ROLE OF DOPAMINE SIGNALLING IN OLFACTORY LEARNING AND IN AUGMENTING MANGANESE MEDIATED NEURODEGENERATION IN *Caenorhabditis elegans*

A THESIS PRESENTED BY

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY

2020

DECLARATION BY THE STUDENT

I, Vishnu Raj, hereby certify that I had personally carried out the work depicted in the thesis entitled, **"Role of Dopamine Signalling in Olfactory Learning and in Augmenting Manganese Mediated Neurodegeneration in *Caenorhabditis elegans*"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: 31/10/2020 **Vishnu Raj**

CERTIFICATE OF THE GUIDE

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This is to certify that Vishnu Raj, of the Division of Molecular Medicine under the Department of Applied Biology, of this Institute has fulfilled the requirements prescribed for the Ph.D degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The thesis entitled, **"Role of Dopamine Signalling in Olfactory Learning and in Augmenting Manganese Mediated Neurodegeneration in *Caenorhabditis elegans*"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: 31/10/2020 **Dr. Anoopkumar Thekkuveettil**

The thesis entitled

ROLE OF DOPAMINE SIGNALLING IN OLFACTORY LEARNING AND IN AUGMENTING MANGANESE MEDIATED NEURODEGENERATION IN *Caenorhabditis elegans*

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of

SREE CHITRA TIRUNAL INSTITUTE FOR
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Dr. Anoopkumar Thekkuveettil (Examiner)

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Abbreviations

5-HT	Serotonin
6-OHDA	6-Hydroxydopamine
AC	Adenylyl cyclase
AD	Alzheimer's Disease
BED	Binge eating disorder
cAMP	Cyclic adenosine monophosphate
C. elegans	*Caenorhabditis elegans*
CNS	Central Nervous System
COMT	Catechol-O-methyltransferase
DA	Dopamine
DAergic	Dopaminergic
DAQ	Dopamine-quinone
DAT	Dopamine Transporter
DOPAC	3,4-dihydroxyphenylacetic acid (DOPAC)
DOPAL	3,4-dihydroxyphenylacetaldehyde
DOPET	3,4-dihydroxyphenylethanol
DS	Dorsal Striatum
GPCR	G protein–coupled receptors
GTP	Guanosine triphosphate
GTPCH	GTP cyclohydrolase
L-DOPA	3,4-dihydroxyphenylalanine
LTD	Long-term depression

LTP	Long-term potentiation
MAO-A	Monoamine oxidase-A
MAO-B	Monoamine oxidase-B
Mn	Manganese
MPTP	1-methyl-4-phenyl1,2,3,6-tetrahydropyridine
NAc	Nucleus Accumbens
PD	Parkinson’s Disease
ROS	Reactive Oxygen Species
SN	Substantia nigra
SNpc	Substantia Nigra pars compacta
SNpr	Substantia Nigra pars reticulata
VMAT-2	Vesicular Monoamine Transporter-2
VTA	Ventral Tegmental Area
WT	Wild-type

SYNOPSIS

Introduction

The fundamental functions of the brain include thinking, feeling, perceiving, learning and memory and altering behaviour. Among them, the formation of memories is critical - without which our capabilities diminish to simple reflexes and stereotypic responses.

Learning is a process of acquiring memory. These memories change the behaviour because of the experience gained during learning or observing an event. Memories we develop on events and facts are known as "declarative memory" that helps in developing consciousness. Memories we develop through learning and experience are known as “procedural memory”. Patients with loss of declarative memory often have procedural memory intact suggesting that both these forms of memories are made through different pathways.

Studies suggest that memory formation needs alterations in the brain. One of the major changes happening in the brain during memory is at the synapses (the region where two neurons communicate) - through changes in synaptic transmission efficiency, called synaptic plasticity. Studies have shown that mutations in synaptic receptors alter memory formation and in turn, changes the connectome function.

The capacity of neurons to create and store information from the immediate experience is a conserved behaviour in all animals. Some memories last for short-term and others for long-term. This ability to sort information into long-term or short-

term memories enables the animals to possess a flexible adaptation towards the ever-changing world.

Even though synaptic involvement in memory has supporting evidence, the biological basis of learning, as well as memory recall, is not well understood - especially the role of various neurotransmitters and the connectomes. Loss of connectome could significantly impact the memory recall pathways. Most common neurodegenerative diseases like Alzheimer's, Huntington's, and Parkinson's have shown memory loss as one of the symptoms.

In this study, I used the model system *Caenorhabditis elegans* to study the role of dopamine (DA) and DAergic neurons in adaptive learning. *C. elegans* is an excellent model for the study because of its well-mapped connectome and established adaptive learning behaviour towards olfactory clues. The aim of the study was to establish the role of DA and its neuronal connectome in learning as well as memory recalling pathways. I also investigated the role of serotonin and acetylcholine in co-regulation of DA function. As a parallel plan, I studied the heavy metal-induced neurotoxicity (manganese) in DA neurons of larvae and adult worms; and assayed for its impact in the learning pathways.

Hypothesis

I hypothesized that both DA neurotransmitter and neurons play a crucial role in learning and memory formation in invertebrates. Additionally, loss of DA neurons by Manganese (Mn) induced toxicity, or genetic mutations will result reduction in memory

formation. To validate this hypothesis, I used an invertebrate *Caenorhabditis elegans* (*C. elegans*) as a model system.

Materials and Methods

All strains of *C. elegans* were maintained on NGM plate with OP-50, an *E. coli* strain, as a food source. The following *C. elegans* strains were used in the study: N2 (Bristol); JC2209 [*olrn-1(ut305) X*]; DCR744 [*cima-1(wy84) IV; wyIs45 X*]; CB1112 [*cat-2(e112)II*]; MT15434 [*tph-1(mg280) II*]; VM487 [*nmr-1(ak4) II*]; LX636 [*dop-1(vs101)*]; LX703 [*dop-3(vs106)*]; LX705 [*dop-1(vs100) dop-3(vs106) X*]; LX734 [*dop-2(vs105) V; dop-1(vs100) dop-3(vs106) X*]; AT7437 [*dat-1::GCaMP-6::mCherry*]; Dat-1::ICE [*dat-1::GFP; dat-1::ICE*]; BZ555 [*egIs1 dat-1pGFP*]; UA44 [*bal11;Pdat-1α-syn::Pdat-1gfp*]; BY200 [*vtIs1(dat-1p::GFP) V*]; MAB300, [*dat-1::GFP(vtIs1)V;smf-2(gk133) X*]; UA57 [*baIs4 (dat-1p::GFP + dat-1p::CAT-2)*].

Chapter-1. This chapter deals with the assessment of the role of DA neurotransmitter and neurons in olfactory learning and memory.

Experiments were carried out using an adaptive memory paradigm - using butanone as the conditioned stimulus and food as the unconditional stimulus. All the worms were age synchronized for each experiment. Training resulted in associated adaptive learning - the worms became attractive towards butanone. For short-term memory, a single training was given; and for long-term memory, seven repeated trainings were given.

Chemotaxis behaviour towards butanone was used to score the learning and memory formation. Three and twenty-four-hour chemotaxis behaviour was recorded for short-term and long term memory, respectively.

The migratory patterns (runs and turns) of the worms were analyzed using maze assay with $CuSO_4$ barriers in NGM plate. This assay was standardized using isoamyl alcohol, an attractant.

The migratory patterns in food and foodless plates, a specific assay for DA dependent behaviour, were recorded using a digital camera (DinoLite). These include body bends and avoidance behaviour towards glycerol. G-75 Sephadex beads were used to induce mechanical stimulation - thus activating DAergic neurons. For exogenous DA assays, NGM plates were made including 20 mM DA.

Activation of DAergic neurons was measured using Calcium imaging studies. For this, I created a transgenic strain containing GCaMP-6, a transient calcium indicator, under dat-1 (DA transporter) promoter using the plasmids pJH3644 and pTH5. Kinetic measurements of fluorescence changes were measured using Leica DMi8 fluorescence microscope.

To rescue the DA neurodegeneration in DAT-1::ICE strain, siRNA mediated silencing of ICE gene was carried out. For this experiment, ICE gene was cloned in pL4440 plasmid and transformed in HT1115 bacteria to express ICE specific siRNA. DAT-1::ICE worms were fed with HT115 expressing siRNA. siRNA mediated rescue of DA neurons was recorded using Leica DMi8 fluorescence microscope.

Tetramisole, an agonist of cholinergic receptors, was used to study acetyl cholinergic receptor density.

Chapter-2. This chapter deals with methods to induce neurotoxicity in DAergic neurons - using exogenous Mn and DA. The percentage of degeneration in DA neurons were measured using fluorescence microscopy. Survival test was carried out with strains maintained on OP-50 after acute exposure to different concentrations of Mn. SWIP assay in the M9 buffer was carried out to test the activity of the DA transporter. All image processing, quantification and data analysis were done using the software Fiji and Graphpad Prism-6.

Major Findings

Chapter-1

The result of short term associated memory (STAM) training indicates that wild type strain developed a significant olfactory adaptive learning and memory as compared to the olfactory memory deficient strains. A similar approach on *cat-2* mutant (tyrosine hydroxylase mutant) strain, deficient in DA neurotransmitter due to the absence of tyrosine hydroxylase (TH) enzyme, showed a significant reduction in adaptive memory formation at all time intervals in comparison to the wild type worms. These strains, however, have the 0th-hour learning intact. The *tph-1* mutant strain, deficient in serotonin synthesis, had no impact in learning and memory formation after STAM training which points to the conclusion that the behaviour observed in TH mutant was not a compensatory effect of serotonin.

The substantial reduction of STAM in DA neurotransmitter deficient strains, lead us to probe whether connectomes involving DA neurons are critical for the learning process. To understand this possibility, I used the strain dat-1::ICE, expressing human caspase interleukin-1β converting enzyme (ICE) transgene under DA transporter dat-1 promoter to ablate the dopamine neurons. The chemotaxis index of short-term adaptive memory of the trained worms at the 0th hour was significantly lower compared to the wild type. The chemotaxis index of 1st hour and 2nd hour was found to be significantly lower when compared to the control. These results indicate that the absence of DA neurons have an impact on learning as well as memory recall. The UA44 strain, carrying human alpha-synuclein, shows normal architecture of CEP neurons on day-1, but on day-3 DA neuron degeneration will become prominent showing puncta and breaks. STAM training on these worms for short term adaptive learning, on day-1 and day-3 stages showed significant variations. Day-1 adult worms had normal chemotaxis index at all time points similar to wild-type worms, but day-3 worms showed a significant reduction in chemotaxis index. This result further underscores the importance of DA neurons in the STAM.

The behaviour patterns of the worms such as body bends and avoidance response showed that the neurodegeneration in dat-1::ICE strain is progressive. The day-1 worms could still be able to produce DA. The Tyrosine hydroxylase (TH) mutants, on the other hand, has a complete absence of DA on day-1 and showed defects in behavioural patterns. These results also indicated that the body bend behaviour needs DA at a low level, but avoidance response needs DA at a higher level.

The maze assay results suggested that the deficiency in learning and memory formation of DAT-1::ICE strain is not due to defect in migration. nmr-1 mutants were

used as the positive control for the assay because they lack the normal migratory pattern to initiate proper runs and turns.

Mechanical stimulation using Sephadex beads-initiated learning even in the absence of food which indicates that DA release during olfactory conditioning has a critical role in learning. To further prove this point, the addition of exogenous DA during olfactory conditioning also resulted in enhanced learning in the absence of food (unconditional stimulus).

To understand the involvement of DA neurons in learning, we used the transgenic strain expressing GCaMP-6 (dat-1::GCaMP-6) under the promoter *dat-1* gene can show a change in fluorescence when calcium ions entering into the neurons during a stimulus. When presented the conditional stimulus, DA neurons evoked the firing patterns in the STAM trained worms compared to untrained worms. The results also showed a delayed response of excitation of DA neurons strongly suggest DA communication might be critical in final decision making rather than olfactory recognition of the stimulus.

Exogenous DA treatment significantly improved memory retention in wild type worms. Tyrosine hydroxylase deficient *cat-2* mutant strain when treated with exogenous DA showed a rescue in learning as well as memory retention similar to that of wild type worms. However, exogenous DA addition in dat-1::ICE strain showed a substantial improvement in early learning but did not show memory recalling.

I then tested the role of DA receptors in olfactory learning. The DA receptor mutants of *dop-1* and *dop-3* (LX636 and LX703 respectively) had a significant reduction in

memory formation similar to tyrosine hydroxylase mutant strain that lacks DA synthesis. Olfactory learning assays on double mutants and triple mutants of *dop-1*, *dop-2* and *dop-3* (LX705 and LX734) showed lack of learning as well as memory recalling. These results, along with the results from the strain DAT-1::ICE with a progressive reduction in DA receptor density due to ageing corroborated the critical role of DA receptors in learning and memory process.

The indirect assay, using tetramisole, was used to assess the cholinergic receptor density using tetramisole. Tetramisole induced a significantly high percentage of paralysis in DAT-1::ICE worms that correlates with high cholinergic receptor density, compared to the wild type control worms. These results indicated a significant correlation between the DAergic neuron and cholinergic receptor density.

Olfactory learning in DAT-1::ICE strains with siRNA mediated silencing of ICE, showed rescue in learning and memory retention . The siRNA treatment also reverted the degeneration of DA neurons in the worms.

Chapter-2

To evaluate the effect of Mn in inducing degeneration of DA neurons, I chose BZ555 (*dat-1::GFP*) strain with GFP reporter under the control of dat-1 promoter to ensure the expression in DA neurons. I tried two approaches. Firstly, L1 larvae were exposed to the Mn ions for 30 minutes to check the effect of the metal ions in development and its late effects in the adult stage (day-1 and above) and secondly, Mn exposure in adult worms (day-1) for 60 minutes to evaluate its immediate effects on DA neurons.

No significant neurodegeneration was observed in L1 larvae exposed to Mn on CEP and ADE neurons in the worms that survived at various concentrations of Mn (0 to 100 mM). However, the percentage of survival showed a concentration-dependent reduction. The dat-1 expression levels were measured after Mn exposure on larvae at day-1, day-2 and day-3 adult worms, as well as 3 hours, 24 hours and 48 hours, post-exposure. No significant change in dat-1 expression was observed compared to the control. However, pre-exposure of exogenous DA at 5 mM and 10 mM concentrations along with 100 mM $MnCl_2$ to L1 worms showed puncta as well as CEP loss in the surviving adult worms.

Four different strains of worms with fluorescently labelled DA neurons were tested for the effect of Mn on adults: BZ555, UA44, MAB300 and BY200. All the strains showed significant neurodegeneration at 100 mM $MnCl_2$. The survival of the strains also showed a significant drop at 50 mM and 100 mM concentration compared to non-exposed worms. Presence of DA escalated the neurodegeneration in these worms compared to $MnCl_2$ (both 50 and 100 mM) alone, and this was also evident in fluorescence intensity analysis.

The role of tyrosine hydroxylase (TH) in Mn neurotoxicity was tested. The TH mutants (*cat-2*) were least sensitive to both Mn and DA treatment but showed a significant drop in body bend numbers in the presence of Mn+DA. TH over synthesis mutants (*cat-2* under *dat-1* promotor) showed high sensitivity to Mn alone treatment. Moreover, the data showed that TH overexpressing worms had lower body bends similar to that of Mn+ DA treatment, indicating that over-stimulation of TH results in enhanced Mn neurotoxicity in adults. Hence, the absence of neurodegeneration in larvae could be due to the down-regulation of TH during Mn exposure. The recovery

of adult worms after exposure to Mn and DA or both, showed downregulation of dat-1 expression. This observation was further strengthened when analyzed the size of cell bodies of DA neurons that showed a significant decrease.

The worms that were exposed to Mn at the L1 stage, compared to the adult stage, showed significant variation in the behavioural assay. The L1 exposed worms behaved better in SWIP assay indicating either they have an efficient clearance of DA (high expression of dat-1), or low DA synthesis (downregulation of TH gene). The DA sensitivity test on worms showed that larval exposure had a significant effect on the receptor level. L1 worms were given repeated exposure to Mn at the adult stage. The data showed that these worms are highly resistant to Mn exposure - only 20% of the worms showed puncta formation compared to 60% when Mn was dosed at the adult stage. These results further indicate the protective mechanism associated with the downregulation of TH. Furthermore, learning and memory were significantly affected in L1 pre-exposed to Mn, indicating a down-regulation of TH, thereby reducing the internal DA level.

Significance of this study

Understanding how the brain translates learning to rewarding experience is a fascinating question. A series of studies have shown that the DAergic system is involved in reward-based behaviour. However, there is a lack of direct evidence to suggest the involvement of DA neurons in memory recalling pathway.

This study indicates that DA neurotransmitter and the DAergic neurons are critical in learning and memory. DA neurons and the connectome associated with these

neurons are critical in memory recall. Based on our data on Mn and DA-mediated toxicity assays, it is evident that DA influences the neurotoxicity and survival of *C. elegans.* It is also evident that larvae were resistant to Mn toxicity through down-regulation of TH and DAT-1 activity. TH downregulation has an immediate impact on the learning pathway. Our study using various mutations and pharmacological approaches in *C. elegans* shows a causal role of DA in memory and indicates that DA neurons play an additive role in the effective processing of cognitive function. Further understanding of these connectomes and its active processing of information will hopefully enrich our understanding of the intrinsic relations between memory, motivation and decision making at cellular levels.

Review of Literature

1. Role of Dopamine in Nervous System

A. Dopamine

Dopamine (DA), also known as 3,4-Dihydroxyphenethylamine, is one of best characterized neurotransmitters (Bäckman *et al*., 2010). It is made up of a benzene ring with two hydroxyl side groups attached to one amine group via an ethyl group (Bäckman *et al*., 2010). DA is an essential catecholamine that functions both in neurons as a neurotransmitter and in non-neuronal cells as an autocrine or paracrine signal (Bäckman *et al*., 2010). The prevailing viewpoint during the 1950s was that DA is an intermediate in the process of synthesising epinephrine and norepinephrine (Carlsson, 1993). Later in 1957, Arvid Carlsson proved that it is not a precursor molecule, but in fact, functions as a neurotransmitter in the central nervous system (CNS) (Yeragani *et al*., 2010). During this period Arvid Carlsson and his colleagues proved that akinetic state caused by reserpine administration was revoked by intravenous injection of the DA precursor 3,4-dihydroxyphenylalanine (L-DOPA) suggesting a critical role of DA in the pathway (Lees, Tolosa and Warren Olanow, 2015).

B. Dopamine Biosynthesis

DA is an important neurotransmitter in the brain and a crucial part of it is synthesized in the mesenteric organs (Eisenhofer *et al*., 1997). The classical pathway for DA biosynthesis takes place in the cytosol of dopaminergic neurons. DA can also be synthesized from dendrites, which similar to cytosolic DA is stored in classical vesicles and smooth endoplasmic reticulum (Elsworth and Roth, 1997). Tyrosine is considered as the precursor for DA synthesis - by phenylalanine getting converted into tyrosine

through the action of phenylalanine hydroxylase (Fernstrom and Fernstrom, 2007). DA synthesis is initiated by the rate-limiting enzyme tyrosine hydroxylase (Elsworth and Roth, 1997) – first producing L-DOPA (dihydroxyphenylalanine) from the precursor tyrosine and this, in turn, converted to DA by the action of aromatic amino acid decarboxylase (Fernstrom, 1983). This enzyme tyrosine hydroxylase is regulated by the phosphorylation of multiple kinases at 4 different serine residues and dephosphorylation by 2 phosphatases (Best, Nijhout and Reed, 2009; Daubner *et al*., 2011). Initial oxidation is strongly regulated and depends on the cofactor tetrahydrobiopterin (BH4), which is synthesised by the action of GTP cyclohydrolase (GTPCH) from guanosine triphosphate (GTP) (Meiser, Weindl and Hiller, 2013).

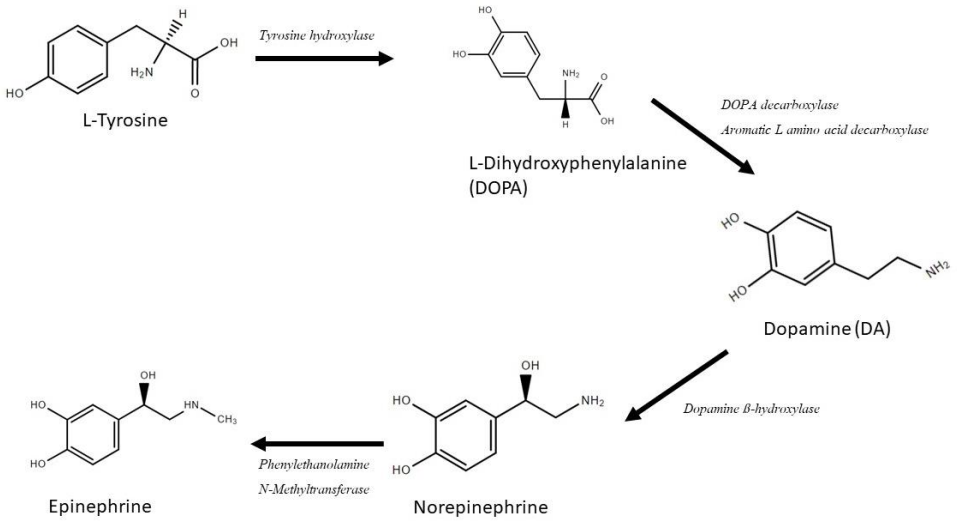

Figure 1. The biosynthetic pathway for the catecholamine neurotransmitters including DA. DA is synthesized from L-tyrosine through a two-step reaction catalysed by tyrosine hydroxylase and DOPA decarboxylase enzymes. Once DA is synthesized it is converted into norepinephrine and epinephrine.

C. Dopamine Packing into synaptic vesicles

Continuous synthesis of DA in the neurons results in auto oxidation and monoamine oxidase mediated free radical generation such as hydroxyl radical (*OH) (Hermida-Ameijeiras, 2004). Therefore, synthesized DA is immediately scavenged by Vesicular Monoamine Transporter-2 (VMAT-2). VMAT-2, a protein located at the synaptic end has two major roles to play: neurotransmission and neuroprotection (Segura-Aguilar *et al*., 2019). VMAT-2 is a member of 12 transmembrane H^{+}-ATPase antiporter, evolutionarily related to the toxin extruding protein family (Fleckenstein and Hanson, 2003; Burman *et al*., 2004). VMAT-2 is responsible for the loading of DA into a synaptic vesicle and its concentration at the synapse increase as it gets ready for vesicular release during neuronal response. VMAT-2 mediated sequestration of DA into synaptic vesicles maintains a low cytosolic concentration that aids in neuroprotection (Croft *et al*., 2005; Pifl *et al*., 2014). A low pH inside this vesicle stabilizes DA thus preventing oxidation (Hnasko *et al*., 2010). A recent work has shown in mice that lower levels of VMAT-2 lead to catecholaminergic neuronal death and symptoms resembling Parkinson's Disease (PD), while higher levels increased synaptic release of DA and protection against dopaminergic neurotoxicants (Lohr *et al*., 2016).

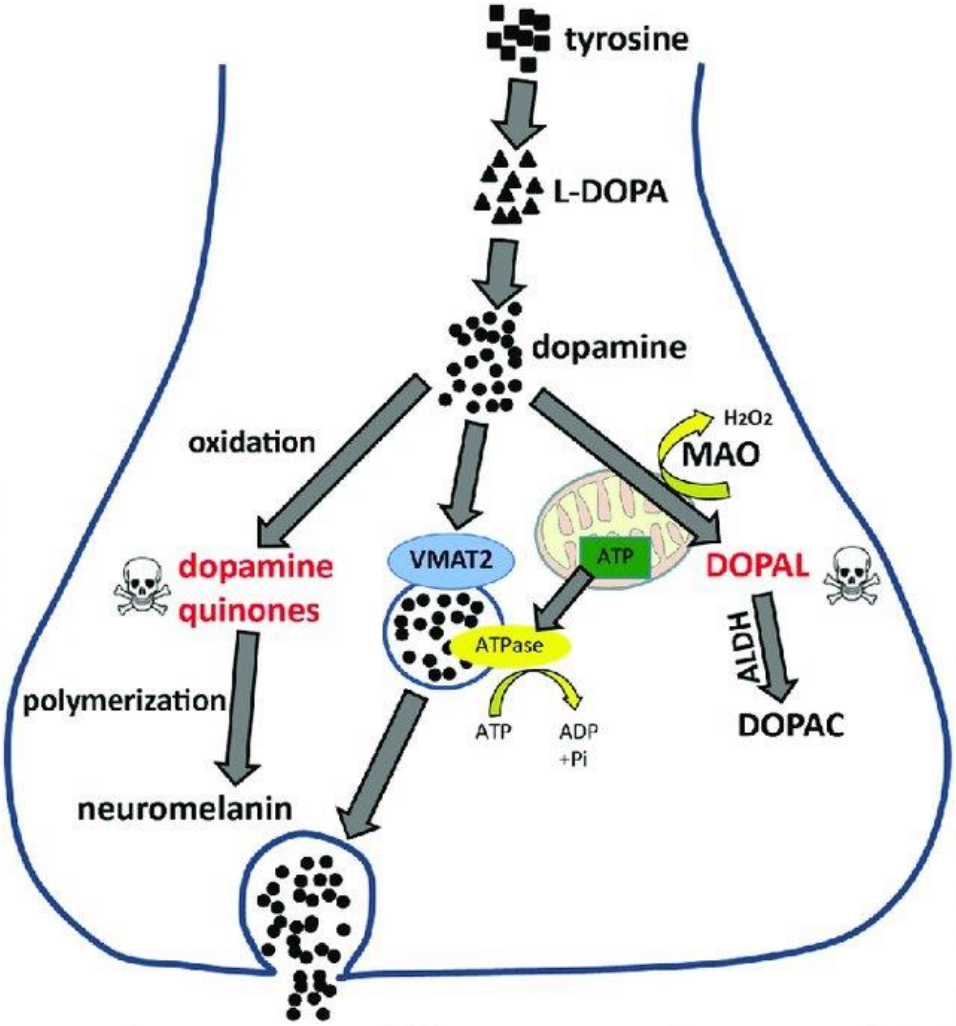

Figure 2: Vesicular Monoamine Transporter mediated packaging of DA into synaptic vesicles. Once DA is synthesized from L-tyrosine by tyrosine hydroxylase it is then transported to axonal region for packaging into synaptic vesicles. VMAT-2 is a member of 12 transmembrane H^+-ATPase antiporter, responsible for the loading of DA into a synaptic vesicle. Figure from Burbulla and Krainc 2019 and reprinted with permission from the Elsevier *Inc* © 2019.

D. Dopamine Release

DA is released in an action potential-dependent manner from both axonal and somatodendritic sites of midbrain dopaminergic neurons in mammalian brain (Gentet and Williams, 2007). Upon receiving an action potential DA is released into the synaptic cleft, by calcium-mediated vesicle release. The extent of DA release is solely

dependent on the neuronal firing rate and pattern (Häusser *et al*., 1995). DA released from somatodendritic sites act to inhibit the action potential output of dopaminergic neurons. This auto inhibition controls the timely release of DA from dopaminergic brain regions(Gentet and Williams, 2007). The major functional roles DA are: controls locomotion and movements (Paus, 2001), helps in cognition, reinforcement, reward (Berridge and Kringelbach, 2008), learning (Drozak and Bryła, 2005) spatial memory function (Luciana, Collins and Depue, 1998), motivation (Depue and Collins, 1999), arousal(Andretic, van Swinderen and Greenspan, 2005), sleep regulation (Grossman *et al*. 2000), feeding, olfaction, hormone regulation (Li, Chen and Smith, 1999).

E. Dopamine Signalling

DA controls several behaviours through the different dopaminergic pathways in the brain (Baik, 2013). Understanding the signalling pathway or communication in the DA system is crucial since a variety of neurological and neuropsychiatric disorders, including schizophrenia, attention deficit hyperactivity disorder, Tourette syndrome obsessive-compulsive disorder, Parkinson's disease, Huntington's disease, and drug addiction, result from impaired DA receptor signalling (Bibb, 2005). The human brain contains two major groups of dopamine neurons. One located in the arcuate nucleus of the hypothalamic median eminence and the other located in the ventral mesencephalon and projects to the forebrain (Matsumoto, 2012). Earlier it was thought that neurotransmitters must involve fast neurotransmission through ligand gated ion channels such as glutamate and GABA (Lodish *et al*., 2000). However, the discovery of DA receptors identified another class of neurotransmission as slow-acting neurotransmitter (Kholodenko, 2006).

F. Dopamine receptors

The major three steps involved in cell signalling are reception, transduction, and final response (Tzlil and Kuruvilla, 2011). In reception, the signalling molecule binds to a receptor protein on the cell membrane or inside the cell. In transduction, receptor protein undergoes a conformational change, which induces downstream cellular response. Generally, this is associated with a series of changes occurring within the cell, and this is called a signal transduction pathway. The final response could be anything from activating the other set of neurons to elicit a physiological response (Rehman and Sharma, 2020).

DA receptors belong to the 7 transmembrane G protein–coupled receptors (GPCRs). GPCRs have the characteristic seven transmembrane regions and three N-linked glycosylation sites. DA receptors express in the central nervous system and peripheral nervous system (Strange, 1993). Pharmacological and biochemical studies show that there are two classes of DA receptors: one group activates adenylate cyclase (AC) and the other group functions independent of AC. Based on the regulation of cyclic AMP DA receptors are classified into two groups: D1 like and D2 like (Kebabian, Petzold and Greengard, 1972). There are 5 different classes of DA receptors have been reported (Bhatia A and Saadabadi A 2020): D1 like receptor family is divided into two subgroups; D1 and D5. Similarly, D2 like receptor family is divided into three subgroups; D2, D3 and D4.

Generally, the D1 receptor family activates AC via the activation of guanosine nucleotide-binding proteins (G proteins) and generates cyclic AMP (cAMP) as the secondary messenger (Sawaguchi and Goldman-Rakic, 1991). D1 receptors induce calcium release as well as are involved in various signal transduction pathways in neuropsychiatric diseases (Figure-3A) (Ha *et al*., 2012). In contrast, the activation of D2 family of DA receptors inhibits the activity of AC and thereby attenuates the generation of cAMP (Wang *et al*., 2018). D2 receptors act either as somatodendritic auto receptors (reduce neuronal excitability) or terminal auto receptors (mostly decrease DA synthesis and its packaging auto receptors) (Figure-3B). Dysfunction of DA receptors leads to pathological conditions, such as hyperprolactinemia, Parkinson's Disease, schizophrenia, Tourette syndrome, attention deficit/hyperactivity disorder, and Huntington disease (Hornykiewicz, 2002; Key *et al*., 2005; van Duijn, 2017). Symptoms associated with these conditions were alleviated using DA receptor agonists and antagonists (Klawans, Goetz and Tanner, 1984; Stanford and Tannock, 2012; Ben-Jonathan, 2020).

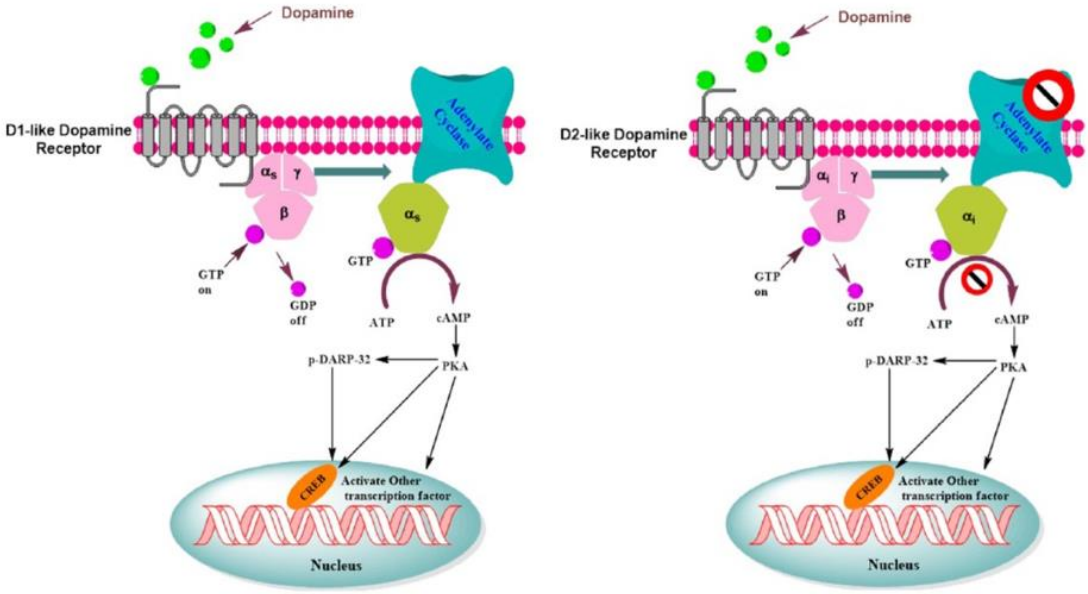

Figure 3: Dopamine receptor functioning D1 receptor class and D2 receptor. DA receptors belong to the 7 transmembrane G protein–coupled receptors (GPCRs). Two classes of DA receptors are generally present: one group activates adenylate cyclase (AC) known as D1-like receptor family and the other group functions independent of AC known as D2-like receptor family. Figure from Mishra *et al*. 2018 and reprinted with permission from the SAGE publications © 2018.

G. Dopamine Metabolism

Degranulation of DA filled synaptic vesicles, upon excitation of DAergic neurons, releases DA into synaptic cleft to further interact with the postsynaptic DA receptors or regulatory presynaptic DA auto receptors (Werkman *et al*., 2006; Zhang and Sulzer, 2012). Prolonged signalling has to be regulated for the efficient signalling process and neuronal homeostasis. This is done through clearance of extracellular DA from the synaptic cleft either by uptake by DAergic neurons or by glial cells (Meiser, Weindl and Hiller, 2013). DA transporter (DAT) present in the synaptic membrane aid in neuronal reuptake of DA and is then sequestrated into the synaptic storage vesicles by the action VMAT-2 (Eriksen, Jørgensen and Gether, 2010).

DA could still accumulate in the cytosol, as a result of leakage from synaptic storage vesicles, and is degraded by action of major two enzymes; catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) (Juárez Olguín *et al*., 2015). Oxidative deamination of DA by MAO leads to the generation of hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL gets oxidized to carboxylic acid 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (ALDH) or further reduced to the 3,4-dihydroxyphenylethanol (DOPET) by alcohol dehydrogenase (ADH). In general, DOPAL is predominantly converted into its oxidized form while reduction to DOPET occurs at a low rate (Eisenhofer, Kopin and Goldstein, 2004).

Glial cells surrounding the DA neurons taken up DA, in the synaptic cleft, and are readily degraded either by MAO or catechol-*O* methyltransferase (COMT). It has been reported that COMT are able to transfer methyl groups from *S*-adenosylmethionine (SAM) to hydroxyl groups of other catecholamines (Kumar and Rai, 2020). 3-*O*-methylation of DOPAC by COMT leads to the generation of homovanillic acid (HVA), one of the major degradation products of DA (Männistö *et al*., 1992; Kaakkola, Gordin and Männistö, 1994). COMT activity is mostly in the glial cells and completely absent in DAergic nigro-striatal neurons.

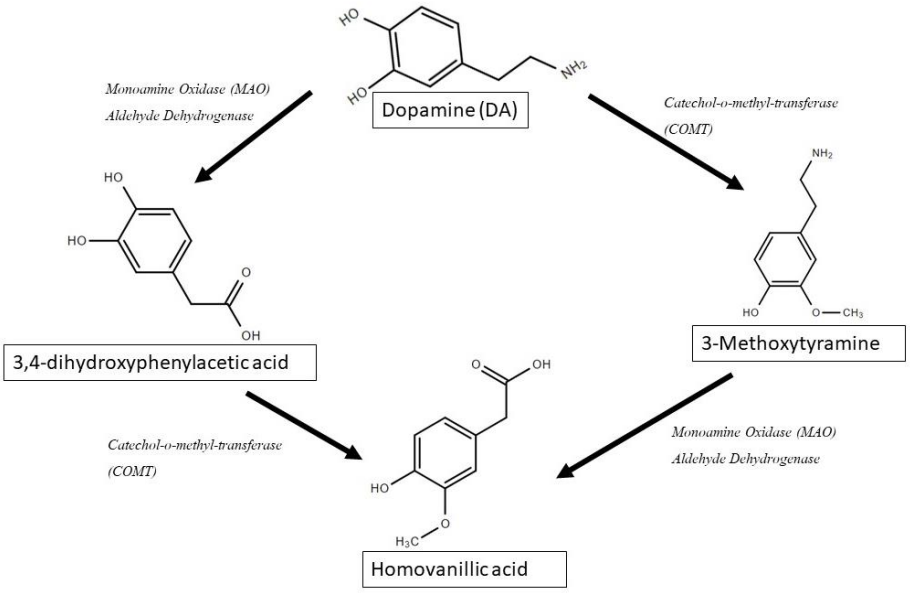

Figure 4: Dopamine metabolism by MAO and COMT pathway. DA accumulate in the cytosol, as a result of leakage from synaptic storage vesicles, is degraded by action of major two enzymes; monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). Oxidative deamination of DA by MAO leads to the generation of hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL gets oxidized to carboxylic acid 3,4-dihydroxyphenylacetic acid (DOPAC) by aldehyde dehydrogenase (ALDH). COMT are able to transfer methyl groups from *S*-adenosylmethionine (SAM) to hydroxyl groups of other catecholamine. 3-*O*-methylation of DOPAC by COMT leads to the generation of homovanillic acid (HVA).

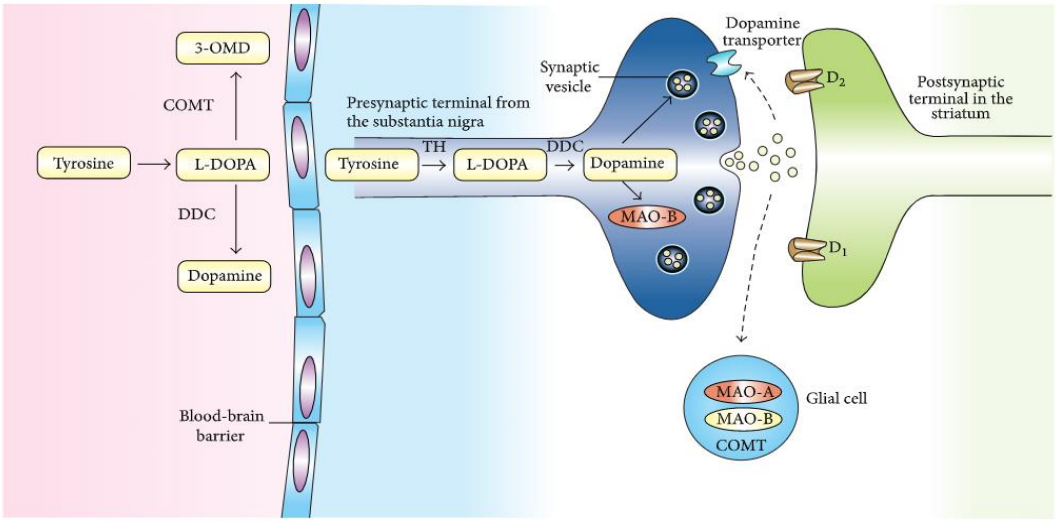

Figure 5: Dopamine metabolism in Dopaminergic neurons and glial cells. Glial cells surrounding the DA neurons taken up DA, in the synaptic cleft, and are readily degraded either by MAO or catechol-*O* methyltransferase (COMT). Figure from Juárez Olguín *et al*., 2016 and reprinted with permission.

H. Dopamine reuptake

Reuptake of extracellular DA in the synaptic cleft to the dopaminergic neurons for its reuse is normally taking place within the neuron. Similarly, DA is reup taken to glial cells for degradation by the MAO and COMT. This reuptake is mainly carried out by the DA transporter (DAT) present on the pre synaptic terminals. It helps in reuptake of extracellular DA for its reuse or metabolization. Through, feedback inhibition with D2 receptors and DAT, dopamine neurons can control their extracellular concentration of DA (Nishijima and Tomiyama, 2016). Thus, prevents the autooxidation of DA and associated toxicity.

I. Dopamine transporter

In the synapse, the activation signal received by the dopamine neurons release DA. Continuous activation or prolonged presence of DA in the synapse is detrimental. Hence once after DA exerts its effects through activation of either postsynaptic or presynaptic DA receptors it is needed to be eliminated from the synaptic cleft. There are special proteins responsible for reuptake of DA into the plasma membrane called DA transporters (DAT) (Miller *et al*., 1999). DAT is a member of the Na^+/Cl^--coupled neurotransmitter transporter family, including the plasma membrane transporters of

serotonin (5-HT) and noradrenaline (Blakely *et al*., 1991; Fritz *et al*., 2002). Blockade in the reuptake of DA may lead to increased extracellular and synaptic DA as well as its increased lifetime leads to extended activation of DA receptors. DAT is exclusively found on dopamine neurons unlike DA synthesizing enzyme tyrosine hydroxylase; TH might be present in other non- dopaminergic catecholamine neurons as well (Miller *et al*., 1999). Hence DAT is called a defining molecule of dopamine neurons. The reuptake is not complete until the involvement of a vesicular monoamine transporter (VMAT2) (Liu, 1992). Once the DAT reuptake DA from the synapse into the plasma membrane or the dopamine neurons, it is then packaged into synaptic vesicle by the help of this member of the toxin-extruding antiporter (TEXAN) gene family VMAT-2 (Schuldiner, Shirvan and Linial, 1995).

It has been proven that VAMT-2 has evolved to sequester toxins into vesicles without causing, and exposing neighbouring cells, to any potential toxicity of the toxin (Liu, 1992). Although, DAT is important for DA neurotransmission, it is also a gateway to substances, and other molecules such as toxic substances. Even, VMAT-2 provides a higher degree of protection against endogenous and exogenous toxins, DAT makes dopamine neurons vulnerable for neuronal injury by transporting toxic substances potentially resembling the structure of DA. Once inside the DAergic neurons these substances can disrupt the cellular homeostasis by disrupting the mitochondria or could react with other vulnerable targets inside the neuronal cell. One classical example is 1-methyl-4-phenyl1,2,3,6-tetrahydropyridine (MPTP), its neurotoxic effects were observed in heroin addicts, as acute form of parkinsonism characterized by slowed movement, gait problems, and other features of idiopathic Parkinson’s disease, which leads to specific death of DAergic neurons in the substantia nigra (Langston *et al*., 1983). Presently the MPTP is used to mimic Parkinson's disease models in monkeys and rodents and other invertebrate models.

The active forms of MPTP such as MPP^+ (produced by the activity of brain monoamine oxidase) are transported through DAT and depends on the DAT expression levels, if more DAT is active then the MPP^+ mediated toxicity would be more and vice versa (Chiba, Trevor and Castagnoli, 1985; Shimada *et al*., 1992).

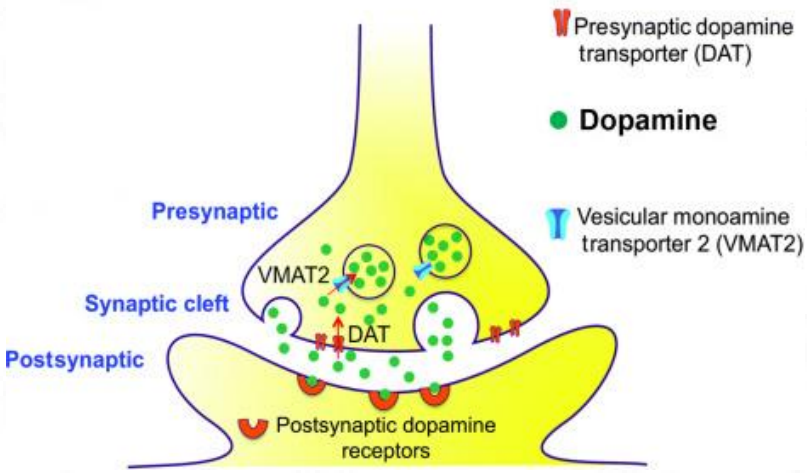

Figure 6: Reuptake of DA by dopamine transporter from synaptic cleft into neuron. Once after DA exerts its effects, it needs to be eliminated from the synaptic cleft. There are special proteins responsible for reuptake of DA into the plasma membrane called DA transporters (DAT), a member of the Na^+/Cl^--coupled neurotransmitter transporter family. DA is then packaged into synaptic vesicle by the help of toxin-extruding antiporter (TEXAN) gene family protein called VMAT-2. Figure from P.C. McHugh *et al*. 2015 and reprinted with permission from the Elsevier *Inc* © 2015.

2. Dopamine Associated Neurological disorders

In the brain, DA is produced in major parts such as substantia nigra, ventral tegmental area, and hypothalamus. Dysfunction or impairment in DA neurotransmitter release, its receptor's function and neurons has been implicated in several neurological

disorders. Following session discusses the major neurological psychiatric disease associated with dopaminergic neuron dysfunction.

A. Schizophrenia

Schizophrenia is a serious mental disorder having its origin in childhood and it is characterized by specific or abnormal reaction to reality. Though it originates at childhood, it may be experienced at later stages of life and have problems similar to hallucinations, delusions and incapability of thinking make difficulty in day-to-day activity. The symptoms or problems associated with schizophrenia may vary, but usually positive symptoms involve delusions (false beliefs that are not par with the reality), hallucinations (hear or see things which never exist), disorganized speech and disorganized motor behaviour (Weinberg, Kirson Weinberg and Arieti, 1955). Negative symptoms include anhedonia, lack of speech and lack of motivation(Weinberg, Kirson Weinberg and Arieti, 1955; Brisch *et al*., 2014). Many evidences point out the involvement of increased DA activity in brain regions and is compelling since it is derived from both human and animal models (McCutcheon, Abi-Dargham and Howes, 2019). However recent studies have shown that alteration in DA concentration and metabolism could be a one of the major precipitating reasons (Howes *et al*., 2017).

Schizophrenia could be the result of more than one disease, rather than a single neurological condition, involving other neurotransmitters such as epinephrine, GABA etc., apart from DA (Berger, 1981). DA hypothesis in schizophrenia states that hyperactivity of dopaminergic neurotransmission results in symptoms associated with the disease. However, later it was modified in the light of many advanced researches

conducted on this area. It now states that a hyperactive DA neurotransmission in the mesolimbic areas and a hypoactive neurotransmission in prefrontal cortex occurs in schizophrenia patients (Weinberg, Kirson Weinberg and Arieti, 1955; Brisch *et al*., 2014). These regions have been associated with dopaminergic neuronal projection and control many functions such as emotion, decision making.

B. Parkinson's Disease

Parkinson's disease (PD) or paralysis agitans is a neurological disorder that mainly affects the motor control functioning of the body. PD patients have progressive degeneration of the substantia nigra (SN) region of the dopaminergic system. This neurodegeneration in the SN will lower the DA level available at the corpus striatum for neurotransmission (Zhang, Wang and Wang, 2019). In human, movement control is achieved by complex interactions of distinctive groups of neurons. One such major group of neurons are dopaminergic neurons in the SN region. Basal ganglia neurons are innervated by the SN neurons and this communication with DA neurotransmitter is responsible for the fine tuning of an organism's locomotion (Stojakovic *et al*., 2017). Hence any events that affect the dopaminergic neurons in the SN will eventually lead to affect the movement of the organism. The major symptoms associated with PD are resting tremor, rigidity, bradykinesia, loss of postural reflexes and lack of movement coordination. The actual cause of cellular death of dopaminergic neurons in PD is not fully understood. Many studies suggest that PD is heterogeneous in nature and both environmental and genetic factors play a role in disease etiology (Triarhou, 2002).

Till date two forms of PD have been identified, sporadic and familial PD (Yang *et al*., 2017). However, the identification of alpha synuclein overexpression (a potential of

cause of dopaminergic neurodegeneration) in familial PD and excess alpha synuclein in sporadic PD give an impression that both forms of PD are interrelated (Wang, 2017). This accumulation of excess alpha synuclein leads to Lewy body formation and eventually accompanies death of dopamine neurons (Rhinn *et al*., 2012) . Detailed molecular mechanisms underlying the dopaminergic neuronal death in PD is given in Fig 7.

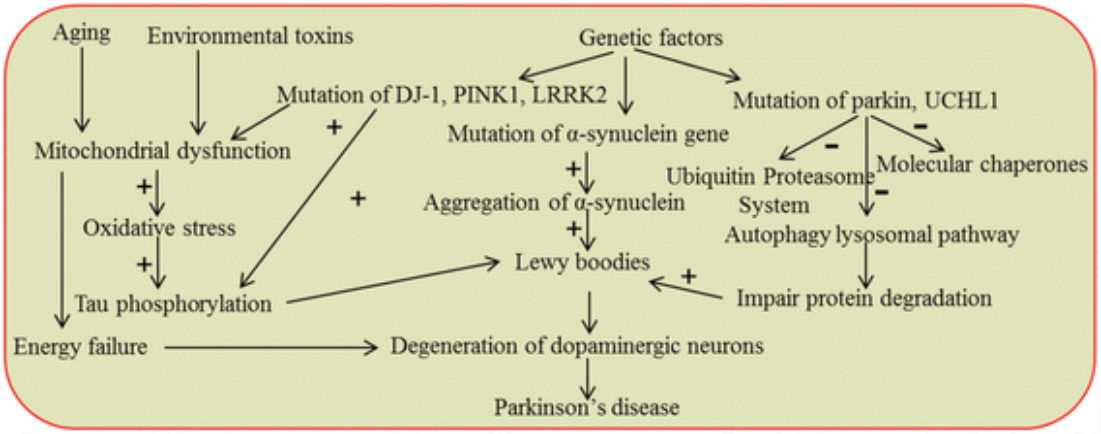

Figure 7: Molecular mechanisms associated with sporadic and familial PD. Two forms of PD have been identified, sporadic and familial. Alpha synuclein overexpression (a potential of cause of dopaminergic neurodegeneration) in familial PD and excess alpha synuclein in sporadic PD give an impression that both forms of PD are interrelated. This accumulation of excess alpha synuclein leads to Lewy body formation and eventually accompanies cell death of dopamine neurons. Figure from Maiti *et al*. 2017 and reprinted with permission.

C. Dementia

Dementia is a general form for memory loss, incapability of problem solving and thinking ability seriously affects cognitive skills. Brain has many distinctive regions; any kind of damage will lead to dementia. The damage to the brain region interferes

with the ability of brain cells to communicate with each other. Most of the changes in the brain that cause dementia are irreversible and get worse over time.

Alzheimer's disease (AD) is the most common form of dementia accounts for 60-80% of all cases (Boyle *et al*., 2019). The other types of dementia include Lewy body dementia, vascular dementia and frontotemporal dementia (Boyle *et al*., 2019). Recently PD patients with high Lewy body deposition have shown severe memory associate problems (Gasca-Salas, 2017). AD is a progressive neurodegenerative disorder, and the role of dopaminergic neurotransmission has been studied in patients with AD since it is involved in emotion and cognition control along with other neurotransmitter abnormalities. Some studies reported that DA and its neurons are involved in the synaptic plasticity mechanisms (Hagena and Manahan-Vaughan, 2016) . In this perspective any synaptic disarrangement or impairment of neurotransmission would results in increased extracellular deposition of amyloid proteins, senile plaque and intracellular fibrillary tangles which result in developing symptoms of dementia. Overall recent reports have been promising the role of dopamine neurons and receptors in association with AD (Kumar and Patel, 2007) .

D. Addiction

Addiction is referred to as the physiological inability to stop or pause consuming a chemical, drug, activity or substance thus causing severe physiological and physical harm. Addiction is a chronic disease that can result from overuse of medications such as opioid painkillers. Addiction often shows symptoms such as engaging in harmful

events, difficulty in relationships, increased risk-taking behaviours. Most important question in neurobiology is why some individuals are more prone to becoming addicted than the others. Recent imaging studies show pre-existing differences in DA circuits might be a critical factor determining this vulnerability among individuals (Koob and Volkow, 2016). This study has noted a reduced level of D2 receptors on its signalling activity compared with non-drug treated controls; suggesting a possible relation between the DA level and reinforcing response seen in addiction. In contrast, long term drug use may abolish DA release in the striatum leading to aberrant functioning of D2 receptors. The reduced activity of D2 receptors eventually affect the activity of orbitofrontal cortex and cingulate gyrus (regions involved in inhibitory control and impulsivity) leads to compulsive drug intake which characterizes addiction (Volkow *et al*., 2007).

E. Binge Eating Disorder

Binge eating disorder (BED) is referred to as consumption of an amount of food that is remarkably larger than most individuals would eat under the same circumstance in a discrete period of time. Individuals with BED are typically noted as overweight or obese, that may eventually lead to develop secondary diseases such as diabetes. Studies on neural correlates associated with this disorder have found the involvement of the dopaminergic system. DA is found to have roles in motivational aspects of feeding (Noble, 2003). Dat-1 transporter gene polymorphisms have been assessed in BED along with antipsychotic drug studies on D2 receptor class. These D2 receptor alterations are also found to be critical in overweight BED patients. Recent works identified other non-dopaminergic targets such as histamine, serotonin, alpha

adrenergic neurons in weight gain associated with typical and atypical antipsychotics (Bello and Hajnal, 2010).

3. Dopamine in Neurodegeneration

DA is the most prevalent catecholaminergic neurotransmitter in the brain (White and Viaud, 1991; Mohebi *et al*., 2019). Dopaminergic neuronal projections are mainly located in substantia nigra (SN), ventral tegmental area and arcuate nuclei. They contribute to the three major dopaminergic pathways in the brain, nigrostriatal pathway, mesocortical pathway and mesolimbic pathway (Figure-8).

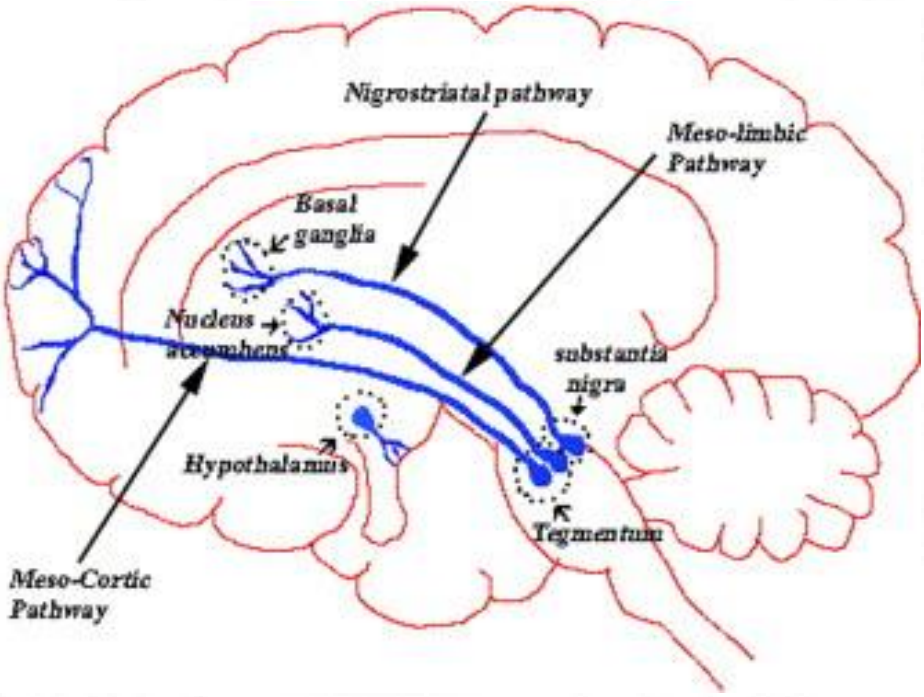

Figure 8: Dopaminergic pathways in the brain. In brain four major dopaminergic pathways have been found they are, nigrostriatal pathway, mesocortical pathway, mesolimbic pathway and tuberoinfundibular pathway. Nigrostriatal pathway transmits dopamine from the substantia nigra pars compacta (SNc) to the basal ganglia/ striatum. Mesocortical pathway transmits DA from are Ventral Tegmental Area (VTA) to the prefrontal cortex. Mesolimbic pathway transmits DA from VTA to the nucleus

accumbens. Tuberoinfundibular pathway transmits dopamine hypothalamus to the pituitary gland. Figure from Chinta and Andersen 2015 and reprinted with permission from the Elsevier Inc © 2015.

Nigrostriatal pathway is responsible for locomotory movements, mesocortical and mesolimbic pathway is responsible for motivation and rewards and finally the tuberoinfundibular pathway is responsible for the secretion of pituitary gland hormones (Luo and Roth, 2000; Chakraborti *et al*., 2019). Current evidence underscores DA can act as a potential neurotoxic agent at high concentrations or in an oxidative environment. This has been observed highly in idiopathic PD, the selective degeneration of the DA (neuromelanin containing) neurons in the substantia nigra pars compacta (SNpC). It is widely believed that the mutual interplay between high DA content and local pro-oxidative environment in the SN promotes the oxidative damage to the dopaminergic neurons, thereby leading to its degeneration (Guo *et al*., 2018). DA can exert their neurotoxic effect via two mechanisms; either through oxidative mechanisms or through non-oxidative mechanisms. Possible mechanisms and molecular events that lead to dopaminergic cell death induced by DA are discussed in subsequent sections.

A. Oxidative Reduction of Dopamine

In the brain region striatum, the maximum concentration of DA amounts nearly 65 µM and at the synaptic terminal it can be as high as 50 µM, even though a large portion of DA is stored in the synaptic vesicles. Aging or any pathological disease state leads to increased turnover or release of DA from the vesicles which leads to the production of reactive oxygen species (known as ROS) mainly H2O2, O2., OH. and toxic quinone

species (Delcambre, Nonnenmacher and Hiller, 2016). This accumulation of toxic ROS compounds finally accounts for the neurodegeneration of the dopaminergic neuron system (Halliwell and Gutteridge, 2015). There are two ways that oxidation of DA could happen; either through enzymatic oxidation or through non-enzymatic oxidation.

a. Enzymatic Oxidation reactions of Dopamine

Oxidative breakdown of DA can occur enzymatically or non-enzymatically. This process of DA breakdown is known as 'autoxidation'. The predominant form for oxidation of DA in the brain is catalyzed by the enzyme monoamine oxidase (MAO) present in the outer mitochondrial membrane. MAO catalyzes the oxidative deamination of DA into 3,4-dihydroxyphenylacetaldehyde (DOPAL) with the concomitant production of H2O2. There are two MAO enzyme classes that have been identified so far, they are; MAO-A and MAO-B (Shih, Chen and Ridd, 1999). MAO-A is predominantly involved in the metabolization of serotonin or noradrenaline and the presence of only a subtle concentration of MAO-A were found in SN. DA is preferentially oxidized by the MAO-B class found in hypothalamus, brain stem, raphe nucleus (Thorpe *et al*., 1987). Besides MAO, other enzymes found in the brain regions are also capable of oxidizing DA namely, lipoxygenase, xanthine oxygenase (which oxidizes DA in the acute presence of H2O2) as well as tyrosinase and prostaglandin H synthase ((Mattammal, Strong, *et al*., 1995; Xu *et al*., 1997)). All these enzymes oxidize dopamine into highly reactive DA quinone (DAQ). However, it is still not clear up to what extent these enzymes are contributing to the oxidation of DA in the brain. Nevertheless, presence of prostaglandin H synthase was found in SN, so as tyrosinase (Mattammal, Strong, *et al*., 1995; Xu *et al*., 1997).

b. Non-enzymatic Oxidation reaction of Dopamine

The main part of the catecholamine DA is its catechol moiety. Since it is a phenolic compound it may undergo oxidation at physiological pH under the influence of oxygen. Corresponding autoxidation of DA leads to the generation of DAQs, similar to enzymatic oxidation of tyrosinase and prostaglandin H synthase. This complex oxygen driven oxidation reaction leads to the generation of semi quinone radical as well. Semiquinone radicals aid in the univalent reduction of oxygen with consequent production of superoxide anion, H2O2 and OH. radicals (Bindoli, Rigobello and Deeble, 1992). At physiological pH this non enzymatic autoxidation reaction is relatively a slow process. It could be accelerated by free transition metals such as iron and manganese. Both these metals lead to the generation of DAQ with the additional products such as H2O2, OH. and ROS (Chevion, Berenshtein and Zhu, 2002). 6-hydroxylation of DAQ via nucleophilic attack of water can lead to the generation of potent neurodegenerative agent 6-OHDA. However, uncatalyzed 6-hydroxylation of DAQ is generally a slow reaction, but this reaction may be enhanced by the presence of metal ions such as manganese or iron at physiological pH. Iron and manganese catalyzed production of 6-OHDA has been demonstrated (Linert *et al*., 1996). 6-OHDA levels have been significantly increased in the PD patients and identified as causative agents of dopaminergic neurodegeneration (Tieu, 2011).

B. Toxicity of Dopamine Derived Oxidation Products

a. Theory of Increased Turnover

DA turnover is an early established theory regarding the potential toxicity of DA on dopaminergic neurons. In PD patients, loss of dopaminergic neurons forces the

residual neurons to elevate their metabolism of DA. This compensatory mechanism of DA metabolism may lead to the generation of toxic ROS molecules in neurons. This theory of compensation is supported by the finding of increased catalysis of L-DOPA formation from tyrosine by the action of enzyme tyrosine hydroxylase (TH) in PD brains (Zigmond, Hastings and Perez, 2002). Hence, it is noteworthy that TH activation leads to an increased production of DA subsequently leads to the generation of ROS. Classically DA turnover is demonstrated as the ratio between metabolites of DA and DA itself (Sossi *et al*., 2002).

b. Dopaldehyde

Increased DA turnover might have reasonably activated MAO activity that may lead to the generation of not only H2O2 but also to the aldehyde DOPAL. This DOPAL undergoes further dehydrogenation to 3,4 dihydroxy phenyl acetic acid (known as DOPAC). As already mentioned, DA is preferably metabolized by MAO-B found in astrocytes. This DOPAL generated is found to be toxic to neurons in-vivo and in-vitro. It has been found that DOPAL uses DA reuptake machinery to enter into the dopaminergic neurons (Mattammal, Haring, *et al*., 1995). High DOPAL concentration due to increased DA turnover contributes to the specific vulnerability of dopaminergic neurons. The mechanism of DOPAL toxicity is associated with its instability and reactivity with -SH groups of proteins or glutathione (GSH). Beside this, aldehyde group of DOPAL can condense with DA to form neurotoxic alkaloids. Since this DOPAL is produced in the outer membrane of the mitochondria it is crucial to have active mitochondrial function to scavenge the toxicity associated. Actively respiring mitochondria shows resistance to DOPAL mediated neurotoxicity. Hence the selective vulnerability of dopaminergic neurons might be a result of interplay between

MAO-B activity, impaired mitochondrial function and elevated DA turnover with increased cellular concentration of DOPAL (Wiemerslage *et al*., 2013).

c. *Dopamine Quinone*

As discussed, DAQ is formed via autoxidation and metal catalyzed oxidation of DA. The oxidative environment owing to the increased iron content, impaired respiratory chain activity and increased prostaglandin H synthase activity leads to the production of DAQ. This highly reactive DAQ contributes significantly to the degeneration of dopaminergic neuronal populations in the brain. The electron deficient nature of DAQ makes it a better candidate for nucleophilic addition reactions leading to the inactivation of vital proteins in the cell (LaVoie and Hastings, 1999). SH groups are the strongest and most ubiquitous nucleophiles in the cell at physiological pH, as a consequence they are very good targets for nucleophilic attack by DAQ. In a cell predominant source of -SH group comes from the amino acid cysteine. The major component of the GSH protein is cysteine, also found in other enzymes such as glyceraldehyde-3-phosphate dehydrogenase and ribonuclease inhibitor proteins. DAQ mediated reaction with SH groups inactivate the protein function which eventually leads to the cell death in neurons (Segura-Aguilar *et al*., 2014).

d. *Dopamine and Alpha synuclein*

Presence of alpha synuclein has been reported in PD patients, in fact it is a major component of Lewy bodies (Schaser *et al*., 2019). Lewy bodies are abnormal aggregations of protein that develop inside neuronal cells, contributing to Parkinson's

disease. Recently it was discovered that DAQ also contributes significantly to alpha synuclein associated toxicity in PD (Zhao *et al*., 2020). However, alpha synuclein did not consist of any -SH groups containing cysteine amino acid. It is believed DAQ reacts to other reactive amino acids like tyrosine or lysine through radical coupling or with nucleophilic attack respectively to form DA-alpha synuclein adducts. These adducts formed by DA- alpha synuclein combination prolong the life time of protofibrils. Protofibrils are usually converted into fibrils deposited in Lewy bodies. Hence the protofibrils are highly reactive whereas fibrils seem to be inert (Goldberg and Lansbury, 2000). This protofibrillar alpha synuclein causes significant permeabilization similar to the action of pore forming protein toxins. This permeabilizing could cause degeneration of dopamine neurons through increased calcium influx, depolarization of the mitochondrial membrane potential and leakage of dopamine from the storage vesicle into the cytoplasm (Volles *et al*., 2001) Moreover, DA dependent neurotoxicity of alpha synuclein was confirmed with overexpression in wild-type or mutant alpha synuclein expressing animals. Overexpression of alpha synuclein in dopaminergic neurons undergo apoptosis through the action of endogenous DA (Jęśko *et al*., 2019).

e. Dopamine and DNA

DNA adducts could be generated by the reactive quinone species derived from DA oxidation. This kind of formation of DNA adducts was observed in HL-60 human leukemia cells containing peroxidase, when DNA was incubated with DA (Lévay and Bodell, 1993). This process of DNA adduct formation could be further enhanced by H_2O_2 and could be prevented by ascorbic acid (Perveen *et al*., 2018). Less DNA adducts formation were observed in the absence of peroxidase free cell lines. Hence

it is believed that quinones or semiquinone radical (DAQ) of DA is solely responsible for the DNA adduct formation. These activated DNA adducts were supposed to be formed via peroxidase or non-enzymatically by metal ions such as iron or manganese (Stokes *et al*., 1996).

f. Dopamine and 6-OHDA

6-OHDA is generally considered as classical neurotoxin for catecholaminergic cells and which is mainly used to model the neurodegenerative events in PD. 6-OHDA was detected in human caudate nucleus and in urine sample of patients treated with L-DOPA (Andrew *et al*., 1993; Tronci and Francardo, 2018). Non enzymatic formation of 6-OHDA is enhanced in an increased oxidative stress condition with elevated cytosolic DA and iron. This has been proved by inhibiting the activity of MAO-A and MAO-B and found to be increasing the cellular concentration of 6-OHDA, by means of increased cytosolic DA concentration. This 6-OHDA formation could be activated by the treatment of DA releasing agents like amphetamine or methamphetamine (Björklund and Dunnett, 2019). The potent toxicity of 6-OHDA can release iron from ferritin in its reduced form Fe2+, which initiates cell damaging processes through Fenton reaction (Jellinger *et al*., 1995). Another important feature of 6-OHDA is it could inhibit the complex-I and complex-IV in the respiratory chain thereby causing mitochondrial impairment.

Overall, DA is able to have neurotoxic effects through enzymatic or non-enzymatic production of ROS and toxic metabolites such as DAQ, DOPAL, 6-OHDA. These compounds can either have both neuroprotective and neurotoxic properties

depending on its iron binding capacity. The final outcome occurs through oxidative modification of vital proteins in the neurons possibly through the activation of apoptosis, necrosis or autophagy (Pedrosa and Soares-da-Silva, 2002).

4. Dopamine in Learning and Memory

DA is a crucial neurotransmitter in reward-based pathways. Many hypotheses have been proposed till date supporting this notion, yet none of this has been left unchallenged. It has been observed in many studies that sparing doses of DA receptor blockers render rewarding stimuli, such as food and water, ineffective (Wise, 2005). DA release in the nucleus accumbens (NAc) has shown significant increase in efficacy of these unconditional stimulus or rewards (Radke *et al*., 2019). DA release is also associated with a memory engram that gives motivational attachments or importance to otherwise neutral environmental stimuli (Radke *et al*., 2019). Nigrostriatal DA is particularly associated with motor functions, but moderate doses of neuroleptic drugs (a drug that binds to DA receptor and blocks the function of DA system-simply known as DA antagonists) decrease the motivation to act to a certain purpose. These DA antagonists did not immediately attenuate the well learned behaviour, rather the response reduced progressively, either on a minute or days' time scale (Wise *et al*., 1978; Wise and Raptis, 1986).

Many hypotheses have been put forth pointing to the importance of DA in reward-based learning (Gardner, Schoenbaum and Gershman, 2018). DA has also been found successful in acting as a reinforcement of stimulus for learning and memory

consolidation establishing motivational foundations for most goal directed behaviours (Bogacz, 2020).

A. Dopamine Hypothesis

When first identified, DA was known to play an important role in food intake, drinking and motivational functions, which strengthens the theory by selective degeneration studies on DA fibers innervated to striatum (known as nigrostriatal pathway). Later it was identified that nigrostriatal dopamine neurons control feeding and drinking while mesolimbic (extension of dopamine neurons from ventral tegmental area to forebrain regions) controls forward locomotion (Radke *et al*., 2019). This is common for most reward seeking behaviours. The development of the DA antagonists paved the way to identify the behaviour towards food and was found to attenuate free feeding behaviour completely (Smith, 1976; McFarland and Ettenberg, 1995). The DA antagonist treated rats or mice did not show learning or response to reward-oriented locomotion (Voiculescu *et al*., 2014). These findings lead to identify the role of DA in the reinforcement pathway.

B. Dopamine in Reinforcement

Reinforcement is the term for imprinting of stimulus association and response habit. Generally, reinforcement is defined as the strengthening of stimulus-stimulus, stimulus-response or stimulus-reward association resulted from accurate timely representation of reward (Shahan, 2017). This hypothesis was tested using the instrumental reinforcement dealing with lever-press for reward and it was found that animals with impaired DA system did not learn the lever-press behaviour (Sackett, Saddoris and Carelli, 2017). Moreover, well trained animals perform normally after being trained and abolish the behaviour after being trained with DA antagonists. This

points out the possibility of memory extinction in response to dopaminergic blockage (Furini *et al*., 2017). This notion that reduction in reinforcement of learned behaviour with the application of DA antagonists were widely accepted, while also been questioned. The major objection to this point was that DA antagonists to some extent caused motoric fatigue like impairment in animals (Furini *et al*., 2017). Overall, the DA hypothesis of reinforcement states that, animals will not develop preferences for places in the environment where they have experienced reward in the absence of a normal functioning dopaminergic neuronal system (Wise and Rompre, 1989).

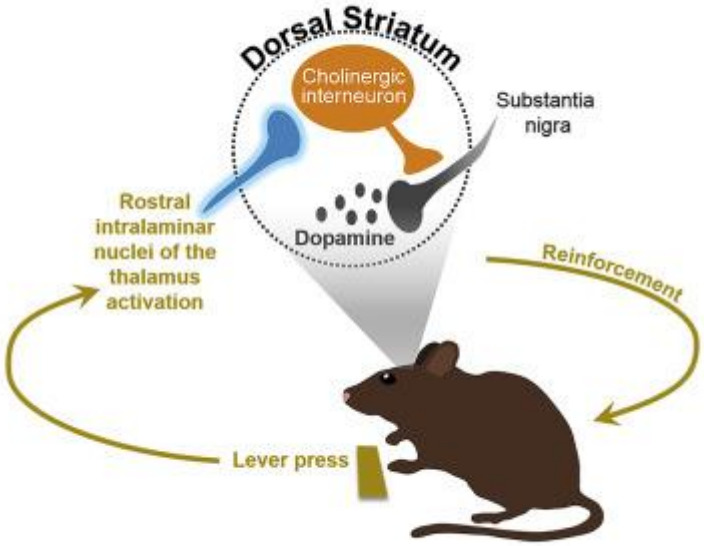

Figure 9: Dopaminergic neurons in the substantia nigra are involved in the positive reinforcement in lever press studies of rat models. Striatal cholinergic interneurons, activated by rostral intralaminar nuclei of the thalamus, enhance the pursuit of reward through local release of DA from dorsal striatum (DS). Activation of the DS cholinergic neurons result in cholinergic interneuron burst firing and eventually leads DA receptor class D2 dependant post-burst pauses in cholinergic interneurons. Figure from Cover *et al*. 2019 and reprinted with permission from the *Cell* © 2019.

Studies have proven that DA is important for most instances as positive reinforcement (Dabney *et al*., 2020). Most of the rewarding stimuli fails to act as effective rewards in DA deficient animals (Rosenzweig, 1967).

C. Hypothesis of Reward

Reinforcement strengthens still-active memory traces of the learned behaviour hence it is called as retroactive effect on learned behaviour. Reward and reward associated stimuli cause motivational arousal and increase the chance of response initiation, even in the absence of primary reward. Generally, “reward” is used to denote the undifferentiated effects of motivational arousal and reinforcement (Rosenzweig, 1967; Everitt, 1991). The reinforcing effect of stimulation given after a response affects the future response of the animal, and usually is long lasting. This means, effects of a reward that is given after a response strengthens the next response even after days.

D. The Dopamine Hypothesis of Conditional Reinforcement

Reward based motivational stimuli do not only elicit a strong behaviour when given prior to the response, but they can also serve as conditional reinforcers when given as a contingent after response. Pairing of light and water to rats will immediately learn to work for the presentation of light when they are thirsty. In such kinds of experiments, addition of amphetamine, a DA agonist, improved response for light (Taylor and Robbins, 1984). Accordingly, DA can modulate the expression of conditional reinforcement as well as an essential component for the establishment of conditional reinforcers. Hence in the first place DA makes a conditional reinforcer through a DA dependent reinforcement. It is then the conditioned reinforcer that causes phasic DA release, for augmenting the effectiveness.

E. The Nucleus Accumbens Hypothesis

Majority of the nucleus accumbens (NAc) hypothesis, i.e., association of reward function with dopamine neurons in the NAc, originated from the brain lesion (a region in an organ or tissue which suffered damage) studies. DA selective lesions in the NAc reduced the rewarding effects of cocaine and amphetamine, but noradrenergic or other lesions on other parts did not (Roberts, Corcoran and Fibiger, 1977). However, such studies claim the role of DA in reward function, but it is not the only DA terminal field that is involved in reward function. Other regions such as nigrostriatal lesion has been implicated in reduced motivational deficits in feeding and drinking, but not the lesions that are restricted to mesolimbic extensions (Smith, Strohmayer and Reis, 1972).

F. Dopamine and Memory Consolidation

Searching for food or water when hungry or thirsty are classified as mostly goal directed motivations. This goal directed motivations can be learned. It is largely done by the initial selective reinforcement of random behaviour with directed stimuli in the environment. Motivations here refer to the motivations to return to the actual rewards we have experienced in the past and to the environmental cues that mark the way to such rewards. DA is important for such motivations, that selectively reinforces associations between the reward and neutral stimuli. Once the stimuli-reward association has been formed, it is stable for a period of time even in the absence of appropriate drive states, such as hunger or thirst (Mendelson, 1966). Once the habit has been established, it remains largely autonomous until the conditional stimuli is removed or devalued through experience. Extinction of the conditional behaviour to the natural stimuli by the association with reward can be resulted from the presentation of repeated unrewarded trials in the absence of drive states (hunger or

thirst) or repeated trials under the influence of dopaminergic antagonists. Therefore, DA appears to be important for learning and memory in most terminal fields of dopaminergic systems such as NAc, nigrostriatal, mesolimbic and mesocortical systems (Heymann *et al*., 2020). Phasic release of DA, induced by the drives and conditional stimuli, from these dopaminergic terminal fields also augment motivation (Cornish and Kalivas, 2000).

G. Dopamine and its role in Memory at the Cellular level

Earlier attempts to link cellular mechanisms with reinforcement was done in relation to the consolidation of long-term memory. Based on this perspective, as pointed earlier, reinforcement is seen as after-effects of learned experience. Reinforcement in its most basic form, is observed as potentiating, imprinting or consolidating memory traces. In Aplysia californica learning at the single neuron level has been demonstrated when a sensory neuron excites a motor neuron and this excitation occurs in the presence of neuromodulatory transmitter (Nargeot and Bédécarrats, 2020). The neuromodulatory transmitter alone could not open or close the ion channels of motor neurons. This is done through a cascade of intracellular signalling events, that finally makes the motor neuron responsive to subsequent excitatory input. Therefore, it strengthens (reinforces, imprints or consolidates) synaptic connection between the sensory neuron and motor neuron (Landauer, 1969; Cornish and Kalivas, 2000).

In mammals two major forms of such learning have been studied; long-term potentiation (LTP) and long-term depression (LTD). LTPs and LTDs have been

studied extensively and found to be a part of dopaminergic brain regions and linked to DA receptor activation. In the hippocampus DA seems to have a reinforcing role. LTPs and LTDs are observed at hippocampal pyramidal cells especially at excitatory synapses. Hippocampal LTP were facilitated by DA D1 receptor agonists and blocked by DA D1 receptor antagonists whereas, LTD is activated by D1 receptor agonists and D2 receptor antagonists and is blocked by D1 antagonists and D2 agonists (Chen *et al*., 1996). LTP and LTD is dependent on DA not only in hippocampus, but also in the dorsal striatum, amygdala and frontal cortex as well.

DA is involved in LTPs and LTDs in ventral tegmental area and LTP is seen in all excitatory synapses on DA containing neurons of substantia nigra (Placzek *et al*., 2016). LTD is particularly observed only in ventral tegmental area and is blocked by binding of DA to D2 receptors. Consequently, D1 or D2 dependent activation has been demonstrated in a number of cortical and limbic sites, but surprisingly exclude the NAc region identified with reward function in most of the behavioural studies (Saal *et al*., 2003).

H. Dopamine and consolidation of Memory

Many behavioural studies have demonstrated the importance of DA in memory consolidation. In behavioural studies DA or DA agonist given after a long-term or short-term learning trial act as reinforcement stimuli. Similar memory consolidation could occur in the brain regions especially in striatum, using D2 agonist as a post-trial injection (Messier and White, 1984). Hence, DA is found to have a distributed role in memory consolidation, reinforcement, imprinting memory traces associated with

different tasks for learning at different brain terminal fields post-trial (White and Viaud, 1991).

5. Invertebrates as a Model for Learning and Memory

Learning and memory are highly interrelated universal attributes of animal kingdom and intensively studied topics in modern brain and cognitive science. That being said, it is crucial for altering the behaviour of an organisms in the face of changing environments and critical for survival and reproduction (Maren, 2008; Guo *et al.*, 2013; Sasakura and Mori, 2013).

In modern neurobiology it is a very daunting task to decipher the neuronal mechanism by which we acquire, store and recall information stored in the form of memory. Use of simple invertebrate model systems has provided a considerable amount of data to have a progress in overall understanding of underlying mechanisms and processes that are involved in this learning and memory pathway (Frost and Megalou, 2009). The invertebrate models have complex neuronal structures involving thousands of neurons and which makes immense possibilities of connections and interactions (Rankin, Beck and Chiba, 1990). These models are widely used for three reasons. Firstly, these invertebrate model systems have displayed many of the learning pathways shown by vertebrates including habituation, dishabituation, sensitization, classical conditioning, second order conditioning, sensory preconditioning, latent inhibition, overshadowing, blocking, context conditioning and operant conditioning (Frost and Megalou, 2009). Secondly, the molecular mechanisms and synaptic

plasticity underlying fundamental memory are highly conserved. Hence, the same type experience dependent plasticity shown by the vertebrate model including paired-pulse facilitation, homosynaptic depression, presynaptic inhibition, presynaptic facilitation, post-tetanic potentiation, long-term potentiation, and long-term depression can be studied (Frost and Megalou, 2009). Thirdly, in invertebrates the number of neurons are very few whereas in some models they are bigger in size thus making its visualization easy and possible to map out the discrete connections (Frost and Megalou, 2009). And a final key reason is that invertebrate model systems possess a large repertoire of sensory behaviour facilitating the learning process (Sasakura and Mori, 2013).

A. Learning: Principles and Paradigms

In the history, Romans have argued in the learning abilities of lower animals. They believed a better understanding of it came up with an operational definition of mind. Cellular models rapidly become an analyzing tool for habituation, sensitization, and classical conditioning (Krasne and Glanzman, 1995). However, critical understanding of the relationship with cellular level and the behavioural strategies of learning remains intricate and ambiguous in mammalian models. Nevertheless, with invertebrate models, this intricate relationship between cellular and behavioural phenomena could be clarified much more effortlessly (Krasne and Glanzman, 1995).

Inference made by the observed relationship between an animal's behaviour with its past experience is called learning (Carew and Sahley, 1986). In a broader way, learning is a byproduct of an animal's experience with one or more stimulus events and its relationship between these stimuli. Recently neurobiologists have adapted many learning paradigms that are known to produce learning in vertebrates to study

invertebrate learning. Here, I briefly summarize key modern learning paradigms used in invertebrate model systems.

a. Non-Associative Learning

We refer to non-associative learning as, those instances, where a stimulus changes behaviour of an animal irrespective of any apparent additional stimulus or event (eg: reward or punishment). In other words, it is a unique learning pattern in which the behaviour and stimulus are not linked or paired. Behaviour in non-associative learning can be changed according to experience with either single stimuli or two stimuli given that they are not temporarily related (Pelley and Le Pelley, 2004). There are two types of it; habituation and sensitization (Blumstein, 2016). Habituation is referred to as the reduction of response towards a repeated stimulus. Whereas sensitization is referred to as the elevated response or strengthening of neurological response to a stimulus when the stimulus was repeatedly given. Studies have shown that habituation and sensitization are mirror images to some extent (Blumstein, 2016).

b. Associative Learning

Associative learning is a fundamental component of adaptive learning and is referred to as the ability of living organisms to perceive or learn relation between two or more events (Jozefowiez, 2012). Associative learning is expressed as it modifies an animal's existing behaviour or creates new behaviours that include conscious or unconscious recognition of a possibility (Christian, 2010). Associative learning can be divided into two distinct categories: classical conditioning and instrumental conditioning or operant conditioning. Classical conditioning is a form of learning where a conditioned stimulus is associated with an unrelated unconditioned stimulus, to

create a new behaviour called conditioned response (eg: Pavlov's experiment). In general, classical conditioning is a formation of association between two stimuli. Whereas, operant conditioning or instrumental conditioning is formation of an association between a behaviour and a stimulus. Operant conditioning occurs through rewards and punishments in order to make an association between a behaviour and consequence (Skinner, 1966). Both classical and operant conditioning are an example of first order conditioning where unconditioned and conditioned stimuli (in classical conditioning) response and reinforcement (in operant conditioning), are paired directly, and is therefore the reason for the learning process.

6. *C. elegans* as a Model System

Caenorhabditis elegans is a simple small free-living soil nematode. It comes under the genus *Caenorhabditis* and has several species under this gene class. The name comes with a mix of Greek and Latin where Caeno means "recent', rhabditis means 'rod like' and elegans means 'elegant' (Frost and Megalou, 2009). One might imagine how this tiny organism could serve as a good model organism. Caenorhabditis elegans was first annotated as Rhabditis elegans by Maupus he collected the animals from humus rich soil from north Africa a place called Algeria. Later, it was categorized in the subgenus Caenorhabditis by Osche. Then Dougherty gave this subgenus with generic status (Nigon and Dougherty, 1950; Fatt and Dougherty, 1963). In 1965, Sydney Brenner came up with *Caenorhabditis elegans* as a model organism. This organism has a great potential and it was able to deliver a large pool of characteristic features that allows us to study different aspects of biology using this tiny animal. Now it has been established as a standard model system to imply a variety of genetic

interventions and that suits understanding of developmental biology, cell biology and neurobiology.

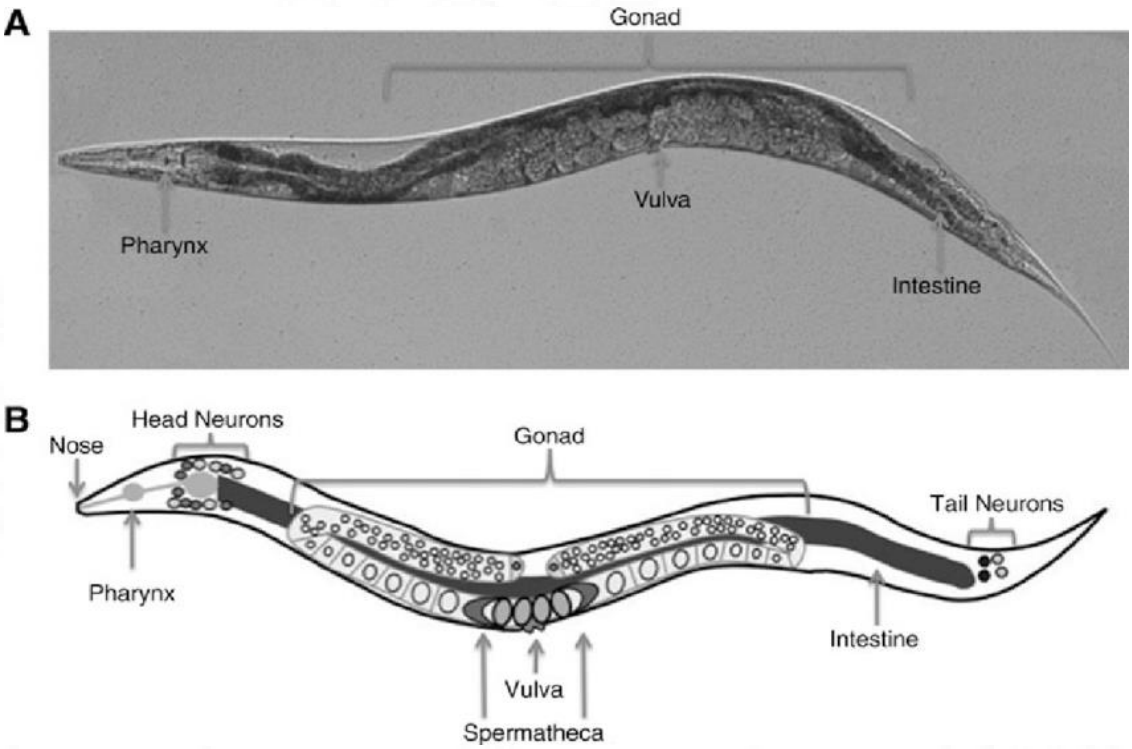

Figure 10: A. Microscopic bright field image of a *C. elegans* hermaphrodite. *C. elegans* has an unsegmented, cylindrical body shape that is tapered at the ends. The image clearly shows different structure of worm's aid in its survival including pharynx, vulva, intestine and overall alignment of the gonad. **B. Diagram depicting the structure of *C. elegans*.** Alignment of the head neurons, nose and tail neurons are noted. Figure from Yen *et al*. 2011 and reprinted with permission from the *Mary Ann Liebert Inc* © 2011.

A. C. elegans as a Model for Learning and memory

Compared to other previously studied invertebrate model organisms *C. elegans* is a simpler model organism, offering the possibility of studying the neurons involved in a given behaviour. *C. elegans* has many characteristics that allow it to become an

excellent model system for learning and memory. Since it is a simple model system, the anatomy of the nervous system has been mapped with the reconstruction of the electron microscopic images of body slices. This further leads to the development of a neuronal wiring diagram, including all of its 302 neurons, engaged in electrical and chemical synapses. Subsequently, laser microsurgery and genetic analysis revealed the function and neurotransmitter of each neuron (White *et al.*, 1986; Rothman, 1989). In early 60's Kandel started working with Aplysia model systems and at the same time Brenner had chosen *C. elegans* to study the development and nervous system. Now this tiny 1mm long transparent nematode became the world's best understood model system. *C. elegans*' short life cycle and ease of cultivation makes it perfect as a laboratory model system (Brenner, 1974). The mode of reproduction is best suited for genetic analysis. This self-fertilizing hermaphrodite can be easily inbred and crossed with male. The availability of thousands of mutants and RNAi constructs makes it a versatile tool for researchers. Adult hermaphrodites are comprises of an invariant cell lineages that consist of 959 cells (Sulston *et al.*, 1983). *C. elegans* with its consistent number of chemical synapses, gap junctions and neuromuscular junctions, is greatly sensitive to the place and surroundings, where they live thus showing many behavioural plasticity. The main paradigm addressed here would be associative learning paradigm since my work is on associative memory training. Hence this section deals with the nematode's ability to learn and remember the environmental stimulus such as taste, smell, temperature and oxygen level. *C. elegans* exhibit remarkable behavioural features of thermotaxis, chemotaxis and aerotaxis to find a more suitable environment. This is done by the exquisite capacity of the nematode learning and remembering the appropriate environment cues. Findings from the gene level, behavioural level and neurocircuitry level underscores the importance of *C.*

elegans as a model system for learning and memory studies (Rankin, Beck and Chiba, 1990).

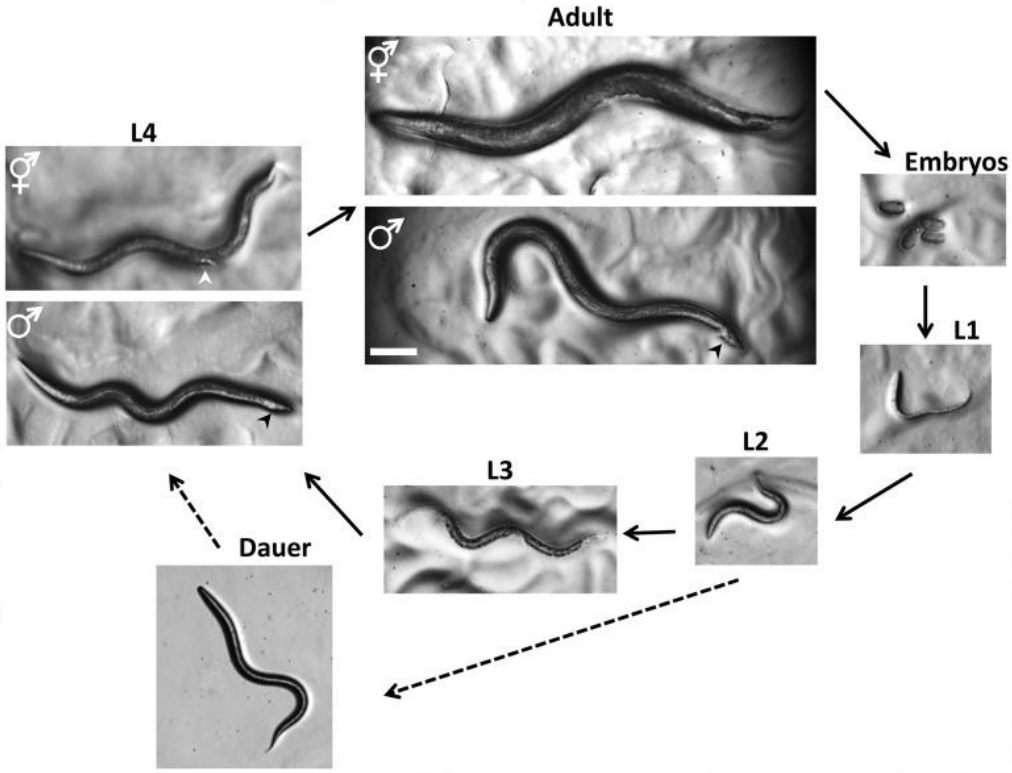

Figure 11: Life cycle of *C. elegans* hermaphrodite. *C. elegans* life cycle at 20^0C. 0 min is fertilization. First cleavage occurs at about 40 min and then it goes through different steps of embryogenesis and finally hatching takes place. From this L1 stage it develops into an adult worm if environment is favorable, if not it will go to a dormant dauer stage. Once the environment become favorable, dauer develop into L4 larval stage then to adult worm. Figure from Corsi *et al*. 2015 and reprinted with permission from the Genetics © 2015.

a. Smell as Conditioned stimulus

C. elegans strains in laboratories are usually reared on bacterial food sources, such as OP-50. These bacterial food sources release certain volatile chemicals or organic components to which *C. elegans* show chemotaxis. One such chemical is diacetyl

which is normally an attractant for *C. elegans*. However, it can be made aversive by pairing it with noxious acetic acid solution (Morrison *et al.*, 1999). Conversely, pre-exposure to butanone in the presence of food made it more attractive to otherwise neutral stimuli (Torayama, Ishihara and Katsura, 2007). It is observed in aged *C. elegans* that benzaldehyde become more attractive because of pairing of the odour with the presence of a food source and is found to be mediated by serotonin suggesting the possibility of multiple mechanisms for stable sensory integration (Torayama, Ishihara and Katsura, 2007; Tsui and van der Kooy, 2008).

It has been demonstrated that *C. elegans* are able to avoid the smell associated with pathogenic bacteria (Beale *et al*., 2006). Generally, *C. elegans* prefer odours associated with known non-pathogenic bacteria. Aversive conditioning to pathogenic bacteria could be achieved by exposing nematodes with pathogenic bacteria for about 3-4 hours. The learning ability could be examined using a choice maze assay with pathogenic and non-pathogenic bacteria only to find that aversive training not only avoided known pathogens but also strongly preferred non-pathogenic bacteria. Hence it suggests the possibility of an attractive and aversive responsed to food preference (Zhang, Lu and Bargmann, 2005).

b. Taste as conditional stimulus

C. elegans show chemotaxis behaviour to salts and various other water-soluble attractants. This strong attractive behaviour to salts is mediated by the ASE gustatory neurons (Bargmann and Robert Horvitz, 1991). The salt NaCl is shown to elicit a residual response in *C. elegans* even in the absence of ASE neurons, because of the presence of ASI, ASG and ADF sensory neurons. Both Na^+ and Cl^- could act as

attractants, where Na^+ is sensed through the left ASE neuron (ASEL) and Cl^- is sensed through the action of right ASE neuron (ASER) (Pierce-Shimomura *et al.*, 2001). In choice assay, *C. elegans* exhibit a random behaviour to diffusive gradients of salt concentrations of Na^+ and Cl^- ions. However, pairing of one of the ions (either Na^+ or Cl^-) with food (positive stimuli) or aversive stimuli (negative stimuli) changed the preference of *C. elegans*. Worms previously exposed to an ion in the presence of food increases its migration towards that ion concentrated area in the choice assay plate. Similarly, aversive training tends to avoid that ion concentrated area on the choice assay. Learned preference and avoidances preferably requires predictive pairing with unconditioned stimulus such as food or aversive stimuli (Wen *et al.*, 1997).

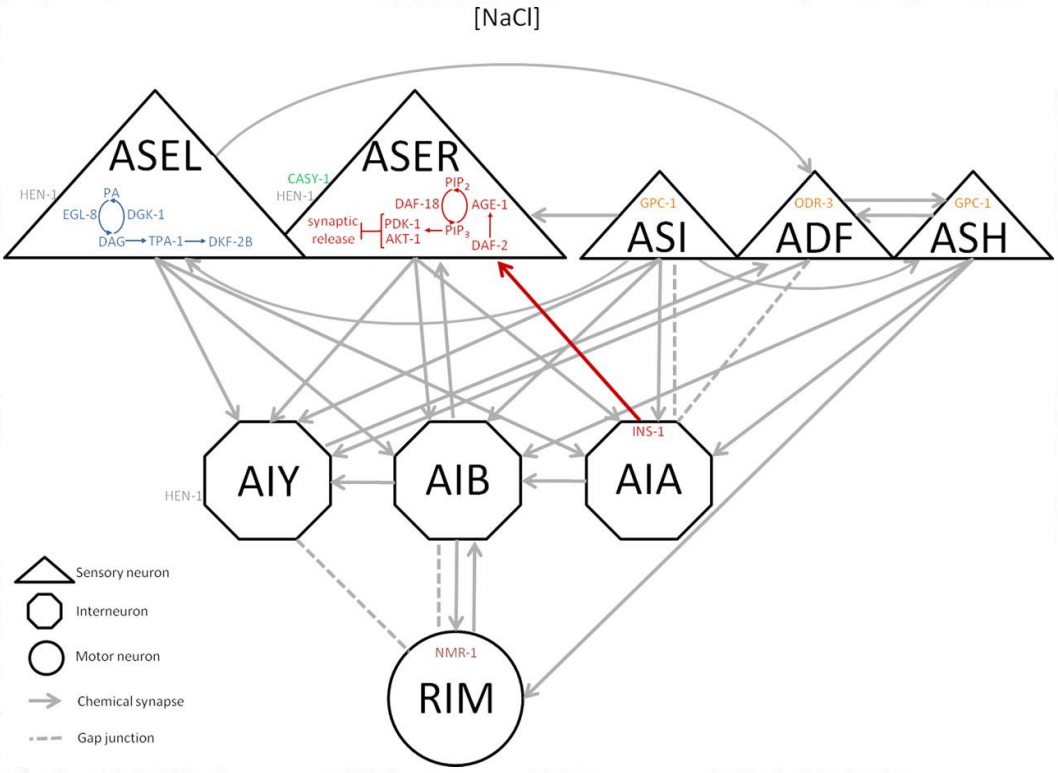

Figure 12: Neurons involved in NaCl aversive response. The network of first level sensory neurons (ASEL & ASER) and second level interneurons (AIY,AIB,AIA) with motor neuron RIM for avoiding response to NaCl. Figure from Ardiel and Rankin 2010 and reprinted with permission from the Cold Spring Harbor Laboratory Press © 2010.

c. Temperature as Conditioned Stimulus

In early 1975, it was observed that worms thermotax to their previous culturing temperature. After reaching to this reared temperature, it will spread isothermally in that specific temperature zone. Later with the help of laser ablation studies, it has been demonstrated AFD neurons are involved as the major thermosensory neuron in the neural circuit for thermotaxis. AFD neuron along with the downstream action of AIY interneuron complete the thermophilic movement in *C. elegans* (Mori and Ohshima, 1995). The sensory neurons, store a residual memory of its own previous cultivation temperature (Kimura *et al.*, 2004). Temperature preference can be changed using classical conditioning methods. It is reported that worms in starved plates tends to disperse more randomly away from their cultivation temperature through a learned understanding of temperature and feeding status of the worm (Hedgecock and Russell, 1975).

d. Oxygen as Conditioned Stimulus

It was demonstrated in *C. elegans* that oxygen preference could also be changed by experience. Worms received in normal laboratory conditions prefer 5%- 12% O_2 when given with oxygen gradient. This preference can be changed by growing nematodes in a low oxygen exposure on rearing condition allows worms aerotaxis to a preferably low oxygen gradient plate 1-7%. Aforementioned aerotaxis behaviour could be a learned association of food with different oxygen gradients (Chang, 2006).

7. Invertebrates as Neurodegenerative Disease Models

Developing human neurodegenerative disease models is quite challenging and is a slow process. In the last five decades, a series of studies on the basics of neurobiology that have been carried out in animal models have shown that there is significant functional conservation at the cellular and molecular levels (Barré-Sinoussi and Montagutelli, 2015). In turn, these observations allowed the use of animal models to investigate human degenerative disease mechanisms or underlying causes (Jucker, 2010). Among these studies, contributions demonstrated by invertebrates in neurodegenerative studies have significantly been accounted (Thompson and Marsh, 2003).

Predominant examples of neurological disorders seen in man include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS) (Manoharan *et al.*, 2016). AD and PD are late-onset loss-of-function neurodegenerative disorders (Dursun *et al.*, 2015). HD and ALS are polyglutamine repeat disorders (Gibbons *et al.*, 2018).

As a neurodegenerative disease model system, many well-known invertebrate model systems have been widely used. Mainly, *Drosophila melanogaster* and *Caenorhabditis elegans* are preferred models because of their short life cycle and genetic tractability. These invertebrate animal models are investigated for the molecular and cellular mechanisms involved in neurodegenerative disease (Thompson and Marsh, 2003).

Approximately 50% of fly genes and 36% of worm genes have similarity to human genes (Culetto, 2000; Rubin, 2000). The estimates based on the basic local alignment search tool (BLAST) indicate that approximately 75% of human disease genes have

orthologs in the fly, and 65% have counterparts in the worm (Sonnhammer and Durbin, 1997; Reiter, 2001).

Early studies on neurodegeneration in invertebrate models focused on the recessive and dominant mutations that cause neuronal apoptosis (Petit *et al.*, 2005), aberrant brain morphology affects membrane vesicle transport, and protein processing (Hay *et al.*, 1997). Dominant mutations in worms lead to neuronal degeneration that mainly includes channel activating mutations (Driscoll and Gerstbrein, 2003; Finley *et al.*, 2003).

A. *C. elegans* as a model for neurodegenerative diseases

C. elegans is a free-living nematode with a short life span of three weeks and short generation cycle of three days. Having a transparent body makes it an excellent model system for visualization of all types of cell lineages at various stages of development, especially the neuronal cells. Optical techniques are widely used to detect the neuronal death and protein inclusions in *C. elegans*. This tiny nematode has a simple nervous system comprising of 302 neurons with unique structure and position with reproducibility from animal to animal (Brenner, 1974; White *et al.*, 1986). Approximately 42% of human disease related genes are having an ortholog in *C. elegans* (Shaye and Greenwald, 2011). In addition, the neuronal gap junctions and synaptic connections between neurons are reproducible and major neurotransmitter systems are conserved among vertebrate and invertebrate (Markaki and Tavernarakis, 2010). Most attractive characteristics of *C. elegans* that contribute to the appeal for neuroscientists is the suitability of experimental approaches that are otherwise not possible in mammalian model systems. For example, forward genetics

can be done using chemical mutagenesis and reverse genetic analysis can be done by means of RNA interference (RNAi) (Gao *et al.*, 2018). For these reasons *C. elegans* has emerged as powerful in vivo model system for studying the neurodegenerative pathological mechanisms (Brenner, 1974; White *et al.*, 1986).

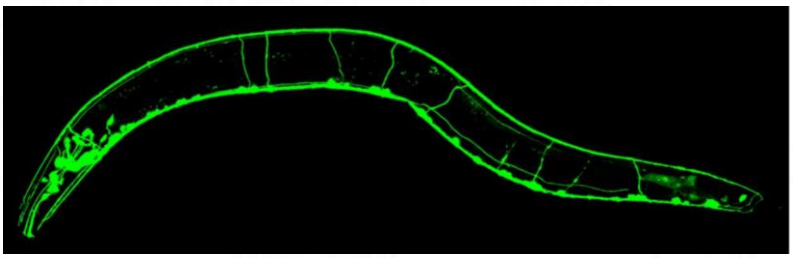

Figure 13: All *C. elegans* neurons are labelled with Green Fluorescent Protein (GFP). The nervous system is very complex organ in *C. elegans*. Out of 959 cells in adult worm 302, almost a third of all the, cells are neurons. These neurons are located inside the pharynx, various ganglia in the head and tail and also along the ventral cord. Fluorescent image showing GFP reporter (sto-6::gfp) labelled on nervous system. Figure from Corsi *et al*. 2015 and reprinted with permission from the Genetics © 2015.

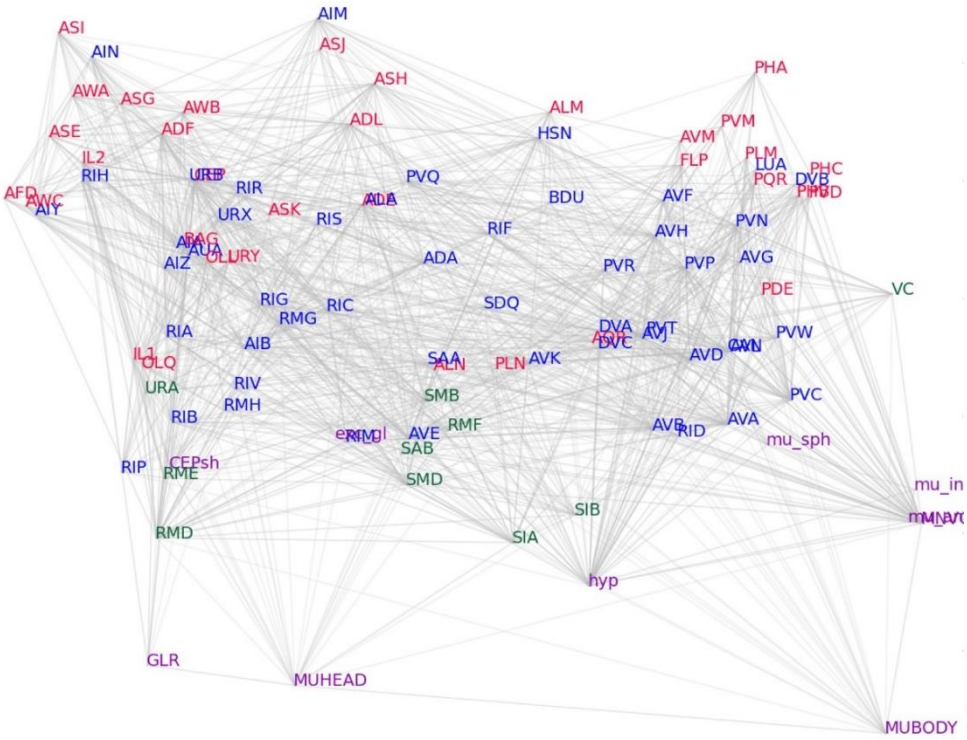

Figure 14: Whole neuronal connectome of *C. elegans* nervous system. Wiring diagram of *C. elegans* is a network of identified neurons labelled by fluorescent markers. This neuronal network is connected through chemical or electrical synapses. In this figure red depicts sensory neurons; blue depicts interneurons and green denotes motorneurons. Figure from Cook *et al*. 2019 and reprinted with permission from the *Springer Nature Limited* © 2019.

Human Disease	Human Genes	*C. elegans* genes
Huntington Disease	*Huntingtin (Htt)*	*n/a*
Alzheimer's Disease	*Amyloid precursor protein (APP)*	*apl-1*
Alzheimer's Disease	*Beta secretase (BACE1)*	*n/a*
Alzheimer's Disease	*Presenilin-1&2 (PS1&PS2)*	*sel-12, hop-1, spe-4*
Alzheimer's Disease	*Microtubule Associated tau (MAPT)*	*ptl-1*
Parkinson's Disease	*PARK1*	*n/a*
Parkinson's Disease	*PARK2*	*pdr-1*
Parkinson's Disease	*PARK5*	*ubh-1*
Parkinson's Disease	*PARK6*	*pink-1*
Parkinson's Disease	*PARK7*	*djr-1.1 &djr-1.2*
Parkinson's Disease	*PARK8*	*lrk-1*
Parkinson's Disease	*PARK9*	*catp-6*
Amyotrophic lateral sclerosis	*SOD-1*	*sod-1*
Spinal Muscular Atrophy	*SMN1/SMN2*	*smn-1*

Table 1: The *C. elegans* ortholog genes associated with human neurodegenerative diseases. Different genes associated to human neurodegenerative diseases are listed along with their *C. elegans* orthologs**.** Table from Dimitriadi and Hart, 2010 and modified with permission from the *Springer Nature Limited* © 2010

Polyglutamine Repeat Diseases

Pathological expansion of the CAG trinucleotide repeats or extension in the coding region of unrelated genes is the major cause of nine neurodegenerative diseases, including both HD and spinocerebellar ataxia (SCA) (Bauer and Nukina, 2009). Each of these neurodegenerative diseases show very distinctive patterns of neuronal degeneration but have an overlapping pattern in humans. The major features of these

diseases include progressive degeneration of neurons, late onset, reduction of life span, impaired motor function, formation of protein aggregates and inclusion bodies throughout the central nervous system (Zoghbi and Orr, 2000; Ross and Shoulson, 2009). HD is the most frequent polyglutamine repeat disease and it is an autosomal dominant disorder featured by a high number of glutamine (polyQ) in the N-terminus of huntingtin protein (Htt) (Caterino *et al.*, 2018).

Even though *C. elegans* is widely used for modelling several aspects of polyglutamine cytotoxicity, it does not contain an Htt ortholog. Expression of human huntingtin exon-1 fragments of various polyQ lengths under osm-10 promoter of *C. elegans* have shown defective cellular uptake at the sensory neuron endings (Faber *et al.*, 1999). The Htt exon-1 expressed nematodes were used to identify polyQ enhancer (pqe-1) gene through genetic screen of enhanced polyQ neurotoxicity (Faber *et al.*, 1999). Cytoplasmic aggregates were evident in the sensory and axonal process of Htt exon-1 expressed neurons. The expression of polyglutamine proteins in the body wall muscle cells showed a significant association with protein aggregates and loss of mobility in *C. elegans* (Garcia *et al.*, 2007).

a. Alzheimer's Disease

Main characteristics of AD is the presence of senile plaques (SPs) and neurofibrillary tangles in affected individuals. The major components of the SPs are the beta amyloid peptide and these peptides are derived from beta amyloid precursor proteins (APP) by the action of beta and gamma secretase (Buoso *et al.*, 2010). This is known as amyloidogenic pathway. One of the major reasons for familial forms of AD pathogenesis are the mutations in APP or Presenilin-1/2 (Kamino *et al.*, 1996). *C. elegans* has APL-1, an APP related gene. However, the APL-1 protein lacks amyloid

beta peptides (Hornsten *et al.*, 2007). Worms with modified genomes expressing neuronal specific Amyloid Beta (Aβ) have been developed (McColl *et al.*, 2009). It has been found that insulin or insulin growth factor (IGF-1) have a role in amyloid beta toxicity (Hornsten *et al.*, 2007; Kitiyanant *et al.*, 2012). Temperature dependent AD models in *C. elegans* have been used to study the gene expression changes upon amyloid beta induction (Cohen *et al.*, 2006; Hassan *et al.*, 2009). Transgenic worm selectively expresses human $A\beta_{42}$ in body wall muscles during temperature upshift was used to study the Aβ accumulation. Lower temperatures (16ºC) were unable to induce Aβ protein however rise in temperature (25ºC) were shown to increase the expression of Aβ has severely affected behaviour (Hassan *et al.*, 2009).

b. Parkinson's Disease

PD is mainly characterized by the progressive neurodegeneration of dopamine neurons due to accumulation of protein inclusions called Lewy bodies (Lang and Lozano, 1998). The main component of the inclusion body is alpha synuclein, which is normally found in nucleus and presynaptic terminals (Goedert, 2001). Mutations or the multiplication (duplications or triplications) of PARK-1 locus that encodes alpha synuclein is the major cause of familial PD (Gandhi and Wood, 2005). In the human genome there are eight PARK gene loci. Out of these eight PARK genes, six PARK orthologs were characterized in *C. elegans* (Harrington *et al.*, 2010). However, *C. elegans* lacks alpha synuclein ortholog (Harrington *et al.*, 2010). *C. elegans* PD models expressing human WT and mutant (A53T) alpha synuclein have been generated to study the role of alpha synuclein in neurodegeneration (Vartiainen *et al.*, 2006). The worms expressing wild type or A53T mutant alpha synuclein under dat-1 promoter leads to significant degeneration of dopamine neurons CEPs, ADEs and

PDEs (Lakso *et al.*, 2004). Worms also found accumulation of alpha synuclein in the cell bodies and neurites of dopaminergic neurons (Vartiainen *et al.*, 2006). Mutations in the leucine rich repeat kinase-2 (LRRK2) gene found to cause autosomal dominant familial PD in humans (Smith *et al.*, 2005). Pan neuronal expression of WT or mutant LRRK2 using snb-1 promoter in worms resulted in substantial loss of dopaminergic neurons similar to PD patients (Saha *et al.*, 2009).

Chapter-1

Introduction

Learning is a process of acquiring memory (Kandel, Dudai and Mayford, 2014). One can define memory as the change in behaviour because of the experience gained during learning or observing an event (Troyer *et al*., 2008). Memories based on events and facts form a type of memory called “declarative memory,” which helps in developing consciousness (Gast, 2018). However, memories we create through learning are generally called “procedural memory”. Both declarative memory and procedural memory are formed through different pathways because patients with loss of declarative memory are found to have procedural memory intact (Quam *et al*., 2018).

The human brain is the most composite entity as we understand till now. The potential to carry out numerous functions is embedded in its complexity. The fundamental functions of the brain include thinking, feeling, perceiving, learning and memory, and behaviour, apart from the major regulatory functions of survival (Dolcos *et al*., 2020). Among them, the formation of memory is critical, without which our capabilities of functioning will be diminished to simple reflexes and stereotypic behaviours (Tyng *et al*., 2017). One can study the process of memory formation using strategies like the role of synapses and their regulatory mechanism in the connectome (Takemura *et al*., 2017).

The neuronal basis of learning and memory is not well understood, especially the role of various neurotransmitters and the connectomes involved. However, the neurotransmitter DA has been shown to play a critical role in both motor and motivational functions. DA antagonists-based studies reported that malfunctioning of the DA system would not initiate reward-oriented behaviours (Volkow *et al*., 2007). A

reward is known as positive reinforcement for the formation of appetitive learning. In mammals, the motivational effectiveness of a particular reward stimulus requires phasic DA release to elevate the response towards it (Wise, 2004). In insects, the neurotransmitter octopamine is involved in reward-based learning, whereas a small population of DA neurons is involved in aversive learning (Schwaerzel *et al*., 2003). Recent evidence has shown the involvement of mushroom bodies in appetitive odour memory formation (Mao and Davis, 2009).

Dopaminergic signalling has been studied in Caenorhabditis elegans. However, the exact role of DA neurons in conditional learning is still unclear. There are limitations in mammalian models to study the role of DA neurons in adaptive learning and memory because of the complexity of the brain. *C. elegans* is an alternate model to study the role of DA neurons in the memory pathway. There are eight DA neurons in the worm. These developmentally distinct dopaminergic neurons are subdivided into three classes based on the morphology: CEP neurons, ADE neurons, and PDE neurons (Sawin, Ranganathan and Horvitz, 2000). In the worm, these developmentally distinct dopaminergic neurons are mapped (Sawin *et al*., 2000). The worms have been shown to have the ability to learn and can form both associative and non-associative memories of both short-term and long-term lasting >24 hours (Kauffman 2010). Reward-based olfactory adaptive learning is found to be highly predictive in these nematodes (Kauffman 2010).

In *C. elegans*, habituation to tap learning was affected when dopaminergic signalling was disrupted. It was found that the functional properties of touch response mediate through the D1 receptor class dop-1 (Bettinger and McIntire, 2004; Kindt *et al*., 2007). Odorant habituation studies in *C. elegans* have shown that DA is essential in making state dependant learning (Bettinger and McIntire, 2004). In *C. elegans*, behavioural

alterations such as ethanol preference depend on dopaminergic and serotoninergic signalling (Lee *et al*. 2009).

However, the role of dopamine in olfactory adaptive learning and memory in nematodes is not well understood. In this study, the results suggest that dopamine and dopaminergic neurons play a significant role in olfactory learning and memory in *C. elegans*.

Materials and Methods

Caenorhabditis elegans Strains

All strains, unless otherwise mentioned, were provided by the Caenorhabditis Genetic Centre (CGC, Minnesota, St. Paul). The following strains were used in this study: The WT strain N2 (Bristol); JC2209 [*olrn-1(ut305) X*]; DCR744 [*cima-1(wy84) IV; wyIs45 X*]; CB1112 [*cat-2(e112)II*]; MT15434 [*tph-1(mg280) II*]; VM487 [*nmr-1(ak4) II*]; LX636 [*dop-1(vs101)*]; LX703 [*dop-3(vs106)*]; LX705 [*dop-1(vs100) dop-3(vs106) X*]; LX734 [*dop-2(vs105) V; dop-1(vs100) dop-3(vs106) X*]; AT7437 [*dat-1::GCaMP-6::mCherry*]. The following strain was a gift from Dr. Andres Villu Marique, University of UTAH Dat-1::ICE [*dat-1::GFP; dat-1::ICE*]. UA44 [*bal11::Pdat-1α-syn::Pdat-1gfp*] was gifted by Randy Blakely, Florida Atlantic University, USA.

Strain	**Genotype**
WT	N2 (Variant. Bristol)
JC2209	[*olrn-1(ut305) X*]
DCR744	[*cima-1(wy84) IV; wyIs45 X*]
CB1112	[*cat-2(e112)II*]
MT15434	[*tph-1(mg280) II*]
VM487	[*nmr-1(ak4) II*]
LX636	[*dop-1(vs101)*]
LX703	[*dop-3(vs106)*]
LX705	[*dop-1(vs100) dop-3(vs106) X*]
LX734	[*dop-2(vs105)V;dop-1(vs100);dop-3(vs106) X*]
AT7437	[*dat-1::GCaMP-6::mCherry*]

Dat-1::ICE	[*dat-1::GFP; dat-1::ICE*]
UA44	[*bal11::Pdat-1α-syn::Pdat-1gfp*]

C. elegans maintenance

Nematodes were cultured in petri dishes having Nematode Growth Medium (NGM) containing OP-50, an *Escherichia coli* (*E.coli*) strain, as a food source. All plates were maintained at 20^0C as previously described (Brenner, 1974). Well-fed young adult worms were (60 hours) used in all of the experiment, unless otherwise mentioned.

Synchronization of C. elegans

Age-synchronized eggs were obtained by isolating embryos from gravid hermaphrodites by hypochlorite treatment according to the standard methods (Stiernagle, 2006). A bleach solution was prepared (20% NaOCl, 1 M NaOH) and gravid adult worms were treated with this. After treatment with this solution for less than 10 min, reaction stopped when worm debris were not visible under naked eye. This solution was washed off for three times following centrifugation at 8000rpm and stored overnight in M9 buffer at 20°C, to hatch all animals into L1 stage. The L1 larvae were transferred to NGM (Nematode Growth Medium) plates previously seeded with OP50 (OD_{600}=0.4) and stored at 20°C for about 48 hr until the worms reach the young adult stage.

Short term Adaptive Memory Training

The short term adaptive training was done as per the protocol mentioned in the paper by Kauffman *et al.*, 2011 with minor modifications. I used Butanone (Himedia, product code-AS053) as conditional stimulus and liquid OP-50 (OD_{600}=0.4-0.6) was used as an unconditional stimulus. Prior to the conditioning step, well-fed young adult worms (day-1) were washed off from NGM media in a centrifuge tube and washed three times with M9 buffer to remove bacterial debris adhered to the worm. Worms were then kept for 1 hr starvation period in the centrifuge tube with an excess of M9 buffer at 20°C. After the starvation period, the worms were transferred to an NGM plate and excess buffer was wiped off with a kimwipe. Immediately, added 400 ul of OP-50 to the plate with worms and 5ul of 10% butanone (prepared with 90% ethanol) was added to the lid and closed the plate. The plate was then sealed with Parafilm and left for 1 hr conditioning period at 20°C (Figure-A).

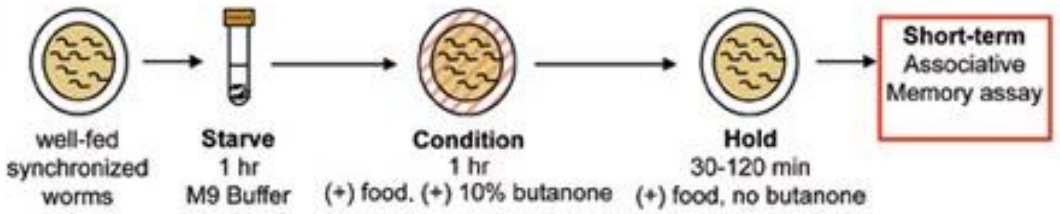

Figure A: Short-term olfactory adaptive training paradigm. Day-1 adult worms were collected from a well-fed plate and then proceeded to starvation followed by conditioning with butanone. Thereafter, the worms were transferred to a food plate until the assay stops. Image was modified from (Kauffman *et al.*, 2011).

Long term Adaptive Memory Training

The protocol for long term adaptive memory formation is similar to the method of Short-term Adaptive Memory training. The only difference is that after the first set of

1 hr starvation and conditioning, the starvation and conditioning steps were given alternatively for 30 min and the cycle was repeated 7 more times (Figure-B) (Kauffman *et al.*, 2011).

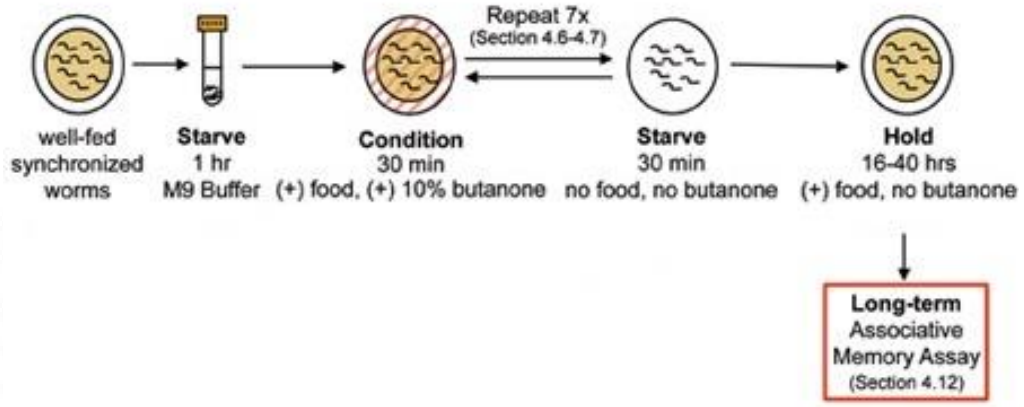

Figure B: Long-term olfactory adaptive paradigm. Similar to the short-term training, the day-1 adult worms were collected and proceeded with starvation and conditioning steps for 7 repeated trials. After the 7th trial the worms were transferred to a food plate until the assay stops. Image was modified from (Kauffman *et al.*, 2011).

Chemotaxis Assay

Olfactory conditioning was assessed as described with some modifications (Kauffman *et al.*, 2011) . Naive and conditioned worms (either short-term or long-term adaptive memory trained) washed off from hold plates were spotted on the center area marked D as shown in the figure 3 on a 10 cm chemotaxis. Excess buffer was wiped off with a kimwipe. 3 ul of butanone was spotted in the test area marked as A and 3 ul of ethanol was spotted in the control area marked as B. Immediately, the lids were closed and plates were kept at 20°C for 20 min. After 20 min, the animals were counted and a chemotaxis index was calculated as described. Chemotaxis Index (C.I.) = (The number of worms at test area − The number of worms at control area)/

Total number of worms (Bargmann, Hartwieg and Robert Horvitz, 1993). The same steps will then continue for assessing CI value for different time points (Figure-C).

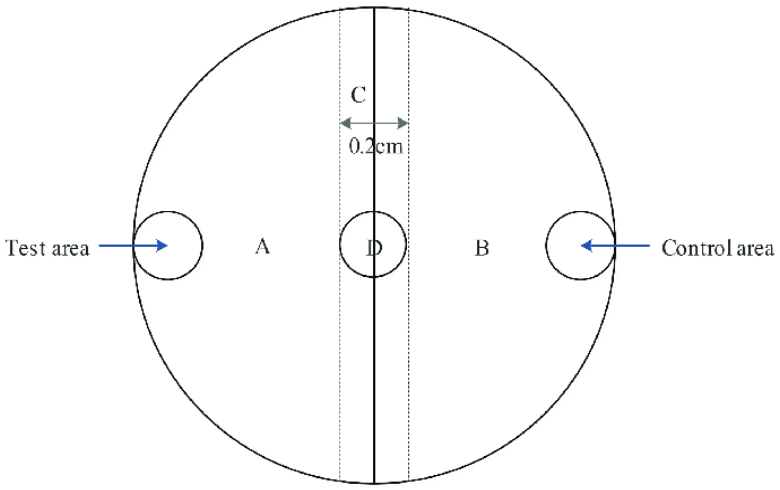

Figure C: Chemotaxis assay plate. The worms are placed on the centre area represented as D. Butanone is dropped on the test area and ethanol is dropped on the control area. Chemotaxis index was calculated based on the number of worms reaching the test area, control area and total number of worms; counted only the worms moved outside the D circle. Image was adopted from (Zhai *et al.*, 2018).

Maze Assay

I performed maze assay to observe the chemotaxis movement of DAT-1::ICE modified from Brockie *et al*. (Brockie *et al.*, 2001). Briefly, isoamyl alcohol (IA) was used as an attractant to find out the chemotaxis movement in both NGM and maze. Mazes were created by painting three times with 15 ul of 200 mM $CuSO_4$ (in d. H_2O) using a paint brush (hair length 0.8) as shown in the figure. A closed maze was developed to prove that $CuSO_4$ acts as a barrier for worm movement. Plates were then allowed to dry at room temperature for 5 min. After drying the plates, nearly 100-200 worms were placed at one end (shown in the figure-D) and 10 ul of IA (1:300

dilution) placed on the other end. The lids were kept closed and the worms were allowed to move freely for 2 hr at room temperature. After 2 hr, the maze index was calculated using the formula: M.I.= (The number of worms at attractant spot − The number of worms at origin)/ Total number of worms.

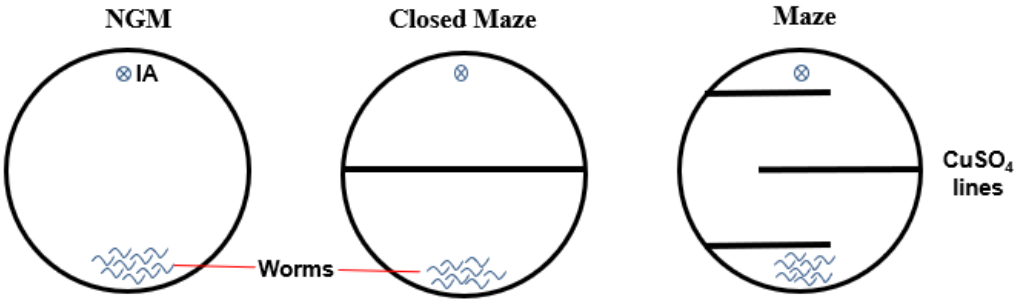

Figure D: Maze assay to assess the migratory behaviour. NGM was used to assess the percentage of WT worms attracted to a given attractant (IA). Closed maze was used to assess the ability of $CuSO_4$ to block the migration of worms. Maze was created by painting $CuSO_4$ on NGM plate to assess the percentage of worms that are able to solve the maze to reach IA by proper runs and turns. In all experimental conditions worms and IA were placed on spots as shown in the figure.

Transgenic strains

In order to study dopaminergic neuronal activity, I created a strain that carries GCaMP-6 under the control of dat-1 promoter which ensures the expression specifically on dopaminergic neurons. The final plasmid was constructed by modification of the plasmid pJH3644 [*Pflp-14::GCaMP6::Cherry] which was gifted by Dr. Mei Zhen, University of Toronto*. Pflp-14 promoter was removed by double digestion with MSc-1 and Pst-1 restriction enzymes. A 1357 bp region comprises dat-1 promoter from the pTH5 (Gift from Dr. Andres Villu Marique, University of UTAH) was amplified. The large fragment from double digestion of pJH3644 was Gibson assembled with the dat-

1 promoter amplified using Gibson primers from pTH5 plasmid construct (see annexure for more details vector creation). The resultant plasmid *pdat-1::GCaMP-6* (*Pdat-1::GCaMP-6::mCherry*) having Dat-1promoter in frame with GCaMP-6 ensures its efficient expression in dopaminergic neurons. Transgenic strains was generated using microinjection method (Mello *et al.*, 1991; Maricq *et al.*, 1995). The transgenic lines having GCaMP-6 under Dat-1 promoter sequence were created by injecting plasmid concentration of 70ng/ul of *pdat-1::GCaMP-6* (Sequence and details attached annexure) to the distal arm of the gonads of young adult worms. The transgenic strains were maintained which ultimately transferred the gene to the next generation extrachromosomally (Mello *et al.*, 1991). The transgenic lines F2 carrying RFP and GFP on dopaminergic neurons were selected and propagated. This strain was named as AT7437 [*dat-1::GCaMP-6::mCherry*]. Cloning details and plasmid sequences are available upon request.

Calcium imaging

The strain AT7437 [*dat-1::GCaMP-6::mCherry*] was placed on a drop of 5% sodium alginate solution to immobilize the animal in a glass slide for imaging. Immediately after the transfer of worms to sodium alginate, 100 ul of 100 mM $CaCl_2$ solution were added to the top of it to make a scaffold. Sodium alginate creates a matrix around the worm allowing only the slightest movement possible which does not interfere with imaging under high magnification. The olfactory stimulus butanone was given by using a small piece of Whatman® filter papers as a flag. Microinjection equipment was used to place the flag containing 10 ul of 10% butanone near the worm, and retracted after 10 second. Leica Microscope Model DMi8 (Leica Microsystems. Wetzlar &

Mannheim, Germany) and objective lens 20X/0.40 (∞/O/C N PLAN) was used to collect the calcium imaging data. Time lapse images were taken at a rate of 2 frames/ second for 2 min.

siRNA mediated silencing

siRNA mediated silencing of ICE were essentially performed using an already established protocol (Ashe *et al.*, 2012). Briefly, siRNA mediated silencing of gene was done by feeding RNAse III resistant *E.coli* HT115 (DE3) expressing double stranded RNA (dsRNA) against a portion of ICE mRNA. The sequence chosen for RNAi has no homology for GFP or any other *C. elegans* genes. A 489 bp of ICE gene, amplified from genomic DNA of strain DAT-1::ICE, was sub-cloned into the plasmid pL4440 having two opposable T7 polymerase promoter sites and was used for in-vivo transcription. Besides, this promoter provides IPTG inducible expression of the phage T7 RNA polymerase (Conte *et al.*, 2015). L1 larvae of DAT-1::ICE strains were plated on to RNAi plates seeded with either empty vector (pL4440) or dsRNA expressing HT115 bacteria and allowed to reach day-1 adult stage. Day-1 adults were observed for gain of fluorescence on dopaminergic neurons under a fluorescence microscopy and used for olfactory adaptive learning assays. Cloning details and plasmid sequences are available upon request (See Annexure for more details).

Tetramisole sensitivity assay

A modified protocol has been used to assess the cholinergic receptor activity (Jospin *et al.*, 2009). I used tetramisole, a racemic mixture and less active form of levamisole, in this assay (Lewis *et al.*, 1980). Day-1 and day-3 old adult hermaphrodites of WT

and DAT-1::ICE were taken from well-fed plates. Worms were then washed three times with M9 buffer to remove OP-50 bacteria, which might otherwise interfere with the experiments. Thereafter, worms were placed on plates containing the drug at concentrations of 0.1mM and 0.5mM (prepared by adding desired volumes of tetramisole into molten agar having a temperature of 50^0C after which the plates were inverted and dried overnight) and the effects on animal movement were observed at 5 min intervals for up to 20min. Absence of body movements observed in response to poking on worms were scored as paralyzed. Percentage of paralyzed animals were plotted against time on X-axis.

Dopamine plate immobilization assay

Exogenous DA assays: A fresh stock solution of 1 M DA-HCl in d.H_2O was prepared each day. The stock solution was wrapped in aluminum foil and stored in a 4°C incubator to minimize oxidation of DA. To make DA-treated plates for worm incubation, 200 ul of the stock solution was added to each 10 cm NGM plate (2%) to make a final concentration of 10 mM DA in the plate. For mock-treatment plates, 200 ul of d.H_2O was added to NGM plates (2%). The solutions were spread onto the plates, and the plates were closed and loosely covered with aluminum foil to dry. Within 15 min, worms were washed off from food plate, washed three times as described above, and then added to the plates for the experimental condition. Assays were performed as described above, except that an aluminum foil was loosely placed over the assay plates for the duration of the assay to limit light exposure

Body Bend Assay

Body bend assays were performed as previously mentioned (Sawin, Ranganathan and Horvitz, 2000). Briefly, the body bend assay was done in the presence and absence of OP-50 and was named as On-food and Off-food respectively. On assay plates 5 animals were placed at the centre and kept for 5 min for acclimatization and allow worm to crawl to the circular OP-50 lawn. After 5 min, videos were recorded using a camera for each worm for 1 min and later body bends were counted manually for 20 sec intervals. For a detailed protocol, refer the protocol paper (Raj and Thekkuveettil,2018).

Avoidance assay

Avoidance assay was done as previously mentioned by Hilliard, Bargmann and Bazzicalupo, 2002. Briefly, a drop of repellent was delivered near the tail of worms on NGM plate with or without OP-50. 0.5 M glycerol stock (dissolved in d.H_2O) was used as repellent in this assay. To make the drop size smaller, an insulin syringe with needle finely polished with sandpaper was used. The drop touches the tail and through capillary action, reaches head amphid sensory organs. Once the chemical reaches the anterior sensory part of the moving worm it may take a reversal (a complete omega turn): which was considered as a positive response. During the assay, an acclimatization time of 20 min was given to worms after its transfer on NGM for both on and off- food experiments and plates were maintained at 20°C. After 20 min, the plate was observed under a stereo microscope and gently applied a drop of 0.5 M glycerol to the tail of the moving worm and its response were recorded as either positive (ones that take an omega turn) or negative (ones that move in the same

direction without any reversal) within 4 seconds. Avoidance index was calculated by dividing the number of positive responses by the total number of trials.

Microscopy

To view the nematodes for picking, transfer, washing etc. a stereo microscope (Magnus Analytics, India) with 10X zooming was used. For fluorescence imaging, Olympus IX51 inverted microscope (Olympus Imaging, Center Valley, PA, USA) that works with image acquisition software NIS Elements-Advanced Research (NIKON) and Rolera XR monochrome camera (QImaging, Canada) was used. Microinjection was done with the help of differential interference contrast DIC facility of Leica Microscope Model DMi8 (Leica Microsystems. Wetzlar & Mannheim, Germany). This microscope was also used for some of the fluorescence imaging as well as in calcium imaging.

Camera

Dino-Lite digital microscope model# AM4115T-GFBW with 5 Megapixels camera and 1.3 Megapixels sensor capable of shooting at 4 frames per second. Sony Alpha a7 III Model Mirrorless Digital Camera with Sony FE 90mm f/2.8 Macro G OSS Lens.

Software

Graphpad Prism version 6 (GraphPad Software Inc.) was used for graphical representation and statistical analysis of the data. The data are presented as Mean + SEM as indicated. Significance was represented as follows *$p<0.05$, **$p<0.01$,

***P<0.001, ****p<0.0001. Image analysis was carried out using Fiji (an open source image processing package based on ImageJ), which was also used for increasing the brightness or contrast of images along with Adobe Photoshop CS2 (Adobe systems, CA, USA). WormLab software from MBF Biosciences for imaging, tracking, and analyzing *C. elegans* movement.

Results

Short-term memory training creates learning and memory in wild-type

A short-term training paradigm was designed, which involves an hour of starvation followed by olfactory signal butanone (conditional stimulus) paired with food (unconditional stimulus) for the next one hour. *C. elegans* sense odour mainly through pairs of sensory neurons, namely AWA, AWB and AWC (Pereira and van der Kooy, 2012). Butanone is sensed through AWC neurons which then communicate with the AIY interneurons to create learning and memory circuits (Walker and Holden-Dye, 2014). Hence, the absence or functional defects of these neurons may result in learning deficiency.

To test whether the short-term adaptive memory training creates learning and memory in WT, I used chemotaxis assy. If a greater number of worms are attracted towards the conditional stimulus (butanone) present on the chemotaxis assay plate, learning was developed. If this behaviour is retained after couple of hours, calculated as chemotaxis index, then the memory formation also found to be good. I checked the learning and memory in WT after the training and found that there is significant learning at 0^{th} hour and memory retention was observed at 1^{st} hour and 2^{nd} hour compared to the naïve worms. I then wanted to test the role of dopaminergic neurons in this reward-associated olfactory learning pathway.

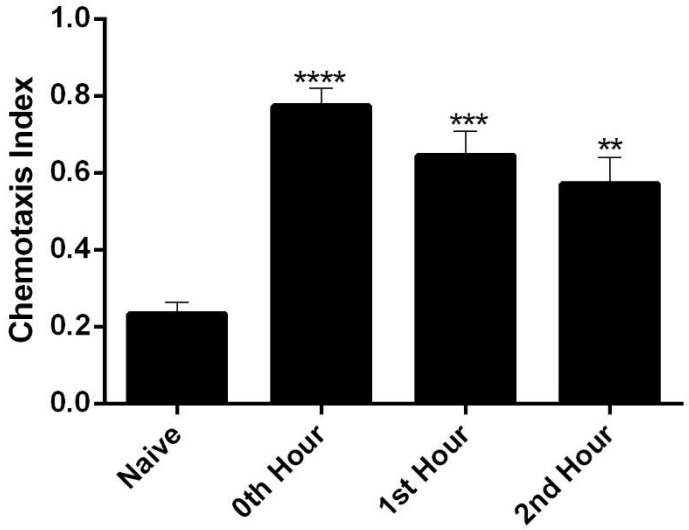

Figure 1: Short term adaptive memory formation in wild-type (WT). Olfactory adaptive training to butanone (conditional stimulus, CS) in presence of OP-50 (unconditional stimulus, UC) significantly increased the attraction towards butanone at the 0^{th} hour in WT ($p<0.0001$) along with 1^{st} hr and 2^{nd} hr memory recalling ($p<0.001$ and $p<0.005$ respectively) compared with the naïve. n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data represented as the mean + S.E.M. Significance indicated: ns- non-significant, * $p< 0.05$, ** $p< 0.005$, ***$p<0.001$ and ****$p<0.0001$.

Dopamine deficient Tyrosine Hydroxylase mutant shows aberrant short-term adaptive memory formation

DA synthesis in *C. elegans* involves an enzyme, Tyrosine hydroxylase (cat-2), and mutants deficient in this enzyme cannot synthesize DA (Sawin *et al* 2000). cat-2 gene is the orthologue of human tyrosine hydroxylase in *C. elegans*. Following the short-term memory training, cat-2 mutant worms significantly reduced adaptive memory formation at all-time intervals compared to the WT worms (Fig 2). It implies that DA plays an essential role in adaptive memory formation. Interestingly, lack of complete

impairment of learning and memory formation in these strains suggests that the DA neurotransmitter is not a prime factor rendering adaptive memory in *C. elegans*.

It has been noted that strain tph-1 mutant (orthologue of human tryptophan hydroxylase 1, provides tryptophan 5-monooxygenase activity- rate-limiting enzyme in the biosynthesis of serotonin), deficient in serotonin synthesis, had learning and memory formation after short term adaptive training, similar to that of WT. However, serotonin is involved in the memory formation of stress-induced vigilance behaviours in the pond snail, Lymnia (Il-Han, Janes, and Lukowiak, 2010). Hence, it is difficult to predict whether learning behaviour observed in cat-2 deficient strain is a partially compensated effect of serotonin. However, lack of DA does hamper the memory storage pathway to a large extend – significantly at the 2nd hour period (Fig 2).

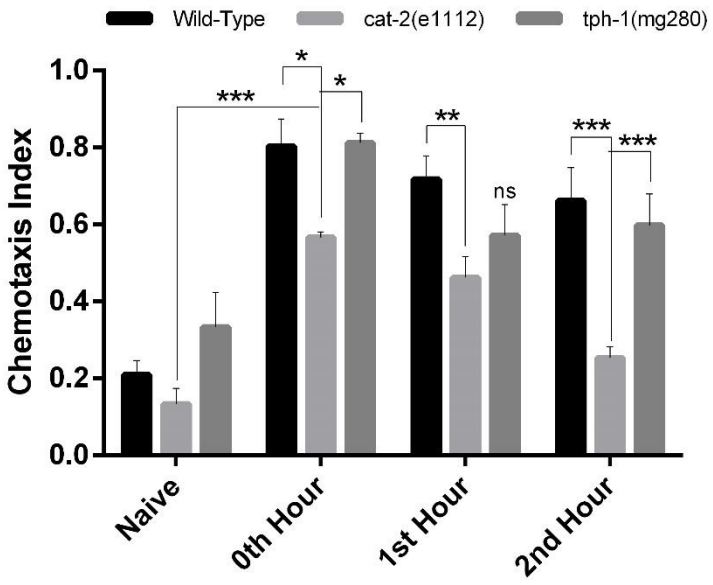

Figure 2: Absence of dopamine neurotransmitter affects short term adaptive memory. Strain deficient in *cat-2* gene (code for tyrosine hydroxylase, a critical enzyme for DA synthesis) show significant learning deficiency in comparison with the WT and strain deficient in *tph-1* (code for tryptophan hydroxylase, a critical enzyme required for serotonin synthesis) at 0^{th} hr ($p<0.05$ for both). *cat-2* mutant also shows significant reduction in memory recall at 1^{st} hr and 2^{nd} hr compared with WT ($p<0.005$ and $p<0.001$ respectively). In addition, at 2^{nd} hr, *cat-2* mutant showed a significant reduction in memory recall compared to the *tph-1* mutant ($p<0.001$) which was not observed at the 1^{st} hr. However, *cat-2* mutant did show a significant increase in CI at 0^{th} hr compared to its own naïve ($p<0.001$). *tph-1* did not show any significant reduction or increase in CI compared to WT at any of the tested time points. n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, *p< 0.05, **p< 0.005 and ***p<0.001.

Dopamine neurons have significant role in short-term and Long-term Memory formation

The significant reduction of STAM in DA- deficient strains leads us to probe whether connectomes involving dopaminergic neurons are critical for the learning process. Dopaminergic neurons (CEP) make a series of both pre- and post-synaptic connections to RIB and RMD neurons, which in turn make several connections to AIY interneuron (Wang *et al*., 2014). An insight on the direct link between DA connectome and learning is of paramount importance because learning defects are observed in Parkinson's disease and Alzheimer's patients where extensive damage to dopaminergic neurons was noted (Germano and Kinsella, 2005; Olson, Lockhart and Lieberman, 2019; Wang and Qi, 2019). To understand the role of dopaminergic neurons in learning and memory, a strain in which the human caspase interleukin-1β converting enzyme (ICE) transgene is incorporated in the promoter of DA transporter (dat-1) was used (Hills *et al*., 2004). The enzyme is expressed along with the dat-1 transporter, and a higher level of this enzyme leads to progressive degeneration of

dopaminergic neurons. As a result, the mutant worm dat-1::ICE the CEP neurons develop normally in early larval stages. But at later larval to early adult stages, a significant amount of degeneration occurs to the neurons (Hills *et al*., 2004). This characteristic feature was exploited to study the role of DA neuron/circuit in learning and memory formation.

The chemotaxis index of short-term adaptive memory trained dat-1::ICE worms at the 0th hour was significantly reduced compared to the WT (Fig 3A), which suggests that the learning process is affected. Chemotaxis index at 1^{st} and 2^{nd} hour, which is also significantly less than that of the WT, points to the lack of memory recall due to loss of dopaminergic neurons. The results suggest that the dopaminergic connectome is essential for maintaining normal learning and memory recalling processes.

The consolidation theory states that long-term memory requires new protein synthesis, whereas short-term memory formation doesn't. Thus, the process of long-term memory formation involves transcription and translation to take place in order to support the long-lasting structural changes in synaptic connections (Zhao *et al*., 2019). In Caenorhabditis elegans, the long-term memory is formed by repeated trials (7 trials), lasts up to 24 hours; in contrast, the short-term memory is formed by single-trial training, which lasts only for a few hours. Since both the memory paradigms are through different biochemical pathways, I checked the long-term memory formation in the absence of dopaminergic neurons. The results showed that in the absence of dopaminergic neurons, impaired recalling of memory at 24^{th} hr and the initial learning at 0^{th} hr itself was disrupted (Fig 3B) compared to the WT.

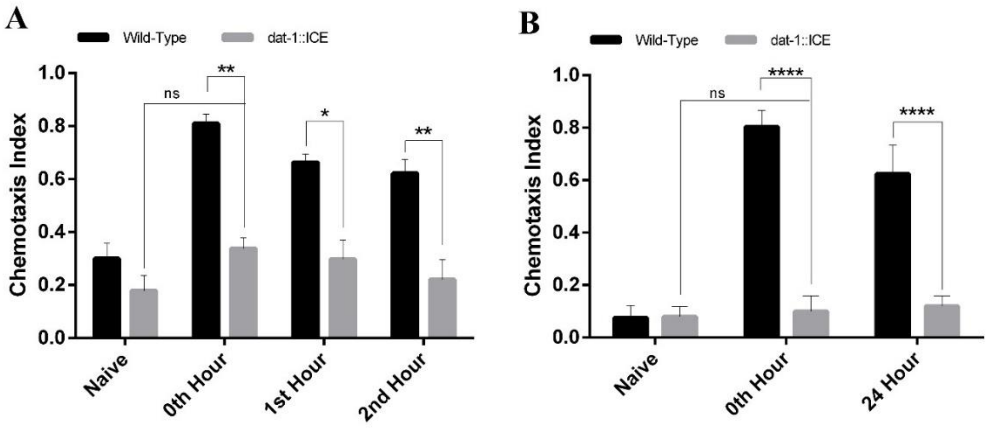

Figure 3: Dopamine neuronal absence affects short-term and long-term adaptive memory. A. dat-1::ICE strain show significant reduction in short-term adaptive learning at 0^{th} hr ($p<0.005$) and memory recalling at 1^{st} and 2^{nd} hr ($p<0.05$ and $p<0.005$ respectively) when compared with WT. dat-1::ICE worms failed to show significant increase in learning compared to its own naïve worms. **B.** dat-1::ICE worms show significant absence of long-term adaptive memory at 0^{th} hr and at 24^{th} hr after training ($p<0.0001$ for both) compared to the WT. dat-1::ICE worms did not show any significant change in learning compared to its own naïve worms. For both experiments n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, *p< 0.05, ** p< 0.005 and ****p<0.0001.

Dopamine controls the locomotory and avoidance behaviour *of C. elegans*

Locomotory and avoidance assays were conducted to understand how DA is important in controlling the behaviour of *C. elegans*. DA is one of the major neurotransmitters modulating the locomotory behaviour in the presence and absence of food (Sawin *et al*., 2000). Further upon validating the behaviour of the worm by using a standardized method of the locomotory assay (Sawin *et al*., 2000). I observed dat-1::ICE strains behaved similarly to WT worms by showing a characteristic slowing

response (50%) in the presence of food. This is mainly due to the surge of DA, whereas cat-2 mutant worms showed a significantly lesser degree (9%) of slowing response (Fig 4A). The deficit in the slowing response of cat-2 mutants was expected since this strain lacks DA (Sawin *et al*., 2000). It was interesting to note that while the lack of DA modified the worm's behaviour in the presence of food, the worms with the absence of CEP neurons showed behaviour similar to that of WT. It could be because the remnant DA neurons can induce a low surge of DA needed for the behaviour. However, the avoidance index of the WT worms to 0.5% glycerol (repellent chemical) (Hilliard *et al*., 2002) showed significantly lower avoidance (74%) in the absence of food (Fig 4B). Both cat-2 and dat-1::ICE worms did not show a significant difference (22% and 36%) in avoidance response on the on-food plate compared to their respective off-food assay plates (Fig 4B). In both cat-2 mutant and dat-1::ICE the avoidance index on the on-food plate was significantly reduced compared to the WT. The conflict of the result of body bends vs. avoidance index of dat1:ICE worms could be due to the level of DA requirement for each assay behaviour. Avoidance response possibly requires an increased surge of DA.

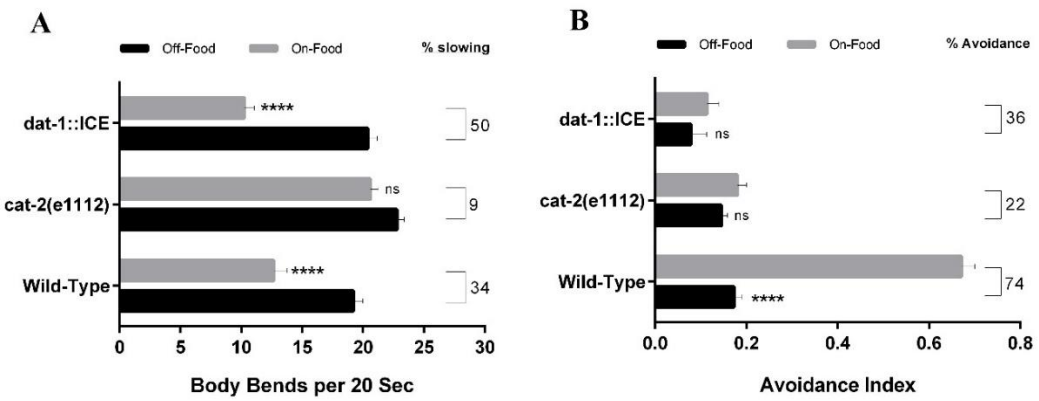

Figure 4: Effect of DA signalling **in locomotory and avoidance** behaviour**. A.** Well-fed worms were transferred to assay plates with or without OP-50, marked as on-food and off-food respectively. WT worms show a 34% of basal slowing response in on-food compared to off-food plate and the difference in body bends was found to be significant ($p<0.0001$). In *cat-2* mutant worm the basal slowing on-food was less compared to the *cat-2* off-food (9%) and with on-food of WT (25% difference) and the difference in off-food with on-food was insignificant ($p>0.05$). However, dat-1::ICE worms showed a significantly higher basal slowing response (50%) compared to the off-food and the difference is significant ($p<0.0001$). $n\geq15$ worms **B.** Freely moving well fed worms on assay plate were exposed to a drop of 0.5 M glycerol as repellent. Off-food behaviour of WT worms showed significant reduction in avoidance to the repellent compared to the on-food ($p<0.0001$). WT worms in off-food shows 74% decrease in avoidance response compared to on-food. Compared to the WT, *cat-2* mutant and dat-1::ICE worms showed a lesser avoidance (22% and 36% respectively) compared to the WT (52% and 38% difference respectively). However, both *cat-2* mutant and dat-1::ICE worms showed significant absence of avoidance towards repellent both in on-food and off-food plates ($p>0.05$). $n\geq5$ trials; each trial contains more than 25 worms; for both experiments statistical analysis was done by two-way ANOVA with Sidak's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, ****$p<0.0001$.

Learning deficiency in worms lacking dopamine neurons is not a cause of migratory issue

Parkinson's and Alzheimer's patients are most often presented with problems associated with movements. A migratory assay was designed to test the possibility of defects in movements manifesting themselves as the learning deficit in dat-1::ICE worms (Fig 5A). The worms must have proper runs and bends to reach the target spot and solve the open maze. Isoamyl alcohol (IA) was used as an attractant to understand the exact proportion of worms that could solve the maze. The WT N2 control animals have shown approximately 50% attraction towards IA (an attractant)

under normal chemotaxis conditions (Fig 5B). However, less than 10% of WT worms can cross the closed maze to reach the IA spot presented at the end of the maze, which indicates that worms cannot cross the CuSO4 barrier (Fig 5B).

The results showed that 50% of dat-1::ICE worms solved the maze similar to WT (Fig 5B). As a positive control, nmr-1 mutants (NMDA-type ionotropic glutamate receptor subunit absent) were used. This mutant lacked the normal migratory pattern to initiate proper runs and turns (Brockie *et al*., 2001) and could not solve the maze (Fig 5B). These results suggest that the learning deficiency observed in dat1:ICE worms is not due to the defect in migratory patterns.

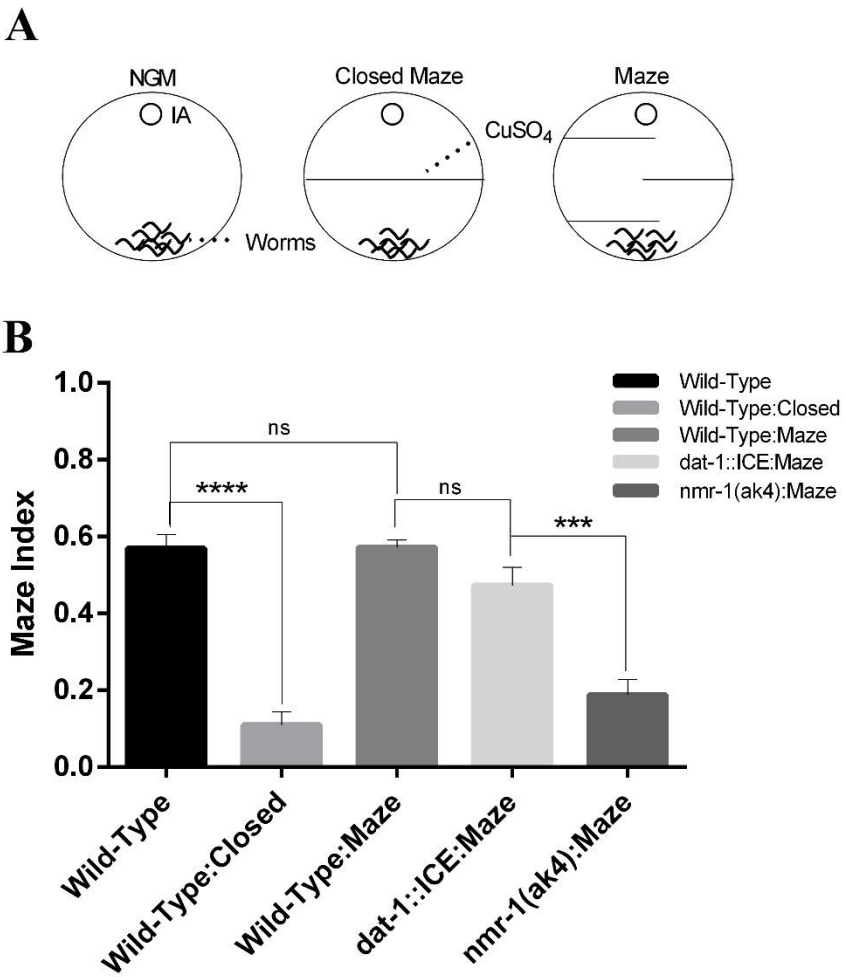

Figure 5: Dopamine neuronal absence does not affect chemotaxis of dat-1::ICE worms on a maze. **A.** $CuSO_4$ solution was scored on NGM plate for creating a maze. The percentage of animal reaching IA (an attractant) spot was calculated after 2 hr of assay time. **B.** Data show 60% of WT reached the IA spot in open maze created without any $CuSO_4$ barrier on NGM. However, only ≤10% of WT worms on closed maze with barrier reached the spot ($p<0.0001$ compared to the WT on open maze). WT worms on maze showed nearly 0.6 maze index (60% of worms) similar to WT on plates without $CuSO_4$. dat-1::ICE worms also showed that ≥50% reach IA spot by solving the maze compared to the WT ($p>0.05$). nmr-1 mutant worms, which act as positive control, showed significantly low percentage worms (<20%) could solve maze in comparison with WT and dat-1::ICE ($p<0.001$). For each experiments n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by one-way ANOVA with Tukey's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, ***$p< 0.001$ and ****$p<0.0001$.

The absence of Dopamine neurons in the worms failed to solve maze assay after short-term training

Maze assay was employed as an alternative learning paradigm, wherein WT and dat-1::ICE strain are trained for short-term adaptive learning in the presence of butanone. Trained WT worms were significantly attracted to the butanone spot at the end of the maze. The WT worms showed a significantly higher maze index after STAM to butanone compare to its own naive (Fig. 6). However, STAM trained dat-1::ICE worms did not show significant improvement in maze index compared to the naïve (Fig 6). These data suggest lack of dopaminergic neurons has an impact on the learning pathway.

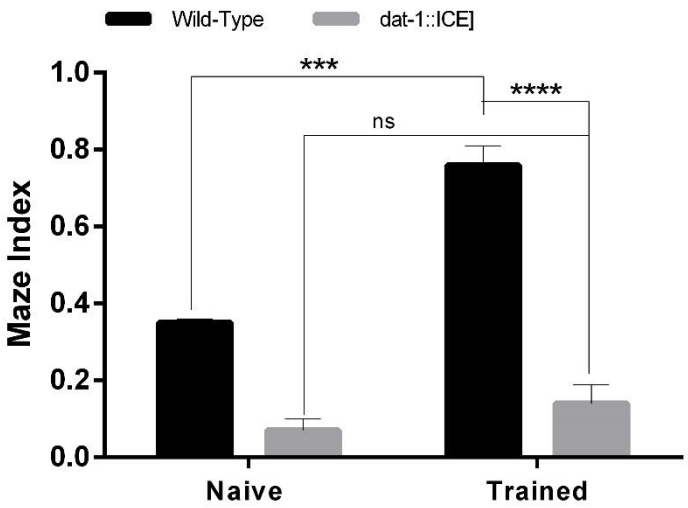

Figure 6: Olfactory training did not improve Maze clearance in dat-1::ICE worms. Short-term adaptive training of dat-1::ICE worms did not significantly increase chemotaxis towards butanone spot through the maze compared to trained WT ($p<0.0001$) and its dat-1::ICE naïve ($p>0.05$). WT worms showed a significantly higher attraction to butanone after the olfactory training compared to its WT naïve ($p<0.0001$). A similar pattern was not observed in dat-1:;ICE worms. n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, *** $p< 0.001$ and ****$p<0.0001$.

Mechanical stimulation of the dopamine neurons evokes learning in wild-type

Dopaminergic neurons are classified under mechanosensory neurons in *C. elegans* (Kindt *et al*., 2007). Upon activation, the dopaminergic neurons are triggered to secrete more DA within the worm, which plays a vital role in the olfactory learning archetype (Vidal-Gadea and Pierce-Shimomura, 2012). Hence, to understand this phenomenon, G-75 Sephadex beads were used to mock as a food source. When the worm hits the beads, its dopaminergic neurons get mechanically stimulated due to the property of mechano-sensation. Eventually, this will elicit a DA surge inside the worm (Kamkin and Kiseleva, 2007).

The worms were trained using G-75 beads instead of food showed significant learning compared to untrained worms (Fig 7). To further prove this effect is due to DA surge in the worms, the worms were trained in the presence of exogenous DA (10 mM) instead of food during the conditioning period. The results showed a similar learning pattern in the worms trained with food or G75 (Fig 7). These results confirm the role of mechanosensory dopaminergic neurons and associated DA release in olfactory associated learning.

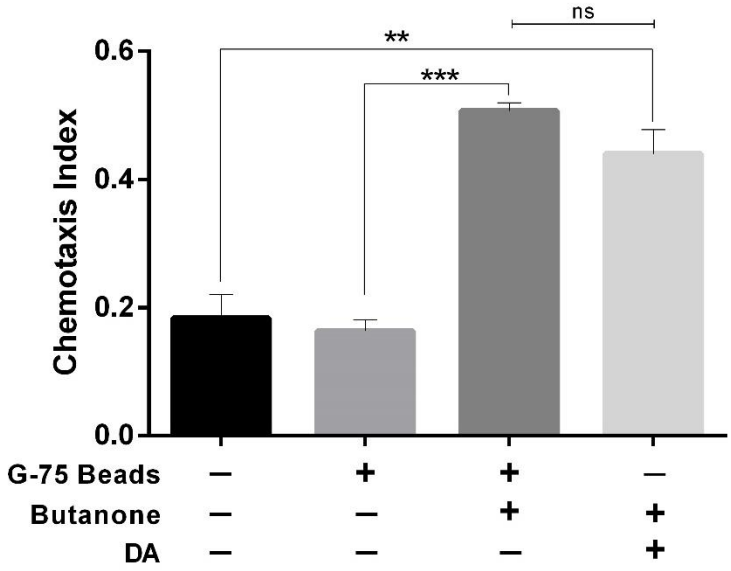

Figure 7: Effect of mechano-stimulation and exogenous dopamine on WT. Mechanosensory dopaminergic neurons were stimulated with G-75 sephadex beads on conditioning plate. Worms trained for short-term memory towards butanone in the presence of G-75 (30mg/ml) beads showed significant increase in learning compared with untrained worms ($p<0.001$) and with the naïve (black bar). Similarly, worms trained with butanone in the presence of exogenous DA (10mM) showed a significant learning compared to the naïve worms without DA ($p<0.005$). Both worms trained on G-75 beads and in presence of DA show similar learning ability ($p>0.05$). For each experiment, n≥3 trails; each trial contains more than 50 worms; statistical analysis was done by one-way ANOVA with Tukey's multiple comparison test. Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, p< **0.005 and p<***0.0001).

Calcium imaging shows dopamine neurons (CEP) respond to the trained olfactory stimulus

A strain expressing GCaMP-6 under dat-1 promoter was generated to confirm the involvement of dopaminergic neurons in the learning pathway. To develop the clone dat-1 promoter (4000 bp) was PCR amplified and cloned to plasmid pJH3644 (flp-14::GcaMP-6::*mCherry*), replacing flp14 using the Gibson assembly cloning method

(see Annexure). The resultant plasmid (pdat-1::GCaMP-6::*mCherry*) was microinjected into the N2 WT worms. The transgenic worms were selected by verifying GFP and RFP fluorescence in dopaminergic neurons (Fig 8A). The strain was named AT7437 (pdat-1::GCaMP-6::*mCherry*). After short-term olfactory adaptive training of AT7437 showed that in the presence of CS stimulus, the CEP neurons showed a delayed increase in fluorescence intensity associated with a transient increase in intracellular calcium influx compared to the naïve (Fig. 8B). After removing CS, the worms continued to show an increased dopaminergic neuronal fluorescence for some time before decreasing (Fig. 8B). These results based on in-vivo calcium transients showed that dopamine neurons are involved in learning and recalling after the short-term adaptive training.

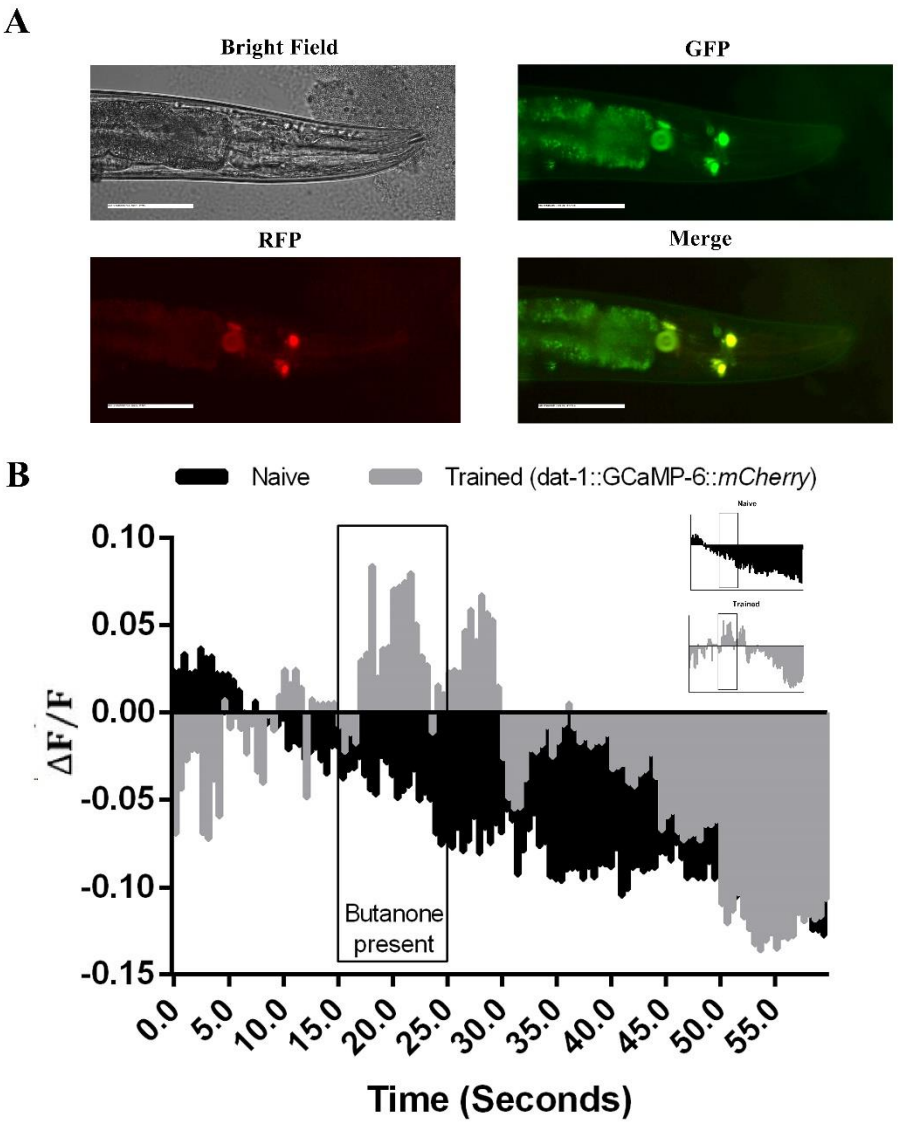

Figure 8: Olfactory training evoke calcium transients in DAergic neurons. A. The F2 transgenic progeny showing the expression of GFP and RFP on dopaminergic neurons. Fluorescence was observed in both CEP and ADE neurons **B.** Calcium imaging of AT7437 (*pdat-1::GCaMP6::mCherry*) shows calcium mediated activation of dopaminergic neurons, when butanone was presented (15th sec), compared with the naïve. This response to conditioned stimulus was continued for a short period of time even after the withdrawal of the stimulus (25th sec) and decreased with time. Untrained naïve worms showed a decrease in response to the CS butanone. Inset, individual representation of naive and trained worms. n≥3 trials; data are represented as the mean and each trial contains more than 5 animals. Scale bar represent 100µM.

siRNA mediated silencing of interleukin beta convertase rescued learning and memory defect in dat-1::ICE worms

siRNA mediated silencing of interleukin beta convertase was done to prevent the DA neurodegeneration in dat-1::ICE strain. dat-1::ICE::RNAi fed worms showed a regain of ADE and CEP dopaminergic neurons compared to empty vector fed control (Fig 9A and 9B). Olfactory learning of dat-1::ICE::RNAi fed worms recovered significant learning at 0th hr and memory recalling at 1st and 2nd hours respectively compared to the empty vector alone fed control (Fig 9C). Dopaminergic neuronal connectome hence is essential in an olfactory associated learning paradigm.

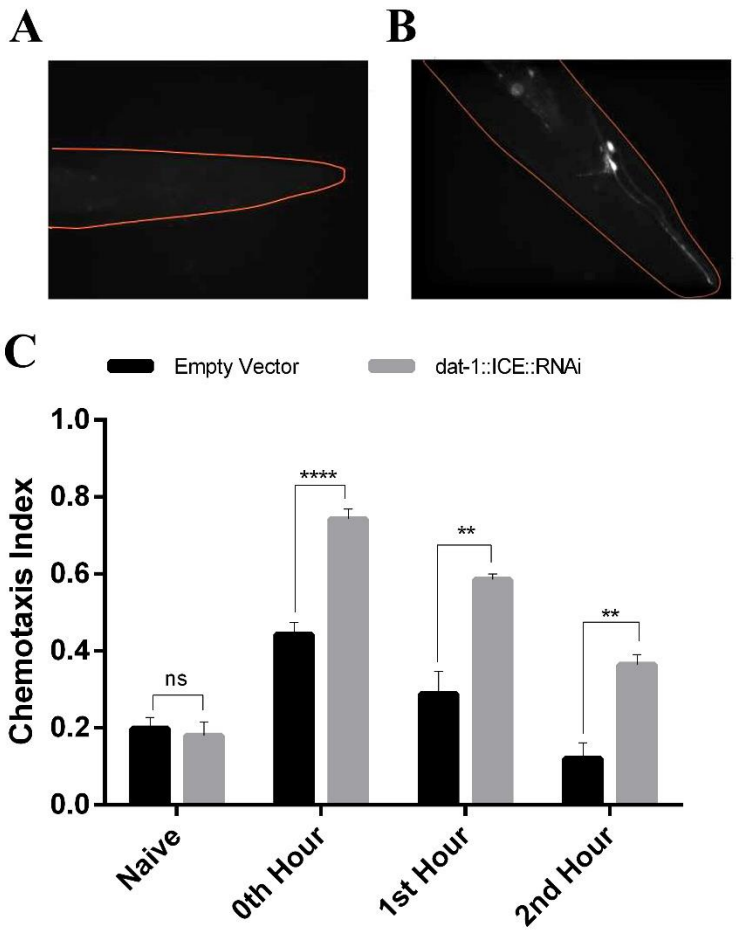

Figure 9: siRNA mediated silencing of ICE in dat-1::ICE worms prevented dopaminergic neurodegeneration and recover/regain learning and memory. A. Empty vector fed worms did not prevent neurodegeneration as observed by the absence of GFP in DAergic neurons. **B.** Recovery of dopaminergic neurons was noted by the presence of GFP in dat-1::ICE::RNAi fed worms. **C.** Short-term adaptive memory training on dat-1::ICE::RNAi fed worms showed significant increase in learning at 0^{th} hr ($p<0.0001$) as well as memory recall at 1^{st} hr and 2^{nd} hr ($p<0.005$) compared with empty vector fed worms. n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Sidak's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, ** $p< 0.005$ and ****$p<0.0001$.

Dopamine neurotransmitter is needed during the early associative phase of learning whereas dopaminergic neurons are essential for memory recall

As discussed earlier, mechanical stimulation and DA presentation during learning have elicited early associated learning in worms. To probe further into the role of the DA pathway in learning and memory paradigms, exogenous DA (10mM) was added during starvation and conditioning steps (along with food) of the training protocol (Ezcurra, Marina *et al*, 2011). A significant improvement in memory retention was observed in WT worms, unlike its untreated control (Fig 10A). cat-2 mutant worms also showed significant improvement in learning and memory retention compared to the non-treated controls (Fig 10B). In dat-1::ICE strains, the addition of exogenous DA (10mM) resulted in a highly significant in learning at 0th hr but no improvement in memory recalling (Fig 10C). To understand which stage of the olfactory training paradigm, DA signalling become critical exogenous DA (10mM) was presented to dat-1::ICE strains either during conditioning or recalling steps of the experiment. The worms exhibited significant learning only when exogenous DA was introduced during

the conditioning step and not during the recalling phase of the experiment (Fig 10D). This result validates the essential function of DA during conditioning.

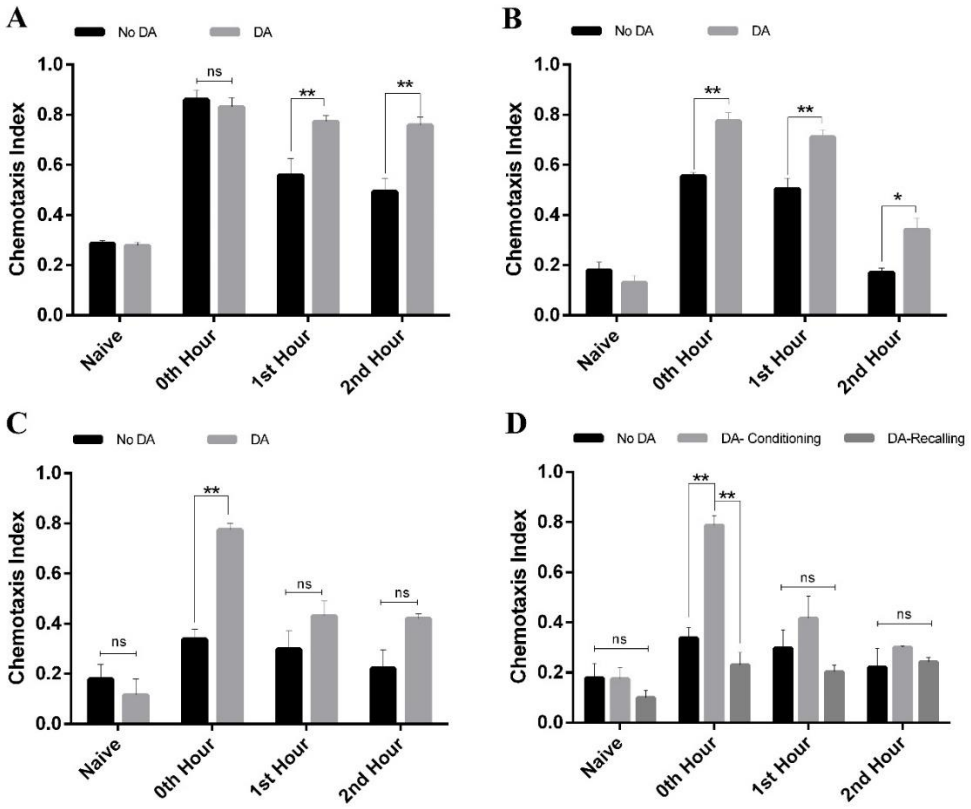

Figure 10: DA enhances olfactory learning and memory recalling. A. Exogenous addition of 10 mM DA during both steps of olfactory training (starvation & conditioning) significantly increased the recalling of memory in WT at 1st hr and 2nd hr ($p<0.005$), whereas, it did not affect the learning at 0th hr ($p>0.05$) compared to non-treated control worms. **B.** DA addition (during starvation & conditioning) improved the 0th hr learning significantly ($p<0.005$) as well as memory recalling at 1st hr and 2nd hr ($p<0.005$ and $p<0.05$ respectively) in *cat-2* mutant worms compared to the non-treated controls. **C.** DA (10mM) addition (during starvation & conditioning) on dat-1::ICE worms significantly improved the 0th hr learning ($p<0.005$) while, memory recalling process remained significantly unaltered ($p>0.05$) compared to the non-treated control worms. **D.** DA (10mM) during the conditioning phase alone is enough to significantly improve the learning in dat-1::ICE ($p<0.005$) but DA given during the recalling phase after being short-term trained did not improve memory recalling ($p>0.05$) compared to non-treated control. n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Sidak's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, *$p< 0.05$ and **$p<0.005$.

Exogenous dopamine-mediated learning and memory formation is mediated through dopamine receptors

DA functions through its receptors and helps in locomotion (Omura *et al*., 2012). As I had previously observed, the exogenous addition of DA improved learning and memory retention (Fig 10). Therefore, I further checked the involvement of DA receptors in short-term adaptive learning and memory by using both single and double DA receptor mutants. After the training, single receptor mutants of dop-1 and dop-3 showed significantly reduced CI value at 0^{th} hour and a significant reduction in CI value at 1^{st} hour and 2^{nd} hour time intervals compared with the WT (Fig 11A). However, these single receptor mutants could not completely reduce CI value at 0th hour compared to their own naïve, similar to cat-2 mutants (Fig 11A).

On the other hand, double receptor mutants (dop-1 &dop-3) showed a significant deficit in CI value after short-term adaptive training at the 0th to 2nd hour time interval (Fig 11B). Olfactory learning assays on double mutants (dop-1 and dop-3) showed a total deficit in learning and memory recall (Fig 11B) which substantiate this hypothesis. These results point out the importance of DA receptors during learning and memory recall.

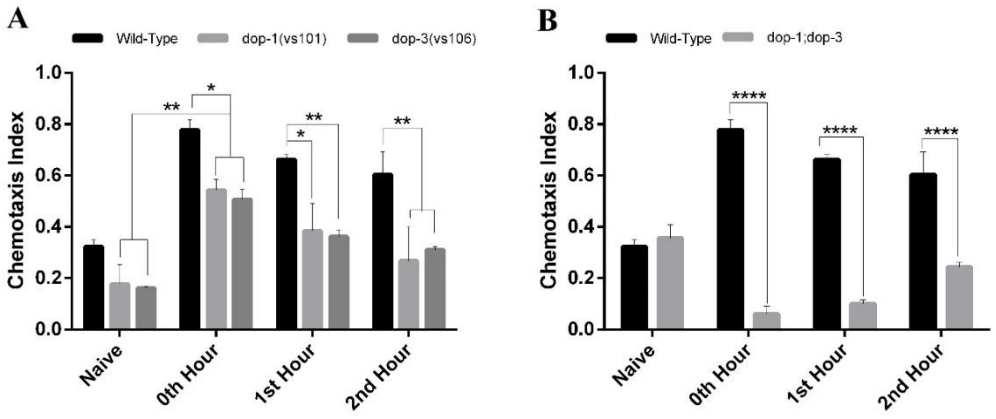

Figure 11: DA receptors are crucial for short-term adaptive memory formation. **A.** DA single receptor mutants of dop-1 and dop-2 were trained for short-term olfactory memory. Dop-1 mutant showed significant reduction in learning at 0^{th} hr ($p<0.05$) and memory recalling at 1^{st} and 2^{nd} hr ($p<0.05$ and $p<0.005$ respectively) compared to the WT. Similarly, dop-3 mutant worms also showed significant reduction in learning at 0^{th} hr ($p<0.05$) and memory recalling at 1^{st} and 2^{nd} hr ($p<0.005$ for both). However, compared to their own naïve worms both dop-1 and dop-3 mutants showed significantly higher learning ($p<0.005$). **B.** Double mutant of DA receptors dop-1 & dop-3 showed significant absence of learning at 0^{th} hr ($p<0.0001$) and memory recalling at 1^{st} hr and 2^{nd} hr ($p<0.0001$ for both) in comparison with WT and own naïve worms. n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, *p< 0.05, **p<0.005, ***p<0.001 and ****p<0.0001.

Progressive dopamine neuronal degeneration makes dat-1::ICE strain respond to exogenous dopamine

My previous results had shown that in dat-1::ICE worms, there was a significant improvement in learning but no memory retention in the presence of exogenous DA. It might be because the neuronal degeneration by human caspase interleukin-1β converting enzyme (ICE) is progressive with the aging of the worm. Therefore, we examined the receptor function of dat-1::ICE worms by observing the percentage of moving worms responding to exogenous DA. A notable increase in the rate of moving worms within DA plate corresponds to lower DA receptor levels/function (Sanyal *et al*., 2004; Allen *et al*., 2011). In the dat-1::ICE strain, a progressive reduction in DA receptor density is seen compared to WT (Fig 12). The percentage of worms moving in the DA plates (20 mM) was significantly higher during day-2 and day-3 of adulthood when compared to that of the WT worms (Fig 12).

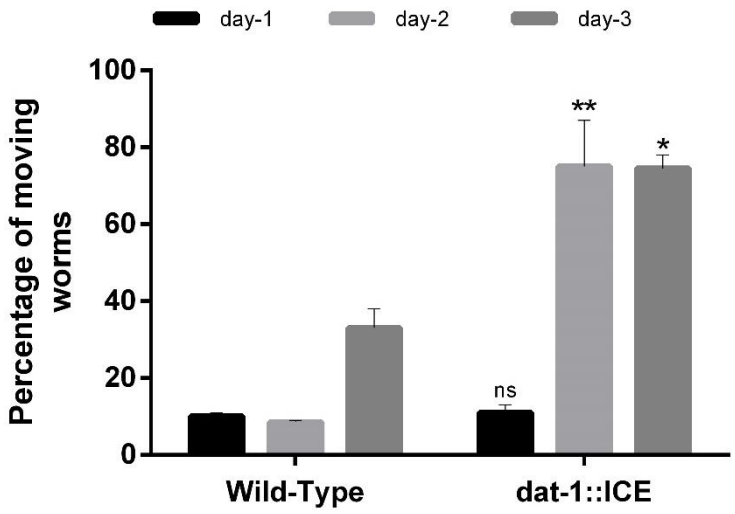

Figure 12: Aging in dat-1::ICE strain reduced DA receptor density. Sensitivity to DA immobilization (20 mM) was tested with day-1 to day-3 worms. A very less percentage of day-1 adults of dat-1::ICE worms were mobile and was similar to that of WT ($p>0.05$). Day-2 and day-3 old dat-1::ICE worms DA plate shows significant increase in percentage of moving animals (less immobilization) compared to the day matched WT ($p<0.005$ and $p<0.05$ respectively). $n\geq3$ trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, *$p< 0.05$ and **$p<0.005$.

Aging reduces the ability of DA mediated learning in dat-1::ICE strain

Further, short-term training was performed with adult dat-1::ICE strains aged day-1,2 and 3, respectively, in the presence and absence of DA. It was observed that only day 1 adults showed a significant increase in memory formation when compared to day-2 and day-3 and DA untreated control groups (Fig 13). The likely reason might be the differences in the receptor density seen at distinct age groups (our previous results). It, in turn, suggests that the neurodegeneration mediated through ICE progresses slowly, and the systemic changes perceived by the worms continue to mature.

Therefore, at day 1, worms initially have higher receptor density, which decreases as worms grow to day-2 and -3 adults.

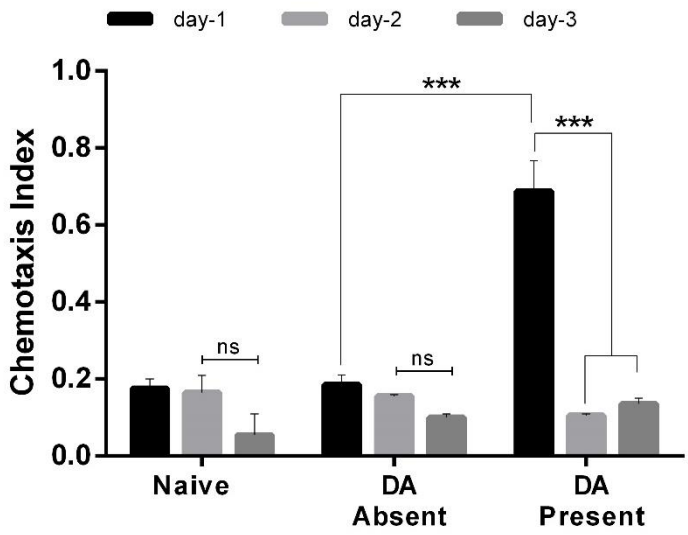

Figure 13: Aging reduces DA mediated learning in dat-1::ICE. Short-term training was performed with adult dat-1::ICE strains aged day-1, 2 and 3. Day-1 adult worms showed significant increase in learning ($p<0.001$) in the presence of DA compared to the age-matched naïve and in the absence of DA after short-term adaptive training. Whereas, day-2 and day-3 worms did not show any signs of learning compared to their age-matched controls ($p>0.05$) and showed significant reduction in learning compared to day-1 worms ($p<0.001$). $n\geq3$ trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Tukey's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant and ***$p<0.001$.

The absence of dopaminergic neurons leads to increased cholinergic receptor activity

Additional changes that occur due to the absence of DA neurons have to be further validated. Moreover, it has been well established that there is a link between cholinergic and dopaminergic neurons (Chuhma *et al*. 2014). It is essential to understand the impact of dopaminergic neuronal degeneration on the cholinergic

system. Therefore, an indirect assay was employed to assess the cholinergic receptor activity based on tetramisole. Tetramisole binds to cholinergic receptors leading to its prolonged activation resulting in paralysis of the worms (Lewis *et al*, 1980). A higher percentage of worms showing paralysis indicates high cholinergic receptor density. A significantly higher percentage of dat-1::ICE worms showed paralysis on day-1 and day-3 than the age-matched WT (Fig 14A and B). These results indicate that an imbalance in dopaminergic neuronal activity results in a rise of cholinergic receptor activity, probably through high receptor density in *C. elegans* in the absence of dopaminergic neurons.

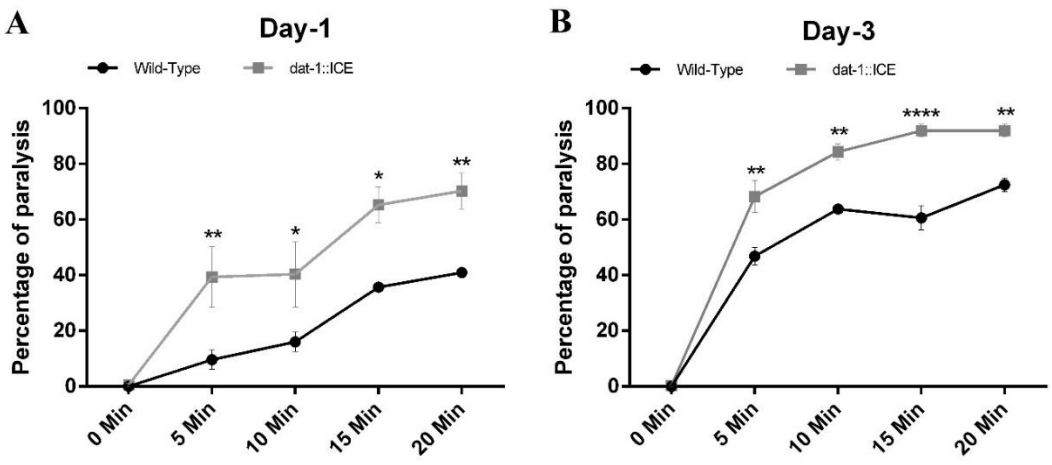

Figure 14: DAergic neuronal absence in dat-1::ICE mutant increased the cholinergic receptor density. A. Day-1 adult worms of dat-1::ICE showed a significantly greater percentage of paralysis in response to 0.1 mM tetramisole at all time intervals ($p<0.005$, $p<0.05$, $p<0.05$ and $p<0.005$ respectively) compared to the WT. **B.** Day-3 adult worms of dat-1::ICE showed significantly larger percentage of paralysis in comparison with WT at all time intervals ($p<0.005$, $p<0.0o5$, $p<0.0001$ and $p<0.005$ respectively). n≥3 trials; each trial contains more than 50 worms; statistical analysis was done by two-way ANOVA with Sidak's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, *p< 0.05, **p<0.005 and ****p<0.0001.

Human alpha-synuclein based dopamine neurodegeneration reduced learning and memory

Alpha-synuclein inclusion bodies can selectively cause degeneration of dopaminergic neurons resulting in Parkinson's disease (Goedert *et al*., 2001). I have taken the strain UA44 (baIn11; Pdat-1:: α-syn, Pdat-1::gfp), which has the human alpha-synuclein gene incorporated in the promoter region of dat-1 transporter expressed in dopaminergic neurons (Ruan *et al*., 2010). As the worm ages, the alpha-synuclein forms inclusion bodies and leads to the degeneration of dopaminergic neurons in *C. elegans* (Vijayan B *et al*, 2019). The dat-1:: α-syn strain showed normal architecture of dopaminergic neurons (ADE and CEPs) on day-1 (Fig 15A). However, on day-3 (Fig 15B), neurodegenerative markers (puncta's and breaks) were observed. To verify if this partial degeneration of dopaminergic neurons affected the learning pathway, the day-1 and day-3 adults of dat-1:: α-syn worms were trained for short-term associated memory. Day-1 adult worms showed a similar chemotaxis index at all time points similar to WT worms which determine normal learning and memory recalling in these worms (Fig 15C). In day-3 adult worms with a defect in neuronal architecture, a significant reduction in 0th-hour learning and the memory was noticed compared to the CI obtained by dat-1:: α-syn day-1 adult worms (Fig 15C). The results from the dat-1::ICE and the day-3 of dat-1:: α-syn worms were similar. These results further add credence to the hypothesis that dopaminergic neurons are involved in the consolidation of learning and memory.

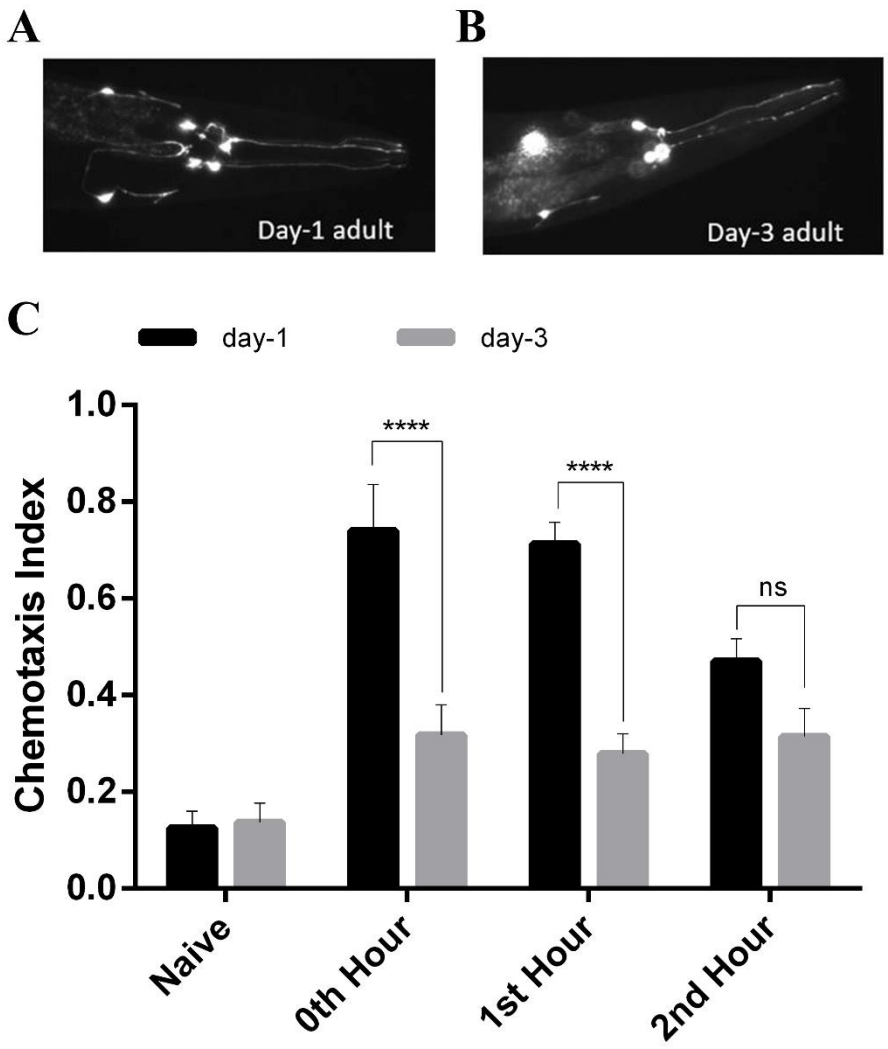

Figure 15: Presence of human alpha-synuclein enhanced DAergic neurodegeneration and affected short-term adaptive olfactory learning. A. Image showing the normal neuronal architecture of dopaminergic neurons ADEs and CEPs by expression of GFP on day-1 dat-1::αsyn mutant strain. **B**. Image showing dopaminergic neuronal degeneration characterized by presence of neurodegenerative markers such as puncta (arrows) and breaks (puncta) in day-3 worms of dat-1:: α-syn. n≥2 trials and each trial contains more than 15 animals. **C.** Day-3 dat-1::αsyn worms showed significantly lesser learning at 0^{th} hr ($p<0.0001$) and memory recalling at 1^{st} hr ($p<0.0001$) compared to the dat-1::αsyn day-1 worms . At 2^{nd} hr, day-3 worms showed a statistically insignificant memory recalling compared to the day-1 ($p>0.05$). For adaptive memory, n≥3 trials; each trial contains more than 50 animals; statistical analysis was done by two-way ANOVA with Sidak's multiple comparisons test; Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant and ****$p<0.0001$.

Discussion

Adaptive learning is an elementary learning process for the animals to predict future outcomes by processing vital environmental cues by experience. Memories, both short-term or long-term memories, are determined by the influence of parameters associated with learning and the parameters experienced during memory retrieval (Buchanan, 2007).

Here, I demonstrate that short-term adaptive training with butanol works better with *C. elegans* to create learning and memory. The sensory neurons (AWC) and interneurons (AIY) are critical in short-term adaptive learning and memory. The role of these neurons in the olfactory recognition pathway has been well studied (Gray, Hill, and Bargmann, 2005). Amphid sensory AWA, AWB, and AWC neuronal pairs in *C. elegans* help to sense odors from the surroundings. In the normal condition, these sensory neuronal pairs are hardwired for repulsion (AWB) or attraction (AWA and AWC) (Bargmann and Horvitz, 1991; Bargmann *et al*., 1993). In a previous study, it has been noted that AWC neurons help in both non-associative and associative learning, suggests distinct forms of learning and memory may use common downstream effectors (Pereira, S. and van der Kooy, D., 2012). AIY interneuron help in the retrieval of imprinted memory through ser-2 receptors (Jin X *et al*., 2016). These results underline the importance of neuronal circuits in creating new learning and adapting to new environmental situations (Sun *et al*., 2019). The absence of DA neurotransmitter resulted in a substantial reduction in learning and memory retrieval in cat-2 DA synthesis gene (an ortholog of human TH gene) mutant worms suggesting the importance of neurotransmitters in passing information for information processing in *C. elegans* (Veletic and Balasingham, 2020). Though neurotransmission is crucial for information outflow, I noted that the absence of DA did not completely attenuate

the learning and memory formation. It could be due to the compensatory mechanism of the serotonergic system during the low level of DA neurotransmission. A similar compensatory action of the serotonergic system was recently observed in MPTP-induced PD models (Ballanger *et al*., 2016). Assuming that serotonin is a crucial part of learning and memory, its absence would have serious consequences. However, serotonin synthesis mutants showed normal learning and memory. Since serotonin absence did not affect learning and memory function, it is conclusive that the learning in cat-2 mutant worms was probably because of a basal level of DA neurotransmitter. Previous studies have shown a low-level reactivity in formaldehyde-induced fluorescence staining in dopaminergic neurons (Lints and Emmons, 1999). Hence, it indicates the absence of the compensatory effect of DA and 5-HT in *C. elegans* as observed in the primates and mammals (Luciana, Collins, and Depue, 1998).

The absence of dopaminergic neurons has significantly affected olfactory learning and memory recalling pathways, almost entirely diminishing adaptive learning ability. Reduction in dopaminergic neuronal activity during reward presentation might hinder the pairing of conditioned stimulus with an unconditioned stimulus. It has been noted in the fly model, as well, that certain groups of dopaminergic neurons enhance reinforcement (Liu *et al*., 2012). Thus, this study demonstrates that the absence of dopaminergic neurons impedes the associative pairing of reward and conditional stimulus. Dopamine neurons otherwise modulate the memory relation via alteration in the strength and direction of synaptic plasticity (Steinberg *et al*., 2013). The DA neurotransmitter and neuronal absence affected adaptive learning and memory and affected the animal's behavior detrimentally. In PD patient’s loss of dopamine neuron in the substantia nigra cause deficit in movement initiation (da Silva *et al*., 2018). We rule out the possibility of a migration defect due to the absence of dopamine neurons

by maze assay and found that worms lacking dopamine neurons can make proper migratory patterns. These findings suggest learning defects observed in the absence of DA neurons are not due to any migratory problem but due to aberrant connectome function associated with loss of dopaminergic neurons.

Further, the importance of dopamine neurons was demonstrated by calcium imaging. Previously, in mice models, it has been shown that dopamine neurons evoked calcium transients in the subpopulation of dopamine neurons in the ventral tegmental area (VTA) in response to Pavlovian fear conditioning (Gore, Soden, and Zweifel, 2014). Similarly, I observed that the dopamine neuronal activation might evoke firing in DA neurons when an olfactory stimulus was presented to trained worms. It suggests that the presence of food acts as a reward during the conditioning stage, which activates dopamine neurons and other downstream associated neurons to establish a positive reinforcement between food and the conditional stimulus (CS) (Steinberg *et al*., 2013). This hypothesis was further validated by the mechanical stimulation of DA and the addition of exogenous DA along with the olfactory stimulus during pairing (conditioning or training) time. G-75 beads activate the dopamine neurons and thus evoke a high DA level inside the organism that probably helps in the association of the CS with that of UC. The same was observed with the exogenous addition of DA, which further adds credence to my theory.

Recent studies on olfactory bulb astrocytes have shown that D1 and D2- class DA receptor antagonists partially reduced the dopaminergic calcium response (Fischer, Scheffler, and Lohr, 2020). I also found that both D1 and D2 like receptors in *C. elegans* play critical roles in olfactory adaptive learning and memory recall. It has been

observed that the cortical circuit exerts its control through the DA concentration and this, in turn, helps proper fine-tuning of cortical networks (Trantham-Davidson *et al*., 2004). The complete absence of learning and memory recall in double mutant and triple mutants suggest partial activation or compensatory action of DA receptors in single receptor mutants through the differential DA concentration in response to the olfactory stimulus. One can alternatively use dopamine receptor blockers such as raclopride or haloperidol to inhibit the activity of these receptors. These antagonists have been shown to inhibit the turning behaviour of *C. elegans* in response to food deprivation (Hills *et al*., 2004). A recent study showed that dopamine signalling is required for non-associative odor learning, such as pre-exposure to 2-nonaone for 1 hour, which enhances the odor avoidance in *C. elegans* (Yamazoe-Umemoto *et al*., 2015). A similar result has been shown in NaCl-based avoidance assay and gustatory plasticity (Hukema, R. K *et al*., 2008).

Learning assays by adding exogenous DA to wild-type and cat-2 mutants improved learning and memory formation. Surprisingly, exogenous addition of DA in dat-1::ICE worms showed a significant increase in olfactory adaptive learning but not on memory recall. Earlier, it has been observed that to elicit memory, dopaminergic neurons in the substantia nigra play a critical role (Kamiński *et al*., 2018). Hence, the presence of DA during the conditioning phase must have initiated the activation of weaker synapses in the mutant. With DA receptor elicited learning, memory recalls requiring intact dopamine neuronal networks (Shivarama, Gopinadhan and Sajikumar, 2016). Further, it was found that the addition of DA during the conditioning stage is crucial in forming learning and memory by making proper pairing food and CS.

In hemiparkinsonian rat models, a reorganization of DA receptor density due to a phenomenon called supersensitivity of DA receptors has been observed (Rangel-

Barajas, Coronel and Florán, 2015). The mRNA coding for DA receptors has been compromised, and the proteins associated with DA signal transduction in the absence of dopamine neurons (Rangel-Barajas, Coronel and Florán, 2015). It was surprising that even in the absence of dopamine neurons, the adult worms can sense the exogenous DA and form learning but not retain memory. However, in mutant worms that do not have dopamine neurons, aging accelerates the attenuation of DA receptors (Karrer *et al*., 2017).

The role of dopamine neurons in learning and memory was further studies using siRNA mediated silencing of ICE gene in dat-1::ICE worms. The silencing of the ICE gene protected the worms from its progressive neurodegeneration during their development. These worms were found to have regained the impaired learning and memory. Worms expressing human alpha-synuclein in CEP neurons were tested as an alternate model for the role of dopamine neurons in memory. Aging accelerated the neurodegeneration of CEP neurons in alpha-synuclein expressing UA44 [bal11;Pdat-1α-syn::Pdat-1gfp] strain, which led to the decline of learning and memory. It is known that DA neurons exhibit phasic activation (burst of action potentials in short bursts) as a “prediction error” signal following reward presentation in classic conditioning (Puig *et al*., 2014). Probably, DA neurons function similarly in *C. elegans*, also affecting the memory decoding pathway. Delayed calcium influx in CEP neurons after the solvent exposure supports this hypothesis. Olfactory adaptive training of strain expressing GCaMP-6 on CEP neurons showed that in the presence of CS stimulus, the dopaminergic neurons evoked a delayed increase in fluorescence intensity associated with a transient increase in intracellular calcium influx compared to the untrained worms. This finding suggests activated dopamine neurons and the

downstream associated neurons are essential to establish positive reinforcement during learning and memory (Steinberg et al., 2013).

It has been noted that during conditional learning in the presence of a stimulus evokes a pause in the neuronal firing activity of cholinergic neurons, and this leads to a subsequent increase in activity of dopaminergic neurons (Aosaki *et al*., 2010). Proper balance in the activity of cholinergic and dopaminergic neurons is required for the functioning of ion channels. This synchronized activity of cholinergic and dopaminergic neurons gets affected in Parkinson's disease (PD) patients due to the lack of the autoinhibition of acetylcholine release through muscarinic receptors (Threlfell *et al*., 2012), resulting in loss of memory. The worms devoid of dopamine neurons showed an increase in sensitivity to tetramisole, a cholinergic receptor agonist. It indicates that the absence of dopamine neurons could lead to an increased expression of cholinergic receptors. Similar results were observed in PD models (Tubert and Murer, 2020).

In summary, this study using various mutations and pharmacological approaches in *C. elegans* reveals the critical role of DA in memory and indicates that DA neurons play a crucial role in the effective processing of cognitive function. Further understanding of these connectomes and their active processing of information will hopefully enrich understanding of the intrinsic relations between memory, motivation, and decision-making at cellular levels.

Chapter-2

Introduction

Manganese (Mn) is an essential mineral virtually present in all diets at low concentrations. Mn is an absolute requirement for amino acid and lipid metabolism and Mn-dependent enzyme families, including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Humans regulate a stable tissue level of Mn by tightly regulating its absorption and excretion (Aschner and Aschner, 2005; Santamaria and Sulsky, 2010). It has long been noted that chronic exposure to Mn may lead to a condition called Manganism, which has symptoms that closely resemble idiopathic Parkinson's disease (iPD), including masked facial expression, gait impairment, and rigidity (Guilarte and Gonzales, 2015). Mn exposed individuals have shown neuronal degeneration, mainly in the dorsal striatum, internal globus pallidus, and substantia nigra pars reticulata (SNpr). The disease is called Mn-induced Parkinsonism (Olanow, 2004; Kwakye *et al*., 2015). Exposure to Mn is not limited to occupational exposure; dietary exposure to Mn is on the rise now. Mn is added to gasoline to increase its octane rating and as a disinfectant in the water. Mn is quite widely used as an additive for parental nutrition and infant supplies (Fell *et al*., 1996; Anyanwu *et al*., 2018).

Dopaminergic neuron degeneration is a hallmark of Parkinson's disease (PD), with its onset associated with genetic susceptibility and aging (Chen P *et al*., 2015). It has been postulated that "dysfunction" of the DA circuit results in movement disorders in manganese exposure, whereas degeneration of dopaminergic neurons occurs in PD (Guilarte and Gonzales, 2015). However, it is generally accepted that individuals exposed to manganese have a higher risk of developing PD (Harischandra *et al*., 2019). The link between metal toxicity and the development of the disease is still

debated. For example, elevated levels of Fe in substantia nigra pars compacta (SNpc) regions of the brain, as well as alterations in serum levels of Fe, Mn, Cu, and Zn in PD patients, have been reported (Zhao *et al*., 2013; Meamar *et al*., 2016). Both Fe and Mn share the same transporters. Due to nutritional deficiency, low levels of Fe can lead to the accumulation of Mn in brain regions such as the striatum, which might lead to the cognitive deficit (Aschner *et al*., 2008). Excessive manganese is known to cause cell death by inducing oxidative stress and mitochondrial dysfunction. Besides, recent studies provide credence to the theory that environmental exposure to toxicants could initiate or propagate neurodegeneration by interfering with disease-associated proteins such as alpha-synuclein and amyloid proteins (Smith *et al*., 2017; Jucker and Walker, 2018). Hence, aberrancy in metal homeostasis could contribute to the pathophysiology of diseases like PD.

Nevertheless, many questions remain unanswered.

1. The involvement of DA neurotransmitter in Mn mediated neurotoxicity

2. How is the DA transporter involved in mediating the toxicity.

In this study, I used *C. elegans* as a model to recapitulate the effect of Mn and DA in dopaminergic neurodegeneration and understand how it affects cognitive ability.

Materials and Methods

Caenorhabditis elegans strains

All strains were maintained in Nematode Growth Medium (NGM) plates with OP50 *E. coli* bacteria as a food source. The strains were maintained at 20˚C. The following strains were used in this study: N2 Bristol wild type; BZ555 [*egIs1 dat-1pGFP*]; UA44 [*bal11;Pdat-1α-syn::Pdat-1gfp*]; gifted by Randy Blakely, Florida Atlantic University, USA. BY200 [*vtIs1(dat-1p::GFP) V*]; and MAB300, [*dat-1::GFP(vtIs1)V;smf-2(gk133) X*]; was gifted by Dr. Micheal Ashner. CB1112 [*cat-2(e112)II*] Gift by Dr. Gert Jansen; UA57 [*baIs4 [dat-1p::GFP + dat-1p::CAT-2*] was gifted by Dr. Cladwell. All strains, unless otherwise mentioned, were provided by the *Caenorhabditis elegans* Centre (CGC, Minnesota).

Strain	**Genotype**
WT	N2 (Variant. Bristol)
BZ555	[egIs1 dat-1pGFP]
CB1112	[*cat-2(e112)II*]
BY200	[vtIs1(dat-1p::GFP) V]
MAB300,	[dat-1::GFP(vtIs1)V;smf-2(gk133) X];
UA44	[*bal11::Pdat-1α-syn::Pdat-1gfp*]
UA57	[baIs4 [dat-1p::GFP + dat-1p::*CAT-2*]

Manganese chloride Preparation

For studying the effect of Manganese on *C. elegans*, different concentrations of $MnCl_2$ solutions were used. A 1 M stock of $MnCl_2$ was prepared in H_2O from which the different concentrations of working solutions were made.

Effect of manganese chloride exposure on larval stage

The L1 larvae of BZ555 were treated with 25 mM, 50 mM and 100 mM $MnCl_2$ for 1hr at 20˚C with intermittent shaking. d. H_2O was used as treatment for control. Post 1 hour, the reaction was stopped and the solution containing worms were centrifuged at 6000 rpm for 1 min and the supernatant was discarded. Worms were washed three times with 1 ml d. H_2O, before transferring to NGM plates seeded with OP50. A set of worms were observed under microscope at 3 hour, 24 hour, 48 hour and 72 hour (indicating the adult stages day 1, day 2 and day 3 of the worm development) after anaesthetizing with sodium azide (25 mM) for neurodegeneration markers such as puncta, breaks, loss of CEP neurons.

Effect of exposure to manganese at adult stage

All strains studied were exposed to 50 mM and 100 mM $MnCl_2$ for 1 hour in 20 µl final volume. The $MnCl_2$ solution was completely removed by transferring the animals to excess d.H_2O and the worms were then transferred to a glass slide and covered with a coverslip. Microscopic observation was done for observing neurodegeneration markers as described before.

Survival Test

Survival after acute $MnCl_2$ treatment was tested in both adults and larval exposure. Larvae exposed to Mn (0 to 100 mM) were transferred to OP-50 plate and

approximate initial numbers were recorded. After 72 hours post exposure, the number of live worms were counted by gently touching the worms by tip of worm picker. For adult worms, 20-25 worms were exposed to 50 mM and 100 mM $MnCl_2$ concentrations at day 1 adult stage. After 1-hr $MnCl_2$ exposure at 20˚C the worms were washed thrice in distilled water and transferred to OP-50 plate and the number of worms were recorded. After 24 hour the number of live worms were counted as mentioned earlier.

Dopamine pre-treatment and $MnCl_2$ exposure

Worms were treated with concentration mentioned either 5mM or10 mM DA (prepared from 1 M stock) for 10 min and then washed 3 times with M9 before exposing them to different concentrations of $MnCl_2$ (0 -100 mM). Both locomotory and fluorescence intensity analysis were carried out as follows.

Effect of Manganese on the worm movement: Locomotory assay

Day 1 adult worms were taken from synchronized population of each strain and then exposed to DA and Mn, as mentioned in the result, for 30 min at 20˚C. The $MnCl_2$ solution was removed by washing the worms thrice with d.H_2O, before transferring them onto NGM plates with or without OP50. The movement of worms on both plates were then recorded using Dino-Lite digital microscope model# AM4115T-GFBW with 5 Megapixels camera and 1.3 Megapixels sensor capable of shooting at 4 frame per second. The worm's locomotion was analysed by measuring body bends made by each worm in 20 sec, which was manually counted and recorded (Sawin *et al* 2000).

Fluorescence intensity analysis

At least 10 worms of treated and un-treated groups were placed on a glass slide containing 25 mM sodium azide in 15 ul M9 buffer final volume. Periodically monitor the worm movement under a stereo microscope and once after the worms were paralysed, a cover glass, covered with petroleum jelly around the corners to avoid desiccation, is placed over the worms. Then images were taken using an inverted microscope Olympus IX51 equipped with Rolera-XR CCD camera (Q Imaging) and image acquisition software NIS Elements-Advanced Research (NIKON). All worms were observed at λex/λem 460-490/520. All image analysis was done by using Fiji open software. The image taken after the respective treatments were loaded onto Fiji (an open software for image analysis- similar to Image J) and measure the intensity based on pixels/ inch. The region of interest was including the CEP and ADE neurons in the head region. Background correction is done by subtracting the average intensity value of 5 readings derived by measuring different areas of background from actual intensity of the region of interest. Final fluorescence intensity selected were background subtracted actual value taken for plotting the graph.

Immobilization assay

Immobilization assay was carried out as mentioned by Felton and Johnson, 2011.Two groups were considered: a control group (no $MnCl_2$) and 50 mM $MnCl_2$ treated group. Each of the two groups were incubated for 40 min at 20˚C in the control plate (no DA) and 20mM DA plate. Control and DA plates were prepared as previously mentioned (Chase, Pepper and Koelle, 2004). No.of mobile, immobile and paralyzed worms were counted from both plates as described by Felton and Jhonson 2011.

Swimming Induced paralysis Assay (SWIP)

SWIP assay was done as previously mentioned (Kudumala, Sossi and Carvelli, 2019). The adult worms were synchronized. The eggs obtained after synchronization were transferred to M9 buffer to hatch into L1 larvae. The larvae were treated with different concentrations of MnCl2 and then transferred to a fresh OP-50 plate for 48 hour to develop into an adult. The adult worms were hand-picked individually and shifted to a centrifuge tube containing 50 ul M9 buffer and allowed it to adapt for 20 min. After that, worm movements were recorded using a Dino-Digital camera. The movement analysis was done using Worm Lab software. Similar protocol was followed for $MnCl_2$ treatment in adult worms.

Learning Index Assay

Learning index assay was similar to the chemotaxis index performed as previously mentioned (Kauffman *et al.*, 2011). $MnCl_2$ Treated and untreated worms were assayed on a chemotaxis plate. After the chemotaxis assay CI values for each time interval were calculated as mentioned (Nishijima and Maruyama, 2017). Learning index (LI) was calculated by subtracting the CI of naive worms (CI_{naive}) from that of conditioned worms ($CI_{conditioned}$) (Amano and Maruyama, 2011).

Microscopy

To view the nematodes for picking, transfer, washing etc. a stereo microscope (Magnus Analytics, India) with 10X zooming was used. For fluorescence imaging, Olympus IX51 inverted microscope, (Olympus Imaging, Center Valley, PA, USA) objective lens 40X/0.60 Ph2/∞/0-2/FN22, that works with image acquisition software NIS Elements-Advanced Research (NIKON) and Rolera XR monochrome camera (QImaging, Canada) was used.

Camera

The Dino-Lite digital microscope model# AM4115T-GFBW with 5 Megapixels camera and 1.3 Megapixels sensor capable of shooting at 4 frames per second. Sony Alpha a7 III Model Mirrorless Digital Camera with Sony FE 90mm f/2.8 Macro G OSS Lens.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc.). The data are presented as Mean + SEM as indicated. Significance was represented as follows *p<0.05, **p<0.01, ***P<0.001, ****p<0.0001.

Results

Dopamine is essential for manganese induced neurodegeneration

The impact of $MnCl_2$ exposure during the L1 larval stage and its effect on the neurodegeneration of dopaminergic (CEP) neurons is unclear. To test this, the L1 larvae of BZ555 strain (expressing GFP on dopaminergic neurons under the dat-1 promoter) was exposed to increasing concentration of $MnCl_2$ (25 mM, 50 mM, and 100 mM) as previously mentioned (Benedetto *et al.*, 2010) (Fig. 1A). Surviving worms after $MnCl_2$ exposure did not show any significant degenerative changes in dopaminergic neurons, including the characteristic neurodegeneration markers such as puncta, blebs, neuronal absence, shrinkage, loss of cell bodies, or dendritic breaks in their adult stage (Fig. 1B-E). However, there was a significant reduction in the survival rate of worms exposed to $MnCl_2$. Treatment at 25 mM, 50 mM and 100 mM concentrations of MnCl2 significantly reduced the survival rate from 60% (25 mM) to less than 40% (100 mM) (Fig. 1F). Treatment at higher concentrations (150 mM and 250 mM) was toxic and resulted in only a few survivors.

To test whether $MnCl_2$ induced any subtle changes on dopaminergic neurons, I measured the expression of dat-1 (DA transporter ortholog) levels in anterior cephalic dopaminergic neurons (CEPs) and anterior deirid bodies (ADEs). However, the results showed no significant changes to the unexposed control in adult worms on days 1, 2, and 3 in all the treatment conditions of 25 mM, 50 mM, and 100 mM $MnCl_2$ (Fig. 1G). To further confirm the absence of early changes in dopamine neuronal degeneration, dat-1 expression levels were quantified in larval stages at 3, 24, and 48 hours after the 100 mM $MnCl_2$ exposure in surviving worms. The results suggested

no significant changes ($p>0.05$) in the dat-1 protein expression levels compared with that of control compared to the larval stages; the adult worms (after 48 hours) showed almost double the expression levels of dat-1 compare to the larvae (Fig. 1H).

I further probed whether a lower expression level of DA in the L1 stage lowered the effect of $MnCl_2$. To test this, I treated the L1 larvae with DA before $MnCl_2$ exposure. The results showed a significant increment of neurodegeneration in surviving worms (Fig. 1I). DA treatment during larval stages alone was causing CEP neurons to degenerate. At 5 mM DA, pre-treatment alone showed a significant increase in neurodegeneration (>40%). But when the worms were $MnCl_2$ exposed after DA pretreatment has shown an additive effect: >60% neurodegeneration compared to 40% in DA alone (Fig. 1I). 10 mM DA treatment alone resulted in more than 50% of worms showing neurodegeneration, and increasing concentration of Mn did not show a significant enhancing effect (Fig. 1I), suggest that the treatment concentration might have reached the saturation point (Roth *et al*, 2013).

Figure 1.

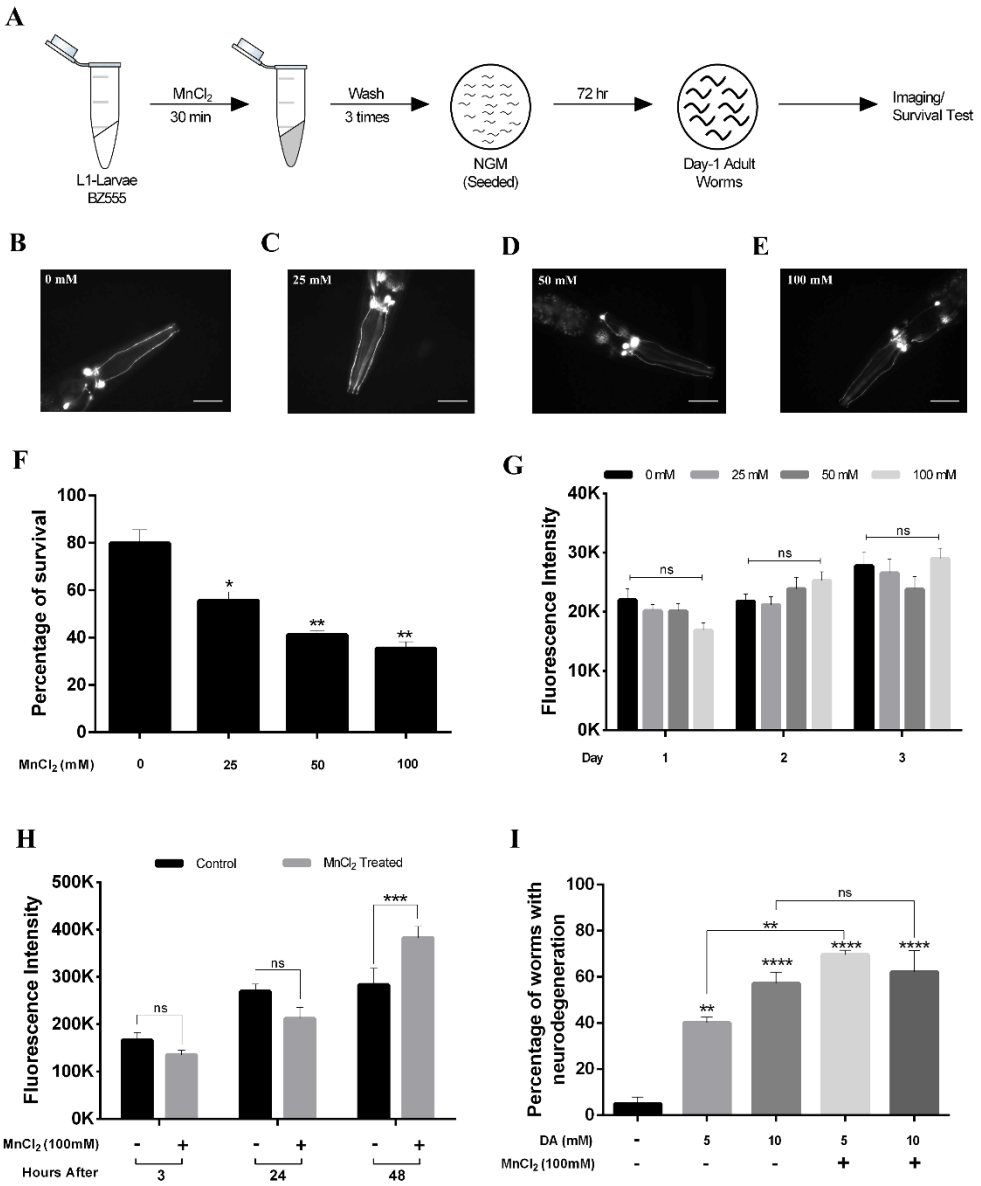

Figure 1: **Larval exposure to $MnCl_2$ shows resistance to neurodegeneration, but exogenous DA enhanced DAergic neurotoxicity. A.** Graphical representation of the protocol for exposure of larval worms to $MnCl_2$. **B-E.** Representative images of worms display continuous GFP on CEP and ADE dopaminergic neurons in dat-1::gfp (BZ555) strain when exposed to $MnCl_2$ at L1 larval stage with concentrations of 0 mM, 25 mM, 50 mM and 100 mM respectively n≥45 worms; scale bar represents 50 µm; exposure 500 ms. **F.** BZ555 larval worms exposed to increasing concentration $MnCl_2$ show significant concentration dependent reduction in survival at 25 mM ($p<0.05$), 50

mM ($p<0.005$) and 100 mM ($p<0.005$) in comparison with the control. n≥2 trials; each trial contains more than 100 worms; statistical analysis was carried out by one-way ANOVA with Dunnett's multiple comparisons test. **G.** Quantification of dat-1::gfp expression of CEP and ADE dopaminergic neurons, of larval worms of BZ555 exposed to different concentrations of $MnCl_2$ at day-1, day-2 and day-3 stages shows non-significant reduction in fluorescence intensity compared to the control of each day ($p>0.05$). n≥ 15 worms; statistical analysis was carried out by two-way ANOVA with Tukey's multiple comparisons test; image exposure was 500ms). **H.** Quantification of dat-1::gfp levels of CEP and ADE dopaminergic neurons after 3 hour, 24 hour and 48 hour of larval worms of BZ555 exposed to 100 mM $MnCl_2$. No significant reduction in fluorescence intensity was observed compared to the control at 3 hour and 24 hour ($p>0.05$), whereas, a significant increase was observed at 48 hour on $MnCl_2$ treated worms compared to control ($p<0.001$). n≥ 15 worms; statistical analysis was carried out by two-way ANOVA with Sidak's multiple comparisons test; image exposure was 700 ms) **I.** BZ555 larval worms pre-exposed to DA (both 5 mM and 10 mM) and followed by 100 mM $MnCl_2$ show neurodegeneration. Compared to the control, both concentration of DA pre-exposure shows significant increase in neurodegeneration ($p<0.005$ and $p<0.0001$). Pre-exposure to either of the concentrations of DA followed by 100 mM $MnCl_2$ exposure further increased the percentage of neurodegeneration observed on day-1 ($p<0.0001$ for both) compared to control. However, only 5mM DA+Mn showed significant increase ($p<0.005$) in neurodegeneration compared to DA alone, but not 10mM DA+MN. n≥3 trials, each trail contains more than 15 worms; statistical analysis was carried out by one-way ANOVA with Tukey's multiple comparisons test. Data are represented as the mean +S.E.M. Significance indicated: ns- non-significant, $p< {}^{*}0.05$, $p< {}^{**}0.005$, $p<{}^{***}0.001$.

To confirm that the presence of DA is critical for $MnCl_2$ to induce neurodegeneration in CEP neurons, I treated the adult worms with $MnCl_2$ in four different strains of worms expressing GFP under dat-1 promoter in CEP neurons: BZ555, UA44, MAB300, and BY200 (Fig 2A). All the strains showed significant changes in CEP neurons after 100 mM Mn treatment (Fig 2B); 50 mM $MnCl_2$ treatment showed that MAB300 and BY200 are more sensitive (>20% showing neuronal changes) compared to the other strains used (Fig 2B). Survivors were significantly low in all the strains tested. 100 mM $MnCl_2$ treatment had a 50-80% mortality rate compared to the 20-40% in 50 mM $MnCl_2$ treatment (Fig 2C), suggesting a dose-dependent effect of $MnCl_2$ to cause

dopaminergic neuronal damage. The DA treatment in adult worms of both MAB300 and BY200 have shown severe dopaminergic neuronal damage when presented alone and subsequently treated with $MnCl_2$ (Fig 2D-G).

The dat-1 expression levels in cell bodies of CEP neurons were measured. The data showed a significant ($p<0.01$) reduction in dat-1 expression levels in worms treated with 100 mM $MnCl_2$. In the presence of 10 mM DA and $MnCl_2$ (both 50 and 100 mM) significant decrease in dat-1 expression levels ($p<0.0001$) was observed (Fig 2H). It could be possible that free DA levels at the synapse cause significant pathological changes in the presence of $MnCl_2$.

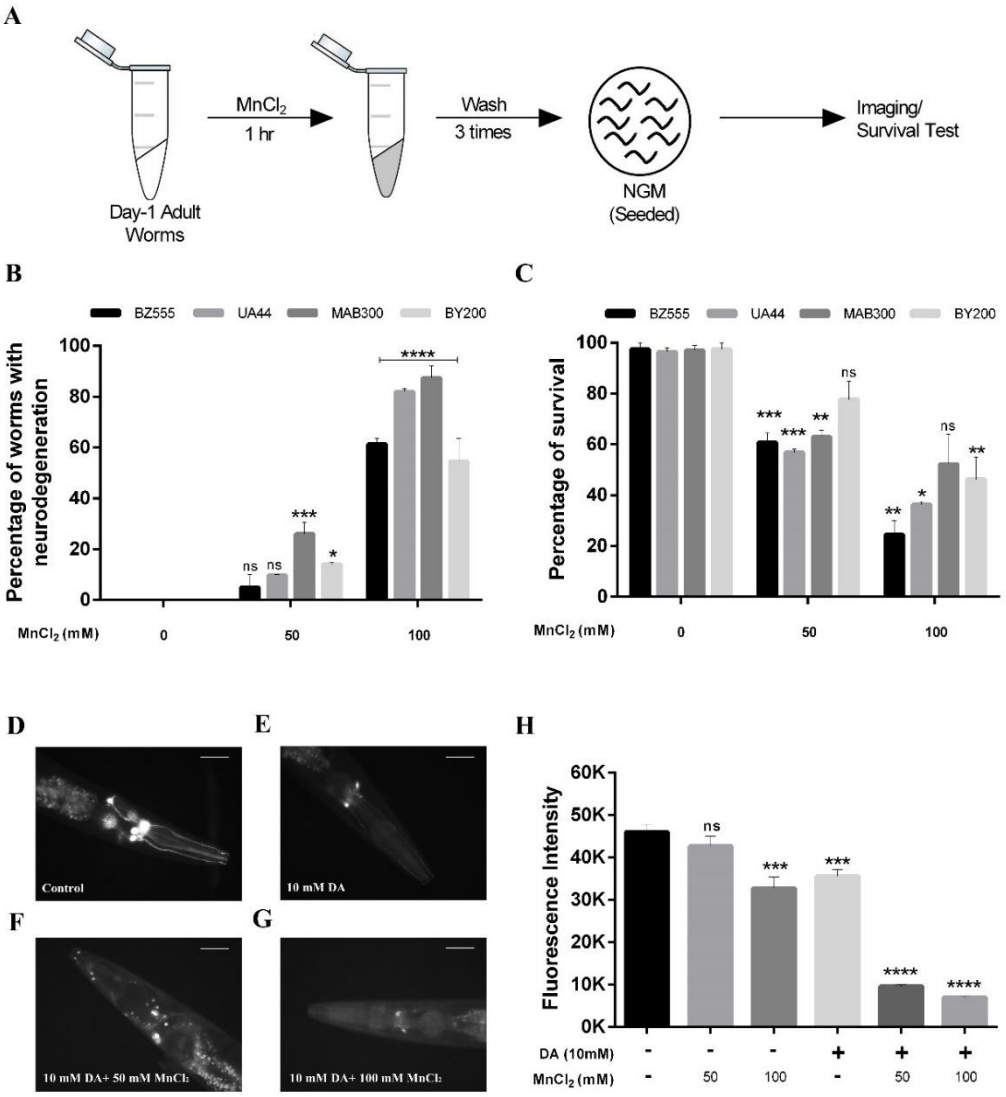

Figure 2. Adult worms showing neurodegeneration in response to $MnCl_2$ and DA exposure. A. Graphical representation of the protocol for exposure of adult worms to $MnCl_2$. **B.** Adult worms of MAB300 and BY200 exposed to $MnCl_2$ for 1hr show a significant increase in neurodegeneration at 50 mM ($p<0.001$ and $p<0.05$ respectively) compared to the control, unlike BZ555 and UA44 strains which were statistically insignificant ($p>0.05$). Worms exposed to 100 mM $MnCl_2$ show significant increase in percentage of animals with neurodegeneration in comparison with the control and 50 mM $MnCl_2$ ($p<0.0001$). Neurodegeneration was scored by the presence of markers such as puncta, blebbing, breaks and loss of CEP neurons. n≥3 trials; each trial contains more 15 worms; statistical analysis was carried out by two-way ANOVA with Tukey's multiple comparisons test. **C.** Adult worms exposed to $MnCl_2$ for 1 hour show concentration dependant reduction in survival. BZ555, UA44 and MAB300 shows significant reduction in survival at 50 mM $MnCl_2$ ($p<0.001$,

p<0.001 and p<0.05 respectively) compared to the control, unlike BY200 which shows no significant (p>0.05) reduction in survival percentage. At 100 mM $MnCl_2$ exposure, strains BZ555, UA44 and BY200 showed significant reduction (p<0.005, p<0.05 and p<0.005 respectively) whereas the strain MAB300 showed no significant (p>0.05) reduction in survival compared to 50 mM $MnCl_2$. In addition, at 100 mM $MnCl_2$ exposure, all strains showed significant reduction in survival (p<0.0001) in comparison with the control. n≥3 trials; each trial contains more than 30 worms; statistical analysis was carried out by two-way ANOVA with Tukey's multiple comparisons test. **D-G.** Representative images of BZ555 CEP and ADE dopaminergic neurons pre-exposed to DA for 10 min followed by 1 hour $MnCl_2$ exposure. **D.** Control worms show continuous display of GFP on CEP and ADE. **E.** Worms pre-exposed to 10 mM DA show reduction in GFP and size of ADE neurons as a marker of neurodegeneration. **F.** 50 mM $MnCl_2$ exposure to worms pre-exposed with DA show neurodegeneration markers such as puncta, breaks on CEP neurons in addition to loss of soma in ADE neurons. **G.** 100 mM $MnCl_2$ exposure to worms pre-exposed with DA show severe neurodegeneration such as loss of CEP neurons and reduced GFP expression on ADE neurons along with shrinkage. n≥3 trials; each trial contains more than 15 worms; scale bar represents 50 µm, exposure 500 ms. **H.** Quantification of dat-1::gfp level of day-1 BZ555 strain on exposure to $MnCl_2$ and DA. 50 mM $MnCl_2$ treated worms did not show any significant reduction (p>0.05) in fluorescence intensity compared to the control. However, 100 mM $MnCl_2$ exposure and 10 mM DA pre-exposure significantly reduced (p<0.001) the GFP fluorescence intensity indicative of neurodegeneration. Pre-exposure to DA followed by $MnCl_2$ exposure further augmented the neurodegeneration shown by significantly reduced (p<0.0001) GFP expression on CEP and ADE neurons. n≥ 15 worms; statistical analysis was carried out by one-way ANOVA with Dunnett's multiple comparisons test. See the materials and method section for details of the strains used. Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, p< *0.05, p< **0.005, p<***0.001 and p<****0.0001).

Dopamine enhances Mn toxicity: Affecting locomotory behaviour

The on-food and off-food behaviors of worms, a characteristic test for the DA function in the worm post-exposure to Mn, were assayed to know whether the worms show altered behaviour in response to Mn induced toxicity (Fig. 3A) (Sawin *et al.*, 2000). The locomotory behaviour of both control and test worms was recorded for 0-24 hours in the presence of DA and Mn (Fig. 3B). A significant slowing down behavior of worms

after 30 minutes of starvation was observed on the on-food plate (Fig. 3B). Worms from the same batch were tested after 30 min, 3 hours, and 24 hours. The on-food behavior recovered from the initial extreme slow-down and was maintained at a lower level than in the off-food; this is mainly due to the surge of DA in the presence of food, which results in significant slowing down behavior of the worm. Under similar conditions in the control worms pre-treated with 10 mM DA, its off-food behaviour also showed a significant reduction in body bends at 0th hour compared to no exposure control (Fig. 3C). After DA was washed off, the worms showed normal recovery from the DA plus starvation shock. It took around 24 hours to reach the on-food behaviour of the control animals (Fig. 3C). Off-food behavior recovered to that of control within 30 minutes after the removal of DA, suggesting DA pretreatment did not severely affect the locomotory behaviour of the worms (Fig. 3C).

However, when worms exposed to 100 mM $MnCl_2$ showed a significant reduction in body bends at 0th hour compared to no exposure control off-food, and on-food body bends, the off-food plate's recovery took less than 3 hours, and the on-food behavior took around 24 hours to recover to the initial state (Fig. 3D). There is no severe damage in the locomotory behavior, except initial time points, suggesting low neurotoxicity in the presence of Mn alone. Pretreatment of DA and subsequent $MnCl_2$ exposure completely immobilized the worms at 0th hour in both on-food and off-food plates (Fig. 3E). These worms showed a slower recovery in off food plate (in less than 3 hours) and completely recovered before 24 hours. However, the on-food behavior showed significantly lower recovery than the no exposure control even after 24 hours (Fig. 3E), suggesting an increased sensitivity to $MnCl_2$ mediated toxicity in the presence of DA in these worms.

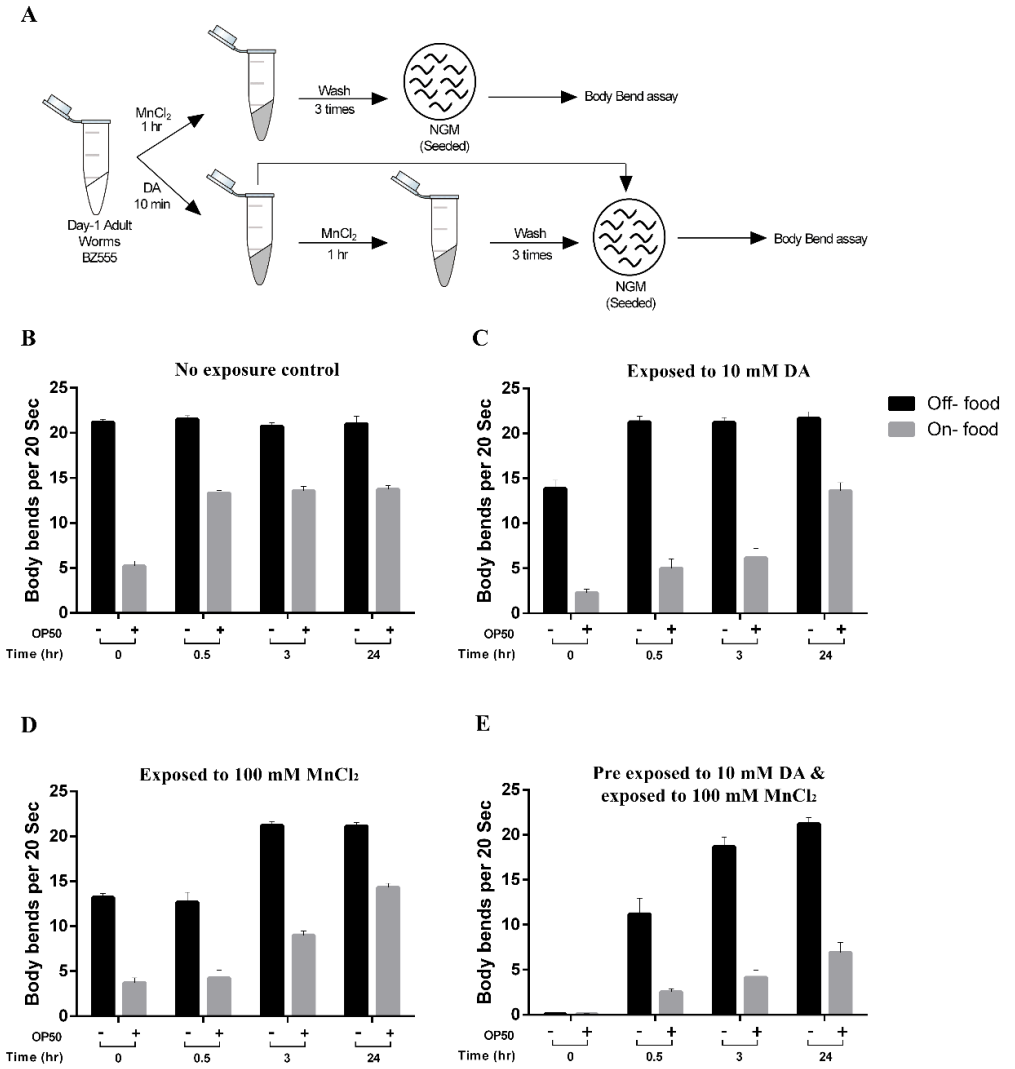

Figure 3. Recovery time kinetics of body bends in off-food and on-food. A. The protocol for exposure of BZ555 adult worms to DA and $MnCl_2$. **B.** No exposure control of adult worms at 0^{th} min (mock treated with d.H2O) show no change in body bends in the absence of food whereas on-food, show reduced body bends due to starvation associated with the mock treatment. This reduction in behaviour was recovered after 30 min to the normal level of on-food behaviour. This level of off-food body bends and recovery trend of on-food, after exposure, considered as normal behaviour. **C.** DA pre-exposure to adults significantly reduced the off-food behaviour and on food behaviour at 0^{th} min ($p<0.0001$ and $p<0.005$ respectively) in comparison with the no exposure control. Off-food behaviour was recovered immediately after 30 min of exposure to DA whereas on-food behaviour takes 24 hours to completely recover it to the level of no-exposure control. **D.** $MnCl_2$ exposure to adults also showed a significant reduction in off-food ($p<0.0001$) and showed non-significant reduction in on-food behaviour at 0^{th} minute ($p>0.05$) in comparison with the no exposure control.

The off-food behaviour was completely recovered to normal by 3 hour post exposure to $MnCl_2$. However, the on-food behaviour takes 24 hours to reach normal level similar to no exposure control. **E.** Pre-exposure to DA followed by $MnCl_2$ exposure to adults significantly lowers both off-food and on-food behaviour at 0^{th} min ($p<0.0001$) compared to the no exposure control. Off-food behaviour slowly recovered to the normal level post 24 hour exposure. However, the on-food behaviour did not recover to normal level even after 24 hour post exposure. n≥15 for all four sets of experiments. All statistical analysis was carried out by two-way ANOVA with Tukey's multiple comparisons test. Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, p< *0.05, p< **0.005, p<***0.001 and p<****0.0001).

Dopamine absence helps to combat $MnCl_2$ mediated neurodegeneration

I wanted to further test the role of DA in $MnCl_2$ induced toxicity. Instead of external addition of DA, I used two strains with altered DA levels: *cat-2* mutant strain (CB1112), deficient in Tyrosine hydroxylase gene, with low DA synthesis and the other strain, having an extra copy of *cat-2* gene (UA57), with its overexpression, leads to increased level of DA synthesis. When these *cat-2* over expressed worms were exposed to Mn, showed significant (22%) reduction in body bends in off-food behaviour compare to the control (Fig 4A). Treatment of *cat-2* over expressed worms with $MnCl_2$ further decreased the body bends by 82% compared to the WT treated with $MnCl_2$, whereas, *cat-2* mutant strain did not show any reduction in body bends. On the other hand, *cat-2* mutant strain showed slightly higher body bends compared with wild type without any treatment. Treatment with DA and $MnCl_2$ alone did not affect the body bend behaviour, but rather showed slightly higher body bends (57 and 63% respectively). However, when presented with DA and $MnCl_2$ together, *cat-2* mutant strain worms showed considerable reduction in body bends (29%). Altogether, *cat-2* mutant strain worms with very low level of DA showed resistance to Mn treatment and *cat-2* over expressed worms with high level of DA showed increased sensitivity to $MnCl_2$ mediated toxicity. In addition to that, *cat-2* over expressed adult worms showed a

significant level (>40%) of neurodegeneration at 50 mM and (>70%) at 100 mM $MnCl_2$ exposure compared to the control (Fig 4B). Neurodegeneration observed in *cat-2* over expressed worms is significantly higher compared to the adult worms of other strains (see Fig 2B). Representative image of *cat-2* over expressed worms showing neurodegeneration at 50 mM and 100 mM $MnCl_2$ treatment (Fig 4C-E).

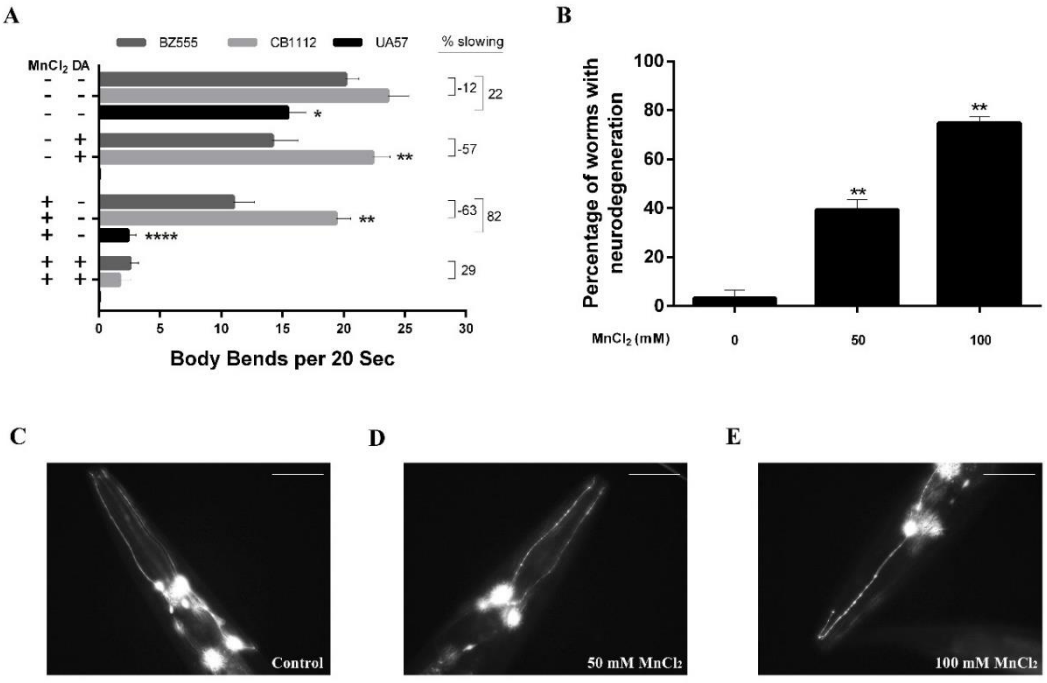

Figure 4. Dopamine enhances neurodegeneration in *cat-2* over expressed and *cat-2* mutant worms. A. Off-food behaviour of the DA synthesis enzyme TH mutants. To assess the activity of DA on DAergic neuronal degeneration I used two strains CB1112 (*cat-2* mutant) and UA57 (*cat-2* over expressed worms). CB1112 is deficient in TH gene (*cat-2*) and UA57 carries an extra copy of *cat-2* gene. No exposure behaviour of both strains was assessed along with BZ555 as control. Off-food body bend behaviour was higher in *cat-2* (12% higher mobility) deficient strain CB1112, whereas *cat-2* over expressing strain UA57 showed significant reduction ($p<0.05$) in body bends observed as nearly 22% slowing, compared to the control. Pre-exposure to DA had significantly increased the body bends in *cat-2* deficient CB1112 ($p<0.005$), observed as 57% increase in mobility compared to BZ555. When $MnCl_2$ exposure alone was given, did not slow down the body bends of CB1112 rather it showed

significantly higher mobility (63%, p<0.005), whereas *cat-2* over expressing UA57 showed significant reduction in body bends (82% slowing down, p<0.0001) when compared to the BZ555. Pre-exposure to DA followed by exposure to $MnCl_2$ reduced body bends of both BZ555 and *cat-2* deficient CB1112 (29% compared to BZ555). n≥15 worms; statistical analysis was carried out by two-way ANOVA with Tukey's multiple comparisons test. **B.** *Cat-2* overexpressing UA57 adult worms exposed to $MnCl_2$ alone show significant increase in percentage of neurodegeneration at both concentrations of 50 mM and 100 mM $MnCl_2$ (p<0.005). Neurodegeneration was scored by the presence of markers such as puncta, blebbing, breaks and loss of CEP neurons. n≥2 trials; each trial contains more than 20 worms; statistical analysis was carried out by one-way ANOVA with Tukey's multiple comparisons test. **C-E.** Representative images of *Cat-2* overexpressing UA57 show neurodegeneration markers on exposure to $MnCl_2$, such as puncta and blebbing at 50 mM and at 100 mM showed breaks and loss of CEP neurons along with other neurodegeneration markers. n≥3 trials; each trial contains more than 15 worms; scale bar represents 50 µm, exposure 500ms. Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, p< *0.05, p< **0.005 and p<****0.0001).

Behavioral alteration due to Mn treatment suggesting change in internal dopamine and dopamine receptors

To test how Mn affects the DA level at the synapse, I performed two tests. SWIP assay is indicative of the level of DA at the synapse because the mean waves taken by the worms will reduce if extracellular DA is higher (Kudumala *et al*, 2017), especially in the synapse (Fig 5A). Pretreatment with DA significantly reduced the body bends, proving that extracellular DA affects behaviour. However, larval worms treated with $MnCl_2$ showed higher mean waves/min than adults treated at 50 mM and 100 mM, pointing at their resistance to neurodegeneration. Though the body bends of larval worms were reduced in the presence of $MnCl_2$ compared to the control, adult worms treated with $MnCl_2$ (50 mM and 100 mM) showed significantly lower mean waves/min than the larval worms. These results suggest that DA levels at the synapse are higher in $MnCl_2$ treated worms, or Mn prevents DA uptake by competitively blocking the DA transporter.

To test the possibility of reduced DA receptor sensitivity, I used the immobilization assay in the presence of DA. This assay was carried out in DA-containing plates to observe the immobilization pattern of the worms. Exogenous DA is received through the DA receptors, and it leads to immobilization of the worm (Sanayal, *et al* 2004; Felton and Johnson, 2011). I used three strains of worms, BZ555, BY200, and MAB300, all expressing GFP under dat-1 promoter in the CEP neurons. All these worms showed a significant reduction in immobilization after 50 mM $MnCl_2$ treatment (Fig 5B), suggesting that sensitivity to exogenous DA in Mn-treated worms is lower than the controls, which is probably due to an aberrant receptor function.

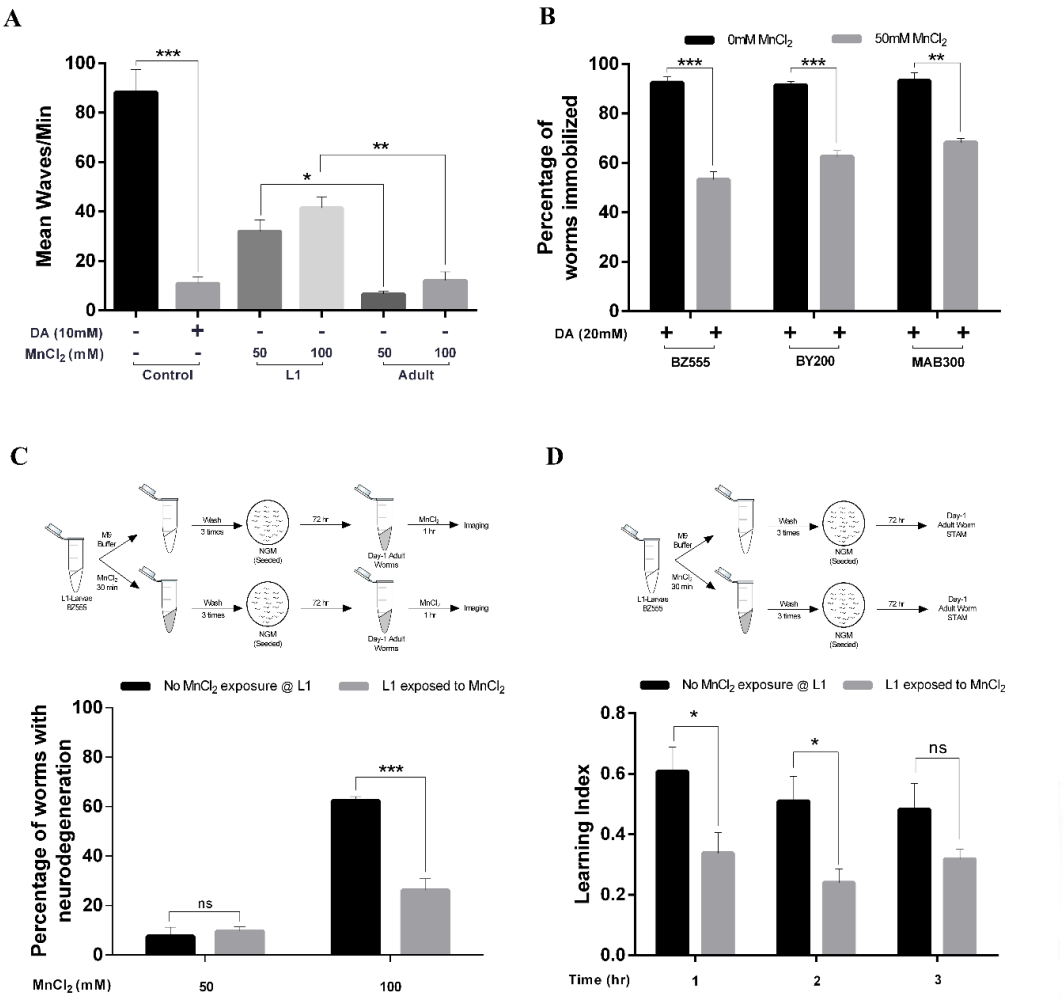

Figure 5. Larval exposure to $MnCl_2$ abolishes short-term associated memory formation in adults. A. SWIP assay of day-1 adult of BZ555 pre exposed to DA significantly affected the mean wave number per minute ($p<0.001$). Larval exposure to $MnCl_2$ did show significant increase in wave number at either concentration of 50 mM and 100 mM $MnCl_2$ compared to day-1 adults at same concentrations ($p<0.05$ and $p<0.005$ respectively). n≥15 worms; statistical analysis was carried out by one-way ANOVA with Tukey's multiple comparisons test. **B.** Immobilization assay on DA plate with larval exposure to strains of BZ555, BY200 and MAB300 to $MnCl_2$ show significant reduction in percentage of worms immobilized, indicating a decreased activity of DA receptors ($p<0.001$, $p<0.001$ and $p<0.005$ respectively). n≥2 trials, each trial contains more than 20 worms; statistical analysis was carried out by two-way ANOVA with Sidak's multiple comparisons test **C.** Graphical representation of the protocol for exposure of BZ555 larval worms to $MnCl_2$ and its re-exposure at adult stage. Worms exposed to 100 mM $MnCl_2$ at L1 stage re-exposed to 50 mM $MnCl_2$ at adult stage show no difference in DAergic neurodegeneration at re-exposure ($p>0.05$) but showed a significant reduction ($p<0.001$) at 100 mM re-exposed worms compared

with the no $MnCl_2$ exposed at L1 control. n≥3 trials; each trial contains more than 15 worms; statistical analysis was carried out by two-way ANOVA with Sidak's multiple comparisons test. **D.** Short-term associated memory training to butanone was given to day-1 adults derived from larval exposure to 100 mM $MnCl_2$ and no exposure control. The data show L1 exposure to $MnCl_2$ have severely affected the learning index at 1st hour ($p<0.05$) as well as recalling of learned behaviour at 2nd hour ($p<0.05$) compared to the no exposure control worms. At 3rd hour larval exposed to $MnCl_2$ worms showed visible reduction in learning index but not significant in comparison with the no exposure control. n≥3 trials; each trial contains more than 50 worms; statistical analysis was carried out by two-way ANOVA with Sidak's multiple comparisons test. Data are represented as the mean + S.E.M. Significance indicated: ns- non-significant, p< *0.05, p< **0.005, p<***0.001.

Mn treated L1 worms show significant resistance to repeated Mn exposure as well as significantly low short term olfactory adaptive learning

The above results indicate a significant change occurring at the cellular level in dopaminergic neurons during Mn exposure. Though the Mn exposure at the larval stage did not show any significant neurodegeneration patterns in the worms, I hypothesized that there might be biochemical modifications at the cellular level, which might have a long-term effect on the worms. Tested this possibility using two approaches: A) the worms exposed to $MnCl_2$ during the larval stage were re-exposed to $MnCl_2$ at their adult stage and were observed for neurotoxicity markers like puncta and breaks. B) Checking the olfactory adaptive learning in the worms to see whether the learning pathways are altered.

Re-exposure to $MnCl_2$ showed that the worms are significantly resistant to DA neurodegeneration (Fig 5C) at 100 mM $MnCl_2$ compared to the non-exposed control. It could be possible that these worms have a low release of DA, as an epigenetic adaptive mechanism, in response to larval exposure of $MnCl_2$. If this is true, then the whole learning pathway in which DA plays a critical role will be affected (unpublished results). Olfactory adaptive learning assay showed that the larval worms exposed to $MnCl_2$ have a significant reduction in learning (1 hour) and memory retention (2nd and 3rd hour) compared to unexposed control (Fig 5D).

Discussion

Animal behaviour is a combinatorial effect of the information processing of the nervous system based on external stimuli. DA is a critical neurotransmitter that drives fundamental functions like movements, habit, reward, attention, and cognition (Nieoullon, 2002; Schultz, 2007). Perturbations in DA levels result in behavioural disorders such as addiction, ADHD, Schizophrenia, manganism, and Parkinson's disease (Mehler-Wex, Riederer and Gerlach, 2006; Cunha *et al*., 2013).

DA signalling is conserved in humans and *C. elegans* and retains almost all DA modulatory genes in the evolution (Vidal-Gadea and Pierce-Shimomura, 2012). Transcriptional regulation of the genes involved in the DA pathway has been well studied in this model system – including DA synthesizing enzyme (cat-2), vesicular monoamine transporter (VMAT-2), DA receptors (dop-1, dop-2, dop-3, and dop-4), DA transporters (dat-1) and a series of regulatory proteins (rnt-1, vt34, etc.) (Robinson *et al*., 2019).

Rats exposed to 100 mg/kg Mn (from postnatal 4 to 28 days) showed impaired learning and memory in Morris and Cincinnati water maze (Amos-Kroohs *et al*., 2017). Children overexposed to Mn develop motor, cognitive, and behavioural deficits (Zoni and Lucchini, 2013). All these data are consistent with my data which showed that larval exposure to Mn in *C. elegans* results in a significant deficit in olfactory adaptive learning and memory in day-1 adults. There is an epigenetic adaptation in the worms because the larval exposure to the Mn reduces the TH activity and DA synthesis.

Similar results were also observed in mammals. In rodents, exposure to Mn during development (from postnatal 1 to 20 days) causes reduced striatal DA levels and cognitive deficit (Tran *et al*., 2002). Early postnatal exposure to Mn (from 28 days before breeding to postnatal 18 days) has shown reduced tyrosine hydroxylase and glutamine synthase mRNA levels in the striatum (Erikson *et al*., 2004).

My results suggest that the presence of DA is essential to induce dopaminergic neuron degeneration. The exact mechanism of the manganese and DA relationship is still not well understood to specify any target of Mn toxicity to dopaminergic neurons. Mn2+ could oxidize to high valence states in an oxygen-rich environment and form Mn3+leading to dopaminergic toxicity through catecholamine oxidation (Sistrunk *et al*., 2007). Scavenging of O2 by Mn^{2+} could oxidize it to Mn^{3+}. The two hydroxy groups in DNA at 3-4-positions of DA and DOPAC allows Mn^{2+} and Mn^{3+} to generate semiquinones and orthoquinone by oxidation of catecholamines. A continuous redox cycling between Mn^{2+} and Mn^{3+} is also believed to be occurring, resulting in DA oxidation product aminochrome (Sistrunk, Ross and Filipov, 2007), which induces acute cell death (Paris and Segura-Aguilar, 2012).

My results suggest that in L1 larvae, the level of DA synthesis is low even though the mRNA expression levels of all DA pathways are similar to that of adult worms. There is supporting evidence to suggest that DA levels are not critical in behaviour at the L1 stage. Cat-2 DA deficient mutants showed similar roaming behaviour to the wild type but were defective in subsequent stages (Stern, Kirst and Bargmann, 2017). Hence, there is a difference in the expression of neurotransmitters as the animal develops,

consistent with the observations that many of the steroid hormones and neuropeptides are expressed during particular development stages (Sisk and Foster, 2004; Truman *et al*, 2005). My present results also show that during the L1 stage, endogenous DA level in the animal is low. Hence, Mn exposure at the L1 stage showed no neurodegeneration as DA is crucial in causing Mn-induced neurodegeneration.

Recovery from the neurodegenerative pattern with partial recovery in behaviour in worms shows that structural recovery follows with delayed functional recovery. These recovery patterns could be due to the short-term exposure to Mn. LUHMES cell lines with acute exposure to rotenone (100 mM 24 hours) showed significant recovery within 7 days (Harris *et al*., 2018). When these cells were re-exposed to study the resilience to rotenone, they showed a significant gene expression profile from that of the first exposure (Harris *et al*., 2018). My results also show a significant difference in response to Mn re-exposure, and worms become resilient to the Mn-induced toxicity.

Similar observations have been made in vervet monkeys treated with Methamphetamine, a psychotic stimulant that releases excess DA levels. These monkeys showed a reversible dopaminergic deficit after 1.5 years (Harvey *et al*., 2000). DA transporter levels show a recovery from 80% loss at one week to 10% at 1.5 years. Moreover, there was a significant recovery in vesicular monoamine transporter-2 and tyrosine hydroxylase levels almost similar to that of the controls (Harvey *et al*., 2000).

The activity of DAT-1 in SWIP assay showed that the early larval exposure to Mn made the animals better adapted. Compared with the adult worms, the larval exposed worms show efficient clearing of DA from the synapse, indicating a better functioning of DAT-1. In addition, the larval exposure to Mn has also affected the DA receptor activity. Sensitivity to DA as analyzed using immobilization assay has shown that larval exposure to Mn has impaired the receptor level functioning. Both these results suggest that Mn could induce a functional reduction in the activity of DAT-1 and DA receptors.

Reduced activity of DAT-1 and receptor functioning providing an advantage for future toxic insult. Acute exposure to Mn on larval worms showed significantly low levels of dopaminergic neurodegeneration. Thus, the epigenetic control of TH and DAT-1 has a vital role in protecting the dopaminergic neurons by decreasing the DA production and thereby, reduce the cellular internalization of Mn (Miller *et al*., 1999).

Even though the reduced expression of TH and DAT-1 has given an advantage for survival, but it might have traded other functions in the organism, such as learning and adapting to a new environment through sensory cues. It is not clear, however, whether such epigenetic modifications are inherited into their subsequent progeny.

Conclusion

The present study demonstrates that DA plays an essential role in olfactory adaptive learning and memory in *C. elegans*. The absence of DA neurotransmitter (cat-2 mutant, an ortholog of human TH gene) resulted in a substantial reduction in learning and memory retrieval. Parallelly, degeneration of DAergic neurons has significantly affected olfactory learning and memory recalling pathways. Similar results have been noted in the fly model, that certain groups of dopaminergic neurons enhance reinforcement (Liu et al., 2012). Hence, the absence of dopaminergic neuronal activation during reward presentation might hinder pairing the conditioned stimulus (CS) with the unconditioned stimulus (UC) during learning and memory. Based on this work and with previously identified neurons in the olfactory learning pathway of *C. elegans*, I deduced a neuronal circuit (Fig 1), which includes CEP neurons, sensory neurons (AWA, AWC), interneurons (AIY, RIA, and RIB), and motor neuron (RMD). Initial sensory stimulus is received by amphid sensory neurons AWA or AWC since both can detect the butanone (Bargmann et al, 1993; Worthy et al, 2018). Both AWA and AWC sensory neurons drive the activation of AIY neurons through chemical synapses. Among these two amphid sensory neurons, AWC has twenty-six synaptic connections with the AIY interneuron. AIY has connection with RIA interneurons. Activation from AWC, in turn, activates AIY and RIA interneurons. Once activated, the RIA interneuron drives the activation of CEP (DA neurons) mechanosensory neurons (Fig 1). Activation of CEP neurons could be either by mechanical stimulation since it is one of the mechanosensory neurons in the nematode (Fig 7 Chapter-1) or through AWA-AWC-AIY-RIA neurons. CEP neurons could activate with RMD motor neurons directly through chemical synapses or indirectly through activation of RIB and RIA interneurons (Pereira, S. and van der Kooy, D., 2012; Pereira L., 2015). This neuronal

circuit model suggests critical involvement of CEP neurons in olfactory learning and memory formation, aid the animal adapt to changing environment through its interaction with amphid sensory neurons, interneurons, and motor neurons.

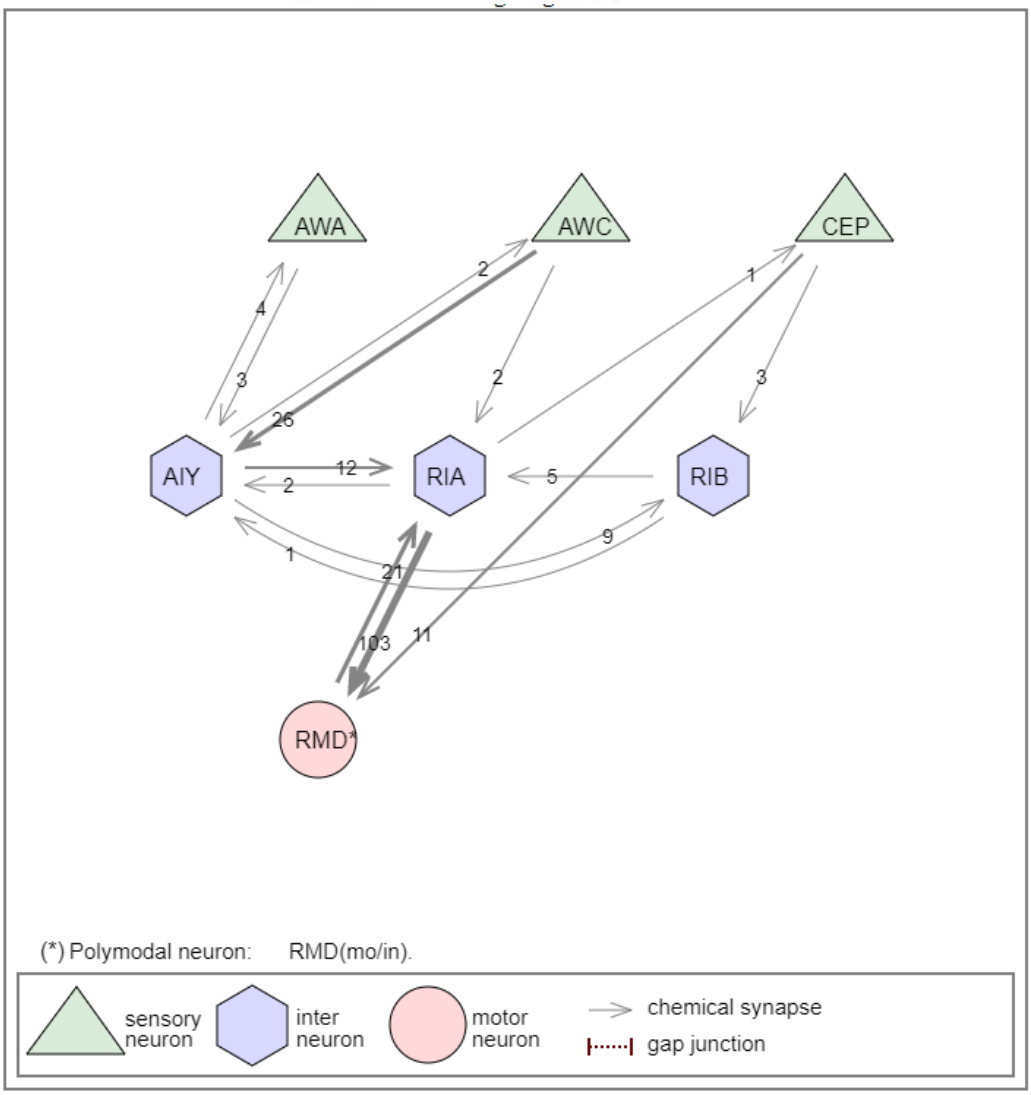

Figure 1: Dopamine neurons play a key role in the learning pathway. AWA and AWC amphid sensory neurons detect an olfactory stimulus, especially butanone in our model. Since AWA and AWC sensory neurons have no synapses with CEP neurons, 1st layer of information is processed plausibly through AIY-RIA interneurons. This activation of AIY-RIA interneurons drives the activation of CEP neuron. CEP neurons interact with RMD motor neurons directly through chemical synapses (11 synapses) or indirectly with RIB and RIA interneurons. Final effector RMD motor neurons take part in migration towards the conditioned stimulus (http://ims.dse .ibaraki. ac.jp/ccep-tool/).

Behavioral studies on tyrosine hydroxylase (cat-2) and dat-1::ICE mutants showed DA and CEP neurons are essential for body bend during exploration and in the avoidance behaviour towards toxic or harmful substances. Recent studies in humans have also suggested tonic levels of DA in the dorsal striatum provide an implicit motor motivational signal, helps in movement (Gepshtein et al., 2014).

Mechanical stimulation during the conditional stimulus, CEP neurons get activated along with the downstream neurons to establish a positive reinforcement between food (UC) and the conditional stimulus (CS) (Steinberg et al., 2013). Dopamine receptors also play a critical role in memory. To abolish memory, it is essential that both dopamine receptors (dop-1 and dop-3) need to be non-functional. These results suggest that both these receptors are functionally redundant and could compensate through partial activation. In the mammalian brain cortical circuit exerts its control through the DA concentration and this, in turn, helps proper fine-tuning of the neuronal network (Trantham-Davidson et al., 2004). Present study results point that dopamine receptors, both D1(dop-1) and D2 (dop-3) like receptors, in *C. elegans* play crucial roles in olfactory adaptive learning and memory.

In addition, progressive CEP neuron degeneration in the dat-1::ICE strain has significantly reduced dopamine receptor levels. This observation was further validated using the learning assays. The day-2 and day-3 old worms showed decreased olfactory learning and memory in dat-1::ICE strain in the presence of exogenous DA. Besides, the progressive degeneration of CEP neurons in dat-1::ICE was found to affect the activity of cholinergic neurons, showing increased sensitivity to tetramisole,

an acetylcholine receptor agonist. These results support the observation that the absence of dopamine neurons leads to increased acetylcholine receptor activity, as seen in PD models (Tubert and Murer, 2020).

Olfactory adaptive training of strain expressing GCaMP-6 on CEP neurons showed that in the presence of CS stimulus, the dopaminergic neurons evoked a delayed increase in fluorescence intensity associated with a transient increase in intracellular calcium influx compared to the untrained worms. This finding suggests activated dopamine neurons and the downstream associated neurons are essential to establish positive reinforcement during learning and memory (Steinberg et al., 2013). siRNA mediated blocking of human caspase interleukin-1β-converting enzyme (ICE) in dat1::ICE worms, reduced the CEP neuron degeneration, and in turn able to rescue the learning and memory defects. Moreover, alpha-synuclein-mediated progressive CEP neurodegeneration resulted in defective olfactory adaptive learning and memory also strongly supplement the importance of CEP neurons in olfactory learning and memory.

Apart from learning and memory, this study discerns an adaptation to chronic levels of Mn during the early development stages in *C. elegans* worms, with resistance to neurotoxicity to repeated Mn exposure. Major alterations are in dopamine synthesis and dopamine receptor expression level. Our data shows that larval exposure to Mn in *C. elegans* results in a significant deficit in olfactory adaptive learning and memory in adult worms. This observation strongly suggests adaptive modification occurs in *C. elegans* worms due to Mn exposure at early stages of development. In essence, this study provides new insights into the functional role of DA-dependent enhancement of

Mn-induced dopaminergic neurodegeneration. Mn exposure during the early development stage had a neuroprotective function towards Mn re-exposure. This protective mechanism could be via transcriptional or post-translational regulation of several candidate genes. These adaptive/epigenetic modifications also result in cognitive impairment in the adult stage indicates alterations in neuronal connectomes or dopamine receptor function. Further understanding these changes would help in deciphering the disease onset both in Manganism and PD.

In summary, the present study using various mutations and pharmacological approaches in *C. elegans* reveals the critical role of DA in memory. It indicates that CEP neurons (the primary DA releasing neurons in the worm) play an effective role in cognitive function. Further understanding of these connectomes and their active processing of information will hopefully enrich our understanding of the intrinsic relations between memory, motivation, and decision making at cellular levels.

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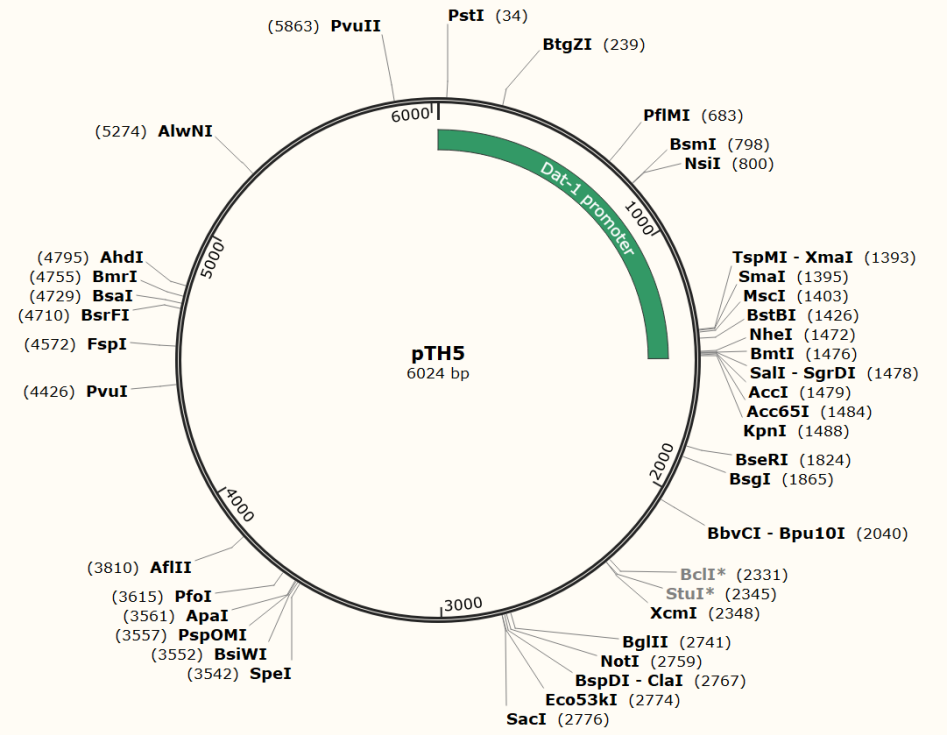
Annexure

Appendices

1. Creating plasmid for dopamine neuronal calcium Imaging (pDAT-1::GCaMP-6)

❖ **Structure of the plasmid pTH5 containing the dat-1 promoter region**



❖ **Sequence of the dat-1 promoter sequence (1357bp)**

TGAAGATGACAAAAATATTCTACATGAGCAAAATCTTCTCATCAGGGCACCCGG

TAG

ATGCTATCTTCTGGTTGCAGAAGATGCTCGTCAGCTAGTTCCCTCCACTT

ACTTGTAAGTTTTTTCTACAAATTTAAAAGAGAAAACCAAAGAAAAATAT

AAAGGAAATGATTATCCTACTCCAACAAAACTCTCCGAAAAATAATTATT

GCTCTAAATCGCATCGCGCTTCGCCGAGCAGCACTTCCCTCACCTACCGA

TCAAAAATAACAGATTACAACGAAACAGGTAGAAAAGAAGTAGAAAATAT

CTAGAAATGAGAGAAAAATTGGTTGATAATAAAAAAAAGTCCGTAAAAAG

ATAGCAATTAAAATTAGAGCGATTGTCCACGAGAAAATTGTGAGAATATT

CAGAGAGTTCTATAAATTTTTGAAAATTTAGCATTTTTATTTCAGCCAAG

ATGTCCTCAACGTCTCATTCCTCATCTCCGAGCCACTGCTATCAAAAATT

CGCCAAAAAGCGGCATCAACCTACGAGAAGTACAAAGTCTTTCTGCCCAC

ACAACCGAATAATTATCTCTGAAATGTTTCTAGTCGTTTTTGTATTTTAA

AGCACATTCCCAATTCTGAATACTTTTCTGAAATCCATGAAATGGAACTT

GAATCCAGTTTTCACTAAAACGACCTCATACACTTTCTCTCGTATCCTCA

AAATATCTATGACATTATCATTAGCTTCGCTAGTTTCATTTCTTTCAAAT

ATTATGCATTCTTAAATTCCGATACCCGCGTGCAAAAGTGCTCTATTGAG

CAACTTTGGGATCATATGTACACACCAATGCCCTTTTCCCAAATCTTTTC

CTGTCCTTTTCTCTAAAAACAATAAATCCATGCCTATTCCAGTATGACCC

CTTTGAAGCAGATATAATCGCACAAACATATACACATAGCTCGGATAAAT

GTAGAAAAAGAAGAAAAGAAGTATAAGTAGATAGATGCTTTCCGGCAATT

ATCCACCGCACCGTAGTCTTCACCAACTGAGACTGCGTCGTTAGGAGACG

CCGACATGATTCAGAAGCAGAATTTGGAAGAAAAACGACGATGATATTGA

GGCTGGCACACATACACCGGAATATTCGACATGCCACCACATCTAGATTC

CAAGGCAATCTCTACCTCTTCCCATTCTTTCGGTTTTTTTGTTCTGACAA

GAAAAGTGGATAGCTACGGGCTCAATGAGCTGATTTTATTTTTAAATATC

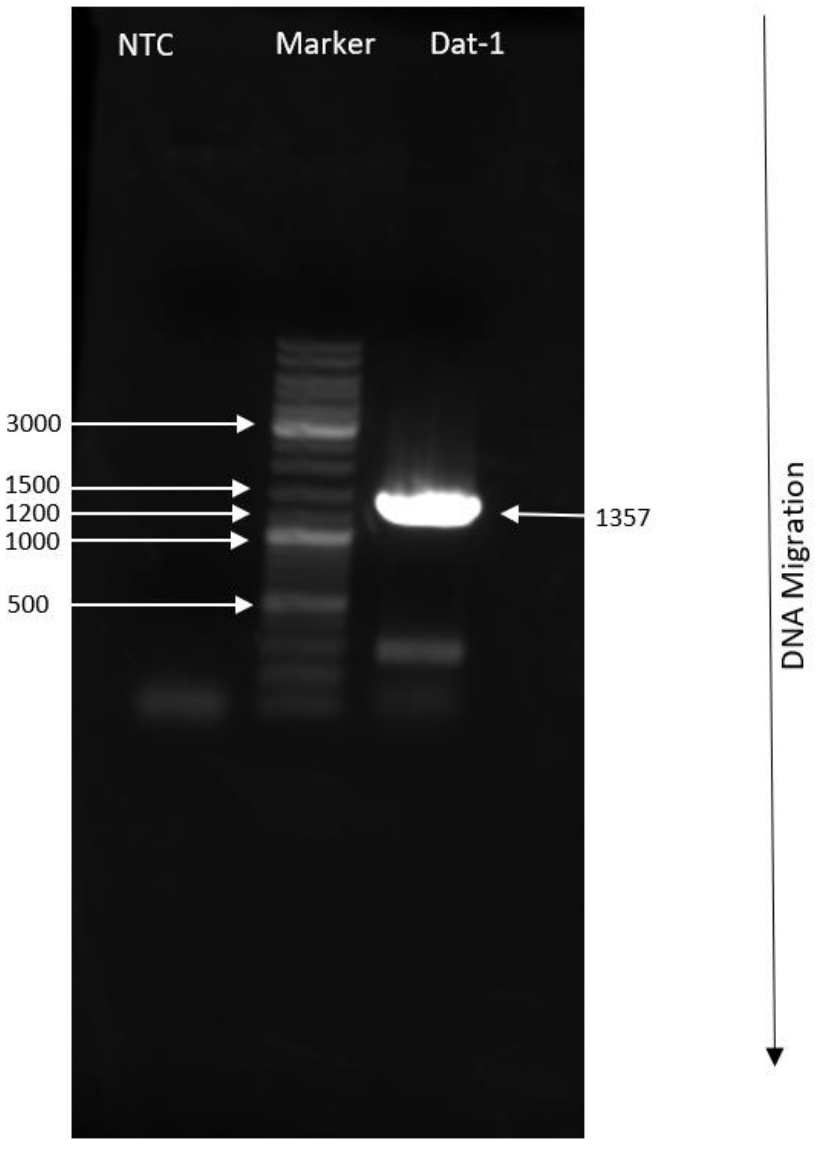
TTAAAACTATACTAGATTCATGTGTTTTCAGGTCCATATTCCAAATTAGT

CGAAAAGCTGATCCCGCTACGGTTTACTCGAATCTCAACAATTTTTAGCC

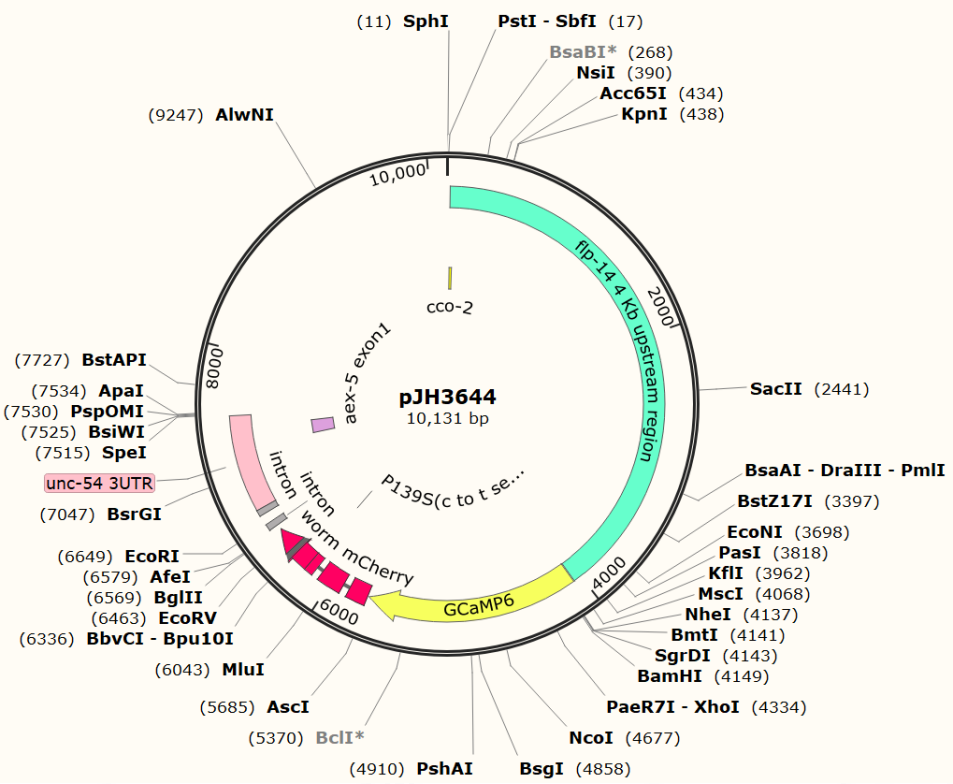
- **The following primers were used for the amplification of dat-1 promoter region from plasmid pTH5. These primers were designed by NEBuilder software (version 2.3.1). A 1357 bp from the dat-1 promoter region were amplified. Restriction enzymes details were used for generating the primers are; 5' end Msc-1 and 3' end Pst-1.**

Dat_1 Fwd	AATAAGCTTGCATGCCTGCATGAAGATGACAAAAATATTCTAC ATG
Dat_1 Rev	CATACCTTTGGGTCCTTTGGGGCTAAAAATTGTTGAGATTC

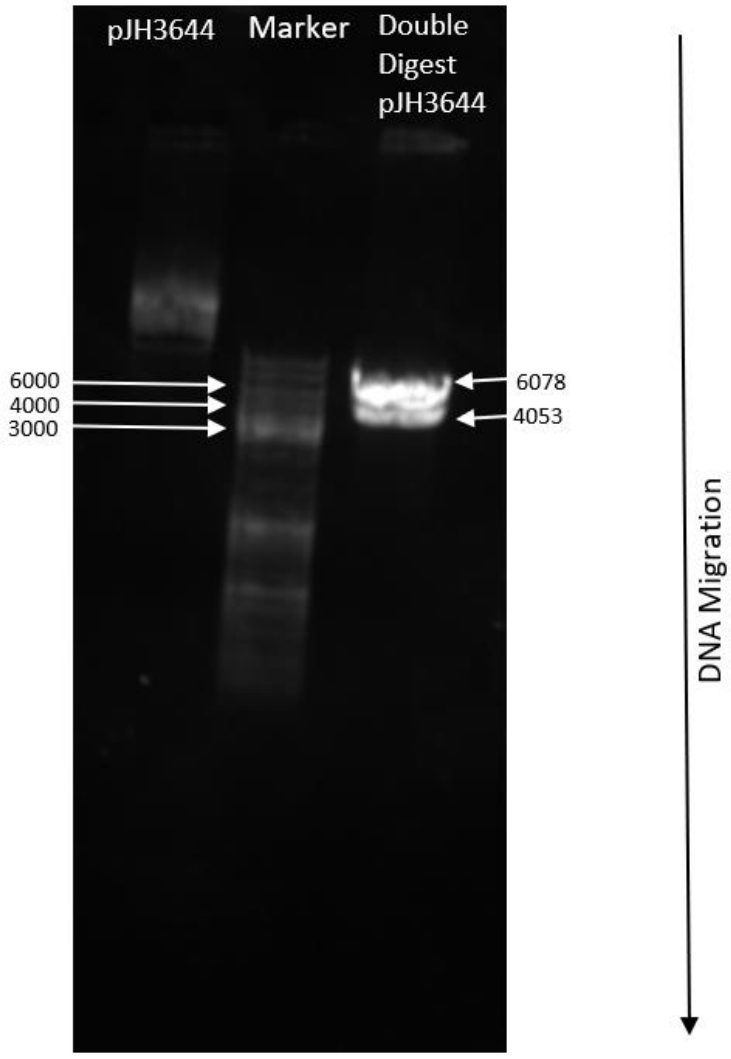
- **PCR amplified dat-1 promoter region by using Gibson primers. The PCR product was gel purified using Qiagen Gel purification Kit (Cat No./ID: 28115).**



❖ **Structure of the plasmid pJH3644 containing GCaMP-6 gene.**



- **pJH3644 plasmid was double digested with restriction enzymes, Msc1 and Pst1 to remove the flp-14 promoter region. Double digestion showed the expected fragment sizes of 6078 and 4053bp. The large fragment 6078 contain GCaMP-6 was gel purified using Qiagen Gel purification Kit (Cat No./ID: 28115).**



❖ **Gibson Assembly reaction was set up using the following reaction mixture.**

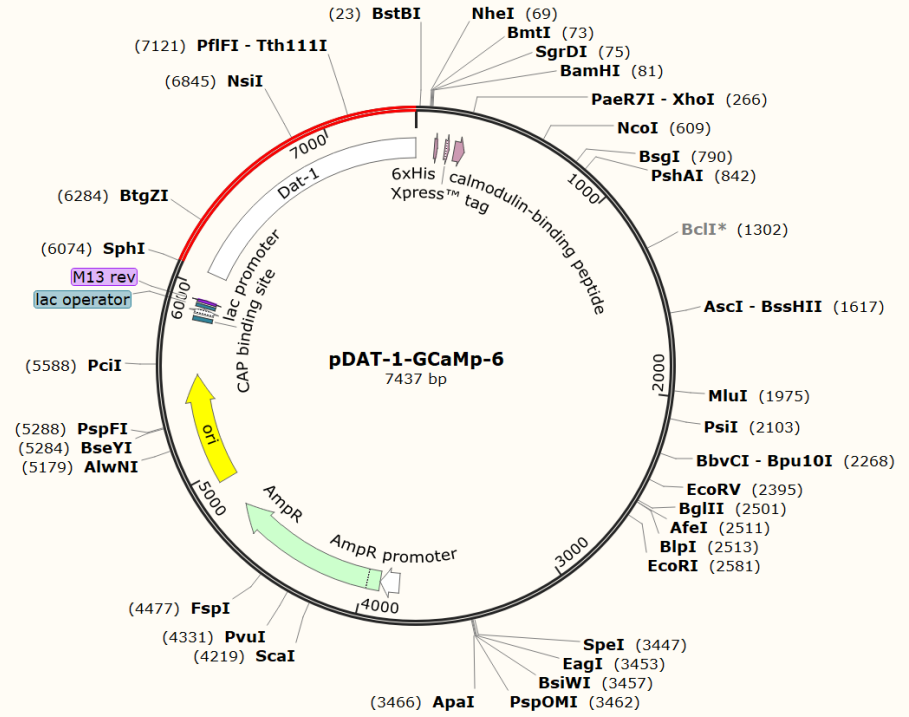
Components of the master mix

	NEB Cat#	Final Rxn Concentration (Gibson 2012)	
PEG-8000		3.75	%
Tris-HCl (pH 7.5)		75	mM
MgCl2		7.5	mM
dGTP		0.15	mM
dTTP		0.15	mM
dATP		0.15	mM
dCTP		0.15	mM
DTT		7.5	mM
NAD		0.75	mM
T5 Exonuclease	M0363S	0.004	U/uL
Phusion Polymerase	M0530S	0.025	U/uL
Taq DNA Ligase	M0208S	4	U/uL
H2O			

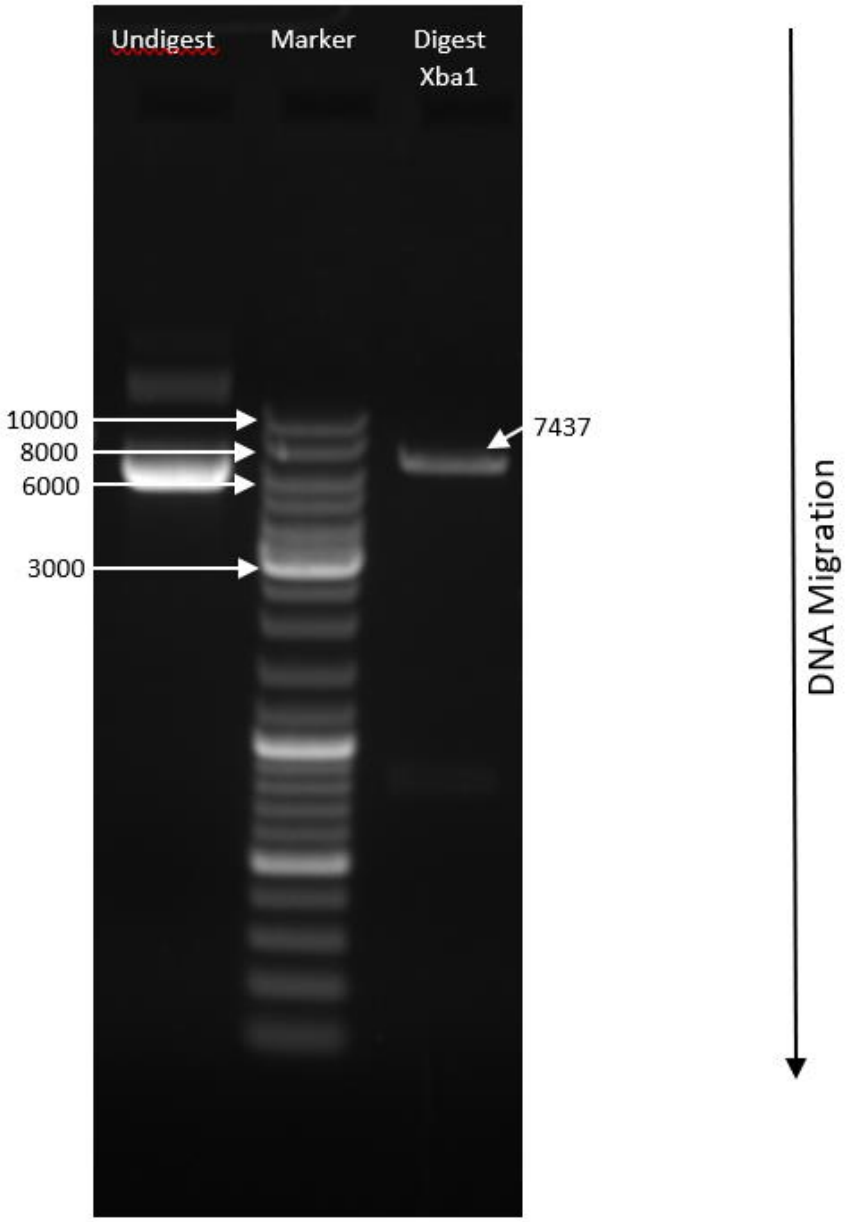
❖ The concentration of dat-1 promoter fragment and the double digested plasmid pJH3644 containing GCaMP-6.

1. Dat-1 Promoter fragment- 150ng
2. pJH3644 fragment -300ng

❖ Plasmid map of the plasmid pDAT-1::GCaMP-6 construct after Gibson assembly.



❖ Conformation of the plasmid pDAT-1-GCamP6 construct after Gibson assembly. Restriction enzyme Xba-1 was used to conform the product size.. Xba-1 digestion linearized the plasmid with the expected size of 7437 bp.



2. Construction of plasmid vector for creating siRNA mediated silencing of human interlukin-1 beta convertase (ICE) mRNA.

- **The mRNA sequence taken for generation of primers for ICE silencing. Genbank Accession ID: MB7507.1.**

GGGAGGAGAGAAAAGCCATGGCCGACAAGGTCCTGAAGGAGAAGAGAAA
GCTGTTTATCCGTTCCATGGGTGAAGGTACAATAAATGGCTTACTGGATGA
ATTATTACAGACAAGGGTGCTGAACAAGGAAGAGATGGAGAAAGTAAAACG
TGAAAATGCTACAGTTATGGATAAGACCCGAGCTTTGATTGACTCCGTTATT
CCGAAAGGGGCACAGGCATGCCAAATTTGCATCACATACATTTGTGAAGAA
GACAGTTACCTGGCAGGGACGCTGGGACTCTCAGCAGATCAAACATCTGG
AAATTACCTTAATATGCAAGACTCTCAAGGAGTACTTTCTTCCTTTCCAGCT
CCTCAGGCAGTGCAGGACAACCCAGCTATGCCCACATCCTCAGGCTCAGA
AGGGAATGTCAAGCTTTGCTCCCTAGAAGAAGCTCAAAGGATATGGAAACA
AAAGTCGGCAGAGATTTATCCAATAATGGACAAGTCAAGCCGCACACGTCT
TGCTCTCATTATCTGCAATGAAGAATTTGACAGTATTCCTAGAAGAACTGGA
GCTGAGGTTGACATCACAGGCATGACAATGCTGCTACAAAATCTGGGGTAC
AGCGTAGATGTGAAAAAAAATCTCACTGCTTCGGACATGACTACAGAGCTG
GAGGCATTTGCACACCGCCCAGAGCACAAGACCTCTGACAGCACGTTCCT
GGTGTTCATGTCTCATGGTATTCGGGAAGGCATTTGTGGGAAGAAACACTC
TGAGCAAGTCCCAGATATACTACAACTCAATGCAATCTTTAACATGTTGAAT
ACCAAGAACTGCCCAAGTTTGAAGGACAAACCGAAGGTGATCATCATCCAG
GCCTGCCGTGGTGACAGCCCTGGTGTGGTGTGGTTTAAAGATTCAGTAGG
AGTTTCTGGAAACCTATCTTTACCAACTACAGAAGAGTTTGAGGATGATGCT

ATTAAGAAAGCCCACATAGAGAAGGATTTTATCGCTTTCTGCTCTTCCACAC

CAGATAATGTTTCTTGGAGACATCCCACAATGGGCTCTGTTTTTATTGGAAG

ACTCATTGAACATATGCAAGAATATGCCTGTTCCTGTGATGTGGAGGAAATT

TTCCGCAAGGTTCGATTTTCATTTGAGCAGCCAGATGGTAGAGCGCAGATG

CCCACCACTGAAAGAGTGACTTTGACAAGATGTTTCTACCTCTTCCCAGGA

CATTAAAATAAGGAAACTGTATGAATGTCTGCGGGCAGGAAGTGAAGAGAT

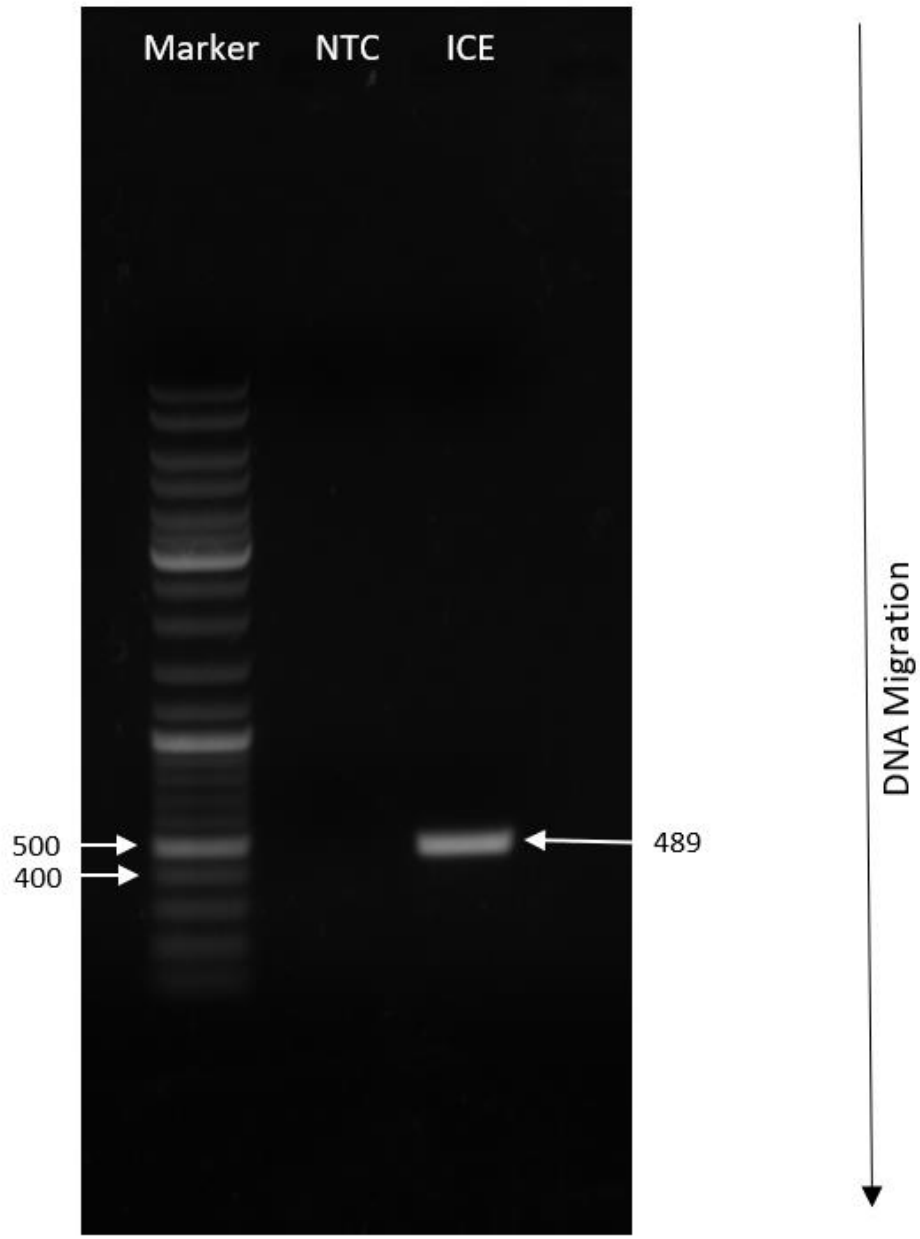
CGTTCTGTAAAAGGTTTTTGGAATTATGTCTGCTGAATAATAAACTTTTTTTG

AAATAATAAATCTGGTAGAAAAATGAAAAAAAAAAAAAAAAA

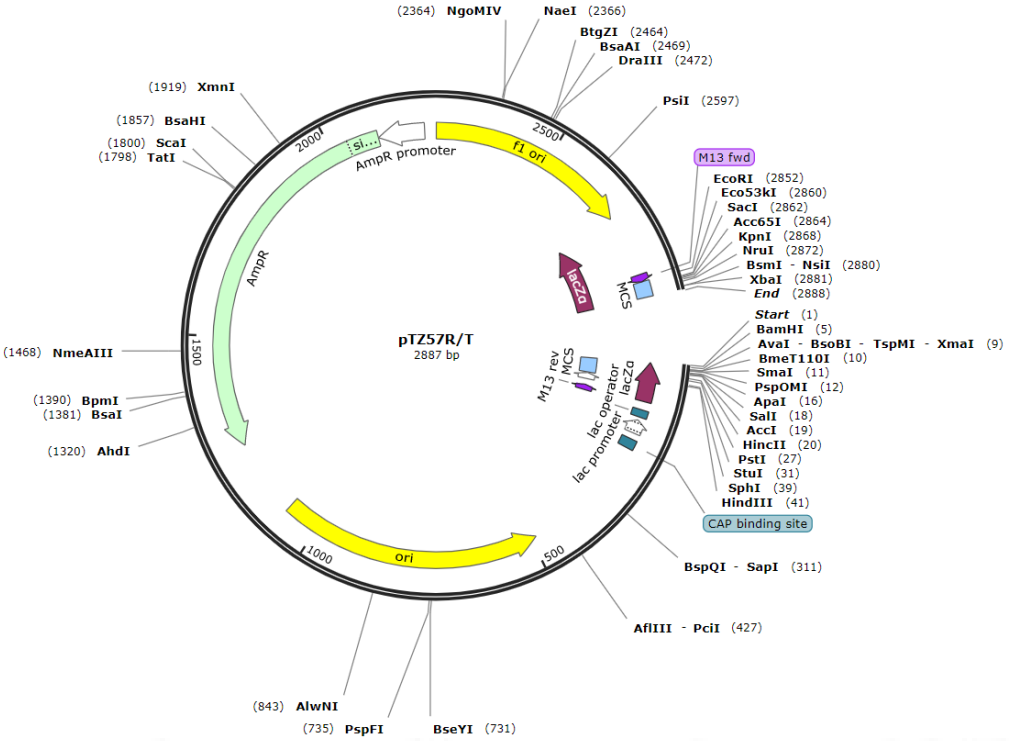
❖ **Primers used for the amplification of 489 bp region of ICE gene.**

ICE_ Fwd	ACAAGACCTCTGACAGCACG
ICE_ Rev	GCATCTGCGCTCTACCATCT

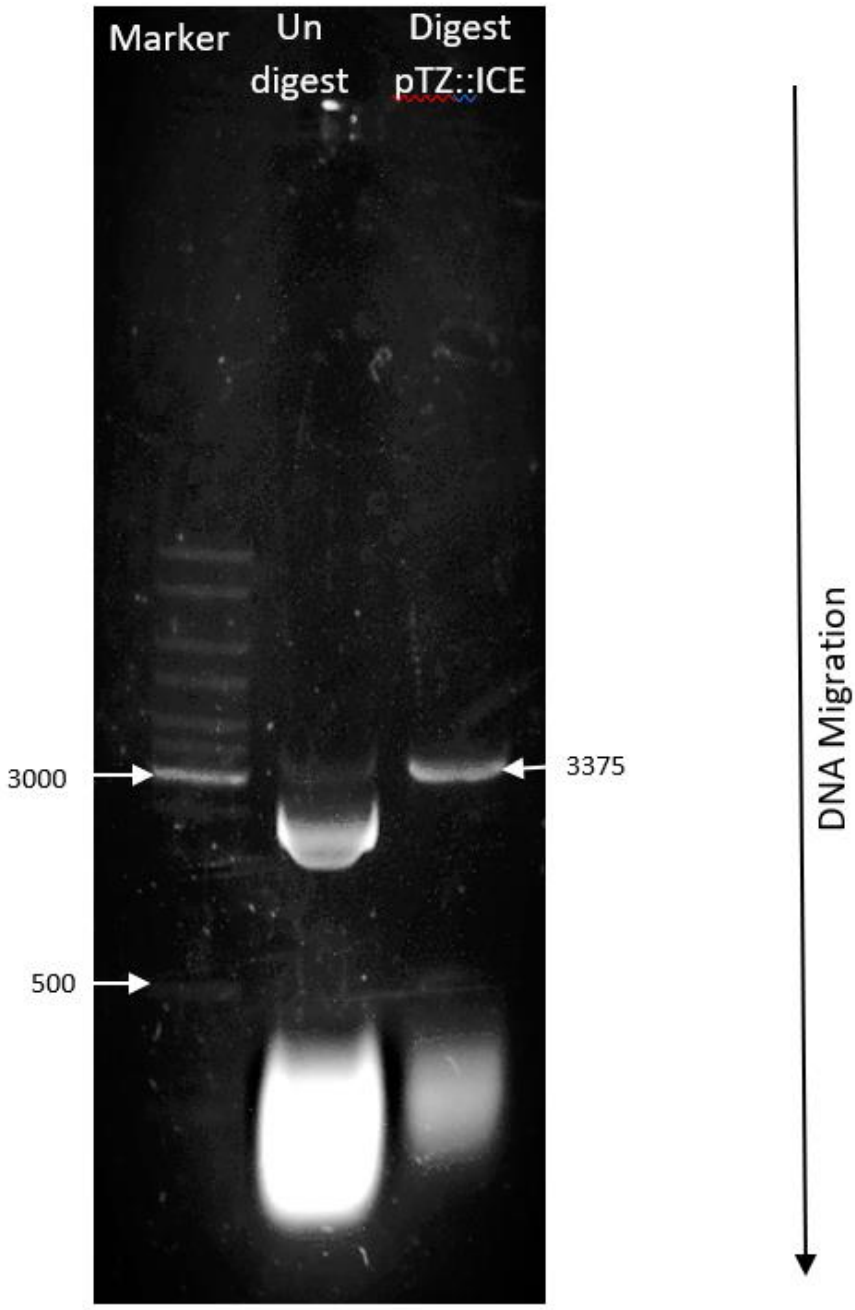
❖ **PCR amplified product of 489 bp. The PCR product is gel purified using Qiagen Gel purification Kit (Cat No./ID: 28115).**



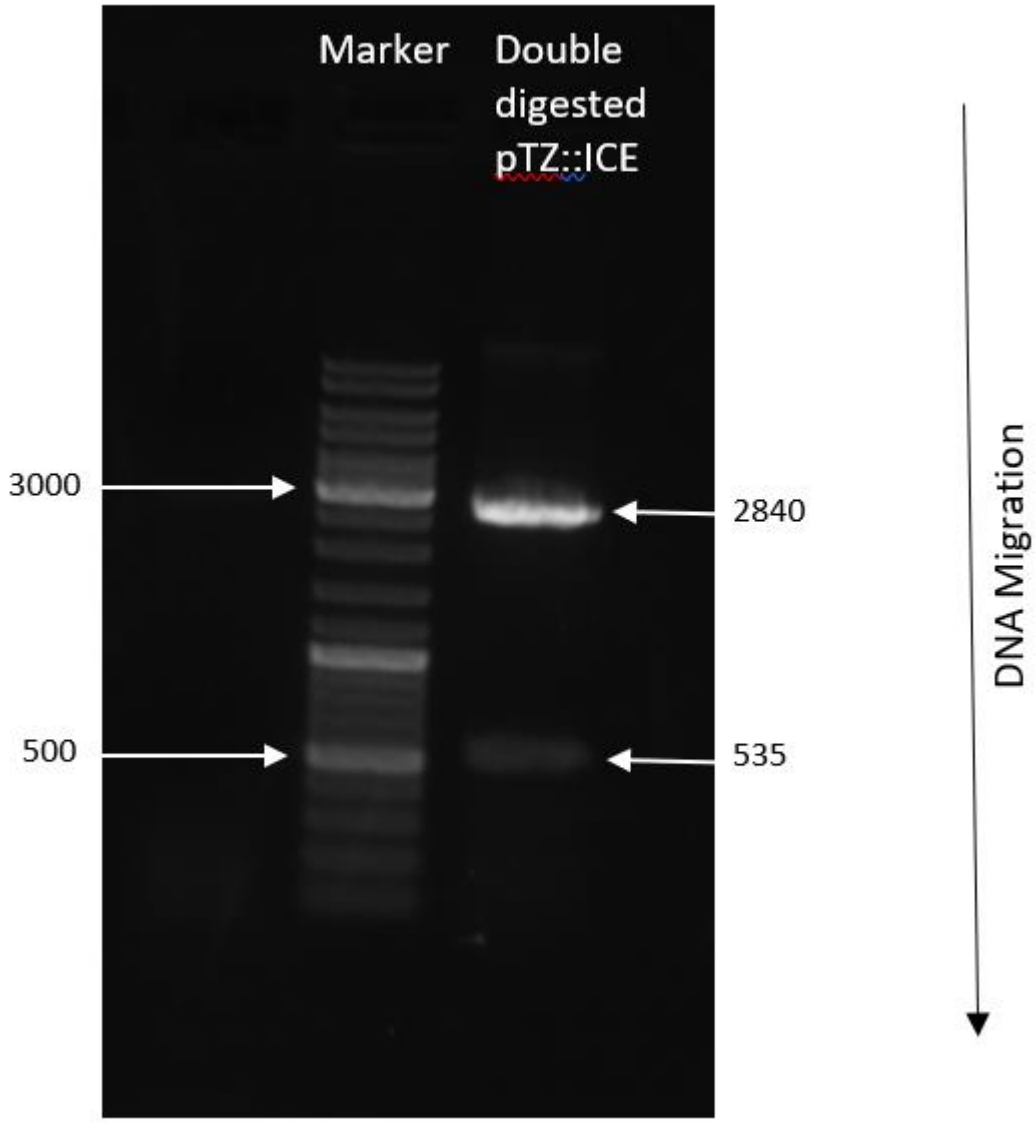
❖ **The PCR product was gel eluted and cloned into the plasmid pTZ57R/T using T/A cloning method.**



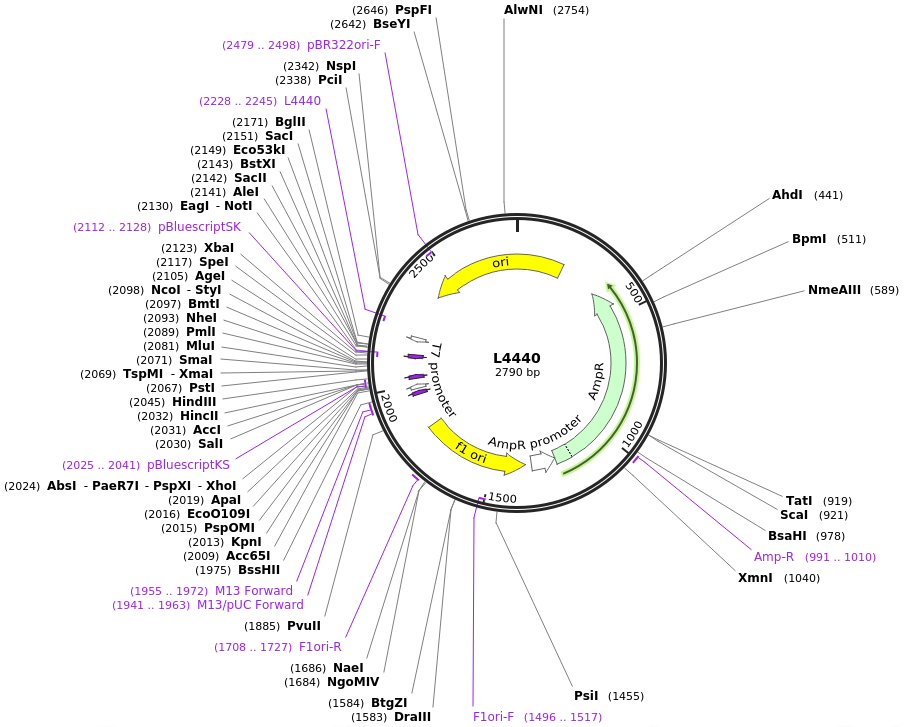
❖ **pTZ::ICE linearising with HindIII restriction enzyme and confirmed the product size 3375bp.**



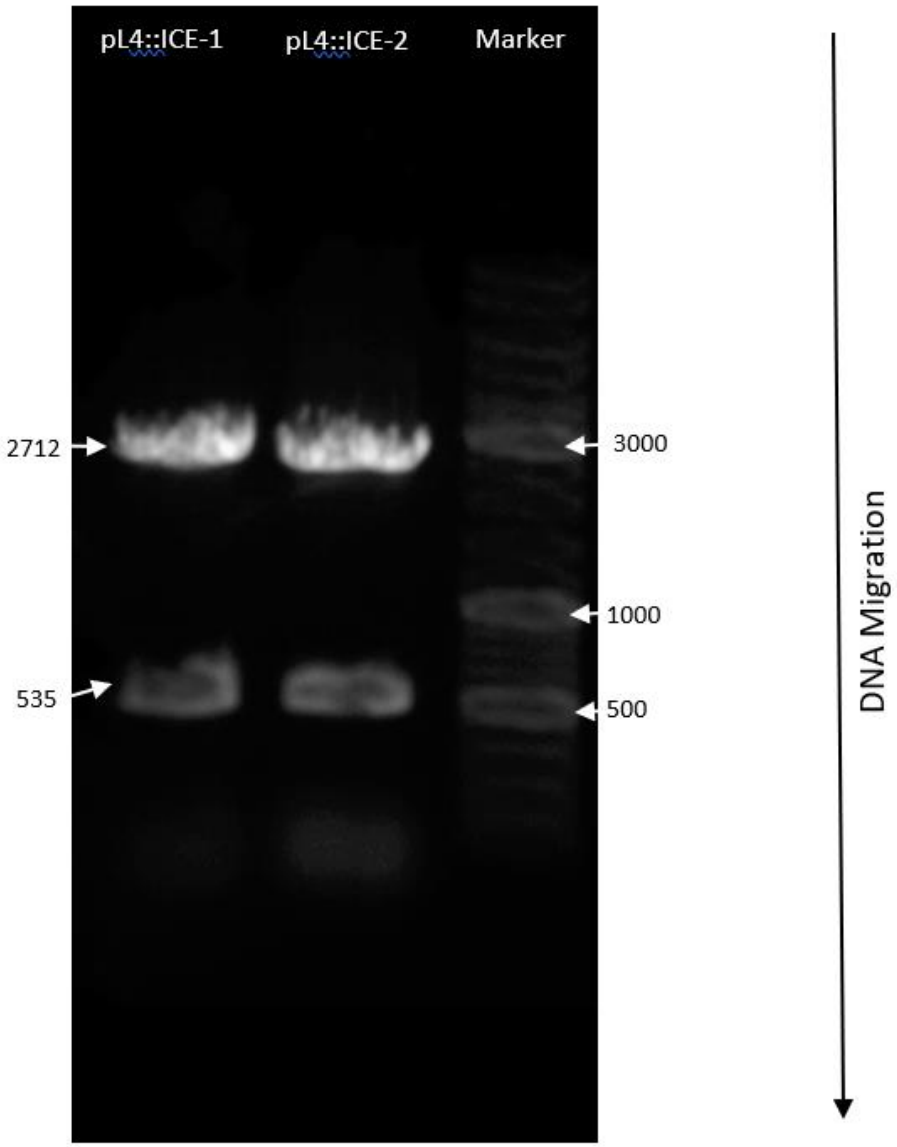
- **The plasmid pTZ::ICE was then double digested using restriction enzymes Xba-1 and HindIII to release the ICE gene fragment. The expected size of the ICE gene fragment is 535 bp. The ICE gene fragment was gel purified using Qiagen Gel purification Kit (Cat No./ID: 28115).**



❖ **The plasmid pL4440 was used to express dsRNA of ICE gene**



- **The ICE gene fragment was cloned pL4440 plasmid that was digested using restriction enzymes, Xba-1 and HINDIII. The resultant plasmid pL4::ICE was conformed using double digestion with Xba-1 and HindIII restriction enzymes. This pL4::ICE plasmid were used for the siRNA mediated silencing of ICE gene.**



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Sources included in the report

SA	**Neuromodulatory Role of Melatonin in Rotenone Induced Parkinson's Disease.docx** Document Neuromodulatory Role of Melatonin in Rotenone Induced Parkinson's Disease.docx (D40498660)	1
J	**PLOS Genetics** URL: ecedbb5f-c6c5-47e5-b9d1-df2ac65bef3f Fetched: 2/24/2019 2:31:30 AM	1
W	URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4479152/ Fetched: 10/30/2020 12:24:00 PM	28