Identification of the Specific Role of the D2-like Dopamine Receptor DOP-2 in the Dopaminergic System of *C. elegans*

by

ROSARIA FORMISANO

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This dissertation is approved by the following members of the Final Oral Review Committee:

Dr. Harbinder S. Dhillon, Committee Chairperson, Department of Biological Science, Delaware State University
Dr. Melissa Harrington, Committee Member, Department of Biological Science, Delaware State University
Dr. Hakeem Lawal, Committee Member, Department of Biological Science, Delaware State University
Dr. Charlie D. Wilson, Committee Member, Department of Biological Science, Delaware State University
Dr. Jeff Caplan, External Committee Member, Delaware Biotechnology Institute, Delaware University

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ABSTRACT

Dopamine signaling plays important roles in many neuronal activities. Dysfunction of the dopaminergic system is thought to be associated with several mental and neurological disorders, such as Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder (ADHD) and drug addition (Ford, 2014). Dopaminergic signaling is highly conserved across the species. In the nematode *C.elegans*, the dopaminergic system controls a variety of behaviors, including locomotion, foraging and learning (Voglis & Tavernarakis, 2008). In this study, the *Caenorhabditis elegans* model was used to understand the auto-receptor functional component of the DOP-2 receptor in modulating dopamine levels in the synaptic cleft. In *C. elegans*, the D2-like dopamine receptor DOP-2 is expressed in all eight of its dopaminergic neurons; it might act as an auto-receptor that modulates dopamine levels at synapses in the nematode. In order to investigate the possible role of DOP-2 as a regulator of dopamine release in the dopamine signaling that takes place at synapses in *C. elegans,* this study focused on analyzing synaptic vesicle release in *dop-2* mutants *in vivo* using FRAP (Fluorescence Recovery After Photobleaching). Results for FRAP experiments showed that *dop-2* deficiency impairs synaptic activity in these worms by increasing the level of dopamine release required for normal dopamine activity in the nematode. In parallel, both behavioral and molecular studies were also used to test for the functional contribution of DOP-2 in the dopamine modulatory mechanism. Using a well-known behavioral assay, the SWimming Induced Paralysis (SWIP), a phenotype which is regulated by exogenous dopamine, it was confirmed that *dop-2* is important to regulate dopamine release. The expression pattern of *dop-2* was also analyzed in this study. *dop-2* expression was observed in both cell bodies and processes of the head dopaminergic neurons

CEP and ADE. On the basis of all these observations, we can conclude that the DOP-2 auto-receptor influences dopamine activity in *C. elegans.*

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LIST OF ABBREVIATIONS

AC	Adenylate cyclase
AMP	Adenosine monophosphate-activated protein
AMPH	Amphetamine
ASIC-1	Acid-sensing ion channel
Ca2+	Calcium
COMT	Catechol-O-Methyltransferase
DA	Dopamine
DAT	Dopamine transporter
DCAA	Aromatic L-amino acid decarboxylase
DOPAC	3, 4 - Dihydroxyphenylacetic acid
FRAP	Fluorescence Recovery After Photobleaching
GPCR	G-protein-coupled receptor
IAA	Isoamyl alcohol
L-DOPA	L-3, 4-dihydroxyphenylalanine
MAO	Mono amine oxidase
pHluorin	pHsensitive green fluorescent protein-based sensor
PKA	Cyclic AMP-dependent protein kinase
PKC	Protein kinase C
ROI	Region of interest
SNAREs	SNAP (soluble NSF attachment protein) receptor
SWIP	SWimming Induced Paralysis
TH	Tyrosine hydroxylase

TM	Transmembrane
TRP	Transient receptor potential channel
VMAT	Vesicular monoamine transporter
wt	Wild type

CHAPTER I:

INTRODUCTION

1.1. Dopamine Transmission in the Mammalian Nervous System

Dopamine belongs to the catecholamine family. Dopamine is synthesized in the substantia nigra and ventral tegmental regions of the brain. It is present in three main axonal pathways: the nitrostriatal pathway, the mesocorticolimbic pathway, and the tuberoinfundibular pathway (Figure 1). The nitrostriatal pathway arises from the substantia nigra pars compacta (SNc) and projects to the dorsal striatum. The nitrostriatal pathway is involved in motor control and its degeneration is associated with Parkinson's disease (Drui et al. 2014). The mesocorticolimbic pathway originates in the Ventral Tegemental Area (VTA) and its projections go to both the nucleus accumbens (NAc) and areas to the prefrontal cortex. The mesocorticolimbic pathway is involved in a variety of functions including motivation, rewards and learning and memory (Linden, 2012). Dysfunction in dopamine signaling is related to neuropsychiatric diseases such as schizophrenia (SZ) and attention-deficit hyperactivity disorder (ADHD). Finally, the tubero-infundibular pathway projects from the hypothalamus to the infundibulum of the posterior pituitary gland where it controls prolactin secretion (Stahl, 2002) (Figure 1).

Dopamine synthesis starts from the conversion of the amino acid L-tyrosine into L-3, 4 dihydroxyphenylalanine (L-DOPA). This reaction is catalyzed by the rate-limiting enzyme tyrosine hydroxylase (TH). L-DOPA is then converted to dopamine by the aromatic L-amino acid decarboxylase enzyme (AADC). Synthesized dopamine is packaged into synaptic vesicles

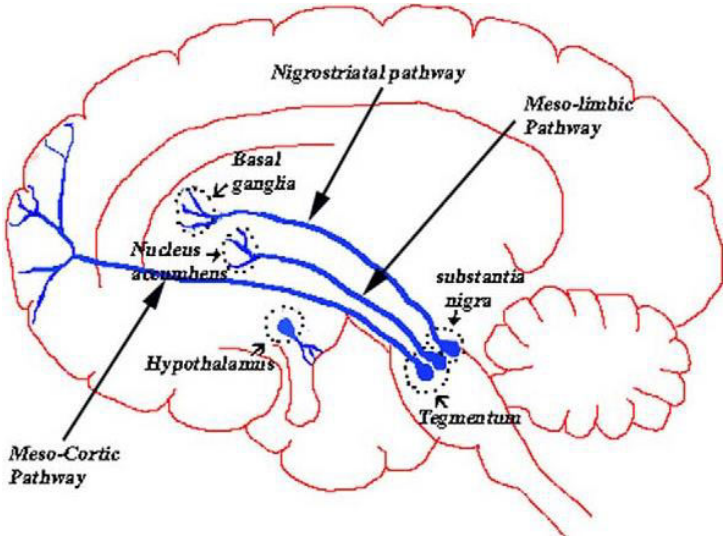


Figure 1. Dopamine pathways in mammalian brain. The nitrostriatal pathway, the mesocorticolimbic pathway, and the tuberoinfundibular pathway. (Source: Chinta and Anderson 2005, The International Journal of Biochemistry and Cell Biology.)

by the vesicular monoamine transporter (VMAT). Upon stimulation, dopamine is released in the synaptic cleft where it binds to dopamine receptors located both pre- and post-synaptically. The dopamine signaling action in the synaptic cleft is terminated by both reuptake into the presynaptic neuron by the dopamine transporter DAT-1 and, enzymatic destruction by the monoamine oxidase (MAO) and the the cathechol-O-methyltransferase (COMT) enzymes. Dopamine is important for many behavioral functions, including cognition, reward and memory. Abnormal dopaminergic signaling has been implicated in several human disorders, such as Parkinson's disease, attention-deficit hyperactivity disorder, addiction to drugs and schizophrenia. However, the complex interactions of dopamine and the causes of these disorders are still unclear.

VERTEBRATE DOPAMINE RECEPTORS

Dopamine exercises its action on many physiological systems through dopamine receptors. In mammals, five dopamine receptors have been identified (D1-D5) and all are members of the rhodopsin-like, class A, seven trans-membrane super family G-protein-coupled receptors (GPCRs) (Missale et al., 1998). Based on signaling properties similarities, the five dopamine receptors are grouped in two classes: the D1-like receptors, which include the D1 and the D5 subtypes, and the D2-like receptors, which include the D2, D3 and D4 subtypes (Neve et al., 2004). D1-like receptors stimulate adenylate cyclase and produce a consequent increase of cyclic AMP. (The first biochemical evidence for a dopamine stimulated adenylate cyclase activity dates to 1972 [Neve et al., 2004]). D1 receptors are highly expressed in the neostriatum, mesolimbic and mesocortical areas. D5 receptors are expressed at low levels in multiple brain regions, including the cortex, hippocampus, hypothalamus, and the dentate gyrus (Beaulieu, Gainetdinov and Sibley, 2011). D2-like receptors, which include the three subtypes D2, D3 and D4, decrease adenylate cyclase activity producing a reduction in cyclic AMP. D2 receptors, which are significantly expressed in the striatum, the nucleus accumbens, the olfactory tubercle, the substantia nigra, the ventral tegmental area and in the hypothalamus, mediate the reward pathway and the voluntary motor control. The D3 receptor has a more limited pattern of expression in the mammalian brain. The highest level of expression of the D3 receptor is found in the limbic areas, the olfactory tubercle, and the islands of Calleja. The D4 receptor has less expression in the brain. However, its expression has been documented in the cortex, amygdala, hippocampus, hypothalamus, globus pallidus, substantia nigra pars reticulata, and thalamus (Beaulieu, Gainetdinov and Sibley, 2011). Dopamine receptors bind to the neurotransmitter dopamine and their sub cellular localization is an important feature for their function.

D1- and D2-like receptors are found at all post-synaptic locations (both axons and dendrites) in dopamine-target neurons in the brain. D1-like type receptors are located only post-synaptically. They are assumed to couple with the G protein G∂s/olf to stimulate adenylate cyclase (AC) with a consequent increase of cyclic AMP levels that ultimately affects cellular excitability (Surmeier et al., 2010).

D2-like receptors can be found both pre and post-synaptically. They couple with the G∂i/o G protein and inhibit adenylate cyclase to modulate different intracellular signaling pathways and reducing the cell excitability (Callier et al., 2003).

VERTEBRATE DOPAMINE D2-AUTORECEPTORS

Pre-synaptically localized receptors, such as the subtypes D2 and D3, are called auto-receptors. It has been suggested that they provide a negative feedback mechanism for the synthesis and release of neurotransmitters at neuronal synapses (Wolf and Roth, 1990). The first dopamine auto-receptors to be discovered were located on the axon terminals of nigrostriatal fibres from rat brain striatum (Farnebo and Hamberger, 1971). Dopamine D2 auto-receptors play an important role in controlling the activity of dopamine neurons by regulating the synthesis, release and uptake of dopamine (Ford, 2014). D2 auto-receptors are considered inhibitory as their activation usually decreases the amount of dopamine released by acting on intracellular calcium signaling (Benoit-Marand, et al., 2001). While the majority of dopamine receptors are found on non-dopamine neurons, dopamine auto-receptors are found exclusively on dopamine neurons, where they can be located on both axons and somatodendrites region (Missale et al., 1998). Even though studies in different cell lines have attributed auto-receptor functions to both D2 and D3 dopamine receptors, genetic and pharmacological studies *in vivo*

have been more controversial, assigning the role of auto-receptor solely to the D2 type (Neve, 2009).

There are two isoforms of D2 auto-receptors: the long form (D2L), and the short form (D2S) resulting from alternative splicing (Ford, 2014). The D2L has an additional 29 amino acid residues inserted in the third cytoplasmatic loop, which has been shown to be important for interactions with effectors (Wang et al., 2000). The fact that the two D2-isoforms present differences in protein structure, expression pattern and molecular effectors might suggests that the two isoforms are likely to have different functions. However, whether there is a difference in function between these two isoforms remains controversial (Ford, 2014). D2 auto-receptors are present on most axonal terminals, where they regulate dopamine activity in two ways (Wolf and Roth, 1990; Benoit-Marand, et al., 2001). In the first case, D2 auto-receptors regulate neurotransmitter vesicular release by either inhibiting the voltage-gated calcium-channels or by hyperpolarizing the voltage-dependent potassium channel Kv1.2 (Cardozo and Bean, 1995; Fulton et al., 2011). In the second case, D2 auto-receptors regulate dopamine by altering either dopamine synthesis and/or its reuptake. Alteration of dopamine synthesis occurs through inhibition of Tyrosine Hydroxylase (TH, the rate-limiting enzyme for the synthesis of dopamine), while alteration of dopamine uptake occurs through an increase of dopamine transporter DAT activity (Wolf and Roth, 1990; Benoit-Marand, et al., 2011).

DOPAMINE SIGNALING INACTIVATION

One of the main contributors of dopamine inactivation by reuptake is the presynaptic dopamine transporter DAT. Studies on mice lacking the dopamine transporter gene present hyper locomotion, cognitive deficits, elevated extracellular dopamine levels and prolonged

dopamine clearance (Giros et al., 1996). In mammals, DAT regulates duration of dopamine action and its diffusion in the extracellular space (Bertolino et al., 2006). DAT is primarily expressed in dopaminergic neurons and belongs to the gene family SLC6A of the Na+/Cl- ion-gradient dependent neurotransmitter transporters (Nianhang et al., 2000). It is localized at the plasma membrane of axons, pre-synaptic axon terminals, and dendrites of dopaminergic neurons. DAT is expressed at high levels in the striatum and in the ventral tegmental area (VTA) of the brain (Nirenberg et al., 1996). Altered DAT expression has been documented in diseases such as Parkinson's (PD), depression, attention deficit hyperactivity disorder (ADHD) and bipolar disorders. DAT consists of 12 transmembrane (TM) spanning helices (TM1-TM12) with large cytoplasmic N- and C-termini which contain sites for post-translational modifications, regulatory motifs and binding sites. Studies have shown that the activity of DAT can be regulated by many molecules, including enzymes, ion channels and receptors. Because of its structure and binding properties, in fact, DAT action can be influenced by different signaling pathways by which neurons can modulate dopamine reuptake (Vaugan and Foster 2013). For example, DAT N-terminus contains serine and threonine residues that may be phosphorylated by protein kinase C (PKC). PKC-mediated proline phosphorylation of DAT threonine 53 may be involved in maintaining DAT transport, while PKC-mediated serine phosphorylation maybe involved in the regulation of DAT expression in the plasma membrane (German et al., 2015).

1.2. Dopamine Transmission in the *C. elegans* Nervous System

CAENORHABDITIS ELEGANS AS AN IDEAL MODEL ORGANISM

The nematode *Caenorhabditis elegans* is often used in biological research as a model system. *C. elegans* is a bacteria-feeding soil nematode whose entire genome, consisting of

100.3 Megabase pairs (Mbp), has been completely sequenced and for which 19,735 protein-coding genes have been predicted (Hillier et al., 2005). This roundworm has a short life cycle ~ 2.5 days at 20°C (Figure 2). It is small in size (1mm long as an adult), has a high rate of

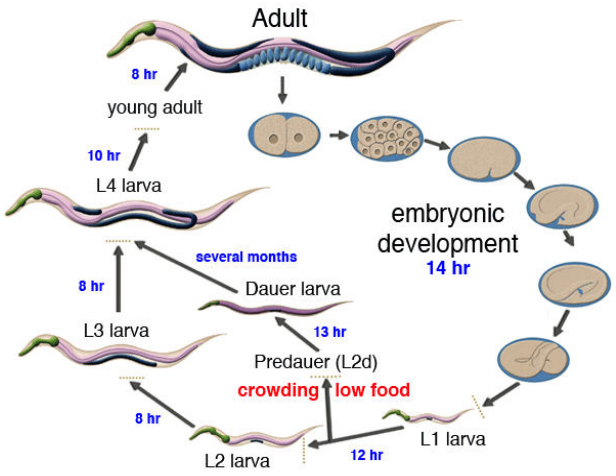


Figure 2. *C. elegans* life cycle. Illustration of *C. elegans* life cycle. In blue are the number of hours the worm spends in a certain stage. (Source: Wormatlas.)

reproduction (300 progeny for each animal) and is easy to maintain in the laboratory (on Petri dishes or liquid media with *E.coli* as a food source at 20°C). *C. elegans* exists either as hermaphrodite (its predominant sex form) or as male. The adult hermaphrodite contains 959 somatic cells; the developmental fate of each of these cells has been completely traced. *C. elegans* has a simple and compact nervous system, containing 302 nerve cells, which have all been identified and whose connectivity has been determined. In addition, the development of sophisticated techniques that allow the small sized *C. elegans* genome to be experimentally manipulated, permits the use of forward and reverse genetic techniques and modern imaging studies.

All these features make *C. elegans* an ideal model organism for the identification of genes involved in a number of important processes, including development, signal transduction, cell death, thermotaxis and olfaction. In addition, a model organism online database, Worm Base, which provides extensive information about *C. elegans* genome, neuroanatomy and development, is available for scientists to consult and use for research studies (Mohri et al., 2005). Because of its transparent body, this animal can be examined at the cellular level. In fact, recent advances in microscopy can be used for *in-vivo* neural circuit analyses in living animals so that it is possible to visually compare differences in protein expression in wild type and mutant *C. elegans* strains. In addition, a combination of modern techniques, such as fluorescence imaging and FRET (fluorescence resonance energy transfer) can be used to monitor and follow real-time molecular interactions of proteins *in vivo*.

THE *C. ELEGANS* NERVOUS SYSTEM

The nervous system of *C. elegans* consists of 302 neurons (118 types) with the entire cell lineage traced (Sulston and Horvitz, 1977). The complete connectivity of all neuronal cells has been drawn by using electron microscopy. The wiring diagram consists of approximately 6400 chemical synapses, 900 gap junctions and 1500 neuromuscular junctions (*Wormatlas*, 2011). The majority of the neurons are located in the head and tail ganglia. The rest of the neurons are located along the ventral cord, the main longitudinal tract that runs along the whole body of the worm. Unlike those of mammals, *C. elegans* neurons are unbranched with a simple morphology of their processes (White et al., 1986). The major synapse-rich region is the nerve ring (the brain of the worm), which is where most of the neuronal activities in the worm take place (Figure 3).

Despite the simplicity of *C.elegans*, its nervous system contains genes that encode

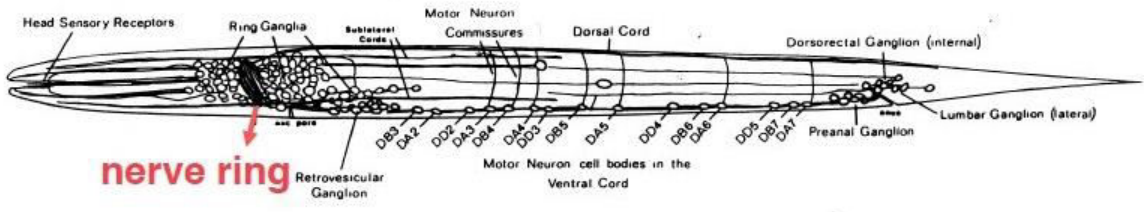


Figure 3. General view of *C. elegans* and its nervous system. View of all cell bodies and process tracts on the midline of the worm. The nerve ring, which is a loop around the pharynx, is indicated in red. The ventral cord runs back from the nerve ring and contains motor neuron cell bodies and processes. (Source: Wormatlas.)

the mammalian brain. Neurotransmitters, neurotransmitter receptors, neurotransmitter synthesis virtually all the key molecular components and neurotransmitter systems that are also found in and release pathways and heterotrimetic GTP-binding protein (G protein) second messenger pathways are highly conserved between *Caenorhabditis elegans* and mammals (Bargmann, 1998). Most ion channels, voltage-gated and ligand-gated ion channels, are similar to vertebrate and all known synaptic components are conserved between worms and mammals (Bargmann, 1998). All this makes *C. elegans* an ideal model organism to study the molecular and genetic basis of evolutionary conserved neuronal processes.

DOPAMINE AND THE DOPAMINERGIC SYSTEM IN *C. ELEGANS*

Dopamine is a major neurotransmitter that plays an important role in regulating neural activities in both vertebrates and invertebrates. This amine is important for many behavioral functions, including cognition, reward and memory. Abnormal dopaminergic signaling has been implicated in several human disorders, such as Parkinson's disease, attention-deficit hyperactivity disorder, addiction to drugs and schizophrenia. However, the complex interactions of dopamine and the causes of these disorders are still unclear.

Together with octopamine, tyramine and serotonin, dopamine is one of the four biogenic amines that modulate many *C. elegans* behaviors in response to environmental changes (Chase and Koelle, 2007). In *C. elegans*, dopamine is synthesized in eight neurons: two anterior deirid neurons (ADEs), two posterior deirid neurons (PDEs) and four cephalic neurons (CEPs). Each of these dopaminergic neurons have ciliated endings embedded in the cuticle, suggesting mechanosensory functions (White et al., 1986). In addition, three pairs of sex-specific dopaminergic neurons, namely the A neurons of rays 5, 7 and 9, have been identified in the male tail of *C. elegans* (Figure 4B).

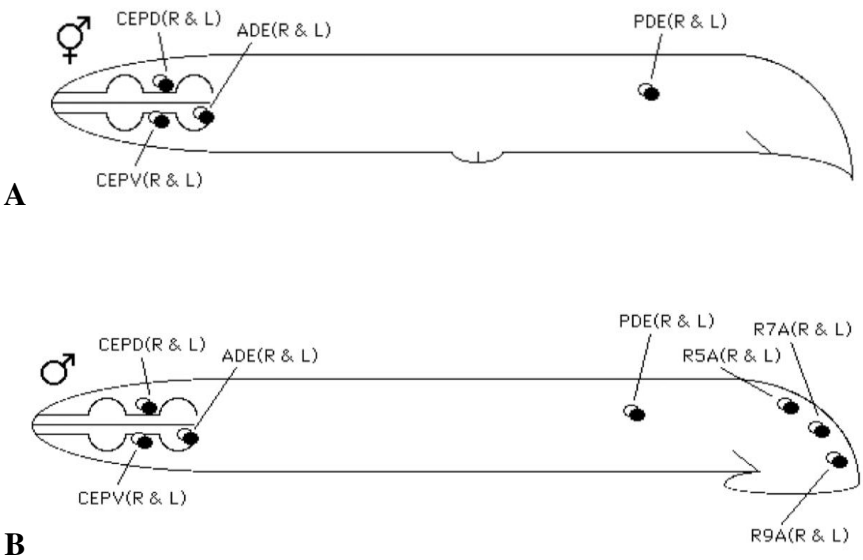


Figure 4. *C. elegans* dopaminergic neurons in both hermaphrodites and males. CEPs: four cephalic neurons, dorsal and ventral, left and right. Found in both hermaphrodites and males. ADEs: anterior deirid neurons (bilaterally symmetric), left and right. Found in both hermaphrodites (**A**) and males (**B**). PDEs: posterior deirid neurons (bilaterally symmetric), right and left, found in both hermaphrodites and males. R5As, R7As, R9As: three pairs of sex-specific dopaminergic neurons (bilaterally symmetric), right and left. Found in the tail of males only. (Source: University of San Diego, Loer Lab.)

As in other animals, dopamine is synthesized from the precursor tyrosine and is released in the synaptic cleft via synaptic vesicles. The gene *cat-2* encodes for the enzyme tyrosine

hydroxylase and *cat-1* encodes for the vesicular monoamine transporter (VMAT) necessary for packing dopamine into synaptic vesicles after it has been synthesized (Duerr et al., 1999). Worm dopaminergic neurons also express *dat-1,* the dopamine transporter that terminates dopamine signaling by reuptake (Figure 5).

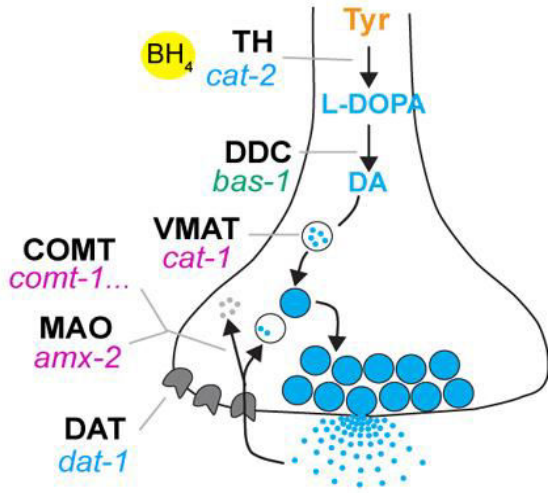


Figure 5. *C. elegans* dopaminergic biosynthesis pathway. Tyrosine is first hydroxylated by tyrosyne hydroxylase TH (*cat-2* in *C. elegans*) to produce L-DOPA. L-DOPA is then decarboxylated by the amino acid decarboxylase (AAAD), encoded by *bas-1* to produce dopamine. Dopamine is then packed into synaptic vesicles by the vesicular monoamine transporter (VMAT) encoded by *cat-1*. (Source: Wormbook.)

Release of dopamine in the synaptic cleft is accompanied by synaptic acidification due to the different pH within the vesicles and the extracellular space. In general, a proton mechanism in the vesicles membrane is used to produce an electrochemical gradient to transport neurotransmitters into vesicles. This process, called vesicles acidification, is powered by the hydrolysis of ATP, and makes the inside of the vesicles more acidic than the cytoplasm (pH ~ 5.5 for synaptic vesicles; pH = 7 for the cytoplasm). When a neurotransmitter is released by exocytosis, this release is associated with a decrease in extracellular pH because of the protons released into the synaptic cleft (Kandal et al., 1991). Maintaining a correct proton concentration inside and outside the cell is critical for correct neuronal functions. Changes in pH can have

different effects on synaptic channels involved in synaptic plasticity. For example, N-methyl D-aspartic acid (NMDA) receptor gated cation channels are inhibited by a decrease of pH. By contrast, an increase of pH can activate a particular kind of ion channel, the acid-sensing-ion-channels (ASICs), which are involved in many brain functions such as learning and memory (Chu and Xiong, 2012). In the nematode, dopaminergic signaling acts at both neurons and muscles and plays an important role in modulating locomotion, egg laying, defecation, food encounter response and learning behavior.

DOPAMINE RECEPTORS IN *C. ELEGANS*

Genes for four dopamine receptors have been predicted in *C. elegans*: DOP-1, DOP-2, DOP-3 and DOP-4. Their gene products are representative of the two classes of dopamine receptors found in mammals: the D1-like and the D2-like receptors. Cloning of cDNAs for DOP-1 and DOP-2 receptors in *C. elegans* have shown that they contain 7-transmembrane domains (TM) and amino acid residues, both of which are thought to be important for DA binding. DOP-1 and DOP-2 receptors also show some characteristic features of G protein coupled receptors, such as consensus phosphorylation sites for protein kinases (Livingstone et al., 1992). DOP-1 and DOP-2 show high homology with D1-like and D2-like receptors, respectively (Gotzes et al., 1994; Hearn et al., 2002). There are three spliced variants for the DOP-1 gene which are not found in humans. DOP-2 also has two spliced variants which are found in mammalian dopamine D2 receptors. Pharmacological studies have shown that the two additional dopamine receptors in *C. elegans*, DOP-3 (a D2-like receptor) and DOP-4 (a D1-like receptor), show DA-induced inhibition and activation of cAMP, respectively (Suo et al., 2004). *dop-1* receptors are expressed in the ALM, AVM, and PLM mechanosensory neurons of *C.*

elegans, while *dop-2* receptor are expressed in all dopaminergic neurons of the worm (Tsalik et al., 2003). D2-like receptors, such as DOP-2, might regulate the release of dopamine at the presynapses (autoreceptors) or might act at the post-synapses (heteroreceptors). Studies have shown that D2-like receptors function as auto-receptors in mammals (Suo et al., 2003) and have been proposed to regulate the release of dopamine from the pre-synaptic neurons as well as its reuptake by the dopamine transporter (DAT) (Williams and Galli, 2006; Voglis and Tavernarakis, 2008).

THE DOP-2 RECEPTOR IN *C. ELEGANS*

The gene *dop-2* (cosmid K09G1.4) from *C. elegans* chromosome V was proved to have amino acid sequence similarities with the vertebrate dopamine D2 receptor (Suo et al., 2003). Three alternative spliced forms of the *dop-2 C. elegans* gene have been found: K09G1.4a, K09G1.4b and K09G1.4c (Suo et al., 2003; Pandey and Dhillon, 2012). All these spliced variants have different sizes in their third intracellular loop. K09G1.4a and K09G1.4b, which have been named CeDOP-2S and CeDOP-2L, respectively, represent the short and long form of the *dop-2* gene. K09G1.4c is named CeDOP-2XL and represents the longest variant (Pandey and Dhillon, 2012). Phylogenetic studies have shown that *C. elegans* DOP-2 has high sequence similarities with both vertebrate and invertebrate species. For example, 51% homology was observed with *Drosophila Melanogaster* D2-like receptors and 42% homology with mammalian D2 receptors (Suo et al., 2003). In *C. elegans*, DOP-2 is the only D2-like receptor expressed in dopaminergic neurons (Preedy, 2016).

Pharmacological studies showed that both CeDOP-2S and CeDOP-2L *C. elegans dop-2* receptor variants have higher binding affinity to dopamine compared to other monoamine

neurotransmitters (Suo et al., 2003). These results are comparable to those made in D2 spliced variants in mammals (Dal Toso et al., 1989). Suo and colleagues also showed that the effect of *C. elegans* DOP-2 on adenylate cyclase was inhibitory just as in mammals.

DOPAMINE-DEPENDENT BEHAVIORS IN *C. ELEGANS*

Release of dopamine at synaptic termini of specific dopaminergic neurons mediates behavioral plasticity in *C. elegans.* Behavior in *C. elegans* is primarly motivated by the instinct to find food. The nematode uses its sensory system to respond to mechanical, chemical and thermal environmental stimuli. The eight dopaminergic neurons in *C. elegans*, two ADE, four CEP and two PDE, are sensory neurons and have been proposed to be mechanosensory (Chase and Koelle, 2007). Studies on mutants deficient in dopamine synthesis or release have shown defects in the ability of the worm to respond to environmental changes and behave accordingly.

Dopamine signaling enables worms to change their locomotion rate in response to the presence or absence of food (a well-defined behavior called basal slowing response). It also allows worms to search efficiently for new sources of food or to modulate locomotion to ensure that they remain in the food area (Chase and Koelle, 2007). Dopamine signaling is also important because it allows *C. elegans* to change its behavior depending on previous experience. This organism, in fact, can learn and remember specific environmental cues, such as changes in odor and temperature associated with the presence of food, and change its behavior accordingly. In addition, dopamine is also required for the suppression of the CREB-dependent gene expression in well-fed animals (Suo et al., 2009) and for the transition between crawling and swimming behavior (Vidal-Gadea et al., 2011). Therefore, abnormal dopamine signaling can

cause a range of defects in the nematode *C. elegans* as well as in humans. Because dopamine signaling is conserved across species, studying the dopamine system in the simple and well described *C. elegans* nervous system can help to understand the molecular mechanisms of dopamine signaling and its effects on behavior.

THE DOPAMINE TRANSPORTER DAT-1 IN *C. ELEGANS*

Javanthi et al. first characterized the dopamine transporter DAT in *C. elegans* in 1998. The *Caenorhabditis elegans* DAT (DAT-1) presents 45% amino acid identity and many functional similarities with the human ortholog DAT (SLC6A3) (Nass, R., et al., 2005). As in mammals, *dat-1* in *C. elegans* is expressed exclusively in the dopaminergic neurons (CEP, ADE, PDE) and has been proved to regulate dopamine neurotransmission by its reuptake from the synaptic cleft (McDonald et al., 2007).

As in humans, *dat-1* function in *C. elegans* is to terminate dopamine signaling by clearing the catecholamine from the extracellular space. Again, as in humans, *dat-1* presents substrate specificity for dopamine compared to other amines. *dat-1* deficiency in the worm results in an accumulation of extracellular dopamine into the synaptic cleft (Hardaway et al., 2012). *dat-1* mutants in *C. elegans* present a swimming-induced paralysis (SWIP) phenotype. This phenotype is probably due to hyper activation of the
dopamine motor neurons exposed to a high quantity of extracellular dopamine (Benedetto et al., 2010).

Today *dat-1* mutants in *C. elegans* are the object of many studies to reveal molecular components of the dopaminergic signaling, which will provide insights into brain disorders.

PREVIOUS STUDIES CONCERNING THE DOPAMINERGIC SYSTEM OF *C. ELEGANS*

Behavioral and molecular studies have been conducted with the aim of identifying genes involved in dopamine neurotrasmission in *C. elegans.* A study performed in Dr. Tavernarakis's laboratory (Institute of Molecular Biology and Biotechnology, Crete, Greece) have identified a key molecule of the molecular mechanism that contributes to modulating dopamine release at synapses in the nematode. This molecule is an acid sensing ion channel called ASIC-1, a member of the DEG/ENaC family, which is activated by extracellular protons. This study proposes that ASIC-1 mediates dopamine release. When dopamine is released in the synaptic cleft, the local pH drops and the resulting increase in acidity activates ASIC-1, which enhances dopamine signaling (Voglis & Tavernarakis, 2008).

My master's degree research focused on further investigating insights into the molecular mechanisms of dopamine signaling. Specifically, I worked on characterizing another gene, *gpa-14,* which we hypothesized to be the putative Gα subunit that transduces the signal on an activated DOP-2 auto-receptor when dopamine is released at synapse. During this study, *gpa-14* and *dop-2* mutants were analyzed both phenotypically and behaviorally. Both stains were found to be normal in terms of body size, shape, growth, movement and locomotion when compared to wild type *N2* worms. A series of behavioral assays, specifically mechanosensation, chemosensation to specific chemicals, habituation and associative learning tests, were used for the behavioral characterization of both *gpa-14* and *dop-2* mutant strains. It was found that *gpa-14* and *dop-2* mutants respond well to tactile and vibrational stimuli (mechanosensation) and that they display normal chemosensation to both soluble and volatile chemicals.

Simple and associative learning capabilities of these two strains were also tested using a habituation assay and a conditioned chemotaxis assay, respectively. Worms normally respond to

the mechanical stimulus of a gentle touch delivered to the body by reversing the direction of their locomotion. After repeated touches, there is a decrease in the response toward the stimulus, due to habituation in the worm. Results from the habituation assay for both *gpa-14* and *dop-2* mutant strains are shown in Figure 6. Both strains show abnormal behavior for this simple form of learning (habituation) and display a similar habituation rate, which is faster compared to that of the wild type *N2* worms.

Associative learning capabilities of *gpa-14* and *dop-2* mutants were also tested by using the conditioning chemotaxis behavioral assay. Animals are able to modulate their behavior based on past experience in order to adapt to environmental changes. This behavioral plasticity is a form of associative learning, which requires an intracellular signaling pathway and subsequent structural changes at the synaptic level. In conditioned chemotaxis, worms that are

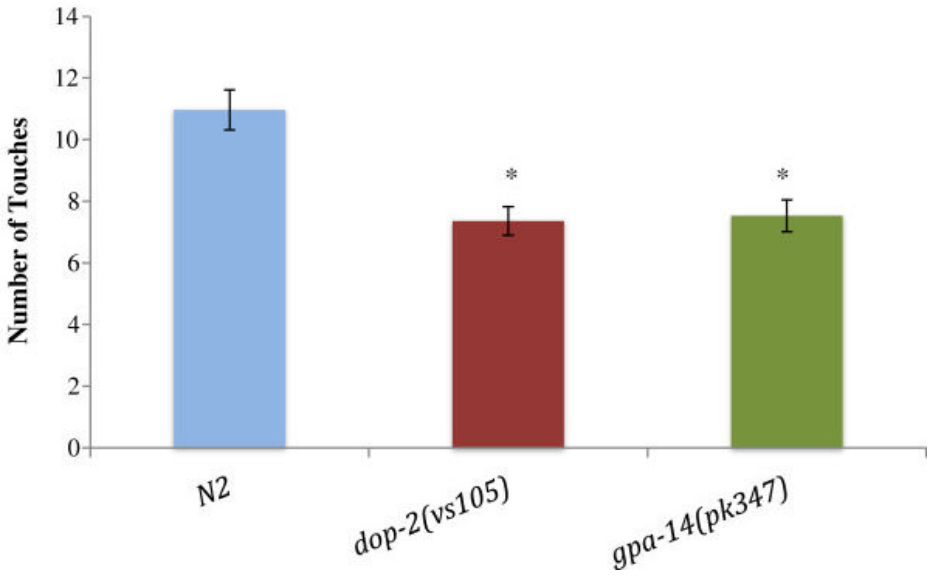


Figure 6. Comparison between the habituation rate of *gpa-14(pk347)* and *dop-2(vs105)* mutants. Worms were assayed on NGM plates in the absence of food (*E.coli*). Stimuli were given every 10 sec. until habituated. Both mutants habituated faster than wild type (*N2*) animals (n = 30, p value < 0.05; two tailed student's t-test). Bars indicate the number of touches needed for habituation for all three strains. Error bars represent SEM values. (Source: Mersha, Formisano et al., 2013. Behavioral and Brain Functions).

usually attracted to a particular compound tend to avoid it after experiencing starvation in the presence of that compound (Colbart and Bargmann, 1995). In order to test the associative learning capability of *gpa-14* and *dop-2* deletion mutants, a chemotaxis-based associative learning assay was performed in which the chemo-attractant isoamyl alcohol (IAA) was paired with starvation. Results from this assay clearly showed a diminished attraction to the IAA after the conditioning period for the wild type *N2* worms (Figure 7), demonstrating the ability of *C. elegans* to learn. On the other hand, the *gpa-14* and *dop-2* mutants continued to be attracted to the IAA, indicating diminished associative learning capability (Figure 7). *gpa-14;dop-2* double mutants were also tested and likewise found to be deficient in associative learning, showing that GPA-14 and DOP-2 operate together in the same pathway, as was hypothesized in this study.

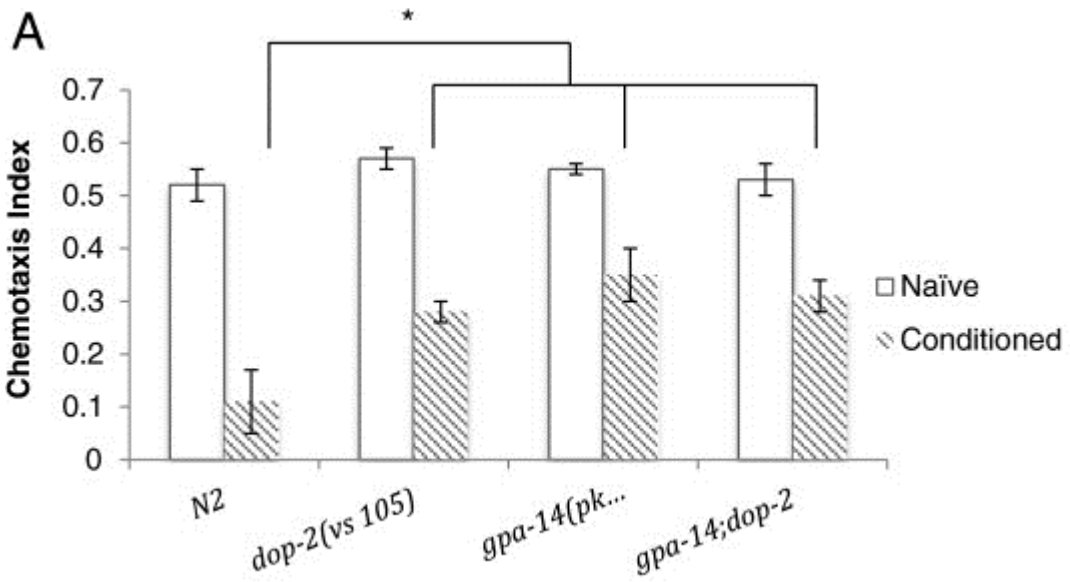


Figure 7. Deletion mutants in *gpa-14(pk347)* and *dop-2(vs105)* present deficit in associative learning capabilities. Learning assay for young adult hermaphrodites of *C. elegans gpa-14, dop-2* and *gpa-14;dop-2* double mutants. Worms were collected, washed and assayed for one "100 fold" dilution of isoamyl alcohol (IAA) chemotaxis directly (naïve) or placed on an NGM plate without food but with IAA for 60 minutes (conditioned). Chemotaxis index was calculated by subtracting the number of worms at the diluent gradient sector from the number of worms at the chemical gradient sector and dividing the result by the total number of worms on the plate. Bars indicate the average chemotaxis index towards IAA for both naïve and conditioned animals. Error bars represent SEM values. (Source: Mersha, Formisano et al., 2013. Behavioral and Brain Functions).

The *in-vitro* physical interaction between GPA-14 and DOP-2 which I was able to show during my master's research was reproduced in successive experiments conducted in our laboratory using the yeast two-hybrid system (Pandey and Dhillon, 2012).

1.3. Hypothesis of this Study

In mammals dopamine binds and activates two different types of receptors: D1-type and D2-type receptors (Ford, 2014). The *C. elegans* DOP-1 receptor shows high sequence similarities with the vertebrate and invertebrate D1-type receptors (Suo et al. 2002) and has been well characterized. However, the *C. elegans* DOP-2 receptor, which presents sequence similarities with the mammalian D2-type receptor (Dal Toso et al. 1989; Giros et al. 1989; Monsma et al. 1989) has received little attention. DOP-2 is expressed exclusively, in the eight dopaminergic neurons of the nematode. This restricted expression to neurons, makes DOP-2 both a receptor and a candidate auto-receptor. However, there is little information on its neuro-regulatory role.

Previous molecular and behavioral studies have suggested that DOP-2 in *C. elegans* participates in the modulation of an experience-dependent behavior through the G∂i subunit *gpa-14* (Pandey and Dhillon, 2012; Mersha, Formisano, et al., 2013). However, the specific function of the *dop-2* gene product on dopaminergic signaling in *C. elegans* remains unclear.

The objective of this work is to investigate the specific *in vivo* role of *dop-2* auto-receptor in regulating neurotransmitter vesicle fusion and release at synapses in the nematode *Caenorhabditis elegans*. Towards this end I propose that DOP-2 acts as an auto-receptor to specifically modulate the release of dopamine by initiating a negative feedback loop at *C. elegans* dopaminergic synapses.

1.4. Specific Aims of this Study

The specific aims of my study are:

I. To use the DA (dopamine) dependent behavior assay known as SWIP (SWimming-Induced Paralysis) *in vivo*, to evaluate the effect of loss of *dop-2* in the *C. elegans* dopaminergic circuit.

II. To analyze the expression pattern and sub-cellular localization of *dop-2* in the *C. elegans* dopaminergic neurons.

III. To determine the effect of *dop-2* mutation on the amount of dopamine released by monitoring the rate of synaptic vesicle exocytosis at dopaminergic synapses using Fluorescence Recovery After Photobleaching (FRAP) in both wild type worms and *dop-2* (*vs105*) mutants.

Chapters II, III and IV will treat each of these aims separately, while Chapter V will draw final conclusions about this study.

CHAPTER II:

EVALUATING THE EFFECT OF LOSS OF *DOP-2* FUNCTION IN THE *C. ELEGANS* DOPAMINERGIC CIRCUIT USING SWIP (SWIMMING-INDUCED PARALYSIS) ASSAY.

2.1. Introduction

The dopaminergic system of *C. elegans* has been proven to be a good model system for dissecting the molecular mechanism that modulates dopamine-dependent behaviors by environmental cues (Vidal-Gadea & Pierce-Shimomura, 2012). In 2007, McDonald and his colleagues described a novel dopamine-dependent behavioral phenotype, Swimming-Induced-Paralysis (SWIP), which emerges in mutants that are deficient in the dopamine transporter DAT-1 when immersed in water. When placed in water, *C.elegans* swim vigorously. However, if *C. elegans* animals that lack the *dat-1* gene are put in water, they sink to the bottom of the well within 5-10 minutes and do not move (McDonald et al. 2007). The function of the dopamine transporter DAT-1 is to terminate dopamine signaling by clearing the catecholamine from the extracellular space. *Dat-1* deficiency in worms results in an accumulation of extracellular dopamine into the synaptic cleft (Hardaway et al., 2012).

Studies have shown that the SWIP phenotype is mediated by the DOP-3 receptor, as the *dop-3* mutation rescues the SWIP phenotype of *dat-1* mutants (Allen et al., 2011). According to this assumption, when *dat-1* mutants are put in water, extra synaptic dopamine accumulates in the synaptic cleft. This accumulation of dopamine overstimulates the D2 like dopamine receptor DOP-3, which is expressed in the cholinergic motor neurons. The overstimulation of DOP-3 leads to an inhibition of acetylcholine release and subsequent paralysis (McDonald et al. 2007).

SWIP is greatly reduced in mutants that cannot synthesize dopamine, such as mutants for the rate limiting enzyme of dopamine biosynthesis *cat-2.*

2.2. Materials and Methods

STRAINS

C. elegans strains were cultured on bacterial lawns of OP50 and maintained at 20˚C using standard methods (Brenner, 1974). The following strains were used in this experiment: *N2*: wild-type Bristol isolate; LX702 *dop-2 (vs105)V;* RB680 *asic-1(ok415)I*, and *dat-1(ok157)III.*

SWIMMING INDUCED PARALYSIS ASSAY

Worms for SWIP were synchronized and 5 to 8 L4 stage animals were taken away from food and placed in 40 *u*l of M9 buffer in a single well of a spot plate. Worms were scored for movement every 2 minutes using a light microscope. The number of paralyzed worms was counted after 20 minutes and reported as a percentage of the total number of animals that were still swimming after 20 minutes. 40 animals per strain were analyzed for this assay. Statistical analysis was carried out using Microsoft Office Excel 2008 software (Microsoft Corporation, Redmond, WA). Comparisons were done using two-tailed Student's *t*-test.

2.3. Results and Discussion

In the attempt to further characterize the *dop-2* gene in terms of its phenotype and in order to confirm the involvement of *dop-2* in the dopaminergic signaling of *C. elegans*, a behavioral analysis was conducted on the *dop-2* mutants. Analysis of *dop-2* mutants was already initiated during my master's degree research. At stated in Chapter I, *dop-2* mutants were

analyzed both phenotypically and behaviorally and were found to be normal in terms of body size, shape, growth, movement and locomotion when compared to wild type *N2* worms. A series of behavioral assays, specifically mechanosensation, chemosensation to specific chemicals, habituation and associative learning tests were used for the behavioral characterization of the *dop-2* (*vs105*) mutant strain. It was found that *dop-2* mutants respond well to tactile and vibrational stimuli (mechanosensation) and that they display normal chemosensation to both soluble and volatile chemicals (Mersha, Formisano,et al., 2013).

Performance of *dop-2* mutants in complex behavioral assays was also tested using the conditioned chemotaxis assay. *C. elegans* are able to modulate their behavior based on past experience in order to adapt to environmental changes. This behavioral plasticity is a form of associative learning, which requires an intracellular signaling pathway and subsequent structural changes at the synaptic level. In order to test the associative learning capability of *dop-2* deletion mutants, a chemotaxis-based associative learning assay was standardized in which the chemo-attractant isoamyl alcohol (IAA) was paired with starvation. In conditioned chemotaxis, worms that are usually attracted to a particular compound tend to avoid it after experiencing starvation in presence of that compound (Colbart and Bargmann, 1995). A comparison between the associative learning capabilities of *dop-2* and *C. elegans* wild type strain (*N2*) during conditioned chemotaxis to isoamyl alcohol showed that *dop-2* has a reduced associative learning capability when compared to wild type worms.

Worms normally respond to the mechanical stimulus of a gentle touch delivered in the body by reversing the direction of their locomotion. After repeated touches, there is a decrease in the response toward the stimulus, due to habituation in the worm. The habituation response of *dop-2* mutants was also assayed in my previous research. It was found that *dop-2* mutants

showed a more rapid habituation than did wild type *N2* worms. Interestingly, both habituation, a simple form of learning, and conditioned chemotaxis, a complex form of learning in *C. elegans* are modulated by dopamine signaling, and studies on dopamine mutant genes such as *asic-1* and *gpa-14* have shown deficits in the rate of habituation and/or associative learning in these mutants compared to that of wild type *N2* (Voglis & Tavernarakis, 2008; Mersha, Formisano,et al., 2013). These results suggest that the genetic lesion (deletion of the *vs105* allele) carried by *dop-2* mutant animals compromises memory and learning capabilities in these mutants. Also, the CEP and ADE dopaminergic neurons, where DOP-2 is expressed, are known to be mechanosensory, since they make synapses with the touch receptor neurons ALM and AVM. It is thought that, when touched, these neurons might modulate the release of dopamine at the synaptic level. This would suggest an involvement of the *dop-2* gene in regulating rate of habituation in the nematode.

Building on these results, which imply an involvement of *dop-2* in the complicated machinery of dopamine signaling, and seeking to further characterize this gene in terms of its phenotype, *dop-2* mutants were tested for the well characterized phenotype SWIP (Swimming Induced Paralysis). As mentioned in the introduction to this chapter, the SWIP phenotype is given by an accumulation of high concentrations of exogenous dopamine in the synaptic extracellular space, which exerts an inhibitory action on the motor neurons with consequent paralysis, as seen in mutants lacking the dopamine transporter *dat-1* (Hardaway et al., 2012). Given the high concentration of exogenous dopamine found in the *dop-2* mutants following my FRAP results (to be presented in Chapter IV), I reasoned that if it was true that there was an accumulation of exogenous dopamine at dopaminergic synapses of *dop-2* mutants, then this strain would also present the SWIP phenotype.

Interestingly, a partial SWIP phenotype in *dop-2* (*vs 105*) mutants was observed compared to wild type *N2* animals (Figure 8). There was no significant statistical difference between the percentage of worms that were still swimming after 20 minutes for the wild type and *asic-1 (ok 415)* mutant worms (Figures 8 and 9).

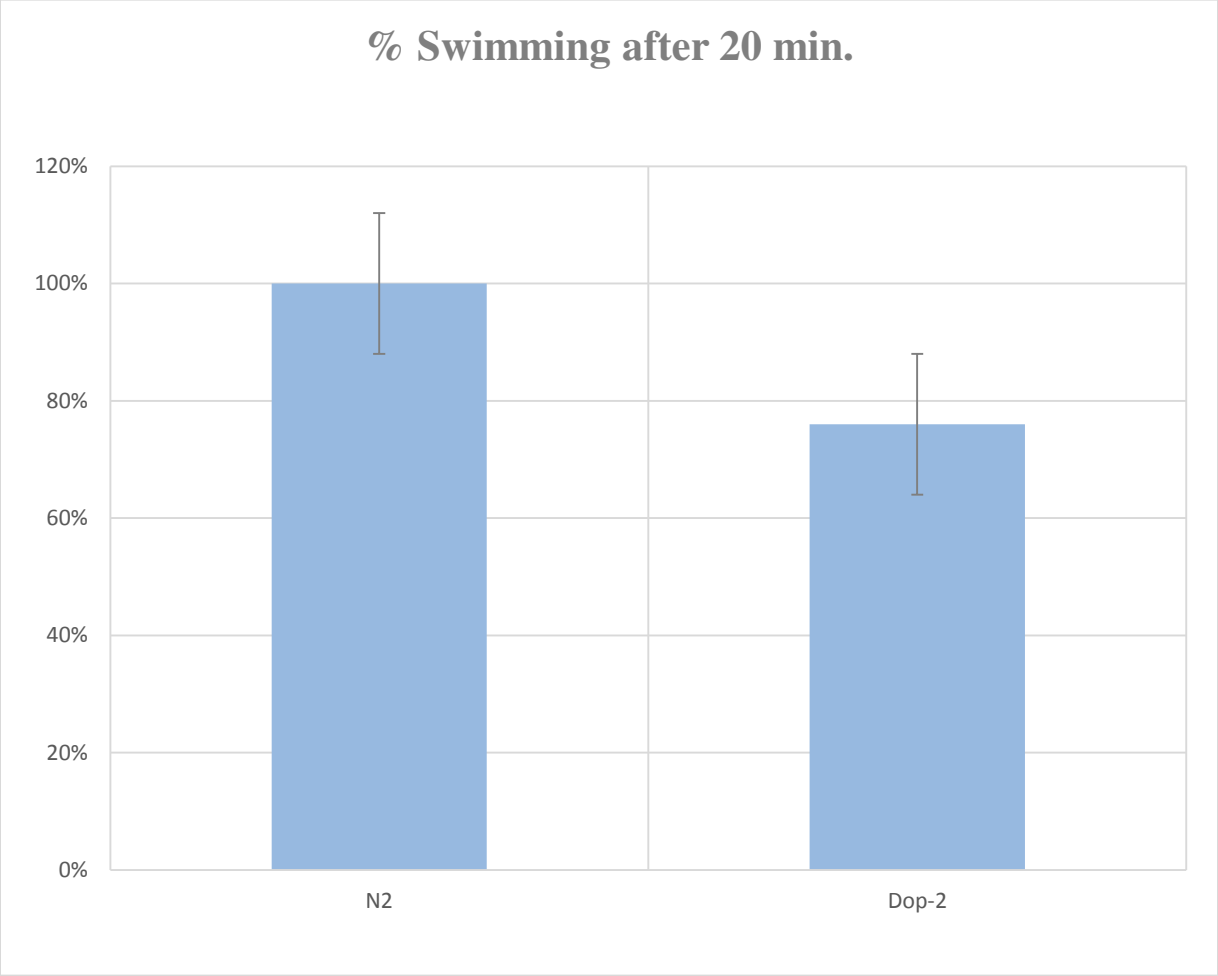


Figure 8. SWIP behavior in wild type and *dop-2* mutants. Wild type (*N2*) animals, when placed in a liquid environment, swim at a regular rate after 20 minutes. *Dop-2* (*vs 105*) mutants display a partial SWIP phenotype with a reduced percentage of number of worms (less than 80%) that are still swimming after 20 minutes. L4 stage worms were assayed in 40*u*L of M9 buffer. (n = 40; p value = 0.005424, t-test). X-axis are the strains. Y-axis is the percentage of worms that are still swimming after 20 minutes. Error bars represent SEM values.

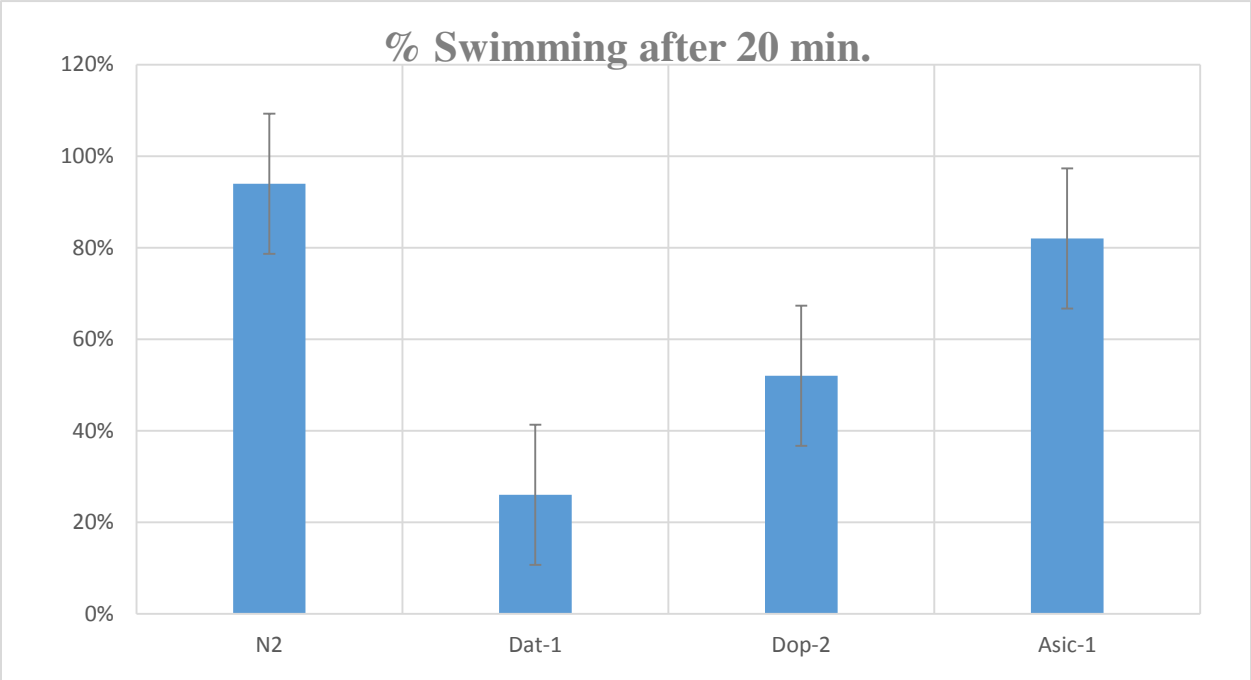


Figure 9. SWIP behavior in wild type, *dop-2, dat-1* and *asic-1* mutants. Comparison of the SWIP phenotype between wild type *N2, dop-2* (*vs 105*), *dat-1* (*ok157)* and *asic-1* (*ok* 415) mutants. As expected, *dat-1* (*ok157)* mutants were those that displayed the higher percentage of paralyzed worms (~ 26%). *Dop-2 (vs 105)* mutants showed a partial SWIP phenotype with a reduced percentage of the number of worms (less than 60%) that were still swimming after 20 minutes. There was no significant statistical difference between the percentage of worms that were still swimming after 20 minutes for the wild type and *asic-1 (ok 415)* mutant worms. L4 stage worms were assayed in 40*u*L of M9 buffer. (n = 46; p value < 0.001, t-test). X-axis are the strains. Y-axis is the percentage of worms that are still swimming after 20 minutes. Error bars represent SEM values.

The partial SWIP phenotype found in *dop-2* mutants strongly support the FRAP results obtained in this study. As dopamine is supposed to be cleared efficiently during swimming (McDonald et al., 2007), the SWIP phenotype in *dop-2* mutants reveals the presence of high levels of dopamine at synaptic clefts which eventually produces paralysis in these worms. This is consistent with previous pharmacological studies on AMPH-induced SWIP for which AMPH, which increases levels of extracellular dopamine, produces SWIP in wild type worms but not in mutants lacking DOP-3, DOP-4 and DOP-2 dopamine receptors (Carvelli, et al., 2010).

The fact that the SWIP phenotype is mediated by the DOP-3 receptor and that both DOP-3 and DOP-2 are D2 like receptors shows the implication of dopamine signaling in the SWIP

behavior of both mutants (Neve et al., 2004). In addition, because D2 like receptors provide a negative feedback loop by inhibiting adenylate cyclase and therefore reducing excitability of the cell (Callier et al., 2003), the result of this assay supports the hypothesis of this study, which aims to assign a neuro-regulatory role to DOP-2.

Finally, in this study it was also observed that the *dop-2* mutation failed to reverse the DA-induced paralysis phenotype of both *dat-1* and *asic-1* animals in the *dop-2;dat-1* and *dop-2;asic-1* double mutants (Figure 10). In fact, both *dop-2;dat-1* and *dop-2;asic-1* double mutants presented a SWIP phenotype similar to that of *dat-1* animals. These results suggest that loss of *dop-2* compromises the ability of *dat-1* mutants to clear DA from the synaptic cleft and can be interpreted as representing almost a complete loss of *dat-1* activity. Loss of *dop-2* and *dat-1* did not have any additive effect on the SWIP phenotype of the *dop-2;dat-1* double mutants ($p > .05$). This result suggests that *dop-2* might mediate dopamine regulation through a *dat-1* dependent mechanism.

On the other hand, as shown in Figure 10, loss of *dop-2* and *asic-1* appears to have an additive effect on the SWIP phenotype of the *dop-2;asic-1* double mutants. In fact, it was found that there was a statistical difference between these mutants and both *dop-2* and *asic-1* animals ($p < .05$). These results suggest that regulation of accumulation of extrasynaptic dopamine is not mediated solely through *dat-1*, and that other genes, such as *dop-2* and/or *asic-1*, might affect levels of exogenous dopamine through a different pathway.

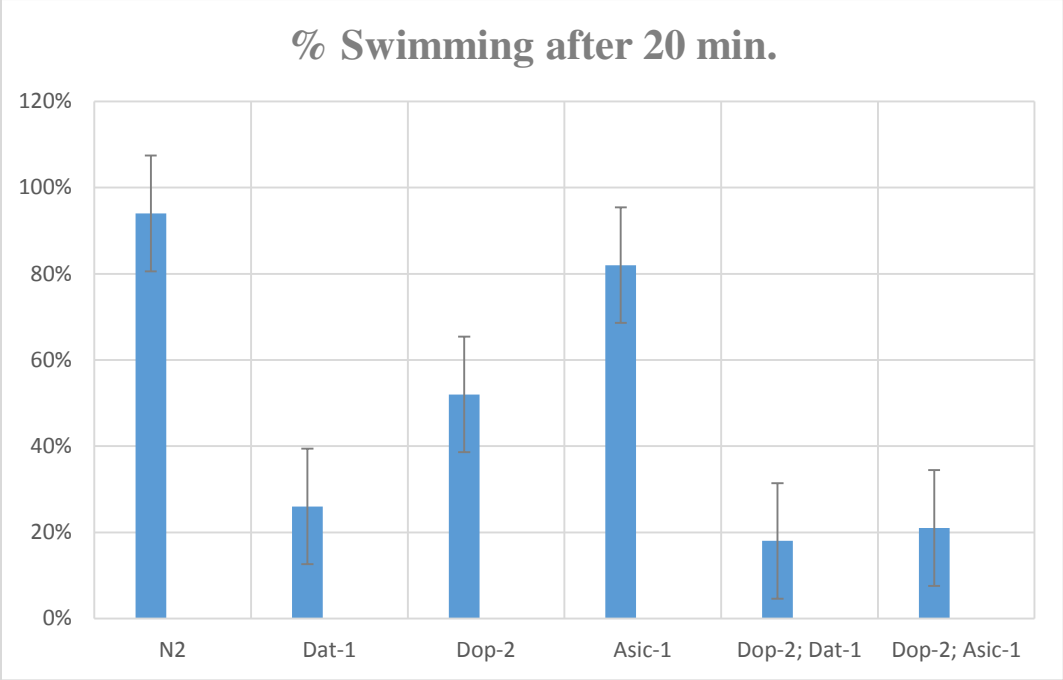


Figure 10. SWIP phenotype in wild type, *dop-2, dat-1, asic-1, dop-2;asic-1* and *dop-2;dat-1* double mutants. Comparison of the SWIP phenotype between wild type *N2, dop-2* (*vs 105*), *dat-1* (*ok157), asic-1* (*ok* 415), *dop-2;dat-1* and *dop-2;asic-1* double mutants. Loss of *dop-2* and *dat-1* appears to have an additive effect on the SWIP phenotype of the *dop-2;dat-1* double mutants. Also *dop-2;asic-1* double mutants exhibit a SWIP phenotype similar to that of *dop-2;dat-1* animals. L4 stage worms were assayed in 40*u*L of M9 buffer. (n = 40; p value < 0.05, t-test). X-axis are the strains. Y-axis is the percentage of worms that are still swimming after 20 minutes. Error bars represent SEM values.

CHAPTER III:

dop-2 EXPRESSION IN THE DOPAMINERGIC NEURONS OF *C. ELEGANS.*

3.1. Introduction

Most studies consider DOP-2 to be a G protein coupled auto-receptor (Ford, 2014; Benoit-Marand, et al., 2001; Neve, 2009). In mammals, dopamine auto-receptors are found exclusively on dopaminergic neurons where they are located at both axons and somatodendrites. It has been proposed that dopamine auto-receptors regulate the firing pattern of dopaminergic neurons and control dopamine activity at axon terminals (Ford, 2014; Missale et al., 1998). Studies on the expression of *dop-2* in *C.elegans* have provided evidence of its expression in dopaminergic neurons (Tsalik, E. L., et al., 2003; Suo et al., 2003). However, expression of DOP-2 protein distribution in these neurons has not been analyzed.

3.2. Materials and Methods

GENERATION OF PLASMID

A plasmid construct containing the 2.6 Kb cDNA fragment of coding sequence of the full length *dop-2* gene was fused to a fluorescent reporter gene using the plasmid pRB952 (pro_{dat-1}::*gfp*::*dat-1*cDNA in pPD 96.41vector) (Figure 11).

The 1845 bp *dat-1*cDNA fragment in pPD 96.41 vector was cut using restriction enzymes Nhe I and Bgl II. The coding region of the full length *dop-2* gene was PCR amplified from the pGEMT-EZ *dop-2* plasmid, using the following oligonucleotides:

GCTAGC ATCGAGGCCGGAGAGACATG and

AGATCT TTAGACATGCGCCTGCTTGTTAC.

These oligonucleotides encoded a NheI and a BglII site suitable for cloning the *dop-2* gene in the pPD 96.41 vector.

Full length *dop-2* cDNA was cloned into the pPD 96.41 vector to create the final pro$_{dat-1}$::*gfp*::*dop-2* cDNA in pPD 96.41vector. The plasmid pro$_{dat-1}$::*gfp*:: *dop-2* cDNA in pPD 96.41 (Fig.3) was verified by DNA sequencing (GENEWIZ, Inc. DNA Sequencing Service. Plainfield, NJ).

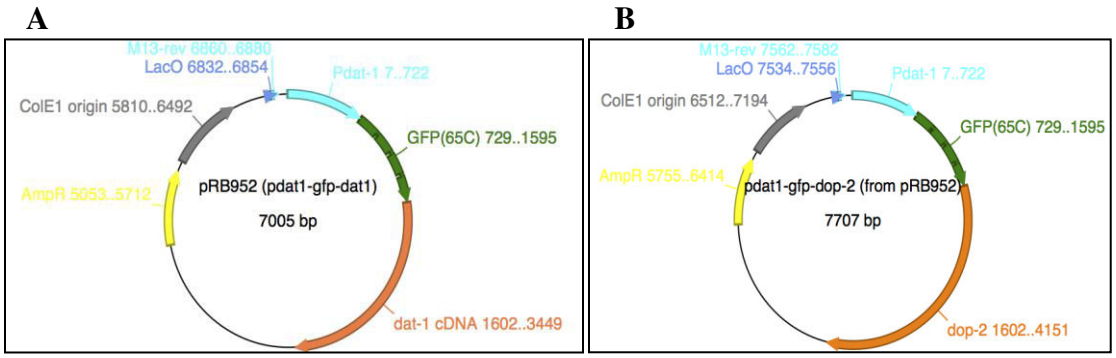


Figure 11. Plasmid Maps. A. pRB952 Plasmid: pro$_{dat-1}$::*gfp*::*dat-1*cDNA in pPD 96.41vector.
B. pro$_{dat-1}$::*gfp*:: *dop-2* cDNA in pPD 96.41vector. (Vector provided by Dr. Randy Blakely, Vanderbilt University Medical Center).

GENERATION OF TRANSGENIC *C. ELEGANS* BY BIOLISTIC TRANSFORMATION

DNA transformation is a common laboratory practice to introduce exogenous DNA into a cell. Transgenic DNA constructs are used to study gene expression and localization, to analyze DNA or RNA regulatory elements, to rescue mutant genes and to study gene structure and function *in vivo.* Exogenous DNA can be either integrated into the organism's genomic DNA or maintained within the organism by the creation of large extrachromosomal DNA arrays.

DNA transformation is an important tool also in studies using *C. elegans*. The first transformation method for use in *C. elegans* was published in 2001 (Praitis, V., et al., 2001). Two methods are available for transformation in *C. elegans*: microinjection and gene

bombardment. In transformation performed by microinjection, exogenous DNA is delivered into the syncytial cytoplasm of the worm germline. This cytoplasm is shared by many germ cell nuclei; therefore, DNA injected here can be delivered to many progeny (Evans, 2006). Microinjection leads to the creation of large extrachromosomal DNA arrays, whose transmission depends on the array size and content of injected plasmid (Mello and Fire, 1995).

In *C.elegans* transformation by gene bombardment, DNA is bound to gold particles which are then shot into the worm using a biolistic instrument called "gene gun." This method allows the formation of low copy-number transgenes, which are randomly integrated in the *C. elegans* genome, as well as high-copy number extrachromosomal arrays (Praitis, V., et al., 2001). The use of the *unc-119* gene, which is usually on the same plasmid as the gene of interest, as a selectable marker, aids in the screening for integrants. The *unc-119* (*ed3*) mutant worms are uncoordinated and do not form the “dauer” stage (a developmentally arrested larval stage formed to survive starvation). Selection for normal movement and dauer stage formation after starvation is a very efficient way to rescue *unc-119* animals carrying integrated transgenes. In this experiment both microinjection and gene bombardment were performed in order to obtain transformed animals.

STRAIN AND PLASMIDS

In this experiment the mutant strain *unc-119* (*ed3*) was used. This spontaneous mutation, which affects locomotion, causes animals to grow slightly dumper than wild type worms and impairs their ability to form the dauer stage (Maduro and Pilgrim, 1995). *Unc-119* (*ed3*) was used as a selectable marker. Two plasmids were used in this experiment: the pDP#MM016b

plasmid, containing full length *unc-119* gene (provided by Katherine Pinter from Dr. Hamza's lab. College Park, MD) and the pro$_{dat\text{-}1}$::*gfp*:: *dop-2* cDNA in pPD 96.41 vector made previously.

WORM PREPARATION

A large number of worms is necessary to perform this experiment. The *unc-119* (*ed3*) mutant worms are difficult to grow on regular NGM plates because their uncoordinated movement prevents them from reaching the food all over the plate. To overcome this problem, egg plates were prepared to grow a large number of worms. Although making egg plates is very time consuming (about 2 to 3 weeks), these plates are better than regular NGM plates because the thick layer of food created by eggs allows *unc-119* mutants to move more easily and reach all the food present on the plate. Worms on egg plates were grown at 20°C until all the food in the plate was consumed (10 to 15 days). In general, five 15cm egg plates are enough to grow worms for one bombardment experiment.

BOMBARDMENT

The experiment of gene bombardment required the following steps:

a) worm preparation

b) DNA preparation

c) preparation of the Bio-Rad PDS-1000/He Hepta System apparatus

d) recovery of worms after bombardment

e) screening for transformants

a) Worm preparation

During this first step, worms were transferred form egg plates to pre-warmed unspotted NGM plates using M9 or S-basal buffer. Plates evenly covered with worms were left to dry on ice for about 30 minutes.

b) DNA preparation

Using a siliconized tube, the DNA mix was prepared according to Table 1.

Table 1. Bombardment DNA mix.

COMPONENTS	***CONCENTRATION***	***VOLUME USED***
Gold Particle	---	100*u*l
pDP#MM016b	0.25*u*g/*u*l	40*u*l
proDAT-1:: GFP:: *dop-2* cDNA	0.9*u*g/*u*l	12*u*l
$CaCl_2$	2.5M	150*u*l
Sperimidine	0.1M	60*u*l

The DNA mix was vortexed, spun and incubated on ice for 30 minutes. After a few washes with 70%, ethanol the mix was re-suspended in 100% ethanol until the time it was ready for use.

c) Preparation of Bio-Rad PDS-1000/He Hepta system

The Bio-Rad PDS-1000/He Hepta System was used to perform bombardment in this experiment. The Bio-Rad PDS-1000/He Hepta System is an apparatus which delivers microparticles toward the target cells by using high-pressure helium gas. Before starting the experiment both the helium tank and the vacuum pressure have to be adjusted according to the type of rupture disk and microcarrier used. The bombardment chamber was washed with ethanol and kimwipes. Microcarriers and rupture disks (900 psi) were dipped in 100% ethanol

and laid on a kimwipe to dry. 20 *u*l of DNA/gold mixture was added to each microcarrier, which were then placed one by one in the microcarrier holder and then into the system. The plate with the worms was placed in the bombardment chamber with the lid off. Pressure was fired until the disk was ruptured. The plate was then removed for recovery.

d) Recovery of worms after bombardment

After bombardment plates were left for recovery at 20°C for about an hour. Once worms were recovered, they were washed out off the plates with S-basal or M9 buffer and distributed on NGM plates with food. These plates were left at room temperature for 2 to 3 weeks before screening for rescued worms. Rescued worms were then used to generate individual lines.

e) Screening procedure

Screening for the *unc-119* rescued phenotype was done by selecting worms with normal movement and dauer stage formation using a dissecting microscope. In addition, two other types of screening were performed in this experiment. In the first type, the presence of transgenes was verified using a confocal microscope in order to visualize the green fluorescent light produced by the expression of the *dop-2* gene. In the second type of screening, the presence of transgenes was verified using single worm PCR. Primers to confirm the presence of transgenes in the apparently transformed worms were designed to target GFP sequences. Single worm PCR was performed by picking one adult worm from the plate and putting it in 5*u*l of worm lysis solution in a PCR tube. Then the tube was put three times in -80º C for 5 minutes each in order to break the cell membrane. A water bath was then performed at 60º C for 60 minutes and at 95º C for 15 minutes. PCR on the lysed worm was then performed using the following primers:

5' GCTGATTGCAGTGCTCGGTAC 3' and

5' TCCTAGATACCAGTCGCCGTTG 3'.

MICROINJECTION

Microinjection is the most widely used technique to generate transgenic *C. elegans*. It requires only a small number of animals and transgenes are selected only a few days after injection. DNA fragments or plasmids are injected in the distal gonad syncytium or directly into unfertilized oocytes. Identification of transgenic animals is facilitated by the use of a marker which is coinjected into the worm together with the DNA of interest.

Microinjection leads to the creation of large extrachromosomal DNA arrays, whose transmission depends on the array size and content of injected plasmid (Mello and Fire, 1995). In transgenic animals carrying exogenous DNA as an extrachromosomal array, genes are expressed similarly to endogenous genes. However, variations of expression of the gene are also seen (mosaic expression).

As our laboratory lacks the instruments necessary to perform microinjection, the experiment was done by Alex Yu in the laboratory of Dr. Catherine Rankin at the University of British Columbia (Vancouver, Canada).

IMAGING

Visualization and localization of *dop-2* expression were performed by using the Fluoview FV10i Olympus Confocal Laser Scanning Microscope. Worms were anesthetized with 10mM levamisole and mounted on a 5% agarose pad. A cover slip was placed on the sample and worms were imaged within 30 minutes of the anesthetic exposure. Animals were observed using

a high magnification water-immersion objective 60X lens. Images were taken both on single plane and in series to repeatedly acquire images in different focus positions.

3.3. Results and Discussion

Results for the analysis of *dop-2* expression in this experiment were derived solely from transgenic worms originated from the microinjection transformation approach. The bombardment transformation approach, which was performed three times, was never successful, due in part to a lack of experience in handling and screening a large number of worms and in part to possible errors in gene cloning.

GFP fluorescence in transgenic *C. elegans* generated by microinjection was observed in both adult worms and in larvae. Variations in GFP fluorescence intensity were observed between worms and worm lines; these variations showed a mosaic pattern of GFP expression, which is typical in microinjection. For the identification of the dopaminergic neurons in transgenes, images of the *C. elegans* dopaminergic neuron distribution and projections from the Wormatlas website were consulted and used as a reference. Figure 12 shows a schematic drawing of DA neurons in *C. elegans* from this website.

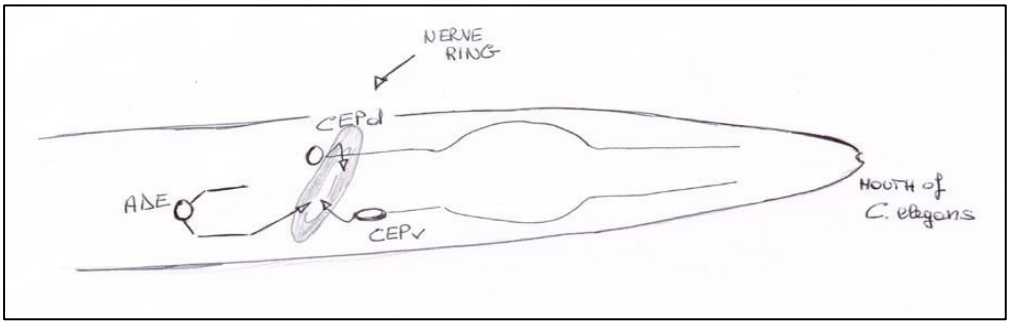


Figure 12. Schematic drawing of DA neurons in *C. elegans.* CEPv, CEPd and ADE dopaminergic neurons are represented with circles. The nerve ring is the gray circle. Dendritic projections are represented as lines. (Source: Wormatlas.)

Expression of GFP::DOP-2 was observed in the somas of the four head dopaminergic neurons CEPs, both ventral and dorsal, along the dendrites, and in the axons localized at the nerve ring (Figure 13). In addition, *dop-2* cDNA expression was seen in the other two cephalic neurons ADEs, left and right, and along ADEs processes ending in the nerve ring (Figures 14 and Figure 15 A and B).

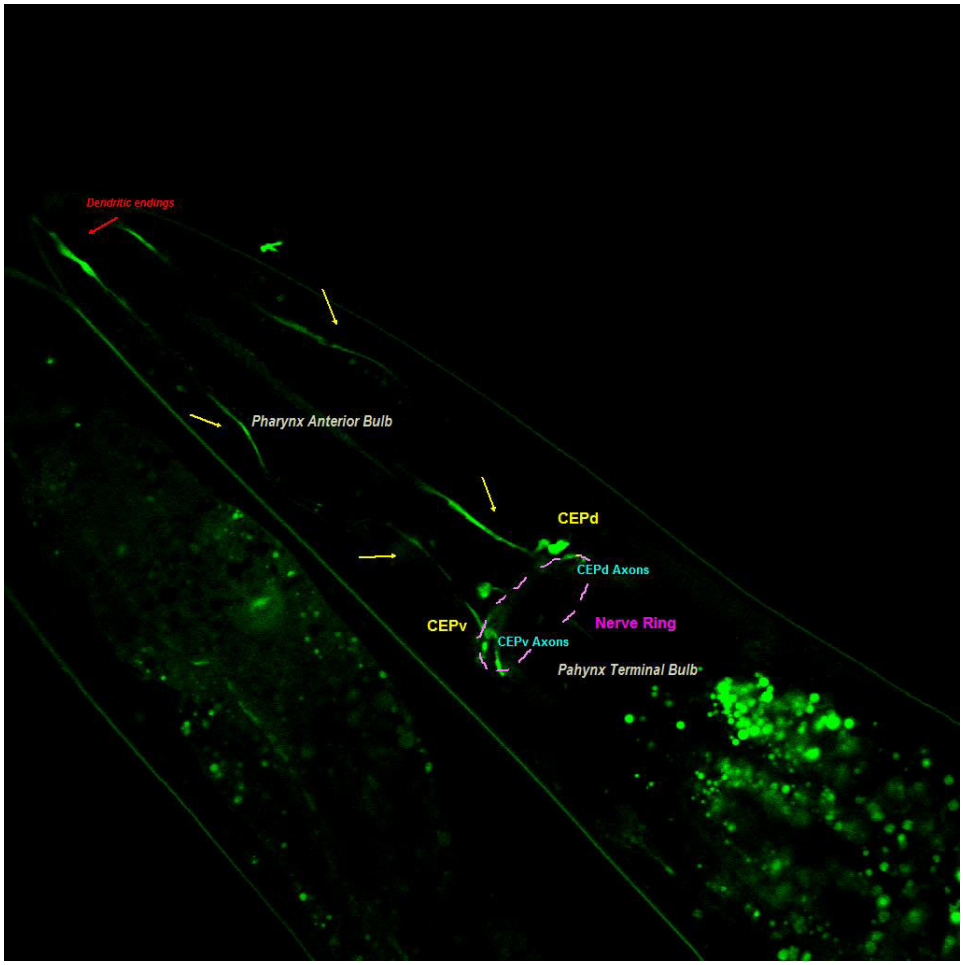


Figure 13. Transgenic animals expressing the *dop-2* cDNA driven by the *pro*DAT-1::GFP promoter in CEP neurons. Confocal image of *dop-2* expression in the CEP dopaminergic neurons in the head of *C.elegans.* Expression is seen in the cell body (highly fluorescent area), in the dendrites (yellow arrows), in the dendritic endings at the tip of the mouth (red arrow) and in the axons at the nerve ring. The Nerve Ring is drawn in pink and includes axons and other nerve endings from the CEP DAergic neurons. All the green dots in the bottom right corner just below the pharynx terminal bulb represent the worm intestine. Worms were immobilized with 10mM levamisole solution on agarose-padded slides and examined under FV10i Olympus Confocal Laser Scanning Microscope. The tip of the head is at the top left of the image.

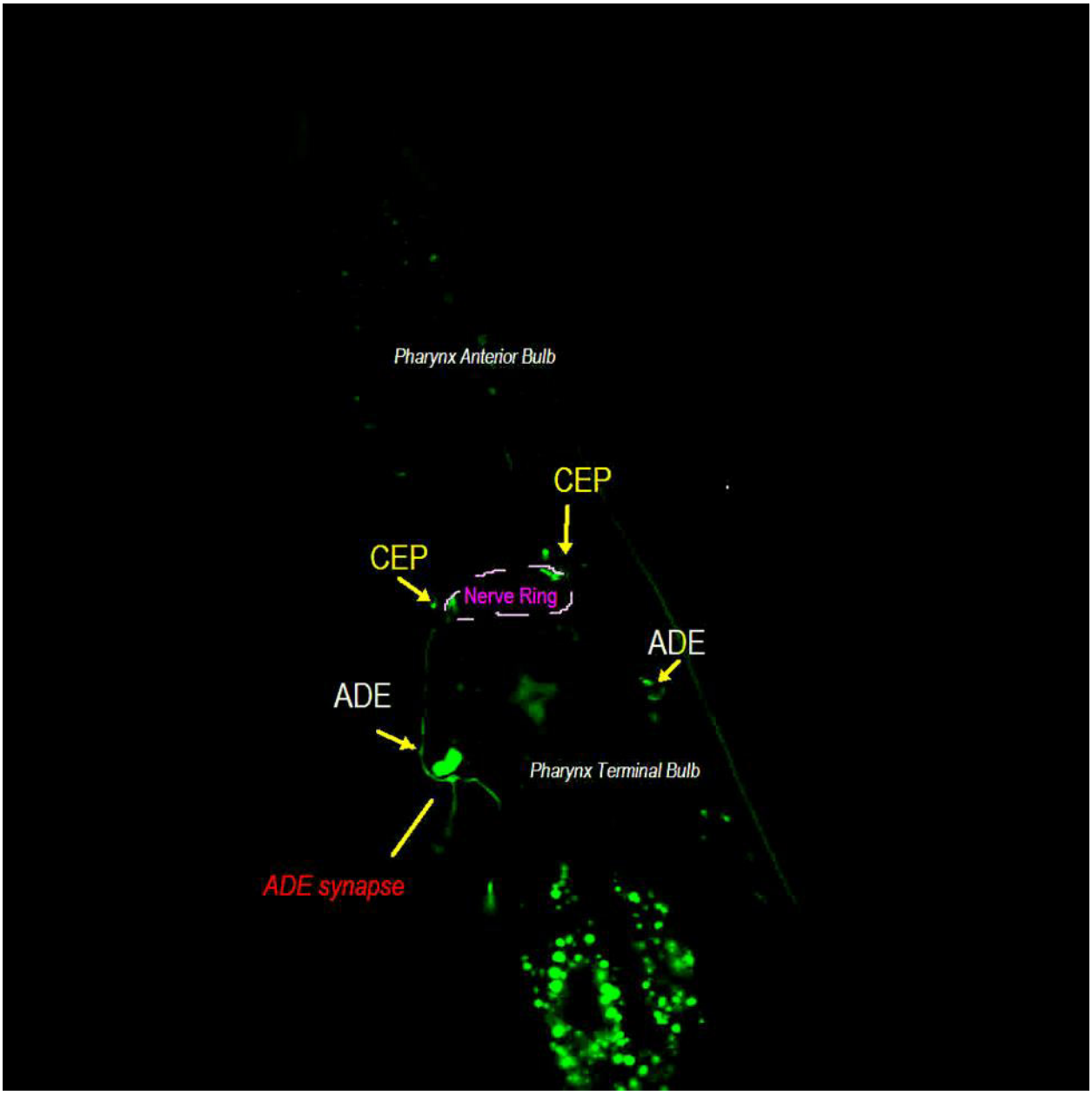
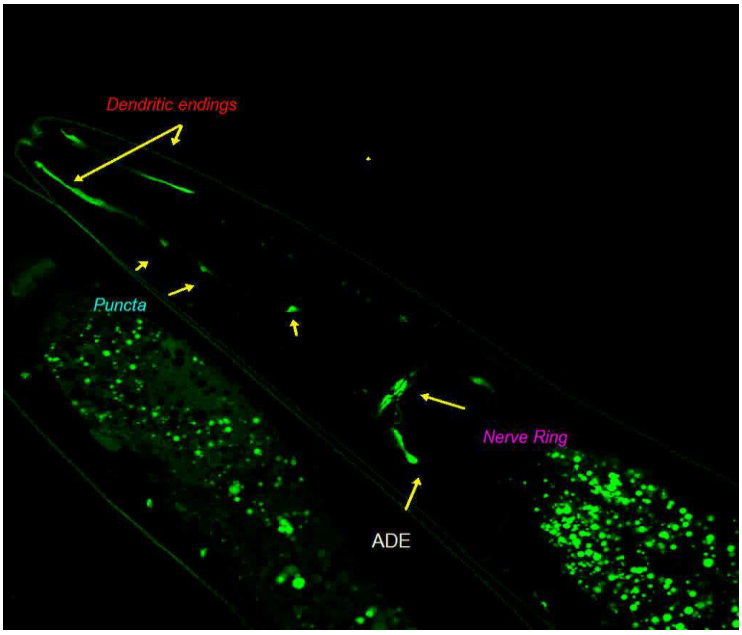


Figure 14. Transgenic animals expressing the *dop-2* cDNA in ADE neurons. Confocal images of *dop-2* expression in the ADE dopaminergic neurons in the head of *C.elegans.* Expression is seen in the cell body and in the ADE processes at the nerve ring (pink dotted lines). ADE synapse is highlighted in red. Worms were immobilized with 10mM levamisole solution on agarose-padded slides and examined under FV10i Olympus Confocal Laser Scanning Microscope. The tip of the head is at the top left of the image.

A



B

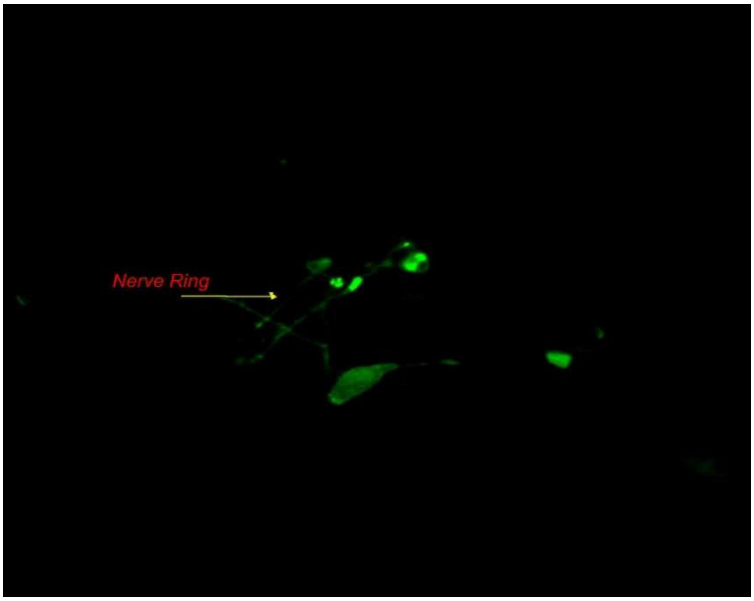


Figure 15. Confocal images of *dop-2* expression in the nervous system of *C. elegans*. Expression is seen in the cell body processes and nerve endings in **A**. The nerve ring is visible in **B.** The tip of the head is at the top left of both images.

dop-2 is also expressed in two additional dopaminergic neurons located in the mid-body section of the worm: the PDE left and right (Altun and Hall, 2011). In this study, however, *dop-2* expression in PDEs was not seen. This was perhaps due to several reasons. To begin with, in microinjection the injected DNA forms extrachromosomal arrays, that are expressed in a mosaic fashion, whose way of transmission does not follow Mendelian laws (Mello and Fire, 1995). Consequently, there is a gradual loss of DNA over generations, which produces mosaic expression of the gene of interest. This would explain why fluorescent signal for *dop-2* expression was not seen in the PDE dopaminergic neurons in these transgenes. Secondly, as mentioned above, the microinjection experiment was performed in Vancouver, B.C., and the transgenes were shipped to our laboratory. Few lines were created from the one plate shipped to us because of the rapid loss of transgenes over generations, which increased the likelihood of observing worms with mosaic expression. If we had been able to create more lines from several plates, we may have been able to observe *dop-2* expression in the PDEs. Indeed the microinjection experiment that produced that plate sent to us had been successful, such that this expression was initially observed in the PDEs.

Expression of *dop-2* was also observed along the processes of dopaminergic neurons, both the CEPs and the ADEs and at synapses (Figure 13 and Figure 14). These results, which localize DOP-2 at the cell bodies and synaptic terminals of the dopaminergic neurons of *C. elegans*, strongly demonstrate the involvement of *dop-2* in dopaminergic functions.

In this study, expression analysis of *dop-2* was carried out with a twofold purpose: a) to confirm its reported expression patterns, and b) to establish its sub-cellular localization so as to correlate its potential function in the nervous system of *C.elegans.* Confocal microscopic examination of *dop-2* transgenic animals expressing the green fluorescent reporter gene revealed

the expression of the gene in the dopaminergic neurons; this result is in line with *dop-2* expression results in previous studies (Suo et al., 2003). In addition, the expression of *dop-2* seen in the processes and in the synaptic areas suggest a direct involvement of this gene in the dopamine signaling system.

CHAPTER IV:

MONITORING DOPAMINE RELEASE *IN VIVO* USING FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP)

4.1. Introduction

Molecular mobility is at the basis of many molecular processes. These processes are important because they participate in the control of intra and inter-cellular signaling by regulating the amplitude and the time of signals (Klein and Waharte, 2010). The importance of monitoring these molecular processes has led to the introduction of accurate methods for quantifying molecular diffusion and concentration of proteins in living cells *in vivo.* Fluorescence Recovery After Photobleaching (FRAP) was introduced in the 1970's as a technique to study the diffusion of fluorescently labeled molecules bound to the plasma membrane of living cells (Axelrod et al., 1976). FRAP is an optical method that relies on the ability to tag the molecule of interest with a fluorescent label. Briefly, in FRAP experiments, a high intensity laser light is used to bleach a region of the sample containing the labeled proteins. A low intensity laser is then used to monitor the recovery of fluorescence due to the movement of molecules from the area outside the bleached region into the bleached one. The recovery of fluorescence of the bleached area as a function of time is then plotted to give the rate of diffusion of the molecular species. Recently, the FRAP technique has increased its popularity due to the discovery of the green fluorescent protein (GFP) which allows prolonged imaging of molecular trafficking *in vivo* (Gonzalez-Gonzalez, et al., 2012).

PHLUORINS AS OPTICAL INDICATORS

Green Fluorescent Proteins (GFP) are stable, non-toxic and non-invasive fluorescent tags which are commonly used as fluorophores to tag living cells (Reits and Neefjes, 2001). Nearly all proteins can be tagged with GFP without compromising the functional properties of the protein itself. In addition, GFP can be bleached without any damaging effect on the cell environment. The wide application of GFP as biological probes and the significant improvements in microscopy have made possible the study of the protein dynamic inside cells (Goulbourne, C.N., et al., 2010). In recent years, GFP family proteins have been engineered to generate a range of mutants with different specificity depending on the application (Gonzalez-Gonzalez, et al., (2012). Among these, the superecliptic pHluorin (SEpHluorin), a pH-sensitive GFP, has been widely used to study molecular processes, such as chemical neurotransmitter release at neuronal synapses (Miesenböck, G., et al., (1998). In SEpHluorin, fluorescence emission is eliminated at low pH by protonation. SEpHluorin tagged molecules are in this way selectively imaged when exposed to more neutral environmental pH. The FRAP technique takes advantage of this genetically encoded optical indicator to measure the rate of synaptic neurotransmitter release during firing in the brain.

The first use of the superecliptic pHluorin as optical indicator for the study of synaptic vesicle release was done by Miesenböck and his colleagues in 1998. Since then, such indicators have been used in many animal models, such as mice, *Drosophila* and *C. elegans* (Voglmaier et at., 2006; Grygoruk et al., 2014; Samuel et al., 2003). Briefly, neurotransmitters, which are stored in specialized endosomes called synaptic vesicles, are released into the synaptic cleft of chemical synapses upon stimulation. Inside their lumen, these vesicles have an acidic pH of ~ 5.6. Following action potential, synaptic vesicles fuse with the plasma membrane, exposing their

lumen to a more neutral pH of ~ 7.4 (Figure 16). The fluorescence change observed during this process is a measure of the rate of vesicle release and can therefore be used as an index of the magnitude of synaptic activity (Sankaranarayanan, S. et al., 2000).

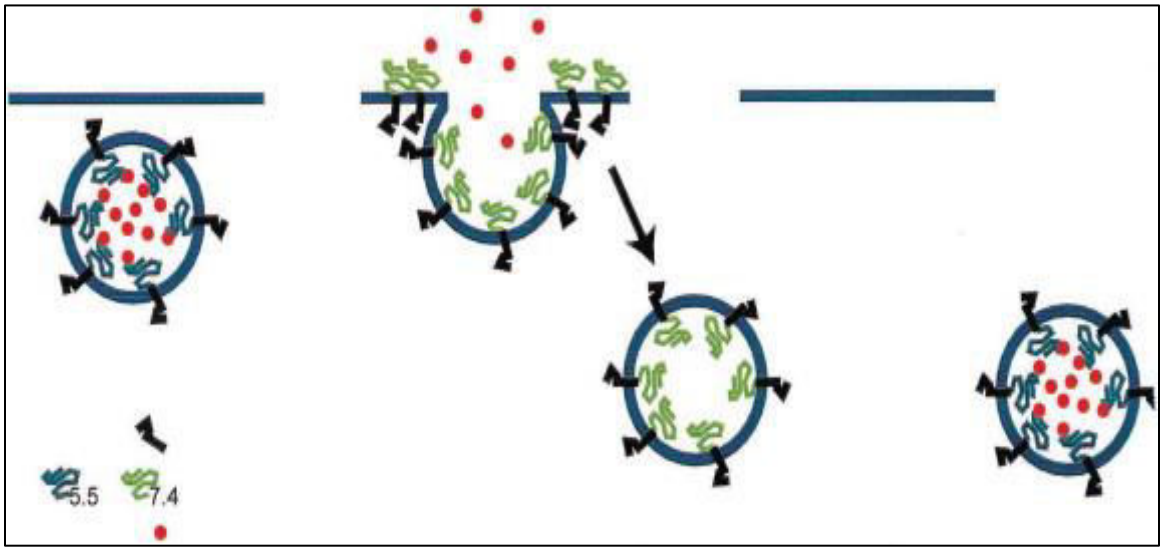


Figure 16. Schematic description of operation of pH-sensitive green fluorescent protein. The pH-sensitive fluorescent proteins are targeted to synaptic vesicles by coupling to the transmembrane domain of the synaptic vesicle protein (synaptobrevin). The pHluorin tag does not fluoresce inside the synaptic vesicle because of the acidic pH (~5.4). Upon stimulation, the vesicle fuses with the plasma membrane, exposing its lumen to the neutral pH 7.4. This makes the pHluorin tags fluorescent again and consequently leads to an increase of fluorescence. (Source: Sankaranarayanan, S. et al., 2000. Biophysical Journal)

4.2. Materials and Methods

STRAINS

The following strains were used in this experiment: *N2* wild-type Bristol, LX702 *dop-2 (vs105)V*, RB680 *asic-1(ok415)I*, *dat-1(ok157)III*, N2*Ex*[p*asic-1*SNB-1::SEpHluorinpRF4] (provided by Dr. Tavernarakis of the Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Crete, Greece), *asic-1(ok415)Ex*[p*asic-1*SNB-1::SEpHluorinpRF4], *dat-1(ok157)Ex*[p*asic-1*SNB-1::SEpHluorinpRF4] and *dop-2(vs105)Ex*[p*asic-1*

SNB-1::SEpHluorinpRF4]. *C. elegans* strains were maintained on NGM (Nematode Growth Media) agar plates and inoculated with strain OP50 of *Escherichia Coli* as a food source and incubated at 20°C.

GENETIC CROSSES

Genetic crosses were performed in order to generate transgenic animals expressing the synaptobrevin-super ecliptic pHluorin reporter fusion. *dop-2 (vs105), asic-1(ok415)* and *dat-1(ok157)* mutant worms were crossed with *N2Ex*[p*asic-1*SNB-1::SEpHluorin], respectively. A genetic cross in *C. elegans* is set up by putting in a plate with a small spot of bacteria L4 stage hermaphrodites and males in a ratio of 1:5 and letting them mate. Generated transgenic animals carrying the SEpHluorin reporter construct were identified by PCR.

IN VIVO FRAP

The FRAP experiments required the following steps:

a) worm preparation

b) microscopy

c) data analysis

d) curve fitting and statistics

a) Preparation of C. elegans strains for imaging

In order to monitor neurotransmitter release at selected synapses, fluorescent strains of interest were grown on small NGM/OP50 plates and maintained in a temperature-controlled incubator at 20˚C. Worms were re-plated every 2 days for experimental needs. With the help of

a platinum wire, L4/young adult worms were picked from plates and mounted on a 5% agarose pad. Animals were then anesthetized with 20mM Levamisol. The use of Levamisol as anesthetic instead of sodium azide is recommended. Levamisol, an acetylcholine agonist that causes permanent contraction of muscle cells, is a mild anesthetic which does not cause oxidative stress in worms the way sodium azide can. A cover slip was placed on the sample and animals were imaged within 30 minutes of anesthetic exposure.

b) Microscopy

A Zeiss LM 710/880 confocal microscope was used for the FRAP experiments. Individual synapses were found first with the 10X objective and then with the 40X objective (C-Apochromat 40x / 1.20 water) to capture images. The region of interest (ROI) was defined and a first image was captured.

To perform the FRAP experiment, three control images of the region of interest (ROI) were taken before photobleaching (one frame every two seconds without any bleach). This is the initial fluorescence intensity before photo-bleaching or pre-bleaching conditions. This provides a reference point for fluorescence recovery. The synaptic region of either CEP or ADE dopaminergic neurons (ROI) was then bleached with high laser power. The number of iterations was 20. The argon laser power was at 15mW in the 488nm wavelength. Emission was filtered with a 500-550nm BP filter. The photobleaching procedure was performed in such a way to reduce the ROI initial fluorescence intensity by 50% or more.

Then 60 images were captured immediately after photo-bleaching (every 2 seconds for 2 minutes after bleaching) to monitor fluorescence recovery. It is important to get the maximum number of time points immediately after photo-bleaching to obtain a good description of the

fluorescence recovery and for further analysis and quantification. A pixel depth of 16-bits was used to measure emission intensity. In the pre and post bleach series of images the laser intensity was attenuated (argon laser power was at 0.15mW in the 488nm wavelength) in order to avoid photo-bleaching. Images were captured using three zoom power. The pinhole was set to 37.3 AU.

c) FRAP data analysis

Using Fiji software, stacks of images were aligned in order to adjust the movement (the region of interest remained in the same position in the image). The align tool used was: 'image'-> 'color' -> 'split channel' -> 'plugins' -> 'registration' -> 'stack registration' -> 'rigid body' and/or 'translation' -> 'crop' -> 'select ROI' -> save as.tiff file. Saved images from Fiji software were opened on Zeiss microscope software ZEN. This is done in order to transfer the adjusted stack of images in the original file. These images were then saved as Final lsm files and used for measuring fluorescence intensity at selected regions. Using the FRAP mode in the ZEN software, fluorescence of the selected synapses was quantified by measuring the fluorescence intensity in three regions: at the synapse of interest F_i; at an unbleached region F_c (which can be another synapse or a cell body region) and at a background region F_b (a non-fluorescent region). Fluorescence intensity values from each region were saved and exported to an Excel spreadsheet. Data from each region of interest was averaged and used to normalize intensity points such that:

$$\text{Normalized Value} = (F_i - F_{b\,Ave}) / (F_i * \text{Bleach Correction})$$

Fluorescence intensity was normalized for background and photobleaching on Excel (Microsoft Corporation, Redmond, USA).

d) Curve fitting and statistics

Curves for each data set were fitted with a one-phase exponential equation. The Student's t-test with a two tail distribution was used with a significant p value > 0.05. One-way ANOVA was used for comparisons of multiple groups followed by the Bonferroni post-hoc test. Both curve fitting and statistical analysis was carried out by using GraphPad Prism 7 software (GraphPad Software Inc. San Diego, USA).

4.3. Results and Discussion

In this study we utilized Fluorescence Recovery After Photobleaching technique (FRAP), to examine the possibility of whether the *C. elegans* DOP-2 auto-receptor acts as a modulator of dopamine release at dopaminergic synapses in the nematode. Neurotransmission involves synaptic vesicles exocytosis during which vesicles containing the neurotransmitter fuse with the presynaptic membrane to release neurotransmitters in the synaptic cleft, ensuring chemical communication between neurons. Visualizing vesicles at synaptic terminals is crucial to elucidating aspects of neurotransmitter release. The specific FRAP methodology used in this study allows us to study neurotransmitter vesicle release by means of a pH-sensitive fluorescent protein-based sensor (pHluorin). In this study, strains were generated in which the synaptic-vesicle-targeted pHluorin gene fusion is driven by an *asic-1* promoter that is expressed in all eight dopaminergic neurons. Individual synapses arising from these neurons were made to undergo fluorescence photobleaching, and fluorescent recovery was recorded in order to monitor synaptic activity. Using a strong excitation argon laser, the fluorescent signal of the GFP protein at a single synapse (region of interest, or ROI), was photobleached at > 50% of its original intensity and recovery was observed by taking time-lapse images. Two additional areas used as control regions were monitored during FRAP experiments. The first control area was a

fluorescent non-bleached region at some distance from the bleached area (either another synapse or a neuron cell body). This "reference region" was monitored to make sure that photobleaching caused by the scanning at each time point did not affect fluorescence recovery during the time of the experiment. The second control area, the "background region", was a non fluorescent region taken outside the bleaching area, whose measurement is important for adjusting the FRAP data to the true zero fluorescence (Figure 17).

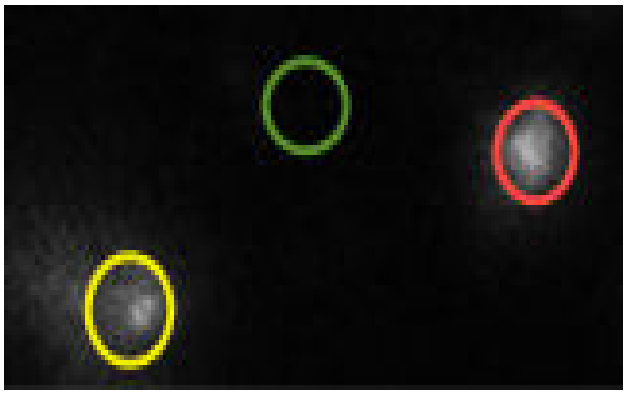


Figure 17. Confocal Image of CEP synapse expressing pHluorin before photobleaching. ROI area is shown as a red circle. The reference region is shown in the yellow circle and the background region in the green circle. (Image is from my FRAP experiments using an LSM 880 Confocal Microscope at the facilities of the Delaware Biotechnology Institute [DBI], Newark, Delaware.)

In the FRAP experiments for this study, time lapse images of synapses labeled with a pH-sensitive green fluorescent protein-based sensor were taken to reveal fusion of dopamine vesicles in the following strains:

N2*Ex*[p*asic-1*SNB-1::SEpHluorinpRF4];

asic-1(ok415)Ex [p*asic-1*SNB-1::SEpHluorinpRF4];

dop-2(vs105)Ex[p*asic-1*SNB-1::SEpHluorinpRF4];

dat-1(ok 157)Ex[p*asic-1*SNB-1::SEpHluorinpRF4].

At first, FRAP data were collected in the wild type *C. elegans* (*N2*) and in the *asic-1* deletion mutant in order to ensure that this FRAP analysis was in line with previously reported studies. Chemical synaptic activity in these two strains had been already analyzed by Voglis & Tavernarakis (2008) using the FRAP technique. The hypothesis of their study was that a specific ion channel, the acid-sensing ion channel (ASIC-1), was responsible for the modulation of the release of dopamine in presynaptic termini in response to an increase of extracellular H+ ions upon neuronal stimulation. Specifically, they hypothesized that ASIC-1 provided positive feedback to enhance dopamine release. Based on this assumption, they predicted that *asic-1* mutants would have a lower rate of dopamine vesicle release compared to the wild type animals. In fact, in their FRAP experiment, they found that in *asic-1* mutant worms the rate of dopamine vesicle release was lower than that in the wild type ones (Figure 18A).

Based on their results, and in order to test our hypothesis, we decided to reproduce Voglis & Tavernarakis's results for two reasons: first, because if our results were the same, they would confirm their findings, and, second, because we would prove that our FRAP technique and analyses were done in the correct way.

When FRAP experiments were performed on dopaminergic synapses of both wild type *N2* worms and *asic-1* mutant animals, a difference in fluorescence recovery between the two *C. elegans* strains was detected, as illustrated in Figure 18B.

The rate of synaptic vesicles exocytosis was measured as a percentage increase (recovery) in fluorescence intensity after photobleaching recorded at individual synapse (y-axis) and represents the movement of synaptic vesicles at synapses.

As revealed by the recovery curves for both strains, the dependence of recovery time on the rate of vesicle release for *asic-1* mutants was lower compared to that for wild type *N2* animals. These results corroborated the findings of Voglis & Tavernarakis's experiments and allowed us to standardize FRAP methodology as well as to provide proof of principle in our initiated FRAP experiments on *dop-2* (*vs 105*) deletion mutants. Figure 19 shows the outcome of FRAP curve for *dop-2(vs 105)* and wild type *N2* animals.

A **B**

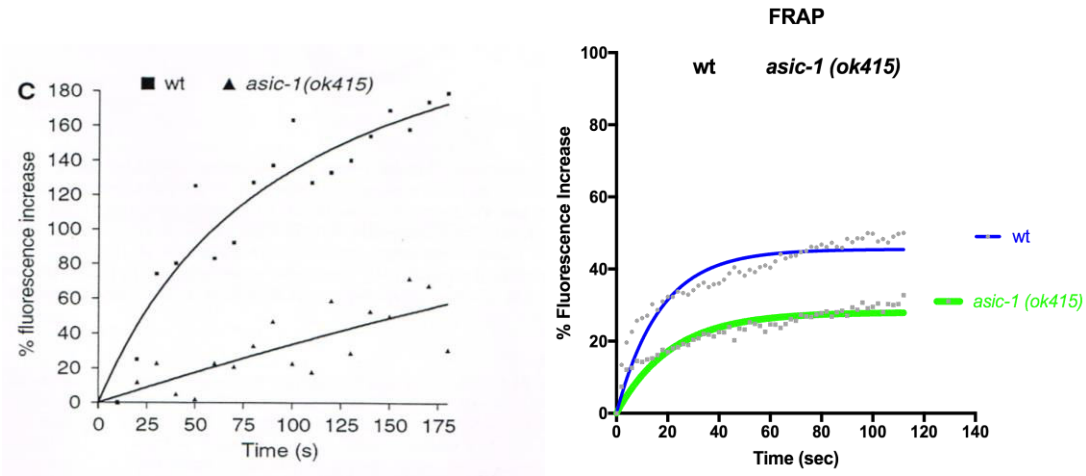


Figure 18. Different percentages of fluorescence increase (% recovery) between wt and *asic-1*(*ok415*) mutants. A. FRAP results comparing percentage of fluorescence increase in wt and *asic-1*(*ok415*) mutant animals from Voglis & Tavernarakis, 2008. **B.** Comparison of the rate of dopamine vesicles release in wt (blue) and *asic-1*(*ok415*) mutant animals (green) from my FRAP experiments. The x-axis is the time course of the experiment, where t = 0 sec. corresponds to the photobleaching. The y-axis is the FRAP measurement. Data were normalized to the first three points in the time-lapse image sequence. Two-tailed t- Student test. P value < 0.0011.

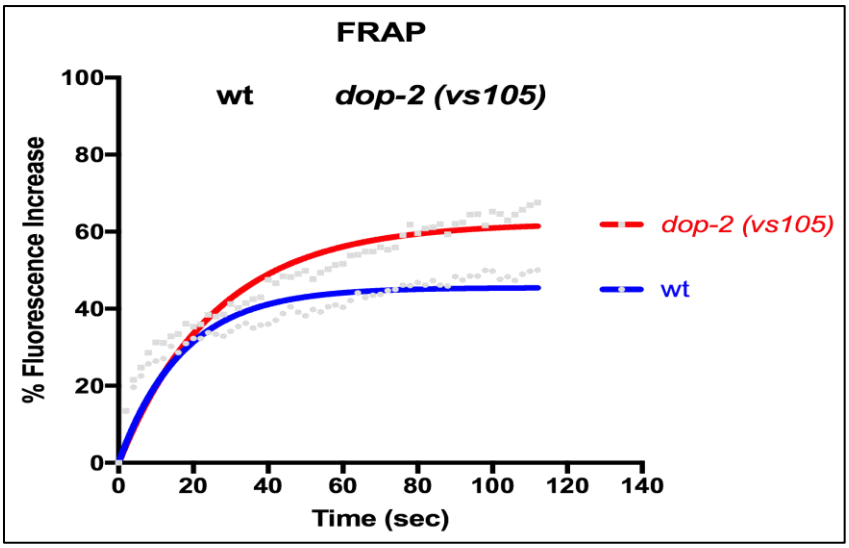


Figure 19. FRAP in *dop-2(vs 105)* mutants. Percentage fluorescence increase (recovery) in wt (blue) and *dop-2(vs 105)* mutant animals (red) from FRAP experiments. The x-axis is the time course of the experiment, where t = 0 sec. corresponds to the photobleaching. The y-axis is the FRAP measurement. Data were normalized to the first three points in the time-lapse image sequence. Two-tailed t- Student test. P value < 0.0052.

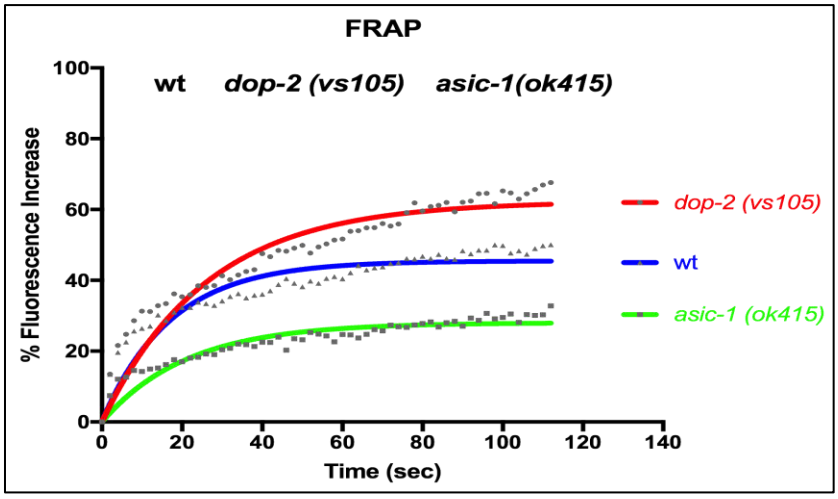


Figure 20. Fluorescence Recovery After Photobleaching in *dop-2(vs 105)* and *asic-1(ok 415)* mutants. Comparison of the rate of dopamine vesicles release in wt (blue), *dop-2(vs 105)* (red) and *asic-1(ok 415)* (green) mutant animals from FRAP experiments. Wild type worms and *asic-1(ok 415)* were used as a positive and negative control respectively. The x-axis is the time course of the experiment, where t = 0 sec. corresponds to the photobleaching. The y-axis is the FRAP measurement. Data were normalized to the first three points in the time-lapse image sequence. One-Way ANOVA and Bonferroni post-hoc test. P value < 0.0001.

As shown in Figures 19 and 20, the percent of fluorescence increase (recovery) for *dop-2(vs 105)* mutants (red line) displays an increase as compared to that of the wild type (blue). For the first 20 seconds after photobleaching, the two curves show a similar trend, while after 20 seconds the *dop-2* curve starts to increase, reaching a higher plateau (~ 60%) compared to that of the wild type (~40%).

The observed change of fluorescence recovery between the two strains may arise from a different mode of vesicle movement that reflects the nature and the dynamic of the molecular process under analysis. As mentioned in the introduction to this chapter, the increase of intensity (recovery) after photobleaching is due to the influx of fluorescent molecules into the ROI area (mobile fraction). It is important to note that not all the molecules that replenish those in the bleached area are free to diffuse. Some can be molecules bound or semi-bound to membranes or to other matrices which are not able to move freely. Therefore, FRAP data can give clues about intercellular processes that take place between molecules, such as interaction between the protein of interest with other proteins or biomolecules. Based on these assumptions, FRAP curves can be of two types: linear and exponential. Linear curves are produced when the exchange rate between bound molecules and free molecules is on the same order as the diffusion rate between bleached and unbleached molecules. Exponential curves are produced when there is a disparity between the exchange rate and the diffusion rate (Breward et al., 2010). Figure 21 shows these two types of curves resulting from FRAP experiments.

Analysis of FRAP curves from *dop-2* mutants and wild type *N2* worms in this study shows that both curves are exponential, meaning that mobility of synaptic vesicles

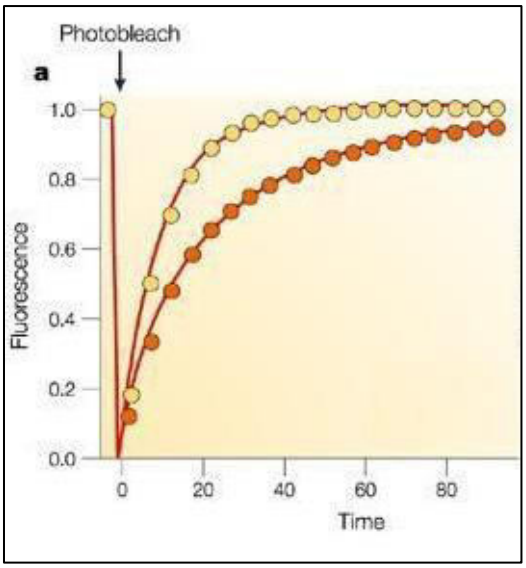


Figure 21. FRAP intensity recovery profiles after photobleaching. Drawing of the recovery curves after photobleaching in FRAP experiments. The red line represents linear recovery, while the green line represents exponential recovery (Source: Breward et al., 2010, Fig. 6a).

in both strains have some constraints which result in vesicles that are not free to relocalize during the time course of the experiment. These results are in line with what is generally thought about conventional synapses, namely, that only a small amount of vesicles is releasable. This is due to the fact that vesicles in the reserve pool are distant from the release site and are immobilized at the cytoskeleton by other proteins. This might also explain why, after photobleaching, the fluorescence recovery never reaches 100 percent.

From the analysis of the FRAP data for *dop-2*, wild type and *asic-1* mutants, the half time $t_{1/2}$, which is the time it takes for the fluorescence intensity to recover to half of the plateau level, was also calculated (Table 2). $T_{1/2}$ is a qualitative measure of how fast synaptic vesicles move into the bleached area during exocytosis (synaptic vesicle fusion). As our results show, there was a statistically significant difference between the $t_{1/2}$ of *dop-2* (22 sec.) and that of wild type (13.5 sec.). This difference shows that the rate of vesicle fusion at *dop-2* mutant synapses is

Table 2. $t_{1/2}$ of fluorescence recovery for wt, *dop-2*, *asic-1* and *dat-1* strains.

$T_{1/2}$ OF FLUORESCENCE RECOVERY (SEC)	
Wild Type *N2*	13.5 sec
dop-2	22.0 sec
asic-1	18.0 sec
dat-1	6.5 sec

slower than in wild type worms, confirming our hypothesis that this might be due to a defect in the speed of either anchoring or membrane fusion in these mutants. On the other hand, no significant difference was found between the $t_{1/2}$ of *dop-2* (22 sec.) and that of *asic-1* (18 sec.).

The FRAP curve for *dop-2* mutants shows an increase in fluorescence compared to wild type *N2* animals. On one hand, this observed increase of fluorescence recovery in *dop-2* mutants might only mean that there is an increase in the rates of exchange and diffusion of labeled vesicles at *dop-2* synapses compared to wild type animals. On the other hand, however, this difference might reflect a dysfunction in the regulatory mechanism at the basis of vesicle release. This suggests that the population of vesicles in *dop-2* mutants might lack the contribution of a regulatory element while the dynamic of the process of vesicle release remains the same. For instance, it is possible that in *dop-2* mutants, the large vesicle reserve pool present at synaptic terminals lacks the cytoskeleton anchoring mechanism. This will result in an increasing mobility of the vesicles that makes them available for neurotransmitter release. This increased mobility could in turn cause either a change in the transport rate of vesicles to the release zone or an imbalance in the exchange rate. According to this view, it is possible that the lack of DOP-2 acts to disturb either the vesicle reserve pool anchoring system or the vesicle axon terminal transport.

Calcium plays a key role in neurotransmitter vesicle exocytosis. Many studies have shown that Ca2+ can trigger vesicle release by interacting with proteins involved to mediate synaptic vesicle fusion such as SNAREs. Indeed, it is thought that Ca2+ influx through voltage gated calcium channels localized at vesicle release sites (active zone) is responsible to initiate vesicle fusion (Riddle, 1997). In addition, other studies have proved that the number of calcium ions that enter the neuron is directly related to the amount of synaptic vesicles released, revealing the importance of Ca2+ in controlling neurotransmitter release. Changes in Ca2+ influx can be modulated by different signals, both pre and post-synaptically (Sudhof, 2008). These observations suggest that enhanced dopamine levels at dopaminergic synapses of *dop-2* mutants might reflect either an abnormal calcium signaling or an alteration in the vesicle fusion/docking mechanism preceding neurotransmitter release.

In the early 1950s, Bernard Katz and his colleagues established that neurotransmitters are released from pre-synaptic terminals in discrete quanta. Synaptic vesicles secrete their contents into the synaptic cleft by fusing with the plasma membrane of the presynaptic terminal and then causing a single quantal release of packets of neurotransmitter (Del Castillo and Katz, 1954). In 1970 John Heuser proved the quantal theory. He conducted biochemical studies that confirmed the correlation between vesicle fusion and quantal transmitter release at frog motor neuromuscular junction (Purves, et al., 2001). However, it is difficult to estimate the concentration of neurotransmitter release, since it is not known how many vesicles are stored in each synapse, how much of the vesicle content is released at time of fusion, or how many vesicles fuse with the pre-synaptic terminal. Electrochemical studies in axonal DA vesicles of healthy mice showed that transmitter content is approximately 30,000 per vesicle but varies from

species to species and from synapse to synapse, suggesting different levels of exocytosic events (Photos et. al., 1998).

The quanta theory provides a possible explanation for the difference in dopamine vesicle release between wild type and *dop-2* deletion mutants found in this study. It can in fact be possible that the DOP-2 auto-receptor modulates the release of dopamine at dopaminergic synapses in *C. elegans* by reducing quanta size (the amount of neurotransmitters per synaptic vesicle), given that D2 auto-receptors are inhibitory. DOP-2 could then work to control the rate-limiting step in the dopamine synthesis by acting on *cat-2*, the homologous tyrosine hydroxylase enzyme (TH) in the nematode. In midbrain dopamine neurons, regulation of TH has been proved to be controlled by both translational and post-translational factors (Pereira and Sulzer, 2012). Post-translational regulation involves phosphorylation of TH, which inhibits its activity producing a decrease in quantal size. Based on these assumptions, one possible explanation for the difference in the rate of dopamine vesicle release between *dop-2* mutants and wild type worms would be that in normal dopaminergic synapses of *C. elegans* DOP-2 activation induces phosphorylation of *cat-2* (the *C. elegans* tyrosine hydroxylase), with the resulting reduction of quanta size. This does not happen in worms with *dop-2* deletion, which in fact have an increase of dopamine release.

An alternative view could be that DOP-2 controls dopamine quantal size by acting on the monoamine vesicular transporter VMAT. VMAT is responsible for the package of transmitters into the synaptic vesicles (Wimalasena 2011). Pharmacological studies on VMAT knockout mice showed that in absence of VMAT exocytosis release is eliminated and that the stores of brain monoamine is dramatically reduced (Takahashi, et al., 1997). VMAT is mainly controlled at the level of protein expression. However, recent studies have shown that also D2-like auto-receptors

can regulate VMAT function, suggesting a role of dopamine receptors in controlling dopamine storage (Edwards, 2007).

As discussed in Chapter II, *dop-2* mutant animals present compromised memory and learning capabilities. The quanta theory provides a good explanation for this deficit. *dop-2* mutants have a higher rate of vesicle fusion compared to wild type *N2* (Figures 19 and 20), which could be the cause of a higher quantal release, consequently causing a high extracellular DA level (See model in Figure 26). High extracellular DA levels would in turn produce saturation of the binding sites of all receptors. As such, the release of dopamine would have no post-synaptic dependent response that could have disrupting effects on synaptic plasticity necessary for learning and memory, for example. This would explain why *dop-2* deletion mutants in *C. elegans* are deficient in learning and memory capabilities. In addition, it would confirm the importance of quantal size in attaining a normal level of extracellular DA during synaptic plasticity.

In this study, the rate of dopamine vesicle release of the dopamine transporter *dat-1* was also investigated using FRAP. Results from FRAP experiments for *dat-1(ok 157)* deletion mutants are shown in Figures 22 and 23.

Examination of the FRAP curve for *dat-1(ok 157)* (pink line) revealed a different rate of recovery compared to that of wild type (blue). Again, the rate of fluorescence recovery was found to be higher for *dat-1* mutants compared to that of the wild type. Like those of the other strains analyzed in this study, this FRAP curve is exponential. In addition, the recovery rate of the *dat-1* curve appeared to be more rapid, with a $t_{½}$ = 6.5 sec. than those of *asic-1* (green), *dop-2* (red) and wild type (blue) (Figure 23 and Table 2). These results indicate that in *dat-1* mutants there is a higher fraction of mobile molecules, suggesting that the function of *dat-1* is not only to

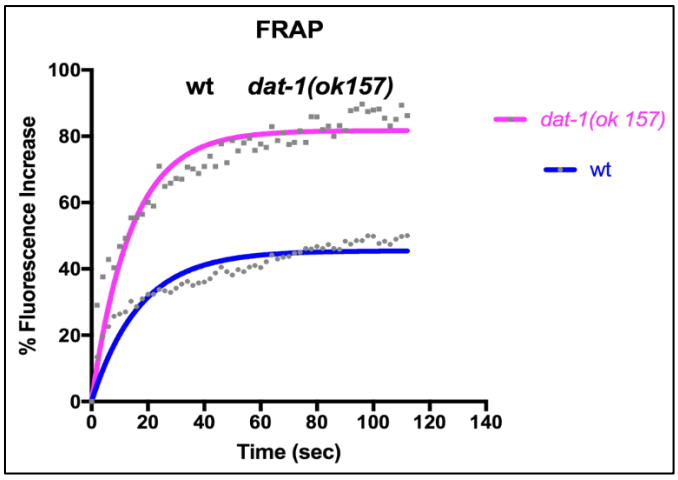


Figure 22. FRAP recording in *dat-1(ok 157)* mutants. Rate of dopamine vesicles release in wild type (blue) and *dat-1*(*ok 157*) mutant animals (pink) from FRAP experiments. The x-axis is the time course of the experiment, where t = 0 sec. corresponds to the photobleaching. The y-axis is the FRAP measurement. Data were normalized to the first three points in the time-lapse image sequence. Two-tailed t- Student test. P value < 0.0001.

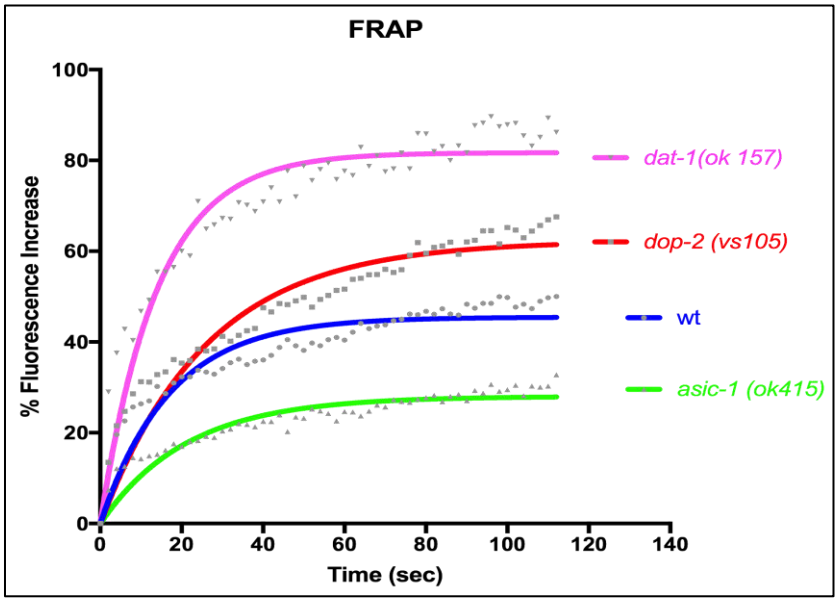


Figure 23. FRAP recording in *dat-1(ok 157), dop-2(vs 105)* and *asic-1(ok 415)* mutants. Comparison of the rate of dopamine vesicles release in wild type (blue), *dat-1(ok 157)* (pink), *dop-2*(*vs 105*) (red) and *asic-1(ok 415)* (green) mutant animals from FRAP experiments. Wild type worms and *asic-1(ok 415)* were used as a positive and negative control respectively. The x-axis is the time course of the experiment, where t = 0 sec. corresponds to the photobleaching. The y-axis is the FRAP measurement. Data were normalized to the first three points in the time-lapse image sequence. One-Way ANOVA and Bonferroni post-hoc test. P value < 0.0001.

terminate dopamine signaling by clearing the catecholamine from the extracellular space but also to modulate dopamine release by acting on the mechanism of synaptic vesicle fusion. In fact, pharmacological studies in mice have shown that, in addition to its more conventional role of dopamine clearance from the extracellular space, DAT also appears to have an active role in DA release. These studies have indeed shown that cocaine and other DAT inhibitors can increase DA release, suggesting a DAT function in restricting vesicle pool mobility and/or release (Sulzer et al., 2016), while other studies propose that DAT blockage increases DA release by mobilizing DA from the synaptic vesicle reserve pool (Venton et al., 2006). Results from our study suggest that because both *dop-2* and *dat-1* present a higher increase of vesicle fusion compared to wild type they might be working together in modulating dopamine release.

To summarize the results of these FRAP experiments and to illustrate the different possible ways in which DOP-2 might function in modulating dopamine release at dopaminergic synapses in *C. elegans,* I propose the model shown in Figure 24. According to this model, *dop-2* is the gene that codes for the dopamine auto-receptor DOP-2, which works to reach the balanced dopamine release necessary to allow the signal to integrate post-synaptically. In general, auto-receptors are located on presynaptic nerve cell terminals and function as part of a feedback loop in the signal transduction pathway. As mentioned in the "Introduction", DOP-2 belongs to the family of the D2 like receptors which inhibit adenyl cyclase activity, providing a negative feedback loop during signal transduction. Based on these assumptions, we would expect DOP-2 to provide a negative feedback loop which decreases the amount of dopamine released at the dopaminergic synapses in *C. elegans*.

The model in Figure 24 shows how DOP-2 auto-receptor might work. Upon stimulation of dopaminergic neurons, dopamine is released in the synaptic cleft. This released dopamine

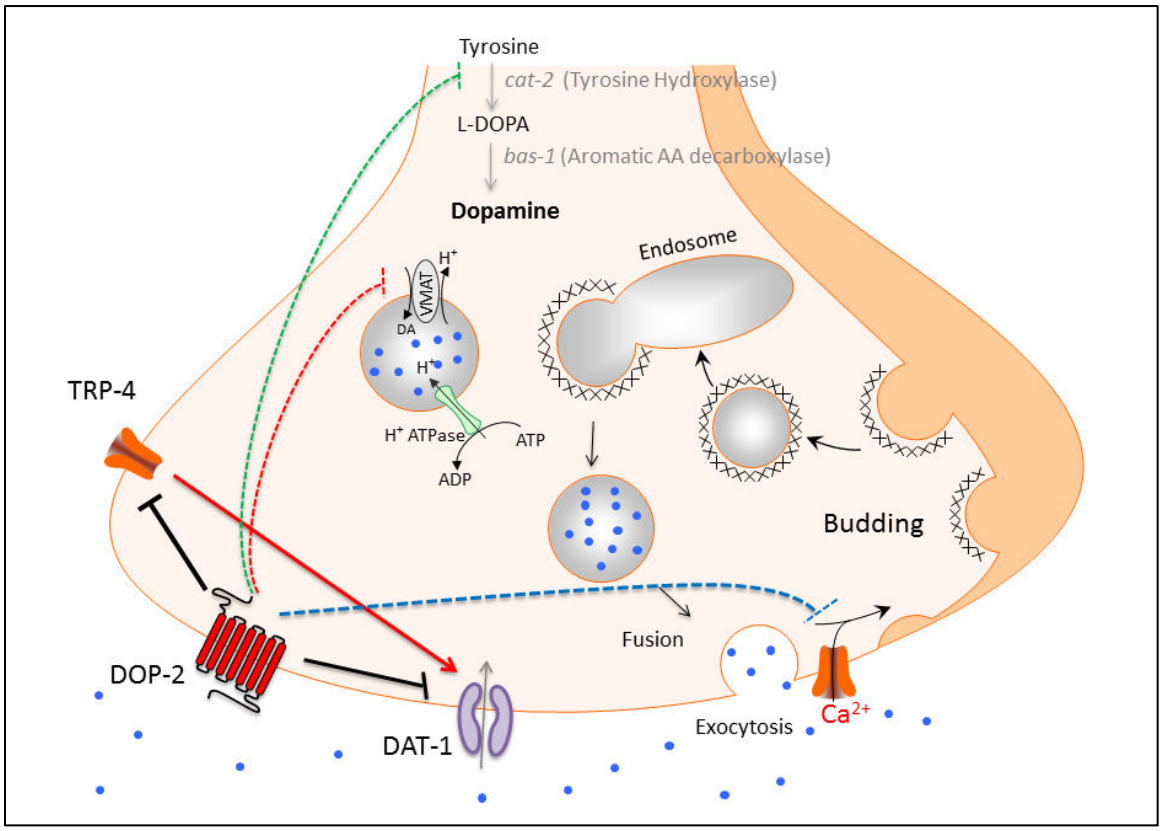


Figure 24. Hypothesized DOP-2 regulatory pathways. DOP-2 auto-receptor could modulate DA release in different ways: 1) by controlling the rate-limiting enzyme of dopamine synthesis *cat-2*; 2) by acting on the monoamine vesicular transporter VMAT; 3) by modulating calcium influx, or 4) by regulating DAT-1 activity.

binds to the DOP-2 auto-receptor, which, through its associated Gα_i protein GPA-14, initiates a signaling pathway which leads to the ultimate goal of balancing dopamine release at synapses in *C. elegans*. This goal can be attained through different regulatory pathways:

1) by acting to control the rate-limiting enzyme of dopamine synthesis *cat-2*, the homologous tyrosine hydroxylase (TH) in the nematode (indicated by the green line in Figure 24);

2) by acting on the monoamine vesicular transporter VMAT, which is responsible for the package of transmitters in the synaptic vesicles, thereby controlling dopamine storage (Figure 24 red line);

3) by modulating calcium influx acting on voltage gated calcium channels localized at vesicle release sites (Figure 24 blue line), which can alter the vesicle fusion/docking mechanism preceding neurotransmitter release, or

4) by regulating DAT-1 DA-release activity through a PKA-mediated phosphorylation of DAT-1 (Figure 24 black line).

Each of these possibilities will now be discussed in detail.

1) Regulation of the rate-limiting enzyme of dopamine synthesis cat-2

Regulation by the DOP-2 auto-receptor of the rate-limiting enzyme for dopamine synthesis TH (*cat-2* gene in the nematode) would occur by inhibition of phosphorylation of TH. As mentioned above, studies have shown that, in normal synapses, PKA activity increases TH activity by phosphorylation of the latter's ser40 residue (Pereira and Sulzer, 2012). The DOP-2 auto-receptor would provide a mechanism of negative regulation on TH in such a way that, when the need for dopamine at synapse is lessened, it would provide a negative feedback mechanism by which it inhibits *cat-2* phosphorylation. In the model shown here, DOP-2, upon activation, may start a G-protein signaling pathway for which cAMP activity of PKA is inhibited, therefore decreasing *cat-2* activity (Figure 25). *dop-2* mutants would miss this mechanism, which would explain why their rate of DA release is higher compared to that of wild type.

2) Regulation of the monoamine vesicular transporter VMAT cat-1.

VMATs are specific membrane integrated transporters localized both at synaptic vesicles and at the plasma membrane, which transfer neurotransmitters from the cytoplasm into synaptic vesicles. VMAT functions is an antiporter, transporting two H+ ions out of the vesicle to

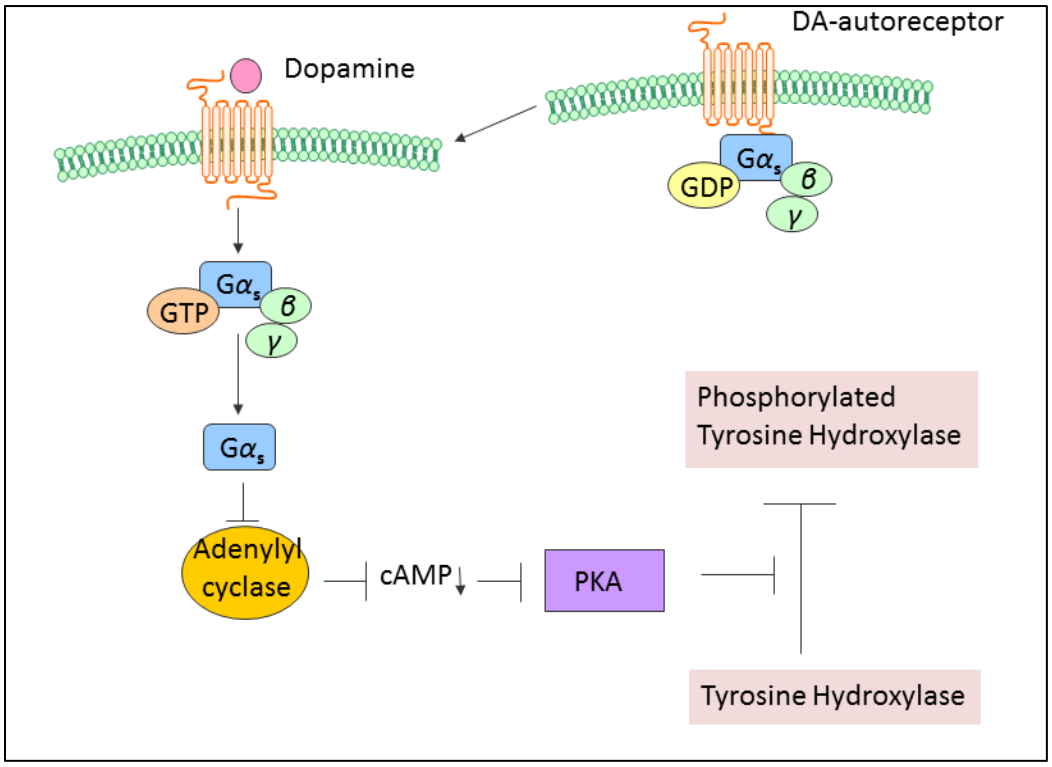


Figure 25. Schematic drawing of the effect of *dop-2* on the rate-limiting enzyme *cat-2* in *dop-2* mutants. When the need for dopamine in the synaptic cleft decreases, and upon DOP-2 activation, the Gα subunit of the GPCR dissociates from the Gβ/Gγ dimer. The Gα subunit inhibits adenylate cyclase (AD) activity on cAMP, causing a decrease in *cat-2* activity.

transport one dopamine molecule into the vesicle. VMAT derives the energy for transport from the H+-ATPase activity which establishes the high intra-vesicular H+ concentration (Wimalasena 2011). The vesicular monoamine transporter can be regulated by post-translational modifications, protein interactions and receptor activation. Post-translational modifications include phosphorylation and glycosylation, which have been proved to be important for vesicular monoamine reuptake and vesicular targeting, respectively (German et al., 2015). Regulation of the vesicular monoamine by protein interaction includes complex formation of VMAT with TH and AADC. The importance of the formation of these complexes is thought to be to prevent damage to the vesicles (Cartier et al., 2010). The mechanism of the regulation of VMAT by receptor activation is not well understood. However, *in vivo*

pharmacological studies have supported the idea that vesicular monoamine is highly sensitive to regulation by both D1 and D2 dopamine type receptors (Sandoval et al., 2002). The FRAP results from this study suggest that the DOP-2 auto-receptor provides a negative regulatory mechanism, which downregulates release of dopamine at synapses, since when deleted there is high rate of dopamine release at synapses in these mutants. In the model proposed above, I suggested that the activation of DOP-2 might influence VMAT activity (*cat-1* in *C. elegans*). In specific, DOP-2 activation may regulate *cat-1* activity by disrupting synaptic intravesicular pH.

Disruption of synaptic intravesicular pH can be caused by altering the H+-ATPase activity. This would produce a deficit in the refilling of dopamine in the synaptic vesicles, which would affect vesicle quantal size. *dop-2* deletion mutants would thus lack this regulatory mechanism and have an increase in quantal size in their synaptic vesicles. (This process is illustrated in Figure 26) An adequate balance of dopamine release at dopaminergic synapse in normal wild type *C. elegans* animals would be attained by a negative feedback regulation provided by activation of the DOP-2 auto-receptor. The DOP-2 regulatory mechanism would act on the activity of the transport system H+-ATPase of the vesicular dopamine transporter (*cat-1*). Mutants for the *dop-2* gene would then have increased vesicular quantal size.

3) Regulation of calcium influx

Calcium influx is necessary for neurotransmitter vesicle release at synaptic terminals. Following an action potential, the pre-synaptic plasma membrane depolarizes, Ca2+ channels open and Ca2+ flows into the nerve terminal to induce exocytosis of synaptic vesicles. It is

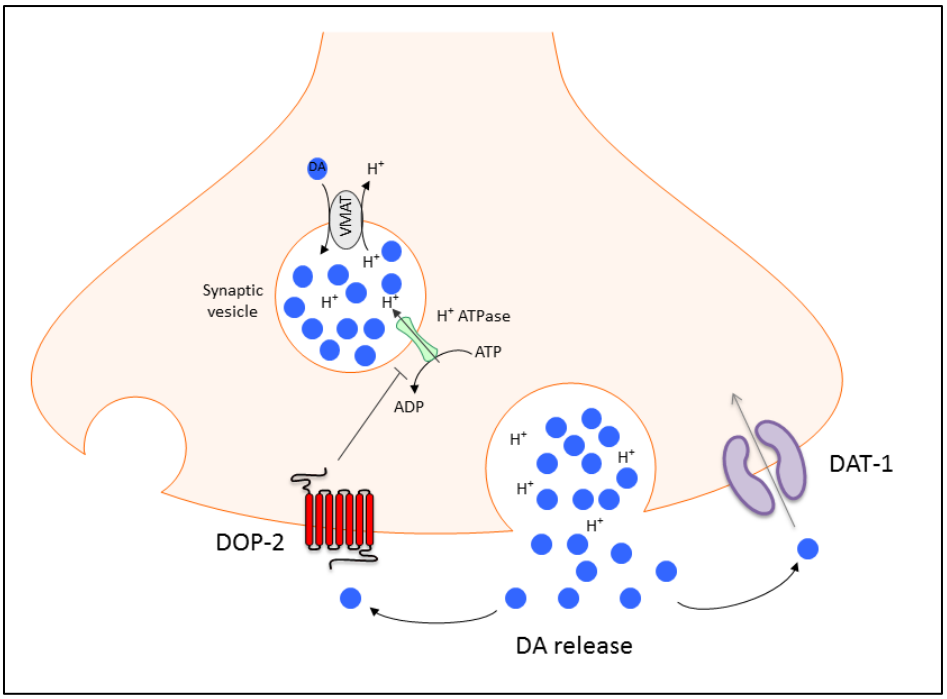


Figure 26. Negative regulation of the vesicular dopamine transporter *cat-1* by the *dop-2* auto-receptor. Adequate balance of dopamine release at dopaminergic synapse in normal wild type *C. elegans* animals is attained by a negative feedback regulation upon activation of the DOP-2 auto-receptor.

thought that calcium mediates the fusion of the synaptic vesiclemembrane with the pre-synaptic plasma membrane. It has been proposed that calcium triggers the formation of the SNARE complex by acting on the calcium-binding synaptic-vesicle protein synaptotagmin. In fact, *C. elegans* mutants in proteins needed for the formation of the SNARE complex show almost no release of dopamine (Richmond, 2005).

In the model in Figure 24, I proposed that the DOP-2 auto-receptor acts as a regulator of calcium entry which in turn regulates the amount of dopamine release. According to this view, DOP-2 would act on the SNARE complex by reducing the affinity of synaptotagmin to calcium, with a resulting decrease of dopamine into the synaptic cleft. Figure 27 illustrates this mechanism. Lacking this mechanism, *dop-2* mutants predictably show an increased vesicles fusion.

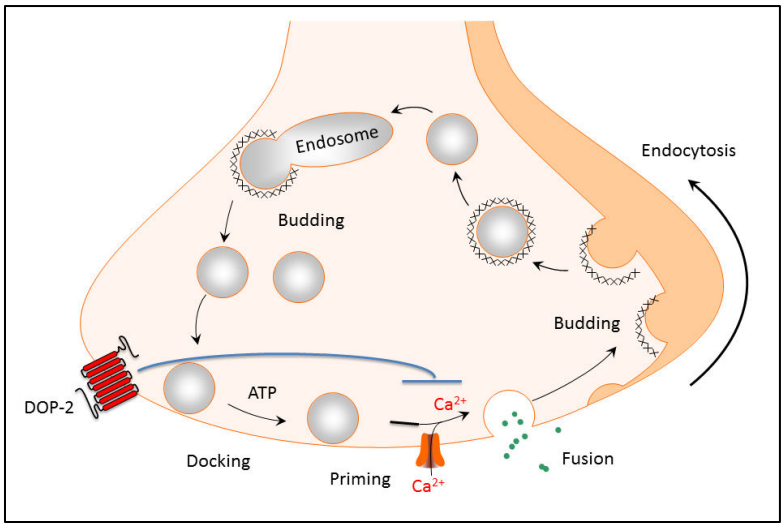


Figure 27. Negative regulation of calcium influx by DOP-2 auto-receptor in wild type *C. elegans.* DOP-2 auto-receptor activation causes a decrease in Ca2+ influx, which prevents dopamine from accumulating in the synaptic cleft, therefore providing a regulatory mechanism for dopamine release. The Ca2+ influx is not regulated in *dop-2* mutants.

4) Regulation of DAT-1 activity

As mentioned in Chapter I, the dopamine transporter DAT provides the first mechanism for clearance of dopamine from the synaptic cleft after release. Mammalian interaction studies have shown that DAT activity can be modulated by the interaction of DAT with many proteins. However, these mechanisms are still under investigation. The physiological mechanism of DAT to remove dopamine is coupled to the translocation of one Cl- and two Na+ ions. Studies on DAT in striatal synaptosomes have revealed a voltage-dependent regulation mechanism for DAT activity, since it has been seen that a depolarized membrane can cause a reduction of DAT activity while a hyperpolarized membrane can cause an increase of DAT activity (Richardson et al., 2015). Therefore, if membrane depolarization leads to a reduction of activity of DAT, there must be some kind of mechanism that induces hyperpolarization, which would increase activity of DAT and re-establish an equilibrium in the reuptake of dopamine by the transporter. Based on this idea, the DOP-2 auto-receptor could act to close an ion channel which is co-expressed at

the pre-synaptic terminal of *C.elegans*, thereby inducing hyperpolarization of the membrane. The ion channel in the nematode is a transient receptor potential channel called TRP 4, which is also expressed in the dopaminergic neurons of *C. elegans.* The resulting hyperpolarization would act on DAT-1 by increasing its activity, thereby equilibrating the level of dopamine at synapse (Figure 28). This passage from depolarization to hyperpolarization of the plasma membrane would be missing in *dop-2* deletion mutants of *C. elegans.*

Although it is known that the primary function of DAT is to clear dopamine from the synaptic cleft after release, this is not its only function. As mentioned above, pharmacological studies in mice have suggested an alternative role of the dopamine transporter DAT, which sees it as a mediator of extracellular DA release. This would imply that part of the DA released at synapses is controlled by DAT (Sulzer et al., 2016). The physiological mechanism of DAT for the release of dopamine into the synaptic cleft is still under investigation. One proposal for explaining this mechanism is that DAT must be phosphorylated at serine 7 by protein kinase C in order for it to release DA (Moritz et al., 2013).

In addition, other studies have suggested an involvement of DAT in restricting vesicle pool mobility and/or release (Sulzer et al., 2016). The presumed mechanism of action of DAT on DA vesicle release is that DAT does so by mobilizing a reserve pool of dopamine-containing synaptic vesicles that would otherwise be anchored to the cytoskeleton structure of the reserve pool. It has been proposed that DAT achieves this by reducing the affinity of proteins, such as synapsin (a protein that segregate synaptic vesicles into the reserve pool), with the synaptic vesicle membrane (Greengard et al., 1993).

As mentioned in Chapter I, because of its structure and binding properties, DAT action can be influenced by different signaling pathways that can be triggered by different molecules

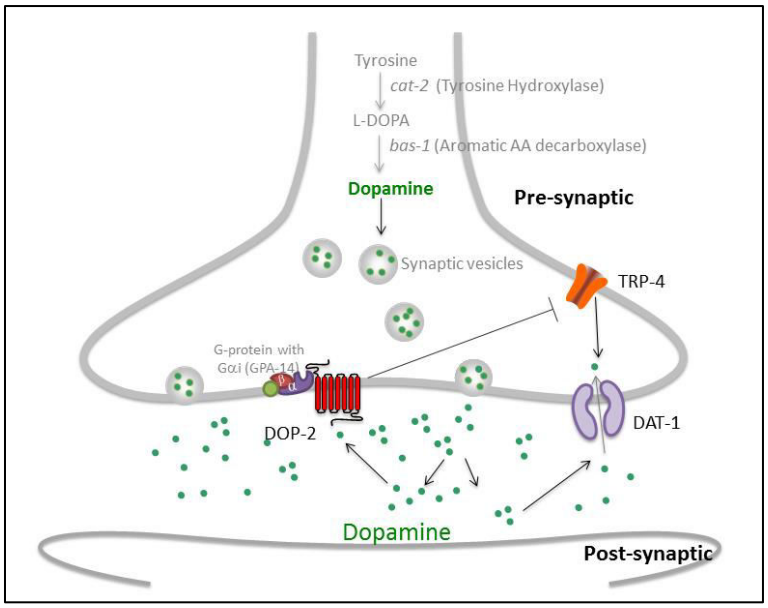
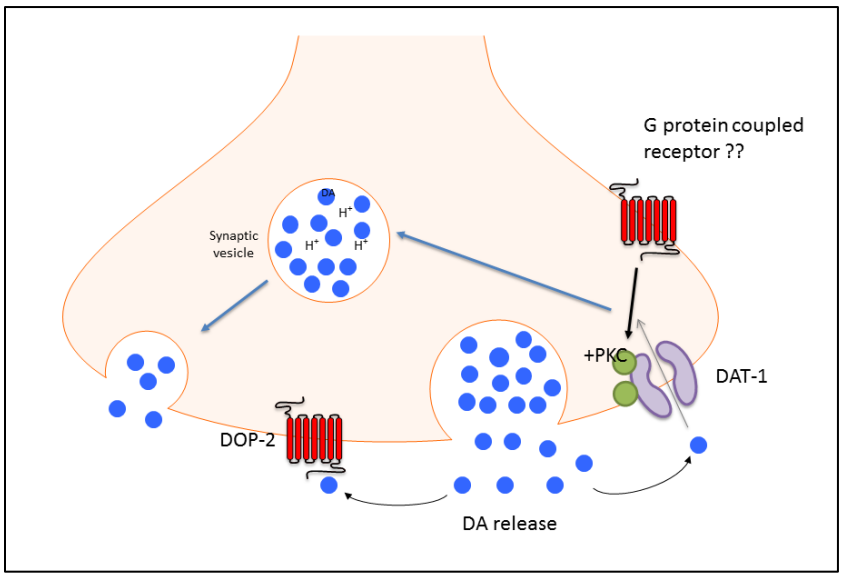


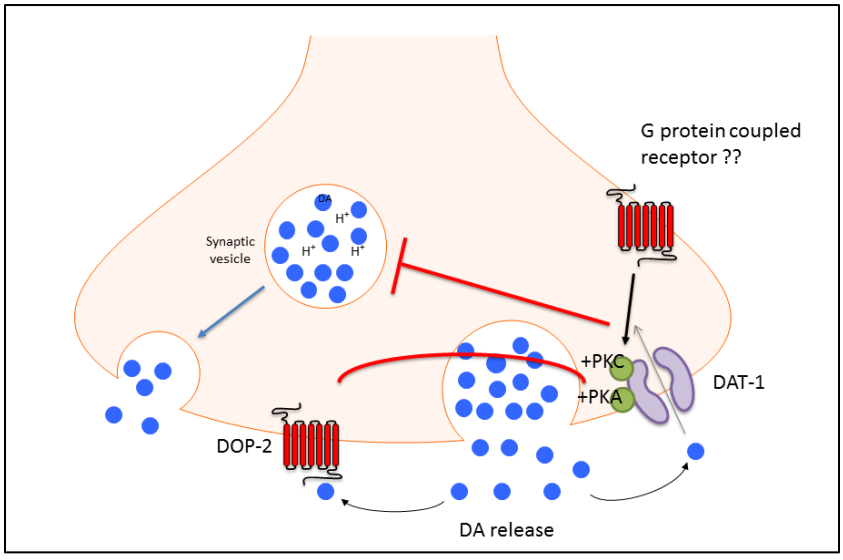
Figure 28. DOP-2 influence on DAT-1 activity. The DOP-2 auto-receptor activates a signaling pathway through which it causes the closure of a transient receptor potential channel called TRP 4. The closure of this channel, which is permeable to Na+ and Ca+ ions, causes hyperpolarization of the pre-synaptic membrane. Hyperpolarization of membrane acts on the dopamine transporter DAT-1 by increasing its activity.

including enzymes, ion channels and receptors (Vaugan and Foster 2013). It has also been proposed that DAT-mediated DA release might occur through a G-protein-coupled receptor expressed in DA neurons together with DAT (Sulzer et al., 2016). Based on these assumptions, the DOP-2 auto-receptor could be the G-protein-coupled receptor that regulates DAT-1 mediated DA release activity. It might be possible that regulation of DAT-1 by the DOP-2 auto-receptor would occur by phosphorylation of another DAT-1 residue site which might be necessary for its DA release function. In normal synapses the mechanism would work as follows: to release the right amount of DA, DAT-1 needs to undergo phosphorylation by PKC. PKC-mediated phosphorylation would increases DA release (Figure 29A). However, when the right level of DA is reached, the DOP-2 auto-receptor would provide an inhibitory action on vesicular DA release by phosphorylating another amino acid residue of DAT-1. The PKA phosphorylation triggered by the DOP-2 auto receptor would produce a decrease in DAT-1 mediated DA release

activity (Figure 29B). PKA-mediated phosphorylation would not occur in *dop-2* mutants even when the correct level of extra synaptic DA is reached.



A



B

Figure 29. DOP-2 regulation of DAT-1 mediated DA release. **A**. PKC induced phosphorylation of DAT-1 causes DA release. **B.** PKC and DOP-2 induced PKA phosphorylation of DAT-1 reduces DA release, providing the correct level of required DA at *C. elegans* synapses.

CHAPTER V:

CONCLUSIONS AND PLAN FOR FUTURE RESEARCH

5.1. Conclusions from this Study

The focus of this study was to investigate the specific *in vivo* role of DOP-2 D2 like receptor in regulating dopamine release at synapses in the nematode *Caenorhabditis elegans*. Specifically, it was proposed that DOP-2 acts as an auto-receptor to modulate the release of dopamine, either directly or indirectly, by initiating a negative feedback loop mechanism at *C. elegans* dopaminergic synapses.

The plan for our experiments was to observe how the *dop-2* mutation could affect the dopamine signaling mechanism that regulates DA levels in the nematode. Three experiments were conducted to test this hypothesis. The first aim of this study was to further characterize *dop-2* mutants phenotypically. To this end, dop-2 mutants were analyzed for the well characterized phenotype SWIP (Swimming Induced Paralysis). SWIP is a phenotype controlled by the D2-like DOP-3 receptor and is typically seen in worms with increased levels of dopamine at synapses, such as mutants for the dopamine transporter *dat-1*. It was observed that *dop-2* mutants showed an intermediate SWIP phenotype between wild type and *dat-1* mutants (Figures 8 and 9). These results indicate that also in *dop-2* mutants there is an accumulation of dopamine in their extracellular synaptic space, probably due to both a high release of dopamine and/or to a decreased activity of *dat-1*. The SWIP phenotype of *dop-2;dat-1* double mutants shows no significant difference from the SWIP phenotype of *dat-1* (Figure 10) suggesting an involvement of *dop-2* in the regulation of dopamine release in response to high levels of exogenous dopamine (Figure 10). The two genes may work either in the same or in a different pathway. Because the

SWIP phenotype is caused by a DOP-3 mediated reduction of acetylcholine release and because of the co-expression of DOP-2 and DOP-3 in the DAergic neurons (Suo et al., 2003), it might be possible that also DOP-2 influences cholinergic activity. Additional experiments are therefore necessary to demonstrate that alternative pathways are in fact involved.

The second aim was to examine expression and sub-cellular localization of *dop-2* in *C. elegans.* Analysis of images of transgenic animals carrying a p*dat*-1::GFP reporter fusion confirmed that *dop-2* is expressed in the dopaminergic neurons of the nematode head, four CEPs and two ADEs (Figures 22, 23 and 24). Expression was seen in both the cell bodies and along the processes, strongly suggesting the involvement of *dop-2* in dopaminergic functions. Expression of *dop-2* in the two mid-body dopaminergic neurons PDE was not seen during this analysis, probably due to mosaic expression of the gene in these transgenic animals.

The third and final aim was to compare the activity of dopaminergic synapses in wild type *N2* and *dop-2* mutants *in vivo*. To this end, synaptic release in dopaminergic neurons of transgenic animals expressing a synaptobrevin-super ecliptic pHluorin reporter fusion (SNB-1::SEpHluorin) under the *asic-1* promoter was monitored by fluorescence recovery after photobleaching (FRAP). Our results showed that the rate of neurotransmitter release at dopaminergic synapses is increased in *dop-2* mutants compared to wild type used as control (Figure 19). The elevated rate of vesicle fusion suggests that *dop-2* mutants lack a mechanism that down-regulates the release of dopamine at synapses. Indeed, D2-like auto-receptors such as *dop-2* have been found to provide a negative feedback loop mechanism by decreasing protein activity, which confirms that *dop-2* functions as an auto-receptor. The observed difference in the rate of dopamine vesicles released at synapses between *dop-2* mutants and wild type *N2* animals provided the background for proposing four different regulatory pathways by which the

DOP-2 auto-receptor could modulate the release of dopamine at synaptic terminals in the nematode. DOP-2 auto-receptor could modulate DA release in different ways: 1) by controlling the rate-limiting enzyme of dopamine synthesis *cat-2*; 2) by acting on the monoamine vesicular transporter VMAT; 3) by modulating calcium influx, or 4) by regulating DAT-1 activity. Figure 24 illustrates these four mechanisms.

5.2. Suggested Plan for Future Research

Even though all the results presented here clearly support the hypothesis of this study, future research to continue this project is needed. Future studies would include the construction of another plasmid vector containing a *dop-2* regulatory sequence fused to a red fluorescence protein to determine *dop-2* sub-cellular localization. This construct could then be used to create transgenic animals expressing *dop-2::*dsRED, whose sub-cellular interaction with *dop-2::*GFP would be investigated. This is necessary for determining the exact *dop-2* expression pattern in the *C. elegans* nervous system and for further confirming the auto-receptor function of *dop-2*.

This research also initiated studies on *dop-2* synaptic activity using the FRAP technique. In this case FRAP was used to determine the effect of DOP-2 on the constitutive release of dopamine. However, it is also important to determine the effect of DOP-2 on evoked dopamine release. Since *dop-2* mutants are defective in memory and learning capabilities, it would be important to understand whether DOP-2 modulates complex dopamine dependent behaviors in *C. elegans.* Therefore, the next step in this research project would be to use FRAP to measure the rate of dopamine vesicle fusion in conditioned *dop-2* mutants to establish whether dopaminergic neuron activity is subject to modulation by training in these mutants. Specifically, synaptic activity should be measured in each specific set of dopaminergic neurons (CEP, ADE

and PDE), because many dopamine-dependent behaviors in *C. elegans* involve only one set of these neurons. This investigation was indeed initiated in this study; however, because the results which I obtained are not sufficient, they have not been included in this thesis.

It would also be important to determine the effect of different combinations of *dop-2* mutations with other dopamine dependent mutants. I proposed that the *dop-2* gene acts as a regulator of dopamine release in *C. elegans* by mediating normal dopaminergic signaling. If this hypothesis is correct, the effect of the *dop-2* mutation on genes whose effect is dopamine dependent will influence their phenotypes. Mutations in both *dop-2* and *dat-1*, for instance, will have different effects in combination than when only one or the other is mutated. This investigation will help to understand the effect of different combinations of mutations between *dop-2* and other genes that are known to be involved in the dopamine signaling pathway in *C. elegans*. Some of these genes could be those that were hypothesized to interact with *dop-2* in the model presented in Figure 24.

Pharmacological analysis of DOP-2 should be also initiated to reinforce our previous findings that mutation in *dop-2* affects dopamine signaling in *C. elegans.* Agonists and antagonists of dopamine receptors should be used as tools to test the effect of the *dop-2* gene on dopamine release in *C. elegans* and to provide significant evidence about the role and the mechanism of this molecule.

Another series of experiments would be required to study the extent to which DOP-2 activation influences TH (*cat-2*), VMAT (*cat-1*), Ca2+ channels and DAT (*dat-1*), respectively. For example, it would be important to determine the *dat-1* regions necessary for *dop-2* coupling. This is important because, as mentioned in Chapter V, *dop-2* can regulate *dat-1* in both its reuptake activity (Figure 28) and/or its DA mediated release activity (Figure 29). Such a study

might indicate that *dop-2* could be involved in both mechanisms of action of *dat-1*. In fact, knowing the site of interaction between these two genes would provide further evidence about their roles of in the regulation of neurotransmitter release.

This study also hypothesized that *dop-2* might control DA vesicle release by acting on the vesicular monoamine transporter *cat-1* (Figure 26). Pharmacological studies on VMAT using the DA agonist AMPH showed a reduced SWIP phenotype in *cat-1* mutants compared to *dat-1* worms, suggesting an involvement of the *cat-1* gene in modulating the release of extracellular DA upon DOP-2 activation (Carvelli et al., 2010). Based on these findings it would be important to further characterize the *cat-1* gene by measuring the rate of vesicle release in these mutants. This would give us a better understanding of its genetic relationship with *dop-2*.

Finally, further studies should be conducted to investigate the role of *dop-2* in regulating calcium entry and therefore vesicle fusion rate. Regulation of calcium influx by the DOP-2 auto-receptor was also hypothesized in this study to be the mechanism at the basis of DA vesicle release at synapse (Figure 27). To test this hypothesis it would be important to initiate studies to measure calcium levels in all the strains mentioned as possible players in the DA vesicle release mechanism.

Results from all the experiments proposed here would both validate our hypothesis and indicate which of the models presented in Chapter IV provides the best description of the mechanism of DA release.

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