.
- Kullyev, A., Dempsey, C. M., Miller, S., Kuan, C.-J., Hapiak, V. M., Komuniecki, R. W., Griffin, C. T., & Sze, J. Y. (2010). A Genetic Survey of Fluoxetine Action on Synaptic Transmission in *Caenorhabditis elegans*. *Genetics*, 186(3), 929–941. <https://doi.org/10.1534/genetics.110.118877>
- Kwasnieski, J. C., Fiore, C., Chaudhari, H. G., & Cohen, B. A. (2014). High-throughput functional testing of ENCODE segmentation predictions. *Genome Research*, 24(10), 1595–1602.
- Lai, C. H., Chou, C. Y., Ch'ang, L. Y., Liu, C. S., & Lin, W. (2000). Identification of novel human genes evolutionarily conserved in *Caenorhabditis elegans* by comparative proteomics. *Genome Research*, 10(5), 703–713. <https://doi.org/10.1101/gr.10.5.703>
- Large, C. R. L., Khanal, R., Hillier, L., Huynh, C., Kubo, C., Kim, J., Waterston, R. H., & Murray, J. I. (2024). *Lineage-resolved analysis of embryonic gene expression evolution in C. elegans and C. briggsae*. *Evolutionary Biology*. <https://doi.org/10.1101/2024.02.03.578695>
- Lee, D., Zdraljevic, S., Cook, D. E., Frézal, L., Hsu, J.-C., Sterken, M. G., Riksen, J. A. G., Wang, J., Kammenga, J. E., Braendle, C., Félix, M.-A., Schroeder, F. C., & Andersen, E. C. (2019). Selection and gene flow shape niche-associated variation in pheromone response. *Nature Ecology & Evolution*, 3(10), 1455–1463. <https://doi.org/10.1038/s41559-019-0982-3>
- Leiz, J., Rutkiewicz, M., Birchmeier, C., Heinemann, U., & Schmidt-Ott, K. M. (2021). Technologies for profiling the impact of genomic variants on transcription factor binding. *Medizinische Genetik*, 33(2), 147–155.

- Lenhard, B., Sandelin, A., & Carninci, P. (2012). Metazoan promoters: Emerging characteristics and insights into transcriptional regulation. *Nature Reviews Genetics*, 13(4), 233–245. <https://doi.org/10.1038/nrg3163>
- Lepack, A. E., Werner, C. T., Stewart, A. F., Fulton, S. L., Zhong, P., Farrelly, L. A., Smith, A. C. W., Ramakrishnan, A., Lyu, Y., Bastle, R. M., Martin, J. A., Mitra, S., O'Connor, R. M., Wang, Z.-J., Molina, H., Turecki, G., Shen, L., Yan, Z., Calipari, E. S., ... Maze, I. (2020). Dopaminylation of histone H3 in ventral tegmental area regulates cocaine seeking. *Science*, 368(6487), 197–201. <https://doi.org/10.1126/science.aaw8806>
- Lesch, K.-P., & Waider, J. (2012). Serotonin in the Modulation of Neural Plasticity and Networks: Implications for Neurodevelopmental Disorders. *Neuron*, 76(1), 175–191. <https://doi.org/10.1016/j.neuron.2012.09.013>
- Lettice, L. A., Heaney, S. J. H., Purdie, L. A., Li, L., de Beer, P., Oostra, B. A., Goode, D., Elgar, G., Hill, R. E., & de Graaff, E. (2003). A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Human Molecular Genetics*, 12(14), 1725–1735. <https://doi.org/10.1093/hmg/ddg180>
- Levo, M., & Segal, E. (2014). In pursuit of design principles of regulatory sequences. *Nature Reviews Genetics*, 15(7), 453–468. <https://doi.org/10.1038/nrg3684>
- Leyva-Díaz, E., & Hobert, O. (2019). Transcription factor autoregulation is required for acquisition and maintenance of neuronal identity. *Development (Cambridge, England)*, 146(13), dev177378. <https://doi.org/10.1242/dev.177378>
- Leyva-Díaz, E., & Hobert, O. (2022). Robust regulatory architecture of pan-neuronal gene expression. *Current Biology: CB*, 32(8), 1715–1727.e8. <https://doi.org/10.1016/j.cub.2022.02.040>
- Lickteig, K. M., Duerr, J. S., Frisby, D. L., Hall, D. H., Rand, J. B., & Miller, D. M. (2001). Regulation of Neurotransmitter Vesicles by the Homeodomain Protein UNC-4 and Its Transcriptional Corepressor UNC-37/Groucho in *Caenorhabditis elegans* Cholinergic Motor Neurons. *Journal of Neuroscience*, 21(6).
- Liu, N., Sun, S., Wang, P., Sun, Y., Hu, Q., & Wang, X. (2021). The Mechanism of Secretion and Metabolism of Gut-Derived 5-Hydroxytryptamine. *International Journal of Molecular Sciences*, 22(15), 7931. <https://doi.org/10.3390/ijms22157931>
- Liu, T., Rechtsteiner, A., Egelhofer, T. A., Vielle, A., Latorre, I., Cheung, M.-S., Ercan, S., Ikegami, K., Jensen, M., Kolasinska-Zwierz, P., Rosenbaum, H., Shin, H., Taing, S., Takasaki, T., Iniguez, A. L., Desai, A., Dernburg, A. F., Kimura, H., Lieb, J. D., ... Liu, X. S. (2011). Broad chromosomal domains of histone modification patterns in *C. elegans*. *Genome Research*, 21(2), 227–236. <https://doi.org/10.1101/gr.115519.110>
- Lloret-Fernández, C., Maicas, M., Mora-Martínez, C., Artacho, A., Jimeno-Martín, Á., Chirivella, L., Weinberg, P., & Flames, N. (2018). A transcription factor collective defines the HSN serotonergic neuron regulatory landscape. *eLife*, 7, e32785. <https://doi.org/10.7554/eLife.32785>
- Loer, C. M., & Kenyon, C. J. (1993). Serotonin-deficient mutants and male mating behavior in the nematode *Caenorhabditis elegans*. *Journal of Neuroscience*, 13(12), 5407–5417. <https://doi.org/10.1523/JNEUROSCI.13-12-05407.1993>

- Loer, C. M., & Rivard, L. (2007). Evolution of neuronal patterning in free-living rhabditid nematodes I: Sex-specific serotonin-containing neurons. *The Journal of Comparative Neurology*, 502(5), 736–767. <https://doi.org/10.1002/cne.21288>
- López-Muñoz, F., Boya, J., & Alamo, C. (2006). Neuron theory, the cornerstone of neuroscience, on the centenary of the Nobel Prize award to Santiago Ramón y Cajal. *Brain Research Bulletin*, 70(4), 391–405. <https://doi.org/10.1016/j.brainresbull.2006.07.010>
- Love, A. C., & Wagner, G. P. (2022). Co-option of stress mechanisms in the origin of evolutionary novelties. *Evolution*, 76(3), 394–413. <https://doi.org/10.1111/evo.14421>
- Maicas, M., Jimeno-Martín, Á., Millán-Trejo, A., Alkema, M. J., & Flames, N. (2021). The transcription factor LAG-1/CSL plays a Notch-independent role in controlling terminal differentiation, fate maintenance, and plasticity of serotonergic chemosensory neurons. *PLOS Biology*, 19(7), e3001334. <https://doi.org/10.1371/journal.pbio.3001334>
- Martire, S., & Banaszynski, L. A. (2020). The roles of histone variants in fine-tuning chromatin organization and function. *Nature Reviews Molecular Cell Biology*, 21(9), 522–541. <https://doi.org/10.1038/s41580-020-0262-8>
- Matys, V., Kel-Margoulis, O. V., Fricke, E., Liebich, I., Land, S., Barre-Dirrie, A., Reuter, I., Chekmenev, D., Krull, M., Hornischer, K., & others. (2006). TRANSFAC® and its module TRANSCOMP®: Transcriptional gene regulation in eukaryotes. *Nucleic Acids Research*, 34(suppl_1), D108–D110.
- Maurange, C., & Gould, A. P. (2005). Brainy but not too brainy: Starting and stopping neuroblast divisions in *Drosophila*. *Trends in Neurosciences*, 28(1), 30–36. <https://doi.org/10.1016/j.tins.2004.10.009>
- McIntire, S. L., Garriga, G., White, J., Jacobson, D., & Horvitz, H. R. (1992). Genes necessary for directed axonal elongation or fasciculation in *C. elegans*. *Neuron*, 8(2), 307–322. [https://doi.org/10.1016/0896-6273\(92\)90297-Q](https://doi.org/10.1016/0896-6273(92)90297-Q)
- Medrano, E., & Collins, K. M. (2023). Muscle-directed mechanosensory feedback activates egg-laying circuit activity and behavior in *Caenorhabditis elegans*. *Current Biology*, 33(11), 2330–2339.e8. <https://doi.org/10.1016/j.cub.2023.05.008>
- Meister, P., Towbin, B. D., Pike, B. L., Ponti, A., & Gasser, S. M. (2010). The spatial dynamics of tissue-specific promoters during *C. elegans* development. *Genes & Development*, 24(8), 766–782. <https://doi.org/10.1101/gad.559610>
- Mello, C. C., & Fire, A. (1995). DNA transformation. *Methods in Cell Biology*, 48, 451–482.
- Mello, C. C., Kramer, J. M., Stinchcomb, D., & Ambros, V. (1991). Efficient gene transfer in *C. elegans*: Extrachromosomal maintenance and integration of transforming sequences. *The EMBO Journal*, 10(12), 3959–3970. <https://doi.org/10.1002/j.1460-2075.1991.tb04966.x>
- Memar, N., Schiemann, S., Hennig, C., Findeis, D., Conradt, B., & Schnabel, R. (2019). Twenty million years of evolution: The embryogenesis of four *Caenorhabditis* species are indistinguishable despite extensive genome divergence. *Developmental Biology*, 447(2), 182–199. <https://doi.org/10.1016/j.ydbio.2018.12.022>

- Methot, S. P., Padeken, J., Brancati, G., Zeller, P., Delaney, C. E., Gaidatzis, D., Kohler, H., van Oudenaarden, A., Großhans, H., & Gasser, S. M. (2021). H3K9me selectively blocks transcription factor activity and ensures differentiated tissue integrity. *Nature Cell Biology*, 23(11), 1163–1175. <https://doi.org/10.1038/s41556-021-00776-w>
- Mignerot, L., Gimond, C., Bolelli, L., Bouleau, C., Sandjak, A., Boulin, T., & Braendle, C. (2024). Natural variation in the *Caenorhabditis elegans* egg-laying circuit modulates an intergenerational fitness trade-off. *eLife*, 12, RP88253. <https://doi.org/10.7554/eLife.88253>
- Miljkovic-Licina, M., Gauchat, D., & Galliot, B. (2004). Neuronal evolution: Analysis of regulatory genes in a first-evolved nervous system, the hydra nervous system. *Bio Systems*, 76(1–3), 75–87. <https://doi.org/10.1016/j.biosystems.2004.05.030>
- Millán-Zambrano, G., Burton, A., Bannister, A. J., & Schneider, R. (2022). Histone post-translational modifications—Cause and consequence of genome function. *Nature Reviews Genetics*, 23(9), 563–580. <https://doi.org/10.1038/s41576-022-00468-7>
- Mogno, I., Kwasnieski, J. C., & Cohen, B. A. (2013). Massively parallel synthetic promoter assays reveal the in vivo effects of binding site variants. *Genome Research*, 23(11), 1908–1915.
- Molina-García, L., Lloret-Fernández, C., Cook, S. J., Kim, B., Bonnington, R. C., Sammut, M., O’Shea, J. M., Gilbert, S. P., Elliott, D. J., Hall, D. H., Emmons, S. W., Barrios, A., & Poole, R. J. (2020). Direct glia-to-neuron transdifferentiation gives rise to a pair of male-specific neurons that ensure nimble male mating. *eLife*, 9, e48361. <https://doi.org/10.7554/eLife.48361>
- Mora-Martínez, C. (2019). *Study of the evolution of terminal differentiation programs using C. elegans serotonergic neurons as a model*.
- Muotri, A. R., & Gage, F. H. (2006). Generation of neuronal variability and complexity. *Nature*, 441(7097), 1087–1093. <https://doi.org/10.1038/nature04959>
- Murphy, D. L., & Lesch, K.-P. (2008). Targeting the murine serotonin transporter: Insights into human neurobiology. *Nature Reviews. Neuroscience*, 9(2), 85–96. <https://doi.org/10.1038/nrn2284>
- Murphy, D. L., & Moya, P. R. (2011). Human serotonin transporter gene (SLC6A4) variants: Their contributions to understanding pharmacogenomic and other functional G×G and G×E differences in health and disease. *Current Opinion in Pharmacology*, 11(1), 3–10. <https://doi.org/10.1016/j.coph.2011.02.008>
- Muschiol, D., & Traunspurger, W. (2007). Life cycle and calculation of the intrinsic rate of natural increase of two bacterivorous nematodes, *Panagrolaimus* sp. And *Poikilolaimus* sp. From chemoautotrophic Movile Cave, Romania. *Nematology*, 9(2), 271–284. <https://doi.org/10.1163/156854107780739117>
- Musser, J. M., Schippers, K. J., Nickel, M., Mizzon, G., Kohn, A. B., Pape, C., Ronchi, P., Papadopoulos, N., Tarashansky, A. J., Hammel, J. U., Wolf, F., Liang, C., Hernández-Plaza, A., Cantalapiedra, C. P., Achim, K., Schieber, N. L., Pan, L., Ruperti, F., Francis, W. R., ... Arendt, D. (2021). Profiling cellular diversity in sponges informs animal cell type and nervous system evolution. *Science (New York, N.Y.)*, 374(6568), 717–723. <https://doi.org/10.1126/science.abj2949>

- Nam, D., & Reineke, E. L. (2017). Timing and Targeting of Treatment in Left Ventricular Hypertrophy. *Methodist DeBakey Cardiovascular Journal*, 13(1), 9–14. <https://doi.org/10.14797/mdcj-13-1-9>
- Neph, S., Vierstra, J., Stergachis, A. B., Reynolds, A. P., Haugen, E., Vernot, B., Thurman, R. E., John, S., Sandstrom, R., Johnson, A. K., & others. (2012). An expansive human regulatory lexicon encoded in transcription factor footprints. *Nature*, 489(7414), 83–90.
- Ng, E. T. H., & Kinjo, A. R. (2024). Plasticity-led and mutation-led evolutions are different modes of the same developmental gene regulatory network. *PeerJ*, 12, e17102. <https://doi.org/10.7717/peerj.17102>
- Noble, T., Stieglitz, J., & Srinivasan, S. (2013). An Integrated Serotonin and Octopamine Neuronal Circuit Directs the Release of an Endocrine Signal to Control *C. elegans* Body Fat. *Cell Metabolism*, 18(5), 672–684. <https://doi.org/10.1016/j.cmet.2013.09.007>
- Olde, B., & McCombie, W. R. (1997). Molecular cloning and functional expression of a serotonin receptor from *Caenorhabditis elegans*. *Journal of Molecular Neuroscience: MN*, 8(1), 53–62. <https://doi.org/10.1007/BF02736863>
- Packer, J. S., Zhu, Q., Huynh, C., Sivaramakrishnan, P., Preston, E., Dueck, H., Stefanik, D., Tan, K., Trapnell, C., Kim, J., Waterston, R. H., & Murray, J. I. (2019). A lineage-resolved molecular atlas of *C. elegans* embryogenesis at single-cell resolution. *Science*, 365(6459), eaax1971. <https://doi.org/10.1126/science.aax1971>
- Padeken, J., Methot, S. P., & Gasser, S. M. (2022). Establishment of H3K9-methylated heterochromatin and its functions in tissue differentiation and maintenance. *Nature Reviews. Molecular Cell Biology*, 23(9), 623–640. <https://doi.org/10.1038/s41580-022-00483-w>
- Panigrahi, A., & O'Malley, B. W. (2021). Mechanisms of enhancer action: The known and the unknown. *Genome Biology*, 22(1), 108. <https://doi.org/10.1186/s13059-021-02322-1>
- Peña, C. J., Bagot, R. C., Labonté, B., & Nestler, E. J. (2014). Epigenetic Signaling in Psychiatric Disorders. *Journal of Molecular Biology*, 426(20), 3389–3412. <https://doi.org/10.1016/j.jmb.2014.03.016>
- Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A., & Bejerano, G. (2013). Enhancers: Five essential questions. *Nature Reviews. Genetics*, 14(4), 288–295. <https://doi.org/10.1038/nrg3458>
- Pereira, L., Kratsios, P., Serrano-Saiz, E., Sheftel, H., Mayo, A. E., Hall, D. H., White, J. G., LeBoeuf, B., Garcia, L. R., Alon, U., & Hobert, O. (2015). A cellular and regulatory map of the cholinergic nervous system of *C. elegans*. *eLife*, 4, e12432. <https://doi.org/10.7554/eLife.12432>
- Perez, L. M., & Alfonso, A. (2020). The conserved ASCL1/MASH-1 ortholog HLH-3 specifies sex-specific ventral cord motor neuron fate in *Caenorhabditis elegans*. *G3: Genes, Genomes, Genetics*, 10(11), 4201–4213.
- Petersen, C., Hermann, R. J., Barg, M.-C., Schalkowski, R., Dirksen, P., Barbosa, C., & Schulenburg, H. (2015). Travelling at a slug's pace: Possible invertebrate vectors of *Caenorhabditis* nematodes. *BMC Ecology*, 15(1), 19. <https://doi.org/10.1186/s12898-015-0050-z>

- Pfennig, D. W. (2021). *Phenotypic Plasticity & Evolution: Causes, Consequences, Controversies* (1st ed.). CRC Press. <https://doi.org/10.1201/9780429343001>
- Pocock, R., & Hobert, O. (2010). Hypoxia activates a latent circuit for processing gustatory information in *C. elegans*. *Nature Neuroscience*, 13(5), 610–614. <https://doi.org/10.1038/nn.2537>
- Poole, R. J., Flames, N., & Cochella, L. (2024). Neurogenesis in *Caenorhabditis elegans*. *Genetics*, 228(2), iyae116. <https://doi.org/10.1093/genetics/iyae116>
- Pourhamzeh, M., Moravej, F. G., Arabi, M., Shahriari, E., Mehrabi, S., Ward, R., Ahadi, R., & Joghataei, M. T. (2022). The Roles of Serotonin in Neuropsychiatric Disorders. *Cellular and Molecular Neurobiology*, 42(6), 1671–1692. <https://doi.org/10.1007/s10571-021-01064-9>
- Ptashne, M. (1986). Gene regulation by proteins acting nearby and at a distance. *Nature*, 322(6081), 697–701. <https://doi.org/10.1038/322697a0>
- Ptashne, M., & Gann, A. (1997). Transcriptional activation by recruitment. *Nature*, 386(6625), 569–577. <https://doi.org/10.1038/386569a0>
- R. Core Team. (2013). R: A language and environment for statistical computing. *Foundation for Statistical Computing, Vienna, Austria*.
- Rada-Iglesias, A., Bajpai, R., Prescott, S., Brugmann, S. A., Swigut, T., & Wysocka, J. (2012). Epigenomic annotation of enhancers predicts transcriptional regulators of human neural crest. *Cell Stem Cell*, 11(5), 633–648. <https://doi.org/10.1016/j.stem.2012.07.006>
- Rajagopal, N., Srinivasan, S., Kooshesh, K., Guo, Y., Edwards, M. D., Banerjee, B., Syed, T., Emons, B. J. M., Gifford, D. K., & Sherwood, R. I. (2016). High-throughput mapping of regulatory DNA. *Nature Biotechnology*, 34(2), 167–174. <https://doi.org/10.1038/nbt.3468>
- Ranganathan, R., Sawin, E. R., Trent, C., & Horvitz, H. R. (2001). Mutations in the *Caenorhabditis elegans* Serotonin Reuptake Transporter MOD-5 Reveal Serotonin-Dependent and -Independent Activities of Fluoxetine. *The Journal of Neuroscience*, 21(16), 5871–5884. <https://doi.org/10.1523/JNEUROSCI.21-16-05871.2001>
- Rao, M., & Gershon, M. D. (2018). Enteric nervous system development: What could possibly go wrong? *Nature Reviews Neuroscience*, 19(9), 552–565. <https://doi.org/10.1038/s41583-018-0041-0>
- Rauluseviciute, I., Riudavets-Puig, R., Blanc-Mathieu, R., Castro-Mondragon, J. A., Ferenc, K., Kumar, V., Lemma, R. B., Lucas, J., Chèneby, J., Baranasic, D., & others. (2024). JASPAR 2024: 20th anniversary of the open-access database of transcription factor binding profiles. *Nucleic Acids Research*, 52(D1), D174–D182.
- Rebeiz, M., Jikomes, N., Kassner, V. A., & Carroll, S. B. (2011). Evolutionary origin of a novel gene expression pattern through co-option of the latent activities of existing regulatory sequences. *Proceedings of the National Academy of Sciences*, 108(25), 10036–10043.
- Reilly, M. B., Tekieli, T., Cros, C., Aguilar, G. R., Lao, J., Toker, I. A., Vidal, B., Leyva-Díaz, E., Bhattacharya, A., Cook, S. J., Smith, J. J., Kovacevic, I., Gulez, B., Fernandez, R. W., Bradford, E. F., Ramadan, Y. H., Kratsios, P., Bao, Z., & Hobert, O. (2022). Widespread employment of conserved *C. elegans* homeobox genes in neuronal

- identity specification. *PLoS Genetics*, 18(9), e1010372. <https://doi.org/10.1371/journal.pgen.1010372>
- Reiter, F., Wienerroither, S., & Stark, A. (2017). Combinatorial function of transcription factors and cofactors. *Current Opinion in Genetics & Development*, 43, 73–81. <https://doi.org/10.1016/j.gde.2016.12.007>
- Rhee, H. S., & Pugh, B. F. (2011). Comprehensive genome-wide protein-DNA interactions detected at single-nucleotide resolution. *Cell*, 147(6), 1408–1419.
- Rhoades, J. L., Nelson, J. C., Nwabudike, I., Yu, S. K., McLachlan, I. G., Madan, G. K., Abebe, E., Powers, J. R., Colón-Ramos, D. A., & Flavell, S. W. (2019). ASICs Mediate Food Responses in an Enteric Serotonergic Neuron that Controls Foraging Behaviors. *Cell*, 176(1), 85–97.e14. <https://doi.org/10.1016/j.cell.2018.11.023>
- Ripoll-Sánchez, L., Watteyne, J., Sun, H., Fernandez, R., Taylor, S. R., Weinreb, A., Bentley, B. L., Hammarlund, M., Miller, D. M., Hobert, O., Beets, I., Vértés, P. E., & Schafer, W. R. (2023). The neuropeptidergic connectome of *C. elegans*. *Neuron*, 111(22), 3570–3589.e5. <https://doi.org/10.1016/j.neuron.2023.09.043>
- Rivard, L., Srinivasan, J., Stone, A., Ochoa, S., Sternberg, P. W., & Loer, C. M. (2010). A comparison of experience-dependent locomotory behaviors and biogenic amine neurons in nematode relatives of *Caenorhabditis elegans*. *BMC Neuroscience*, 11(1), 22. <https://doi.org/10.1186/1471-2202-11-22>
- Riveiro, A. R., & Brickman, J. M. (2020). From pluripotency to totipotency: An experimentalist's guide to cellular potency. *Development*, 147(16), dev189845. <https://doi.org/10.1242/dev.189845>
- Roeder, R. G. (1996). The role of general initiation factors in transcription by RNA polymerase II. *Trends in Biochemical Sciences*, 21(9), 327–335.
- Roux, A. E., Yuan, H., Podshivalova, K., Hendrickson, D., Kerr, R., Kenyon, C., & Kelley, D. (2023). Individual cell types in *C. elegans* age differently and activate distinct cell-protective responses. *Cell Reports*, 42(8), 112902. <https://doi.org/10.1016/j.celrep.2023.112902>
- Sammut, M., Cook, S. J., Nguyen, K. C. Q., Felton, T., Hall, D. H., Emmons, S. W., Poole, R. J., & Barrios, A. (2015). Glia-derived neurons are required for sex-specific learning in *C. elegans*. *Nature*, 526(7573), 385–390. <https://doi.org/10.1038/nature15700>
- Sanyal, A., Lajoie, B. R., Jain, G., & Dekker, J. (2012). The long-range interaction landscape of gene promoters. *Nature*, 489(7414), 109–113. <https://doi.org/10.1038/nature11279>
- Sarkar, S. (2004). From the Reaktionsnorm to the Evolution of Adaptive Plasticity: A Historical Sketch 1909–1999. In T. J. DeWitt & S. M. Scheiner (Eds.), *Phenotypic Plasticity: Functional and Conceptual Approaches* (p. 0). Oxford University Press. <https://doi.org/10.1093/oso/9780195138962.003.0002>
- Sawin, E. R., Ranganathan, R., & Horvitz, H. R. (2000). *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron*, 26(3), 619–631. [https://doi.org/10.1016/s0896-6273\(00\)81199-x](https://doi.org/10.1016/s0896-6273(00)81199-x)
- Schafer, W. (2005). Egg-laying. *WormBook*. <https://doi.org/10.1895/wormbook.1.38.1>

- Schedl, T., & Kimble, J. (1988). Fog-2, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. *Genetics*, 119(1), 43–61.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, 9(7), 676–682. <https://doi.org/10.1038/nmeth.2019>
- Schneider, T. D., & Stephens, R. M. (1990). Sequence logos: A new way to display consensus sequences. *Nucleic Acids Research*, 18(20), 6097–6100. <https://doi.org/10.1093/nar/18.20.6097>
- Schulenburg, H., & Félix, M.-A. (2017). The Natural Biotic Environment of *Caenorhabditis elegans*. *Genetics*, 206(1), 55–86. <https://doi.org/10.1534/genetics.116.195511>
- Segal, E., Raveh-Sadka, T., Schroeder, M., Unnerstall, U., & Gaul, U. (2008). Predicting expression patterns from regulatory sequence in *Drosophila* segmentation. *Nature*, 451(7178), 535–540.
- Serrano-Saiz, E., Pereira, L., Gendrel, M., Aghayeva, U., Bhattacharya, A., Howell, K., Garcia, L. R., & Hobert, O. (2017). A Neurotransmitter Atlas of the *Caenorhabditis elegans* Male Nervous System Reveals Sexually Dimorphic Neurotransmitter Usage. *Genetics*, 206(3), 1251–1269. <https://doi.org/10.1534/genetics.117.202127>
- Serrano-Saiz, E., Poole, R. J., Felton, T., Zhang, F., De La Cruz, E. D., & Hobert, O. (2013). Modular control of glutamatergic neuronal identity in *C. elegans* by distinct homeodomain proteins. *Cell*, 155(3), 659–673. <https://doi.org/10.1016/j.cell.2013.09.052>
- Shen, B., Zhang, W., Zhang, J., Zhou, J., Wang, J., Chen, L., Wang, L., Hodgkins, A., Iyer, V., Huang, X., & Skarnes, W. C. (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nature Methods*, 11(4), 399–402. <https://doi.org/10.1038/nmeth.2857>
- Shlyueva, D., Stampfel, G., & Stark, A. (2014). Transcriptional enhancers: From properties to genome-wide predictions. *Nature Reviews Genetics*, 15(4), 272–286. <https://doi.org/10.1038/nrg3682>
- Shyn, S. I., Kerr, R., & Schafer, W. R. (2003). Serotonin and Go Modulate Functional States of Neurons and Muscles Controlling *C. elegans* Egg-Laying Behavior. *Current Biology*, 13(21), 1910–1915. <https://doi.org/10.1016/j.cub.2003.10.025>
- Siersbæk, R., Rabiee, A., Nielsen, R., Sidoli, S., Traynor, S., Loft, A., Poulsen, L. L. C., Rogowska-Wrzesinska, A., Jensen, O. N., & Mandrup, S. (2014). Transcription Factor Cooperativity in Early Adipogenic Hotspots and Super-Enhancers. *Cell Reports*, 7(5), 1443–1455. <https://doi.org/10.1016/j.celrep.2014.04.042>
- Siletti, K., Hodge, R., Mossi Albiach, A., Lee, K. W., Ding, S.-L., Hu, L., Lönnerberg, P., Bakken, T., Casper, T., Clark, M., Dee, N., Gloe, J., Hirschstein, D., Shapovalova, N. V., Keene, C. D., Nyhus, J., Tung, H., Yanny, A. M., Arenas, E., ... Linnarsson, S. (2023). Transcriptomic diversity of cell types across the adult human brain. *Science*, 382(6667), eadd7046. <https://doi.org/10.1126/science.add7046>

- Simon, J. M., Giresi, P. G., Davis, I. J., & Lieb, J. D. (2012). Using formaldehyde-assisted isolation of regulatory elements (FAIRE) to isolate active regulatory DNA. *Nature Protocols*, 7(2), 256–267.
- Singh, V. K., Saini, A., Kalsan, M., Kumar, N., & Chandra, R. (2016). Describing the Stem Cell Potency: The Various Methods of Functional Assessment and In silico Diagnostics. *Frontiers in Cell and Developmental Biology*, 4. <https://doi.org/10.3389/fcell.2016.00134>
- Skene, P. J., & Henikoff, S. (2017). An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. *Elife*, 6, e21856.
- Slack, J. (2021). *Stem cells: A very short introduction* (2nd ed.). Oxford University Press.
- Slatko, B. E., Taylor, M. J., & Foster, J. M. (2010). The Wolbachia endosymbiont as an anti-filarial nematode target. *Symbiosis*, 51(1), 55–65. <https://doi.org/10.1007/s13199-010-0067-1>
- Slattery, M., Riley, T., Liu, P., Abe, N., Gomez-Alcala, P., Dror, I., Zhou, T., Rohs, R., Honig, B., Bussemaker, H. J., & others. (2011). Cofactor binding evokes latent differences in DNA binding specificity between Hox proteins. *Cell*, 147(6), 1270–1282.
- Sloat, S. A., Noble, L. M., Paaby, A. B., Bernstein, M., Chang, A., Kaur, T., Yuen, J., Tintori, S. C., Jackson, J. L., Martel, A., Salome Correa, J. A., Stevens, L., Kiontke, K., Blaxter, M., & Rockman, M. V. (2022). Caenorhabditis nematodes colonize ephemeral resource patches in neotropical forests. *Ecology and Evolution*, 12(7), e9124. <https://doi.org/10.1002/ece3.9124>
- Smith, J. J., & Kratsios, P. (2024). Hox gene functions in the *C. elegans* nervous system: From early patterning to maintenance of neuronal identity. *Seminars in Cell & Developmental Biology*, 152–153, 58–69. <https://doi.org/10.1016/j.semcdb.2022.11.012>
- Smith, R. P., Taher, L., Patwardhan, R. P., Kim, M. J., Inoue, F., Shendure, J., Ovcharenko, I., & Ahituv, N. (2013). Massively parallel decoding of mammalian regulatory sequences supports a flexible organizational model. *Nature Genetics*, 45(9), 1021–1028. <https://doi.org/10.1038/ng.2713>
- Smith, Z. D., & Meissner, A. (2013). DNA methylation: Roles in mammalian development. *Nature Reviews Genetics*, 14(3), 204–220.
- Sommer, R. J. (2006). *Pristionchus pacificus*. In *WormBook: The Online Review of C. elegans Biology* [Internet]. WormBook. <https://www.ncbi.nlm.nih.gov/books/NBK19735/>
- Spitz, F., & Furlong, E. E. (2012). Transcription factors: From enhancer binding to developmental control. *Nature Reviews Genetics*, 13(9), 613–626.
- Stefflova, K., Thybert, D., Wilson, M. D., Streeter, I., Aleksic, J., Karagianni, P., Brazma, A., Adams, D. J., Talianidis, I., Marioni, J. C., Flicek, P., & Odom, D. T. (2013). Cooperativity and Rapid Evolution of Cobound Transcription Factors in Closely Related Mammals. *Cell*, 154(3), 530–540. <https://doi.org/10.1016/j.cell.2013.07.007>
- Sterken, M. G., Snoek, L. B., Kammenga, J. E., & Andersen, E. C. (2015). The laboratory domestication of *Caenorhabditis elegans*. *Trends in Genetics: TIG*, 31(5), 224–231. <https://doi.org/10.1016/j.tig.2015.02.009>

- Stevens, L., Félix, M.-A., Beltran, T., Braendle, C., Caurcel, C., Fausett, S., Fitch, D., Frézal, L., Gosse, C., Kaur, T., Kiontke, K., Newton, M. D., Noble, L. M., Richaud, A., Rockman, M. V., Sudhaus, W., & Blaxter, M. (2019). Comparative genomics of 10 new *Caenorhabditis* species. *Evolution Letters*, 3(2), 217–236. <https://doi.org/10.1002/evl3.110>
- Stevens, L., Rooke, S., Falzon, L. C., Machuka, E. M., Momanyi, K., Murungi, M. K., Njoroge, S. M., Odinga, C. O., Ogendo, A., Ogola, J., Fèvre, E. M., & Blaxter, M. (2020). The Genome of *Caenorhabditis bovis*. *Current Biology*, 30(6), 1023–1031.e4. <https://doi.org/10.1016/j.cub.2020.01.074>
- Stormo, G. D., & Zhao, Y. (2010). Determining the specificity of protein-DNA interactions. *Nature Reviews. Genetics*, 11(11), 751–760. <https://doi.org/10.1038/nrg2845>
- Sulston, J. E., Albertson, D. G., & Thomson, J. N. (1980). The *Caenorhabditis elegans* male: Postembryonic development of nongonadal structures. *Developmental Biology*, 78(2), 542–576. [https://doi.org/10.1016/0012-1606\(80\)90352-8](https://doi.org/10.1016/0012-1606(80)90352-8)
- Sulston, J. E., & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Developmental Biology*, 56(1), 110–156. [https://doi.org/10.1016/0012-1606\(77\)90158-0](https://doi.org/10.1016/0012-1606(77)90158-0)
- Sulston, J. E., Schierenberg, E., White, J. G., & Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Developmental Biology*, 100(1), 64–119. [https://doi.org/10.1016/0012-1606\(83\)90201-4](https://doi.org/10.1016/0012-1606(83)90201-4)
- Susoy, V., & Samuel, A. D. (2023). Evolutionarily-conserved behavioral plasticity enables context-dependent performance of mating behavior in *C. elegans*. *bioRxiv*, 2023–04.
- Swoboda, P., Adler, H. T., & Thomas, J. H. (2000). The RFX-Type Transcription Factor DAF-19 Regulates Sensory Neuron Cilium Formation in *C. elegans*. *Molecular Cell*, 5(3), 411–421. [https://doi.org/10.1016/S1097-2765\(00\)80436-0](https://doi.org/10.1016/S1097-2765(00)80436-0)
- Sze, J. Y., Victor, M., Loer, C., Shi, Y., & Ruvkun, G. (2000). Food and metabolic signalling defects in a *Caenorhabditis elegans* serotonin-synthesis mutant. *Nature*, 403(6769), 560–564. <https://doi.org/10.1038/35000609>
- Sze, J. Y., Zhang, S., Li, J., & Ruvkun, G. (2002). The *C. elegans* POU-domain transcription factor UNC-86 regulates the *tph-1* tryptophan hydroxylase gene and neurite outgrowth in specific serotonergic neurons. *Development*, 129(16), 3901–3911. <https://doi.org/10.1242/dev.129.16.3901>
- Tamagawa, K., Dayi, M., Sun, S., Hata, R., Kikuchi, T., Haruta, N., Sugimoto, A., & Makino, T. (2024). Evolutionary changes of noncoding elements associated with transition of sexual mode in *Caenorhabditis* nematodes. *Science Advances*, 10(37), eadn9913. <https://doi.org/10.1126/sciadv.adn9913>
- Tang, Y., Nyengaard, J. R., De Groot, D. M., & Gundersen, H. J. (2001). Total regional and global number of synapses in the human brain neocortex. *Synapse (New York, N.Y.)*, 41(3), 258–273. <https://doi.org/10.1002/syn.1083>
- Taskiran, I. I., Spanier, K. I., Dickmanken, H., Kempynck, N., Pančíková, A., Ekşi, E. C., Hulselmans, G., Ismail, J. N., Theunis, K., Vandepoel, R., Christiaens, V., Mauduit, D., & Aerts, S. (2024). Cell-type-directed design of synthetic enhancers. *Nature*, 626(7997), 212–220. <https://doi.org/10.1038/s41586-023-06936-2>

- Taylor, S. R., Santpere, G., Weinreb, A., Barrett, A., Reilly, M. B., Xu, C., Varol, E., Oikonomou, P., Glenwinkel, L., McWhirter, R., Poff, A., Basavaraju, M., Rafi, I., Yemini, E., Cook, S. J., Abrams, A., Vidal, B., Cros, C., Tavazoie, S., ... Miller, D. M. (2021). Molecular topography of an entire nervous system. *Cell*, *184*(16), 4329–4347.e23. <https://doi.org/10.1016/j.cell.2021.06.023>
- Thomas, C. G., Woodruff, G. C., & Haag, E. S. (2012). Causes and consequences of the evolution of reproductive mode in *Caenorhabditis* nematodes. *Trends in Genetics*, *28*(5), 213–220. <https://doi.org/10.1016/j.tig.2012.02.007>
- Toker, I. A., Lev, I., Mor, Y., Gurevich, Y., Fisher, D., Houriz-Zeevi, L., Antonova, O., Doron, H., Anava, S., Gingold, H., & others. (2022). Transgenerational inheritance of sexual attractiveness via small RNAs enhances evolvability in *C. elegans*. *Developmental Cell*, *57*(3), 298–309.
- Towbin, B. D., González-Aguilera, C., Sack, R., Gaidatzis, D., Kalck, V., Meister, P., Askjaer, P., & Gasser, S. M. (2012). Step-Wise Methylation of Histone H3K9 Positions Heterochromatin at the Nuclear Periphery. *Cell*, *150*(5), 934–947. <https://doi.org/10.1016/j.cell.2012.06.051>
- Valenstein, E. S. (2002). The Discovery of Chemical Neurotransmitters. *Brain and Cognition*, *49*(1), 73–95. <https://doi.org/10.1006/brcg.2001.1487>
- van der Burg, K. R. L., Lewis, J. J., Brack, B. J., Fandino, R. A., Mazo-Vargas, A., & Reed, R. D. (2020). Genomic architecture of a genetically assimilated seasonal color pattern. *Science*, *370*(6517), 721–725. <https://doi.org/10.1126/science.aaz3017>
- Vandel, J., Cassan, O., Lèbre, S., Lecellier, C.-H., & Bréhélin, L. (2019). Probing transcription factor combinatorics in different promoter classes and in enhancers. *BMC Genomics*, *20*(1), 103. <https://doi.org/10.1186/s12864-018-5408-0>
- Venters, B. J., Wachi, S., Mavrich, T. N., Andersen, B. E., Jena, P., Sinnamon, A. J., Jain, P., Roller, N. S., Jiang, C., Hemeryck-Walsh, C., & others. (2011). A comprehensive genomic binding map of gene and chromatin regulatory proteins in *Saccharomyces*. *Molecular Cell*, *41*(4), 480–492.
- Verdin, E., & Ott, M. (2015). 50 years of protein acetylation: From gene regulation to epigenetics, metabolism and beyond. *Nature Reviews Molecular Cell Biology*, *16*(4), 258–264. <https://doi.org/10.1038/nrm3931>
- Vidal, B., Gulez, B., Cao, W. X., Leyva-Díaz, E., Reilly, M. B., Tekieli, T., & Hobert, O. (2022). The enteric nervous system of the *C. elegans* pharynx is specified by the *Sine oculis*-like homeobox gene *ceh-34*. *eLife*, *11*, e76003. <https://doi.org/10.7554/eLife.76003>
- Vigne, P., Gimond, C., Ferrari, C., Vielle, A., Hallin, J., Pino-Querido, A., Mouridi, S. E., Mignerot, L., Frøkj, C., Boulín, T., Teotónio, H., & Braendle, C. (2021). A single-nucleotide change underlies the genetic assimilation of a plastic trait. *SCIENCE ADVANCES*.
- Waddington, C. H. (1953). Genetic Assimilation of an Acquired Character. *Evolution*, *7*(2), 118–126. <https://doi.org/10.2307/2405747>
- Waddington, C. H. (1957). *The Strategy of the Genes*. Routledge. <https://doi.org/10.4324/9781315765471>

- Waggoner, L. E., Zhou, G. T., Schafer, R. W., & Schafer, W. R. (1998). Control of Alternative Behavioral States by Serotonin in *Caenorhabditis elegans*. *Neuron*, 21(1), 203–214. [https://doi.org/10.1016/S0896-6273\(00\)80527-9](https://doi.org/10.1016/S0896-6273(00)80527-9)
- Walker, D. M., Cates, H. M., Heller, E. A., & Nestler, E. J. (2015). Regulation of chromatin states by drugs of abuse. *Current Opinion in Neurobiology*, 30, 112–121. <https://doi.org/10.1016/j.conb.2014.11.002>
- Wang, B. B., Müller-Immergluck, M. M., Austin, J., Robinson, N. T., Chisholm, A., & Kenyon, C. (1993). A homeotic gene cluster patterns the anteroposterior body axis of *C. elegans*. *Cell*, 74(1), 29–42. [https://doi.org/10.1016/0092-8674\(93\)90292-X](https://doi.org/10.1016/0092-8674(93)90292-X)
- Wang, C., Vidal, B., Sural, S., Loer, C., Aguilar, G. R., Merritt, D. M., Toker, I. A., Vogt, M. C., Cros, C. C., & Hobert, O. (2024). A neurotransmitter atlas of *C. elegans* males and hermaphrodites. *eLife*, 13, RP95402. <https://doi.org/10.7554/eLife.95402>
- Wang, J., Zhuang, J., Iyer, S., Lin, X., Whitfield, T. W., Greven, M. C., Pierce, B. G., Dong, X., Kundaje, A., Cheng, Y., Rando, O. J., Birney, E., Myers, R. M., Noble, W. S., Snyder, M., & Weng, Z. (2012). Sequence features and chromatin structure around the genomic regions bound by 119 human transcription factors. *Genome Research*, 22(9), 1798–1812. <https://doi.org/10.1101/gr.139105.112>
- Wang, Y., Liu, H., & Sun, Z. (2017). Lamarck rises from his grave: Parental environment-induced epigenetic inheritance in model organisms and humans. *Biological Reviews*, 92(4), 2084–2111. <https://doi.org/10.1111/brv.12322>
- Weirauch, M. T., Yang, A., Albu, M., Cote, A. G., Montenegro-Montero, A., Drewe, P., Najafabadi, H. S., Lambert, S. A., Mann, I., Cook, K., & others. (2014). Determination and inference of eukaryotic transcription factor sequence specificity. *Cell*, 158(6), 1431–1443.
- White, J. G., Southgate, E., Thomson, J. N., & Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 314(1165), 1–340. <https://doi.org/10.1098/rstb.1986.0056>
- White, M. A., Myers, C. A., Corbo, J. C., & Cohen, B. A. (2013). Massively parallel in vivo enhancer assay reveals that highly local features determine the cis-regulatory function of ChIP-seq peaks. *Proceedings of the National Academy of Sciences of the United States of America*, 110(29), 11952–11957. <https://doi.org/10.1073/pnas.1307449110>
- Whitfield, T. W., Wang, J., Collins, P. J., Partridge, E. C., Aldred, S. F., Trinklein, N. D., Myers, R. M., & Weng, Z. (2012). Functional analysis of transcription factor binding sites in human promoters. *Genome Biology*, 13, 1–16.
- Wiles, E. T., & Selker, E. U. (2017). H3K27 methylation: A promiscuous repressive chromatin mark. *Current Opinion in Genetics & Development*, 43, 31–37. <https://doi.org/10.1016/j.gde.2016.11.001>
- Wingender, E., Schoeps, T., & Dönitz, J. (2013). TFClass: An expandable hierarchical classification of human transcription factors. *Nucleic Acids Research*, 41(Database issue), D165–170. <https://doi.org/10.1093/nar/gks1123>
- Winnier, A. R., Meir, J. Y.-J., Ross, J. M., Tavernarakis, N., Driscoll, M., Ishihara, T., Katsura, I., & Miller, D. M. (1999). UNC-4/UNC-37-dependent repression of motor neuron-specific genes controls synaptic choice in *Caenorhabditis elegans*.

- Genes & Development*, 13(21), 2774–2786.
<https://doi.org/10.1101/gad.13.21.2774>
- Woodruff, G. C., & Phillips, P. C. (2018). Field studies reveal a close relative of *C. elegans* thrives in the fresh figs of *Ficus septica* and disperses on its *Ceratosolen* pollinating wasps. *BMC Ecology*, 18(1), 26. <https://doi.org/10.1186/s12898-018-0182-z>
- Xie, Y., Moussaif, M., Choi, S., Xu, L., & Sze, J. Y. (2013). RFX Transcription Factor DAF-19 Regulates 5-HT and Innate Immune Responses to Pathogenic Bacteria in *Caenorhabditis elegans*. *PLOS Genetics*, 9(3), e1003324. <https://doi.org/10.1371/journal.pgen.1003324>
- Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L., & Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the *Drosophila* mesoderm. *Genes & Development*, 12(15), 2354–2370. <https://doi.org/10.1101/gad.12.15.2354>
- Yan, J., Enge, M., Whittington, T., Dave, K., Liu, J., Sur, I., Schmierer, B., Jolma, A., Kivioja, T., Taipale, M., & Taipale, J. (2013). Transcription Factor Binding in Human Cells Occurs in Dense Clusters Formed around Cohesin Anchor Sites. *Cell*, 154(4), 801–813. <https://doi.org/10.1016/j.cell.2013.07.034>
- Yang, J.-H., Li, J.-H., Jiang, S., Zhou, H., & Qu, L.-H. (2013). ChIPBase: A database for decoding the transcriptional regulation of long non-coding RNA and microRNA genes from ChIP-Seq data. *Nucleic Acids Research*, 41(D1), D177–D187.
- Yu, J., Vogt, M. C., Fox, B. W., Wrobel, C. J. J., Fajardo Palomino, D., Curtis, B. J., Zhang, B., Le, H. H., Tauffenberger, A., Hobert, O., & Schroeder, F. C. (2023). Parallel pathways for serotonin biosynthesis and metabolism in *C. elegans*. *Nature Chemical Biology*, 19(2), 141–150. <https://doi.org/10.1038/s41589-022-01148-7>
- Zarkower, D. (2006). Somatic sex determination. *WormBook: The Online Review of C. Elegans Biology [Internet]*.
- Zauner, H., & Sommer, R. J. (2007). Evolution of robustness in the signaling network of *Pristionchus* vulva development. *Proceedings of the National Academy of Sciences*, 104(24), 10086–10091. <https://doi.org/10.1073/pnas.0610799104>
- Zeng, H., & Sanes, J. R. (2017). Neuronal cell-type classification: Challenges, opportunities and the path forward. *Nature Reviews Neuroscience*, 18(9), 530–546. <https://doi.org/10.1038/nrn.2017.85>
- Zhang, F., Bhattacharya, A., Nelson, J. C., Abe, N., Gordon, P., Lloret-Fernandez, C., Maicas, M., Flames, N., Mann, R. S., Colón-Ramos, D. A., & Hobert, O. (2014). The LIM and POU homeobox genes *ttx-3* and *unc-86* act as terminal selectors in distinct cholinergic and serotonergic neuron types. *Development (Cambridge, England)*, 141(2), 422–435. <https://doi.org/10.1242/dev.099721>
- Zhang, M., Chung, S. H., Fang-Yen, C., Craig, C., Kerr, R. A., Suzuki, H., Samuel, A. D. T., Mazur, E., & Schafer, W. R. (2008). A Self-Regulating Feed-Forward Circuit Controlling *C. elegans* Egg-Laying Behavior. *Current Biology*, 18(19), 1445–1455. <https://doi.org/10.1016/j.cub.2008.08.047>
- Zhao, C., & Emmons, S. W. (1995). A transcription factor controlling development of peripheral sense organs in *C. elegans*. *Nature*, 373(6509), 74–78. <https://doi.org/10.1038/373074a0>

- Zhao, Z., Boyle, T. J., Bao, Z., Murray, J. I., Mericle, B., & Waterston, R. H. (2008). Comparative analysis of embryonic cell lineage between *Caenorhabditis briggsae* and *Caenorhabditis elegans*. *Developmental Biology*, 314(1), 93–99. <https://doi.org/10.1016/j.ydbio.2007.11.015>
- Zheng, C., Karimzadegan, S., Chiang, V., & Chalfie, M. (2013). Histone methylation restrains the expression of subtype-specific genes during terminal neuronal differentiation in *Caenorhabditis elegans*. *PLoS Genetics*, 9(12), e1004017. <https://doi.org/10.1371/journal.pgen.1004017>

RESUMEN

Introducción

El sistema nervioso es uno de los tejidos más complejos de un organismo, entender cómo se genera a través del desarrollo y cómo ha evolucionado es un gran reto. En esta tesis pretendemos arrojar luz sobre estas cuestiones explorando cómo los cambios en las redes reguladoras contribuyen a la aparición de nuevas identidades neuronales y sus implicaciones evolutivas.

Durante el desarrollo embrionario, las células restringen su potencial para convertirse en diferentes tipos celulares, un concepto conocido como potencia, a medida que se comprometen con un linaje celular específico (Singh et al., 2016). En el caso de la especificación del destino de las células neuronales, este proceso está guiado por una serie de decisiones estrictamente reguladas. En primer lugar, las células deben comprometerse con un destino neuronal, un paso en el que influyen señales externas, como los morfógenos. Estas señales activan las redes reguladoras de genes (GRN, gene regulatory network), que a su vez inducen la expresión de genes proneurales. Esta activación conduce a la formación de neuroblastos que, en última instancia, se especializan en linajes neuronales específicos. A medida que las neuronas maduran, adquieren sus características estructurales, como axones, dendritas y sinapsis. Por último, durante la diferenciación terminal, las neuronas expresan genes efectores que definen su identidad molecular, dotándolas de propiedades únicas y funciones especializadas (Davidson, 2006; Hobert, 2005; Wenick y Hobert, 2004).

Las GRNs intervienen no sólo en el compromiso de una célula con un determinado linaje, sino también en su diferenciación terminal, y todos los pasos están regulados por la regulación espacial y temporal de diferentes genes. Esta regulación está integrada por módulos cis-reguladores (CRMs, *cis-*

regulatory modules) que consisten en grupos de motivos de secuencias cortas, reconocidos por diversas proteínas de unión al ADN, como los factores de transcripción (TFs, transcription factors) que conforman los elementos *trans*-reguladores (Davidson, 2006). Aunque la visión simplificada de la regulación transcripcional implica la unión de un TF a una secuencia específica de unión a factores de transcripción (TFBD, transcription factor binding site), la realidad es más compleja. Los TF reconocen motivos cortos de 4-8 pares de bases que aparecen con frecuencia en todo el genoma. Por lo tanto, la actividad combinatoria de múltiples TF es crucial para lograr la especificidad en la regulación génica (Reiter et al., 2017). Además, la unión de los TF a sus motivos está condicionada por diferentes características como la posición del nucleosoma, la accesibilidad de la cromatina, las marcas de histonas o incluso la presencia de otros TF.

Se ha descubierto que la accesibilidad de la cromatina desempeña un papel importante en la modulación de la expresión génica a lo largo del desarrollo, la diferenciación y en respuesta a estímulos ambientales (Berger, 2007). Los diferentes estados de «abierto» a «cerrado» se rigen por múltiples factores, entre ellos los complejos de remodelación de la cromatina, la metilación del ADN, las variantes de las histonas o las modificaciones postraduccionales (PTM, post-transcriptional modification) de las histonas. Las PTMs son el mecanismo más estudiado en el que las modificaciones covalentes de las histonas, incluyendo la metilación, acetilación, fosforilación o la recientemente identificada serotonilación, afectan a la estructura de la cromatina, haciéndola más o menos accesible a la maquinaria de transcripción (Millán-Zambrano et al., 2022). Es el caso de la metilación en el noveno residuo de lisina de la histona 3 (H3K9me), que se encuentra en la heterocromatina y se relaciona con una acción restrictiva sobre la expresión génica (Bonn et al., 2012), y recientemente también se ha descrito que tiene un papel importante en el silenciamiento de la

expresión de genes efectores de linajes celulares no deseados (Padeken et al., 2022).

Esta interacción entre la regulación génica y la accesibilidad de la cromatina proporciona un marco para comprender los mecanismos epigenéticos, es decir, los procesos de regulación que influyen en la expresión génica sin alterar la secuencia de ADN subyacente. Los mecanismos epigenéticos añaden una capa de plasticidad que permite la diversidad fenotípica y facilita las respuestas adaptativas a los cambios ambientales.

En el contexto de la evolución, la plasticidad fenotípica permite a los organismos responder dinámicamente a entornos cambiantes, creando oportunidades para que surjan nuevos rasgos. Existen dos marcos evolutivos clave: la evolución guiada por mutaciones (EML), en la que las mutaciones genéticas hereditarias están sujetas a la selección natural; y la evolución guiada por la plasticidad (EPL), en la que la propia plasticidad fenotípica impulsa el cambio evolutivo (Pfennig, 2021). Un ejemplo histórico de PLE es el concepto de «asimilación genética» acuñado por Waddington (1953), según el cual los fenotipos inducidos por el medio ambiente se codifican genéticamente con el tiempo (Waddington, 1953). Esta perspectiva evolutiva pone en relieve la compleja relación entre el desarrollo, la regulación genética y el potencial adaptativo de los organismos.

Centrándonos en la evolución de la diversidad celular, una característica importante para su estudio es la clasificación de los tipos celulares basada en sus perfiles moleculares, que es crucial para identificar tipos celulares homólogos entre especies y desentrañar las relaciones evolutivas entre ellos. El estudio de los factores de transcripción conservados y las firmas moleculares es especialmente útil para rastrear el linaje y la divergencia funcional de los tipos celulares (Arendt, 2008; Arendt et al., 2016). Varios mecanismos impulsan

la aparición de nuevos tipos celulares. 1) la segregación funcional que se produce cuando un tipo de célula hermana conserva una función perdida por la otra, 2) la divergencia de funciones que se produce cuando las células hermanas evolucionan hacia papeles distintos, a menudo facilitada por la duplicación de genes y la subsiguiente subfuncionalización. 3) la adquisición de funciones nuevas que pueden dar lugar a tipos celulares completamente nuevos, normalmente mediante cambios en la regulación génica o la cooptación de redes génicas existentes y, por último, 4) la fijación de un estado transitorio que es similar a la asimilación genética, en la que un fenotipo temporal e inducible se convierte en un rasgo permanente y constitutivo, contribuyendo a la diversidad celular.

Dada la compleja interacción entre plasticidad y evolución, así como la aparición de nuevos tipos celulares, en nuestro proyecto aprovecharemos la simplicidad del organismo *C. elegans* y la profunda caracterización del linaje de todas las células de su organismo; linaje que comparte con el resto de las especies de *Caenorhabditis*; para contribuir a desvelar los mecanismos fundamentales que impulsan la innovación evolutiva.

Nos vamos a centrar en el fenotipo serotoninérgico diferencial en las motoneuronas VC4 y VC5 entre *C. angaria*, en el que estas neuronas son serotoninérgicas y colinérgicas, y *C. elegans*, donde en principio son colinérgicas, pero muestran un fenotipo serotoninérgico parcial, para tratar de desentrañar los mecanismos evolutivos que impulsan esta diferencia. En *C. elegans* existen dos tipos de neuronas serotoninérgicas, las que sintetizan 5-HT y expresan las enzimas necesarias para su síntesis (TPH-1, BAS-1, CAT-4) y las que la recaptan y sólo expresan el recaptador de serotonina MOD-5/SERT y el transportador vesicular de monoaminas CAT-1/VMAT. Entonces, considerando el papel de estas neuronas junto con las neuronas HSN en el comportamiento

de puesta de huevos (Schafer, 2005), estudiaremos también la relevancia del fenotipo serotoninérgico en VC4 y VC5 en la regulación de ese comportamiento.

Esta tesis se basa en el trabajo presentado por el Dr. Carlos Mora en su tesis, de la que podemos concluir que 1) Las neuronas VC4 y VC5 en el grupo de especies de *Angaria* han adquirido un fenotipo serotoninérgico, a través de la recaptación de serotonina HSN, probablemente debido a la expresión constitutiva del transportador de 5-HT MOD-5 en estas neuronas; 2) En *C. elegans* VC4 y VC5 tienen un fenotipo serotoninérgico parcial (expresan *cat-1*), que está controlado por el gen proneural *hlh-3* y el factor de transcripción homeodominio *unc-4* 3) Las modificaciones epigenéticas en *C. elegans* previenen el fenotipo serotoninérgico completo en VC4 y VC5 probablemente a través de la represión directa o indirecta del locus *mod-5* (Mora-Martínez, 2019)

Objetivos

En esta tesis, pretendemos estudiar los cambios moleculares evolutivos que tuvieron lugar en el grupo de especies de *Angaria* en relación con los fenotipos serotoninérgicos de las neuronas VC4 y VC5 y cuál podría ser la relevancia funcional en el comportamiento del rasgo adquirido. En cuanto a la represión en *C. elegans* del fenotipo serotoninérgico de VC4 y VC5, queremos explorar si las señales ambientales pueden liberar esa represión, lo que demostraría que el rasgo es realmente plástico. Por último, también buscamos explorar los mecanismos moleculares de la evolución de otros fenotipos neuronales, centrándome específicamente en las neuronas CA del macho.

En concreto, el proyecto de mi tesis doctoral se subdivide en cuatro objetivos principales:

- 1) Caracterización molecular de los cambios en las redes de regulación neuronal que subyacen al fenotipo serotoninérgico en las VC4 y VC5 de *C. angaria*.
- 2) Análisis del comportamiento de puesta de huevos de diferentes cepas de *C. angaria* y *C. elegans* para comprender el papel fisiológico del rasgo adquirido en las especies de *Angaria*.
- 3) Estudio de la posible naturaleza plástica del fenotipo serotoninérgico VC4 y VC5 en *C. elegans*: identificación de desencadenantes ambientales y relevancia funcional.
- 4) Descripción de la aparición de otro rasgo novedoso en especies de *Angaria*: el fenotipo serotoninérgico de neuronas CA específicas del macho.

Metodología y resultados

Regulación génica del fenotipo serotoninérgico de las neuronas VC4 y VC5 de *C. angaria* y *C. elegans*

Cambios en las regiones cis-reguladoras son responsables del fenotipo serotoninérgico de las neuronas VC4 y VC5 de *C. angaria*

En primer lugar, exploramos las bases moleculares del fenotipo serotoninérgico observado en las neuronas VC4 y VC5 de *C. angaria* centrándonos en los principales genes de las neuronas recaptadoras de serotonina, *cat-1* y *mod-5*. El Dr. Mora ya descubrió que las diferencias en el fenotipo 5-HT se debían a cambios en las regiones cis-reguladoras. Analizó la actividad de las regiones reguladoras de *mod-5* y *cat-1* del genoma de *C. elegans* y *C. angaria* en *C. elegans* y en *C. angaria* y descubrió que 1) El CRM de *cat-1* de *C. angaria* se activa en las VC4 y VC5 de *C. elegans*; 2) El CRM de *mod-5* de *C. angaria* también se activa en las VC4 y VC5 de *C. elegans* 3) ninguno de los CRMs probadas de *mod-5* de *C. elegans*, o el reportero endógeno de *mod-5*, introducido por CRISPR se expresan en las neuronas VC4 y VC5 de *C. elegans*.

Luego analizamos las construcciones reporteras de *cat-1* y *mod-5* de *C. elegans* y *cat-1* de *C. angaria* en animales transgénicos de *C. angaria*. Encontramos que ninguno de los reporteros de *cat-1* (*C. elegans* o *C. angaria*) conducía a la expresión de gfp en las VC4 o VC5 en *C. angaria*, pero ambos eran activos en el resto de las neuronas sintetizadoras de monoaminas (ADF, NSM, CEPV, CEPD, ADE, AIM, PDE, HSN, RIC y RIM) excepto en RIH y en AIM con el reportero de *cat-1* de *C. elegans*. La falta de expresión de *cat-1* en las VC4 y VC5 de *C. angaria* podría sugerir que el CRM que impulsa la expresión de *cat-1* en estas neuronas en *C. angaria* podría encontrarse fuera de la región analizada. En el caso de *C.*

elegans mod-5, no pude ver la expresión en VC4 y VC5, pero estaba activa en NSM, ADF, y AIM. Estos resultados sugieren que las regiones reguladoras de *mod-5* de *C. elegans* no son activas en las neuronas VC4 o VC5 de *C. angaria*.

También confirmamos la expresión de *cat-1* en las neuronas VC4 y VC5 de *C. angaria* realizando una inmunotinción contra el anticuerpo *cat-1* de *C. elegans* (VMAT) (ambas especies comparten un 76% de identidad), que dio una tinción positiva en estas neuronas.

Finalmente, para apoyar el modelo cis-regulador con otras especies, el Dr. Mora realizó cepas reporteras de *C. elegans* portadoras de las regiones reguladoras de *mod-5* de dos especies adicionales del grupo *Angaria*, *C. castelli* y *C. quiockensis* y encontró expresión de gfp en las neuronas VC4 y VC5 de *C. elegans* con ambas construcciones. Después me centré en una especie fuera del grupo *Angaria* pero filogenéticamente cercana a *C. elegans*, la especie *C. virilis*, y las regiones reguladoras de *mod-5* no pudieron conducir la expresión de GFP en las neuronas VC4 y VC5, que sin embargo, fue activa en las neuronas NSM. En conjunto, estos resultados indicaron que las neuronas VC4 y VC5 de *C. angaria*, así como otras especies del grupo *Angaria*, adquirieron el fenotipo serotoninérgico a través de cambios en las regiones cis-reguladoras de *mod-5* localizadas en el primer intrón de este locus.

El homeodominio UNC-4/UNCX activa directamente la expresión del potenciador de *mod-5*/SERT de *C. angaria* en las neuronas VC4 y VC5 de *C. elegans*

A continuación, exploramos el factor de transcripción que podría estar regulando la expresión de *mod-5*. Como ya sabíamos que UNC-4 era esencial para la expresión de *cat-1* en *C. elegans* exploramos su requerimiento para la activación del CRM de *mod-5* de *C. angaria*, debido a las dificultades de trabajar con *C. angaria* exploramos la expresión de *C. angaria mod-5* en una cepa de *C.*

elegans que porta ese constructo. El Dr. Mora comprobó que la expresión del reportero de *C. angaria mod-5* se reducía significativamente en los mutantes *unc-4* de *C. elegans*. Luego demostramos la acción directa de UNC-4 sobre el CRM de *mod-5* de *C. angaria* mutando los tres sitios putativos de unión de UNC-4 de la región promotora mínima de ese CRM. Luego los mutamos por separado mediante mutagénesis dirigida y descubrimos que el tercer sitio era el más funcional. También estudié la funcionalidad de los sitios de unión de UNC-4 en el contexto endógeno de *C. angaria* y ningún *C. angaria mod-5/SERT* enhancer is sufficient to induce VC4 and VC5 serotonergic phenotype in *C. elegans*.

El potenciador de *mod-5* de *C. angaria* es suficiente para inducir el fenotipo serotoninérgico en las VC4 y VC5 de *C. elegans*

A continuación, introdujimos el primer intrón de *mod-5* de *C. angaria*, que era el que portaba el potenciador activo en VC4 y VC5, en el contexto endógeno de *C. elegans* mediante CRISPR, en una cepa que contenía un reportero endógeno de *mod-5*. Los gusanos mostraron una tinción fuerte y penetrante en VC4 y VC5, sin embargo, no fuimos capaces de ver la expresión del reportero endógeno en las neuronas VC4 o VC5 en *C. elegans*. También descubrimos que el potenciador de *C. angaria mod-5* introducido en el contexto de *C. elegans* dependía de UNC-4 para su activación y la subsiguiente tinción de serotonina.

Bajos niveles del TF homeodominio UNC-4/UNCX restringen el fenotipo serotoninérgico VC4 y VC5 en *C. elegans*.

El Dr. Mora también descubrió que en *C. elegans*, la expresión de *mod-5* en las neuronas VC4 y VC5 está reprimida por la vía H3K9me, y era dependiente de la actividad UNC-4, sin embargo, las construcciones de *mod-5* en *C. elegans* no mostraban expresión en VC4 y VC5 en *C. elegans*. Estudios previos sobre la actividad de los potenciadores en las distintas especies de *Drosophila* revelaron que las secuencias inicialmente consideradas inactivas a menudo muestran una actividad residual, lo que sugiere que los potenciadores subóptimos pueden

evolucionar hasta convertirse en regiones reguladoras robustas (Rebeiz et al., 2011). Entonces propusimos que el intrón 1 de *C. elegans* podría ser subóptimo para la activación por UNC-4. Para explorar esto, sobre expresamos *unc-4* bajo un promotor de choque térmico y descubrimos que restauraba la expresión de GFP en el reportero *mod-5* de *C. elegans* y aumentaba la tinción de serotonina en las neuronas VC4 y VC5, confirmando la actividad potenciadora residual del locus *mod-5* de *C. elegans*. También descubrimos que la sobreexpresión de *unc-4* inducida por choque térmico en el estadio L4 aumentaba significativamente la expresión de GFP en comparación con la inducción en el estadio de adulto joven, lo que pone de manifiesto la sensibilidad temporal de la activación de *mod-5*.

La señalización de H3K9me y la sobreexpresión de UNC-4/UNCX parecen actuar en paralelo

Por último, exploramos si el fenotipo de los mutantes epigenéticos podría estar mediado por el aumento de los niveles de expresión de *unc-4*. Analicé los niveles de expresión endógena de *unc-4* (utilizando un alelo marcado con CRISPR) entre los gusanos de tipo salvaje y mutantes de *hpl-2*. Sin embargo, contrariamente a nuestra hipótesis inicial, descubrí que los niveles de expresión de *unc-4* se reducían significativamente en este fondo mutante, lo que indica que el fenotipo serotoninérgico en VC4 y VC5 observado tanto en la sobreexpresión de *unc-4* como en los mutantes H3K9me parece ser independientes entre sí.

En conjunto, hemos encontrado que 1) el fenotipo serotoninérgico de las especies de Angaria en VC4 y VC5 está mediado por cambios en las regiones cis-reguladoras de *mod-5*, 2) *unc-4* activa directamente el locus de *mod-5* en *C.*

angaria, 3) el potenciador *mod-5* de *C. angaria* es suficiente para inducir el fenotipo serotoninérgico de las neuronas VC4 y VC5 en *C. elegans*, 4) la sobreexpresión de *unc-4* puede inducir el fenotipo serotoninérgico en las neuronas VC4 y VC5 de *C. elegans* incluso en el contexto de la represión de H3K9me de tipo salvaje, y 6) la activación del fenotipo serotoninérgico en las neuronas VC4 y VC5 de *C. elegans* de mutantes de la vía H3K9me o de la inducción de *unc-4* son independientes entre sí.

Consecuencias conductuales del fenotipo serotoninérgico de las neuronas VC4 y VC5 en *C. angaria*

*Estudio comparativo del número de huevos puestos en *C. elegans* y *C. angaria**

Para investigar las implicaciones funcionales del fenotipo serotoninérgico en las neuronas VC4 y VC5 en *C. angaria*, estudiamos su efecto en el comportamiento de puesta de huevos. Estas neuronas, junto con las HSN, controlan el proceso de puesta de huevos, siendo la serotonina (5-HT) uno de los principales neurotransmisores involucrados. Nuestra hipótesis fue que las neuronas VC4 y VC5 serotoninérgicas podrían recaptar serotonina de las HSN, controlando las contracciones de los músculos de la vulva y, por ende, la puesta de huevos.

En primer lugar, estudiamos el número total de huevos puestos diariamente durante 3 días consecutivos (periodos de 24 h, 48 h y 72 h). Dado que *C. angaria* y *C. elegans* son especies diferentes, aprovechamos cepas previamente generadas en el proyecto para comparar la misma especie, pero con la única diferencia del fenotipo serotoninérgico en las neuronas VC4 y VC5. Por lo tanto, comparamos animales *C. elegans* silvestres N2 con una cepa transgénica de *C. elegans* que porta un constructo para expresar el cDNA de *mod-5* en sus

neuronas VC ('angarizados'). En el caso de *C. angaria*, comparamos animales control con una cepa mutante de *mod-5* de *C. angaria* ('elenganizados'). Es importante mencionar que *C. angaria* es una especie dióica, lo que significa que necesita aparearse para producir descendencia, mientras que *C. elegans* es hermafrodita.

En *C. elegans*, los animales transgénicos que expresaban *mod-5* en las neuronas VC4 y VC5 ('angarizados') pusieron significativamente menos huevos en general en comparación con los silvestres, lo que respalda la hipótesis de que la recaptación de 5-HT por parte de las neuronas VC4 y VC5 controla las contracciones de la vulva. En *C. angaria*, los animales silvestres pusieron menos huevos que *C. elegans*; sin embargo, contrariamente a las expectativas, los mutantes de *mod-5* ('elenganizados') también mostraron defectos significativos en la puesta de huevos en todas las etapas en comparación con los silvestres. También cuantificamos los huevos retenidos dentro de los gusanos 24 horas después de alcanzar la etapa de adulto joven. Encontramos que tanto los animales transgénicos de *C. elegans* como los mutantes de *mod-5* de *C. angaria* retuvieron más huevos que sus respectivos controles.

Posteriormente, estudiamos el comportamiento de puesta de huevos en *C. quiockensis*; que comparte el fenotipo serotoninérgico en VC4 y VC5 y también es dióico; y en *C. briggsae*; una especie hermafrodita que carece de dicho fenotipo. Observamos que mostraban patrones específicos de puesta de huevos según la especie, con *C. quiockensis* poniendo menos huevos, predominantemente en las primeras 24 horas después del apareamiento, y *C. briggsae* poniendo más huevos.

En general, aunque los resultados sugieren que la expresión de *mod-5* en las neuronas VC4 y VC5 afecta el comportamiento de puesta de huevos, las

implicaciones funcionales específicas del fenotipo serotoninérgico siguían sin estar claras.

Estudio de la dinámica de puesta de huevos en *C. elegans* y *C. angaria*

Para refinar el análisis de la puesta de huevos, realizamos grabaciones individuales de gusanos durante intervalos de 6 horas, 24 horas después del apareamiento o de alcanzar la etapa de adulto joven. El comportamiento de puesta de huevos se modeló como un proceso rítmico de dos estados, con fases activas de puesta de huevos con eventos variables (en periodos de 1-2 minutos) y fases inactivas sin puesta de huevos de duraciones variables, como ya se había descrito para la cepa N2 de *C. elegans* (Waggoner et al., 1998). Definimos algunos conceptos importantes: cada huevo puesto se denomina evento de puesta de huevos, y el tiempo entre dos eventos de puesta de huevos es un intervalo. Si el tiempo entre dos eventos de puesta de huevos es mayor a 120 segundos, el intervalo se considera inactivo. Alternativamente, todos los eventos de puesta de huevos separados por menos de 120 segundos pertenecerán a la misma ventana activa de puesta de huevos, y el tiempo entre dos eventos dentro de una ventana activa se denominará intervalo activo.

Estas grabaciones se analizaron en los cuatro genotipos mencionados: *C. elegans* silvestre, *C. elegans* con expresión de *mod-5* en las neuronas VC4-VC5, *C. angaria* silvestre y mutantes de *mod-5* en *C. angaria*.

Los resultados en *C. elegans* mostraron que los gusanos silvestres exhibieron un comportamiento bifásico, con intervalos activos e inactivos claramente definidos. En contraste, *C. angaria* carecía de este patrón bifásico, mostrando eventos de puesta de huevos más aleatorios. Mientras que *C. elegans* con expresión de *mod-5* en las neuronas VC ponía menos huevos y tenía intervalos activos y ventanas de puesta de huevos más cortas en comparación con los

silvestres, no se observaron diferencias significativas en la dinámica de puesta de huevos entre *C. angaria* silvestre y los mutantes de *mod-5*.

En general, los datos respaldan la hipótesis de que *mod-5* en las neuronas VC modula la dinámica de puesta de huevos en *C. elegans* al acortar los intervalos activos y reducir el número de huevos puestos por ventana. Sin embargo, no se identificaron diferencias conductuales atribuibles al fenotipo serotoninérgico de las neuronas VC4 y VC5 en *C. angaria*.

Inducción de la puesta de huevos en medios con alta concentración de serotonina

Para explorar el papel del fenotipo serotoninérgico de VC4 y VC5 en la modulación de respuestas a serotonina exógena, realizamos ensayos de puesta de huevos, exponiendo a los gusanos a 10 mM de 5-HT durante 2 horas y contabilizando el número de huevos puestos. A 10 mM de 5-HT, *C. elegans* silvestre mostró una fuerte inducción de puesta de huevos, como se había descrito en la bibliografía (Horvitz et al., 1982), mientras que los *C. elegans* transgénicos con expresión de *mod-5* en las neuronas VC exhibieron una respuesta significativamente reducida. En *C. angaria*, los gusanos silvestres no respondieron a la 5-HT exógena de manera similar a resultados previos en nuestro laboratorio (Mora-Martínez, 2019), pero los mutantes de *mod-5* mostraron un aumento en la puesta de huevos, destacando el papel de *mod-5* en atenuar esta respuesta.

Redujimos la concentración de 5-HT e incluimos la cepa de *C. elegans* que lleva el intrón 1 de *mod-5* de *C. angaria*. Encontramos que tanto *C. elegans* con el intrón como los *C. elegans* con expresión de *mod-5* en las VC muestran un número significativamente menor de huevos puestos bajo el estímulo de 5-HT en comparación con los silvestres. Sin embargo, en *C. angaria* no se observaron diferencias significativas.

Estos hallazgos sugieren que el fenotipo serotoninérgico en las neuronas VC4 y VC5 contribuye a modular las respuestas a la serotonina exógena, protegiendo potencialmente a los gusanos de fluctuaciones ambientales de 5-HT.

Las neuronas VC4 y VC5 de la cepa *C. elegans* que contienen el primer intrón de *mod-5* de *C. angaria*, pueden recaptar serotonina del medio ambiente.

Dado que identificamos un fenotipo conductual directamente atribuible a la presencia del fenotipo serotoninérgico en VC4 y VC5 en ambas especies de *Caenorhabditis*, propusimos una posible explicación. Considerando que la estructura de la vulva, los músculos vulvares y las terminaciones de las neuronas VC4 y VC5 están expuestos al ambiente externo, y que la serotonina es crucial para las contracciones musculares de la vulva impulsadas por las HSN (Desai et al., 1988), planteamos la hipótesis de que las neuronas VC4 y VC5 podrían recaptar serotonina externa. Este mecanismo podría prevenir que la serotonina ambiental interfiera con la señalización endógena de las HSN hacia los músculos vulvares. Para probar esto, utilizamos cepas de *C. elegans* con el intrón 1 de *mod-5* de *C. angaria* en un fondo mutante *bas-1* (para eliminar toda la 5-HT interna) y encontramos que las neuronas VC4 y VC5 pueden recaptar serotonina de ambientes ricos en serotonina.

En resumen, esta sección de resultados permite concluir que 1) las especies con VC4 y VC5 completamente serotoninérgicas tienden a poner menos huevos que los gusanos con cantidades variables o sin 5-HT en estas neuronas; 2) la dinámica de puesta de huevos de *C. angaria* no sigue el patrón bimodal encontrado en *C. elegans*; 3) El destino serotoninérgico de VC4 y VC5 en *C. angaria* desempeña un papel en el control del comportamiento de puesta de huevos en entornos con altas concentraciones de 5-HT; 4) la inducción

serotoninérgica de VC4 y VC5 en *C. elegans* protege contra la inducción de puesta de huevos provocada por serotonina exógena y 5) las neuronas VC4 y VC5 que expresan *mod-5* pueden recaptar serotonina del medio externo.

El rasgo serotoninérgico de VC4 y VC5 puede representar una adaptación en *C. angaria*, que habita en ambientes ricos en serotonina, para evitar que la serotonina ambiental altere la dinámica de puesta de huevos.

Caracterización de la plasticidad del fenotipo serotoninérgico de VC4 y VC5 en *C. elegans*

Estudio del fenotipo serotoninérgico en las VC4 y VC5 de aislados silvestres de *C. elegans*

Durante los ensayos de tinción con 5-HT, observamos variabilidad en los fenotipos serotoninérgicos de las neuronas VC4 y VC5 en la cepa N2 de *C. elegans*. Sin embargo, esta es una línea adaptada al laboratorio, con divergencias conocidas de las poblaciones silvestres. Para investigar si esta variabilidad refleja condiciones naturales, realicé tinciones con 5-HT en 18 aislados silvestres genéticamente diversos de *C. elegans* obtenidos del *Caenorhabditis* Natural Diversity Resource (CaeNDR). Las cepas mostraron diferencias notables, que iban desde niveles mínimos de tinción en VC4 y VC5 hasta frecuencias altas (80%), con una gran variabilidad incluso en poblaciones sincronizadas entre réplicas de la misma cepa. De manera inesperada, también se observaron porcentajes altos de tinción en réplicas de N2, lo que sugiere posibles disparadores ambientales como la humedad, la temperatura o contaminantes. Estos hallazgos destacan contribuciones genéticas y ambientales significativas a la plasticidad del fenotipo serotoninérgico de VC4 y VC5 en *C. elegans*.

Comparativa de las dinámicas de puesta de huevos entre N2 y el aislado silvestre CB4856.

Para investigar la influencia de la variabilidad genética en las dinámicas de puesta de huevos en *C. elegans*, comparamos la cepa N2 con el aislado silvestre CB4856, que presenta un alto porcentaje de serotonina en VC4 y VC5, mediante análisis en video de seis horas. Observamos que CB4856 mostró pendientes menos pronunciadas en intervalos cortos, menor frecuencia de puesta de huevos y períodos inactivos más largos en comparación con N2. Los gusanos CB4856 pusieron menos huevos en general, con ventanas de puesta de huevos más cortas, menos ventanas activas y menos huevos por ventana. Estos rasgos se asemejan a los animales transgénicos N2 que expresan *mod-5* en las neuronas VC4 y VC5, aunque CB4856 mostró menos ventanas de puesta de huevos que la cepa transgénica.

Estos hallazgos sugieren que las dinámicas de puesta de huevos en aislados silvestres como CB4856 están moldeadas por mecanismos regulatorios genéticos y serotoninérgicos distintos, enfatizando el papel de la diversidad genética natural en la modulación de este comportamiento.

El apareamiento como desencadenante del fenotipo serotoninérgico VC4 y VC5 en *C. elegans* y el comportamiento de puesta de huevos en hermafroditas cruzadas

Tras observar la variabilidad y plasticidad en el fenotipo serotoninérgico de las neuronas VC4 y VC5 entre isotipos de *C. elegans* y réplicas, exploramos posibles disparadores ambientales para este fenotipo. Se investigó inicialmente el estrés, sin encontrar inducción significativa mediante estresores clásicos como inanición, alta temperatura, hipoxia o infección patogénica (Mora-Martínez, 2019).

Sin embargo, los estímulos mecanosensoriales, particularmente los asociados al apareamiento se propusieron como posibles desencadenantes debido a la

respuesta reportada de las neuronas VC4 y VC5 a estímulos mecánicos (Medrano & Collins, 2023) y el aumento en la producción de machos bajo estrés. Para probar esto, realizamos tinciones con 5-HT en las cepas N2 silvestre y *him-5(e1490)* (con altas proporciones de machos). Aunque no se observaron diferencias significativas en la intensidad de 5-HT en VC4 y VC5 entre genotipos, se notó una tendencia hacia una mayor tinción en los mutantes *him-5(e1490)*, lo que sugiere un posible vínculo entre el apareamiento y la activación serotoninérgica. Sin embargo, no se pudieron identificar hermafroditas apareadas, lo que podría haber diluido los resultados; este aspecto será evaluado en el futuro.

Considerando estos resultados preliminares, decidimos estudiar el comportamiento de puesta de huevos de hembras hermafroditas apareadas en comparación con hembras no apareadas para determinar si el apareamiento induce cambios en el comportamiento relacionados con el fenotipo serotoninérgico de VC.

Cuantificamos el número de huevos puestos por hembras hermafroditas auto fecundadas y apareadas de N2 durante intervalos de 24 horas a lo largo de tres días. Las hembras auto fecundadas pusieron más huevos durante las primeras 24 horas, mientras que las hembras apareadas mostraron una puesta de huevos retrasada, liberando más huevos en el segundo día y manteniendo una mayor producción de huevos 72 horas después de la etapa de adulto joven. Este patrón probablemente refleja la influencia de la disponibilidad de esperma debido al cruce con machos.

Para obtener una visión más detallada de las dinámicas de puesta de huevos, realizamos grabaciones de seis horas de duración. Encontramos que el apareamiento reduce la frecuencia y duración de las ventanas de puesta de huevos, aumenta los intervalos inactivos y altera las dinámicas de

comportamiento de puesta de huevos. Sin embargo, el número de huevos puestos por ventana de puesta de huevos permanece constante. Se necesitan más experimentos con configuraciones alternativas para probar esta hipótesis y aclarar los mecanismos subyacentes, incluida la posible implicación de la inducción serotoninérgica de VC4 y VC5 y la liberación de 5-HT de HSN.

Aunque el apareamiento parece influir en el comportamiento de puesta de huevos y la tinción con 5-HT de VC4 y VC5, nuestros resultados fueron inconclusos. Decidí explorar otros disparadores, en particular entornos con alta concentración de 5-HT, que fueron importantes para controlar la puesta de huevos en *C. angaria*. Por lo tanto, cultivé *C. elegans* N2 en medios semisólidos con alta concentración de 5-HT para investigar este aspecto.

El crecimiento en un ambiente de alta concentración de 5-HT induce el fenotipo serotoninérgico en las neuronas VC4 y VC5 de *C. elegans*.

Observamos que la exposición a altas concentraciones externas de 5-HT inducía un fenotipo serotoninérgico en las neuronas VC4 y VC5 de *C. elegans*. Sin embargo, encontramos que los animales que vivieron solo una generación en un entorno con 10 mM de 5-HT adquirieron un fenotipo serotoninérgico robusto en estas dos neuronas. Estos resultados sugieren que el destino serotoninérgico de las neuronas VC4 y VC5 es plástico e influenciado por los niveles ambientales de 5-HT.

Además, se confirmó la capacidad de las neuronas VC4 y VC5 para recaptar 5-HT externa en una cepa mutante *bas-1*, que carece de síntesis de 5-HT. Esta capacidad de recaptación era evidente incluso sin producción interna de serotonina.

Sin embargo, no se observó una plasticidad similar en las especies *C. briggsae* y *C. tropicalis*, lo que sugiere que esta plasticidad podría no ser generalizada en el

género *Caenorhabditis*. Esto plantea la posibilidad de una asimilación genética en el grupo de especies *Angaria*, donde los fenotipos serotoninérgicos constitutivos están presentes. Se necesitan más experimentos para explorar la base genética de esta plasticidad y si podría desencadenarse en otras especies o bajo diferentes condiciones.

El fenotipo serotoninérgico de VC4 y VC5 en *C. elegans* crecidos en ambientes con alto contenido de 5-HT es dependiente de UNC-4 y MOD-5.

Dado que encontramos la necesidad de *unc-4* y *mod-5* para el fenotipo serotoninérgico en VC4 y VC5 en *C. angaria*, investigamos el papel de estos dos genes en el fenotipo serotoninérgico de VC4 y VC5 en *C. elegans* expuestos a 5-HT externo. Descubrimos que UNC-4 es necesario para la adquisición de este fenotipo, ya que su ausencia condujo a una disminución significativa en la intensidad de la tinción con 5-HT. De manera similar, MOD-5 es necesario para recaptar 5-HT, pero incluso en mutantes *mod-5* se observó tinción residual, lo que sugiere un mecanismo alternativo de recaptación. Al cruzar mutantes *mod-5* con mutantes *bas-1* (incapaces de sintetizar serotonina), demostramos que aún ocurre algo de recaptación de serotonina, lo que indica una vía de recaptación complementaria, independiente de MOD-5. Estos hallazgos sugieren que tanto UNC-4 como MOD-5 son esenciales para la respuesta de VC4 y VC5 a 5-HT ambiental, con mecanismos adicionales implicados en la recaptación de 5-HT.

El fenotipo serotoninérgico plástico de *C. elegans* VC4 y VC5 tiene un papel protector frente a la inducción de la puesta de huevos debido a la 5-HT exógena.

Tras descubrir la plasticidad del fenotipo serotoninérgico en las neuronas VC4 y VC5 en *C. elegans*, investigamos la importancia conductual de este fenotipo. Encontramos que los gusanos criados en entornos serotoninérgicos estaban

protegidos de la inducción de puesta de huevos causada por la exposición aguda a 5-HT. Específicamente, los gusanos criados en medios serotoninérgicos pusieron menos huevos cuando se expusieron a 5-HT en comparación con aquellos criados en condiciones de control, lo que indica una respuesta protectora. Experimentos adicionales mostraron que UNC-4 y MOD-5 son necesarios para esta respuesta conductual. Los mutantes UNC-4 mostraron una respuesta leve a 5-HT, mientras que los mutantes MOD-5 carecieron de la respuesta protectora por completo, destacando el papel esencial de MOD-5 en amortiguar los efectos de 5-HT externa.

En conjunto, estos hallazgos me permiten concluir que 1) el fenotipo serotoninérgico en las neuronas VC4 y VC5 es un rasgo plástico en *C. elegans*; 2) las neuronas VC4 y VC5 se vuelven serotoninérgicas cuando los gusanos se cultivan durante una generación en entornos con alta concentración de 5-HT; 3) VC4 y VC5 en *C. elegans* son capaces de recaptar 5-HT del exterior; 4) el fenotipo serotoninérgico en VC4 y VC5 inducido por la exposición a 5-HT exógeno no se observa en otras dos especies analizadas del género *Caenorhabditis* 5) el fenotipo serotoninérgico en las neuronas VC4 y VC5, cuando se cultiva con 5-HT exógeno, depende de *unc-4* y, en parte, de *mod-5* y 6) La exposición a 5-HT induce una respuesta protectora frente a la inducción de puesta de huevos causada por la exposición aguda a 5-HT, y esta respuesta también depende de *unc-4* y *mod-5*.

Análisis del fenotipo serotoninérgico de las neuronas específicas de los machos CPs y CAs en *C. elegans* y *C. angaria*

En el capítulo final de mi tesis, examiné el fenotipo serotoninérgico de las neuronas CP y CA en machos de *C. elegans* y *C. angaria*, que son homólogos a las neuronas VC en hembras. Las CPs en *C. elegans* son serotoninérgicas, mientras

que las CAs son principalmente colinérgicas, aunque muestran una tinción de serotonina poco confiable. En los machos de *C. angaria*, tanto las CPs como las CAs se tiñen para serotonina, lo que nos llevó a investigar si la regulación del fenotipo serotoninérgico de las CAs es similar a la de las neuronas VC.

En *C. angaria*, las CAs requieren *mod-5* para la recaptación de serotonina. Realicé una tinción de serotonina en ejemplares silvestres de *C. angaria* y en mutantes *mod-5(vlc45)*, y evalué las CPs y CAs de los machos. Encontramos una reducción significativa en el número de CAs teñidas en los mutantes; sin embargo, algunas neuronas mostraron tinción residual de serotonina, lo que sugiere mecanismos compensatorios.

Aprovechamos las microinyecciones realizadas en *C. angaria* para estudiar la actividad de los constructos reporteros de *cat-1* y *mod-5* de *C. elegans*, así como el constructo reportero de *cat-1* de *C. angaria*. Descubrimos que todos los reporteros eran activos en las neuronas CP serotoninérgicas, pero ninguno lo era en las CAs. Planteamos realizar un análisis de las regiones regulatorias de *mod-5* en *C. angaria* para identificar si el CRM mínimo de *mod-5* en *C. angaria*, que es activo en las neuronas VC4 y VC5, también es activo en las CAs. Además, la falta de expresión del reportero de *cat-1* de *C. angaria* en las CAs sugiere que el potenciador activo en estas neuronas se encuentra fuera de las regiones estudiadas en este reportero.

También verificamos la expresión de *mod-5* a partir del genoma de *C. elegans* y *C. angaria* en las neuronas CA y CP de *C. elegans*. La expresión del reportero endógeno *mod-5 (vlc47)* de *C. elegans* en las neuronas CPs y CAs solo se observó en las CPs. Posteriormente, estudiamos la actividad del reportero *mod-5* de *C. angaria* (que es activo en las neuronas VC4 y VC5 de ambas especies) en machos de *C. elegans*, y encontramos que tanto las CPs como las CAs activan este reportero, aunque la expresión era más prevalente en las CPs. Sin embargo, es

importante mencionar que el número de gusanos evaluados fue muy bajo (n=20), por lo que la tinción debería repetirse. En resumen, estos resultados, aunque preliminares, son compatibles con un modelo cis-regulatorio para la evolución del rasgo de recaptación de 5-HT en las neuronas CA de *C. angaria*, homólogas a las neuronas VC4 y VC5 en el hermafrodita.

Por último, exploré el papel de las CAs serotoninérgicas en el comportamiento de apareamiento, específicamente en el apareamiento en espiral, que es único en las especies del grupo Angaria (Kiontke et al., 2011). Descripciones previas sobre el papel de las CPs en el comportamiento de apareamiento en *C. elegans* demostraron que la ablación de las CPs o de sus precursores (incluidas las CAs) estaba relacionada con un pobre comportamiento de apareamiento, específicamente el movimiento de giro alrededor del hermafrodita en la búsqueda de la vulva (movimiento en espiral). También se describió la importancia de la serotonina (5-HT) para la curvatura de la cola exhibida por los machos durante el apareamiento (Kiontke et al., 2011). Más recientemente, se ha reportado que los machos de *C. elegans* pueden mostrar un apareamiento en espiral cuando se colocan en medios semi-líquidos, lo que sugiere que este comportamiento es plástico (Susoy & Samuel, 2023).

Para comenzar a explorar el posible papel de las CAs serotoninérgicas (junto con las CPs) en el comportamiento de apareamiento en espiral en *C. angaria* y posiblemente en el comportamiento aprendido de *C. elegans*, estudiamos la curvatura de la cola tanto en condiciones de laboratorio como en presencia de 5-HT exógena. En *C. elegans*, tanto la cepa silvestre como la cepa con el primer intrón de *mod-5* de *C. angaria* mostraron curvatura de la cola cuando fueron expuestos a 5-HT exógena, sin diferencias significativas entre las cepas. En el caso de *C. angaria*, al igual que en *C. elegans*, encontramos un bajo porcentaje

de curvatura de la cola en machos silvestres en condiciones basales. Sin embargo, los mutantes *mod-5(vlc45)* mostraron un porcentaje significativo de machos con la cola curvada incluso en condiciones de control, lo que podría significar una sobreactivación de los músculos de la pared corporal debido a la liberación de serotonina de las CPs en ausencia de recaptación mediada por *mod-5*. En concordancia con esta hipótesis, la 5-HT exógena induce curvatura en *C. angaria*, pero no aumenta aún más la curvatura en los mutantes *mod-5(vlc45)*. En conclusión, de esta última parte de mi tesis podemos concluir que 1) las neuronas CAs en *C. angaria* han adquirido un fenotipo de recaptación de 5-HT dependiente de *mod-5*; 2) el reportero de *mod-5* de *C. angaria* se expresa en las CAs de *C. elegans*, lo que respalda la existencia de un mecanismo cis-regulatorio en la adquisición evolutiva de este rasgo; 3) el primer intrón del locus *mod-5* de *C. angaria* no es suficiente para recapitular todo el fenotipo serotoninérgico en las CAs de *C. elegans* y 4) los machos mutantes *mod-5(vlc45)* de *C. angaria* muestran un mayor porcentaje de colas curvadas en condiciones de control, lo que podría significar una sobreactivación de los músculos de la pared corporal.

Conclusiones

- El fenotipo serotoninérgico en VC4 y VC5 de *C. angaria* está mediado por cambios en las regiones *cis*-reguladoras de *mod-5* que están directamente controladas por la acción de UNC-4.
- El potenciador (enhancer) de *mod-5* en *C. angaria* es suficiente para inducir el fenotipo serotoninérgico de las neuronas VC4 y VC5 en *C. elegans*.
- En *C. elegans* silvestre, tanto los niveles moderados de UNC-4 como el silenciamiento epigenético del locus *mod-5* reprimen el fenotipo serotoninérgico en VC4 y VC5 y la sobreexpresión de UNC-4 es suficiente para activar, aunque a niveles bajos, el potenciador del intrón 1 de *mod-5* de *C. elegans*.
- La activación del fenotipo serotoninérgico en las neuronas VC4 y VC5 de *C. elegans* de mutantes de la vía H3K9me o de la inducción de *unc-4* son independientes entre sí.
- El fenotipo serotoninérgico en las neuronas VC4 y VC5 es un rasgo plástico en *C. elegans* inducible por altas concentraciones de 5-HT ambientales, pero esta plasticidad no se observa en dos especies adicionales del género *Caenorhabditis*, *C. briggsae* y *C. tropicalis*.
- VC4 y VC5 en *C. elegans* son capaces de recaptar 5-HT del ambiente externo.
- El fenotipo serotoninérgico en VC4 y VC5 inducido por cultivo con 5-HT exógena depende de *unc-4* y, en parte, de *mod-5*.
- La exposición a 5-HT en el cultivo hace que los gusanos adquieran cierta protección frente a la inducción de puesta de huevos producida por la

exposición aguda a 5-HT. Esta respuesta también depende de *unc-4* y *mod-5*.

ANNEX

Annex 3.1. Strains.

Strains	Specie	Genotype
N2	<i>C. elegans</i>	wildtype
RGD1	<i>C. angaria</i>	wildtype
NFB2196	<i>C.angaria</i>	mod-5(vlc45)
AF16	<i>C. briggsae</i>	wildtype
JU2745	<i>C. quiockensis</i>	wildtype
JU1373	<i>C. tropicalis</i>	wildtype
JU1247	<i>C. castelli</i>	wildtype
JU1968	<i>C.virilis</i>	wildtype
QG122	<i>C. kamaaiina</i>	wildtype
PX506	<i>C. remanei</i>	wildtype
JU1771	<i>C. doughertyi</i>	wildtype
NKZ35	<i>C. inopinata</i>	wildtype
QX1211	<i>C. elegans</i>	wildtype
JU1581	<i>C. elegans</i>	wildtype
MY23	<i>C. elegans</i>	wildtype
ED3017	<i>C. elegans</i>	wildtype
DL200	<i>C. elegans</i>	wildtype
JU258	<i>C. elegans</i>	wildtype
MY16	<i>C. elegans</i>	wildtype
JJ11398	<i>C. elegans</i>	wildtype
ED3049	<i>C. elegans</i>	wildtype
DL238	<i>C. elegans</i>	wildtype
JU1580	<i>C. elegans</i>	wildtype
CB4856	<i>C. elegans</i>	wildtype
CX11314	<i>C. elegans</i>	wildtype
LKC34	<i>C. elegans</i>	wildtype
EG4725	<i>C. elegans</i>	wildtype
JU1440	<i>C. elegans</i>	wildtype
JU775	<i>C. elegans</i>	wildtype
JU782	<i>C. elegans</i>	wildtype
PHX7715	<i>C. elegans</i>	mod-5(vlc47 syb7715)I
NFB2676	<i>C. elegans</i>	him-5(e1490)
NFB1966	<i>C. elegans</i>	vlcEx1103[cat-1promC1::mod-5cDNA; elt-2::gfp; ttx-3::mCherry]
PHX1658	<i>C. elegans</i>	unc-4(syb1658[unc-4::GFP]) II.
NFB2782	<i>C. elegans</i>	unc-4(syb1658[unc-4::GFP]) II; hpl-2(tm1489) III
MT7988	<i>C. elegans</i>	bas-1(ad446) III.

VC1528	<i>C. elegans</i>	<i>unc-4(gk705)II</i>
MT8944	<i>C. elegans</i>	<i>mod-5(n822) I</i>
COP1365	<i>C. elegans</i>	<i>mod-5(knu383)</i>
MT8944	<i>C. elegans</i>	<i>mod-5(n822) I.</i>
NFB2803	<i>C. elegans</i>	<i>bas-1(ad446)III; mod-5(vlc47 syb7715)I</i>
NFB2844	<i>C. elegans</i>	<i>mod-5(knu383), bas-1(ad446)</i>
NFB2223	<i>C. elegans</i>	<i>vlcEx1195[sp12 castelli mod-5prom2(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2225	<i>C. elegans</i>	<i>vlcEx1197[sp12 castelli mod-5prom2(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2224	<i>C. elegans</i>	<i>vlcEx1196[sp12 castelli mod-5prom2(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2205	<i>C. elegans</i>	<i>vlcEx1177[sp38 quiockensis mod-5prom2(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2206	<i>C. elegans</i>	<i>vlcEx1178[sp38 quiockensis mod-5prom2(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2207	<i>C. elegans</i>	<i>vlcEx1179[sp38 quiockensis mod-5prom2(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2397	<i>C. elegans</i>	<i>vlcEx1257(ln1mod-5sp13::GFP); rol-6</i>
NFB2398	<i>C. elegans</i>	<i>vlcEx1258(ln1mod-5sp13::GFP); rol-6</i>
NFB2399	<i>C. elegans</i>	<i>vlcEx1259(ln1mod-5sp13::GFP); rol-6</i>
NFB2176	<i>C. elegans</i>	<i>vlcEx1158[angaria mod-5prom5(50ng/uL); rol-6su1006(100ng/uL)]</i>
NFB2177	<i>C. elegans</i>	<i>vlcEx1159[angaria mod-5prom5(50ng/uL); rol-6su1006(100ng/uL)]</i>
NFB2227	<i>C. elegans</i>	<i>unc-4(gk705)II; vlcEx1158[angaria mod-5prom5(50ng/uL); rol-6su1006(100ng/uL)]</i>
NFB2228	<i>C. elegans</i>	<i>unc-4(gk705)II; vlcEx1159[angaria mod-5prom5(50ng/uL); rol-6su1006(100ng/uL)]</i>
NFB2208	<i>C. elegans</i>	<i>vlcEx1180[angaria mod-5prom9(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2209	<i>C. elegans</i>	<i>vlcEx1181[angaria mod-5prom9(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2210	<i>C. elegans</i>	<i>vlcEx1182[angaria mod-5prom9(50ng/uL); rol-6 su1006(100ng/uL)]</i>
NFB2567	<i>C. elegans</i>	<i>vlcEx1331 (ANmod5prom9_3HD mut(50ng/ul), rol-6)</i>
NFB2568	<i>C. elegans</i>	<i>vlcEx1332 (ANmod5prom9_3HD mut(50ng/ul), rol-6)</i>
NFB2569	<i>C. elegans</i>	<i>vlcEx1333 (ANmod5prom9_3HD mut(50ng/ul), rol-6)</i>
NFB2601	<i>C. elegans</i>	<i>vlcEx1342(ANmod-5 prom9_HD#1 mut(50ng/ul), rol-6)</i>

NFB2602	<i>C. elegans</i>	<i>vlcEx1343 (ANmod-5 prom9_HD#1 mut(50ng/ul), rol-6)</i>
NFB2590	<i>C. elegans</i>	<i>vlcEx1338 (ANmod-5 prom9_HD#2 mut (50ng/ul), rol-6)</i>
NFB2591	<i>C. elegans</i>	<i>vlcEx1339 (ANmod-5 prom9_HD#2 mut (50ng/ul), rol-6)</i>
NFB2592	<i>C. elegans</i>	<i>vlcEx1340 (ANmod-5 prom9_HD#3 mut (50ng/ul), rol-6)</i>
NFB2593	<i>C. elegans</i>	<i>vlcEx1341(ANmod-5 prom9_HD#3 mut (50ng/ul), rol-6)</i>
NFB593	<i>C. elegans</i>	<i>vlcEx321(mod-5prom3::gfp (50ng/ul),rol-6)</i>
NFB2620	<i>C. elegans</i>	<i>vlcEx1347(mod5prom3::gfp; rol-6;unc-4:hs)</i>
NFB2621	<i>C. elegans</i>	<i>vlcEx1348(mod5prom3::gfp; rol-6;unc-4:hs)</i>
NFB2622	<i>C. elegans</i>	<i>vlcEx1349(mod5prom3::gfp; rol-6;unc-4:hs)</i>
NFB2617	<i>C. elegans</i>	<i>vlcEx1344(rol-6; unc-4::hs); mod-5(vlc47[mod-5::T2A::mNeonGreen]) I</i>
NFB2618	<i>C. elegans</i>	<i>vlcEx1345(rol-6; unc-4::hs); mod-5(vlc47[mod-5::T2A::mNeonGreen]) I</i>
NFB2619	<i>C. elegans</i>	<i>vlcEx1346 (mod5prom3::gfp; rol-6;unc-4:hs)</i>
NFB2668	<i>C. elegans</i>	<i>mod-5(knu383); vlcEx1347(mod5prom3::gfp; rol-6;unc-4:hs)</i>
NFB2669	<i>C. elegans</i>	<i>mod-5(knu383);vlcEx1348(mod5prom3::gfp; rol-6;unc-4:hs)</i>
NFB2843	<i>C. elegans</i>	<i>mod-5(vlc47 syb7715); unc-4(gk705)</i>

Annex 4.1. *C. elegans* and *C. angaria* constructs in *C. angaria*

<i>C. elegans</i> constructs in <i>C. angaria</i>			
Strain	RGD1	RGD1	RGD1
Species	<i>C. angaria</i>	<i>C. angaria</i>	<i>C. angaria</i>
Background	<i>wild type</i>	<i>wild type</i>	<i>wild type</i>
DNA array	<i>C.ele cat-1</i>	<i>C. ele mod-5</i>	<i>C. ele mod-5</i>
reporter	<i>prom1</i>	<i>prom3</i>	<i>prom3</i>
line/genotype	Ex line	F1 scored, no Ex lines	Ex line
N heads	30	12	31
N vulvas	30	12	31
NSM GFP ON	53	23	61
ADF GFP ON	45	16	19
CEPV GFP ON	52	0	
CEPD GFP ON	46	0	
RIC GFP ON	24	0	
RIM GFP ON	22	0	
RIH GFP ON	0	0	0
AIM GFP ON	0	0	15
ADE GFP ON	41	0	
HSN GFP ON	22	0	
PDE GFP ON	46	0	
VC4-5 GFP ON	2	0	0
% VC4-5 GFP ON	3.3333333	0	0

C. angaria* constructs in *C. angaria

Strain	RGD1	RGD1	RGD1
Species	<i>C. angaria</i>	<i>C. angaria</i>	<i>C. angaria</i>
Background	<i>wild type</i>	<i>wild type</i>	<i>wild type</i>
DNA array	<i>C. ang cat-1</i>	<i>C. ang cat-1</i>	<i>C. ang mod-5</i>
reporter	<i>prom1</i>	<i>prom1</i>	<i>prom5</i>
line/genotype	F1 scored, no Ex lines	Ex line	F1 scored, no Ex lines
N heads	27	31	28
N vulvas	27	31	20
NSM GFP ON	42	62	40
ADF GFP ON	32	56	30
CEPV GFP ON	24	58	
CEPD GFP ON	14	54	
RIC GFP ON	16	26	
RIM GFP ON	12	34	
RIH GFP ON	0	0	0
AIM GFP ON	0	0	30
ADE GFP ON	20	49	
HSN GFP ON	27	50	
PDE GFP ON	12	58	
VC4-5 GFP ON	4	0	39
% VC4-5 GFP ON	7.4074074	0	97.5

Annex 4.2. *mod-5* intron 1 from other species in *C. elegans*

C. castelli constructs in *C. elegans*

Strain	NFB2223	NFB2225	NFB2224
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wild type	wild type	wild type
DNA array	<i>C.castelli</i> (sp.12) <i>mod-5</i>	<i>C.castelli</i> (sp.12) <i>mod-5</i>	<i>C.castelli</i> (sp.12) <i>mod-5</i>
Reporter	<i>prom2</i>	<i>prom2</i>	<i>prom2</i>
Array	<i>vlcEx1195</i>	<i>vlcEx1197</i>	<i>vlcEx1196</i>
line/genotype	L1	L3	L2
N heads	30	31	30
N vulvas	30	31	30
NSM GFP ON	55	46	50
ADF GFP ON	0	0	0
RIH GFP ON	0	0	0
AIM GFP ON	0	3	0
HSN GFP ON	0	0	0
VC1-3 GFP ON	0	0	0
VC4-5 GFP ON	41	37	38
VC6 GFP ON	0	0	0
% VC4-5 GFP ON	68.33	59.68	63.33
mean lines		63.7814	

C. quiockensis constructs in *C. elegans*

Strain	NFB2205	NFB2206	NFB2207
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wild type	wild type	wild type
DNA array	<i>C. quiockensis</i> (sp.38) <i>mod-5</i>	<i>C. quiockensis</i> (sp.38) <i>mod-5</i>	<i>C. quiockensis</i> (sp.38) <i>mod-5</i>
Reporter	<i>prom2</i>	<i>prom2</i>	<i>prom2</i>
Array	<i>vlcEx1177</i>	<i>vlcEx1178</i>	<i>vlcEx1179</i>
line/genotype	L1	L4	L6
N heads	30	32	30
N vulvas	30	31	30
NSM GFP ON	59	55	57
ADF GFP ON	55	61	57
RIH GFP ON	9	14	9
AIM GFP ON	48	59	53
HSN GFP ON	0	0	0
VC1-3 GFP ON	0	0	0
VC4-5 GFP ON	17	27	9
VC6 GFP ON	0	0	0
% VC4-5 GFP ON	28.33	43.55	15
mean lines		28.9606	

C. virilis constructs in *C. elegans*

Strain	NFB2397	NFB2398	NFB2399
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wild type	wild type	wild type
DNA array	<i>C.virilis</i> (sp.13) mod-5	<i>C.virilis</i> (sp.13) mod-5	<i>C.virilis</i> (sp.13) mod-5
Reporter	prom 2	prom 2	prom 2
Array	vlcEx1257	vlcEx1258	vlcEx1259
line/genotype	L1	L2	L3
N heads	50	52	54
N vulvas	50	52	54
NSM GFP ON	100	104	108
ADF GFP ON	0	0	0
RIH GFP ON	0	0	0
AIM GFP ON	0	0	0
HSN GFP ON	0	0	0
VC1-3 GFP ON	0	0	0
VC4-5 GFP ON	0	2	0
VC6 GFP ON	0	0	0
% VC4-5 GFP ON	0	1.92	0
mean lines		0.64103	

Annex 4.3. UNCX binding sites mutated

Carlos Mora results. *unc-4* is essential for *Cang_mod5prom5::gfp* expression in *C. elegans*.

Strain	NFB2176	NFB2177	NFB2227	NFB2228
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wildtype	wildtype	<i>unc-4(gk705)</i>	<i>unc-4(gk705)</i>
DNA array/allele	<i>C.angaria mod-5prom5</i>	<i>C.angaria mod-5prom5</i>	<i>C.angaria mod-5prom5</i>	<i>C.angaria mod-5prom5</i>
Reporter	<i>prom5</i>	<i>prom5</i>	<i>prom5</i>	<i>prom5</i>
Line	L1	L2	L1	L2
N heads	32	32	0	0
N vulvas	32	31	38	38
N NSM	60	56	not scored	not scored
N ADF	0	0	not scored	not scored
N AIM	4	4	not scored	not scored
N HSN	0	0	0	0
N VC1-3	30	26	0	0
N VC4-5	59	52	0	0
N VC6	19	13	0	0
N with unknown	28	24	0	0
%VC4-5 mean	92.19	83.87	0	0
		88.03		0

***Cang_mod5prom9* WT expression in *C. elegans*.**

Strain	NFB2208	NFB2209	NFB2210
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wildtype	wildtype	wildtype
DNA array/allele	<i>vlcEx1180</i>	<i>vlcEx1181</i>	<i>vlcEx1182</i>
Reporter	<i>C.angaria mod-5prom9</i>	<i>C.angaria mod-5prom9</i>	<i>C.angaria mod-5prom9</i>
Line	L1	L2	L3
N heads	33	33	35
N vulvas	33	33	35
N NSM	0	2	0
N ADF	0	2	3
N AIM	0	0	0
N RIH	0	0	0
N HSN	0	0	0
N VC1-3	6	9	1
N VC4-5	58	66	70
N VC6	2	3	1
N with unknown	1	8	0
%VC4-5	87.8787879	100	100
mean	95.959596		

Cang_mod-5prom9_3HD* expression in *C. elegans

Strain	NFB2567	NFB2568	NFB2569
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	<i>wildtype</i>	<i>wildtype</i>	<i>wildtype</i>
DNA array/allele	<i>vlcEx1331</i>	<i>vlcEx1332</i>	<i>vlcEx1333</i>
Reporter	<i>C.angaria mod-5prom9_3HD</i>	<i>C.angaria mod-5prom9_3HD</i>	<i>C.angaria mod-5prom9_3HD</i>
Line	L1	L2	L3
N heads	32	31	36
N vulvas	32	31	36
N NSM	0	0	0
N ADF	2	2	2
N AIM	0	0	0
N RIH	0	0	0
N HSN	0	0	0
N VC1-3	6	2	0
N VC4-5	4	0	0
N VC6	1	3	0
%VC4-5	6.25	0	0
mean	2.08333333		

Cang_mod-5prom9 HD mutated separately in *C. elegans*

Strain	NFB2209	NFB2210	NFB2601	NFB2602
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wildtype	wildtype	wildtype	wildtype
DNA array/allele	<i>vlcEx1181</i>	<i>vlcEx1182</i>	<i>vlcEx1342</i>	<i>vlcEx1343</i>
Reporter	<i>C.angaria mod-5 prom9</i>	<i>C.angaria mod-5 prom9</i>	<i>C. angaria mod-5 prom9_HD#1</i>	<i>C. angaria mod-5 prom9_HD#1</i>
Line	L2	L3	L1	L2
N heads	34	30	30	31
N vulvas	34	30	30	31
N NSM	0	0	0	0
N ADF	1	3	0	0
N AIM	0	0	0	0
N RIH	0	0	0	0
N HSN	0	0	0	0
N VC1-3	9	1	0	0
N VC4-5	68	60	47	36
N VC6	3	1	0	0
%VC4-5	100	100	78.3333333	58.0645161
mean	100		68.1989247	

Species	NFB2590	NFB2591	NFB2592	NFB2593
Background	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
DNA array/allele	<i>wildtype</i>	<i>wildtype</i>	<i>wildtype</i>	<i>wildtype</i>
Reporter	<i>vlcEx1338</i>	<i>vlcEx1339</i>	<i>vlcEx1340</i>	<i>vlcEx1341</i>
Line	<i>C. angaria mod-5 prom9_HD#2</i>	<i>C. angaria mod-5 prom9_HD#2</i>	<i>C. angaria mod-5 prom9_HD#3</i>	<i>C. angaria mod-5 prom9_HD#3</i>
	2		3	
N heads	L1	L2	L1	L2
N vulvas	33	32	32	34
N NSM	33	32	32	34
N ADF	0	0	0	0
N AIM	4	2	0	0
N RIH	0	0	0	0
N HSN	0	0	0	0
N VC1-3	0	0	0	0
N VC4-5	1	1	0	0
N VC6	39	36	1	2
%VC4-5	59.0909091	56.25	1.5625	2.94117647
mean	57.670454		2.2518382	
			4	

Cang_mod-5prom9 WT and 3HD expression in *C. angaria*

Species	<i>C. angaria</i>	<i>C. angaria</i>
Background	wildtype	wildtype
DNA array/allele	F1 scored, no Ex lines	F1 scored, no Ex lines
Reporter	<i>C.angaria mod-5prom9</i>	<i>C.angaria mod5 prom9_3HD</i>
Line	Transgenic F1 scored	Transgenic F1 scored
N heads	28	35
N vulvas	28	35
N NSM	0	0
N ADF	0	0
N AIM	0	0
N RIH	0	0
N HSN	0	0
N VC1-3	0	0
N VC4-5	39	0
N VC6	0	0
N with unknown	0	0
VC4-5 percentage	69.6428571	0

Annex 4.4. 5HT staining in *C. elegans*, *C. angaria* and *C. elegans* (*vlc47 syb7715*), and *C. elegans* (*vlc47 syb7715*); *unc-4(gk705)* mutant

Strain	PHX7715	N2	RGD1
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. angaria</i>
Genotype	<i>mod-5(vlc47 syb7715)</i>	<i>wild type</i>	<i>wild type</i>
N vulvas	40	40	40
VC4-5 5HT	79	10	80
%VC4-5 5HT	98.75	12.5	100

Strain	PHX7715	NFB2843
Species	<i>C. elegans</i>	<i>C. elegans</i>
Genotype	<i>mod-5(vlc47 syb7715)</i>	<i>mod-5(vlc47 syb7715); unc-4(gk705)</i>
N vulvas	42	44
VC4-5 5HT	84	0
%VC4-5 5HT	100	0

Annex 4.5. *mod-5* expression with UNC-4 induction

Figure

4.7A

Strain	NFB593	NFB2619	NFB2620	NFB2621	NFB2622
DNA array	<i>mod-5p3::gfp;</i> <i>rol-6</i>	<i>hsp::unc-4;</i> <i>mod5</i> <i>p3::gfp; rol-6</i>	<i>hsp::unc-4;</i> <i>mod5</i> <i>p3::gfp; rol-6</i>	<i>hsp::unc-4;</i> <i>mod5p3::gfp</i> <i>; rol-6</i>	<i>hsp::unc-4;</i> <i>mod5p3::gfp</i> <i>; rol-6</i>
Reporter	<i>mod-5</i> <i>prom3</i>	<i>mod-5</i> <i>prom3</i>	<i>mod-5</i> <i>prom3</i>	<i>mod-5</i> <i>prom3</i>	<i>mod-5</i> <i>prom3</i>
Array		<i>vlcEx1346</i>	<i>vlcEx1347</i>	<i>vlcEx1348</i>	<i>vlcEx1349</i>
Background	Wild type	Wild type	Wild type	Wild type	Wild type
line/genotype	L1	L1	L2	L3	L4
N vulvas	30	29	17	19	33
VC4/5	0	34	21	26	48
%VC4/5	0	58.620	61.7647	68.4210	72.727

Figure

4.7B

Strain	NFB1719	NFB2617	NFB2618
DNA array		<i>hsp::unc-4</i>	<i>hsp::unc-4</i>
Reporter	<i>vlc47[mod-5::T2A::mNG]</i>	<i>vlc47[mod-5::T2A::mNG]</i>	<i>vlc47[mod-5::T2A::mNG]</i>
Array		<i>vlcEx1344</i>	<i>vlcEx1345</i>
Background	Wild type	Wild type	Wild type
line/genotype		L1	L2
N vulvas	30	29	17
VC4/5	0	0	0
%VC4/5	0	0	0

Figure

4.7C

Strain	NFB2617	NFB2618	NFB2745	NFB2746
DNA array	<i>hsp::unc-4</i>	<i>hsp::unc-4</i>	<i>hsp::unc-4, rol-6, ttx-3</i>	<i>hsp::unc-4, rol-6, ttx-3</i>
Reporter	vlc47[<i>mod-5::T2A::mNG</i>]	vlc47[<i>mod-5::T2A::mNG</i>]	vlc47[<i>mod-5::T2A::mNG</i>]	vlc47[<i>mod-5::T2A::mNG</i>]
Array	vlcEx1344	vlcEx1345	vlcEx1378	vlcEx1379
Background	Wild	Wild	<i>hpl-2</i>	<i>hpl-2</i>
line/genotype	L1	L2	L1	L2
N vulvas	30	32	31	30
VC4/5	0	0	0	0
%VC4/5	0	0	0	0

Annex 4.6 5HT staining with UNC-4 induction.

Figure		4.7 D							
		4.8							
Strain		NFB2620			NFB2621			NFB2622	
DNA array		<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>			<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>			<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>	
Reporter		<i>mod5prom3::gfp</i>			<i>mod5prom3::gfp</i>			<i>mod5prom3::gfp</i>	
Array		<i>vlcEx1347</i>			<i>vlcEx1348</i>			<i>vlcEx1349</i>	
Background		Wild type			Wild type			Wild type	
line/genotype		L1			L2			L3	
N vulvas	Heat Shock	41	41	50	40	46	52	41	49
HSN		82	not scored	not scored	80	not scored	not scored	82	not scored
VC4/5		32	30	26	39	20	30	44	30
%VC4/5		39.02	36.58	26	48.75	21.73	28.8	53.65	30.61
Mean per line				33.86			33.11		42.13
Mean / experiment				33.86			33.11		42.13
N vulvas	NO Heat Shock	40	49	51	42	50	50	41	50
HSN		80	not scored	not scored	84	not scored	not scored	82	not scored
VC4/5		2	6	4	0	0	8	8	0
%VC4/5		2.5	6.12	3.92	0	0	8	9.75	0
Mean per line				4.18			2.6		4.87
Mean per experiment				4.18			2.66		3.78

Figure	1.7 E					
Strain	NFB2620					
DNA array	<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>					
Reporter	mod5prom3::gfp					
Array	vlcEx1347					
Background	Wild type					
line/genotype	L1					
N vulvas	Heat Shock	31	Heat Shock	31		31
HSN	Worms with array	not scored	Worms	not scored	NO heat shock	not scored
VC4/5		36	W/O array	8		6
%VC4/5		58.06		12.90		9.67

Figure		4.7 F			
Strain	NFB2617			NFB2618	
DNA array	<i>hsp::unc-4,rol-6</i>			<i>hsp::unc-4,rol-6</i>	
Reporter	<i>mod-5(vlc47[mod-5::T2A::mNeonGreen]) I</i>			<i>mod-5(vlc47[mod-5::T2A::mNeonGreen]) I</i>	
Array	<i>vlcEx1344</i>			<i>vlcEx1345</i>	
Background	Wild type			Wild type	
line/geno type	L1			L2	
N vulvas	Heat shock	49	36	49	47
HSN		98	72	98	94
VC4/5		16	26	10	27
%VC4/5		16.32	36.11	10.20	28.72
Mean per line			26.21		19.46
N vulvas	NO heat shock	47	45	52	53
HSN		94	90	102	90
VC4/5		0	4	0	3
%VC4/5		0	4.44	0	2.83
Mean per line			2.22		1.41

Figure

4.7 G

Strain	Heat Shock	NFB593			NFB2620			NFB2621		
DNA array		<i>mod5prom3::gfp; rol-6</i>			<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>			<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>		
Reporter		<i>mod5prom3::gfp</i>			<i>mod5prom3::gfp</i>			<i>mod5prom3::gfp</i>		
Array					<i>vlcEx1347</i>			<i>vlcEx1348</i>		
Background		Wild type			Wild type			Wild type		
line/ genotype					L1			L2		
N vulvas		50	50	52	45	50	52	43	50	52
VC4/5		5	10	5	32	44	18	29	48	16
%VC4/5		5	10	4.80	35.55	44	17.30	33.72	48	15.38

Figure

4.7 H

Strain	Heat shock	NFB2668		NFB2669	
DNA array		<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>		<i>hsp::unc-4;mod-5prom3::gfp,rol-6</i>	
Reporter		<i>mod5p3::gfp</i>		<i>mod5p3::gfp</i>	
Array		<i>vlcEx1347</i>		<i>vlcEx1348</i>	
Background		<i>mod-5(knu383)</i>		<i>mod-5(knu383)</i>	
line/genotype		L1		L2	
N vulvas		50	50	49	49
VC4/5		2	2	3	1
%VC4/5		2	2	3.06	1.02
Mean per line		2		2.04	
N vulvas	NO heat shock	53	50	51	50
VC4/5		2	2	0	0
%VC4/5		1.88	2	0	0
Mean per line		1.94		0	

Annex 4.7. *mod-5* expression in VC4 and VC5 upon UNC-4 induction between L4 and YA

Strain		NFB593	NFB2620	NFB2621
DNA array		<i>mod5prom3::gfp; rol-6</i>	<i>hsp::unc-4; mod5prom3::gfp;rol-7</i>	<i>hsp::unc-4; mod5prom3::gfp;rol-8</i>
Reporter Array		<i>mod5p3::gfp</i>	<i>mod5p3::gfp vlcEx1347</i>	<i>mod5p3::gfp vlcEx1348</i>
Background		Wild type	Wild type	Wild type
N vulvas		30	26	29
VC4/5	L4	0	42	52
%VC4/5		0	80.76	89.65
N vulvas		30	28	29
VC4/5	Young adult	0	31	30
%VC4/5		0	55.35	51.72

**Annex 4.8 UNC-4 fluorescence levels in *hpl-2* mutants. CTCF
(corrected total cell fluorescence)**

Strain	Background	Reporter
PHX1658	wildtype	<i>unc-4(syb1658[unc-4::GFP])</i>
NFB2782	<i>hpl-2(tm1489)</i>	<i>unc-4(syb1658[unc-4::GFP])</i>

PHX1658		NFB2782	
1217.23606	2729.18859	161.2433	832.153873
564.04116	2241.45346	239.205972	1842.61688
1289.07216	2025.0822	882.206616	1021.16588
1326.01675	812.753979	791.788506	470.07808
3032.11744	397.825559	1477.16227	1191.94565
814.783662	951.72458	1252.60067	820.387508
1586.20444	558.264542	1451.57208	957.592851
1589.92443	1041.81385	1159.6184	902.314178
2910.91465	1375.28681	683.890896	2283.56636
1686.19994	2485.35307	833.370346	21.80966
1421.20849	2435.59996	837.926735	1535.85737
2059.10463	1102.24705	1095.85075	842.077214
1679.17575	1318.40803	818.387695	1448.42622
1849.13723	205.864637	1021.6817	2047.68709
1034.64036	1148.73086	1196.27384	1944.20818
293.302651	2627.83393	1546.72478	2028.4207
904.099901	1081.99158	933.371435	2353.08365
861.927329	2088.23331	1564.25753	2462.87291
3260.84212	1166.106	199.176148	166.108923
3620.59117	1218.42673	1438.71919	792.883
659.935706	1130.35674	3017.05812	894.643099
1090.48257	2083.8889	2125.49292	724.36187
2926.95501	1860.85154	190.380627	1256.49516
2740.87255	1289.95317	-102.897267	866.934623
2177.24846	1052.40179	290.3932	830.480462
2994.67344	1187.48607	945.459267	710.283309
2274.68362	580.079183		
2442.53572	1279.98044		
1505.89528			

Annex 4.9. Number of eggs laid at 24, 48 and 72h after YA

<i>C. elegans</i> WT N2			<i>C. elegans mod-5</i> VCs			N2 x N2 <i>him-5</i>		
24h	48h	72h	24h	48h	72h	24h	48h	72h
41	104	68	24	--	--	95	129	127
58	100	87	106	127	24	102	124	136
38	109	73	--	--	--	93	63	48
40	99	100	55	34	22	106	121	122
35	106	55	35	130	41	101	132	122
57	94	123	111	98	29	104	111	112
33	101	43	82	101	19	102	100	147
46	124	97	45	49	30	93	103	123
51	97	61	106	99	21	96	121	138
94	103	61	53	69	30	100	105	95
120	95	65	112	81	13	170	140	118
134	88	49	74	115	18	118	132	77
110	114	39	85	86	39	180	171	-
105	115	35	118	85	8	96	99	190
130	111	72	111	97	45	169	167	130
119	118	76	104	89	18	109	122	78
126	121	45	120	124	29	119	126	65
108	97	43	100	96	30	81	126	71
127	87	46	170	41	12	86	123	125
103	101	36	132	96	13	92	95	40
111	102	63	121	120	43	102	126	83
142	101	65	68	1	0	95	140	91
148	105	30	118	80	0	105	98	86
136	132	29	98	63	0	65	128	73
159	114	33	46	1	0	120	115	142
132	141	29	12	4	0	97	151	70
135	128	24	97	13	0	109	0	
160	128	36	84	41	0	121	110	77
132	102	51	82	21	0	113	106	51
158	138	35	76	41	0	137	92	49
115	84	36	0	44	0			
147	109	20	106	90	28			
156	118	54	120	85	33			
148	93	30	106	111	61			
124	99	74	113	70	26			
146	93	61	94	96	63			
128	101	60	60	48	40			
161	100	60	34	53	21			
113	124	63	115	110	24			
109	103	83	80	90	41			
148	90	13	109	97	36			
159	103	35	53	102	45			
121	84	56	108	112	38			
114	115	28	94	111	24			
116	125	30	90	101	56			
140	102	25	81	121	52			
130	135	24	62	-				
122	138	81	110	96	21			
109	143	65	68	102	45			
123	120	31	105	97	30			
108	108	77						
125	127	61						
129	103	41						

<i>C.angaria</i> WT			<i>♀C.ang mod-5(vlc45)</i> x <i>C.ang</i> WT σ		
24h	48h	72h	24h	48h	72h
64	22	1	53	7	0
80	50	0	46	1	0
74	69	1	-	-	-
60	29	0	21	0	-
74	22	0	55	7	0
61	31	11	0	0	0
47	40	6	12	1	-
71	17	0	71	12	0
55	67	14	60	12	0
53	-	-	-	-	-
51	36	0	73	8	-
88	3	0	46	1	0
94	14	0	110	37	0
79	31	0	79	5	1
18	--	--	99	23	0
84	23	0	3	0	0
90	1	0	0	2	0
51	56	0	91	5	0
37	6	0	93	0	3
62	35	0	42	16	0
56	1	0	42	0	0
63	29	0	60	3	0
92	30	0	50	3	0
91	7	1	91	1	0
56	2	0	49	2	0
14	5	3	11	-	-
63	1	0	16	0	0
59	0	-	43	10	0
34	0	0	14	0	1
72	6	0	1	10	0
102	26	0	0	0	0
105	18	0	78	2	0
70	52	0	63	30	1
1	-	-	56	0	0
76	31	0	72	15	0
77	23	0	71	2	0
99	35	0	44	3	0
117	0	0	70	33	0
98	5	0	0	9	0
			23	3	-
			26	5	0

<i>C. quiocensis</i>			<i>C. briggsae</i>		
24h	48h	72h	24h	48h	72h
12	4	0	79	99	64
1	-	-	67	45	14
50	1	2	79	0	0
64	1	0	84	87	43
56	5	0	0	0	0
63	12	1	84	86	53
15	0	1	79	100	76
53	0	3	100	92	56
54	0	0	93	78	48
15	3	0	0	0	0
8	1	-	114	113	66
2	0	-	111	114	90
30	17	1	108	113	82
64	2	1	94	127	68
56	3	2	97	118	81
13	1	0	94	122	69
11	41	0	112	--	
12	0	0	123	65	3
71	22	0	104	108	51
55	4	0	109	113	75
1	1	-	88	84	62
44	18	-	126	119	105
5	0	-	126	94	64
0	0	-	140	130	76
4	0	-	70	93	58
65	0	-	103	105	105
4	0	-	117	96	46
0	0	-	96	110	48
2	0	-	137	106	46
			35	1	75
			104	105	0
			101	110	75

Annex 4.10. Eggs retained

<i>C. elegans</i> WT N2	<i>C. elegans mod-5</i> VCs	<i>C. angaria</i> WT	♀ <i>C.ang mod-5(vlc45)</i> x <i>C.ang</i> WT♂
16	17	2	0
12	21	2	1
12	22	2	2
11	20	2	6
18	19	2	7
12	17	9	2
14	27	1	3
15	26	2	1
13	15	0	5
24	21	1	3
14	19	4	9
19	22	1	16
21	19	8	3
28	21	3	4
10	19	2	1
17	21	3	10
20	27	4	3
19	21	4	1
14	20	6	11
18	19	2	2
23	20	3	14
17	18	0	3
16	26	2	7
17	24	2	6
15	21	1	2
16	33	2	3
16		2	3
13		0	
15		5	
17		1	

Annex 4. 11. Egg laying dynamics

Strain	Species	Background	Condition	Array	n worms
N2	<i>C. elegans</i>	wild type		<i>Ex cat-1promC1:mod-5-T2A-GFP</i>	7
NFB1966	<i>C. elegans</i>	wild type			8
RGD1	<i>C. angaria</i>	wild type	mated		10
NFB2196	<i>C. angaria</i>	<i>mod-5 (vlc45)</i>	mated		6
N2	<i>C. elegans</i>	wild type	mated		10
CB4856	<i>C. elegans</i>	wild type			9

Number of eggs

<i>C.elegans</i> WT	<i>C. elegans</i> <i>mod-5</i> VCs	<i>C. angaria</i> WT	<i>C. angaria mod-5(vlc45)</i>	<i>C.elegans</i> WT N2 mated	<i>C. elegans</i> WT CB4856
45	46	15	19	31	14
62	40	22	10	48	15
54	29	18	22	36	28
47	37	14	19	42	20
49	33	15	18	28	24
57	32	24	19	28	15
57	46	29		30	16
	37	31		27	21
		11		34	18
		12		36	

Number of egg laying windows

<i>C. elegans</i> WT	<i>C. elegans</i> <i>mod-5</i> VCs	<i>C. angaria</i> WT	<i>C. angaria mod-5(vlc45)</i>	<i>C. elegans</i> WT N2 mated	<i>C. elegans</i> WT CB4856
17	19	13	18	12	12
17	11	21	10	15	14
16	14	13	18	12	7
14	16	12	17	13	11
20	14	13	17	11	13
20	15	17	16	9	13
18	16	21		11	10
	16	28		9	11
		10		13	12
		11		18	

Eggs per egg laying window

<i>C.elegans</i> WT		<i>C. elegans</i> <i>mod-5</i> VCs		<i>C. angaria</i> WT			<i>C. angaria</i> <i>mod-5(vlc45)</i>		<i>C.elegans</i> WT N2 mated		<i>C. elegans</i> WT CB4856	
4	3	2	1	2	1	1	2	1	3	3	1	2
2	1	3	3	1	1	1	1	1	5	1	1	1
3	3	5	3	1	2	2	1	1	2	1	1	2
1	3	2	3	1	1	1	1	1	3	3	1	2
2	3	3	1	1	1	2	1	1	2	2	1	1
1	1	2	3	1	2	1	1	1	2	2	1	1
4	4	2	1	1	2	1	1	1	3	4	1	2
2	7	1	2	1	2	1	1	1	2	1	1	1
3	3	3	3	1	1	1	1	1	1	3	1	3
2	4	5	1	2	1	1	1	1	2	5	1	4
2	5	2	3	1	1	1	1	1	2	2	2	2
1	5	1	2	1	1	1	1	1	4	4	2	2
1	5	3	1	1	1	1	1	1	7	2	1	2
3	4	4	1	1	2	1	1	1	2	2	3	1
5	2	1	1	1	1	1	1	1	4	3	2	1
6	5	1	1	1	1	2	1	1	4	3	2	1
3	3	1	1	1	1	1	1	1	2	3	1	1
1	4	2	2	1	2	1	1	1	3	5	1	1
5	2	3	2	1	1	1	1	1	3	4	1	1
6	7	2	2	2	1	1	1	2	1	5	1	1
5	2	4	2	1	1	1	1	1	1	4	1	4
3	1	4	2	1	1	1	1	2	5	2	1	1
5	3	7	2	1	2	1	1	1	4	3	1	1
3	4	3	1	1	2	1	1	1	1	1	4	5
5	1	3	3	1	1	2	1	1	5	1	2	1
5	5	4	3	1	2	1	1	2	5	2	1	1
5	1	1	4	1	1	1	1	1	1	4	4	1
3	4	3	4	1	2	1	1	1	2	2	3	1
3	4	5	1	1	1	2	1	1	3	4	5	2
3	2	4	1	1	2	1	1	1	1	2	2	1
3	5	2	6	1	1	1	1	2	5	3	3	2
3	5	4	2	1	1	2	1	1	2	4	2	2
2	4	1	5	1	1	1	1	1	3	1	2	1
4	4	3	1	1	1	1	1	1	5	3	1	1
2	3	3	8	2	1	2	2	1	6	2	1	3
5	3	2	1	1	1	2	1	2	2	7	2	1
1	4	2	3	1	1	2	1	1	2	3	1	2
5	1	3	4	1	2	1	1	1	3	3	4	1
2	3	2	3	2	1	2	1	1	2	1	1	1
6	1	1	4	1	1	1	1	1	1	3	2	2
2	4	1	2	2	1	1	2	1	2	4	2	2
3	4	1	1	1	1	1	1	1	4	1	1	4
3	1	2	1	1	1	1	2	1	2	1	2	1
1	1	2	1	1	1	1	1	1	5	1	1	1
2	4	4	3	2	1	2	1	1	4	4	1	1
1	6	3	3	1	1	1	2	1	2	1	1	2
4	2	2	3	2	1	1	1	1	4	4	2	2
5	2	1	2	1	1	1	1	2	2	1	1	2
6	2	5	2	1	1	1	1	2	8	2	1	1

<i>C.elegans</i> WT	<i>C. elegans mod-5</i> VCs	<i>C. angaria</i> WT	<i>C. angaria mod-5(vlc45)</i>	<i>C.elegans</i> WT N2 mated
1 2	4 2	1 1 1	3 1	3 1
1 2	4 2	1 1 1	3 6	1 2
1 1	1 3	1 1 1	2 5	1
1 4	2 2	1 1 2	3 4	
3 1	3 1		1 1	
2 1	1 3		1 2	
5 5	1 1		4 2	
4 4	1 4		2 1	
1 4	2 3		2 1	
2 2	1 3		1 1	
2 1	2 3		3 2	
1 3	6		2 4	
			2	

Egg laying window length

<i>C. elegans</i> WT		<i>C. elegans mod-5</i> VCs		<i>C. elegans</i> WT N2 mated		<i>C. elegans</i> WT CB4856	
122.5	111.11	5.55	20	50	0	0	0
10	22.22	2.77	5	55	25	0	0
52.5	0	16.66	5	0	10	0	0
0	0	5.55	0	35	0	0	0
0	0	8.33	5	0	75	0	140
0	0	2.778	0	0	0	0	0
142.5	102.277	0	0	50	0	0	0
67.5	68.181	0	30	0	0	0	130
65	131.81	16.66	35	0	95	0	0
0	54.545	113.90	50	5	0	0	0
2.5	0	2.7781	0	60	20	0	0
0	0	0	0	180	0	0	0
0	27.272	2.778	25	110	30	0	0
65	0	16.668	5	5	105	50	0
107.5	38.636	0	50	10	0	10	0
80	0	0	0	20	0	80	15
57.5	56.818	0	90	10	0	0	0
0	36.363	2.778	0	5	35	0	0
153.859	150	8.334	10	35	0	0	90
105.778	25	7.692	15	0	5	0	0
59.620	45.454	51.92	5	0	0	0	0
90.392	93.181	3.846	15	80	0	0	0
80.776	84.090	61.546	10	10	0	0	0
32.695	40.909	9.6162	0	0	55	40	0
0	45.45	55.774	0	100	90	65	0
19.23	13.888	15.385	0	120	145	0	0
63.467	50	0	10	0	0	65	0
5.769	97.222	21.155	15	0	5	5	70
0	5.555	98.085	5	115	0	110	35
30.7719	94.444	40.388	5	0	0	10	0
1008904	0	4.167	0	70	0	60	0

<i>C. elegans</i> WT		<i>C. elegans mod-5</i> VCs		<i>C.elegans</i> WT N2 mated		<i>C. elegans</i> WT CB4856	
7.692	58.333	16.668	0	5	0	55	5
57.697	19.444	0	0	20	0	0	0
96.162	113.888	4.167	20	90	0	0	0
167.5	11.111	8.334	10	90	0	0	0
75	108.333333	4.167068	0	0	60	0	0
122.5	63.888	0	5	5	90	0	0
55	0	6.2506	0	5	0	130	0
132.5	16.6666	8.334	85	10	0	0	0
5	0	0	60	0	20	20	0
50	97.222	0	65	15	0	25	0
7.5	77.777	0	20	15	0	0	
50	136.111	4.167	105.778	0	175	55	
7.5	88.888	0	15.385	100	5	0	
0	0	86.546	0	55	10	0	
55	0	7.6929	7.692	10	20	0	
57.5	27.777	1.92324	0	30	20	5	
0	97.222	0	5.7697	5	55	0	
65	22.222	11.539	3.8464	60	10	0	
0	0	94.239	0	80	0	115	
69.230	0	88.469	23.0789	15	5	0	
76.923	0	0	1.9232	5	0	0	
0	0	13.46	0	80	0	85	
113.46	0	59.620	0	35	0	0	
55.76	8.3333	0	0	0	85	0	
103.846	0	0	0	0	90	0	
7.69230	0	0	0	135	5	0	
86.5384	41.6666	92.3159	5	10	30	5	
55.7692	0	0	0	0	0	70	
146.153	42.3076	55.7741	5.769	90	0	0	
0	3.84615	71.1601		0	5	0	
				10		0	

Egg laying window length

	<i>C. angaria</i> WT				<i>C. angaria mod-5(vlc45)</i>		
0	0	0	65	8.92	0	0	
0	40	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	6.81746	0	0	0	
0	0	0	0	0	0	0	
0	15	0	0	0	0	0	
0	0	0	0	0	0	0	
0	0	0	0	0	65		
0	5	0	0	0	0		
32.5	0	0	0	0	105		
0	0	0	70	0			
0	25	0	0	0	0		
0	30	0	0	0	0		
0	110	0	0	0	25		
0	0	0	30	0	0		
0	0	0	0	0	0		
0	0	0	0	0	0		
0	0	0	30	0	0		
0	0	0	0	0	0		
67.307	100	0	0	0	90		
0	0	0	25	0	0		
0	0	0	25	0	0		
0	0	30	65	0	0		
0	30	0	0	0	0		
0	0	5	75	0	0		
0	0	0	0	0	0		
0	0	0	0	0	0		
0	0	0	0	0	0		
0	25	0	0	0	0		
0	10	0	0	0	0		
0	0	0	0	0	0		
0	65	0	6.817	0	0		
0	0	0	0	0	0		
0	45	0	0	0	0		
52.777	0	0	0	15	0		
0	60	0		0	25		
0	0	0		0	0		
0	0	30		0	0		
22.22	0	0		0	50		
0	0	0		0	0		
61.111	0	30		35	0		
0	0	0		0	0		
0	0	0		15	0		
0	45	25		0	60		
38.888	0	25		0	0		

Active interval length

<i>C. elegans</i> WT				<i>C. elegans</i> <i>mod-5</i> VCs			<i>C. elegans</i> WT N2 mated				<i>C. elegans</i> WT CB4856
110	8.33	0	0	5.56	30	4.17	50	5	35	5	0
2.5	58.33	82.5	13.89	2.78	15	0	0	0	0	80	0
10	5.56	22.5	50	0	0	71.16	10	0	0	5	10
10.	2.78	62.5	19.44	8.33	5	5.77	5	10	5	5	40
10.	33.33	5	8.33	2.78	5	9.62	40	5	5	20	10
42	16.67	7.5	69.44	5.56	10	3.85	0	5	0	5	80
0	19.44	22.5	5.56	0	5	3.85	0	5	10	0	0
70	36.11	40	8.33	5.56	5	1.92	10	5	15	0	40
15	5.56	70	19.44	5.56	5	0	25	5	0	5	0
57	11.11	52.5	9.65	2.78	0	7.69	0	10	15		65
67.5	11.11	0	0	2.78	40	3.85	0	5	0		30
65	50	55	25	0	5	0	0	5	0		10
0	11.11	0	0	5.56	5	0	50	45	5		25
0	81.52	92.5	30.77	11.11	5	5.77	0	5	40		5
2.5	2.78	0	0	2.78	5	88.47	5	0	0		0
0	30.56	40	75	108.35	20	84.62	60	5	55		25
65	33.33	5	0	2.78	5	3.85	10	5	0		0
5	16.67	0	28.85	0	50	0	55	0	10		65
10	13.89	20	5.77	2.78	0	13.46	115	0	45		20
70	30.56	27.5	1.92	0	5	25	15	5	10		10
22.5	52.78	2.5	0	2.78	5	34.62	20	0	10		60
32.5	13.89	7.5	36.54	0	0	92.32	0	0	20		0
0	11.11	10	50	8.33	10		25	5	0		55
32.5	33.33	0	46.15	8.33	5		0	5	5		0.
0	19.44	15	9.62	2.78	5		50	75	15		0
15	16.67	17.5	61.54	8.33	0		5	0	5		25
57.5	52.78	7.5	84.62	0	5		5	15	10		20
0	8.33	0	0	7.69	5		5	10	5		85
50	19.44	7.5	3.85	3.85	5		0	0	5		20
5.77	38.89	2.5	0	44.23	10		5	10	20		25
90.39	63.89	52.5	15.38	3.85	10		15	0	0		55
7.69	47.22	0	26.92	1.92	0		0	0	70		5
7.69	0	2.5	0	1.92	5		10	0	10		115
0	5.56	55	69.44	0	10		0	75	5		0
17.31	16.67	10	0	3.85	0		5	0	10		0
80.78	0	27.5	19.44	1.92	9.62		5	0	5		0
0	79.55	0	0	1.92	36.54		30	0	20		70
5.77	22.73	27.5	27.78	53.85	0		5	0	60		0
0	68.18	0	0	0	3.85		65	0	10		0
53.85	54.55	46.15	0	0	1.92		10	10	60		35
0	52.27	23.08	33.33	3.85	101.93		0	30	0		0
0	13.64	13.46	2.78	5.77	3.85		0	5	85		0
90.39	11.36	63.46	0	3.85	11.54		10	0	5		0
0	54.55	0	61.11	51.93	3.85		0	50	0		5
23.08	0	0	22.22	3.85	3.85		0	0	0		0
50	0	9.62	0	5.77	3.85		5	0	50		110
7.69	0	1.92	0	5.77	5.77		95	20	0		30
32.70	27.27	19.44	0	13.46	3.85		0	0	10		55
0	38.64	0	0	7.69	0		5	30	90		0
19.23	0	22.22	8.33	1.92	9.62		25	0	0		65
0	56.82	0	0	3.85	13.46		90	10	5		10
57.70	36.36		0	90.39	1.92		0	0	15		0

<i>C.elegans</i> WT				<i>C. elegans</i> <i>mod-5</i> VCs		<i>C.elegans</i> WT N2 mated			<i>C. elegans</i> WT CB4856	
5.77	0	5.77	9.09	1.92	5	0	95	0	0	0
5.77	25	75.01	31.82	25	20	115	5	0	15	15
0	27.27	7.69	2.27	1.92	5	0	25	65	0	0
0	97.73	7.69	43.18	13.46	5	25	5	90	90	90
30.77	0			4.17	0	40	5	20	0	0
0	18.18			12.5	5	5	0	25	85	85
13.46	0			4.17	0	0	0	35	0	0
0	6.82			0	0	5	0	100	0	0
7.69	0			4.17	25	15	0	10	5	5
25	29.55			0	5	5	10	0	70	70
23.08	4.55			4.17	5	5	0	20	0	0
30.77	11.36			4.17	25	0	45	20	0	0
0	0			4.17	5	40	0	50	0	0
7.69	61.36			0	5	45	0	0	0	0
7.69	31.82			4.17	55.77	5	10	10	0	0
0	6.82			2.08	3.85	0	0	5	0	0
50	77.27			8.33	21.16	50	80	0		

<i>C. angaria</i> WT		<i>C. angaria mod-5(vlc45)</i>	
0	60	8.93	
32.5	20	15	
67.31	0	35	
52.78	5	15	
22.22	0	25	
61.11	5	50	
38.89	40	60	
75	15	90	
6.82	5	65	
6.82	25	105	
70	30	25	
30	110		
30	100		
25	30		
25	25		
65	10		
0	65		
0	45		
15	60		
5	45		
10	30		
5	5		
0	5		
10	55		
5	5		
70			

Inactive interval length

<i>C.elegans</i> WT			<i>C. elegans</i> VC4-VC5prom:: <i>mod-5</i>			<i>C.elegans</i> WT N2 mated			<i>C. elegans</i> WT CB4856			
555	1080.7	1125	388.94	1319.3	126	365	121	227	219	160	148	206
	7			5	0	0	0	0	5	5	5	5
1030	1613.4	458.33	1975.2	898.16	435	147	194	187	292	267	755	190
	6		5			0	0	5	0	5		5
1175	1746.1	844.44	1541.8	1829.0	145	147	214	137	141	950	116	318
	5		6	1		5	0	5	0		5	0
287.50	1621.1	547.22	1577.9	1840.5	121	114	140	169	810	235	145	625
	5		8	5	0	0		5			5	
1712.5	2092.3	1358.3	1164.0	638.52	170	154	182	144	180	194	143	187
0	1	3	4			0	5	0	5	0	0	0
1090	1853.8	1308.3	1261.2	276.95	270	132	327	200	202	615	295	124
	5	3	7			0	0		0			5
152.50	1138.4	761.11	408.39	346.18	272	204	138	230	173	241	168	163
	6				0	0	0		5	5	5	5
2972.5	928.85	1427.7	1650.2	2752.1	102	765	130	147	149	299	570	109
0		8	1	7	5		0	0	5	5		0
240	1646.1	1108.3	694.53	1075.1	116	188	950	860	186	130	111	205
	5	3		0	0	5			5	0	0	
1185	1367.3	713.89	1502.9	815.46	500	102	228	180	130	175	625	119
	1		7			5	0		5			5
232.50	382.69	1350	141.68	286.56	740	199	227	205	183	384	685	152
						5	5	5	0	5		5
932.50	846.15	1525	966.79	3708.0	185	318	485	165	159	222	555	178
				2	0	0			0	5		5

<i>C.elegans</i> WT			<i>C. elegans</i> VC4-VC5prom:: <i>mod-5</i>			<i>C.elegans</i> WT N2 mated			<i>C. elegans</i> WT CB4856			
1610	1746.1 5	944.44	1250.16	1532.83	235	760	1300	1950	526 5	207 5	120 5	143 0
2327.5 0	1050	294.44	194.47	1577.06	1265	1860	2295	765	272 0	735	800	263 0
1860	369.44	1175	1075.14	3398.38	1315	1635	3145	760	185 0	383 5	380	193 5
1122.5 0	300	1413.8 9	1189.04	965.47	770	220	2440	1150	985	317 0	885	115 5
144.24	1047.2 2	2430.5 6	1291.83	934.70	945	1215	4940	1570	160 0	150	100 0	294 5
286.56	3475	2002.7 8	425.05	125.01	2750	1115	4550	1520	193 0	143 5	330	197 0
1915.5 6	150	1395.4 5	2673.31	644.29	1060	650	1175	1610	122 0	444 0	100 5	185
813.53	1316.6 7	1431.8 2	1211.65	1067.40	496.20	805	2725	2260	650	625	105 0	306 5
865.46	1388.8 9	852.27	3246.44	2100.19	761.61	1720	1825	860	135 0	412 0	690	153 5
1084.7 1	886.11	125	1938.63	432.73	194.25	2115	1280	1505	255 5	555 0	970	308 0
1221.2 6	302.78	1445.4 5	609.67	2270	2529.07	805	3240	1895	700	159 5	335	188 0
1313.5 8	744.44	1565.9 1	1188.57	490	2225.20	1180	205	1245	105 5	920	940	166 0
1227.0 3	1000	1170.4 5	1742.46	865	1111.64	2505	150	1655	200 0	176 5	690	950
578.90	433.33	1645.4 5	1890.55	1730	610	1190	2580	3040	357 0	187 5	460	295
871.23	1436.1 1	852.27	1159.72	1020	385	2355	2575	975	211 5	165 0	930	188 5
694.29	1769.4 4	2710	2050.18	1150	640	2295	1950	1055	236 5	130	101 0	230
3006.0 4	1391.6 7	172.50	1514.73	305	1490	4095	1750	720	219 5	240	215	5
1915.5 6	897.22	905	1664.74	1180	2230	955	1195	930	124 5	266 0	124 0	5
1184.7 2	897.22	190	1231.37	2425	1630	965	1120	2515	209 5	182 0	735	0
1000.0 9	244.44	927.50	3131.55	1415	1814.76	3345	1885	2820	635	236 0	109 5	221
890	579.55	1297.5 0	860.50	390	3280	1650	850	635	590	130	665	358
782.50	1688.6 4	250	1818.93	1470	1415	1810	4170	675	398 0	332 5	425	323
2825	172.73	1312.5 0	1031.35	1945	1712.67	685	3135	710	201 5	150	156 5	
1080	2697.7 3	1120.4 5	1027.18	2305		850	1755		340	825	400	
2307.5 0	975	1175	1279.29	1355		145	2250		169 5	600	303 0	
695	602.27		583.39	1410		1480	1395		119 5	755	162 5	
2085	122.73		1237.62	1175		2375	1385		122 0	127 0	800	

Inactive interval length

<i>C. angaria wild type</i>				<i>C. angaria mod-5(vlc45)</i>	
342.5	1455	1124.88	625	1462.62	120
1602.5	1430	161.35	1870	1748.36	1415
1727.5	295	2070.24	1245	332.17	1860
942.5	1685	1270.32	1635	1076.87	945
2212.5	570	1097.61	2305	3119.90	305
1727.5	1110	2467.92	1485	1284.03	1340
1617.5	625	1313.5	755	1492.98	1615
2337.5	685	952.17	1165	878.64	1760
2042.5	555	1960	755	319.67	175
1345	1205	2340	1030	2550.21	2770
847.5	800	570	1490	982.22	1485
2230	380	1625	625	764.35	645
144.23	885	2240	950	846.5	1155
930.77	1000	1895	2065	1321.54	255
251.92	330	1955	1905	2237.68	1090
1984.62	1005	1770	3180	542.90	875
157.69	1050	325	1419.44	155.37	150
1369.23	690	3420	331.78	475	2085
1786.54	970	135	2092.96	1670	1930
244.23	335	1850	661.29	515	1190
1313.46	940	1360	670	3705	985
186.54	690	360	820	120	835
1292.31	460	1785	1880	3040	1530
146.15	930	2180	1660	1590	1040
1819.23	1010	1880	1777.78	955	1300
205.77	215	1705	2736.11	1270	365
1430.77	1240	335	955	295	1965
148.08	735	1170	645	525	325
1921.15	1095	1125	1535	1535	3805
292.31	665	430	3080	1695	1795
1303.85	425	1830	694.44	780	145
515.38	1565	1610	2591.67	975	165
672.22	400	165		1625	1150
1358.33	3030	1670		400	1205
591.67	1625	1355		1240	1765
1886.11	800	1665		400	355
3222.22	2215	1300		1925	1815
905.56	1885	785		1320	1860
1791.67	3065	1100		1165	1435
				2170	1385
				1530	925
				265	1920
				1770	170
				645	
				1150	
				1555	
				1660	

Annex 4.12. Egg-laying in worms exposed to 5-HT

C. elegans strains 5mM 5-HT					
CONTROL			5mM 5-HT		
C. elegans N2	C. elegans mod-5 VCs	C. elegans intron (vlc47 syb7715)	C. elegans N2	C. elegans mod-5 VCs	C. elegans intron (vlc47 syb7715)
0	0	0	6	0	10
0	0	0	7	2	6
0	0	0	10	0	0
0	0	0	5	0	6
0	0	0	3	0	0
7	0	4	5	0	13
1	0	2	8	4	0
0	0	6	9	0	14
1	0	3	1	0	1
2	0	1	9	1	6
0	0	0	11	2	0
0	0	0	6	0	0
0	0	1	9	0	7
0	0	0	1	0	0
0	0	0	6	0	0
0	0	0	16	0	8
0	0	0	10	0	0
0	0	0	8	0	10
0	0	0	10	1	1
0	0	0	2	0	4
0	0	0	0	0	4
0	0	0	2	2	0
0	0	0	3	0	7
0	0	0	4	0	0
0	0	0	7	0	4
0	0	0	0	1	3
0	0	0	8	0	5
0	0	0	8	0	0
0	0	0	7		1
0		4	5		6
0		0	3		2
0		0	0		9
0		0	2		3
1		0	0		1
5		0	0		5
		0			0

C. angaria strains 5mM 5-HT

CONTROL		5mM 5-HT	
<i>C. angaria</i> RGD1	<i>C. angaria mod-5(vlc45)</i>	<i>C. angaria</i> RGD1	<i>C. angaria mod-5(vlc45)</i>
4	5	5	5
2	7	3	11
5	3	6	0
3	6	9	9
3	0	6	1
5	2	4	0
5	3	5	3
0	0	0	3
4	5	4	5
1	0	4	6
0	3	0	10
4	0	5	4

Annex 4.13. VCs reuptake exogenous 5-HT

Strain	PHX7715	NFB2803		NFB2803	
Species	<i>C. elegans</i>	<i>C. elegans</i>		<i>C. elegans</i>	
Genotype	<i>mod-5(vlc47 syb7715)</i>	<i>bas-1(ad446); mod-5(vlc47 syb7715)</i>		<i>bas-1(ad446); mod-5(vlc47 syb7715)</i>	
Transgene	<i>C.ang intron 1 mod-5::T2A::mNG</i>	<i>C.ang intron 1 mod-5::T2A::mNG</i>		<i>C.ang intron 1 mod-5::T2A::mNG</i>	
Growth conditions	Control	Control		5HT	
N vulvas	40	48	50	49	51
N VC4-5	80	0	0	98	88
% ON VC4-5	100	0	0	100	86.2745098
Mean	100		0		93.1372549

Annex 4.14. Wild isolates VC4 and VC5 serotonergic phenotype

<i>C. elegans</i> strains	Not synchronized		Synchronized 1		Synchronized 2	
	VC 4/5 ON	%VC4- 5ON	VC 4/5 ON	%VC4- 5ON	VC 4/5 ON	%VC4- 5ON
QX1211	0	0	56	66.67		
JU1581	8	12.50				
MY23			32		11	12.50
ED3017	12	21.43	21	26.58	30	35.71
N2	16	21.62	24	24.24	45	50
DL200	24	33.33	18	21.18		
JU258	30	44.12	57	72.15	49	56.98
MY16	32	44.44	66	78.57	35	39.77
JJ11398	33	47.14	42	51.22	41	43.62
ED3049	38	54.29	74	89.16	47	55.95
DL238	40	57.14	56	65.12	14	16.67
JU1580	41	56.94	35	38.89		
CB4856	50	69.44	61	72.62	55	58.51
CX11314	53	75.71	37	43.02	70	79.55
LKC34	54	75	25	29.76	63	70
EG4725	56	77.78	60	70.59	50	56.82
JU1440	57	71.25	27	32.53	72	82.76
JU775	59	84.29	60	75		
JU782	57	81.43	65	79.27	33	35.87

Annex 1.15. 5-HT intensity analysis in VC4 and VC5 between N2 and *him-5(e1490)* (corrected total cell fluorescence)

Strain	Background
N2	wildtype
NFB2676	<i>him-5(e1490)</i>

N2		<i>him-5(e1490)</i>	
2279,14747	2923,14327	13917,2898	366,236
4090,20967	2243,0159	-619,636233	386,1834
1051,0728	2502,0825	5494,6435	1380,047
1186,2795	2142,48267	1080,262	2796,54277
481,6777	1622,7324	353,3851	2189,32083
1872,6752	2119,4364	3112,8592	951,0548
2061,0283	2023,2069	1934,151	1525,6036
1550,01477	4997,19767	124,871867	3619,622
1320,9425	313,0817	1966,6495	1014,48033
1482,65507	-272,9044	2493,5365	1135,2552
-193,154933	44,3608	889,228	745,539733
106,8465	2745,58107	-886,8194	1599,67373
1105,6125	2145,973	-231,3976	497,775367
1681,68197	2520,81373	489,192533	1328,98823
247,558667	2793,1572	5840,25133	-22,8784333
324,21	1334,157	1460,9046	1388,652
2981,3327	3964,0844	1722,06353	1277,90467
1022,0679	3003,8308	2616,47267	-199,2646
250,2454	1091,7242	3351,363	1259,0092
1495,796	289,5612	7635,65617	
537,7882	1897,4466	4563,68073	
-780,782733	1946,1756	7003,3596	
366,2571	1018,24133	3318,6336	
289,1908	1525,28477	824,637	
93,6324	1858,06573	1865,2375	
37,3384	1629,99827	2408,6555	
-24,2175	1121,8944	2028,0944	
-185,576667	1835,0264	-2100,60303	
3819,1734	3126,309	6410,05073	
3698,1716	584,8387	3098,77267	
1120,2114	1360,63347	507,193333	
2042,6879	4818,116	438,739833	
1424,4058	394,461333	9291,1209	
11,5306	822,279333	7699,722	
1295,3132	314,0274	-430,7921	
3995,04933	350,5046	-115,2916	
1203,68637	2501,7214	5945,2823	
660,462	1453,1824	2862,715	
1551,5172	-180,462667	554,770867	
1498,342	190,2188	2216,84747	
808,791	498,2888	1009,43467	
921,694767	340,6104	488,695933	
842,486167	1574,4706	16880,3376	
451,5806	1382,12743	12762,2398	
2608,7394	4350,29053	4617,31887	
4450,91387	2904,8356	3028,37727	
1383,45537		1551,403	

Annex 4.16. Worms cultured in 10mM 5-HT

Strain	N2						N2			
Genotype	<i>C. elegans</i> wildtype						<i>C. elegans</i> wildtype			
Growth conditions	control			5-HT 10mM			control		5-HT 10mM	
Generation	3rd						6th			
N vulvas	40			40			40		40	
N ON VC4-5	39			70			41		78	
%ON VC4-5	48.75			87.5			51.25		97.5	
N FAINT VC4-5	1			0			15		0	
% FAINT VC4-6	1.25			0			18.75		0	

Species	<i>C. elegans</i>									
Genotype	wildtype						<i>bas-1(ad446)</i>			
Growth conditions	control			5-HT 10mM			control		5-HT 10mM	
Generation				1st						
N vulvas	52	53	51	35	50	52	48	50	49	51
N ON VC4-5	34	6	3	68	87	89	0	0	98	85
%ON VC4-5	32.6923 077	5.66037 736	2.94117 647	97.1428 571	87	85.5 7692 31	0	0	100	83.3333 333
N FAINT VC4-5	7	16	4	0	4	7	0	0	0	3
%FAINT VC4-5	6.73076 923	15.0943 396	3.92156 863	0	4	6.73 0769 23	0	0	0	2.94117 647
Mean ON	13.7646 205			89.9065 934			0		91.6666 667	
Mean FAINT	8.58222 583			3.57692 308			0		1.47058 824	
N heads									49	49
N ON NSM									75	40
N ON RIH									17	0
N ON AIM									63	22

Annex 4.17. Intensity analysis of worms cultured in 5-HT

N2 control		N2 5-HT 10mM	
1889,86067	8128,59317	157756,36	23416,41
6401,52	6847,06517	18543,7497	12927,311
3681,4976	1211,64213	148183,116	169937,061
1653,016	1143,46077	225562,158	178642,529
7960,92267	8071,6746	95920,558	61814,6767
327,436067	-325,078933	81671,3393	23327,28
3893,15813	112,7878	79326,4615	36652,7605
5412,50387	27,342	111593,208	43804,5191
	1459,1084	30923,982	8744,8219
4575,6176	5571,29067	60405,4259	7161,23533
213,9135	-497,719833	93040,5843	102181,811
1031,62497	-1778,9249	52595,6052	78357,6564
5560,0442	527,7248	36831,27	54701,1668
6928,0024	224,306933	28778,9891	65385,2634
242,257667	104,0828	40885,7405	36846,7597
221,79	1250,50253	26070,6737	23305,4206
-252,229467	6291,14893	221303,992	52861,8248
201,989033	658,819	152628,157	66827,8417
731,0064	821,1046	221921,687	96790,4446
1254,65303	3648,932	182566,507	
2817,8785	225,622333	118869,836	
982,710667	280,115833	96845,0546	
6584,22833	24,6938	52058,7024	
5740,99613	6281,90193	33465,8678	
423,4292	8997,1598	7196,51947	
247,9965	5102,679	17917,8404	
131,5512		19227,2943	
-49,6925		2017,68367	
2754,63907		2996,73367	
6082,81917		95862,1806	
8472,6934		122675,256	
3546,6244		20525,5776	
8327,281		57722,3234	
8419,10043		41178,4887	
561,665833		25837,7033	
69,1451333		72978,8148	
2856,90393		17667,06	
1617,26033		106669,256	
-19,1411		198439,44	
61,4863		6189,3584	
4424,7139		7225,56733	
1583,86267		41016,8084	
9469,8172		28920,948	
3747,24873		48571,7487	
79,0562667		76998,0964	
1323,3636		84364,736	
-1452,55933		34367,3263	
5008,59		158158,086	
11323,8643		176368,585	
3404,4142			

Annex 1.18. 5-HT staining in other species cultured in 10mM of 5-HT

Strain	AF16						JU1373					
Species	<i>C. briggsae</i>						<i>C. tropicalis</i>					
Genotype	wildtype						wildtype					
Growth conditions	control			5-HT 10mM			control			5-HT 10mM		
Generation	1st	1st	1st	1st	1st	1st	1st	1st	1st	1st	1st	1st
N vulvas	40	41	40	40	41	40	42	40	40	41	40	38
N ON VC4-5	0	0	0	0	0	0	0	0	0	0	0	0
%ON VC4-5	0	0	0	0	0	0	0	0	0	0	0	0

Annex 1.19. 5-HT staining in *mod-5(n822)*, *mod-5(knu383)* and *unc-4(gk705)* cultured in 10mM of 5-HT

Strain	VC1528		MT8944		COP1365	
Species	<i>C. elegans</i>		<i>C. elegans</i>		<i>C. elegans</i>	
Genotype	<i>unc-4(gk705)</i>		<i>mod-5(n822)</i>		<i>mod-5(knu383)</i>	
Growth conditions	contr ol	5-HT 10mM	control	5-HT 10mM	contr ol	5-HT 10mM
Generation	1st	1st	1st	1st	1st	1st
N vulvas	40	40	43	42	43	40
N ON VC4-5	0	14	4	30	0	68
%ON VC4-5	0	17.5	4.65116279	35.7142857	0	85

Annex 1.20. Intensity analysis of 5-HT staining in different strains cultured in 10mM of 5-HT

Strain	N2	COP1365	MT8944	VC1528	MT7988	NFB2844
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wildtype	<i>mod-5(knu383)</i>	<i>mod-5(n822)</i>	<i>unc-4(gk705)</i>	<i>bas-1(ad446)</i>	<i>bas-1(ad446), mod-5(knu383)</i>

WT	WT 5-HT	<i>mod-5(knu383)</i> Control	<i>mod-5(knu383)</i> 5-HT	<i>mod-5(n822)</i> Control	<i>mod-5(n822)</i> 5-HT
-67.41	899.75	98.29	762.39	94.37	97.37
24.42	1369.88	-95.66	498.38	40.15	29.32
51.78	2466.48	63.62	695.54	-23.24	14.32
-55.03	893.09	77.76	160.87	1.48	73.43
70.91	1897.85	22.69	240.63	406.52	57.05
-41.31	1566.22	33.19	365.13	263.20	1263.97
16.31	12625.81	182.33	415.05	-25.50	1210.96
166.39	22693.74	136.57	123.84	5.95	16.38
734.08	1666.69	166.22	967.38	84.58	7.01
71.88	677.83	433.42	809.44	68.08	195.68
-5.83	170.28	27.95	555.06	54.85	110.25
-113.45	135.10	56.84	727.15	157.94	3.93
19.26	4016.38	-151.32	753.67	471.25	-36.42
130.65	6176.07	80.91	645.62	62.60	967.91
-41.55	29138.59	134	460.04	-117.70	1910.03
-84.26	17745.84	-35.30	110.07	-23.40	3441.24
-46.52	6737.09	-14.68	304.79	-88.57	1032.97
-46.02	6439.96	88.72	396.37	269.63	5575.01
6.16	5489.66	-12.20	248.26	414.32	2687.52
-29.69	5060.62	112.66	339.51	212.21	4349.31
48.62	2712.08	159.40	-75.42	82.18	6.35
90.78	964.36	100.07	8.57	-9.57	0.22
127.75	2634.67	46.02	280.33	60.99	2900.87
-112.53	2240.42	-79.18	189.15	57.05	1772.88
102.62	11167.35	27.68	447.61	445.41	160.20
-198.61	5753.17	58.65	412.49	61.52	-71.19
17.85	-530.32	107.84	188.22	149.44	3313.40
-14	-538.80	9.44	380	98.66	3621.61
29.43	6847.08	93.29	591.38	238.82	2038.78
-35.23	5745.36	-59.22	686.78	-50.40	2615.75
52.86	1636.76	112.91	139.47	-77.46	580.08
19.63	2743.91	35.15	236.69	-74.51	-46.58
35.13	3885.64	8.40	773.91	26.96	199.47
10.90	3556.96	-34.31	589.55	98.61	440.72
42.66	18898.79	32.84	1931.76	42.10	3189.85
-12.21	6249.59	37.45	798.37	-2.29	1397.12
-70.57		123.12	883.33	49.19	2456.31
125.38		56.30	635.50	5.50	623.45
-82.41		27.95	1093.41	35.39	3348.08
-223.84		-51.70	-73.88	6.14	2049.63
76.88		7.42	2197.41	12.93	2713.58
1.26		13.07	1648.50	141.28	3082
-48.97		55.96	1121.52	80.25	778.37
-80.72		-60.57	561.23	81.08	629.79
122.58		100.37	587.23	338.43	1000.94
220.52		-12.42	268.03	33.83	577.54
37.73		42.75	917.40	238.39	
73.33		27.43	629.71	-43.59	
-43.01		136.37	628.82	103.24	
-21.12		105.73	413.54	-113.56	
		-0.86	177.53		
		-51.80	160.31		
		339.95	778.81		
		-1011.97	1061.13		

unc-4(gk705) Control	unc-4(gk705) 5-HT	bas-1(ad446) Control	bas-1(ad446) 5HT	bas-1(ad446), mod-5(knu383) Control	bas-1(ad446), mod-5(knu383) 5-HT
244.17	974.46	35.26	1118.14	49.12	4614.20
336.76	2018.97	5.95	4368.99	104.39	2787.93
-156.08	1066.87	151.81	2458.29	-4.54	1206.69
86.12	23.29	3.71	2358.73	-18.90	687.53
10.55	248.54	-8.59	1093.98	10.13	384.73
46.28	170.89	-78.56	1796.76	17.80	230.79
50.42	256.88	38.52	1040.93	73.16	4843.98
-32.93	1036.03	-1.33	327.39	-48	4700.99
25.03	-319.16	52.97	586.04	96.83	918.73
-30.35	333.45	111.49	67.15	0.27	1357.21
961.23	1659.08	-1.39	393.73	114.66	126.16
-0.15	42.94	-24.68	939.34	-10.38	109.07
-77.69	516.80	-15.51	2689.59	-62.50	362.81
152.61	112.85	17.96	1177.31	42.90	117.26
10.59	21.92	-4.66	1500.22	33.20	386.95
86.07	1858.89	26.90	2393.42	20.20	1056.62
42.48	-161.42	-9.08	609.15	50.28	-0.54
148.32	312.23	-16.03	1892.70	0.45	63.55
143.37	961.98	-89.19	2525.69	-5.45	149.93
-8.36	1217.31	-181.67	1592.95	4.08	29.55
-356.69	1137.42	92.76	1178.06	-546.01	22.43
45.11	1183.24	53.14	773.13	-92.47	41.78
41.87	4033.37	15.73	1484.86	-85.12	1923.23
-236.04	1326.88	31.94	888.37	-78.83	1386.24
-188.29	5.93	-82.01	3955.37	18.38	806.28
503.51	-96.90	117	1677.05	-47.98	689.74
391.04	23.81	88.46	334.69	-31.48	958.59
30.59	13.79	-0.26	223.88	75.47	743.11
-141.21	252.42	-25.05	2680.62	51.58	552.62
305.09	-359.37	-16.57	1742.27	-27.81	497.42
1003.36	-31.57	-258.11	243.70	15.27	2867.01
-222.23	-232.45	-55.91	1266.58	21.07	3142.62
-73.84	351.66	30.59	2069.89	-288.67	501.24
-300.36	275.93	19.82	2185.62	639.46	687.12
-562.85	627.07	19.23	528.40	-28.65	1317.27
825.41	1212.71	17.46	268.97	24.57	-34.22
172.32	919.14	148.05	85.07	21.89	1698.99
554.97	107.60	-85.81	-17.82	-8.66	1848.95
578.53	982.20	-5.66	641.45	38.41	294.85
333.38	470.27	-22.08	797.95	-66.17	316.77
-137.92	220.29	5.55	1054.29	63.74	208.79
40.64	1768.89	9.94	230.07	217.71	70.60
152.76	1057.49	19.64	853.03	-3.83	1148.23
5.71	152.90	40.84	2059.04	11.64	1024.70
358.34	658.47	-62.47	1395.79		310.48
258.17	421.85	-61.45	765.62		1577.49
135.95	468.23	-25.01	1820.32		1007.24
-128.16	373.69	23.32	1619.58		1119.43
-50.07	73.95	-48.25			
-73.32	-221.18	-8.02			
136.18	528.29				
335	-51.38				
183.63	1514.57				
374.75	-141.22				
763.78	501.83				
	181.39				
	539.57				

Annex 4.21. Eggs laid in worms cultured in 5-HT and exposed to 5-HT

Strain	N2	COP1365	VC1528
Species	<i>C. elegans</i>	<i>C. elegans</i>	<i>C. elegans</i>
Background	wildtype	<i>mod-5(knu383)</i>	<i>unc-4(gk705)</i>

Control conditions											
CONTROL						5mM 5-HT					
<i>C. elegans</i> wildtype		<i>C. elegans mod-5 (knu383)</i>		<i>C. elegans unc-4(gk705)</i>		<i>C. elegans</i> wildtype		<i>C. elegans mod-5 (knu383)</i>		<i>C. elegans unc-4(gk705)</i>	
0	0	0	2	0	0	2	3	3	12	0	4
0	0	0	2	0	0	1	5	3	3	8	4
0	0	0	0	0	0	19	7	7	6	9	4
0	0	0	0	0	0	4	0	0	9	7	4
5	0	3	0	0	0	12	0	10	6	4	0
1	0	0	0	0	0	9	9	8	11	5	4
0	0	0	0	0	0	0	6	8	5	8	0
0	0	2	0	0	2	0	3	6	7	4	4
1	0	0	2	0	0	9	6	6	8	0	
0	0	0		0		21	9	4		4	
0	0	0		0		19	3	8		0	
1	0	0		0		10	2	1		0	
0	1	2		0		5	3	9		11	
0	0	0		0		4	2	3		0	
0	1	0		0		6	2	4		0	
0	0	0		0		3	4	8		0	
0	0	0		0		0	2	2		0	
3	0	1		0		0	2	8		12	
0	0	0		2		5	2	6		1	
3	4	1		0		2	3	3		2	
0	2	2		0		3	3	4		11	
0	0	0		0		0	1	3		9	
0	0	0		0		6	6	9		2	
0	0	2		4		6	4	4		1	
0	0	0		0		0	1	8		4	
0	0	0		0		0	6	3		2	
0	0	0		0		0	3	3		4	
0	0	0		0		2	5	4		5	
0	0	0		0		0	3	0		1	
0	0	0		0		0	4	3		0	
0	0	0		0		0	0	5		0	
0	0	0		0		4	4	3		0	
0	0	0		0		6	10	7		5	
0	0	0		0		6		5		4	
0	0	1		1		0		7		4	
4	0	1		1		0		8		0	
0	0	0		0		0		1		4	
0	2	0		0		0		2		6	
0		3		0		4		4		2	

Grown in 5-HT 10mM

CONTROL				5mM 5-HT			
<i>C. elegans</i> wildtype		<i>C. elegans</i> <i>mod-5</i> (<i>knu383</i>)	<i>C. elegans</i> <i>unc-4</i> (<i>gk705</i>)	<i>C. elegans</i> wildtype		<i>C. elegans</i> <i>mod-5</i> (<i>knu383</i>)	<i>C. elegans</i> <i>unc-4</i> (<i>gk705</i>)
2	2	7	0	0	0	11	1
1	3	0	0	0	0	7	0
19	2	0	0	0	1	4	0
4	2	0	0	9	0	7	0
12	4	4	0	0	1	3	4
9	2	0	0	0	0	9	0
0	2	0	0	4	0	5	1
0	2	0	0	0	0	8	0
9	3	0	0	2	0	13	0
21	3	3	0	1	3	15	0
19	1	0	0	0	2	1	3
10	6	0	0	0	1	12	4
5	4	0	0	0	0	10	0
4	1	1	0	5	0	5	0
6	6	0	0	0	0	4	0
3	3	0	0	0	0	2	1
0	5	0	2	0	1	1	1
0	3	0	0	8	0	4	9
5	4	0	6	0	0	13	0
2	0	0	0	1	1	8	3
3	4	0	0	0	1	5	6
0		4	0	0		8	0
6		2	0	4		0	3
6		0	0	1		6	4
0		0	0	0		0	4
0		2	0	0		6	3
0		0	0	0		5	0
2		0	0	0		6	6
0		0	0	0		11	0
0		4	1	0		0	0
0		0	0	0		0	7
4		1	0	1		6	3
6		0	0	0		3	0
6		0	0	6		6	4
0		0	0	0		5	6
0		0	0	1		11	0
0		0	0	2		0	0
0		0	0	0		0	2
4		0	0	1		1	0
10		0	0	0		6	0
3		0	0	0		8	3
5		7		0		4	
7		0		0		5	
0		0		0		9	
0		0		0		7	
9		0		0		3	
6		0		0		6	
3		0		0		5	
6				0			
9				0			
3				0			

Annex 4.22. 5-HT staining males

Strain Specie Background N worms	N2		PHX7715		RGD1		NFB2196	
	<i>C. elegans</i> wildtype		<i>C. elegans</i> <i>vlc47 syb7715</i>		<i>C. angaria</i> wildtype		<i>C. angaria</i> <i>vlc45</i>	
	40		20		50		50	
	n neurons	%	n neurons	%	n neurons	%	n neurons	%
CP1 5-HT	40	100	20	100	50	100	49	98
CP2 5-HT	40	100	20	100	50	100	48	96
CP3 5-HT	40	100	20	100	50	100	49	98
CP4 5-HT	39	97.5	20	100	50	100	49	98
CP5 5-HT	40	100	20	100	50	100	49	98
CP6 5-HT	40	100	20	100	49	98	49	98
CA1 5-HT	0	0	8	40	43	86	8	16
CA2 5-HT	0	0	4	20	37	74	8	16
CA3 5-HT	0	0	5	25	45	90	10	20
CA4 5-HT	0	0	4	20	35	70	8	16
CA5 5-HT	0	0	1	5	24	48	1	2
CA6 5-HT	0	0	0	0	19	38	3	6

Annex 4.23. *C. elegans* and *C. angaria*

C. elegans constructs in *C. angaria*

Strain	RGD1	RGD1
Species	<i>C. angaria</i>	<i>C. angaria</i>
Background	wild type	wild type
DNA array	<i>C. ele cat-1</i>	<i>C. ele mod-5</i>
reporter	<i>prom1</i>	<i>prom3</i>
line/genotype	Ex line	Ex line
N worms	32	24
CP1-6 GFP ON	175	128
CA1-6 GFP ON	0	26
% CP1-6	91.1458333	88.8888889
% CA1-6	0	3.38541667

C. angaria constructs in *C. angaria*

Strain	RGD1	RGD1
Species	<i>C. angaria</i>	<i>C. angaria</i>
Background	wild type	wild type
DNA array	<i>C. ang cat-1</i>	<i>C. ang cat-1</i>
reporter	<i>prom1</i>	<i>prom1</i>
line/genotype	F1 scored, no Ex lines	Ex line
N worms	15	31
CP1-6 GFP ON	31	181
CA1-6 GFP ON	0	0
% CP1-6	34.4444444	97.311828
% CA1-6	0	0

Annex 4.24. *mod-5* expression in *C. elegans* and *C. angaria* CPs and CAs

Strain	NFB1719		NFB2176	
Species	<i>C. elegans</i>		<i>C. elegans</i>	
Background	<i>wildtype</i>		<i>wildtype</i>	
DNA array	<i>C.elegans mod-5</i>		<i>C.angaria mod-5</i>	
reporter	CRISPR <i>mod-5(vlc47)</i>		<i>prom5</i>	
	n neurons	%	n neurons	%
N worms	30		31	
CP1 GFP	13	43.3333333	25	80.6451613
CP2 5-HT	18	60	20	64.516129
CP3 5-HT	25	83.3333333	26	83.8709677
CP4 5-HT	25	83.3333333	25	80.6451613
CP5 5-HT	28	93.3333333	30	96.7741935
CP6 5-HT	23	76.6666667	25	80.6451613
CA1 5-HT	0	0	15	48.3870968
CA2 5-HT	0	0	18	58.0645161
CA3 5-HT	0	0	16	51.6129032
CA4 5-HT	0	0	14	45.1612903
CA5 5-HT	0	0	11	35.483871
CA6 5-HT	0	0	4	12.9032258

Annex 4.25. Tail curling in males exposed to 5-HT 5mM

Male curling				
Strains	CONTROL		5mM 5-HT	
	<i>C. elegans</i> N2	<i>C. elegans</i> intron (<i>vlc47 syb7715</i>)	<i>C. elegans</i> N2	<i>C. elegans</i> intron (<i>vlc47 syb7715</i>)
Worms curled	2	3	9	12
TOTAL WORMS	12	16	15	14

Male curling				
Strains	CONTROL		5mM 5-HT	
	<i>C. angaria</i> RGD1	<i>C. angaria</i> mod-5(<i>vlc45</i>)	<i>C. angaria</i> RGD1	<i>C. angaria</i> mod-5(<i>vlc45</i>)
Worms curled	1	22	22	25
TOTAL WORMS	19	26	22	26

