Understanding the role of DOP-2, a dopamine autoreceptor, in ethanol dependent locomotion of *Caenorhabditis elegans*

A thesis

Submitted for the degree of

Doctor of Philosophy

By

Anuradha Singh

Department of Biological Sciences

Indian Institute of Science Education and Research (IISER)

Mohali- 140306

MAY 2021

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH MOHALI

Sector 81, SAS Nagar, Mohali

PO Manauli, Punjab 140306, India

DECLARATION

The work presented in this thesis entitled "**Understanding the role of DOP-2, a dopamine autoreceptor, in ethanol dependent locomotion of *Caenorhabditis elegans***" has been carried out by me under the supervision of Dr. Kavita Babu at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

(Anuradha Singh)

Date:

Place:

In my capacity as supervisor of the candidate's doctoral thesis work, I certify that above statements by the candidate are true to the best of my knowledge.

(Dr. Kavita Babu)

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

Table of Contents

List of Figures

List of Tables

List of Appendix Tables

Acknowledgements

Today, at the completion of my thesis work, I experience immense joy and gratitude, for all the people who helped me throughout this journey. I feel blessed that I came across such great people who helped me to grow on both personal and professional fronts. I am immensely grateful to them and would like this opportunity to thank them from core of my heart, as they are one's whose support made this possible.

Firstly, I would like to pay my sincere gratitude to my PhD. Supervisor Dr. Kavita Babu for believing in me and providing me the opportunity to work under her guidance. I am extremely grateful to her for the counsel and advice she provided at the time of need and her mentorship. From the day, I joined her lab, I admire her, for the great person she is, providing a great environment and freedom to work even for a new student like me. She was always there to help me at every step, let it be discussions for the project, funding, presentations, seminars and writing research articles. Although now she has moved to IISc, to her new lab, still she makes sure to be there guiding and supporting me. She has always raised my spirits, being happy even at my smallest success, and be optimistic that however hard the times are, we will find a way out. And the most important thing I learnt from her is that whatever we do or achieve, remember to be a good person, as she is. It has been a wonderful experience to be her student, that will help me through my career and life.

I express my immense gratitude to my doctoral committee members, Dr. Lolitika Mondal and Dr. Rajesh Ramachandran for their suggestions, and timely review for the progress of my work. They have always encouraged me to do better and provided important scientific inputs with regard to my work. Apart from the work, they have also helped and encouraged me in shaping my presentations and writing skills. I am immensely thankful to them for their time and involvement in my work.

I would also like to thank Dr. Samarjit Bhattacharya, for sharing his insights for my work, during the presentations and seminars. I thank him for the constructive criticism that is necessary for keeping the work in check and widen my research from various perspective.

I would also like to thank the *C. elegans* Genetics Center (CGC), National BioResource Project (NBRP) and Addgene for all the reagents provided for completing my thesis work successfully. A special thanks to the *C. elegans* research community for all the reagents that helped me improve my work and add significance to my story (mentioned in Appendix tables). I am obliged and proud to be the part of such a generous community.

I would also like to express my sincere gratitude to Dr. Anindya Ghosh-Roy from NBRC, Manesar and his student Harjot Kaur, for letting me work in their lab and helping me with an important experiment for the thesis, that helped in adding significance to my work.

I would also like to acknowledge one special person who was the driving force for me to join PhD. and purse research. I would immensely like to thank Dr. Ashim Mukherjee and his lab member especially Dr. Ankita Singh, at M.H.G., B.H.U., for providing me the first taste of research. In their lab I developed interest in research work and a spirit to work for things that intrigued you the most. I am thankful to them for shaping my thoughts and imagination's in the right direction.

I would also like to thank all the faculty of Department of Biological Sciences of IISER Mohali for patiently listening to my presentations throughout the course of PhD. I am thankful for their valuable comments which really helped me to make improvements in my work.

I would also like to thank wholeheartedly to my lab post doc Dr. Pratima Sharma Pandey. I am forever indebted to her for the trust and belief she showed in me as a colleague. She has always guided and helped me throughout the work and helped me learn things in the new lab. She has always encouraged, guided, and worked along with me to troubleshoot all the problems I faced during this journey. I look up to her for both personal and professional advice. I admire her working spirit how well she manages everything and her dedication for science.

I am also thankful to the entire IISER family, administration, library, instrumentation, store and purchase sections, mess staff, canteen staff and hotel caretakers for their cooperation in the smooth running of my mind and PhD.

I am extremely grateful to have the most incredible lab members. Their active interest in my work, useful suggestions, and constructive remarks, during lab seminars and in general provided a cordial environment in the lab. I find myself lucky to enter the lab, at a high time as a junior, getting a lot of help from my senior lab members to shape my project. The lab was lucky to have two wonderful post docs, Dr. Yogesh Dahiya and Dr. Pratima Sharma Pandey, who have been a continuous support system for me. They have introduced me to different techniques and helped with the project and thesis work. I have worked alongside with Dr. Pratima while, Yogesh Sir has helped me learning difficult techniques like FRAP, for my thesis work. I am very thankful

to them for their help, love and support in and out of the lab. I am thankful to my senior Dr. Shruti Thapliyal for being there with suggestions whenever I needed her. My sincere thanks to Dr. Ashwini Bharadwaj, being a very helpful senior, apart from instances of his leg pulling sessions in the lab. He was great at providing insights to the project and suggesting experiments that helped me for the completion of my work. I would also like to thank my seniors Dr. Pallavi Sharma and Dr. Vina Tikyani, for helping me learning techniques in the lab, and being there for me when needed. I immensely thankful to my senior Dr. Nagesh Kadam, for helping me whenever required both personally and professional, he was more like a colleague to me than a senior, to whom I could look up to in the difficult times. I am also thankful to my junior, Umer, who entertains us with the tales of his beautiful land Kashmir. He is a very keen and intelligent student, who also loves to help everyone in need. I am thankful to him for his help during writing my thesis, as he introduced and helped me to make figure using a new software. I am also thankful to my lovable juniors Anupreet, Sukant, Akansha, Varun and Shagun for their help and co-operation when needed. Thanks to Ankit who works hard to maintain the lab and arranges things on time.

Words are never enough to express one's gratitude towards their family. My parents have always been my support system, none of this would have been possible without their support. Their immense unwavering faith in me, and freedom to pursue my dreams and their encouragement has helped me fulfil it. No words can describe the sacrifices that you made for me, all along your life for my dreams to come true, I hope, I have made you proud. Special thanks my siblings Anurag and Abhijit, who will always be close to my heart for their unconditional love and support and the bond owe share as a family that keeps us together in all tough and happy times. Last but not the least, I wish to thank all whose name could not be included but are fondly remembered.

Above all, I am grateful to almighty God, Whose grace and blessings permeate each and every aspect of my life.

Anuradha Singh

List of Publications

The work described in the Thesis has been published in PLOS GENETICS

1. Pandey P*., **Singh A*.,** Kaur H., Ghosh-Roy A., Babu K. Increased dopaminergic neurotransmission results in ethanol dependent sedative behaviors in *Caenorhabditis elegans*. PLOS GENETICS. Feb 2021. https://doi.org/10.1371/journal.pgen.1009346.

2. Anuradha Singh, Pratima Pandey and Kavita Babu. Ethanol Induced Sedative (EIS) Behavior Assay, to determine increased Dopamine and Acetylcholine defects in *C. elegans*. Bio Protocol, *In Press*.

3. Pratima Pandey, Umer Bhat, **Anuradha Singh**, Aishwarya Joy, Varun Bihari, Nagesh Kadam and Kavita Babu. EXP-1 and STR-2 receptors regulate chemosensation and dauer formation in a DAF-7 dependent manner. ENEURO. March 2021. DOI: https://doi.org/10.1523/ENEURO.0473-20.2021.

⁎ **Co-first authors**

ABBREVIATIONS

Weights and measures

%	Percent
µmol, nmoles, mmoles,	micromole, nanomoles, millimoles,
°C	Degree centigrade
bp, kb	Base pair, kilobase, Megabase
kDa	Kilodalton
rpm	Revolutions per minute
RT	Room temperature
sec, min, h	Second, minute, hour
µg, mg, g	microgram, milligram, gram
µl, ml, L	Microliter, milliliter, liter,
µM, mM, M,	micromolar, millimolar, molar
mV,V	Millivolt,Volt

Symbols

~	Approximately
=	Equal to
α	Alpha
β	Beta
γ	Gamma
Δ	Delta

Techniques

PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR

Chemicals

Amp	Ampicillin
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
dNTPs	2'-deoxyadenosine 5'-triphosphate
DTT	dithiothreitol; Cleland's reagent
EDTA	Ethylenediamine-tetra-acetic acid
BDM	2, 3-butanedione monoxamine

Miscellaneous

aa	amino acids
ACh	Acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
AUDs	Alcohol Use Disorders
Ca^{2+}	Calcium ion
CGC	*C. elegans* Genetics Center
CPG	Central Pattern Generator

C-terminal	Carboxy- terminal
DNA	Deoxyribonucleic acid
DA	Dopamine
DAT	Dopamine Transporter
DNC	Dorsal Nerve Cord
EIS	Ethanol Induced Sedative Behavior
EtOH	Ethanol
FRAP	Fluorescence Recovery after Photobleaching
GABA	Gamma- aminobutyric acid
GFP	Green Fluorescent Protein
LB	Luria Bertani
NAC	Nucleus Accumbens
NCBI	National Center for Biotechnology Information
NGM	Nematode Growth Medium
NMDA	N-methyl-D-aspartate
NMJ	Neuro-Muscular Junction
NT	Neurotransmitter
N-terminal	Amino- terminal
RNA	Ribonucleic acid
RP	Reverse primer
SEM	Standard error mean
TE	Tris chloride and EDTA
TRP	Transient Receptor Potential Channel
VNC	Ventral Nerve Cord
VP	Ventral Palladium
VTA	Ventral Tegmental Area
WT	Wild Type

SYNOPSIS

General Introduction

Caenorhabditis elegans as a model organism, is popular for its powerful genetic tools, short generation time, concise nervous system and easily performable behavioural assays. It has already been established as a model for studying the various aspects of neuroactive drugs (Schafer, 2004; Giacomotto and Ségalat, 2010; Alaimo J T et al., 2003; Hawkins et al., 2015). These animals encounter Ethanol (EtOH) in their natural habitat in the form of rotten fruits and thus can be expected to have an evolutionarily developed neuronal circuitry that can navigate and tolerate the presence of EtOH. The *C. elegans* dopaminergic (DA) system is very compact, with merely eight DA neurons as compared to ~500,000 DA neurons in mammalian systems. *C. elegans* DA receptors belong to two subfamilies similar to the classification of mammalian DA receptors, these include the D1-like and D2-like receptors (Suo et al., 2003). EtOH shows its effect in a concentration dependent manner, acting as a stimulant at lower concentration and depressant at higher concentration. Studies in *C. elegans* have reported that here too, EtOH administration shows dose dependent decline in the locomotor activity of the animal with increasing levels of EtOH exposure, which is similar to the depressive effects seen in other animal systems (Davies et.al., 2003; Hawkins et al., 2015). The internal dose of EtOH responsible for this behaviour is similar to the rodent and mammalian systems, indicating that there exist similar molecular targets (Lee et.al., 2009). The molecular mechanisms responsible for these behaviours are yet to be fully investigated to identify targets for reliable treatments of chronic alcohol exposure.

Here we have focused our studies on deciphering the neuronal circuitry that could play a role in regulating locomotory behaviour under the influence of ethanol. My thesis provides evidence to show that:

- DOP-2 autoreceptors acts to regulate dopamine release and the loss of this receptor leads to an ethanol induced sedative behaviours.
- Increased dopamine acts to regulate locomotion through NLP-12 neuromodulation in the presence of EtOH.

DOP-2 autoreceptor acts to regulate dopamine release and the loss of this receptor leads to an Ethanol Induced Sedative behaviors

Introduction

C. elegans dopaminergic signalling has been shown to be involved in regulating a variety of behaviours such as egg laying, defecation, habituation and associative learning (McDonald et al., 2006; Sawin et al., 2000; Voglis and Tavernakaris, 2008). Most importantly it has been shown to regulate locomotory behaviours like basal slowing and gait transition (Vidal-Gadea and Pierce-Shimomura, 2012).

The five dopaminergic receptors present in *C. elegans*, belong to two subfamilies D1 and D2 similar to their mammalian counterparts. While the expression of these receptors is found on different neuronal and non-neuronal cells, the D2 class receptor DOP-2 is only present on dopaminergic neurons making it a good candidate for DA autoregulation. However, its deletion doesn't show any behavioral defect, as reported for other DA receptors such as DOP-1 and DOP-3 where they have been shown to function antagonistically to regulate the locomotion of *C. elegans* under normal condition (Sawin et al., 2000; Chase et al., 2004). In mammals the D2 receptor has been shown to play important role in the regulation of dopaminergic signalling through feedback inhibition, that in turn controls synthesis, release and reuptake of dopamine (Ford, 2014). Studies in mammalian systems implicate these D2 receptors (D2R) functions as being affected by alcohol (Thanos et al., 2005).

The mechanism of how DOP-2 autoreceptors functions in *C. elegans* is still unknown, as is information on how alcohol could be affecting behaviour through this receptor. Here we have characterized the role of a dopaminergic autoreceptor DOP-2 in the context to movement related behaviors in the presence of EtOH. We have screened dopaminergic receptors and pathway mutants in an EtOH-based assay and found that the D2 autoreceptor mutants, *dop-2*, display a novel locomotory phenotype that we refer to as Ethanol Induced Sedative (EIS) behaviour.

Results and Conclusions

In this study we have characterized the role of a dopaminergic autoreceptor DOP-2 in the context of locomotory behavior in *C. elegans*. DOP-2 is expressed presynaptically in all the dopaminergic neurons where it acts as an autoreceptor. We show here that mutants in *dop-2* shows no obvious locomotory defects. It has been proposed that DOP-2 may be involved in regulating multiple aspects neuromodulation, where this autoreceptor could act to regulate several different behaviours instead of one specific behavior and the loss of this receptor could be compensated by other regulatory molecules (Ford, 2014). This suggests that we may see a phenotype in *dop-2* mutants under non wild-type or stressed conditions. Ethanol has been shown to modulate behavior and function in mammals and invertebrates through the dopaminergic system where it has been shown to increase dopamine release through the reward pathway in mammals (Yim and Gonzales, 2000; Weiss et al., 1993 and Di Chiara, 2002). We have performed experiments to test for behavioral defects in *dop-2* mutants in the presence of ethanol (EtOH). On chronic exposure to EtOH (2-16 hr), we found that *dop-2* mutant animals showed locomotory defects. The EtOH-based assay was performed as previously detailed at 400 millimolar (mM) concentration of EtOH (Davies et al., 2003). We performed a timeline-based analysis of both wild-type (WT) animals and *dop-2* mutant *C. elegans*, and observed that, both the strains paralyze upon exposure to ethanol within 10-12 min, but the WT animals start recovering from EtOH exposure after 30-40 min as shown in a previous study (Davies et al., 2003). However, the *dop-2* mutant animals do not show recovery from paralysis until 120 min. As the WT animals fully recover and start moving forming sinusoidal tracks in 60 min, *dop-2* mutant animals show a locomotory defect even at the 120 min time point, and we found very distinct concentric tracks, where the animals moved in circles by dragging their body. We also observed that while the WT *C. elegans* largely left the plate by 16 hr post EtOH exposure, the *dop-2* mutant animals stayed on the plates and continued showing the locomotory defective behavior. We quantitated the behavior at the120 min time point, by tabulating the number and the amplitude of body bends in *dop-2* mutant animals along with WT control *C. elegans*. We observed a significant decrease in both these parameters in the *dop-2* mutant animals when compared to the WT control animals. We have termed this behavior as an Ethanol Induced Sedative (EIS) behavior. Our experiments also suggested that the

posterior region of the animals was more severely affected in comparison to the anterior region. We screened multiple dopaminergic pathway mutants through this EtOH assay and observed no obvious observable behavioral defect in the other mutant lines screened. We performed multiple control experiments including quantifying the number and amplitude of body-bends in both WT and *dop-2* mutant animals in the absence of EtOH, here we found that the *dop-2* mutants showed a behavior similar to WT animals. We next went on to rescue the *dop-2* mutant phenotype using DOP-2 expressed under its own promoter. Our rescue experiments showed complete rescue of the *dop-2* mutant phenotype indicating that the locomotory defects seen in *dop-2* mutants in the presence of EtOH were indeed due to loss of *dop-2* function.

To understand how DA levels are involved in affecting the EIS behavior and the function of DOP-2 as an autoreceptor in *C. elegans*, we utilized *cat-2* mutant animals. CAT-2 encodes for a tyrosine hydroxylase required for the synthesis of DA from tyrosine, these mutants have significantly decreased levels of DA (Alaimo J T et al., 2003). Upon screening through the EtOH assay these mutants showed a behavior similar to WT animals, and in the double mutants of *cat-2; dop-2* showed complete suppression of EIS behavior, thus decreasing DA levels suppressed the EIS behavior observed in *dop-2* mutant animals. Previous studies show that the D2 autoreceptor exerts a negative regulatory role for maintaining the synaptic levels of dopamine (Benoit-Marand et al., 2001; Schmitz et al., 2002). To examine the role of DOP-2 in maintaining dopamine levels, we observed the behavior of WT animals on EtOH after treating the animals with exogenous DA. The WT *C. elegans* on exposure to exogenous DA in the presence of EtOH show the EIS like behavior that was observed in *dop-2* mutants, while just exogenous DA exposed animals show normal movement. These results indicate that the EIS behavior could be due to increased levels of DA in *dop-2* mutant animals.

We also performed Fluorescence Recovery After Photobleaching (FRAP) experiments to substantiate our finding that DOP-2 regulates the levels of DA at the synapse in the presence of EtOH. We bleached the DA neuron specific synapses and quantitated the percentage recovery after bleaching over 2 min, we observed a faster recovery in *dop-2* mutants in the presence of EtOH, compared to *dop-2* and WT animals in the absence of

EtOH or WT animals in the presence of EtOH. These data suggest that DOP-2 acts to regulate the DA levels at the synapse, and its absence leads to increase in the levels of DA in the presence of EtOH. In mammals it has been previously shown that DOP-2 regulates the activity of at DA Transporter (DAT), by regulating its surface expression (Lee et al., 2007). This prompted us to quantitate the cell surface expression of the DAT-1 transporter, we found a slight but significant decrease in DAT-1 expression in *dop-2* mutants in the presence of EtOH. These results indicate that DOP-2 might be functioning by regulating the expression of DAT-1 transporter on the cell surface. Together these data allow us to gain more insight into DOP-2 function at the synapse.

Increased dopamine acts to regulate locomotion through NLP-12 neuromodulation in the presence of EtOH

Introduction

As indicated in the previous section, the posterior region of *C. elegans* was more severely affected in the EIS behavior when compared to the anterior region of the worm. Since in the posterior region of the animal, there is only a single pair of dopaminergic neurons, the PDE neurons, we focused our attention on this neuron pair. It has been previously reported that PDE makes direct synaptic contact with the DVA neuron (Bhattacharya et al., 2014). DVA is known to modulate locomotion both positively and negatively, hence providing a unique mechanism whereby a single neuron can fine-tune motor activity (Li et al., 2006). DVA is an interneuron that has connections with both motor neurons and interneurons and relays information for locomotion through motor circuits (Gray et al., 2005; Bhattacharya et al., 2014; Hu et al., 2011). Thus, it is possible that dopamine secreted from PDE could be involved in regulating the movement of *C. elegans* through DVA in the presence of EtOH. The DVA neuron expresses the DA receptor DOP-1, a D1-like excitatory receptor (Bhattacharya et al., 2014). In Drosophila and mammals, it has been demonstrated that D1-like DA receptors promote EtOH-induced disinhibition (Kong et al., 2010; Abraho et al., 2011). In this chapter I will describe our experiments that show that DOP-2 functions through the DOP-1 receptor in the DVA neuron to allow for normal locomotory function in the presence of EtOH. Further, our results indicate that DOP-2 functions through the

neuropeptide NLP-12 and its receptor CKR-1 to allow for normal locomotion through the release of Acetylcholine at the neuromuscular junction.

Results and Conclusions

The EIS behaviour described in the previous section showed a distinct enhancement in the posterior region in comparison with the anterior region. This allowed us to hypothesise that the PDE neurons could be involved in this behavior. PDE neurons are the only posterior dopaminergic neuron, they have been shown to have processes ending in the posterior region of *C. elegans* (Brenner, 1986). Upon ablating the PDE neurons we observe suppression of the EIS behaviour in *dop-2* mutant animals. Increase in dopamine release in the reward pathway as a result of ethanol exposure, has been shown to lead to the activation of locomotion activating acetylcholine receptors (Soderpalm et al., 2000; Kamens and Phillips, 2008; Kamens et al., 2009). Further, in *C. elegans* dopaminergic receptors DOP-1 and DOP-3 have been shown to act antagonistically to control locomotion through the cholinergic pathway (Chase et al., 2004). Previous studies have shown that the hypercontracted state observed on EtOH exposure is due to increased acetylcholine at the NMJ (Hawkins et al., 2015). In order to test the role of acetylcholine in the EIS behavior, we first analysed cholinergic mutants and found that none of them showed the EIS phenotype. We next tested for the EIS phenotype in the presence of increased Acetylcholine levels. This was achieved by exposing WT animals to aldicarb followed by ethanol exposure, WT animals showed a phenotype similar to that seen in *dop-2* mutants in this assay. These results suggest that dopamine mediates its action through increased acetylcholine at NMJ, leading to muscle excitation and hypercontraction.

The PDE sensory neuron sends strong synaptic connections to the DVA neuron (Brenner, 1974; Sawin et al., 2000), DVA is an interneuron that regulates locomotion (Li et al., 2006). Previous work has shown that the DOP-1 receptors present on DVA are involved in sensing dopamine released by PDE (Bhattacharya et al., 2014). We found that *dop-1* receptor mutants did not show an obvious phenotype on ethanol exposure and the *dop-2; dop-1* double mutants showed complete suppression of the EIS phenotype seen in *dop-2* mutant animals. Further, DOP-1 receptors on DVA have been shown to regulate the activation of the neuropeptide NLP-12 where activation of DOP-1 allows for release of NLP-12

(Bhattacharya et al., 2014) NLP-12 in turn activates a downstream signalling cascade, where NLP-12 binds to its receptor CKR-2 (cholecystokinin like receptor) on the cholinergic motor neurons and regulate the release of acetylcholine (Hu et al., 2011; Bhattacharya et.al., 2014), increase in the levels of acetylcholine at the NMJ excite the muscles and maintain a hypercontracted state (Hu et al., 2011; Davies et al., 2015).

We went on to test if DOP-2 functioned through NLP-12 to allow for increased ACh release in cholinergic neurons. To elucidate the role of NLP-12 in regulating the EIS behavior, we overexpressed NLP-12 and found that these animals showed an EIS phenotype that was indistinguishable from *dop-2* mutant animals. We also found that loss of *nlp-12* did not show an EIS phenotype and that *nlp-12* mutants completely suppressed the *dop-2* EIS phenotype, indicating that DOP-2 could be functioning through NLP-12. We further went on to find that DOP-2 functioned through the NLP-12 receptor CKR-2 and that the EIS phenotype seen in DOP-2 mutants was likely through increased muscle activation, as overexpression of the acetylcholine receptor, ACR-16 on body-wall muscles, showed an EIS phenotype similar to that seen in *dop-2* mutant animals.

Taken together, our results indicate that DOP-2 in PDE mediates the EIS phenotype through the DOP-1 receptor present on DVA postsynaptic to PDE through modulating the release of NLP-12 neuropeptide that in turn controls locomotion by controlling acetylcholine levels.

Studies have shown that it is difficult to assign specific roles to neuromodulators like DOP-2, as they have roles in a wide array of behaviours and many of its functions could be transient and subtle. Moreover, there could be other molecules that function in the absence of *dop-2* in the WT scenario, making it difficult to assign specific functions for DOP-2 through behavioural analysis of *C. elegans* mutants. Since our experiments were performed in the presence if EtOH, we were able to clearly bring out the phenotypes associated with *dop-2* mutants in the presence of alcohol. Our studies have gone on to identify the underlying molecules and circuit that are involved in DOP-2 functioning in EtOH. In conclusion, my thesis provides important insights into the role of DOP-2 in the *C. elegans* nervous system.

References

Abrahao, K.P., Quadros, I.M., and Souza-Formigoni, M.L. (2011). Nucleus accumbens dopamine D (1) receptors regulate the expression of ethanol-induced behavioural sensitization. Int J Neuropsychopharmacol *14*, 175-185.

Alaimo, J.T., Davis, S.J., Song, S.S., Burnette, C.R., Grotewiel, M., Shelton, K.L., Pierce-Shimomura, J.T., Davies, A.G., and Bettinger, J.C. (2012). Ethanol metabolism and osmolarity modify behavioral responses to ethanol in C. elegans. Alcohol Clin Exp Res *36*, 1840-1850.

Benoit-Marand, M., Borrelli, E., and Gonon, F. (2001). Inhibition of dopamine release via presynaptic D2 receptors: time course and functional characteristics in vivo. The Journal of neuroscience: the official journal of the Society for Neuroscience *21*, 9134-9141.

Bhattacharya, R., Touroutine, D., Barbagallo, B., Climer, J., Lambert, C.M., Clark, C.M., Alkema, M.J., and Francis, M.M. (2014). A conserved dopamine-cholecystokinin signaling pathway shapes context-dependent Caenorhabditis elegans behavior. PLoS genetics *10*, e1004584.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Chase, D.L., Pepper, J.S., and Koelle, M.R. (2004). Mechanism of extrasynaptic dopamine signaling in Caenorhabditis elegans. Nat Neurosci *7*, 1096-1103.

Davies, A.G., Pierce-Shimomura, J.T., Kim, H., VanHoven, M.K., Thiele, T.R., Bonci, A., Bargmann, C.I., and McIntire, S.L. (2003). A central role of the BK potassium channel in behavioral responses to ethanol in C. elegans. Cell *115*, 655-666.

Di Chiara (2002). Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. Behavioural brain research 137 (1-2), 75-114.

Dillon J, Andrianakis I, Mould R, Ient B, Liu W, James C. (2013) Distinct molecular targets including SLO-1 and gap junctions are engaged across a continuum of ethanol concentrations in *Caenorhabditis elegans*. FASEB J 27:4266–4278.

Ford, C.P. (2014). The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. Neuroscience *282*, 13-22.

Giacomotto, J., and Segalat, L. (2010). High-throughput screening and small animal models, where are we? Br J Pharmacol *160*, 204-216.

Gray, J.M., Hill, J.J., and Bargmann, C.I. (2005). A circuit for navigation in Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America *102*, 3184-3191.

Hawkins, E.G., Martin, I., Kondo, L.M., Judy, M.E., Brings, V.E., Chan, C.L., Blackwell, G.G., Bettinger, J.C., and Davies, A.G. (2015). A novel cholinergic action of alcohol and the development of tolerance to that effect in Caenorhabditis elegans. Genetics *199*, 135-149.

Hu, Z., Pym, E.C., Babu, K., Vashlishan Murray, A.B., and Kaplan, J.M. (2011). A neuropeptide-mediated stretch response links muscle contraction to changes in neurotransmitter release. Neuron *71*, 92-102.

Kong, E.C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M.R., Heberlein, U., Bainton, R.J., Hirsh, J., and Wolf, F.W. (2010). A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in Drosophila. PloS one *5*, e9954.

Lee, J., Jee, C., and McIntire, S.L. (2009). Ethanol preference in C. elegans. Genes Brain Behav *8*, 578-585.

Li, W., Feng, Z., Sternberg, P.W., and Xu, X.Z. (2006). A C. elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. Nature *440*, 684-687.

McDonald, P.W., Jessen, T., Field, J.R., and Blakely, R.D. (2006). Dopamine signaling architecture in Caenorhabditis elegans. Cell Mol Neurobiol *26*, 593-618.

McKay, Raizen D, Gottschalk A, Schafer WR, Avery L (2004). eat-2 and eat-18 are required for nicotinic neurotransmission in the C. elegans pharynx. Genetics 166: 161-169.

Mitchell, P., Mould, R., Dillon, J., Glautier, S., Andrianakis, I., James, C., Pugh, A., Holden-Dye, L., and O'Connor, V. (2010). A differential role for neuropeptides in acute and chronic adaptive responses to alcohol: behavioural and genetic analysis in Caenorhabditis elegans. PloS one *5*, e10422.

Salamone JD, Correa M (2002). Motivational views of reinforcement: implications for understanding the behavioral functions of nucleus accumbens dopamine. Behav Brain Res 137:3–25.

Sawin, E.R., Ranganathan, R., and Horvitz, H.R. (2000). C. elegans locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. Neuron *26*, 619-631.

Schafer, W.R. (2004). Addiction research in a simple animal model: the nematode Caenorhabditis elegans. Neuropharmacology *47 Suppl 1*, 123-131.

Schmitz, Y., Schmauss, C., and Sulzer, D. (2002). Altered dopamine release and uptake kinetics in mice lacking D2 receptors. The Journal of neuroscience: the official journal of the Society for Neuroscience *22*, 8002-8009.

Soderpalm B., Ericson M., Olausson P., Blomqvist O., Engel J. A., 2000. Nicotinic mechanisms involved in the dopamine activating and reinforcing properties of ethanol. Behav. Brain Res. 113: 85–96.

Suo, S., Sasagawa, N., and Ishiura, S. (2003). Cloning and characterization of a Caenorhabditis elegans D2-like dopamine receptor. J Neurochem *86*, 869-878.

Thanos, P.K., Rivera, S.N., Weaver, K., Grandy, D.K., Rubinstein, M., Umegaki, H., Wang, G.J., Hitzemann, R., and Volkow, N.D. (2005). Dopamine D2R DNA transfer in dopamine D2 receptor-deficient mice: effects on ethanol drinking. Life Sci *77*, 130-139.

Topper, S.M., Aguilar, S.C., Topper, V.Y., Elbel, E., and Pierce-Shimomura, J.T. (2014). Alcohol disinhibition of behaviors in C. elegans. PloS one *9*, e92965.

Voglis, G., and Tavernarakis, N. (2008). A synaptic DEG/ENaC ion channel mediates learning in C. elegans by facilitating dopamine signalling. The EMBO journal *27*, 3288-3299.

Vidal-Gadea, G.A., and Pierce-Shimomura, J.T. (2012). Conserved role of dopamine in the modulation of behaviour. Commun Integr Biol. 5(5): 440–447.

Weiss, F., Parsons, L.H., Schulteis, G., Hyytia, P., Lorang, M.T., Bloom, F.E., and Koob, G.F. (1996). Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats. The Journal of neuroscience: the official journal of the Society for Neuroscience *16*, 3474-3485.

Yim, H.J., and Gonzales, R.A. (2000). Ethanol-induced increases in dopamine extracellular concentration in rat nucleus accumbens are accounted for by increased release and not uptake inhibition. Alcohol *22*, 107-115.

CHAPTER I

Review of literature

PART I. GENERAL INTRODUCTION

Caenorhabditis elegans is a transparent microscopic animal, about 1mm in length (Riddle et al., 1997). Its natural habitat is soil or decomposing organic matter, whereas in lab it is grown on nematode growth media (NGM), and fed on bacteria, *Escherichia coli*. It was classified into a separate species by Maupas in 1900 and established as a model organism by Sydney Brenner in 1965. *C. elegans* has a transparent body and a very concise nervous system of 302 neurons, making it an excellent model system for the field of neurobiology. It has a short life cycle, that lasts for three days at 20^{o}C. With four larval stages (L1, L2, L3 and L4) that molt to give an adult form. It has a brood size of 300-350 animals, with majority of the worms being hermaphrodite and less than 1% being males. Males can be differentiated by differences in their tails which has a hook like appendage and smaller body size when compared to hermaphrodites.

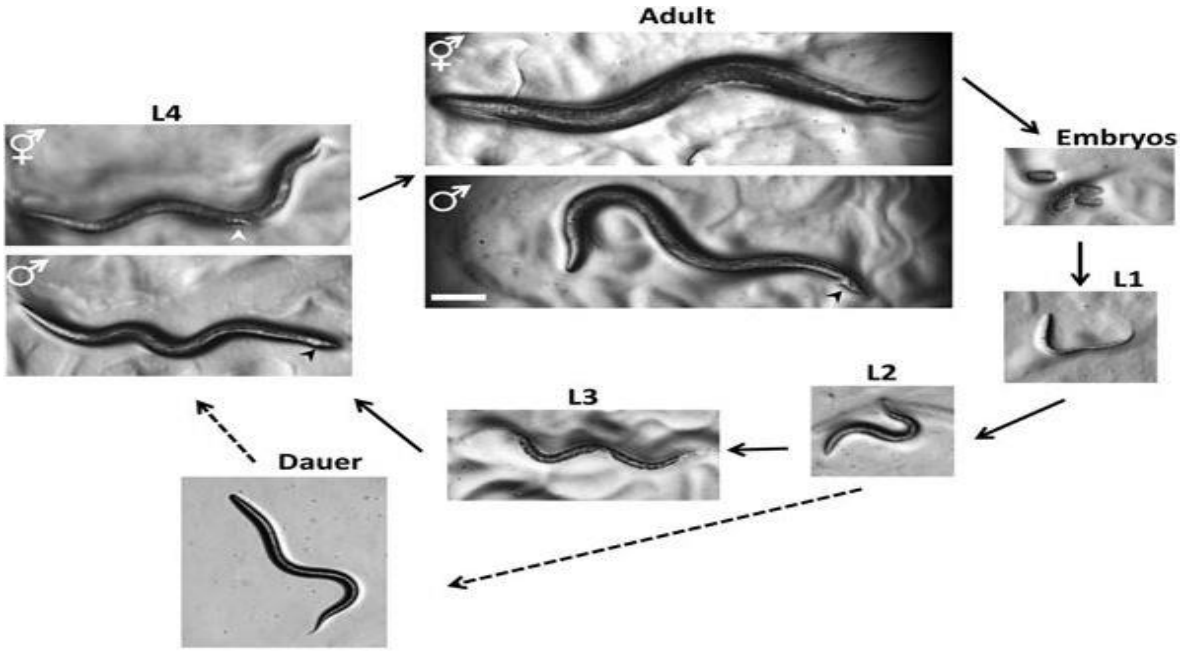


Figure 1.1. Representation of *C. elegans* life cycle.

C. elegans life cycle consists of four larval stages L1, L2, L3 and L4 before reaching adult stage. In the absence of food after L1 stage they get transformed into dauer stage. The individual sexes can be distinguished at the L4 stage as males and hermaphrodites (Image taken from wormbook).

C. elegans was the first multicellular organism to have its whole genome sequenced. Although the genome of *C. elegans* is smaller in comparison to mammals, about 30 times smaller, yet it has been shown to encode for more than 20,000 protein coding genes, similar to that of the human genome which encodes for around 25,000 protein coding genes. *C. elegans* has a completely mapped nervous system, with just 302 neurons and 52 glial and supporting cells. Further, the complete connectome of neurons was constructed from serial sections obtained from electron micrographs, along with the complete lineage map for each neuron. The major neurotransmitters (NT) released through these neurons are similar to the NT found in mammals like excitatory acetylcholine and glutamate, inhibitory gamma amino butyric acid (GABA) and monoamines such as dopamine (DA), serotonin and octopamine. Its ease of maintenance and long-term storage by freezing and storing in liquid nitrogen makes its more amenable to work with. Some other important features that make it an attractive model to work with are, its transparent body, that allows to perform live tracking of neuronal activity through calcium imaging and visualization of protein localization.

C. elegans is a well-established model for studying the effects of various drugs and neuroactive compounds, as it can modulate its behavior in response to the external environment, by adapting to the chronic exposure of a drug. An integrative analysis of the effect of drug can be done by studying the behavior of the organism, identifying the circuits affecting the behavior and the proteins through which the drug is acting. A high level of conservation between *C. elegans* to humans allow us to look for the conserved molecular targets.

Part II. DOP-2 autoreceptor regulates dopamine levels and its loss leads to an Ethanol Induced Sedative behaviour

1.1. Locomotion

Locomotion is the most fundamental animal behavior, that an organism uses for activities like food search, escape response, migration from a repulsive condition etc. Invertebrates have provided a great model system to understand the motor circuits, based on fact that they have a simple nervous system and defined motor patterns. *C. elegans* has posed as an important model for better understanding of motor control, with a simple nervous system that is capable of regulating complex behaviors. The body wall muscle in *C. elegans* is innervated by 75 motor neurons that regulate the forward and backward locomotion and understanding it leads us towards the mechanism and circuit of the sensory inputs generating an appropriate behavioral output. *C. elegans* moves by generating a thrust that propagates in the form of body bend along the body. Two locomotory patterns are observed in *C. elegans* swimming and crawling. During swimming the animal forms alternating "C" shaped confirmations, enabling navigation and when on a solid surface the wavelength is shortened to the body length producing "S" shaped wave. Thus, the motor control here is being shaped by interactions between the animal to its external environment through proprioception.

A central pattern generator (CPG) is needed for induction of locomotion, it is a group of neurons which on activation spontaneously generate rhythmic patterns leading to activation of neurons responsible for regulating locomotory behavior in the absence of any external cue (Brown, 1914, 1911). CPG need command input to generate the pattern, and also modulates the behavior. In many model systems neuromodulators like dopamine (DA) and serotonin regulate the CPG based modulation of locomotion (Clemens, 2012; Ray et al., 2003; Pictone and Sillar, 2016). In *C. elegans* also these biogenic amines are involved in regulating these locomotor patterns. Crawling is induced by DA in a liquid medium with viscosity similar to water, serotonin induces swimming in shallow liquid (Vidal-Gadea et al., 2011). Thus, biogenic amines have been shown to modulate locomotion in response to external cues and environment and are involved in direct modulation of locomotor behavior.

1.1.1. Neurotransmitter mediated regulation of locomotion

The fine tuning between different neurotransmitter systems in *C. elegans* shapes and regulates its locomotory behavior. The two classes of cholinergic motor neurons differentially regulate both forward and backward locomotion, by stimulating muscle contraction and through the activation of GABAergic motor neurons, that are involved in inhibiting the contraction of the collateral muscle to generate a locomotor pattern. Acetylcholine also regulates generation of locomotory behavior in several organisms (Sholomenko et al., 1991; Dubuc et al., 2008; Smetana et al., 2010; Ryczko and Dubuc, 2013). In mammals too it acts to control locomotor activity by providing inhibitory feedback inputs important for generating spinal locomotor pattern (Jordan et al., 2014). The GABAergic motor neurons also regulate locomotion, but their regulatory pattern changes throughout the life cycle of *C. elegans*, compared to the regulation by cholinergic motor neurons (Zhen and Samuel, 2015). GABAergic motor neurons are mostly inhibitory in nature, involved in the inhibition of body wall muscles helping the worm to propagate in a sinusoidal wave like pattern, also helping in the change in posture and directions (Zhen and Samuel, 2015). It also acts an inhibitory NT in the CNS of the mammalian brain, where it has important role in regulating locomotion behavior. Changes in the receptor function and increase in extra synaptic levels of GABA leads to loss of motor control and its hyperactivation suppresses the DA neurons involved in regulating motor behavior in mammals (Wahab et al., 2019). Thus both cholinergic and GABAergic NT system have important role in regulating the locomotory behavior in both mammals and *C. elegans*.

1.1.2. Biogenic amines in regulation of locomotion

Serotonin is an important biogenic amine neurotransmitter (NT) linked with depression in humans (Cowen, 2008). It is challenging to understand the signaling in human brain due to its underlying complex network, expressing 14 different types of receptors, classified into ion channels and G protein coupled receptors (Millan et al., 2008), where it can act both through classical synapses and also in a paracrine manner, making it complex to understand. *C. elegans* also uses serotonin as a NT, known synaptic connections and ease of forward genetics makes it an excellent model to study the proteins and circuits involved in mediating serotonin response. Serotonin is synthesized in six neurons in *C. elegans*; NSM, HSN, ADF, VC4/5, AIM and RIH (Horwitz et al., 1982; Jafari et al., 2011). Mutants in serotonin synthesis show locomotion defects, reduced speed i.e., enhanced

slowing behavior (Sawin et al., 2000). Further serotonin has been shown to regulate this slowing behavior through extra synaptic signaling, being released from the neurosecretory motor neurons (NSM) cells. It diffuses to distant target cells and acts through its receptors targeting the G protein signaling components through a G protein coupled receptor SER-1 and its target G protein GOA-1, in the head neurons and through serotonin gated chloride channels present on GABAergic motor neurons on the body wall muscles providing yet another mechanism for slow locomotion (Gurel et al., 2012).

Dopamine (DA) is an important NT that regulates locomotory behavior and cognition through G protein coupled receptors. They modulate the synaptic activity in mammalian systems. The DA neuron projections ascend from the substantia nigra pars compacta in the mammalian brain to the basal ganglia. The basal ganglia projects onto the mesencephalic locomotor region (MLR), a vertebrate brain stem region involved in regulating locomotion (Ryczko and Dubuc, 2017). It is this network whose function disrupts upon DA neuron degeneration in Parkinson's disease leading to motor dysfunction (Ryczko and Dubuc, 2017). DA has been shown to regulate locomotor activity through its receptors. The D1 like receptors promote locomotor activity, while the D2 like have a slowing effect on the locomotor frequency of animals (Barriere et al., 2004). DA also acts as a central pattern generator (CPG) in invertebrate system and mediate its effect through the D1 and D2 like receptors, that have opposing roles on spinal CPG and regulation of locomotion, as observed in mammals (Clemens et al., 2012). In *C. elegans* too DA regulates the locomotory behavior through two types of DA receptors, D1-like and D2-like receptors similar to their mammalian counterparts. It is responsible for the gait change from crawling to swimming in *C. elegans* as in other higher organisms (Mesce and Pierce-Shimomura, 2010). All the dopaminergic (DAergic) neurons are mechanosensory in nature thus it has also been shown to modulate the behavior of the organism in response to external cues by proprioception (Vidal-Gadea and Pierce-Shimomura, 2012). The external cues being food or mating and the external environment of the organism. Changes in the external cues and environment are judged by the animal and it adjusts its internal condition according to it, to generate an appropriate behavioral response. A classic example is change of locomotory state, like from swimming to crawling, regulated by the DA system in *C. elegans* along with regulation of locomotory state in other animal systems (Mesce and Pierce-Shimomura, 2010; Vidal-Gadea et al., 2011; Abdelhack, 2016).

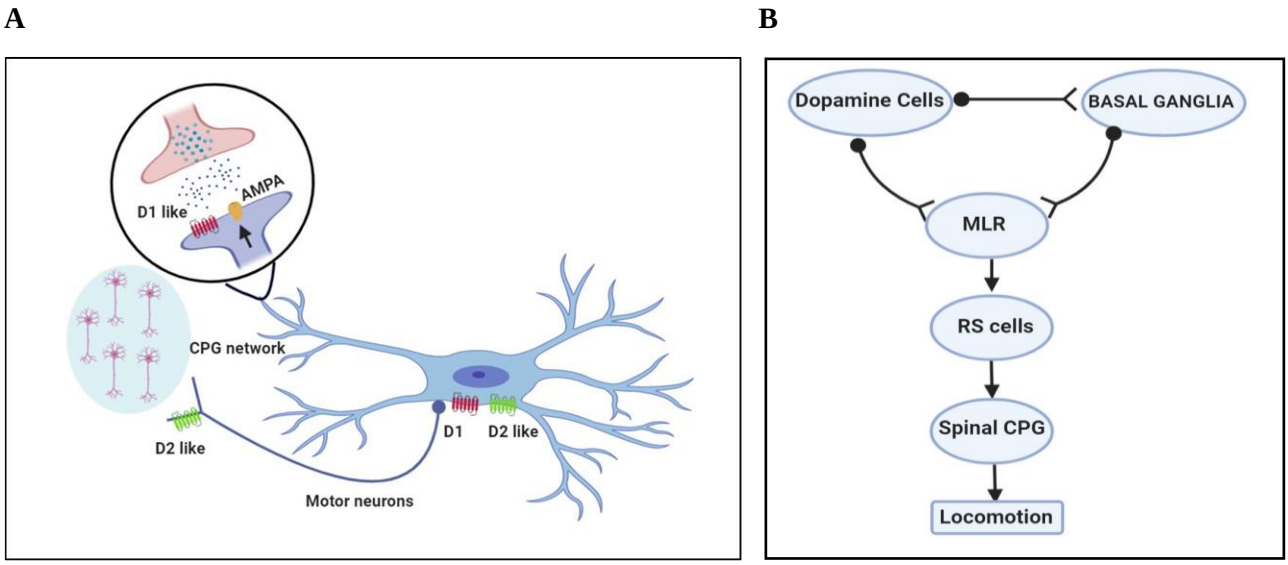


Figure 1.2. Effect of dopamine on locomotion

Dopamine (DA) regulates the locomotion circuit, by modulating the synaptic components and intrinsic properties of the known components of the locomotor network. A. DA exerts its effect through the motor and interneurons that converge at the spinal locomotor network, to maintain the locomotor activity. DA has also been shown to increase AMPA conductance through the D1 like receptor mechanism, D2 like receptors also regulate the excitation of the locomotor network. B. This figure represents the connectivity between the DA cells, the basal ganglia and the mesencephalic locomotor region (MLR), the reticulospinal cells (RS) and the central pattern generator (CPG) for regulating locomotion [Adapted from (Ryczko and Dubuc, 2017; Sharples et al., 2014)].

1.2. Effect of ethanol on locomotion

Among all the drugs of abuse ethanol is a widely and commonly used abusive drug, with adverse social and behavioral effects. It acts as a wide yet low specificity central nervous system (CNS) depressant. On chronic exposure, at lower doses it leads to a euphoria like condition, while higher dose may result in relaxation and sedation, which can affect the CNS and lead conditions like coma and respiratory failure. Thus, excessive alcohol intake is detrimental to human health (reviewed in Sukhes Mukherjee, 2013). Many studies have focused on understanding the mode of action and dependency of this drug. Behavioral responses to alcohol and susceptibility to alcohol use disorders (AUDs) vary since they are dependent upon environmental, physiological and genetic differences amongst individuals (Prescott and Kendler, 1999; Schuckit and Smith, 1996). However, it is still unclear how alcohol functions to modulate various behaviors, making it is important to identify and analyze target gene/s and molecular pathways which function to modulate behavioral phenotype/s upon alcohol intake. AUDs require functioning through multiple synaptic molecules including acetylcholine (ACh), GABA, glutamate, Dopamine (DA), neuropeptide-Y related pathways and ligand gated channels (Reviewed in (Bettinger and Davies, 2014; Harris and Trudell et al., 2008; Spanagel, 2009; Pierce-Shimomura et al., 2014). Ethanol (EtOH) has both positive, its intake has been shown to increase DA release which in turn induces reward pathways producing euphoria like condition and brings about disinhibition of behaviors (Correa et al., 2003; Baik, 2013), and negative reinforcing effects, relief from anxiety and leading to psychological and physiological dependence. Physiological dependence on EtOH develops with complicated medical conditions, depending on the effects of acute and chronic intoxication. The common symptoms of withdrawal from EtOH abuse are convulsions, coma and death, which is treated with the help of drugs with same site of action as EtOH, example of such compound is benzodiazepines (Schuckit, 2009 and reviewed in Lewis, 1996).

EtOH has been shown to influence the motor activity of organisms across different species depending on its dose (Wolf et al., 2002; Petri and Korpi, 1993; Davies et al., 2003). In mammals EtOH has been shown to stimulate locomotion at lower dose, while it depresses locomotion at higher concentration (Philips and Shen, 1996). The invertebrate system also provides great insight into the effects of EtOH, among them, *C. elegans* acts a well-established model for studying alcoholism (Reviewed in Engleman et al., 2015). It adapts to the external environment to generate appropriate behavior, like response to a drug exposure. Several studies have investigated the role

of acute and chronic EtOH exposure on locomotory behavior of the animal. Davies et al., show a dose dependent (100 – 500 mM) decline in the frequency of locomotion and egg laying and flattening of the body bends (Davies et al., 2003; Kapfhamer et al., 2008; Eckenhoff and Yang, 1994). Concentration dependent effect of EtOH in liquid medium also affects locomotion of the worm, by inhibiting the thrashing behavior (Mitchell et al., 2007). Graham et al., show that unc-18, involved in cholinergic synaptic transmission, provides sensitivity to EtOH, these animals are hyperactive at 22mM concentration, and show dose dependent decline in locomotion as previously observed at 200 – 500 mM conc. (Graham et al., 2008).

Although ethanol induced psychomotor control has been characterized previously, the mechanism and molecular effectors underlying such control still need a better understanding. And this behavior is also of utmost importance as other drugs of abuse such as cocaine, morphine and diazepam also share similar locomotor effects. The most important NT systems involved mediating the locomotor effects of EtOH are the GABAergic system, Glutamatergic and the DAergic system.

1.2.1. Neurotransmitter mediated effects of EtOH

1.2.1.1. Glutamate

A lot of studies have investigated the role of glutamate in the stimulant effects of drugs, associated with the reward pathway through interaction with NT and neuromodulatory systems in the mesocorticolimbic, amygdala and the reward circuitry of the brain (Chandler, 2003; Spanagel, 2009). It is a major excitatory neurotransmitter in brain and an important component for the development of alcohol dependence. Ethanol affects the glutamatergic system by interacting with NMDA receptors, hence mediating behavioral and physiological effects (Hoffman et. al., 1989). Acute EtOH administration causes behavioral defects, mediating its action by inhibiting calcium influx through the NMDA receptors and in turn altering calcium dependent signaling, while tolerance to EtOH is associated with overexcitation of these receptors and enhanced calcium influx on EtOH withdrawal (Chandler, 2003). As glutamatergic neurotransmission functions to shape experience dependent brain plasticity, it plays an important role in mediating long lasting effects of EtOH. Glutamatergic system has also been shown to influence the mesolimbic DAergic pathway, and it is the DAergic system that is activated in response to alcohol administration. The glutamatergic neurons project from different regions of the brain to the ventral tegmental area (VTA) and nucleus accumbens (NAC) and once stimulated they lead to induction of DA release

in these regions. Some glutamatergic neurons are also identified in the VTA itself that influence the DA activity through receptors other than those described previously (Spanagel, 2009).

Glutamatergic signaling in *C. elegans* is involved in mediating response to EtOH exposure. A glutamate receptor gene *glr-2* is involved in EtOH response, as its transcription was significantly increased on EtOH exposure at different time points, and the response to EtOH was found reversible, as the levels were back to normal on removal from EtOH (Know et al., 2003). The glutamatergic signaling required for reversal behavior is also found to be affected by EtOH conditioning, thus regulating one more aspect of locomotion (Brockie et al., 2001; Hills et al., 2004).

1.2.1.2. GABA

The GABAergic system is involved in regulating the activating effects of EtOH, as studies have shown that administration of GABA antagonist counteract EtOH induced locomotor activation (Biswas and Carlson, 1978). GABAergic system has been shown to influence motor activity in response to drug abuse through the DAergic system. Previous studies have described the role of both DA and GABAergic system in regulating the locomotor activity in response to psychomotor stimulants (reviewed in Baik, 2013; Salgado and Kaplitt, 2015; Pena et. al., 2015). DA transmission from the NAC is influenced from the VTA, leading to enhanced DA release, which is the main effecter of the psychomotor stimulant drugs influencing the locomotion of an organism (Hubner and Koob, 1990). Projections from the GABAergic neurons in the NAC region extend to the ventral palladium (VP) and from here the neurons project through different structure in the VTA-NAC region influencing DA transmission (Philips and Shen, 1996). In *C. elegans unc-25* and *unc-47* mutant, involved in GABAergic synaptic transmission, are deficient in synthesis and transport of GABA, show resistance to EtOH induced hypercontraction behavior (Hawkins et al., 2015).

1.2.1.3. Acetylcholine

The nicotinic acetylcholine receptors have emerged as an important target of EtOH in the reward pathway and for developing EtOH dependence. These receptors are expressed on both pre-and post- synaptic neurons exerting their effect by modulating the release of acetylcholine. EtOH has been shown to modulate the expression of nAChR and release in the brain mesolimbic pathway

(Dopico and Lovinger; 2009), affecting the mesolimbic DA signaling, thus the reward pathway and dependence behavior (Blomqvist et al., 1993). The VTA nAChR is linked with negative reinforcing properties of EtOH such as self-administration of the drug (Ericson et al., 1998; reviewed in Wu et al., 2014). A study in the invertebrate system, *C. elegans* has also shown role of specific nACh levamisole sensitive receptor in the hypercontraction behavior observed on EtOH exposure i.e., the EHC behavior (Hawkins et al., 2015).

1.2.2. Role of biogenic amines in regulating EtOH mediated behaviors

1.2.2.1. Serotonin

Serotonin acts as a communicating NT in the brain. EtOH has been linked with activating the reward circuitry in the brain and serotonin plays a very important role in mediating that effect in the brain. Several studies have reported an increase in brain serotonin levels, pointing towards enhanced signal transduction (Reviewed in LeMarquand et al., 1994). EtOH also affects the serotonergic receptors, by enhancing their activity causing modification in their protein structure (Lovinger and Zhou 1994). It also interacts with different NT systems in the brain, most important is the DA system, leading to increase in EtOH mediated activation of the DA neurons in the VTA region of the brain (Brodie et al., 1995). In *C. elegans* too serotonin is required for developing EtOH preference behavior. The serotonin synthesis pathway mutant is defective in developing EtOH preference, indicating an important role of serotonin in EtOH mediated behavior (Lee et al., 2009).

1.2.2.2. Dopamine

Ethanol mediates its effect by activating the mesolimbic DA pathway, and DA is involved in the control of spontaneous locomotory responses through this pathway. This links regulation of EtOH induced behavioral responses with DA. Several studies provide evidence that EtOH leads to an increase in the levels of DA in the striatum, NAC and VTA (Reviewed in Baik, 2013) (Imperato and Chiara, 1986). Reduction in the levels of DA has been reported on exposure to high doses of EtOH, leading to a sedative effect (Imperato and Chiara, 1986). EtOH leads to increase in DA synthesis and findings also revel the effect of EtOH on the synthesis and metabolism of DA and also the activity of DA neuron (Alari et. al., 1987; Carlsson and Lindquist, 1973). All these effects

of EtOH are mediated on the presynaptic DA neuron leading to an increase in the levels of DA and through second messenger on the postsynaptic neurons. The effect of EtOH is finally mediated through the G protein coupled DA receptors (Rabin and Molinoff, 1981). DA has also been shown to stimulate locomotion when exposed to lower concentration of EtOH, an effect dependent upon the varying specificities of DA receptor ligand to the different receptor subtypes (Liljequist et al., 1981; reviewed in Phillips and Shen, 1996). Studies have shown that DA antagonist on binding to D1 and D2 receptors reduced the ethanol stimulated activity, also affecting the baseline locomotor activity (Liljequist et al., 1981). Studies show that EtOH mediated activation of DA pathway also involves the central nicotinic acetylcholine receptors (Blomquist et al., 1992). Both EtOH and nicotine activate the mesolimbic DA system, and nicotine exerts its effect through the nicotinic acetylcholine (nAChR) receptors (Clarke et al., 1988). EtOH has been shown to enhance DA release in the NAC and DA synthesis in the limbic area mainly through the activation of central nACh receptors (Blomqvist et al., 1993). A study in the invertebrate system, *C. elegans* has also shown role of specific nACh levamisole sensitive receptor in the hypercontraction behavior observed on EtOH exposure i.e., the EHC behavior (Hawkins et al., 2015). Thus, the DA pathway emerges as an important mediator for studying the effects of EtOH, as it is directly and indirectly activated through GABAergic and nACh receptors on EtOH administration. Glutamatergic system also leads to the induction of DA release in the VTA and the NAC regions of the mammalian brain, either directly or indirectly through its receptor activation. Thus, different pathways converge to modulate the levels of DA in brain, on EtOH administration, leading to various behavioral and physiological changes, both on acute and chronic exposure. In the invertebrate system too, DA regulates several EtOH dependent behaviors. Reduction in EtOH induced locomotor activity, is observed in DA synthesis mutants of *Drosophila* (Bainton et al., 2000). In *C. elegans* also *cat-2* a DA synthesis pathway mutant, is resistant to developing EtOH preference (Lee et al., 2010). A D1 like receptor *dop-4* is involved in regulating the disinhibition of locomotor behavior i.e., from swimming to crawling (Topper et. al, 2014). Making the DA pathway an important signaling pathway that needs be studied from the perspective of EtOH abuse.

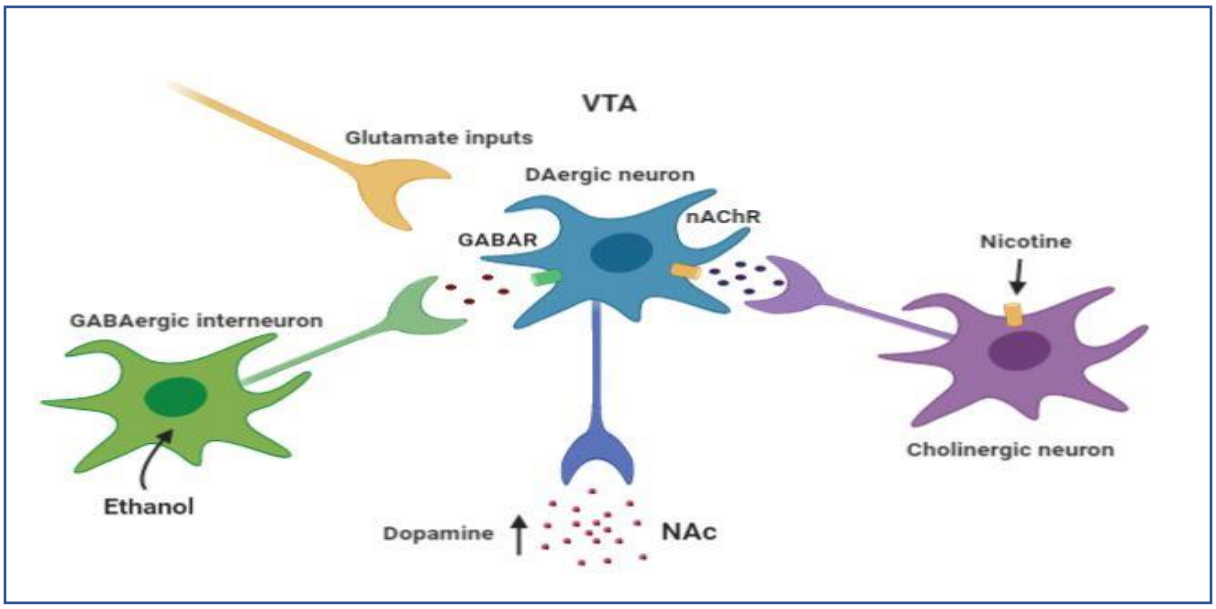


Figure 1.3. Effect of EtOH on brain neurotransmitter systems

Ethanol inhibits GABAergic transmission by promoting the $GABA_A$ receptors, in the ventral tegmental area (VTA), thus disinhibiting the VTA DA system. The VTA DA neurons release DA in the nucleus accumbens (NAC) and activate the reward process upon drug abuse. Glutamatergic neurons send projections on VTA and mesolimbic DA neurons, leading to their activation and enhanced DA release. EtOH also mediates its effect by binding to nicotinic acetylcholine receptors, either directly on the DA neurons or through the cholinergic system.

1.3. Dopaminergic signaling pathway

1.3.1. Dopamine Synthesis Pathway

Dopamine (DA) synthesis starts from amino acid phenylalanine, it proceeds to tyrosine and DOPA to DA. The rate limiting enzyme for DA synthesis is Tyrosine Hydroxylase, another important enzyme in the synthesis is DOPA decarboxylase, that decarboxylates DOPA to form DA. It is known as a ubiquitous signaling molecule, expressed across different taxa. DA acts to modulate behavior in response to external cues and environment, most importantly with respect to movement related behaviors, such as gait transition and locomotion patterns across species (Vidal-Gadea and Pierce-Shimomura, 2012). It acts as an important neurotransmitter in the mammalian brain, regulating diverse functions like, locomotion, cognition, emotion, reward and motivation (Missale et.al., 1998). DA neurons in mammals are mainly found in the nigrostriatal and mesolimbic regions, where they act to regulate diverse function.

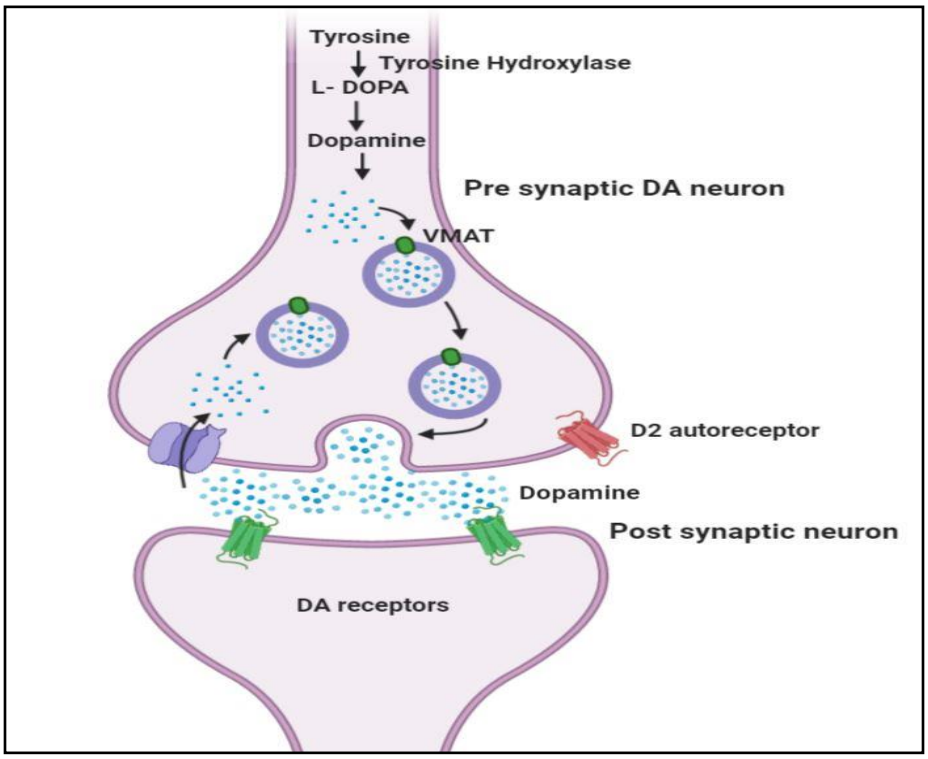


Figure 1.4. The DA pathway

DA is synthesized from tyrosine, converted to L- 3,4 dihydroxyphenylalanin (L-DOPA) by the enzyme tyrosine hydroxylase. Decarboxylation of L-DOPA produces DA. DA is then incorporated into the vesicles by the vesicular monoamine transporter-2 (VMAT-2). The vesicle fuse to the membrane and release their contents into the synaptic cleft. DA mediates its effect by binding to the post synaptic receptors and the extra DA is transported back with the help of the DA transporter (DAT). Presynaptic D2 receptors play role in autoregulation of the DA neurons, through feedback mechanism [Adapted from (Bravo et. al., 2014)].

1.3.2. The mammalian dopaminergic system

DA mediates its diverse effect by binding to its two distinct type of G-protein coupled receptors (GPCRs), the D1-like and D2-like class of DA receptor, with five different subtypes (D1, D2, D3, D4 and D5). The characterization is through pharmacological and biochemical differences between the receptor subtypes. The distinction is based on the similarity in the transmembrane domains of the receptors as it is highly conserved in the D1-D5 receptor subtypes belonging to the D1-like receptors and among D1, D2 and D3 receptor subtypes belonging to the D2-like receptors. Based on stimulating adenyl cyclase, where D1-like receptors D1 and D5 are stimulatory and D2-like receptors D2, D3 and D5 are inhibitory in nature (Martel and McArthur, 2020).

The analysis of DA receptors revealed that they are members of G protein coupled receptor family and contain seven transmembrane domains. The D1 like receptors have high homology within their TM domain, ligand binding site, with variable affinities for binding DA. D2 like receptors are pharmacologically more different from each other when compared to D1 like receptors.

1.3.3. Expression

DA receptors are widely expressed in the mammalian brain mainly in the DA neurons in the substantia nigra pars compacta, the VTA and hypothalamus, along with their expression in non-neuronal cells. The D1 receptors are localized in the entopeduncular nucleus, substantia nigra pars reticulata, striatum, hypothalamus, thalamus, limbic system localized on the GABAergic neurons. The D5 receptor is mainly localized in the hippocampus, lateral mamillary nucleus, lateral thalamus, striatum, cerebral cortex. D1 receptors are most abundant in nature, whereas D5 receptors have restricted expression. D1 and D5 receptors are coexpressed on some neurons of prefrontal area and hippocampus, having both pre- and post-synaptic localization. While both D1

and D5 receptors are largely present on GABAergic neurons, D5 receptor expression is also found on the cholinergic interneurons. The D2 like receptor D2 is mainly found in the striatum, nucleus accumbens in the GABAergic neurons. It has also been reported to be expressed in DA neurons found in the ventral tegmental area, hypothalamus and substantia nigra pars compacta. The D3 receptors are found on the limbic areas of the NAC and also in the VTA area on the DA neurons, but the expression is less in comparison to the D2 receptors. D3 receptors are also present in the regions with almost negligible contacts with DAergic axons, suggesting the extrasynaptic role of DA. The D4 receptors are highly expressed in the hippocampus, amygdala, frontal cortex and the hypothalamus, on the GABAergic interneurons. It has also been found to be significantly expressed in the retina (reviewed in Missale et al., 1998 and Beaulieu and Gainetdinov, 2011).

1.3.4. Structure and mode of action

The DAergic receptors belong to the GPCRs superfamily. Mode of action for the DAergic receptors, has always been of great interest, how they act to activate different complements of G proteins and other effectors independent of G protein signaling event. The GPCRs mediate their action through subset of G proteins classified as Gαs, Gαi, Gαq and $G\alpha_{12}$. The G protein is made up of three subunits, alpha, beta and gamma, but its classification is based on the nature of its alpha subunit. The binding event triggers a sequence of process, leading to release of GDP bound to the alpha subunit, binding of GTP and dissociation of the alpha subunit from rest of the complex that is now active to transduce the signal. The activation of Gαs leads to the stimulation of adenyl cyclase (AC), the Gαi inhibits cAMP production, and the free βγ complex can lead to the activation of other signaling pathways. The D1-like receptors can also mediate their effect through other G proteins like Golfα and in some cases to the inhibitory Giα/Goα (reviewed in Missale et al., 1998 and Beaulieu and Gainetdinov, 2011). The D2 like receptors play an inhibitory role and mediate their action by the inhibition of adenyl cyclase. The D2 presynaptic receptor, acts as an autoreceptor to regulate the release of DA. This receptor is specifically located on DAergic neurons and plays an important role in regulating the DA system through feedback inhibition regulating the synthesis, release and transport of DA. These autoreceptors are located on the soma dendrites of the mid brain DA neurons in the VTA and substantia nigra pars compacta region, they have also been shown to inhibit the excitation of DA neurons by activation of a hyperpolarizing potassium GIRK current. Autoreceptors work in a feedback loop, once activated through the potassium

conductance or the regulated gene expression of DA membrane transporter or expression of tyrosine hydroxylase. Activation of these receptors leads to reduction in DA neuron excitation and its release of DA. These receptors regulate DA neuron activity by inhibiting the vesicular DA release, on being released DA activates these autoreceptors to regulate its probability of frequent presynaptic stimulation, which helps in regulating DA release during prolonged bursts of action potential. D2 receptors can also exert regulation decreasing the synthesis of DA, through the inhibition of tyrosine hydroxylase. It is achieved through the inhibition of Ac and reduction in the phosphorylation in the regulatory domain of TH. This process also affects the filling of vesicles and thus distribution and expression of vesicular monoamine transporter (VMAT) is also affected. DAT DA transporter is responsible for clearance of DA from the extracellular space. D2 autoreceptors also exert regulation by increasing DAT activity. But this mechanism is activated only under high levels of DA, and selectively deleting D2 autoreceptors have no effect on DAT activity. It might only be activated during hyperactivation of D2 receptors or in a state of prolonged stimulation (reviewed in Beaulieu and Gainetdinov 2011).

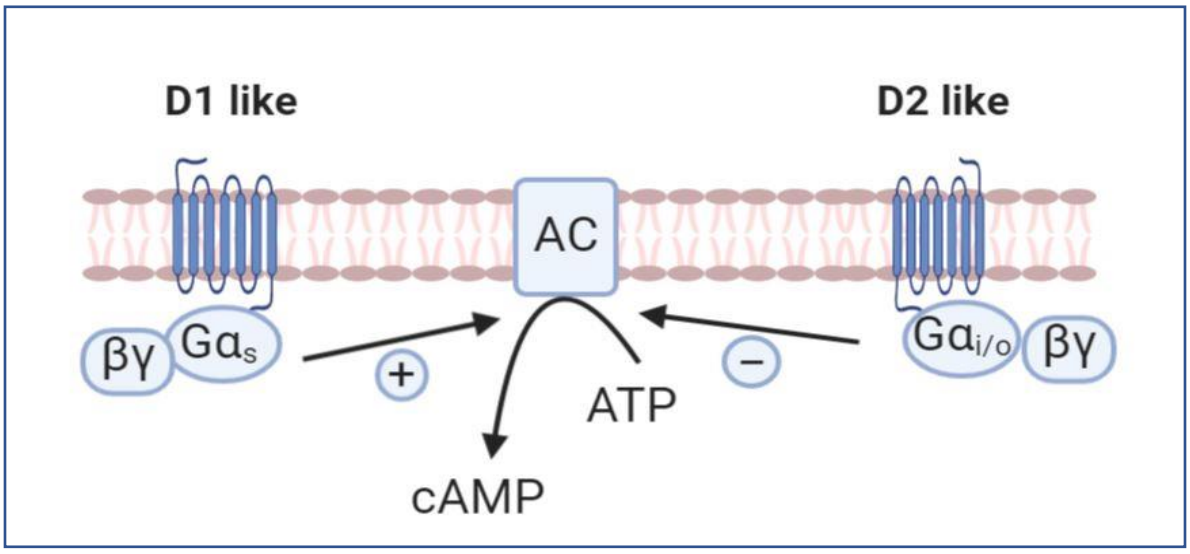


Figure 1.5. DA receptor

Both D1 and D2 like DA receptors are seven transmembrane G protein coupled receptors (GPCRs). They possess an extracellular amino terminus and seven putative membranes spanning alpha helixes connected to intracellular and extracellular protein loops. D1 like receptors are excitatory bound to stimulatory G protein, while the D2 like receptors are inhibitory in nature bound to inhibitory G protein, mediating their action by regulating the activity of cAMP [Adapted from (Tocris Bioscience, 2020)].

1.3.5. Function

DA regulates and modulates important aspects of locomotion, by binding to its receptors. Previously it has been reported to regulate the forward locomotion through the activation of its D1, D2 and D3 receptors present in the ventral striatum area (Missale et al., 1998). The D2 autoreceptor activation leads to decrease in the release of DA, modulating the locomotor activity, which is decreased significantly, while the postsynaptic D2 receptor activation leads to increased locomotor activity (Beaulieu and Gainetdinov 2011). Although the D1 and D3 receptors themselves do not have a significant contribution in regulating locomotion, their synergistic interaction helps in shaping the forward locomotion (White et al., 1988). The D3 receptor plays an inhibitory role in regulation of locomotion (Sibley, 1999; Joseph et al., 2002).

DA has always been related with the drugs of abuse, as they seem to mediate their action by enhancing the release of DA. The administration of these psychostimulants and drugs acts through the mesolimbic area to increase the release of DA (Adinoff, 2004). Both D1 and D2 receptor are involved in regulating this behavior (Missale et al., 1998). The most potent stimulants are the opiates, the mechanism of action is through disinhibition of the DA system through the inhibition of GABAergic neurons in the vicinity, which normally act to maintain the neighboring DA neurons under inhibition (Adinoff, 2004). The DA neurons in the mesolimbic system synapse on the dendritic spines of GABAergic neurons in NAC, both D1 and D2 type receptor are expressed on these neurons. The mesolimbic DA system gets inputs from different neurotransmitter systems, the cholinergic from brainstem, glutamatergic from the cortical structures and the GABAergic inputs from the nucleus accumbens, giving rise to the endogenous pleasure circuitry, which is activated by natural rewarding stimuluses, but also through the drugs of abuse (Philips and Shen, 1996). Alcohol is one of the most widely abused drugs, the GABAergic, cholinergic and serotonergic systems modulate ethanol excitation of the VTA. The dose of ethanol has regulatory effect on the release of DA from the striatal region, high concentration dampens while low doses increase DA via midbrain, while chronic ethanol exposure produces adaptations in DA release through the opioid system. An increase in the density of D1 DA receptors, has been observed on treatment with EtOH. Several studies have shown correlation between D2 receptor density and administration of psychostimulants and drugs of abuse. The blockade of D2 receptors lead to reduction in ethanol induced stimulation (Volkow et al., 1999,1996, Novak et al., 2000; Stefanini

et al., 1992; Philips et al., 1998). Studies have also shown that long term alcohol abuse leads to changes in gene expression and the receptor complexes for D2 receptors (Volkow et al., 1996, reviewed in Hui and Gang, 2014). Decrease in the binding efficiency of D2 receptors has been observed in the NAC of alcohol preferring rats when compared non-alcohol preferring ones, and upregulation of D2 receptor in alcohol preferring rats leads to reduction in alcohol consumption and preference, which was also found effective in the case of cocaine abuse (Thanos et al., 2004; Thanos et al., 2002).

Table 1.1. Mechanism and Function of mammalian DA receptors [Adapted from (Mishra et al., 2018)].

Receptors	Type	Mechanism	Function
D1	Gs coupled	Increased cAMP by activation of adenylate cyclase	Locomotion, memory, attention, impulse control, and regulation of renal function
D5	Gs coupled	Increased Adenylate cyclase	Cognition, Decision making, attention, motor learning and renin secretion
D2	Gi coupled	Increased cAMP by activation of adenylate cyclase	Attention, locomotion, sleep, memory and learning
D3	Gi coupled	Decrease in Adenylate cyclase	Attention, locomotion, cognition, impulse control, sleep
D4	Gi coupled	Decrease in Adenylate cyclase	Impulse control, cognition, attention, sleep

1.4. *C. elegans* Dopaminergic system

The *C. elegans* DA system is very compact, with merely eight DA neurons as compared to ~500,000 neurons in the ventral midbrain of humans (Sulston et al., 1975) and reviewed in (Hegarty et al., 2013).

1.4.1. Expression

Six DA neuron are found in the anterior region, with two pair of CEPs and a pair of ADE neurons and a pair of PDE neuron in the posterior region. While male have six additional DA neurons located in the tail. High conservation is found in the synthesis pathway and other molecular players of the DA signaling pathway in *C. elegans* to those in mammals. These neurons are usually mechanosensory in nature as their dendrites have microtubule containing cilium, which are involved in detecting movement and external cues like food. The CEP neuron has a single long dendrite running from the cell body near the nerve ring throughout the length of the head and ends where the cilia penetrates the cuticle near the nose of the worm. The ADE dendrites travel all the length into the deirid sensilla penetrating it with their ciliated endings, the cell body of ADE is located behind the second bulb of pharynx. The axons of CEP and ADE neurons are directed towards the nerve ring and the dorsal pair of CEP neuron receives some synaptic inputs from ADE. The cell body of PDE neuron is found on the posterior side of the vulva, its axon processes extend near the nerve ring and enter the nerve cord. It sends and receives synaptic connections from and to other neurons (Reviewed in Nass and Blakely, 2003).

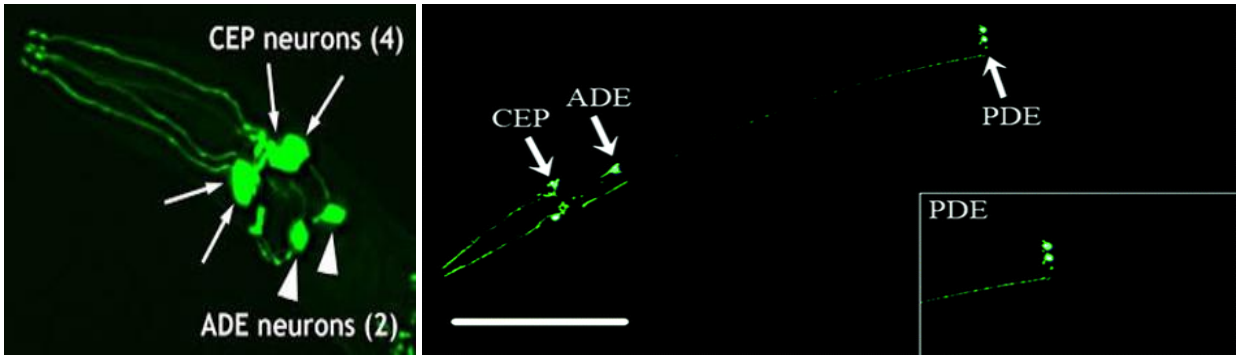


Figure 1.6. Dopaminergic Neurons in *C. elegans*.

Representative figure and image of *C. elegans* dopaminergic system [Image taken from (Doitsidou et al., 2018)].

1.4.2. The *C. elegans* DA pathway

The DA pathway is remarkably conserved in *C. elegans* from the mammalian system. The neurons express genes encoding similar synthesis pathway enzymes the tyrosine hydroxylase (TH/*cat-2*), dopa decarboxylase (*bas-1*), the transporters VMAT vesicular monoamine transporter (*cat-1*), DA membrane transporter (*dat-1*), and along with the receptors and autoreceptor (*dop-2*). The DA neurons have been shown to function both through classical synapses and extrasynaptically, being released in the body cavity. All the DA neurons are mechanosensory in nature expressing TRP-4 a mechanoreceptive channel, that helps in sensing stress arising due to the external surface, on and off food, modulating the locomotory behavior of the worms (Sawin et al., 2000; Li et al., 2006). DA mediates its action by binding to its wide variety of receptors that are expressed abundantly in both neuronal and non-neuronal cells. The DA receptors are comprised of seven transmembrane superfamily of G-protein coupled receptors (GPCRs) and similar to their mammalian counterparts, they are divided into D1-like and D2-like receptors, (DOP-1 and DOP-4) and (DOP-2 and DOP-3) respectively (Suo et al., 2003). While the D1 like receptors are expressed both in neuronal and non-neuronal cells like the muscles and glial cells, the D2 like receptors have expression only on the neurons exerting a neuromodulatory role (Suo et al., 2003). DOP-2 a D2 like DAergic autoreceptor is also expressed on the DA neurons, functioning in a negative feedback loop. DA regulates several important behaviors by binding to these receptors, such as locomotion, food response, mating behavior, learning and memory and egg laying. Among all these behaviors the regulation of locomotion through DA is observed across different species and in *C. elegans* too DA regulates different aspects of locomotory behaviors.

1.4.3. Function

A classic example of modulation of behavior by DA in response to external cues observed in *C. elegans* is the adjustment of speed i.e., is the basal slowing behavior observed in the presence of food and alteration in locomotion behavior off food (Sawin et al., 2000). DA modulates the locomotion behavior of the animal so that it remains in food enriched area and regulates two important aspects of the foraging behavior, the local and global search behavior (Hills et al., 2004).

Most of the behaviors controlled by DA are revealed by the analysis of *cat-2* mutant animals, as CAT-2 encodes for a rate limiting step enzyme tyrosine hydroxylase, and thus these mutants have significantly decreased levels of DA. Yet contradictory to this statement the *cat-2* mutants still have significant levels of DA, thus there are still a wide array of DA regulated behaviors other than those observed in *cat-2* mutants. DA functions to modulate the locomotory behavior of the worm, a classical example is the antagonistic action of the DOP-1 and DOP-3 receptors coexpressed in the motor neurons, they act to modulate the locomotion rate. DOP-1 receptors also play an important role in habituation behavior, loss of DOP-1 causes defects in animals' ability to habituate to a stimulus, here tapping response (Sanyal et al., 2004). DA also helps to modulate and shape behavior by analyzing the external environment and its internal physiological state, like the animals show rapid habituation to the escape response in the presence of food, as the mechanosensory DA neurons help in sensing (Kindt et al., 2007). DA also modulates several behaviors in *C. elegans* by fine tuning between DA and serotonin, such as egg laying, pharyngeal pumping, and swimming (Horvitz et al., 1982; Weinshenker et al., 1995). Also age-related changes in the locomotion behavior have been linked with DA/serotonin balance in the older animals (Murakami et al., 2008). DA is involved in the process of gait transition in higher organisms, in *C. elegans* also we observe a similar pattern, D1 like receptors are shown to be regulating these two distinct forms of locomotory gaits, swimming and crawling, yet another example of how important DA is for maintaining normal movement behavior in animals (Vidal-Gidea et al., 2011). Thus, DA is known to regulate the motor outputs of an animal in context dependent manner. The D1 and D2 like receptors regulate important aspects of locomotory behavior, but the role of D2 autoreceptor is still unclear, as of how it contributes to shape the motor outputs. The D2 autoreceptor signals through Gαi, acting in a negative feedback loop to regulate the release of DA. DOP-2 is involved in regulating the associative learning and habituation behavior of the animals (Voglis and Tavernakaris, 2008; Mersha et al., 2013).

Table 1.2. Mechanism and Function of *C. elegans* DA receptors [Adapted from (Sharma et al., 2013)].

Receptors	Type	Mechanism	Function
DOP-1	Gαs	Increased cAMP by activation of adenylate cyclase	Habituation to tapping, basal slowing behavior, gait transition
DOP-4	Gq	Increased Adenylate cyclase	Avoidance response, disinhibition of EtOH induced behaviors
DOP-3	Gαo-coupled	inhibit the activation of adenylyl cyclase	Basal slowing behavior, negative regulation of locomotion
DOP-2	Gαi/o	inhibit the activation of adenylyl cyclase	Associative learning, anterior touch habituation

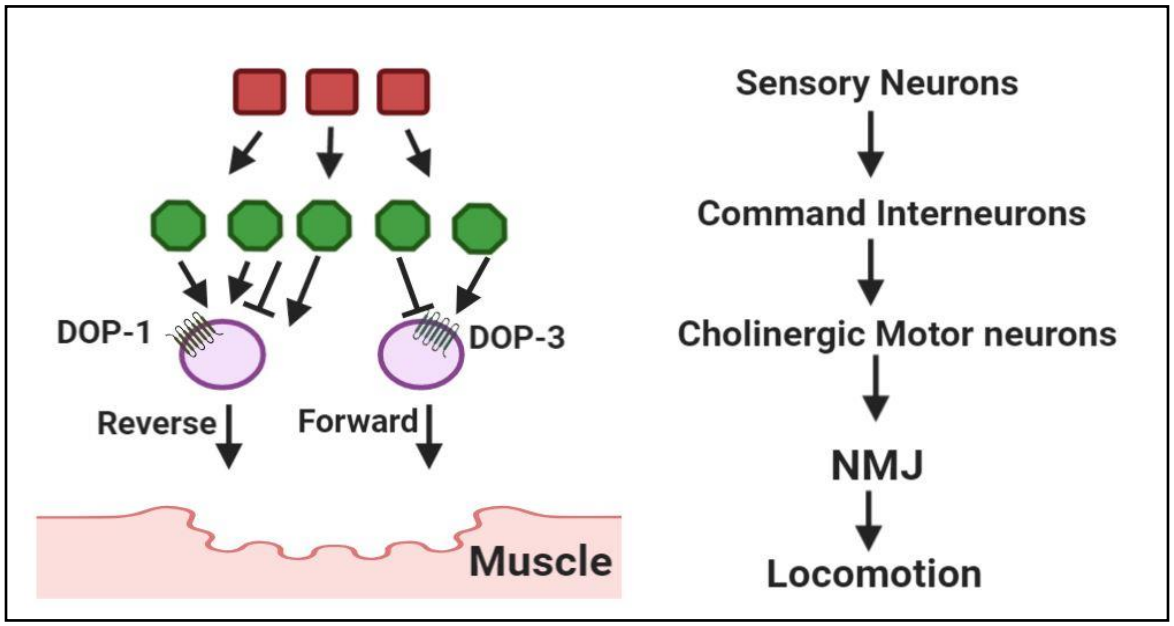


Figure 1.7. Schematic of DA mediated regulation of locomotion

Many sensory neurons innervate the command interneurons, which integrate these signals on cholinergic motor neurons, activating them, to regulate the forward and backward locomotion. The DOP-1 and DOP-3

receptors coexpressed on the cholinergic motor neurons regulate locomotion through their antagonistic regulation, excitatory and inhibitory nature respectively [Adapted from (Chase et. al., 2004)].

1.5. Dopamine and alcohol

D2 auto receptors in mammals are involved in altering the effects of drugs of abuse, they are involved in regulating the behavior of the animals in the presence of EtOH, cocaine and morphine. Most of these drugs of abuse lead to increase in the extracellular levels of DA, whose exposure leads to long term changes like addiction. In *C. elegans* too the DA receptors are involved in regulating drug induced behaviors. The D1 like receptor DOP-4 is involved in regulating disinhibition of behaviors on EtOH exposure, similar D1 receptor function is also observed in the mammalian system, involved in locomotor disinhibition (Melendez et. al., 2002), similar effects were observed in Drosophila (Kong et. al., 2010). A lot of studies have reported a correlation between D2 receptors and alcohol consumption and development of alcoholism (Kraschewski et al., 2009; Lu et al., 2001; Thanos et al., 2005; Volkow et al., 2006). Moreover, studies have also shown that D2 receptor levels are enhanced in alcoholics and that attenuation in the ligand activation of D2 receptors drives craving and relapse of alcoholism (Feltmann et al., 2018; Volkow et al., 2013). The DOP-2 autoreceptor itself does not shows any locomotory behavior regulation, as it is likely that behaviors associated with deletion of neuromodulatory molecules are not easily observable in native conditions since they are required to modulate multiple behaviors and not one specific behavior (Ford, 2014). As ethanol (EtOH) has been shown to increase the release of DA from the mammalian ventral tegmental area and also increased DA levels were found in the nucleus accumbens (Imperato and Di Chiara, 1986; Weiss et al., 1996; Yim and Gonzales, 2000).

1.6. *C. elegans* as a model for alcoholism

C. elegans is a well-established model for studying the conserved molecular targets for altered behavioral response on EtOH exposure. Studies have reported that EtOH administration shows dose dependent decline in the locomotor activity of the worms with increasing levels of EtOH exposure, which is similar to the depressive effects of EtOH observed in other animal systems (Davies et al., 2003; Alaimo et al., 2012; Hawkins et al., 2015). The internal dose of EtOH found responsible for this behavior is similar to that observed in mammalian systems, i.e., 20 to 30mM internal EtOH concentration in *C. elegans* corresponding to the intoxication levels in mammals 0.1%, i.e., 21.7mM, indicating that there might exist similar molecular targets (Lee et al., 2009). EtOH has also been shown to regulate learning that is state dependent through the DA pathway

(Lee et. al., 2009). Several molecular targets of EtOH have been identified in *C. elegans*, about 50 genes have been identified and their orthologs have been found in humans that have implications on AUDs (Grotewiel and Bettinger, 2015). One of the major targets identified is the SLO-1 BK potassium channel, *slo-1* mutants show resistance to the effects of ethanol. Ethanol mediates its effect by activating conductance through these potassium channels, that are encoded by *slo-1* gene, at similar concentrations found intoxicating in mammals (Davies et al., 2003). Several studies have shown that EtOH activates and has a direct effect on these BK channels in mammalian system (Dopico et al., 1996,1998) and also in Drosophila (Cowmeadow et al., 2005). The BK potassium channel is activated in *C. elegans* at 20-100mM concentration and show a state similar to EtOH intoxicated animals. It mediates the effect of acute EtOH exposure, which in turn leads to the activation of these channels and depresses neuron firing (Davies et. al., 2003). A neuropeptide receptor gene npr-1 has been shown to provide tolerance against EtOH, by Davies in 2004, based on recovery from EtOH induced decrease in the locomotor activity. Tolerance is developed on long term exposure to a given concentration of EtOH, differentiated into chronic and acute tolerance dependent on the exposure sessions. It may be defined as gradual decrease in response to EtOH administration on prior exposure. While the WT Bristol strain shows tolerance after 50 minutes (min) of EtOH exposure, the *npr-1* mutant strain shows greater tolerance over the same time (Davies et al., 2004). Since both loss of D2-like receptors and EtOH tend to increase DA levels, we went on to test *dop-2* deletion mutants for movement defect/s in the presence of EtOH and found a robust behavior involving decrease in body bends and the flattening of the body bends which we have termed Ethanol Induced Sedative (EIS) behavior.

Table 1.3. Known molecular targets of EtOH in *C. elegans* [Adapted from (Khanh, 2018)].

Molecular Targets	Effect	Studies Conducted
SLO-1 BK potassium channel	Locomotion and egg laying	Davies et. al., 2003
SLO-1 BK and SK channels	Modulation of activity of neurons involved on reward and reinforcement behavior	Brodie et. al., 2007
EGL-3, NPY and CRF	Ethanol withdrawal	Mitchell et. al., 2007 Davies et. al., 2004
SER-4 and SER-7 serotonin receptors tph-1 and ser-4	EtOH mediated gustatory plasticity Locomotion	Wang et. al., 2011
slo-1, rab-3 and unc-64 unc-79	Resistance to EtOH Hypersensitivity to EtOH	Davies et. al., 2012
ADH encoding gene sodh-1 and ALDH encoding gene alh-6 or alh-13	Hypersensitivity to EtOH	Alaimo et. al., 2012
D1 like receptor DOP-4	EtOH induced disinhibition of crawling	Topper et. al., 2014
T3521 mutation in BK channel	Conserved role of BK channels across species	Davis et. al., 2014
nAChR EAT-6 (Na/K ATPase)	Ethanol induced excitation (EHC) Tolerance against EtOH effect (EHC)	Hawkins et. al., 2015
SWI/SNF chromatin remodeling complex	Altered sensitivity and tolerance against EtOH	Mathies et. al., 2015

Part III. NEUROPEPTIDE MEDIATED REGULATION OF BEHAVIORS IN *C. elegans*

1.7. Neuropeptide signaling

Neuropeptides acting as neurotransmitters (NT) are short stretches of amino acids, involved in modulating synaptic activity either through classical synapses or extrasynaptically. They can also act extrasynaptically as a short-range NT signal or a hormone acting on site other than nervous system, mediating regulation at different levels. There are about 113 genes that encode for over 250 unique neuropeptides, in *C. elegans* (Li et al., 1999). These neuropeptides are divided into three subfamilies, the FMRF amide- like peptide (FLPs) that consist of 71 different peptides (Li, 2005), the insulin like peptides comprising of 40 peptides (Li et al., 2003), and a third family called neuropeptide like proteins, the largest group comprising of about 155 neuropeptides (Janssen et al., 2010).

Precursor neuropeptides undergo posttranslational modifications at their N - and C - terminus to give rise to a mature peptide (Sieburth et al., 2005). These mature peptides are then packed and stored in the dense core vesicles (DCV), released at the site of action either at the synapse or extrasynaptically in a paracrine manner. A kinesin motor protein UNC-104 along with some other trafficking proteins like IDA-1 and UNC-116, is involved in the transportation of these DCVs (Hall et al., 1991; Zahn et al., 2004). These DCV are scattered all over the nerve terminus compared to the NT vesicles that are confined to the synaptic zones (Salio et al., 2006). These DCVs release their content with the increase in calcium current all across the nerve ending, during stimulation, releasing around 18000 signaling molecules (Salio et al., 2006). A proteolytic enzyme like NEP-1 neprilysin clean these neuropeptides after their release, as they are not recycled back like NT (Sieburth et al., 2005). Here, I discuss about a neuropeptide NLP-12, that has emerged as a major regulator of locomotion under different conditions in *C. elegans*.

1.8. Neuropeptides along with NT modulate different behaviors

Neuronal connectivity in the brain is shaped through different context and the internal state of the organism, which basically represents the neuromodulators. Also, a single network can be modified in several ways by these modulators to generate a number of related behaviors. The complexity of synaptic plasticity and intrinsic properties observed in vertebrate neuronal cells is almost similar to the invertebrate system. Locomotion is one of the many behaviors influenced by

neuromodulation, with several variants like forward, backward, walking and running. *C. elegans* is a great model organism to study these neuromodulatory molecules, with a compact nervous system, consisting of classical NTs, about 100 neuropeptides and biogenic amines modulators as DA, serotonin, tyramine and octopamine. In *C. elegans* these biogenic amines modulate different behaviors, serotonin and DA regulate food related behaviors like feeding, egg laying and the locomotory behavior. The locomotory behavior is dependent upon the availability of food and feeding state of the organism, regulated by DA, serotonin and neuropeptide modulation.

As discussed previously neuromodulators act to shape the behavior of an organism in response to an external cue or the internal physiological state. It is very complex to understand this system how it acts across circuits to regulate these behaviors. Neuropeptides often act by modulating the signaling across synapses, thus altering the neuronal circuit activity and excitability. They have been shown to modulate motor pattern across different species through neuromodulation along with other NTs (Marder, 2012; Nusbaum and Blitz, 2012). In *C. elegans* too a neuropeptide NLP-12 shows context dependent modulation of foraging behavior. The locomotory behavior of the worm is highly influenced by the availability of food. Classical example of food modulated behavior comes from the DAergic system, the basal slowing behavior, where the animal slows down its movement on encountering a food patch. DA receptors DOP-1 and DOP-3 function antagonistically to regulate this behavior (Chase et al., 2004). It also shows changes in locomotory behaviors such as turning frequency, dispersal on unavailability of food where it switches from its area restricted search behavior to global search, for survival (Hill et al., 2005). Thus *C. elegans* locomotory behavior is context dependent and is highly shaped by the availability of food.

1.9. Neuropeptide NLP-12 mediated modulation of behavior

NLP-12 is a *C. elegans* neuropeptide and a homolog of mammalian Cholecystokinin (CCK). It modulates the behavior of organism by altering the motor circuits involved in regulating the body wall muscles. It also modulates the local search behavior, dependent on context like varying oxygen levels and food availability (Bhattacharya et al., 2004; Hums et al., 2016). NLP-12 has been also shown to regulate locomotion through proprioception and control the posture of worm (Hu et al., 2011; Hu et al., 2015). Further studies have explored the mechanism through which NLP-12 mediates such regulation. NLP-12 acts through two distinct GPCR receptors CKR-1 and

CKR-2 and influences the area restricted search behavior (ARS). ARS is characterized by rapid change in the trajectories to restrict the animal in their present environment. A study by Bhattacharya et. al. has shown DA mediated modulation of NLP-12 neuropeptide signaling that regulates the foraging behavior of the worm. This study shows the role of D1 like DA receptor DOP-1 in regulating the NLP-1/CCK signaling in a context dependent manner. NLP-12 expression is restricted only to a mechanosensory interneuron DVA, that plays role in both positive and negative regulation of locomotion.

DVA is a mechanosensory interneuron, that regulates locomotion through proprioception. It has been shown to both positively and negatively modulate locomotion. It is stretch sensitive neuron, with TRP-4 mechanoreceptive TRP (Transient Receptor Protein) channels. A Ca^{2+} mediated positive regulator of DVA promotes the muscle contraction, which is then sensed by TRP-4 channels present on DVA neuron acting as a negative regulator and prevents further muscle contraction, thus maintaining a fine tuning of motor activity through proprioception (Li et al., 2006). Later, Hu et al. have shown a mechanosensory feedback system, where the stretch sensitive neuron DVA is involved in regulating locomotion through proprioception. DVA secretes a neuropeptide NLP-12 in response to muscle contraction, it then binds to its receptor CKR-2 present on cholinergic motor neurons, leading to enhanced cholinergic transmission at the NMJs. Thus regulating the locomotor behavior through this feedback loop (Hu et al., 2011). Bhattacharya et al., show that DVA also modulates locomotion and control body posture of the animal through sensory cues such as food, which activates the cholinergic motor neurons through L-AChR receptors, leading to muscle contraction, sensed by mechanoreceptive TRP-4 channels (Bhattacharya et al., 2014).

DVA is situated postsynaptic to a posterior DAergic neuron PDE, whose role has been reported in food sensing and is positioned just presynaptically to both motor neurons and interneurons involved in regulating locomotion (White et al., 1986). PDE sends very strong synaptic connections on DVA, mediating its action through the DOP-1 receptors present on DVA neuron. DA has also been shown to act extrasynaptically, thus inputs from anterior DAergic neurons CEP and ADE may also be contributing to the behavior, as all the three DA neurons are involved in modulating the foraging behavior of worm. The olfactory neuron AWC also plays an important role in food sensing and feeds this sensory information of food availability through

mechanosensory DAergic neurons, thus modulation of foraging behavior through DVA neuron is dependent on both direct and indirect inputs from the mechanosensory DA neurons (Bhattacharya et al., 2004, 2005). A recent study has shown DA mediated regulation of foraging behavior, that affects locomotion through the action of two distinct neuropeptides FLP-1 and NLP-12/ CCK pathway, acting from AVK and DVA neurons respectively (Oranth et al., 2018). Thus DVA emerges as an important regulator of locomotion in all these studies, modulating the movement behavior directly by mechanosensation or through signals received from other neurons like the DAergic neurons that shapes the locomotory behavior.

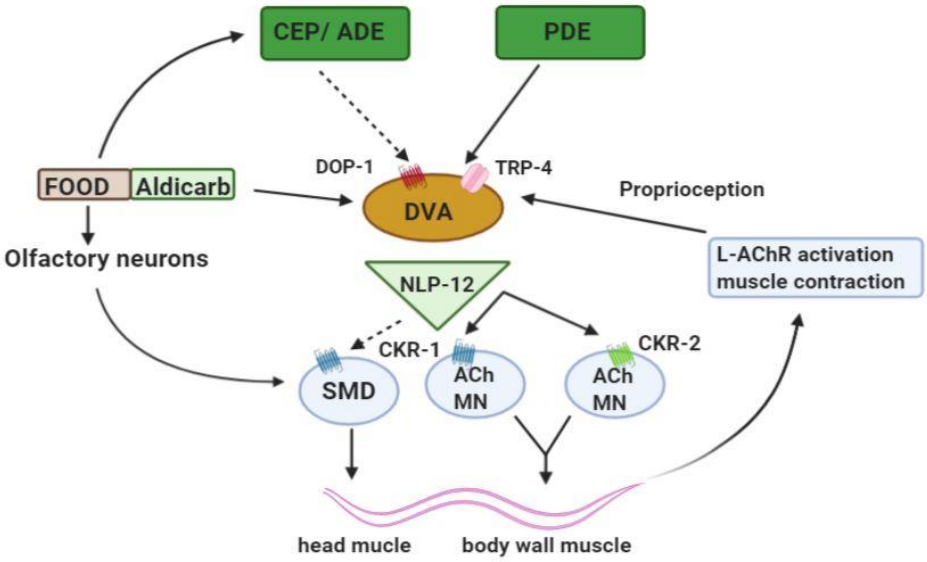


Figure 1.8. DVA and NLP-12 circuitry: modulation of locomotory behavior

DVA modulates locomotion through a neuropeptide NLP-12, specific to it, under different conditions. A cholinesterase inhibitor (aldicarb) has been shown to induce synaptic potentiation by NLP-12, released by activation of DVA, a stretch sensitive neuron, expressing mechanosensory TRP-4 channels, acting in a proprioceptive feedback loop (Hu et. al.; 2011). DVA receives synaptic inputs from a DAergic neuron PDE, and extrasynaptic actions of DA are represented by dashed line, from CEP and ADE head DAergic neurons, involved in regulating foraging behavior, once activated they activate the downstream signalling pathway through DVA interneuron, releasing NLP-12, which bind to its receptors CKR-1 and CKR-2 present on cholinergic motor neurons, thus modulating Ach release and excitation of body wall muscles [Adapted from (Bhattacharya et al., 2014; Ramachandran et al., 2020, Hu et al., 2011)].

CHAPTER II

Materials and Methods

SECTION A: Materials

2.1. Chemicals and reagents

Molecular and analytical grade chemicals used for this study were obtained from commercial sources. The components of Nematode Growth Media (NGM) and other fine reagents were obtained from Sigma Aldrich USA, Merck India, HI Media India and Difco, USA. Primers were designed manually or using IDT software, map construction and visualization was done using Snapgene software, which were then synthesized from IDT and Eurofins. All the enzymes used for restriction cloning (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Red Taq DNA polymerase and other modifying enzymes), their buffers and DNA ladders were purchased from New England Biolabs Inc, USA and Biochem, India. The Miniprep plasmid and Gel-extraction kits were obtained from Qiagen, USA or Bioneer, Korea. Important chemicals used for the study, Ethanol (molecular assay grade), Dopamine (DA), Tetramisole hydrochloride (Levamisole), Aldicarb PESTANAL and 2,3-butanedione monoxamine (BDM) were procured from Fischer Scientific, USA.

2.2. Strains and plasmids

The plasmids and strains used in this study are listed in Table 2.1 and 2.2. The *C. elegans* strains used in this study were mostly obtained from Caenorhabditis elegans Genetics Centre and the plasmids were obtained from Addgene. The strains and plasmids that are obtained from other labs are mentioned in Table 2.1 and 2.2.

Table 2.1. List of plasmids used in this study.

S. No.	Plasmid No.	Plasmid	Source
1	pBAB911	*dop-2p::dop-2::cfp*	Rene Garcia Lab
2	pBAB912	*nlp-12p*::GFP	This Study
3	pBAB913	*nlp-12p*::NLP-12	This Study
4	pBAB914	*nlp-12p*::*sl2*::*wrm*Scarlet	This Study
5	pBAB915	*nlp-12p::dop-1::* sl2::*wrm*Scarlet	This Study
6	pBAB916	*gpa-14p::dop-2*	This Study
7	pBAB917	*gpa-16p::dop-2*	This Study

Table 2.2. List of strains used in this study.

Strain	Genotype	Comments
BAB912	*dop-2(vs105)V 4X* outcrossed (original strain LX702)	CGC strain
BAB913	*dop-3(vs106)X 2X* outcrossed (original strain LX703)	CGC strain
BAB914	*dop-1(vs100)X 2X* outcrossed (original strain LX645)	CGC strain
BAB915	*cat-2(n4547)II 3X* outcrossed (original strain MT15620)	CGC strain
PR1152	*cha-1(p1152)IV (outcrossed 4X)*	CGC strain
BAB916	*acr-16(ok789)V 2X* outcrossed (original strain RB918)	CGC strain
BAB917	*slo-1(eg142)V 2X* outcrossed (original strain BZ142)	CGC strain
BAB918	*dat-1(ok157)III 3X* outcrossed (original strain RM2702)	CGC strain
LSC32	*ckr-2(tm3082)III (outcrossed 8X)*	CGC strain
BAB920	*ckr-1(ok2502)I 2X* outcrossed (original strain RB1923)	CGC strain
BAB919	*nlp-12(ok335) 3X* outcrossed (original strain RB607)	CGC strain
nuIs299	*myo-3p*::*acr-16*::*gfp*	Josh Kaplan Lab
IR724	*asic-1*::*snb-1*::SEpHlourin	Blakely Lab
BY834	*dat-1p*::*dat-1*::*gfp*	Blakely Lab
BZ555	egls1(*dat-1p*::gfp)	CGC strain
BAB900	*dop-1(vs100); dop-2(vs105)*	This study
BAB901	*cat-2(n4547); dop-2(vs105)*	This study
BAB902	*egls1*(*dat-1p*::*gfp*); *dop-2(vs105)*	This study
BAB903	*dat-1p*::*dat-1*::GFP; *dop-2(vs105)*	This study

BAB904	*asic-1*::*snb-1*::SEpHluorin; *dop-2(vs105)*	This study
BAB905	*dop-2p*::DOP-2::CFP;*dop-2* (IndEx905)	This study
BAB906	*nlp-12p*::*nlp-12* (IndEx906)	This study
BAB907	*nlp-12p*::*dop-1*::*wrm*Scarlet; *dop-1(vs100); dop-2(vs105)* (IndEx907)	This study
BAB908	*nlp-12p*::*dop-1*::*wrm*Scarlet;*dop-1(vs100)* (IndEx908)	This study
BAB909	*myo-3p*::*acr-16*::*gfp*; *cat-2(n4547)*	This study
BAB910	*cha-1(p1152); dop-2(vs105)*	This study
BAB911	*ckr-2(tm3082); dop-2(vs105)*	This study
BAB921	*ckr-1(ok2502); dop-2(vs105)*	This study
BAB922	*ckr-2(tm3082); nlp-12p*::NLP-12 (IndEx906)	This study
BAB923	*gpa-16p*::DOP-2*; dop-2* (IndEx909)	This study
BAB924	*gpa-14p*::DOP-2*; dop-2* (IndEx910)	This study
BAB925	*nlp-12(ok335); dop-2(vs105)*	This study

Table 2.3. List of oligonucleotides used in this study.

Primer Code	Sequence	Comment	Gene
PRS37	CCCTTGAATGGCCTCCACC	Genotyping Forward External	*dop-2*
PRS38	CAGTACTCCGGTACCGAGCAC	Genotyping Forward Internal	*dop-2*
PRS39	CTCGGGAGCACTTGTGAGAG	Genotyping Reverse External	*dop-2*
PRS23	TCACAGATGTCCGTTTTCCA	Genotyping Forward External	*acr-16*

PRS24	TCAATGATTCCGAGTGACGA	Genotyping Reverse External	*acr-16*
PRS 314	GTGCCTGGAGGAGCGCAAATATTGG	Genotyping WT Forward	*slo-1*
PRS 315	GTGCCTGGAGGAGCGCAAATATTAA	Genotyping Mutant Forward	*slo-1*
PRS 316	GGACTTGCCCTGCGGTCCCGAATAC	Genotyping Reverse	*slo-1*
PRS 322	CCAAATTAGTCGAAAAGCTGATCCCGC	Genotyping Forward External	*dat-1*
PRS 323	GTGATCCTTGCCTGGGGGCTTC	Genotyping Forward Internal	*dat-1*
PRS 324	GAAGCCCCCAGGCAAGGATCAC	Genotyping Reverse External	*dat-1*
PRS 332	GGAATAGGAACCATAGAAGATCTCC	Genotyping Forward External	*cat-2*
PRS 333	CGATGACTGTGACACCGCGAGG	Genotyping Reverse External	*cat-2*
PRS 334	GGCCGAGAACTGATAACCCAGC	Genotyping Reverse Internal	*cat-2*
PRS 340	GGACCCAAACATGCCACAGTGATATGG	Genotyping Forward External	*dop-1*
PRS 341	GAAGATTCAGGCGAGTTGCATTCGC	Genotyping Reverse External	*dop-1*
PRS 342	GAATGCTCGTCTAAAGTCACGATTG	Genotyping Forward Internal	*dop-1*
PRS 343	GGTGTTCGCAATATTTGCGAAGACG	Genotyping Forward External	*dop-3*
PRS 344	CCATCAGCGTGCTTTACTCGTTCAC	Genotyping Reverse External	*dop-3*
PRS 345	GTGACGGTTTGTAGAGATCGTTCTC	Genotyping Forward Internal	*dop-3*
PRS587	GTGTGCCGAGAAAAGTCCACTG	Genotyping Forward	*cha-1*
PRS588	CTCGATCCGGTTGAATATTGTACG	Genotyping WT Reverse	*cha-1*

PRS588	CTCGATCCGGTTGAATATTGTAAA	Genotyping Mutant Reverse	*cha-1*
AS7	ACCTGGCGGGGGAAACAACA	Genotyping Forward External	*ckr-2*
AS8	CCGTCAGCGAAATCTATCGT	Genotyping Reverse External	*ckr-2*
AS5	CACTCCATCACTACGACAGT	Genotyping Forward Internal	*ckr-2*
PRS 473	CCCCCCGGGATGAACGATTTGCAATGG CC	Cloning Forward XmaI	*dop-1* cDNA
PRS 474	CCCCCCGGGCTATTCCGGAATGGTTTC CTCG	Cloning Reverse KpnI	*dop-1* cDNA
AS1	AACTGCAGGGCCGAGACGAATCCGGA GG	Cloning Forward PstI	*nlp-12p*
AS4	CGGGATCCGCATTTTGTCGGAGGCAAT TG	Cloning Reverse BamHI	*nlp-12p*
AS3	CGGGATCCGAAAATGTGTCGCTTCGAG AC	Cloning Reverse BamHI	NLP-12
PRS 605	ACATGCATGCCCTCTCTTCGTCTTCTTC TTC	Cloning Forward SphI	*gpa-14p*
PRS606	CCCCCCGGGCTCAACTATAATGTCCTG AAATAC	Cloning Reverse XmaI	*gpa-14p*
PRS607	ACATGCATGCGATTCTTAGCATTTTTGCC	Cloning Forward SphI	*gpa-16p*
PRS608	CCCCCCGGGCTGCAAAAAAATTTGGG ATTTTTAGC	Cloning Reverse XmaI	*gpa-16p*
PRS609	CAGTATCTTATTCCAGAGCATCG	Genotyping Forward External	*ckr-1*
PRS610	GAGAATTAATTGGGAGGATGAAC	Genotyping Reverse External	*ckr-1*
PRS611	CACGTGTGACTCCACGAAAG	Genotyping Forward Internal	*ckr-1*
PRS317	AGAGACATGGAATGTCTCCC	Cloning Forward NheI	*dop-2*
PRS318	GGTGGTAAAGTCATTGTTCGTCCGCCT AC	Cloning Reverse XmaI	*dop-2*

SECTION B: Methods

2.3. *C. elegans* strain maintenance.

Animals were maintained according to standard procedure as mentioned by Brenner (1974), at 20^{o}C using OP50 *Escherichia coli* as food, seeded on nematode growth media plates. The N2 Bristol strain were used as Wild Type (WT) control animals in the study. The animals were maintained in the laboratory at 20^{o}C and 16^{o}C conditions respectively, along with long term storage by making stocks stored in liquid nitrogen. The Sodium hypochlorite solution was used to synchronise worms, for the desirable stage used for behavioural assays and experiment in the study, and this treatment also significantly removes any bacterial and fungal contamination.

2.4. *C. elegans* genomic DNA isolation.

Worms were synchronized and grown on OP50 plates till adult stage. They were collected from these plates using 1ml of M9 buffer into microcentrifuge tubes (MCT), pelleted using gravity for 2-3 min, after with the supernatant was discarded, and the worms were again resuspended in M9, this step was repeated two-three times to wash thoroughly, to remove food (OP50). The final worm pellet was resuspended in 600 µl of Cell lysis solution containing Tris, EDTA and SDS, tubes were inverted to mix thoroughly, the above solution was exposed to three simultaneous freeze / thaw cycles using liquid nitrogen. 6 µl of proteinase K was added mixed by inverting the tubes, the tubes were then incubated at 55^{o}C for 1 hour (hr) for proper lysis. 3 µl of RNase A solution was added, mixed, and incubated at 37^{o}C for 30 min to degrade RNA in the cell lysate. 200 µl of protein precipitation solution of ammonium acetate was added and mixed by vortexing vigorously for 20 seconds (sec), tubes were incubated on ice for 5 min, a white pellet is formed, centrifuge at 4^{o}C for 3 min at 13,000 revolutions per minute (rpm). Decant the supernatant to MCT containing 600 µl of iso- propanol, mix by gently inverting the tubes for 50 times, precipitated genomic DNA is visible as thin white thread. Pellet the DNA by centrifugation at 13,000 rpm for 1 min. Wash the small white pellet using 600 µl of 70% ethanol (EtOH), by inverting the tubes, again pellet the DNA by centrifugation at 13,000 rpm for 1 min. Completely remove the supernatant and dry the pellet by keeping tubes at 37^{o}C with lid opened foe 5-10 min. Add 30 µl of (TE) Tris and EDTA buffer, incubate the tubes at 65^{o}C for 45-60 min till the DNA pellet dissolves. Store the purified genomic DNA at -20^{o}C.

2.5. Constructs and transgenes

All constructs were generated using pPD95.75 as the backbone with standard restriction digestion cloning procedure (Russell, 2001). Transgenic lines were generated by microinjecting desired plasmid as described earlier (Mello et al., 1991; Mello and Fire, 1995).

nlp-12 promoter used in pBAB911 was cloned by amplifying a 355-base pair (bp) upstream region of nlp-12 gene from genomic DNA using (AS1) AACTGCAGGGCCGAGACGAATCCGGAGG and (AS4) CGGGATCCGCATTTTGTCGGAGGCAATT primers and cloned into pPD95.75 vector using PstI and BamHI sites. pBAB913 was generated by cloning Pnlp-12 into exp-1::*sl2*::wrm Scarlet vector using PstI and XmaI sites.

pBAB912 contains 1.7 kb of whole genomic region of nlp-12 (promoter and gene) amplified from genomic DNA using (AS1) AACTGCAGGGCCGAGACGAATCCGGAGG PstI and (AS3) CGGGATCCGAAAATGTGTCGCTTCGAGAC and cloned into pPD95.75 vector using PstI and BamHI sites, for generating *nlp-12* overexpression lines.

For *dop-2* rescue experiment *Pdop-2*::DOP-2::mCherry construct obtained from Rene Garcia Lab was injected into *dop-2* mutant animals.

(pBAB914) For *dop-1* rescue experiment *dop-1* cDNA (1.2kb) was cloned under *nlp-12* promoter in pBAB913 under XmaI and KpnI sites.

The pBAB916 and pBAB917 constructs contains *gpa-14 (*1.9 kb) and *gpa-16* (2.8 kb) promoters respectively amplified from genomic DNA and cloned upstream of the *dop-2* cDNA sequence amplified from the *dop-2p*::*dop-2::cfp* construct obtained from Rene Garcia Lab and cloned into the *pPD49.26* vector under NheI and XmaI sites. The promoters were cloned upstream using SphI and XmaI sites.

2.6. Behavioral Assay

Ethanol induced sedative behavior assay

C. elegans were synchronized by bleaching and grown on nematode growth media (NGM), maintained in well fed condition for the assay. The EtOH preexposure plates were prepared using

unseeded plates i.e., without food plates that were dried for 3 hr under airflow in the hood followed by EtOH exposure, 400mM EtOH (condition used for all the behavioral assays) (different concentrations were tried to observe the effect of EtOH as mentioned by (Davies *et.al.*; 2003) was spread on the plates after drying, plates were sealed with parafilm to avoid any loss and EtOH was allowed to equilibrate for 2hr at 20 degrees. Adult animals with one or two eggs were transferred first to without food plates for 15 to 20 sec to get rid of the food so that food doesn't interferes the behavior, then again transferred to the EtOH pre-exposure plates. 10 animals were used for each set and the experiment was set up in triplicate. Animals show coiling behavior and get paralyzed within 10 min of EtOH exposure, recovery starts in all the strains except *dop-2* after 30 min, all the strains used for assay along with WT show EIS (Ethanol induced sedative) like behavior initially as the movement starts but regain their normal locomotory behavior after 60 min except *dop-2* mutant shows EIS behavior, thus the behavior was analyzed after 2 hr since the transfer, videos were recorded as (10 frames/ sec) and observed for 1 min on AxioCam MRm (Carl-Zeiss), using micromanager software.

Detailed Protocol

Ethanol Induced Sedative Behavior: An assay to investigate increased dopamine signaling in *C. elegans*

Abstract

Dopamine (DA) signaling affects locomotion, feeding, learning and memory in *C. elegans*. Various assays have been developed to study the proteins involved in these behaviors. However, these assays show behavioral output only when there is major change in the DA levels. Thus, we decided to design an assay where we could observe behavioral output even with low levels of alteration in DA levels. To achieve this, we combined movement assay with ethanol that is also known to function through DA pathway. We successfully utilized this assay to assign function for a DA autoreceptor, DOP-2. This assay correlates the increase in DA levels due to ethanol and movement obstruction due to dry surface into a circular sedative behavior that we designated as Ethanol Induced Sedative (EIS) behavior.

Keywords: EtOH (Ethanol), *C. elegans*, Dopamine (DA), sedative behavior

Background

Alcohol is a widely abused drug with a plethora of associated diseases and adversely impacts societal functioning. Multiple studies have focused on unravelling the mode of action and effect of this drug. However, the neuronal mechanisms underlying alcohol susceptibility and disinhibition are not clear. Studies across various species have demonstrated that alcohol intake increases the release of the neurotransmitter DA that induces the reward pathway (Baik, 2013; Imperato and Di Chiara, 1986; Weiss *et al*., 1993). Although *C. elegans* does not mimic all the complexities of mammalian system, it has been successfully modelled for studying alcohol dependent neuronal behaviors. Studies show that *C. elegans* display diverse aspects of alcohol responses (Davies *et. al.*, 2003, 2004). Previously *C. elegans* investigations revealed that there is a dose dependent decline in the locomotor activity upon acute, chronic alcohol exposure (~400-500mM) due to its depressive effects (Davies *et. al.*, 2003; Lee *et. al*., 2009). The DA system in *C. elegans* is involved in feeding, movement, learning, and memory and like signals through two receptor subfamilies D1-like and D2-like receptors. The D2-like receptor, DOP-2, exhibited no obvious phenotype when analyzed for DA dependent behaviors although it was expressed in all the dopaminergic neurons and is predicted to be an autoreceptor (Chase *et.al.*, 2004). We devised an assay utilizing the EIS behavior observed in mutants of *dop-2* to investigate the neuronal circuitry that was involved in regulating locomotory behavior under the influence of EtOH (Pandey *et. al.*, 2021).

Materials and Reagents

1. 60mm Petri dishes (60 mm: Tarsons, catalog number: 460061)
2. Spreader (Tarsons, catalog number: 920081)
3. 99.99% platinum wire (Sigma-Aldrich, catalog number: 267201)
4. *C. elegans*: N2 (Wild type worms) (University of Minnesota, Caenorhabditis Genetic Center) and *dop-2(vs105)*
5. Escherichia coli OP50 (University of Minnesota, Caenorhabditis Genetic Center)
6. Ethanol (EtOH) (Fisher chemical, catalog number: UN1170)
7. Cholesterol (SRL Sisco Research Laboratories, catalog number: 54181)
8. Calcium chloride dihydrate (CaCl2.2H2O) (Sigma-Aldrich, catalog number: C3306)

9. Magnesium sulfate (MgSO4) (Sigma-Aldrich, catalog number: M7506)

10. Potassium phosphate, monobasic (KH2PO4) (Sigma-Aldrich, catalog number: P5379)

11. Potassium phosphate, dibasic (K2HPO4) (Sigma-Aldrich, catalog number: P8281)

12. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)

13. Bacto-agar (HiMedia Laboratories, catalog number: GRM026)

14. Bacto-peptone (BD, catalog number: 211677)

15. 400mM EtOH (see Recipes)

16. 5 mg/ml cholesterol (see Recipes)

17. 1 M CaCl2 stock solution (see Recipes)

18. 1 M MgSO4 stock solution (see Recipes)

19. 1 M KPO4, pH 6.0 stock solution (see Recipes)

20. Nematode growth medium (NGM) agar plates (see Recipes)

Equipment

1. Pipettes (Eppendorf, model: Research® plus, catalog number: 2231000224)

2. 2 L glass conical flask (DWK Life Sciences, DURAN, catalog number: 2121763)

3. Autoclave (Equitron-7431 SLEFA)

4. Microscope (ZEISS, model: Stemi 2000 C)

Software

1. GraphPad Prism v6 (GraphPad Software)

2. Image J

Procedure

1. *C. elegans* strains were synchronized by bleaching the worms and synchronized on 60mm NGM plates
2. Prepare and seed regular 60mm plates for maintenance of *C. elegans* on food i.e., E. coli OP50 (used for seeding) under sterile conditions in a bacterial hood and let it grow at 37 degree overnight.
3. Prepare fresh 60mm (NGM) plates containing 8ml media, at least one day before the assay, and store at 4 degree until used.
4. On the day of assay, take out the plates from 4 degree and dry them in the laminar airflow for 3-4 hr, with lid open.
5. Spread 400mM (196 microliter) ethanol on dried 60mm plates with 8ml media, and control plates without ethanol (dried plates) are prepared for the assay.
6. Ethanol preexposure plates are sealed with parafilm to avoid loss of ethanol and kept at 20 degrees to allow for ethanol to equilibrate.
7. The ethanol preexposure plates are now ready and can be used for the assay.
8. Transfer 10 worms of each genotype from food plate to an unseeded plate (to get rid of the food) for 15-20 sec, and then transfer the worms to ethanol preexposure assay and control plates, leave the plates undisturbed for 2hr, at 20 degrees.
9. Observe the worms after 2-hr interval. Count the number of worms on the plates and make 1-min videos for each animal to observe the locomotory behavior by counting number of body bends (anterior/posterior) and the amplitude of body bends to characterize the behavior.
10. We do a timeline-based analysis of the behavior, starting from exposure to EtOH at different time points starting from 30 min, 60min, 120min, 300min and ON (16hr). WT *C. elegans* show normal behavior (off food) after recovery from EtOH induced paralysis

(movie 1). While the DAergic autoreceptor mutant shows an Ethanol Induced Sedative (EIS) behavior (movie 2).

11. Recovery from the EIS behavior can also be observed by transferring the mutant animals to normal NGM plates with food, after 2hr from the EtOH assay. We observe recovery in the mutant animals after 1hr from being transferred to normal conditions.

12. Here, *dop-2* an autoreceptor mutant, DOP-2 has been reported to regulate the release of dopamine in the DA neurons. The *dop-2* mutant shows a novel Ethanol Induced Sedative behavior on ethanol exposure in above mentioned conditions.

Data Analysis

1. Calculate the number of anterior/posterior body bends separately in a one-min video at 2hr. time point.
2. Software: GraphPad Prism v6.
3. At least 10 worms are used for each assay plate, set up the experiment in triplicate for each strain
4. Statistical analysis: A one way-ANOVA is used to determine statistical *P*-values.

Notes

1. Drying the NGM plates perfectly is important for equilibration of ethanol and visualization of tracks.
2. Seal the plates with parafilm to avoid loss of ethanol.

Recipes

1. 5 mg/ml cholesterol

Add 500 mg of cholesterol in 100 ml of 95% ethanol and mix by rotating on a rotator at room temperature. It takes a few hr to dissolve

Store at 4 °C

2. 1 M $CaCl_2$ stock solution

Dissolve 14.7 g of $CaCl_2 \bullet 2H_2O$ in 100 ml ddH_2O and autoclave for 15 min at 121 °C

Store at 4 °C

3. 1 M $MgSO_4$ stock solution

Dissolve 12.04 g of $MgSO_4$ in 100 ml ddH_2O and autoclave for 15 min at 121 ℃

Store at 4 ℃

4. 1 M KPO_4, pH 6.0 stock solution

a. Mix 108.3 g KH_2PO_4 and 35.6 g K_2HPO_4 in 500 ml ddH_2O
b. Adjust the pH to 6.0 by adding NaOH, finally make up the volume to 1 L
c. Aliquot the solution and autoclave for 15 min at 121 ℃
d. Store at 4 ℃

5. Nematode growth medium (NGM) agar plate

a. Add 3 g NaCl, 16 g Bacto-agar, and 2.5 g Bacto-peptone in 975 ml of ddH_2O in a 2 L flask
b. Autoclave for 50 min at 121 ℃
c. Let the NGM agar cool to 55-60 ℃. Add 1 ml of 5 mg/ml cholesterol, 1 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, and 25 ml of 1 M KPO_4, pH 6.0

6. 400mM Ethanol

Spread 196 microliter of ethanol on 8ml media in a 60mm plate.

Seal the plate with parafilm and let it equilibrate at 20 ℃

2.6. Exogenous Dopamine Assay

For exogenous DA application, 1M freshly prepared DA was used as previously described by (Chao *et.al.*; 2014), DA was spread on EtOH preexposure plates for EtOH + DA set and on unseeded dry plate for DA exposure set. WT and *cat-2* mutants were used for the assay, different concentration of DA was used for WT and *cat-2* mutant, 40mM and 80mM respectively as *cat-2* is defective in DA synthesis and has very low levels of endogenous DA compared to WT animals, plates were protected from light and used within 10 min for the assay. Different assay conditions were used for WT animals to show that the effect was due to increased DA concentration and not just DA or EtOH alone, three sets of conditions were used for WT animals as follows – DA exposure, EtOH exposure, DA + EtOH exposure. Animals were transferred from unseeded plates to the assay plate, behaviour was analysed making 1 min videos (10 frames/sec) after 2hr EtOH exposure.

2.7. EtOH Based Aldicarb Assay

This assay is modified in observation from the aldicarb assay to study the behavior of worms on EtOH exposure, may be due to increased levels of acetylcholine, aldicarb assay was performed as described previously by (Mahoney et al.; 2006), plates with 1mM aldicarb (sigma-Aldrich;33386) were prepared 1day prior to the assay and dried, worms were transferred to aldicarb plates and then to EtOH preexposure plates once they reach the stage of paralysis on aldicarb i.e. 60 min, the behavior was analyzed after 2hr since the transfer, videos recorded (10 frames/ sec) and observed for 1 min on AxioCam MRm (Carl-Zeiss), using micromanager software.

2.11. Liquid Hypercontraction Assay

Young adult worms were used for this assay, animals were grown on standard NGM plates, they were first picked on unseeded plates to get rid of food, and then transferred to 400mM solution of ethanol prepared in M9 buffer, the behaviour of animals was observed as the time of paralysis.

2.12. Pharyngeal Pumping Assay

The feeding behavior of worm is comprised of rhythmic contractions of the pharynx, a neuromuscular controlled organ, the bacteria is drawn up to the isthmus into the terminal bulb, where it is crushed by the grinder, and this is called pumping. This pumping movement is clearly

visible under the microscope and the number of pumps per min can be counted manually. The worms were exposed to EtOH in the presence of food, and the number of pumping episodes were counted per min. This assay was performed to observe the effect of EtOH on pharyngeal pumping behaviour of worm. Animals were grown on standard NGM plates, plates for the assay were prepared following the same procedure of EtOH assay, plates were then seeded and used for the assay. Young adult worms were transferred to the assay plates one worm was observed at a time for five times the pumping behaviour was observed and counted for a min using hand counter, the assay was repeated with at least six to seven worms of each strain.

2.13. Egg Laying Assay

Worms were synchronized by bleaching and transferred to seeded NGM plates, young adult worms were used for the egg laying assay. These worms were transferred to the EtOH assay plates following the assay protocol described previously. Single worm was assayed per plate to count the number of eggs laid in the time frame of 16hr our assay timeline. Number of eggs laid on fresh food plates were also counted after 16hr used as control for EtOH assay condition.

2.8. Microscopy

The DAT-1::GFP imaging experiment was performed on Leica Sp8 confocal microscope using Argon laser at 10% gain. Young adult animals immobilized using 30mg/ml BDM (2,3-ButaneDione monoxime) on 2% agarose. All the image quantitation was done taking whole cell body expression using FIJI. Experiments were performed both with control (without EtOH) and experimental animals (with EtOH exposure).

2.9. Ablation

PDE and DVA ablation experiments were performed to test the dependence of the behavior on these neurons, tagged with *dat-1*::GFP and *nlp-12*::scarlet-red respectively. L-2 staged worms were used for the ablation experiment, as loss of function is more effective in early stages (Avery and Horvitz, 1987, 1989; Bargmann and Horvitz, 1991a). During ablation and imaging the worms were immobilized using 5% agarose pad in 10mM levamisole hydrochloride (Sigma 10380000) anesthesia or with 0.1-um-diameter polystyrene beads (00876-15; Polystyrene suspension). Bruker Corporations ULTIMA setup was used to perform two-photon imaging and Ablation simultaneously as mentioned in (Basu et al.; 2017). A 60X water immersion objective was used for ablation and imaging experiment, GFP was imaged using 920 nanomolar (nm) and scRed using

1040 nm laser, shot for 20ms pulsed femtosecond IR laser [pulse width 80fs, irradiation pulse width: 20ms, laser point spread function (PSF) 400nm and Z axis PSF-1.5um] was used for all ablation experiments. Animals were then checked for proper ablation by checking the fluorescence. These animals were now allowed to grow and recover till they reach the assay stage, the animals were then transferred to unseeded plates followed by transfer to ethanol preexposure plates. Videos were recorded (10 frames/sec) and behavior was analyzed by quantitating the anterior/posterior body bends and amplitude using FIJI after 2hr of EtOH exposure. The results were plotted using graph pad prism using standard error of mean (SEM) and evaluated using one – way ANOVA.

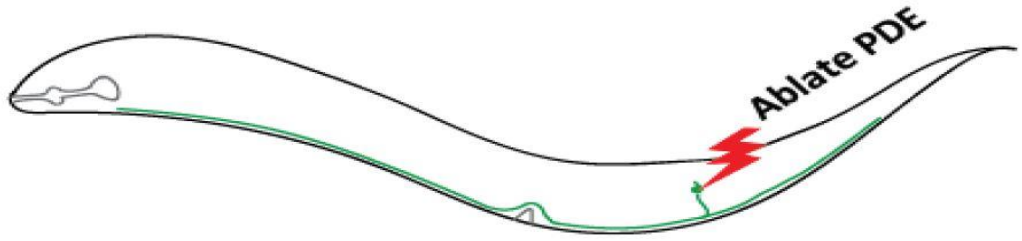


Figure 2.1. Illustrative diagram of PDE neuronal ablation in *C. elegans*.

2.10. FRAP

The increased extracellular release kinematics of DA in presence of EtOH based on DA vesicle fusion, was analyzed, and observed using DAergic neurons (PDE) tagged with synaptobrevin pHlourin as previously described by (Samuel *et.al.*; 2003, Tavernarakis and Voglis; 2008, Blakely et.al.; 2015). Late young adult animals were mounted on 2% agarose pad and paralysed using 0.05% levamisole. FRAP experiments were performed on Leica SP8 inverted confocal microscope. PDE synapses were analyzed, processes were identified by Synaptobrevin::SEpHluorin fluorescence, bleaching was done using 488nm argon laser, 80% bleach power, 5-10 sec to an intensity of 10-15% of original fluorescence value. Fluorescence was monitored at every 10 sec for 2 min and analysis was done using FIJI. Percentage recovery was calculated at each time point. 20 synapses were analyzed per genotype. Data was plotted using GraphPad Prism, analyzed using non-linear regression plot, one phase association exponential equation was used.

CHAPTER III

DOP-2 autoreceptor acts to regulate dopamine release and an ethanol induced behaviour

Abstract

The dopamine (DA) system in *C. elegans* is involved in the animals feeding, movement and learning and memory and functions through two types of receptor subfamilies D1-like and D2-like receptors. DOP-2, a D2-like family autoreceptor was found to have minimal role if any when studied for DA dependent behaviors and the functions of this receptor are still largely unknown. In this study, we have used an ethanol (EtOH) based assay to screen the dopaminergic (DAergic) pathway mutants. We found that the DOP-2 autoreceptor mutant showed an ethanol induced sedative behavior (EIS), the animal moves in circles with decrease in body bends and amplitude. This behavior is DOP-2 dependent as we were able to rescue the behavior putting back DOP-2 in the mutant animals and observed that they were behaving like Wild Type (WT) animals, thus this behavior is DOP-2 specific. The mechanism of how the DOP-2 autoreceptor functions in *C. elegans* is still largely unknown, although in the mammalian system the D2 autoreceptor functions in feedback inhibition on activation to regulate the levels of DA by regulating its synthesis, release and reuptake at the NMJ. On exposure to exogenous DA in EtOH assay conditions, WT animals behaves like *dop-2* mutant animals, while the *cat-2* (synthesis pathway) mutants with low or negligible levels of DA, showed a behavior similar to WT animal on EtOH exposure. Our FRAP analysis also strengthens the statement as we observed significantly faster recovery of DA in *dop-2* mutants compared to WT animals. Thus, the DOP-2 dependent EIS behavior is regulated by increase in synaptic DA concentrations.

3.1. Introduction

DA is a well-known neurotransmitter (NT) molecule conserved across different taxa. It is involved in modulating the behavioral outputs in response to the external environment and cues i.e., mostly the maintenance of locomotion and gait transition (Vida-Gadea and Pierce-Shimomura, 2012). In *C. elegans* DA has been shown to modulate locomotion in the presence and absence of food, and the basal slowing behavior on food encounter (Chase et.al., 2004). Thus, DA here also regulates locomotion like in other organisms. The highly concise nervous system of *C. elegans* make it an ideal model to study regulation of behaviors by DA. The DAergic receptors in *C. elegans* are divided into two subfamilies like their mammalian counterparts, D1 and D2 class receptors. The D1 class comprises of excitatory receptors DOP-1 and DOP-4 and D2 class comprises of inhibitory receptors DOP-2 and DOP-3 (Suo et.al., 2002, 2003). As discussed previously the most classical example of modulation of behavior in response to external cues by DA signaling is by D1 and D2 receptors, DOP-1 and DOP-3, that are coexpressed on the mechanosensory and motor neurons and act antagonistically to regulate locomotion upon food encounter (Chase et.al., 2004). While most of these receptors express on different neuronal and non-neuronal cells, the D2 like DOP-2 receptor expresses presynaptically on all the DAergic neurons (Suo et.al., 2002, 2003), making it a perfect candidate for autoregulation of DA, as in mammals the D2 autoreceptor functions in a negative feedback loop to regulate the release of DA (Ford, 2014). Most of the DA receptors regulate movement related behaviors, the D2 class receptor DOP-2 deletion shows no such defect (Allen et al., 2011; Chase et al., 2004; Sawin et al., 2000). As it is often observed that behaviors associated with deletion of neuromodulatory molecules are not very prominent, since different molecular players might be involved in regulating the behavior (Ford, 2014). Hence we decided to use an external cue to try and enhance the defect seen in these mutants.

Several drugs of abuse mediate their effect through the DA system, leading to enhanced DA release. One of the most widely abused and easily available drug among them is EtOH. In mammals it has been shown to increase DA release by activating the reward pathway, from the ventral tegmental area (VTA) and increased DA levels were found in the nucleus accumbens (NAC) (Imperato and Di Chiara, 1986; Weiss et al., 1996; Yim and Gonzales, 2000). *C. elegans* is already a well-established model for studying various aspects of ethanol abuse. As it encounters alcohol in its natural habitat (rotten fruits) and hence could have evolutionary developed neuronal circuitry for alcohol sensitivity (Khanh, 2018). Dose dependent decline in locomotor activity on

EtOH exposure has been observed in *C. elegans* on increasing concentrations, like the depressive effects of EtOH observed in mammals (Alaimo et al., 2012; Davies et al., 2003; Hawkins et al., 2015). The internal dose of intoxication is similar for mammals and *C. elegans*, indicating towards, similar molecular targets (Lee et al., 2009). Several molecular targets of EtOH have already been identified in *C. elegans* such as the BK potassium channel SLO-1, a neuropeptide receptor NPR-1 and it also brings about disinhibition of behaviors, also observed in mammals regulated by D1 like receptor DOP-4 (Davies et al., 2003; Davies et.al., 2004; Topper et.al., 2014). The DOP-2 autoreceptor belongs to the D2-like class of receptors, present on the DAergic neurons, playing important roles in regulation of the DAergic system through feedback inhibition, in turn controlling synthesis, release and reuptake of DA (Ford, 2014). Several studies in the mammalian system report the role of these D2 receptor in implicating the effects of alcohol (Kraschewski et al., 2009; Lu et al., 2001; Thanos et al., 2005; Volkow et al., 2006). Moreover, studies have also shown that D2 receptor levels are enhanced in alcoholics and that attenuation in the ligand activation of D2 receptors drives craving and relapse of alcoholism (Feltmann et al., 2018; Volkow et al., 2013).

Here we devised an EtOH dependent assay and initially screened all the DAergic receptors and pathway mutants in this assay. We found that a D2 autoreceptor mutant *dop-2* specifically displayed a novel locomotory phenotype when exposed to high levels of EtOH, where worms slowly dragged their body in concentric circles and referred as ethanol induced sedative (EIS) behavior.

3.2. Results

3.2.1. DOP-2 mutants show increased sensitivity to EtOH

In *C. elegans*, the DA pathway is widely known to modulate egg laying, defecation, basal slowing, habituation, and associative learning (Sawin et al, 2000; McDonald et al, 2006; Voglis and Naktarias., 2004). DA receptors DOP-1 and DOP-3 function antagonistically to regulate movement. Another receptor DOP-2 is expressed presynaptically in all the DA neurons and has the potential to function as an autoreceptor. It's mutants do not show any defect that can explain its autoreceptor function. It is possible that under normal conditions its absence is compensated by

other regulatory molecules in the cell and hence the phenotypes were not very prominent. Modulation of behaviour and function through the DAergic system upon exposure to drugs of abuse such as ethanol has been established by many mammalian and invertebrate studies (Kameda et al., 2007; Siciliano CA et al., 2019). Thus, we speculated that exposing *dop-2* worms to EtOH may serve as a better strategy to screen for *dop-2* defects. In this study we have screened the DAergic receptor mutants through an EtOH based liquid hypercontraction assay (Hawkins et al., 2015) at 200 mM of concentration of EtOH made up using M9 buffer, at different time points up to 30 min at an interval of every 5 min to observe the behavior, we observed only *dop-2* mutants showed a phenotype in this assay. The *dop-2* mutants showed faster paralysis when compared to other DA receptor mutants and wild type (WT) animals in this EtOH based Liquid hypercontraction assay. Thus, these mutants showed enhanced sensitivity to EtOH, we quantitated and plotted the behavior as the time of paralysis observed in the DAergic receptor mutants (Fig. 3.1. A). Next, we wanted to check that if loss of *dop-2* affects the function of all muscles or specifically affects locomotion through its effect on body wall muscles. We decided to address this question by looking at the posterior muscle function by quantitating egg-laying that requires the posterior muscles and the anterior muscle function by quantitating anterior pharyngeal pumping that requires anterior muscle function. The egg laying muscles have been shown to be regulated by multiple NT including DA (Weinshenker et al., 1995), and pharyngeal pumping has been shown to be regulated by serotonin (reviewed in Ishita et al., 2020), yet studies have shown that serotonergic behaviors could also be modulated by DA (reviewed in Vidal-Gadea and Pierce-Shimomura, 2012). On observing the behaviours, we found significantly decreased egg laying over 16 hr by *dop-2* mutants treated with ethanol when compared to WT control animals under similar conditions, while the untreated WT and *dop-2* mutant animals in normal conditions showed no significant differences in the egg laying behaviors (Fig. 3.1. D, C). No difference was observed in the pharyngeal pumping behavior, in *dop-2* mutant animals treated with EtOH when compared to WT control animals (Fig. 3.1. B). These results suggest that *dop-2* mutants show increased sensitivity to EtOH exposure, affecting the body wall muscle function.

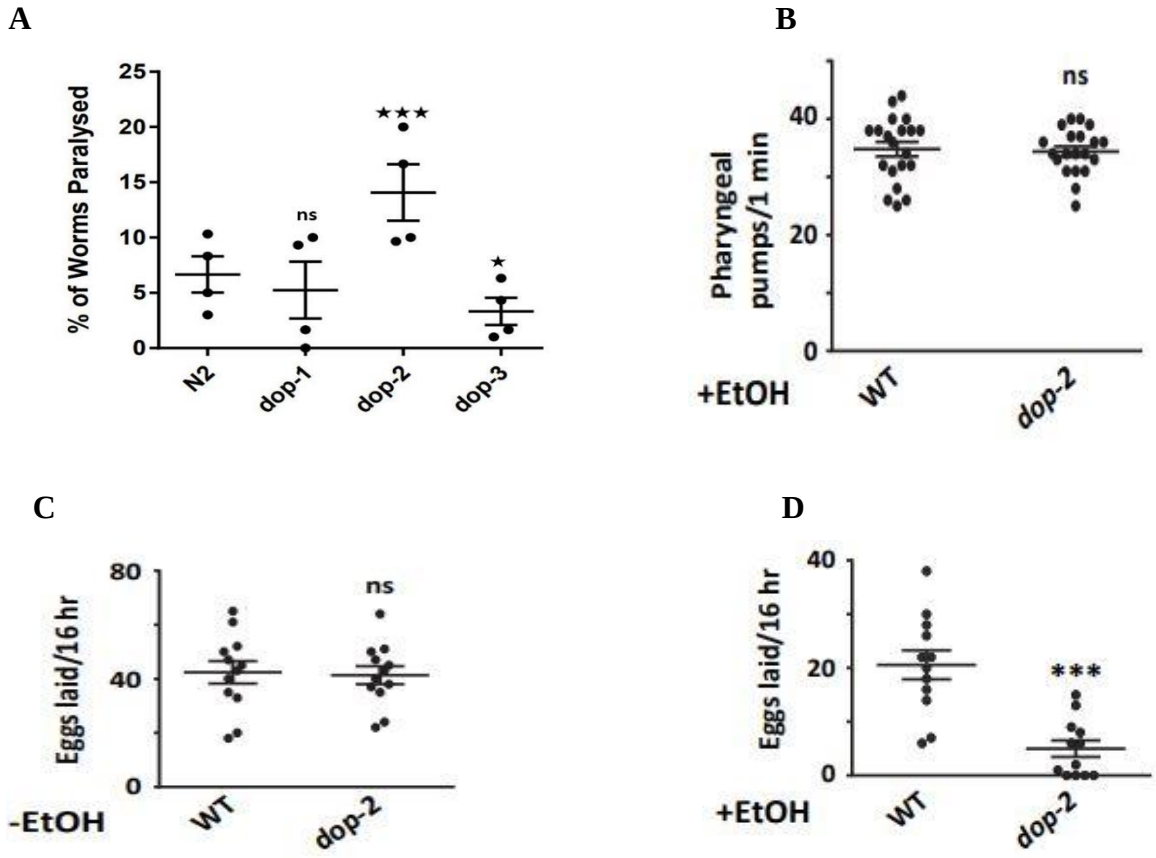


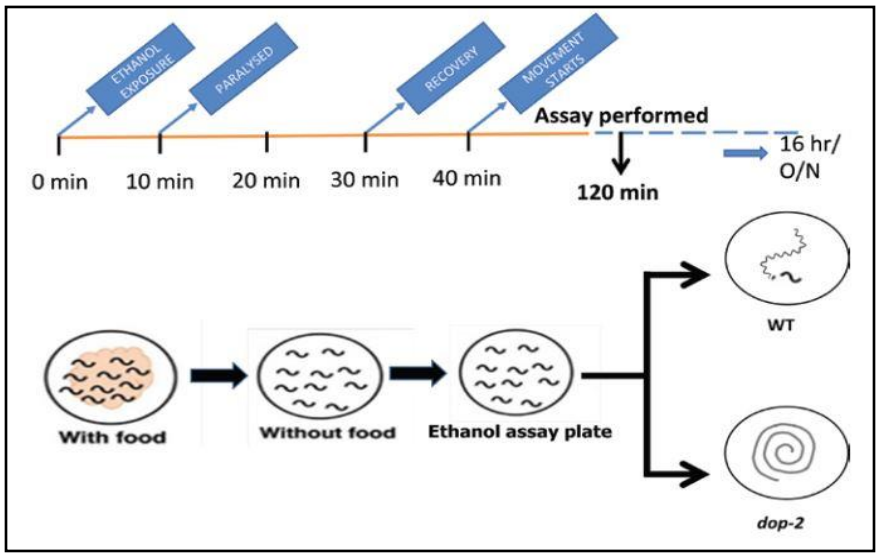
Figure 3.1. EtOH affects locomotory behavior and egg laying in dop-2 mutant animals.

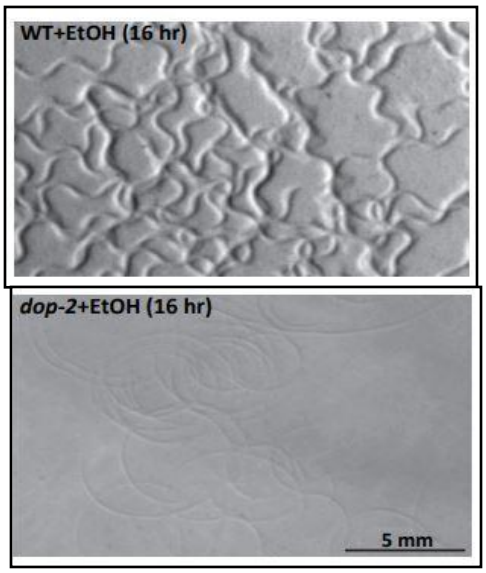
A. *dop-2* mutants display increased sensitivity to EtOH, compared to WT and other DA receptor mutants, in the EtOH based liquid hypercontraction assay, the dot plot represents number of animals paralysed for each genotype in the given time, the behavior was analysed for 30 min. B. No defect was observed in the pharyngeal pumping behavior on comparing WT and *dop-2* mutant animals, the behavior was scored for 1 min for each animal. C. No defect was observed in the egg laying behavior of *dop-2* mutant animals compared to WT, the behavior was observed on food and observed for 16 hr. D. Significant defect in the egg laying behavior was observed in the *dop-2* mutant animals, compared to WT, in the presence of EtOH, the behavior was observed in the absence of food in the EtOH assay and observed for 16 hrs. Error bars represent $\pm$S.E.M and the p-values were calculated using student t - test and one-way ANOVA using Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

3.2.2. Ethanol (EtOH) exposure affects movement of Dopamine Autoreceptor, *dop-2* mutants

The previous results from the EtOH based liquid hypercontraction assay showed an increased sensitivity to EtOH in *dop-2* mutant animals, but we were not able to quantitate and specify the behavior observed. Hence, we devised an assay, where worms were exposed to high concentration of EtOH (400mM). When *dop-2* mutant worms were exposed to 400mM EtOH, worms showed a unique behavior where worms kept moving in circles and dragged the posterior part of their body (Fig. 3.2.1. A). During this movement we observed that there was slowing of movement and flattening of body bends. Previously, it was shown that worms show flattening of the body bends at higher concentration (400 mM) of EtOH (Davies, 2004). This effect on forward movement was quantitated and it showed that there was clear distinction between the number of anterior and posterior body bends and the amplitude of body bends (Fig. 3.2.1.D, E). To remove any possibility that the behaviour could be affected by the dried surface of the NGM plates we performed control experiments in which both *dop-2* and WT animals were assayed on the dried NGM plates in the absence of ethanol. There was no defect in the body bends or flattening of bends were not observed (Fig. 3.2.1. D, E).

A **B**





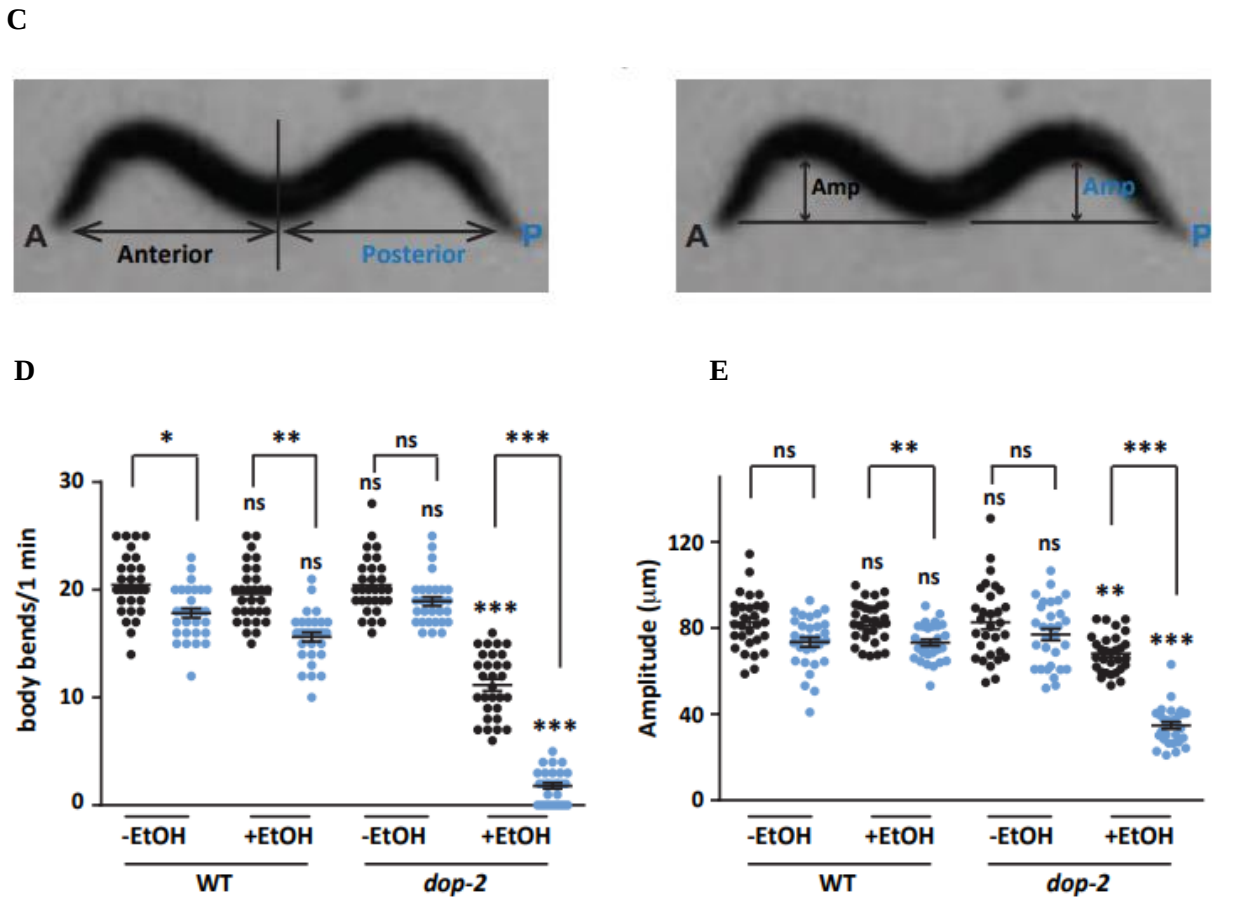


Figure 3.2.1. Analysis of movement defects upon exposure to 400 mM ethanol

A. An illustration of the timeline and experimental procedure for ethanol (EtOH) treatment in *C. elegans*. The time points showing paralysis and recovery of WT animals are indicated on the time-line. Experiments to test the number of body bends and amplitude of body bends were performed at approximately the two-hr time point that is indicated in the time-line, WT animals recover and show normal movement while *dop-2* mutant animals show movement defect, move forming circular tracks. B. Images of tracks from plates with WT and *dop-2* animals treated with EtOH and left overnight (16 hr) on the plate that was imaged. *C. elegans* body bends in the anterior (A) and posterior (P) regions marked by a partition and the amplitude of body bends shown by a double-sided arrow indicating amplitude (Amp) in the anterior and posterior regions. D. Quantitative analysis of number of body-bends for WT and *dop-2* mutant animals, both with and without EtOH treatment (n=10, N=3 and F = 201, DF = 7). E. Quantitative analysis of amplitude of body-bends for WT and *dop-2* mutant animals, both with and without EtOH treatment (n=10, N=3 and F = 61.2 DF = 7). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype and treatment condition. Error bars represent $\pm$S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

Next, we wanted to understand the physiological and genetic basis of the behavior. We screened for the DAergic synthesis pathway and receptor mutants. After performing EtOH assay for these components we observed that none of the mutants showed *dop-2* like behaviour upon EtOH exposure (Fig. 3.2.2. A, B). Recent studies from various model organisms have found that BK channel (calcium-sensitive potassium channels) proteins play an important role in modulating the behavioral effects of EtOH (Bettinger JC and Davies AG, 2014). Mutant of *slo*-1 BK potassium channel was tested for the behaviour but none other than *dop-2* mutants showed this peculiar locomotion defect i.e., the worm moving in concentric circles, with its posterior half being more affected and basically dragged for anterior region and we designated it as Ethanol Induced Sedative (EIS) behaviour. Quantitation of the behaviour showed a decrease in overall body bends and more significantly there was decrease in the posterior body bends in case of *dop-2* mutants (Fig. 3.2.2. A). The reduction in the amplitude of body bends was significant when comparing *dop-2* mutants to WT worms (Fig. 3.2.2. B). Thus *dop-2* mutants showed reduction in the posterior body bends and its amplitude. When we performed our assays, it was quite evident that during the initial phase of assay both WT and *dop-2* mutant strains paralyzed in 10-12 min and then slowly started recovering from paralysis. During the recovery phase WT worms showed mild EIS like behaviour but recovered from the behavior with in 1hr and showed normal locomotion behavior. For all experiments we performed quantitation of locomotory behaviour at 2hr post EtOH exposure. Wild type and other DA synthesis pathway and receptor mutant worms when observed for behaviour showed no significant change in the no. of body bends (Fig. 3.2.2. A) and the amplitude of body bends (Fig. 3.2.2. B). Together these results suggest that *dop-2* mutants have higher, prolonged sedative response to EtOH. Our results suggest that Ethanol induced sedative (EIS) behavior is a *dop-2* dependent, to further confirm that this is indeed the case we made transgenic rescue lines with DOP-2 cloned under its own promoter *Pdop-2*::DOP-2 and used this line for our ethanol assays. We observed that putting back *dop-2* in the mutants rescued the EIS behaviour. Rescued worms showed no significant difference in their behaviour upon comparing the no. of body bends and the amplitude of body bends with WT (Fig. 3.2.2. C, D).

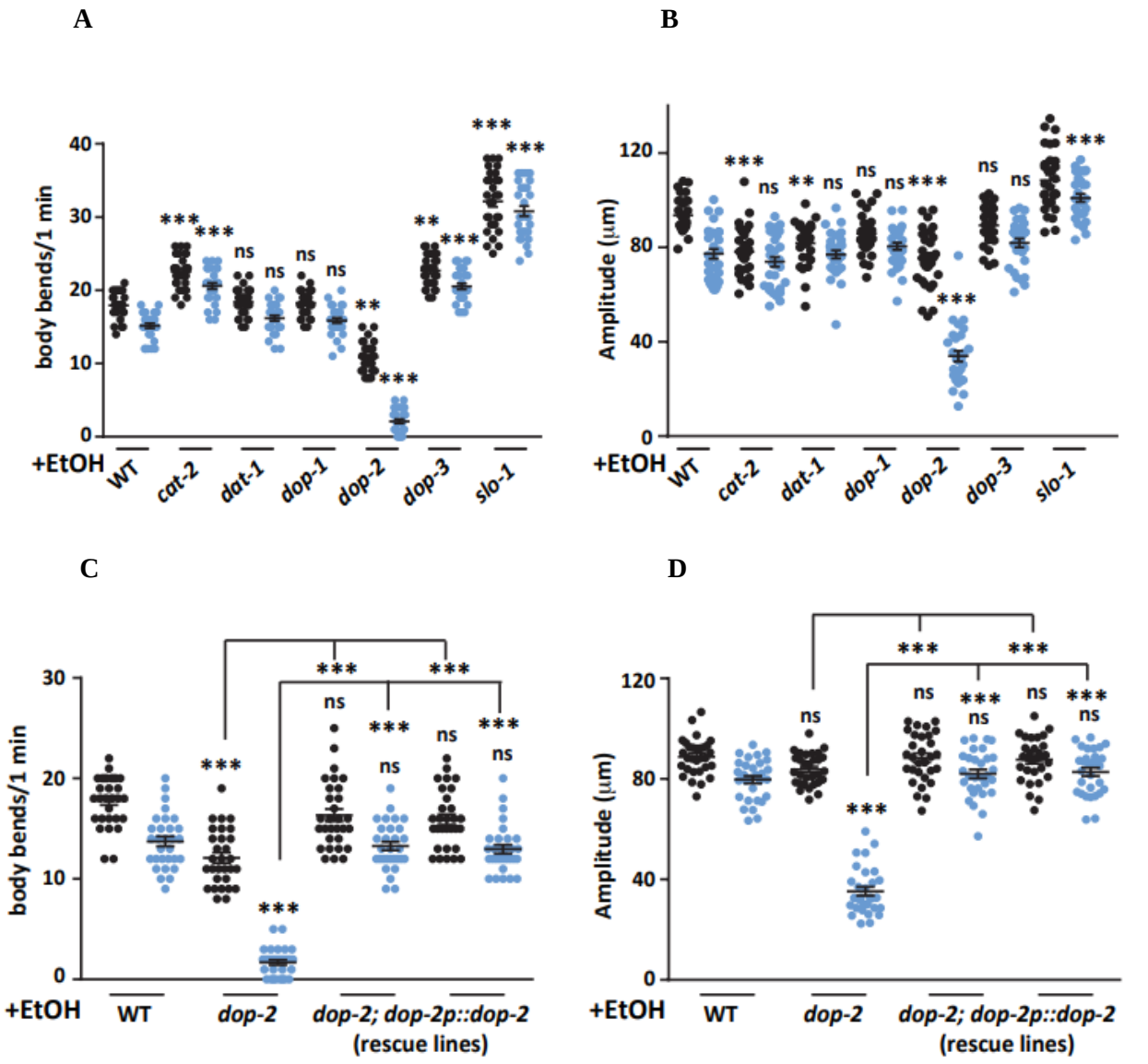


Figure 3.2.2. Analysis and rescue of dop-2 specific EIS behavior.

A. Graph of number of body bends (anterior body bends are shown in black and posterior body bends are shown in blue in all graphs) in wild type (WT), *cat-2*, *dat-1*, *dop-1*, *dop-2*, *dop-3* and *slo-1* animals upon Ethanol (EtOH) treatment, (n=10, N=3 and F = 66.8, DF = 13). B. Graph of amplitude of body bends (anterior amplitude of body bends are shown in black and posterior amplitude of body bends are shown in blue in all graphs) for WT, *cat-2*, *dat-1*, *dop-1*, *dop-2*, *dop-3* and *slo-1* mutants upon EtOH treatment, (n=10, N=3 and F = 82.2, DF = 13). C. Graph indicating body bend measurements for rescue of the *dop-2* behavior using transgenic expression of *dop-2* under the *dop-2* promoter. Anterior body bends are shown in black and posterior body bends are shown in blue in all graphs (n=10, N=3 and F = 109, DF = 7). D. Graph indicates amplitude of body bend measurements for WT, *dop-2* and rescue lines. Anterior amplitude of body bends are shown in black and posterior amplitude of body bends are shown in blue in all graphs (n=10, N=3 and F = 128, DF = 7).

3.2.3. Time-course based analysis of EIS behavior and Recovery

We observed that the *dop-2* mutant animals do not recover from the EIS behavior even after chronic EtOH exposure of 16hr, while the WT animals recover and largely leave the assay plate in search of food by 16hr. Thus, we decided to observe and quantitate the EIS behavior in control and *dop-2* mutant animals and found that at the initial time point of exposure to EtOH the *dop-2* mutants show a behavior similar to WT animals. The WT and *dop-2* mutants are completely paralyzed at 10-12 min of exposure, as shown in previous studies for WT animals (Davies and Mclntire, 2004) (Fig 3.3. A, B, C, D). While the WT animals start recovering from the behavior within 30 min, the *dop-2* mutants do not show recovery from paralysis till 120 min. The mutants do not show full recovery from the effect of ethanol and show locomotion defect where they are not able overcome the flattening effect on their body bends, especially the posterior region seemed more affected. Further we observed the behavior for 16hr referred as O/N and found that the while the WT animals show normal behavior of worms under starvation condition as shown by previous studies, the *dop-2* mutant animals do not show recovery from the EIS behavior (Fig 3.3. A, B, C, D). Next, we wanted to study the recovery of *dop-2* mutant animals, after removing them from the assay condition to normal condition in the presence of food. This was done at 120 min time point when we start observing the EIS behavior in *dop-2* mutants, these animals start showing recovery from the EIS behavior after 60 min of being transferred to the food plates in the absence of EtOH from the assay plates (Fig 3.3. E, F). Thus, our results here show that the EIS behavior is both DOP-2 and EtOH dependent behavior, and *dop-2* mutants show increased sensitivity to EtOH.

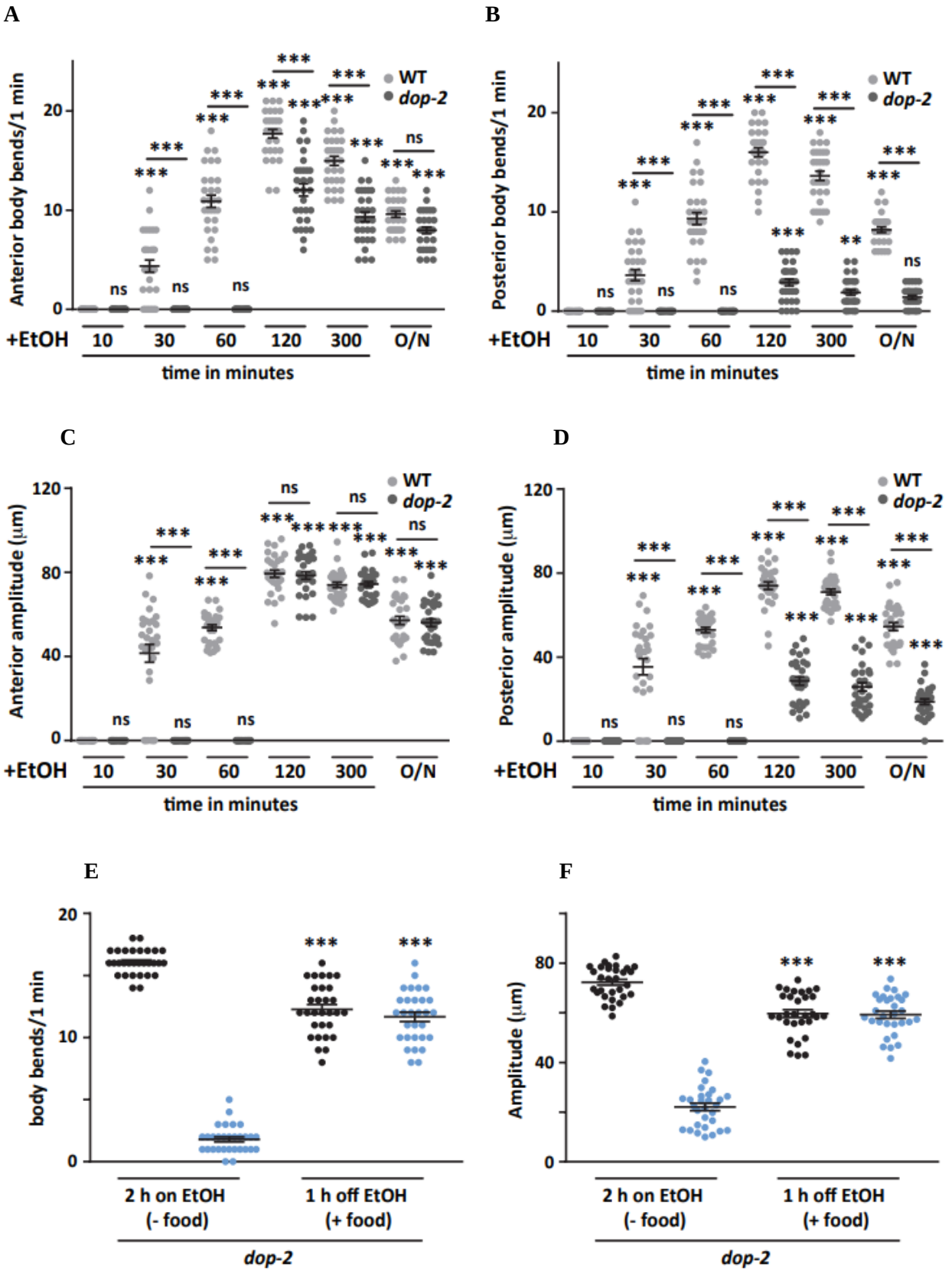

A
B
C
D
E
F
WT
dop-2
Anterior body bends/1 min
Posterior body bends/1 min
Anterior amplitude (µm)
Posterior amplitude (µm)
body bends/1 min
Amplitude (µm)
+EtOH
10
30
60
120
300
O/N
time in minutes
ns
***
**
2 h on EtOH
(- food)
1 h off EtOH
(+ food)
dop-2

Figure 3.3. Time dependent analysis and recovery of dop-2 dependent EIS behavior.

A. Number of anterior body bends quantified from WT and *dop-2* mutant upon EtOH treatment at 10 minutes (min), 30 min, 60 min, 120 min, 300 min and overnight (16 hr) time points (n=10, N=3 and F= 239, DF= 11). B. Number of posterior body bends quantified from WT and *dop-2* mutant animals during an EtOH assay at 10 min, 30 min, 60 min, 120 min, 300 min and overnight (16 hr) time points (n=10, N=3 and F= 272, DF= 11). C. Amplitude of anterior body bends quantified from WT and *dop-2* mutant animals upon EtOH treatment at 10 min, 30 min, 60 min, 120 min, 300 min and overnight (16 hr) time points (n=10, N=3 and F= 372, DF= 11). D. Amplitude of posterior body bends quantified from WT and *dop-2* mutant animals upon EtOH treatment at 10 min, 30 min, 60 min, 120 min, 300 min and overnight (16 hr) time points (n=10, N=3 and F= 263, DF=11). The same videos of moving animals were used to quantitate both number of body bends and the amplitude of body bends for each genotype. E. Graph of number of body bends quantitated for *dop-2* mutant animals under different conditions including 2 hr on EtOH (-food) and the same animals transferred to NGM plates with food to study the recovery of EIS behavior in *dop-2* mutant animals, observed and quantitated after 1 hr on food, (n=10, N=3 and F= 406, DF = 3). F. Graph of amplitude of body bends quantitated for *dop-2* mutant animals under different conditions including 2 hr on EtOH (-food) and the same animals transferred to NGM plates with food to study the recovery of EIS behavior in *dop-2* mutant animals, observed and quantitated after 1hr on food, (n=10, N=3 and F= 226, DF = 3). Error bars represent $\pm$S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

3.2.4. Dopamine is required for EIS behaviour and exogenous DA applied to WT can induce the behaviour

DOP-2 functions on DAergic neuron, thus the EIS behavior may be dependent on DA. D2 like autoreceptors have been shown to modulate the levels of DA through the regulation of transporters and the components of DA synthesis pathway (Ford, 2014). In this manner it regulates the release of DA and maintains a basal level of synaptic DA. Till now there is no clear evidence showing the function of DOP-2 autoreceptor in such a regulatory role. EIS behaviour provided us with an experimental model to investigate function of DOP-2 in the sedative movement during exposure to high levels of EtOH. Thus, we examined how the DA levels and DA synthesis pathway components might affect this behaviour. Thus, to address this we utilized *cat-2* mutants in EtOH assay. Since CAT-2 gene encodes for tyrosine hydroxylase and synthesizes DA from tyrosine, a rate limiting step in DA biosynthesis. The *cat-2* mutant utilized is an allelic deletion mutant and was reported to have 20-30% of basal levels of DA in the system (Lints and Emmons 1999; Sanyal et.al., 2004). We performed EtOH assay with this mutant of *cat-2*. Upon observation and quantitation of *cat-2* mutant behaviour it was quite evident that decreased levels of DA are not involved in the sedative, sluggish behaviour shown by *dop-2* (Fig. 3.4.1. A, B). Previously it was shown that in case of *cat-2* mutants increased locomotor activity is observed as compared to WT

animals (Sawin et al, 2000; Sanyal et al., 2004). Now we wanted to know where in the pathway is DOP-2 functioning so we generated *cat-2; dop-2* double mutant and performed EtOH assay. We observed that this double mutant showed a similar kind of behavior as seen in *cat-2* mutant worms (Fig. 3.4.1. A, B). This evidence indicated that *cat-2* mutation is functioning upstream of DOP-2 and which is quite relevant since it is involved in the synthesis of DA and it results in the suppression of *dop-2* EIS behaviour.

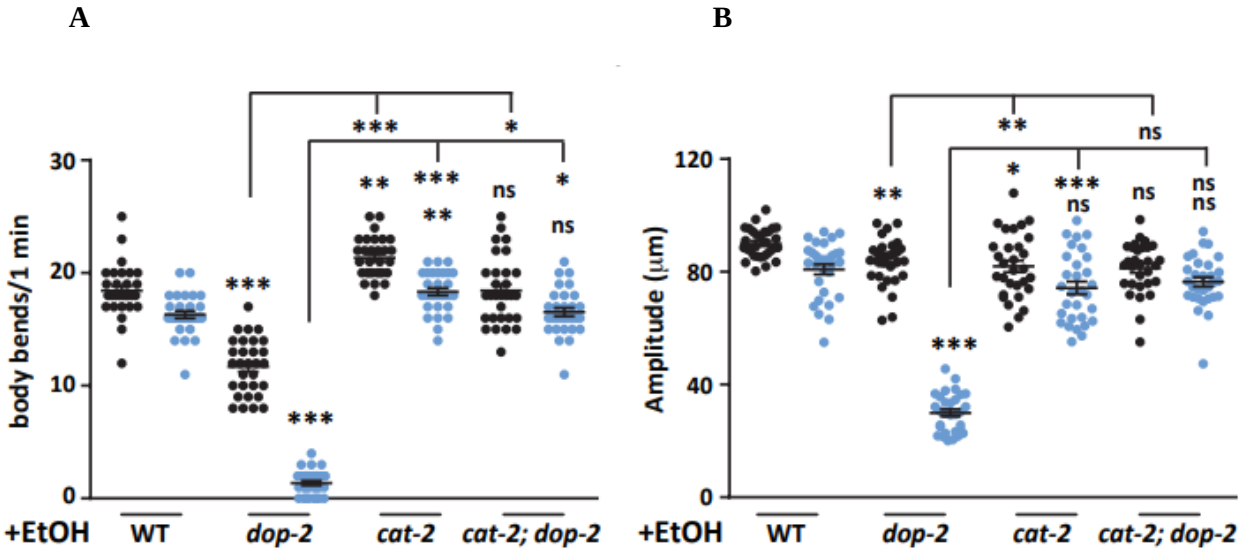


Figure 3.4.1. A DA pathway mutant *cat-2* suppresses the *dop-2* dependent EIS behavior.

A. Graph represents the number of body bends in WT, *dop-2, cat-2 and cat-2; dop-2* mutants in the presence of EtOH. Anterior body bends are shown in black and posterior body bends are shown in blue in all graphs (n=10, N=3 and F = 265, DF = 7). B. Quantitation of the amplitude of body bends in WT, *dop-2*, *cat-2 and cat-2; dop-2* strains in the presence of EtOH. Anterior amplitude of body bends are shown in black and posterior amplitude of body bends are shown in blue in all graphs (n=10, N=3 and F = 121, DF = 7). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

Thus, the suppression of behaviour observed in *cat-2* mutants is due to decreased levels of DA and opposite condition could be responsible for the EIS behaviour. There are reports in the mammalian system indicating the negative regulatory role of D2 autoreceptor in regulating the synaptic levels of DA (Benoit-Marand et al., 2001, Rouge-Pont et al., 2002, Schmitz et al., 2002, Lindgren et al., 2003). We provided the animals with exogenous DA in the EtOH assay and observed the behaviour of WT animals. We provided the animals with two conditions i.e., DA in the presence and absence of EtOH to rule out any possibility that the behavior is only DA dependent and EtOH plays no role in regulating the behavior. We noted that the WT animals on DA exposure showed no prominent modification in their behaviour when compared for the body bends (Fig. 3.4.2. A) and amplitude of body bends (Fig. 3.4.2. B). Whereas, when increased levels of DA accompanied EtOH on the assay plates, the exposed animals showed distinct EIS behaviour with marked reduction in the no. of body bends and the amplitude of body bends as compared to WT worms exposed to EtOH without DA (Fig. 3.4.2. A, B). These results suggest that DA is responsible for EIS behaviour in *C. elegans*, the deletion of *dop-2* leads to the absence of DOP-2 auto-regulation. The elevated release of DA goes unregulated in its absence since release in response to EtOH will be substantially high but compensatory mechanism of DOP-2 is not present. This increased synaptic DA potentiates activity through the post-synaptic connections. Thus, WT worms recapitulated the EIS of *dop-2* worms upon exposure to exogenous DA in the presence of EtOH.

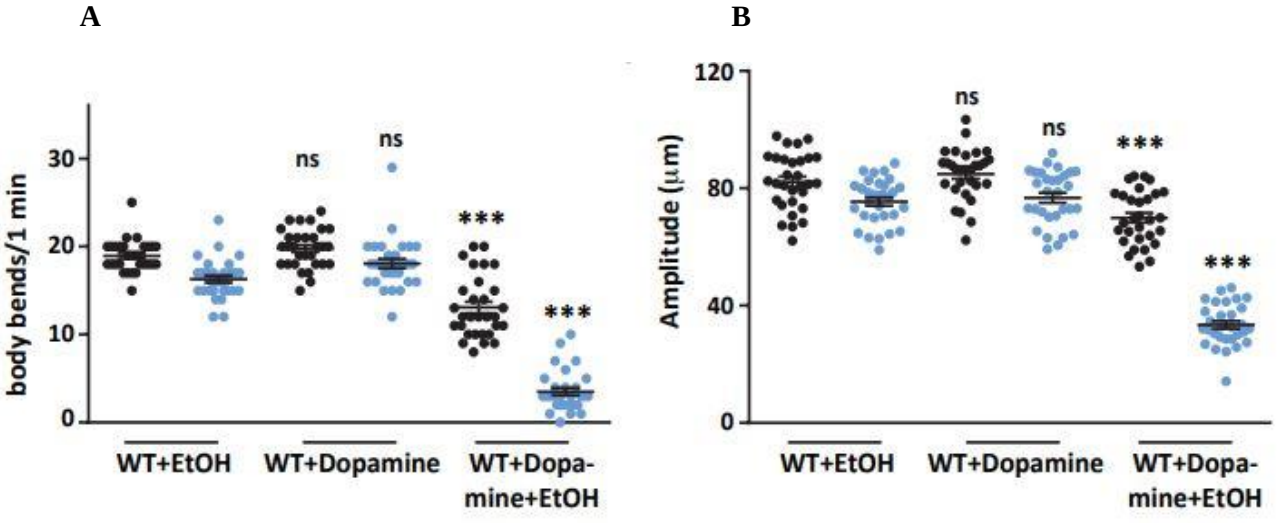


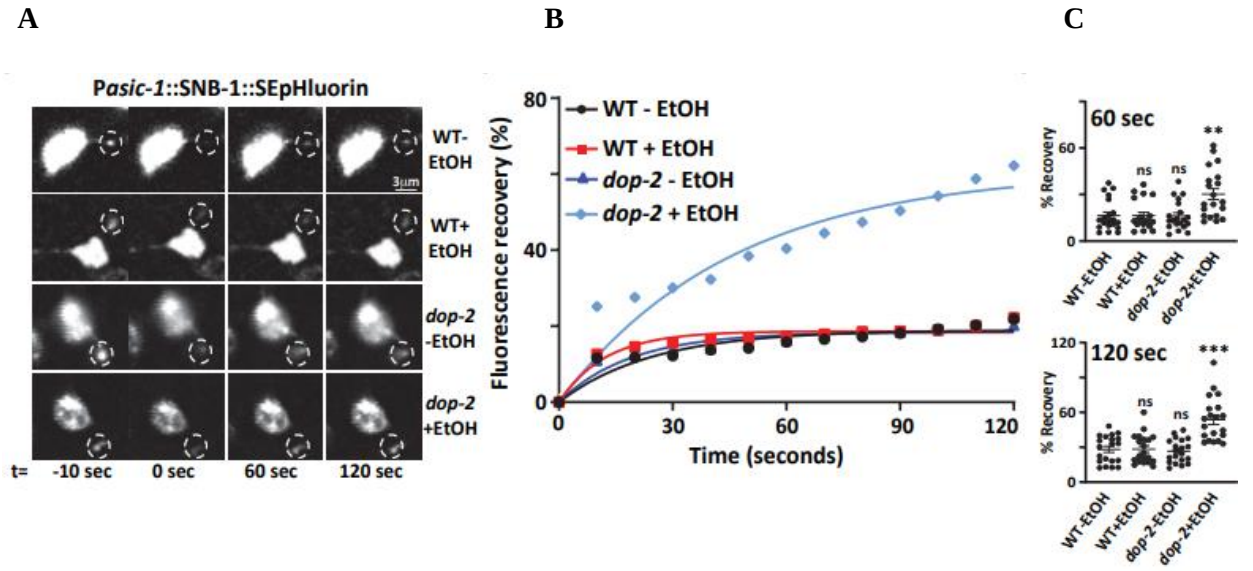
Figure 3.4.2. WT animals show dop-2 like EIS behavior on exposure to exogenous DA.

A. Graph representing the number of body bends in WT animals under different conditions (+ EtOH, + DA and + DA + EtOH), (n=10, N=3 and F = 169, DF = 5). B. Graph shows the amplitude of body bends in WT *C. elegans* under different conditions (+ EtOH, + DA, + DA + EtOH), (n=10, N=3 and F = 141, DF = 5). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

3.2.5. Mutants of *dop-2* worms show increased dopamine release in the presence of EtOH

Our above results indicate that there is increased DA release in *dop-2* mutants in the presence of EtOH. However, DAT-1 is present on the DA neurons that recycles DA back into the neuron. This led us to think that in the absence of *dop-2*, DAT-1 could not function optimally. We reasoned that if the reuptake mechanism was affected then *dat-1* (DA transporter) deletion mutant should also show EIS like phenotype since DA levels should be higher than WT, but that wasn't the case (Fig. 3.2.2. A, B). Prior reports indicate that D2 like receptors are involved in the surface localization of DAT-1. This was studied by utilizing DAT-1::GFP translational fusion line. We performed imaging and quantitated the cell surface expression of DAT-1 transporter in PDE neuron in the presence and absence of EtOH in the WT and *dop-2* mutant backgrounds (Fig. 3.5. D). We observed a slight reduction in cell surface expression of DAT-1 in *dop-2* mutants when exposed to EtOH (Fig. 3.5. E). Thus, our results till now indicate that in case of *dop-2* mutant there is increased synaptic DA as in case of *dat-1* mutant. DOP-2 might be regulating the levels by not allowing for more release. However, in the absence of *dop-2*, DA release is more but DAT-1 expression on the surface is reduced below optimal levels and thus there is more DA levels at the synapse. To further strengthen our observation, we used FRAP (optical recording of fluorescence recovery after photobleaching) as a tool to directly examine the DA release and synaptic activity (Samuel et al., 2003; Miesenbock et al., 1998). Here we used synaptobrevin-super ecliptic pHluorin reporter fusion (SNB-1::SEpHluorin) expressed under DAergic asic-1 promoter (Voglis and Tavernarakis, 2014). PDE neuron synapses were examined with pH sensitive GFP, "superecliptic pHluorin," attached to a vesicular protein SNB-1. The fluorescence was bleached in the synapses of PDE neuron and with exocytosis pH sensitive fluorescence recovered and the

rate of recovery at the bleached area showed the measure of DA release in this case. Increased release of DA can be monitored by the rate of recovery in PDE synapses post bleach (Fig 3.5. A). We observed a significant fast rate of recovery in the case of *dop-2* mutant animals in presence of EtOH (Fig. 3.5. B) and increased rate of recovery at 60 sec and 120 sec time points (Fig. 3.5. C). Thus, in the presence of EtOH *dop-2* mutant animals show increased synaptic release of DA since the regulatory mechanism of the neuron is lost. Since *dop-2* is also involved in regulating the surface expression of *dat-1* thus reuptake of increased DA is inefficient in the absence of *dop-2* and which results in unchecked accumulation of DA at the synapse.



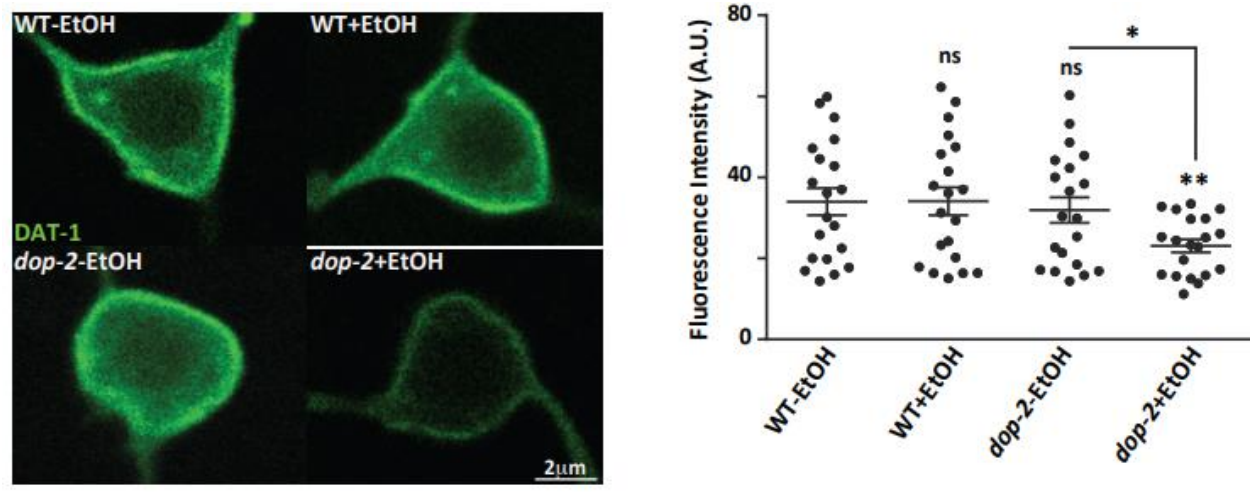


Figure 3.5. Mutants in *dop-2* show increased dopamine release in presence of ethanol.

A. Fluorescence recovery after photobleaching (FRAP) was performed on the PDE neuron synapses labeled with P*asic-1*::SNB-1::SEpHluorin. The images represent a PDE neuron synapse before bleaching (-10 seconds (sec)), followed by bleaching (0 sec) and post bleach at 60 sec and 120 sec. B. Quantitation of rate of recovery taking the mean of all the recovery data points of each *C. elegans* in WT and *dop-2* mutant backgrounds with and without (+/- EtOH) treatment over 120 sec FRAP time course. Data represents 20-22 synapses per genotype. C. Dot plots from FRAP data for percentage recovery were plotted for 60 sec (n=10, N=2 and F = 16.7, DF = 3) and 120 sec (n=10, N=2 and F = 20.3, DF = 3) time points respectively as we observed a sharp rise in percentage recovery at these time points. D. Representative images of DAT-1::GFP expression in WT and *dop-2* mutant background with and without EtOH treatment. E. Whole cell fluorescence quantification of DAT-1::GFP in PDE neurons for WT and *dop-2* mutants with and without EtOH, (n=10, N=2 and F = 3.09, DF = 3). Error bars represent $\pm$S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

3.3. Discussion

DOP-2 belongs to a family of D2-like inhibitory receptors. They negatively regulate the release of DA by feedback inhibition of DA release from presynaptic terminals due to lowered cell firing (Mercuri et al., 1997; De Mei et al., 2009; Ford, 2014;). In *C. elegans* investigations have reported various physiological behaviours for the DA pathway mutants. DOP-2 is only present on the DA neurons and this makes it a very good candidate for autoregulation of DA. However, *dop-2* deletion doesn't show defects in DA dependent behaviours as has been reported for other DA receptors such as DOP-1and DOP-3 (Sawin et al., 2000; Chase et al., 2004; Allen et al., 2011). It is often found that the behaviours associated with deletion of neuromodulatory molecules are not very prominent since there are several different molecular players involved in regulating the behavior. EtOH has been shown to increase DA release from the ventral tegmental area and increased levels were found in nucleus acccumens when measured by microdialysis (Yim and Gonzales, 2000; Weiss et al., 1993; Imperato and Di Chiara, 1986). Since both *dop-2* mutants and EtOH tend to increase DA levels, thus it prompted us to test *dop-2* deletion mutants for movement defect/s in the presence of EtOH and served as a very useful strategy. We saw very interesting results with chronic exposure (2 hr to 24 hr) of *dop-2* worms to EtOH. After 2 hr when worms were quantitated for body bends and their amplitude, it was evident that there was drastic decrease in both. Upon analysis of plates with chronic exposure of *dop-2* mutants, we found distinct concentric tracks with least signs of sinusoidal movement. It was noticeable that

here most of the worms stayed on the plates even after 24 hr whereas in case of other strains, only few worms if any were left on the plates. Thus, it can be inferred that food search behaviour became totally defective or they had lost any desire to feed. Their movement wasn't the cause for the reason not to escape as there were tracks all over the plates even on the sides but they preferred to be on the plates. In future it will be interesting to study various behaviours of the worms once they have been removed from the EtOH assay plates. Previously it has been reported that worms show tolerance towards acute EtOH exposure and after recovery they exhibit various forms of disinhibitions in the behaviours (Mitchell et al., 2010; Topper et al., 2014). In *C. elegans* it's the first time that effect of EtOH has been studied for extremely chronic conditions (400mM for 24 hr). The lethargic/sedative pattern found was the consequence of increased DA levels. EtOH perturbed DA levels in *dop-2* mutants to higher levels as shown in our FRAP studies. To substantiate our results, we also applied DA exogenously and EtOH assay revealed that WT worms behaved like *dop-2* loss of function mutants. We also found that this defect in movement wasn't due to the SLO-1 channels, reported as the major target of EtOH in *C. elegans* (Davies et al., 2004). We used loss of function mutant of SLO-1 and it showed increased locomotion, whereas we haven't tested O/E mutants, but we expect the behaviour would differ since they have expression mainly in the anterior neurons and muscles. Here we reveal the fact that EtOH affects the worm movement through DA and the unique phenotype of *dop-2* mutant along with FRAP studies substantiates the fact that DOP-2 is the regulator of DA levels at the synapse. High concentrations of EtOH are known to affect the pharyngeal pumping in *C. elegans* (Dillon et al., 2013). We were also quite curious to know if *dop-2* mutants showed defects in the pharyngeal pumping. Since the sedative behaviour of *dop-2* mutant was so prominent at 400mM conc., we assumed that it's pharyngeal pumping is also affected. However, to our surprise it had no quantitative effect on the rate of pharyngeal pumping. Further, EtOH based liquid hypercontraction behaviour was also comparable to WT and showed increased sensitivity in *dop-2* animals. So, we conclude that *dop-2* mutant alone could increase the DA levels to certain extent only since there exists compensatory mechanism to alleviate the increased synaptic DA but EtOH pushes it to extreme levels and shows the phenotype.

The synaptic levels of DA are maintained by the activities of DA transporter (DAT-1), that recycles the DA back to the cell (Benoit-Marand et al., 2002) in conjunction with DA autoreceptor (DOP-2). DA transporter (DAT) is responsible for DA clearance from the extracellular space (Schmitz et al., 2003, Ford et al., 2010). In mammals the activity of DAT can be controlled by D2-autoreceptors by regulating their surface expression (Cass and Gerhardt, 1994, Dickinson et al., 1999, Mayfield and Zahniser, 2001, Schmitz et al., 2002, Wu et al., 2002, Benoit-Marand et al., 2011). This occurs at least partially via an increase in DAT cell surface expression after D2-receptor activation (Mayfield and Zahniser, 2001). This was shown by external application of saturating amounts of DA, it is not clear whether levels of DA in vivo rise to high enough levels to engage this mechanism during periods of physiologically relevant activity (Ford et al., 2014). D2-antagonists or D2 deletion could not alter DAT dependent DA uptake (Benoit-Marand et al., 2001, Benoit-Marand et al., 2011; Bello et al., 2011; Anzalone et al., 2012). Thus D2-autoreceptor dependent DAT regulation could not be shown clearly (Kennedy et al., 1992, Beckstead et al., 2007). Our FRAP experiments conducted in the presence of increased EtOH (400mM) and in the absence of *dop-2* showed that there was increase in DA levels being released. *dop-2* mutants themselves do not show any behavioral defect, the behavior is observed only in the presence of EtOH, known to enhance the release of DA. While in Wild Type animals the increased DA levels lead to activation of DOP-2 autoreceptor and engage a negative feedback mechanism to regulate the release of DA, which is absent in the *dop-2* receptor mutants, leading to accumulation of DA at the synaptic cleft. DAT-1 is required to maintain the synaptic levels of DA, by recycling the dopamine back to the cell and thus clearance of DA from the extracellular space. Thus, overexpression of DAT should be able to suppress the EIS phenotype observed in *dop-2* mutants. Further we studied the DAT-1 membrane expression by utilizing DAT-1::GFP fusion protein localization and found that in *dop-2* mutant it was low as compared to WT and also when exposed to EtOH. Thus, we showed that DAT-1 surface transport is dependent upon DOP-2. In *dop-2* mutants exposed to high levels of EtOH, DA levels increased at the synapse since DAT-1 was also functioning sub optimally. This provided further support to the idea that DOP-2 regulates the DA levels at the synapse.

CHAPTER IV

Increased dopamine acts to regulate locomotion through NLP-12 neuromodulation

Abstract

As we previously report in our study, the role of D2 like autoreceptor DOP-2 in regulating a EtOH induced sedative behavior (EIS), we use this EtOH assay to delineate the neuronal circuitry that is involved in regulating the EIS behavior. The posterior part of the worm seemed to more severely affected than the anterior region, and our results validate this, as we observe partial rescue from the behavior on expressing DOP-2 in posterior neuron but not in the anterior dopaminergic (DAergic) neuron. We go on to show through our ablation studies that the DOP-2 autoreceptor functions on PDE, a posterior DAergic neuron to regulate the levels of dopamine (DA) at the synapse. PDE forms unidirectional synapses on a mechanosensory neuron DVA. We further show that increased DA at the synapse in *dop-2* mutant mediate its action by activating the downstream circuitry, by binding to D1 like receptor DOP-1 expressed on DVA neuron. Activation of DVA through the DOP-1 receptor leads to enhanced release of a DVA specific neuropeptide NLP-12, that binds to its cholecystokinin like receptor CKR-2 present on cholinergic motor neuron, thus modulating, here leading to increase in the release of a neurotransmitter acetylcholine (Ach), that leads to muscle excitation and hypercontraction, leading to the EIS behavior.

4.1. Introduction

Animals tend to respond to the changes in external environment by appropriately modulating their behavior by changing their internal state (Bhattacharya et al., 2014). DA a highly conserved neurotransmitter (NT) has been shown to regulate such behavior across species, most significantly the movement related behavior and change in state. DA mediates its action by binding to its widely expressed receptors, the D1 and D2 like receptors, that allow for fine tuning of behavioral output in response to an external cue (Vidal-Gadea and Pierce-Shimomura).

C. elegans is a well-established model for studying the DA signaling pathway, there are eight DA neurons in hermaphrodite, two pairs of CEP, a pair of ADE in the head and a pair of PDE DAergic neuron in the posterior region. DA acts through classical synapses as well extrasynaptically to regulate different behaviors. The D1 and D2 like receptors DOP-1 and DOP-3 are coexpressed on same neuron and act antagonistically to regulate locomotion (Chase et al., 2004). In the previous chapter we have characterized a EtOH induced sedative behavior (EIS) specific to a D2 like receptor DOP-2, whose deletion normally does not show any defect, as it acts as an autoreceptor and is specifically expressed on DAergic neurons. Our results indicate towards the fact that the posterior region is more severely affected than the anterior.

PDE is the only DAergic neuron present in the posterior region of the worm. It is mechanosensory in nature as other DAergic neurons and is involved in regulating the basal slowing behavior of the worm in context to food (Sawin et al., 2000). PDE has also been shown to regulate food search behavior, and locomotion through a neuropeptide NLP-12, secreted specifically from a mechanosensory interneuron DVA (Bhattacharya et al., 2014). PDE send strong synapses to DVA, which integrates the sensory information received from PDE to accordingly modulate the behavior. DVA itself is a mechanosensory neuron, regulating locomotion through proprioception. It regulates locomotion through a mechanosensory feedback mechanism, by modulating the levels of acetylcholine, through NLP-12 (Hu et al., 2011, Bhattacharya et al., 2014). PDE has been shown to function through DOP-1 receptors present on DVA, leading to the activation of downstream circuitry, shown to regulate the local search behavior in context to food, and on immediate removal from it (Bhattacharya et al., 2014; Hills et al., 2004). All the previous studies show that the final behavioral modulation is mediated by a neuropeptide NLP-12 secreted from DVA, by binding to its cholecystokinin like receptor CKR-2 and CKR-1. These receptors are expressed on the

cholinergic motor neurons, regulating activation and release of acetylcholine at the NMJ (Hu et al., 2004; Bhattacharya et al., 2014; Ramachandran et al., 2020).

Overall, our work implicates the role of *dop-2* in the EtOH induced sedative phenotype by demonstrating that 1. Mutants in *dop-2* show increased DA release in the PDE neuron upon chronic EtOH treatment. 2. The increased DA released from the PDE sensory neuron may cause increased release of the neuropeptide, NLP-12 from the DVA interneuron. However, our data does not preclude the possibility that other DAergic neurons could also be involved in this process. These neurons may function through as yet unidentified mechanisms to allow for the EIS phenotype seen in *dop-2* mutant animals. 3. Increased signaling through NLP-12 could cause increased cholinergic neuronal function and result in the observed EIS phenotype.

4.2. Results

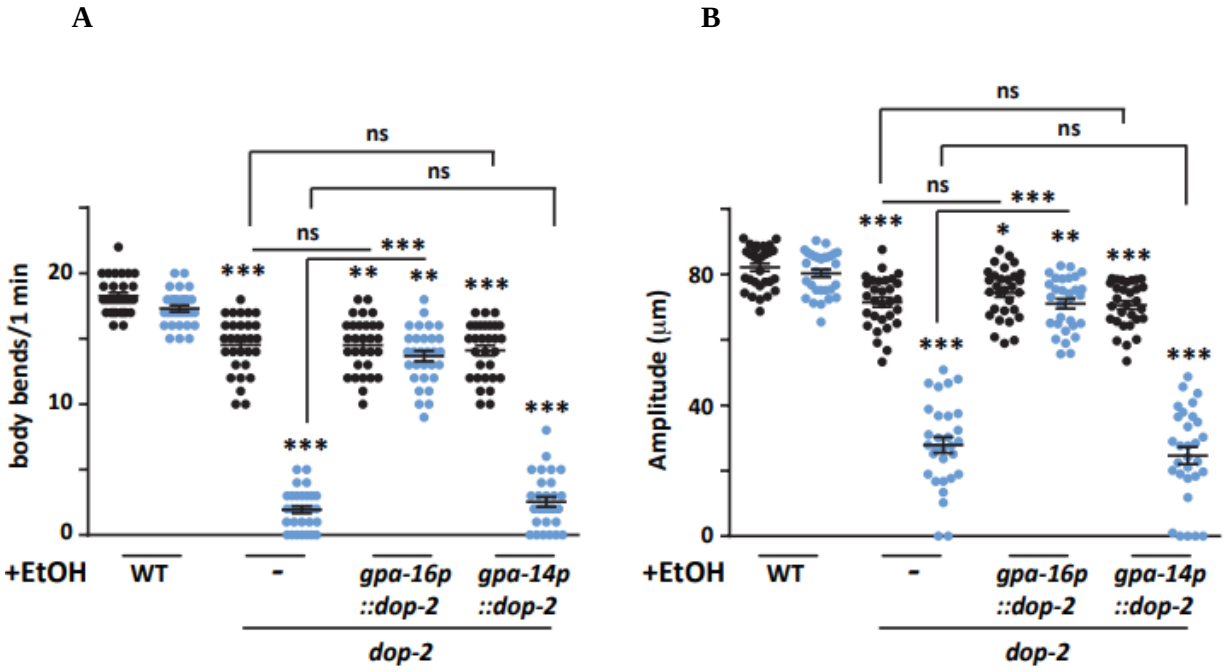
4.2.1. EIS behaviour is DOP-2 dependent and is modulated through dopaminergic PDE neuron

There are eight DAergic neurons in *C. elegans* hermaphrodite, the two pair of CEP and a pair of ADE in the head and a pair of PDE neurons in the posterior half of the *C. elegans* (Sulston et al., 1975). All these DAergic neurons are mechanosensory in nature and control the basal slowing behavior (Sawin et al., 2000). Our behavioural experiment points toward the fact that the posterior half of the animal is more affected than the anterior. The only DAergic neuron with sensory endings at the posterior half is PDE and it is also involved in harsh touch behaviour and context dependent modulation of movement (Shawn Xu et al., 2011; Bhattacharya et al., 2014). In a study by Voglis and Tavernarakis, 2004, it was indicated that DOP-2 may also modulate the DA release from these neurons. In a more recent work, it was shown that PDE neuron through synaptic signalling via DVA interneuron regulates movement in worms. Thus, it is possible that the EIS behaviour could be due to the defective release of DA from DAergic neurons and since the defect was more prominent in the posterior half of the worm thus it encouraged us to investigate the role of PDE in the EtOH dependent behaviour of *dop-2* mutant.

We were not able to find a PDE specific promoter to perform the rescue experiments, we decided to do rescue using two different promoters, which were expressed in posterior PDE and anterior ADE DAergic neurons, along with their expression in other neurons. We used the gpa-14 promoter

(expressed in subset of neurons along with anterior DAergic neuron ADE but not in PDE, posterior DAergic neuron (Jansen et al., 1999)) and gpa-16 promoter (expressed in subset of neurons along with the posterior PDE neurons but not the anterior DAergic neurons (Jansen et al., 1999)). After screening the gpa-16 and gpa-14 expressing animals through the EtOH assay we observed that gpa-16 promoter (PDE expressing) largely rescued the EIS phenotype observed in the *dop-2* mutant animals (Fig. 4.1 A, B), while animals expressing DOP-2 under gpa-14 promoter in *dop-2* background showed behavior like *dop-2* mutant animals (Fig. 4.1 A, B).

To further elucidate the role of PDE in the movement defect, we ablated PDE neuron during early larval stage (Fig 4.1. C). Here we used P*dat-1*::GFP DAergic marker strain. These worms with ablated PDE neuron were used for EtOH assay. We observed that on exposure to EtOH the PDE ablated WT animals displayed no obvious defects in the no. of body bends and the amplitude of body bends (Fig. 4.1 D, E). The *dop-2* mutant mock worms showed the EtOH induced behaviour as before. In contrast, the *dop-2* mutant worms with PDE ablated neuron surprisingly lost the behaviour and behaved like WT animals with normal no. of body bends and amplitude (Fig. 4.1 D, E). These results implicate that EIS is DOP-2 dependent and DOP-2 functions in PDE neuron to regulate this behaviour.



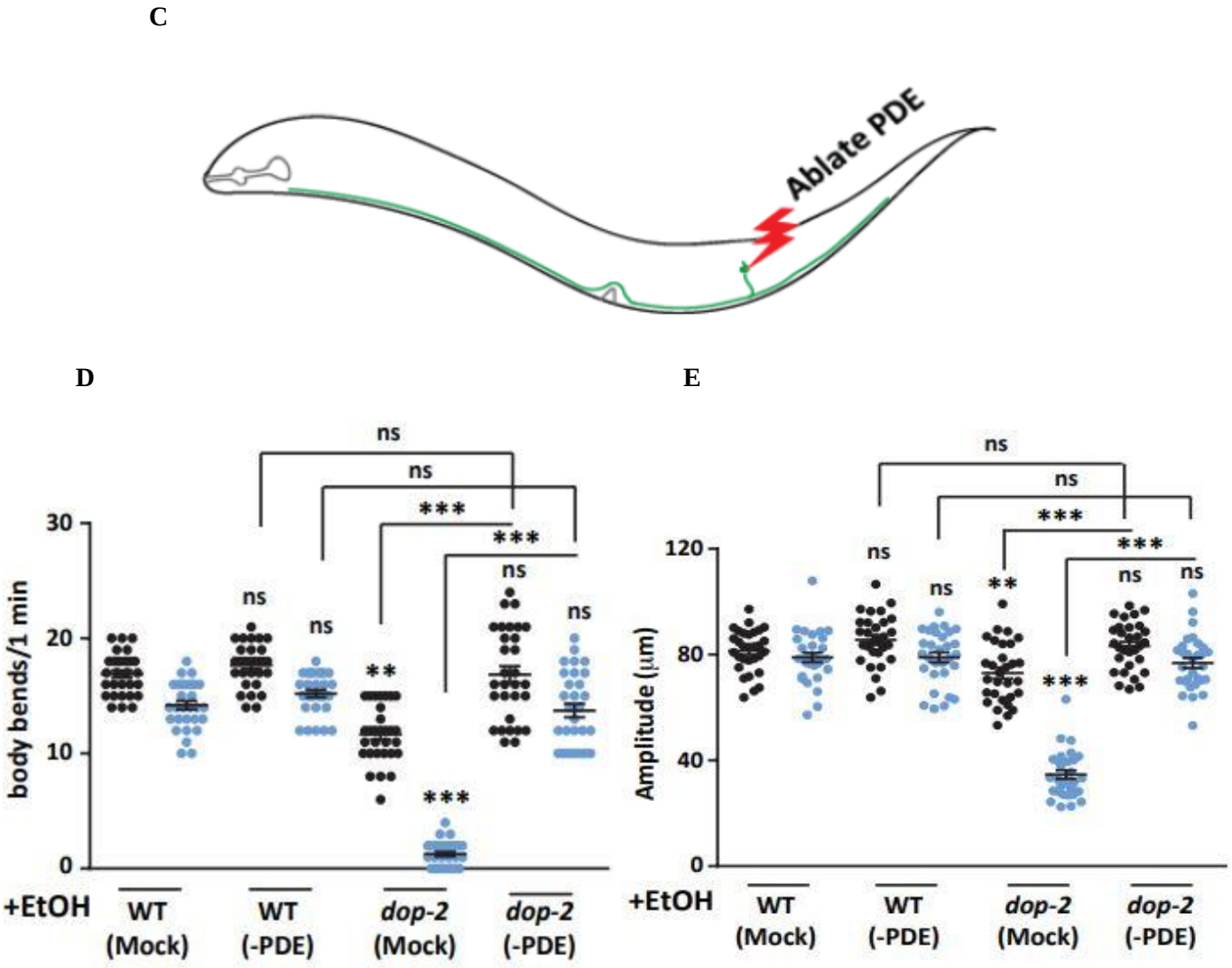


Figure 4.1. DOP-2 functions in the PDE neuron for the Ethanol Induced Sedative (EIS) behavior.

A. Graph representing the number of body bends quantitated from WT, *dop-2*, *dop-2*; *gpa-16p::dop-2* and *dop-2*; *gpa-14p::dop-2* lines. Anterior body bends are shown in black and posterior body bends are shown in blue in the graph (n=10, N=3 and F=336, DF=7). B. Graph representing amplitude of body bends quantitated from WT, *dop-2*, *dop-2*; *gpa-16p::dop-2* and *dop-2*; *gpa-14p::dop-2* lines. C. Illustration of PDE neuron ablation. D. Quantitation of the number of body bends in mock ablated and PDE ablated animals, (n=10, N=3 and F = 140, DF = 7). E. Quantitation of the amplitude of body bends in mock ablated and PDE ablated *C. elegans*, (n=10, N=3 and F = 84.5, DF = 7). All experiments in this figure were performed in the presence of EtOH (+EtOH). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

4.2.2. DOP-2 functions through DOP-1 present in the DVA neuron

Till now our results showed that EIS behaviour is modulated by NT DA. PDE has been found to be responsible for DA effect but how this is leading to defects in movement of the EtOH intoxicated worms needs to be delineated. In a study by Bhattacharya et al., 2014 it was shown that the DA released from PDE neuron can activate DOP-1 present on DVA neuron. DVA neuron upon activation results in the release of neuropeptide NLP-12 that results in the activation of downstream motor neurons. PDE forms strong synaptic connections with sensory interneuron DVA (Horwitz et al., 2000). We ablated the DVA neuron to show that it is the neuron in the downstream circuitry regulating the behaviour, but the DVA ablated animals are already defective in locomotion (Fig. 4.2. A, B) as previously shown (Li et al., 2006). Thus, we studied how DAergic receptor, DOP-1 deletion can affect the movement of the worms. After performing EtOH assay we found that no significant difference was observed on comparing *dop-1* mutant behaviour to WT. Next we made *dop-1;dop-2* double mutant and observed that it showed same kind of behaviour as *dop-1* mutant in the assay with no significant change in the body bends and amplitude (Fig. 4.2. C, D). Thus, *dop-1* deletion was able to suppress the *dop-2* mutant EIS behaviour. If deletion of *dop-1* is obstructing the DA signal from PDE then we reasoned that restoring it back should overcome that hindrance. We cloned the *dop-1* gene under DVA specific promoter *nlp-12* and injected it into *dop-1; dop-2* double mutant background to see if it can restore the EIS phenotype of *dop-2* mutants. It was observed that restoring back *dop-1* in the double mutant, rescued the EIS behaviour with characteristic decrease in body bends and amplitude as compared to WT (Fig. 4.2. C, D). Our results indicate that the EIS is dependent upon DA released from PDE and DOP-1 receptor present on DVA neuron.

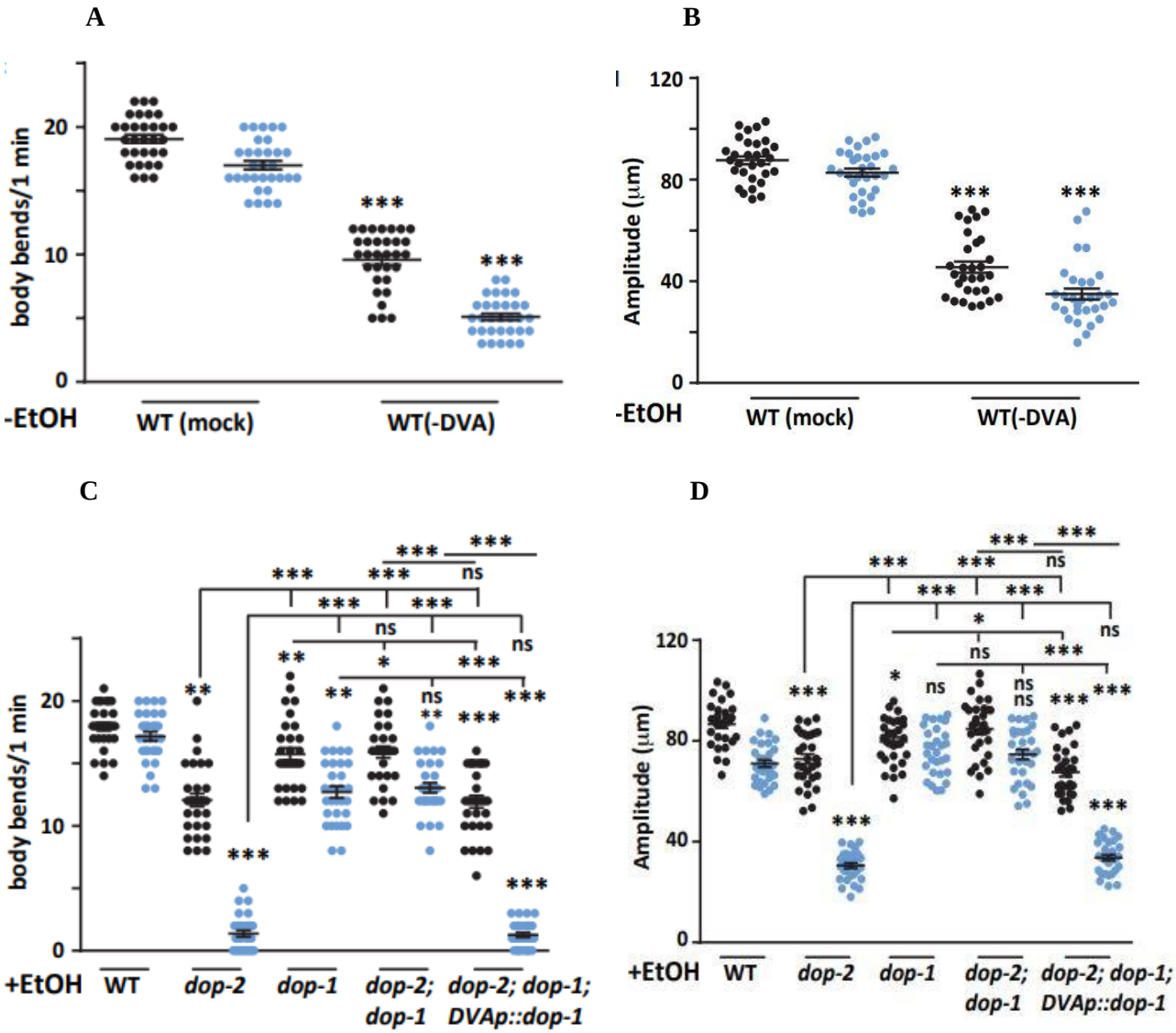


Figure 4.2. DOP-2 functions through the DOP-1 in the DVA neuron.

DVA ablation affects locomotion in WT animals A. Graph representing the number of body bends in mock and DVA ablated WT animals not treated with EtOH, (n=10, N=3 and F = 368, DF = 3). B. Graph representing the amplitude of body bends in mock and DVA ablated WT animals not treated with EtOH, (n=10, N=3 and F = 192, DF = 3). C. Graph shows number of body bends in WT, *dop-2*, *dop-1*, *dop-2; dop-1* double mutants and the *dop-1* rescue line (*dop-2; dop-1*; *DVAp::dop-1*) on EtOH treatment. Anterior body bends are shown in black and posterior body bends are shown in blue in all graphs (n=10, N=3 and F = 212, DF = 9). D. Graph shows amplitude of body bends in WT, *dop-2*, *dop-1*, *dop-2; dop-1* double mutant and the *dop-1* rescue line (*dop-2; dop-1*; *DVAp::dop-1*) on EtOH treatment. Anterior amplitude of body bends are shown in black and posterior amplitude of body bends are shown in blue in all graphs (n=10, N=3 and F = 105, DF = 9). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way

ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

4.2.3. DVA functions through NLP-12 to regulate the EIS behavior

DVA has been shown previously to function through neuropeptide NLP-12. NLP-12 release potentially activates the downstream postsynaptic cholinergic motor neurons by binding to its receptors, CKR-2 (cholecystokinin like receptor). Interestingly, it has been shown previously that NLP-12 secretion is directly correlated with the speed of the worm (Hu et al., 2011). Hence, NLP-12 might also be required for EIS behaviour. We overexpressed NLP-12, O/E animals showed EIS behaviour with decrease in body bends and amplitude as seen in *dop-2* mutants (Fig. 4.3.1. C, D) whereas *nlp-12* mutants behaved like WT animals with no significant change in body bends and amplitude (Fig. 4.3.1. A, B). Previous studies have reported that NLP-12 modulates the motor neurons and movement of the animals. Although increased NLP-12 has been shown to stimulate the speed when observed on food, whereas in the presence of EtOH the worms move slowly and drag their posterior body. The decrease in speed in our case could be due to the fact that there is no food stimulation available moreover we are quantitating the phenotype after being exposed to EtOH in the absence of food for 2hr. Hyperexcitation of muscles may be responsible for decreased shallow body bends of posterior region (Davies et al., 2003). It indicates that the increased levels of NLP-12 and ACh or decreased GABA could be responsible for paralysis of the posterior body. This part is just being dragged since anterior part is not that paralyzed.

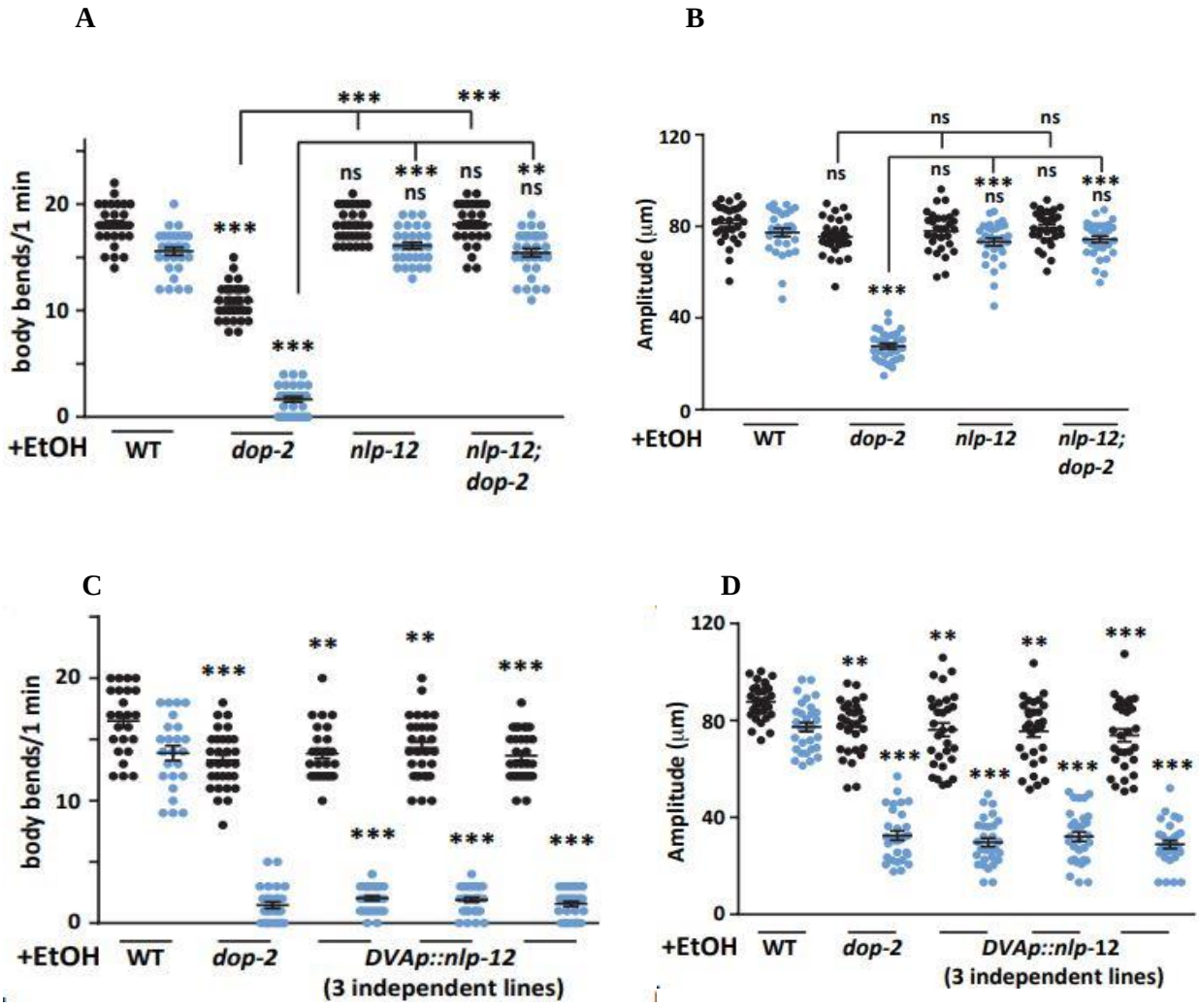


Figure 4.3.1. DOP-2 functions through NLP-12 in the DVA neuron.

A. Quantitation of number of body bends in WT, *dop-2*, *nlp-12* and *nlp-12; dop-2* animals upon EtOH treatment, (n=10, N=3 and F = 295, DF = 7). B. Quantitation of amplitude of body bends in WT, *dop-2*, *nlp-12* and *nlp-12; dop-2* animals upon EtOH treatment, (n=10, N=3 and F = 134, DF = 7). C. Quantitation of number of body bends in WT, *dop-2* and three NLP-12 overexpression (OE) lines upon EtOH treatment, (n=10, N=3 and F = 302, DF = 9). The NLP-12(OE) lines do not show significant differences when compared to the *dop-2* mutant strain. D. Quantitation of amplitude of body bends in WT, *dop-2* and three NLP-12(OE) lines upon EtOH treatment, (n=10, N=3 and F = 141, DF = 9). The NLP-12(OE) lines do not show significant differences when compared to the *dop-2* mutant strain. The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

As control experiment in accordance with prior studies we tested the behavior of WT and NLP-12 OE animals without EtOH and food, while the WT animals show immediate changes in their locomotory patterns when removed from food, i.e., the local search behavior, but in this behavior changes to long runs and dispersal resulting in decreased amplitude (Bhattacharya et al., 2014; Gray et al., 2005) on prolonged food deprivation. We go on to test the NLP-12 OE animals in above conditions and found that these animals showed behavior similar to what was observed before, increased amplitude within first 30 min off food (Bhattacharya et al., 2014; Ramachandran et al., 2020) and Fig. 4.3.2. A). And as our timeline for the experiment of ethanol assay is 120 min, we have quantitated the amplitude of body bends at this time point and observed a very substantial increase in the amplitude at 120 min time point (Fig. 4.3.2. A), while the *dop-2* mutant animals showed a behavior similar to WT animals in the absence of EtOH (Fig. 4.3.2. A). Our results indicate that in *dop-2* mutant, increased levels of DA activate DOP-1 receptors on PDE membrane. This leads to secretion of higher levels of NLP-12 neuropeptide that controls increased motor signalling and hence paralysis of posterior region of worms that is evident in the EIS behaviour of *C. elegans*. Thus, we have successfully shown that DOP-2 is an autoreceptor that regulates DA release from DA neurons. The DA released from PDE directly activates DVA neuron through its synaptic connections and it further connects to motor neurons to modulate movement. It is such a fascinating scenario where wired signalling can be easily distinguished from non-wired signalling and also shows that the intensity of synaptic signalling is more efficient and focused.

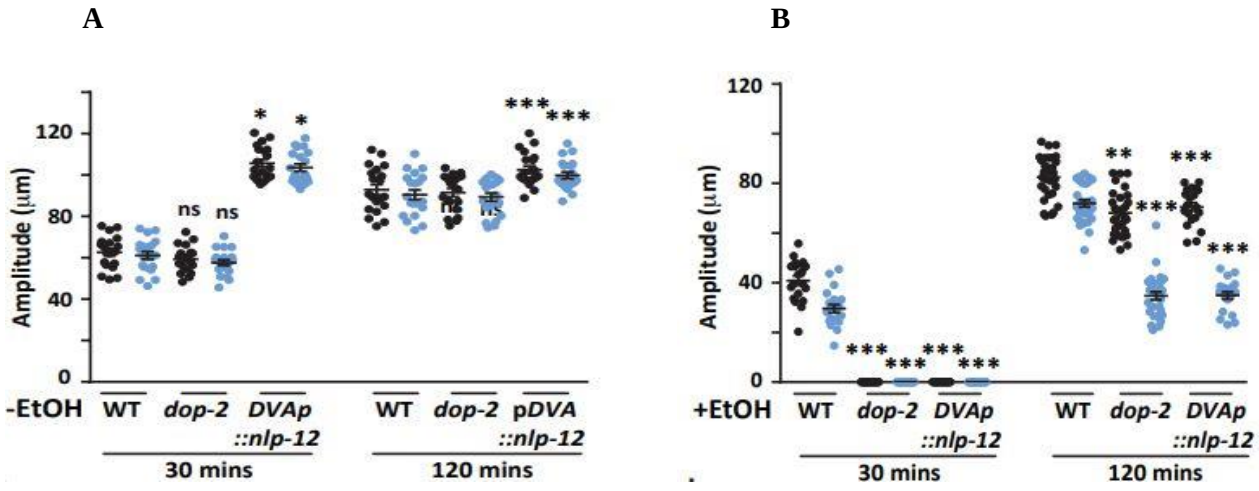


Figure 4.3.2. EIS like behavior observed in NLP-12 OE animals is specific to effects of EtOH.

A. Quantitation of the amplitude of body bends (anterior body bends are shown in black and posterior body bends are shown in blue in all graphs) from WT, *dop-2* mutant animals and an NLP-12 overexpression line where NLP-12 is expressed in DVA neurons. These experiments were performed at 30 and 120 min off food without EtOH treatment, (n=10, N=2 and F = 104, DF = 11). B. Quantitation of the amplitude of body bends (anterior amplitude of body bends are shown in black and posterior amplitude of body bends are shown in blue in all graphs) from WT, *dop-2* mutant animals and an NLP-12 overexpression line where NLP-12 is expressed in DVA neurons. These experiments were performed at 30 and 120 min off food with EtOH treatment, (n=10, N=2 and F = 522, DF = 11). Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

4.2.4. Increased levels of DA stimulate acetylcholine (Ach) signalling resulting in EIS behaviour

Previous studies have shown that DA DOP-1 (D1-like) and DOP-3 (D2-like) receptors regulate locomotion in *C. elegans*. (Chase et al., 2004; Omura et al., 2012). Studies also indicated that the hypercontracted state observed in case of EtOH exposure in the animals is due to increased acetylcholine at the NMJ (Davies et. al., 2015). All these studies prompted us to evaluate if DA is involved in regulating locomotion through the cholinergic pathway. Initially we investigated if decreased levels of ACh can display the behaviour so we started with screening of cholinergic pathway and receptor mutants and found no significant changes in the behaviour on EtOH exposure after prolonged exposure (Fig. 4.4.1. A, B).

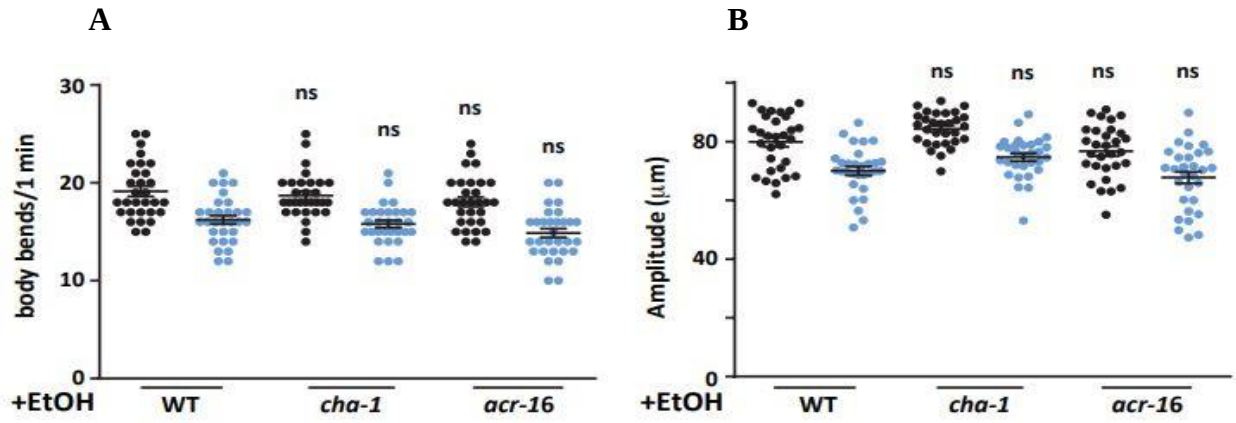


Figure 4.4.1. Mutants in cholinergic pathway do not show movement defect on EtOH exposure.

A. Graph indicating the number of body bends quantitated from WT, *cha-1* and *acr-16* mutants upon EtOH treatment (n=10, N=3 and F = 15.0, DF = 5). B. Graph indicating amplitude of body bends quantitated from WT, *cha-1* and *acr-16* mutants upon EtOH treatment (n=10, N=3 and F = 10.8, DF = 5).

Then, we manipulated aldicarb assay to serve our purpose for defect in neuromuscular signaling (Mahoney et al., 2006). Here worms were exposed to 100mM aldicarb followed by 400mM EtOH exposure. We found that WT animals exposed to the above-mentioned assay conditions showed a behavior similar to *dop-2* mutant animals and observed significant reduction in no. of body bends and amplitude in WT animals (Fig. 4.4.2. A, B). Acetylcholine synthesis pathway mutant, *cha-1* was used as a control and it is reported to show resistance to aldicarb (Rand and Russel 1984). These animals when exposed to EtOH didn't show the defect in movement that we were expecting (Fig. 4.4.2. A, B).

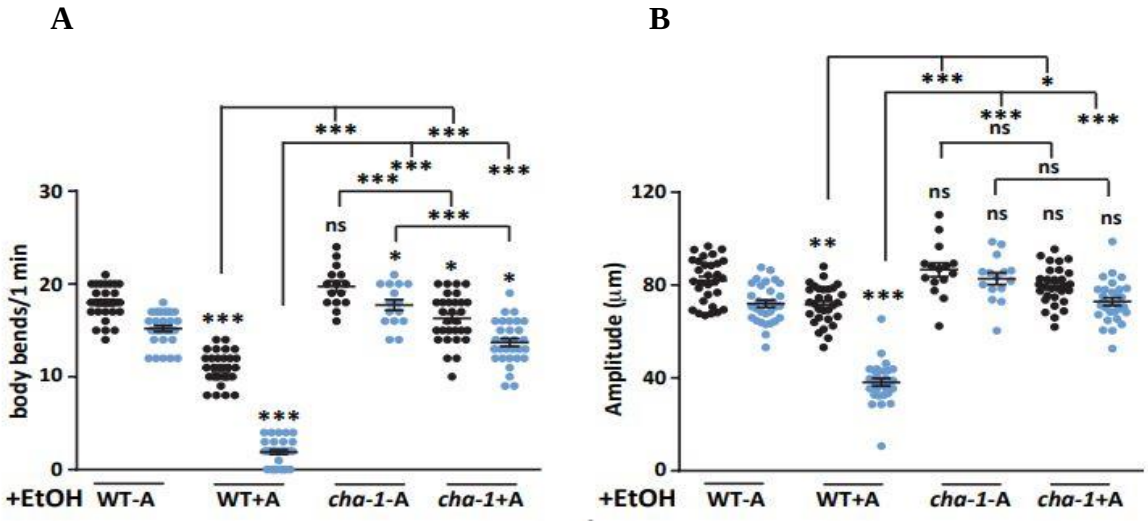


Figure 4.4.2. Increased acetylcholine signaling causes increased sensitivity to ethanol in WT animals.

A. Graph of number of body bends for WT C. elegans not treated with aldicarb (WT-A), WT animals treated with aldicarb (WT+A), cha-1 mutants not treated with aldicarb (cha-1-A) and cha-1 mutants treated with aldicarb (cha-1+A) on EtOH plates. Anterior body bends are shown in black and posterior body bends are shown in blue in all graphs (n=10, N=3 and F = 209, DF = 7). B. Graph indicates amplitude of body bends for WT C. elegans not treated with aldicarb (WT-A), WT animals treated with aldicarb (WT+A), cha-1 mutants not treated with aldicarb (cha-1-A) and cha-1 mutants treated with aldicarb (cha-1+A) on EtOH

plates. Anterior amplitude of body bends are shown in black and posterior amplitude of body bends are shown in blue in all graphs (n=10, N=3 and F = 77.3, DF = 7). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

Therefore, we though that may be *dop-2* EIS behavior is an outcome of increased cholinergic signaling at the postsynaptic cholinergic motor neurons. ACh, signals mainly through the nicotinic ACR-16 receptors present on the post synaptic membrane. In *acr-16* mutant there is about 85% decrease in response to ACh compared to WT animals (Touroutine et al., 2005). To delineate the Ach signalling in EIS behaviour we screened both *acr-16* mutant and ACR-16 O/E lines. Experimental analysis of behaviour revealed that while the *acr-16* mutant shows a similar behaviour like WT whereas ACR-16 O/E animals partially mimic the EIS behaviour upon comparing their body bends and amplitude (Fig. 4.4.3. A, B). To confirm that ACR-16 OE is functioning through the DAergic pathway to cause the EIS behavior, we made ACR-16 OE line and DAergic synthesis pathway mutant *cat-2*, double mutant line and when we screened these animals through the EtOH assay we observed that the EIS phenotype observed in ACR-16 OE animals was completely supressed in low levels of DA (Fig. 4.4.3. A, B). This result indicates that ACR 16 OE is functioning downstream of the DAergic pathway to give the EIS phenotype.

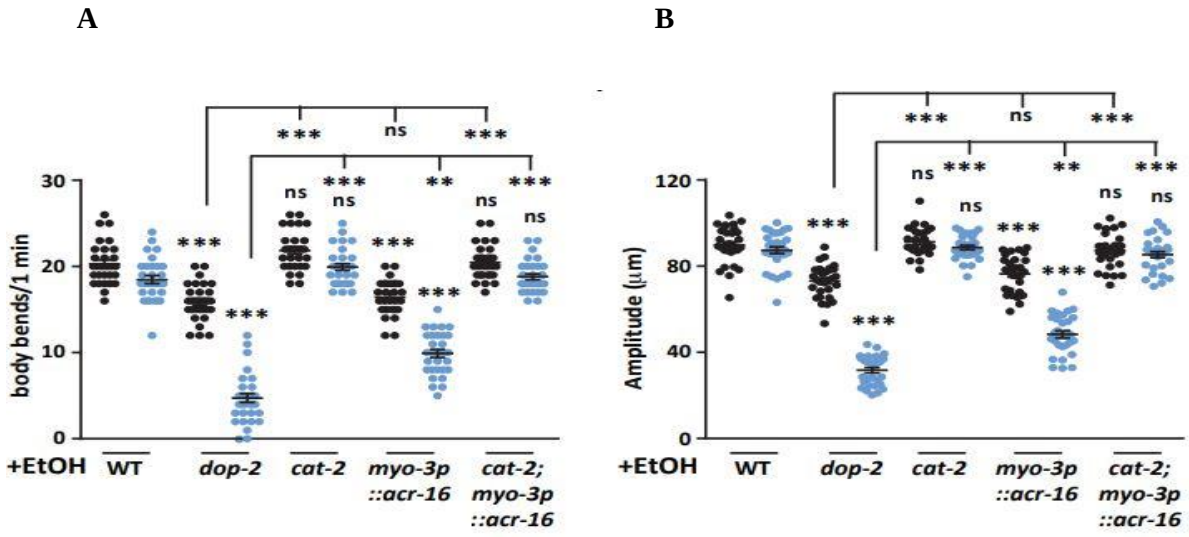


Figure 4.4.3. Increase in Ach is dependent on the DAergic pathway.

A. Quantitation of number of body bends from WT, *dop*-2, *cat-2*, *myo-3p::acr-16::gfp* line and the *cat-2; myo-3p::acr-16::gfp* lines on EtOH, (n=10, N=3 and F = 28.2, DF = 5). B. Quantitation of amplitude of body bends from WT, *dop*-2, *cat-2*, *myo-3p::acr-16::gfp* and the *cat-2; myo-3p::acr-16::gfp* lines on EtOH, (n=10, N=3 and F = 32.4, DF = 5). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

We have also tested ACR-16 OE animals along with WT, *dop-2* and *cha-1* mutant animals for any movement related defect in the absence of EtOH and found that their no significant defects in the movement related behavior in these animals both in the presence and absence of aldicarb, except *cha-1* mutant animals show resistance to aldicarb and show increased number of body bends in the presence of aldicarb as known previously (Fig. 4.4.4. A, B). Hence, elevated muscle excitation through increased acetylcholine is playing a significant role in this behaviour in the animals.

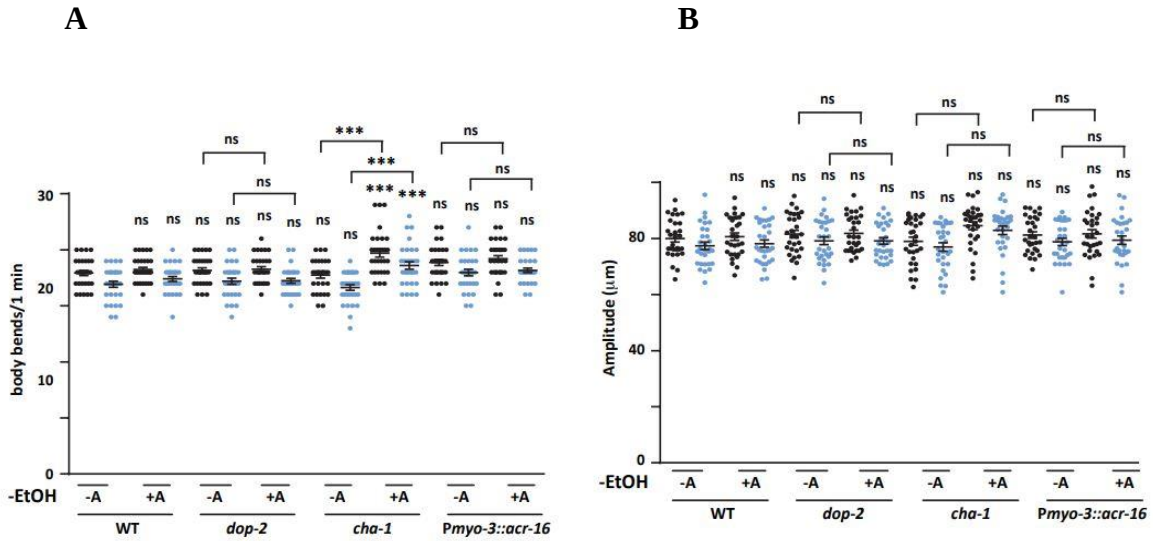


Figure 4.4.4. EIS behavior is both Ach and EtOH dependent.

A. Graph indicating the number of body bends quantitated from WT, *dop-2*, *cha-1* and *myo-3p::acr-16* under with aldicarb (+A) and without aldicarb (-A) conditions (n=10, N=3 and F =10.1, DF =15). B. Graph indicating amplitude of body bends quantitated from WT, *dop-2*, *cha-1* and *myo-3p::acr-16* under with aldicarb (+A) and without aldicarb (-A) conditions (n=10, N=3 and F =2.30, DF =15). The same videos of moving animals were used to quantitate both number of body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "ns" indicates not significant in all graphs.

4.2.5. NLP-12 functions through its Cholecystokinin like receptor CKR-2 to regulate the EIS behavior

Previous studies have shown that NLP-12 peptides function through their receptor CKR-2 in cholinergic motor neurons (Hu et al., 2011; Janssen et al., 2008; Peeters et al., 2012). To test if CKR-2 is involved in the EIS behavior seen in *dop-2* mutants, we tested both *ckr-2* and *ckr-2; dop-2* double mutants in the EtOH assay. Our data indicate that *ckr-2* mutants behaved like WT animals and the *ckr-2; dop-2* double mutants completely suppressed the *dop-2* mutant phenotype and showed a behavior similar to *ckr-2* and WT animals (Fig 4.5.1. A, B). These data indicate that the increased DA in *dop-2* mutants, signals through DOP-1, increasing NLP-12 that functions through CKR-2.

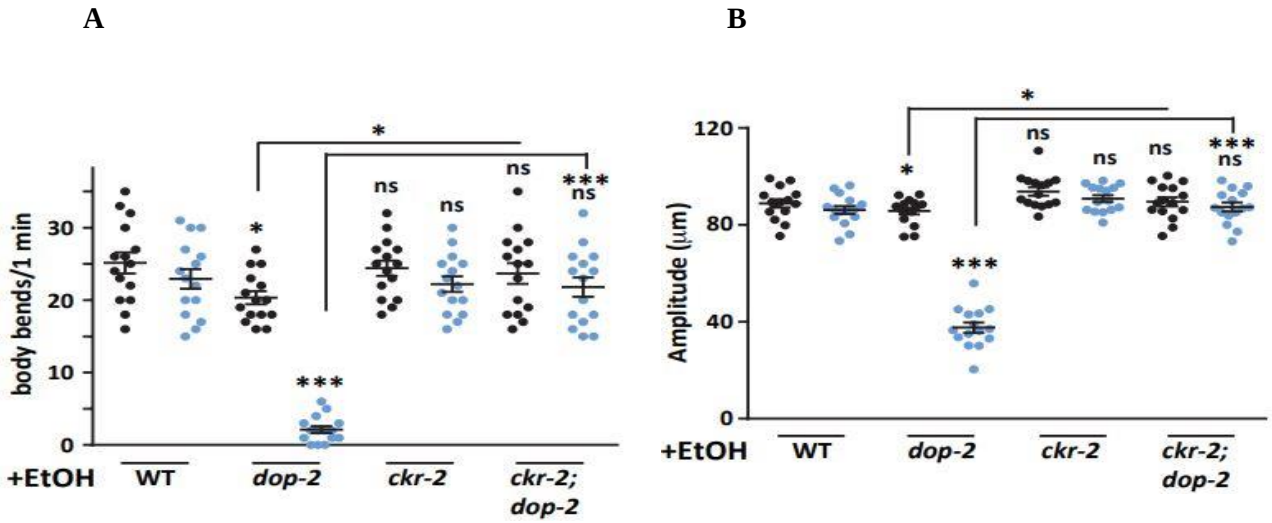


Figure 4.5.1. Loss of CKR-2 completely suppresses the dop-2 dependent EIS behavior.

A. Quantitation of number of body bends in WT, *dop-2*, *ckr-2* and *ckr-2; dop-2* animals upon EtOH treatment, (n=5, N=3 and F = 40.8, DF = 7). B. Quantitation of amplitude of body bends in WT, *dop-2*, *ckr-2* and *ckr-2; dop-2* animals upon EtOH treatment, (n=5, N=3 and F = 111, DF = 7). All experiments in this figure were performed in the presence of EtOH (+EtOH). The same videos of moving animals were used to quantitate both number body bends and the amplitude of body bends for each genotype. Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

Studies have shown that CKR-2 is expressed in motor neurons in the head and along the body of the animal (Hu et al., 2011; Ramachandran et al., 2020). Here we speculate DOP-2 could be functioning through CKR-2 present on cholinergic motor neurons that are in proximity to the DVA neuron. In order to test if the EIS phenotype caused by NLP-12(OE) is also functioning through CKR-2, we made mutants of the NLP-12(OE) line with the *ckr-2* mutation and tested these animals for the EIS phenotype. Here again we found that *ckr-2* suppressed the EIS phenotype caused by NLP-12(OE) (Fig. 4.5.2. A, B)

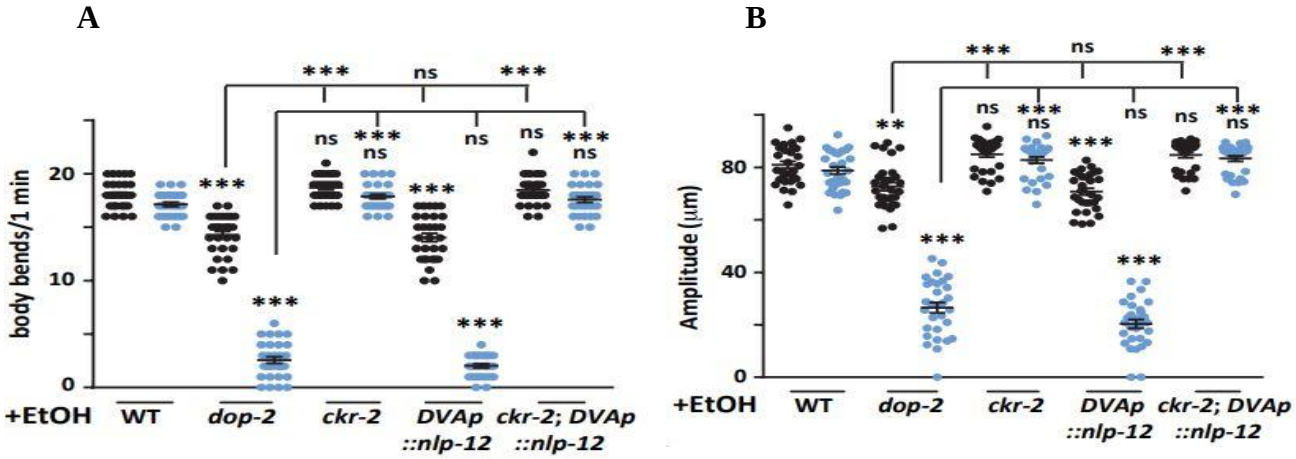


Figure 4.5.2. Loss of CKR-2 suppresses the EIS behavior observed in NLP-12 OE animals.

A. Graph representing the number of body bends quantitated from WT, *dop-2*, *ckr-2*, *DVAp::nlp-12* and *ckr-2*; *DVAp::nlp-12* treated with EtOH, (n=10, N=3 and F=576, DF=9). B. Graph representing the amplitude of body bends quantitated from WT, *dop-2*, *ckr-2*, *DVAp::nlp-12* and *ckr-2*; *DVAp::nlp-12* animals treated with EtOH, (n=10, N=3 and F=306, DF=9).

In a recent study preprint Ramachandran et al. (2020), it has been suggested that NLP-12 also functions through the CKR-1 receptor. Hence, we went on to test *ckr-1* mutants for suppression of the *dop-2* EIS phenotype. We found that *ckr-1* mutants did not show a phenotype upon exposure to EtOH for 2 hr and the *ckr-1; dop-2* double mutants behaved just like the *dop-2* single mutant animals (Fig. 4.5.3. A, B). These data suggest that *dop-2* is not functioning upstream of *ckr-1* for the EIS phenotype.

Our data thus far suggests that DA released from PDE signals through DOP-1 receptors in the DVA interneuron, which in turn releases either one or both NLP-12 peptides in this circuitry and is responsible for the EIS behavior in *dop-2* mutants treated with EtOH.

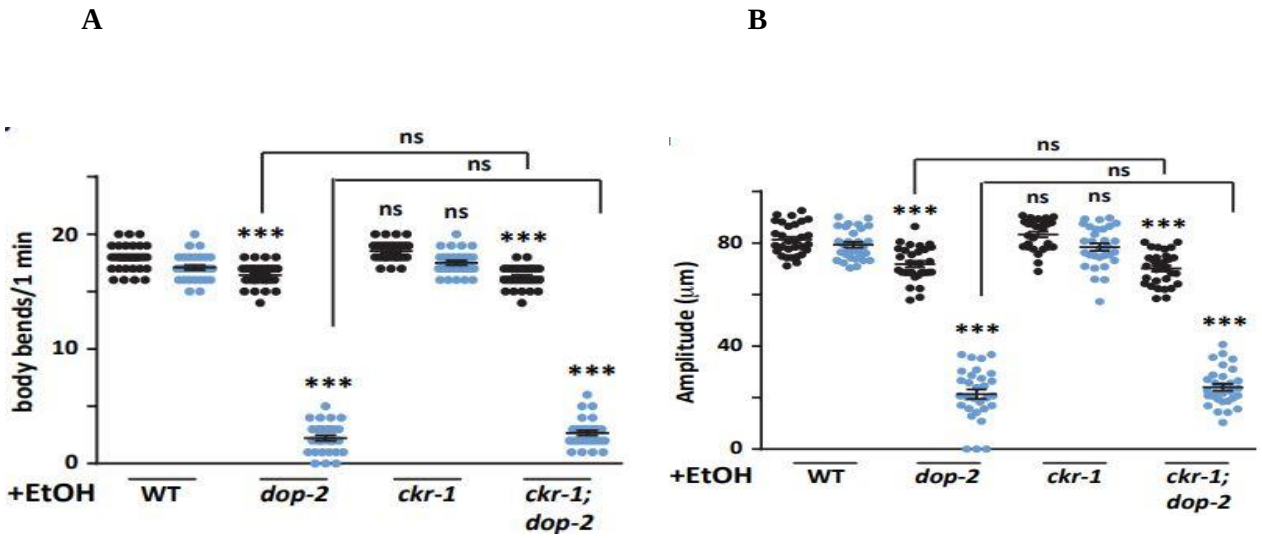


Figure 4.5.3. NLP-12 functions through its receptor CKR-2 but not CKR-1.

A. Graph representing number of body bends quantitated from WT, *dop-2*, *ckr-1* and *ckr-1*; *dop-2* animals treated with EtOH, (n=10, N=3 and F=1193, DF=7). B. Graph representing amplitude of body bends quantitated from WT, *dop-2*, *ckr-1* and *ckr-1*; *dop-2* animals treated with EtOH, (n=10, N=3 and F= 383, DF=7). Error bars represent ±S.E.M., "n" represents the number of animals and "N" represents the number of replicates. The p-values were calculated using one-way ANOVA and Tukey-Kramer multiple comparison test; "*" indicates $p<0.05$, "**" indicates $p<0.01$, "***" indicates $p<0.001$ and "ns" indicates not significant in all graphs.

Discussion

We observed the decrease in the body bends and the amplitude only in the posterior part of the worms in *dop-2* mutants. DA signaling like many other monoamines was found to be extrasynaptic in *C. elegans* since the receptors are not postsynaptic to the DA neurons. This type of signaling is slow, wireless, and acting on many receivers thus the effect of increased DA levels due to *dop-2* mutation will dampen by the time it reaches motor neurons. However, in case of PDE neuron there exists chemical, wired, unidirectional synapses with DVA (the regulator of posterior and forward movement) neuron, thus it is the best candidate neuron to study the effect of DOP-2 and its function. It's interesting to understand the connection of PDE to DVA, since monoamines such as DA are known to function largely extrasynaptically (De-Miguel et al., 2012; Fuke et al., 2012). Bentley et al., 2016, also showed in their computational studies that there exists no or very less overlap between monoamine extrasynaptic connections with wired and gap junction networks which are highly overlapping. The extrasynaptic signaling is well suited since DA and mostly other monoamines participate in signaling physiologically important states that essentially involves the entire organism, such as food availability and food dependent movement behaviors. However, the presence of PDE neuron in the posterior region and connected directly to movement controlling neuron is relevant for the escape behaviour that is also regulated by DA. Thus, during danger, the surge in DA is instantly relayed and the escape behaviour is initiated. It is important for such a circuitry to exist in the hind region of the worm as anterior part is already quite apt to sense danger due to presence of many other sensory neurons such as olfactory neurons, nociceptive neurons and mechanosensory neurons. Thus, it became clear that the anterior DA circuitry functions to control quite distinct behaviours from the posterior since their target neurons have very different functions. Previously also it was reported through various studies that the effect of ethanol on locomotion behaviour is quite distinct from other effects of EtOH (Hawkins et al., 2014).

Since in the posterior region of the worm, there is only one pair of DAergic neuron PDE, so we focused on that neuron and performed studies around that neuron. It was reported previously that this neuron makes direct synaptic contact with the DVA neuron (Bhattacharya et al., 2012). DVA is known to modulate locomotion both positively and negatively providing a unique mechanism whereby a single neuron can fine-tune motor activity (Li et al., 2006). DVA is an interneuron that has connections with both motor neurons and interneurons and relays information for locomotion

through motor circuit (Gray et al., 2005; Bhattacharya et al., 2012). Thus, it provides for a very good reason that DA secreted from PDE is regulating the movement of the worm through DVA. This particular neuron has DA receptor DOP-1, a D1-like excitatory receptor (Bhattacharya et al., 2014). In *Drosophila* and mammals, it has been demonstrated that D1-like DA receptors promote EtOH-induced disinhibition (Kong et al., 2010; Abraho et al., 2011). It is to be noted that DOP-3 (inhibitory) DA receptor is not present along with DOP-1. Since both DOP-1 and DOP-3 have overlapping presence in most of the motor neurons that receives extrasynaptic input from DA and thus the compensatory effect is prevalent. We got prominent behaviour since DOP-1 is excitatory in function and hyperactivates the downstream movement circuit. We checked for the synaptic connections of ADE and CEP neurons and found that these neurons do not form any synapses with the movement controlling neurons. These DAergic neurons are present in the head region of the worm; however, the anterior part of the worms wasn't affected in *dop-2* mutants. Thus, it indicates that: 1. The increased release of DA from the anterior worm region is responsible for various behaviours thus single defect wasn't highlighted. 2. DA secreted from anterior neurons didn't find the synaptic partners directly so effect was not prominent. 3. Anterior DA neurons are more involved in the modulatory responses other than the visibly distinguishable behaviours.

To analyse that the *dop-2* behaviour with defects in the posterior region of the worm is specifically PDE neuron dependent, we ablated PDE neuron in *dop-2* mutant background and found that the phenotype was lost. NLP-12 and DOP-1 are the two molecules important for movement regulation through DVA. This was tested by introducing *dop-1* mutation in *dop-2* deletion background and *dop-2* dependent EIS behaviour was lost. However, when DOP-1 was introduced back specifically in the DVA neuron, it showed the EIS behaviour. This supported our idea that increased DA is signaling through DOP-1 in DVA neuron. In prior study it was shown that movement induces NLP-12 release from DVA neurons and enhances ACh release at NMJs (Hu Z et al., 2011). Thus, PDE releases DA that signals through DOP-1 (excitatory) receptor present on DVA and is involved in regulating NLP-12 release to alter the functioning of motor neurons. Thus, increased levels of NLP-12 in the EtOH background should also show the phenotype. Since NLP-12 is solely secreted from DVA, we overexpressed NLP-12 in DVA neuron and found that it could recapitulate the *dop-2* mutant behaviour. We also tested the *nlp-12* mmutants in the presence of high conc of EtOH but didn't find the phenotype. It was previously shown that worms lacking NLP-12 have decreased locomotion (Zhitao Hu et al., 2011). However, in our assay the behavior was not found. Thus,

these results support the model that *dop-2* mutants exposed to EtOH increases DA signaling through DOP-1. It affects the secretion of NLP-12 from DVA and demonstrate a behavioural phenotype that may be due to increased muscle synapse activity. As reported previously by Bhattacharya et al., 2012 that NLP-12 mediated effects on the motor pattern require intact L-AChR signaling at the NMJ. Several studies in the past suggested that neuropeptides regulate transmission at cholinergic NMJs in *C. elegans*. Many other neuropeptide mutants (deletion) were found to be aldicarb resistant, indicating that neuropeptides can regulate synaptic transmission (Kass et al., 2001; Jacob and Kaplan, 2003; Sieburth et al., 2005; Sieburth et al., 2007; Speese et al., 2007; Husson and Schoofs, 2007; Edwards et al., 2009; Sumakovic et al., 2009). So, we utilized aldicarb, an acetylcholine esterase inhibitor that would increase levels of ACh at the NMJ. We found that WT animals when exposed to 1mM aldicarb showed the behaviour, suggesting a role of ACh signaling in the behaviour. Thus, the PDE neuron positioned posteriorly releases increased DA in *dop-2* mutants in the presence of EtOH and causes body muscle contraction, through altered cholinergic transmission.

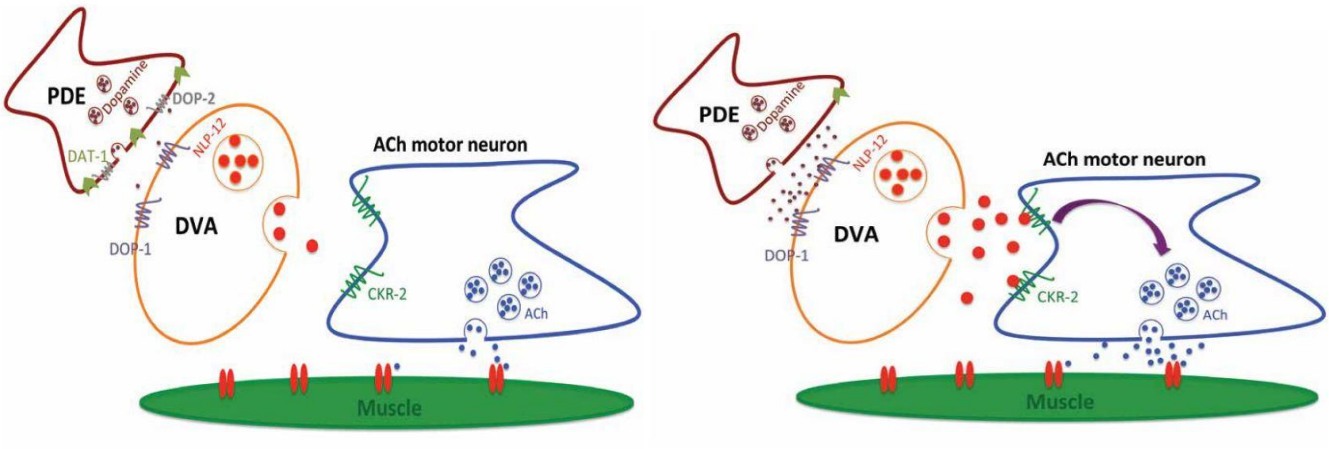


Figure 4.6. Proposed model for DOP-2 functioning in the presence of EtOH.

(a) DOP-2 DA autoreceptors functions in the posterior DA neurons (PDE) to regulate DA levels, in the presence of EtOH. (b) Loss of the *dop-2* autoreceptor leads to unregulated release of DA in the presence of EtOH. The increased levels of DA activate the function of DOP-1 receptors present on the DVA neuron causing increased DVA activation. This in turn may cause increased release of the neuropeptide NLP-12, which in turn could cause increased cholinergic signaling at the NMJ by binding to the CKR-2 receptor in motor neurons and activating the cholinergic motor neurons that are in close proximity to the DVA neuron. This model is based on this work and prior studies (Bhattacharya et al., 2014; Hu et al., 2011).

Significance of the study

In this study we utilized the dopamine autoreceptor mutant and investigated the underlying mechanism of chronic alcohol exposure. Till now the studies haven't focused much on dopamine autoreceptor DOP-2. The unique phenotype of *dop-2* mutants made various important revelations that this phenotype was functioning in a coordinated manner by employing dopamine, neuropeptide and motor signaling. We were able to deduce the function for DOP-2 autoreceptor. Till now it was shown to have minimal function if any.

The outcome of this study was being able to move from behavior to molecules to a circuit using the *C. elegans* model system. We were able to delineate the pathway through which DOP-2 functions upon EtOH exposure that resulted in extremely slow movement of *dop-2* mutant animals. Our experiments gave evidence to the fact that increased DA leads to muscle contractions through acetylcholine. Our studies provide important evidence since it is always difficult to assign specific role to neuromodulators such as DOP-2, since they have role in wide array of behaviours which can be very transient and subtle. Thus, to identify their role in locomotion, we needed to use ethanol stress to be able to see an obvious phenotype.

In our future studies our efforts will be to understand the post withdrawal effects of this chronic ethanol exposure. It will also be interesting to study the sensitivity and tolerance that can be developed to acute levels of EtOH after chronic exposure. We hope to understand the effect of ethanol on next generation behaviours to check for heritable effects of chronic EtOH intake.

Appendix

Appendix Table 1: List of movies used in this thesis

Sr No.	Movie No.	Details
1	Movie 1	WT (+EtOH) 120 min
2	Movie 2	*dop-2* (+EtOH) 120 min
3	Movie 3	WT mock ablated (PDE) (+EtOH) 120 min
4	Movie 4	WT PDE ablated (+EtOH) 120 min
5	Movie 5	*dop-2* mock ablated (PDE) (+EtOH) 120 min
6	Movie 6	*dop-2* PDE ablated (+EtOH) 120 min
7	Movie 7	WT + Dopamine (-EtOH) 120 min
8	Movie 8	WT + Dopamine (+EtOH) 120 min
9	Movie 9	NLP-12 overexpression (+EtOH) 120 min
10	Movie 10	WT DVA ablated (-EtOH) 120 min
11	Movie 11	WT + aldicarb (+EtOH)

Appendix Table 2: Reagents used in the thesis

Lab	Reagent Strain/Plasmid	Citation
Blakely Lab	IR725	Hardaway et al., 2015
Blakely Lab	BY834	Hardaway et al., 2015
Kaplan Lab	nuls299	Babu et al., 2011
Rene Garcia Lab	*dop-2p::dop-2::cfp*	Correa et. al., 2012

Bibliography

Bibliography

Abrahao, K.P., Quadros, I.M., and Souza-Formigoni, M.L. (2011). Nucleus accumbens dopamine D(1) receptors regulate the expression of ethanol-induced behavioural sensitization. Int J Neuropsychopharmacol *14*, 175-185.

Abdelhack, M. (2016). Dopaminergic neurons modulate locomotion in Caenorhabditis elegans. BioRxiv.

Alaimo, J.T., Davis, S.J., Song, S.S., Burnette, C.R., Grotewiel, M., Shelton, K.L., Pierce-Shimomura, J.T., Davies, A.G., and Bettinger, J.C. (2012). Ethanol metabolism and osmolarity modify behavioral responses to ethanol in C. elegans. Alcohol Clin Exp Res *36*, 1840-1850.

Alari, L., Sjoquist, B., and Lewander, T. (1987). Ethanol-induced hypothermia and biogenic amine metabolites. Drug and Alcohol Dependence 19-4: 369-373.

Allen, A.T., Maher, K.N., Wani, K.A., Betts, K.E., and Chase, D.L. (2011). Coexpressed D1- and D2-like dopamine receptors antagonistically modulate acetylcholine release in Caenorhabditis elegans. Genetics *188*, 579-590.

Anzalone, A., Lizardi-Ortiz, J.E., Ramos, M., De Mei, C., Hopf, F.W., Iaccarino, C., Halbout, B., Jacobsen, J., Kinoshita, C., Welter, M., *et al.* (2012). Dual control of dopamine synthesis and release by presynaptic and postsynaptic dopamine D2 receptors. The Journal of neuroscience: the official journal of the Society for Neuroscience *32*, 9023-9034.

Babu, K., Hu, Z., Chien, S.C., Garriga, G., and Kaplan, J.M. (2011). The immunoglobulin super family protein RIG-3 prevents synaptic potentiation and regulates Wnt signaling. Neuron *71*, 103-116.

Baidya, M., Genovez, M., Torres, M., and Chao, M.Y. (2014). Dopamine modulation of avoidance behavior in Caenorhabditis elegans requires the NMDA receptor NMR-1. PloS one *9*, e102958.

Baik, J.H. (2013). Dopamine signaling in reward-related behaviors. Front Neural Circuits *7*, 152.

Barriere, G., Mellen, N., Cazalets, J.R. (2004). Neuromodulation of the locomotor network by dopamine in the isolated spinal cord of newborn rat. Eur J Neurosci. 19:1325–1335.

Basu, A., Dey, S., Puri, D., Das Saha, N., Sabharwal, V., Thyagarajan, P., Srivastava, P., Koushika, S.P., and Ghosh-Roy, A. (2017). let-7 miRNA controls CED-7 homotypic adhesion and EFF-1-mediated axonal self-fusion to restore touch sensation following injury. Proceedings of the National Academy of Sciences of the United States of America *114*, E10206-E10215.

Beaulieu, J.M., and Gainetdinov, R.R. (2011). The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol Rev *63*, 182-217.

Beckstead, M.J., and Williams, J.T. (2007). Long-term depression of a dopamine IPSC. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 2074-2080.

Bello, E.P., Mateo, Y., Gelman, D.M., Noain, D., Shin, J.H., Low, M.J., Alvarez, V.A., Lovinger, D.M., and Rubinstein, M. (2011). Cocaine supersensitivity and enhanced motivation for reward in mice lacking dopamine D2 autoreceptors. Nat Neurosci *14*, 1033-1038.

Benoit-Marand, M., Ballion, B., Borrelli, E., Boraud, T., and Gonon, F. (2011). Inhibition of dopamine uptake by D2 antagonists: an in vivo study. J Neurochem *116*, 449-458.

Benoit-Marand, M., Borrelli, E., and Gonon, F. (2001). Inhibition of dopamine release via presynaptic D2 receptors: time course and functional characteristics in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience *21*, 9134-9141.

Benoit-Marand, M., Jaber, M., and Gonon, F. (2000). Release and elimination of dopamine in vivo in mice lacking the dopamine transporter: functional consequences. Eur J Neurosci *12*, 2985-2992.

Bettinger, J.C., Carnell, L., Davies, A.G., and McIntire, S.L. (2004). The use of Caenorhabditis elegans in molecular neuropharmacology. Int Rev Neurobiol *62*, 195-212.

Bettinger, J.C., and Davies, A.G. (2014). The role of the BK channel in ethanol response behaviors: evidence from model organism and human studies. Front Physiol *5*, 346.

Bhardwaj, A., Thapliyal, S., Dahiya, Y., and Babu, K. (2018). FLP-18 Functions through the G-Protein-Coupled Receptors NPR-1 and NPR-4 to Modulate Reversal Length in Caenorhabditis elegans. The Journal of neuroscience : the official journal of the Society for Neuroscience *38*, 4641-4654.

Bhattacharya, R., and Francis, M.M. (2015). In the proper context: Neuropeptide regulation of behavioral transitions during food searching. Worm *4*, e1062971.

Bhattacharya, R., Touroutine, D., Barbagallo, B., Climer, J., Lambert, C.M., Clark, C.M., Alkema, M.J., and Francis, M.M. (2014). A conserved dopamine-cholecystokinin signaling pathway shapes context-dependent Caenorhabditis elegans behavior. PLoS genetics *10*, e1004584.

Blomqvist, O., Engel, J.A., Nissbrandt, H., and Soderpalm, B. (1993). The mesolimbic dopamine-activating properties of ethanol are antagonized by mecamylamine. European Journal of Pharmacology 249, 2: 207-213.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Brown, T.G. (1911). The Intrinsic Factors in the Act of Progression in the Mammal. roc R Soc Lond B Biol Sci 84 308–319. Brown, T.G. (1914). On the nature of the fundamental activity of the nervous centres; together with an analysis of the conditioning of rhythmic activity in progression, and a theory of the evolution of function in the nervous system. J Physiol 48, 18-46.

Bunzow, J.R., Van Tol, H.H., Grandy, D.K., Albert, P., Salon, J., Christie, M., Machida, C.A., Neve, K.A., and Civelli, O. (1988). Cloning and expression of a rat D2 dopamine receptor cDNA. Nature *336*, 783-787.

Carvelli, L., McDonald, P.W., Blakely, R.D., and DeFelice, L.J. (2004). Dopamine transporters depolarize neurons by a channel mechanism. Proceedings of the National Academy of Sciences of the United States of America *101*, 16046-16051.

Carlsson, A., Lindqvist, M. (1973). Effect of ethanol on the hydroxylation of tyrosine and tryptophan in rat brain in vivo. J Pharm Pharmacol. 25:437-440.

Cass, W.A., and Gerhardt, G.A. (1994). Direct in vivo evidence that D2 dopamine receptors can modulate dopamine uptake. Neurosci Lett *176*, 259-263.

Chase, D.L., Pepper, J.S., and Koelle, M.R. (2004). Mechanism of extrasynaptic dopamine signaling in Caenorhabditis elegans. Nat Neurosci *7*, 1096-1103.

Chandler, L.J. (2003) Ethanol and brain plasticity: receptors and molecular networks of the postsynaptic density as targets of ethanol. Pharmacol Ther 99: 311-326.

Cianciulli, A., Yoslov, L., Buscemi, K., Sullivan, N., Vance, R.T., Janton, F., Szurgot, M.R., Buerkert, T., Li, E., and Nelson, M.D. (2019). Interneurons Regulate Locomotion Quiescence via Cyclic Adenosine Monophosphate Signaling During Stress-Induced Sleep in Caenorhabditis elegans. Genetics 119.302293.

Clarke, P.B.S., Jakubovic, A., Fibiger, H.C. (1988). Anatomical analysis of the involvement of mesolimbocortical dopamine in the locomotor stimulant actions of d-amphetamine and apomorphine. Psychopharmacology. 96:511–20.

Clemens, S., Belin-Rauscent, A., Simmers, J., and Combes, D. (2012). Opposing modulatory effects of D1- and D2-like receptor activation on a spinal central pattern generator. J. Neurophysiol. 107, 2250–2259. doi: 10.1152/jn.00366.201

Correa, P. A., LeBoeuf, B., and Garcia, L.R. (2012). C. elegans dopaminergic D2-like receptors delimit recurrent cholinergic-mediated motor programs during a goal-oriented behavior. PLoS genetics *8*, e1003015.

Correa, P.A., Gruninger, T., and Garcia, L.R. (2015). DOP-2 D2-Like Receptor Regulates UNC-7 Innexins to Attenuate Recurrent Sensory Motor Neurons during C. elegans Copulation. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 9990-10004.

Corsi, A.K., Wightman, B., and Chalfie, M. (2015). A Transparent Window into Biology: A Primer on Caenorhabditis elegans. Genetics *200*, 387-407.

Cowen, P.J. (2008). Serotonin and depression: pathophysiological mechanism or marketing myth? Trends Pharmacol Sci. 9:433-6.

Cowmeadow, R.B., Krishnan, H.R., and Atkinson, N.S. (2005). The slowpoke gene is necessary for rapid ethanol tolerance in Drosophila. 29(10):1777-86.

Davies, A.G., and McIntire, S.L. (2004). Using C. elegans to screen for targets of ethanol and behavior-altering drugs. Biol Proced Online *6*, 113-119.

Davies, A.G., Pierce-Shimomura, J.T., Kim, H., VanHoven, M.K., Thiele, T.R., Bonci, A., Bargmann, C.I., and McIntire, S.L. (2003). A central role of the BK potassium channel in behavioral responses to ethanol in C. elegans. Cell *115*, 655-666.

De Mei, C., Ramos, M., Iitaka, C., and Borrelli, E. (2009). Getting specialized: presynaptic and postsynaptic dopamine D2 receptors. Curr Opin Pharmacol *9*, 53-58.

Dickinson, S.D., Sabeti, J., Larson, G.A., Giardina, K., Rubinstein, M., Kelly, M.A., Grandy, D.K., Low, M.J., Gerhardt, G.A., and Zahniser, N.R. (1999). Dopamine D2 receptor-deficient mice exhibit decreased dopamine transporter function but no changes in dopamine release in dorsal striatum. J Neurochem *72*, 148-156.

Dopico, A.M., Anantharam, V., and Treistman, S.N. (1997). Ethanol Increases the Activity of Ca++ -Dependent K+(mslo) Channels: Functional Interaction with Cytosolic Ca++. JPET 284:258–268.

Dopico, A.M. and Treistman, S.N. (1996). Ethanol has opposite effects on the activity of two cloned large conductance, Ca++-activated K1 (bslo and mslo) channels (Abstract). Soc Neurosci 22 Pt 1:351.

Dubuc, R., Brocard, F., Antri, M., Fenelon, K., Gariepy, J. F., Smetana, R., et al. (2008). Initiation of locomotion in lampreys. *Brain Res. Rev*. 57, 172–182.

Eddison, M., Guarnieri, D.J., Cheng, L., Liu, C.H., Moffat, K.G., Davis, G., and Heberlein, U. (2011). arouser reveals a role for synapse number in the regulation of ethanol sensitivity. Neuron *70*, 979-990.

Edwards, S.L., Charlie, N.K., Richmond, J.E., Hegermann, J., Eimer, S., and Miller, K.G. (2009). Impaired dense core vesicle maturation in Caenorhabditis elegans mutants lacking Rab2. J Cell Biol *186*, 881-895.

Engleman, E.A., Katner, S.N., and Neal-Beliveau, B.S. (2016). Caenorhabditis elegans as a Model to Study the Molecular and Genetic Mechanisms of Drug Addiction. Prog Mol Biol Transl Sci. 137: 229–252.

Feltmann, K., Borroto-Escuela, D.O., Ruegg, J., Pinton, L., de Oliveira Sergio, T., Narvaez, M., Jimenez-Beristain, A., Ekstrom, T.J., Fuxe, K., and Steensland, P. (2018). Effects of Long-Term Alcohol Drinking on the Dopamine D2 Receptor: Gene Expression and Heteroreceptor Complexes in the Striatum in Rats. Alcohol Clin Exp Res *42*, 338-351.

Ford, C.P. (2014). The role of D2-autoreceptors in regulating dopamine neuron activity and transmission. Neuroscience *282*, 13-22.

Formisano, R., Mersha, M.D., Caplan, J., Singh, A., Rankin, C.H., Tavernarakis, N., and Dhillon, H.S. (2020). Synaptic vesicle fusion is modulated through feedback inhibition by dopamine auto-receptors. Synapse *74*, e22131.

Giacomotto, J., and Segalat, L. (2010). High-throughput screening and small animal models, where are we? Br J Pharmacol *160*, 204-216.

Gray, J.M., Hill, J.J., and Bargmann, C.I. (2005). A circuit for navigation in Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America *102*, 3184-3191.

Grotewiel, M. and Bettinger, J.C. (2015). Drosophila and Caenorhabditis elegans as Discovery Platforms for Genes Involved in Human Alcohol Use Disorder. Alcohol Clin Exp Res. 39(8): 1292- 311.

Gurel, G., Gustafson, M.A., Pepper, J.S., Horvitz, H.R., and Koelle, M.R. (2012). Receptors and Other Signaling Proteins Required for Serotonin Control of Locomotion in *Caenorhabditis elegans*. Genetics 192(4): 1359-1317.

Hardaway, J.A., Sturgeon, S.M., Snarrenberg, C.L., Li, Z., Xu, X.Z., Bermingham, D.P., Odiase, P., Spencer, W.C., Miller, D.M., 3rd, Carvelli, L., *et al.* (2015). Glial Expression of the Caenorhabditis elegans Gene swip-10 Supports Glutamate Dependent Control of Extrasynaptic Dopamine Signaling. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 9409-9423.

Harris, R.A., Trudell, J.R., and Mihic, S.J. (2008). Ethanol's molecular targets. Sci Signal *1*, re7.

Hawkins, E.G., Martin, I., Kondo, L.M., Judy, M.E., Brings, V.E., Chan, C.L., Blackwell, G.G., Bettinger, J.C., and Davies, A.G. (2015). A novel cholinergic action of alcohol and the development of tolerance to that effect in Caenorhabditis elegans. Genetics *199*, 135-149.

Hegarty, S.V., Sullivan, A.M., and O'Keeffe, G.W. (2013). Midbrain dopaminergic neurons: a review of the molecular circuitry that regulates their development. Developmental biology *379*, 123-138.

Hoffman, P.L., Rabe, C.S., Moses, F., Tabakoff, B. (1989). N-methyl-d-aspartate receptors and ethanol inhibition of calcium flux and cyclic GMP production. Journal of Neurochemistry. 52:1937–1940.

Horwitz, H.R., Chalfie, M., Sulston, J.E., and Evans, P.D. (1982). Serotonin and octopamine in the nematode Caenorhabditis elegans. Science 28;216(4549).

Hubner, C. B., & Koob, G. F. (1990). The ventral pallidum plays a role in mediating cocaine and heroin self-administration in the rat. Brain Research, 508(1), 20–29.

Hu, Z., Pym, E.C., Babu, K., Vashlishan Murray, A.B., and Kaplan, J.M. (2011). A neuropeptide-mediated stretch response links muscle contraction to changes in neurotransmitter release. Neuron *71*, 92-102.

Hu, Z., Vashlishan-Murray A.B., and Kaplan, J.M. (2015). NLP-12 Engages Different UNC-13 Proteins to Potentiate Tonic and Evoked Release. J Neurosci. 35(3): 1038–1042.

Hums, I., Riedl, J., Mende, F., Kato, S., Kaplan, H.S., Latham, R., Sonntag, M., Traunmuller, L., and Zimmer, M. (2016). Regulation of two motor patterns enables the gradual adjustment of locomotion strategy in Caenorhabditis elegans. Elife *5*.

Hui, M.A. and Gang, Z.H.U. (2014). The dopamine system and alcohol dependence. j.issn. 26(2): 61-68.

Imperato, A., and Di Chiara, G. (1986). Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J Pharmacol Exp Ther *239*, 219-228.

Ishita, Y., Chihara, T., and Okumura, M. (2020). Serotonergic modulation of feeding behavior in Caenorhabditis elegans and other related nematodes. Neurosci Res *154*, 9-19.

Jacob, T.C., and Kaplan, J.M. (2003). The EGL-21 carboxypeptidase E facilitates acetylcholine release at Caenorhabditis elegans neuromuscular junctions. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 2122-2130.

Jafari, G., Xie, Y., Kullyev, A., Liang, B., and Sze, J.Y. (2011). Regulation of Extrasynaptic 5-HT by Serotonin Reuptake Transporter Function in 5-HT-Absorbing Neurons Underscores Adaptation Behavior in *Caenorhabditis elegans*. The Journal of Neuroscience 31(24): 8948-8957.

Jansen, G., Thijssen, K.L., Werner, P., van der Horst, M., Hazendonk, E., and Plasterk, R.H. (1999). The complete family of genes encoding G proteins of Caenorhabditis elegans. Nature genetics *21*, 414-419.

Janssen, T., Meelkop, E., Lindemans, M., Verstraelen, K., Husson, S.J., Temmerman, L., Nachman, R.J., and Schoofs, L. (2008). Discovery of a cholecystokinin-gastrin-like signaling system in nematodes. Endocrinology *149*, 2826-2839.

Jordan, L.M., McVagh, J.R., Noga, B.R., Cabaj, A.M., Majczynski, H., Slawinska, U., Provencher, J., leblond, H., and Rossignol, S. (2014). Cholinergic mechanisms in spinal locomotion—potential target for rehabilitation approaches. fncir. 00132.

Joseph, J.D., Wang, Y.M., Miles, P.R., Budygin, E.A., Picetti, R., Gainetdinov, R.R., Caron, M.G., and Wightman, R.M. (2002). Dopamine autoreceptor regulation of release and uptake in mouse brain slices in the absence of D(3) receptors. Neuroscience 112:39 – 49.

Kameda, S.R., Frussa-Filho, R., Carvalho, R.C., Takatsu-Coleman, A.L., Ricardo, V.P., Patti, C.L., Calzavara, M.B., Lopez, G.B., Araujo, N.P., Abilio, V.C., *et al.* (2007). Dissociation of the effects of

ethanol on memory, anxiety, and motor behavior in mice tested in the plus-maze discriminative avoidance task. Psychopharmacology (Berl) *192*, 39-48.

Kass, J., Jacob, T.C., Kim, P., and Kaplan, J.M. (2001). The EGL-3 proprotein convertase regulates mechanosensory responses of Caenorhabditis elegans. The Journal of neuroscience: the official journal of the Society for Neuroscience *21*, 9265-9272.

Kennedy, R.T., Jones, S.R., and Wightman, R.M. (1992). Dynamic observation of dopamine autoreceptor effects in rat striatal slices. J Neurochem *59*, 449-455.

Kindt, K.S., Quast, K.B., Giles, A.C., De, S., Hendrey, D., Nicastro, I., Rankin, C.H., and Schafer, W.R. (2007). Dopamine mediates context-dependent modulation of sensory plasticity in C. elegans. Neuron. 55(4): 662-76.

Koeltzow, T.E., Xu, M., Cooper, D.C., Hu, X.T., Tonegawa, S., Wolf, M.E., and White, F.J. (1998). Alterations in dopamine release but not dopamine autoreceptor function in dopamine D3 receptor mutant mice. The Journal of neuroscience: the official journal of the Society for Neuroscience *18*, 2231-2238.

Kong, E.C., Woo, K., Li, H., Lebestky, T., Mayer, N., Sniffen, M.R., Heberlein, U., Bainton, R.J., Hirsh, J., and Wolf, F.W. (2010). A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in Drosophila. PloS one *5*, e9954.

Kraschewski, A., Reese, J., Anghelescu, I., Winterer, G., Schmidt, L.G., Gallinat, J., Finckh, U., Rommelspacher, H., and Wernicke, C. (2009). Association of the dopamine D2 receptor gene with alcohol dependence: haplotypes and subgroups of alcoholics as key factors for understanding receptor function. Pharmacogenet Genomics *19*, 513-527.

Lee, F.J., Pei, L., Moszczynska, A., Vukusic, B., Fletcher, P.J., and Liu, F. (2007). Dopamine transporter cell surface localization facilitated by a direct interaction with the dopamine D2 receptor. The EMBO journal *26*, 2127-2136.

Lee, J., Jee, C., and McIntire, S.L. (2009). Ethanol preference in C. elegans. Genes Brain Behav *8*, 578-585.

Lewis, M.J. (1996). Alcohol reinforcement and neuropharmacological therapeutics. Alcohol Suppl.;31(1):17-25.

Li, W., Feng, Z., Sternberg, P.W., and Xu, X.Z. (2006). A C. elegans stretch receptor neuron revealed by a mechanosensitive TRP channel homologue. Nature *440*, 684-687.

Li, W., Kang, L., Piggott, B.J., Feng, Z., and Xu, X.Z. (2011). The neural circuits and sensory channels mediating harsh touch sensation in Caenorhabditis elegans. Nat Commun *2*, 315.

Liljequist, S., Berggren, U., Engel, J. (1981). The effect of catecholamine receptor antagonists on ethanol-induced locomotor stimulation. *J Neural Transm* 50: 57–67.

Lu, R.B., Lee, J.F., Ko, H.C., and Lin, W.W. (2001). Dopamine D2 receptor gene (DRD2) is associated with alcoholism with conduct disorder. Alcohol Clin Exp Res *25*, 177-184.

Mahoney, T.R., Luo, S., and Nonet, M.L. (2006). Analysis of synaptic transmission in Caenorhabditis elegans using an aldicarb-sensitivity assay. Nat Protoc *1*, 1772-1777.

Marder, E. (2012). Neuromodulation of neuronal circuits: back to the future. Neuron 4;76(1):1-11.

Martel, J.C. and McArthur, S.C. (2020). Dopamine Receptor Subtypes, Physiology and Pharmacology: New Ligands and Concepts in Schizophrenia. Front. Pharmacol. 10.3389.

Mayfield, R.D., and Zahniser, N.R. (2001). Dopamine D2 receptor regulation of the dopamine transporter expressed in Xenopus laevis oocytes is voltage-independent. Mol Pharmacol *59*, 113-121.

McDonald, P.W., Jessen, T., Field, J.R., and Blakely, R.D. (2006). Dopamine signaling architecture in Caenorhabditis elegans. Cell Mol Neurobiol *26*, 593-618.

Mello, C., and Fire, A. (1995). DNA transformation. Methods in cell biology *48*, 451-482.

Melendez, R.I., Rodd-Henricks, Z.A., Engleman, E.A., Li, T.K., McBride, W.J., et al. (2002) Microdialysis of dopamine in the nucleus accumbens of alcohol-preferring (P) rats during anticipation and operant self-administration of ethanol. Alcohol Clin Exp Res 26: 318–325.

Mesce, K.A. and Pierce-Shimomura, J.T. (2010). Shared strategies for behavioral switching: understanding how locomotor patterns are turned on and off. Front. Behav. Neurosci., 10.3389.

Mercuri, N.B., Saiardi, A., Bonci, A., Picetti, R., Calabresi, P., Bernardi, G., and Borrelli, E. (1997). Loss of autoreceptor function in dopaminergic neurons from dopamine D2 receptor deficient mice. Neuroscience *79*, 323-327.

Mersha, M., Formisano, R., McDonald, R., Pandey, P., Tavernakaris, N., and Singh, H. (2013). GPA-14, a Gαi subunit mediates dopaminergic behavioral plasticity in C. elegans. Behav brain Funct. 9: 16.

Miesenbock, G., De Angelis, D.A., and Rothman, J.E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature *394*, 192-195.

Millan, M.J., Marin, P., Bockaert, J., and LA Cour, C.M. (2008). Signaling at G-protein-coupled serotonin receptors: recent advances and future research directions. Cell Press 9:29.

Missale, C., Nash, S. R., Robinson, S. W., Jaber, M., Caron, M. G. (1998). Dopamine receptors: from structure to function. *Physiol. Rev.* 78 189–225

Mitchell, P., Mould, R., Dillon, J., Glautier, S., Andrianakis, I., James, C., Pugh, A., Holden-Dye, L., and O'Connor, V. (2010). A differential role for neuropeptides in acute and chronic adaptive responses to alcohol: behavioural and genetic analysis in Caenorhabditis elegans. PloS one *5*, e10422.

Mitchell, P.H., Bull, K., Glautier, S., Hopper, N.A., Holden-Dye, L., and O'Connor, V. (2007). The concentration-dependent effects of ethanol on Caenorhabditis elegans behaviour. Pharmacogenomics J *7*, 411-417.

Murakami, H., Bessinger, K., Hellmann, J., Murakami, S. (2007). Manipulation of serotonin signal suppresses early phase of behavioral aging in *Caenorhabditis elegans.* Neurobiol Aging. 29:1093–100.

Nass, R., Blakely, R.D. (2003). The Caenorhabditis elegans Dopaminergic System: Opportunities for Insights into Dopamine Transport and Neurodegeneration. Annual Review of Pharmacology and Toxicology. Vol. 43:521-544

Nowak, K.L., McBride, W.J., Lumeng, L., Li, T.K., Murphy, J.M. (2000). Involvement of dopamine D_2 autoreceptors in the ventral tegmental area on alcohol and saccharin intake of the alcohol-preferring P rat. *Alcohol Clin Exp Res*. 24476- 483

Nusbaum, M.P. and Blitz, D.M. (2012). Neuropeptide modulation of microcircuits. Curr Opin Neurobiol. 22(4):592-601.

Omura, D.T., Clark, D.A., Samuel, A.D., and Horvitz, H.R. (2012). Dopamine signaling is essential for precise rates of locomotion by C. elegans. PloS one *7*, e38649.

Oranth, A., Schultheis, C., Tolstenkov, O., Erbguth, K., Nagpal, J., Hain, D., Brauner, M., Wabnig, S., Steuer Costa, W., McWhirter, R.D., *et al.* (2018). Food Sensation Modulates Locomotion by Dopamine and Neuropeptide Signaling in a Distributed Neuronal Network. Neuron *100*, 1414-1428 e1410.

Pandey, P., and Harbinder, S. (2012). The Caenorhabditis elegans D2-like dopamine receptor DOP-2 physically interacts with GPA-14, a Galphai subunit. J Mol Signal *7*, 3.

Peeters, L., Janssen, T., De Haes, W., Beets, I., Meelkop, E., Grant, W., and Schoofs, L. (2012). A pharmacological study of NLP-12 neuropeptide signaling in free-living and parasitic nematodes. Peptides *34*, 82-87.

Pena, I.D., Gevorkiana, R., and Shi, W.X. (2015). Psychostimulants affect dopamine transmission through both dopamine transporter-dependent and independent mechanisms. Eur J Pharmacol. 764: 562–570.

Petri, P. and Korpi, E.R. (1993). Voluntary ethanol drinking increases locomotor activity in alcohol-preferring AA rats. Pharmacology Biochemistry and Behavior 44(1):127-32.

Phillips, T.J., Shen, E.H. (1996). Neurochemical bases of locomotion and ethanol stimulant effects. Int Rev Neurobiol 39: 243–282.

Phillips, T.J., Brown, K.J., Burkhart Kasch, S., Wenger, C.D., Kelly, M.A., Rubinstein, M., Grandy, D.K. Low MJ Alcohol preference and sensitivity are markedly reduced in mice lacking dopamine D_2 receptors. *Nat Neurosci* 1998;1610- 615

Pictonei, L.D. and Sillar, K.T. (2016). Mechanisms underlying the endogenous dopaminergic inhibition of spinal locomotor circuit function in Xenopus tadpoles. Scientific Reports 6: 35749.

Prescott, C.A., and Kendler, K.S. (1999). Genetic and environmental contributions to alcohol abuse and dependence in a population-based sample of male twins. Am J Psychiatry *156*, 34-40.

Rabin, R.A., Malinoff, P.B. (1983). Multiple sites of action of ethanol on adenylate cyclase. J Pharmacol Exp Therap. 227:551-556.

Ramachandran, S., Banerjee, N., Bhattacharya, R., Touroutine, D., Lambert, C.M., Schoofs, L., I., B., and M., F.M. (2020). A conserved neuropeptide system links head and body motor circuits to enable adaptive behavior. bioRxiv.

Rand, J.B., and Russell, R.L. (1984). Choline acetyltransferase-deficient mutants of the nematode Caenorhabditis elegans. Genetics *106*, 227-248.

Ray, L.D., Brocard, F., Bourcier-Lucas, C., Auclair, F., Lafaille, P., Dubac, R. (2003). Nicotinic activation of reticulospinal cells involved in the control of swimming lampreys. Eur J Neurosci 17: 137-148.

Rivet, J.M., Audinot, V., Gobert, A., Peglion, J.L., and Millan, M.J. (1994). Modulation of mesolimbic dopamine release by the selective dopamine D3 receptor antagonist, (+)-S 14297. Eur J Pharmacol *265*, 175-177.

Riddle,D.L., Blumenthal, T.E., Meyer, B.J., and Priess, J.R. (1997). Introdyction to *C. elegans*. WormBase.

Rouge-Pont, F., Usiello, A., Benoit-Marand, M., Gonon, F., Piazza, P.V., and Borrelli, E. (2002). Changes in extracellular dopamine induced by morphine and cocaine: crucial control by D2 receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 3293-3301.

Russell, J.S.A.D. (2001). Molecular Cloning: A Laboratory Manual.

Ryczko, D., and Dubuc, R. (2013). The multifunctional mesencephalic locomotor region. *Curr. Pharm. Des*. 19, 4448–4470.

Ryczko, D. and Dubuc, R. (2017). Dopamine and the Brainstem Locomotor Networks: From Lamprey to Human. Front Neurosci. 11:295.

Samuel, A.D., Silva, R.A., and Murthy, V.N. (2003). Synaptic activity of the AFD neuron in Caenorhabditis elegans correlates with thermotactic memory. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 373-376.

Salgado, S. and Kaplitt, M.G. (2015). The Nucleus Accumbens: A Comprehensive Review. Stereotact Funct Neurosurg. 93:75-93.

Sawin, E.R., Ranganathan, R., and Horvitz, H.R. (2000). C. elegans locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. Neuron *26*, 619-631.

Schafer, W.R. (2004). Addiction research in a simple animal model: the nematode Caenorhabditis elegans. Neuropharmacology *47 Suppl 1*, 123-131.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., *et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nature methods *9*, 676-682.

Schmitz, Y., Schmauss, C., and Sulzer, D. (2002). Altered dopamine release and uptake kinetics in mice lacking D2 receptors. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 8002-8009.

Schuckit, M.A., and Smith, T.L. (1996). An 8-year follow-up of 450 sons of alcoholic and control subjects. Arch Gen Psychiatry *53*, 202-210.

Sharples, S.A., Koblinger, K., Humphreys, J.M., and Whelan, P.J. (2014). Dopamine: a parallel pathway for the modulation of spinal locomotor networks. fncir. 8:55.

Sibley, D.R. (1999). New insights into dopaminergic receptor function using antisense and genetically altered animals. Annu Rev Pharmacol Toxicol 39:313–341.

Siciliano, C.A., Mauterer, M.I., Fordahl, S.C., and Jones, S.R. (2019). Modulation of striatal dopamine dynamics by cocaine self-administration and amphetamine treatment in female rats. Eur J Neurosci.

Sieburth, D., Ch'ng, Q., Dybbs, M., Tavazoie, M., Kennedy, S., Wang, D., Dupuy, D., Rual, J.F., Hill, D.E., Vidal, M., *et al.* (2005). Systematic analysis of genes required for synapse structure and function. Nature *436*, 510-517.

Sieburth, D., Madison, J.M., and Kaplan, J.M. (2007). PKC-1 regulates secretion of neuropeptides. Nat Neurosci *10*, 49-57.

Smetana, R., Juvin, L., Dubuc, R., and Alford, S. (2010). A parallel cholinergic brainstem pathway for enhancing locomotor drive. *Nat. Neurosci*. 13, 731–738.

Sholomenko, G. N., Funk, G. D., and Steeves, J. D. (1991). Avian locomotion activated by brainstem infusion of neurotransmitter agonists and antagonists. I. Acetylcholine excitatory amino acids and substance P. *Exp. Brain Res*. 85, 659–673.

Spanagel, R. (2009). Alcoholism: a systems approach from molecular physiology to addictive behavior. Physiological reviews *89*, 649-705.

Speese, S., Petrie, M., Schuske, K., Ailion, M., Ann, K., Iwasaki, K., Jorgensen, E.M., and Martin, T.F. (2007). UNC-31 (CAPS) is required for dense-core vesicle but not synaptic vesicle exocytosis in Caenorhabditis elegans. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 6150-6162.

Strader, C.D., Fong, T.M., Tota, M.R., Underwood, D., and Dixon, R.A. (1994). Structure and function of G protein-coupled receptors. Annu Rev Biochem *63*, 101-132.

Stefanini, E., Frau, M., Garau, M., GGarau, B., Fadda, F., Gessa, G.L. (1992). Alcohol-preferring rats have fewer dopamine D_2 receptors in the limbic system. *Alcohol Alcohol*. 27127- 130

Sukhes, M. (2013). Alcoholism and its effects on the central nervous system. Curr Neurovasc Res. 10(3):256-62.

Sulston, J., Dew, M., and Brenner, S. (1975). Dopaminergic neurons in the nematode Caenorhabditis elegans. J Comp Neurol *163*, 215-226.

Sumakovic, M., Hegermann, J., Luo, L., Husson, S.J., Schwarze, K., Olendrowitz, C., Schoofs, L., Richmond, J., and Eimer, S. (2009). UNC-108/RAB-2 and its effector RIC-19 are involved in dense core vesicle maturation in Caenorhabditis elegans. J Cell Biol *186*, 897-914.

Suo, S., Culotti, J.G., and Van Tol, H.H. (2009). Dopamine counteracts octopamine signalling in a neural circuit mediating food response in C. elegans. The EMBO journal *28*, 2437-2448.

Suo, S., and Ishiura, S. (2013). Dopamine modulates acetylcholine release via octopamine and CREB signaling in Caenorhabditis elegans. PloS one *8*, e72578.

Suo, S., Sasagawa, N., and Ishiura, S. (2002). Identification of a dopamine receptor from Caenorhabditis elegans. Neurosci Lett *319*, 13-16.

Suo, S., Sasagawa, N., and Ishiura, S. (2003). Cloning and characterization of a Caenorhabditis elegans D2-like dopamine receptor. J Neurochem *86*, 869-878.

Tao, L., Porto, D., Li, Z., Fechner, S., Lee, S.A., Goodman, M.B., Xu, X.Z.S., Lu, H., and Shen, K. (2019). Parallel Processing of Two Mechanosensory Modalities by a Single Neuron in C. elegans. Dev Cell *51*, 617-631 e613.

Thanos, P.K., Rivera, S.N., Weaver, K., Grandy, D.K., Rubinstein, M., Umegaki, H., Wang, G.J., Hitzemann, R., and Volkow, N.D. (2005). Dopamine D2R DNA transfer in dopamine D2 receptor-deficient mice: effects on ethanol drinking. Life Sci *77*, 130-139.

Thanos, P.K., Taintor, N., Rivera, S.N., Umegaki, H., Ikari, H., Roth, G., Ingram, D.K., Hitzemann, R., Fowler, J.S., Gatley, J., Wang, G.J., Volkow, N.D. (2004). DRD2 gene transfer into the nucleus accumbens of the alcohol preferring (P) and non preferring (NP) rats attenuates alcohol drinking. Alcohol Clin Exp Res. 28:720–728

Thanos, P.K., Taintor, N.B., Alexoff, D., Vaska, P., Logan, J., Grandy, D.K. (2002). In vivo comparative imaging of dopamine D2 knockout and wild-type mice with (11)C-raclopride and microPET. J Nucl Med. 43:1570–7.

Topper, S.M., Aguilar, S.C., Topper, V.Y., Elbel, E., and Pierce-Shimomura, J.T. (2014). Alcohol disinhibition of behaviors in C. elegans. PloS one *9*, e92965.

Touroutine, D., Fox, R.M., Von Stetina, S.E., Burdina, A., Miller, D.M., 3rd, and Richmond, J.E. (2005). acr-16 encodes an essential subunit of the levamisole-resistant nicotinic receptor at the Caenorhabditis elegans neuromuscular junction. The Journal of biological chemistry *280*, 27013-27021.

Vashlishan, A.B., Madison, J.M., Dybbs, M., Bai, J., Sieburth, D., Ch'ng, Q., Tavazoie, M., and Kaplan, J.M. (2008). An RNAi screen identifies genes that regulate GABA synapses. Neuron *58*, 346-361.

Vidal-Gadea, A.G., and Pierce-Shimomura, J.T. (2012). Conserved role of dopamine in the modulation of behavior. Communicative & integrative biology *5*, 440-447.

Voglis, G., and Tavernarakis, N. (2008). A synaptic DEG/ENaC ion channel mediates learning in C. elegans by facilitating dopamine signalling. The EMBO journal *27*, 3288-3299.

Volkow, N.D., Tomasi, D., Wang, G.J., Telang, F., Fowler, J.S., Logan, J., Maynard, L.J., and Wong, C.T. (2013). Predominance of D2 receptors in mediating dopamine's effects in brain metabolism: effects of alcoholism. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 4527-4535.

Volkow, N.D., Wang, G.J., Begleiter, H., Porjesz, B., Fowler, J.S., Telang, F., Wong, C., Ma, Y., Logan, J., Goldstein, R., *et al.* (2006). High levels of dopamine D2 receptors in unaffected members of alcoholic families: possible protective factors. Arch Gen Psychiatry *63*, 999-1008.

Volkow, N.D., Wang, G.J., Fowler, J.S., Logan, J., Gatley, S.J., Wong, C. (1999). Reinforcing effects of psychostimulants in humans are associated with increases in brain dopamine and occupancy of D2 receptors. *J Pharmacol Exp Ther*. 291: 409–415.

Volkow, N.D., Wang, G.J., Fowler, J.S., Logan, J., Hitzemann, R, Ding, Y.S., Pappas, N., Shea, C., Piscani, K. (1996). Decreases in dopamine receptors but not in dopamine transporters in alcoholics. *Alcohol Clin Exp Res*. 201594- 1598

Wahab, D.Y.A.A., Gau, C.H., Zakaria, R., Karuppan, M.K.M., A-rahbi, B.S., Abdullah, Z., Alrafiah, A., and Abdullah, J.M. (2019). Review on Cross Talk between Neurotransmitters and Neuroinflammation in Striatum and Cerebellum in the Mediation of Motor Behaviour. BioMed Research International. 1767203

Weinshenker, D., Garriga, G., and Thomas, J.H. (1995). Genetic and pharmacological analysis of neurotransmitters controlling egg laying in C. elegans. The Journal of neuroscience : the official journal of the Society for Neuroscience *15*, 6975-6985.

Weiss, F., Parsons, L.H., Schulteis, G., Hyytia, P., Lorang, M.T., Bloom, F.E., and Koob, G.F. (1996). Ethanol self-administration restores withdrawal-associated deficiencies in accumbal dopamine and 5-hydroxytryptamine release in dependent rats. The Journal of neuroscience : the official journal of the Society for Neuroscience *16*, 3474-3485.

White, J.G., Southgate, E., Thomson, J.N. and Brenner, S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. Philosophical Transactions of the Royal Society of London B. Biological Sciences *314*, 1-340.

White, F.J., Bednarz, L.M., Wachtel, S.R., Hjorth, S., and Brooderson, R.J. (1988). Is stimulation of both D1 and D2 receptors necessary for the expression of dopaminemediated behaviors? Pharmacol Biochem Behav 30:189 –193.

Wolf, F.W., Rodan, A.R., Tsai, L.T.Y., and Heberlein, U. (2002). High-Resolution Analysis of Ethanol-Induced Locomotor Stimulation in Drosophila. JNeurosci. 22-24-11035-11044.

Wu, Q., Reith, M.E., Walker, Q.D., Kuhn, C.M., Carroll, F.I., and Garris, P.A. (2002). Concurrent autoreceptor-mediated control of dopamine release and uptake during neurotransmission: an in vivo voltammetric study. The Journal of neuroscience : the official journal of the Society for Neuroscience *22*, 6272-6281.

Yim, H.J., and Gonzales, R.A. (2000). Ethanol-induced increases in dopamine extracellular concentration in rat nucleus accumbens are accounted for by increased release and not uptake inhibition. Alcohol *22*, 107-115.

Zhen, M. and Samuel, D.T. (2015). C. elegans locomotion: small circuits, complex functions. Current Opinion in Neurobiology. 33:117–126

Zhou, Q.Y., Grandy, D.K., Thambi, L., Kushner, J.A., Van Tol, H.H., Cone, R., Pribnow, D., Salon, J., Bunzow, J.R., and Civelli, O. (1990). Cloning and expression of human and rat D1 dopamine receptors. Nature *347*, 76-80.