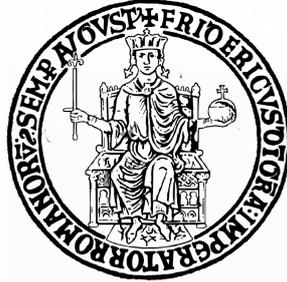


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Identification of new genes involved in Dopaminergic system using the animal model Caenorhabditis elegans

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ABSTRACT

Dopamine (DA) is a neurotransmitter that plays a number of very important roles in humans and other animals, such as regulating movement, behavior and cognition. Excess and deficiency of DA have been associated with a number of neurological diseases, including Parkinson's disease and drug addiction. To date the molecular mechanisms and the key elements involved in these disorders have not completely discovered. In *C. elegans*, studies aimed at understanding the molecular mechanisms regulating the DAergic system have taken advantage either of chemical treatments or of engineered transgenes. Unfortunately these approaches are not feasible to study the loss of function of a gene, while classic genetic mutations can cause pleiotropic effects or lethal phenotypes in mutant animals, hampering the study of the role played by a gene of interest in DAergic system. In this work I used *C. elegans* as animal model and a powerful variant of RNA-interference approach, to dissect the role played specifically and only in DAergic system by two candidate genes, overcoming the technical limitations previously described for loss of gene function, such as lethality or pleiotropic effects. With this approach I therefore reduced the function of *unc-64* gene, the ortholog of the mammalian component of the core synaptic vesicle fusion machinery Syntaxin1A, only in the *C. elegans* DAergic system. Using a well known behavioral assay *in vivo*, the SWimming-Induced Paralysis (SWIP), which is known to be regulated by endogenous DA uptake by the DA transporter DAT-1, I confirmed that the presence of *unc-64* in DAergic circuit is important to guarantee a correct neurotransmitter reuptake. Moreover I showed that the SWIP effect, produced in the nematode after treatment with a psychostimulant such as Amphetamine (AMPH), strongly depends on the presence of *unc-64* specifically in DAergic circuit, together with DAT-1 and DA receptors. This demonstrates that *unc-64* is a novel key modulator of DA reuptake mechanism. I then investigated the role of *unc-63* gene, the ortholog of the mammalian alpha 6 Cholinergic nicotinic receptor CHRNA6, in the *C. elegans* DAergic system. Using microscopy analysis I demonstrated a specific expression of *unc-63* gene in the nematode DAergic neurons. DAergic related behaviors are not impaired *in vivo* by loss of *unc-63*, thus demonstrating that *unc-63* does not exert a main role in regulating the DA signaling in this neuronal circuit. Rather, through a well established drug treatment, I showed that *unc-63* presence specifically in the DAergic

system is necessary to mediate the toxic action produced by the nicotinic agonist DMPP (1,1-dimethyl-4-phenylpiperazinium), during larval development.

In conclusion, all these results validated *C. elegans* as a powerful animal model where to identify new genes playing a role in Daergic neurons and demonstrated all the power of our innovative variant of the RNAi approach, which, by the knock-down a gene of interest only in these neurons, allows to discover new genetic modifiers of Daergic neurotransmission.

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CHAPTER I

Dopaminergic neurotransmission in the human central nervous system

Dopamine (DA) is the most abundant catecholamine of the central nervous system and is highly conserved among species [1, 2]. DA is involved in the modulation of a variety of physiological functions ranging from locomotion, motivation, learning and memory to hormone secretion, reward and thermoregulation [3-5]. Defects in the DAergic neurotransmission have been linked to severe pathologies of the central nervous system. For example, the decrease in the levels of DA in the brain leads to a neurodegenerative pathology known as Parkinson's disease, characterized by severe impairments such as rigidity, inability to direct movements and loss of coordination. The altered DA signaling observed in human neurological diseases is often related to dysfunction of genes involved in the biosynthesis, transport, release and reuptake of the DA. However the mechanism of DA signaling is very sophisticated and therefore it is regulated by other several key elements of the DAergic system. The identification of new elements involved in DAergic neurons function may contribute to a better understanding of the mechanisms that, when altered, cause dysfunctions in DAergic circuit.

DA is present in three major axonal pathway: the nigrostriatal, the mesocorticolimbic and tuberoinfundibular pathways (Figure 1) [6, 7]. Projections constituting the nigrostriatal pathway arise from the midbrain DAergic cell bodies located in substantia nigra pars compacta (SNpC) and innervate caudate and putamen (dorsal striatum) (Figure 1). The nigrostriatal pathway is involved in the control of movement and its degeneration is associated with Parkinson's disease [8]. The mesocortical and mesolimbic pathways arise from DAergic neurons present in the midbrain Ventral Tegmental Area (VTA) (Figure 1).

Both of these DAergic systems are involved in emotion related behaviors, including motivation and reward [9]. Mesolimbic DAergic projections are involved in a broad range of functions including novelty detection, learning and memory. The major DA pathway, the tuberoinfundibular tract, projects from the hypothalamus to the infundibulum of the posterior pituitary [10]. Here, DA release inhibits the release of the hormone prolactin, which is important in the reproductive cycle.

A loss of cortical DA signaling results in a variety of disease states, ranging from

Attention-Deficit Hyperactivity Disorder (ADHD) to Schizophrenia (SZ). Moreover drugs of abuse, including opiates, cocaine, amphetamine and nicotine, are also involved in mesolimbic system for their psychomotor effects. Di Chiara and Imperato in 1988 discovered the interesting link between drugs of abuse and DA release in the Nucleus Accumbens (NAc), reporting that rats administered with drugs typically abused by humans, resulted in an increase in DA concentrations in both the NAc and the striatum [11].

Taken together, all these studies revealed the main districts of the Central nervous system involved in regulating the DA neurotransmission that, when altered, can lead to the onset of human neurological disease.

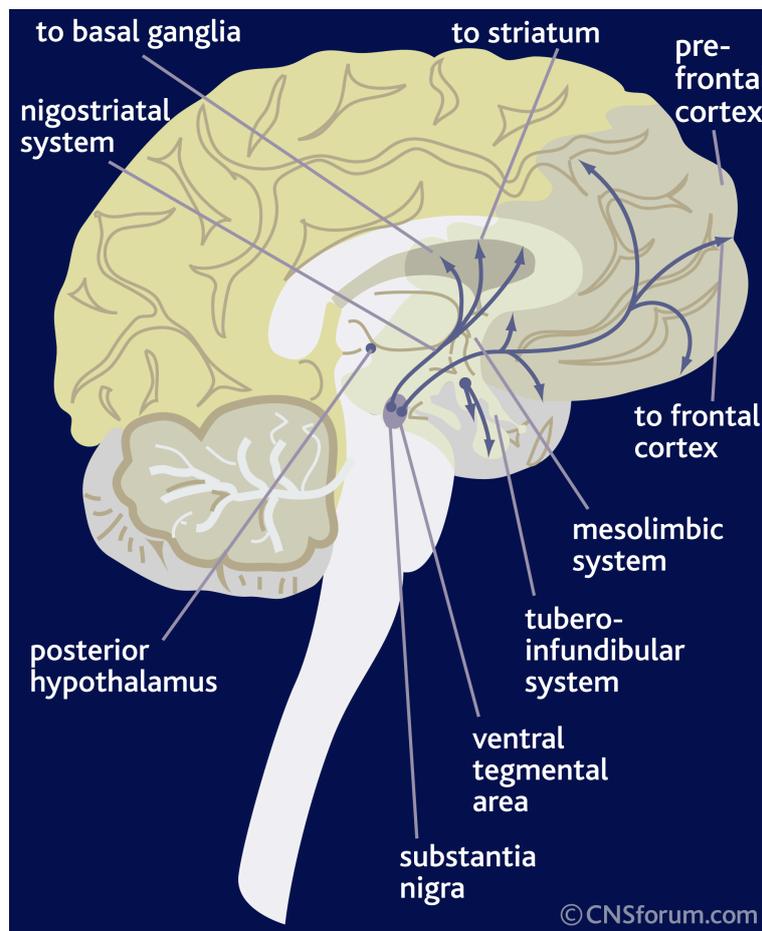


Figure 1. Dopaminergic projections in the mammalian brain

Illustration of major DA projections in the central nervous system. The nigrostriatal pathway originates in the substantia nigra and projects to the striatum. The mesolimbic projections originate in the VTA and project both to the nucleus accumbens (not shown) and areas in the prefrontal cortex. The final system is the tuberoinfundibular system which projects from the hypothalamus to the pituitary (Figure from www.cnsforum.com)

Biosynthesis of dopamine

DA is synthesized from L-tyrosine in a pathway that includes Tyrosine Hydroxylase (TH) and Aromatic Acid DeCarboxylase (DCAA) enzymes (Figure 2). TH is the initial rate limiting factor since it hydroxylates the L- tyrosine to L-DOPA, thus playing a key role in DA synthesis. Similar to other catecholamines DA is produced both in cell bodies and in the nerve terminals. Once it is synthesized, DA is packaged into synaptic vesicles by a Vesicular MonoAmine Transporter (VMAT) and it is released into the synaptic cleft where binds to DAergic receptors and activates various signaling pathways in target cells (Figure 2). The DA signaling is controlled by receptors that have been classified into two sub-families, D1- and D2-like, on the basis of their biochemical and pharmacological properties (Figure 2). D1-like family includes D1 and D5 receptors, whereas D2-like includes D2, D3 and D4 receptors. D1-like receptors are coupled to G_s proteins and their activation results in an increase in cAMP concentration. D2-like receptors are coupled to G_i proteins and their activation results in a decrease in cAMP levels [12]. DA signaling is terminated by the reuptake of DA into the presynaptic neuron by the DA Transporter (DAT). The DA that is reabsorbed by DAT is then repackaged into vesicles by VMAT and is ready for another subsequent round of release (Figure 2). The DA biosynthetic cycle ends with the inactivation of neurotransmitter by two different enzymes, the MonoAmine Oxidase (MAO) and the Catechol O-MethylTransferase (COMT) from which the main final metabolites of DA are produced: HomoVanillic Acid (HVA) and 3,4-DihydroxyPhenylAcetic acid (DOPAC) [13].

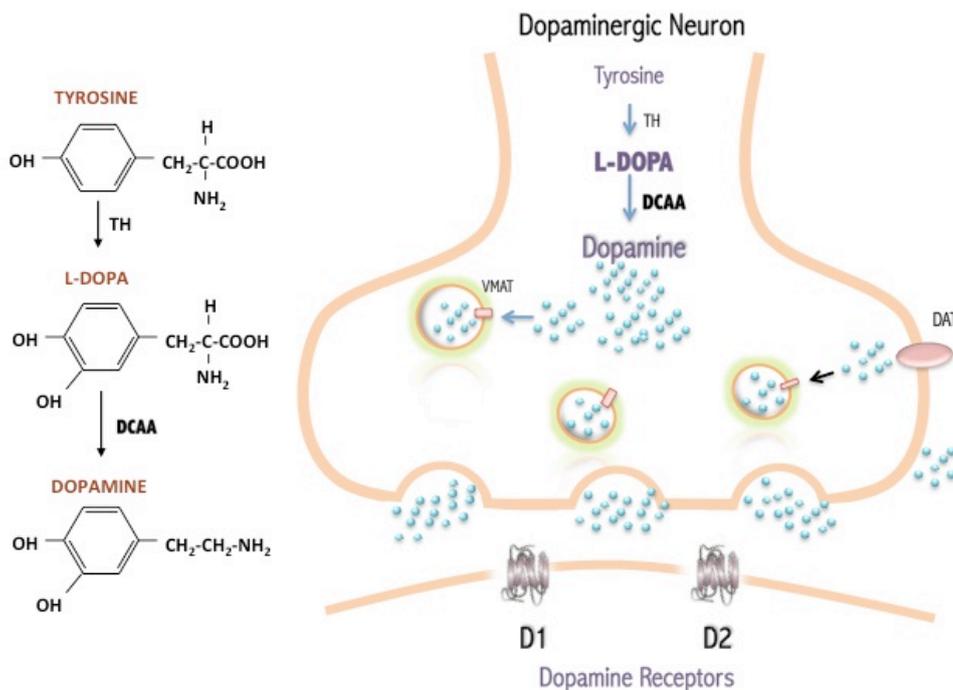


Figure 2. Biosynthesis of dopamine and dopaminergic synapse

Synthesis of DA from L-DOPA in the DAergic nerve terminals. DCAA, aromatic acid decarboxylase; DAT, DA transporter, TH, Tyrosine Hydroxylase; D1, D1-like DA receptor; D2, D2-like DA receptor (Figure from [14]).

Dopamine transporter (DAT): a critical enzyme for dopamine regulation

An important characteristic of mammalian DAergic neurons is the presence of the DA Transporter DAT. It is an integral plasma membrane phosphoprotein belonging to the SCL6 family of transporters, a family which also includes transporters for the biogenic amines serotonin and norepinephrine (SERT and NET respectively), as well as for the inhibitory transmitters GABA and glycine [15]. DAT is a specific marker of DAergic neurons and in the human brains it localizes to axons and dendrites of mesencephalic and hypothalamic DA neurons, which innervate the striatum and frontal cortex, and that directly or indirectly regulates locomotor activity, cognition, emotion, reward, and neuroendocrine function [16-18]. DAT is responsible for the reuptake of DA in the presynaptic neuron following release into the synaptic cleft [15, 19, 20]. In detail DAT coordinates the spatial and temporal action of DA by actively clearing the amine from the extracellular space. Although diffusion and enzymatic degradation may partially govern the synaptic concentration of DA, several studies have established DAT as key

element responsible for DA signalling in the brain, by mediating rapid clearance of DA from the synaptic clefts [21, 22].

Interestingly the control of DA signalling can be altered by the actions of psychostimulants such as AMPH (Amphetamine) that, interacting with DAT, attenuate DA clearance and increase DA release. The final result of AMPH abuse is to elevate the extracellular concentration of DA and, as a consequence, enhance DA signaling [23, 24]. However all the key molecular players involved in the behavioral effects induced by AMPH are still unknown. Because of the importance of DAT in DA neurotransmission, it is likely that DAT is under strict regulatory control, but the mechanism of this regulation is poorly understood [25-27]. Dysregulation of DAT activity, which leads to inappropriate DA clearance, is known to play an important role in the etiology of DAergic related disorders such as schizophrenia, depression, and Parkinson's disease [28, 29]. Researchers have therefore focused on elucidating signaling mechanisms involved in DAT trafficking to and from the membrane, in order to try to better understand the molecular mechanisms underlying DAergic related diseases. It has been therefore discovered that the cytosolic N-terminus of DAT is important for translocation to the plasma membrane or retention at the plasma membrane while the C-terminus is important for constitutive and PKC-mediated endocytosis of DAT [30-32]. Moreover the N-terminus of DAT represents the site of many protein-protein interactions that can regulate DAT surface expression and activity, thus modulating the DA clearance [33]. One of the main regulatory partner of DAT is the neuronal SNARE (Soluble N-ethylmaleimide Associated protein REceptor) protein Syntaxin1A (Syn1A), which is involved also in the molecular machinery of DA release [34].

The contribution of SNARE protein Syn1A in dopaminergic system

Syn1A is a nervous system-specific protein, belonging to the Syntaxin superfamily. It possesses a single C-terminus transmembrane domain, a SNARE domain (known as H3), and an N-terminus regulatory domain (Habc). Syn1A binds Synaptotagmin in a calcium-dependent fashion and interacts with voltage dependent calcium and potassium channels, via the C-terminus H3 domain. It is mainly implicated in the regulation of

exocytosis by mediating the fusion of the vesicular membrane with the plasma membrane to release vesicular contents into the extracellular space.

Moreover, several studies have drawn explicit attention to the physical and functional interaction of Syn1A with plasma membrane ion channels [35-38] and neurotransmitter transporters [39-45]. In particular, several studies have demonstrated a direct interaction of Syn1A with the cytoplasmic N-terminus of neurotransmitter transporters, including the GABA transporter (GAT1) [46], the serotonin transporter (SERT) [42], the norepinephrine transporter (NET) [44, 47] and the DA transporter (DAT) [48, 49]. These interactions allow Syn1A to play a key role both to regulate transport activity and channel properties of the neurotransmitter transporters [39, 44, 50].

Regarding DAT, previous studies demonstrated a direct interaction between Syn1A and human DAT (hDAT) in a heterologous expression system and with native DAT in murine striatal synaptosomes [49]. Interestingly, in a different animal model such as the nematode *C. elegans*, Carvelli et al. suggested that *C. elegans* Syn1A (UNC-64) binds the N-terminus of *C. elegans* DAT (DAT-1) and its loss may suppress the DAT1 channel-like activity [40]. In addition, they observed that fusion of green fluorescent protein (GFP) to the DAT-1 N-terminus disrupted DAT1 interaction with UNC-64 and caused a swimming induced paralysis [40], a behavioural phenotype previously observed in DAT-1 deficient nematodes [51]. Whether the defect in GFP::DAT-1 animals was directly caused by perturbed DAT-1/UNC-64 interactions cannot be discerned. These studies, demonstrated that Syn1A may regulate DAT reverse transport and ion channel activity, supporting the idea that this SNARE protein could represent an important element in DAergic circuit to guarantee a correct DA reuptake. However the specific function of Syn1A in this neuronal circuit has not been completely clarified.

The contribution of nicotine acetylcholine receptors in dopaminergic system

As previously described, in the human brain DA plays a role in motor control, motivation, arousal, cognition, and reward. Regarding the motor control, DA released from the midbrain DAergic neurons exerts its role together with acetylcholine (ACh), produced by the cholinergic circuit. Interesting the striatal DAergic and Cholinergic

systems play an overlapping role in regulating central nervous system functions linked to motor activity and that are relevant in diseases such as Parkinson's disease [52-54]. The extensive colocalization of DA and ACh in the nigrostriatal pathway most likely underlies the functional interdependence of these two systems [52, 54, 55]. In particular ACh though the cholinergic transmission modulates striatal DA levels by stimulating nicotinic acetylcholine receptors (nAChRs). nAChRs are Cys-loop ligand-gated ion channels formed by pentameric complexes made up of combinations of a number of different subunits. The subunits composing nAChRs, possess two adjacent cysteines in the ACh binding site referred to as α subunits, whereas those with no such motif are referred to as non- α subunits. nAChRs can be divided into two subfamilies, according to the co-assembly of the subunits that influence their physiological and pharmacological profiles. In particular there are homopentamers composed of α -subunits and heteropentamers, characterized by the combination of α - and β -subunits in different ratios [56, 57]. A single subunit is composed of about 600 amino acids and has four separate transmembrane segments (TM1-TM4) with a large N- and a small C-termini facing the synaptic cleft (Figure 3) [58, 59]. The ligand-binding sites are located at the interfaces of the N-terminal hydrophilic domain of α -subunit and its adjacent α/β subunit (Figure 3) [56].

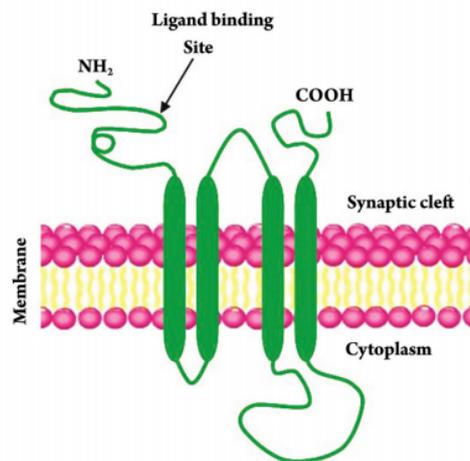


Figure 3. Structure of nAChRs

One subunit of the nAChR contains a large N- and a small C-terminal extracellular domains, four transmembrane domains (M1-M4), and a long cytoplasmic loop between M3 and M4 (Figure from [60]).

The ion channel (mainly permeable to cations as Na⁺ and Ca²⁺) can be opened only when the receptor is activated by endogenous acetylcholine (ACh) or exogenous ligand (e.g. nicotine) binding to the ligand binding site (Figure 3) [58, 59]. Furthermore, the β -subunits also largely contribute to the physiological and pharmacological properties (such as desensitization, inward rectification, and functional rundown) of the receptors [61, 62].

Mammals possess 17 subunits composing the nAChRs, characterized as either “muscle” (α 1, β 1, γ , δ , and ϵ) or “neuronal” (α 2–10, β 2–4) subtypes [63-65]. Neuronal nAChRs are widely distributed throughout the central nervous system (CNS) and are mainly involved in motor control [66]. In addition some of these nAChRs seem to be concentrated in DAergic system in the brain, thus researchers have focused their studies in identifying genes coding for the subunits composing these receptors, to better understand their functions in DAergic circuit under physiological and pathological conditions.

In particular, the α 6-subunit, encoded by CHRNA6 gene, has been shown to have a potential role in this neuronal circuit. It can be found with other subunits in heteromeric forms (i.e. α 6 β 2- and α 4 α 6 β 2-nAChRs) and is highly expressed in visual and catecholaminergic pathways, including the DAergic nigrostriatal pathway [67]. In order to study its function in DAergic circuit, have been conducted experiments characterized by the induction of a damage in the regions of nigrostriatal system of rodent and monkey, that include α 6-subunit combined with other subunits in heteromeric forms (α 6 α 4 β 2 β 3 and α 6 β 2 β 3). Interestingly they observed that, after nigrostriatal damage, there is a loss of α 6-subunit that correlates with a decline in DA release, suggesting a biologically relevant association of these subunits in the DAergic circuit. Therefore being particularly susceptible to nigrostriatal damage, α 6-subunits may represent an important target for the treatment of neurodegenerative disorders including Parkinson’s disease [68, 69].

Even if researchers have just begun to explore the involvement of α 6-subunit in DAergic circuit, the informations reached to date reinforce the idea that they may play an important role in this neuronal circuit.

CHAPTER II

Dopaminergic neurotransmission in *Caenorhabditis elegans* nervous system

In order to understand the complex mechanisms of the nervous system, neuroscientists rely on different types of animal models that include vertebrates like rodents [70-72], chick [73, 74] and *Xenopus* [75] and invertebrates like *Caenorhabditis elegans* [76], *Drosophila* [77], *Ascaris* [78, 79] and cephalopods [80]. The decision to use a particular animal model is based on its validity and simplicity in conducting experimental studies for answering specific biological questions.

In my thesis, I was interested in investigating the role of Syn1A and CHRNA6 in DAergic system and therefore it has been necessary to use a model that not only conserves an high homology with human DAergic pathway, but that also displays interesting specific DA-mediated behaviors and shows an accessible nervous system. One such model is the nematode *Caenorhabditis elegans*.

***Caenorhabditis elegans*: a powerful animal model**

Caenorhabditis elegans (*C. elegans*) is a free-living, soil nematode found in temperate climates [81]. It has been used as a valuable biological model since Nobel Prize-winning Sydney Brenner used the nematode to perform genetic screens for the purpose of unveiling mutations that alter its movement [82]. Since then, *C. elegans* has been extensively used because of its small size, rapid generation time, short lifespan, simple and measurable behaviors, extensive biological characterization, genetic tractability and for the high degree of conservation of gene sequences [81]. With adult worms being approximately 1 mm in length, a large number of worms can be grown in a very small space, most often on agar plates containing *Escherichia coli* (*E. coli*), which *C. elegans* consumes as food (Figure 4) [82].

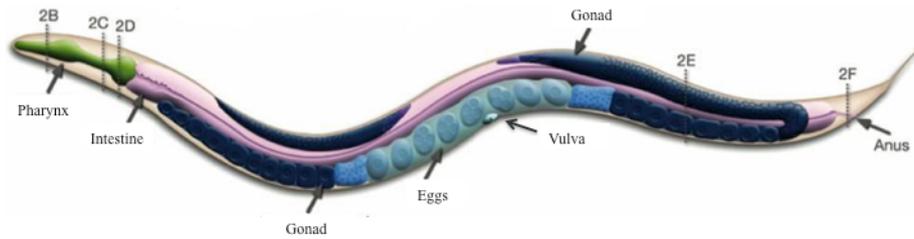


Figure 4. The nematode *C. elegans*.

Hermaphrodite, anterior is to the left, ventral is down (Figure adapted from www.wormatlas.org).

C. elegans proceeds through its life cycle in approximately three days and has a lifespan of about three weeks. Under normal conditions (when food is present and temperature is near to 20°C), the worm passes through 4 larval stages consisting of L1, L2, L3, L4 and after that it develops into young adults capable of laying their own eggs [81, 83]. Moreover this small worm is transparent allowing the observation of cells and features within the entire organism without the need to kill it or permeabilise and fixate [81]. In this way it can be used as an *in vivo* system while maintaining many beneficial characteristics of an *in vitro* system.

Despite its simplicity, *C. elegans* presents many similarities with other multicellular organisms including complex organ structures, a rich array of sophisticated behaviors (social, sexual, and learning behaviors) and a 60-80% of homology with human genes [84]. Although the worm presents limitations compared with more complex organisms, it contributed to understand many fundamental conserved mechanisms that have been used to investigate many aspects of animal development and translated to vertebrates and humans. Examples are the programmed cell death [85, 86], the mechanisms mediated by RNA interference (RNAi) [87], and the use *in vivo* of the Green Fluorescent Protein (GFP), which were described by the Nobel Prize winners B. Horvitz, A. Fire, C. Mello and M. Chalfie, respectively.

Many large scale genetic screens using classical genetics and RNAi techniques have been performed to identify new genes, phenotypes, and interacting proteins [88-90]. All these features make *C. elegans* a powerful tool in genetic and genomic studies.

Finally the nematode presents also several useful characteristics as animal model for studying neurobiology, since the wiring diagram of the nervous system consists of a defined set of 302 neurons, which are well mapped and characterized and the main

interactions between them are very well known [91].

The *C. elegans* nervous system

The ~100 MB genome of *C. elegans* codes for ~20,000 protein-coding genes many of which are required for the function of the most complex and well-characterized organ in the nematode, the nervous system. It contains 302 neurons of 118 subtypes [92, 93], 6393 chemical synapses, 890 electrical junctions, and 1410 neuromuscular junctions [94]. *C. elegans* represents the only organism whose entire nervous system has been completely reconstructed by electron microscopy (EM) [91]. Most neurons have a simple morphology with a single process emanating from the cell body which, in most of the cases, is located in the anterior head region, surrounding the elongated bilobed structure of the pharynx. The remaining cell bodies are located along the ventral, lateral or posterior tail regions (Figure 5). The major synaptic activity in the animal is in the nerve ring or “worm brain”, a region of high neuronal process density that might function as a central information exchange in the nervous system of *C. elegans* (Figure 5) [91].

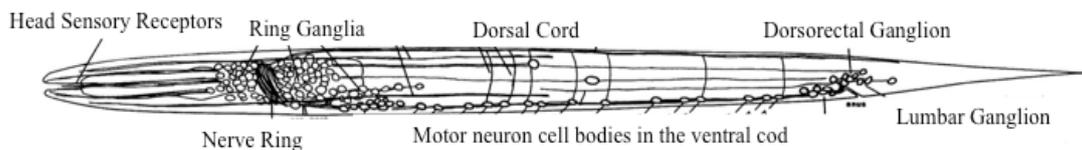


Figure 5. A general view of the L1 larva nervous system

The main region of neurons location is the nerve ring, which is a loop around the pharynx. The ventral cord runs back from this and contains motor neuron cell bodies in addition to processes. Those ventral cord motor neurons, that do not send a commissure around the left side of the body to the dorsal cord, send one commissure to the right side. There are four small ganglia in the tail: the preanal ganglion (not highlighted), the dorsorectal ganglion, and two lumbar ganglia, one on each side. Anterior is to the left, ventral is down (Figure adapted from www.wormatlas.org).

The structure and connectivity of neurons in the *C. elegans* nervous system are reminiscent of the postganglionic neurons of the autonomic nervous system, that form synapses en passant at multiple places along target tissues, contain non-traditional pre-

synaptic specializations, and have almost no post-synaptic specialization [91]. Despite the relative anatomical simplicity of the *C. elegans* nervous system, it contains many of the known signaling components and neurotransmitter systems found in the mammalian nervous system. It contains acetylcholine, glutamate, γ -aminobutyric acid (GABA), serotonin (5-HT), dopamine (DA), and neuropeptides [95], among other chemical messengers. Neurotransmitter-specific transporters (membrane and vesicular) and receptors (including G-protein-coupled) are also highly conserved with their mammalian counterpart. Most ligand-gated and voltage-gated ion channels are present within the worm, with the notable exception of the voltage-gated ion channel [95]; however, the voltage-gated Ca^{++} channels are conserved and, as in mammals, can generate the necessary action potentials for neurotransmission. Moreover all the known synaptic components involved in synaptic vesicle plasma membrane interactions and exocytosis, including syntaxins, synaptotagmin, synaptobrevin, and SNAP-25, are highly conserved between worms and mammals [95].

For this reason many researchers have turned to this model organism for studying the molecular and genetic basis of neuronal behaviors that, when altered, may cause neurodegenerative disorders, including Parkinson's and Alzheimer's diseases [96].

Dopaminergic neurons in *C. elegans*

As previously mentioned the *C. elegans* nervous system has many neurotransmitters, neuromodulators, and signaling pathways that are evolutionarily conserved including the DA system [95, 97]. DAergic neurons were initially identified by Sulston and coworkers, who used the catecholamine-specific technique of formaldehyde-induced fluorescence (FIF) to reveal 8 DA neurons in the hermaphrodite with an additional set of 6 DA neurons specific to males (Figure 6) [97]. DAergic cell bodies and processes were visualized using fluorescence microscopy, confirmed as DA by alumina absorption and thin-layer chromatography (TLC). Based on FIF micrographs and worm DA content as well as the estimated volume of the cell bodies and processes, the concentration of DA in the nerve endings is predicted to be very similar to the concentration within mammalian varicosities [97].

DAergic neurons are believed to be mechanosensory neurons because the microtubule-

containing cilium at the end of the dendrites are embedded in the subcuticle and may detect movement or food [98-100]. The most anterior of these cell pairings are the CEPs (two dorsal and two ventral) containing a single long dendrite that extends from the cell body near the nerve ring, through the length of the head where the ciliated endings enter the cuticle near the nose of the animal (Figure 6) [91, 98]. The ADE cell bodies are located behind the second bulb of the pharynx, and the dendrites contain ciliated endings that travel into the deirid sensilla (Figure 6). The axons of both CEPs and ADEs are directed into the nerve ring, and the dorsal pair of CEPs may receive some synaptic input from ADEs. The cell bodies of PDEs are located posterior to the vulva, and, as with other DA neurons, the dendrites contain ciliated endings that enter the sensillum (Figure 6). The PDE axons enter the nerve cord, where the anterior process extends to near the nerve ring, and receive en passant synaptic connections from other neurons [91, 101]. The male tail contains 3 extra sets of DA containing neurons required for mating [102, 103].

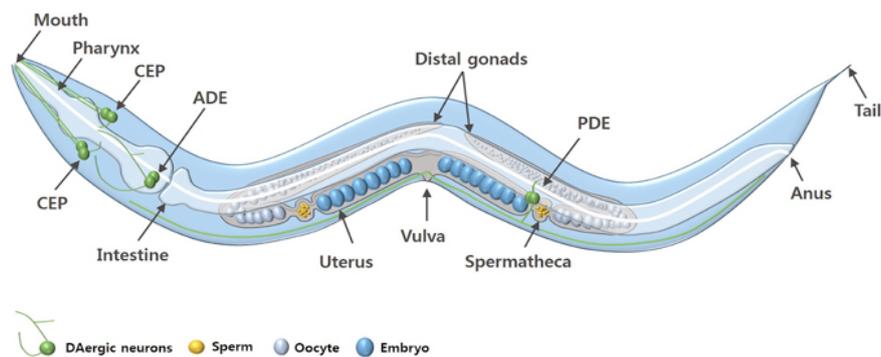


Figure 6. The *C. elegans* dopaminergic neurons

The picture shows an adult *C. elegans* hermaphrodite, with key anatomical features and the DAergic neurons (green). The DAergic neurons include four cephalic (CEP) neurons, two anterior deirid (ADE) neurons, and two posterior deirid (PDE) neurons. Males have six additional DAergic neurons located in the tail (not shown) (Figure from [104]).

The distinctive morphology and anatomical positioning of DAergic neurons in *C. elegans* makes it an attractive model to study neurodegeneration, particularly in relation to Parkinson Disease [105, 106]. Nass and Blakely demonstrated the use of *C. elegans* as a model in 2001, by administering the neurotoxin, 6-hydroxydopamine (6-OHDA) [106]. Due to the similarity in structure to neurotransmitter DA, 6-OHDA is accumulated into the DAergic neurons by pre-synaptic DA transporter, thereby causing

cytotoxic damage and cell death. Effective neurodegeneration was scored by examining whether the dendritic extensions of the CEP neurons were intact from the cell body to the tip after OHDA treatment [106]. This method of evaluating intact dendrites, in addition to noting any change in cell shape and/or size, was adopted for subsequent studies of DA neuron degeneration [107-109].

Genes involved in dopaminergic signaling in *C. elegans*

The biosynthesis of the catecholamine DA in the nematode shares notable features with mammalian neurons and starts from the Tyrosine Hydroxylase enzyme (encoded by the *cat-2* gene). CAT-2 converts tyrosine to 1-DihydroxyPheny-l-Alaninelevadopa (L-DOPA) that is then converted by BAS-1 (aromatic amino acid decarboxylase, encoded by the *bas-1* gene) to DA [2, 97, 102] (Figure 7). Cytosolic DA is packaged into synaptic vesicles by CAT-1 (vesicular monoamine transporter, encoded by the *cat-1* gene) [110] where it is stored until it is secreted upon neuronal depolarization (Figure 7). Synaptic DA is taken up by DA transporter DAT-1 (encoded by the *dat-1* gene) that, through the reuptake of the neurotransmitter terminates the DA signaling [111] (Figure 7).

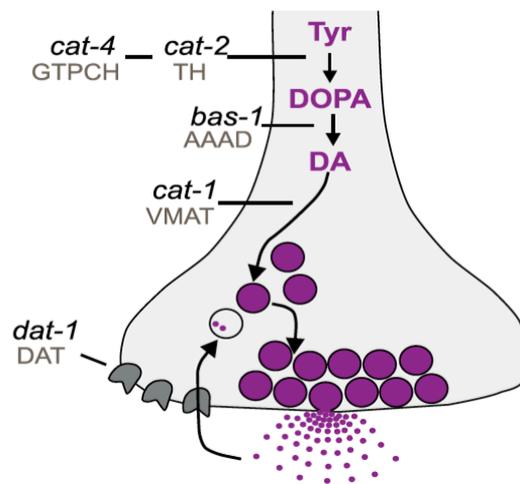


Figure 7. Schematic representation of a dopaminergic neuron synapse

The biosynthesis of the catecholamine DA in the nematode starts from CAT-2 that converts tyrosine to L-DOPA that is then converted by BAS-1 to DA. Cytosolic DA is packaged into synaptic vesicles by CAT-1 where it is stored until it is secreted upon neuronal depolarization. Synaptic DA is taken up by DA transporter DAT-1 that, through the reuptake of the neurotransmitter terminates the DA signaling. AAAD: aromatic L-amino acid decarboxylase; bas-1: Biogenic

Amine Synthesis related 1, cat: abnormal CATEcholamine distribution, DA: dopamine, dat: DopAmine Transporter, GTPCH: GTP cyclohydrolase, TH: Tyrosine hydroxylase, Tyr: tyrosine, VMAT: vesicular monoamine transporter (Figure from [112]).

Sulston and coworkers isolated several mutants deficient in DAergic pathway, revealing that the *C. elegans* DAergic system is fundamentally very similar to vertebrates. For example, *cat-1* animal mutants (*catecholamine* deficient) have a dramatic reduction in DA content yet over a threefold increase in L-DOPA and are unable to load DA into synaptic vesicles [97]. Duerr and coworkers confirmed these results when they identified the mutation in the *C. elegans* orthologue of mammalian Vesicular MonoAmine Transporters (VMATs) [110].

Also *cat-2* mutants (*catecholamine* deficient) have been identified by Sulston, and they have a complete loss of DA, as detected by FIF and TLC [97]. Lints and Emmons subsequently identified the gene *cat-2* via BLAST searches and gene sequencing, and they found that it encodes a polypeptide, CAT-2, with 50% amino acid identity to tyrosine hydroxylase (TH) [2].

Finally as in mammals two types of DAergic receptors can be identified in *C. elegans*: D1-like receptor (increases cAMP level after DA stimulation) that includes DOP-1 and DOP-4, and D2-like receptor (decreases cAMP level after DA stimulation) that includes DOP-2 and DOP-3 [2, 97, 102, 113-116].

Dopamine-related behaviors in *C. elegans*

Similarly to vertebrates, DA produced in the nematode represents one of the main neurotransmitter and it plays a crucial role as neuromodulator, by altering the intrinsic properties of neurons within circuits, both presynaptically and postsynaptically [117].

In *C. elegans*, DA controls through a sophisticated signaling a wide range of behaviors, including mechanical stimuli, foraging behavior, and food-dependent modulation of locomotion [99, 113, 114, 118]. Similarly to humans, DA signaling mechanisms in the nematode are regulated by environmental factors and by various essential components encoded by genes involved in DA synthesis, release, reuptake, and response [97, 119-121]. Similarly to humans where altered DA signaling is involved in several disorders,

in the nematode defective signaling impairs the control that DA exerts on behaviors previously mentioned.

For example, when healthy non-starved worms move into an area of the culture plate with food bacteria, wild-type (N2) L4 larval stage nematodes drastically slow their locomotion speed. This behavior is known as “Basal Slowing” response. It was found that the detection of the bacterial lawn is mediated by the eight DAergic neurons (two pairs of each CEPV, CEPD, ADE and PDE neurons), but it also depends on functional DA signaling. Indeed genetic mutants of the *cat-2* gene (*catecholamine deficient*), which encodes for tyrosine hydroxylase enzyme for the biosynthesis of DA, do not exhibit basal slowing response, indicating that this behavior is DA-mediated [99, 109]. Exogenous DA restores basal slowing response in *cat-2* mutants as well as in the *cat-4;bas-1* double mutants (an animal which lacks catecholamines altogether), suggesting that a lack of endogenous DA is responsible for this behavior [99].

As mentioned before, DA signaling mechanisms does not depend only on DA biosynthesis but also on other processes that include DA packaging, release and reuptake. The presynaptic DA Transporter (DAT-1), encoded by the *dat-1* gene in the nematode, as well as in human, is of particular importance in reuptaking DA from the synaptic cleft and therefore in modulating the neurotransmitter signal [111, 122]. McDonald et al using *C.elegans* demonstrated that loss of *dat-1* elicits a DA-dependent locomotory phenotype known as Swimming Induced Paralysis (SWIP) [51]. They observed that wild-type L4 stage worms swim at a continuous rate for 10 min when forced to engage in vigorous motor activity in water, while *dat-1* mutant worms initiate normal swimming, but then paralyze over the next six minutes. This paralysis is mainly mediated by DAT-1 activity and also by DOP-3 receptor, because in water animals have to exert maximal motor activity, which needs an efficient clearance of DA mediated by the DA transporter activity. Indeed in *dat-1* genetic mutants there is an extrasynaptic DA accumulation and an over-stimulation of DOP-3 receptors on the cholinergic motor neurons, that leads to an inhibition of acetylcholine release and consequent paralysis.

The *unc-64* gene in *C. elegans*

In 1998 Nonet demonstrated that *unc-64* gene in *C. elegans*, corresponding to cosmid T07H5, showed an high similarity to the vertebrate neuronal synaptic protein syntaxin1A and *Drosophila* Syx1A [123]. *unc-64* gene maps on Chromosome III and it is composed of 8 exons spanning 5 kb. Its protein product exhibits 95,5% of homology with human counterpart and consists of 291 amino acids [123].

In the nematode UNC-64 is as an essential component of the core synaptic vesicle fusion machinery and it is required for normal locomotion [123]. It is expressed in the nervous system and secretory tissues. In particular it is widely distributed in neurons of the nerve ring (Figure 8A) and in the ventral (Figure 8B) and dorsal cord. In neurons, the protein is ubiquitously distributed and similarly to vertebrates it has been also found in the nematode synaptic vesicles [124-126]. Outside the nervous system the protein is expressed in the intestine, the cells of the vulva and in the spermatheca (Figure 8C-D) [123].



Figure 8. Expression of *unc-64* syntaxin

Whole adult wild-type hermaphrodite worms fixed and stained with anti-UNC-64 primary antibodies and visualized with FITC-conjugated antibodies. (A) Lateral view of the head region showing immunoreactivity in the nerve ring, dorsal cord, and pharyngeal nervous system. (B) Ventral view of the midbody region showing expression in ventral cord axons, neuronal cell bodies, and in commissural and sublateral processes. (C) A lateral view of the midbody showing UNC-64 immunoreactivity on the basolateral surface of the intestine. (D) A lateral view of the vulva showing fluorescence in the cells of the vulva (Figure adapted from [123]). Scale bar in all panels is 20µm.

Down-regulation of UNC-64, using classical RNA interference to decrease the endogenous levels of protein product and investigate its role in the nematode, leads to growth rate and locomotion defects [127].

All six *unc-64* genetic mutants isolated so far exhibit locomotory abnormalities. The two mutants allele *js115* and *js116* are lethal, arrest development just after hatching and

die as L1 larvae. In detail *js115* animals (Figure 9B) are completely paralyzed and rarely maintain a sinusoidal posture such as in the wild type animals (Figure 9A) and *js116* animals exhibit very slow motor movements and tend to adopt a coiled position (Figure 9C). In addition the pharyngeal pumping and motor defecation are completely abolished in these mutants. Hypomorphic mutants *js21*, *e246*, *md130* and *md1259* present milder locomotory defects, whereas pharyngeal pumping and motor defecation are relatively normal [128].

In summary, a variety of defective phenotypes have been found in all *unc-64* mutants isolated and in animals where the gene is knocked-down by classic RNA interference approach. These defects and in particular the defective locomotion are very severe and do not allow to use these genetic models to perform more subtle behavioral assays to specifically explore the role of *unc-64* gene in DAergic circuit.

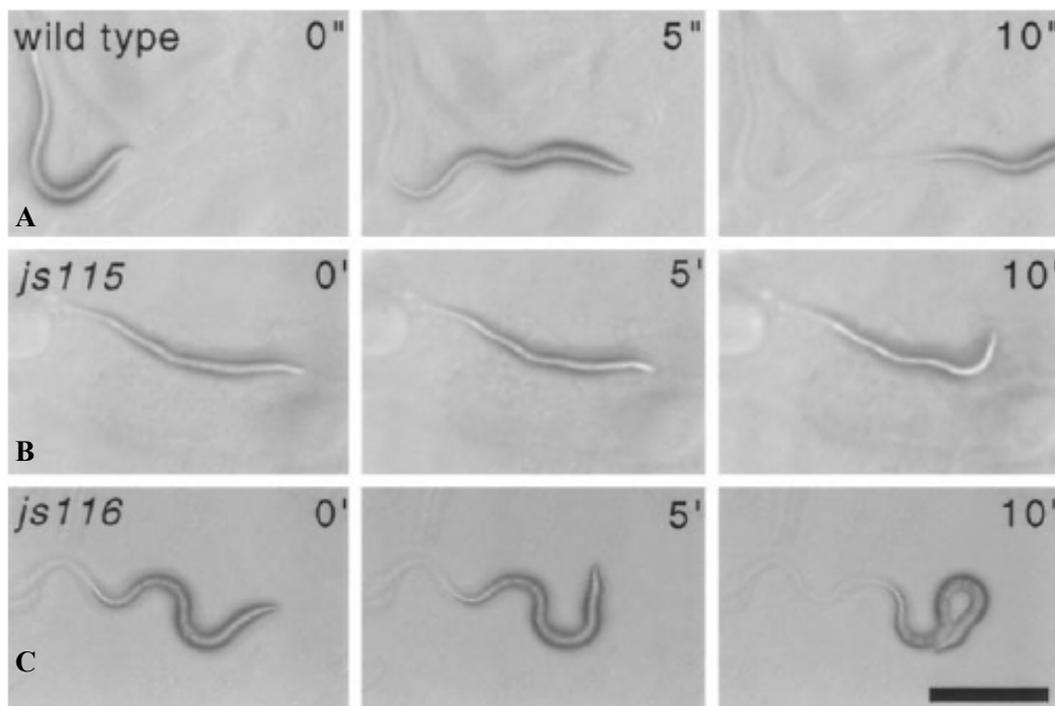


Figure 9. Phenotype of lethal *unc-64* syntaxin mutants

Bright field images of first larval stage animals of wild-type, *unc-64(js115)*, and *unc-64(js116)* animals on an *E. coli* bacterial lawn at the indicated time intervals (wild-type in seconds and mutants in minutes) (Figure from [123]). Scale bar is 100 μ m.

The *unc-63* gene in *C. elegans*

In *C. elegans* *unc-63* gene corresponds to YAC vector Y72E2 and shows homology with human gene encoding for an $\alpha 6$ -subunit of a levamisole-sensitive nicotinic acetylcholine receptor, encoded by CHRNA6 gene. *unc-63* gene maps on chromosome I and it is composed of 10 exons spanning 7.5 kb [129]. Its protein product exhibits 35% of homology with human counterpart, consists of 502 amino acids and possesses motifs common to Cys-loop ligand-gated ion channels [129], including an N-terminal signal peptide of 23 amino acids [130], 4 transmembrane regions, and the Cys-loop [64]. Stretches of amino acids in loops A–F, which are involved in ligand binding, are also conserved. In loop C, there are two adjacent cysteines, defining UNC-63 as a nAChR α subunit (Figure 10) [129].

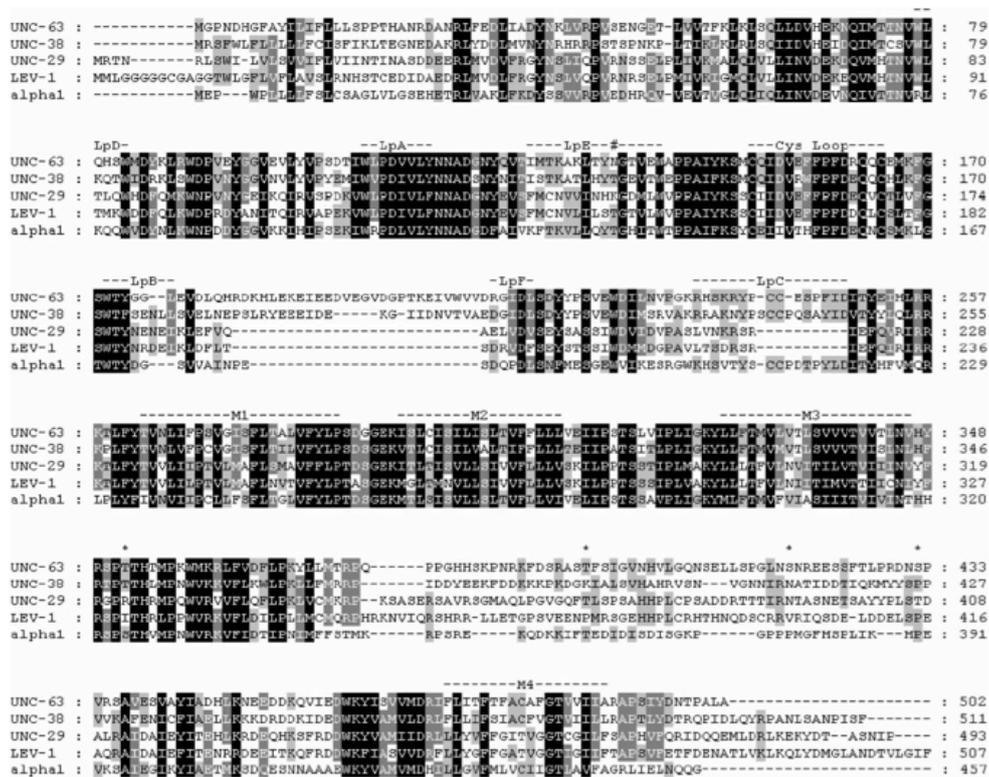


Figure 10. Alignment of UNC-63 and other *C. elegans* AchR with human muscular α -nAChR

The overall similarity between the human muscular α -nAChR subunit (alpha1) and the nematode sequences is 35,3%. Homology is particularly significant in those regions that are thought to be functionally significant. The protein alignment includes UNC-38, LEV-1, UNC-29, and the human muscular α -nAChR subunit (Figure from [129]).

In the nematode, UNC-63 is required for normal locomotion and regulation of egg-laying behavior, and functions as a subunit of a ligand-gated ion channel that likely mediates fast actions of acetylcholine at neuromuscular junctions and in the nervous system [129].

It is expressed in all body wall muscles, in vulval muscles and in many cells of the nervous system, including motor neurons in the ventral nerve cord and neurons in the head, posterior lateral, pre-anal, and lumbar ganglia (Figure 11A-D) [129].

Down-regulation of UNC-63 by classical RNA interference leads to defects in locomotion and egg laying [127].

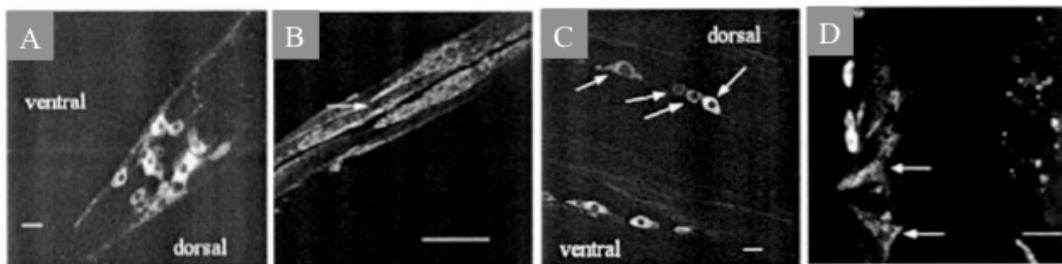


Figure 11. Expression pattern of UNC-63

Transgenic young adult animals showing a typical expression pattern of UNC-63 protein tagged to GFP. Neurons in the anal ganglia (A), and body wall muscle cells (arrow in B) are fluorescent. Scale bar is 5 μ m and 50 μ m respectively (anterior is on the right). Four neurons of the posterior lateral ganglion and some vulval muscles cells (arrows in C-D) also express UNC-63::GFP. Scale bar is 5 μ m and 10 μ m respectively. (anterior is on the left) (Figure adapted from [129]).

Moreover several genetic mutants have been generated and they all exhibit several defective phenotypes. The two null mutants alleles *x13* and *x18* exhibited several defective phenotypes including a strongly uncoordinated locomotion and levamisole resistance [129], whereas the null mutant allele *x37* in addition to these phenotypes also presents swimming defects and resistance to the toxicity caused during post-embryonic development by the nicotinic receptor agonist DMPP (1,1-dimethyl-4-phenylpiperazinium) [131, 132]. Other hypomorphic mutants alleles showed less severe uncoordinated locomotion (alleles *b404* and *x26*) and swimming defects (*x26*) [132, 133].

Also in this case the defective locomotion observed using both classical RNA

interference and genetic mutations, makes it impossible to study UNC-63 function in DAergic circuit. In particular, uncoordinated locomotion can hamper the analysis of DA-mediate behavioral assays, which are necessary to explore the specific role of this gene in DA neuronal circuit.

MATERIAL AND METHODS

Cultivation and Strains

All *Caenorhabditis elegans* strains were cultivated under standard conditions at 20°C on NGM plates seeded with *Escherichia coli* strain OP50, as described by Brenner [82], except for worms that were grown on *Escherichia coli* strain HB101 as a food source. Worms were grown in non-crowded conditions. The wild-type *C. elegans* strain used was N2, Bristol variety. Other strains used include *dat-1(ok157)III*; *cat-2(e1112)II*; *unc-63(x37)I*; *sid-1(qt9)V*; *otIs181[dat-1::mCherry; ttx-3::mCherry]III*; *otIs199[cat-2::GFP; rgef-1(F25B3.3)::dsRed; rol-6(su1006)]*; *vtIs1 [pdat-1::gfp, rol-6(su1006)]*; *sEx14685[punc-63::gfp; dpy(+)]*, *dpy-5(e907)I*, that were provided by the *Caenorhabditis Genetics Center* (CGC, University of Minnesota, USA).

Transgenic lines, and relative genotype, generated in this work, were as follows.

To express of *gfp* in DAergic neurons with *pcat-2*:

gbEx519 [*pcat-2::gfp* 50 ng/uL; *podr-1::rfp*]

To express of *gfp* in DAergic neurons with *pdat-1*:

gbEx570 [*pdat-1::gfp* 50 ng/uL; *podr-1::rfp*]

To knock-down the *gfp* in DAergic neurons with *pcat-2*:

gbEx525 [*pcat-2::gfp* RNAi sas 50 ng/uL; *podr-1::rfp*]; *vtIs1* [*pdat-1::gfp*; *rol-6(su1006)*]

To knock-down the *gfp* in DAergic neurons with *pdat-1*:

gbEx572a [*pdat-1::gfp* RNAi sas 50ng/uL; *pelt-2::rfp*]; *otIs199* [*pcat-2::gfp*; *prgef-1::dsRed*; *rol-6 (su1006)*]

gbEx572b [*pdat-1::gfp* RNAi sas 50ng/uL; *pelt-2::rfp*]; *otIs199* [*pcat-2::gfp*; *prgef-1::dsRed*; *rol-6 (su1006)*]

gbEx572c [*pdat-1::gfp* RNAi sas 50ng/uL; *pelt-2::rfp*]; *otIs199* [*pcat-2::gfp*; *prgef-1::dsRed*; *rol-6 (su1006)*]

gbEx617a [*pdat-1::gfp* RNAi sas 50ng/uL; *pelt-2::rfp*]

gbEx617b [*pdat-1::gfp* RNAi sas 50ng/uL; *pelt-2::rfp*]

To knock-down the *dat-1* in DAergic neurons with *pdat-1*:

gbEx584a [*pdat-1::dat-1* RNAi sas 50ng/uL; *podr-1::rfp*; *pcat-2::GFP*]

gbEx584b [*pdat-1::dat-1* RNAi sas 50ng/uL; *podr-1::rfp*; *pcat-2::GFP*]

gbEx584c [*pdat-1::dat-1* RNAi sas 50ng/uL; *podr-1::rfp*; *pcat-2::GFP*]

To knock-down the *cat-2* in DAergic neurons with *pdat-1*:

gbEx574a [*pdat-1::cat-2* RNAi sas 50ng/uL; *podr-1::rfp*]

gbEx574b [*pdat-1::cat-2* RNAi sas 50ng/uL; *podr-1::rfp*]

gbEx574c [*pdat-1::cat-2* RNAi sas 50ng/uL; *podr-1::rfp*]

To knock-down the *unc-63* in DAergic neurons with *pdat-1*:

gbEx582a [*pdat-1::unc-63* RNAi sas 50ng/uL; *pelt-2::rfp*]

gbEx582b [*pdat-1::unc-63* RNAi sas 50ng/uL; *pelt-2::rfp*]

gbEx582c [*pdat-1::unc-63* RNAi sas 50ng/uL; *pelt-2::rfp*]

To knock-down the *unc-64* in DAergic neurons with *pdat-1*:

gbEx585a [*pdat-1::unc-64* RNAi sas 50ng/uL; *podr-1::rfp*; *pcat-2::GFP*]

gbEx585b [*pdat-1::unc-64* RNAi sas 50ng/uL; *podr-1::rfp*; *pcat-2::GFP*]

gbEx585c [*pdat-1::unc-64* RNAi sas 50ng/uL; *podr-1::rfp*; *pcat-2::GFP*]

To co-localize *unc-63* with DAergic neurons:

otIs181[*dat-1::mCherry*; *ttx-3::mCherry*]; *sEx14685*[*punc-63::gfp*; *dpy(+)*]

gbEx569a[*punc-63::unc-63-SL2-GFP*; *rol-6(su1006)*]; *otIs181*[*dat-1::mCherry*; *ttx-3::mCherry*]

To express *unc-63^{wt}* in muscles and neurons with *pmyo-3*:

gbEx563a [*pAF54 pmyo-3::unc-63wt*; *podr-1::RFP*]

To express *unc-63^{wt}* in neurons with *punc-63*:

gbEx559a [*pAF68 punc-63::unc-63wt*; *podr-1::RFP*]

To rescue the function of *unc-63^{wt}*, expressed in muscles and neurons, in *unc-63(x37)* background:

gbEx563a [*pAF54 pmyo-3::unc-63wt*; *podr-1::RFP*]; *unc-63(x37)*

To rescue the function of *unc-63^{wt}*, expressed in neurons, in *unc-63(x37)* background:

gbEx559a [*pAF68 punc-63::unc-63wt*; *podr-1::RFP*]; *unc-63(x37)*

To remove systemic spreading of RNAi:

gbEx582b [*pdat-1::unc-63* RNAi sas; *pelt-2::rfp*]; *sid-1(qt9)*

Genomic DNA extraction

Nematode genomic DNA was extracted using the following protocol: worms were washed and harvested from NGM plates. Lysis buffer (0.1 M TrisCl pH 8.5, 0.1 M NaCl, 50 mM EDTA pH 8.0, 1% SDS) with 20 mg/mL of Proteinase K was added to harvested worms and incubated at 60°C for 1 hr. Proteinase K was then inactivated by incubation for 20 min at 95°C and 2-3 μ L of the lysates were directly used for PCR reactions.

Construction of transgenes for neuron specific knock-down

The construction of transgene for neuron specific knock-down were made by PCR fusion as previously described [134]. Genomic sequences, corresponding to the target gene and to the chosen specific promoter, are amplified separately.

For the *cat-2* promoter a 600 bp fragment, upstream of the ATG, was amplified using the following primers:

Pf *cat-2*: ataataaaactgcgtggcgtg

Pr *cat-2*: ctctccaattttcaagggg

Nested primer used for the second step was:

Pf*2 *cat-2*: cgtgttgtaagaacgtgcttgatcg

For the *dat-1* promoter a 795 bp fragment, upstream of the ATG, was amplified using the following primers:

Pf *dat-1*: aaagtctttctgcccacacaa

Pr *dat-1*: agtaaaccgtagcgggatcag

Nested primer used for the second step was:

Pf* *dat-1*: cgacctatacactttctctcg

For the *C. elegans* target genes, I amplified from genomic DNA the same exon rich regions that have been used for plasmid libraries prepared for RNAi by feeding experiments [135-137]. For *gfp* expression and knocking-down I amplified the fragment to be fused from A. Fire plasmids pPD95.75 and L4417, respectively.

Primers used to amplify the *unc-63* region (2480 bp) were:

Tfa *unc-63* sas: tggcgggtatttgaattctcc

Tfs *unc-63* sas: tgagttttgccccttttg

Tr* *unc-63* sas : TGATCCGCTATATAGGCAACACT

Tf* *unc-63* sas: TGTTTGAAAAAGTTTCCACCTGT

Primers used to fuse the *unc-63* fragment to *dat-1* promoter were:

Tf *pdat-1::unc-63* sas: ctgatcccgtctacggtttactTGTTTGAAAAAGTTTCCACCTGT

Tr *pdat-1::unc-63* sas: ctgatcccgtctacggtttactTGATCCGCTATATAGGCAACACT

Primers used to amplify the *unc-64* region (1999 bp) were:

Tfa1 *unc-64* sas: ctttcgtgctgagacctgtc

Tfs *unc-64* sas: AATGCCAGGAATATACTGAATGAG

Tr* *unc-64* (1) sas: CTCAATTCGATCAACCATCTCTC

Tf* *unc-64* (1) sas: AGAGATTCGTGGAAGTGTGGATA

Primers used to fuse the *unc-64* fragment to *dat-1* promoter were:

Tf *pdat-1::unc-64* sas: ctgatcccgtctacggtttactAGAGATTCGTGGAAGTGTGGATA

Tr *pdat-1::unc-64* sas: ctgatcccgtctacggtttactCTCAATTCGATCAACCATCTCTC

Primers used to amplify the *gfp* region (890 bp) for knocking-down were:

Tfa *gfp* sas: gttgtaaaacgacggcagt

Tfs *gfp* sas: GGCCGATTCATTAATGCAG

Tf* *gfp* sas: tcaactataGGGAGACCGGCA

Tr* *gfp* sas: tcactatagggcgaattggg

Primers used to fuse the *gfp* fragment to *dat-1* promoter were:

Tf *pdat-1::gfp* sas: ctgatcccgtctacggtttactTCACTATAGGGAGACCGGCA

Tr *pdat-1::gfp* sas: ctgatcccgtctacggtttactTCACTATAGGGCGAATTGGG

Primers used to fuse the *gfp* fragment to *cat-2* promoter were:

Tf *pdat-2::gfp* sas: cccttgaaaaattggaagagTCACTATAGGGAGACCGGCA

Tr *pdat-2::gfp* sas: cccttgaaaaattggaagagTCACTATAGGGCGAATTGGG

Primers used to amplify the *dat-1* region (890 bp) were:

Tfs *dat-1* sas: TTCGAACCTGATCTCAACCC

Tfa *dat-1* sas: TGCAGTTGGTGCCTACAGg

Tf* *dat-1* sas: AAGCAAATGCACCGAACTCT

Tr**dat-1*sas: AGCTCCAGCAAAACTTCCAA

Primers used to fuse the *dat-1* fragment to *dat-1* promoter were:

Tf *pdat-1::dat-1* sas: ctgatcccgtctacggtttactAAGCAAATGCACCGAACTCT

Tr *pdat-1::dat-1* sas: ctgatcccgtctacggtttact AGCTCCAGCAAAACTTCCAA

Primers used to amplify the *cat-2* region (1092 bp) were:

Tfa *cat-2* sas: caagctcttgatccgtga

Tfs *cat-2* sas: acaatctgctgaacgcctt

Tf* *cat-2* sas: GAAATTCTCGATTTTCGCCA

Tr* *cat-1* sas : CTTCTTTGCACAACCCGAAT

Primers used to fuse the *cat-2* fragment to *dat-1* promoter were:

Tf *pdat-1::cat-2* sas: ctgatcccgtacggtttactGAAATTCTCGATTTTCGCCA

Tr *pdat-1::cat-2* sas: ctgatcccgtacggtttactCTTCTTTGCACAACCCGAAT

All the Tf and Tr primers had at their 5'-end 20-21 additional nucleotides complementary to 3' end of the promoter, which drove the knocking-down.

A mixture of sense and anti-sense PCR fusion product at the concentration of 50ng/uL was microinjected together with co-transformation markers *podr-1::RFP*, expressed in the AWB and AWC neurons (kind gift from C. Bargmann, Rockefeller University, USA) or *pelt-2::RFP*, expressed in intestinal cells (kind gift from J.D. McGhee, University of Calgary, USA) at the concentration of 30ng/uL, into the gonad of N2 animals using standard microinjection.

Microinjection

Transgenic animals are generated by injecting appropriate DNA fragments or plasmids into the distal gonad syncytium or directly into the developing oocytes. Injected DNA forms large extrachromosomal arrays incorporating between 50 and 300 copies, which are inherited by the progeny of injected animals. Identification of transgenic individuals is facilitated by the use of several available transformation markers, which are co-injected along with the DNA of interest. In the present work I used the two fluorescent co-transformation markers *podr-1::RFP* and *pelt-2::RFP*, previously described (see above). A varying number of F1 progeny that carry the desired extrachromosomal array can thereby be identified by characteristic phenotypes associated with each transformation marker. Extrachromosomal arrays are inherited in a non-Mendelian manner to subsequent generations. Typically, a varying percentage (between 5 and 80%) of next-generation progeny will carry the extrachromosomal array [138].

Genetic crosses

A genetic cross in *C. elegans* is usually carry out by using non-starved animals at the L4 stage. Hermaphrodites and males in a 5:1 ratio, respectively, are transferred to a new plate that contains only a minimal surface area of bacteria to encourage physical proximity and thus increase the likelihood of mating. A paternal marker was used to select the non-self F1 progeny that was moved to a new fresh plate to identify our strain of interest.

Construction of transgenes for rescue experiments

The construction of transgenic lines for rescue experiments of *unc-63* function was performed by microinjection of plasmid DNA into the gonad, using standard microinjection [138] and afterwards by performing genetic crosses. In the case of *unc-63* project, N2 animals were injected with a DNA mixture cointaining plasmid pAF54[pmyo-3::*unc-63*] or pAF68[punc-63::*unc-63*] at the concentration of 30ng/uL together with a co-transformation marker *podr-1::RFP* at the concentration of 30ng/uL. The two transgenes obtained, *gbEx563a* [pAF54 *pmyo-3::unc-63wt*; *podr-1::RFP*] or *gbEx559a* [pAF68 *punc-63::unc-63wt*; *podr-1::RFP*] were then crossed as previously described (see above) with a *unc-63(x37)* mutant strain. The two transgenic homozygous strains obtained were:

- *gbEx563a* [pAF54 *pmyo-3::unc-63wt*; *podr-1::RFP*]; *unc-63(x37)*
- *gbEx559a* [pAF68 *punc-63::unc-63wt*; *podr-1::RFP*]; *unc-63(x37)*

The presence of *unc-63(x37)* allele was confirmed by performing the SWIP assay (see below) and by sequencing the appropriate region of genomic DNA. Plasmids pAF54 and pAF68 were a kind gift from J.L. Bessereau (Paris, France).

Construction of transgenes with no systemic spreading of RNAi

The construction of a transgenic line without systemic spreading of RNAi was performed by microinjection of DNA into the gonad, using standard microinjection [138] and afterwards by performing genetic crosses. N2 animals were injected with a DNA mixture cointaining (*pdat-1::unc-63* RNAi sas) at the concentration of 50ng/uL,

together with a co-transformation marker *pelt-2::RFP* at the concentration of 30ng/uL. The transgene obtained *gbEx582b* [*pdat-1::unc-63* RNAi sas; *pelt-2::rfp*] was crossed as previously described (see above) with a *sid-1(qt9)* mutant strain. The transgenic homozygous strain obtained is *gbEx582b* [*pdat-1::unc-63* RNAi sas; *pelt-2::rfp*]; *sid-1(qt9)*. The presence of *sid-1(qt9)* allele was confirmed by PCR genotyping and by sequencing the appropriate region of genomic DNA from the transgenic strain.

Construction of transgenes for microscopy analysis

The construction of transgenic lines for microscopy analysis was performed by microinjection of plasmid DNA into the gonad, using standard microinjection [138], or by genetic crosses. In the first case *otIs181*[*dat-1::mCherry* + *ttx-3::mCherry*] transgenic animals were injected with a DNA mixture containing plasmid *pAF66* (*punc-63::unc-63-SL2-GFP*) at the concentration of 30ng/uL together with *pRF4* plasmid [*rol-6(su1006)*], as co-transformation marker at the concentration of 50ng/uL. The double transgenic strain obtained was *gbEx569a*[*punc-63::unc-63-SL2-GFP*; *rol-6(su1006)*]; *otIs181*[*pdat-1::mCherry*; *ttx-3::mCherry*]. Plasmids *pAF66* was a kind gift from J.L. Bessereau (Paris, France).

In the second case the *sEx14685*(*punc-63::gfp*) transgenic animals was crossed as previously described (see above) with *otIs181*[*dat-1::mCherry*; *ttx-3::mCherry*] transgenic animals. The transgenic homozygous strain obtained was *sEx14685*(*punc-63::gfp*); *otIs181*[*dat-1::mCherry*; *ttx-3::mCherry*]. The presence of the two transgenes was confirmed by selecting animals at the stereomicroscope using GFP and *mCherry* fluorescence filters.

Microscopy and imaging

Animals were mounted and anesthetized with 0.01% tetramisole hydrochloride (Sigma-Aldrich) on 4% agar pads. As regarding the analysis of GFP expression and knocking-down on DAergic neurons, animals were visualized using Zeiss Axioskop microscopes. Confocal microscopy images were obtained with a confocal microscopy Leica (TCS SP2 AOBS filter-free spectral laser scanning) and were formatted using LCS (Leica confocal software).

Swimming Induced Paralysis (SWIP) assay

SWIP assay was done as previously described [51]. All worms assayed were selected as hermaphrodites at L3 or L4 stage, 24 hours before the execution of the experiments. Animals were placed in 40 uL of water with or without AMPH in a Pyrex Spot Plate and scored for movement for 10 minutes. In this study, we used an intermediate concentration of AMPH (0,5 mM) that is known to be physiologically relevant for long treatment periods such as 10 minutes [139]. The number of paralyzed worms was reported as a percentage among the total number of animals observed in each test. At least 3 trials were conducted on each strain.

Data were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA).

Basal Slowing Response (BSR) assay

Basal slowing response assays were done as previously described [114]. Young egg-laying adults, 18-22 hours post L4 stage at 20° C, were assayed. The locomotion rates of young adult animals were quantified by counting the number of body bends completed in 5 consecutive 20-second intervals in the presence and the absence of HB101 bacteria. Data were collected for 6 animals per condition for a total of 30 measurements per condition. Percent of slowing was calculated by dividing the difference between locomotion rates on and off food by the locomotion rate off food. Data were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA).

Nicotinic agonist resistance assay

1,1-Dimethyl-4-phenylpiperazinium (DMPP) (Sigma) was dissolved in water and added to 55°C-equilibrated NGM agar just before plates were poured, as previously described [131]. Gravid adult worms were sacrificed to recover the eggs that were carefully transferred on 0,75mM DMPP-containing plates and incubated for 24 hours (@20°C). After 24 hours the number of L1 larvae was scored and the assay was concluded when surviving L3, L4 and dauer larvae were scored, after 3 days of development.

Data were analyzed using Prism software (GraphPad Software, Inc., San Diego, CA).

Statistical analysis

Primer of Biostatistics and GraphPad Prism softwares were used for statistical analyses. The statistical significance was determined using Mann-Whitney or T-student or Compare two proportions tests. Standard error of mean was used to estimate variation within a single population.

AIM OF THE PROJECT

The neurotransmitter DA, produced by the DAergic system, controls several behaviors in the nervous system, that when altered may cause neuronal disorders. A correct balance of the DA levels mainly depends on the elements constituting the DA-biosynthesis pathway, including the initial rate limiting enzyme Tyrosine Hydroxylase (TH) and the DA Transporter (DAT). However other key elements have been also identified that seem to participate to DAergic neurotransmission, including the SNARE protein syntaxin1A (Syn1A) and the $\alpha 6$ -subunit of the nicotin acetylcholine receptors (CHRNA6). During my PhD thesis, I used the nematode *Caenorhabditis elegans* as a model system to explore, *in vivo*, the role in the DAergic circuit of *unc-64* and *unc-63* genes, homologues to CHRNA6 and syntaxin1A. I chose these two genes because of evidences in literature that suggest their potential involvement in DAergic system. By studying these genes we want to contribute to a better understanding of their role in DAergic system and of their possible involvement in pathologic conditions.

EXPERIMENTAL PLAN

During my PhD thesis, I used the advantages of the nematode *C. elegans* as animal model to explore in details the role of *unc-64* and *unc-63* genes, the mammalian syntaxin1A and CHRNA6 homologues, *in vivo*.

In *C. elegans* several experimental models have been generated aimed at understanding the complex mechanisms regulating the DAergic system. These models include chemical treatments and engineered transgenes, which unfortunately are not feasible to study the loss of function of a gene [140]. In addition classic genetic mutations can cause pleiotropic effects or lethal phenotypes in mutant animals that can hamper the study of DAergic system. This was true both for *unc-64* (Syn1A) and *unc-63* (CHRNA6) genes.

In the case of *unc-64*, its down regulation by classical RNA interference leads to growth rate and locomotion defects [127]. So *unc-64* genetic mutants can not be used to perform DA-related behavioral assays *in vivo*. In particular, *unc-64* null mutants exhibit locomotory abnormalities that lead to a complete paralysis, while hypomorphic mutants still present severe locomotory abnormalities [123].

Also for *unc-63*, downregulation by RNA interference leads to severe locomotion defects [127]. Moreover severe locomotory abnormalities are observed in *unc-63* mutants, together with other phenotypes which includes swimming defect, levamisole and DMPP resistance [129, 131-133].

Therefore *unc-63* and *unc-64* genetic models obtained using genetic mutations or classic RNA interference are not appropriate to investigate the role of these two genes in *C. elegans* DAergic circuit, which requires specific subtle behavioral assays. To overcome these limitations, I took advantage of a powerful RNAi technique developed in the laboratory where I Have been doing my PhD work [134] to set up efficient transgenic models of DAergic specific knock-down of *unc-63* and *unc-64* genes.

The discovery of RNAi has provided a powerful reverse genetic tool that has enormously increased the range of *C. elegans* genes whose function can be rapidly analyzed [87, 88]. However there are several limitations to the use of RNAi to address the function of genes in specific cells, especially in neurons [141]. First, direct delivery of dsRNA to worms, by injection, feeding or soaking [142] results in systemic RNAi, which affects many or all the cells in the organism. Thus it is not possible, with this

approach, to dissect the role exerted by a gene of interest in specific cells or group of cells. Moreover essential genes are obviously very difficult to study. Second, some late acting genes and most of the genes expressed in neurons are largely resistant to RNAi knocking-down [141, 142]. In *C. elegans*, a transgene driven genetic interference has been described as a possible way to overcome some of the limitations of classical RNAi obtained by direct delivery of dsRNA to the animals [141]. However each of these techniques present different limitations. The method described by Tavernarakis *et al.* (2000), based on the generation of a harpin dsRNA transgene driven by a heat-shock promoter, cannot be utilized for cell-specific knock-down and there are also problems related to the stability of the harpin in the plasmid [141, 143]. Modifications of this approach, using cell-specific promoters, have also been described but the efficiency of knocking-down in neurons and the cell-autonomy of the effects obtained have either not been addressed or have produced conflicting results [141, 144, 145]. Finally an inducible genetic interference has also been obtained with sense and antisense RNAs transcribed as separate molecules and not as a harpin plasmid [146]. However also in this case, an ubiquitously heat-shock promoter was used and thus the knock-down of gene function was not targeted to specific cells.

The innovative RNAi technique developed in the laboratory by Esposito *et al.* (2007) enables to efficiently reduce the function of a chosen gene in selected *C. elegans* neurons. This neuron-specific RNAi knocking-down method is transgene driven and is based on the expression, under a cell-specific promoter, of sense and antisense RNA molecules corresponding to a fragment of the gene of interest (for this reason it was called RNAi sas). The sense and antisense fragments are easily amplified and can be mixed and injected as such to transform animals, making this approach relatively fast and easy (Figure 12) [134]. It has been demonstrated that gene knocking-down obtained with RNAi sas is efficient, heritable and cell-autonomous. The RNAi sas has been used with success to knock-down several genes in different neuronal classes and to investigate their role in development and in the survival of selected neurons, as well as in the behavioral response they control [147-151].

In conclusion this powerful RNAi approach allows to dissect the role played by a gene of interest, specifically in chosen neurons in otherwise wild type animals, eliminating the onset of lethal effects due to the use of classic genetic mutations or the difficulty of

studying the gene loss of function by using chemical treatments or RNA interference approaches previously described.

Using this innovative RNAi strategy I reduced the function of the two candidate genes selectively in DAergic *C. elegans* neurons, thus generating transgenic animals which were viable and able to move. Afterwards I performed molecular and behavioral studies on these transgenic animals to dissect the role of *unc-64* and *unc-63* genes in DAergic circuit.

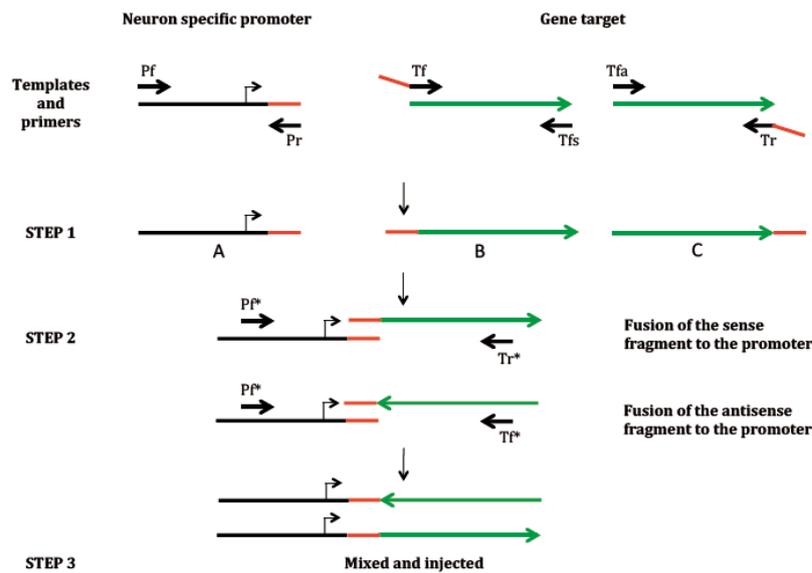


Figure 12. Construction of transgenes for neuron specific knock-down.

The construction of transgene for neuron specific knock-down includes three steps. In the first step, genomic sequences corresponding to the target gene and to the chosen specific promoter are amplified separately. The neuron specific promoter (black line) is amplified with primers Promoter forward (arrow Pf) and Promoter reverse (arrow Pr) obtaining the product A. An exon rich fragment of the target gene (green line) is amplified in two different reactions to obtain the sense and antisense fragments. The sense fragment (product B) is amplified with primers Target forward (arrow Tf) and Target forward sense (arrow Tf_s). The antisense fragment (product C) is amplified with primers Target forward antisense (arrow Tfa) and Target reverse (arrow Tr). The Tf and Tr primers had at their 5' end 20-21 additional nucleotides complementary to 3' end of the of promoter (red line). In the second step A is fused by amplification to B or C, separately, by using the nested primers Pf* and either Tr* or Tf*. These two reactions yield DNA fragments in which the target gene fragment can be transcribed by the specific promoter in the sense and antisense orientation. In the third step the fusion fragment are mixed in equimolar amounts and injected, together with a visible marker (not shown), in recipient animals.

RESULTS

Set up of a specific and efficient knock-down of genes of interest in dopaminergic neurons

To dissect the role played by a gene of interest, specifically in DAergic neurons in otherwise wild type animals, we have to obtain transgenic animals injected with DNA constructs made of a specific DAergic promoter fused to a part of the genomic region of the target gene, to be amplified in sense and antisense direction. Moreover we need to prepare the appropriate positive and negative controls, where genes known to play a function (positive control genes) and not playing a role (negative controls) in DAergic neurons are knocked-down with the same approach and their phenotypes are compared to the corresponding genetic mutants.

In order to knock-down in DAergic neurons the different genes of interest, I tested the efficiency of the promoters of two genes, *cat-2* and *dat-1*, hence named *p_{cat-2}* and *p_{dat-1}*. These promoters have been described in literature to be specific to DAergic neurons and we decided to compare their efficiency by using them to express green fluorescent protein (GFP) reporter gene and then to separately knock-down the same reporter gene in DAergic neurons. *dat-1* gene encodes for DAT Transporter and is required to regulate synaptic DA signaling by controlling extracellular DA levels (Figure 7). A promoter spanning 0.7 kb of *dat-1* upstream region fused to *gfp* causes an intense gene reporter expression, restricted to DAergic neurons [106]. *cat-2* gene encodes for Tyrosine Hydroxylase (TH) and is the rate-limiting enzyme required to produce L-Dopa in DA biosynthesis. In this case, 1.5 kb of the presumptive *cat-2* promoter were used to drive *gfp* expression and an intense expression of the reporter only in DAergic neurons was observed [2, 97, 102]. Moreover Flames and Hobert in 2009 demonstrated the existence of small *cis*-regulatory modules (CRM) in each DAergic promoter, necessary to drive expression specifically in *C. elegans* DAergic neurons [152]. Based on these informations, I generated PCR constructs by fusing the *gfp* gene to the promoter regions of *cat-2* (0.6 kb) or *dat-1* (0.8 kb), which include the *cis*-regulatory modules (CRM). These PCR fusions were injected in wild type animals and I obtained two different transgenic strains expressing *p_{dat-1}::gfp* and *p_{cat-2}::gfp*, respectively. I carried out analysis at the compound fluorescence microscope to

determine the expression pattern of the two promoters in DAergic neurons. As shown in Figure 13 both promoters were expressed in all DAergic neurons and only in them (data not shown). Moreover the percentage of CEP, ADE and PDE visible neurons was different for the two promoters, with *pdat-1* expressed in 90%, 93% and 92% of expected neurons, respectively, and *pcat-2* in 64%, 81% and 31%, respectively.

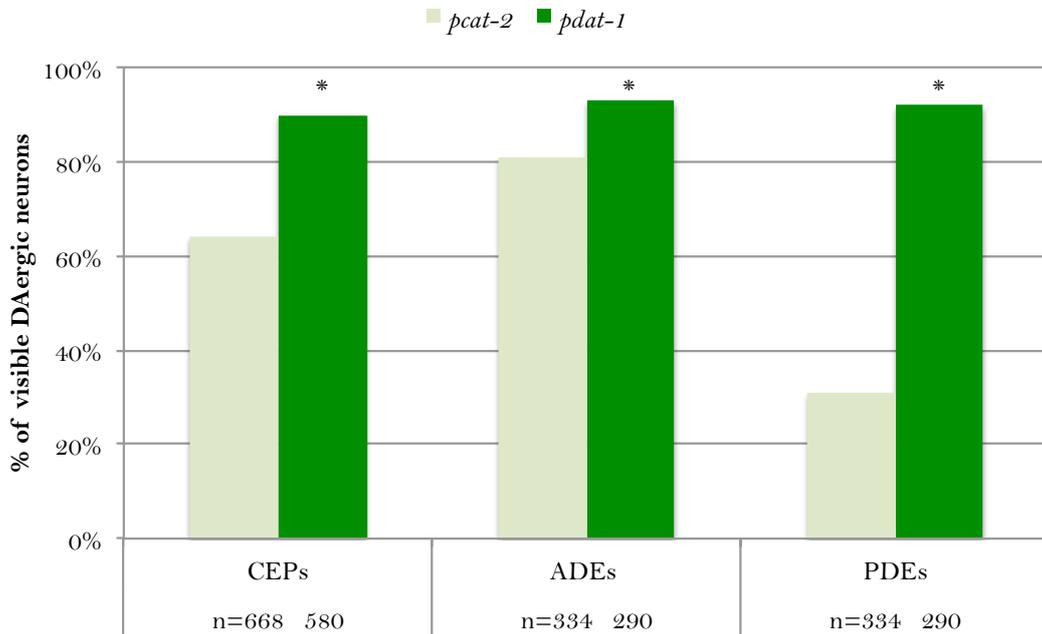


Figure 13. Efficiency of the two dopaminergic promoters in driving GFP expression in dopaminergic neurons
Quantification of CEPs, ADEs and PDEs visible neurons, among the expected ones in transgenic animals expressing GFP gene under *pdat-1* (green bar) or *pcat-2* (light green bar). Neurons were quantified at L4 larval stage, using compound fluorescence microscopy. n is the number of neurons expected to be visible (8 per worm). Asterisks represent statistical difference between two bars by using Compare two proportions non parametric test (* $p < 0.001$ significantly different from *pcat-2*).

Then I examined the ability of the two promoters to knock-down a reporter gene in DAergic neurons. With this aim I used the RNAi sas technique [134] described above, to reduce the expression of *gfp* in the same neurons but in a different transgenic background where GFP is constitutively expressed. To this aim I drove the expression of fragments corresponding to a part of the *gfp* gene, in sense and antisense direction, under the control of *pdat-1* or *pcat-2*. The two PCR constructs obtained were injected in two different integrated transgenic strains, in which the *gfp* is expressed in DAergic

neurons under the control of *pcat-2* and *pdat-1*, respectively. A “complementary” approach, i.e. *pdat-1::gfp(RNAisas)* silencing construct injected in *pcat-2::GFP* transgenic strain for GFP expression and viceversa, was used to avoid any disturbance of the same promoter on gene knock-down and on gene expression. Compound fluorescence microscope analysis was used on determining a possible different ability between the two promoters to knock-down the *gfp*. In particular after *gfp* knock-down using *pdat-1*, only 12%, 6% and 29% of CEP, ADE and PDE neurons, respectively, were still visible, while in the controls that constitutively express the *gfp* in DAergic neurons 100%, 94% and 99% of CEP, ADE and PDE neurons are usually visible. Differently, 91%, 90% and 90% of CEP, ADE and PDE neurons, respectively, were visible when using *pcat-2* (Figure 14).

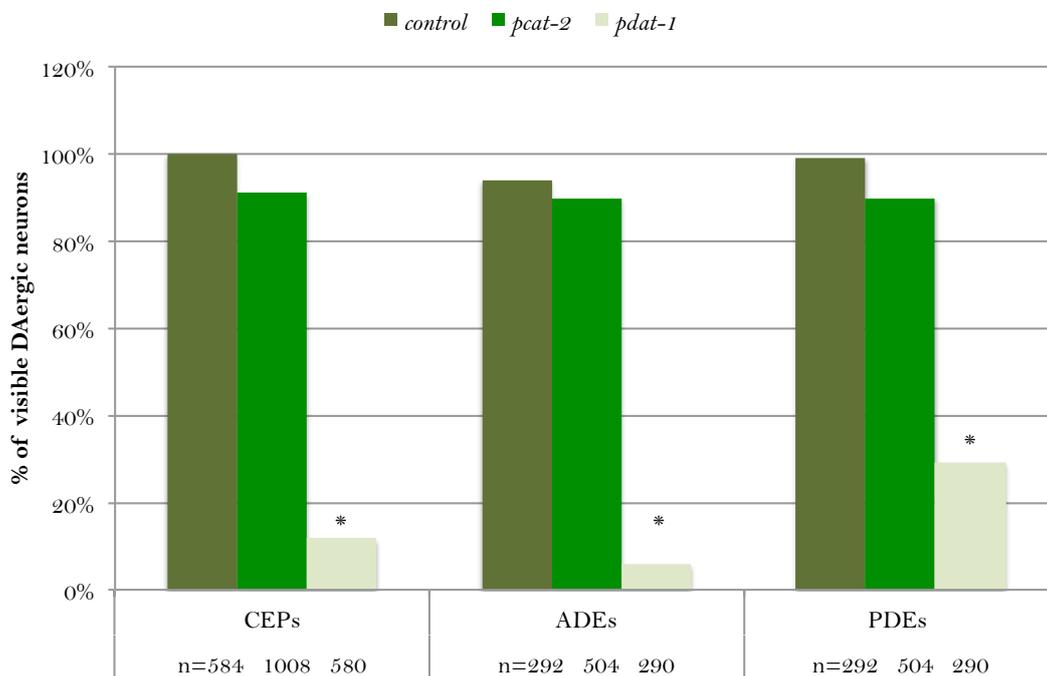


Figure 14. Efficiency of the two specific promoters in knocking-down GFP expression in dopaminergic neurons

Quantification of CEPs, ADEs and PDEs visible neurons, among the number of neurons expected to be present in transgenic strains with two interfering constructs, *pdat-1::gfp(RNAisas)* or *pcat-2::gfp(RNAisas)*, and GFP constitutively expressed. The *gfp* specific knock-down in DAergic neurons induces a reduction in the number of visible neurons that is stronger with *pdat-1* (green bar) than *pcat-2* (light green bar). Neurons visible in controls are represented by a dark green bar. Neurons were quantified in L4 larval stage animals under compound fluorescence microscopy. n is the number of neurons expected to be present. Asterisks represent statistical difference from *pcat-2* and control by using Compare two proportions non parametric statistical test (* $p < 0.001$ significantly different from

pcat-2 and control).

In conclusion all these analysis revealed that *pdat-1* is much more efficient in driving in DAergic neurons the expression and the knocking-down of a *gfp* reporter than *pcat-2*. Thus I used this promoter for further experiments and to generate transgenic models of DA specific knock-down of the two genes of interest, *unc-63* and *unc-64*.

Before generating these transgenic models, I evaluated the ability of *pdat-1* to knock-down in DAergic neurons genes normally expressed in the same neurons (positive control genes). With this purpose, I chose *dat-1* and *cat-2* genes, known to have an essential role in the DA pathway.

The nematode *C. elegans* is an animal model with a simple but sophisticated DAergic nervous system. In particular a variety of DA-mediated behavioral assays have been identified. In order to confirm the knock-down of the positive control genes *cat-2* and *dat-1* in DAergic system, I performed two of these behavioral assays, which are the Basal Slowing Response (BSR) and the SWimming Induced Paralysis assay (SWIP).

The Basal Slowing Response (BSR) assay tests one of the main effects of DA release from DAergic neurons in response to food. As demonstrated from Sawin *et al.*, wild-type animals slow down their rate of locomotion when they encounter a bacterial lawn, whereas *cat-2(e1112)* mutant animals, defective for tyrosine hydroxylase and therefore for DA synthesis, do not exhibit a slowing response because they lack endogenous DA [99]. To obtain a *cat-2* knock-down by using the RNAi sas technique, I generated transgenic animals expressing sense and antisense corresponding to *cat-2* sequence under the control of *pdat-1* promoter. Afterwards I used the basal slowing response assay to evaluate the ability of the technique to efficiently knock-down *cat-2* gene in DAergic neurons. As reported in Figure 15, the transgenic animals lacking *cat-2* in DAergic neurons exhibited a defective basal slowing response similar to *cat-2(e1112)* null mutant animals, whereas the negative control, represented by transgenic animals lacking *gfp* in the same neurons using the same approach, reduced its locomotion rate in response to bacterial food, behaving similarly to wild-type animals. These results demonstrated that the DAergic specific knock-down of *cat-2* gene, using *pdat-1* and this novel RNAi approach, is effective and is able to reproduce the specific phenotype observed in *cat-2(e1112)* mutant animals.

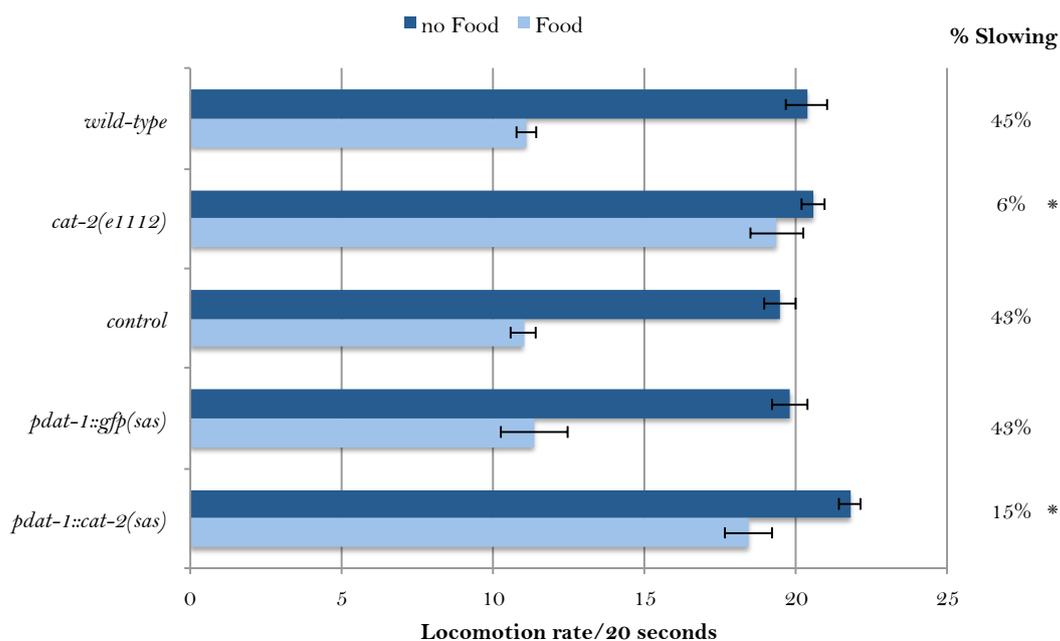


Figure 15. *cat-2* knock-down in dopaminergic neurons causes a defect in Basal Slowing Response

Transgenic animals lacking *cat-2* in DAergic neurons exhibit a defective slowing response (*pdat-1::cat-2(sas)*), similar to *cat-2(e1112)* mutant animals and different from the negative controls: wild-type animals (first row), non transgenic animals (*control*) and negative controls (*pdat-1::gfp(sas)*). Locomotory rate was counted for 5 young adult worms per strain, for five intervals each of 20-seconds, with a total of 30 independent measurements per condition. Dark-blue bars indicate locomotion rate among 20 seconds in the absence of food, and blue bars indicate locomotion rate in the presence of food. Error bars indicate SEM between independent experiments. Asterisks indicate values significantly different from the 45% of slowing seen in the wild type (Student's t-test: *P <0.001). The percentage of slowing in the presence of bacteria for each strain is shown on the right.

Another DAergic specific behavior in the nematode is the specific locomotory response to an excess of extrasynaptic DA neurotransmitter. As described by McDonald *et al.* 2007, this behavior consists in a paralysis subsequent to vigorous swimming or thrashing, termed “SWimming-Induced Paralysis” (SWIP) that is dependent on endogenous DA release as well as re-uptake by DAT-1 transporter function. In fact 50% of *dat-1(ok157)* null mutant animals, which are defective for DA reuptake into presynaptic neurons, display a paralytic phenotype when placed in water for 10 minutes [51]. To generate a suitable *dat-1* knock-down as positive control by using the RNAi sas technique, I generated transgenic animals expressing sense and antisense fragments corresponding to *dat-1* gene sequence under the control of *pdat-1*. Afterwards I performed the SWIP assay to evaluate the ability of the approach to efficiently knock-

down *dat-1* in DAergic neurons. This analysis revealed that transgenic animals lacking *dat-1* in DAergic neurons showed an intermediate SWIP response, compared to *dat-1(ok157)* mutant animals, with 30% of the animals becoming paralyzed after 10 minutes of swimming in water versus 55% in the genetic mutant. The negative controls, as above, showed a SWIP response similar to the wild-type strain (below 10%) (Figure 16). In conclusion also this result confirmed that the use of *pdat-1* and of RNAi sas approach are able to efficiently reduce the function of a target gene in DAergic neurons, generating the same phenotype observed in mutant animals.

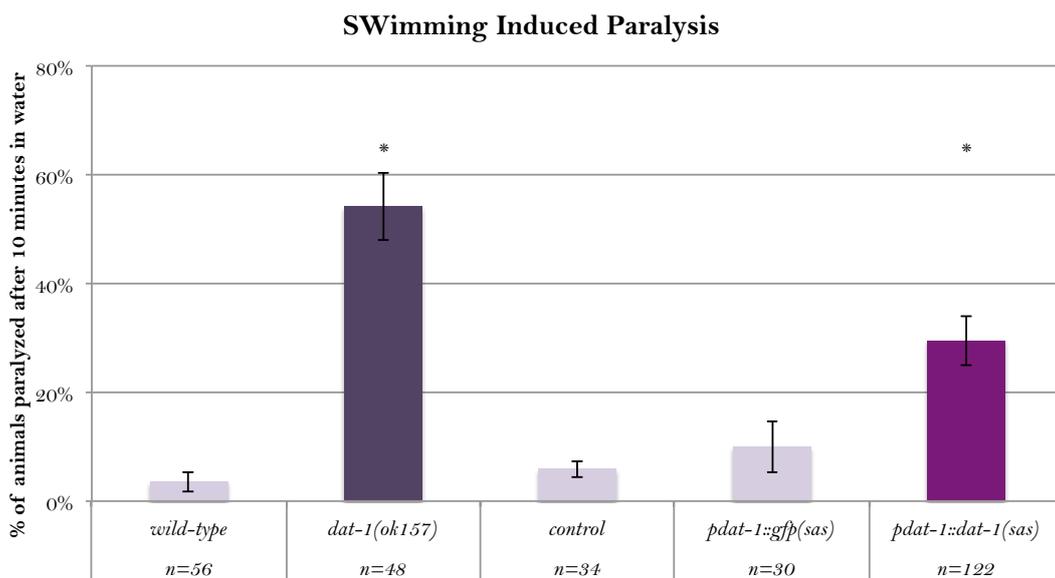


Figure 16. The *dat-1* knock-down in dopaminergic neurons induces SWIP

After knocking-down *dat-1* specifically in DAergic neurons, the 30% of transgenic animals became paralyzed (purple bar), similarly to *dat-1(ok157)* mutant animals (plum bar). Error bars indicate SEM between independent experiments. Statistical significance was assessed by Compare two proportions (* $p < 0.05$, significantly different from wild-type, control and *pdat-1::gfp(sas)*). n is the number of L4 animals observed.

Cell specific knock-down of *unc-64* gene

UNC-64 is an essential component of the core synaptic vesicle fusion machinery in the nematode and it is required for normal locomotion [123]. Previous works have shown that in *C. elegans* UNC-64, similarly to what happens in mammals [48, 49], interacts with the N-terminus of DAT-1 [40]. Moreover it has been observed that fusing the GFP to DAT-1 N-terminus prevented DAT-1 interaction with UNC-64, causing a swimming

induced paralysis (SWIP) [40], the behavioural phenotype observed in DAT-1 deficient nematodes [51].

In order to explore the role of UNC-64 in *C. elegans* DAergic system, I focused on the effects of its knock-down in DAergic neurons on motor behavior. To study the role of Syntaxin 1A in *C. elegans* DAergic circuit, I generated a transgenic model, using the RNAi sas technique, to knock-down the *C. elegans* Syn1A homologue, *unc-64*, in DAergic circuit. The DAergic specific knock-down of *unc-64* gene was obtained by injecting the interfering construct, prepared as above, by PCR-fusing the *pdat-1* promoter to sense and antisense fragments corresponding to *unc-64* gene sequence.

All the transgenic lines obtained from this microinjection were viable, did not present developmental defects and abnormal locomotion. Therefore by using the RNAi sas technique I overcame the problems related to the classical genetic and RNAi approaches, that cause the onset of several defective phenotypes and did not allow to perform behavioral assays, which are essential to explore the role of *unc-64* gene in DAergic circuit.

Behavioral analysis

I carried out the SWIP assay on transgenic animals where *unc-64* is knocked-down in DAergic neurons, and observed that 24% of them showed a locomotion paralysis when placed in water for 10 minutes, similarly to *dat-1(ok157)* null mutant animals (Figure 17). Moreover this intermediate SWIP response is also very similar to the positive control, represented by animals lacking *dat-1* in the same neurons, that presented 30% of paralysed animals (Figure 17). This result demonstrated that the knock-down of *unc-64* specifically in DAergic neurons is able to increase the extrasynaptic endogenous DA levels and cause a SWIP. This demonstrates that *unc-64* presence is important in DAergic system to guarantee a correct DA reuptake.

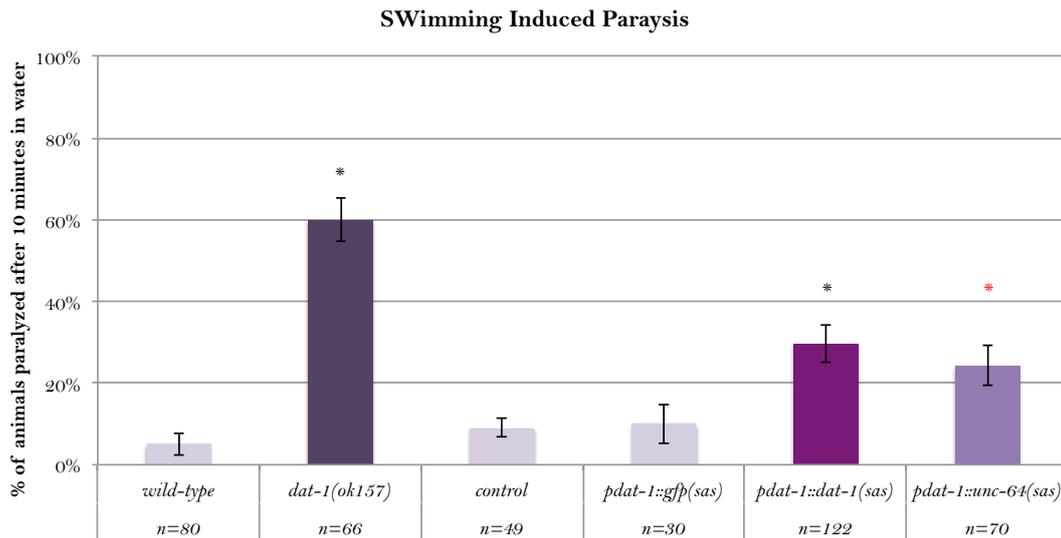


Figure 17. The effects of dopaminergic specific knock-down of *unc-64* in SWIP response

After knocking-down *unc-64* in DAergic neurons, the 24% of transgenic animals became paralyzed after 10 minutes in water (lilac bar), similarly to *dat-1(ok157)* null mutant (plum bar) and *pdat-1::dat-1(sas)* (purple bar). Error bars indicate SEM between independent experiments. Statistical significance was assessed by using Compare two proportions statistical test (* $p < 0.05$ significantly different from wild-type, control and *pdat-1::gfp(sas)*; * $p < 0.05$ significantly different from wild-type). n is the number of L4 animals observed.

A dysfunction in DA reuptake has been implicated in the effects of several psychostimulants of abuse, such as Amphetamines (AMPH). Having as a substrate DAT-1 protein, AMPH elevates extracellular DA levels by antagonizing DA clearance and stimulating DAT-mediated DA efflux. The final result of AMPH is to elevate the extracellular concentration of DA and, as a consequence, to alterate DA signaling [23, 153, 154]. To date all the key molecular players involved in the mechanism of AMPH are still not identified.

Carvelli et al. showed that the treatment of *C. elegans* wild-type animals for 10 minutes with 0,5 mM of AMPH induced a significant SWIP with respect to *dat-1(ok157)* null mutant animals that were resistant, confirming that AMPH induces also in *C. elegans* an accumulation of extracellular DA and that this phenomenon requires DAT-1 expression [155]. They also demonstrated that AMPH-Induced SWIP is mediated by DA storage, DA synthesis, and DA receptors signaling, including DOP-2, DOP-3, and DOP-4 receptors [155].

In an attempt to verify if UNC-64 is one of the key elements of DAergic signaling

involved in the behavioral effects induced by AMPH, in collaboration with the Carvelli Laboratory, we measured AMPH-induced SWIP in transgenic animals where *unc-64* is specifically knocked-down in DAergic neurons. Interestingly, when transgenic animals lacking *unc-64* in DAergic neurons were tested in the presence of AMPH, a strong and significant reduction in SWIP was observed, as compared to wild-type animals and negative controls (Figure 18). Moreover this reduction in SWIP response is similar to *dat-1(ok157)* null mutant animals and to transgenic animals where *dat-1* is specifically knocked-down in DAergic neurons, suggesting that the knock-down of the *unc-64* gene in DAergic system is sufficient to decrease the capacity of the transgenic animals to paralyze upon exposure to AMPH (Figure 18). These data suggest that AMPH-induced SWIP response not only depends on DAT-1 protein but also on the UNC-64 activity, specifically in DAergic system.

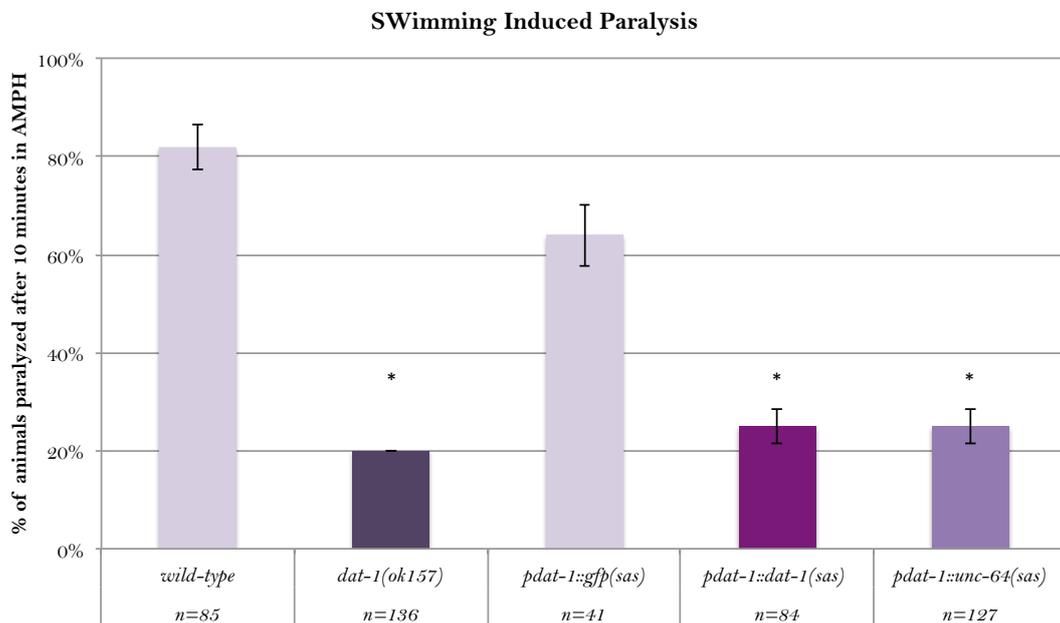


Figure 18. The effects of dopaminergic specific knock-down of *unc-64* in AMPH response

AMPH treatment of transgenic animals where *unc-64* is knocked-down in DAergic neurons, induces a reduction in SWIP response (lilac bar) similar to *dat-1(ok157)* null mutant (plum bar) and *dat-1* knock-down (purple bar), and not observed in wild-type and negative control (*pdat-1::gfp(sas)*). Error bars indicate SEM between independent experiments. Statistical significance was assessed by using Compare two proportions test (* $p < 0.01$, significantly different from wild-type and *pdat-1::gfp(sas)*). n is the number of L4 animals observed.

UNC-63 involvement in dopaminergic system

UNC-63 plays an important role in cholinergic transmission at the nematode neuromuscular junction [129]. To explore the possible role of UNC-63, the $\alpha 6$ subunit of the nicotin Acetylcholine receptor in *C. elegans* DAergic circuit, I initially needed to demonstrate its expression in this specific neuronal system.

UNC-63 is expressed in dopaminergic neurons

Previous works revealed that it is expressed in body wall muscles, in head muscles and in many neurons in the head and in the ventral cord of the nematode [129] (Figure 11). To date it has never been described a specific expression in DAergic neurons. To determine a possible role of *unc-63* in DAergic circuit, I initially needed to determine its expression pattern. With this purpose, I used transgenic animals, available from the *C.elegans* stock center, in which a *gfp* reporter was fused to *unc-63* promoter (*punc-63*), allowing its expression both in muscles and in neurons (*punc-63::gfp*) [156]. Afterwards I checked the possible expression pattern in DAergic neurons by crossing transgenic animals bearing *punc-63::gfp* transgene with a reporter strain that expresses *mCherry* fluorophore in all the DAergic neurons, under the control of *dat-1* promoter (*pdat-1::mCherry*). I analysed these double transgenic strains with confocal microscopy and I observed that, apart from the expression in body wall muscles and in other cells of the nervous system, GFP was expressed also in DAergic neurons, co-localizing with *mCherry* in cell bodies (Figure 19). This result demonstrates that a nicotinic acetylcholine receptor (nAChR) containing the UNC-63 subunit in the nematode, may have an important function in regulating the *C. elegans* DAergic system. To confirm this result I also made a second analysis by observing, at the confocal microscope, a second type of transgenic animals obtained by injecting a second plasmid which has been previously used to determine the *unc-63* expression profile by a different research group [131]. This plasmid was injected in the reporter strain *pdat-1::mCherry* as above. Again a co-localization of *GFP* with *mCherry* was visible in DAergic neurons (not shown).

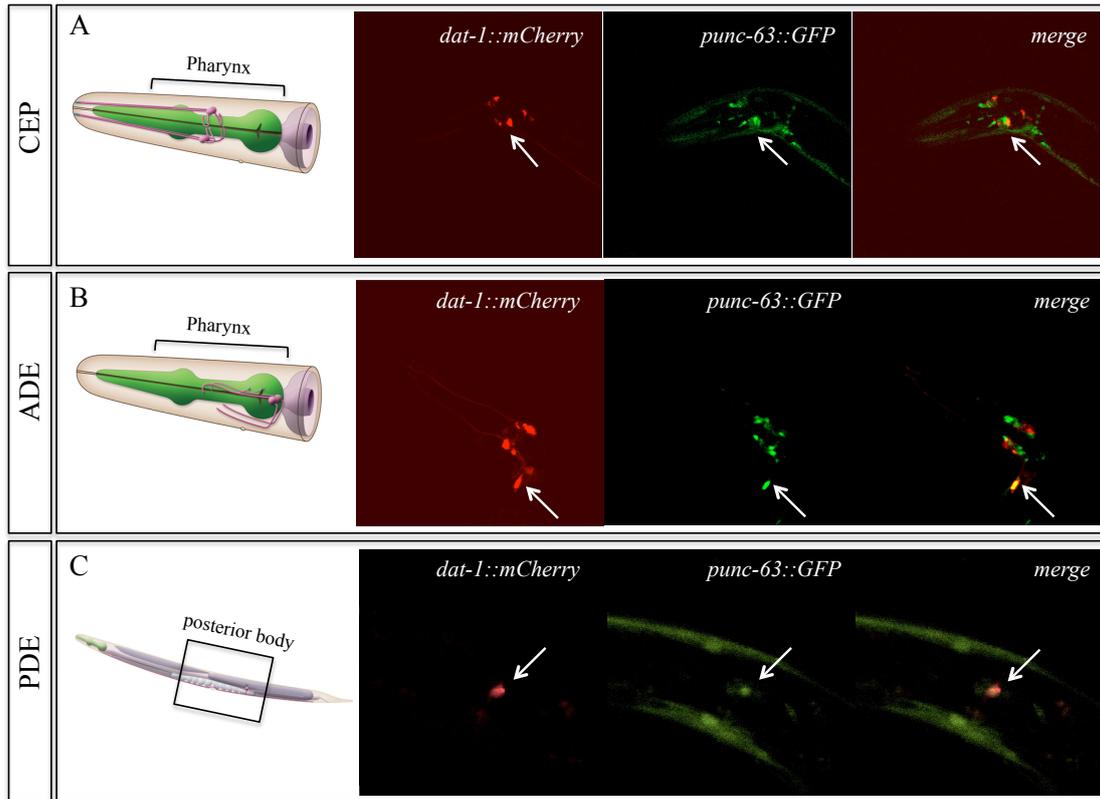


Figure 19. Expression pattern of *unc-63* in dopaminergic circuit

Columns from left to right show: a diagram of DAergic neurons (WormAtlas 2008); animal region with the expression of a DAergic neuron-specific marker *pdat-1::mCherry* (Rhodamine filter); the GFP expression under *punc-63* promoter (GFP filter); merge of the two previous images. (A) Left panel: diagram of CEP neurons in the head (WormAtlas 2008); middle left panel: expression of *mCherry* in CEP neurons; middle right panel: GFP expression under *punc-63* promoter in muscles and neurons of the head; right panel: merge of the two previous images shows that GFP and *mCherry* colocalize in CEP neurons (images were captured at 630x magnification). (B) Middle panel, from left to right: diagram of ADE neurons in the head (WormAtlas 2008); head regions containing ADE neurons visualized by the expression of a DAergic neuron-specific marker *pdat-1::mcherry*; GFP expression under *punc-63* promoter in neurons of the head; merge of the two previous images shows that GFP and *mCherry* colocalize in ADE neurons (images captured at 630x magnification). (C) Lower panel, from left to right: diagram of PDE neurons at mid body (WormAtlas 2008); PDE neurons visualized by the expression of a DAergic neuron-specific marker *pdat-1::mcherry*; GFP expression under *punc-63* promoter in a neuron at midbody; merge of the two previous images shows that GFP and *mCherry* colocalize in PDE neurons (images captured at 1000x magnification). All the animals were examined with a confocal microscopy Leica equipped with epifluorescence. Anterior is to the left, ventral is down in all pictures.

Behavioral analysis of *unc-63* interfered animals

Once revealed that *unc-63*, the homolog to the human CHRNA6 gene, is also expressed in DAergic neurons, I explored its functional role in the same neurons by generating transgenic animals lacking the gene in DAergic neurons using the RNAi technique. The DAergic specific knock-down of *unc-63* gene was obtained, as above, by fusing the *pdat-1* promoter to sense and antisense fragments corresponding to *unc-63* gene sequence.

All the transgenic lines obtained from this microinjection were viable and did not present abnormal locomotion. Therefore I again overcame the problems related to the classical genetic approaches, that because of severe defective phenotypes observed in *unc-63* genetic mutants, did not allow to perform behavioral assays, which are essential to explore the role of *unc-63* gene in DAergic circuit.

Transgenic animals lacking *unc-63* in DAergic neurons were analysed for behavioral assays known to be DA-mediated.

Firstly, I verified the response to food in transgenic animals, by performing the Basal Slowing Response assay. As shown in Figure 20, these transgenic animals exhibit a normal rate of locomotion, similar to the wild-type and the negative control. This result suggests that the specific knocking-down of *unc-63* in DAergic circuit does not down-regulate the DA biosynthesis, since interfered animals still maintain their ability to detect the presence of food.

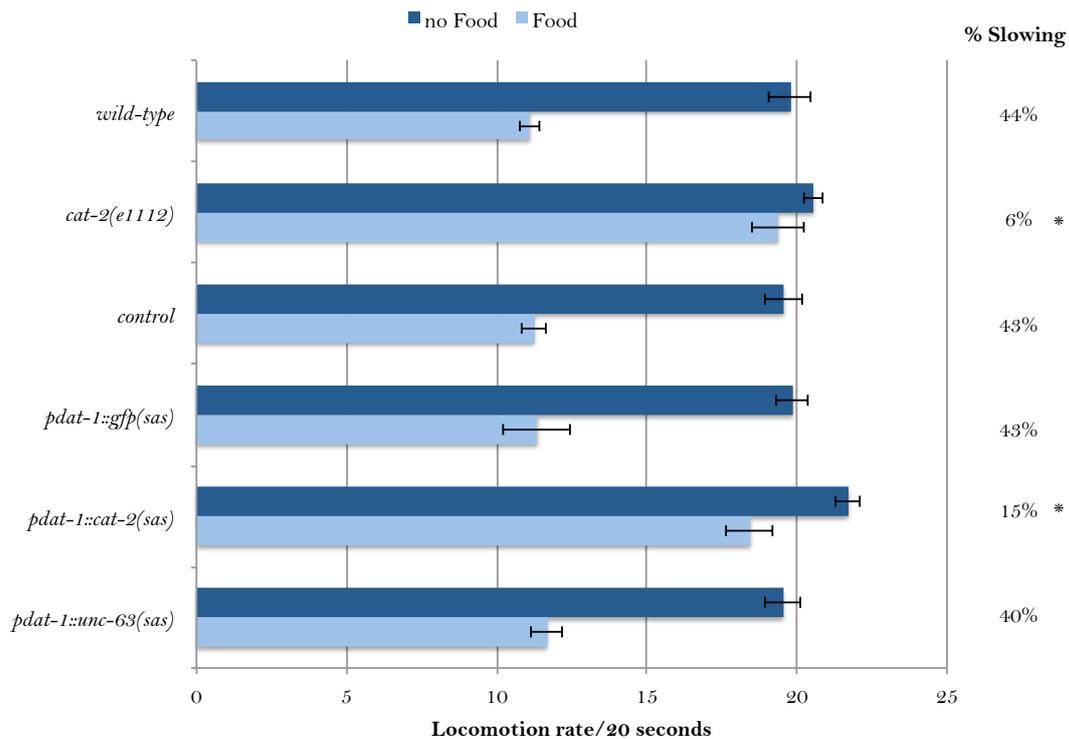


Figure 20. The effect of dopaminergic specific knock-down of *unc-63* in the Basal Slowing Response

After knocking-down *unc-63* specifically in DAergic neurons, transgenic animals exhibit normal slowing response (*pdat-1::unc-63(sas)*), similarly to wild-type animals. Locomotory rate was counted for 5 young adult worms per strain, for five intervals each of 20-seconds, with a total of 30 independent measurements per condition. Dark-blue bars indicate locomotion rate among 20 seconds in the absence of food, and blue bars indicate locomotion rate in the presence of food. Error bars indicate SEM between independent experiments. Asterisks indicate values significantly different from the 44% of slowing seen in the wild type (Student's t-test: * $p < 0.001$). The percentage of slowing in the presence of bacteria for each strain is shown on the right.

I also performed the SWIP assay, to verify if *unc-63* specific loss in DAergic neurons may enhance the DA signaling in terms of increasing extrasynaptic neurotransmitter levels. As shown in Figure 21, only 18% of transgenic animals lacking of *unc-63* in DAergic neurons became paralyzed, when placed in water for 10 minutes. This percentage was not significantly different from the wild-type and the negative controls, suggesting that the contribution of *unc-63* is not essential in increasing the extrasynaptic DA levels in *C. elegans* DAergic system.

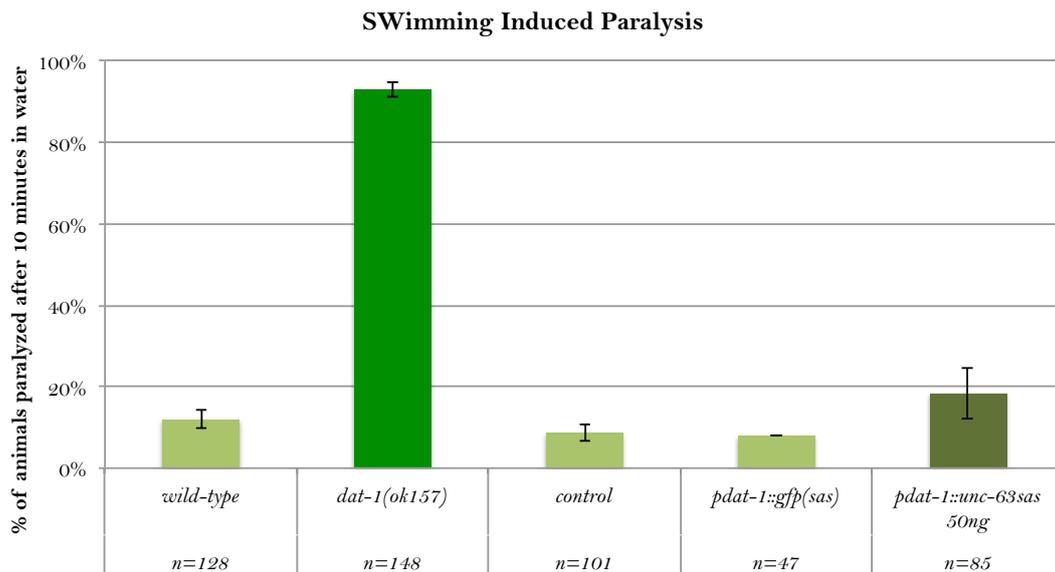


Figure 21. The effect of dopaminergic specific knock-down of *unc-63* in the SWIP response

After knocking-down *unc-63* specifically in DAergic neurons, a very low percentage of transgenic animals became paralyzed after 10 minutes in water (dark green bar), non significantly different from wild-type, control and *pdat-1::gfp(sas)* (light green bars). Error bars indicate SEM between independent experiments. Statistical significance of the difference was assessed by using Compare two proportions test. n is the number of L3 animals observed.

UNC-63 is required in dopaminergic neurons to mediate DMPP-induced toxicity during *C. elegans* larval development

Given that the previous DAergic specific behavioral assays revealed that UNC-63 subunit does not have a role in modulating DA levels in the nematode DAergic system, I explored whether it may play a different function in this neuronal system.

Previous research demonstrated that the UNC-63 subunit mediates, during post-embryonic development, the toxicity to DMPP (1,1-dimethyl-4-phenylpiperazinium), a nicotinic receptor agonist known to be able to activate most vertebrate nAChR subtypes [131, 157-159]. The *C. elegans* larvae normally get to adulthood through a discontinuous development through four larval stages (L1 to L4), each terminated by a molt. This molt cycle is tightly coordinated with the postembryonic cellular divisions. Ruaud and Bessereau discovered that manipulating nicotinic neurotransmission by chronically exposing animals to DMPP during post-embryonic development cause a lethal phenotype at L2 to L3 molt [131]. In particular it has been observed the exposure of a defective cuticle during the L2 stage that remains at the subsequent

molt, resulting in a lethal overlap of these two developmental stages. They also discovered that the action of the nicotinic agonist was not due to general toxicity, but rather was specific and was mediated through UNC-63 containing receptors, because *unc-63(x37)* null mutants were partially resistant to the stage-specific lethality of DMPP [131]. Partial resistance was explained by functional redundancy between nAChR subunits. Moreover the DMPP sensitivity was not mediated by muscle tissue, but rather by neurons expressing UNC-63 receptor [131]. To date the neuronal pathways mediating this effect have not been determined.

By using this novel paradigm introduced by Ruaud and Bessereau, I investigated whether DAergic system may be one of the neuronal pathways through which UNC-63 mediates the toxic effects produced by DMPP exposure. To this end I initially performed rescue experiments aimed at confirming that the DMPP sensitivity was mediated by neurons expressing UNC-63 and not by muscles. The rescue experiments were made by expressing the *unc-63* full-length cDNA in muscles through a muscle specific promoter (*pmyo-3*) or both in muscles and in neurons through the *unc-63* endogenous promoter (*punc-63*). The *punc-63* promoter has been previously used to determine the *unc-63* expression profile (see paragraph above) and together with *pmyo-3* promoter they have also been used for rescue experiments in the DMPP-resistance assay [131]. The two different rescuing constructs were microinjected in wild-type animals and then transferred in *unc-63(x37)* null mutants. The transgenic animals obtained by rescuing the function of *unc-63* in both muscle and in neurons, as tested after DMPP exposure, were significantly less resistant to the nicotinic receptor agonist, with 17% of survival worms (Figure 22). Differently transgenic animals in which the function of *unc-63* is rescued only in muscles, still presented a DMPP sensitivity similar to the *unc-63* null mutant, with 41% of survival worms (Figure 22).

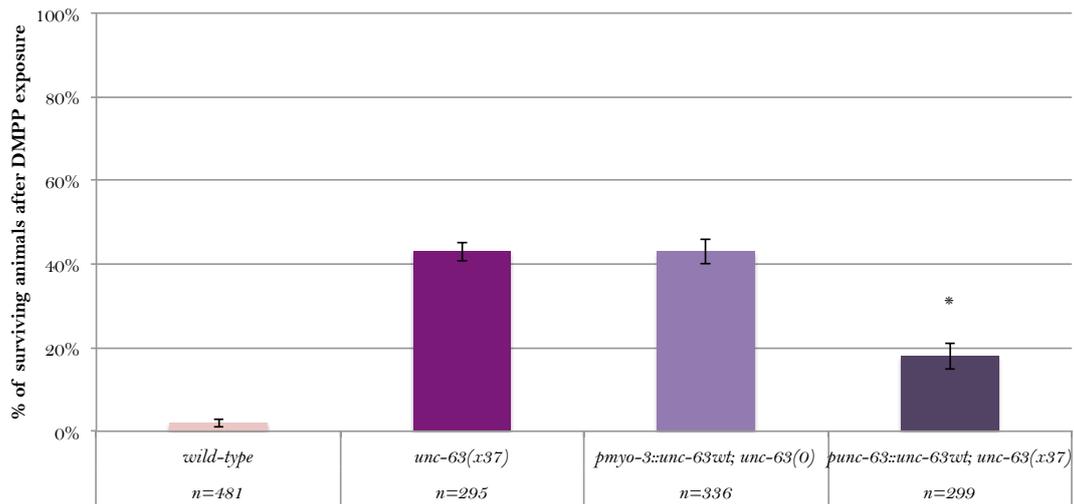


Figure 22. Neurons expressing UNC-63 receptor are required for the DMPP toxicity

Survival on 0.75 mM DMPP. Muscular expression of UNC-63 does not rescue *unc-63(x37)* DMPP resistance (lilac bar), while muscular and neuronal expression (plum bar) produce a strain which is significantly less resistant to DMPP than *unc-63(x37)* (purple bar) (* $p < 0.05$, Mann-Whitney test). Error bars indicate SEM between independent experiments. n is the number of animals observed.

Once confirmed that the expression of the UNC-63 subunit in neurons, and not in muscles, is required to trigger the action of DMPP, I examined the response to the nicotinic agonist of transgenic animals in which the function of *unc-63* is knocked-down specifically in DAergic neurons. As reported in Figure 23, these animals showed a significant DMPP resistance, similar to the *unc-63(x37)* null mutant, with 45% of survival worms. This was different from the negative control, represented by transgenic animals lacking *gfp* in the same neurons, with 8% of survival worms and a DMPP sensitivity similar to wild-type animals (Figure 23). Interestingly these data indicate that the DAergic specific knock-down of *unc-63* is sufficient to induce a partial DMPP resistance, thus strongly supporting the idea that its presence in DAergic neurons is required to mediate the DMPP lethal effect in the nematode.

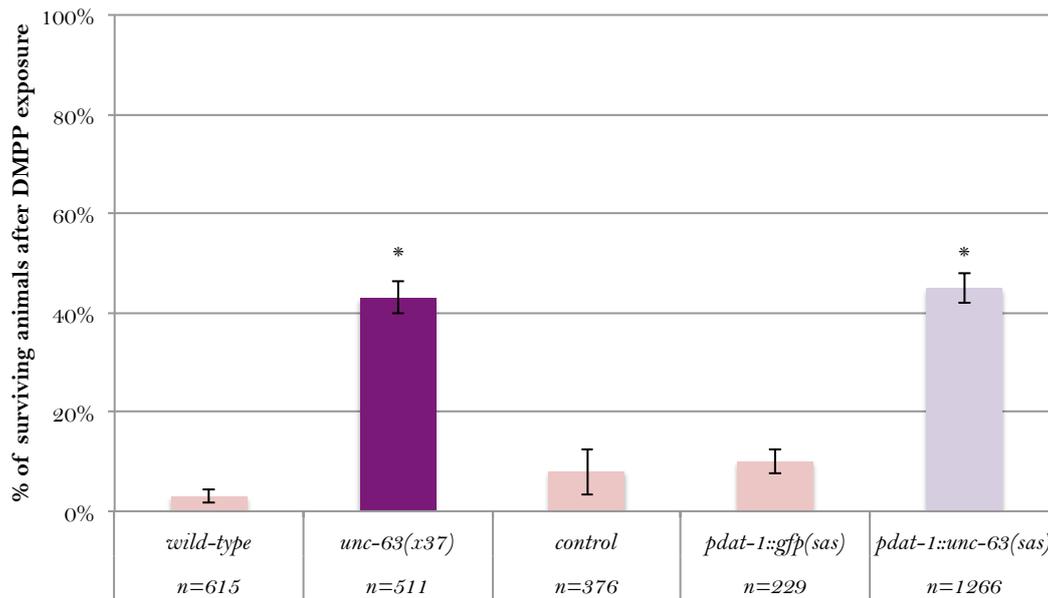


Figure 23. The effect of dopaminergic specific knock-down of *unc-63* on DMPP resistance

Survival on 0.75 mM DMPP. DAergic knocking-down of *unc-63* (light violet bar) causes a DMPP resistance similar to null mutant (purple bar). (* $p < 0.05$, Mann-Whitney test, significantly different from wild-type, control and *pdat-1::gfp(sas)*). Error bars indicate SEM between independent experiments. n is the number of animals observed.

To further confirm this hypothesis, and to demonstrate that knocking-down is confined to DAergic neurons, I transferred transgenic constructs in a *sid-1* mutant background, deficient for systemic RNAi. In fact *sid-1* encodes a dsRNA-channel conserved in humans and mice, responsible for the trafficking of RNA knocking-down molecules into all cells and therefore it is required for systemic RNAi [160]. It has been demonstrated that mutations in *sid-1* gene, such as allele *qt9*, are completely defective for systemic RNAi, whereas they are fully functional for cell-autonomous RNAi [160]. Thus to prevent systemic spreading of RNAi, I exposed to DMPP transgenic animals obtained by transferring the DAergic specific interfering construct of *unc-63* in *sid-1(qt9)* mutant background. As reported in Figure 24 transgenic animals still present a partial resistance to DMPP similar to *unc-63* null mutant, with 30% of surviving worms. This is different from *sid-1(qt9)* mutant strain, that showed a sensitivity to DMPP similar to the wild-type animals with 9% of survival worms (Figure 24). Altogether, these data confirm the idea that the partial DMPP resistance observed in UNC-63 mutants is mediated by the *unc-63* loss of activity specifically in DAergic neurons, indicating that this neuronal circuit is the target required to protect animals from DMPP

toxicity during larval development.

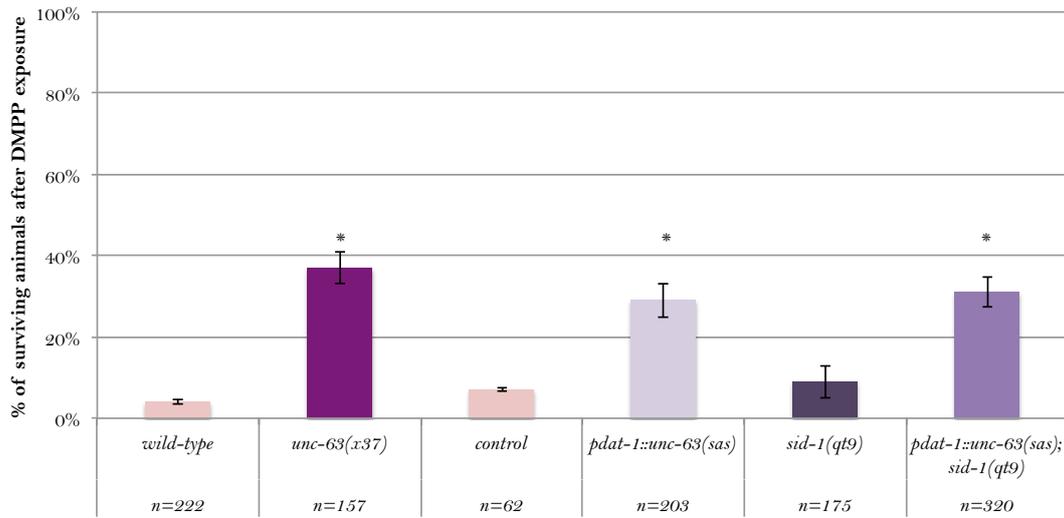


Figure 24. Dopaminergic system represents the neuronal tissue through which UNC-63 mediates the toxic effect produced by the DMPP exposure

Survival on 0.75 mM DMPP. *unc-63* knocking-down in DAergic neurons in *sid-1* mutant background (lilac bar) showed a resistance to DMPP similar to *unc-63(x37)* null mutant (purple bar), to *unc-63(RNAi sas)* (light lilac bar) and significantly different from control and wild-type (pink bars) and from *sid-1* (plum bar). (* $p < 0.05$, Mann-Whitney test, significantly different from wild-type, control and *sid-1(qt9)*). Error bars indicate SEM between independent experiments. n is the number of animals observed.

DISCUSSION

DA is one of the main neurotransmitter of the central nervous system, responsible for several physiological functions including locomotion, motivation and learning [3-5]. Alterations in the DA levels may lead to neuronal disorders of which the molecular mechanisms and the key elements responsible are not completely understood. I focused my PhD project in carrying out a genetic analysis on DAergic system, to identify some of these elements, which may help to better understand the complex mechanisms regulating this neuronal system. In order to perform specific studies on these novel key elements in DAergic system, it has been necessary to use an animal model that is amenable for genetic manipulations, and that conserves an high homology with human DAergic pathway and allows to analyse specific behaviors which are known to be mediated by DA. One such model is the nematode *Caenorhabditis elegans*. This animal, with its powerful genetics and an easily accessible nervous system has been used by researchers as a model to investigate neurodegeneration [161] and to understand the molecular mechanisms underlying DAergic system development and regulation [140].

In the present study, I explored the role in the *C. elegans* DAergic system of *unc-64* and *unc-63* genes, orthologs of the human genes Syntaxin1A and CHRNA6. I have chosen these genes because there are strong evidences that they may play a role in DAergic system which have never been fully demonstrated [49, 68, 69]. Previous studies on *unc-64* and *unc-63* genes in *C. elegans* have utilised genetic mutants or classic RNA interference technique [123, 127, 129, 131-133]. These models present some limitations, making it difficult to dissect their possible role in DAergic system. In particular uncoordinated locomotion due to muscle dysfunction and growth defects hamper the analysis of DA-mediated behaviors, indispensable to explore the possible role they may play in this neuronal circuit. Therefore, I used a powerful RNAi strategy, named RNAi sense and antisense (RNAi sas), to generate two transgenic models where the function of *unc-63* or *unc-64* genes, was selectively knocked-down in *C. elegans* DAergic neurons. To obtain efficient transgenic models, it has been necessary to initially choose a DAergic-specific promoter, such as *pdat-1*, able to specifically and efficiently drive both the expression and the knock-down of a reporter-gene (such as

gfp) in these neurons only. *pdat-1* promoter has been used also to knock-down genes to be used as positive controls (*cat-2* and *dat-1*). By obtaining an highly efficient and specific knock-down for these control genes in DAergic neurons, I obtained the first model of DA-neurons specific RNA-interference ever. After having set up the best experimental conditions, I generated transgenic models lacking *unc-64* or *unc-63* only in DAergic-neurons. Importantly, these animals are viable and able to move, allowing us to overcome the limitations of classical approaches such as defects in growth and in locomotion. These models allow to study the role played by the genes of interest in selected DA neurons in a very fast, direct and simple way.

Characterization of the phenotypes caused by *unc-64/Syntaxin1A* knock-down in dopaminergic neurons

Several studies have recognized the SNARE protein Syntaxin1A as one of the regulatory partner of DA transporter DAT, that can regulate reverse transport and ion channel activity through its direct interaction with DAT N-terminus [40, 49]. The molecular mechanisms of protein-protein interaction has been demonstrated using the yeast-two-hybrid approach and fusion proteins *in vitro* [48, 49], while in *C. elegans* an N-terminal tag of DAT-1 disrupt the interactions between CeDAT-1/hDAT and CeUNC-64/hSyntaxin1A [40]. These studies support the hypothesis that Syntaxin1A may represent an important key element in DAergic circuit to guarantee a correct DA reuptake, a role that has never been completely clarified.

During my PhD project I have explored the role of *unc-64* gene in the *C. elegans* DAergic system *in vivo*, using a genetic approach. I took advantage of the RNAi as technique to generate a transgenic model where the function of *unc-64* gene was knocked-down specifically in DAergic neurons. Transgenic animals obtained with this approach have been used to perform *in vivo* the SWimming Induced Paralysis (SWIP) assay, which allows to explore a behaviour associated with an excess of synaptic DA [51]. Interestingly we have demonstrated that the lack of *unc-64* in DAergic system induces a paralysis similar, albeit less penetrant, to *dat-1(ok157)* null mutant, demonstrating for the first time *in vivo* that its presence in this neuronal system is necessary to modulate a function similar to the DA transporter and to guarantee a

correct neurotransmitter reuptake.

Treatment of *C. elegans* with psychostimulants such as Amphetamine (AMPH), induces a SWIP phenotype in the nematode, that requires DAT-1 correct expression. The key molecular players involved in this behaviour are still unclear. In an attempt to verify if UNC-64 may be one of these key elements, in collaboration with Carvelli's Laboratory, we have measured AMPH-induced SWIP in transgenic animals where *unc-64* is specifically knocked-down in DAergic neurons. Interestingly we have shown that animals lacking UNC-64 specifically in DAergic neurons have a decreased sensitivity to AMPH treatment, similarly to DAT-1 mutants. Our results strongly support *unc-64* as a novel key mediator in DAergic circuit of the AMPH-induced SWIP, together with DAT-1 and DAergic receptors.

Characterization of the phenotypes caused by *unc-63*/hCHRNA6 knock-down in *C. elegans* dopaminergic system

It has been recognised that the striatal DAergic and cholinergic systems play an overlapping role in regulating central nervous system functions, linked to motor activity and that are relevant in diseases such as Parkinson's disease [52-54]. In particular Acetylcholine (ACh) modulates striatal DA levels by stimulating nicotinic acetylcholine receptors (nAChRs). Interestingly, $\alpha 6$ subunit of nAChRs, encoded by CHRNA6 gene, is highly expressed in DAergic nigrostriatal pathway [67]. Moreover the induction of a damage in the nigrostriatal system of rodent and monkey, produces the loss of $\alpha 6$ -subunit that correlates with a decline in DA release, thus suggesting a biological relevance of this subunit in DAergic circuit [68, 69].

For this reason I explored the role of *unc-63* gene, the ortholog of the mammalian CHRNA6, in the *C. elegans* DAergic system. Firstly, it has been necessary to determine the possible expression of UNC-63 in this neuronal system. With this aim, I have analysed transgenic animals expressing the *gfp* under the control of an *unc-63* promoter, to visualize tissues and neurons in which *unc-63* is normally expressed. In addition the same animals express a red fluorescent reporter under the control of *pdat-1* which allows to easily visualize and recognize DAergic neurons. Interestingly, this analysis revealed that *unc-63* is also localized in DAergic neurons, supporting the hypothesis

that nAChRs containing the UNC-63 subunit may have a role in *C. elegans* DAergic system. After demonstrating the expression of *unc-63* in DAergic neurons, I have used the RNAi technique to generate a *C. elegans* model in which *unc-63* is selectively knocked-down in the same neurons. In this way I had the opportunity to study *in vivo* its role in this neuronal circuit. I tried to identify behavioural alterations *in vivo* arising from its specific knock-down in DAergic neurons, by performing the Basal Slowing Response (BSR) and the SWIP assays [51, 99]. In both cases the specific knock-down of *unc-63* in DAergic neurons does not cause any phenotype, suggesting that *unc-63* loss does not severely reduce or enhance the DA levels. From these results I concluded that UNC-63 subunit does not exert a major role in regulating the DA signalling in *C. elegans* DAergic system. Thus I have explored if it may have a novel function in the same neuronal system.

Previous studies on the nematode have demonstrated that illegitimate activation of nAChRs containing the UNC-63 subunit by the nicotinic agonist DMPP (1,1-dimethyl-4-phenylpiperazinium), induced a lethal heterochronic phenotype during the second larval stage. The primary target of DMPP seems to be neuronal, since the loss of expression of the nAChR subunit UNC-63 in neurons partially protects the animals from DMPP toxicity [131]. Using the experimental paradigm described above, I have shown that the lack of *unc-63* in DAergic system is able to produce a partial resistance to the nicotinic agonist DMPP, as observed in the null mutant, supporting for a specific role in the mechanism of DMPP toxicity during larval development. Importantly these results have allowed for the first time to recognize the DAergic system as the main neuronal target required to protect the animals from the DMPP toxicity.

FUTURE PERSPECTIVES

Results obtained have demonstrated the advantages of using *C. elegans* as an animal model and of the RNAi approach, to explore novel roles of *unc-64* and *unc-63* genes in DAergic neurons.

In order to complete the first part of the study on *unc-64* gene, we will perform HPLC (High-Performance Liquid Chromatography) analysis, in collaboration with Dr. Leo (Italian Institute of Technology, Genova), to detect DA levels in transgenic animals lacking *unc-64* in DAergic neurons. We expect that as observed in *dat-1* null mutants [51], we should observe an enhancement of DA accumulation in transgenic animals. Moreover in collaboration with the research group of Dr. Carvelli (University of North Dakota) we will perform electrophysiological studies on *C. elegans* embryonic culture to evaluate the synaptic activity of DAergic neurons after knocking-down *unc-64*. We expect to see a defect in DA uptake after *unc-64* knock-down, as observed in *dat-1* null genetic mutants.

Moreover, using the same approach, I have shown that the main role of UNC-63 in DAergic system is not related to the control of DA signaling. Results obtained strongly suggest instead that its presence in this neuronal tissue is required to modulate the sensitivity to the nicotinic agonist DMPP during larval development.

To complete the study on the role of *unc-63* on DAergic system, I will characterize the morphology of DAergic neurons, in order to identify possible morphological defects related to its specific knock-down in these neurons. Moreover it may be interesting, to generate and characterize new transgenic models, by using the RNAi approach, that lack others nAChRs in order to identify the receptor complex mediating nicotine effects in DAergic neurons.

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