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Loss-of-function mutants in a *C. elegans* dopamine auto-receptor gene are aberrant in associative learning and habituation

by

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ABSTRACT

Neurotransmitters and their receptors play pivotal roles in the normal functioning of the nervous system including learning and memory, and associated pathological states. For example, inapt dopaminergic transmission can cause various behavioral disorders, such as Parkinson's disease, attention-deficit hyperactivity disorder, addiction to drugs and schizophrenia. The precise role of dopamine in behavior including learning and memory is not well understood, as well as in diseases is not fully clear. Recent work in *C. elegans* has shown that dopamine release in behavioral plasticity is modulated in part through a dopamine auto-receptor DOP-2 in the participating pre-synaptic neurons. The DOP-2 sequence is similar to D2-type mammalian dopamine receptors with characteristic seven trans-membrane structure known to act through G-protein coupled pathways. We are testing the hypothesis that the dopamine auto-receptor acts through an identified Gα-subunit. Our methods include behavioral analysis, genetically-crossed transgenic animal generation and con-focal microscopy. Data-mining the *C. elegans* expression database has revealed that *dop-2* shares expression overlap with *gpa-14*, which codes for a Gα-subunit. We have carried out a detailed characterization of the *dop-2* of loss-of-function mutant. Results from our experiments indicate an interaction between the respective gene products of *dop-2* and *gpa-14*.

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LIST OF ABBREVATIONS

7TM	Seven transmembrane
ADE	Anterior deirid neurons
ASIC-1	Acid sensing ionic channel
BLAST	Basic Linear Alignment and Search Tool
C. elegans	*Caenorhabditis elegans*
cAMP	Cyclic adenosine monophospate
CEP	Cephalic neurons
CGC	Caenorhabditis Genetics Center
CRE	cAMP response element
CREB	cAMP response element-binding protein
DAT	Dopamine transporters
DOP-2	Dopamine receptor-2
E.coli	*Escherichia coli*
EGL-30	Egg laying-30
$G\alpha_s$	G-protein alpha stimulator
$G\alpha_i$	G-protein alpha inhibitory

GDP	Guanosine diphosphate
GOA-1	G-protein O alpha-1
GPA-12	G-protein alpha -12
GPA-14	G-protein alpha -14
GPCR	G-protein coupled receptors
GSA-1	G-protein subunit alpha
GTP	Guanosine triphosphate
IAA	Isoamyl alcohol
LTD	Long term depression
LTP	Long term potentiation
MAP	Mitogen-activated protein
NaN3	sodium azide
N2	Wild type
NGM	Normal growth media
OP-50	*E.coli* strain
PCR	Polymerase chain reaction
PDE	Posterior deirid neurons
PKA	Protein Kinase A
SEM	Standard error of mean

I. INTRODUCTION

Learning and Memory

Many organisms are born with the essential innate information they need to survive already hardwired in their brain. Human babies are born with limited information required for survival, and most of our new skills are acquired through experience. Learning is generally defined as the process of acquiring new knowledge or modifying the knowledge that already exists (Kandel et al., 1991; Okano et al, 2000). In order to survive and reproduce, living things must not only learn to adapt to their ever changing environment, but they must also store the information gathered for future recall. This process of storing and recalling information is known as memory (Bansal and Parle, 2010).

In non-associative learning, the simplest form of learning, the organism's response towards a harmless but repeated stimulus is tested (Kandel et al., 1991). Habituation and sensitization are classified as non-associative learning. During habituation, the organism is exposed to a repeated stimulus. Exposure to this harmless stimulus will result in reduced response towards the stimulus. This phenomenon explains why a person learns to ignore the sound of a pendulum clock after few minutes of exposure. However, in sensitization, the organism exhibits an increase of attention towards the stimulus. In the more complex associative learning, the animal learns to

associate one stimulus with another (Kandel et al., 1991). Perhaps the most famous example of associative learning was presented by Ivan Pavlov, in which dogs were trained to associate the sound of a bell with food. Whenever the dogs heard the sound of the bell, they salivated in anticipation of food (Pavlov, 1928).

The details of habituation were studied by Eric Kandel using the marine sea slug *Aplysia californica* (Yan-You et al., 2005). Stimulus was delivered to the siphon resulting in the withdrawal of the siphon and the gills. After repeated exposure to this stimulus, the organism eventually stopped withdrawing its siphon and gills. This experiment indicated that repeated stimulation causes excitatory interneurons to produce weaker synaptic potentials causing the motor neurons to drastically reduce their firing rates (and eventually stop). This decrease in the synaptic potentials is caused by reduction in the number of transmitter vesicles released into the synapse (Kandel et al., 1991).

Once new information is acquired, memory is created as neural correlates that allow access to the information for future recall. Three levels of memory have been described in temporal terms. Short-term memory has a very limited capacity for storing information, usually 7 plus or minus 2 items (Miller , 1955). It allows recall only after a few minutes and involves biochemical changes in key synapses (Kandel et al., 1991). Short-term memory and intermediate-term memory are often considered synonymous; they both involve long-term depression (LTD). Long term depression is defined as a reduction in synaptic transmission induced by low frequency stimulation of the neurons (Bliss and Lomo, 1973). Long-term memory correlates with long term potentiation (LTP), which is defined as an increase in communication between two nerve cells induced by high frequency stimulation of the neurons (Formisano, 2009).

Long-term memory involves three processes; gene expression, the synthesis of new proteins and the growth of new synaptic connections (Kandel et al., 1991). In order for long-term memory to take place, protein kinase A (PKA) recruits mitogen-activated protein (MAP) kinase and enters the nucleus. Once in the nucleus, PKA phosphorylates and activates the transcription factor CREB-1. The activated CREB-1 then binds to the promoter element CRE (MAP kinase blocks the inhibitory actions of CREB-2). This leads to the initiation of two major events. First, the genes turned on as a result of CREB result in the production of the enzyme ubiquitin carboxyterminal hydroxylase. This ensures uninterrupted production of PKA, ensuring the continuity of the previously stated pathway. Secondly, the transcription factor C/EBP coding genes get turned on causing the activation of genes needed for proteins associated with the growth of new synaptic neurons.

Numerous studies have shown that appropriate acquisition and recall of learning and memory are strongly controlled by various neurotransmitters, in particular dopamine, glutamate, norepinephrine and serotonin (Chase and Koelle, 2007; Sawin et al, 2000). Experiments with *Aplysia californica* sensory neurons have shown that activation of serotonin receptors in post-synaptic neurons initiate a G-protein mediated signal transduction cascade that increases intracellular cAMP and Ca++ levels, which in turn initiate molecular cascades that can influence both short-term and long term synaptic changes. The changes in synaptic efficacy caused by cAMP are, in part, due to increased release of glutamate, an excitatory neurotransmitter that acts through an inhibitory Gα subunit (Nicholls et al., 2006). The cellular correlate of memory in mammals is defined

by long term potentiation, characterized by synaptic strengthening caused by high frequency stimulation of specific chemical synapses (Staubli and Lynch, 1987).

Studies with mammalian hippocampal neurons have confirmed the involvement of serotonin and glutamate in the cAMP mediated learning pathway. Mammalian studies have also revealed a role for another neurotransmitter, dopamine, which is required for memory stabilization possibly through a cytoplasmic polyadenylation element binding protein (Theis et al., 2003; reviewed in Barco et al., 2006).

Dopamine

Dopamine is a modulatory monoamine neurotransmitter responsible for regulating neuronal plasticity in both vertebrates and invertebrates. In addition to learning and memory, dopamine is also associated with movement, reward and cognition. Abnormal transmission of dopamine is involved with disorders like Alzheimer's disease, Parkinson's disease, schizophrenia and drug addiction (Jenner et al., 1992; Koob, 1992; Nestler et al., 1996; Hietala et al., 1995)

Dopamine is a catecholamine neurotransmitter found in both vertebrates and invertebrates. In mamals, it is mainly produced in the substantia nigra and ventral tegmental area. So far, in humans, five dopamine receptors (D1, D2, D3, D4 and D5) have been identified. It is synthesized from the amino acid L-tyrosine and packed into vesicles before getting released in the synapse. Once it reaches the synapse, it can get degraded by the enzyme monoamine oxidase or get taken back up by the dopamine transporter (DAT). Although the mechanism of reuptake for dopamine is well understood, the factors controlling the release of dopamine into the synapse remain unknown.

Dopamine acts through dopamine receptors, which belong to the family of seven-transmembrane G protein-coupled receptors that act through cAMP and Ca^{++} signal transduction mechanisms. Dopamine receptive neurons have a high concentration of receptors localized to the dendritic spine region, whose morphological plasticity is known to be regulated through development and experience (Wei-Dong et al., 2009). Dopamine receptors are classified into two families, the D1- and D2-types. The D1-receptors are coupled to $G\alpha_s$ G-proteins and stimulate adenylate cyclase which increases the production of cAMP, and activates protein kinase A (PKA) and subsequent post-synaptic downstream signaling systems. In general, the D2-receptors act antagonistically to the D1 receptors in terms of cAMP/PKA-dependent signaling, and are also known to modulate intracellular Ca^{2+} dependent signaling processes through $G\alpha_i$. Synergistic effects of D1 and D2 type receptors have also been reported for certain behaviors in mice (McNamara et al., 2003). Re-uptake of dopamine from the synaptic cleft is achieved through the dopamine transporter DAT in pre-synaptic neurons, which also express D2 auto-receptors. The mechanism that leads to dopamine release and the signal transduction processes mediated by dopamine receptors are poorly understood. The D2 auto-receptors have been proposed to regulate the release of dopamine from the pre-synaptic neurons as well as reuptake by DAT (Williams and Galli, 2006; Voglis and Tavernarakis, 2008).

C. elegans as a model to study dopaminergic modulation in behavioral plasticity

Ever since the description of *Caenorhabditis elegans* (*C. elegans*) as a potential multicellular lab model in 1974 by Sydney Brenner, it has been widely used for

biological research. *C. elegans* is a transparent, soil dwelling, nematode worm that feeds on bacteria. Their entire genome of 100.3Mbp was the first completely sequenced genome of a multicellular organism in 1998. It has been estimated that it has 19,735 protein coding open reading frames (Hillier et al, 2005).

C. elegans has two sexes, hermaphrodite and male. Although hermaphrodite is the predominant sex form, males compromise 0.05% of the population. After the hermaphrodites lay the eggs, it goes through four cycles (L1-L4). The normal life cycle of *C. elegans* lasts for approximately 2.5 days when grown at 20°C. On average, each hermaphrodite lays about 300 eggs. In the laboratory, the organism is maintained on solid agar with *E.coli* (OP 50 strain) as a food source.

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 (White et al 1986).

In addition to the favorable laboratory traits mentioned above, mutant *C. elegans strains* can be easily acquired from The Caenorhabditis Genetics Center (CGC). Worm Base, a *C. elegans* online database, also provides extensive information about *C. elegans* genome, neuroanatomy and development. It is available for scientists to consult and use for research studies (Mohri et al., 2005). When stored at -80°C with 15% glycerol, the worms can survive for several years, and revived when required.

Learning and memory in *C. elegans*

In C. *elegans,* behavior is strongly influenced by changes in the environment. As a result, it has the ability to associate change in the environment with the presence or

absence of food. Its ability to learn through association makes *C. elegans* an ideal organism to study the molecular changes that take place during learning and memory.

C. elegans' ability to learn can be tested through habituation (non-associative learning) and chemotaxis (associative learning). Administering repeated mechanical tap stimuli could test habituation by counting the number of taps it takes for the animal to stop responding. Associative learning, such as classical conditioning and differential conditioning, can be tested by using assays in which worms are conditioned to specific chemicals that are paired with the presence or absence of food and then assaying the worms for changes in chemotactic response towards the conditioned chemical (Saeki et al., 2001; Law et al., 2004, Formisano, 2009).

Role of dopamine C. *elegans*

Similar to mammals, dopamine has been implicated in the regulation of learning and memory in *C. elegans.* In addition, dopamine also plays an important role in modulating locomotion, egg laying, defecation and food encounter responses. In the adult hermaphrodite *C. elegans*, dopamine is synthesized in eight neurons (Figure 1): 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, male worms also have three pairs of sex-specific dopaminergic neurons in their tails, the A neurons of rays 5, 7 and 9

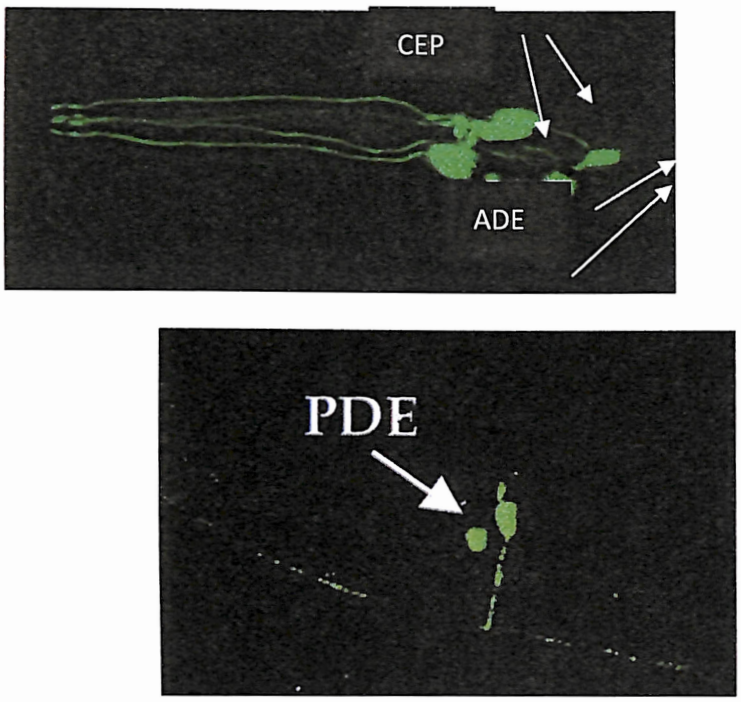

Figure 1. *C.elegans* dopaminergic neurons. The GFP fluorescence indicates cephalic neurons (CEP), anterior deirid neurons (ADE) and posterior deirid neurons found in the tail region (Nass et al., 2001)

Although there might be additional factors that regulate the release of dopamine into the synapse, a previous study has implicated that the acid sensing ionic channel, ASIC-1 plays a significant role in regulating the release of dopamine. While dopamine is stored in the vesicles, it is accompanied by H^+ ions. During the release of dopamine into the synapse, H^+ ions are also released, resulting in the acidification of the synapse (Voglis and Tavernarakis, 2008). The acidification of the synapse activates the pre-synaptic ASIC-1 channels. The activated ASIC-1 channels facilitate the influx of Na^+, which in turn modulate future synaptic vesicle release (Figure 2).

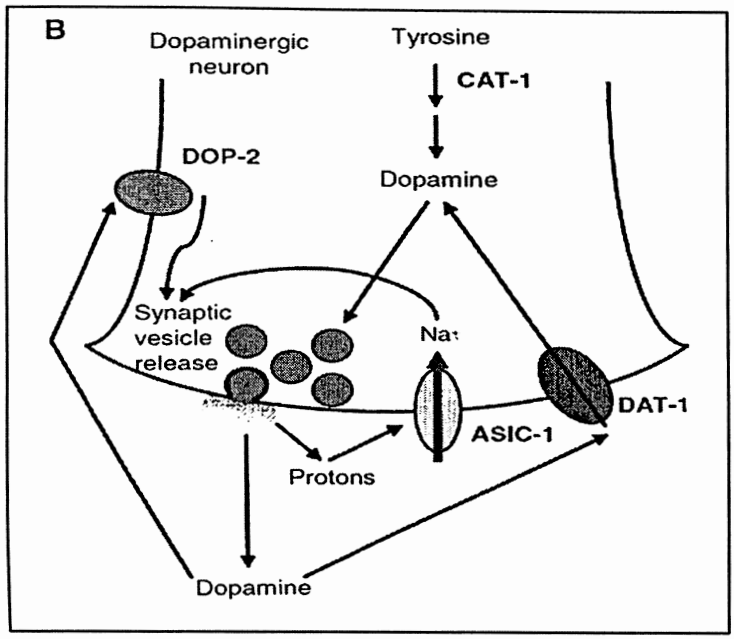

Figure 2 Modulation of dopamine release by the presynaptic Acid Sensing Ionic Channel-1 (ASIC-1). The acidification of the synapse activates the autoreceptor promoting further dopamine release (Voglis and Tavernarakis, 2008).

Based on sequence similarity, *C. elegans* have four dopamine receptors; DOP-1, DOP-2, DOP-3 and DOP-4. Previous work done by (Suo et al., 2003) compared the amino acid sequence of DOP-2 and showed that it resembles D2-like receptors rather than D-1 like receptors. In mammals, D2 receptors have been shown to act as presynaptic autoreceptors (Mercuri et al., 1997; L'hirondel et al., 1998). The DOP-2 receptor is found on the membrane of the dopamine releasing neurons and can therefore function as an auto-receptor. It has also been known to play a critical role in the feedback loop which modulates the amount of dopamine that is released by the presynaptic component of a synapse.

Similar to other 7-TM receptors, dopamine receptors are also known to act through G-protein coupled pathways. G-proteins, frequently used for cell signaling, have

three subunitis: α, β and γ. When ligands bind to the seven-transmembrane G protein coupled receptors (GPCRs) a conformational change occurs inside the cell membrane activating the heterotrimeric G-proteins. Once activated, the Gα subunit exchanges GDP (guanosine diphosphate) for GTP (guanosine triphosphate) and dissociates from the Gβγ complex to activate the secondary messenger system cAMP.

The *Caenorhabditis elegans* genome encodes for 21 Gα, 2 Gβ and 2 Gγ genes (Jansen et al., 1999; Cuppen et al., 2003). The Gα subunits play the most diverse roles in *C. elegans* (Formisano, 2009). The Gβ and the Gγ subunits seem to function together with the Gα subunits in the G protein signaling pathway and to have some regulatory functions as well (Bastiani and Mendel, 2006). In *C. elegans* there is one α subunit which is homologous to each of the four mammalian Gα subunits: Gs, Gi/Go, Gq, and G12. The four homologous genes for the subunits mentioned earlier (*gsa-1, egl-30, goa-1 gpa-12*), encode protein products that are involved in the regulatation of muscle activity such as locomotion, pharyngeal pumping, egg laying, and cell death (Bastiani and Mendel, 2006) and are broadly expressed in the nematode. The remaining 17 Gα subunits are unique to *C. elegans;* they do not present enough similarities with mammalian G proteins and therefore have not been classified yet. They are predicted to code for subunits that might regulate perception and chemosensation in the nematode and are mostly expressed in chemosensory neurons (Formisano, 2009).

Characteristics	Mutated Gene		
	dop-2	*gpa-14*	*dop-2;gpa-14*
Body Size and Shape	Normal	Normal	Normal
Growth	Normal	Normal	Normal
Movement	Normal	Normal	Normal
Egg Laying	Normal	Normal	Normal

Table 1. phenotypic analysis of *dop-2* and *gpa-14* mutants

Hypothesis

We hypothesized that the dopamine autoreceptor DOP-2 modulates dopamine concentration in the synaptic cleft via interaction with the Gα subunit GPA-14. Our experimental approach included behavioral analysis, genetically-crossed transgenic animal generation and confocal microscopy. Our results showed that both *dop-2* and *gpa-14* deletion mutants show similar deficiencies in learning and memory.

II. MATERIALS AND METHODS

Strains

All *C. elegans* were obtained through the Caenorhabditis Genetic Center, University of Minnesota, Minneapolis, MN. Worms were grown in nematode growth media (NGM) plates at 20˚C as described in (Brenner, 1974; Hope, 1999). The *E. coli* strain OP50 was used as a food source. N2; wild type Bristol isolate; *dop-2 (vs105)V; BO207.3* and *gpa-14(*pk342, strain NL788*)*were the strains used throughout the study.

Behavioral Assays

For each experiment, well fed, three-day old synchronized young adult worms were used. Special attention was given to make sure that the worms were not over crowded or starved.

Habituation assays: In preparation for the assay both non seeded and seeded Normal Growth Media (NGM) plates prepared fresh the night before the assay and left overnight at room temperature. Approximately 10 worms were transferred to the new NGM plates. Using an eyelash hair, the worm was tapped on the head. In response to this stimulus, the worms typically move backwards. The number of times the animal moves backward until it no longer responds to the stimulus was counted. The experiment was repeated with 5mM exogenous dopamine hydrochloride (ACROS-organics, New Jersey, USA). 60 µl of 5mM dopamine was spread on the NGM plates and allowed 5-10 minutes to dry. The experiment was conducted as described above

Chemotaxis assays: chemotaxis plates were prepared the night before the assay and kept at room temperature for 1 hour before use as described by (Bargmann et al, 1991). Animals were collected with M9 buffer and washed two more times before the assay. For conditioning, the animals were exposed to 3 µl of isoamyl alcohol for 90 minutes. In order to immobilize the animals 2µl of 1M sodium azide (NaN_3) was placed on the trap and gradient points 10 minutes before the start of the assay. Worms were placed at the starting point equidistant to both the trap and gradient points. 1/100 isoamyl alcohol diluted in 100% ethanol was placed on the gradient while 100% ethanol was placed on the trap point. Plates were left undisturbed for one hour and then put at - 10°C for 10 minutes. Chemotaxis index was calculated by subtracting the number of worms at the trap point from the number of worms found at the gradient point and dividing it by the total number of worms found on the plate. Worms located at the starting point were excluded from counting, as these worms were most likely dead or severely injured during the washing process. Additional chemotaxis assays were conducted using 5mM exogenous dopamine. The worms were grown and/or conditioned in the presence of 5mM exogenous dopamine.

Basal slowing assays: NGM plates were prepared fresh the night before the assay. The *E. coli* strain OP50 was spread in a ring with a diameter of 1cm as described by (Sawin et al., 2000). Plates were incubated at 37° C. Plates were left at room temperature for approximately an hour before starting the experiment. Synchronized, young adult worms were placed in the middle of the *E. coli* ring. After 5 minutes, the number of body bends in 20s was counted for each animal. The experiment was also conducted without OP50.

Generating Males

Five well-fed L_4 worms transferred to NGM plates seeded with OP50. They were then heat shocked at 31.5° C for 6-7 hours. After 2-3 days, three males were picked and transferred to a plate with at least two hermaphrodites.

Generating Mutant Strains

Five *gpa-14* males were transferred and allowed to mate with one *dop-2* hermaphrodite. Once the worms become young adults, 5-10 worms were transferred to a new plate and allowed to lay eggs. After laying eggs, a single hermaphrodite ecliptic worm was removed and its mutation was confirmed by PCR.

Single Worm PCR

DNA from a single worm was extracted using proteinase K. 1 µl 20mg/ml proteinase case was dissolved with 95 µl 1x PCR buffer. Each worm was lysed in 5µl proteinaseK-buffer solution. After freezing and thawing for five minutes, worms were lysed in the PCR machine using the following setting.

65˚C for 60-90 min

95˚C for 15 min

Once DNA was extracted, PCR was used to amplify 3kb N2 and 850 bp of the coding regions of *gpa-14.* The following primers were used:

Fwd: 5'-CCC AGC CGA AAT GGA GAA GG-3'

Rev: 5'-CGT TGC CCG GAA A- 5'

The following settings were used for PCR:

1. 94°C, 4 min
2. 94°C, 30 sec
3. 60°C, 30 sec
4. 72°C, 3 min
5. Steps 2, 3 and 4 repeated for 30 cycles.
6. 72°C, 7 min

For *dop-2,* PCR was used to amplify 350bp of N2 and 230bp of the coding regions of *dop-2.* The following primers were used:

Fwd: 5'- AAC GAT TCC TTG CGA TTC TG- 3'

Rev: 5'- AAA GGA CTT CAC TGC ACG AC-3'

The following settings were used for PCR:

1. 94°C, 4 min
2. 94°C, 30 sec
3. 58°C, 30 sec

4. 72°C, 1 min

5. Steps 2, 3 and 4 repeated for 30 cycles.

6. 72°C, 7 min

PCR products were confirmed by gel electrophoresis.

Sequence analysis

The DNASTAR Laser Gene seqbuilder software was used to analyze deletion sequences.

Statistical analysis

For behavioral assays, statistical analysis was carried out using the Microsoft Office 2007 Excel software (Microsoft Corporation, Redmond, WA). The Student paired *t*-test with a two tails distribution was used with a significant p value > 0.05

III. RESULTS

Sequence analysis of *dop-2*

In order to characterize the role of dopamine auto-receptors in the learning pathway of *C. elegans,* we selected *gpa-14* and *dop-2* loss of function mutants. The cosmid K09G1.4 from *C. elegans* chromosome V contains a 6 kb open reading frame. Here, three alternate splice variants are predicted with primary transcripts of 6381bp containing 14 to 15 exons (Figure 3). These sequences were then compared using Basic Linear Alignment and Search Tool (BLAST) and analyzed using one-to-one sequence alignment (using EMBOSS pair-wise alignment) of both the *C. elegans dop-2* and the human D2 sequence (Altschul et al., 1997; Figure 4). The *C. elegans dop-2* sequence showed approximately 40% similarity.

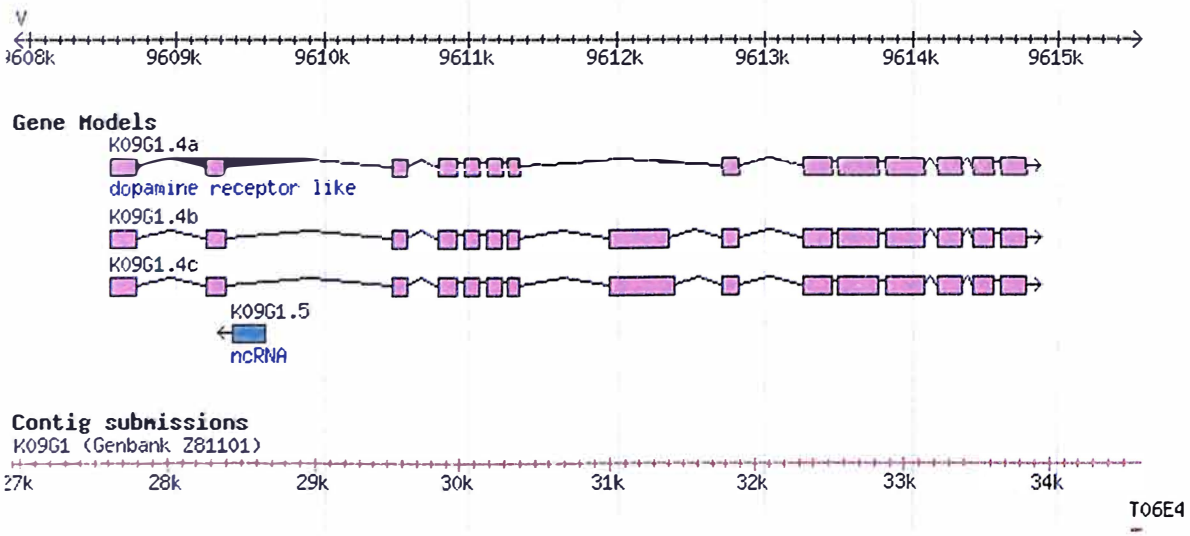

Figure 3. Gene model of K09G1.4 (Wormbase)

Comprehensive phenotypic and behavioral analysis of *gpa-14* and *dop-2* deletion mutants

The *gpa-14* (pk342, strain NL788) and *dop-2* (*vs105*) mutant strains were examined for visual and gross phenotypic abnormalities. Compared to wild type *N2* worms, these strains exhibited normal body size, shape, growth, movement and locomotion (Table 1).

Behavioral characterization of the mutant strains was carried out using a series of behavioral assays, specifically habituation, associative learning chemotaxis and basal slowing.

```
# Aligned_sequences: 2
# 1: CeDOP-2
# 2: HumD2
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 719
# Identity:     178/719 (24.8%)
# Similarity:   278/719 (38.7%)
# Gaps:         290/719 (40.3%)
# Score: 574.5
#
#
#=======================================

CeDOP-2             1 MEAGETWNVSLEWPPPSLDLSTITQTPSTIVGSGIPLN----------     38
                      |:.     ::|.|....|:....::          |.|
HumD2               1 MDP-----LNLSWYDDDLERQNWSR----------PFNGSDGKADRPHYN     35

CeDOP-2            39 -YAGLSLIVIPLITLLGNLLVIISVLRYRALQSAINFLILGLAVADLLVA     87
                       ||.|..::|.:| :.||:||.::|.|.:|||:..|:||:.|||||||||
HumD2              36 YYATLLTLLIAVI-VFGNVLVCMAVSREKALQTTTNYLIVSLAVADLLVA     84

CeDOP-2            88 IIVMPYAVYVYVTNGDWYLGNLMCDIYMASDVCCSTASILLLAVISFDRY    137
                      .:|||:.||:.|. |:|....:.|||::..||...|||||.|..||.|||
HumD2              85 TLVMPWVVYLEVV-GEWKFSRIHCDIFVTLDVMMCTASILNLCAISIDRY    133

CeDOP-2           138 RAVSLPIQYSRQSQNVKRVWTLIAVIWLVSLTLASPMVFGVNVRPPDANP    187
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HumD2             134 TAVAMPMLYNTRYSSKRRVTVMISIVWVLSFTISCPLLFGLN----NADQ    179
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YECRFYNAEFSILSSMISFVIPCFLVLFVYIRIIIALKKREKAAKMRREK    237
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HumD2            180 NECIIANPAFVVYSSIVSFYVPFIVTLLVYIKIYIVLRRRRKRVNTKRSS    229

CeDOP-2          238 NTI-AHGLTMRPDTGEEQVDEEAAGRIVAGPDEREFGNSSTPRSSLESLS    286
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HumD2            230 RAFRAH---------------------LRAPLK---GNCTHP--------    247

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                  |::.:         ||...:               |:|   |:.:
HumD2            248 EDMKL---------CTVIMK---------------SNG---SFPV-----    265

CeDOP-2          337 FRNLSRNYSTKHHRKVVKVNRGNSRNNSRTASITNQSDDALIPAIIRTIS    386
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HumD2            266 -----------NRRRVEAARRAQELEMEMLSS-----------------    286

CeDOP-2          387 RKSPRLFRRDKTDIKKHSMILANPITEPPKEYRRVSMPIHPTNSQTETET    436
                                          |.||:..|  ..||.|::.|.
HumD2            287 -------------------------TSPPERTR--YSPIPPSHHQL----    305

CeDOP-2          437 ISASRDIENLPTTTISRSTTANSAELLGSPDDFEKFPALITETVLEDVLA    486
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HumD2            306 --------TLP--------DPSHHGLHSTPDS----PA------------    323

CeDOP-2          487 ETREGCFMQPTVSFALTVREMEGNALNNLKGCSVESSRRVSQVDPPLAIQ    536
                           :|         |..|:|.:
HumD2            324 --------KP---------EKNGHAKD-----------------------    333

CeDOP-2          537 ILTRPSLPHLDLQRMDSIGTTCSSKTRADSLRSVDSKGSKKSNRNGIAVK    586
                           |..:.::..|.|..:.|||. ||:::.
HumD2            334 --------HPKIAKIFEIQTMPNGKTRT-SLKTMS---------------    359

CeDOP-2          587 LVKRAIKHEHSLKRKVSKAQRKEKRATKTLGVVVGVFLVCWVPFFVINIL    636
                             :||:|  |:|||:||:.|.:|:|||::||:|||:.:||
HumD2            360 ------------RRKLS--QQKEKKATQMLAIVLGVFIICWLPFFITHIL    395

CeDOP-2          637 NAVCILLNKDSCQVGYDLFFYCTWIGYMNSFMNPIIYTIFNTEFRRAFKS    686
                  |..|      .|.:...|:...||:||:||.:||||||.||.|||:||..
HumD2            396 NIHC------DCNIPPVLYSAFTWLGYVNSAVNPIIYTTFNIEFRKAFLK    439

CeDOP-2          687 IIFGRNSTRHHFSNKQAHV    705
                  |:..
HumD2            440 ILHC---------------    443
```

Figure 4. One-to-one sequence alignment between *C. elegans dop-2* sequence and the human D2 receptor sequence. The sequence from *C. elegans* shows approximately 40% similarity to the human D2 receptor.

The animals' ability to respond to a repeated stimulus was tested using habituation assay as stated previously. Compared to the wildtype N2, both *dop-2* and *gpa-14* mutants

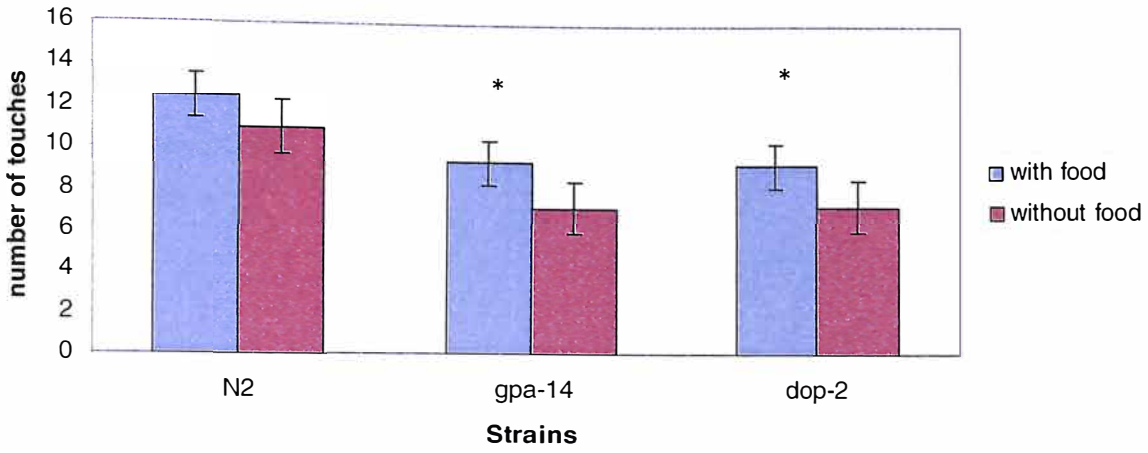

Figure 5 Habituation assay for wild type control worms (*N2*) and mutants *dop-2* and *gpa-14*. Worms were assayed on NGM plate with and without food (*E.coli*). Stimuli were given every 5 sec. until habituated. Both *gpa-14* and *dop-2* mutants habituate faster than wild type (*N2*) animals both in the presence (blue) and absence of food (red) (p value = < 0.0001, t-test). Bars indicate the number of touches needed for habituation for all three *C. elegans* strains tested (n=60 for each in six experiments). Error bars represent SEM values.

Performance of *gpa-14* and *dop-2* mutants in complex behavioral assays was also tested in a chemotaxis-based associative learning assay in which the chemo-attractant isoamyl alcohol was paired with starvation (Formisano, 2009). 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). First, we challenged the worms with 1:100 isoamyl alcohol (no conditioning). The worms showed strong attraction towards isoamyl alcohol (Figure 6).

When the conditioned *dop-2* and *gpa-14* mutants were challenged with isoamyl alcohol, they exhibited reduced avoidance (Figure 6). Contrary to previous work done in our lab, this result indicates that the absence of DOP-2 and GPA-14 compromise the worms' associative learning capability to isoamyl alcohol conditioned chemotaxis.

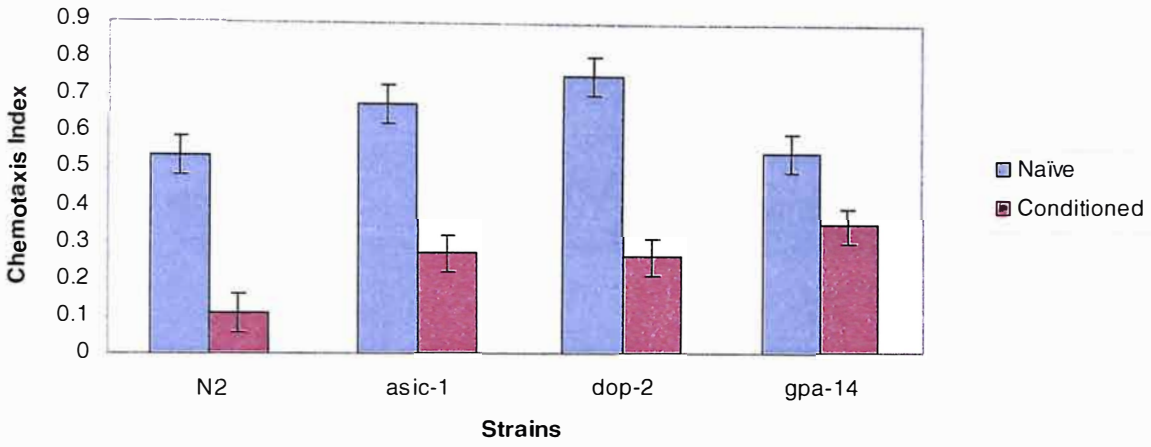

Figure 6. Associative learning chemotaxis assay for young adult hermaphrodites of *C. elegans dop-2* and *gpa-14* mutants. Worms were collected, washed and assayed for isoamyl alcohol chemotaxis on NGM plate without food. Blue bars represent naïve, unconditioned worms while red bars represent worms conditioned with isoamyl alcohol for 90 minutes before assay. Bars indicate the average chemotaxis index towards isoamyl alcohol. *asic*-1 mutants were assayed as negative control Error bars represent SEM values (n=450 for each strain in four experiments; p= 0.054 for naives and p=0.169 for conditioned , t-test).

In addition to learning and memory, dopamine also mediates movement and food sensing behaviors. When *C. elegans* encounter food, they move more slowly (Sawin et al., 2000). This reduction in locomotory rate can be tested using basal slowing assay.

Here, the worms are washed free off their original food source before being re-introduced to a new food source. Once they are transferred to a new plate with fresh *E.*

coli, their body bends are counted for the duration of 20 seconds (Figure 7). Our tests showed that there is no significant difference between N2's and the deletion mutants' locomotory rates. The same results were also seen when the test was repeated in the presence of food

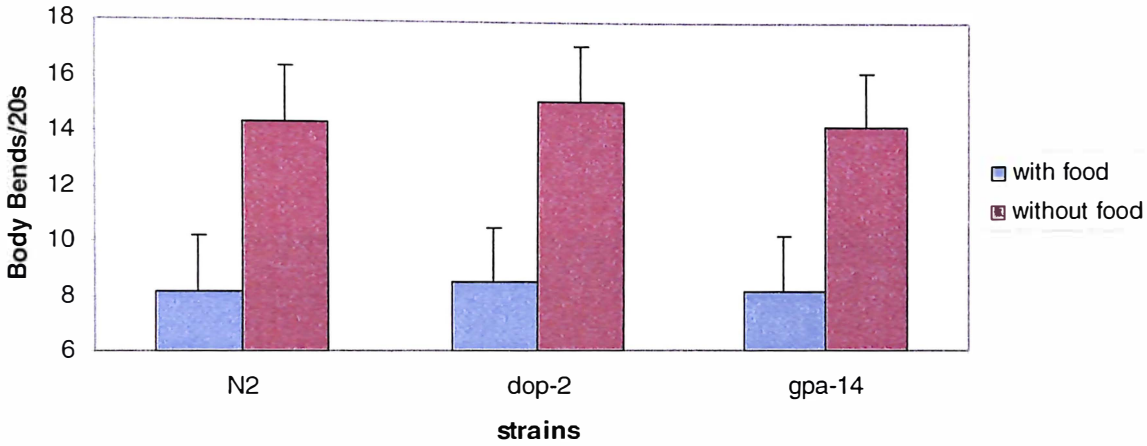

Figure 7 Basal slowing assays for young adult hermaphrodite *dop-2* and *gpa-14* mutant strains. 3 days old synchronized worms were transferred to plates with *E. coli* rings. Body bends of each individual worm were counted for 20 seconds. Basal slowing assay with food is represented by the blue bars while basal slowing assay without food is represented by the red bars. Bars indicate the average body bends/20 in three experiments (n=60 for each strain; P=0.067 without food and P=0.178 with food). SEM represented by y-error bars

Generation of mutants

In order to test the cumulative effects double deletion, *gpa-14 x dop-2* double mutants were generated and maintained (Figure 8A). After our initial PCR screening, we maintained lines with heterozygous lines (*dop-2* deletion 230bp; *gpa-14* deletion 390bp). After allowing the worms to reproduce we performed more PCR screening to isolate lines with stable *dop-2* and *gpa-14* deletions (Figure 8B).

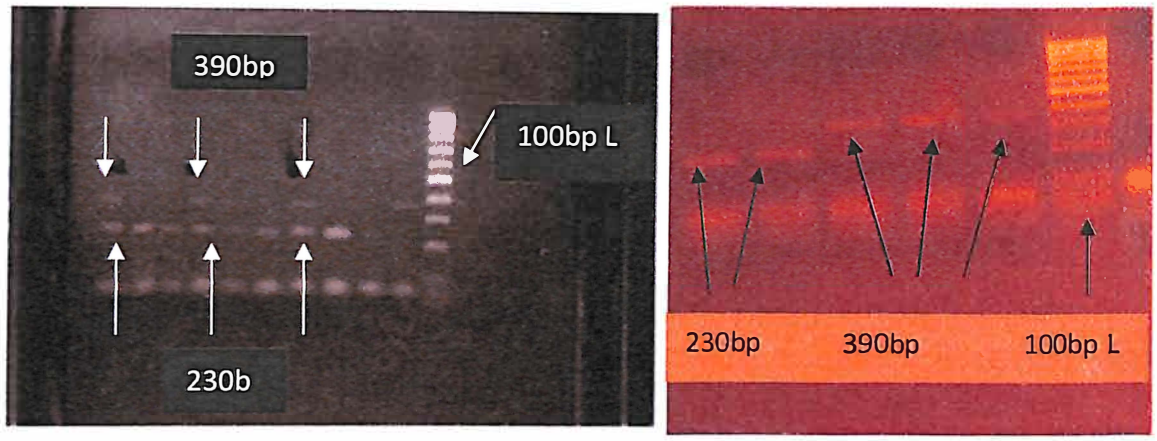

Figure 8 Agarose gel electrophoresis confirmation of *gpa-14 x dop-2* double mutants. Five *gpa-14* males were crossed with two *dop-2* hermaphrodites. Mutants were confirmed using PCR (A) PCR confirmation of F_1 progenies. Strains 1,4 and 8 (represented by lanes 1,4 and8) were maintained (B) After allowing the worms to reproduce additional PCR was performed to isolate stable lines with both *gpa-14* and *dop-2* deletion (represented by lanes 3,4 and 5).

Phenotypic analysis of the *gpa-14*; *dop-2* double mutant showed no abnormalities. They showed no aberration in overall morphology, growth or movement. However, behavioral assays showed learning deficiencies similar to the *dop-2* and *gpa-14* deletion

mutants. The double mutants' habituation was significantly faster than N2 (Figure 9). The associative learning chemotaxis assay also showed no cumulative abnormalities in the double mutants (Figure 10).

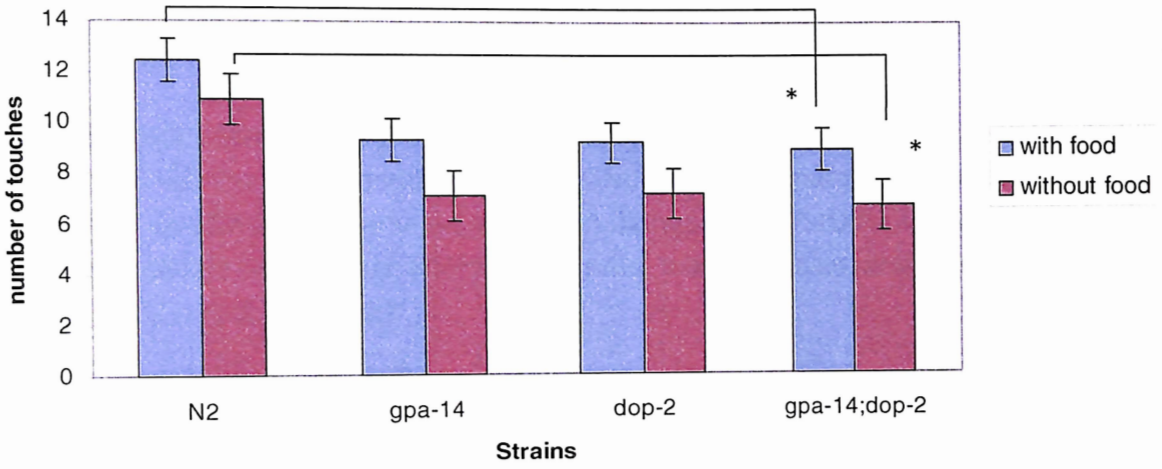

Figure 9 Habituation Assay for *gpa-14*; *dop-2* double mutants. Three-day old synchronized worms were assayed on NGM plate with and without food (*E.coli*). Stimuli were given every 5 sec. until habituated. *gpa-14* x *dop-2* double mutants habituate faster than wild type (*N2*) animals both in the presence (blue) and absence of food (red). They also exhibit similar habituation rates as *dop-2* and *gpa-14* mutants (p value = < 0.0001, t-test). Bars indicate the number of touches needed for habituation for all three *C. elegans* strains tested (n=60 for each in six experiments). Error bars represent SEM values.

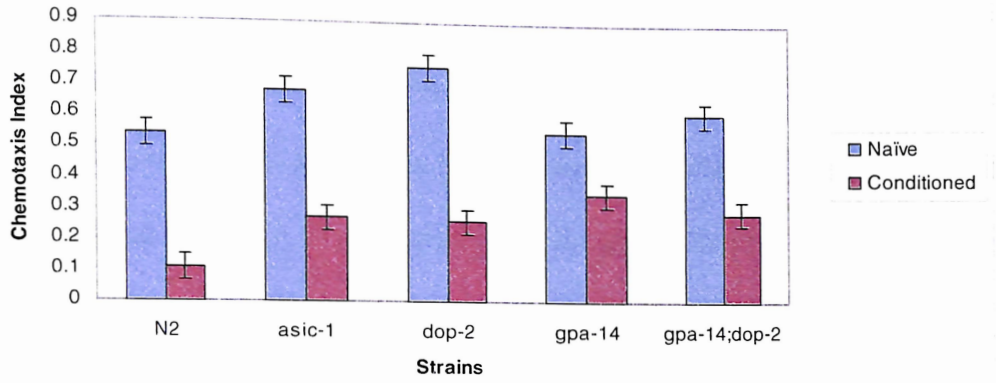

Figure 10 Associative learning chemotaxis assay for *gpa-14* x *dop-2* double mutants. Synchronized worms were conditioned on NGM plates with pure isoamyl alcohol for 90 minutes. The conditioned worms were then challenged with 1:100 isoamyl alcohol for 1 hour. Bars indicate the average chemotaxis index towards the attractant. Error bars represent SEM. (p=0.78, t-test)

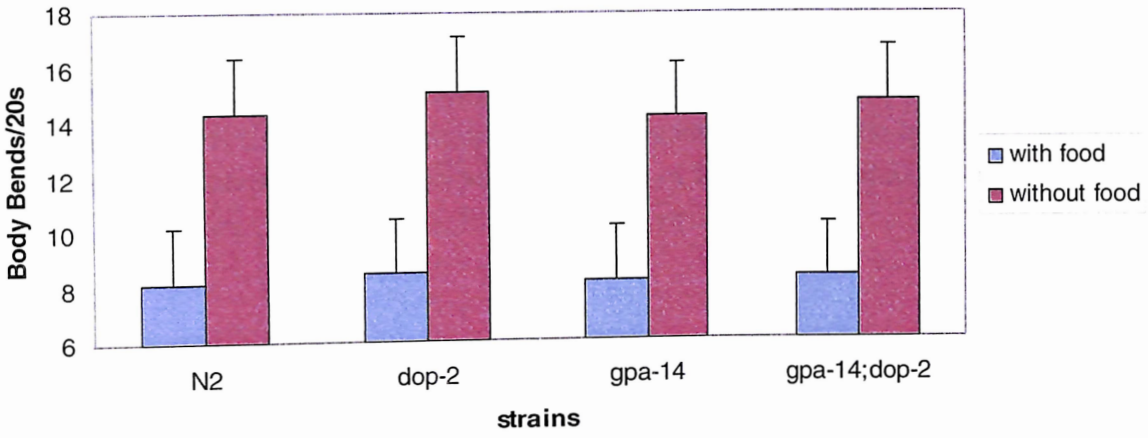

Figure 11 Basal slowing assays for *dop-2; gpa-14* double *mutant* strains. Synchronized worms were washed and transferred to NGM plates with *E.*coli ring. The body bends of each individual worm were counted for 20 seconds. Bars represent the average bodybends/20seconds. SEM represented by the Y-error bars (N=40, P =0.80).

Exogenous dopamine rescue

We envisaged that the absence of DOP-2 and GPA-14 results in reduced level of dopamine in the synapse causing several behavioral aberrations. The figures provided above have illustrated that these loss of function mutants indeed show deficiency in learning and behavior. Thus, we rescued these deficiencies using exogenous dopamine.

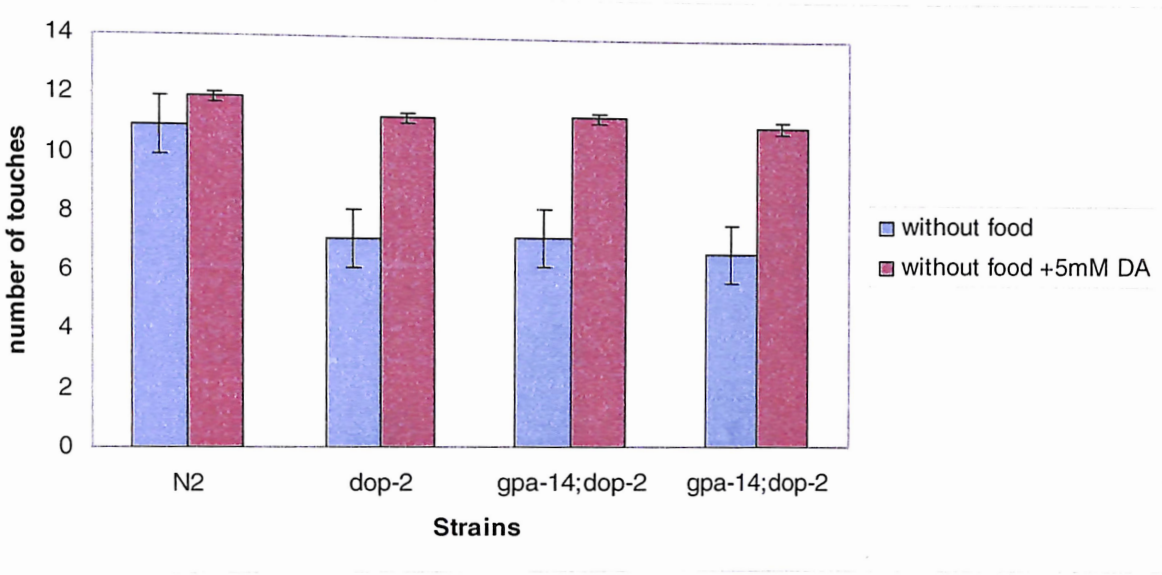

Figure 12 Habituation assay with 5mM exogenous Dopamine. Worms assayed on NGM plates in the presence of 5mM dopamine. Worms were tapped on the head every 5 seconds until habituated. Bars indicate the number of touches required for habituation. Y-error bars represent SEM. (n=40; P>0.05 for all strains)

When habituation was done in the presence of exogenous dopamine, the habituation rate went down. Comparison of the rate of habitation of the mutants to that of N2 showed no statistically significant difference (Figure 12). Our conditioned associative learning chemotaxis assays for *dop-2* and *gpa-14* mutants showed that these double mutants failed to associate isoamyl alcohol with starvation. In order to distinguish if low

levels of dopamine cause this abnormality, we conditioned and tested the worms in the presence of 5mM exogenous dopamine (Figure 13). The exogenous dopamine was able to rescue their deficiency. They were able to show a diminished attraction to isoamyl alcohol after the conditioning period. We also grew the worms on NGM plates + 5mM dopamine (Figure 14). Again, the exogenous dopamine was able to rescue their learning deficiency, showing that dopamine is required for conditioned associative learning.

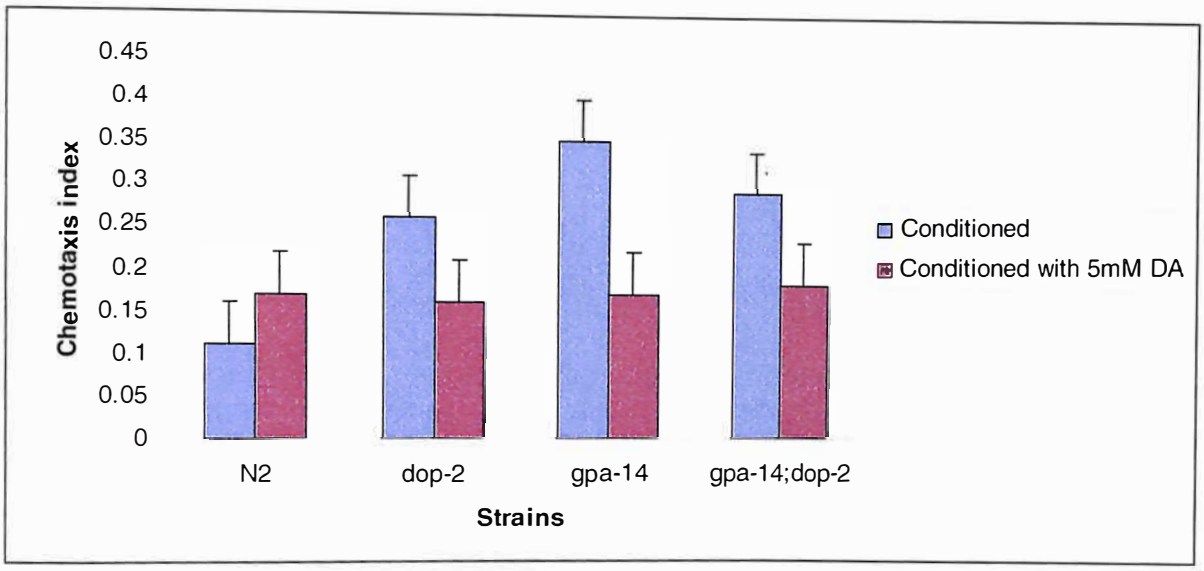

Figure 13 Associative learning chemotaxis assay with 5mM dopamine. Before being challenged with 1:100 isoamyl alcohol, worms were conditioned with pure isoamyl alcohol without the presence of food. The worms were conditioned and tested with the presence of 5mM exogenous dopamine. Bars indicate the average chemotaxis index towards the attractant. Error bars represent SEM (n=450 for each strain, $p<0.001$)

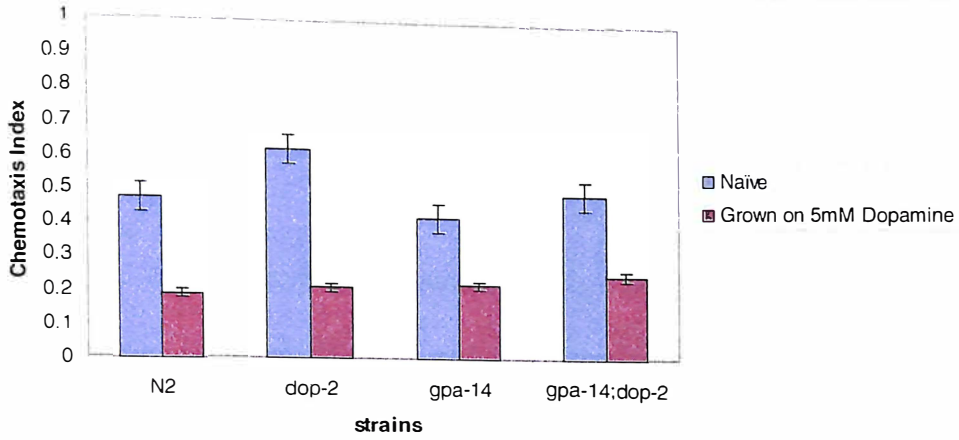

Figure 14 Associative learning assay for worms grown on NGM plates with 5mM exogenous dopamine. Worms were conditioned and tested on normal chemotaxis plates without dopamine. Bars indicate the average chemotaxis index for three experiments. SEM represented by y-error bars (n=300, $p<0.001$).

IV. DISCUSSION

The neurotransmitter dopamine plays a central role in learning and memory. Dopamine is a catecholamine neurotransmitter found in both vertebrates and invertebrates. Abnormal transmission of dopamine is associated with several neurological disorders. The soil dwelling nematode *Caenorhabditis elegans* (*C. elegans*) is an ideal organism to study the molecular changes that take place during learning and memory. Their behavior is strongly influenced by changes in the environment. Dopamine also plays a major role in modulating neuronal plasticity in *C. elegans.* DOP-2, an autoreceptor of interest is a seven transmembrane receptor that acts through G-protein coupled pathways. Studies have shown that dopamine levels are modulated by DOP-2 and ASIC-1. In order to unravel the working partners of DOP-2, our lab conducted data mining. Based on expression pattern overlap, work was initiated on characterizing mutants in shortlisted candidate genes particularly GPA-14, a Gα protein that shows expression overlap with both DOP-2 and ASIC-1 (Formisano, 2009).

We hypothesized that the dopamine autoreceptor DOP-2 modulates dopamine concentration in the synaptic cleft via interaction with the Gα protein, GPA-14. As a result, both *dop-2* and *gpa-14* deletion mutants show similar deficiencies in learning and memory.

In order to test our hypothesis, we conducted a series of behavioral assays including habituation, associative learning (chemotaxis-based) and basal slowing. Our behavioral assays showed that *gpa-14* and *dop-2* share similar behavioral abnormalities.

This was a clear indication that GPA-14 and DOP-2 work together to regulate synaptic dopamine level. Although we had planned to conduct FRAP to test the level of dopamine being released in the synapses of all 8 dopaminergic neurons, we were unable to do so due to technical and time constraints.

Behavioral analysis of *gpa-14* and *dop-2* mutants

Visual examination of both *gpa-14* and *dop-2* loss-of-function showed no abnormalities in body size, shape and movement compared to wildtype. In addition to its role in learning and memory, dopamine has been reported to regulate food encounter responses (Sawin et al, 2000). In order to see if the absence of *dop-2* or *gpa-14* affects the food encounter response, basal slowing assay was conducted. We found that the absence of these two genes had no effect on their food encounter response. This could mean that, although the level of dopamine is reduced in these mutants, it is not significant enough to affect their food encounter response. Another possibility is that basal slowing is not as sensitive to dopamine levels as mechanosensation and chemosensensation (Voglis & Tavernarakis, 2008).

Abnormalities in response towards a single touch stimulus were tested using habituation. Previous studies have shown that dopamine deficiency results in faster

habituation rates while abundance in dopamine results in slower habituation rates (Sanyal et al, 2004; Kindt et al, 2007; Voglis & Tavernarakis, 2008). We found that both *dop-2* and *gpa-14* mutants have almost identical habituation rates. Compared to N2, their habitation rate was significantly faster.

The reduction in the responses of the mechanosensory neurons AVL and AVM is believed to be responsible for the fast habituation rates seen in *gpa-14* and *dop-2* (Kindt et al, 2007). These mechanosensory neurons make synapses with the dopaminergic neurons CEP and ADE. When a continuous stimulus is present, the amount of dopamine released into the synapse of the CEP and ADE neurons is reduced. This causes the excitatory interneurons to send weaker signals to the motor neurons. As a result, the firing rate of the motor neurons drastically decreases and eventually dies. When there is no activity in the motor neurons, the worms become unable to move away from the stimulus and learn to ignore it (Kandel et al., 1991). To further prove this, we gave the worms exogenous dopamine. The extra supplement of dopamine was able to restore the habituation rate back to normal proving that the reduction in dopamine level is responsible for the fast habituation rate.

A chemotaxis assay was used to detect abnormalities in the animals' ability to sense and respond to chemical stimulus. Generally, *C. elegans* move towards chemical compounds that resemble the smell of food. Iso-amyl alcohol (water soluble), is one of the chemical that is widely used for conducting chemotaxis assays. Chemoattractants are detected by the neurons ADF, ASE, ASG, ASI, ASJ and ASK located in the amphid organs which are directly or indirectly exposed to the environment (Bargmann et al.,

2006). Previous studies have shown that ablation of the ASE neurons reduces but does not abolish chemotaxis behavior in *C. elegans.* However ablation of the ASE, ASI, ADF and ASG neurons abolishes the chemotaxis effect completely (Bargmann et al., 1991). This indicates that not the all of the neurons mentioned above play the same role in sensing chemicals. Although it is believed to play a minor role, the gpa-14 gene is expressed in the ASI, ASJ and ASK neurons (Bargmann et al., 2006).

Our chemotaxis assay showed that both *gpa-14* and *dop-2* mutants were defective in chemoreception when compared to N2. After being conditioned with iso-amyl alcohol in the absence of food, the mutants failed to move away from the iso-amyl alcohol. This could indicate that ASI, ASJ and ASK neurons play a bigger role than previously thought. Even though gpa-14 is expressed in 50% of the chemosensory neurons, it is significant enough to result a significant behavioral abnormality.

The CEP, ADE, PDE are believed to be mechanosensory neurons, based on the data we collected, they might also play a role in chemosensation. Both DOP-2 and GPA-14 are expressed in the ADE dopaminergic neurons, leading us to speculate that there might be a possible convergence of the mechanosensation and chemosensation pathways in these neurons.

Based on the behavioral data we have collected, it is safe to say that the DOP-2 autoreceptor works with the GPA-14 G-protein coupled receptor to modulate further release of dopamine (Figure 15). It is pertinent to mention here that experiments primarly performed by other colleagues in our lab have established the physical interaction

between the two in yeast two-hybrid and in-vitro binging assays

(Pandey, Dhillon et al., manuscript under review).

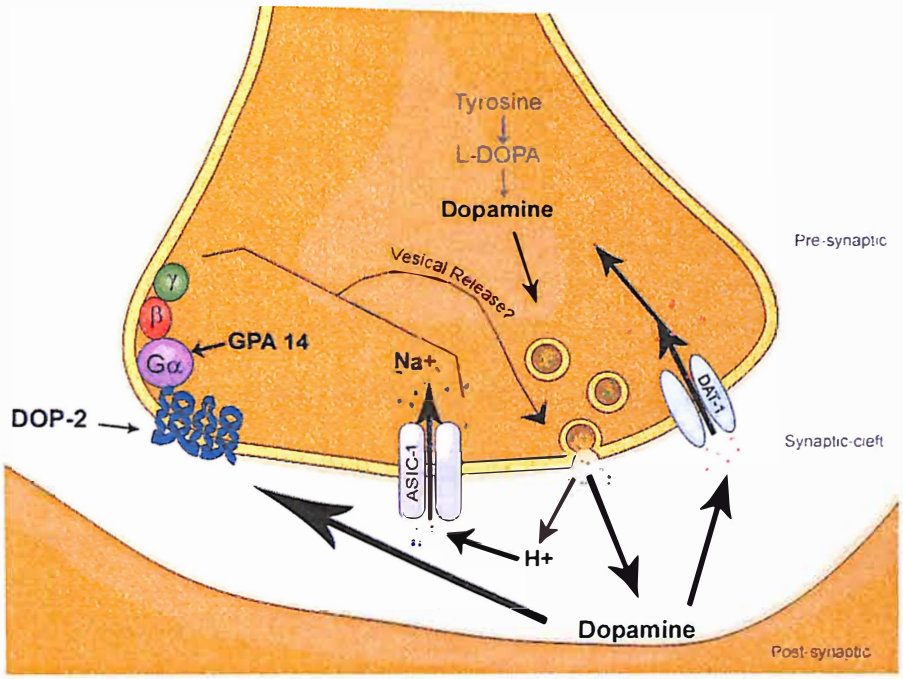

Figure 15. DOP-2 and GPA-14 interactions in the ADE dopaminergic neurons (Developed from Tavernarakis and Voglis 2008; drawing by Tim Pierpont).

During the course of my experiments I was able to generate two interesting transgenic strains in mutant backgrounds of interest, namely the *gpa-14(pK 342);* N2*Ex* [p_{asic-1} SNB-1::SEpHluorinpRf4] and *dop-2 (v105);* N2*Ex* [p_{asic-1} SNB-1::SEpHluorinpRf4]. It will be interesting to carry out further studies with these strains using FRAP which will provide an accurate measure of the synaptic dopamine levels along with the rate of dopamine getting released into the synapse. In return, we expect the FRAP experiments to conclusively confirm our findings that the level of dopamine is reduced in both *dop-2* and *gpa-14 mutants.*

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Laboratory Skills

Growth and maintenance of *C. elegans,* DNA isolation, PCR, Gel electrophoresis, behavioral assays, fluorescence imaging, western blot, yeast two hybrid, ELISA, collection and analysis of patient data, analysis of chest x-ray and blood work results

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